

International Congress of Immunology 2016

IMMUNOTHERAPY: HARNESSING THE POWER OF THE IMMUNE SYSTEM

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Abstract Book

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Oral Abstracts | Monday, 22 August 2016

30 Minute Oral | 08:30 - 10:15

B Cells

Novel aspects of B cell response to vaccination and viral infection

*Batista, F. Gaya, M, Martinez, N.
Ragon Institute*

B lymphocytes form an integral part of the immune system via the production of specific antibodies and by establishing immunological memory enabling a swift and effective response to pathogenic assault. To fully understand the processes whereby this is achieved, it is essential to gain a comprehensive understanding of the events involved in B cell activation and antibody production. This is initiated by the encounter with and acquisition of cognate antigen by the B cell receptor (BCR); processes occurring primarily in secondary lymph nodes (SLOs) such as the lymph node (LN) and which are regulated by complex systems. The functionality of these systems is strictly dependent on maintaining the architecture of the SLOs. During this talk I will describe how the LN structure enables the early encounter of B cells with antigen. Antigenic properties such as the size and nature of the pathogen affect the specialized lymph node antigen delivery systems that exist in readiness to deliver pathogen-derived antigen to B cells. This variability in the types of encounter enables the most appropriate response for that particular antigen to occur, giving the maximal protection to the host. Lastly, the consequences of the disruption of the LN architecture, and the help provided by other cells such as iNKTs, on the immune response will also be described. Their critical role in the immune response is evident following infection, which causes a temporary disruption in the cellular organization of lymphoid organs and lack of essential cytokines leading to a reduction in immune responses.

Tolerance & Transplantation

Immunoregulation in Transplantation

*Wood, K., Bushell, A., Harden, P., Issa, F., Hester, J.
University of Oxford*

Immune regulation is fundamental to any immune response to ensure that it is appropriate for the perceived threat to the host. Strategies for the induction of specific unresponsiveness to donor alloantigens currently under investigation in the clinic take advantage of two of the major mechanisms for the induction of tolerance to self antigens – deletion and immunoregulation/suppression. We have demonstrated that human regulatory T cells expanded *ex vivo* can protect human allografts (skin, islets and vessels) from rejection. Treg migrate to the allograft and function *in situ*. Donor alloantigen reactive Treg are more effective on a per cell basis than polyclonal Treg. Together with other leukocyte populations, including regulatory T cells,

B cells and macrophages as well as myeloid derived suppressor cells and dendritic cells, Treg contribute to the regulation of immune responses *in vivo* after organ transplantation. The identification and characterisation of Treg that can control immune responsiveness to alloantigens has opened up exciting opportunities for new therapies in transplantation. Phase 1/2a clinical trials are in progress – www.onestudy.org.

A new approach to GVHD based on biomarkers

*Ferrara, J.
Icahn School of Medicine at Mount Sinai*

Acute graft versus host disease (GVHD) is the major toxicity of allogeneic bone marrow transplantation. Recent research has identified and validated unexpected biomarkers that predict outcomes. This session will review the use of large and informative biorepositories to create biomarker signatures that predict severe GVHD prior to its clinical onset and that can guide preemptive GVHD therapy. This session will also review mechanistic studies that have illuminated unexpected interactions between the innate and adaptive immune systems in the GI tract that have led to both conceptual breakthroughs and the discovery of novel therapeutic approaches.

Cancer Immunotherapy

Immunotherapy for persisting viral infection and associated cancer

*Frazer, I.¹, Mattarollo, S.¹, Leggatt, G.¹, Wells, J.¹, Tuong, K.¹, Kuo, P.¹, Jazayeri, S.¹, Lambert, P.², Chandra, J.^{1,3}
¹The University of Queensland, Australia, ²The University of Madison, Wisconsin, ³Admedus Immunotherapies*

While vaccines have proven effective at preventing virus infections, immunotherapy for persisting cancer associated viral infections and their associated cancers has proven less tractable. Using a model where a viral antigen, the E7 protein of human papillomavirus (HPV)16, is expressed in grafted mouse skin, we have determined mechanisms preventing effective immunotherapy of persisting HPV infection. In this model, the effector functions of vaccine induced cytotoxic T cells are locally inhibited by multiple mechanisms initiated by hyper-proliferative epithelium associated with HPV infection, and mediated via inflammasome activation, IL-18 and IFN γ . Intra-dermal delivery of an E7 specific polynucleotide vaccine encoding ubiquitin conjugated viral proteins can overcome inhibition of effector T cell function in this model. A herpes virus (HSV) glycoprotein D immunotherapy using the same strategy has, in clinical trial, induced a cell mediated immune response, and is currently under evaluation in persistently HSV-2 infected subjects.

Autoimmunity

1906

Insulin-degrading enzyme deficiency protects from type 1 diabetes by regulating autoantigenicity and proliferation of pancreatic beta cells

Bessard, M.-A.¹, Moser, A.¹, Waeckel-Enée, E.¹, Chhuon, C.², Lipecka, J.², Kim, J.¹, Guenette, S.³, Santamaria, P.⁴, Wong, F.S.⁵, Diana, J.¹, Guerrero, C.², Unanue, E.⁶, van Endert, P.¹

¹INSERM U 1151, Paris, France, ²INSERM US 24, Paris, France,

³Massachusetts General Institute for Neurodegenerative Diseases, Charlestown, United States, ⁴University of Calgary, Julia McFarlane Diabetes Research Centre, Calgary, Canada, ⁵University of Cardiff, Cardiff, United Kingdom, ⁶Washington University School of Medicine, Division of Immunobiology, Saint Louis, United States

Type 1 diabetes is the result of the destruction of pancreatic beta cells by autoreactive T cells. Proinsulin as an autoantigen with beta cell-restricted expression triggers and sustains the autoimmune CD4+ and CD8+ T cell response and islet inflammation. We hypothesized that insulin-degrading enzyme (IDE), a protease genetically associated with type 2 diabetes possessing very high insulin affinity, might be involved in proinsulin processing and presentation. We find high expression of IDE and an increased number of autoantigenic insulin B chain fragments in IDE-deficient beta cells of non-obese diabetic (NOD) mice, and normal to increased stimulation of insulin-specific CD8 and CD4 T cells by IDE-deficient islets. This suggests that IDE physiologically degrades (pro)insulin in beta cells. However, surprisingly, IDE-deficient NOD mice are more resistant to diabetes transfer by T cells specific for insulin but not for another key autoantigen, harbor fewer diabetogenic splenocytes and display strongly reduced diabetes incidence. Moreover, IDE-deficient islet grafts are more resistant to autoimmune rejection. Seeking to explain the apparent paradox between normal to increased insulin presentation and resistance to the diabetogenic action of insulin-specific T cells, we find that IDE deficiency results in upregulated beta cell regeneration in response to autoimmune inflammation. Diabetes protection in IDE-deficient mice most likely result from moderately increased beta cell stress recently shown to induce beta cell proliferation. Thus IDE acts both in processing of the key autoantigen in murine type 1 diabetes and as a regulator of beta cell stress, ultimately enhancing autoimmune pathology and diabetes.

Redemption or Revolt of Forbidden Clones: Mutations, Autoantibodies and CTLA4-Ig Therapy

Goodnow, C.¹, Reed, J.¹, Burnett, D.¹, Brink, R.¹, Christ, D.¹, Schofield, P.¹, Perotti, S.², Enders, A.², Ziegler, J.³, Wainstein, B.³, Roscioli, T.^{1,3}, Gray, P.³

¹Garvan Institute of Medical Research, Darlinghurst NSW, ²John Curtin School of Medical Research, Canberra ACT, ³Sydney Children's Hospital, Randwick, NSW

Many B cells in the pre-immune repertoire carry antibodies that bind to self-antigens. Some are deleted before these antibodies can be tested for binding foreign antigens, but others are

carried on anergic B cells. Tolerance by B cell clonal anergy is enigmatic since the B cells exist in a potentially reversible state, balanced between activation and apoptosis, posing the risk of autoimmunity. Here I will describe a clinical case of LRBA deficiency resulting in life-threatening autoimmune cytopenias that rapidly resolved by treatment with CTLA4-Ig (Abatacept) following diagnosis by whole genome sequencing. By producing a mouse avatar of the case, we confirm that LRBA-deficiency leads selectively to low CTLA4 on Tregs and other T cells. Autoantibody secretion in LRBA or CTLA4 deficiency may be explained by our earlier findings that failure to dampen CD86 expression on anergic B cells allows them to escape FasL-mediated killing by helper T cells and be reactivated to form huge numbers of plasma cells. I will present evidence that physiological reactivation of anergic cells in humans and mice yields precursors for germinal centre cells that hypermutate their antibody variable segments away from self-reactivity. This represents a mechanism for actively acquired tolerance that has been hypothesised in the past but not taken seriously. IgD, which is the main antigen receptor displayed on anergic B cells before any reactivation, helps to keep anergic cells alive in the pre-immunerepertoire by attenuating their response to self-antigens.

Oral Abstract Sessions

10:30 - 12:10

Transcription Factors

2131

The dual role of Bcl6 in the regulation of the differentiation and function of follicular cytotoxic T cells

Chen, Y.¹, Leong, Y.A.¹, Man, K.², Ong, H.¹, Wu, D.³, Kallies, A.², Yu, D.¹

¹Monash University, Department of Biochemistry and Molecular Biology, Melbourne, Australia, ²Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia, ³University of North Carolina, Chapel Hill, United States

Follicular helper T (T_{fh}) cells are a subset of CD4⁺ helper T cells that express the chemokine receptor CXCR5 and migrate into B-cell follicles. T_{fh} cells support germinal centre response for antibody affinity maturation and memory formation. As the master transcription factor for T_{fh} differentiation, Bcl6 is required for CXCR5 expression on CD4⁺ T cells. Recently, my lab identified a subset of CD8⁺ cytotoxic T cells that express CXCR5 and localize to B-cell follicles in a model of viral infection. CXCR5⁺ CD8⁺ T cells are required to control the infection of T_{fh} cells and termed follicular cytotoxic T (T_{fc}) cells. As in T_{fh} cells, we found Bcl6 was also upregulated in T_{fc} cells. The loss of Bcl6 led to the defects of T_{fc} differentiation while overexpression of Bcl6 promoted the differentiation of T_{fc} cells. Importantly, we also found Bcl6 suppresses the cytotoxic function by inhibiting the expression of genes that encode cytotoxic molecules such as Granzyme B. In HIV infections, T_{fh} cells serve as the major CD4 T cell compartment for HIV-1 infection, replication, and production. The discovery of the dual role of Bcl6 in the regulation of T_{fc} differentiation and function might provide an explanation why CD8⁺ T cells are less effective to control the infection of T_{fh} cells in B-cell follicles than non-T_{fh} cells in T-cell zone.

2075

Mapping regulatory networks in human regulatory T cells by chromatin conformation capture

Sadlon, T.^{1,2}, Bandara, V.¹, Brown, C.¹, Beyer, M.³, Schultze, J.³, Bent, S.¹, Forrest, A.⁴, Barry, S.²

¹Molecular Immunology, Robinson Research Institute, Adelaide, Australia, ²Women's and Children's Health Network, Gastroenterology, Adelaide, Australia, ³LIMES, University of Bonn, Bonn, Germany, ⁴Henry Perkins Institute for Medical Research, Genomics, Perth, Australia

Regulatory T cells (T_{reg}) play a key role in tolerance and immune homeostasis. Our research has revealed that T_{reg} function and stability is orchestrated by gene networks regulated by FOXP3 and microRNAs. It is now evident that coordinated gene regulation occurs in a cell specific manner to bring together regulatory elements and coding regions, and this is conformation dependant. While bioinformatics can

predict targets of transcription factors with some accuracy, and genomics datasets can now identify functional motifs in chromatin, such as super enhancers and lncRNAs, the targets of these regions cannot be predicted by linear annotation models. Conformation capture can determine which non coding elements interact with T_{reg} specific genes, and we can superimpose on this our FOXP3 binding site data. This analysis will reveal the conformation dependant transcriptional regulation of T_{reg} genes, and will also allow for the first time annotation of SNPs from autoimmune diseases to functional targets. As proof of principle we have used SATB1, a key FOXP3 repressed gene in T_{reg} (1), as a conformation capture target. Using 4Cseq we have identified a T cell specific activation induced FOXP3 responsive super enhancer over 300Kb upstream, and this region includes 5 enhancer elements. We now confirm that this enhancer is T cell activation dependant, is repressed by FOXP3, and overlaps a number of IBD/Colitis SNPs from GWAS datasets, confirming the power of this approach. The functional impact of autoimmune SNPs on SATB1 expression is now under investigation

1) Beyer M, et al. Nature immunology 2011;12(9):898-907.

768

A combinatorial threshold model for effector differentiation of CD8⁺ T cells mediated by Blimp-1 and T-bet

Masson, F.^{1,2,3}, Xin, A.^{2,3}, Liao, Y.^{2,3}, Preston, S.^{2,3}, Guan, T.⁴, Gloury, R.^{2,3}, Olshansky, M.^{2,5}, Lin, J.-X.⁶, Li, P.⁶, Speed, T.P.^{2,7}, Smyth, G.K.^{2,7}, Ernst, M.^{1,8}, Leonard, W.J.⁶, Pellegrini, M.^{2,3}, Kaech, S.^{4,9}, Nutt, S.L.^{2,3}, Shi, W.^{2,7}, Belz, G.T.^{2,3}, Kallies, A.^{2,3}

¹Olivia Newton-John Cancer Research Institute, Cancer Inflammation Laboratory, Heidelberg, Australia, ²Walter & Eliza Hall Institute, Parkville, Australia, ³University of Melbourne, Department of Medical Biology, Parkville, Australia, ⁴Yale University School of Medicine, Department of Immunobiology, New Haven, United States, ⁵University of Melbourne, Department of Computing and Information Systems, Parkville, Australia, ⁶National Heart, Lung and Blood Institute, NIH, Bethesda, United States, ⁷University of Melbourne, Department of Mathematics and Statistics, Parkville, Australia, ⁸La Trobe University, School of Cancer Medicine, Heidelberg, Australia, ⁹Howard Hughes Medical Institute, Chavry Chase, United States

T cell responses are guided by cytokines that induce transcriptional regulators, which ultimately control the differentiation of effector and memory T cells. However, it is unknown how their activities are coordinated and integrated during this process. In the present study, we used broad transcriptional profiling of antigen-specific CD8⁺ T cells to systematically dissect the relative contributions and interdependency of two major drivers of CD8⁺ effector T cell differentiation, Blimp-1 and T-bet. Furthermore, we unraveled how IL-2 impacts on transcriptional changes during effector cell differentiation *in vivo*. We showed that Blimp-1 acts as a signal integration node for IL-2 and pro-inflammatory cytokines, in particular IL-12, which overlap to initiate effector differentiation. Moreover, while deficiency in either Blimp-1 or T-bet left effector function partially intact, combined ablation of both factors resulted in loss of expression of effector molecules and an inability to control systemic viral infection. Importantly,

T-bet/Blimp1 double-deficiency resulted in the derepression of several molecules usually associated with IL-17 producing CD8⁺ T (Tc17) cells and severe immune pathology. Overall, our data reveal two major pathways of effector differentiation governed by the availability of Blimp-1 and T-bet and suggest a model for cytokine-induced transcriptional changes that combine quantitatively and qualitatively to promote robust effector CD8⁺ T-cell differentiation.

757

The role of SATB1 in virus-specific CD8⁺ T cell mediated immunity

Nuessing, S., Russ, B.E., Grant, E.J., Kedzierska, K., Turner, S.J. University of Melbourne, Peter Doherty Institute for Infection and Immunity, Microbiology and Immunology, Melbourne, Australia

CD8⁺ T cells are a critical component of the immune response to viral infection such as the influenza virus. Upon virus recognition immunologically naïve T cells undergo a program of proliferation and functional differentiation resulting in a large pool of effector cells secreting cytotoxic molecules and pro-inflammatory cytokines required for viral clearance. Importantly, a long-lived pool of virus-specific memory T cells survives after resolution of the infection and responds more readily following re-infection, allowing faster viral clearance and providing the basis of protective T cell-mediated immunity.

Changes to the structure of DNA-protein complexes, within responding T cells underpin the differences in gene transcription that confer the functional and phenotypic differences between naïve, effector, and memory T cells. SATB1 is a master regulator of chromatin structure, and as such, a key regulator of transcription. We have shown that *Satb1* is expressed in naïve T cells, down-regulated as naïve cells differentiate to effector and then memory, in both mice and human. Additionally, characterization of mice bearing a dysfunctional *Satb1* gene revealed a depletion of T cells bearing the CD44^{hi} signature, a gene up-regulated after T cell activation. On this basis, we hypothesize that SATB1 regulates CD8⁺ T cell differentiation. To address this hypothesis, we use next generation sequencing methods to characterize the genome-wide distribution of SATB1 binding at each stage of T cell differentiation. Combining these data with transcriptomic analyses of the wild-type and *Satb1* mutant mice will provide insights into the role of SATB1 in mediating virus-specific CD8⁺ T cell differentiation.

1665

Transcriptional networks that establish B cell identity

Ikawa, T.¹, Miyai, T.¹, Kawamoto, H.²

¹RIKEN Center for Integrative Medical Sciences, Yokohama, Japan,

²Kyoto University, Institute for Frontier Medical Sciences, Kyoto, Japan

B lymphocytes are generated from hematopoietic stem cells (HSCs) through a successive series of lineage restriction processes. Although many transcription factors (TFs) and epigenetic regulators have been implicated in controlling the B cell fate specification, exact mechanisms remain elusive. We have recently established a culture system that can

examine gene regulatory networks of HSCs differentiating into B cell lineage (Ikawa et al. 2015). We overexpressed Id3 protein fused with ERT2 (Estrogen receptor) protein whose nuclear translocation is induced by 4-hydroxytamoxifen (4-OHT) in hematopoietic progenitors and cultured them in B cell differentiation condition. B cell development of Id3-transduced cells was blocked at an early developmental stage and the cells grew enormously with maintaining multipotency in the presence of 4-OHT. Virtually all cells became CD19⁺ B cells by simply withdrawing 4-OHT within 7 days. We then performed RNA-Seq analysis at multiple time points in this system. The expressions of “master” TFs such as Ebf1 and Pax5 were dramatically upregulated after 48hrs of induction. Notably, the sequential upregulation of TFs before the onset of the key TF program was found. The earliest responding TFs such as Egr1 and Klf4 had a peak at 0.5-2hrs followed by a continuous stream of TFs within 24hrs. Knockdown of these genes led to the defected B cell generation both in vivo and in vitro. Taken together, our findings identified a genome-wide view of the dynamic transcriptional waves in B cell fate determination.

1387

Hierarchical regulation of enhancer establishment and gene expression by transcription factors during mononuclear phagocyte development

Kurotaki, D.¹, Nakabayashi, J.², Nishiyama, A.¹, Sasaki, H.¹, Kaneko, N.¹, Ozato, K.³, Suzuki, Y.⁴, Tamura, T.¹

¹Yokohama City University Graduate School of Medicine, Department of Immunology, Yokohama, Japan, ²Yokohama City University, Advanced Medical Research Center, Yokohama, Japan, ³NICHD, NIH, Bethesda, United States, ⁴Graduate School of Frontier Sciences, University of Tokyo, Department of Computational Biology, Chiba, Japan

Monocytes and dendritic cells (DCs) are mononuclear phagocytes essential for innate and adaptive immunity. These phagocytes develop from hematopoietic stem cells via intermediate progenitors, such as granulocyte-monocyte progenitors (GMPs), monocyte-DC progenitors (MDPs), and common monocyte progenitors (cMoPs) or common DC progenitors (CDPs). However, the molecular mechanism underlying their differentiation potential remains incompletely understood. Recent studies suggest that promoter-distal enhancers are key for cell fate decision. In this study, we performed chromatin immunoprecipitation-sequencing (ChIP-seq) analysis of enhancer-related histone modifications (H3K4me1 and H3K27ac) in GMPs, MDPs, cMoPs, CDPs, monocytes, DCs, and neutrophils. We found that monocyte- and DC-specific enhancers were gradually established at progenitor stages prior to gene expression. DNA motif analysis implicated that these enhancers were regulated by combinations of lineage-determining transcription factors such as PU.1, RUNX, C/EBP, and IRF. Interestingly, the motifs specifically enriched in the mononuclear phagocyte lineage were the IRF binding motifs. Among IRFs, IRF8 is known to be highly expressed from the MDP stage and necessary for MDP-to-CDP and cMoP-to-monocyte transitions. Somewhat unexpectedly, however, global gene expression patterns were comparable

between the remaining *Irf8*^{-/-} progenitors and their wild-type counterparts. Nevertheless, the enhancer landscapes of *Irf8*^{-/-} progenitors were significantly distinct from those of wild-type cells, with the enhancer landscapes of *Irf8*^{-/-} GMPs, MDPs, and cMoPs all remaining similar to that of wild-type GMPs. IRF8 was required particularly for the establishment of enhancers common in monocytes and DCs. These results illustrate that enhancer establishment and gene expression are dynamically and hierarchically regulated by transcription factors during mononuclear phagocyte development.

1506

Batf2/Irf1 induces inflammatory responses in classically activated macrophages, lipopolysaccharides, and mycobacterial infection

Roy, S.^{1,2}, Guler, R.^{3,4}, Parihar, S.P.^{3,4}, Schmeier, S.⁵, Kaczowski, B.^{1,2}, Nishimura, H.^{1,2}, Shin, J.W.^{1,2}, Negishi, Y.^{1,2}, Ozturk, M.^{3,4}, Hurdoyal, R.^{3,4}, Kubosaki, A.¹, Kimura, Y.¹, de Hoon, M.J.L.^{1,2}, Hayashizaki, Y.^{2,6}, Brombacher, F.^{3,4}, Suzuki, H.^{1,2}

¹RIKEN Center for Life Science Technologies, Yokohama, Japan, ²Riken Omics Science Center, Yokohama, Japan, ³University of Cape Town/Institute of Infectious Disease and Molecular Medicine (IDM), Division of Immunology, Cape Town, South Africa, ⁴International Centre for Genetic Engineering and Biotechnology (ICGEB), Cape Town Component, Cape Town, South Africa, ⁵Massey University, Institute of Natural and Mathematical Sciences, North Shore City, New Zealand, ⁶Riken Preventive Medicine and Diagnosis Innovation Program (PMI), Yokohama, Japan

Basic leucine zipper transcription factor Batf2 is poorly described, whereas Batf and Batf3 have been shown to play essential roles in dendritic cell, T cell, and B cell development and regulation. Batf2 was drastically induced in IFN- γ -activated classical macrophages (M1) compared with unstimulated or IL-4-activated alternative macrophages (M2). Batf2 knockdown experiments from IFN- γ -activated macrophages and subsequent expression profiling demonstrated important roles for regulation of immune responses, inducing inflammatory and host-protective genes *Tnf*, *Ccl5*, and *Nos2*. *Mycobacterium tuberculosis* (Beijing strain HN878)-infected macrophages further induced Batf2 and augmented host-protective Batf2-dependent genes, particularly in M1, whose mechanism was suggested to be mediated through both TLR2 and TLR4 by LPS and heat-killed HN878 (HKTb) stimulation experiments. *Irf1* binding motif was enriched in the promoters of Batf2-regulated genes. Coimmunoprecipitation study demonstrated Batf2 association with *Irf1*. Furthermore, *Irf1* knockdown showed downregulation of IFN- γ - or LPS/HKTb-activated host-protective genes *Tnf*, *Ccl5*, *Il12b*, and *Nos2*. Batf2 deficiency in mice resulted in resistance to *M. tuberculosis* infection with reduced immunopathology. Conclusively, Batf2 is an activation marker gene for M1 involved in gene regulation of IFN- γ -activated classical macrophages, as well as LPS/HKTb-induced macrophage stimulation, possibly by Batf2/*Irf1* gene induction. Taken together, these results underline the role of Batf2/*Irf1* in inducing inflammatory responses in *M. tuberculosis* infection.

2879

The effects of transgenic expression of a T-box molecule, eomesodermin, in naive T cells on their activation and exhaustion status

Eshima, K., Misawa, K., Noma, H., Iwabuchi, K.
Kitasato University School of Medicine, Department of Immunology, Sagami-hara, Japan

A T-box family transcription molecule, Eomesodermin (Eomes), controls effector function of CD8⁺ CTLs. Eomes is expressed in effector or memory CD8⁺ T-cells, but is also found in "exhausted" cells, although its precise roles there remain to be fully delineated. In order to determine the relevance of Eomes in T-cell activation statuses, we developed Eomes-transgenic mouse using human CD2 promoter/enhancer. In Eomes-Tg mouse, the number of peripheral T-cells was smaller than that in wild type mice. This was accompanied with decreased expression of CD127 and Bcl-2 in Tg mature T-cells, which is frequently observed in exhausted T-cells. It was also noticed that higher proportion of peripheral CD8⁺ T-cells in Eomes-Tg mice expressed some of exhausted T-cells markers, such as, PD-1, LAG-3, or TIM-3, suggesting that part of exhausted T-cell phenotypes may result from higher expression of Eomes. As for other activation markers, expression of CD44, CD122, Ly-6C was also up-regulated on Eomes-Tg CD8⁺ T-cells. By utilizing Tg-TCR, it was suggested that optimal expression of many of these markers, except for CD122, still requires TCR-stimulation. Transgenic expression of Eomes alone may not be adequate for provision of functional activity to CD8⁺ T-cells, either, since CD44^{lo+} CD8⁺ T-cells from Eomes-Tg did not produce IFN- γ or exert cytotoxicity until antigenically primed. Collectively, these results imply that Eomes induction alone may be insufficient for proper CTL differentiation and that Eomes expression without TCR stimulation might induce exhausted phenotypes.

2692

Deciphering the importance of H3K27me3 demethylation during early CD8⁺ T cell responses against influenza A virus

Li, J., Olshansky, M., Turner, S.
University of Melbourne, Department of Microbiology & Immunology, Melbourne, Australia

The acquisition and maintenance of cytotoxic CD8⁺ T cell (CTL) function in response to influenza virus infection is partly regulated by co-ordinated changes in chromatin structure, histone modifications and binding of transcription factors. Developmental or lineage-defining genes in stem cells and CD4⁺ T cells are bivalently marked by the repressive trimethylated lysine 27 (H3K27me3) and activating trimethylated lysine 4 (H3K4me3) on the H3 histone. In naive CD8⁺ T cells, we have also shown that a number of bivalent genes rapidly resolve to a transcriptionally permissive state when differentiated into virus-specific effector and memory T cells (Russ *et al.*, 2014). To understand how the early removal of the repressive mark, H3K27me3, defines immediate transcriptional responses of bivalent genes, naive CD8⁺ T cells were stimulated with the SIINFEKL peptide for 3, 5 and 24hrs. Whole-genome H3K27me3 chromatin-immunoprecipitation-sequencing (ChIP-seq) and

RNA-seq at each time point showed that distinct cohorts of gene expression, including bivalently marked genes, *Tbx21* (encoding T-bet) and *Irf4*, are transcribed as early as 3hrs post-activation. Importantly, inhibition of H3K27me3 demethylation perturbed the immediate expression of these transcription factors along with early activation markers *i.e.* CD62L and CD69. Therefore, the timely resolution of chromatin bivalency by H3K27me3 demethylation, significantly shapes early CTL gene expression programs through the activation of these transcription factors. These molecular signatures potentially represents a novel regulatory step involved in the formation of effector and memory CD8⁺ T cells.

Emerging Technologies

1831

Next-generation detection of antigen-responsive T cells using DNA barcode-labeled peptide-major histocompatibility complex I multimers

Bentzen, A.K.¹, Marquard, A.M.², Lyngaa, R.¹, Saini, S.K.¹, Andersen, S.R.¹, Donia, M.³, Svane, I.M.³, Thor Straten, P.³, Szallasi, Z.², Jakobsen, S.N.⁴, Eklund, A.C.², Hadrup, S.R.¹

¹Section for Immunology and Vaccinology, Technical University of Denmark, Frederiksberg, Denmark, ²Center for Biological Sequence Analysis, Technical University of Denmark, Lyngby, Denmark, ³Center for Cancer Immune Therapy, Copenhagen University Hospital, Herlev, Denmark, ⁴Immudex, Copenhagen, Denmark

Identification of antigenic peptides recognized by T cells is important for understanding and treating immune related diseases. Current cytometry-based approaches are limited to simultaneous screening of T cell reactivity towards 10-100 distinct peptide specificities, which poorly match the large diversity of T cell recognition in humans. Consequently it has been impossible to comprehensively analyze T cell responsiveness in cancer, infectious and autoimmune diseases. We present and validate a novel technology that enables parallel detection of numerous different peptide-MHC responsive T cells in a single sample using >1000 different peptide-MHC multimers labeled with individual DNA barcodes. After isolation of MHC multimer binding T cells their recognition are revealed by amplification and sequencing of the MHC multimer-associated DNA barcodes. The relative frequency of the sequenced DNA barcodes originating from a given peptide-MHC motif relates to the size of the antigen-responsive T cell population. We have demonstrated the use of large panels of >1000 DNA barcoded MHC multimers for detection of rare

T cell populations of virus and cancer-restricted origin in various tissues and compared with combinatorial encoding of fluorescent-labeled MHC multimers. Finally, we have demonstrated that this technology can be applied for multiplex T cell detection both in limited biological samples, such as uncultured tumor material, and for simultaneous assessment of target recognition and functional capability of T cells. This technology enables true high-throughput detection of antigen-responsive

T cells and will advance our understanding of immune recognition from model antigens to genome-wide immune

assessments on a personalized basis.

3509

Multiplexed spatially resolved protein detection from FFPE samples for oncology and immunotherapy

Rhodes, M.¹, Warren, S.¹, Jung, J.¹, Merritt, C.¹, Webster, P.¹, Dunaway, D.¹, Mills, G.², Tumei, P.³, Beechem, J.¹

¹NanoString Technologies, Seattle, United States, ²MD Anderson Cancer Center, Houston, United States, ³University of California, Los Angeles, Los Angeles, United States

Traditional immunohistochemistry approaches to staining proteins within FFPE tissue are suboptimal because they only evaluate a few targets simultaneously, rely on signal amplification, and require subjective assessment of staining intensity. NanoString has developed a method for determining abundance and localization of a large number of proteins (potentially up to 800-plex) from FFPE samples using non-amplified digital counting. Tissue sections are stained with antibodies conjugated to UV-cleavable barcodes and proteins are enumerated by counting UV-released barcodes with the the nCounter® Analysis System. This allows the quantification of proteins from either a whole slide (to facilitate biomarker development in highly reproducible format) or from small regions of interest within the tumor (to permit the discovery of novel biology).

Here, we present two applications of the technology. First, breast cancer samples are stained with a oncocktail of barcode labeled antibodies including HER2, ER, PR, EGFR, histone, and others. Regions of interest are selected and barcodes from the cocktail of antibodies quantified. The robustness of the system is demonstrated by digital counts which are proportional to the illuminated area and strong concordance between visual intensity of HER2 staining and the HER2 digital counts. In the second example, a 30+ cocktail of antibodies recognizing immunologically relevant targets (including cell lineage markers and immune checkpoints) are used to stain tumors and elucidate patterns of immune cell distribution and activation states.

This new technology enables highly multiplexed characterization of protein expression and localization on both tumor and infiltrating immune cells and facilitates the development of novel biomarkers.

2351

Proof of concept for MHC allelic replacement by CRISPR-Cas9 assisted cassette exchange

Kelton, W., Waindok, A., Pogson, M., Parola, C., Reddy, S.
ETH Zürich, BSSE, Basel, Switzerland

Allogeneic cellular transplantation is widely employed to treat various genetic diseases and hematological malignancies. Locating suitable donors for these procedures is often challenging, as MHC/HLA gene alleles need to be matched in order to prevent transplant rejection. We report here a proof of concept study for an ex-vivo CRISPR-Cas9-based approach for the precise exchange of MHC alleles (~5kb) at the native genomic locus. For initial evaluation, the method was performed

on immortalized mouse antigen-presenting cells (RAW264.7 macrophages) expressing high levels of surface MHC (H2-Kd). Genomic exchange at the H2-K locus was achieved via co-transfection of cells with a plasmid carrying Cas9 and guide RNA targeting the H2-Kd locus and a homology donor repair template encoding an alternate MHC allele (H2-Kb). Following homology directed repair, modified cells were isolated by flow cytometry based on expression of new MHC. Engineered cells expressing the alternate H2-Kb allele were able to present a model antigen peptide (OVA-SINFEKLL) and activate a cognate T cell hybridoma line, demonstrating they were active for downstream immune functions. We envision this approach could be used in the future to improve MHC/HLA matching in cellular transplantation.

2716

Editing the genome in cells and mice using CRISPR/Cas9 technology

Herold, M.J.^{1,2}, Aubrey, B.J.^{1,2,3}, Kelly, G.L.^{1,2}, Kueh, A.J.^{1,2}, Brennan, M.S.^{1,2}, O'Connor, L.^{1,2}, Milla, L.^{1,2}, Wilcox, S.^{1,2}, Tai, L.¹, Strasser, A.^{1,2}

¹The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia, ²Melbourne University, Department of Medical Biology, Melbourne, Australia, ³Royal Melbourne Hospital, Department of Clinical Haematology, Melbourne, Australia

CRISPR/Cas9 mediated genome engineering provides an easy and rapid way to edit genes *in vitro* and *in vivo*. Initial experimental strategies utilised proved to have a low efficiency and were not broadly applicable to all cell types. In order to overcome this hurdle and to allow for efficient modification of genes in the haematopoietic system, we have developed a novel drug-inducible lentiviral system to deliver the CRISPR/Cas9 platform to cells permitting efficient genome engineering *in vitro* and *in vivo*. Additionally we have recently also implemented the CRISPR/Cas9 technology for producing genetically modified mice and to use it as a screening tool for the identification of novel cell death regulators.

336

Cancer-specific T cell receptor isolation by single cell PCR for cancer immunotherapy

Hibbert, L., Teng, M., Ryan, R., Baker, D., Clark, V., Williams, L., Conlon, L., Hale, K., Davis, S., Weigand, L., Paston, S., Molloy, P., Vuidepot, A., Hassan, N., Jakobsen, B.

Immunocore Ltd, Abingdon, United Kingdom

Malignant cells may be recognised by T cells binding cell surface Class I HLA (Human Leukocyte Antigen)-peptide complexes presenting disease-associated epitopes. Many cancer patients have been shown to generate CD8 cytotoxic T cell responses to tumour-associated antigens. However this is often insufficient for the immune system to clear tumours, resulting in progression of cancer. This is in part due to the low avidity of these T cells as well as the ability of cancer cells to develop escape mechanisms to avoid destruction by T cells. To overcome these issues, we have engineered novel, bi-functional protein therapeutics

termed ImmTACs (Immune mobilising monoclonal TCR Against Cancer) which re-direct the immune system to target and destroy tumour cells with a high degree of potency and specificity. An ImmTAC comprises a high affinity 'monoclonal' T cell Receptor (mTCR) targeting a cancer-associated HLA-peptide complex, fused to an anti-CD3 scFv domain which activates an anti-tumour T cell response.

In order to produce ImmTACs, we have developed an integrated in-house process leading to the isolation of TCRs specific for validated cancer epitopes. High affinity ImmTACs are then generated through affinity maturation by phage display. We have developed a method of rapidly identifying TCR chains from T cell clones using targeted amplification from single cells. We describe the steps leading to cloning of wild type TCRs with single cell PCR. We present data to illustrate the successful isolation of TCRs as a result of this procedure.

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A scalable multiplex assay enabling assessment of TCR specificity to hundreds of self- and pathogen-derived antigens

Klinger, M., Taniguchi, R., Hu, J., Hayes, T., Wittkop, T., Asbury, T., Moorhead, M., Emerson, R., Sherwood, A., Robins, H., Faham, M. Adaptive Biotechnologies Corp, South San Francisco, United States

Monitoring antigen-specific T-cells is critical for the study of immune responses and development of biomarkers and immunotherapeutics. We developed and validated a novel multiplex assay (MIRA, or Multiplexed Identification of TCR Antigen specificity) that combines conventional immune monitoring techniques and TCR repertoire sequencing to assess T-cell specificity to large numbers of query antigens. MIRA is a sensitive assay enabling detection of antigen-specific TCR clonotypes well below the limit of detection of conventional immune monitoring assays including flow cytometry and ELISPOT. Here we report the results from a scaled-up version of the assay using 270 different query peptide antigens (159 self- and 111 pathogen-derived). We identified >500 TCR clonotypes at frequencies as low as 1 per million T-cells that were specific to 41 query antigens from 6 healthy HLA-A*02-positive individuals. Most of the antigen-specific TCRs identified recognized one of 27 different peptides derived from a variety of pathogens including CMV, EBV, Flu, Rotavirus, HSV, mTB, WNV and HIV. A subset of antigen-specific TCRs recognized one of 14 different peptides derived from self including MART1, RCC, BCL-2, MAGE, STEAP1, KLK4, CAMEL and MOG. These data support the notion that escape and survival of self antigen-specific T-cells occurs without causing overt autoimmunity in healthy individuals. We show MIRA can be used to assess TCR specificities to hundreds of query antigens simultaneously. The assay is highly scalable and easily modified to accommodate thousands of additional query antigens. This technology may be used to monitor T-cell specificity to antigens relevant to infection, autoimmunity and cancer.

3224**Small airway-on-a-chip: a novel microphysiological system to study human lung inflammation *in vitro***

Hajipouran Benam, K.¹, Villenave, R.¹, Lucchesi, C.¹, Hubeau, C.², Varone, A.¹, Ferrante, T.C.¹, Weaver, J.C.¹, Bahinski, A.¹, Hamilton, G.A.¹, Ingber, D.E.¹

¹Harvard University, Wyss Institute for Biologically Inspired Engineering, Boston, United States, ²Pfizer Inc, Cambridge, United States

Development of new therapeutics for lung inflammatory and infectious diseases, such as chronic obstructive pulmonary disease (COPD) and respiratory viral infections, which are associated with significant morbidity and mortality, has been hindered by challenges to study organ-level complexities of lung inflammation *in vitro*.

Here, we applied a microengineering technological approach known as 'organ-on-a-chip' to create a human lung small airway-on-a-chip that supports full differentiation of a pseudostratified mucociliary bronchiolar epithelium composed of cells isolated from normal or diseased donors underlined by a functional microvascular endothelium, which experiences continuous blood-like fluid flow. Airway chips lined with well-differentiated COPD epithelia recapitulated features of the disease including selective cytokine hypersecretion, increased neutrophil recruitment, and clinical exacerbations by exposure to pathogen-mimetic compounds. Using this robust *in vitro* method for modeling human lung inflammatory disorders, it was possible to detect synergistic effects of lung endothelium and epithelium on cytokine secretion, identify new biomarkers of disease exacerbation, and measure responses to anti-inflammatory compounds that inhibit cytokine-induced recruitment of circulating neutrophils under physiological vascular shear. Importantly, the 'synthetic biology' nature of our tissue engineering approach allowed us to independently control and vary key system parameters that influence organ-level lung mucosal inflammation.

Thus, the human small airway-on-a-chip offers a powerful complement to animal models for both analyzing human pathophysiology and carrying out preclinical drug evaluation.

1127**The GARD assay for potency assessment of skin sensitizing chemicals**

Forreryd, A., Zeller, K., Lindberg, T., Albrekt, A.-S., Chawade, A., Lindstedt, M.

Lund University, Department of Immunotechnology, Medicin Village, Lund, Sweden

Allergic Contact Dermatitis (ACD) is caused by adverse immune reactions in the skin and develops upon repeated exposure to chemical haptens. To reduce exposure, efforts are being made to develop assays for identification of such compounds. We have previously developed an assay (GARD) based on a biomarker signature of 200 mRNAs, identified by transcriptomics of a myeloid cell-line stimulated with reference chemicals (n=38). GARD classifies unknown compounds binary, as either skin sensitizers or non-sensitizers, with an accuracy estimated to

89% (on 39 test chemicals). The aim of the current study is to broaden applicability domain of GARD to include also potency assessments.

We utilized the versatility of analyzing complete transcriptomes of cells, divided reference samples into potency groups, (CLP-labeling: no cat, weak=1B, strong=1A) and identified an alternative signature for prediction of sensitizing potency, using Random Forest (RF). To shed additional light into the molecular mechanisms, we also performed a pathway analysis.

A signature comprising 30 genes was identified. The performance of the signature was validated by cross-validation and estimated to an accuracy of 85% (no cat), 83% (1A) and 79% (1B). We also found a correlation between metabolic and cell-cycle associated pathways and sensitizing potency.

Combining the original and new signature, we present a testing strategy with an ability not only to identify sensitizing chemicals, but rather to perform a more complete risk assessment. Ongoing work focuses on expanding reference chemicals with an additional 50 chemicals to improve predictive performance, and to elucidate molecular mechanism involved in sensitization potency.

1711 **α HER2/CD3 bifunctional RNA engineered human T cells specifically eliminate HER2+ gastric cancer**

Luo, F., Qian, J., Yang, J., Deng, Y., Zheng, X., Liu, J., Chu, Y.
Fudan University, Shanghai, China

Genetically engineered T cells therapy is a promising strategy in cancer immunotherapy. One successful strategy is chimeric antigen receptor (CAR)-T cells therapy, but CARs-restricted on cell surface and side effects of retrovirus are partial limitations for CAR-T therapy. An alternative strategy is bispecific T cell engager (BiTE), but the therapeutic potential of BiTEs is still limited by the short half-life of antibodies, the lack of endogenous effector T cells in patients with advanced cancer, and severe adverse effects. In this study, we developed a novel secretable human epidermal growth factor receptor 2 (HER2)-targeting BiTE, α HER2/CD3. These α HER2/CD3 RNA engineered human T cells persistently secreted α HER2/CD3 fusion proteins, which were released to help engineered T cell to exhibit HER2-specific activity, or redirect bystander T cells to HER2+ cancer cells and even inhibit HER2+ cancer cells proliferation directly. Additionally, HER2+ tumor-bearing mice treated with the secretable α HER2/CD3 RNA engineered T cells got a significant tumor growth inhibition and prolonged survival without observed adverse effect. Thus, the secretable α HER2/CD3 T cells have the characteristics of high potency, long term and low toxicity, which might offer an attractive HER2-targeting immunotherapy for solid tumors.

Treg 1

2600

Nr4a receptors promote completion of Treg cell developmental program and prevent conversion of labile Treg precursors into pathogenic cells

Sekiya, T., Yoshimura, A.

Keio University School of Medicine, Department of Microbiology and Immunology, Tokyo, Japan

Recently, our group reported that Nr4a family of nuclear orphan receptors plays crucial roles in Treg cell development. In this study, we investigated molecular mechanisms for Treg cell development in the thymus, by focusing on the roles mediated by Nr4a. First, we tried to distinguish Nr4a-dependent and -independent Treg cell developmental programs. As a result, we found that several Treg cell-developmental programs were strictly dependent on Nr4a, including suppression of majority of inflammatory cytokine genes, and induction of *Ikzf4* (Eos). On the other hand, induction of many genes in the Treg-signature gene set, including *Ctla4* and *Ii2ra*, were revealed to be independent of Nr4a function. Even Foxp3 was induced, albeit at a reduced level, in Nr4a-deficient Treg precursor (preTreg) cells. However, the Foxp3 expression in Nr4a-deficient preTreg cells was so transient and unstable, that the expression disappeared within one day, even under a supporting condition for Foxp3 expression. Next, we analyzed cooperation between Nr4a and other Treg-developmental factors, including IL-2/CD25 and TNF superfamily (TNFSF)/TNF receptor super family (TNFRSF). By an in vitro system, we revealed that Nr4a were indispensable for IL2/CD25- and TNFSF/TNFRSF-mediated conversion of Treg precursor into Tregs. On the other hand, it was also suggested that Nr4a alone could not induce Tregs, but requires cooperation with IL2/CD25 and TNFSF/TNFRSF. Finally, we found that Nr4a-deficient preTreg cells have a potential to induce autoimmunity. Reflecting the diverse spectrum of self-reactive TCR repertoire of Tregs, Nr4a-deficient preTreg cells elicited autoimmunity against diverse array of self-antigens, including both ubiquitous and tissue-specific ones.

3469

Human Foxp3-negative follicular regulatory T cells control IgE responses

Canete, P.F.¹, Sweet, R.A.¹, Papa, I.¹, Gonzalez-Figueroa, P.¹, Ohkura, N.², Cuenca, M.¹, Silva-Cayetano, A.¹, Ohms, S.J.³, Barry, E.⁴, Grimbaldeston, M.⁵, Sakaguchi, S.², Cook, M.C.^{1,6}, Vinuesa, C.G.¹

¹JCSMR/Australian National University, Immunology and Infectious Disease, Canberra, Australia, ²WPI Immunology Frontier Research Center, Osaka University, Laboratory of Experimental Immunology, Osaka, Japan, ³JCSMR/Australian National University, ACRF Biomolecular Resource Facility, Canberra, Australia, ⁴Centre for Cancer Biology, University of South Australia & SA Pathology, Cytokine Receptor Laboratory, Adelaide, Australia, ⁵Centre for Cancer Biology, University of South Australia & SA Pathology, Mast Cell Laboratory, Adelaide, Australia, ⁶Canberra Hospital, Translational Research Unit, Canberra, Australia

Antibody responses to most infectious and food protein

antigens depend on help to B cells from specialised T follicular helper (Tfh) cells. A subset of Foxp3+ regulatory T cells (Tregs) has been described in mice, with a prominent role in repressing germinal center reactions that are critical for memory B cell formation and long-lived antibody responses. These specialised Tregs co-opt the Bcl-6-dependent Tfh differentiation pathway in order to access the B cell-rich follicles and have therefore been designated as T follicular regulatory (Tfr) cells. Little is known about the ontogeny or function of human Tfr cells. Here we identify a unique Bcl-6-expressing follicular regulatory T cell in human secondary lymphoid tissue, that lacks Foxp3 expression and the thymic-imprinted Foxp3 methylation pattern, but shares expression of key Treg molecules. These cells, designated Tfr2 cells, are the predominant source of T cell-derived IL-10 in human tonsil. Whereas IL-10 alone promotes B cell terminal differentiation into plasma cells, IL-10-producing Tfr2 cells suppress human B cell differentiation and profoundly limit IgE production. Intriguingly, Tfr2 cells only exert their effects in the presence of Tfr2 cells, at least in part through repressing Tfh-derived IL-21 and CD40L. Tfr2 cells are enriched at human oral-associated lymphoid tissues; continuous exposure to food antigens at these sites make Tfr2 cells an ideal candidate to suppress food allergies.

3642

Selective control of regulatory T cells through TCR signaling molecule

Tanaka, A.¹, Nishikawa, H.¹, Noguchi, S.^{1,2}, Morikawa, H.¹, Takahashi, N.², Sakaguchi, N.¹, Sakaguchi, S.¹

¹Osaka University, Immunology Frontier Research Center, Suita, Japan, ²Akita University, Department of Hematology, Nephrology, and Rheumatology, Akita, Japan

Regulatory T (Treg) cells are essential for the active maintenance of immunological self-tolerance and homeostasis. On the other hand, Treg cells infiltrate tumors and hinder effective anti-tumor immune responses in patients, requiring a method to selectively control Treg cells in tumors without eliciting autoimmunity. Here we show that in Treg cells, proximal TCR signaling molecules including Lck and ZAP-70 are regulated specifically to maintain low levels of gene expression and/or basal kinase activity. This Treg-specific repression renders Tregs, especially terminally differentiated and highly suppressive Foxp3+ effector Treg (eTreg) cells, vulnerable to inhibition of Lck and leads to selective depletion of eTreg cells in humans. Imatinib, a tyrosine kinase inhibitor of the oncogenic BCR-ABL fusion protein specifically expressed in chronic myelogenous leukemia (CML) cells, inhibits tyrosine phosphorylation of Lck, as an off-target effect. Long-term imatinib-treated CML patients in complete molecular remission showed selective depletion of eTreg cells whereas those failed in molecular remission did not. The former concurrently exhibited a general increase in the number of effector- or memory-type CD8+ T cells producing multiple cytokines. In vitro, imatinib induced apoptosis predominantly in eTreg cells, augmenting CD8+ T-cell responses against various tumor antigens in healthy individuals and cancer patients. Imatinib is, therefore, able to attenuate TCR signaling intensity more profoundly in eTreg cells than in other T cells, rendering

the former more susceptible to signal-deprived apoptotic cell death. Thus, small molecules that can selectively deplete eTreg cells via exploiting Treg-specific functions are instrumental in augmenting anti-tumor immunity in various cancers.

2314

Pharmacological inhibition of acid sphingomyelinase or genetic ablation enhances CD4⁺ Foxp3⁺ regulatory T cell activity

Hollmann, C.¹, Reuter, D.¹, Werner, S.¹, Avota, E.¹, Müller, N.¹, Japtok, L.², Kleuser, B.², Becker, K.A.³, Gulbins, E.³, Schneider-Schaulies, J.¹, Beyersdorf, N.¹

¹University of Wuerzburg, Institute for Virology and Immunobiology, Wuerzburg, Germany, ²University of Potsdam, Institute of Nutritional Science, Nuthetal, Germany, ³University of Duisburg-Essen, Institute for Molecular Biology, Essen, Germany

The acid sphingomyelinase (human: ASM, mouse: Asm) hydrolyses sphingomyelin into phosphocholine and ceramide which generates signalling platforms and affects viral pathogenicity. CD4⁺ Foxp3⁺ regulatory T (Treg) cells depend on CD28 signalling for their survival and function, a receptor that has previously been shown to activate the Asm/ASM. In line with these findings we observed that Treg cells of wild-type (wt) mice displayed higher basal and CD28-induced Asm activity and, thus, contained more ceramide than conventional CD4⁺ T (Tconv) cells. Investigating Asm-deficient mice we could show that the frequency of Treg cells among CD4⁺ T cells is increased and their suppressive activity is enhanced compared to wt mice - at least partially due to a higher turnover of the effector molecule CTLA-4. Similar to anti-CD28 antibody-mediated expansion of Treg cells in wt mice, these changes in Treg cell frequencies and/ or function in the Asm-deficient mice were associated with more infected neurons using a CNS infection model with recombinant measles virus. Of clinical importance, pharmacological inhibition of Asm in mouse splenocytes *in vitro* and in mice *in vivo* led to higher frequencies of Treg cells among CD4⁺ T cells, as did ASM inhibition in primary human peripheral blood mononuclear cells (PBMC). ASM inhibitors should, thus, be considered as potential immunomodulatory agents for the therapy of inflammatory and autoimmune disorders. This study was supported by a grant from the DFG (FOR2123/P02).

1170

TCR signal strength regulates Akt substrate specificity to induce alternate Th and Treg differentiation programs

Morel, P.A.¹, Hawse, W.F.¹, Sheehan, R.P.², Boggess, W.C.³, Faeder, J.R.²

¹University of Pittsburgh, Immunology, Pittsburgh, United States, ²University of Pittsburgh, Computational and Systems Biology, Pittsburgh, United States, ³University of Notre Dame, Notre Dame, United States

T regulatory (Treg) cells are induced following stimulation of naïve CD4 T cells with low dose antigen to an extent that is negatively correlated with signaling via the Akt/mTOR pathway. Strong TCR signals induce high levels of Akt activity that inhibit

development of Treg by poorly understood mechanisms. Here, we show that high dose stimulation of T cells results in the phosphorylation of Akt on two regulatory sites, Serine (S) 473 and Threonine (T) 308, whereas low dose stimulation results in only T308 phosphorylation. Mathematical modeling shows that the dual phosphorylation of Akt on S473 and T308 is controlled by a feedback loop involving PTEN, mTORC2 and the transcription factor FoxO1 that creates a sharp activation threshold with respect to antigen dose and stimulus duration. Using mass spectrometry to analyze phosphorylated Akt substrates at different levels of stimulation, we find profound differences in the substrates phosphorylated, suggesting that a switch in substrate specificity coupled to the phosphorylation status of Akt may lead to alternative cell fates. Proteins differentially phosphorylated by these two states of Akt include RNA splicing factors, and we find changes in the splice variant expression levels of key TCR signaling proteins, such as CD3 ζ and CD45 that correlate with the observed differences in cell fate. Knockdown of specific splicing factors changed the ratios of Th versus Treg cells induced. Together, this work demonstrates that alternative splicing can affect the outcome of T cell fate decisions and identifies alternate Akt-mediated signaling networks that drive CD4⁺ T cell differentiation.

1532

Evaluation of Galectin-9 blocking mono-clonal antibodies as novel immune-checkpoint inhibitors via the targeting of regulatory T cells in cancer

Mustapha, R.¹, Mrizak, D.¹, Renaud, S.¹, Barjeon, C.², de Launoit, Y.¹, Pancré, V.¹, Moralès, O.¹, Busson, P.², Delhem, N.¹

¹CNRS, UMR 8161, Institut de Biologie de Lille, Lille, France,

²Université Paris-sud, CNRS UMR 8126 and Institut Gustave Roussy, Villejuif, France

The immune system has the potential to recognize and eliminate cancer cells but is held back by certain inhibitory pathways. Regulatory T cells (Tregs) are key players in these pathways. They are a subpopulation of T lymphocytes whose role is to inhibit the immune response in order to maintain immunological homeostasis. However, the Treg population is often upregulated in cancer patients where it promotes tumor. This has made Tregs an appealing target for immunotherapeutic approaches such as anti-CTLA-4 and anti-PD-1. Galectin-9 (Gal-9) a b-galactoside binding lectin has been described as an immunosuppressive molecule which is expressed by both cancer cells and Tregs in order to suppress the anti-tumoral immune response. In this study we first aimed to confirm the role of Gal-9 in Treg-mediated-immuno-suppression. Then we tested the effects of Gal-9 blocking on Treg function and subsequently the anti-tumoral immune response. Following Treg isolation from healthy donors, we have proven via QPCR, ELISA and flow cytometry that Gal-9 is highly expressed and secreted by Tregs with respect to conventional T cells. Moreover, the anti-Gal-9 antibody significantly inhibited the immuno-suppressive function of Tregs in mixed leukocyte reaction proliferation tests. We have also demonstrated that Gal-9 blocking in PBMC culture promotes the secretion of Th1 cytokines TNF-a and IFN-g. Finally, we successfully used the anti-Gal-9 antibody to inhibit

the suppressive function of human nasopharyngeal carcinoma (hNPC) derived exosomes and to significantly limit the growth of xeno-transplanted hNPC tumors in immuno-deficient mice that were previously reconstituted with a human immune system.

1582

Toll-like receptor 9 is required for the maintenance of CD25⁺FoxP3⁺CD4⁺T_{reg} cells during *Listeria monocytogenes* infection

Dolina, J., Lee, J., Schoenberger, S.

La Jolla Institute for Allergy and Immunology, San Diego, United States

It has long been appreciated, but not understood, that the CD8⁺ cytotoxic T lymphocyte (CTL) dependence on CD4⁺ T cell help (T_h) is conditional; needed for some immunogens but not others. One explanation for this phenomenon envisions T_h requirement as an intrinsic property of the pathogen itself rather than its introduction to the immune system. Here we show that dependence of the optimal CD8⁺

T cell response to *Listeria monocytogenes* (*Lm*) on CD4⁺ T cells is a function of the immunogen dose used for priming, with low dose *Lm* (LD; 50 or 10³ CFU WT or Δ ActA, respectively) inducing a primary antigen-specific CTL response profoundly dependent on CD4⁺ T_h cells while that induced by high dose *Lm* (HD; 4×10³ or 10⁶ CFU WT or Δ ActA, respectively) is significantly inhibited by CD4⁺CD25⁺FoxP3⁺ regulatory T cells (T_{reg}). The T_h-independence of HD immunization is not overcome by additional antigen but instead involves the inflammatory response to more bacteria. Evaluation of various TLR pathways as the relevant sensing mechanism showed that TLR2 is required for CTL responses to LD immunization, and that HD immunization in the absence of TLR9 results in a simultaneous loss of CD25⁺FoxP3⁺CD4⁺ T_{reg} cells and increase in conventional CD4⁺ T_h cells and CTLs. Our data thus reveal that the CTL response to the same pathogen is determined by distinct roles for CD4⁺ T cells as helpers versus regulators based on immunogen dose and demonstrate a previously undescribed role for TLR9 in the regulation of CD4⁺ T_h and T_{reg} cells.

2281

High-content cytotoxic assays reveal the biological activity of pathological and therapeutical cytotoxic T lymphocytes

Guipouy, D.^{1,2,3,4}, Gertner-Dardenne, J.⁴, Belmonte, N.⁴, Dupré, L.^{1,2,3}

¹Centre de Physiopathologie de Toulouse Purpan, Inserm UMR 1043, Toulouse, France, ²Université Toulouse III Paul Sabatier, Toulouse, France, ³CNRS, UMR 5282, Toulouse, France, ⁴TxCell, Valbonne-Sophia Antipolis, France

The killing efficiency of cytotoxic T cells is usually evaluated by measuring the percentage of dead target cells resulting from a 4-hr incubation with an excess of cytotoxic T cells. However, the conventional assays fail to capture the ability of these cells to sustain killing over prolonged time. We have recently developed a combination of flow cytometry- and microscopy-based assays to provide kinetic measurements of target cell elimination by human CD8⁺ T cells (Vasconcelos *et al.*, Cell Rep. 2015,11:1474). We here developed these assays further to assess in depth the

cytotoxic activity of

T cells in two clinically-relevant settings: i) CD8⁺ T cells isolated from Wiskott-Aldrich syndrome (WAS) patients, ii) antigen-specific type-1 regulatory CD4⁺ T cells (Ovasave[®], TxCell), which are currently tested in refractory Crohn's Disease patients (Phase IIb). Although WAS CD8⁺

T cells were able to kill target cells in 4hr, their ability to sustain killing over 24hr was strongly impaired. The defect of WAS CD8⁺ T cells to control target cells over time was linked to an inability to assemble stable synapses, resulting from abnormal actin cytoskeleton organization and LFA-1 activation. Our assays also revealed that a potential mechanism of action of therapeutic regulatory T cells relied on the elimination of myeloid cell via a cytotoxic mechanism. Interestingly, these unconventional cytotoxic

T cells developed a robust cumulative killing activity over 24hr. Our study highlights the advantage of applying flow cytometry- and microscopy-based assays to characterize, with high sensitivity, the kinetics of the cytotoxic activity of clinically-relevant T lymphocytes.

Allergy 1

1245

The Nedd4-2-Ndfip1 axis is essential to curtail mast cell-dependent IgE-mediated anaphylaxis *in vivo*

Yip, K.H.¹, Kolesnikoff, N.¹, Hauschild, N.¹, Lopez, A.F.^{1,2}, Galli, S.J.³, Kumar, S.^{1,2}, Grimbaldston, M.A.^{1,2}

¹Centre for Cancer Biology, University of South Australia and SA Pathology, Adelaide, Australia, ²University of Adelaide, School of Medicine, Adelaide, Australia, ³Stanford University, Departments of Pathology and of Microbiology and Immunology, Stanford, United States

Cross-linkage of the IgE receptor (FcεRI) by specific antigen ligation on mast cells (MCs) plays a critical role in the pathological process of IgE-dependent allergic disorders, such as anaphylaxis and asthma. Restraint of intracellular signal transduction pathways that promote release of MC-derived pro-inflammatory mediators is necessary to dampen activation and restore homeostasis. In the course of studying the molecular basis regulating IgE-mediated MC function, we discovered that the ubiquitin ligase, Nedd4-2, is a major gatekeeper of MC activation and the adapter molecule Ndfip1 (Nedd4 family interacting protein 1) has a role in this process.

Herein, we show that loss of Nedd4-2 or Ndfip1 activity in MCs causes an increase in the magnitude and duration of IgE-induced pro-inflammatory mediator release *in vitro*. A closer inspection of the signalsome activities of FcεRI engagement in *Nedd4-2^{-/-}* and *Ndfip1^{-/-}* MCs compared to the wild-type counterparts, revealed that p-Syk polyubiquitination by Nedd4-2/Ndfip1 proximal to FcεRI aggregation was disrupted, leading to exacerbated calcium mobilization and prolonged propagation of multiple downstream signaling events including those of the ERK1/2 and NF-κB pathways. The biological significance of these investigations is highlighted further in an IgE-mediated MC-dependent passive cutaneous anaphylaxis mouse model that employed the adoptive transfer of *Nedd4-2^{-/-}* and *Ndfip1^{-/-}* MCs

into conditional MC-deficient *Cpa3-Cre; Mcl-1^{fl/fl}* mice. In this *in vivo* setting, loss of MC-Nedd4-2 or -Ndfip1 resulted in a striking sustained inflammatory influx at the reaction site. Collectively, our findings reveal a previously unknown negative regulatory mechanism whereby Nedd4-2 and Ndfip1 are essential to restrain MC function.

1908

Humanized allergic NOD-SCID IL-2R γ ^{-/-} mice as *in vivo* model for novel allergy vaccines

Vizzardelli, C.¹, Nagl, B.¹, Neunkirchner, A.², Zimmann, F.¹, Kitzmüller, C.¹, Jahn-Schmid, B.¹, Pickl, W.F.², Bohle, B.¹

¹Medical University Vienna, Department of Pathophysiology and Allergy Research, Vienna, Austria, ²Medical University Vienna, Institute of Immunology, Vienna, Austria

Birch pollen allergy affects around 100 million people worldwide. The only treatment curing IgE-mediated allergy with long-term benefit is allergen-specific immunotherapy (AIT). Unfortunately, AIT is not successful in all treated patients. Therefore, one major focus of allergy research is the development of AIT vaccines with improved efficacy. To study such vaccines in an *in vivo* model imitating the human allergic response we humanized NOD-SCID IL-2R γ ^{-/-} (NSG) mice with cells from birch pollen-allergic patients. Briefly, PBMC were injected intraperitoneally (i.p.) together with birch pollen extract (BPE). After seven days, mice were boosted once i.p. with BPE. In addition, mice were humanized with BPE-specific T cell lines (TCL) expanded from PBMC of birch pollen-allergic patients. NSG-mice were challenged thrice intranasally (i.n.) with BPE or PBS. Thereafter, airway hyperresponsiveness (AHR) and lung inflammation was monitored. Human CD45⁺ cells were assessed in lungs and spleens by flowcytometry.

Mice humanized with PBMC and BPE-specific TCL and challenged with BPE showed significantly higher AHR and higher numbers of eosinophils, neutrophils, and basophils, in bronchoalveolar-lavage-fluids (BALF) than mice challenged with PBS. Human CD45⁺ cells were detected in lungs and spleens in both humanization conditions. As expected, higher percentages of CD4⁺T cells were found in mice humanized with BPE-specific TCL. NSG-mice humanized with cells from birch pollen-allergic donors developed allergic lung inflammation to birch pollen. We will now use this *in vivo* model for testing of novel vaccines for birch pollen allergy developed in our laboratory.

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3791

Role of interleukin-36 γ in regulating lung inflammation

Tay, H., Yang, M., Hsu, A., Nguyen, T.-H., Plank, M., Maltby, S., Bartlett, N., Hansbro, P., Foster, P.

The University of Newcastle, Immunology & Microbiology, Newcastle, Australia

Respiratory infections are thought to be one of the major risk factors that trigger the steroid resistant asthma exacerbations. In these patients innate inflammatory mediators and cells are

linked to pathogenesis and disease severity. Our investigations have identified novel roles of interleukin (IL)-36 family cytokines in regulating airway inflammation and alterations in lung function during infection and pathogen-induced exacerbation of asthma. We found that the expression of IL-36 γ was significantly increased by pathogen-associated molecular patterns derived from bacteria and viruses in macrophages. Administration of recombinant IL-36 γ *in vivo* leads to increase inflammatory genes expression (gene profiling) and recruitment of innate immune cells that are linked to asthma pathogenesis. We next sought to determine the importance of IL-36 γ in asthma using a well-characterised murine model of allergic airway disease. Mice were sensitised and challenged with ovalbumin (OVA) to induce hallmark features of asthma. During allergen challenge, recombinant IL-36 γ was administered intranasally and pulmonary function was assessed. Mice treated with IL-36 γ became insensitive to corticosteroid therapy. In conclusion, we show that infections that trigger asthma exacerbations are linked to IL-36 production and that delivery of this cytokine to the airways results in steroid-resistant airway hyperresponsiveness and neutrophilic inflammation. Our results suggest that IL-36 γ could be a potential therapeutic target for neutrophilic and severe asthmatics.

2813

Parvalbumin from Atlantic cod interacts with bronchial epithelial cells and induces differential expression of cytokines

Kalic, T.¹, Ellinger, I.¹, Gepp, B.¹, Radauer, C.¹, Waltl, E.², Niederberger, V.², Breiteneder, H.¹

¹Medical University of Vienna, Department of Pathophysiology and Allergy Research, Vienna, Austria, ²Medical University of Vienna, Department of Otorhinolaryngology, Vienna, Austria

Background: Inhalation of aerosolized fish allergens and fish matrix components is often associated with severe IgE-mediated reactions in sensitized individuals. We explored interactions of bronchial epithelial cells with the major codfish allergen Gad m 1 and the cod food matrix.

Methods: We used the human bronchial epithelial cell line 16HBE14o-. Polarized cells were treated with natural Gad m 1 with or without the codfish-derived food matrix. Fluorescently labelled allergen was detected by confocal microscopy. mRNA levels of IL-6 and CCL2 were determined by qRT-PCR. Concentration of cytokines in the basolateral cell culture medium was measured by the Luminex assay.

Results: Apical exposure of the cells to Gad m 1 resulted in binding of the allergen to the lateral membrane domain (below ZO-1 level). The allergen was not internalized by the cells nor detected in the basolateral medium. IL-6 mRNA and protein levels were increased by 30% after treatment with codfish matrix but not with the allergen alone. The CCL2 concentration was 50% higher in the basolateral medium of samples treated with Gad m 1 compared to fish matrix-treated samples and the negative control.

Conclusion: The major codfish allergen Gad m 1 interacts with the plasma membrane of bronchial epithelial cells. This interaction leads to changes in gene and protein expression

of IL-6 and CCL2, which are further modulated by food matrix components and may play a role in allergic reactions to fish via inhalation. Supported by the Austrian Science Fund doctoral program W1248-B1 and grants SFB 4608 and 4613.

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The ORMDL3-ceramide axis may be a novel therapeutic target for the control of allergic airway disease

Sturgill, J.¹, Oyeniran, C.², Conrad, D.³, Spiegel, S.²

¹Virginia Commonwealth University, Family and Community Health, Richmond, United States, ²Virginia Commonwealth University, Biochemistry, Richmond, United States, ³Virginia Commonwealth University, Microbiology and Immunology, Richmond, United States

Asthma, defined as a chronic inflammatory condition characterized by episodic shortness of breath with expiratory wheezing and cough, is a serious health concern. The WHO estimates that asthma affects more than 230 million people worldwide. There is a strong genetic component to asthma and numerous genome-wide association studies have identified ORM (yeast)-like protein isoform 3 (ORMDL3) as an asthma associated gene. Surprisingly however, the mechanism by which ORMDL3 contributes to asthma pathogenesis is not well understood. The yeast ortholog of ORMDL3 is a negative regulator of serine palmitoyltransferase (SPT), the rate limiting step in de novo ceramide synthesis, yet elevations of ceramide rather than its reduction have been linked to lung inflammation. Thus, we examined the role of ORMDL3 in asthma immunopathology. Consistent with its role in yeast, we show that decreasing expression of ORMDL3 in lung epithelial cells and macrophages increases ceramide and conversely, modest increases in ORMDL3 decrease ceramide levels. In a house dust mite (HDM) mouse model of allergic airway disease, allergen challenge induced expression of ORMDL3 and resulted in a concomitant increase in lung ceramide. Intriguingly, the use of specific drugs, which inhibit ceramide synthesis, prevented HDM-induced airway hyperreactivity (AHR) and suppressed airway inflammation. Nasal administration of the orally available FDA approved prodrug FTY720/fingolimod reduced both ORMDL3 expression and ceramide production while mitigating airway inflammation, hyperreactivity, and mucus hypersecretion in HDM challenged mice. Thus the ORMDL3-ceramide pathway may be a novel therapeutic target for the control of allergic asthma.

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Effect of CTLA4-Ig on steroid resistant asthma model

Kouyama, S.¹, Yamaguchi, M.¹, Ohtomo-Abe, A.¹, Kamide, Y.¹, Hayashi, H.¹, Watai, K.¹, Mitsui, C.¹, Sekiya, K.¹, Tsuburai, T.¹, Fukutomi, Y.¹, Taniguchi, M.¹, Ohtomo, T.², Kaminuma, O.³, Mori, A.¹

¹National Hospital Organization, Sagami-hara National Hospital, Clinical Research Center, Sagami-hara, Japan, ²National Hospital Organization, Sagami-hara National Hospital/Tokyo University of Pharmacy and Life Science, Department of Pharmacotherapeutics, Hachioji, Japan, ³University of Yamanashi, The Center for Life Science Research, Chuo, Japan

Rationale: To investigate the role of helper T (Th) cells in steroid resistant (SR) asthma, steroid sensitive (SS) and SR Th clones were selected *in vitro*, and adoptively transferred into unprimed mice. Effect of CTLA4-Ig was analyzed both *in vitro* and *in vivo*.

Methods: For *in vitro* evaluation, ovalbumin (OVA) reactive Th clones were cultured with antigen presenting cells and OVA in the presence of various concentrations of dexamethasone (DEX). Proliferative responses of Th clones were measured by ³H-thymidine incorporation. For *in vivo* assessments, unprimed BALB/c mice were transferred with Th clones, challenged with OVA, and administered with DEX subcutaneously. Bronchoalveolar lavage fluid (BALF) was obtained 48 hr after challenge, and the number of infiltrating cells was differentially counted. CTLA4-Ig was administered through nasal inhalation or venous injection.

Results: SS and SR clones were selected based on the effect of DEX on the proliferative responses of antigen-stimulated Th clones. Airway infiltration of eosinophils and lymphocytes of mice transferred with SS clones were effectively inhibited by the administration of DEX. In contrast, those of mice transferred with SR clones were not significantly inhibited by DEX. Administration of CTLA4-Ig significantly suppressed the proliferation of DEX-treated SR clones *in vitro*, and eosinophil infiltration of SR asthma model transferred with SR clones *in vivo*.

Conclusions: Steroid sensitivity of Th clones assessed *in vitro* was consistent with that of adoptively transferred asthma model assessed *in vivo*. Costimulatory signal mediated through CD28 is crucial for the induction of steroid resistance both *in vitro* and *in vivo*.

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Are house dust mites (HDM) potential carriers of bacteria responsible for the induction of sensitisation to microbial "allergens"?

Dzoro, S.¹, Mittermann, I.¹, Nehr, M.², Hirschl, A.², Wikberg, G.³, Johansson, C.⁴, Lundeberg, L.³, Scheynius, A.⁵, Valenta, R.¹

¹Medical University of Vienna, Department of Pathophysiology and Allergy Research, Vienna, Austria, ²Medical University of Vienna, Division of Clinical Microbiology, Clinical Institute of Laboratory Medicine, Vienna, Austria, ³Karolinska University Hospital, Dermatology and Venereology Unit, Stockholm, Sweden, ⁴Karolinska Institute and Karolinska University Hospital, Translational Immunology Unit, Department of Medicine, Solna, Stockholm, Sweden, ⁵Karolinska Institute and Karolinska University Hospital, Translational Immunology Unit, Department of Medicine Solna, Stockholm, Sweden

Introduction: IgE reactivity to various *Staphylococcus aureus* and *Escherichia coli* antigens can be found in up to 25% of atopic dermatitis (AD) patients. A genomic analysis recently described *S. aureus* and *E. coli* species as abundant bacteria within the house dust mite (HDM) microbiome. We therefore investigated the role of mites as potential carriers for IgE sensitisation to microbial elements in AD patients.

Materials and methods: Sera from 179 AD patients, was analysed for IgE reactivity to a comprehensive panel of HDM allergens by chip analysis, and to *S. aureus* and *E. coli* by immunoblotting. Selected sera were additionally tested on the

mite ImmunoCAP. Inhibition experiments were conducted by pre-incubating patient sera with bacterial extracts, prior to HDM immunoblot or ImmunoCAP assay. Rabbit antibodies raised against *S. aureus* and *E. coli* antigens were tested for reactivity to blotted HDM extract.

Results: The frequency of IgE sensitisation to bacterial antigens was significantly higher in HDM allergic patients compared to allergic subjects without HDM allergy. Specific antibody probes detected *S.aureus* and *E.coli* antigens in immune-blotted HDM extract. Furthermore, pre-incubation of sera from HDM allergic patients with bacterial extract, inhibited IgE binding to bands in blotted HDM extract, and reduced IgE binding to HDM extracts in ImmunoCAP measurements.

Conclusion: House dust mites are potential carriers of bacteria responsible for the induction of IgE sensitisation to microbial antigens.

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Virus infected human mast cells have a unique and potent type 1 interferon response and selectively respond to interferon activation

Portales, L.¹, Oldford, S.², Haidl, I.D.¹, Marshall, J.S.¹

¹Dalhousie University, Microbiology and Immunology, Halifax, Canada, ²Dalhousie University, Medicine, Halifax, Canada

Mast cells are frequent at mucosal surfaces and have a sentinel role in infection. They have been implicated in the pathogenesis of asthma where viral infections are associated with disease exacerbation. Type 1 interferons (IFNs) are critical for the early response to viral infection. We therefore examined human mast cell production of, and response to, type 1 IFNs.

Using reovirus, which infects multiple cell types, we analyzed the IFN response of cord blood derived human mast cells and structural cells. Mast cells expressed a wide range of IFNs including IFN β , IFN α 1, IFN α 2, IFN α 4, IFN α 6 and IFN α 8 upon infection. In contrast, structural cells, such as normal lung fibroblasts had a highly restricted pattern of IFN expression. When peripheral blood NK cells were treated with supernatants from virus activated mast cells they demonstrated significantly greater CD69 expression and cytotoxicity than those treated with uninfected mast cell supernatants. These responses were dependent on type 1 IFNs. *In vivo*, virus infected mast cells also significantly enhanced both NK cell activation and recruitment. Human mast cell responses to IFN α 2 were also examined. Significant production of several cytokines was observed following IFN treatment, including VEGF-A and IL-1 receptor antagonist, in the absence of mast cell degranulation.

These data suggest that mast cells contribute substantially to the IFN response to viral infection at mucosal surfaces, and that such responses activate NK cells. The subsequent mast cell response to IFNs might potentially aid in tissue remodeling and regulation of inflammation.

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The effect of early postnatal colonisation of newborns by probiotic vaccine Colinfant New Born on allergy incidence and immune system characteristics

Hrdy, J.¹, Sukenikova, L.¹, Novotna, O.¹, Petraskova, P.¹, Borakova, K.², Prokesova, L.¹

¹First Faculty of Medicine, Charles University in Prague, Institute of Immunology and Microbiology, Prague, Czech Republic, ²Institut for the Care of Mother and Child, Paediatric Unit, Prague, Czech Republic

Allergies belong to the most common diseases with steadily increasing incidence. Probiotics are believed to prevent or reduce allergy development. Nevertheless, the mechanism of their beneficial effect is poorly understood. Decreased allergy incidence was observed 5, 10, 20 years after initial supplementation of newborns with Colinfant New Born (*E. coli* O83:K24:H31). To reveal the mechanism of the action, immune characteristics of peripheral blood regulatory T cells (Tregs) of probiotic colonized and noncolonized children of allergic mothers (high risk children) and noncolonized children of healthy mothers were compared by flow cytometry. The capacity of *E. coli* O83:K24:H31 to promote maturation of dendritic cells (DC) and induce immune responses was tested in vitro by coculture of probiotic primed DC derived from cord blood precursors with naive CD4+. Functional characteristics of Tregs (MFI of FoxP3, intracellular presence of regulatory cytokines IL-10, TGF- β) were decreased in the allergic group. Probiotic colonized children have increased regulatory potency of Tregs (MFI of FoxP3, regulatory cytokines) in comparison to noncolonized children. *E. coli* O83:K24:H31 promotes maturation of cord blood derived DC. The beneficial effect of probiotics on newborn immature immune system could be, at least partially, explained by the modulating immune function of Tregs. Although we detected increased proportion of Tregs in peripheral blood of allergic children, their functional properties were decreased in comparison with Tregs of healthy children. We suggest increased proportion of Tregs in allergic children reflects an effort to compensate impaired function of Tregs. This work was supported by AZV CR15-26877A and PRVOUK P25/LF1/2.

Lymphocyte Signalling

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Soluble CD52 binds the damage-associated molecular pattern protein HMGB1 to mediate T-cell suppression through the Siglec-10 receptor

Bandala-Sanchez, E.¹, Ngui, K.¹, Stone, N.L.¹, Pearce, L.A.², Adams, T.A.², Harrison, L.C.¹

¹Walter and Eliza Hall Institute of Medical Research, Population Health and Immunity Division, Parkville, Australia, ²Commonwealth Scientific and Industrial Research Organisation (CSIRO), Materials Science & Engineering, Melbourne, Australia

Introduction: Activated T cells release the GPI-anchored glycopeptide CD52, which suppresses bystander T cells by binding the inhibitory receptor, sialic acid binding Ig-like lectin-10 (Siglec-10) (1). Siglec-10 was reported to bind another

GPI glycoprotein, CD24, in the presence of the damage-associated molecular pattern (DAMP) protein, high-mobility group box 1 (HMGB1) (2).

Objective: To determine the role of HMGB1 in Siglec-10 binding and action of CD52.

Methods: Binding of recombinant CD52-Fc to HMGB1 and Siglec-10-Fc proteins was performed in microtiter plates and by surface plasmon resonance. Native proteins associated with CD52-Fc were analysed by immunoprecipitation and Western blotting of lysed human Jurkat line and human blood CD4⁺ T cells.

Results: T-cell suppression by CD52-Fc *in vitro* depended on the presence of native HMGB1 in serum and did not occur in serum-free medium. Intact HMGB1 or its cytokine-inducing Box B domain, but not its anti-inflammatory Box A domain, bound to soluble CD52 with a K_d of ~70nM. Binding to HMGB1 significantly enhanced binding of CD52 to Siglec-10. This interaction impaired phosphorylation of T-cell receptor (TCR)-associated tyrosine kinases Lck and Zap70, after TCR crosslinking by anti-CD3 monoclonal antibody. CD52-Fc was recovered from T cells in a complex with HMGB1 and Siglec-10, and the SH2 domain-containing tyrosine phosphatase SHP1.

Conclusion: CD52 exerts a concerted immunosuppressive effect first by capturing HMGB1, abrogating its pro-inflammatory effect, followed by binding to the Siglec-10 receptor to inhibit TCR phosphorylation.

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Role of Galectin-3 in influenza virus infection by regulating host immune responses and IL-1 β production

Chen, Y.-J.¹, Wang, S.-F.², Chen, H.-Y.^{1,3}, Liu, F.-T.^{1,3}

¹IBMS, Academia Sinica, Taipei, Taiwan, Republic of China,

²Kaohsiung Medical University, Department of Medical Laboratory Science and Biotechnology, Kaohsiung, Taiwan, Republic of China,

³UC Davis Medical Center, Department of Dermatology, Sacramento, United States

H5N1 Virus induces cytokine production and excessive inflammatory responses that contribute to the pathogenesis of human H5N1 disease. Galectin-3 is a β -galactoside-binding animal lectin and has been reported to participate in the host response to microbial infections. Here we investigated the role of endogenous galectin-3 in H5N1 virus-induced host immune responses. We observed that galectin-3 knockout (Gal3KO) mice were less susceptible to H5N1 infection compared to wild-type (WT) mice, whereas the viral loads in the lungs were comparable between the two genotypes. Moreover, we found that H5N1-infected Gal3KO mice exhibited a lower degree of lung inflammation, including neutrophil infiltration. In addition, the levels of the proinflammatory cytokine IL-1 β in both bronchoalveolar lavage fluid and lungs were significantly reduced in H5N1-infected Gal3KO mice. It has been reported that influenza virus induces IL-1 β production through the NLRP3 inflammasome pathway. We showed that NLRP3 and IL-1 β mRNA levels and NLRP3 inflammasome components and pro-IL-1 β protein levels were comparable between Gal3KO and WT bone marrow-derived macrophages (BMMs) infected

with H5N1. However, IL-1 β secretion and ASC oligomerization, an indicator of inflammasome activation, were decreased in Gal3KO BMMs compared to WT BMMs, in response to H5N1 infection. In addition, the presence of galectin-3 enhanced the IL-1 β processing in HEK293T cells containing reconstituted NLRP3 inflammasome complex and thus autonomously secreting IL-1 β . Combined, our results suggest that galectin-3 may enhance the pathological effects of H5N1 virus infection by promoting the host inflammatory response and that galectin-3 may positively regulate IL-1 β production by promoting NLRP3 inflammasome activation.

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Themis regulates cytokine signals in mature CD8⁺ T cells

Brzostek, J., Zhao, X., Mehta, M., Gascoigne, N.

National University of Singapore, Department of Microbiology and Immunology, Singapore, Singapore

Themis plays a critical role in regulation of T cell receptor (TCR) signal strength during thymic development, through interaction with phosphatase Shp1¹. Germline deletion of Themis leads to severe perturbation of mature T cell numbers and phenotype, but this is likely to result from the abnormal thymic development. We have developed a conditional knockout mouse to investigate the role of Themis in peripheral T cells.

Post-selection deletion of Themis reduces CD8⁺ T cell numbers in the periphery. Themis-deficient lymphocytes show unimpaired responses to TCR stimulation. However, Themis-deficient CD8⁺ T cells show reduction in proliferative responses to common γ chain cytokines. Since Themis interacts with Shp1, a negative regulator of IL-4 signalling in peripheral T cells², we investigated the role Shp1 in controlling common γ chain cytokine responses in CD8⁺ T cells. T-cell specific deletion of Shp1 enhances CD8⁺ T cell responses to common γ chain cytokines. This opposing effect of Themis and Shp1 deficiency on cytokine responses suggests that Themis acts as a positive regulator of cytokine signals, though binding to and sequestering the negative regulator Shp1 from the cytokine signalling networks. We are currently investigating this proposed molecular mechanism and the physiological significance of Themis-dependent regulation of cytokine signals the context of T cell responses to bacterial infections.

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Interactomics and structure-function analysis of the RLTPR protein underpin its essential role for costimulation via CD28 in mouse and human T cells

Malissen, M., Roncagalli, R., Cucchetti, M., Bergot, E., Jarmuzynski, N., Gregoire, C., Goncalves Menoita, M., Liang, Y., Malissen, B. CIML, Marseille, France

RLTPR comprises concatenated leucine-rich repeats (LRR) and mice harboring a mutation - called *Basilic* - in one of those repeats mimicked fully a CD28 deficiency.

The lymphoid lineage-specific actin-uncapping protein RLTPR is essential for costimulation via CD28 and the development of regulatory T cells. RLTPR colocalizes with CD28 at the immune synapse and couples CD28 to the Carma1 cytosolic effectors. However, the precise molecular function of RLTPR in CD28 costimulation and role in humans remains elusive. We have used quantitative mass spectrometry and T cells from mice in which a tag for affinity purification was knocked in the *Rltpr* gene to determine the composition and dynamics of the signaling complex that formed around RLTPR following T cell stimulation. We demonstrated that a physical association existed between RLTPR and upstream (CD28) and downstream (CARMA1) components of the CD28 pathway. Proteins not associated before with the CD28 pathway were also identified in the RLTPR interactome. By developing RLTPR deficient mice, we showed that the *Basilic* mutation corresponded to a null mutation, thereby establishing the functional importance of the LRR domain. Using Crispr-Cas9-based gene editing of Jurkat T-cell line, we demonstrated that RLTPR is also essential in human for costimulation via CD28. Our findings underpin the similar role exerted by RLTPR in human and mouse T cells and point in a new direction regarding its mode of action during CD28 costimulation.

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"Microsynapse" composed of focal adhesion molecules surrounding TCR microcluster is essential for T cell activation

Saito, T.^{1,2}, Hashimoto-Tane, A.¹

¹RIKEN Center for Integrative Medical Sciences, Laboratory for Cell Signaling, Yokohama, Japan, ²Osaka University, WPI Immunology Frontier Research Center, Suita, Japan

Immunological synapse (IS) formed at the interface between T cells and antigen-presenting cells represent hallmark of initiation of acquired immunity. T cell activation is initiated at T cell receptor (TCR) microclusters (MCs), in which TCR and signaling molecules assemble at the interface prior to IS formation. We found that each TCR-MC was transiently bordered by a ring structure made of integrin and focal adhesion molecule in early phase of activation, which is similar structure to mature immunological synapse (IS) in micro-scale and we named "Microsynapse". The micro adhesion-ring in the microsynapse is composed of LFA-1, focal adhesion molecules such as paxillin and Pyk2, and myosin II (MyoII), and is supported by F-actin core and MyoII activity through LFA-1 outside-in signals. The formation of microsynapse and the adhesion-ring was transient and especially sustained upon weak TCR stimulation to recruit LAT and SLP76. Perturbation of the microsynapse induced impairment of TCR-MC development and resulted in impaired cellular signaling and T cell activation and functions. Thus, the microsynapse composed of the core TCR-MC and surrounding micro adhesion-ring is a critical structure for initial T cell activation through integrin outside-in signals.

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TRAF adaptors limit IL-6 receptor signaling through an unexpected binding to the signaling transducer receptor gp130

So, T.¹, Nagashima, H.¹, Okuyama, Y.¹, Hayahi, T.¹, Asao, A.¹, Kawabe, T.¹, Yamaki, S.¹, Nakano, H.², Croft, M.³, Ishii, N.¹

¹Tohoku University Graduate School of Medicine, Microbiology and Immunology, Sendai, Japan, ²Toho University School of Medicine, Biochemistry, Tokyo, Japan, ³La Jolla Institute for Allergy and Immunology, Immune Regulation, La Jolla, United States

There is growing evidence that TNF receptor-associated factors (TRAFs) are recruited to unconventional cytokine receptors and control their key signaling pathways in lymphocytes. We have found that TRAF5 expressed in CD4⁺ T cells constitutively binds to a cytoplasmic region of the signal transducing receptor gp130 through its C-terminal TRAF domain and inhibits the recruitment and activation of STAT3 mediated by IL-6. This function of TRAF5 significantly impacts IL-6-driven Th17 cell differentiation. However, it is not clear how TRAF5 inhibits the gp130-STAT3 signaling axis and whether other TRAF family molecules contribute to this process. We found that amino acid residues 774-798 in gp130, which contain TRAF-binding consensus motifs, were critical not only for association with TRAF5 but also with TRAF2. gp130 expressed on naive CD4⁺ T cells gradually decreased after T cell activation and its expression reached a minimum level at 48 h. Whereas TRAF5 rapidly disappeared from activated naive T cells within 4 h, TRAF2 was stably expressed in primed T cells during the course of Th17 development. These results demonstrate that both TRAF2 and TRAF5 inhibit the early signaling activity of the IL-6 receptor complex in naive CD4⁺ T cells and that TRAF2 works as a negative regulator even in the later stage of Th17 development. Accordingly, shRNA-mediated *Traf2*-knockdown in differentiating *Traf5*^{-/-} CD4⁺ T cells strongly promoted IL-6-driven Th17 differentiation. We propose a novel signaling mechanism of Th17-lineage commitment that is spatiotemporally regulated by the adaptor proteins TRAF2 and TRAF5.

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A structured pathway for T cell receptor signal transmission through the membrane

Krshnan, L.^{1,2}, Park, S.³, Im, W.³, Call, M.^{1,2}, Call, M.^{1,2}

¹Walter & Eliza Hall Institute of Medical Research, Structural Biology, Parkville, Australia, ²The University of Melbourne, Parkville, Australia, ³University of Kansas, Department of Molecular Biosciences and Center for Computational Biology, Lawrence, United States

The octameric T cell receptor (TCR)-CD3 complex signals the recognition of antigenic peptide:MHC ligands on target cells by initiating a cascade of intracellular biochemical events beginning with phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) on CD3 and ζ cytoplasmic tails by the src-family kinase Ick. How ligand-sensing is communicated across the cell membrane is an important unresolved problem in T cell biology, and a lack of structural information on the intact receptor complex stands as a major

barrier to the development of specific mechanistic models. Both extracellular and intracellular structural transitions have been implicated in TCR "triggering," but little is known about whether specific structures within the transmembrane (TM) regions of the receptor complex are required to support the coupling of these events through the lipid bilayer. We have recently shown that the TCR $\alpha\beta$ TM domains form a closely associated coiled-coil interface that provides a structured core for the TCR $\alpha\beta$ -CD3 $\delta\epsilon$ -CD3 $\gamma\epsilon$ - $\zeta\zeta$ complex in the membrane. This interface is mediated by a network of inter-helical hydrogen bonds between TM sequences that are absolutely conserved across vertebrate evolution and in both $\alpha\beta$ and $\gamma\delta$ forms of the TCR. Receptors with mutations at the key polar residues were expressed normally but exhibited a severe defect in antigen-triggered IL-2 production. These data support a model in which extracellular alterations propagate through the structured TCR/CD3 TM domains to initiate intracellular signalling and provide a structural rationale for the ability of T cells to respond to a single antigenic peptide:MHC ligand through a receptor-intrinsic conformational mechanism.

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Quantitative analysis of Vav1 signalosome in primary CD4 T cells identify several new Vav1 partners including CD226

Gaud, G.¹, Roncagalli, R.², Gonzalez De Pedro, A.³, Malissen, B.², SAOUDI, A.¹

¹INSERM U 1043, CNRS UMR 5282, Toulouse, France, ²CIML, Marseille, France, ³IPBS, Toulouse, France

A tightly controlled balance of immune cell signaling is necessary for proper immunity. A dysfunction of this balance has been observed in several immune mediated diseases. Vav1 is a signaling molecule involved in T cell development and functions. Recently, our team showed that Vav1 is involved in several auto-immune diseases such as multiple sclerosis and myasthenia gravis. However, the molecular mechanisms by which Vav1 controls these phenotypes are still poorly understood.

In the present study, we analyzed the composition and dynamic of Vav1 signalosome in primary CD4

T cells by generating a novel animal model where Vav1 was tagged at its C-terminus with OneStrepTag (Vav1OST). Following *in vitro* stimulation of primary CD4 T lymphocytes, Vav1 was pull-down at different time-points, and its interactome was analyzed by mass spectrometry, allowing the characterization of Vav1 interactors and their binding kinetics following stimulation. This approach allowed us to describe the first spatial and temporal vav1 interactome and to identify numerous new Vav1 partners. We analyzed the major biological processes of these interactors and as expected most of them are involved in TCR signaling. This proteomic study also leads us to identify 4 co-receptors involved in immunological synapse formation. Among them, CD226 is involved in CD4 T cell proliferation and differentiation. We demonstrate here for the first time that Vav1 is a crucial effector of CD226 signaling in CD4 T cells.

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Engagement of TCR with foreign and self antigens trigger distinct signaling pathways to generate different T cell responses

Shiokawa, M.¹, Ishikawa, E.¹, Ogata, M.², Saito, T.³, Yamasaki, S.¹

¹Division of Molecular Immunology, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan, ²Department of Biochemistry and Proteomics, Mie University Graduate School of Medicine, Tsu, Japan, ³Laboratory for Cell Signaling, RIKEN Center for IMS, Yokohama, Japan

Mature T cells are activated by recognizing foreign antigens (Ags) through T cell receptor (TCR). Although it was widely believed that endogenous self Ags do not activate mature T cells, recent studies have demonstrated that they can induce T cell maintenance in the periphery. However, it remains unclear how these distinct responses are generated through the same receptor. To address this issue, we compared the contribution of Erk, a pivotal MAP kinase for cell growth and survival, to these distinct responses. By establishing mature T cell-specific Erk1/2-deficient mice, we found that mature T cells could survive in the periphery without Erk. Moreover, homeostatic T cell proliferation was observed in the absence of Erk, suggesting that Erk is not essential for T cell maintenance induced by self Ags. In contrast, OVA-specific responses, such as proliferation or cytokines production, were abrogated by loss of Erk. Thus, T cell responses induced by self/foreign Ags are regulated differentially, at least concerning the requirement of Erk. Next, to examine the correlation of peptide affinity and Erk dependency, OT-I TCR Tg T cells transferred into TAP-deficient mice were stimulated with peptides with different affinities and examined T cell responses. High-affinity peptide SIINFEKL could induce vigorous proliferation *in vivo*, whereas low-affinity peptides induced survival without proliferation. Again, we found that the former response was largely dependent on Erk. Collectively, these results suggest that the strength of TCR signal may determine different mature T cell responses through distinct signaling pathways.

Autoimmunity 1

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Rogue germinal center B cells: unconventional lymphocytes released by FAS inactivation that drive IgE and autoantibody production

Butt, D.¹, Chan, T.¹, Bourne, K.¹, Hermes, J.¹, Strasser, A.², Tangye, S.¹, Phan, T.¹, Rao, V.K.³, Brink, R.¹

¹Garvan Institute of Medical Research, Sydney, Australia, ²The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia, ³National Institute of Allergy and Infectious Diseases, NIH, Bethesda, United States

The mechanistic links between genetic variation and autoantibody production in autoimmune disease remain obscure. Inactivating mutations in the death receptor FAS or its ligand (FASL) cause Autoimmune Lymphoproliferative Syndrome (ALPS) and lupus-like autoimmune diseases. FAS is thought to safeguard against autoantibody production by

delivering death signals to self-reactive B cells that arise in the germinal centre (GC). Nevertheless, studies performed over the past two decades to elucidate a role for FAS in the regulation of GC B cell selection, differentiation and self-tolerance have yielded conflicting results. To definitively address the role of FAS in regulating GC B cell responses, we utilised a high resolution *in vivo* mouse model, specifically the adoptive transfer and challenge of anti-hen egg lysozyme (SW_{HEL}) B cells lacking expression of FAS. Surprisingly, the results indicated that FAS was in fact not required for the deletion of self-reactive B cells that arise in the GC reaction. Instead, it was discovered that FAS functions by preventing the development of a previously unrecognized lymphocyte population designated "rogue" GC (GCr) B cells. GCr B cells somatically mutate and survive despite losing antigen reactivity and differentiate into large populations of plasma cells with unconventional specificities, including autoantibody-secreting clones. Significantly, IgE⁺ plasma cells were particularly increased, leading us to identify a major cohort of ALPS patients with a hyper-IgE phenotype. Data presented will reveal that GCr B cells are a major driver of autoantibody production and provide a mechanistic explanation for the linked production of IgE and autoantibodies in autoimmune disease.

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ES-62, an immunomodulator secreted by the filarial nematode *Acanthocheilonema viteae* resets the effector:regulatory B cell balance in the MRL/Lpr model of systemic lupus erythematosus

Harnett, M.M.¹, Rodgers, D.T.¹, Pineda, M.A.¹, Mc Grath, M.A.¹, Al-Riyami, L.², Harnett, W.²

¹University of Glasgow, Glasgow, United Kingdom, ²University of Strathclyde, Glasgow, United Kingdom

Systemic lupus erythematosus (SLE) is an autoimmune condition characterized by high titers of anti-nuclear antibodies (ANA) where deposition of the resulting immune complexes (IC) initiates inflammatory pathologies with the kidneys, joints and skin commonly affected. Existing therapies rely heavily on generalised immunosuppression with recent disappointing trials of B cell-targeting biologics underscoring the urgent need for safer, effective therapies. The striking inverse relationship between active filarial nematode infection and SLE recently reported in an endemic area of India is consistent with that observed between parasitic worm infection and autoimmune disease, globally. This has been proposed to reflect the actions of immunomodulators that are secreted by parasitic worms to promote their survival by dampening inflammation but without seriously immunocompromising the host. Interest has therefore now focused on exploiting worm-derived immunomodulators to develop therapies for such inflammatory disorders. We have shown one such immunomodulator, ES-62, to protect against kidney and joint damage in the MRL/Lpr model of SLE. In particular, ES-62 suppresses the production of ANA and consequent IC-mediated development of proteinuria. Such protection correlates with induction of a hypo-responsive phenotype of effector B cell and an increase in the levels of IL-10-producing MZP-like "regulatory" B cells, and can be mimicked by the adoptive transfer of B cells from ES-62-treated MRL/Lpr mice.

Successful suppression of collagen-induced arthritis, a model of rheumatoid arthritis, is also associated with ES-62 resetting the effector:regulatory B cell balance, suggesting that this provides a unifying homeostatic mechanism for the therapeutic actions of this immunomodulator in autoimmune disease.

2572

Pathogenicity of anti-HMGCR auto-antibodies in necrotizing autoimmune myopathy

Bergua, C.¹, Chiavelli, H.¹, Drouot, L.¹, Jouen, F.^{1,2}, Benveniste, O.^{3,4}, Boyer, O.^{1,2}

¹Normandy University, Inserm U 905, Rouen, France, ²Rouen University Hospital, Department of Immunology, Rouen, France,

³Pitie-Salpetriere University Hospital, Department of Internal Medicine, Paris, France, ⁴UPMC, Inserm U 974, Paris, France

Background and objective: Necrotizing autoimmune myopathies (NAM) are a newly recognized group of severe acquired myopathies characterized by prominent myofiber necrosis with little inflammation. NAM may be associated to auto-antibodies (aAbs) against the statins target 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR). Since its expression is ubiquitous rather than muscle-specific, the role of anti-HMGCR aAbs in NAM remains elusive. We investigated the pathogenic role of anti-HMGCR aAbs in a new *in vivo* mouse model.

Methods: Recombinant human rhHMGCR was coupled to fluorescent beads and anti-HLMGCR aAb level were assayed by Luminex. IgGs purified from anti-HMGCR aAb-positive patients were transferred to C57BL/6 or Rag-/- mice. Alternatively, mice were immunized with rhHMGCR plus adjuvant. The requirement for complement was studied using C3-/- mice.

Results: Injection of anti-HMGCR positive IgGs resulted in significant decrease in muscle strength. This was associated with moderate myofiber necrosis with myophagocytosis, fetal myosin re-expression and hIgG deposits. The muscle holoproteome (day 8 post-transfer) was profoundly modified, with increase in muscle regeneration- and metabolism-related proteins. The effect was transient in immunocompetent mice and prolonged in immunodeficient animals. Immunization with rhHMGCR led to reduced muscle strength. In complement-deficient mice, anti-HMGCR positive IgGs did not transfer disease.

Conclusion: Anti-HMGCR aAbs are directly involved in disease pathogenesis. IgG deposits and absence of disease in C3-/- mice indicate a role for the classical pathway of complement. These results support further evaluation of plasma exchange and/or B cell-targeted therapy in anti-HMGCR-associated NAM.

3891

Cellular misfolded proteins rescued from degradation by MHC class II molecules are targets for autoantibodies in autoimmune diseases

Jin, H.^{1,2}, Tanimura, K.^{2,3}, Arase, N.^{2,4}, Lanier, L.L.⁵, Arase, H.^{1,2}

¹Osaka University, Laboratory of Immunochemistry, World Premier International (WPI) Immunology Frontier Research Center, Suita,

Japan, ²Osaka University, Department of Immunochemistry, Research Institute for Microbial Diseases, Suita, Japan, ³Kobe

University, Department of Obstetrics and Gynecology, Graduate School of Medicine, Kobe, Japan, ⁴Osaka University, Department of Dermatology, Graduate School of Medicine, Suita, Japan, ⁵UCSF, Department of Microbiology and Immunology and the Cancer Research Institute, San Francisco, United States

MHC class II allelic polymorphisms are strongly associated with susceptibility to many autoimmune diseases. In addition, abnormal MHC class II molecules are often observed in autoimmune-diseased tissues. However, it has remained unclear how MHC class II genes control susceptibility to autoimmune diseases. Recently, we found that MHC class II molecules function as a molecular chaperon to transport cellular misfolded ER proteins to the cell surface intact, without processing to peptides, by associating with them via their peptide-binding groove.

Here, we show that sera from patients of autoimmune diseases such as rheumatoid arthritis (RA) contain autoantibodies specific to misfolded proteins complexed with MHC class II molecules. A strong correlation between autoantibody binding to misfolded proteins complexed with certain HLA-DR alleles and the odds ratio for that allele's association with RA was observed ($r = 0.81$; $P = 4.6 \times 10^{-5}$). Indeed, association of autoantigens with aberrantly expressed MHC class II molecules was detected in autoimmune diseased tissues but not in normal tissues. Similarly, we found that β 2-glycoprotein I (β 2GPI) complexed with MHC class II molecules is a major target for autoantibodies in antiphospholipid syndrome (APS).

These findings suggest that misfolded proteins complexed with MHC class II molecules are involved in autoimmune diseases as targets for autoantibodies. Because cellular misfolded proteins are promptly degraded in the cells, immune cells might not be tolerized with the cellular misfolded proteins. Therefore, cellular misfolded proteins rescued from degradation by aberrantly expressed MHC class II molecules might induce autoantibody production, which results in autoimmune diseases.

2068

Targeting KCa1.1 channels inhibits the invasiveness of fibroblast-like synoviocytes and attenuates models of rheumatoid arthritis

Tanner, M.R.¹, Huq, R.¹, Tajhya, R.B.¹, Pennington, M.W.², Laragione, T.³, Gulko, P.S.³, Beeton, C.¹

¹Baylor College of Medicine, Molecular Physiology and Biophysics, Houston, United States, ²Peptides International, Inc., Louisville, United States, ³Icahn School of Medicine at Mt. Sinai, Medicine / Rheumatology, New York, United States

Highly-invasive fibroblast-like synoviocyte (FLS) play a central role in the pathogenesis of rheumatoid arthritis (RA). Currently, no RA therapy has been developed to specifically target FLS.

We have found that highly-invasive FLS from patients with RA and from rats with the pristane-induced and collagen-induced arthritis models of RA express higher levels of the KCa1.1 potassium channel at their plasma membrane when compared to FLS from patients with osteoarthritis or from healthy rats, respectively. Selectively blocking this channel inhibits RA-FLS, including decreasing proliferation and release of cytokines,

chemokines, angiogenic factors, and proteases. Importantly, we have found that the presence and activity of KCa1.1 at the plasma membrane of RA-FLS is both necessary and sufficient to regulate the *ex vivo* invasiveness of RA-FLS. This is accomplished through the role of KCa1.1 as a regulator of calcium homeostasis in RA-FLS, with downstream effects on Akt activity and the plasma membrane expression and activation of β_1 integrins.

Pharmacological inhibition of KCa1.1 after onset of clinical signs of arthritis significantly reduces joint inflammation, bone and cartilage damage, and synovial hyperplasia in both the collagen-induced and pristane-induced rat models of RA. FLS from blocker-treated animals also have reduced *ex vivo* invasiveness and proliferation compared to those from vehicle-treated animals.

These studies indicate the importance of KCa1.1 as a novel target for RA and emphasize the potential efficacy of directly inhibiting FLS in reducing the severity of this debilitating disease.

3167

Glycerophospholipids regulate immunity and inflammation via induction of myeloid-derived suppressor cells

Singh, R.^{1,2,3}, Prasad, P.¹, Tran, C.¹, Halder, R.¹

¹UCLA, Medicine / Rheumatology / Autoimmunity and Tolerance Laboratory, Los Angeles, United States, ²UCLA, Jonsson Comprehensive Cancer Center, Los Angeles, United States, ³UCLA, Pathology and Laboratory Medicine, Los Angeles, United States

CD1d presents lipid antigens to T-cells. Glycosphingolipid (GSL) antigens such as β GluCer, and glycerophospholipid (GPL) antigens such as phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS), have been eluted and identified by mass spectrometry as natural human CD1d ligands, and PC and PE have been eluted from murine CD1d. Here, we report that the normal immune repertoire contains $\alpha\beta$ T-cells that recognize abundant self-GPLs in a CD1d-restricted manner. CD1d-tetramers loaded with GPLs, including PA, PC, PI, PS, phosphatidylethanolamine (PE), and BMP (bis(monoacylglycero)phosphate), identify 0.4-4% T-cells in the lymphoid organs of wild-type and iNKT-deficient Ja18^{-/-} mice but not in CD1d^{-/-} mice. GPL-reactive T-cells don't recognize GSL-loaded tetramers and don't respond to α GalCer, suggesting that GPL-reactive T-cells are distinct from iNKT cells. GPL-reactive T-cells expand, express CD69, and produce cytokines upon *in vivo* priming. However, self-GPL antigens potently inhibited the proliferation and cytokine production by invariant natural killer T-cells via induction of monocytic myeloid-derived suppressor cells (M-MDSC) that inhibited invariant natural killer T-cells in an IL-10-dependent manner. Treatment with a GPL as well as adoptive transfer of GPL-modified M-MDSC ameliorated liver inflammation in the Con A hepatitis model, whereas it reversed the iNKT cell-mediated protection against tumor in the melanoma lung metastasis model. These observations support a new role for self-GPLs to help maintain homeostasis between the diverse populations of lipid-reactive T-cells. These observations have important implications for immune pathogenesis and intervention in conditions associated with altered lipid metabolism and inflammation.

4108

Role of the TNF-family cytokine TL1A and its receptor DR3 in systemic autoimmunity and glomerulonephritis*Meylan, F., Hayes, E., Ferdinand, J., Farley, T., Richoz, N., Gabay, O., Siegel, R.**NIH/NIAMS, Immunoregulation Group, Bethesda, United States*

DR3 (Tnfrsf25) is a death domain-containing tumor necrosis factor receptor found primarily on activated T cells and innate lymphocytes. Interaction with its ligand the TNF-family cytokine TL1A plays critical roles in diverse autoimmune disease models in facilitating T cell accumulation at the site of inflammation while the systemic response was unaffected. To determine whether DR3 could play a role in systemic autoimmunity, we studied the role of DR3 in the MRL/lpr mouse model of lupus. lpr mice have a mutation in the TNF family receptor Fas (CD95, Tnfrsf6) that impair its expression, causing a systemic autoimmune disease that mimics systemic lupus erythematosus (SLE) observed in humans. DR3 deficient lpr mice developed identical degrees of lymphadenopathy, splenomegaly compared to control littermates. Accumulation of autoantibodies was also not dependent on DR3. However, DR3 deficiency significantly protected MRL/lpr mice from IgG and C3 glomerular immune complex deposition and DR3 deficient kidneys had decreased medullary T cell accumulation, decreased periglomerular and medullary macrophage infiltrates, reduced proteinuria and reduced renal pathology. In addition, DR3-deficient CD4+ T cell transfer in a chronic graft-versus-host disease (GVHD) model showed similar levels of autoantibodies and immunoglobulin class switching than with wild-type CD4+ T cells but decreased proteinuria and reduced renal pathology. These findings show that DR3 specifically promotes renal pathology in the MRL/lpr mouse model of lupus nephritis and in chronic GVHD without affecting systemic autoimmunity. Blockade of TL1A-DR3 interactions may be a novel strategy to counter nephritis and other organ damage in human SLE.

748

Inflammatory circuit of Th17 cells, fibroblast-like synoviocytes, and ILCs in the development of autoimmune arthritis*Hirota, K.^{1,2}, Ito, Y.¹, Hashimoto, M.³, Watanabe, H.¹, Kondoh, G.¹, Tanaka, A.², Yasuda, K.², Kopf, M.⁴, Potocnik, A.J.⁵, Stockinger, B.⁶, Sakaguchi, N.², Sakaguchi, S.^{1,2}**¹Institute for Frontier Medical Science, Kyoto University, Kyoto, Japan, ²iFReC, Osaka University, Osaka, Japan, ³Kyoto University, Kyoto, Japan, ⁴Institute of Integrative Biology, Zurich, Switzerland, ⁵Institute of Immunology and Infection, University of Edinburgh, Edinburgh, United Kingdom, ⁶Francis Crick Institute, Mill Hill Laboratory, London, United Kingdom*

Proinflammatory cytokine and cellular networks at sites of inflammation play a pivotal role in immune-mediated diseases such as rheumatoid arthritis. Yet, inflammatory cascades in target tissues downstream of Th17-mediated autoimmune responses remain largely obscure. Here, we show in an animal model of rheumatoid arthritis, SKG mice, that GM-CSF from fibroblast-like synoviocytes (FLS), but not from Th17 cells is a key inflammatory

mediator downstream of arthritogenic Th17 responses to initiate autoimmune arthritis. The effector cytokine IL-17, but not GM-CSF from Th17 cells led to induction of an inflammatory profile and drove GM-CSF production in FLS. Intriguingly, synovial tissue inflammation expands innate lymphoid cells (ILCs) which produce large amounts of inflammatory cytokines in inflamed joints and the selective depletion of ILCs significantly ameliorated the severity of autoimmune arthritis, demonstrating that synovial ILCs contribute to the tissue inflammation. Fate mapping of synovial ILCs using IL-17-iCre R26ReYFP SKG mice revealed that inflammatory synovial ILCs were not derived from the group 3 ILCs which are generally associated with Th17 immunity. Together, these data indicate that arthritogenic Th17 cells orchestrate a cellular network and inflammatory mediators in the development of autoimmune arthritis.

2352

TLR induced IL-1beta production in monocytes and dendritic cells in T1D patients*Sediva, A.¹, Kayyserova, J.¹, Zentsova, I.¹, Paračková, Z.¹, Hromádková, H.¹, Katina, S.², Šumník, Z.³, Koloušková, S.³, Štechová, K.⁴
¹Motol University Hospital, Department of Immunology, Prague, Czech Republic, ²Masaryk University, Institute of Mathematics and Statistics, Brno, Czech Republic, ³Motol University Hospital, Department of Pediatrics, Prague, Czech Republic, ⁴Motol University Hospital, Department of Internal Medicine, Prague, Czech Republic*

Background: IL-1 is a cytokine with an important role in a pathogenesis of type1 diabetes (T1D) with a direct damaging effect on pancreatic beta cells. IL-1 signature is typical particularly for recent onset T1D and IL-1 blockade for T1D was tested in clinical trials. However, the initial event leading to an increased IL-1 secretion in T1D is not precisely known.

Methods: The IL-1 production in peripheral blood mononuclear cells (PBMCs) upon stimulation with TLR 3,4,7,9 ligands (LPS, polyI:C, ssRNA and CpG 2216) was tested in a cohort of 70 patients with recent onset T1D, 25 with long term T1D, 65 their relatives and 68 healthy controls. We have further specified the cellular source of IL-1 secretion within PBMC population.

Results: In comparison to controls, T1D patients and relatives, but particularly long-term patients, secreted increased amount of IL-1beta upon stimulation with TLR ligands. While monocytes are a dominant source of LPS induced IL-1beta in controls, both myeloid (mDCs) and plasmacytoid dendritic cells (pDCs) contributed significantly to IL-1 beta production in T1D (increase of IL-1beta producing cells upon LPS stimulation p 0.015 for mDCs, 0.0016 for pDCs). Such increase of IL-1beta production is surprising particularly for pDCs as LPS induced notable IL-1 production in pDCs despite their weak TLR4 expression.

Conclusion: The pattern of IL-1 beta secretion is different in T1D patients compared to controls. TLR ligands 4, 7 and 9 induced significantly more IL-1 beta production in T1D patients. Additionally, TLR stimulation alone was sufficient for IL-1beta production by blood DCs.

Vaccines 1

2243

Using a “prime-and-trap” vaccine strategy to generate liver T_{RM} and protect mice from malaria liver-stage infection

Ng, W.Y.¹, Fernandez-Ruiz, D.¹, Ma, J.¹, Zaid, A.^{1,2}, Wong, Y.C.³, Lau, L.S.¹, Mollard, V.⁴, Cozijnsen, A.⁴, Collins, N.¹, Li, J.^{5,6}, Davey, G.M.¹, Holz, L.^{1,2}, Tay, S.S.³, Tan, P.S.⁶, Bowen, D.G.³, Koch-Nolte, F.⁷, Björn, R.⁷, Carbone, F.R.¹, Crabb, B.S.⁶, Lahoud, M.^{6,8}, Cockburn, I.A.⁹, Mueller, S.N.^{1,2}, Bertolino, P.³, McFadden, G.I.⁴, Caminschi, I.^{6,8}, Heath, W.R.^{1,2}

¹Peter Doherty Institute for Infection and Immunity at The University of Melbourne, Dept. of Microbiology & Immunology, Melbourne, Australia, ²The ARC Centre of Excellence in Advanced Molecular Imaging, University of Melbourne, Melbourne, Australia, ³Liver Immunology Group, Centenary Institute and AW Morrow Gastroenterology and Liver Centre, University of Sydney and Royal Prince Alfred Hospital, Sydney, Australia, ⁴The School of Biosciences, University of Melbourne, Melbourne, Australia, ⁵Peter Doherty Institute for Infection and Immunity at The University of Melbourne, Melbourne, Australia, ⁶Macfarlane Burnet Institute for Medical Research & Public Health, Melbourne, Australia, ⁷Institute of Immunology, University Medical Centre Hamburg-Eppendorf, Hamburg, Germany, ⁸Monash University, Clayton, Australia, ⁹John Curtin School of Medical Research, Australian National University, Dept. of Immunology and Infectious Disease, Canberra, Australia

Malaria is a deadly disease that causes more than 600,000 deaths each year with the majority being children below the age of five. Tissue resident memory T cells (T_{RM}) are a recently discovered subset of non-circulating memory T cells that reside permanently in peripheral tissues after the resolution of infection and can accelerate the clearance of pathogens upon reinfection. Recently, our laboratory has identified a population of T_{RM} in the liver after immunization with irradiated *Plasmodium berghei* ANKA (PbA) sporozoites, an effective form of vaccination. We then asked whether liver T_{RM} could be efficiently generated through the use of a novel vaccination strategy termed “prime and trap”: a two-stage approach that involves activation of plasmodium-specific CD8⁺ T cells in the spleen using Clec9A-targeted antigen (prime), followed by trapping in the liver through local antigen expression using adeno-associated virus vector (trap). By tracking responses of PbA-specific TCR transgenic CD8⁺ PbT-I cells, the prime-and-trap vaccination strategy was shown to form large numbers of liver T_{RM} and to elicit high level of sterile protection that persisted for more than 6 months. Follow-up studies have extended this work to natural malaria antigens and endogenous T cells, revealing protective immune response against malaria. Our findings describe a highly promising novel vaccination approach that confers protection against murine malaria.

1930

A novel R848-conjugated inactivated influenza virus vaccine is safe and efficacious in a neonate nonhuman primate model

Holbrook, B.¹, Kim, J.¹, Blevins, L.¹, Jorgensen, M.¹, Kock, N.¹, D'Agostino Jr, R.¹, Aycock, S.T.¹, Hadimani, M.², King, S.B.², Parks, G.¹,

Alexander-Miller, M.³

¹Wake Forest School of Medicine, Winston-Salem, United States, ²Wake Forest University, Winston-Salem, United States, ³Wake Forest School of Medicine, Microbiology and Immunology, Winston-Salem, United States

Influenza virus infection of neonates poses a major health concern, often resulting in severe disease and hospitalization. Compounding this issue is the lack of vaccines for this at-risk population. Thus, development of an effective vaccine is an urgent need. To address this, we have tested a novel TLR 7/8 agonist (R848) conjugated influenza virus vaccine. The use of the intact virion represents a step forward in conjugate vaccine design as it provides multiple antigenic targets allowing for elicitation of a broad immune response. Immunogenicity and efficacy were tested in a nonhuman primate neonate, a model which is highly relevant for human infants with regard to immune development, TLR function, and physiology. Our results show that this vaccine induces high level virus-specific antibody and cell mediated immune responses in neonates. These responses promote increased virus clearance and reduced lung pathology following challenge compared to a non-adjuvanted virus vaccine. Surprisingly, the addition of a second TLR agonist (flagellin) did not enhance vaccine protection, suggesting combinations of TLR that provide increased efficacy must be determined empirically. These data support further exploration of this new virus conjugate vaccine approach as a platform for use in the at-risk neonate population to induce protection against influenza as well as other viral diseases.

2847

Protective efficacy of tuberculosis-specific T Cell immunity in the lung after aerosol delivery of adenoviral vaccines in nonhuman primates

Darrah, P.¹, Roederer, M.¹, Flynn, J.², Scanga, C.², Coleman, M.T.², DiFazio, R.², Lin, P.², Evans, T.³, Laddy, D.³, Anantha, R.³, Limbach, M.P.³, Temmerman, S.⁴, Demoitie, M.-A.⁴, Seder, R.¹

¹NIH, Vaccine Research Center, NIAID, Bethesda, United States, ²University of Pittsburgh School of Medicine, Pittsburgh, United States, ³Aeras, Rockville, United States, ⁴GlaxoSmithKline, Rixensart, Belgium

The magnitude, quality, breadth, and location of T cell responses are critical factors in determining protection against infections such as HIV, malaria, and tuberculosis (TB). For TB, developing a vaccine that prevents pulmonary infection may require an immunization route that elicits a high frequency of T cells directly in the lung. Accordingly, simultaneous intramuscular and aerosol (IM/AE) rAd5-TB (a recombinant adenovirus serotype 5 expressing *Mycobacterium tuberculosis* [Mtb] antigens) was delivered to BCG-primed rhesus macaques. Intradermal BCG priming induced low-level TB-specific CD4 T cell cytokine responses in blood and BAL. IM/AE boosting with rAd5-TB generated robust TB-specific CD4 and CD8 effector T cells in BAL that produced primarily IFN- γ , with subsets that also produced TNF and/or IL-2. Twelve weeks post-boost, animals were challenged intrabronchially with a low dose

(~10 CFU) of Mtb Erdman, monitored non-invasively using PET/CT scanning, and evaluated for pathology and bacterial burdens at a 6-month end point. Remarkably, the presence of a high frequency of TB-specific effector T cells in the lung after IM/AE rAd5-TB immunization did not improve outcome measures after challenge compared to animals that received only BCG. These data show that while IM/AE immunization was an effective approach for inducing T cells directly in the lung (and periphery), rAd5 vectors with the tested TB antigens were not sufficient to enhance protection over BCG alone in rhesus macaques. Studies are underway to determine mechanisms for the limited efficacy of rAd5-TB vaccines in this model.

3188

Characterising the lymphatic immune responses induced by sub-cutaneous and intramuscular injection of the human vaccine adjuvant AS01

de Veer, M.¹, Neeland, M.², Collignon, C.³, Didierlaurent, A.³

¹Monash University, Physiology, Clayton, Australia, ²Murdoch Childrens Research Institute, Parkville, Australia, ³GSK Vaccines, Rixensart, Belgium

AS01 is a liposome-based Adjuvant System incorporating the immunostimulant 3-O-desacyl-4'-monophosphoryl lipid A (MPL) and the saponin QS-21. AS01 is included in two candidate vaccines that have demonstrated clinical efficacy, i.e. the RTS,S malaria vaccine and the herpes zoster vaccine. AS01 has been shown to enhance adaptive immunity via activation of the innate immune system, however the respective contribution of the injection site and the draining lymph node remain unclear. We report the development of an ovine lymphatic cannulation model to investigate the *in vivo* trafficking of cells within the lymphatic network following intramuscular administration of an AS01-adjuvanted vaccine. Injections were given to sheep using a vaccine dose and volume comparable to that administered in humans. We show that AS01 induced the transient migration of neutrophils into the tissue draining lymph and out of the lymph node that rapidly resolved within 24 hours. This was followed by the recruitment of antigen-positive monocytes and MHCII^{high} dendritic cells into muscle-draining afferent lymph within 48 hours of vaccination. This initial innate response correlated with the generation of an adaptive response in the local lymph node, characterised by the production of blast cells, IFN γ -producing T cells and antigen-specific antibodies in efferent lymph within 5 days of vaccination. This study reports the development a surgical cannulation model that was used to quantitate lymphatic cellular migration following intramuscular or subcutaneous vaccination with AS01. It adds to our understanding of the immunogenic function of AS01 for the development of safe and effective human vaccine formulations.

1808

Immunogenicity and biodistribution of nanoparticles *in vivo*

Tsirikis, P.¹, Wilson, K.², Xiang, S.², Wei, W.³, Ma, G.³, Selomulya, C.¹, Plebanski, M.²

¹Monash University, Clayton Campus, Chemical Engineering, Clayton, Australia, ²Monash University Central Clinical School,

Melbourne, Australia, ³National Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing, China

Nanoparticles have been widely used in vaccine design as both adjuvants and antigen delivery vehicles. In a seminal study, 40-50 nm nanoparticles with conjugated antigen were shown to induce high antibody titers and IFN- γ production in mice but with no added inflammatory stimuli. Subsequent research has shown that similar levels of immunogenicity can be achieved via the co-injection of naked 40-50 nm nanoparticles adjuvants and larger 500 nm nanoparticles with conjugated antigen. Furthermore, recent works indicate that particle shape can also influence the immune response. As such, we investigate the influence of surface morphology using 40-50 nm smooth and rough surfaced nanoparticle adjuvants and report their differential immunogenicity via ELISA, ELISpot and flow cytometry. Further, we determine the biodistribution of fluorescent 40-50 nm nanoparticle adjuvants with smooth and rough surfaces and larger 500 nm nanoparticles. Nanoparticle size is shown to be a discriminating factor in lymph node drainage, using a Carestream FX PRO *in vivo* imaging system and fluorescence microscopy of lymph nodes sectioned *ex vivo*. To elucidate the safety profile of this vaccine construct, we also investigate the biodistribution of nanoparticles within the major organs. The outcomes from this study provide key design criteria in the development of novel nanoparticle immunotherapeutics for the treatment of disease.

3016

Modulation of host immune responses using influenza viruses expressing functional miR-150

Xia, Y., Izzard, L., Ye, S., Stambas, J.

Deakin University, School of Medicine, Geelong, Australia

Current influenza vaccines offer considerable protection for the majority of the population. However, vaccine efficacy amongst high risk populations, such as the elderly, is variable. Novel approaches are therefore required to improve vaccine efficiency in these susceptible populations. MicroRNAs (miRNAs) play important roles in the regulation of immune responses by modulating gene expression. Our work describes the generation and delivery of engineered influenza viruses with the ability to express defined Immunomodulatory miRNAs. MicroRNA-150 (miR-150) has been shown to be involved in the development of T cell and B cell. Furthermore, evidence suggests that circulating miR-150 can be actively transported to target cells where it can influence gene expression. In this study, we first investigated the ability of recombinant influenza viruses to deliver immunomodulatory miR-150, and alter cytokine production *in vitro*. miR-150 was inserted into an artificial intron between the NS1 and NEP genes of the PR8 H1N1 (PR8-150) and X31 H3N2 (X31-150) influenza A viruses and was rescued using reverse genetics. Mature miR-150 expression was confirmed using qPCR and by knockdown of its target gene, c-Myb, using dual-luciferase. Moreover, cytokine mRNA expression levels (IL-6, IFN- β , CXCL10 and CCL5) were enhanced following infection with PR8-150 in A549 cells. Mice infected intranasally

with recombinant X31-150 virus showed significantly higher numbers of neutrophils, macrophages and influenza specific CD8+ T cells post infection compared to X31 wild type controls. These results demonstrate that recombinant influenza viruses can be used to deliver functional miRNAs with the ability to modulate immunity both *in vitro* and *in vivo*.

4219

Novel mannose- α (1 \rightarrow 2)-mannose decorated liposomes for dendritic cell targeted nanovaccines: strong humoral and cell immune response against *Brucella ovis*

Pappalardo, J.S.¹, Chodilef, M.M.¹, Mignaqui, A.C.¹, Marcellino, R.¹, Zamit, A.L.², Salmaso, S.³, Levchenko, T.S.⁴, Torchilin, V.P.⁴, Robles, C.A.¹

¹National Institute for Agricultural Technology (INTA), Bariloche Research Station (EEA Bariloche) - Animal Health Group (GSA), Bariloche, Argentina, ²National Institute for Agricultural Technology (INTA), Agribusiness Research Center (CIA), Hurlingham, Argentina, ³University of Padova, Department of Pharmaceutical and Pharmacological Sciences, Padova, Italy, ⁴Northeastern University, Center for Pharmaceutical Biotechnology and Nanomedicine, Boston, United States

Dendritic Cells (DC), are the only professional antigen-presenting cells due to their unique ability to activate naïve T lymphocytes, thus initiating the adaptive immune response. DC-targeted vaccination is a promising strategy for the prevention of certain infectious diseases and cancer. The activation of DC for this purpose is attractive because of their ability to present epitopes through the MHC class I and II, and to activate the cytotoxic CD8+ T cell responses, among others.

Mannose is a saccharide that belongs to the pathogen-associated molecular patterns which can be exploited to decorate nanovehicles such as liposomes to target their cargo to DC.

Brucella ovis is a pathogen that generates big losses in the sheep industry. To overcome *B. ovis* infection, it is necessary to generate a strong humoral and cell mediated immune response.

Here we report the use of a liposomal formulation decorated with Mannose- α (1 \rightarrow 2)-Mannose through a PEG spacer and afterwards loaded with *B. ovis* antigens as a novel vaccine against *B. ovis* infection. The experimental vaccine was able to trigger a strong humoral response in mice and rams up to 6 months post vaccination. In addition, it was able to generate specific IFN γ secretion 12 months after vaccination in rams.

These results show that the liposomal platform targeted to DC using Mannose- α (1 \rightarrow 2)-Mannose is a promising nanovaccine to elicit a strong humoral and cell response. For this reason, it can be considered as a strategy to be explored for DC-targeted vaccination with a variety of antigens, for veterinary and human applications.

3574

Quercetin exhibits enhanced Th1/Th2 immune response and CD11c⁺ dendritic cell infiltration in peritoneum of ovalbumin immunized mice

Singh, D., Tanwar, H.

Defence Institute of Physiology and Allied Sciences, Immunomodulation, New Delhi, India

Quercetin, one of the most abundant of plant flavonoids, has been studied with a great deal of attention over the last several decades mainly for its properties in inflammation and allergy. In this study, we are reporting for the first time the *in vivo* immunostimulatory activity of quercetin in ovalbumin immunized Balb/c mice. Administration of quercetin (50mg/kg body weight) along with ovalbumin antigen showed increased ova specific serum IgG antibody titres in comparison to the control group ($p < 0.05$). Quercetin administration not only showed predominance of Th2 immune response by increasing the IgG1 antibody titres, but also increased the infiltration of CD11c⁺ dendritic cells in the mouse peritoneum and also increased LPS activated IL-1 β and nitric oxide (NO) production by peritoneal macrophages. Expression of Oct-2, Tbx21 and GATA-3 proteins also increased in splenocytes of quercetin administered mice. Quercetin also did not cause any haemolysis in human RBCs. Overall; our findings strongly demonstrate the novel *in vivo* immunostimulatory and adjuvant potentials of quercetin.

Keywords: Quercetin, Flavonoids, Immunomodulators, Dendritic cells, Antibody titre.

1488

Single shot booster vaccination against diphtheria does not induce sufficient long-term protection, particularly in elderly people

Grasse, M., Weinberger, B., Meryk, A., Grubeck-Loebenstern, B.

University of Innsbruck/Institute for Biomedical Aging Research, Immunological Division, Innsbruck, Austria

Immunization is one of the most successful health intervention against infectious diseases. However, the efficacy of vaccination is reduced in old age. Our study analyzed specific immune responses following a booster vaccination containing tetanus and diphtheria toxoid in healthy elderly (>60y; n=87) and young volunteers (25-40y; n=46). Long term protection was evaluated for 27 elderly and 17 young adults 5 years later. In addition, antigen-specific T-cells producing a panel of cytokines were quantified.

Before the vaccination 9% of the older and none of the young individuals had tetanus-specific antibody levels below the protective limit. The booster induced sufficient protection in both age groups for the following 5 years. The protection against diphtheria was almost equal before the vaccination (52% for the elderly and 48% for the young donors). Antibody concentrations increased significantly 4 weeks after vaccination, but dropped substantially over 5 years leaving again 54% (elderly) and 24% (young) below protective antibody levels. Thus, compared to the elderly young adults have a significantly better, but still insufficient maintenance of diphtheria-specific

antibodies. We found correlations between diphtheria-specific antibodies and diphtheria-specific T-cells producing different cytokines. Among those were GM-CSF-producing T-cells which we detected in a lower frequency in elderly compared to young adults.

In conclusion, our findings demonstrate that a booster vaccination induce long-lasting immunity against tetanus but not diphtheria, particularly in elderly people. GM-CSF might be useful as an adjuvant to improve diphtheria vaccination. We set up a mouse model to further investigate this question.

Tumour Immunology 1

2952

Increasing antitumor efficacy of checkpoint blockade by targeting to the tumor microenvironment

Ingram, J., Dougan, M., Rashidian, M., Linnebacher, A., Ploegh, H. Whitehead Institute for Biomedical Research, Cambridge, United States

Disruption of immune checkpoint interactions by antibodies has delivered on its promise: first in treating metastatic melanomas, and now also in non-small cell lung cancer. Not all tumors respond to treatment and therefore diagnostic tools to predict responsiveness are an important goal, especially with the severe immune side effects of checkpoint blockade. To address these challenges, we developed VHHs--variable region domains derived from camelid heavy chain-only antibodies--that serve as modular binding agents, not only to image PD-L1 and CTLA-4 non-invasively, but also to counteract their inhibitory activity *in vivo*. We exploit the modifiability of VHHs to direct therapeutic payloads to the tumor microenvironment through linkage to anti-PD-L1, achieving enhanced antitumor efficacy. High affinity anti-PD-L1 VHHs hold promise as a tool to re-engineer the tumor microenvironment.

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Induction of stem cell-like memory T cells by cancer vaccine correlates to enhanced antitumor effect

Wu, S., Peng, Y., Wang, L., Zhu, W., Wu, J., Xie, J., Celis, E., He, Y. Augusta University (formerly Georgia Regents University, Medicine and Cancer Center, Augusta, United States

To understand why cancer vaccine-activated immune responses often failed to generate antitumor effect, we studied the antitumor effects of two alpha fetoprotein (AFP) epitope (AFP₂₁₂- and AFP₄₉₉-) specific CD8 T cells recently identified in our lab. We found that both T cells could recognize AFP+ tumor cells. However, while the AFP₄₉₉-specific CD8 T cells can be re-activated *in vitro* to generate potent CTL activity against AFP+ tumor cells, the AFP₂₁₂-specific CD8 T cells activated by the same vaccine in the same mouse have a very limited CTL activity after *in vitro* stimulation. Importantly, only the AFP₄₉₉-specific, but not the AFP₂₁₂-specific, CD8 T cells can prevent AFP+ tumor challenge *in vivo*. Mechanistically, majority of the AFP₂₁₂-specific CD8 T cells were dead or undergoing apoptosis after persistent *in vitro* AFP₂₁₂ peptide stimulation, a scenario

existed in the tumor microenvironment. However, the AFP₄₉₉-specific CD8 T cells were enriched by AFP₄₉₉ peptide stimulation and maintain their killing activity while the AFP₂₁₂-specific CD8 T cells were eliminated and functionally suppressed by long-term Ag stimulation. Surprisingly, only the AFP₄₉₉-specific CD8 T cells contain a population of cells that resemble to the newly discovered stem-like memory T cells. We propose that the stem-like memory T cells may provide the constant source for generating functional fresh and young effector cells to generate antitumor effect. The possible reasons and mechanism for generating stem-like memory T cells by cancer vaccines will be discussed, providing new clues and rationale for designing more effective cancer vaccines to achieve antitumor effect.

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Effector regulatory T cells are associated with disease-free survival in colorectal cancer

Ward Hartstonge, K.¹, McCulloch, T.¹, Kamps, A.¹, Cretney, E.², Munro, F.³, McCall, J.³, Kemp, R.¹

¹University of Otago, Microbiology and Immunology, Dunedin, New Zealand, ²Walter & Eliza Hall Institute, Melbourne, Australia, ³University of Otago, Department of Surgical Sciences, Dunedin, New Zealand

Analysis of tumour infiltrating T cells in colorectal cancer is superior to the current staging at predicting disease-free survival. The Immunoscore has been proposed as a way to incorporate T cell infiltrates into the current staging by measuring CD3+ and CD8+ T cells. Unlike in many cancers, high tumour infiltrating regulatory T cells (Tregs) are associated with good patient outcomes in colorectal cancer. Effector Tregs are potently suppressive in mice. Effector Tregs are present in human colorectal cancer but their role in patient outcome is not known.

Immunofluorescence was used to analyse immune cell infiltrates in early stage (II) colorectal cancer patients and to compare those with recurrent and non-recurrent disease (n=33). CD3 and CD8 were used for the Immunoscore. FoxP3, Blimp-1 and CD3 were used to identify effector regulatory T cells.

Patients with a high Immunoscore (high T cell infiltrate) had increased disease-free survival than patients with a low Immunoscore (low T cell infiltrate, Log-rank test p>0.001). The ability to predict patient outcome was improved by measuring the infiltrate of CD4+FoxP3+Blimp-1+ cells (effector regulatory T cells, Log-rank test p>0.001). Patients with a low Immunoscore but high infiltrate of CD4+FoxP3+Blimp-1+ cells at the invasive margin had increased disease-free survival than those with a low Immunoscore and a low infiltrate of CD4+FoxP3+Blimp-1+ cells.

These results indicate that effector Tregs may play an important role in patient outcome in colorectal cancer. Incorporation of effector Tregs into the Immunoscore may help to identify patients at high risk of recurrent disease.

3898**Antigen-specific regulatory T cell suppression of the CD4+ T cell anti-tumour response**

Terry, A., Bolton, H., Guy, T., Fazekas de St Groth, B., Shklovskaya, E. Centenary Institute, University of Sydney, T Cell Biology, Camperdown, Australia

Regulatory T cells (Tregs) play a dominant role in suppression of anti-tumour responses, evident by the promising results seen in patients treated with immunotherapies targeting Tregs (eg. Ipilimumab). The exact cell targets and mechanism by which Tregs suppress anti-tumour responses have yet to be fully established. We have developed a model to study the effect of tumour-specific Tregs on the anti-tumour responses of CD4 T cells and the tumour microenvironment.

B16.F10 tumours expressing a model neoantigen (HELMCC) grew rapidly in immunodeficient mice but were rapidly rejected after adoptive transfer of naive MCC-specific CD4 T cells. Co-transfer of MCC-specific Tregs but not polyclonal Tregs prevented rejection of tumours. Suppression of the CD4 T cell response was associated with a significant reduction in DC costimulation, T cell proliferation and cytokine production. Depletion of MCC-specific Tregs (derived from FoxP3.DTR mice) rescued the T cell response only when performed early after co-transfer (day 3 post transfer), before T cells had differentiated.

In order to establish the effect of Tregs on the CD4 T cell response in immunosufficient mice, FoxP3.DTR mice were used as tumour hosts. Only when MCC-specific T cells were transferred into Treg-depleted hosts when Treg number was at the lowest and DC costimulation was highest were T cells able to mount an effective response, expressing IFN γ +TNF α +IL-2+, controlling tumour growth and even inducing endogenous host CD4 T cell cytokine production.

In summary, CD4 T cells can mount potent anti-tumour responses but only if priming occurs under Treg-deficient conditions. This is therefore relevant to the design of adoptive-cell immunotherapeutics.

1528**Dysregulated repair processes in inflammation and colorectal cancer: pathogenic regulatory T cells to blame?**

Franchini, F.^{1,2}, West, N.^{1,2}, Royston, D.J.³, Powrie, F.M.^{1,2}

¹Kennedy Institute of Rheumatology, University of Oxford, Oxford, United Kingdom, ²Translational Gastroenterology Unit, Nuffield Department of Clinical Medicine, Oxford, United Kingdom,

³University of Oxford, Department of Cellular Pathology, Oxford, United Kingdom

There is now abundant evidence that chronic inflammation can promote tumorigenesis and patients with inflammatory bowel disease have an increased risk of developing colitis-associated cancer (CAC). Although inflammatory pathways that drive colitis are well-described much less is known about the cellular and molecular mechanisms that induce and sustain cancerous lesions in the intestine.

Transfer of regulatory T cells (Tregs) from spleens of 129.IL-10^{-/-} mice accelerated and increased the tumorigenesis in 129.RAG^{-/-} recipients infected with *Helicobacter hepaticus*, while

transfer of WT Tregs led to no phenotype. In less than 3 months, mice transferred with IL10^{-/-} Tregs showed macroscopic tumour lesions in the colon and invasive carcinomas were confirmed by histological analysis. This project focuses on deciphering the molecular pathway by which a defective Treg response driven by a microbial insult promotes tumorigenesis.

Amphiregulin (AREG) is a key member of the EGF family we show that interleukin-10 deficient Tregs upregulate AREG as well as TGF- β at the transcriptional level compared to WT Tregs, which lead us to hypothesize that IL10^{-/-} Tregs could promote cancer development through a dysregulated repair response. In addition, we showed that transcripts for tissue remodelling factors as well as Sox4,

a master regulator of epithelial-mesenchymal transition (EMT), are upregulated in tumour tissue compared to inflamed tissue controls.

Altogether, the results of this study may lead to the identification of key pathways involved in tumorigenesis, which could be relevant for novel therapeutic targets.

3258**The effects of targeting both PD-1 and CD96 on tumour immunity, autoimmunity and immune homeostasis**

Harjunpaa, H.M.^{1,2}, Blake, S.J.¹, Liu, J.^{1,2}, Allen, S.¹, Smyth, M.J.^{1,2}, Teng, M.W.L.^{1,2}

¹QIMR Berghofer Medical Research Institute, Brisbane, Australia,

²University of Queensland, Brisbane, Australia

Tumour cells have developed multiple mechanisms to suppress anti-tumour immunity. One important mechanism is delivered via immune checkpoint receptors. Indeed, immunotherapy targeting immune checkpoint receptors, particularly PD-1 has shown significant efficacy and durable responses against a variety of different cancers. Given that anti-PD-1 will be used as the backbone in combination therapies due to its efficacy and safety, there is a search to determine which other pathways can synergize with anti-PD-1 to increase anti-tumour efficacy. CD96 is the least known member of immune checkpoint receptors and binds ligands of the nectin and nectin-like family. It is expressed on T cells and NK cells following activation and has been shown to suppress NK cell activity. This study aims to characterize the effects of targeting both PD-1 and CD96 on tumour immunity, development of autoimmunity and immune homeostasis. For this purpose, a novel PD-1/CD96 double knock-out (DKO) mouse was generated in our laboratory. Differences in frequencies and numbers of NK cells and Tregs in the PD-1/CD96 DKO mouse were observed compared to wild type or either PD-1 or CD96 single knock-out mouse. From our results, PD-1/CD96 DKO mice displayed improved anti-tumour efficacy compared to wild type, PD-1 or CD96 single knock-out mice in some tumour models. Importantly, PD-1/CD96 DKO mice that were aged for more than a year did not develop any overt signs of autoimmunity. Our findings suggest targeting PD-1 and CD96 in combination will be a safe and effective strategy to improve anti-tumour efficacy.

1718**Alpha fetoprotein immune modulation via DC and NK cells***Butterfield, L.¹, Santos, P.¹, Shi, J.¹, Vujanovic, L.¹, Delgoffe, G.²**¹University of Pittsburgh, Medicine, Surgery and Immunology, Pittsburgh, United States, ²University of Pittsburgh, Immunology, Pittsburgh, United States*

Alpha fetoprotein (AFP) is an oncofetal antigen expressed by over 50% of hepatocellular carcinoma (HCC) tumors. Previous studies proposed an immunoregulatory role for AFP. To examine the effects of AFP on dendritic cell (DC) and NK cell differentiation and function, peripheral blood cells were cultured in the presence of cord blood-derived "normal" AFP (nAFP) or HCC "tumor"-derived AFP (tAFP), and cellular phenotype and function were assessed. We have previously shown that monocytes cultured in vitro with tAFP differentiated into DC that retained a monocytic morphology, were phenotypically immature and failed to induce robust T cell proliferative responses. Here, we further examine nAFP and tAFP-induced effects on: 1) CD1 family surface expression; 2) cytokine and chemokine production; 3) glycolysis and oxidative phosphorylation metabolism; and 4) viability. In DC, we show that surface expression of CD1a, CD1b, CD1c and CD1d are reduced by nAFP and are further reduced by tAFP. TAFP-DC had decreased secretion of chemokines CCL1, CCL2, CCL3, CCL4, CCL17, CCL20 and CCL22 and we observe a significant defect in mitochondrial oxidative phosphorylation in both nAFP and tAFP-DC as early as 1 day after tAFP exposure. In NK cells, we show that these cells also take up AFP, show a short term skewed activation and cytokine secretion profile, but over time, have decreased viability. These DC and NK cell effects appear to be mediated by both AFP protein and a LMW binding partner. Together, these data implicate AFP as a key mediator of immune modulation and suppression.

2714**Treating cancer one interferon at a time***Buzzqai, A.¹, Foley, B.¹, Hart, P.¹, Robinson, B.², Fear, V.², Waithman, J.^{1,2}**¹Telethon Kids Institute, University of Western Australia, Perth, Australia, ²National Centre of Asbestos Related Disease, University of Western of Australia, Perth, Australia*

Why our bodies produce 14 subtypes of type I interferons (IFNs) is poorly understood. Only the IFN- α 2 subtype has been investigated as a possible treatment option for certain cancers. We investigated if other individual subtypes have the potential to arrest tumour growth and/or enhance anti-tumour immunity. We transduced the murine melanoma cell line B16 with five individual IFN subtypes (B16_{IFN_v}, B16_{IFN_w}, B16_{IFN_x}, B16_{IFN_y}, B16_{IFN_z}) and grafted them onto mice. We found that wild-type mice inoculated with B16_{IFN_v} or B16_{IFN_w} have significant delayed tumour growth. When mice lacking the receptor for IFN (IFNAR^{0/0}) are inoculated with B16_{IFN_v} or B16_{IFN_w} tumours grow at the same rate as B16 control cells. This result provides strong evidence that type I IFNs indirectly mediate tumour control by harnessing anti-tumour immunity.

Strikingly, cohorts of either wild-type or IFNAR^{0/0} mice inoculated with B16_{IFN_x}, B16_{IFN_y} or B16_{IFN_z} cells never develop a palpable tumour. This

intriguing result suggests that these IFNs can directly exert their effect on tumour cells through a yet undefined mechanism.

In conclusion, our data provides compelling evidence that the family of 14 type I IFNs did not evolve solely to coordinate efficient immunity as the literature currently suggests. Instead, we postulate that multiple type I IFN subtypes exist to enable an individual to confront malignancies, such as cancer, through at least two distinct pathways:

- 1) either indirectly through immune modulation or
- 2) directly by arresting development and growth.

2858**Intravital visualization of brain metastasis immune surveillance reveals organ-specific regulation by the neuronal chemokine fractalkine***Nwajei, F.¹, Shanmugasundaram, M.¹, Zal, M.A.¹, Gabrusiewicz, K.², Wu, W.¹, Heimberger, A.², Zal, T.¹**¹University of Texas MD Anderson Cancer Center, Department of Immunology, Houston, United States, ²University of Texas MD Anderson Cancer Center, Department of Neurosurgery, Houston, United States*

Behind the blood-brain barrier, brain and other central nervous system (CNS) tissues are typically excluded from the immune surveillance by lymphocytes. As such, the CNS has been considered as immune privileged. Longitudinal intravital multiphoton microscopy via thinned skull windows revealed that single cell micrometastases of otherwise lethal MCA cancer progressed within the brains of syngeneic immune competent mice initially, but then became infiltrated by myeloid and T cell subsets followed by immune-mediated regression. In contrast, MCA brain metastases progressed lethally when the adaptive immunity was compromised such as by Rag deficiency or CD8 depletion. Interestingly, the neuronal chemokine fractalkine, which is uniquely recognized by the CX3CR1 chemokine receptor expressed by the microglia and various immune cell subsets, was highly upregulated around brain tumor borders, and brain MCA tumors were not rejected in CX3CR1-deficient mice. The contextual tracking of T cell motility patterns and cell subset depletion experiments revealed that T cell surveillance of brain metastases was cancer type-dependent and that it was organized largely by the CD11c⁺ CX3CR1⁺ monocytic dendritic cell (DC) subset rather than by the brain-resident CX3CR1-high microglia. Our results show that the blood-brain barrier is easily breached for T cell surveillance of brain invading cancers in a manner that is organ-specific such as through the recruitment of monocytic DC by the neuronal chemokine fractalkine. In extension, our study suggests that the phenomenon of organ specificity of tumor metastasis that is explained by the "seed and soil" concept should be re-considered as also involving organ-dependent immune regulation.

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IFN-lambda production of CD8 cDCs in response to DNA-viruses, transfected dsDNA or cyclic-di-nucleotides depends on STING

Lauterbach, H.¹, Bathke, B.¹, Sanos, S.¹, Chaplin, P.¹, Suter, M.², O'Keefe, M.³, Hochrein, H.¹

¹Bavarian Nordic, Research Immunology, Martinsried, Germany,

²University of Zurich, Zurich, Switzerland, ³Monash University, Department Biochemistry & Molecular Biology, Melbourne, Australia

CD8 α ⁺ conventional dendritic cells (CD8 cDCs) and plasmacytoid dendritic cells (pDC) are major producers of IFN-lambda (IL-28/29). Previously, we have shown that upon stimulation with dsRNA murine CD8 cDCs were the exclusive source of IFN-lambda *in vivo* and *in vitro* via a TLR3/TRIF dependent recognition pathway. A finding confirmed for the human equivalent cDCs expressing CD141 (BDCA3). In contrast, pDCs ignored dsRNA but produced large amounts of IFN-lambda in response to TLR7 or TLR9 stimuli via MyD88.

Here, we demonstrate that both, the CD8 cDCs and pDCs, are able to produce IFN-lambda in response to DNA viruses such as HSV-1 or poxviruses. Our analysis of the pattern recognition receptors or adaptor molecules involved revealed that pDCs mainly use TLR9 and MyD88 for the detection of DNA viruses, whereas CD8 cDCs depend on the adaptor molecule STING but not on MyD88. The transfection of CD8 cDCs with different forms of dsDNA or cyclic-di-nucleotides such as c-di-GMP or c-GAMP induced, similar to the infection with DNA viruses, large amounts of IFN-lambda in a STING dependent way.

Thus, the immune system produces IFN-lambda in response to DNA via two different DC subsets (CD8 cDCs and pDCs), different pattern recognition receptors (cytoplasmic DNA receptors and TLR9) and adaptor molecules (STING and MyD88) as well as downstream signaling components (IRF3 and IRF7). We hypothesize, that this widespread redundancy counteracts viral or other pathogen encoded inhibitory mechanisms, possibly acting on the specific DC subsets, the pattern recognition receptors and adaptor molecules or other signaling components.

1107

Type I interferon signalling suppresses anti-parasitic CD4⁺ T cell responses during visceral leishmaniasis

Kumar, R.^{1,2,3}, Bunn, P.², Singh, N.³, Sundar, S.³, Engwerda, C.²

¹Netaji Subhas Institute of Technology (University of Delhi), Div. of Biotechnology, Delhi, India, ²QIMR Berghofer Medical Research Institute, Immunology & Infection Lab, Brisbane, Australia,

³Banaras Hindu University, Dept. of Medicine, Varanasi, India

Many pathogens, including viruses, bacteria, and protozoan parasites, suppress cell mediated immune responses through activation of type I Interferon (IFN-1) signalling. However the role of IFN-1 during *Leishmania donovani* infection causing visceral leishmaniasis (VL) is not well known. Here we report that IFN-1 play an important role in the pathogenesis of VL by

impairing parasite clearance and suppressing pro-inflammatory cytokine production. Mice lacking type-1 IFN signalling (B6. IFNAR1^{-/-} mice) had enhanced pro-inflammatory cytokine production and better control of parasite burden in liver as well as spleen compared to wild type C57BL/6 mice. IFN-1 signalling suppressed the CD4⁺ derived IFN- γ production and prevented Th1 response from controlling parasite replication. Experiment using bone marrow chimeric mice revealed that IFN-1 did not suppress CD4⁺ T via directly acting to this cell type. Using conventional dendritic cell (cDC) specific IFN-1 deficient mice (CD11c Cre-IFNAR^{fl/fl}), we observed that IFN-1 hampered CD4⁺ T cells response acting via dendritic cells. Studies in VL patients supported these findings and showed enhanced accumulation of mRNA encoding type I IFN signature genes in peripheral blood mononuclear cells (PBMCs) that were reduced following successful drug therapy. Critically, we also showed, using a whole blood assay, that blockade of type-1 IFN signalling enhanced antigen specific IFN- γ production, and that this response was HLA-II restricted. Together, these results identify type-1 IFN signalling pathways as a potential therapeutic target to treat VL by stimulating anti-parasitic CD4⁺ T cell responses.

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MKP5 regulates RIG-I and STING mediated IRF3-type I interferon response in response to virus infection

James, S.J.¹, Jiao, H.¹, Teh, H.-Y.¹, Takahashi, H.¹, Yamamoto, N.¹, Dong, C.², Zhang, Y.¹

¹National University of Singapore, Microbiology & Immunology, Singapore, Singapore, ²Tsinghua University School of Medicine, Beijing, China

Detection of cytosolic nucleic acids from viral pathogens induces the production of type I interferons (IFNs) including IFN α and IFN β , which are critical for immune response to virus infection. Cytosolic RNA sensor RIG-I is important for recognition of RNA viruses including Sendai virus and influenza virus leading to IRF3 activation and type I IFN expression. Cytosolic DNA sensor STING, on the other hand, is essential for the induction of type I IFNs in response to cytosolic DNA of pathogen- and self-origin. However, how RIG-I and STING mediated IRF3-type I IFN response is negatively regulated is unclear. MAPK phosphatases (MKPs), also known as dual-specificity phosphatase (DUSPs), are cysteine-based protein tyrosine phosphatases that dephosphorylate phosphotyrosine, phosphothreonine, and phosphoserine residues in their substrates. They are originally identified as negative regulator of MAP kinases. It has been shown that members of this protein family also target other important signalling molecules such as FOXO1 and STATs. In this study, we demonstrate that RIG-I and STING stimulation induce the expression of MKP5. MKP5 protein interacts with RIG-I-MAVS-IRF3 or STING-MAVS-IRF3 signaling complexes to inhibit IRF3 activation by dephosphorylation of IRF3 in response to RNA virus or DNA virus infection. Our study reveals a critical function of MKP5 in negative regulation of IRF3-type I IFN axis in response to virus infection, which may be explored by viruses to evade host immunity. This function of MKP5 may also be important for the host to regulate the intensity of antiviral immune response to avoid uncontrolled immunopathology.

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In vivo visualizing the IFN- β response required for tumor growth control in a therapeutic model of poly A:U administration

Maccioni, M.¹, Brinkmann, M.², Araya, P.¹, Jablonska, J.², Roselli, E.¹, Nocera, D.A.¹, Nuñez, N.¹, Lienenklaus, S.², Kröger, A.², Weiss, S.²
¹CIBICI-CONICET, Departamento de Bioquímica Clínica. Facultad de Ciencias Químicas. Universidad Nacional de Córdoba, Córdoba, Argentina, ²Molecular Immunology, Helmholtz Centre for Infection Research, HZI, Braunschweig, Germany

The crucial role that endogenously produced IFN- β plays in eliciting an immune response against cancer has recently started to be elucidated. Endogenous IFN- β has an important role in immune surveillance and control of tumor development. Accordingly, the role of TLR agonists as cancer therapeutic agents are being revisited via the strategy of intra/peritumoral injection with the idea of stimulating the production of endogenous type I IFN inside the tumor. Polyadenylic-polyuridylic acid (poly A:U) is a dsRNA mimetic explored empirically in cancer immunotherapy long time ago with little knowledge regarding its mechanisms of action. In this work, we have in vivo visualized the IFN- β required for the anti-tumor immune response elicited in a therapeutic model of poly A:U administration. Here, we have identified the role of host type I IFNs, cell populations that are sources of IFN- β in the tumor microenvironment and other host requirements for tumor control in this model. One single peritumoral dose of poly A:U was sufficient to induce IFN- β , readily visualized in vivo. IFN- β production relied mainly on the activation of the transcription factor IRF3 and the molecule UNC93B1, indicating that TLR3 is required for recognizing poly A:U. CD11c+ cells were an important, but not the only source of IFN- β . Host type I IFN signaling was absolutely required for the reduced tumor growth, prolonged mice survival and the strong anti-tumor specific immune response elicited upon poly A:U administration. These findings add new perspectives to the use of IFN- β inducing compounds in tumor therapy.

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Serum interleukin 38 is associated with disease severity and organ involvement in systemic lupus erythematosus

Rudloff, L.^{1,2}, Godsell, J.³, Nold-Petry, C.A.^{1,2}, Harris, J.³, Hoi, A.³, Morand, E.F.³, Nold, M.F.^{1,2}
¹Hudson Institute of Medical Research, Ritchie Centre, Clayton, Australia, ²Monash University, Department of Paediatrics, Melbourne, Australia, ³Monash University, Centre for Inflammatory Diseases, Monash Medical Centre, Melbourne, Australia

Aims: Interleukin (IL)-38 is an IL-1 cytokine family member. Gene polymorphisms in IL-38 are associated with inflammatory diseases, and recombinant IL-38 inhibits IL-17 and IL-22. Intriguingly, both IL-17 and IL22 play a role in systemic lupus erythematosus (SLE), a severe autoimmune disease. We therefore set out to investigate IL-38 in SLE.

Methods: IL-38 and IL-10 were quantified in serum from SLE patients at admission (baseline, 142 patients) and two subsequent clinic visits (115/142). Serum of 28 healthy donors served as controls. Moreover, we silenced IL-38 in PBMC from

healthy volunteers by siRNA (siIL-38) and measured IL-6.

Results: IL-38 was detectable in 59 out of 345 patient samples (17.1%). IL-38 abundance in SLE samples was significantly higher compared to controls. Patients with active disease had 18.4-fold higher serum IL-38 than patients with non-active disease. Importantly, IL-38 was associated with increased risk of renal and CNS disease, and when IL-38 was detectable at baseline, patients had a 1.7-fold increased risk of developing persistently active disease. Remarkably, siIL-38-treated PBMC from healthy volunteers produced up to 30-fold more IL-6 than control-transfected cells when stimulated with CpG or imiquimod. Similarly, in SLE patients, the anti-inflammatory IL-10 was 5-fold higher when IL-38 was detectable, suggesting that IL-38 may be protective in SLE.

Conclusions: This study is the first to demonstrate the anti-inflammatory activity of endogenous IL-38. We reveal IL-38 as the first mediator that exhibits an association with markers of SLE disease activity, renal and CNS involvement; IL-38 may thus become the first biomarker for SLE.

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A new function for TRAF1 in regulation of TLR signaling underlies the role of TRAF1 in rheumatic disease

Abdul Sater, A.¹, Edilova, M.¹, Clouthier, D.¹, Mbanwi, A.¹, Kremmer, E.², Watts, T.¹
¹University of Toronto, Immunology, Toronto, Canada, ²Institut für Molekulare Immunologie, Deutsches Forschungszentrum für Gesundheit und Umwelt (GmbH), München, Germany

TNF receptor (TNFR) associated factor 1 (TRAF1) is a signaling adaptor known for its role in TNFR-induced cell survival. Here we show that monocytes from healthy donors with a rheumatoid arthritis-associated SNP in the TRAF1 gene express less TRAF1 protein but produce higher levels of inflammatory cytokines in response to LPS. TRAF1, via its MATH domain, binds to HOIP and HOIL1, components of the linear ubiquitination (LUBAC) complex, to interfere with the recruitment and linear ubiquitination of NF- κ B essential modifier (NEMO). This results in decreased NF- κ B activation and cytokine production, independently of TNF- α . Consistently, TRAF1^{-/-} mice show increased susceptibility to endotoxin-induced septic shock. These findings reveal an unexpected role for TRAF1 in regulation of TLR signaling, providing an explanation for increased inflammation associated with a disease associated TRAF1 SNP.

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Elevated pressure promotes EndoMT-induced fibrosis via a caveolin-1 dependent mechanism

Shihata, W.A.^{1,2}, Andrews, K.L.¹, Sampson, A.K.¹, Fang, L.¹, Murphy, A.J.¹, Kaye, D.M.¹, Chin-Dusting, J.P.F.¹
¹Baker IDI Heart and Diabetes Institute, Melbourne, Australia, ²Monash University, Melbourne, Australia

Introduction: A major risk-factor for cardiac fibrosis is high blood pressure, which we have previously shown to induce endothelial activation *via* a caveolin-1 (cav-1) dependent mechanism. Endothelial-to-mesenchymal transition (EndoMT)

is a key player in the development of cardiac fibrosis. Here, we examine the hypothesis that increased pressure promotes EndoMT-induced fibrosis via a cav-1 dependent mechanism.

Methods: Human umbilical vein endothelial cells (HUVECs) or cav-1 knockdown (cav-1 KD) cells were treated with TGF β 1 (10ng/mL) and TGF β 2 (10ng/mL), known inducers of EndoMT, or pressurised to 120 mmHg for up to 5 days. Wild-type and cav-1^{-/-} mice were used to confirm our findings *in vivo*.

Results: TGF β 1/2-treated HUVECs had increased expression of fibroblast genes, vimentin and alpha-smooth muscle actin (α SMA), and decrease of the endothelial gene, CD31. A similar pattern was observed when HUVECs were exposed to a hypertensive pressure (120mmHg), suggesting that exposure to increased pressure promotes EndoMT. This was also accompanied by an increase in both MMP2 and MMP9 activity, consistent with the premise that pressure induces the breakdown of collagen type IV, essential for the progression of EndoMT. Interestingly, cav-1 KD cells were protected from these pressure-induced changes. In angiotensin II (AngII) and noradrenaline (NA) mouse models of hypertension, we found that Cav-1^{-/-} mice compared to WT mice were protected from perivascular fibrosis and cardiac hypertrophy and failed to increase vimentin and α SMA gene expression.

Conclusions: Hypertension induces EndoMT via a cav-1 dependent mechanism and may be responsible for the end-organ fibrosis observed in the context of high blood pressure.

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Targeting IL-4 receptor alpha mediated signaling to treat fibroproliferative diseases: the case of chronic schistosomiasis

*Nono Komgwep, J., Ndlovu, H., Brombacher, F.
University of Cape Town/Institute of Infectious Disease and
Molecular Medicine, Cape Town, South Africa*

Fibroproliferative diseases represent a leading cause of mortality. Despite their pathophysiological differences, this group of diseases associates with activation of myofibroblast, deposition of collagen and fibrosis, a cascade of events that can be initiated and amplified by type 2 cytokines. Central to the production of type-2 cytokines is the host signaling via the IL-4 receptor alpha (IL-4Ra). A pathogenic role has therefore been suggested for the signaling via IL-4Ra, principally mediated by IL-13 and poorly IL-4 in fibroproliferative diseases like allergy and schistosomiasis. However, the therapeutic value of targeting IL-4Ra has only been successfully addressed in the former but not the latter disease. Utilizing a new and tractable murine model of inducible cessation of IL-4Ra mediated signaling, we now report that cessation of IL-4Ra mediated signaling abrogates already initiated type-2 immune responses in murine models of helminth infections uncovering an unprecedented role for IL-4Ra mediated signaling in the maintenance of type-2 immune responses. Importantly, in the advanced fibrotic model of chronic schistosomiasis, cessation of IL-4Ra mediated signaling considerably reduced the egg-surrounding collagen as well as the total collagen content in the liver without affecting the viability of the infected animals. Overall, our data indicate that targeting IL-4Ra could be a viable option for the mitigation of

type-2 immunopathologies and the treatment of associated morbidities like fibrosis. Since our findings could prove equally attractive for the management of other fibroproliferative diseases, further avenues of investigation on the wide anti-fibroproliferative potential of our approach will be discussed.

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Autocrine and paracrine IL-2 signals collaborate to regulate distinct phases of CD8 T cell memory

Yuzefpolskiy, Y.¹, Penny, L.A.², Baumann, F.M.², Smith, K.A.³, Sarkar, S.^{4,5}, Kalia, V.^{4,5}

¹Penn State University, Seattle, United States, ²Penn State University, University Park, United States, ³Weill Cornell Medical College, New York, United States, ⁴University of Washington School of Medicine, Seattle, United States, ⁵Seattle Children's Research Institute, Seattle, United States

Proliferation is a defining feature of clonal cytotoxic T lymphocyte (CTL) responses upon activation. However, how proliferative events during primary expansion relate to divergent CD8 T cell fate outcomes remains poorly defined. Emerging evidence from our laboratory and others in the field suggest that death-fated effectors undergo more pronounced proliferation compared to memory-fated cells during antigen-driven T cell expansion. Adoptive transfer of activated CD8 T cell-subsets, purified based on their extent of proliferation, showed that *in vivo* differentiation of CD8 T cells and memory fate are linked to proliferation. We found that the less-divided effector CTL subset preferentially contributed to the central memory lineage. Interestingly, these less-divided memory precursor cells were also programmed to produce more IL-2 compared to their more-divided terminal effector counterparts. How do memory-fated cells that make their own IL-2 (a pro-proliferative cytokine) proliferate less and how does this make them better fit for the long-lived memory lineage? To answer this question, we generated CD8 T cells that could be conditionally ablated for *il2*. We ablated IL-2 specifically in antigen-specific CD8 T cells, during distinct phases of effector and memory differentiation. Our data showed that while paracrine IL-2 is critical for driving the early *in vivo* proliferative burst, the programmed life-long autochthonous IL-2 in memory-fated cells was necessary for antigen-independent maintenance, and robust secondary expansion of memory cells. Thus, autocrine and paracrine IL-2 signals collaborate during distinct stages of T cell differentiation to direct diverse CTL lineages through effects on proliferation.

45 Minute Oral

12:30:00 - 13:15:00

Th Differentiation

Molecular mechanisms of human T helper cell differentiation

Lahesmaa, R.

University of Turku

Regulation of the immune response is central for human health. In healthy individuals there is an appropriate balance between the adaptive and innate arms of the immune response. T cells, in particular, orchestrate the adaptive arm of the immune system and are required for key immune functions. T helper (Th) cell subsets with distinct cytokine secretion profiles and function play a crucial role in host defense. Pathological imbalances in these lymphocyte subsets contribute to allergy, chronic inflammatory and autoimmune diseases as well as in cancer. Effector cell functions are suppressed by natural or induced T regulatory cells (Treg and iTreg, respectively). Understanding the gene regulatory pathways controlling T-cell differentiation into functionally distinct subsets is important to understand the pathogenesis of immune-mediated diseases and to develop better therapies. Our efforts have focused on the detailed studies of transcriptional and epigenetic regulation of the early stages of primary human Th cell differentiation. Genome wide systems level approaches followed by functional studies have revealed new factors and molecular mechanisms contributing to the regulation of Th cell lineage specification in human. Our recent results will be presented and discussed.

Dendritic Cells

Toward an integrative biology of T cells and dendritic cells

Malissen, M., Roncagalli, R., Henri, S., Dalod M., Luche, H.

Centre d'Immunologie de Marseille-Luminy

T cells probe the surface of dendritic cells (DCs) in search of cues reflecting the antigenic and inflammatory status of the body tissues. It remains a daunting task to understand how T cell activation is regulated through the summation of a multitude of positive and negative inputs and how their integration contributes to the unfolding of appropriate T cell responses. One of our major objectives is to understand how mutations that reduce TCR signaling output paradoxically lead to severe immune pathologies in both the mouse and human species. More specifically, we would like to elucidate the mechanisms through which during physiological, antigen-driven T cell responses some signaling "hub" used by the TCR leads first to activation of intracellular signaling pathways and then exerts with a temporal delay a feedback inhibition that leads to rapid attenuation of the whole TCR signaling pathway. In the absence of such negative feedback, T cell responses evolves into chronic pro-inflammatory T cell responses that perpetuate themselves

in a TCR-independent manner and induce the production of massive amounts of autoantibodies. After giving an overview of the major actors of T cell activation, we will present some recent genetic and proteomic approaches that we have developed to tackle the complexity of T cell activation under physiological conditions and at the systemic levels.

Innate Lymphoid Cells

Regulatory mechanisms for ILC2 functions in allergic inflammation

Koyasu, S.¹, Kabata, H.², Koga, S.¹, Moro, K.¹

¹RIKEN Center for Integrative Medical Sciences, ²Keio University School of Medicine

Innate lymphoid cells (ILC) are classified into three groups, ILC1, ILC2 and ILC3 based on their ability to produce distinct sets of cytokines. We have identified a previously unidentified lymphocyte population producing large amounts of type 2 cytokines, which we named Natural Helper (NH) cells. We identified NH cells in lymphoid clusters in adipose tissues, which we termed fat-associated lymphoid cluster (FALC). NH cells produce Th2 cytokines constitutively without any stimulation, and support the self-renewal of B1 cells and IgA production by B cells. Stimulation by IL-33 or helminth infection activates NH cells to produce large amounts of IL-5 and IL-13, which induce eosinophilia and goblet cell hyperplasia, both of which play an important role in anti-helminth immunity and pathophysiology of allergic diseases. NH cells are now considered to be a member of group 2 innate lymphoid cells (ILC2) that are tissue-resident lymphoid cells present in various tissues. We have shown that NH cells are involved in the steroid resistance of allergic airway inflammation. Steroid resistance is induced by thymic stromal lymphopoietin (TSLP) through Stat5-dependent signals. In addition, we have identified interferon- α/β , interferon- γ and IL-27 as negative regulators of NH cell functions in vivo. These cytokines suppress NH cell proliferation and functions through Stat1-dependent signals. Thus, NH cell functions are differentially controlled by distinct sets of Stat proteins. We will present our recent work on the regulatory mechanisms of NH cells in allergic inflammation.

13:30:00 - 15:10:00

Reproductive Immunology

428

Roles of iNKT and dendritic cells in myometrium for the induction of miscarriages by α -galactosylceramide

Negishi, Y.^{1,2}, Ichikawa, T.^{1,2}, Shimizu, M.¹, Takeshita, T.², Takahashi, H.¹

¹Nippon Medical School, Microbiology and Immunology, Tokyo, Japan, ²Nippon Medical School, Obstetrics and Gynecology, Tokyo, Japan

Innate immunity plays a crucial role in the control of pregnancy. When IL-12 (0.2 μ g/mouse) was administered to syngeneic-mated pregnant mice (C57BL/6 (♀ x ♂)) on day 7.5 of gestation (Gd 7.5), significant fetal loss was observed. Such IL-12 generally secreted by innate DEC-205+ dendritic cells (DCs) and invariant natural killer T (iNKT) cells expressing IL-12-receptor can be activated by the externally added IL-12. In addition, intraperitoneal (i.p.) administration of 2 μ g of α -galactosylceramide (α -GalCer), known to activate iNKT cells, on Gd 7.5 induced miscarriages in both syngeneic-mated pregnant C57BL/6 mice and allogeneic-mated pregnant mice (C57BL/6 (♀) x BALB/c (♂)). On the other hand, miscarriages were not induced in iNKT cells-deficient Ja18-/- mice by the IL-12 or α -GalCer treatment. Surprisingly, the percentage of both DEC-205+ DCs and CD1d-restricted NK1.1+ iNKT cells was far higher in the myometrium of pregnant mice treated i.p. with α -GalCer than it was in the decidua. The IL-12 secreted from α -GalCer-activated DEC-205+ DCs then stimulates the secretion of various cytokines including IL-2, IL-4, IFN- γ , TNF- α , perforin, and granzyme B from the NK1.1+ iNKT cells, leading to miscarriages in the pregnant mice. These findings may provide a new notion about the importance of myometrium rather than decidua in the regulation of pregnancy as well as the mechanism for miscarriages through activated innate DEC-205+ DCs and NK1.1+ iNKT cells in the myometrium of pregnant mice.

3972

Protection of mother and fetus against bacterial infection induced complications via prophylactic treatment during pregnancy with a microbial-derived immunomodulator

Scott, N.M.¹, Jones, A.C.¹, Lauzon-Joset, J.-F.¹, Mincham, K.T.¹, Troy, N.M.¹, Prescott, S.L.^{1,2}, Robertson, S.A.³, Bosco, A.¹, Holt, P.G.¹, Strickland, D.H.¹

¹Telethon Kids Institute, The University of Western Australia, Subiaco, Australia, ²The University of Western Australia, School of Pediatrics and Child Health, West Perth, Australia, ³Robinson Research Institute, University of Adelaide, Adelaide, Australia

Maternal bacterial infection can be linked to serious pregnancy complications, including increased mortality and morbidity for mother and child, fetal growth restriction, and preterm delivery. These outcomes are also associated with a detrimental impact on child growth and development during early life, and significantly increase risk of non-communicable disease during

adulthood.

Safe effective novel treatments to protect against infection and inflammation induced complications could provide exciting opportunities for improving pregnancy outcomes. Pro-biotics, and other agents classified generally as immunomodulators have shown great promise for prevention of inflammatory diseases. OM-85, a cocktail lysate of several heat-killed respiratory bacterial antigens is one such compound, which is currently in use for attenuation of infection-associated airway inflammatory symptoms in human infants.

Using a pregnant mouse model with LPS as an inflammatory bacterial challenge, we have demonstrated that OM-85 markedly reduces risk for fetal loss and growth restriction. Here, we have conducted RNASeq analysis of gestational tissues, which has revealed that OM-85 treatment attenuates the LPS-driven activation of TNF and IL1 driven pro-inflammatory gene networks in maternal gestational tissues, but preserves interferon-mediated networks.

These results suggest that OM-85 has potential as a novel, safe, effective treatment strategy to protect against bacterial infection-induced inflammatory complications during pregnancy.

3149

Vitamin-D and fetomaternal immunity: effects on uterine natural killer cells

Jeffery, L.¹, Tamblin, J.^{1,2}, Susarla, R.¹, Moss, P.³, Hewison, M.¹, Kilby, M.²

¹University of Birmingham Institute of Metabolism and Systems Research, College of Medical and Dental Sciences, Birmingham, United Kingdom, ²Birmingham Women's Foundation Hospital, Edgbaston, Birmingham, United Kingdom, ³University of Birmingham, Institute of Immunology and Immunotherapy, College of Medical and Dental Sciences, Birmingham, United Kingdom

Vitamin-D deficiency is prevalent in pregnant women. Active vitamin-D (1,25(OH)₂D₃) exerts important non-classical immune-regulatory effects and the maternal placenta (decidua) appears a potential target. CD56+ uterine natural killer cells (uNKs) are the most prominent decidual immune cell during early pregnancy. Given their critical role in fetal implantation and placentation, we hypothesised uNKs are a local source of 1,25(OH)₂D₃ and a pivotal immunomodulatory target for vitamin-D.

CD56+ NK cells were isolated from 1st trimester decidua (uNKs) and matched maternal peripheral blood (pNKs), and stimulated with IL-2, IL-15, and IL-12 with or without 1,25(OH)₂D₃ or 25(OH)D₃. At 24-hours, regulators of vitamin-D signalling and metabolism: 1- α -hydroxylase (CYP27B1), 24-hydroxylase (CYP24A1), vitamin-D-receptor (VDR), were measured by qPCR and IFN- γ , a marker of NK function, by qPCR, flow-cytometry and ELISA. 1,25(OH)₂D₃ was measured by liquid-chromatography-tandem-mass-spectrometry (LC-MS/MS).

Both uNKs and pNKs expressed an intracrine vitamin-D metabolic system, characterised by CYP27B1, CYP24A1 and VDR. Cytokine stimulation up-regulated vitamin-D-activating CYP27B1 and VDR in both subsets (p < 0.05), whilst vitamin-D-deactivating CYP24A1 remained unchanged. Detection of 1,25(OH)₂D₃ in 25(OH)D₃-treated stimulation cultures further confirmed the

functional induction of the system upon stimulation. Feedback regulation was also observed as 1,25(OH)₂D₃ increased CYP24A1 (~44-fold(uNKs), 85-fold(pNKs)).

In NK function studies, 1,25(OH)₂D₃ reduced IFN-γ expression by uNK cells only, suggesting differential vitamin-D sensitivity in the NK subsets. Overall, these data show that uNKs have a functional vitamin-D system, and may be more sensitive to 1,25(OH)₂D₃ than their peripheral counterparts. Vitamin-D metabolism by uNKs and their response to it is a possible mechanism through which vitamin-D affects pregnancy outcomes.

3338

Antigen-specific protective immunity in *Chlamydia trachomatis* genital infection is dependent on effector cell microRNAs

Arulanandam, B.P., Gupta, R., Arkatkar, T., Keck, J.P., Castillo, K., Yu, J.J., Chambers, J.P., Guentzel, M.N.
University of Texas at San Antonio, South Texas Center for Emerging Infectious Disease and Center of Excellence in Infection Genomics, San Antonio, United States

Anti-chlamydial immunity involves efficient presentation of chlamydial antigens (Ag) to effector cell populations resulting in Ag-specific immune responses. There is however, limited information on inherent underlying mechanisms that regulate these events. Previous reports from our laboratory have established that selected microRNAs (miRs) function as molecular regulators of immunity and contribute to disease pathogenesis following intravaginal (i.vag) *Chlamydia trachomatis* (Ct) infection. In the current study, we investigated immune cell type-specific miRs and the role in Ag-specific immune responses. We observed significant up-regulation of a cohort of miRs in Ct infected antigen presenting cells (APC) and Ag-specific CD4⁺ T-cells isolated from Cm infected mice. *Ex vivo* co-cultures of miR-agonist and antagonist treated APC and Ag-specific CD4⁺ T-cells resulted in significant regulation of Ct-specific IFN-γ production. *In vivo* miR depletion studies in Ct infected mice demonstrated a role for regulation of Ag-specific immune responses, IFN-γ production and disease pathogenesis. Additionally, these miRs were significantly regulated in intranasally vaccinated mice protected against a genital Ct infection and in cohorts of Ct-infected women with inflammation and reproductive pathology. Taken together, our data indicates a role for a cohort of miRs in immune effector cells, namely APC and CD4⁺ T cells in regulating Ag-specific protective immunity in Ct infected mice and the translational relevance in human cohorts. Since Ag-specific CD4⁺ T cells and IFN-γ are critical for protection against genital Ct, we are currently using 'omics-level' approaches to determine involved targets and pathways regulated by these miRs.

2312

Maternal asthma exacerbation before mating offers strong protection against offspring asthma

Dähling, S.¹, Blois, S.M.¹, Klopffleisch, R.², Conrad, M.L.¹

¹Charité Medical University, Berlin, Germany, ²Free University Berlin, Berlin, Germany

Background: Prenatal environmental exposures during critical developmental time points can influence allergy susceptibility later in life. Mouse models demonstrate that placental transfer of antibodies from sensitized mothers protects against offspring asthma. Moreover, maternal asthma exacerbation over the entirety of pregnancy results in protective effects against offspring asthma severity. It is not known, however, which gestational time points are important for offspring asthma protection.

Methods: To examine the importance of particular gestational time points with regard to offspring asthma susceptibility, mother mice were sensitized with PBS or casein then challenged at one of the following time points: before mating, gestation day (gd) 11.5-13.5 or gd 14.5-16.5. Experimental asthma was subsequently induced in four week old offspring using the antigen ovalbumin. Offspring asthma severity was assessed by bronchoalveolar lavage (BAL) differential cell counts and cytokines, lung inflammation and mucous production, and serum ovalbumin specific antibodies.

Results: The strongest protective effects against asthma were observed in offspring from mothers challenged before mating, followed by mothers challenged from gd 14.5-16.5. Allergic offspring from these mothers had significantly fewer BAL leukocytes; due to significantly fewer eosinophils and lymphocytes, and significantly less IL-4, IL-13, IL-12p40, IL-12p70, GM-CSF, KC and eotaxin than allergic offspring from control mothers. No difference was seen in OVA specific antibodies. No differences were observed in offspring lung inflammation or mucous production, likely due to the short length of the maternal challenge.

Conclusion: Significant protection against asthma was observed in offspring from allergic mothers challenged before mating or from day 14.5-16.5 of gestation.

3862

Repeated priming with male seminal fluid expands regulatory T cell populations to build pregnancy tolerance in mice

Robertson, S.A., Zhang, B., Wahid, H.H., Moldenhauer, L.M.

University of Adelaide, Robinson Research Institute, Adelaide, Australia

Female tolerance of the semi-allogeneic fetus in pregnancy requires regulatory T (Treg) cells and is maximised when populations of paternal alloantigen-reactive peripheral Treg cells are expanded by contact with seminal fluid from the conceiving male partner at conception. Here, we investigated whether repeated exposure to seminal fluid can sequentially expand the Treg cell pool. Female C57Bl/6 (B6) female mice were mated once or four times to B6 males, Balb/c or Balb/b males, followed by low dose RU486 administration to prevent pregnancy. Uterus

and para-aortic lymph nodes were recovered at day 3.5 post-coitum (pc) after the final mating. Using immunohistochemistry and flow cytometry for the Treg cell transcription factor Foxp3, we demonstrated the expansion in uterine Treg cells was 6-fold greater at day 3.5 pc after 4 prior exposures compared to a single exposure to Balb/c males, and similar increases were evident in lymph nodes. In contrast, no increase was induced by repeated mating with B6 or Balb/b males, indicating requirement for MHC alloantigens. Seminal fluid, as opposed to neuroendocrine or physical responses to mating were necessary, since mating to males surgically rendered deficient in sperm and/or seminal plasma failed to elicit a Treg cell increase. Collectively, these data provide evidence that repeated seminal fluid contact acts to expand the Treg cell pool during early pregnancy, and that sperm-associated or plasma constituents of seminal fluid elicit the Treg cell response. These data provide a mechanistic explanation for the link between duration of sexual cohabitation and protection from pregnancy complications in women.

1306

Effect of TGF- β treatment on Treg cells in adverse pregnancy caused by *Toxoplasma gondii* infection

Hu, X.¹, Zhan, S.¹, Zhang, H.¹, Li, Z.¹, Zhao, M.², Liu, X.¹, Zhan, R.², Liu, Z.²

¹Binzhou Medical University, Department of Immunology, Yantai, China, ²Binzhou Medical University, Department of Medicine & Pharmacy Research Center, Yantai, China

Aims: To examine the effect of TGF- β treatment on the differentiation and function of Treg cells and to improve adverse pregnancy caused by *T. gondii* infection.

Methods: *T. gondii*-infected pregnant mouse was treated with TGF- β or TGF- β -neutralizing antibodies, sacrificed on gestational day (gd) 7 and pregnancy outcomes were observed. Treg cell number, and the expression of pSmad3, CTLA-4 and PD-1 were analyzed by flow cytometry. Histopathological changes of placentas were assessed using HE staining. IL-10 and TNF- α levels in placenta were measured using ELISA.

Results: TGF- β treatment improved *T. gondii*-induced adverse pregnancy outcomes, while TGF- β neutralization demonstrated more serious adverse pregnancy outcomes compared with infected controls. TGF- β treatment increased Treg cell number, while TGF- β neutralization decreased Treg cell number. Pathological histology revealed that TGF- β treatment alleviated hemorrhage and partly restored the uterine spiral arteries of the placenta, while TGF- β neutralization led to serious hemorrhage and more dilated uterine spiral arteries of placenta compared with infected controls. pSmad3 expression in CD4⁺ cells and CTLA-4 and PD-1 levels on Treg cells were up-regulated by TGF- β treatment but down-regulated by TGF- β neutralization. IL-10 levels increased, TNF- α levels decreased, and ratio of IL-10/TNF- α increased after TGF- β treatment, while IL-10 levels decreased, TNF- α levels increased, and ratio of IL-10/TNF- α decreased after TGF- β neutralization.

Conclusion: Our data indicated that TGF- β treatment could up-regulate the differentiation and function of Treg cells through TGF- β /Smad3 signaling pathway and could improve adverse pregnancy outcomes caused by *T. gondii* infection.

Keywords: TGF- β , pSmad3, CTLA-4, PD-1, CD4⁺Foxp3⁺Treg cells, *Toxoplasma gondii*, pregnancy outcome

3540

Circulating blood macrophages transmit *Chlamydia* infection to the testis

Bryan, E.R.¹, Kollipara, A.¹, Armitage, C.W.¹, Redgrove, K.A.², McLaughlin, E.A.², Carey, A.J.¹, Beagley, K.W.¹

¹Queensland University of Technology, Institute of Health and Biomedical Innovation, Brisbane, Australia, ²University of Newcastle, Faculty of Science and IT, Newcastle, Australia

Introduction: *Chlamydia* research has focused almost entirely on females and this has resulted in male disease being underestimated and understudied. The purpose of this study was to determine the mechanism of *Chlamydia* transmission from the lower to the upper male reproductive tract and the impact of *Chlamydia* colonisation of the upper tract leading to deregulation of spermatogenesis and infertility.

Methods/results: We infected 6 week old, male, BALB/c mice that had either undergone vasectomy or not. We found that two weeks post-infection, *Chlamydia* was detected by PCR in the testes and whole blood of all mice. There were histological changes to testes and epididymis, with pathological tubular destruction occurring in infected mice. To determine if macrophages served as a source of testicular infection we showed that isolated primary macrophages were susceptible to infection for up to 4 hours and produced viable progeny. Co-culture of *Chlamydia*-infected RAW264.7 macrophages with TM3 Leydig, TM4 Sertoli and GC-1 Germ cells showed that progeny from macrophages were able to infect each of these cell types, which all contained fragmented DNA resultant from infection.

Conclusion: The presence of *Chlamydia* in the testes of vasectomised mice indicates an alternate route of ascending infection to the classical urethral-epididymal-testicular route. Further investigation is required to establish if infected macrophages home to the testes or travel to many distant sites. Regardless, this is a probable route of transmission of *Chlamydia* to the testes. The consequential testicular infection compromises male infertility through damage to spermatogenesis support cells and tubule structure.

3616

Uterine regulatory T cells prepare a tolerogenic environment for embryo implantation and imprint maternal-fetal tolerance during early gestation

Florez, L.M.¹, Ruocco, M.G.¹, Courau, T.¹, Fourcade, G.¹, Nehar-Belaid, D.¹, Klatzmann, D.^{1,2}

¹Sorbonne Universités, UPMC Univ Paris 06, INSERM, Immunology-Immunopathology-Immunotherapy, Paris, France, ²AP-HP, Hôpital Pitié-Salpêtrière, Biotherapy (CIC-BTi) and Inflammation-Immunopathology-Biotherapy Department (i2B), Paris, France

Regulatory T cells (Tregs) are a key component to successful gestation. Indeed, depletion of Tregs leads to rejection of allogeneic fetuses in mice and correlative human studies

revealed low Treg numbers associated with recurrent spontaneous abortion in women. However, the role of Tregs at the maternal-fetal interface remains poorly understood.

In this work, we combine flow cytometry, transcriptomics and confocal as well as intravital two-photon imaging to study the phenotype and function of uterine Tregs (uTregs).

We found that in the non-pregnant uterus uTregs localize in close proximity to the uterine glands and increase in proportion during the estrus cycle preparing the uterus for fecundation. Moreover, we show a spatio-temporal regulation of uTregs during pregnancy that re-localize in the pregnant uterus and interact with cells of the innate and adaptive immune system. Indeed, the proportion of uTregs significantly increases as early as 2 days after embryo implantation. Our data indicate that effector CD4 and CD8 cells also increase in numbers in the pregnant uterus towards mid-gestation. However, uTregs present a strikingly activated phenotype suggesting that they are able to mediate local immune suppression in order to protect the fetus of the effector T cells. Furthermore, contrasting with the well-described pro-inflammatory role of mast cells, our results reveal mast cells as important mediators in the Treg-dependent establishment of maternal-fetal tolerance.

Together, these results outline another adaptive/innate cells cross-talk selected during evolution of the mammalian adaptive immune system to protect the developing semi-allogenic fetus.

4101

Characterization and functionality of MAIT cells at the fetomaternal interface

Solders, M.^{1,2}, Gorchs, L.¹, Erkers, T.¹, Nava, S.¹, Ringdén, O.¹, Magalhaes, I.³, Kaipe, H.^{1,4}

¹Karolinska Institutet, Laboratory Medicine, Stockholm, Sweden,

²Karolinska University Hospital, Center for Allogeneic Stem Cell Transplantation, Stockholm, Sweden,

³Karolinska Institutet,

⁴Karolinska University Hospital, Clinical Immunology and Transfusion Medicine, Stockholm, Sweden

During pregnancy, the maternal immune system must tolerate the allogeneic fetus while maintaining immunity against pathogens. We investigated the presence, distribution, phenotype and functionality of the innate-like mucosal-associated invariant T (MAIT) cells at the fetomaternal interface. After uncomplicated term deliveries through caesarian sections, paired samples of lymphocytes were isolated from peripheral blood (PB), intervillous blood (IVB), decidua parietalis (DP), and umbilical cord blood (UCB) (n=8).

The proportion of MAIT cells within the CD3+ T-cell population was significantly higher in IVB (median 5.4%, range 2.7-19.2) compared to PB (1.7%, 0.7-7.7), DP (0.7%, 0.5-7.8), and UCB (0.1%, 0.1-10.4). The expression of Ki-67 in MAIT cells was low in IVB, suggesting that MAIT cells are recruited rather than locally proliferating. MAIT cells with an effector memory phenotype (CD45RA⁺CCR7⁻) were enriched in IVB compared to PB, DP, and UCB. MAIT cells from DP expressed significantly higher levels of both activation and exhaustion markers (CD69, CD25, HLA-DR, PD-1) compared to both PB and IVB. Upon *E. coli* stimulation, MAIT cells from IVB showed a significantly higher expression

of interferon- γ and granzyme B compared to PB, DP, and UCB. Unstimulated MAIT cells in IVB had a higher endogenous expression of perforin compared to PB.

To conclude, we show that MAIT cells are present in the placenta and in cord blood, and that they are enriched in placental blood. The increased frequency of highly reactive MAIT cells in the IVB may be a physiological way to maintain bacterial immunity in the immunosuppressed environment at the fetomaternal interface.

Computational Immunology & Systems Biology

3895

The earliest lineage priming regulators leading to the dendritic cell subtypes

Schreuder, J.¹, Kocovski, N.¹, Tran, J.¹, Tian, L.¹, Ritchie, M.¹, Naik, S.^{2,3}

¹The Walter and Eliza Hall Institute of Medical Research, Molecular Medicine, Melbourne, Australia, ²The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia, ³The University of Melbourne, Medical Biology, Melbourne, Australia

The different dendritic cell subtypes are pivotal players in the recognition of pathogens and activation of the adaptive immune response and include plasmacytoid DCs (pDCs) and the two conventional DCs (cDC); cDC1 and cDC2. They are derived from hematopoietic progenitors. However, prior evidence has demonstrated that, at the single cell level, these progenitors are heterogeneous in their fate and biased towards certain DC subtypes. These findings raise the question as to whether 'lineage bias' occurs either stochastically, or through environmental mechanisms, or alternatively through undefined intrinsic molecular programs. For the latter hypothesis, one cannot both assess the transcriptome of a single progenitor and test its fate - as one test necessarily destroys the chance to test the other. To overcome this, we performed clone-splitting experiments where the daughters of a single progenitor early during expansion stage were separated in three: some daughters for testing fate in duplicate, and others for RNA-seq. By computationally comparing the transcriptome to fate, per clone, for dozens of clones, we made several findings:

- 1) DC fate bias is pre-existing and intrinsic to most proposed 'multipotent' progenitors,
- 2) fate bias was the norm, not the exception, and
- 3) we identified novel molecular programs by linear regression models that correlated gene expression with fate bias.

These included known factors, as well as novel transcription factors and epigenetic regulators. These findings have important implications as to how stem and progenitor cells generate fate diversity, and at which point (and through which mechanisms) in the developmental pathway these arise.

267

Characterisation of the T cell receptor repertoire following immunisation*Chain, B.¹, Best, K.², Cinelli, M.¹, Friedman, N.³, Mark, M.³, Reich-Zeliger, S.³, Sun, Y.⁴, Shave-Taylor, J.⁴, Shifrut, E.³*¹*UCL, Infection and Immunity, London, United Kingdom, ²UCL, CoMPLEX, London, United Kingdom, ³Weizmann Institute, Immunology, Rehovot, Israel, ⁴UCL, Computer Sciences, London, United Kingdom*

The T cell repertoire consists of an ensemble of alpha and beta T cell receptor (TCR) sequences which characterise a blood or tissue sample at a particular time. We understand in detail the molecular interaction between an individual T cell receptor and its cognate peptide/MHC antigen target, but the impact of antigen exposure on the repertoire as a whole is less well understood. The problem is compounded because the stochastic nature of TCR generation results in different repertoires between individuals. We approach the problem from the perspective of classification of repertoires after immunisation with different antigens in a space indexed by all possible TCRs (we focus on CDR3 (complementarity determining region 3) sequences, which play a major role in antigen recognition). We reduce dimensionality by decomposing each CDR3 into sets of short amino acid motifs. From a biophysical perspective, these motifs may capture conserved contact points between TCR and MHC/peptide complex. The frequency of each motif in a sample of TCRs after immunisation with a given antigen defines the repertoire in a new feature space. Finally, we use support vector machines and other “weak learner” algorithms to identify sets of features which correctly classify repertoires from different immunisations. The results suggest that immunisation induces widespread changes in the TCR repertoire which are distributed over a large number of individual TCRs, and are mainly private and differ between individuals immunised with the same antigen.

1155

Analysis of antigen-specific responses by high throughput sequencing of the T cell receptor repertoire*Oakes, T.¹, Popple, A.L.², Heather, J.¹, Singleton, H.², Best, K.¹, Williams, J.³, Dearman, R.J.², Kimber, I.², Chain, B.¹*¹*University College London, London, United Kingdom, ²University of Manchester, Manchester, United Kingdom, ³Salford Royal NHS Foundation Trust, Salford, United Kingdom*

Probing the T cell receptor (TCR) repertoire can provide a snapshot of a person's immune status, providing information on the clonality of T cells, and may therefore be a useful biomarker to monitor immune responses.

We have developed a reliable and economical amplification protocol that can be used to characterise the TCR repertoire using high-throughput sequencing and pipeline for the downstream analysis. Introduction of barcodes, which label every cDNA molecule before amplification allow for correction of PCR bias, and PCR and sequencing error. The protocol can be used to sequence TCR repertoires of diverse types of samples; including whole or FACS fractionated blood or tissue and *in vitro* cultured cells.

We were able to identify putative antigen-specific T cells in patients with allergic contact dermatitis after comparing the *ex vivo* TCR repertoire to allergen-stimulated *in vitro* samples (12/NW/0602). Longitudinal blood samples from alopecia patients treated with DCP to stimulate hair growth, before and after sensitisation, were also analysed to identify DCP-reactive T cells (14/EE/1067). The frequencies of allergen-specific clones will be used to parametrise mathematical models of skin sensitisation to improve our ability to predict safe levels of human exposure to chemical allergens.

We have been able to show that our protocol and analysis pipeline to study the TCR repertoire is robust and that we are able to identify putative antigen-specific T cells from different sets of samples. Crucially, we show the importance of single molecule barcodes to allow quantitative as well as qualitative information on the TCR repertoire.

1682

Persisting fetal clonotypes influence the structure and overlap of adult human TCR repertoires*Pogorelyy, M.¹, Elhanati, Y.², Marcou, Q.², Sycheva, A.¹, Komech, E.¹, Britanova, O.^{3,4,5}, Chudakov, D.^{3,4,5}, Mamedov, I.¹, Lebedev, Y.¹, Mora, T.⁶, Walczak, A.²*¹*Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Laboratory of Comparative and Functional Genomics, Moscow, Russian Federation, ²Ecole Normale Supérieure, Laboratoire de Physique Théorique, Paris, France, ³Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Laboratory of Genomics of Adaptive Immunity, Moscow, Russian Federation, ⁴Pirogov Russian National Research Medical University, Moscow, Russian Federation, ⁵Masaryk University, Central European Institute of Technology, Brno, Czech Republic, ⁶Ecole Normale Supérieure, Laboratoire de Physique Statistique, Paris, France*

To ensure protection against the large diversity of pathogens, the adaptive immune system maintains a large set of diverse T-cell receptors. This diversity is generated through the stochastic process of V(D)J-recombination, which makes the independent production of the same sequence twice relatively rare. Yet, unrelated individuals do share a substantial fraction of their receptors. These common receptors, which make up the “public” repertoire, and usually correspond to large clonotypes. Although convergent recombination has been proposed to explain these public clones, the origin of their large sizes is debated. The formation of the TCR repertoire starts in prenatal development, during which the enzyme inserting random nucleotides is initially absent, producing a subset of T-cells with limited diversity. Here, by analysing deep sequencing T-cell repertoire data from monozygotic twins, who share blood cells during pregnancy, and comparing to unrelated individuals and to data-driven models of stochastic recombination, we show that T-cell clones generated before birth can survive and maintain high abundances in adult organism for decades. By profiling the T-cell repertoire in the umbilical cord and in peripheral blood of individuals of various ages, combining deep sequencing with advanced statistical methods, we show the number of clones with no inserted nucleotides is high at birth, and decays slowly with age. Taken together, our results suggest that large,

low diversity public clones are created during pregnancy, and survive over long periods, providing the basis of a robust public repertoire.

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2553

Regulatory T cells suppress effector T cell proliferation by limiting division destiny

Dowling, M.R., Hawkins, E.D., Marchingo, J.M., Heinzl, S., Hodgkin, P.D. The Walter and Eliza Hall Institute of Medical Research, Immunology, Parkville, Australia

CD4+CD25+FOXP3+ regulatory T cells (Tregs) play a critical role in immune homeostasis, however the precise mechanisms by which they exert their regulatory function remains contentious. Many different mechanisms of Treg suppression have been demonstrated including stimulatory cytokine deprivation, inhibitory cytokine production, direct cell-contact dependent mechanisms, and limiting access to antigen-presenting cells. The relative importance of different mechanisms remains unclear. Recent work has established the Cyton model as a quantitative framework for understanding the kinetics of effector T cell proliferation, revealing a role for independent regulation of division times, death times, and cessation of division ('division destiny'). Furthermore, using this quantitative approach, independent signals through the T cell receptor, costimulatory signals and cytokines have been shown to sum independently to determine the size of the T cell response (Marchingo et al Science 2014).

We used this mathematical modeling approach to study the effect of Tregs on the kinetics of effector CD4+ T cell (Teff) proliferation. No effect on division times or the rate of death was observed, instead we demonstrate that T regs exhibit their inhibitory function by reducing division destiny. We then compared this quantitative kinetic effect of Tregs to the effect of various interventions aimed at mimicking proposed mechanisms of Treg action. We found that the effect of Tregs on effector T cells was most closely reproduced by blocking interleukin-2, supporting a mechanism where Tregs reduce cytokine availability to Teffs. Thus, mathematical modeling can provide novel mechanistic insights into lymphocyte signal integration in the presence of multiple competing pathways.

775

Computer model analysis of the difference between F759 and wild type mice in rheumatoid-like arthritis emergence

Yamada, S.¹, Yoshimura, A.², Atsumi, T.³, Murakami, M.³

¹Okayama University of Science, Department of Intelligent Mechanical Engineering, Okayama, Japan, ²Keio University School of Medicine, Tokyo, Japan, ³Hokkaido University, Sapporo, Japan

Rheumatoid arthritis (RA) is a common autoimmune disease characterized by chronic inflammation in joints. In order to analyze the RA emergence, we developed its computer model by using F759 mice. F759 mouse is an IL-6-mediated RA model dependent on excess activation of NFκB in synovial fibroblasts. These mice have an amino acid substitution, Y759F, in gp130.

Because phosphorylated Y759 is the binding site of SOCS3, which creates a negative feedback loop in the IL-6 signaling pathway, IL-6-mediated STAT3 activation is enhanced in F759 mice and is followed by the spontaneous development of a RA-like disease with age.

The model contains the activation of NFκB and JAK/STAT signaling pathway in the synovial cells in joints and the cytokine production by the infiltrated Th-17 and macrophages. The reactions to mimic the RA-like disease in the model are described in the differential equations, and are solved mathematically by using Runge-Kutta-Gill method.

F759 joint synovial cells show an enhanced activation of NFκB in the presence of IL-17 and IL-6 followed by excess expressions of chemokines, IL-6 and epregrin in the joints, which are considered to be essential to emerge the RA-like disease. The effects of several types of antibodies and inhibitors were reproduced by this computer model. The sensitivity analysis showed that the IL-6-STAT3 signaling was more important than the NFκB signaling. We will investigate the RA emergence by comparing simulation results with experimental results. This work is supported by JSPS KAKENHI Grant Number 25330356.

4178

Utilization of SWIFT cluster templates to register (normalize) fluorescence intensity in flow cytometry data at two levels of resolution

Mosmann, T.¹, Rebhahn, J.¹, Quataert, S.¹, Sharma, G.²

¹University of Rochester Medical Center, Center for Vaccine Biology and Immunology, Rochester, United States, ²University of Rochester, Electrical and Computer Engineering, Rochester, United States

Variation in flow cytometry data can result from technical issues (drift in cytometer settings or staining protocols), or from true biological diversity. Technical variation often obscures subtle biological differences in datasets. Manual methods for minimizing variation are subjective and time-consuming. Automated methods for selectively reducing different contributions to variation would improve subsequent manual or automated analysis. Semi-automated approaches (relying on histogram peaks or manual gates) have helped, and we now propose two fully-automated, objective registration (fluorescence normalization) methods based on SWIFT cluster templates (data-derived Gaussian model descriptions of the multidimensional data).

'Per-channel' registration compares cluster locations between the target and the sample to be registered, reconciling 'global' differences between samples while maintaining local fluorescence differences. This objectively uses information from all clusters and can correct global artifacts introduced by variations in staining or cytometer settings that cause 'batch' differences between assay days.

'Per-cluster' registration iteratively aligns all clusters independently with the corresponding target template clusters, moving cells in overlap regions according to their relative probability of belonging to each cluster. This registers cells more completely, and can remove individual e.g. genetic variation in cluster positions. Per-cluster registration improves the accuracy

of measuring the NUMBER OF CELLS/CLUSTER across many samples, but may remove real biological FLUORESCENCE INTENSITY differences between samples. Thus the optimal registration method depends on the experimental design. Our two methods can be blended with a specified weight, to remove only batch variations, or additionally remove individual intensity differences if cells/cluster are the most important experimental outcome.

3877

Distinctive differences in lymphocyte receptor repertoire and in cell frequencies results in an individual immune system

Rubelt, E.¹, Bolen, C.R.¹, Vander Heiden, J.², Marie McGuire, H.³, Gadala-Maria, D.², Euskirchen, G.¹, Mamedov, M.¹, Swan, G.E.⁴, Dekker, C.L.⁵, Levin, M.⁶, Cowell, L.G.⁶, Pulendran, B.⁷, Kleinstein, S.H.², Davis, M.M.¹

¹Stanford University School of Medicine, Stanford, United States,

²Yale University, New Haven, United States, ³University of Sydney, Sydney, Australia, ⁴Stanford University School of Medicine, Stanford Prevention Research Center, Stanford, United States,

⁵Stanford University School of Medicine, Department of Pediatrics (Infectious Diseases), Stanford, United States, ⁶University of Texas Southwestern Medical Center, Department of Clinical Sciences, Dallas, United States, ⁷Emory University, Atlanta, United States

The adaptive immune system's ability to protect the body requires a coordinated response from different components of the immune system. B and T cells with their highly diverse antigen receptor repertoire play a crucial role in the adaptive immune response.

To investigate the immune response, it is essential to first understand how individuals initially differ in their immune system. However, the influence of individual genetic and epigenetic differences on these repertoires is not typically measured. By leveraging the unique characteristics of B, CD4⁺ T, and CD8⁺ T lymphocyte subsets from monozygotic twins, we quantify the impact of heritable factors on both the V(D)J recombination process and on thymic selection. We show that the resulting biases in both V(D)J usage and N/P addition lengths, which are found in naïve and antigen experienced cells, contribute to significant variation in the CDR3 region. Moreover, we show that the relative usage of V and J gene segments is chromosomally biased, with approximately 1.5 times as many rearrangements originating from a single chromosome. These data refine our understanding of the heritable mechanisms affecting the repertoire. We continued the investigation into individual flu vaccine responses as an investigation of how repertoire selection are modified in the context of an ongoing immune response. Combined with a broad immunophenotyping CyToF panel, we can comprehensively show how individuals differ before and after vaccine challenging as well as uncovered remarkable similarities in specific aspects of the immune responses.

The data generated by these studies will be stored in *ImmPort*.

3022

Large-scale network analysis reveals the architecture of antibody repertoires

Miho, E., Greiff, V., Menzel, U., Reddy, S.T.

ETH Zurich, Biosystems Science and Engineering, Basel, Switzerland

Humoral immunity is achieved by a vast ensemble of distinct antibody clones; however, the comprehensive structure of an individual's immune repertoire has remained elusive. Specifically, it is unknown how antibody repertoire structure evolves throughout B-cell development. Network analysis has been used in the last decade to characterize relations in complex systems: we have leveraged the power of network analysis to express the similarity relation between each clone to all other clones in immune repertoire networks. In order to capture expansively the structure of entire antibody repertoire networks, we established a high-performance computing platform. It enables the construction of antibody repertoire networks up to the scale of naïve murine B-cell populations ($\approx 10^6$ estimated clones). We generated and analyzed ≈ 600 million clonal sequences from various stages of the B-cell lineage, pre-B cells, naïve B-cells and memory plasma cells, of unimmunized and immunized cohorts. Our results indicate that throughout B-cell development, the similarity relation among clones is power-law distributed: antibody repertoires are dominated by relatively few clonal hubs which concentrate large numbers of clonal variants. Interestingly, these hubs incorporate clones of higher frequency suggesting their targeted selection. Our large-scale network study provides unprecedented quantitative insight into the processes of antibody clonal selection, expansion and evolution across B-cell ontogeny.

Immunity to Viruses 1

3986

Categorical analysis of the complex associations of T cell antigen specificity and T cell exhaustion in chronic HBV infection

Cheng, Y.¹, Leong, M.-L.¹, Chen, J.¹, De Sessions, P.², Lloyd Hibberd, M.², Poidinger, M.¹, Bertoletti, A.³, Lim, S.G.⁴, Newell, E.¹

¹Singapore Immunology Network (SIgN), A*STAR, Singapore, Singapore, ²Genome Institute of Singapore, A*STAR, Singapore, Singapore, ³Duke-NUS Graduate Medical School, Singapore, Singapore, ⁴National University Health System, Singapore, Singapore, Singapore

Chronic hepatitis B virus (CHB) infection is associated with T cell dysfunction and persistent yet highly variable viral burden. Here, coupling mass cytometry and combinatorial tetramer strategy, we probed 563 different HLA-A*1101-restricted T-cell specificities spanning the entire HBV genome on CD8⁺ T cells from patients across four clinical stages. Antigen-specific T cells were simultaneously profiled using 26 differentiation and coinhibitory markers (PD-1, TIM-3, LAG-3, CTLA-4, TIGIT, 2B4, CD160, BTLA, and HVEM). Using high-dimensional analysis (t-SNE) and a novel analysis platform (One-SENSE) that we recently developed, our data shows that the exhaustion profiles of antigen-specific T cells are highly complex and associated

with T-cell differentiation and function in CHB infection. The remarkable heterogeneity of CD8⁺ T cells suggested the diverse usages of coinhibitory markers in different memory-effector subsets are influenced by the development of CHB. T-cells specific for numerous novel HBV epitopes also displayed variable profiles of coinhibitory markers during the progression of natural persistent infection. HBV_{core178}-specific CD8⁺ T cells correlated with improved clinical outcome by their upregulated frequency and distinct memory phenotype, with high PD-1 and 2B4. HBV_{pol106}-specific T cells had higher 2B4 and TIGIT, but lower BTLA. In addition, the heterogeneous exhaustion profiles of HBV_{pol106}-specific T cells linked to the progression of disease. These results together indicate that a highly complex exhaustion profile of HBV-specific T cells exists during a natural chronic infection and challenges the current model of hierarchical T cell exhaustion. The frequencies and differentiation profiles of HBV-specific CD8⁺ T cells may be useful for predictive value.

3406

Antigen-driven specialization and differentiation of T cells in chronic infections

Utzschneider, D.T.^{1,2}, Alfei, F.², Pinschewer, D.D.³, Zehn, D.²

¹University of California San Diego, Cellular & Molecular Medicine, La Jolla, United States, ²Centre Hospitalier Universitaire Vaudoise (CHUV), University of Lausanne, Lausanne, Switzerland, ³University of Basel, Division of Experimental Virology, Department of Biomedicine, Basel, Switzerland

Chronic infections induce T cells showing impaired cytokine secretion and up-regulated inhibitory receptor expression such as PD-1. What determines the acquisition of this chronic phenotype and how it impacts T cell function remained vaguely understood. Using newly generated recombinant antigen variant-expressing chronic LCMV strains, we uncovered that T cell differentiation and the acquisition of a chronic "exhausted" phenotype depends critically on the frequency of TCR engagement and less significantly on the strength of TCR stimulation. In fact, we noted that low level antigen exposure promotes the formation of T cells with an acute phenotype in chronic infections. Unexpectedly, we found that T cell populations with an acute or chronic phenotype are maintained equally well in chronic infections and undergo comparable primary and secondary expansion. Thus, our observations contrast the view that T cells with a typical chronic infection phenotype are severely functionally impaired and rapidly transition into a terminal stage of differentiation. Instead, our data unravel that T cells primarily undergo a form of phenotypic and functional differentiation in the early phase of a chronic LCMV infection without inheriting a net survival or expansion deficit and we demonstrate that the acquired chronic phenotype transitions into the memory T cell compartment.

1912

Spatiotemporally distinct interactions with DC subsets facilitates CD4 and CD8 T cell activation to localized viral infection

Hor, J.L.^{1,2}, Whitney, P.G.¹, Zaid, A.^{1,2}, Brooks, A.G.¹, Heath, W.R.^{1,2}, Mueller, S.N.^{1,2}

¹University of Melbourne, Department of Microbiology & Immunology, Melbourne, Australia, ²The ARC Centre of Excellence in Advanced Molecular Imaging, University of Melbourne, Melbourne, Australia

The dynamics of when and where CD4 T cells provide help for CD8 T cell priming, and which dendritic cells (DCs) activate CD4 T cells *in vivo* following localized infection are poorly understood. Using a cutaneous herpes simplex virus infection model combined with intravital 2-photon imaging of the draining lymph node (LN) to concurrently visualize pathogen-specific CD4 and CD8 T cells, we found that early priming of CD4 T cells involved clustering with migratory skin DC. CD8 T cells did not interact with migratory DC and their activation was delayed compared to CD4 T cells, instead requiring later interactions with LN-resident XCR1⁺ DC. CD4 T cells interacted with these late CD8 T cell clusters on resident DC. Together, these data reveal asynchronous T cell activation by distinct DC subsets and highlight the key role of XCR1⁺ DC as the central platform for CTL activation and the delivery of CD4 T cell help.

3186

IL-15 and TGF- β promote the selective retention of virus-specific resident memory T cells in human lymphoid organs

Woon, H.¹, Braun, A.², Li, J.², Smith, C.³, Edwards, J.¹, Sierro, F.¹, Feng, C.¹, Khanna, R.³, Elliot, M.⁴, Tangye, S.⁵, Rickinson, A.⁶, Gebhardt, T.², Britton, W.¹, Palendira, M.⁷

¹Centenary Institute, Camperdown, Australia, ²Peter Doherty Institute for Infection and Immunity at The University of Melbourne, Melbourne, Australia, ³QIMR Berghofer Medical Research Institute, Brisbane, Australia, ⁴Royal Prince Alfred Hospital, Camperdown, Australia, ⁵Garvan Institute of Medical Research, Darlinghurst, Australia, ⁶University of Birmingham, Edgbaston, United Kingdom, ⁷Centenary Institute, Newtown, Australia

Tissue-resident memory T (Trm) cells are required for protection at sites of reinfection in mice. The characteristics of these cells and the mechanisms that control their retention in human tissues are poorly understood. Here we describe two distinct subsets of memory CD8⁺ T cells, identifiable as CD69⁺CD103⁺ and CD69⁺CD103⁻, that are retained within human lymphoid tissues in the absence of recent T cell stimulation. These two subsets are not only distinct in their phenotype and transcriptional profile, but also anatomically separated within the tissue environments, with the majority of CD69⁺CD103⁺ cell population positioned along the epithelial barrier. Our study also reveals for the first time that IL-15 and TGF- β co-operate to extinguish expression of sphingosine-1-phosphate receptor, required for T cell exit from tissues, and its transcriptional activator, Kruppel-like factor 2 (KLF2). Therefore these locally produced cytokines can potentiate the strategic positioning of Trm cells at barrier surfaces. Interestingly, Epstein-Barr virus-

specific (but not cytomegalovirus-specific) CD8⁺ memory T cells preferentially accumulate in the tonsils and acquire a CD69⁺CD103⁺ phenotype, ensuring their retention at the epithelial sites where Epstein-Barr virus is likely to replicate. This strategic positioning of pathogen-specific Trm cells could be critical to prevent reactivation of persisting viral infections in humans.

1231

Migration and transcriptional regulation of T_{FH} cell differentiation

Watson, L.-R.¹, Binek, A.¹, Wang, Z.², Mackay, C.³, Moon, J.⁴, Kedzierska, K.², Good-Jacobson, K.⁵, Belz, G.⁶, Groom, J.¹

¹The Walter and Eliza Hall Institute of Medical Research, Department of Immunology and Molecular Immunology, Parkville, Australia, ²University of Melbourne, Department of Microbiology & Immunology, Melbourne, Australia, ³Pfizer Inc, Inflammation & Immunology, Cambridge, United States, ⁴Massachusetts General Hospital and Harvard Medical School, Center for Immunology and Inflammatory Diseases, Charlestown, United States, ⁵Monash University, Department of Microbiology, Clayton, Australia, ⁶The Walter and Eliza Hall Institute of Medical Research, Department of Molecular Immunology, Parkville, Australia

The differentiation of CD4⁺ T cells into specialised helper subsets, such as T_{H1}, T_{H2}, T_{H17}, T_{FH} and T_{REG}, is essential to orchestrate flexible immune responses. Functional differentiation of CD4⁺ T cells into

T helper subsets is regulated by environmental factors, and instructed by master transcription factors. Recent studies have highlighted the plasticity that occurs between these effector subsets to fine-tune responses. In addition, within these lineages, some further specialisation can occur, such as the context-specific tailoring of T_{REG} cell function. Similarly, we hypothesize that T_{FH} differentiation varies depending on the infectious onslaught, in order to optimize B cell antibody production and isotype switching, and establish protection from vaccine and infection. We have developed a novel influenza model to track antigen-specific CD4⁺ T cell responses by imaging and detection of I-A^b natural repertoire cells. Our data reveal a CXCR3-dependent differentiation pathway for T_{FH} cells following influenza infection. This pathway can be overcome when CD4⁺ T cells are deficient in the transcription factor T-bet. Thus, we have uncovered unique migration and transcriptional requirements for the differentiation of T_{FH} cells, which impact the humoral responses to varying pathogens.

862

The role of monocyte apoptotic cell disassembly in influenza A virus infection

Atkin-Smith, G., Duan, E., Zanker, D., Chen, W., Poon, I.
La Trobe University, Bundoora, Australia

Everyday billions of cells die through apoptosis and thus it is essential that cell debris is rapidly cleared to avoid pathological events such as cardiovascular and autoimmune disease. The fragmentation of an apoptotic cell into apoptotic

bodies, via a process termed apoptotic cell disassembly, is proposed to facilitate cell clearance and communication through the trafficking of biomolecules (e.g. DNA and RNA). Recently we have characterised a novel membrane protrusion, coined beaded-apoptopodia, which facilitates apoptotic body generation through fragmentation. Importantly, we have characterised pharmaceutical compounds which can manipulate this process. As apoptotic bodies can transport biomolecules we asked whether apoptotic cell disassembly is involved in the pathogenesis of Influenza A virus (IAV). Firstly we demonstrated that IAV can induce THP-1 monocyte apoptosis and promote the generation of beaded-apoptopodia and apoptotic bodies *in vitro*. Additionally IAV induces monocyte apoptosis and apoptotic body generation *in vivo*. Secondly, IAV proteins including hemagglutinin and nucleoprotein were found in beaded-apoptopodia and apoptotic bodies derived from infected THP-1 monocytes. Apoptotic bodies purified from IAV-infected cells induced apoptosis when incubated with uninfected host cells, whereas control apoptotic bodies (UV treatment) did not. This indicates that after initial viral infection, apoptotic bodies may propagate infection. Lastly when blocking apoptotic cell disassembly by pharmaceutical manipulation, viral propagation to uninfected host cells was inhibited. Overall data suggests that monocyte apoptotic cell disassembly may play an important role in the pathogenesis of IAV and unveils novel therapeutic strategies to target viral spread.

2057

Study of rhinovirus-specific antibody responses in children under 4 years using the PreDicta microarray

Niespodziana, K.¹, Stenberg-Hammar, K.^{2,3}, Cabauatan, C.R.¹, Napora-Wijata, K.¹, Vacal, P.¹, Gallerano, D.¹, Lupinek, C.¹, Ebner, D.⁴, Schleder, T.⁴, Harwanegg, C.⁴, Melén, E.^{5,6}, Söderhäll, C.⁷, van Hage, M.⁸, Hedlin, G.^{2,3}, Valenta, R.¹

¹Medical University of Vienna, Institute of Pathophysiology and Allergy Research, Division of Immunopathology, Vienna, Austria, ²Karolinska University Hospital, Astrid Lindgren Children's Hospital, Stockholm, Sweden, ³Karolinska University Hospital, Department of Women's and Children's Health, Stockholm, Sweden, ⁴Phadia Austria GmbH, Part of Thermo Fisher Scientific ImmunoDiagnostics, Vienna, Austria, ⁵Karolinska Institutet, Institute of Environmental Medicine, Stockholm, Sweden, ⁶Sachs' Children's Hospital, Södersjukhuset, Stockholm, Sweden, ⁷Karolinska Institutet, Department of Biosciences and Nutrition, and Center for Innovative Medicine (CIMED), Stockholm, Sweden, ⁸Karolinska Institutet and University Hospital, Clinical Immunology and Allergy Unit, Department of Medicine, Stockholm, Sweden

Rhinovirus (RV) infections are major triggers of acute exacerbations of asthma and chronic obstructive pulmonary disease (COPD) in both children and adults. The association of rhinovirus infections with exacerbations of respiratory disease is mainly based on the demonstration of the presence of virus at the onset of exacerbation. However, there are currently no serological tests available which would allow detecting specificities of antibody responses against RV epitopes as a result of infection. We, therefore, developed a high resolution antibody assay based on recombinant proteins, fragments and

synthetic peptides derived from VP1 N-terminal fragment of the most diverse RV strains. Using this microarray, we measured RV-specific IgG antibodies in serum samples from 120 preschool children collected during an acute episode of wheeze and at follow-up visit after approximately 12 weeks. We demonstrated that extremely small sample volumes were sufficient to detect RV-specific IgG antibodies to a broad panel of micro-arrayed RV antigens and that it was possible to discriminate between group- and partially strain-specific antibody responses. Furthermore, we found that the number of reactive VP1 N-terminal peptides was age-dependent. The number of peptides recognized by RV-specific IgG antibodies at the acute and the follow-up visit in the youngest group of children, 6-8 months, was significantly lower than in children above 1 year. Our results thus suggest that the microarray will be useful to identify the most relevant and clinically important RV strains involved in exacerbations of respiratory diseases and will provide a rational basis for the design of a RV vaccine.

3468

Bone marrow monocyte priming and pathological CNS infiltration are independently mediated by local IFN- γ production in WNV encephalitis

Niewold, P., Ashhurst, T., van Vreden, C., King, N.
University of Sydney, Sydney, Australia

During murine West Nile virus (WNV) encephalitis, bone marrow (BM)-derived Ly6C^{hi} inflammatory monocyte infiltration of the central nervous system (CNS) causes lethal immunopathology. Recently, NK cell-derived IFN- γ has been shown to prime Ly6C^{hi} monocytes in the BM prior to their emigration during anti-parasitic responses in C57BL/6 mice. To determine if this pathway plays a role in viral infection, we compared the pathogenesis of WNV encephalitis in C57BL/6 and SJL/J mice, since the latter have reduced NK cell numbers and IFN- γ responses. Using 18-colour flow cytometry, we found that CNS infiltration of Ly6C^{hi} monocytes was 2-fold lower in SJL/J than C57BL/6 mice. Depletion of IFN- γ in WNV-infected C57BL/6 mice reduced both efflux of monocytes from the BM and monocyte influx into the brain, significantly improving clinical disease scores. Furthermore, in NK cell-depleted C57BL/6 mice, Ly6C^{hi} monocyte accumulation in the BM was similar to IFN- γ -neutralised mice. However, migration of monocytes into the brain in these mice was not reduced, suggesting that a CNS source of IFN- γ influences brain infiltration. Indeed, T cells were the main IFN- γ -producing subsets in the encephalitic brain; although numbers of CNS-infiltrating T cells and NK cells were not statistically different, there were 13-fold more IFN- γ ⁺ CD4⁺ and CD8⁺ T cells than IFN- γ ⁺ NK cells. Therefore, we propose a 2-step model where monocytes undergo NK-cell-driven IFN- γ -mediated priming prior to BM egress, followed by local T cell-driven IFN- γ -dependent entry into the brain. IFN- γ , critical in viral eradication, thus exacerbates WNV disease by driving CNS infiltration of pathological inflammatory monocytes.

3774

HLA-B polymorphisms and their influences on immunity

Raghavan, M., Geng, J., Yarzabek, B., Silva, G.
University of Michigan Medical School, Ann Arbor, United States

Major histocompatibility complex (MHC) class I polymorphisms are shown to influence outcomes in a number of infectious diseases, cancers and inflammatory diseases. In human immunodeficiency virus (HIV) infections, among all genetic factors known to influence progression to acquired immunodeficiency syndrome (AIDS), the strongest associations link to human MHC class I genes (human leukocyte antigens (HLA)). There are three sets of genes that encode human MHC class I proteins. These are the HLA-A, HLA-B and HLA-C genes, with each locus being highly polymorphic, and the HLA-B locus being the most variable. Each HLA class I protein binds to distinct sets of peptide antigens, which are presented to CD8 T cells. While it is generally assumed that HLA-disease associations link to the peptide binding characteristics of individual HLA class I molecules, it remains largely unknown whether and how differences in assembly characteristics of HLA class I molecules could influence immunological outcomes. Polymorphisms at the HLA-B locus profoundly influence the intracellular assembly characteristics of HLA-B molecules. Based on these findings, we used quantitative flow cytometry to compare HLA-B cell surface expression levels in primary human lymphocytes in a large cohort of healthy donors. Our findings indicate allele and donor dependent variations in HLA-B cell surface expression, the molecular mechanisms of which are being defined. Such differences are expected to contribute to the known influences of HLA-B allelic variations upon disease outcomes.

B Cells 1

3035

A mucosal-derived systemic IgM B-cell memory response in non-immunized mice

Le Gallou, S., Fritzen, R., Thai, L.-H., Weller, S., Weill, J.-C., Reynaud, C.-A.
Université Paris Descartes Faculté de Médecine, Institut Necker-Enfants Malades - INSERM U 1151/CNRS UMR 8253, Paris Cedex, France

The AID-Cre-EYFP mouse (Dogan et al., 2009) allows the irreversible labelling of B cells engaged in an immune response upon tamoxifen ingestion. We observed a systemic response in absence of immunization, with EYFP⁺ B cells detectable at different frequencies in Peyer's patches, spleen and bone marrow up to 6 months after a 2-weeks tamoxifen-labelling episode. Plasma cells and memory B cells were present, with 80 % of the EYFP⁺ plasma cells being IgA⁺ and 70-80% of EYFP⁺ memory B cells being IgM⁺. Both subsets had mutated Ig genes, with mutation frequencies 2-3 times higher in IgA⁺ than in IgM⁺ B cells. Adoptive transfers showed that EYFP⁺ B cells have a B2, not a B1, origin. A large reduction in the memory and plasma cell IgA compartment was observed in germ-free animals, while the IgM response was still present, but was almost unmutated. Hybridomas were generated for the spleen IgM memory subset,

and harbored specificities against the commensal flora, as well as against human bacterial isolates. Ig sequencing revealed clonal relationships between Peyer's patch EYFP⁺ memory B cells and spleen IgM and IgA subsets, as well as with bone marrow plasma cells. BrdU labeling pulse-chase experiments and kinetics of germinal center EYFP⁺ B cell maintenance in Peyer's patches suggested that the systemic compartment is constantly renewed from a stable pool of mucosal B cell clones. This population represents a new layer of pre-activated B cells, generated in the gut, persisting through continuous replenishment, and ensuring a systemic survey for bacterial antigens.

3786

IL21 signaling in the B-cell response to antigen

Tarlinton, D.¹, Zotos, D.², Fung, I.², O'Donnell, K.², Light, A.²

¹Monash University, Immunology and Pathology, Prahran, Australia, ²Walter & Eliza Hall Institute of Medical Research, Parkville, Australia

The B-cell response to protein antigens initially generate short-lived, extra-follicular plasma cells; long-lived early memory B-cells that are not affinity matured; and germinal centers (GC). GC produce high affinity memory B-cells and long-lived, bone marrow resident plasma cells. We and others have previously reported that the Tfh-derived cytokine IL21 in the GC sustains B-cell proliferation, allowing affinity maturation to proceed, and promotes high affinity plasma cells in the bone marrow. These activities of IL21 on GC B-cells were direct rather than altering the development or behaviour of Tfh. We found also that IL21 receptor (IL21R) deficiency reduced the frequency of extrafollicular plasma cells and enhanced the appearance of early, pre-GC memory B cells. Collectively these results identified multiple roles for IL21 signaling in the B-cell response, acting either at multiple stages via multiple mechanisms or alternatively affecting a single, pre-GC stage, the consequences of which unfolded in the subsequent different pathways.

To define further the functions of IL21 in the B-cell response, we tracked antigen specific B cells during the very early stages of the response and have determined cell location and in vivo B-cell proliferation. We find that absence of IL21R affects B cell migration into GC through what appears to be a chemokine mediated mechanism. In addition IL21R deficiency leads to significantly reduced proliferation at a discrete stage of GC development. These results refine our understanding of IL21 action on antigen activated B-cells, identifying multiple points of action with their corresponding implications for B-T cell interactions.

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Dynamic changes in E-protein activity orchestrate germinal center and plasma cell development

Gloury, R., Zotos, D., Nutt, S., Tarlinton, D., Kallies, A.

The Walter and Eliza Hall Institute of Medical Research, Molecular Immunology, Melbourne, Australia

The interplay between transcription factors of the E protein family and their antagonists, the Id proteins, is important for

the regulation of lymphocyte development and differentiation. In the B cell lineage, E2A in particular is essential for early B cell commitment. However, despite being expressed at high levels throughout B cell development, E2A is dispensable for the generation of mature B cells and plasma cells in peripheral lymphoid organs.

Using a number of genetic approaches we have now further examined the role of E and Id proteins in B cells. We have found that Id3 was expressed at high levels in resting B cells and was downregulated in a division dependent manner after B cell activation. This suggested that tight Id3-mediated regulation of E protein activity might be important for B cell activation. Indeed, our studies have shown that the down-regulation of Id3 in B cells is essential for releasing E2A and E2-2, which in a redundant manner are required for antigen-induced B cell differentiation, being essential to both germinal centre formation and plasma cell differentiation. Thus, mice deficient in both E2-2 and E2A lacked any humoral immune response. Applying RNA-seq and ChIP-seq technologies we have identified critical target genes of E protein activity in B cells and uncovered a new layer of the gene regulatory network, which controls B cell responses.

3094

A novel motif in activation induced deaminase (AID) is necessary for targeting its mutagenic activity in B cells but is dispensable for E coli mutagenesis

Methot, S.P.^{1,2}, Litzler, L.C.^{1,3}, Eranki, A.K.¹, Fifield, H.⁴, Patenaude, A.-M.¹, Cote, J.-F.¹, Verdun, R.E.⁵, Larijani, M.⁴, Di Noia, J.M.^{1,2,3}

¹Institut de Recherches Cliniques de Montreal, Mechanisms of Genetic Diversity, Montreal, Canada, ²McGill University, Experimental Medicine, Montreal, Canada, ³University of Montreal, Biochemistry, Montreal, Canada, ⁴Memorial University of Newfoundland, Medicine, St. Johns, Canada, ⁵University of Miami Miller School of Medicine, Gerontology and Geriatric Medicine, Miami, United States

Activation-Induced deaminase (AID) introduces mutations and initiates DNA breaks at the Immunoglobulin genes (*Ig*), thereby initiating antibody somatic hypermutation (SHM) and class switch recombination (CSR) in activated B cells. As a side effect, AID can also damage hundreds of other genomic locations. Most AID is cytoplasmic and must be imported into the nucleus, where it associates with thousands of genomic locations. Many questions remain about the genomic targeting of AID, including whether there are mechanisms regulating its ability to deaminate or not after occupying a given locus.

Our work has identified a structurally defined arginine-rich motif in AID that is necessary for its mutagenic activity in B cells. Substituting any of these residues prevents SHM and CSR in B cells. Strikingly, these mutations do not affect the ability of AID to bind to or deaminate DNA *in vitro*, or to mutate the *E coli* genome. They do not affect AID subcellular trafficking, RNA association, SPT5 binding or nuclear chromatin association; yet, all these mutations abrogate the interaction of AID with the *Igh* locus. Furthermore, these mutants seem to lose the ability to damage the eukaryotic genome altogether, as judged by their lack of cellular toxicity when forced to accumulate inside the nucleus.

Our results identify a novel functional domain in AID that either mediates its specific recruitment to mutation-susceptible loci in B cells, or mediates a "licensing" step of AID mutagenic activity at the chromatin. This finding also has implications for the mutagenic activity of the APOBEC enzymes in vertebrates.

169

Investigating the role of the Bcl2 family member A1/Bfl1 in lymphocyte homeostasis and function

Tuzlak, S.¹, Sochalska, M.¹, Haschka, M.¹, Strasser, A.², Herold, M.², Villunger, A.¹

¹Medical University Innsbruck, Div. of Developmental Immunology, Innsbruck, Austria, ²WEHI, Molecular Genetics of Cancer Division, Melbourne, Australia

The Bcl2 family controls lymphocyte development and homeostasis by regulating mitochondrial apoptosis in response to a large set of cues, including self- or foreign peptides/antigens, cytokine-deprivation or metabolic stress. Anti-apoptotic Bcl2 proteins such as Bcl2 itself, Bclx or Mcl1 have established roles in lymphocyte development and survival but the importance of A1/Bfl1 in these processes remains ill-defined.

We have used conditional RNAi in vivo to explore the role of A1/Bfl1 in immune cell homeostasis. These studies suggest rate-limiting roles in mast cells as well as B cell homeostasis and activation. We observed that A1/Bfl1 expression is placed under control of the B cell receptor (BCR) and the SYK/BTK kinase module. Tonic, as well as activation-induced BCR-signalling thereby seems to control A1 levels to antagonize Bim-dependent apoptosis.

Future analysis will aim at exploring B cell dependent immunity in RNAi vs. conditional knock-out mice lacking A1 selectively in lymphocytes.

876

Serine/threonine phosphatase PP4 is required for class switch recombination through recruitment of gammaH2AX

Chen, M.-Y.¹, Chen, Y.-P.¹, Tan, T.-H.^{1,2}, Su, Y.-W.¹

¹National Health Research Institutes, Immunology Research Center, Miaoli County, Taiwan, Republic of China, ²Baylor College of Medicine, Department of Pathology & Immunology, Houston, United States

PP4 is a serine/threonine phosphatase, which regulates a number of crucial cellular physiology. We have previously reported that genetic ablation of PP4 in mature B cells by CD23 promoter-mediated cre-loxp system impairs immunoglobulin (Ig) class switch recombination (CSR). However, the mechanism behind PP4 for CSR is unknown. In those mutant mice, the basal levels of serum Igs of all isotypes including IgM were strongly reduced. To address whether the impaired CSR in PP4-deficient B cells was derived from the reduced IgM production, we generated PP4 conditional knockout mice by deaminase activation-induced deaminase (AID) promoter-mediated cre. As expected, the basal level of serum IgM in AID/cre/PP4^{F/F} mice was comparable to control mice. Despite of that, the basal levels of all switched Ig isotypes were drastically reduced in AID/cre/

PP4^{F/F} mice. After challenged with T cell-independent antigen TNP-Ficoll, AID/cre/PP4^{F/F} mice failed to mount antigen-specific IgG3 and IgG1. The results revealed that with normal mature B cells and serum IgM, CSR remained defect in AID/cre/PP4^{F/F} mice. Upon stimulation by LPS plus IL-4, PP4-deficient B cells exhibited increased ATM phosphorylation on S1987, suggesting severe DNA damage during CSR. Consistently with this, PP4-deficient B cells had increased p53-foci and phospho-p53-foci on S15 upon stimulation by DNA damaging drug etoposide *in vitro*. Although ATM-p53 axis was activated in PP4-deficient B cells, upon stimulation by etoposide the mutant B cells showed reduced gammaH2AX-foci in intensity and duration. We thus propose that PP4 is required for CSR through efficient recruitment of gammaH2AX, which is essential for S-region repair.

2542

Selective regulation of noncoding RNA by mediating AID-interacting molecule GANP

Maeda, K.¹, Singh, S.K.², Shimoda, M.^{1,3}, Akira, S.¹, Sakaguchi, N.⁴

¹IFReC, RIMD, Osaka University, Laboratory of Host Defense, Suita, Japan, ²IFReC, Osaka University, Laboratory of Host Defense, Suita, Japan, ³Kumamoto University, Graduate School of Medical Sciences, Immunology, Kumamoto, Japan, ⁴IFReC, Osaka University, Suita, Japan

GANP is a component of RNA export transcription/export-2 (TREX-2) complex in mammalian cells. GANP interacts to activation-induced cytidine deaminase (AID) and plays important role for affinity maturation of antibodies in B-cells. We explore how GANP controls the interaction of translation machinery with RNA transcripts in B-cells. We found that GANP physically interacts with eIF4E under the phosphorylation-dependent manner in the cytoplasm of B-cells. Phosphorylation of eIF4E increases its affinity for capped mRNA and favors its entry into translation initiation complexes. GANP also interacts with cap structure at the 5'-end of mRNAs and recruits cap-dependent translation initiation complex. Remarkably, GANP-depletion reduced the non-coding RNAs of particular clusters that are known to be essential for B-cell differentiation and activation. GANP is presumably interacted with the metabolism of particular RNA species transcribed from the B-cell genome throughout initial transcription, ribonucleoprotein assembly, RNA export and the later regulation processes. GANP might play a critical role in regulation of selective RNA for maturation of GC B-cells.

3893

Unraveling the function of human IgD in individuals with heterozygous mutations in IGHD

Nechvatalova, J.^{1,2}, Bartol, S.J.W.³, Chovancova, Z.^{1,2}, Vlkova, M.^{1,2}, van Zelm, M.C.^{3,4}

¹St Anne's Faculty Hospital, Clinical Immunology and Allergology, Brno, Czech Republic, ²Masaryk University, Faculty of Medicine, Brno, Czech Republic, ³Erasmus MC, Dept. Immunology, Rotterdam, Netherlands, ⁴Monash University, Dept. Immunology and Pathology, Melbourne, Australia

Surface immunoglobulin D (IgD) is co-expressed with IgM on naive B cells. Still, the role of surface IgD remains enigmatic >50

years after its discovery. We here identified four members of one family who carried heterozygous nonsense mutations in exon 1 of *IGHD*. We judged this a unique setting to study the role of surface IgD in B-cell homeostasis and antibody responses. Blood B-cells were analyzed with multi-color flow cytometry. IgD⁺ and IgD⁻ naive mature B cells were FACS-purified for replication history analysis using KRECs. IgG and IgA transcripts were sequenced to study somatic hypermutations (SHM). IgD allele usage was determined with a restriction enzyme assay. All *IGHD* heterozygous individuals had normal numbers of B cells and serum immunoglobulins, and did not show signs of an immunodeficiency. IgD⁺ and IgD⁻ naive mature B cells were present in equal numbers and showed similar immunophenotypes. Only CD79b expression was lower in the IgD⁻ subset, reflecting the absence of IgD. The replication history of IgD⁻ naive mature B cells was normal. IgA and IgG-switched alleles were equally derived from the wildtype and mutant alleles, and carried similar mutation frequencies between transcripts derived from IgD-wildtype and IgD-mutant alleles. Human IgD⁻ B cells are not out-competed by IgD⁺ B cells *in vivo*, and surface IgD is not required for naive B-cell homeostasis and antibody maturation. Thus, the evolutionary conservation of IgD might be more related to the function of secreted rather than surface IgD.

1559

The role of gene conversion in the diversification of murine IgM plasma cells

Dale, G.¹, Bohannon, C.², Antia, R.², Jacob, J.¹

¹Emory School of Medicine, Emory University, Atlanta, United States, ²Emory University, Atlanta, United States

Humoral immunity is marked by long-lived antibody responses against pathogens. The duration of this response is dependent on long-lived plasma cells that are primarily located in the bone marrow and are of IgG isotype. However, we have identified a population of antigen specific, long-lived IgM plasma cells that reside in the red pulp of the spleen and display an atypical mutation profile. Following immunization of mice with 4-Hydroxy-3-nitrophenyl (NP) conjugated to chicken gamma globulin (CGG) (NPCGG) in alum, we isolated plasma cells (CD138⁺ B220⁻) and sequenced them via 454 pyrosequencing for μ heavy chain. Out of a total of 292 IgHV186.2 (which encode NP-specific antibodies) IgM sequences, we observe at least 58 that exhibited shared tracts of mutations (≥ 3 mutations per tract). We find that these mutations are AID dependent and primarily occur in the framework regions of the V_H gene - unlike IgG plasma cells, where mutations are predominantly located within the complementarity determining regions (CDRs). Upon further analysis, we find strong evidence that these mutations appear to be shared between multiple unique IgM plasma cell clones isolated from different individual animals. Additionally, these tracts match other highly homologous V_H genes in both position and identity. Taken together, we posit that these mutations in IgM plasma cells are not generated through murine somatic hypermutation pathways as described extensively in the literature but are, in fact, generated through templated mutagenesis using highly homologous V_H genes through gene conversion.

2043

Analysis of the IgE repertoire in healthy, non-allergic individuals by massive parallel sequencing

Koning, M., Trollmann, I., Griffioen, M., Veelken, H.

Leiden University Medical Center, Department of Hematology, Leiden, Netherlands

The largest reported IgE repertoire comprises merely thirty immunoglobulin sequences. We investigated the healthy IgE repertoire in comparison to IgM and IgG.

Peripheral blood CD19⁺ cells from six healthy donors were divided into aliquots of 2×10^6 cells. For each donor, mRNA was amplified by ARTISAN PCR (bias-free isotype-specific 5'RACE protocol) from 5 aliquots per IgE, IgG, and IgM isotype in parallel. PacBio sequencing of the resulting 15 amplicon libraries per donor yielded a median of 284 (range: 100-743) full-length sequences/aliquot and 27500 sequences in total.

A median of 261 (range: 175-358) unique IgE sequences were obtained per donor. 9.1-61.8% of individual IgE sequences were represented in >2 aliquots of the same donor. Only 1.4-7.8% IgM and 5.7-16.3% IgG sequences, respectively, were identified in multiple aliquots. Whereas 29VDJ sequences were found as both IgM and IgG isotype, all IgE sequences were restricted to their isotype. The median number of somatic mutations per sequence differed significantly between IgE (median 5.6%), IgM (0.3%), and IgG (8.3%). Intraclonal sequence diversity in IgE clones was predominantly due to silent FR mutations. IGHV3-11, IGHV3-9, and the IGHV3 family were significantly overrepresented in IgE compared to IgM/IgG. IGHV3-30, IGHV3-33, IGHV4-59, and IGHV2, 4, 5, and 6 families were underrepresented.

The peripheral IgE repertoire is less diverse than IgM/IgG and displays a strong IGHV bias and an intermediate mutation load without ongoing affinity maturation. IgE and IgG/IgM repertoires apparently do not overlap. These data provide a reference to investigate the IgE repertoire in allergy, parasitology, or desensitization.

NK cells 1

3127

NK cell receptor inhibition determines NK cell cytolytic potential through accumulated granular load

Goodridge, J.¹, Clancy, T.¹, Jacobs, B.¹, Pfeifferle, A.², Brech, A.³, Skarpen, E.³, Stenmark, H.³, Malmberg, K.J.¹

¹Institute for Cancer Research, Norwegian Radium Hospital, Department of Immunology, Oslo, Norway, ²Karolinska Institute, CIM, Stockholm, Sweden, ³Institute for Cancer Research, Molecular Cell Biology, Oslo, Norway

Discrimination of self from non-self through the continuous selection of effector specificity is the backbone of multicellular immunity. For natural killer (NK) cells this specificity is achieved by unique combinations of variable germ-line receptors that recognize self-MHC antigens. The expression of NK cell receptors and their inhibitory interaction with self-ligands is a key determinant for the development of pre-primed effector responses, a process termed NK cell education. This calibration of effector potential to self-MHC allows for rapid sensing of

discontinuity in the level of MHC expression during infection, cellular stress or tumor transformation, whilst operating within a framework of overall tolerance to normal tissues. While NK cell education is firmly established across species, the underlying mechanism connecting inhibitory signaling to the development of intrinsic functional potential has yet to be resolved. We describe here the first stable imprint of NK cell education, the granular accumulation of granzyme B that occurs in primary NK cells under specific inhibitory self-interaction. This accumulation underlies the development of natural killer cell effector potential, traced first in the regulatory shift from inducible to constitutive effector transcription that occurs during NK cell differentiation, then shaped by surface signaling through killer cell immunoglobulin-like receptors as maturing NK cells interact continuously with the MHC environment. The development of a granular effector phenotype that is influenced directly by inhibitory interactions provides insight into the possible role cytolytic granules play in potentiating effector signals, as a function of granular intensity and as a means to cultivate NK cell specificity.

4212

Targeting regulators of natural killer cell homeostasis in cancer immunotherapy

Delconte, R.B.¹, Kolesnik, T.B.¹, Rautela, J.¹, Smyth, M.J.², Nicholson, S.E.¹, Huntington, N.D.¹

¹The Walter and Eliza Hall Institute of Medical, Parkville, Australia,

²Queensland Institute of Medical Research, Brisbane, Australia

Natural Killer (NK) cells are cytotoxic cells found throughout the body and are integral in the identification and destruction of malignant cells. This activity is governed by the cytokine interleukin (IL)-15 and the presence of foreign and self-ligands. We have identified CIS (Cytokine-inducible SH2-containing protein; *Cish* gene) as the critical negative regulator of IL-15 signalling in NK cells. *Cish* was rapidly induced in response to IL-15 and deletion of *Cish* rendered NK cells hypersensitive to IL-15, as evidenced by superior proliferation, survival, IFN- γ production and cytotoxicity towards tumours. This was associated with enhanced JAK/STAT signalling in *Cish*-deleted NK cells. *Cish*^{-/-} mice were resistant to melanoma, prostate and breast cancer metastasis *in vivo*, and this was intrinsic to NK cell activity. This study has uncovered a potent checkpoint in NK cell-mediated tumour immunity and holds promise for novel immunotherapies directed at blocking CIS function.

3563

Novel function of NK cell to regulate cancer-associated inflammation

Hayakawa, Y.

Institute of Natural Medicine, University of Toyama, Division of Pathogenic Biochemistry, Toyama, Japan

Although many studies show the importance of NK cells as a direct anti-tumor effector cell to control tumor growth, the immuno-regulatory function of NK cells to control cancer-associated inflammation, which facilitate tumor progression,

remains unknown. By using *in vivo* bioluminescent imaging, we monitored the exact behavior of cancer cells controlled by NK cells. Consistent with previous observations, depletion of NK cells resulted in facilitating *in vivo* cancer cell proliferation and IFN- γ was a dominant effector molecule to control *in vivo* cancer cell proliferation. Importantly, depletion of NK cells did not affect cancer cell proliferation in mice lacking IL-17, which has been known as an important cytokine for triggering neutrophilic inflammation. Furthermore, depletion of neutrophils diminished such facilitation of *in vivo* cancer cell proliferation in NK cell-depleted mice. These findings imply a novel function of NK cell as a regulatory cell to suppress tumor-promoting neutrophilic inflammation. We would like to further discuss our current effort on investigating the mechanism by which NK cell regulates tumor-promoting neutrophilic inflammation.

1770

Extent of inflammatory stimuli influences the response of functionally distinct natural killer cell subsets

Aguilar, E.G.¹, Zamora, A.E.¹, Sungur, C.M.¹, Murphy, W.J.^{1,2}

¹University of California, Davis, Dermatology, Sacramento,

United States, ²University of California, Davis, Internal Medicine, Sacramento, United States

Natural Killer cells are lymphocytes of the innate immune system that play roles in both viral infection and cancer. NK cells can be divided into distinct subsets based on expression of inhibitory receptors capable of binding to MHC class I molecules. Those that are capable of binding to self-MHC are termed licensed NK cells, and are the classic cytotoxic effector cell. Those that cannot bind to self-MHC are termed unlicensed, and are thought to be hyporesponsive in terms of cytotoxicity. The functional consequences of licensing outside of cytotoxicity remain unclear, with some reporting distinct effector roles for each subset during viral infection. Here, we show differences in the NK subset response to MCMV infection, dependent on the dose of virus used to challenge mice. With a lower dose of virus, differences in the localization of NK subsets after infection were noted, as well as differences in the extent of the virus-specific T cell responses and viral titer in mice depleted of specific NK subsets. When a higher dose of virus was used, these differential effects were abrogated and associated with an overall expansion of both licensed and unlicensed virus-specific Ly49H⁺ NK cells. Collectively, these results suggest that the inherent functional differences between the licensed and unlicensed NK cell subsets may be overcome depending on the extent of the inflammatory stimuli.

4610

Human NK cell maturation requires CD56-mediated motility and formation of the developmental synapse

Mace, E.¹, Gunesch, J.¹, Dixon, A.², Orange, J.¹

¹Baylor College of Medicine, Houston, United States, ²Rice

University, Houston, United States

Natural killer cells are critical for the control of viral infection and malignancy and hold tremendous promise in the field of

immunotherapy. Despite their importance, human NK cell development is poorly understood. It is thought that NK cells are derived from CD34⁺ precursors that ultimately generate the two subsets of NK cells found within peripheral blood, CD56^{bright} and CD56^{dim}. Each has unique phenotypic and functional properties, although the role of CD56 on human NK cells has not been described. Both subsets can be recapitulated in vitro from precursors using stromal cells and cytokines. While it is known that stromal cell contact is required, the nature of these interactions is not known.

To dissect the contacts that drive human NK cell maturation, we performed high resolution live cell confocal microscopy of developing NK cells with stromal cells. We found that NK cells undergo substantive motility on stroma, and that this motility is driven by CD56 itself. Further, we identify a novel immunological synapse, which we term the developmental synapse, formed between developing NK cells and stroma. **Formation of the developmental synapse leads to polarization and downstream signaling, and blocking its formation leads to aberrant NK cell development.** Stromal cell contact also regulates expression of L-selectin, which cooperates in NK cell motility. Together, our data shows for the first time a functional role of CD56 in human NK cell homeostasis. It also introduces the developmental synapse, a novel bona fide immunological synapse that drives human NK cell development.

3320

Natural killer (NK) cells and $\gamma\delta$ T cells differentially modulate Th17-driven disease in ankylosing spondylitis

Wythe, S., Griseri, T., Freidin, A., Demetriou, P., Samson, S., Mccann, F., Horwood, N.

University of Oxford, Kennedy Institute of Rheumatology, Oxford, United Kingdom

Ankylosing spondylitis (AS) is characterised by chronic joint inflammation, inflammatory bowel disease, and inappropriate new bone formation at the entheses. However, the immunological factors linking gastrointestinal inflammation with joint pathology are not well delineated. Natural Killer (NK) cells are recruited to these inflammation sites. As such we hypothesized that NK cells form a bridge linking gastrointestinal inflammation and joint pathology. To test this hypothesis we used the recently characterised BALB/c ZAP-70W163C-mutant (SKG) mouse model of AS, in which disease is initiated by a single intraperitoneal (i.p.) injection of beta-1,3-glucan (curdlan). Mice were depleted of NK cells (anti-asialo GM1; 2x/wk; i.p) and arthritis, enthesitis and weight scored weekly. Inflammation was observed in both the ileum and the joint following challenge, and NK cell numbers were increased in both locations. NK-depletion exacerbated weight loss and ileitis score, correlating with increased numbers of IL-22-producing $\gamma\delta$ T cells, Th17 cells, and neutrophils. In the joint, arthritis, enthesitis and new bone formation were all ablated and correlated with a significant reduction in gdT cells and neutrophils. In contrast, $\gamma\delta$ T cell depletion (clone GL3; 2x/wk; i.p) increased disease in both ileum and joint. These data identify a protective role for NK cells and $\gamma\delta$ T cells in ileitis and suggest that NK-gdT cell cross-regulation may be an important factor in determining

morbidity. This study highlights novel cellular targets for the treatment of this complex multifactorial human disease.

443

Human lung NK cells are predominantly constituted by highly differentiated hypofunctional CD69⁺CD56^{dim} cells

Marquardt, N.¹, Kekäläinen, E.¹, Wilson, J.N.¹, Ivarsson, M.A.¹, Mjösberg, J.¹, Säfholm, J.², Manson, M.L.², Adner, M.², Al-Ameri, M.³, Bergman, P.³, Orre, A.-C.³, Dahlén, B.², Dahlén, S.-E.², Ljunggren, H.-G.¹, Michaëlsson, J.¹

¹Karolinska Institutet, Department of Medicine, Stockholm, Sweden, ²Karolinska Institutet, Institute of Environmental Medicine, Stockholm, Sweden, ³Karolinska University Hospital, Department of Molecular Medicine and Surgery, Stockholm, Sweden

Background: In contrast to the extensive knowledge about human NK cells in peripheral blood, relatively little is known about NK cells in human lung. Knowledge about the composition, differentiation, and function of human lung NK cells is critical to better understand their role in diseases affecting the lung, including asthma, COPD and cancer.

Objective: We sought to analyze and compare the phenotypic and functional characteristics of human NK cells in lung and peripheral blood at the single cell level.

Methods: NK cells in human lung tissue and matched peripheral blood from a total of 132 subjects were analyzed by 16-color flow cytometry.

Results: CD56^{dim}CD16⁺ NK cells made up the vast majority of NK cells in human lung, had a more differentiated phenotype, and more frequently expressed educating KIR compared to NK cells in peripheral blood. Despite this human lung NK cells were hyporesponsive, even after priming with IFN- α . Most NK cells in lung lacked expression of CD69, a marker of tissue residency, whereas the majority of T cells and CD127⁺ innate lymphoid cells in lung expressed CD69. The small detectable population of tissue-resident CD69⁺ NK cells in the lung consisted of immature CD56^{bright}CD16⁻ NK cells and less differentiated CD56^{dim}CD16⁺ NK cells.

Conclusion: We here identify and characterize the major NK cell populations in the human lung. Our data introduce the concept that the majority of NK cells in the lung dynamically move between the blood and lung, rather than residing in the lung as *bona fide* tissue-resident CD69⁺ NK cells.

1915

Harnessing NK cells to target chronic lymphocytic leukemia: design and efficacy of novel trispecific immunoligands

Vyas, M., Pogge von Strandmann, E., Innate Immunity Group University Clinic Cologne, University of Cologne, Cologne, Germany

Chronic lymphocytic leukemia (CLL) is the most prominent B cell malignancy among adults in the Western world and characterized by a clonal expansion of B cells. There is emerging evidence that natural killer (NK) cells play a pivotal role in the immunosurveillance of CLL. Currently available mAbs are partially efficient in CLL and new agents that are more specific and effective are needed.

Our approach is to design bi- and tri-specific recombinant proteins (immunoligands) which simultaneously bind to tumor cells and NK cells resulting in NK cell-mediated killing of the former while bypassing their immune-escape mechanisms. One such trispecific immunoligand (ULBP2-aCD19-aCD19) aims to target CD19 antigen on CLL cells while a second arm activates NK cells through ULBP2, a ligand for the activating receptor NKG2D. *In vitro* data with ULBP2-aCD19-aCD19 revealed an enhanced NK cell-dependent lysis of CLL cell line and primary CLL cells in allogenic and autologous settings. This effect could be successfully replicated in an immunodeficient mouse model (NSG) as a part of *in vivo* analysis.

On the other hand, novel trispecific immunoligands are being analysed for their ability to enhance the efficacy of NK cell activation. This design includes a single chain (scFv) recognizing CD19 and two arms engaging different NK cell receptors CD16 (via anti-CD16 scFv) and NKG2D (via ULBP2). Based on the evidence that activating receptors on NK cells can synergize to enhance NK cell effector functions, we hypothesize that such trispecific immunoligands will be more potent immunotherapeutic agents.

2638

Selective modulation of Natural Killer (NK) cell phenotypes through chromatin modifying mechanisms

Cribbs, A.^{1,2,3}, Feldmann, M.², Soderstrom, K.³, Oppermann, U.³

¹University of Oxford, DPAG, Oxford, United Kingdom, ²Kennedy Institute of Rheumatology, Oxford, United Kingdom, ³Botnar Research Centre, Oxford, United Kingdom

Background: Natural killer (NK) cells are sentinels of the innate immune system by monitoring cell surfaces for the expression of MHC class I molecules and stress markers. For example, NK cells are critical for immune defence against malignant or virus infected target cells by engaging activating receptors, such as NKp30, leading to a cytolytic response.

Aims: Our research aims are to identify epigenetic mechanisms controlling immune cell functions. To understand NK cell function we use chemical and knockdown tools to study “readers, writers and erasers of a histone code” that regulate gene transcription.

Results: A focused epigenetic compound screen, comprising 60+ potent and selective epigenetic inhibitors, identified Jumonji-type histone demethylases and the BET bromodomains to play a fundamental role in regulating NK cell effector function. We show that JMJD3/UTX and BRD2/4 regulate a number of inflammatory cytokines, most notably pro-inflammatory IFN- γ . Inhibition of BRD2/4, but not JMJD3/UTX, resulted in inhibited NK cell-mediated cytotoxicity in a model system using K562 target cells. Transcriptome analysis revealed that this was accompanied by a robust down-regulation of key genes, in particular NKp30, further highlighted by antibody blocking experiments, revealing a critical role for NKp30 in mediating target killing.

Conclusion: Our results suggest that BET inhibitors exert deleterious effects on NK cell function by downregulating NKp30, which may weaken immune surveillance. Overall, our results show that both Jumonji histone demethylases and BET

bromodomains are important in regulating NK cell function, with BET bromodomains being key regulators of NK cell mediated cytotoxic killing of tumour cells.

Autoimmunity 2

3776

Regulation of immune response genes by endogenous retroviruses

Loetsch, C.^{1,2}, Warren, J.¹, Parsons, J.¹, Buske, F.¹, Dinger, M.¹, King, C.^{1,2}

¹Garvan Institute of Medical Research, Darlinghurst, Australia, ²St. Vincent's Clinical School, University of New South Wales, Sydney, Australia

Type 1 diabetes (T1D) is an autoimmune disease caused by a combination of genetic and environmental factors. Results from genome-wide association studies and sero-epidemiological studies have linked infections with human enteroviruses, such as coxsackievirus B4 (CVB4), with an increased risk of T1D. Despite their relative abundance within mammalian genomes (8-10%), the role of endogenous retroviruses (ERVs) in the development of virus-induced chronic inflammation and autoimmunity remains unknown. We hypothesize that endogenous retroviruses can actively shape immune responses to exogenous virus infections and may thereby contribute to inappropriate responses against the host tissue.

Taking a genome-wide approach, we analysed the response of pancreatic target tissue to inflammation resulting from both pancreatrophic virus infection (Coxsackievirus B4) and spontaneous autoimmunity (NOD mice). Tissue specific upregulation of ERV expression was observed in the pancreatic beta cells of CVB4-infected mice and prolonged ERV up-regulation was exclusively observed in diabetes-prone mice. Viral response gene pathways were similarly overrepresented in CVB4 infected and pre-diabetic uninfected NOD mice. Beyond this, viral infection and spontaneous autoimmune inflammation induced the expression of endogenous retroviruses (particularly *ERV1* elements) and retroviral sensors (e.g. *Aim2* and *Irf16*). Feature analysis revealed that genomic positions of endogenous retroviruses positively correlated with promoter regions of CVB4-induced genes, while ERV expression within down-regulated genes was associated with enhancers, TF-binding sites and open chromatin regions.

Taken together, our findings suggest that ERVs can contribute to host-specific immune responses through both immune recognition and transcriptional regulation, and support their involvement in the development of chronic inflammation and autoimmune disease.

1082

A single epitope is recognized by anti-IFN γ autoantibodies in patients with mycobacterial disease: clinical implications

Lin, C.-H.¹, Chi, C.-Y.², Ding, J.-Y.¹, Shih, H.-P.¹, Lo, C.-C.¹, Ku, C.-L.¹

¹Chang Gung University, Taoyuan, Taiwan, Republic of China,

²China Medical University Hospital, Taichung, Taiwan, Republic of China

Autoantibodies (AutoAbs) against interferon-gamma (IFN γ)

underlying mycobacterial diseases are an emerging medical issue in Southeast Asia. The origin of these AutoAbs is unclear. However, a vast majority of patients share the human leukocyte antigen (HLA) class II DRB1*15:02/16:02-DQB1*05:01/05:02 haplotype, suggesting a common T-cell- and B-cell-dependent mechanism underlying the production of IFN γ -specific AutoAbs. Herein, we report that these AutoAbs recognize a major epitope (P₁₂₁₋₁₃₁) in the C-terminus of IFN γ , a region critical for IFN γ receptor (IFN γ R) activation. AutoAbs to this epitope demonstrate neutralizing activity against IFN γ , preventing signaling via its receptor. Interestingly, the primary structure of this epitope is 100% homologous to a stretch of environmental fungus *Aspergillus* protein Noc2. We show that AutoAbs from patients can react with Noc2. In *in vivo* studies, rats immunized with *Aspergillus* Noc2 peptide developed antibodies against human IFN γ , and rats immunized with human IFN γ peptide developed antibodies against *Aspergillus* Noc2. Moreover, we generated epitope-erased IFN γ (EE-IFN γ), which has a modified major neutralizing epitope region that reduces 38% of its binding affinity with anti-IFN γ AutoAbs but not with the IFN γ receptor. EE-IFN γ can activate the IFN γ R downstream signaling pathway in the presence of anti-IFN γ AutoAbs *ex vivo*. In conclusion, we identified a shared, critical B-cell epitope of anti-IFN γ AutoAbs in patients and propose a molecular mimicry model in which the production of anti-IFN γ AutoAbs is triggered by the *Aspergillus* Noc2 protein. Moreover, EE-IFN γ may exhibit therapeutic potential for patients with anti-IFN γ AutoAbs.

2639

Isolation and characterization of hypocretin-reactive CD4⁺ T cells in narcoleptic patients

Latorre, D.¹, Armentani, E.¹, Kallweit, U.², Manconi, M.³, Khatami, R.⁴, Bassetti, C.L.², Sallusto, F.¹

¹Center of Medical Immunology, Institute for Research in Biomedicine, Università della Svizzera Italiana, Bellinzona, Switzerland, ²University Hospital, Neurology Department, Bern, Switzerland, ³Ospedale Regionale di Lugano (EOC), Lugano, Switzerland, ⁴Center for Sleep Research and Sleep Medicine, Clinic Barmelweid, Barmelweid, Switzerland

Narcolepsy-cataplexy is a rare sleep-wake disorder that manifests in genetically predisposed individuals and is caused by the selective loss of neuronal cells of the posterior hypothalamus that produce the neuropeptide hypocretin. Genome-wide association studies showed a strong association with HLA-DQB1*06:02 and other genes involved in immune modulation, supporting the notion that narcolepsy is a T cell-mediated autoimmune disease. However, attempts to identify hypocretin-specific T cells in narcoleptic patients have been so far unsuccessful. In this study we combined antigenic stimulation, T cell cloning, and TCR deep sequencing to characterize the T cell response in narcoleptic-cataplectic HLA-DQ0602-positive patients. T cells were isolated from blood and, when available, CSF, and either directly stimulated with overlapping peptides covering the entire hypocretin precursor protein (prepro-hypocretin) or expanded polyclonally to generate T cell libraries that were subsequently interrogated for reactivity against

prepro-hypocretin peptides as well as putative cross-reactive antigens. Hypocretin-reactive CD4⁺ T cell clones were identified in all patients analyzed so far, but not in HLA-DQ0602-positive control donors. The clones use different TCR α/β and recognize several regions of the prepro-hypocretin protein. Next-generation TCR sequencing was also performed on total T cells from blood and CSF in order to gather information on narcoleptic patients' TCR repertoire, to search for shared clonotypes, and to define expansion and localization *in vivo* of hypocretin-reactive T cell clones. Collectively, our data demonstrate for the first time the existence of expanded hypocretin-reactive CD4⁺ T cells in narcoleptic patients.

435

Stimulation of the melanocortin-1 receptor leads to direct neuroprotection via orphan nuclear 4 receptor in inflammatory neurodegeneration

Sucker, N.¹, Herrmann, A.M.², Faber, C.³, Wiendl, H.², Luger, T.A.¹, Meuth, S.G.², Loser, K.¹

¹University of Münster, Department of Dermatology, Münster, Germany, ²University of Münster, Department of Neurology, Münster, Germany, ³University of Münster, Institute for Clinical Radiology, Münster, Germany

In inflammation-associated progressive neurodegenerative disorders, e.g. multiple sclerosis (MS), inflammatory infiltrates, including Th1 and Th17 cells, cause demyelination and axonal/neuronal damage. Regulatory T cells (Treg) control the activation and infiltration of autoreactive T cells into the central nervous system (CNS). However, in experimental autoimmune encephalomyelitis (EAE) and MS, Treg function is severely impaired. Here we demonstrated that Nle4-D-Phe7- α -melanocyte-stimulating hormone (NDP-MSH), by binding to the melanocortin-1 receptor (MC-1R), induced functional Treg, which efficiently inhibited EAE progression. Strikingly, NDP-MSH also prevented immune cell infiltration into the CNS by restoring the integrity of the blood-brain barrier and exerted strong, long-lasting direct neuroprotective effects in an active (MOG-immunization) and spontaneous EAE model (Devic mice). Moreover, NDP-MSH prevented excitotoxic neuronal cell death in isolated mouse as well as human neuronal cells and re-established action potential firing in embryonic hippocampal neurons. Gene expression studies performed in brain and spinal cord tissue from MOG-immunized and NDP-MSH-treated mice revealed that neuroprotection by NDP-MSH was mediated via signaling through MC-1R, phosphorylation of the cAMP response element-binding protein (CREB) and subsequent activation of the orphan nuclear 4 receptor NR4A1 in mouse and human neurons. NDP-MSH has recently received European Medicines Agency (EMA) approval for the treatment of erythropoietic porphyria and thus, our novel data might support its use in treating neuroinflammatory and neurodegenerative diseases, e.g. relapsing-remitting MS.

2544**Macrophage derived ROS regulate arthritis and psoriasis-like dermatitis in ZAP deficient SKG mice***Guerard, S., Holmdahl, R., Wing, K.**Karolinska Institutet, MBB, Stockholm, Sweden*

A key issue in immunology is to reveal the interplay of factors that lead to multi-factorial diseases such as rheumatoid arthritis (RA) and psoriasis. ZAP70 deficient SKG mice develop a T cell-dependent arthritis that mimics RA when injected with mannan, a polysaccharide from yeast. However, injection of mannan also induces a T cell independent psoriasis like disease, even in wild type mice, which is dramatically enhanced by lack of reactive oxygen species (ROS). To investigate the role of ROS in T cell-driven autoimmunity, we introduced the *Ncf1^{m1j}* mutation in the SKG mouse model to prevent ROS burst by phagocytes. SKG mice were susceptible to both IL-17 mediated T cell driven arthritis and acute psoriasis-like dermatitis. *In vivo* imaging showed higher ROS concentrations in joints of arthritic SKG mice compared to naïve and wild type mice, which demonstrate an association between ROS and joint inflammation. However SKG.*Ncf1^{m1j/m1j}* mice, which lack ROS, developed more severe arthritis and psoriasis-like dermatitis that could not be attributed to either T cell or B cell responses. However, autoimmunity drastically reverted to baseline level when ROS production was introduced in macrophages under the human CD68 promoter. This clearly demonstrates the critical role of macrophage-derived ROS in regulating aberrant inflammation in tissue. Thus, despite the T cell dependency of arthritis in the SKG model, the lack of ROS enhanced autoimmunity through T cell independent mechanisms.

3191**The role of anti-inflammatory cytokine interleukin-27 in atherosclerosis***Peshkova, I., Fatkhullina, A., Koltsova, E.**Fox Chase Cancer Center, Philadelphia, United States*

Atherosclerosis is a lipid-driven chronic inflammatory disease. Cytokines are important mediators of inflammation and atherosclerosis. While pro-inflammatory cytokines were extensively investigated, little is known about the role of anti-inflammatory cytokines in regulation of vascular inflammation. We tested whether immunoregulatory IL-27R signaling controls inflammation in mouse models of atherosclerosis. We found that atherosclerosis-prone mice with hematopoietic deficiency of IL-27R (*Ldlr^{-/-}* mice reconstituted with bone marrow from *Il27ra^{-/-}*) or global deficiency (*Il27ra^{-/-} x ApoE^{-/-}*) developed significantly larger atherosclerotic lesions compared to controls. Aortas of *Il27ra^{-/-}* mice contained more CD45⁺ leukocytes and CD4⁺ T cells, which produced pro-atherogenic cytokines IL-17A and TNF- α . These cytokines normally suppressed by IL-27, regulated the expression of CCL2 and other chemokines, which in turn led to accumulation of myeloid CD11b⁺ and CD11c⁺ cells in atherosclerotic aortas. Two-photon live imaging revealed enhanced interactions between antigen presenting cells and CD4 T cells in the aortas of *Il27ra^{-/-}* mice accompanied by enhanced T cell proliferation. Moreover, macrophages in *Il27ra^{-/-}* aortas also demonstrated enhanced ability to produce

pro-inflammatory cytokines, including IL-1. The blockade of IL-1R signaling, however, strongly suppressed atherosclerosis in *Il27ra^{-/-}* but not control mice, suggesting regulatory role of IL-27 in IL-1 production in atherosclerosis.

Overall, our data demonstrate that IL-27R signaling in atherosclerosis is required to control function of antigen presenting cells modulating subsequent T cell activation in the aortas. Moreover, it controls macrophage activation and pro-inflammatory cytokine production. These mechanisms altogether curb pathogenic T cell lineage differentiation and, thus, atherosclerosis, suggesting potent anti-atherogenic role of IL-27.

4167**T cell tolerance in the pancreatic islets is mediated by MerTK during type 1 diabetes***Lindsay, R.^{1,2}, Tracy, D.¹, Friedman, R.^{1,2}**¹National Jewish Health, Biomedical Research, Denver, United States, ²University of Colorado Denver, Immunology and Microbiology, Denver, United States*

Type 1 diabetes (T1D) is a T cell mediated autoimmune disease that results from destruction of pancreatic islet β -cells. Curiously, for years following disease onset β -cell containing islets with T cell infiltration can still be found, suggesting a tolerance checkpoint following islet infiltration. While T cells are required for T1D progression, CD11c⁺ cells also infiltrate the islets. We depleted CD11c⁺ cells to determine their role in maintaining T cell tolerance in the islets. CD11c depletion resulted in increased CD8 T cell motility arrest and effector function. MerTK⁺ antigen presenting cells are associated with T cell tolerance induction following apoptotic cell uptake. We identified MerTK expression on a subset of islet-infiltrating CD11c⁺ cells. To test if MerTK mediates tolerance in the islets, we treated asymptomatic non-obese diabetic (NOD) with pre-existing islet infiltration and non-autoimmune C57BL/6 mice with UNC2025, a MerTK/Flt3 inhibitor. Blood glucose remained normal in C57BL/6 mice. However, in 50% of NOD mice, MerTK/Flt3 inhibition resulted in extremely rapid diabetes onset. The rapid disease onset occurred prior to Flt3-dependent population shifts in the islets, suggesting a MerTK rather than Flt3 mediated effect. Within 24 hours of MerTK inhibition, islet-antigen specific CD4 and CD8 T cells arrested motility in the islets, and CD8 T cell activation increased in the islets. Thus, inhibition of MerTK signaling resulted in a break in T cell tolerance and rapid islet destruction. These results suggest that CD11c⁺ cells are involved in a MerTK-dependent tolerance checkpoint during T1D, which maintains T cell tolerance in the islets.

1305**CD4⁺ T cells from the pancreatic islets of an organ donor who had type 1 diabetes recognize epitopes formed by transpeptidation***Elso, C.¹, So, M.¹, Tresoldi, E.¹, Kumar, N.¹, DeLong, T.², Haskins, K.², Mannerling, S.¹**¹St. Vincent's Institute of Medical Research, Melbourne, Australia,**²Barbara Davis Center for Childhood Diabetes, University of Colorado, Denver, United States*

Type 1 diabetes (T1D) is an autoimmune disease caused by the T-cell mediated destruction of the insulin-producing beta cells. Current evidence suggests that HLA-DQ8 and/or HLA-DQ2 restricted CD4⁺ T-cell responses against (pro)insulin, play an important role in the pathogenesis of T1D. However, the antigens/epitopes recognized by pathogenic CD4⁺ T cells have not been identified. The possibility that autoimmune responses target epitopes formed by posttranslational modification of self-proteins has gained support in several autoimmune diseases, but it remains unclear if a similar mechanism operates in type 1 diabetes. Recently CD4⁺ T cells that cause autoimmune diabetes in the NOD mouse model were shown to recognize a novel form of posttranslational modification - epitopes formed by the fusion of two beta-cell proteins. To investigate if these epitopes, termed hybrid insulin peptides (HIPs), are recognized by human CD4⁺ T cells in T1D we tested CD4⁺ T-cell clones isolated from the residual pancreatic islets of an organ donor who suffered from T1D. Two clones responded to an epitope formed by the fusion of proinsulin with IAPP2, another protein found in the granules of beta cells. The response to this epitope is restricted by HLA-DQ8. Preliminary data suggests that CD4⁺ T-cell responses to this epitope can be detected in the peripheral blood of HLA-DQ8⁺ individuals with T1D, but not those without T1D. These observations support the hypothesis that autoimmune human CD4⁺ T-cell responses in T1D target epitopes formed by protein-protein fusion and may explain how these putatively pathogenic T cells avoid central tolerance.

3770

Granzyme A-deficiency breaks immune tolerance and promotes organ-specific autoimmune disease through a type I interferon-dependent pathway

Mollah, Z.¹, Quah, H.S.¹, Graham, K.¹, Jhala, G.¹, Krishnamurthy, B.¹, Trapani, J.², Bird, P.³, Brodnicki, T.¹, Kay, T.¹, Thomas, H.¹

¹St Vincent's Institute, Immunology and Diabetes, Fitzroy, Australia,

²Peter MacCallum Cancer Centre, East Melbourne, Australia,

³Monash Biomedicine Discovery Institute, Clayton, Australia

Granzyme A is a protease implicated in the degradation of intracellular DNA. Nucleotide complexes are known triggers of innate immunity and systemic autoimmunity, but a role in organ-specific autoimmune disease has not been demonstrated. To investigate whether such a mechanism could be an endogenous trigger for autoimmunity, we examined the impact of granzyme A deficiency in the NOD mouse model of autoimmune diabetes. NOD.Gzma^{-/-} mice developed diabetes earlier than wild-type NOD mice. In addition, granzyme A deficiency was able to break tolerance in NOD.PI mice that are tolerant to proinsulin and never develop diabetes, resulting in insulin autoantibody production and diabetes. Single-stranded DNA was observed more frequently in the cytoplasm of immune cells in NOD.Gzma^{-/-} mice compared with NOD mice, suggesting that granzyme A is required for efficient degradation of aberrant DNA. Consistent with increased nucleic acid sensing, islets from NOD.Gzma^{-/-} mice had increased expression of IFN-response genes. Diabetes returned to the expected rate in NOD.Gzma^{-/-} mice that also lacked type I IFN receptor expression. Our data suggest a new mechanism for the etiology of autoimmune diabetes, where

accumulation of aberrant cytoplasmic DNA in innate immune cells results in excessive IFN production by these cells within islets. This is the first indication of an *in vivo* role for granzyme A in maintaining immune tolerance.

HIV 1

3230

Neutrophils mediate potent and rapid anti-HIV antibody-dependent functions

Worley, M.¹, Kelleher, A.D.^{2,3}, Kent, S.J.^{1,4}, Chung, A.W.¹

¹University of Melbourne, Peter Doherty Institute for Infection and Immunity, Department of Microbiology and Immunology, Melbourne, Australia, ²University of New South Wales / Kirby Institute, Sydney, Australia, ³St. Vincent's Centre for Applied Medical Research, Immunovirology Laboratory, Sydney, Australia, ⁴Monash University, Melbourne Sexual Health Centre and Department of Infectious Diseases, Melbourne, Australia

Background: Functional antibodies have been shown to mediate innate immune effector responses such as antibody dependent cellular cytotoxicity (ADCC) and their importance in HIV protection was highlighted by the RV144 HIV vaccine trial. However, the majority of studies have focused on antibody activation of NK cells and monocytes, while neutrophils remain understudied. Neutrophils are of particular importance as they are abundantly present in the mucosal sites of HIV transmission, constitute 40-70% of white blood cells and can respond rapidly to stimuli. We investigated the repertoire of antibody dependent functions in HIV infected patients and examined any differences between long-term slow progressors (LTSP) and progressors.

Methods: Fresh human blood neutrophils, NK and monocytes were evaluated for gp120 envelope-specific ADCC using HIV-specific antibodies. In addition, antibody-dependent phagocytosis (ADP) and ADCC activity was evaluated using plasma IgG from 19 LTSP and 14 HIV progressors.

Results: Neutrophils readily mediated HIV-specific ADCC and ADP and significant correlations were observed for LTSP $R_5=0.626$ ($p=0.004$) and progressors $R_5=0.653$ ($p=0.014$). Neutrophils mediated ADCC rapidly having 2 fold greater responses than NK cells and similar responses to monocytes. Similar levels of ADP and ADCC were observed in neutrophils between the LTSP and progressors patient groups.

Conclusion: HIV-specific IgG can activate neutrophils to mediate ADP and ADCC against HIV-1 envelope protein. As both of these functions are highly correlated, the same Fc receptors/antibodies may be utilised to mediate these responses. The rapid action and high magnitude of ADCC by neutrophils highlights their potential importance early in HIV infections.

2613

"HIV-1 controllers"- what's their secret?

Madhavi, V.¹, Wines, B.², Emery, S.³, Amin, J.³, Kelleher, A.³, Hogarth, M.², Stratov, I.^{1,4}, Kent, S.^{1,4,5}

¹University of Melbourne, Peter Doherty Institute for Infection and Immunity, Microbiology and Immunology, Melbourne, Australia, ²Burnet Institute, Centre for Biomedical Research, Melbourne,

Australia, ³Kirby Institute for Infection and Immunity in Society, University of New South Wales, Sydney, Australia, ⁴Melbourne Sexual Health Clinic, Carlton, Melbourne, Australia, ⁵Alfred Hospital, Infectious Diseases Unit, Prahran, Melbourne, Australia

Objectives: HIV-1 elite controllers (ECs) offer a unique opportunity to explore protective immune effector mechanisms. HIV-1-specific antibody-dependent cellular cytotoxicity (ADCC) has been shown to play an important role in protection from HIV-1 infection in various studies including the RV144 vaccine trial. However, evidence for HIV-1 Env and non-Env antigen-specific ADCC role in protection from disease progression among HIV-1-infected individuals including ECs is limited.

Methods: We recruited 22 HIV-1 ECs (< 20 copies RNA/ml) and 44 HIV-1 progressors (>10,000 copies RNA/ml) in order to compare possible immune correlates of HIV-1 control among ECs. We measured HIV-1 Env-specific Fc-mediated antibody effector functions using a novel ELISA-based Fc-receptor-binding assay and rapid fluorometric ADCC-mediated killing assay (RFADCC). We also measured HIV-1 Vpu-specific ADCC responses using Fc-receptor-binding assays.

Results: We found high levels of HIV-1 gp140-specific FcγRIIIa-binding antibodies in ECs compared to progressors ($p=0.001$). ADCC-mediated killing of HIV-1 gp140-coated targets was higher in ECs than progressors. FcγRIIIa binding of antibodies to Vpu was found to be higher in ECs compared to progressors ($p=0.002$). We have mapped Vpu-specific FcγRIIIa-binding antibodies to a 13-mer linear peptide (Vpu19) and found that ECs have higher FcγRIIIa-binding antibodies against Vpu19 compared to progressors ($p=0.004$).

Conclusions: Our data suggests role for HIV-1 Env- and Vpu-specific ADCC in elite immune control of HIV-1. Furthermore, Vpu19 is a common ADCC epitope in ECs. Attempts to isolate monoclonal ADCC antibodies are currently underway. Our findings have implications for understanding a role of ADCC in HIV-1 control and use of ADCC monoclonal antibodies as immunotherapeutics.

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Gut microbiome and immune activation in mucosal SHIV transmission

Sui, Y., Dzutsev, A., Venzon, D., Frey, B., Trinchieri, G., Berzofsky, J. National Cancer Institute, National Institute of Health, Bethesda, United States

It is unknown whether the gut microbiome affects HIV transmission. In our recent SHIV vaccine study, in which 42% vaccine efficacy ($p = 0.025$) was achieved, we found that the naïve rhesus macaques from two different sources had significantly different rates of infection against repeated low-dose intrarectal challenge with SHIVSF162P4 virus. Exploring causes, we found that the more susceptible group of 7 macaques (N7) in the original shipment (as well as the 21 vaccinated animals in pre-immunization rectal biopsies) had significantly more activated CD4⁺CCR5⁺Ki67⁺ T cells in the rectal mucosa than the more resistant group of 11 macaques from a different source. The prevalence of pre-challenge activated rectal CD4 T cells in the naïve macaques correlated inversely

with the number of challenges required to infect. The groups also differed significantly by principal component analysis of activation markers, with only N7 matching the vaccinated group. Because the two naïve groups came from different sources, we hypothesized that their microbiomes may differ and might explain the activation difference. Indeed, after sequencing 16s rRNA, at the phylum level and genus level, we found differences between the two naïve groups that correlated with immune activation status. For example, significantly lower ratios of *Bacteroides* to *Prevotella*, and significantly lower levels of *Firmicutes* were found in the susceptible cohort. These parameters also inversely correlated with high levels of immune activation in the rectal mucosa. Thus, host-microbiome interactions might influence the HIV/SIV mucosal transmission through effects on mucosal T-cell activation.

3825

Ablating cellular inhibitors of Apoptosis (cIAPs) specifically sensitizes HIV-infected CD4⁺ T cells to death, reducing viraemia and immune attrition in humanized mice

Allison, C.¹, Preston, S.¹, Mota, T.², Stutz, M.¹, Cheong, K.², Lewin, S.², Pellegrini, M.¹

¹Walter and Eliza Hall Institute of Medical Research (WEHI), Infection and Immunity, Melbourne, Australia, ²Peter Doherty Institute for Infection and Immunity, Melbourne, Australia

Background: Inhibitor of Apoptosis (IAP) molecules are pro-survival proteins, which confer resistance to inflammatory death-inducing molecules, such as TNF and TRAIL. IAP down-regulation thereby enhances cellular susceptibility to apoptosis upon exposure to such stimuli. Modulating IAP expression during chronic infection is a novel strategy to eliminate infected cells.

Methods: We treated HIV-infected primary human CD4⁺ T cells *in vitro* with a IAP antagonist, *birinapant*, which selectively induces the degradation of cellular IAPs, cIAP1 and cIAP2. These experiments were complemented with *in vivo* birinapant treatment of HIV-infected humanised (CD34⁺ human hematopoietic-reconstituted NOD.SCID.IL2gnull) mice. Plasma and cellular viral titres were assessed by qPCR.

Results: Birinapant induced the rapid and preferential killing of infected over uninfected CD4⁺ T cells, and a concomitant reduction in infectious virus. This heightened sensitivity is most likely due to amplified TNF signaling, as we observed enhanced TNF receptor expression on HIV-infected CD4⁺ T cells. We found that treating humanised mice with as few as two doses of birinapant one week after HIV infection induces sustained reductions in viral load. We hypothesise that this is achieved by the rapid apoptosis of infected cells early after treatment, thereby limiting viral spread and disease progression.

Conclusion: This is the first time that modulating IAP expression has been shown to be effective for the selective killing of HIV-infected cells. Our humanized mice data affirms the utility of this approach *in vivo*, warranting further investigation into the ability of cIAP inhibition to sensitize all HIV-infected cells, particularly the latent reservoir, to apoptosis.

4117

Overexpression of total-STAT1 during lymphopenia-induced proliferation leads to diminished CD4 T cell survival: implications in HIV infection

Le Saout, C.¹, Luckey, M.², Villarino, A.³, Smith, M.¹, Myers, T.⁴, Hasley, R.¹, Park, J.-H.², JJ, O.³, Lane, H.C.¹, Catalfamo, M.¹

¹NIH, NIAID, CMRS/Laboratory of Immunoregulation, Bethesda, United States, ²NIH, NIAMS, Bethesda, United States, ³NIH, NCI, CCR, Experimental Immunology Branch, Bethesda, United States, ⁴NIH, NIAID, Genomic Technologies Section/Research Technologies Branch, Bethesda, United States

During HIV infection the CD4 T cell pool is in constant homeostatic pressure because of its depletion. This effect is evident by the association between the rate of proliferating CD4 T cells and CD4 counts, which is also observed in healthy volunteers. In contrast, such associations are not observed in the CD8 T cell pool of patients or healthy controls suggesting that CD4 and CD8 T cell pools are differentially regulated and/or intrinsic characteristics contribute to the homeostatic regulation of these pools. In a model of adoptive T cell transfer into RAG^{-/-} mice, T cells undergoing IL-7 dependent lymphopenia-induced proliferation (LIP), upregulate total STAT1 (t-STAT1) expression leading to an IL-7-dependent STAT1 signaling in addition to STAT5. In patients with HIV infection t-STAT1 expression levels in CD4 T cells correlate with the degree of lymphopenia and showed phosphorylation of STAT1 in addition to STAT5. The IL-7-dependent STAT1 signaling is not very well described. To address the potential involvement of this pathway in human disease, we generated a STAT1 transgenic (STAT1-Tg) mouse to understand the contribution of IL-7-dependent STAT1 activation on T cell homeostasis in the setting of lymphopenia. RNAseq data of *in vitro* IL-7 stimulated CD4 cells showed IFN- γ associated genes. *In vivo*, CD4 T cells from STAT1-Tg mice upregulate the levels of t-STAT1 when undergoing LIP. In these conditions, CD4 T cells showed diminished survival and accumulation in the lymphoid organs. This mechanism maybe involved in regulating the expansion of the CD4 T cell pool under lymphopenic conditions.

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Prime-boost immunisation strategies with recombinant HIV vaccines induces vaginal mucosa HIV-specific CD8 T-cells

Tan, H.-X.¹, Alcantara, S.¹, Amarasena, T.¹, Ranasinghe, C.², Kent, S.¹, De Rose, R.¹

¹The University of Melbourne, at the Peter Doherty Institute for Infection and Immunity, Department of Microbiology and Immunology, Melbourne, Australia, ²John Curtin School of Medical Research, Australian National University, Division of Immunology and Genetics, Canberra, Australia

Cervico-vaginal HIV-1 infection remains localised for several days, providing a short window for immune clearance before systemic dissemination. We explore vaccine regimens using recombinant vectors expressing HIV-1 proteins to induce HIV-specific CTLs in the vaginal mucosa.

Utilising recombinant influenza viruses encoding whole HIV-1 p24 protein (Flu-p24), we performed heterosubtypic (H1N1-H3N2) intranasal prime and intravaginal boost vaccinations

in BALB/c mice. HIV-specific CTLs were induced in the vaginal mucosa at 6% of total vaginal CD8⁺T-cells. At 14-days post-boost, HIV-specific CTLs were present at 1 in every 1000 nucleated cell of the vaginal mucosa, as quantified by immuno-fluorescence microscopy. HIV-specific CTLs were present at 30-days post-boost, albeit with a 2-fold reduction vs. 14-days post-boost. Spatial analysis revealed that 70% of CD8⁺ T-cells induced were localised in the epithelial lining of the vaginal mucosa. 40% of CD8⁺ T-cells maintained CD103⁺ expression at 14- and 30-days post-boost. The concurrent expression of CD103⁺ and epithelial localisation indicate that a proportion of these cells are vaginal-resident CTLs.

We investigated an alternative heterologous prime-boost intranasal vaccination, combined with an intravaginal treatment to enhance vaginal CTL recruitment. Mice were primed with a Gag-expressing fowlpox virus, followed a Flu-p24 booster. To enhance recruitment or “pull” CTLs into the vaginal mucosa, an inflammatory stimulus (nonoxynol-9) was concurrently administered intravaginally during boosting. Nonoxynol-9 induced a 1.5-fold increase in HIV-specific CTLs vs. a mock intravaginal treatment

($p < 0.05$).

These mucosal prime-boost strategies establish frontline vaginal-resident CTLs and may induce heightened local surveillance for protection against vaginal HIV-1 exposure.

1552

CX3CR1+ CD8 T cells promote monocyte activation and cardiovascular risk

Freeman, M.¹, Panigrahi, S.¹, Mudd, J.^{1,2}, Sieg, S.¹, Funderburg, N.³, Lederman, M.¹, Zidar, D.⁴

¹Case Western Reserve University, Cleveland, United States, ²National Institute of Allergy and Infectious Diseases, Bethesda, United States, ³Ohio State University, Columbus, United States, ⁴University Hospitals, Cleveland, United States

HIV-infected individuals sustain elevated inflammation that is linked to increased risk of cardiovascular disease despite virus suppression with antiretroviral therapy (ART). This inflammatory state is accompanied by expansion of circulating memory CD8 T cells. Also seen is increased platelet activation and sustained increases in proportions of inflammatory and procoagulant monocytes that is similar to the changes seen in uninfected persons with acute coronary syndromes. Recently we and others identified co-localization of macrophages and CD8⁺ T cells in the aortic endothelium of SIV-infected macaques. Because inflammatory cytokines such as IFN γ can exert a “priming” influence on monocytes, leading to exaggerated production of cytokines such as TNF in response to LPS stimulation, we hypothesized that TCR-stimulated CD8 T cells could exert a pro-atherosclerotic effect via paracrine actions on monocytes. Here we identify a population of circulating memory CD8 T cells that express CX3CR1 (fractalkine receptor) that is enriched in ART-treated HIV-infected subjects. CX3CR1⁺ CD8 T cells are the major IFN γ -producing CD8 T cell population and have elevated expression of the platelet-binding receptor PSGL-1 and the thrombin-activated receptor PAR-1, suggesting that these endothelial homing CD8 T cells could interact with coagulation

elements. Additionally, TCR-stimulated CD8 T cells enhance monocyte activation and cell surface tissue factor expression that is dependent on the inflammatory cytokine TNF *in vitro*. Thus, we have identified a link among activated CD8 T cells, platelets, and monocytes that could provide a mechanistic pathway for elevated cardiovascular risk in ART-treated HIV infection.

3305

Immunological efficacy of therapeutic vaccination in chronically HIV-1 infected patients is impacted by the process and the route of vaccine administration

Brezar, V.^{1,2,3}, Hani, L.^{1,2,3}, Surenaud, M.^{1,2,3}, Lacabartz, C.^{1,2,3}, Lelièvre, J.D.^{1,2,3,4}, Lévy, Y.^{1,2,3,4}, Seddiki, N.^{1,2,3}

¹INSERM U 955 Eq 16, Créteil, France, ²Université Paris-Est Créteil, Faculté de Médecine, Créteil, France, ³Vaccine Research Institute (VRI), Créteil, France, ⁴AP-HP, Hôpital H. Mondor - A. Chenevier, Service d'Immunologie Clinique et Maladies Infectieuses, Créteil, France

The role of regulatory T cells (Tregs) in vaccination has been poorly investigated. We have recently reported that vaccination with ex vivo-generated dendritic-cells (DC) loaded with HIV-lipopeptides (LIPO-5-DC vaccine) given subcutaneously to HIV-infected patients was well tolerated and highly immunogenic (Y. Levy et al, EJI 2014).

We show here that vaccinees (n=14) who displayed lower levels of HIV-specific CD4⁺CD134⁺CD25⁺CD39⁺FoxP3⁺ Tregs responded better to the LIPO-5-DC vaccine. After vaccination, the frequency of HIV-specific Tregs decreased (from 69.3 at week -4 to 31.7% at week 16) and inversely correlated with HIV-specific IFN- γ -producing cells (r=-0.64, p=0.002).

We evaluated LIPO-5-specific Tregs responses in another therapeutic immunization study (ANRS093) that combined two different vaccines (recombinant ALVAC-HIV (vCP1433) and Lipo-6T (HIV-1 lipopeptides)). In contrast to the former, this vaccine was not loaded on DC but was administered to patients intramuscularly (n=18). Our data revealed that the frequency of LIPO-5-specific Tregs CD4⁺CD134⁺CD25⁺CD39⁺FoxP3⁺ was similar to baseline (34.22 at week 0 and 40.4% at week 16) and there was no skewing from regulatory to effector phenotype as we observed in the LIPO-5-DC vaccination. Importantly, we also observed that HIV-specific CD4⁺CD134⁺CD25⁺CD39⁺FoxP3⁺ correlated positively with viral load after treatment interruption (ATI).

Taken together our data suggest that the process and the route of therapeutic immunization with HIV-lipopeptides in chronically infected patients impacts on the quality of the responses by shifting the balance between regulatory and effector responses thus leading to efficiency of viral control.

2956

Bad romance: defining the molecular link between *Neisseria gonorrhoeae* and HIV

Xu, S.X.¹, Leontyev, D.², Kaul, R.², Gray-Owen, S.D.¹

¹University of Toronto, Molecular Genetics, Toronto, Canada,

²University of Toronto, Department of Medicine, Toronto, Canada

Human immunodeficiency virus (HIV) is a sexually transmitted infection affecting millions of people worldwide. With no existing cure and development of resistance against current antiviral drugs, there is a pressing need to reduce transmission of the virus and limit disease progression to acquired immune deficiency syndrome (AIDS). Clinical observations show that gonorrhea infection increases HIV shedding and transmission, and enhances an individual's susceptibility to HIV infection upon exposure. While *in vitro* studies of interactions between HIV and *Neisseria gonorrhoeae* (Ngo) have elucidated processes with the potential to drive transmission, these have not been considered *in vivo* because HIV and Ngo are both human-restricted pathogens. Here, we describe the development and initial findings from the first animal model of HIV and Ngo co-infection, using immunodeficient NSG mice reconstituted with a human immune system via hematopoietic stem cell transplantation. While it had not previously been appreciated, we observe that systemic HIV infection results in local shedding of virus in the female genital tract of humanized mice, reflecting the phenomenon seen in humans. While plasma levels of virus were not notably impacted by vaginal Ngo infection, the co-infected mice showed elevated shedding of HIV into the genital tract. Ongoing work aims to reveal the relative contribution of Ngo-derived factors and infection-induced inflammatory responses to this localized increase in HIV within the co-infected mucosa. Moreover, this model lays the foundation for future work aiming to intervene in the pathogenic synergy between these two devastating pathogens.

T Cell Memory

1137

T cell exhaustion is reinforced by progressive de novo DNA methylation programming

Youngblood, B.¹, Ghoniem, H.¹, Abdelsamed, H.¹, Carter, R.¹, Hale, S.², Ahn, E.², Im, S.², Ahmed, R.²

¹St Jude Children's Research Hospital, Immunology MS 351, Memphis, United States, ²Emory University School of Medicine, Atlanta, United States

Antigen-specific CD8 T cells play a critical role in controlling chronic infections and cancer, but progressively lose their effector functions during prolonged antigen exposure. Repression of CD8 T cell effector functions, commonly referred to as T cell exhaustion, limits the ability of the immune system to purge the chronic pathogen from the host. It has recently become recognized that CD8 T cell exhaustion programs can be reinforced and heritably maintained. Therefore in order to develop and/or improve current therapeutic approaches that utilize host antigen-specific T cells to treat chronic infections or cancer a major challenge for the field is to identify mechanisms that stabilize T cell exhaustion. We have recently found that epigenetic modifications acquired in pathogen-specific CD8 T cells during prolonged antigen exposure reinforce T cell exhaustion. Using the LCMV model system of chronic viral infection we investigated the role of Dnmt3a mediated de novo DNA methylation in regulating CD8 T cell exhaustion. Strikingly, conditional deletion of Dnmt3a in activated CD8 T

cells blocked the cells from becoming exhausted. Longitudinal analysis of whole-genome methylation programming of WT and Dnmt3a cKO CD8 T cells from chronically infected animals reveals progressive acquisition of Dnmt3a-dependent DNA methylation programs in genes, including interferon gamma, that are coupled to the progressive decline of effector functions. These results have significant implications for therapeutic strategies that utilize reactivation of host pathogen-specific CD8 T cells to control chronic viral infections or cancer and provide a nucleotide-resolution map of epigenetic programs progressively acquired during T cell exhaustion.

2390

IL-15 complexes induce migration of resting memory CD8 T cells into mucosal tissues

Marzo, A., Sowell, R., Rogozinska, M.

Rush University Medical Center, Chicago, United States

CD8 T cells are key players in our immune system's defense against infectious diseases and tumors. However, we do not fully understand how CD8 T cells enter and take up residence in mucosal tissues, to protect against mucosa-acquired infections. We sought to identify potential targets to therapeutically enhance CD8 T cell responses within these sites of frequent pathogen exposure. Our study reveals a novel mechanism by which IL-15, a cytokine responsible for the maintenance of memory CD8 T cells, also elicits migration of resting memory CD8 T cells to mucosal tissues. Interleukin-15 (IL-15) is an essential cytokine known to promote T cell survival and activate the effector function of memory phenotype CD8 T cells. Blocking IL-15 signals also significantly impacts tissue specific effector and memory CD8 T cell formation. Here we demonstrate that IL-15 influences the generation of memory CD8 T cells by first promoting their accumulation into mucosal tissues and secondly by sustaining expression of Bcl-6 and T-bet. We show that the mechanism for this recruitment is largely dependent on mTOR and its subsequent inactivation of FoxO1. Last, we show that IL-15 complexes delivered locally to mucosal tissues without re-infection is an effective strategy to enhance establishment of Tissue Resident Memory (T_{REM}) CD8 T cells within mucosal tissues. This study provides mechanistic insight into how IL-15 controls the generation of memory CD8 T cells and influences their trafficking and ability to take up residence within peripheral tissues.

2982

Distinct mechanisms govern resident memory T cell differentiation and survival in different tissues

Wynne-Jones, E., Freestone, D., Davies, B., Newman, D.M., Carbone, F.R., Mackay, L.K.

The University of Melbourne, Microbiology and Immunology, Parkville, Australia

Tissue-resident memory T (Trm) cells are a recently defined population of memory T cells that persist in peripheral tissues after infection, and contribute to effective local immunity. Trm cells have been identified in a number of peripheral tissues

including epithelial and solid organs. Transcription factors governing the development and maintenance of these cells are just beginning to be uncovered. Our data demonstrate that the transcriptional regulation of Trm cells is distinct to memory T cell subsets in the circulation, in that an essential process during Trm formation in the skin and lung is the down-regulation of the T-box transcription factors Eomes and T-bet. Further, we found that some residual T-bet expression was required for Trm cell survival in these organs. Intriguingly, we found that the dependence of Trm cells on transcription factors varied according to anatomic location. In CD103⁺ CD69⁺ Trm cells, expression of the transcription factor T-bet was absolutely required in some organs, whereas in other organs it was dispensable. Lack of T-bet also resulted in altered cytokine production, with Trm cells making a switch to produce IL-17. Additionally, we found that Trm cells in different tissues have distinct cytokine requirements for their survival. These data demonstrate adaptation of Trm cells to specific tissue microenvironments, and highlight the importance of studying these cells in a variety of tissues. In future, such insights will inform approaches used to target Trm cells in various clinical settings.

4196

Intravascular surveillance by terminally differentiated effector and memory CD8⁺ T cells

Barreiro, O., Loughhead, S., Gerlach, C., Wanders, L., Zhang, Z., von Andrian, U.H.

Harvard Medical School, Department of Microbiology and Immunobiology, Boston, United States

Following infection, pathogen-specific T cells proliferate and divide in a heterogeneous manner, giving rise to effector and memory T cells with distinct migratory patterns. This migratory division of labor ensures that the host is efficiently scanned for ongoing or recurring infections. Recent evidence suggests that the presumed migratory behavior of the classical effector memory T cell (Tem) subset needs revision, as Tem appear to be unexpectedly absent from the extravascular compartment in non-lymphoid tissues. Here, we report that terminally differentiated effector and memory T cells adhere to and patrol dermal endothelium, while less differentiated T cells do not. This behavior is preferentially observed in arterioles and migration tends to be retrograde to blood flow. Furthermore, patrolling T cells survey endothelium for antigen (Ag) as injection of cognate Ag results in their immediate arrest. Together, this suggests that terminally differentiated effector and memory T cells engage in a migratory behavior that supports protective scanning of specific vascular beds.

2942

The role of IL-36 in the immune response to influenza infection

Wein, A.¹, Dunbar, P.¹, McMaster, S.¹, Hayward, S.¹, Denning, T.², Kohlmeier, J.¹

¹Emory University School of Medicine, Atlanta, United States,

²Georgia State University, Institute for Biomedical Sciences, Atlanta, United States

The IL-36 family of cytokines is a recently discovered and little-understood relative of IL-1. IL-36 activation plays an important role in the development of psoriasis, tumor rejection, and asthma; however, its role in respiratory viral infection has not been studied. We have found that IL-36 family cytokines are upregulated during influenza infection in mice and the main source of IL-36g is neutrophils. Mice lacking IL-36g or IL-36R have increased viral titers following influenza infection and mice deficient in IL-36g show delayed viral clearance. In addition, mice without IL-36g or IL-36R have increased pathology during influenza infection compared to WT mice. During the early response to influenza, there is a defect in innate immune cell recruitment to the lungs, particularly neutrophils. Following clearance of infection, virus-specific CD8+ T cell memory generation and recall is impaired. These results point to important and diverse roles for IL-36 cytokines in the immune response to influenza as well as the generation of long-lived cellular immunity to respiratory virus infections.

832

Stage-specific role for FoxO1 in the maintenance of T-cell memory

Suresh, M., Tejera, M.M., Neldner, B.

University of Wisconsin-Madison, Pathobiological Sciences, Madison, United States

During CD8 T-cell responses to acute viral infections, naïve CD8 T cells are activated to expand and differentiate into effector cells and a subset of effector cells further differentiate into a self-renewing population of memory CD8 T cells. Specific transcription programs guide the successive progression of responding CD8 T cells through various differentiation states (naïve → effector → memory) during a T-cell response. We and others have identified FoxO1 as a key transcription factor that regulates the differentiation of memory CD8 T cells. However, the temporal requirement for FoxO1 during various phases of the CD8 T-cell response remains unknown. In this study, by inducible ablation of FoxO1 in mixed bone marrow chimeras, we investigated the temporal and cell-intrinsic requirement for FoxO1 in the generation and maintenance of T-cell memory. We find that full expression of key surface molecules including CD44, CD62L and CD127 in memory CD8 T cells required continuous activity of FoxO1. Likewise, FoxO1 plays a vital and non-redundant role in maintaining the polyfunctionality of memory CD8 and CD4 memory T cells and inducing full expression of TCF-1 through out the T-cell response. Additionally, during the contraction phase, FoxO1 represses the expression of effector molecules such as granzyme B and facilitates effector-to-memory transition and functional maturation of memory T cells. Taken together, these findings suggest a non-redundant stage-specific role for FoxO1 in the transcriptional regulation of effector-to-memory transition and maintenance of T-cell memory.

4441

How environment alters immunity: comparing immune systems of children from low- and high-income countries

Wagar, L.E.¹, Bolen, C.R.¹, Sigal, N.², Haque, R.³, Parsonnet, J.⁴, Petri Jr, W.A.⁵, Davis, M.M.^{1,2,6}

¹Stanford University, Department of Microbiology and Immunology, Stanford, United States, ²Stanford University, Institute for Immunity, Transplantation and Infection, Stanford, United States, ³International Center for Diarrhoeal Disease Research, Department of Enteric and Respiratory Infections, Dhaka, Bangladesh, ⁴Stanford University, Department of Medicine, Division of Infectious Diseases and Geographic Medicine, Stanford, United States, ⁵University of Virginia, Department of Microbiology, Immunology and Cancer Biology, Charlottesville, United States, ⁶Howard Hughes Medical Institute, Stanford, United States

The first five years of a child's life are a period of both rapid immune development and increased risk of morbidity and mortality due to infectious etiologies. Lifelong health depends on successful tuning of responses against a wide variety of pathogens and innocuous environmental stimuli encountered during this time. However, relatively little is currently known about the environmental effects on the development of the pediatric immune system. We aimed to understand how a high pathogen burden early in life could alter immune responses and the development of lymphocyte memory. To this end, we performed a high dimensional, 49-parameter mass cytometry (CyTOF) analysis to deeply phenotype unstimulated and ex-vivo stimulated PBMCs from children under five years old from Dhaka, Bangladesh and the San Francisco Bay Area, USA. We find that very early in life (less than one year old), Bangladeshi and American children's lymphocytes are phenotypically and functionally very similar. However, beginning at one year, there is considerable variation in the ability of Bangladeshi children's T, B, and NK cells to respond to restimulation, with many children producing poor pro-inflammatory cytokine responses. We also find that T cell differentiation and cytokine responses within the Bangladeshi cohort correlate with very early CMV infection and recurrent diarrheal illness early in life. These data suggest that early environmental exposure can have a substantial impact on pediatric immune development, and that this factor should be considered when implementing immunization and health strategies in low-income countries.

1003

Local boosting with 4-1BBL induces long-lived lung parenchymal and circulating memory populations that protect against influenza infection

Zhou, A.C., Wagar, L.E., Wortzman, M.E., Watts, T.H.

University of Toronto, Immunology, Toronto, Canada

CD8 T cell immunity against conserved internal viral proteins can confer heterotypic protection against influenza infection when antibody responses are lost due to viral mutations. However, even the induction of flu-specific T cell memory by live infection is transient, particularly in the lung, and immune senescence has been observed with age. To induce long-lived protective cellular immunity, we stimulated the TNFR-family

member 4-1BB in the airways to boost secondary CD8 T cell responses in a prime-boost immunization regimen. Mice were infected with influenza A/HK-X31 and boosted intranasally one-month later with replication-deficient adenovirus encoding influenza nucleoprotein (NP) alone (Ad-NP) or NP with 4-1BBL (Ad-NP-4-1BBL). Local boosting with Ad-NP-4-1BBL induced remarkably long-lived polyfunctional NP-specific CD8 T cell memory populations both within the lung parenchyma and the systemic circulation. Mixed bone marrow chimeras showed this effect to be CD8 T cell intrinsic through the 4-1BB-4-1BBL pathway. Ad-NP-4-1BBL protected mice from lethal heterotypic flu challenge for 9-11 months post-boost at a dose where Ad-NP had minimal efficacy. This may be due to local lung-parenchymal CD103⁺CD69⁺ and CD103⁺CD69⁺ memory populations that persisted for months after Ad-NP-4-1BBL-boost, but not after Ad-NP where parenchymal memory cells were undetectable after 60 days. In addition, Ad-NP-4-1BBL induced a long-lived effector-like circulating CD62L-TCF-1^{lo}T-bet^{hi}Eomes^{lo} memory population that exhibited high IL-7R expression, which might contribute to the longevity of the response. Further investigation of these long-lived tissue and circulating effector memory populations can inform vaccine design and identify early biomarkers of an effective boost.

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2623

Long-lived resident CD4⁺ Th2 memory cells cause allergen-induced exacerbations of allergic asthma

Kazemi, S., Bošnjak, B., Altenburger, L.M., Mokrović, G., Epstein, M.M. Medical University of Vienna, Department of Dermatology, DIAID, Experimental Allergy Lab., Vienna, Austria

Allergic asthma is a chronic disease characterized by lung inflammation and allergen-induced exacerbations. Using a mouse model that mimics the relapsing-remitting nature of allergic asthma, we discovered that long-term Th2 memory CD4⁺ T cells reside in the lungs of mice recovered from the initiation of disease. They are found in long-lived lung inflammatory infiltrates for the entire lifetime of the mouse and immediately respond to secondary allergen challenge causing the features of allergic asthma. To further characterize recovered mice, we administered anti-CD4 (GK1.5) antibodies to *in vivo* deplete CD4⁺ T cells with the aim of abrogating allergen-induced disease relapse. After depletion and following administration of a fluorescence-labeled anti-CD4 (RM4-4), we found a population of 'protected' cells located within lung inflammatory infiltrates that resisted depletion and were allergen-specific Th2 memory cells. Furthermore, the protected cells resisted FTY720 treatment, expanded within lung infiltrates upon allergen rechallenge and were responsible for relapses of allergic asthma up to 636 days following disease initiation. Taken together, our data reveal a crucial pathogenic population of long-lived resident Th2 memory cells that cause allergen-induced asthma relapses, explain why most patients with severe allergic asthma do not respond to anti-CD4 treatment, and provide an important target for therapeutic intervention.

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Unraveling the dynamics of fate in emergency haematopoiesis using cellular barcoding

Lin, D., Gao, J., Hodgkin, P., Naik, S. WEHI, Melbourne, Australia

Recent single cell tracking studies demonstrate a great deal of fate heterogeneity within the haematopoietic stem and progenitor cell population under steady-state conditions. However, the regulatory mechanisms of single cell fate during emergency conditions such as infection or cytokine exposure (where specific cell types increase in number) are largely unknown. This can theoretically occur either through greater clonal expansion of pre-existing progenitors, and/or emergency recruitment of progenitors. The latter might occur at the expense of other lineages through 'lineage diversion' or via recruitment of otherwise dormant progenitors.

To understand which of these scenarios accounts for fate's highlight the intricacies of how individual progenitors modify their behavior during demand-adapted haematopoiesis.

3253

Increased Src kinase activity leads to chronic obstructive pulmonary disease (COPD) and lung cancer

Lau, M.¹, Tsantikos, E.¹, Maxwell, M.¹, Duan, E.², Ernst, M.³, Hibbs, M.⁴ ¹Monash University, Immunology, Melbourne, Australia, ²La Trobe University, Melbourne, Australia, ³Olivia Newton-John Cancer Research Institute, Melbourne, Australia, ⁴Monash University, Melbourne, Australia

Members of the Src family of kinases especially Hck, Lyn and Fgr, which are highly expressed in myeloid cells, are known for their roles in innate immunity. Increased Lyn and Hck activity elevates the magnitude of a classical innate immune response elicited by LPS. However, inappropriate activity by Src kinases can result in other severe consequences. Specifically in the lung, the increase of Hck activity in Hck^{F/F} mice results in spontaneous lung inflammation and multiple phenotypes resembling COPD. Increased Lyn activity in Lyn^{up/up} mice not only causes COPD traits but also increases lung cancer susceptibility. Lung inflammation in Lyn^{up/up} mice was found to be driven by the lung epithelium. Although not considered an immune cell, epithelial cells are especially important in lung immunity against infection and injury. Further characterisation showed early sign of epithelial to mesenchymal cell transition and upregulation of EGF-receptor expression in the lung, providing a mechanism for cancer progression. The pathways that predispose COPD patients to lung cancer is still a mystery and our study has uncovered a possible underlying mechanism. By contrast, lung inflammation and COPD traits in Hck^{F/F} mice were immune cells driven. However, depleting neutrophils alone resolved airspace enlargement but not mucus cell metaplasia. This suggests that different traits of COPD are contributed by different processes of the multi-faceted lung inflammation in Hck^{F/F} mice. As the roles of Src kinases in COPD development is only recently appreciated, our unique mouse models has allow

better understanding of this emerging new signaling networks that drives COPD.

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Interleukin-3 amplifies acute inflammation and is a potential therapeutic target in sepsis

Weber, G.F.^{1,2}, Chousterman, B.G.², He, S.², Fenn, A.M.², Nairz, M.², Anzai, A.², Brenner, T.³, Uhle, F.³, Iwamoto, Y.², Robbins, C.S.², Noiret, L.², Maier, S.L.⁴, Zönnchen, T.⁴, Rahbari, N.N.⁴, Schölch, S.⁴, Klotasche-Von Ameln, A.⁵, Chavakis, T.⁵, Weitz, J.⁴, Hofer, S.³, Weigand, M.A.³, Nahrendorf, M.², Weissleder, R.², Swirski, F.K.²

¹Universitätsklinikum Erlangen, FAU Erlangen-Nürnberg, Department of Surgery, Erlangen, Germany, ²Massachusetts General Hospital/Harvard Medical School, Center for Systems Biology, Boston, United States, ³Universitätsklinikum Heidelberg, Ruprecht-Karls-Universität Heidelberg, Department of Anaesthesiology, Heidelberg, Germany, ⁴Universitätsklinikum Dresden, Technische Universität Dresden, Department of Surgery, Dresden, Germany, ⁵Universitätsklinikum Dresden, Technische Universität Dresden, Department of Clinical Pathochemistry, Dresden, Germany

Purpose: Sepsis is a frequently fatal condition characterized by an uncontrolled host reaction to microbial infection claiming millions of lives world wide every year. In the initial, inflammatory phase, the immune system overproduces cytokine-secreting leukocytes that can cause organ damage and death. Recent clinical trials targeting inflammatory mediators have failed, reflecting an urgent need for a better fundamental understanding of sepsis' pathophysiology.

Methods: In a translational approach we subjected IL-3^{-/-} and wild type (wt) mice to Cecal Ligation and Puncture (CLP), a mouse model of experimental sepsis. After extensive *in vivo* and *ex vivo* phenotyping we enumerated the emergency myelopoiesis using *in vivo* adoptive transfer and *in vitro* experiments. We identified the source of interleukin (IL)-3 using flow cytometric, RT-PCR and immunofluorescence analysis. Finally, we determined the importance of Interleukin-3 in human sepsis in an independent retrospective and prospective clinical trial.

Results: Here we show that IL-3 is an essential inducer of inflammation in sepsis. We show that innate response activator (IRA) B cell produced IL-3 amplifies myelopoiesis of Ly-6C^{high} monocytes and neutrophils, and potentiates the cytokine storm in sepsis. Compared to IL-3^{-/-} mice, wt animals develop multi-organ damage and succumb to infection more rapidly and in larger numbers. In patients with sepsis, high plasma IL-3 levels associate with high mortality even after adjusting for prognostic indicators.

Conclusions: Altogether, this study enriches our understanding of immune activation, and identifies IL-3 as an orchestrator of emergency hematopoiesis and potential therapeutic target for the treatment of sepsis.

1364

SMG1 is a regulator of toll-like receptor responses

James, A.¹, Luff, J.², Ho, U.³, Quek, H.², Lavin, M.², Roberts, T.^{1,4}

¹Western Sydney University, Ingham Institute for Applied Medical Research, Sydney, Australia, ²The University of Queensland, Centre for Clinical Research, Brisbane, Australia, ³QIMR Berghofer Medical Research Institute, Brisbane, Australia, ⁴Western Sydney University, Molecular Medicine Research Group, Sydney, Australia

Cells can stimulate innate immune responses by recognising the presence of conserved components of bacteria or viruses such as lipopolysaccharide, double-stranded RNA or unmethylated DNA. A key family involved in recognition of these molecules is the toll-like receptors (TLR). Activation of TLRs leads to a pro-inflammatory response characterised by activation of cell signalling cascades and cytokine production. Here we have identified the protein kinase Suppressor of Morphogenesis in Genitalia 1 (SMG1) as a regulator of TLR responses. SMG1 is a member of the PI3-Kinase like kinase family and has known roles in post-transcriptional regulation of gene expression and in DNA damage response. SMG1 has also been identified as a tumour suppressor potentially due to regulation of inflammatory pathways. SMG1 regulates both cellular signalling in response to TLR activation and also RNA metabolism. Loss of SMG1 has no effect on MAP kinases activation but enhanced NF- κ B dependent gene expression. Down-regulation of SMG1 also prevented regulation by nonsense mediated decay during an inflammatory response. Further SMG1 loss also affected feedback signalling via Type I interferon receptors and the subsequent expression of interferon stimulated genes.

2000

GM-CSF-induced expansion of phagocytes is sufficient to trigger demyelination and neurological deficits

Spath, S.¹, Komuczki, J.¹, Hermann, M.², Pelczar, P.^{2,3}, Schreiner, B.¹, Becher, B.¹

¹University of Zurich, Institute of Experimental Immunology, Zurich, Switzerland, ²University of Zurich, Institute of Laboratory Animal Science, Zurich, Switzerland, ³University of Basel, Center for Transgenic Models, Basel, Switzerland

Elevated frequencies of GM-CSF-producing helper T (Th) cells are consistently found in multiple sclerosis (MS) patients and GM-CSF expression is a non-redundant feature of pathogenic Th cells in preclinical models of MS. GM-CSF activates an inflammatory signature in monocytes, and their progeny are the most abundant cellular infiltrate in acute MS lesions. To model deregulated GM-CSF levels, we generated a transgenic mouse line allowing the induction of GM-CSF expression in mature, peripheral Th cells. This antigen-independent GM-CSF release induced severe neurological deficits with almost 100% penetrance, accompanied by the infiltration of inflammatory monocyte-derived phagocytes into the brain stem and spinal cord. These tissue-invading phagocytes contacted neuronal cell bodies and nerve fibers, produced reactive oxygen species (ROS) and caused demyelination. We propose that the CNS is particularly vulnerable to the infiltration of inflammatory phagocytes driven by deregulated GM-CSF production.

3066 HGK/MAP4K4 deficiency induces TRAF2 stabilization and Th17 differentiation leading to T-cell-mediated type 2 diabetes

Chuang, H.-C., Tan, T.-H.

National Health Research Institutes, Zhunan, Taiwan, Republic of China

Proinflammatory cytokines play important roles in insulin resistance. Here we report that T-cell-specific conditional HGK (MAP4K4) knockout (T-HGK cKO) mice developed systemic inflammation and insulin resistance, which was ameliorated by either IL-6 or IL-17 neutralization. HGK directly phosphorylated TRAF2 at Ser-35, leading to lysosomal degradation of TRAF2 and subsequent inhibition of IL-6 production. HGK kinase activity was decreased and TRAF2 protein levels were increased under TCR signaling, suggesting that HGK maintains a resting state of T cells. Thus, HGK knockout T cells constitutively displayed overexpression of TRAF2 and overproduction of IL-6.

IL-6-overproducing HGK knockout T cells were attracted to adipose tissue by the CCL20-CCR6 axis; the accumulation of HGK knockout T cells in adipose tissue was blocked by CCR6 knockout. In adipose tissue, IL-6 secreted from HGK knockout T cells enhanced the levels of the adipokine leptin. The data derived from T-cell-specific leptin receptor/HGK double knockout mice or IL-6 KO/HGK cKO mice demonstrated that IL-6-overproducing HGK knockout T cells further differentiated into Th17 cells by a synergistic effect of leptin and IL-6. Adoptive transfer experiments further showed that these IL-6/IL-17 double-positive T cells were pathogenic cells for insulin resistance. Clinical samples from type 2 diabetes (T2D) patients were used to study the clinical relevance. Notably, HGK levels fell and IL-6 levels increased in T cells from T2D patients. The clinical significance of HGK-deficient T cells will be presented in the meeting. Taken together, HGK plays important roles in the generation of adipose-tissue Th17 cells and the pathogenesis of T-cell-mediated T2D.

3686 Matrix Metalloproteinase-7 increases IL-8 release from primary lung fibroblasts derived from patients with/without chronic lung transplant rejection

Jaffar, J.^{1,2}, Symons, K.¹, Phan, T.^{1,2}, O'Hehir, R.^{1,2}, Glaspole, I.¹, Westall, G.^{1,2}

¹Monash University, Alfred Hospital, Allergy, Immunology and Respiratory Medicine, Melbourne, Australia, ²Monash University, Immunology and Pathology, Melbourne, Australia

Fibroblasts are the main producers of extracellular matrix (ECM) in the body and when dysregulated, turn normal wound healing into fibrosis, as seen in chronic graft rejection. In lung transplant patients with chronic graft rejection, pro-inflammatory interleukin (IL)-8 cytokine levels are elevated in bronchoalveolar lavage (BAL) and coincide with fibrosis. Matrix Metalloproteinase (MMP)-7 is an ECM degrading enzyme which has been shown to be elevated in fibrotic lung disease. However, its effect on lung fibroblasts is unknown. Primary lung fibroblasts from patients with and without

chronic rejection were stimulated with MMP7 with/without the pro-fibrotic cytokine transforming growth factor (TGF)- β 1. Expression of MMP7/IL-8 in blood, BAL, lung tissue, and fibroblasts was measured in healthy lungs and from patients undergoing transplantation for graft rejection. Transplant patients were followed post operation and repeat blood and BAL collection was performed at 3, 6, 12 months.

MMP7 increased IL-8 release into culture supernatant by lung fibroblasts at 72 hours post stimulation. MMP7 augmented TGF β 1-induced activation of fibroblasts as measured by α -smooth muscle actin production. MMP7 was detected in lung tissue and mainly colocalized with epithelial cells.

IL-8 is a major chemotactic factor for neutrophils, which hone to sites of wound healing in the lung. MMP7 is upregulated quickly after injury by epithelial cells. Patients with idiopathic pulmonary fibrosis, a lethally progressive disease, have elevated IL-8 and MMP7 levels. This is the first study to show that MMP7 may play a role in chronic lung rejection driven by increased IL-8 production by lung fibroblasts.

1014 Altered response of macrophages to an inflammatory stimulus in an allergic environment

Herbert, C., Chia, N., Garthwaite, L., Kumar, R.K.

UNSW Australia, Inflammation and Infection Research, School of Medical Sciences, Sydney, Australia

Exacerbations of asthma are associated with the production of interleukin-33 (IL-33) by airway epithelial cells, which activates many immune cells, including macrophages. The presence in an asthmatic airway of T_H2 cytokines, which are strongly associated with allergy, may contribute to the exaggerated airway inflammation. We hypothesised that the production of pro-inflammatory cytokines by IL-33-stimulated macrophages would be enhanced in an allergic cytokine environment, and that drugs which promote the resolution of inflammation, including resolvin E1 (RvE1), would suppress this enhanced response. Further, we hypothesised that these enhanced responses would be associated with altered expression of regulatory microRNAs (miRNAs). RAW264.7 cells were cultured with T_H2 cytokines (IL-4/IL-13) for 48 hrs, then stimulated with IL-33 for 4 hrs. In other experiments, cells were treated with RvE1 concurrently with IL-33. Pro-inflammatory mediators were assessed using quantitative real-time PCR (RT-PCR). Expression of miRNAs was explored using microarray analysis, RT-PCR and miRNA-mRNA prediction databases. In cells pre-treated with IL-4 and IL-13, expression of mRNA for *Ccl3*, *Ccl5*, *Ccl17*, *Ccl24* and *Il1b* was significantly elevated following IL-33 stimulation. RvE1 suppressed the enhanced production of *Ccl3*, *Ccl5*, *Ccl24* and *Il1b*. There was up-regulation of miR-155-5p and down-regulation of miR-106b-3p in response to T_H2 cytokines and IL-33 stimulation; these miRNAs are predicted to regulate aspects of allergic inflammation. We conclude that macrophages may contribute to the exaggerated airway inflammation in exacerbations of allergic asthma, and that RvE1 has potential as a therapeutic agent that targets macrophages. The mechanisms which regulate inflammatory cytokine production in asthma warrant further investigation.

1909

An alternatively spliced IL-15 isoform modulates cell signaling and promotes skin lesions in herpes simplex virus-1 zosteriform mouse model

Ku, C.-C., Chang, Y.-H., Lin, Y.-J., Chien, Y.

National Taiwan University, Taipei, Taiwan, Republic of China

Interleukine15 (IL-15) is the member of IL-2 family cytokines, signaling through the common γ chain (γ_c) and the shared IL-2 receptor β (IL-2/IL-15R β) to activate Jak3/Stat5, Ras/MAPK and PI3K/Akt pathways. While IL-15 is best known to support memory CD8⁺ T cells and NK cells, our previous study demonstrated novel functions of an alternatively spliced IL-15 isoform that has a partial deletion in exon 7 of the IL-15 gene (IL-15 Δ E7). IL-15 Δ E7 failed to activate phosphorylation of STAT5, reduced the level of IKK β phosphorylation and delayed the activation of JNK in *in vitro* experiments. These results show that IL-15 Δ E7 induces less optimal activation in treated cells. Ectopic expression of IL-15 Δ E7 in C57/BL6 wild type (WT) mouse skin profoundly blocked neutrophil infiltration to inflamed skin induced by abrasion, sodium dodecyl sulfate or imiquimod treatment, suggesting a regulatory role of IL-15 Δ E7 in mediating proinflammatory response. Furthermore, infection of IL-15 Δ E7 ectopically expressed mouse skin with HSV-1 resulted in a prolonged and earlier re-emerged zosteriform rash compared with mock expressed WT mice. While HSV-1 significantly induced expression of genes that encode for neutrophils recruiting (*Cxcl1*, *Cxcl2*, *Cxcl3* and *Cxcl5*), macrophages recruiting (*Ccl2*) chemokines, proinflammatory cytokines (*Il6*, *Il1b*) and HSV-1 induced T cell activation factor (*Tnfsf14*), they were all suppressed in HSV-1 infected IL-15 Δ E7-expressed skin. These results suggest that IL-15 Δ E7 modulates immune cell chemotaxis and anti-HSV-1 immunity in skin and affects reactivation of HSV-1 from the innervating neurons. The naturally occurring IL-15 Δ E7 serves as a potential therapeutic agent for treating recurrent cutaneous HSV-1 infection and inflammatory diseases.

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A new Toll-like receptor adaptor protein that provides specificity to inflammatory cytokine production from macrophages

Luo, L.¹, Bokil, N.¹, Lansdaal, N.¹, Wall, A.¹, Marceline, F.¹, Guo, Z.¹, Alexandrov, K.¹, Ross, I.¹, Hibbs, M.², Stow, J.¹, Sweet, M.¹

¹Institute for Molecular Bioscience, The University of Queensland, Brisbane, Australia, ²Monash University, Department of Immunology, Melbourne, Australia

Toll-like receptors (TLRs) relay downstream pro-inflammatory signalling responses through well-characterized adaptor proteins that contain Toll/Interleukin-1 receptor (TIR) domains. However, many cell types express TLRs and TIR-containing adaptor proteins, and the molecular mechanisms that enable heightened and selective TLR-inducible inflammatory responses in innate immune cells are not well understood. Herein, we describe a novel member of the transmembrane adaptor protein (TRAP) family, which is exclusively expressed by macrophages, dendritic cells and B cells. Using recombinant proteins, we show

that this non-TIR adaptor protein directly interacts with the TLR4 TIR domain. In macrophages, the interaction between this cell-surface adaptor and TLR4 is agonist (lipopolysaccharide, LPS)-induced. Using both gain- and loss-of-function approaches in primary macrophages, we demonstrate that the adaptor is essential for LPS-inducible IL-6 and IL-12 production, whereas this pathway does not regulate the production of other TLR-inducible inflammatory or regulatory cytokines. Moreover, the adaptor does not control TNF-inducible IL-6 and IL-12 production, thus demonstrating its selective role in TLR signalling. Mutations in the adaptor that abolish the interaction with TLR4 also abrogate LPS-inducible cytokine production. Mechanistically, the adaptor recruits the Lyn tyrosine kinase to enable agonist-induced TLR4 tyrosine phosphorylation, and subsequent pro-inflammatory signalling responses. We thus define a new pathway that provides exquisite specificity to inflammatory cytokine outputs downstream of TLRs in innate immune cells. Given its specific cytokine targets (IL-6 and IL-12), this pathway is likely to be critical in shaping adaptive immune responses.

Mini Oral Sessions

15:30:00 - 16:30:00

NK Cells

739

Loss of DNAM-1 ligand expression by acute myeloid leukemia cells renders them resistant to NK cell killing

Kearney, C., Ramsbottom, K., Voskoboinik, I., Darcy, P., Oliaro, J.
The Peter MacCallum Cancer Centre, Melbourne, Australia

Acute myeloid leukemia (AML) is associated with poor natural killer (NK) cell function through aberrant expression of NK cell activating receptors and their ligands on tumor cells. These alterations are thought to promote formation of inhibitory NK-target cell synapses, in which killer cell degranulation is attenuated. Allogeneic stem cell transplantation can be effective in treating AML, through restoration of NK cell lytic activity. Similarly, agents that augment NK cell activating signals within the immunological synapse may provide some therapeutic benefit. However, the receptor-ligand interactions that critically dictate NK cell function in AML remain undefined. Here we demonstrate that CD112/CD155 expression is required for DNAM-1-dependent killing of AML cells. Indeed, the low, or absent, expression of CD112/CD155 on multiple AML cell lines resulted in failure to stimulate optimal NK cell function. Importantly, isolated clones with low CD112/155 expression were resistant to NK cell killing while those expressing abundant levels of CD112/155 were highly susceptible. Attenuated NK cell killing in the absence of CD112/CD155 originated from decreased NK-target cell conjugation. Furthermore, we reveal by time-lapse microscopy, a significant increase in NK cell 'failed killing' in the absence of DNAM-1 ligands. Consequently, NK cells preferentially lysed ligand-expressing cells within heterogeneous populations, driving clonal selection of CD112/CD155 negative blasts upon NK cell attack. Taken together, we identify reduced CD155 expression as a major NK cell escape mechanism in AML and an opportunity for targeted immunotherapy.

2520

Killer cell immunoglobulin-like receptor 3DL1 polymorphism defines distinct hierarchies of human leukocyte antigen recognition

Saunders, P.¹, Pymm, P.², Rossjohn, J.^{2,3}, Brooks, A.¹, Vivian, J.^{2,3}

¹Melbourne University, Melbourne, Australia, ²Monash University, Biochemistry, Clayton, Australia, ³ARC Centre of Excellence in Advanced Molecular Imaging, Monash University, Clayton, Australia

The interaction between killer cell Immunoglobulin-like Receptor 3DL1 (KIR3DL1) and HLA-class I molecules has been linked to NK cell control of viral infections and malignancy. However due to the vast polymorphism of the genes encoding both its HLA-I ligands and the receptor itself, a mechanistic

understanding of how these receptor/ligand interactions impact disease outcomes remains unclear. KIR3DL1 tetramers representative of the two main inhibitory KIR3DL1 lineages (-*005 and -*015) together with an interlineage recombinant (*001) were screened for reactivity against a comprehensive panel of HLA-I molecules. This revealed distinct hierarchies of preferred HLA-I ligands for each KIR3DL1 allotype, with KIR3DL1*005 recognising a wider array of HLA-I ligands than either the KIR3DL1*015 or -*001. These differences in binding were also reflected in functional assays utilising NK cell clones expressing specific KIR3DL1 allotypes. Intriguingly, while the polymorphic differences between KIR3DL1*001, *005 and *015 were remote from the KIR3DL1-HLA-I interface, the structures of the three KIR3DL1-HLA-I complexes showed that the broader specificity of KIR3DL1*005 correlated with an altered juxtapositioning of the D1-D2 domains and increased mobility within the ligand binding site, conferring a greater tolerance for disparate ligands. Collectively, we provide a molecular basis underpinning the impact of KIR3DL1 polymorphism on HLA-I recognition.

3675

Plundering the non-classical MHC to find buried treasure

Andrews, D.¹, Nguyen, A.¹, Goodall, K.¹, Berry, R.^{2,3}, Brooks, A.⁴, Rossjohn, J.^{2,3,5}, Sullivan, L.⁴

¹Monash University, Central Clinical School, Immunology and Pathology, Melbourne, Australia, ²Monash University, Infection and Immunity Program and Department of Biochemistry and Molecular Biology, Biomedicine Discovery Institute, Clayton, Australia, ³Monash University, ARC Centre of Excellence in Advanced Molecular Imaging, Clayton, Australia, ⁴University of Melbourne, Peter Doherty Institute for Infection and Immunity, Microbiology and Immunology, Melbourne, Australia, ⁵Cardiff University School of Medicine, Institute of Infection and Immunity, Cardiff, United Kingdom

Recognition is central to the development and activation of immune cells and the Major Histocompatibility Complex (MHC) acts as a gatekeeper for these processes. Given the central role of the MHC, understanding the mechanisms by which it interacts with immune cells is key to our knowledge of immunity. One population of cells heavily dependent upon the MHC is Natural Killer (NK) cells. As a member of the frontline immune response, these cells can respond rapidly to pathogens by killing infected targets, with early experiments leading to the assumption that this was their only role in immunity. However, we now understand that the signals controlling NK cell activation are highly complex and that their role in immunity exceeds killing. The mouse non-classical MHC encodes genes that exhibit site-specific expression or unique presentation capabilities and we are exploring these molecules as a means to identify new pathways of NK cell regulation. Among these molecules our studies of H2-Q10 have identified 2 new immune processes including site-specific regulation of NK cell development and interaction with the CD8αα homodimer. Given the emerging role of NK cells in clinically relevant situations such as transplant rejection and viral infection, understanding how NK cells function is of paramount importance to allow us to harness them more effectively in the future.

11 Intratumoral natural killer (NK) cells possess inhibitory phenotype and suppress Th1 effector function

Paul, S., Lal, G.

National Centre for Cell Science, Infection and Immunity, Pune, India

Spontaneous cytolytic function of peripheral NK cells control tumor growth and metastasis. NK cells were used as adoptive cellular therapy to control tumor growth, but clinical trials did not show very encouraging results. The cellular and molecular mechanism that controls the function of intratumoral NK cells is not well defined.

B16F10 melanoma cells (1×10^6 cells/mouse) were subcutaneously injected into C57BL/6 mice. At day 5, CD11b⁺CD27⁺CD3⁺NK1.1⁺ NK cells were selectively recruited into the tumor microenvironment, and these NK cells showed increased CD62L expression and reduced proliferation. Intratumoral NK cells showed significantly reduced expression of activating receptors (NKG2D, Ly49D and Ly49H), cytokine receptors (IL-21R, IL-6R α , IFN- γ R, CD122 and CD25), perforin, IFN- γ and GM-CSF; significantly increased IL-10 and inhibitory receptors (NKG2A and Ly49A), and reduced cytolytic function compared to splenic population. Depletion of NK cells with intravenous injection of anti-NK1.1 mAb reduced the frequency of effector Th1 and Th17 cells in spleen, lymph nodes and tumor. Purified NK cells co-cultured with naive CD4 T cells increased the differentiation into Th1 lineage, and this was dependent on NK cell produced IFN- γ . Since, intratumoral NK cells showed reduced activating receptor, we treated NK cell and CD4 T cell co-culture with anti-NKG2D, anti-Ly49D or anti-Ly49H antibody, and observed enhanced differentiation of Th1 cells.

Inflamed tumor microenvironment selectively recruits a specific subset of NK cells, and regulates the differentiation of effector Th1 cells. Understanding cellular and molecular mechanism of intratumoral NK cell function will help design better anti-tumor therapeutic strategies.

949 Idelalisib promotes anti CD20-mediated ADCC by inhibiting immunosuppressive ROS production in monocytes

Akhiani, A.A.¹, Hallner, A.¹, Werlenius, O.², Aurelius, J.¹, Martner, A.¹, Hellstrand, K.¹, Thorén, F.B.¹

¹University of Gothenburg, Sahlgrenska Cancer Center, Gothenburg, Sweden, ²University of Gothenburg, Sahlgrenska Cancer Center, Department of Medicine, Gothenburg, Sweden

Leukemic cells from patients with chronic lymphocytic leukemia (CLL) display increased phosphatidylinositol 3-kinase (PI3K) activity. The PI3K- δ selective inhibitor idelalisib, alone or in conjunction with anti-CD20 antibodies, was recently approved in the treatment of CLL. Idelalisib is assumed to act by promoting apoptosis in CLL cells, but alternative or supplementary mechanisms are conceivable. Natural killer (NK) cells are pivotal effector cells in the ADCC reaction against anti-CD20-coated target cells, including CLL cells. NK cells are highly susceptible to inhibition exerted by reactive oxygen species (ROS), which are produced by monocytes and other myeloid cells. For the present study, we aimed to determine the impact of PI3K inhibition on

ROS production in human peripheral blood monocytes. It was observed that therapeutic antibodies against CD20 (rituximab and ofatumumab) induced substantial ROS production from monocytes, which in turn triggered extensive cell death in co-cultured NK cells. Idelalisib, at low concentration (0.1 μ M), efficiently reduced the anti-CD20-induced ROS production and prevented NK cell apoptosis (13 ± 2.7 vs $83 \pm 2.5\%$, Mean \pm SEM, MO:NK1:2 ratio). Similar NK cell-protective effects were observed using the pan-PI3K inhibitor Ly294002, inhibitors of ROS formation and reagents neutralizing extracellular ROS. In addition, NK cells were co-cultured overnight with monocytes and assayed for anti-CD20-mediated ADCC against B-lymphoblastoid cells. In these experiments, monocytes strongly inhibited ADCC by producing ROS. Idelalisib efficiently restored anti-CD20-mediated ADCC and NK cell degranulation against lymphoblastoid cells. Our results suggest that idelalisib, by reducing formation of immunosuppressive ROS, improves the NK cell capacity to exert ADCC, which may contribute to its anti-leukemic properties.

3478

Preparation of TERT-modified human NK cells

Streltsova, M.A., Erokhina, S.A., Kanevskiy, L.M., Kovalenko, E.I. Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry RAS, Moscow, Russian Federation

Genetic modification of NK cells with TERT may significantly increase the efficiency of generation of these cells for the anti-cancer treatment. Preliminary choice of NK cells with desired functional properties may provide basis for development of personalized adoptive immunotherapy. The goal of this work is the transduction of telomerase gene into NK cells for optimization of their propagation for immunotherapeutic purposes. Retroviral vector system with TERT was selected. Transfection was performed using calcium phosphate-based method. For transduction procedure we used 24-well plates pretreated with RetroNectin solution. TRAP assay was used for qualitative detection of telomerase activity in the cells. Transduction of tumor cell lines HEK293T and Jurkat were performed to validate the models. Cell fractions expressing reporter protein were 20-30% and 12-25% for HEK293T and Jurkat cells, respectively. Transduced cells showed a higher level of telomerase activity than non-transduced ones. NK cells were isolated from peripheral mononuclear cells of healthy volunteers by magnetic separation. For NK cell transduction we used inhibitor of 3-phosphoinositide-dependent kinase-1 BX795. We had not been able to transduce native NK cells. However, NK cells stimulated with IL-2 and K562 cells bearing membrane-bound IL-21 were successfully transduced (30-50% of whole cell population). Effective genetic modification of NK cells with TERT will make easier an accumulation of NK cells for potential immunotherapy.

This work was supported by Russian Science Foundation (grant #16-15-00309).

HIV

1124

Influenza vaccine induces anti-nuclear and anti-double strand DNA IgG antibodies and their relates to levels of microbial translocation in antiretroviral-treated aviremic HIV-infected patients

Luo, Z.¹, Martin, L.¹, Kilby, J.M.¹, Liu, H.², Jin, P.², Stroncek, D.², Jiang, W.¹

¹Medical University of South Carolina, Chareleston, United States,

²National Institutes of Health, Bethesda, United States

The mechanism of autoantibody (autoAb) induction in HIV disease is unknown. In this study, blood samples from healthy controls (n = 16) and ART-treated aviremic HIV+ patients (n = 26) receiving 2013-2014 influenza vaccinations were analyzed pre-vaccination (D0), on day 7 (D7) and 14 (D14) post-vaccination. Microbial translocation was tested for LPS and total bacterial 16S rDNA by limulus amebocyte assay and qPCR respectively. ANA (OD) and anti-dsDNA (IU/ml) IgG were evaluated by ELISA. Microarray was analyzed in purified B cells on D0. Data are presented as median (IQR). Results. At baseline, there was increased plasma LPS [pg/mL, 18.0 (13.3-22.0) vs 13.4 (9.82-15.2), P=0.006] in patients. Bacterial 16S rDNA tended to be higher in patients compared with controls [copies/μL, 18.5 (9.4-25.2) vs 5.3 (0-34.7), P = 0.18]. B cells of patients exhibited increased TLR2 and 4 expression and NF-κB pathway activation by microarray. Plasma ANA on D0 and D14 were 0.31 (0.25-0.41) and 0.58 (0.31-1.29) vs 0.32 (0.23-0.40) and 0.31 (0.24-0.38); and anti-dsDNA IgG levels on D0 and D14 were 15.2 (8.06-23.7) and 20.2 (13.0-50.7) vs 15.3 (12.3-37.1) and 15.5 (13.0-36.7), in patients and controls respectively. Only patients showed significant increases of ANA and anti-dsDNA IgG post-vaccination (P < 0.001). Direct correlations between plasma LPS and ANA (r=0.48, P=0.01) and between plasma 16S rDNA and anti-dsDNA IgGs (r=0.44, P=0.04) were found only in patients. Conclusion. B cell function is not fully recovered by ART, as reflected by producing autoAbs in response to vaccinations and its correlates with levels of microbial translocation.

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The role of chemokines in the natural resistance of baboons to SIV infection

Obregon-Perko, V.^{1,2}, Parodi, L.M.², Hodara, V.L.^{2,3}, Giavedoni, L.D.^{2,3}

¹University of Texas Health Science Center at San Antonio, Microbiology & Immunology, San Antonio, United States, ²Texas Biomedical Research Institute, Virology & Immunology, San Antonio, United States, ³Southwest National Primate Research Center, Texas Biomedical Research Institute, San Antonio, United States

Simian immunodeficiency viruses (SIV) naturally infect over 40 species of African non-human primates (NHP) where they usually cause a chronic non-pathogenic infection. Asian macaques, however, progress to simian AIDS when experimentally infected. Baboons, an abundant African NHP, are not naturally infected with SIV. Previous work has demonstrated that baboons are resistant to chronic SIV infection *in vivo*, but mechanisms underlying resistance remain unknown. Identifying molecules

involved in baboon natural resistance to SIV could impact the development of next-generation HIV therapies and preventions. Peripheral blood mononuclear cells (PBMC) and CD4 cells were isolated from baboon and rhesus macaque whole blood. Cells were stimulated for 48 hr and infected with SIVmac251. Flow analysis revealed that before infection, baboon and rhesus PBMC had similar levels of CD4 T cells, CD4 CCR5 T cells, surface CD4, and surface CCR5. Baboon PBMC were susceptible to SIV infection, but replication kinetics were delayed and viral loads were lower compared to rhesus PBMC. SIV infection in baboons was not altered by natural STLV co-infection, but susceptibility to infection increased with age of the cell donor. Infection of isolated baboon CD4 cells progressed faster than in baboon PBMC. Compared to rhesus, baboon PBMC produced higher levels of MIP-1α, MIP-1β, and RANTES before and during infection; RANTES levels were markedly lower in CD4 cultures. Inhibition of these chemokines in baboon PBMC resulted in higher SIV loads. These chemokines suppress HIV/SIV infection by competing for binding to CCR5 and thus may contribute to SIV resistance in baboons.

3835

Immune response to *Mycobacterium tuberculosis* in patients with human immunodeficiency virus vertical infection

Alves, P.C.M.¹, Castelhana, M.V.¹, Macedo, V.S.¹, Arrym, M.P.¹, Mazzola, T.N.¹, Guimaraes, F.², Silva, M.T.N.³

¹University of Campinas/Faculty of Medical Sciences, Center for Investigation in Pediatrics, Campinas, Brazil, ²University of Campinas/Women's Hospital 'Prof Dr José Aristodemo Pinotti' -CAISM, Campinas, Brazil, ³University of Campinas/Faculty of Medical Sciences, Department of Pediatrics and Center for Investigation in Pediatrics, Campinas, Brazil

Background: HIV replication control by combined antiretroviral therapy (cART) reduces by 80% the chance of *Mycobacterium tuberculosis* (MTB) coinfection, but the incidence of cases in HIV patients is still higher than in healthy people. In this scenario, the aim of this study was to enlighten the cellular immune responses against mycobacterial antigens in HIV- infected patients.

Methods: Six vertically HIV-infected patients under cART and without plasma HIV-1 replication, recruited at the University of Campinas Clinical Hospital (UNICAMP, Brazil), and nine healthy controls participated in this study. *Mycobacterium bovis* (BCG) and MTB lysates and MTB recombinant ESAT-6 antigen were used to stimulate peripheral blood mononuclear cell cultures. Lymphoproliferation (measured by Percent Divided - PD, Proliferation Index - PI, and Division Index - DI) and IL-5, IL-17 and IFN-γ frequency among T cell subsets were assessed by flow cytometry.

Results: In MTB lysate-stimulated cultures, controls showed higher PD of CD3+ (p=0.006), TCR-γδ+ (p=0.034) and T CD4+CD45RO+ cells (p=0.035), PI of CD3+ cells (p=0.034), and DI of CD3+ (p=0.001), TCR-γδ+ (p=0.034), T CD4+CD45RO+ (p=0.025) and T CD8+CD45RO+ cells (p=0.05) than the patient group. In BCG lysate-stimulated cultures, there were higher PD values of CD3+ (p=0.029), TCR-γδ+ (p=0.045), T

CD4+CD45RO+ ($p=0.034$) and T CD8+CD45RO+ cells ($p=0.027$), DI of T CD8+CD45RO+ ($p=0.025$) and TCR- $\gamma\delta$ + cells ($p=0.05$), and frequency of IFN- γ +TCR- $\gamma\delta$ + cells ($p=0.05$) from controls in comparison with the patient group.

Conclusions: Even with HIV-1 control, HIV-infected patients still revealed lower T cell responses to mycobacterial antigens, which could set them at risk of HIV/TB co-infection.

1073

HIV-1 Nef down-regulated CD1a lipid antigen presentation in immature dendritic cells through hemopoietic cell kinase (Hck) and p21-activated kinase 2 (PAK2)

Shinya, E., Shimizu, M., Owaki, A., Okura, S., Takahashi, H. Nippon Medical School, Microbiology and Immunology, Tokyo, Japan

Dendritic cells (DCs) might be a key factor in *in vivo* pathogenesis of HIV-1 infection, so that they could provide a promising strategy to control and eventually overcome the fatal infection. DCs specifically express CD1s, the non-MHC lipid antigen (Ag)-presenting molecules. Simultaneously, HIV-1 Nef down-regulates CD1 expression besides MHC in immature DCs. Moreover, CD1d-restricted CD4⁺ NKT cells are infected by HIV-1, reducing the number of these cells in HIV-1-infected individuals. To understand the exact role of DCs and CD1-mediated immune response during HIV-1 infection, Nef down-regulation of CD1a-restricted lipid/glycolipid Ag presentation in iDCs was analyzed. We demonstrated the involvement of Nef in association with hemopoietic cell kinase (Hck) and p21-activated kinase 2 (PAK2), and found that Hck, which is expressed strongly in iDCs, augmented this mutual interaction. Hck might be another therapeutic target to preserve the function of DCs in the course of HIV-1 infection, which are potential reservoirs of HIV-1 even after antiretroviral therapy.

1494

Complement-opsonized HIV-1 overcomes restriction in dendritic cells

Posch, W.¹, Steger, M.¹, Moris, A.², Diaz-Griffero, F.³, Lass-Flörl, C.¹, Keppler, O.⁴, Wilflingseder, D.¹

¹Medical University of Innsbruck, Division of Hygiene and Medical Microbiology, Innsbruck, Austria, ²INSERM U 1135, Center for Immunology and Microbial Infections, Paris, France, ³Albert Einstein College of Medicine, Department of Microbiology and Immunology, New York, United States, ⁴University of Frankfurt, Institute of Medical Virology, Frankfurt, Germany

DCs express intrinsic cellular defense mechanisms to specifically inhibit HIV-1 replication. Thus, DCs are productively infected only at very low levels with HIV-1, and this non-permissiveness of DCs is suggested to go along with viral evasion. We now illustrate that complement-opsonized HIV-1 (HIV-C) efficiently bypasses SAMHD1 restriction and productively infects DCs including BDCA-1 DCs. Efficient DC infection by HIV-C was also observed using single-cycle HIV-C, and correlated with a remarkable elevated SAMHD1 T592 phosphorylation but not SAMHD1 degradation. If SAMHD1 phosphorylation was

blocked using a CDK2-inhibitor HIV-C-induced DC infection was also significantly abrogated. Additionally, we found a higher maturation and co-stimulatory potential, aberrant type I interferon expression and signaling as well as a stronger induction of cellular immune responses in HIV-C-treated DCs. Collectively, our data highlight a novel protective mechanism mediated by complement opsonization of HIV to effectively promote DC immune functions, which might be in the future exploited to tackle HIV infection.

4537

Sequential dysfunction and progressive depletion of *Candida albicans*-specific CD4 T cell response in HIV-1 infection

Hu, H.¹, Liu, F.¹, Fan, X.¹, Ferguson, M.², Hou, W.³, Sun, J.¹, Soong, L.¹
¹University of Texas Medical Branch, Microbiology and Immunology, Galveston, United States, ²University of Texas Medical Branch, Division of Infectious Disease, Galveston, United States, ³Wuhan University School of Medicine, Wuhan, China

Loss of immune control over opportunistic infections can occur at different stages of HIV-1 (HIV) disease, among which mucosal candidiasis caused by the fungal pathogen *Candida albicans* (*C. albicans*) is one of the early and common manifestations in HIV-infected subjects. The underlying immunological basis is not well defined. We have previously shown that compared to cytomegalovirus (CMV)-specific CD4 cells, *C. albicans*-specific human CD4 T cells are highly permissive to HIV *in vitro*. Here, based on an antiretroviral treatment (ART) naïve, HIV infection cohort (RV21), we investigated longitudinally the impact of HIV on *C. albicans*- and CMV-specific CD4 immunity *in vivo*. We found a sequential dysfunction and preferential depletion for *C. albicans*-specific CD4 response during progressive HIV infection. Compared to Th1 (IFN- γ , MIP-1 β) functional subsets, the Th17 functional subsets (IL-17, IL-22) of *C. albicans*-specific CD4 T cells were more permissive to HIV *in vitro* and impaired earlier in HIV-infected subjects. Infection history analysis showed that *C. albicans*-specific CD4 T cells were more susceptible to HIV *in vivo*, harboring modestly but significantly higher levels of HIV DNA, than CMV-specific CD4 T cells. Longitudinal analysis of HIV-infected individuals with ongoing CD4 depletion demonstrated that *C. albicans*-specific CD4 response was preferentially and progressively depleted. Taken together, these data suggest a potential mechanism for earlier loss of immune control over mucosal candidiasis in HIV-infected patients and may provide new insights into pathogen-specific immune failure in AIDS pathogenesis.

3482

Influence of host genetic factors on HIV in North Indian population

Mehra, N. All India Institute of Medical Sciences, National Chair, New Delhi, India

Genomic analyses of viremic controllers, elite controllers, exposed uninfected individuals, rapid and slow progressors have revealed correlates of HIV/AIDS vulnerability in a population

specific manner. We aimed to dissect the largely unknown genetic propensity to HIV infection in the North Indian population. Here we present our data about the influence of genes that regulate HIV cell entry (chemokine coreceptors like CCR5, CCR2 and their ligands like CCL3L1), as well as others that influence viral replication and dynamics, including tripartite interaction motif 5 a (TRIM5 α), apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G (APOBEC), T cell/ transmembrane, immunoglobulin and mucin (TIM) family proteins as well as pro and anti inflammatory cytokines. Our major findings are i) Protective CCR5 delta 32 mutation is rare (< 1%), and CCR5 promoter variants were found associated with susceptibility and development of AIDS, ii) No association of CCR2 64I, CCL3L1 copy numbers and APOBEC3B deletion (29.5 kb) with HIV susceptibility and/or resistance, iii) Among the observed seven TIM-1 exon-4 haplotypes, significantly higher CD4 counts were observed in D3-A +ve HIV patients, iv) TRIM5 α -exon 2 variant 43Tyr-allele and haplotypes carrying this allele were associated with resistance to HIV infection and v) Significantly higher allelic frequencies of IL-1 α -889 T and IL-4 -1098 T were observed in HIV patients, while IL-1 α -889 CC, IL-4 -1098 GG and IL-6 nt565 AA genotypes were observed significantly lower as compared healthy uninfected controls. The study represents distribution of immunogenetic variants and their influence on HIV/AIDS outcome in North Indian population.

Immunity to Viruses 1

2969

Broad $\alpha\beta$ TCR cross-strain recognition of influenza A viruses

Quiñones-Parra, S.M.¹, Uldrich, A.P.¹, Boon, A.², Rimmelzwaan, G.³, Godfrey, D.I.¹, Rossjohn, J.⁴, Gras, S.⁴, Kedzierska, K.¹

¹University of Melbourne, Dept of Microbiology and Immunology, at the Peter Doherty Institute for Infection and Immunity, Melbourne, Australia, ²Washington University, School of Medicine, St. Louis, United States, ³Erasmus Medical Centre, Dept of Viroscience, Rotterdam, Netherlands, ⁴Monash University, Dept of Biochemistry and Molecular Biology, School of Biomedical Sciences, Melbourne, Australia

Respiratory infections caused by influenza viruses are a global health concern and persistent pandemic threat. Current antibody-based influenza vaccines are greatly undermined by the antigenic adaptation of influenza viruses. In contrast, protective CD8⁺ cytotoxic T lymphocytes (CTL) recognize more conserved antigenic determinants and thus, provide potential for broadly-protective vaccines. However, although to a less extent compared to antibody epitopes, CTL immunity can also be abrogated by selection of viral escape variants. Thus, $\alpha\beta$ TCR recognition of multiple pHLA-I influenza variants may circumvent immune escape and consequently broaden protective CTL immunity against antigenically distinct and newly-emerged influenza viruses. However, the molecular bases of $\alpha\beta$ TCR cross-strain recognition remain poorly defined. Here, we describe $\alpha\beta$ TCR signatures displaying a spectrum of cross-reactivity towards the hypervariable HLA-B*3501-NP₄₁₈ influenza epitope. Probing against twelve NP₄₁₈ peptide variants occurring in nature, we revealed a remarkable extent

of functional cross-reactivity, with each $\alpha\beta$ TCR recognizing between 5 and 9 peptides presented by HLA-B*3501. Functional avidity analysis showed that fine specificity is determined by the $\alpha\beta$ TCR clonotype and that the cross-reactive capacity comes at the expense of compromising pHLA-I functional avidity. Furthermore, alanine scan experiments showed that a solvent exposed Asp at p5 and an Iso at p7 in the peptide, act as a major pivots for $\alpha\beta$ TCR recognition. Our study illustrates an unrecognized breadth in viral pHLA-I recognition by single $\alpha\beta$ TCRs with the potential to provide broad CTL immunity against multiple IAVs and presents insights into the molecular basis of $\alpha\beta$ TCR cross-recognition of influenza viruses.

3018

To the bat MHC! Antigen processing and presentation during viral infection

Woon, A.¹, Wynne, J.², Dudek, N.¹, Ng, J.³, Baker, M.², Wang, L.³, Purcell, A.¹

¹Monash Biomedicine Discovery Institute, Infection and Immunity Program, Biochemistry & Molecular Biology, Clayton, Australia, ²CSIRO AAHL, Health and Biosecurity, Geelong, Australia, ³Duke-NUS Graduate Medical School, Program in Emerging Infectious Diseases, Singapore, Singapore

Bats are a major reservoir of emerging infectious diseases and harbour deadly viruses such as Ebola, SARS, MERS and Hendra virus without clinical signs of disease. Vaccines are not available against many of these viruses and a better understanding of the bat immune system could aid vaccine development.

We hypothesise that the bat adaptive immune system contributes to the ability of bats to co-exist asymptotically with viruses. One of the key adaptive immune responses is the presentation of antigens by major histocompatibility complex (MHC) class I molecules to CD8⁺ T cells. We sought to further understand antigen processing and presentation in bats by characterising the first bat MHC class I molecules using a proteomic approach.

Using LC-MS/MS, we determined that bat MHC class I molecules, like their human and mouse homologues, associate with a peptide-loading complex. Furthermore, we also characterised the first peptide repertoires of bat MHC class I molecules. These peptides ranged from 8-15 amino acid residues and motif analysis of the endogenous peptides revealed strong amino acid biases at various anchor positions. Lastly, we were able to identify Hendra virus-derived peptides from infected bat cells which also display this binding motif, suggesting these motifs may be exploited to predict epitopes for vaccine development and immunological studies.

This study is the first characterisation of bat MHC class I molecules and their respective peptide repertoires. Our results provide fundamental insights into the antigen processing and presentation pathways of bats, which ultimately can be used to understand viral control.

1546

Innate mediators of amphibian immunity provide protection against human influenza viruses

Holthausen, D.¹, George, S.², Jacob, J.¹

¹Emory University, Atlanta, United States, ²Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, India

Frogs and toads are incredible reservoirs of innate immune peptides. Amphibians secrete host defense peptides from their skin as a response to stress. This ancient form of innate immunity acts to protect the amphibians against microbial pathogens. The quantity and scope of secreted peptides dwarfs mammalian analogues, accounting for a considerable portion of all known host defense peptides. The non-invasive and harmless methods for frog peptide collection, along with the abundance and breadth of these peptides, makes them excellent choices for novel peptide drug therapies. Studies have shown that these peptides can effectively neutralize enveloped viruses, gram-negative and gram-positive bacteria, mycobacteria, fungi, and even cancerous or transformed cells. Because of the nature of antimicrobial peptides, often targeting the most critical and conserved aspects of a micro-organism, they may prove a vital alternative to conventional drugs plagued by pathogen resistance. Given the untapped potential of these peptides for anti-viral therapies, we assessed the antiviral activity of novel host defense peptides from the skin of the Indian fungoid frog, *Hylarana malabarica*. During our analyses, we isolated several peptides from *H. malabarica* that show anti-viral activity against human influenza viruses. Our studies indicate that these peptides from *H. malabarica* demonstrate anti-influenza activity *in vitro*, and also show potential as an *in vivo* anti-viral therapy.

1851

Effects of human papilloma virus oncoprotein E7 on antigen presentation and potential involvement of keratinocyte exosomes

Budhwani, M.¹, Jemon, K.², Ly, K.¹, Hibma, M.¹

¹University of Otago, Department of Pathology, Dunedin, New Zealand, ²University Teknologi Malaysia, Faculty of Biosciences and Medical Engineering, Johor, Malaysia

High-risk human papillomavirus (HPV) infection may lead to the development of several human cancers that cause significant mortality worldwide. HPV type 16 (HPV16) is the most common cancer-causing genotype. The HPV16 E7 oncoprotein is expressed in epidermal keratinocytes and has been implicated in evasion of host immunity. The aim of this study is to determine the regulatory effects of E7 on antigen presentation.

To test these effects, E7 or E7 cloned in reverse (E7rev), and ovalbumin (Ova) genes were delivered to mouse skin cells *in vivo*, and their expression controlled by the keratin 14 promoter, and the responses to Ova were measured. We found that co-expression of E7 in the Ova expressing cells strongly suppressed the CD8⁺ T-cell proliferative response towards Ova. We also found that E7 reduced ova uptake by skin Langerhans cells (LCs) and co-stimulatory marker expression of lymph node dendritic cells. Surprisingly, we did not observe any effect on the reduced T-cell proliferation to Ova following depletion of LCs and

langerin positive skin dermal DCs, suggesting that these cells are not involved in activation the the CD8 T cell response to Ova. Instead we found that exosome-like particles produced from E7-expressing mouse keratinocytes significantly reduced co-stimulatory marker expression on DCs, and proliferation of ova specific T-cells *in vitro*.

In conclusion, we provide evidence for E7 regulation of CD8T cell responses, and of E7-exosome mediated antigen presentation via dendritic cells. These regulatory effects may contribute to HPV evasion of the host immune response.

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Natural H3N2 influenza A infection in humans expands memory B cells specific for the hemagglutinin stalk domain

Tesini, B.¹, Halliley, J.¹, Ellebedy, A.², Kanagaiah, P.¹, Anderson, C.¹, Dediego, M.¹, Chaves, F.¹, Wang, J.¹, Krammer, F.³, Yang, H.¹, Zand, M.¹, Ahmed, R.², Treanor, J.¹, Topham, D.¹, Sangster, M.¹

¹University of Rochester, Rochester, United States, ²Emory University, Atlanta, United States, ³Icahn School of Medicine at Mt. Sinai, New York, United States

The B cell response to influenza infection and vaccination is profoundly influenced by the composition of an individual's memory B cell (MBC) pool. However, changes to the MBC pool induced by exposure to influenza in different forms are not well characterized. Here, we analyzed virus-specific B cell responses in 8 subjects infected with H3N2 influenza A virus (confirmed by PCR) during the 2012-13 season. Peripheral blood mononuclear cells and plasma were collected at the time of presentation with an influenza-like illness and on days 3, 10, and 28 thereafter. Hemagglutination inhibiting antibody titers against the prevalent H3N2 strain A/Victoria/361/2011 increased 2-4-fold in all subjects by day 28. Circulating IgG plasmablasts (PBs) specific for the Vic/11 H3 and the viral nucleoprotein commonly peaked on day 3. Approximately 25% of H3-specific IgG PBs bound the conserved stalk domain. IgA PBs included the same specificities, but numbers were low and variable. A wave of recently proliferated, non-antibody secreting B cells peaked on day 10. These included virus-specific cells and likely represented newly formed MBC precursors. In most subjects, H3 (head and stalk domain)- and nucleoprotein-specific IgG MBC frequencies progressively increased from days 3 to 28. Increases in virus-specific IgA MBC frequencies were smaller and variable. Overall, we demonstrate that the specificities of MBC populations expanded by natural H3N2 infection reflect the early virus-specific PB response. Importantly, this includes IgG and IgA MBCs specific for the HA stalk domain, a target of broadly neutralizing antibodies that are poorly induced by inactivated H3N2 vaccination.

2364**Suppression of dendritic cell necroptosis by pandemic influenza virus***Hartmann, B., Albrecht, R., Marjanovic, N., Sealfon, S.**Icahn School of Medicine at Mt. Sinai, New York, United States*

The public health risk of a new pandemic influenza virus (IAV) approaching the scope and mortality of the devastating 1918 strain makes it important to identify virus-host mechanisms unique to pandemic strains. Human dendritic cell infection by two different H1N1 seasonal IAV strains (1991 and 1999) induces widespread RNA degradation and programmed cell death beginning at 4 h after infection. Characteristic of necroptosis, the cell death showed nuclear condensation and fragmentation that was caspase independent and RIP3 kinase dependent. Notably, infection with the pandemic 1918 and 2009 H1N1 IAV strains did not induce dendritic cell RNA degradation and necroptosis. Furthermore, necroptosis induced in dendritic cells by polyI:C exposure was suppressed by concomitant infection with the pandemic 2009 IAV. These results indicate that infection with the pandemic viruses not only fails to induce the dendritic cell necroptosis seen with the seasonal IAV strains, but that it actively suppresses necroptosis. Virus chimera studies implicate the HA virus segment as mediating the necroptosis inhibition by the pandemic IAV strains. Necroptosis leads to the release of intracellular components which serve as danger associated molecular patterns (DAMP), making this a highly immunogenic cell death mechanism. Consistent with this view, we find that the suppression of virus-induced necroptosis by the pandemic IAV reduces T cell activation. These studies show that pandemic viruses are unique in suppressing a key immunological danger signal. The suppression of the generation of DAMPs from infected immune cells may contribute to the pathogenicity of pandemic H1N1 IAV viruses.

4464**Targeting the NLRP3 inflammasome is a viable option for the treatment of pathogenic influenza virus infection***Tate, M.¹, Ong, J.¹, Dowling, J.¹, Robertson, A.², Cooper, M.², Mansell, A.¹**¹Hudson Institute of Medical Research, Centre for Innate Immunity and Infectious Diseases, Melbourne, Australia, ²The University of Queensland, Institute for Molecular Bioscience, Brisbane, Australia*

Fatal influenza A virus (IAV) infections in humans, such as those resulting from spill over of virus from birds, are associated with excessive production of pro-inflammatory cytokines and chemokines. The emergence of novel avian H7N9 IAV in humans with over 700 confirmed cases (38% mortality) and associated hypercytokinemia, highlights the need to identify the molecular mechanisms that drive these excessive immune responses.

We investigated the role of the NLRP3 inflammasome in modulating disease during IAV infection of mice. Using the NLRP3 inhibitor MCC950, we reveal for the first time that NLRP3 plays a biphasic role in modulating IAV disease. Early intranasal MCC950 treatment rendered mice more susceptible to PR8 (H1N1) and HKx31 (H3N2) infection; however, late inhibition of NLRP3 reduced local and systemic inflammation and protected against severe disease. Importantly, MCC950 did not alter

viral loads in the respiratory tract of mice. Late treatment with MCC950 was associated with significantly reduced levels of a number of pro-inflammatory cytokines including IL-1 β in the airways and sera. In addition, we have identified which cell populations in the lung MCC950 can penetrate.

Our study is the first to define the role of the NLRP3 inflammasome during pathogenic IAV challenge as both protective and detrimental. In particular, our data indicates NLRP3 plays a role in amplifying the production of local and systemic pro-inflammatory cytokines during severe IAV infections. These data provide the first evidence that temporally therapeutically targeting the NLRP3 inflammasome may be a clinical option for reducing inflammation associated with severe IAV infections.

Allergy 1

1524**Local remodeling, mast cell activation and sphingosine-1-phosphate elevation precede atopic dermatitis***Wedman, P., Aladhami, A., Chumanevich, A., Fuseler, J., Oskeritzian, C.**University of South Carolina School of Medicine, Pathology, Microbiology and Immunology, Columbia, United States*

Atopic dermatitis (AD) is an inflammatory skin disease whose pathogenic mechanisms remain unclear. Chronic lesions have been extensively characterized however few studies have investigated the initial phases of AD, including early skin alterations prior to any visible lesion. We recently reported a novel method of computer-assisted image analysis for *in situ* measurement of mouse skin mast cells (MC) activation. We applied similar morphometric approaches to quantify local MC activation status and skin remodeling after a single epicutaneous exposure to antigen/ovalbumin (OVA), compared to saline controls in a human AD-like preclinical model. Remarkably, epidermal and dermal thickening was substantiated after a single OVA application, also resulting in early cellular infiltration in the vascularized hypodermis layer of the skin. Furthermore, skin remodeling correlated with augmented local chemokine production, MC activation, and increased levels of a potent sphingolipid metabolite sphingosine-1-phosphate (S1P) that we have shown could activate MC-derived chemokine production. Importantly, cell infiltration was severely mitigated in mice genetically ablated for sphingosine kinase 1, predominant enzyme isoform producing S1P, and in MC-deficient *Kit^{W-sh/W-sh}* mice. We conclude that MC and S1P could initiate the development of AD by driving early inflammatory infiltration and skin remodeling through their contribution to local chemokine production, offering perhaps new prophylactic approaches for this disease whose treatment management still remains a clinical challenge.

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Perinatal exposure to GOS/inulin prebiotics prevent food allergy by promoting tolerance and protecting intestineBouchaud, G.¹, Castan, L.^{1,2}, Chesné, J.², Braza, F.², Aubert, P.^{3,4}, Neunlist, M.^{3,4}, Magnan, A.^{2,4,5}, Bodinier, M.¹¹INRA, UR 1268 BIA, Nantes, France, ²INSERM, UMR 1087, Institut du Thorax, Nantes, France, ³INSERM, UMR 913, Institut des Maladies de l'Appareil Digestif (IMAD), Faculté de Médecine, Nantes, France, ⁴DHU 2020 Médecine Personnalisée des Maladies Chroniques, Nantes, France, ⁵CHU de Nantes, Service de Pneumologie, Nantes, France

Aims: Food allergies (FA) are increasing and prevention strategies are nonexistent. They are linked to imbalance of microbiota and immune system. Fibers as prebiotics have been proposed to restore this balance and seem to be one alternative to prevent or reduce allergies, particularly during infancy. In this context, we evaluated early nutritional intervention with prebiotics, via the mother during pregnancy and lactation (perinatal), to prevent food allergic risk in mice.

Method: Pregnant and lactating mice were exposed or not to GOS/Inulin prebiotics. Then, pups were intraperitoneally sensitized and then orally exposed to wheat allergen to mimic FA. Phenotypic and blood markers of allergy, immune cells and microbiota were analyzed.

Results: In mice exposed to GOS/Inulin prebiotics via their mothers, we observed a decrease of FA symptoms associated with a fall of allergic markers (IgE), a rise of tolerance markers (IgA) and a protection from the alteration of intestinal permeability and epithelium. Immunity was also modified by prebiotics as shown by the decrease of IL-4 and IL-5 productions and the increase of IFN- γ and TGF- β secretions associated with a rise of T regulatory cells in gut lymphoid organs. Prebiotics also induced a strong modification of mother mice intestinal microbiota especially for *Lactobacillus* that was the only one being transferred to pups.

Conclusion: Prebiotics supplemented diet during perinatal period protects pups against FA by decreasing levels of allergic markers, clinical symptoms and intestinal permeability. Moreover, prebiotics also modulate immune reaction during allergy toward tolerance and modify intestinal microbiota.

2428

Stromal interaction molecule-1/2 plays essential role in basophil activation and development of IgE-mediated chronic allergic inflammationYoshikawa, S.¹, Oh-hora, M.², Adachi, T.³, Yamanishi, Y.¹, Karasuyama, H.¹¹Tokyo Medical and Dental University, Immune Regulation, Tokyo, Japan, ²Kyushu University, Division of Molecular Immunology, Fukuoka, Japan, ³Tokyo Medical and Dental University, Department of Immunology, Tokyo, Japan

Cross-linking of the high-affinity IgE receptors (Fc ϵ RI) in mast cells increases intracellular Ca²⁺ concentration through store operated Ca²⁺ entry (SOCE), leading to mast cell degranulation. Recent report suggested that stromal interaction molecule 1 (STIM1), which is a sensor of ER Ca²⁺ stores that couples

depletion of Ca²⁺ from ER stores with SOCE influx, is essential for mast cell degranulation. However, the function of STIM1 in basophil is unknown. To address this issue, we here established transgenic mice expressing a tamoxifen-inducible improved-Cre recombinase under the control of the basophil-specific promoter (*Mcpt8*^{CreERT2}), and crossbred them with mice carrying loxP-flanked *Stim1* and *Stim2*. The tamoxifen-induced STIM1/2 depletion in basophils resulted in impaired generation of SOCE influx *in vitro* in response to the stimulation with IgE plus antigens. Moreover, STIM1/2-deficient basophils also displayed impaired degranulation and production of IL-4 and IL-6 *in vitro* in response to the IgE/antigen stimulation, suggesting that STIM1/2 is essential for the Fc ϵ RI-mediated basophil activation. Importantly, mice with targeted deletion of STIM1/2 in basophils failed to develop IgE-mediated chronic allergic inflammation (IgE-CAI), a prototype of basophil-mediated allergic response. Although the number of basophils in peripheral blood was comparable in those and wild-type mice, the basophil recruitment to the skin lesions was severely impaired in the former. Taken together, STIM1/2 appears to play an essential role in the recruitment and activation of basophils.

2712

Bronchoepithelial cell-derived prostaglandin D₂ inhibits eosinophilic lung inflammationMaehara, T.¹, Nakamura, T.¹, Aritake, K.², Urade, Y.², Murata, T.¹¹University of Tokyo, Graduate School of Agriculture and Life Science, Department of Animal Radiology, Tokyo, Japan,²University of Tsukuba, International Institute for Intergrative Sleep Medicine, Ibaraki, Japan

Objective: Eosinophilic lung inflammation is a hallmark of asthma. Although the concentration of prostaglandin D₂ (PGD₂) is elevated in the allergic lung tissue, its contribution in disease progression remains unclear. We attempted to clarify the role of PGD₂ in ovalbumin (OVA)-induced allergic lung inflammation using hematopoietic PGD synthase (H-PGDS) deficient mice.

Results: Repeated inhalation of OVA induced eosinophil infiltration which was accompanied by the increase in mRNA expressions of eosinophilic chemoattractant including TNF- α , IL-5, RANTES and Eotaxin-1 in WT mouse lung. Gene deficiency of H-PGDS enhanced both the OVA-induced eosinophilic infiltration and mRNA expressions of these cytokines/chemokines. Continuous administration of a PGD₂ receptor agonist attenuated the OVA-induced allergic lung inflammation in WT mice. Immunostaining showed that bronchoepithelial cells strongly expressed H-PGDS and TNF- α in the allergic WT mouse lung. In cultured bronchus tissue of WT mice, a treatment of TNF- α increased the mRNA expression of RANTES and Eotaxin-1. H-PGDS deficiency promoted the TNF- α induced mRNA expressions of these chemokines, which were inhibited by PGD₂ receptor agonism.

Conclusion: Bronchoepithelial cell-derived PGD₂ acts as a negative regulator by inhibiting the expression of chemoattractants in eosinophilic lung inflammation.

3222

Genome-derived proteomics reveals the hidden allergens of the pacific oyster (*Crassostrea gigas*)

Nugraha, R.^{1,2,3}, *Kamath, S.D.*^{1,2,3}, *Martina, K.*^{1,2,3,4}, *Rolland, J.M.*^{5,6}, *O'Hehir, R.E.*^{5,6}, *Zenger, K.R.*⁷, *Lopata, A.L.*^{1,2,3}

¹College of Public Health, Medical and Veterinary Science, James Cook University, Townsville, Australia, ²Centre for Biodiscovery and Molecular Development of Therapeutics, James Cook University, Townsville, Australia, ³Australian Institute of Tropical Health and Medicine, James Cook University, Townsville, Australia, ⁴National Measurement Institute, Melbourne, Australia, ⁵Monash University, Department of Immunology and Pathology, Melbourne, Australia, ⁶Alfred Hospital and Central Clinical School, Monash University, Department of Allergy, Immunology and Respiratory Medicine, Melbourne, Australia, ⁷College of Marine & Environmental Sciences, James Cook University, Townsville, Australia

The increasing production and consumption of molluscs is associated with a rise in prevalence of mollusc allergy worldwide, currently ranging from 0.15% to 1.3% of the general population. However, the elucidation of mollusc allergens still lags behind other seafood groups such as fish and crustacean. Genomic data have been utilized previously for improved identification of non-food allergens by performing similarity searching using the BLAST program. Based on the published genome of the Pacific oyster (*Crassostrea gigas*) we aimed to identify novel allergens using a similar genomic approach.

Eleven novel putative and potentially cross-reactive Pacific oyster allergens were discovered using *in silico* analyses. The allergenicity of these proteins was characterized by a combination of immunoassays and transcriptome-derived proteomic analyses. Immunoblotting of Pacific oyster extract showed that many proteins gave a positive reaction with IgE antibodies from patients with confirmed mollusc allergy. The identity of these IgE-reactive proteins was determined by mass spectrometry. Some of the putative and potentially cross-reactive allergens are confirmed to be novel allergens.

Using an integrated genomic approach, this study was able to characterise food allergens from the Pacific oyster not described elsewhere. These newly identified allergens and knowledge of their gene structure will facilitate development of improved diagnosis and specific immunotherapy for oyster allergy.

Treg

2706

Human intrahepatic T_{reg} are functional, require IL-2 from effector cells for survival and are susceptible to FAS Ligand mediated apoptosis

*Jeffery, H.C.*¹, *Chen, Y.-Y.*¹, *Hunter, S.*¹, *Bhogal, R.*¹, *Birtwistle, J.*², *Braitch, M.K.*¹, *Roberts, S.*¹, *Ming, M.*¹, *Hannah, J.*¹, *Thomas, C.*¹, *Adeli, G.*¹, *Hubscher, S.*^{1,3}, *Syn, W.-K.*⁴, *Afford, S.C.*¹, *Lalor, P.*¹, *Adams, D.H.*^{1,3}, *Oo, Y.H.*^{1,3}

¹University of Birmingham, Centre for Liver Research & NIHR Biomedical Research Unit, Institute of Immunology and Immunotherapy, Birmingham, United Kingdom, ²University of Birmingham, Clinical Immunology Service, Birmingham, United Kingdom, ³University Hospital of Birmingham NHS Foundation

Trust, Birmingham, United Kingdom, ⁴Institute of Hepatology, London, United Kingdom

Regulatory T cells (T_{reg}) suppress T effector cell proliferation and maintain immune homeostasis. Autoimmune liver diseases (AILD) persist despite high frequencies of T_{reg} in the liver suggesting that the local hepatic microenvironment might affect T_{reg} stability, survival or function. We hypothesized that interactions between T_{reg} and endothelial cells during recruitment and then with epithelial cells within the liver affect T_{reg} stability, survival and function. We explored the function of T_{reg} after migration through human hepatic sinusoidal endothelium (post-endothelial migrated (PEMT_{reg})) and the effect of subsequent interactions with cholangiocytes and local proinflammatory cytokines on the survival and stability of T_{reg}. We demonstrate by ELISA that the intrahepatic microenvironment is enriched with proinflammatory cytokines but is deficient in IL-2, and confirm that activated CD4 and CD8 T cells are the main source of IL-2 in the inflamed liver. Migration through endothelium into the inflamed liver microenvironment did not affect T_{reg} stability, however functional capacity was reduced. Furthermore, the addition of exogenous IL-2 enhanced PEMT_{reg} phosphoSTAT5 signaling compared with PEMCD8. Liver infiltrating T_{reg} reside close to FAS Ligand expressing bile ducts. T_{reg} from diseased livers expressed high levels of CD95 and co-culture with cholangiocytes or their supernatants induced preferential apoptosis of T_{reg} compared to CD8 effector cells and this apoptosis was inhibited by IL-2 or blockade of CD95. These results provide a mechanism to explain T_{reg} dysfunction in inflamed tissues and suggest that IL-2 supplementation, particularly if used in conjunction with T_{reg} therapy, could restore immune homeostasis in inflammatory and AILD.

1081

CD28 expression by donor CD4⁺ Foxp3⁺ regulatory T cells is required for long-term, but not short-term suppression of acute graft versus host disease in mice

Uri, A., *Werner, S.*, *Hünig, T.*, *Kerkau, T.*, *Beyersdorf, N.*

University of Würzburg, Institute for Virology and Immunobiology, Würzburg, Germany

Acute graft versus host disease (aGvHD) is a major cause of transplant-related death after hematopoietic stem cell (HSC) transplantation and is induced by mature T cells in the graft. In this study, we investigated the role of CD28 co-stimulation in CD4⁺ conventional (CD4⁺CD25⁻, Tconv) and regulatory (CD4⁺CD25⁺, Treg) T cell expansion and function during aGvHD. We used tamoxifen-inducible Cre CD28^{fllox/-} mice (iCD28KO mice) to delete CD28 on either donor CD4⁺ Tconv or Treg in the C57BL/6 into BALB/c HSC transplantation model. Treatment of the recipient mice with tamoxifen from day 0 to day +3 after transplantation, efficiently deleted CD28 on the donor T cells. When we transferred CD4⁺ Tconv only, CD28 deletion on these cells only transiently reduced inflammation but did not prolong survival of the recipient mice, as compared to mice that received CD28-sufficient CD4⁺ Tconv. To test the impact of CD28 deletion on donor Treg in aGvHD, we co-transplanted wild type CD4⁺ Tconv with either CD28 sufficient or iCD28KO Treg.

Both, CD28-sufficient and -deficient Treg protected recipient mice from aGvHD-induced lethality until three weeks after transplantation. However, remission of aGvHD symptoms and long-term survival was highly dependent on CD28 expression by Treg. Together this suggests that in humans therapeutic blockade of CD28, which has been shown to protect mice from aGvHD, should also be limited to the early phase of the disease. This work was supported by a grant from the "Deutsche José Carreras Leukämie-Stiftung".

3180

CD4+ T cell homing in cystic fibrosis peripheral blood

Mulcahy, E.¹, Asad, S.², McGuire, H.², Roddam, L.¹, Beggs, S.³, Cooley, M.¹, Fazekas de St Groth, B.²

¹University of Tasmania, School of Medicine, Hobart, Australia,

²Centenary Institute, Newtown, Australia, ³Royal Hobart Hospital, Hobart, Australia

Cystic fibrosis (CF) is the most common life-limiting single-gene disease, which results from mutations of *CFTR*. *CFTR* is expressed on immune cells including APCs, monocytes and lymphocytes and lack of expression on lymphocytes has been reported to result in abnormal immune responses. A nonresolving hyperinflammatory Th2/Th17-skewed immune response is also characteristic of CF. We therefore hypothesised that altered homing of T cells may play a role in the pathogenesis of CF lung disease.

PBMC isolated from people with CF and healthy age-matched controls were stained for CD4, CD25, CD127 and CD45RO for identification of naive and memory tregs, and for the Th1, Th2 and Th17 homing markers CXCR3, CCR4 and CCR6 for multicolour flow cytometric analysis.

CXCR3+, CCR4+ and CCR6+ memory Treg cell percentages were all decreased in CF blood compared with controls while CCR6+, but not CXCR3+ or CCR4+ memory effector CD4 cells were decreased. No difference was seen between CF and control in any naive CD4 or Treg cell populations. We hypothesise that decreased levels of memory cells in blood is due to their homing to the major site of infection, the lungs, resulting in an imbalance of regulatory and effector subsets homing to the lungs. The ongoing inflammation in CF also suggests that those CXCR3+, CCR4+ and CCR6+ Tregs homing to lungs may not function efficiently to reduce the damaging responses. These alterations in CCR6+ CD4 and Treg subsets support that CF is dominated by Th17 responses that could be targeted to reduce lung inflammation and damage.

3878

Interleukin-21 limits regulatory T cell populations by modulating responsiveness to Interleukin-2

Jandl, C.^{1,2}, Liu, S.M.^{1,2}, Fernandez De Cantete Nieto, P.³, Warren, J.¹, Hughes, W.E.¹, Vogelzang, A.^{1,2}, Webster, K.^{1,2}, Craig, M.⁴, Uzel, G.⁵, Tangye, S.^{1,2}, Dent, A.⁶, Vinuesa, C.³, Sprent, J.^{1,2}, King, C.^{1,2}

¹Garvan Institute of Medical Research, Immunology Division, Sydney, Australia, ²St. Vincent's Clinical School, UNSW Australia, Department of Medicine, Sydney, Australia, ³John Curtin School of Medical Research, Australian National University, Division of

Immunology, Canberra, Australia, ⁴The Children's Hospital at Westmead, Institute of Endocrinology and Diabetes, Sydney, Australia, ⁵National Institute of Allergy and Infectious Diseases, National Institute of Health, Laboratory of Clinical Infectious Diseases, Bethesda, United States, ⁶Indiana University School of Dentistry, Department of Microbiology and Immunology, Indianapolis, United States

Interleukin (IL)-2 dependent T follicular regulatory (Tfr) cells control IL-21 producing T follicular helper cells and B cells during the germinal center (GC) reaction, but how regulation is contained within the GC niche to facilitate antibody production has remained unknown. Here, we report a marked increase of both total regulatory T cells (Tregs) and Tfr cells in peripheral blood of human patients with a loss of function mutation in the IL-21 receptor. In mice, IL-21 similarly inhibited the expansion of Tregs and Tfr cells following T dependent immunization. The negative influence of IL-21 on Tfr cells was cell intrinsic and associated with decreased expression of the high affinity IL-2 receptor (CD25). Bcl6, expressed in abundance in Tfr cells, inhibited CD25 expression and IL-21 mediated inhibition of CD25 was Bcl6-dependent. Parallel immunization of *Il6*^{-/-} mice demonstrated that IL-6, whilst equally able to induce Bcl6, was not effective at inhibiting Tfr cell expansion. Genome wide analyses of differentially expressed genes in CD4+ T cells in response to IL-21 uncovered a cache of genes that negatively regulate FoxP3 expression. These findings identify a mechanism whereby IL-21 reinforces humoral immunity; inhibiting the expansion of Tfr cells by limiting responsiveness to IL-2 and stabilizing FoxP3.

4192

Bach2 is required for sustained CD4 T cell responses

Sidwell, T.¹, Vasanthakumar, A.^{1,2}, Thelemann, C.^{1,2}, Kallies, A.^{1,2}

¹Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia, ²University of Melbourne, Department of Medical Biology, Melbourne, Australia

To characterise the function of Bach2 in CD4 T cells *in vivo*, we utilised the SMARTA T cell receptor, specific for a lymphocytic choriomeningitis (LCMV) MHCII epitope. Congenically marked wild type (WT) and Bach2-deficient CD4+ SMARTA T cells were co-transferred into the same recipient mice and their responses to infection compared. Bach2-deficient antigen-specific responders initially resembled control cells in activation status, capacity for effector differentiation and division. However, following the peak of the response there was more than ten-fold loss of *Bach2*^{-/-} responder cells compared to controls, a defect that persisted into the memory phase. Further characterisation at and around the time of loss indicated that *Bach2*^{-/-} responder cells and WT controls were similar in cell cycling, metabolic fitness and effector differentiation.

To study this defect in a more controlled manner, we cultured WT and Bach2-deficient CD4 T cells *in vitro*. Bach2-deficient T cells initially upregulated markers of activation similar to WT controls, but progressed more slowly through division. Furthermore, Bach2-deficient cells expressed dramatically increased amounts of effector molecules, including IFN γ , IL-

17 and IL-10, paralleled by derepression of transcription factor Blimp1. In addition we observed a severe impairment of the *Bach2*^{-/-} CD4 T cells in Foxp3 upregulation. A loss of these cells *in vitro* at a similar time post stimulation to *in vivo* cells indicates an underlying requirement for *Bach2* for the survival of CD4 T cells in allowing their continued response to antigen and their subsequent memory generation. Study on the molecular basis of this requirement is ongoing.

2789

Low dose Proleukin promotes enhanced regulatory phenotype with selective induction of phosphoSTAT5 and prosurvival Bcl-2 in blood and liver Treg in autoimmune liver disease

*Jeffery, H.*¹, *Jeffery, L.E.*², *Adams, D.H.*^{1,3}, *Oo, Y.H.*^{1,3}

¹University of Birmingham, Centre for Liver Research & NIHR Biomedical Research Unit, Institute of Immunology and Immunotherapy, Birmingham, United Kingdom, ²University of Birmingham, Institute of Metabolism and Systems Research, Birmingham, United Kingdom, ³University Hospital of Birmingham NHS Foundation Trust, Birmingham, United Kingdom

CD4⁺CD25^{high}CD127^{low}FOXP3⁺ regulatory T cells (Treg) play a central role in maintaining peripheral self-immune tolerance. Functional impairment in Treg and/or imbalances in Treg-to-Teffector cell ratios contribute to the pathogenesis of autoimmune diseases. Treg depend on IL-2 for their survival and function via high expression of CD25 (IL-2R α). Studies in graft-versus-host disease, HCV-induced vasculitis and type-1 diabetes have indicated that low dose IL-2 therapy might help to control autoimmune diseases. The potential to use low dose IL-2 in adults with autoimmune liver diseases (AILD) (Type-1 Autoimmune Hepatitis and Primary Biliary Cholangitis) is unknown and was investigated in this study. Peripheral blood and liver-infiltrating lymphocytes from patients with AILD were treated with doses of clinical grade IL-2 (Proleukin) in the range 0-1000IU/ml and phosphorylation of STAT5, and expression of markers of function and survival analysed. A very low dose of Proleukin, < 5IU/ml, induced STAT5 phosphorylation in Treg but not in other lymphocyte subsets or natural killer cells. This dose of Proleukin stimulated a series of phenotypic changes in blood and liver Treg of patients, including up-regulation of regulatory functional markers CTLA-4, FOXP3 and CD25 and enhanced expression of the anti-apoptotic protein, Bcl-2, without inducing noticeable activation or upregulating survival factors in effector T-cells. The IL-2 level in the human inflamed liver is near undetectable. We report that low dose Proleukin therapy could selectively enhance Treg function and survival to restore tolerance in AILD and consideration of these findings will be important in the ongoing design of GMP Treg therapy for AILD.

1454

The use of Basiliximab as induction therapy in heart-transplanted children deregulates the regulatory T-cell population (Treg), compromising the natural mechanisms of tolerance of transplanted organs

*López-Abente, J.*¹, *Camino, M.*², *Gil, N.*², *Panadero, E.*², *Clemente, M.*³,

*Martínez-Bonet, M.*⁴, *Urschel, S.*⁵, *West, L.*⁶, *Pion, M.*¹, *Correa-Rocha, R.*¹
¹Gregorio Marañón Health Research Institute (IISGM), Laboratory of Immune-Regulation, Madrid, Spain, ²Hospital General Universitario Gregorio Marañón, Pediatric Cardiology Division, Madrid, Spain, ³Gregorio Marañón Health Research Institute (IISGM), Cell Culture Unit, Madrid, Spain, ⁴Gregorio Marañón Health Research Institute (IISGM), Laboratory of Molecular Immunobiology, Madrid, Spain, ⁵University of Alberta / Stollery Children's Hospital, Pediatric Cardiac Transplantation, Edmonton, Canada, ⁶University of Alberta, Alberta Transplant Institute, Edmonton, Canada

Immunosuppressors are used prior and after transplantation to avoid immune rejection of the transplanted organ. However, the lack of specificity of these immunosuppressive drugs produces pleiotropic effects on the whole immune system and potentially on regulatory T cells (Tregs). Tregs suppress inappropriate immune responses, are crucial for the maintenance of immune tolerance, and could be notably relevant to prevent rejection in transplanted children. Between these immunosuppressors, Basiliximab is a monoclonal antibody against the α -chain (CD25) of IL-2 receptor. CD25 allows Tregs to detect the excessive proliferation of effector cells and induce their proliferation and survival. Therefore, although Basiliximab induction immunotherapy could prevent early graft rejection, it could also impede the possible tolerance of the transplanted organ.

We performed an exhaustive analysis of immune subsets including Tregs in heart-transplanted children treated with Basiliximab (n=2) or not treated (n=3). We show that the use of Basiliximab during the first 4 days post-transplant completely blocks CD25 on Tregs for at least three months. We investigated whether this block could "blind" Tregs and alter their functionality and survival by treating *in vitro* PBMC from healthy donors with Basiliximab (n=6). Basiliximab produced a marked Treg deregulation inducing a decrease in Foxp3 expression, a reduction in IL-10 production and a diminution in Treg proliferation under α -CD3/ α -CD28 activation. Therefore, the use of Basiliximab induction in transplanted patients could compromise Treg-mediated tolerance during the first three months post-transplant, which is the period with highest incidence of acute rejection, and could contribute to deregulation of the immune homeostasis.

Immunity to Bacteria & Fungi 1

3477

Gender dimorphism in the immunomodulatory role of beta 2-glycoprotein I in sepsis

El-Assaad, E.^{1,2}, *Kizny Gordon, A.*^{2,3}, *Zhou, S.*^{1,2}, *Qi, M.*^{1,2}, *Beretov, J.*^{1,4}, *Chen, G.*^{1,2}, *Giannakopoulos, B.*^{1,2}, *Krilis, S.*^{1,2}

¹University of New South Wales, St George and Sutherland Clinical School, Faculty of Medicine, Sydney, Australia, ²St George Hospital, Department of Infectious Disease, Immunology and Sexual Health, Sydney, Australia, ³St George Hospital, Department of Anatomical Pathology, South Eastern Area Laboratory Service, Kogarah, Australia, ⁴St George Hospital, Department of Anatomical Pathology, South Eastern Area Laboratory Service, Sydney, Australia

Lipopolysaccharide (LPS) from gram-negative bacteria is a key molecule implicated in the pathogenesis of sepsis. In human sepsis, females have a lower incidence and a less severe presentation than males. However, the mechanisms underlying this gender dimorphism are not clear. In this study, we examined the role of beta 2-glycoprotein 1 (β 2GP-1), an abundant plasma LPS binding protein, in male, female wild type (WT) and β 2GP-1^{-/-} mice challenged with LPS or *E. coli* intravenously.

In WT mice, baseline levels of total β 2GP-1 were higher in male mice and the levels decreased following challenge with LPS or *E. coli*. This is an identical response seen in male human volunteers injected intravenously with LPS. Six hours post LPS or *E. coli* challenge, female and male β 2GP-1^{-/-} mice produced different signatures of pro and anti-inflammatory cytokines. Male β 2GP-1^{-/-} challenged with LPS produced higher levels of the pro-inflammatory cytokines TNF, MCP-1 and IL-12p70 than female β 2GP-1^{-/-} mice. Female mice challenged with *E. coli* produced higher levels of anti-inflammatory cytokine IL-10 whilst male mice produced higher levels of IL-6 and MCP-1. Female WT mice cleared *E. coli* more efficiently from the circulation than male mice. However, in the absence of β 2GP-1 this efficiency is lost and in addition, the sex difference in bacterial clearance is diminished. These findings support a role for β 2GP-1 in modulating the gender-specific susceptibility to bacterial-sepsis, not previously reported, and present potential for targeted treatments.

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Langerin⁺ CD8a⁺ dendritic cells play a protective role early after systemic bacterial infection in mice

Prendergast, K.^{1,2}, Petersen, T.¹, Hermans, I.¹, Kirman, J.³

¹Malaghan Institute of Medical Research, Wellington, New Zealand, ²University of Sydney, Sydney, Australia, ³University of Otago, Microbiology and Immunology, Dunedin, New Zealand

Residing in the marginal zone of the murine spleen, langerin⁺ CD8a⁺ dendritic cells (DC) are known to be able to cross-prime CD8⁺ T cells and produce IL-12. Given these qualities, we hypothesised that langerin⁺ CD8a⁺ DC might be critical drivers of adaptive responses to blood-borne bacterial infections. Using intravenous *Mycobacterium bovis* bacille Calmette Guerin (BCG) as a model of blood-borne bacterial infection, we investigated the impact of depleting langerin⁺ CD8a⁺ DC in mice that express the diphtheria toxin receptor under the control of the langerin promoter. We found bacterial numbers in the spleen increased when langerin⁺ CD8a⁺ DC were depleted during the first week of intravenous BCG infection; this was associated with delayed CD8⁺ T cell responses and reduced serum levels of IL-12p40. *In vivo* depletion of CD8⁺ T cells or IL-12 neutralisation did not induce a similar increase in bacterial numbers, suggesting langerin⁺ CD8a⁺ DC were exerting bacterial control by another mechanism. Further experiments revealed depletion of langerin⁺ CD8a⁺ DC led to increased bacterial numbers in the spleen as early as 30 mins after BCG infection. Our data reveal, that in addition to initiating CD8⁺ T cell responses and IL-12 production, langerin⁺ CD8a⁺ DC mediate early control of systemic bacterial infection.

865

A potential role for bacterial superantigens in tonsillar hypertrophy

Radcliff, F.¹, Clow, F.¹, Mahadevan, M.², Waldvogel-Thurlow, S.³, Proft, T.¹, Douglas, R.³, Fraser, J.¹

¹University of Auckland, Molecular Medicine and Pathology, Auckland, New Zealand, ²Gillies Hospital, Auckland, New Zealand, ³University of Auckland, Department of Surgery, Auckland, New Zealand

The tonsils are lymphoid organs that act as sentinels for the nasopharyngeal region and can be prone to recurrent infection, particularly throughout early childhood. We hypothesise that some cases of tonsillar hypertrophy may be driven by production of bacterial superantigens (SAG), potent activators of selected TCR V β subsets that are primarily produced by *Staphylococcus aureus* and Group A Streptococcus (GAS). To date we have collected tonsil tissue from 81 patients (median age = 4 years) undergoing a tonsillectomy for recurrent tonsillitis (RT) or Obstructive Sleep Apnoea (OSA). *S. aureus* was cultured from the tonsil surface and/or tissue of ~50% (40/81) of patients and GAS from 10% (8/81) of patients. The tonsillar CD4⁺ and CD8⁺ TCRV β repertoire was assessed by flow cytometry and skewing identified in 30% (25/81) of patients, 70% (18/25) of which had RT. *S. aureus* and GAS were cultured from 11/25 and 2/25 of these patients. All bacterial isolates are being profiled for the presence of SAG genes by multiplex PCR and mitogenic activity by proliferation assays to determine whether there is a match between TCR V β skewing patterns and selected SAG in some patients. Fluorescence microscopy is being employed to determine whether bacteria, particularly *S. aureus*, are present in a location where they can interact with T cells and drive inflammation. If a causal association between the presence of *S. aureus* and tonsillar hypertrophy is established, this could lead to a revision of current treatment practices for this illness.

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Lung tissue resident T cell immune responses to *Mycobacterium tuberculosis* infection in humans

Ogongo, P.^{1,2}, Leslie, A.¹

¹Kwa-Zulu Natal Research Institute for TB and HIV, Durban, South Africa, ²Institute of Primate Research, Tropical and Infectious Diseases, Nairobi, Kenya

Pathogen specific lymphocytes accumulate in the tissue where they are needed to fight off infection and, importantly, these responses are not necessarily represented in the circulation. Data from animal models of *M. tuberculosis* infection show that even within the lung, immune response differ between different TB granuloma, and these differences correlate with local TB control. However, data on human adaptive immune responses to TB infection come mostly from the study of blood. We hypothesise that adaptive immune responses to *M.tb* infection in the lung are different from immune responses in circulation during infection. We aim to learn the phenotype, function and antigen specificity of TB adaptive immune response that can control *M.tb* at the level of individual lesion and correlate these to responses that are detectable in circulation. Working

with tuberculosis infected human lung samples processed under BSL3 procedures, the immune profiles in the tissue are investigated by an array of immunological techniques including mass cytometry, cell sorting by conventional flow cytometry, as well as histology. Matched blood from same individuals are also processed for PBMC and acquired similarly. Initial results show that CD103 is a better marker of activation of lung resident T cells than CD69. Lung resident T cells are multifunctional producing a mixture of TNF-alpha, IL-2 and IFN-gamma and also produce low levels of IL-17. We observe that CD8+ specifically produce high levels of IFN-gamma. The overall findings of this study will inform better tuberculosis vaccine design strategies to augment tuberculosis control efforts.

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ManLAM of mycobacterium tuberculosis negatively regulates anti-TB immunity

Zhang, X.-L., Pan, Q., Yuan, C., Sun, X.-M.

Wuhan University School of Medicine, Immunology, Wuhan, China

Many pathogens-surface glycans have been implicated as an immunosuppressive epitopes and might contribute to chronic or latent infection and immune invasion. Mannose-capped lipoarabinomanan (ManLAM) is a lipoglycan serving as a major cell wall component in Mycobacterium tuberculosis (M.tb). Here, we demonstrated a novel ManLAM mediated immune evasion mechanism by inhibitory effects on the polarizations of M1 macrophages and Th1 cells. Our data suggest that BM2, an aptamer specifically against ManLAM with low and no toxicity, holds a potential application as BCG immunoadjuvant and provides a new strategy for TB vaccine design.

Accumulating evidences suggest that B cells contribute significantly to shaping and modulating the immune response to intracellular microbes. Here we report that IL-10-producing B (B10) cells are increased in peripheral blood mononuclear cells (PBMC) from patients with active pulmonary tuberculosis (TB). We also demonstrate a novel mechanism in which M.tb ManLAM exploits B10 cells to negatively regulate anti-TB immunity.

1075

A forward genetic screen for zebrafish genes involved in pneumococcal infection

Saralahti, A.¹, Harjula, S.-K.¹, Partanen, E.¹, Rantapero, T.¹, Granberg, K.¹, Lohi, O.², Nykter, M.¹, Rounioja, S.³, Parikka, M.¹, Rämetsä, M.^{1,4,5}

¹University of Tampere, BioMediTech, Tampere, Finland, ²University of Tampere, Tampere Center for Child Health Research, Tampere, Finland, ³Fimlab Laboratories, Tampere, Finland, ⁴Tampere University Hospital, Department of Pediatrics, Tampere, Finland, ⁵Oulu University Hospital, Department of Children and Adolescents, Oulu, Finland

Streptococcus pneumoniae (pneumococcus) is a major human pathogen and one of the leading causes of pneumonia, septicemia, and meningitis. The complex interactions occurring between the pneumococcus and the immune system are only partly understood and, therefore, the optimal treatment and

prevention methods are lacking. Previously, we showed that zebrafish (*Danio rerio*) are valuable hosts in the study of the innate immune response against pneumococcus. In the present study, we have employed the zebrafish embryo model for the forward genetic screen to identify novel host genes involved in pneumococcal infection. Using the gene-breaking transposon based mutagenesis method, we generated 150 zebrafish families with unknown mutations and screened these families for the altered susceptibility for systemic pneumococcal infection. With this screen, we were able to reveal 21 mutant families that are hypersusceptible to pneumococcal infection and, recently, we identified the insertion sites in these families by Next Generation Sequencing (NGS). NGS revealed several genes and intergenic regions with a potential, previously unknown, role in pneumococcal infection. Now, the role of these genomic sites is assessed in zebrafish embryos using the CRISPR/Cas9 site-directed mutagenesis and the role of these sites in the defense against pneumococcus is characterized further. Eventually, the screen is likely to expand our understanding of the innate immune response associated with pneumococcal diseases.

2053

Distinct immunological signature for *M.tuberculosis* infected children with and without overt disease

Dreesman, A.¹, Smits, K.¹, Corbière, V.¹, Dirix, V.¹, Debulpaepe, S.¹, Libin, M.¹, De Schutter, I.², Singh, M.³, Locht, C.⁴, Malfroot, A.², Mascart, F.¹

¹Université Libre de Bruxelles, Laboratory of Vaccinology and Mucosal Immunity, Brussels, Belgium, ²Universitair Ziekenhuis Brussel, Department of Pediatric Pulmonology, Brussels, Belgium, ³Lionex Diagnostics and Therapeutics, Braunschweig, Germany, ⁴Inserm U 1019, Institut Pasteur de Lille, Lille, France

Pediatric tuberculosis remains a global health problem associated with high morbidity and mortality. Its clinical spectrum differs from that of adults, as the majority of disease in children results from direct progression of the primary infection. A better understanding of protective immune responses is required to develop improved vaccines, to provide accurate diagnostic tests and to discover innovative therapeutic approaches. We characterized *Mycobacterium tuberculosis*-specific functional T cell subsets induced by two mycobacterial antigens (early-secreted-antigen-6 (ESAT-6), heparin-binding haemagglutinin (HBHA)) by flow cytometry, in 87 children exposed to *M. tuberculosis* in a low TB incidence country. They were classified as active tuberculosis (aTB), infection without overt disease (latent TB infection, LTBI), or uninfected. aTB cases were characterized by higher proportions of TNF- α ^{single+} CD4⁺T cells ($p < 0.05$ and trend in response to ESAT-6 and HBHA, respectively). In contrast, the proportions of IFN- γ ^{single+} CD4⁺ T cells were higher in children with LTBI compared to those with aTB ($p = 0.001$ and $p < 0.05$ for ESAT-6 and HBHA, respectively). The proportion of HBHA-induced IL-17^{single+} CD4⁺T cells was also higher in children with LTBI ($p < 0.05$). In contrast to the existing immunodiagnostic tests that are unable to discriminate between aTB and LTBI, we identify here distinct immunological signatures for children infected with *M. tuberculosis* at different stages of the disease. Our results provide the proof of concept

for the development of new, stage-specific diagnostic tests, that may help to target treatment of childhood tuberculosis to those that need it most.

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The role of the CXCL9/CXCR3 axis in mediating T-cell skewing in chronic lymphocytic leukemia

Hanna, B.S., Röbner, P.M., Lichter, P., Seiffert, M.

German Cancer Research Center (DKFZ), Molecular Genetics, Heidelberg, Germany

Chronic lymphocytic leukemia (CLL) is a malignancy of mature CD5⁺ B-cells that is characterized by a dysfunctional immune system. Disease development in CLL patients and mouse models is associated with skewing of T-cells towards antigen-experienced and exhausted phenotypes. However, the mechanisms governing these changes remain largely elusive. Serum analysis of the E μ -TCL1 mouse model of CLL has previously shown upregulation of various T-cell chemoattractants such as CXCL9 and CXCL16. Gene expression profiling and intracellular flow cytometric analysis further confirmed that monocytes are the major source of the increased CXCL9 in leukemic mice. In addition to impacting on CLL development, myeloid cell depletion using liposomal clodronate was associated with downregulation of serum CXCL9 levels. Concomitantly, decreased numbers of antigen-experienced T-cells were detected following myeloid cell depletion, suggesting a role of monocyte-derived CXCL9 in regulating T-cell skewing in CLL. Flow cytometric analysis revealed that higher percentages of T-cells in leukemic mice express the corresponding receptor, CXCR3. In addition, CXCR3 was mainly expressed on memory CD8⁺ T-cells with significantly lower levels on the PD-1^{hi} population. Furthermore, CXCR3⁺ CD8⁺ T-cells showed remarkable differences in the expression of several transcriptional regulators, activation markers and cytokines compared to PD-1^{hi} T-cells. Moreover, CLL development in CXCR3^{-/-} mice induced significantly lower numbers of memory T-cells, yet the numbers of PD-1^{hi} cells remained largely unaffected. Collectively, these data suggest that the CXCL9/CXCR3 axis specifically contributes to memory T-cell accumulation in CLL. The molecular mechanisms that regulate CXCR3 expression on various T-cell populations in CLL are currently under investigation.

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The tumor antigen NGcGM3 is a human CD1d ligand capable of mediating B cell and NKT interaction

Gentilini, M.V., Perez, M.E., Fernandez, P., Fainboim, L., Arana, E.

INIGEM (University of Buenos Aires-CONICET), Buenos Aires, Argentina

The ganglioside NGcGM3 is a tumor antigen (Ag). Some people have circulating Ag specific IgGs, capable of complement mediated cytotoxicity against NGcGM3 positive cells, relevant for tumor surveillance. Considering the Ag's chemical nature, we postulated it as a candidate ligand for CD1d. We aimed to assess whether the immune mechanism involved in the generation of

anti-NGcGM3 Abs entailed an interaction between B cells (Bc) and invariant NKT cells (iNKT).

We set up a cell free Ag presentation assay using a recombinant CD1d-IgG1 fusion on NGcGM3-coated plates and tested whether there were differences in the binding of the fusion and an IgG1 isotype control. Differences between them were reproducibly significant ($p < 0.01$), which was also true for differences with uncoated control wells. The results were confirmed through alternative biochemical assays including competitive ELISA with a known CD1d ligand (IC₅₀ 1.2 mmol/L). Using FACS, we demonstrated that human Bc present NGcGM3 in a CD1d context and paraformaldehyde treatment of cells expressing CD1d affects the presentation. Finally, by co-culturing primary human Bc with autologous iNKT and measuring Ki-67 expression, we detected a significant increment in the proliferation of both cell populations when Ag was on the medium, which was abrogated by blocking CD1d.

Our findings identify a novel, endogenous, human CD1d ligand, which is sufficiently competent to stimulate iNKT. We postulate that CD1d-restricted Bc presentation of NGcGM3 drives effective iNKT activation, a mechanism involved in the antitumor response that has not been previously described for humans, which may contribute to understanding anti-NGcGM3 occurrence.

1240

Highly suppressive regulatory T cells frequency in peripheral blood of breast cancer patients and their association with exhausted T CD8 lymphocytes

Martín Manzo, M.V.^{1,2}, Lara Gutiérrez, C.A.^{3,4}, Vargas De León, C.^{2,5}, Acuña Tovar, M.^{4,6}, Gutiérrez Reyes, G.², Zentella Dehesa, A.^{7,8}, Carrero Sánchez, J.C.⁹, Pérez García, A.^{2,10}, Lázaro León, J.M.⁴, Hernández Ruiz, J.^{2,11}

¹Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México, Ciudad de México, Mexico, ²Laboratorio de Hígado, Páncreas y Motilidad, Facultad de Medicina, Universidad Nacional Autónoma de México, Unidad Experimental, Ciudad de México, Mexico, ³Doctorado en Investigación en Medicina, Instituto Politécnico Nacional, Ciudad de México, Mexico, ⁴Unidad de Oncología Médica, Hospital General de México 'Dr. Eduardo Liceaga', Ciudad de México, Mexico, ⁵Maestría en Ciencias de la Salud, Instituto Politécnico Nacional, Ciudad de México, Mexico, ⁶Maestría en Ciencias Médicas, Odontológicas y de la Salud, Universidad Nacional Autónoma de México, Ciudad de México, Mexico, ⁷Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Departamento de Medicina Genómica y Toxicología Ambiental, Ciudad de México, Mexico, ⁸Instituto Nacional de Ciencias Médicas y Nutrición 'Salvador Zubirán', Departamento de Bioquímica, Ciudad de México, Mexico, ⁹Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Departamento de Inmunología, Ciudad de México, Mexico, ¹⁰Cirugía Experimental, Hospital General de México 'Dr. Eduardo Liceaga', Ciudad de México, Mexico, ¹¹Dirección de Investigación, Hospital General de México 'Dr. Eduardo Liceaga', Ciudad de México, Mexico

Tregs and exhausted T CD8 cells play an important role in immune evasion mechanisms exploited by cancer. These cells

have been found in tumor and peripheral blood of breast cancer (BrCA) patients. TIM-3 and PD-1 are inhibitory molecules upregulated in exhausted T CD8 cells and in highly suppressive Tregs located in blood and tumor of cancer patients. We compared the expression profile of PD-1 and TIM-3 in Tregs and T CD8⁺ in the peripheral blood of 7 healthy women vs. 14 stage III breast cancer patients; and studied the correlation between Tregs and exhausted T CD8⁺ subpopulations. We obtained 15 ml of peripheral blood; mononuclear cells were separated by ficoll density gradient, stained with monoclonal antibodies and analyzed by flow cytometry. PD-1⁺TIM-3⁺Tregs were increased in BrCA patients vs. controls ($p=0.01$) and correlated positively with PD-1⁺TIM-3⁺ memory and TEMRA T CD8⁺ ($r=0.56$); negatively with PD-1⁺TIM-3⁺ memory T CD8⁺ ($r=-0.56$). TIM-3⁺ (PD-1⁻ and PD-1⁺) Tregs correlated positively with PD-1⁻ TIM-3⁺ ($r=0.64$) and PD-1⁻TIM-3⁺ TEMRA T CD8⁺ ($r=0.56$). PD-1⁻TIM-3⁻ Tregs were decreased in BrCA vs. controls ($p=0.04$). PD-1⁺TIM-3⁻ Tregs were not significantly different, but in BrCA patients they correlated positively with PD-1⁺TIM-3⁻ TEMRA T CD8⁺ ($r=0.78$); negatively with PD-1⁻TIM-3⁻ TEMRA T CD8⁺ ($r=-0.60$), PD-1⁻TIM-3⁺ memory ($r=-0.60$) and TEMRA T CD8⁺ ($r=-0.70$). Our data reveal that PD-1⁺TIM-3⁺ Tregs are elevated in BrCA patients' blood and are associated with the exhausted T CD8⁺ lymphocytes, suggesting that Tregs and exhausted T CD8 populations may be interacting between them, contributing to immune evasion mechanisms in BrCA patients.

1819

Positive feedback loop between B cells/plasma cells and macrophages promote M2 macrophage-elicited hepatoma progression

Kuang, D.-M.

Sun Yat-sen University, School of Life Sciences, Guangzhou, China

Tumor progression has been recognized as the product of an evolving crosstalk between different cell types within tumor and its stroma. However, the crosstalk between humoral immunity and innate immunity within human cancer microenvironments is poorly defined. We show that infiltration of plasma cells orchestrates the differentiation and function of protumorigenic macrophages in human hepatocellular carcinoma (HCC): specialized polarized immunoglobulin G (IgG)-secreting plasma cells in HCCs trigger the differentiation of protumorigenic M2b polarized macrophages. Reciprocally, autocrine interleukin 6-elicited-interferon-inducible protein 10 (IP-10) and membrane-bound intercellular cell adhesion molecule-1/vascular cell adhesion molecule (ICAM-1/VCAM) expressed by macrophages selectively induces long-lived IgG-secreting plasma cells, forming a positive feedback loop, in coculture systems and mice. Plasma cell infiltration determines macrophage-associated clinical outcome of HCC patients. These findings suggest that bidirectional communications between macrophages and B cells in cancer microenvironments support humoral immunity-elicited myeloid cell suppression and disease progression.

2208

Anti-VISTA antibody enhances the anti-tumor immune response in mice with mammary tumors

Jensen, S.M.¹, Afentoulis, M.¹, Wegmann, K.W.¹, Campion, L.², Snyder, L.A.², Fox, B.A.¹

¹Earle A Chiles Research Institute, Portland Providence Medical Center, Portland, United States, ²Janssen Research and Development, LCC, Spring House, United States

The recent success of a number of immune checkpoint inhibitors in clinical cancer trials has spurred on research for other potential checkpoint targets. VISTA, V-domain Ig suppressor of T cell activation, is a novel checkpoint inhibitor that has shown the ability to suppress T cell proliferation and functionality. We examined the antitumor efficacy of an anti-mouse VISTA antibody (13F3) in established orthotopic murine mammary tumors (FAT) in female FVB/NJ mice. Mice bearing palpable tumors were treated with the anti-VISTA antibody every other day for 6 total doses, with the first treatment starting at day 11 post-tumor challenge. Treatment with anti-VISTA resulted in a statistically significant reduction in mean tumor growth. T cells from tumor draining lymph nodes of anti-VISTA treated mice secreted more IFN γ when stimulated with tumor compared to T cells from non-treated mice. Depletion of CD8⁺ cells, but not CD4⁺ cells, prior to treatment abrogated the therapeutic anti-tumor response. Employing multicolor immunohistochemistry we detected T cell infiltration of both CD4⁺ and CD8⁺ T cells into tumors. These studies demonstrate that an anti-VISTA antibody can augment the anti-mammary tumor immune response leading to a reduction in tumor growth.

3190

APOBEC gene family expression and activity in chronic lymphocytic leukemia: APOBEC3F splice variants correlate with patient outcome

Chu, C.C.^{1,2,3}, Vergani, S.¹, Yan, X.-J.¹, Dhayalan, A.¹, Patten, P.E.M.⁴, MacCarthy, T.⁵, Yuan, C.⁵, Barrientos, J.C.^{1,2}, Kolitz, J.E.^{1,2}, Allen, S.L.^{1,2}, Rai, K.R.^{1,2}, Chiorazzi, N.^{1,2,3}

¹Feinstein Institute for Medical Research, Northwell Health, Manhasset, United States, ²Hofstra Northwell School of Medicine, Department of Medicine, Hempstead, United States, ³Hofstra Northwell School of Medicine, Department of Molecular Medicine, Hempstead, United States, ⁴King's College London, Department of Haemato-Oncology, London, United Kingdom, ⁵Stony Brook University, State University of New York, Department of Applied Mathematics and Statistics, Stony Brook, United States

Human chronic lymphocytic leukemia (CLL) is an accumulation of clonal CD5⁺CD19⁺ B-lymphocytes, which may become more aggressive by acquisition of genome-wide somatic gene mutations and cytogenetic aberrations. Large-scale genomic ultra-deep sequencing of CLL cells has detected mutational signatures consistent with activity of the APOBEC (apolipoprotein B mRNA editing enzyme, catalytic polypeptide) family of cytidine deaminases, including activation-induced deaminase (AID) and APOBEC3 genes. This suggests that expression of APOBEC family members may lead to CLL progression. To test this, we correlated APOBEC family member gene expression

in CLL cells by microarray and quantitative real time PCR with patient outcome (time to first treatment and overall survival). Expression differences in AID, APOBEC3B, APOBEC3F and APOBEC3H in CLL cells exhibited correlations with worse patient outcome, whereas APOBEC3G did not. APOBEC3A levels were very low and difficult to measure. Interestingly, expression of a truncated splice variant of APOBEC3F exhibited an inverse correlation with worse patient outcome. We hypothesize that expression of truncated APOBEC3F may interfere with APOBEC3 family member mutational activity. To test mutational activity in CLL, we activated CLL cells by xenograft transfer into NOD-scid IL2Ry^{null} mice and analyzed mutational signatures in the expressed immunoglobulin variable region (IGHV). Induced IGHV mutational signatures consistent with AID, but little APOBEC3 activity, were found. This suggests that mutational activity of APOBEC3 genes in IGHV is inhibited, although their activity outside of IGHV cannot be excluded. Furthermore, these data support the hypothesis that AID mutation activity in CLL could lead to adverse consequences.

3455

Regulation of inflammatory tumor microenvironments by SphK1/S1P signaling

Guo, B.¹, Ogretmen, B.², Li, Z.¹

¹Medical University of South Carolina (MUSC), Department of Microbiology and Immunology, Charleston, United States, ²Medical University of South Carolina (MUSC), Department of Biochemistry and Molecular Biology, Charleston, United States

Tumor growth and progression are influenced by the interaction between tumor cells and inflammatory microenvironments. Sphingolipids, specifically ceramide and sphingosine 1-phosphate (S1P), are bioactive signaling molecules, which regulate cellular survival, proliferation, migration in innate and adoptive immune systems. Emerging evidence indicates that S1P also plays important roles in the development of various tumors, including breast cancer, through its ability to promote tumor cell growth, and invasion. In human cancer patients, increased expression of sphingosine kinase 1 (SphK1), a kinase for S1P synthesis, is correlated with poor prognosis and high incidence of diseases recurrence. While most of studies have focused on the role of sphingolipids in proliferation and survival of cancer cells, how the SphK1/S1P pathway modulates inflammatory microenvironments and tumor immunity is poorly understood. Our preliminary data have demonstrated that the interaction of sphingolipids and inflammasomes modulates tumor microenvironment and promote tumor metastasis. Inflammasomes are newly recognized innate immune sensors critical for active IL-1 β production and inflammatory response. Our results show that S1P induced inflammasome activation and IL-1 β production. We found that mice deficient for SK1 or inflammasome components had much reduced tumor growth and lung metastasis. Interestingly, we also found that IL-1 induced expression of SK1 in macrophages and tumor cells. Together, our results support a novel model whereby sphingolipids and inflammasome/IL-1 pathways are functional linked to induce inflammation and tumor development.

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1217

The potential of inflammatory responses to contribute to the development of transfusion-related acute lung injury (TRALI)

Sultana, A.^{1,2,3}, Meka, D.^{1,3}, Dean, M.M.^{1,3}, Simonova, G.^{1,4}, Christensen, A.-M.^{1,3}, Flower, R.L.^{1,2,3}, Tung, J.-P.^{1,2,3,4}

¹Australian Red Cross Blood Service, Research and Development, Kelvin Grove, Australia, ²University of Queensland, School of Medicine, Brisbane, Australia, ³Queensland University of Technology, Faculty of Health, Brisbane, Australia, ⁴The Prince Charles Hospital, The Critical Care Research Group, Chermside, Australia

Transfusion-related acute lung injury (TRALI) is a significant cause of post-transfusion morbidity and mortality. TRALI develops via a two-insult mechanism: the first insult being the patient's underlying morbidity, and the second insult being transfusion-related. Biological response modifiers (BRMs) that accumulate during blood product storage and anti-leucocyte antibodies are considered causative. Recruitment and activation of neutrophils and monocytes is implicated in TRALI; however, the precise mechanisms remain uncertain.

An in vitro transfusion model was developed in which blood from healthy volunteers, incubated with or without lipopolysaccharide (LPS; first insult), was exposed to either a BRM (soluble CD40 ligand (sCD40L), 5-hydroxyeicosatetraenoic acid (HETE), 12-HETE, 15-HETE or interleukin (IL)-8) or an antibody (antibody targeting human leucocyte antigen (HLA) class II). Cytometric bead array, intracellular staining and flow cytometry were used to investigate overall, neutrophil-specific and monocyte-specific inflammatory responses (i.e. changes in concentration or expression of a panel of chemokines and cytokines).

Without LPS, inflammatory responses were suppressed following exposure to BRMs or anti-HLA-II, suggesting that less ill patients may have an increased risk of infection following transfusion of these factors. In contrast, in an induced inflammatory state (i.e. with LPS), exposure to anti-HLA-II, IL-8, 12-HETE or 15-HETE resulted in pro-inflammatory changes, suggesting that the transfusion of these factors may contribute to TRALI development in severely ill patients.

This study modelled how transfusion-related inflammation may contribute to the development of TRALI. This improved understanding may contribute to the identification of strategies to reduce the risk of TRALI and improve blood transfusion safety.

4538

IL-27 induces T cell stemness and restrains their effector functions in vivo

Liu, Z.¹, Zhu, J.², Liu, J.-Q.¹, Wu, L.¹, Zhu, X.¹, Bai, X.-F.^{1,2}

¹Ohio State University, Columbus, United States, ²Shanghai Jiaotong University School of Medicine, Shanghai, Shanghai, China

Interleukin-27 (IL-27) is a heterodimeric cytokine that is composed of two subunits, i.e. Epstein-Barr virus (EBV)-induced gene 3 (EBI3) and IL-27p28 (also known as IL-30). IL-27 is known

to induce the expression of a range of inhibitory molecules such as IL-10 and PD-L1 in T cells. Our previous *in vitro* study has revealed that IL-27 also induces T cell expression of Sca-1/Ly-6A, a cell surface glycoprotein that controls self-renewal capacity of stem cells. In this study, we have examined if IL-27 induces Sca-1 expression in T cells *in vivo* and its functional relevance. We found that IL-27-deficient mice (deficient for either P28 or EBI3) had profound reduction of Sca-1 expression in naïve and memory T cells. In contrast, *in vivo* delivery of IL-27 using adeno-associated viral vectors (AAV) strongly induced the expression of Sca-1 in naïve and memory T cell populations in a Stat1-dependent manner. In IL-27-deficient mice, ConA-induced liver injury was significantly enhanced, with reduced expression of Sca-1, PD-L1 and IL-10 in liver T cells. In contrast, *in vivo* delivery of IL-27 by AAV induced the expression of Sca-1, PD-L1 and IL-10 in liver T cells and significantly protected mice from ConA-induced liver injury. Thus, IL-27 induces T cell “stemness” but at the same time restrains their immediate effector functions *in vivo*.

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A new GM-CSF-dependent pathway in arthritis

Achuthan, A., Lee, M.-C., Saleh, R., Frye, A., Fleetwood, A., Cook, A., Hamilton, J.

University of Melbourne, Dept of Medicine, Parkville, Australia

The socioeconomic burden of inflammatory diseases, such as rheumatoid arthritis, is enormous both in terms of direct and indirect healthcare costs. Clinical trials in rheumatoid arthritis targeting the cytokine, granulocyte macrophage-colony stimulating factor (GM-CSF) are showing promise although its mode of action remains largely unknown. Increased macrophage numbers in the synovial fluid from arthritic knee joints is highly correlated with the severity of the disease. The interferon regulatory factor (IRF) family of transcription factors are important in controlling expression of genes involved in immune functions. Their key role in controlling gene expression in monocytes/macrophages has recently become a major focus of research.

We report here that GM-CSF induced IRF4 expression, while suppressing IRF8 in primary human monocytes. We found that the chemokine CCL17 expression was induced in GM-CSF-treated human monocytes and largely dependent on IRF4 transcription factor. Significantly, a recent study reported that synovial fluid from patients with rheumatoid arthritis had elevated levels of CCL17 as compared to healthy controls. Interestingly, CCL17 gene is clustered together with CCL22 and CX3CL1 on human chromosome 16q13. The transcriptional regulation of these three chemokines by GM-CSF in human monocytes and their role in *in vivo* arthritis models will be presented and discussed.

137

Enhanced expression of BAFF receptor (BR3) on peripheral monocytes contributes production of IgG by B cells through IL-6 signaling in patients with primary Sjögren's syndrome

Yoshimoto, K., Ishioka, E., Nishikawa, A., Suzuki, K., Takeuchi, T.

Keio University School of Medicine, Tokyo, Japan

Background and purpose: We found that the expression level of BAFF receptor (BR3) on pSS monocytes was significantly elevated compared with normal monocytes, and that BAFF robustly increased IL-6 production by pSS monocytes *in vitro*. We also found that the proportion of BR3 positive monocytes (BR3+/CD14+) was positively and significantly correlated with the serum IgG level in pSS patients. These data suggest a possible involvement of monocytes in IgG production. In this study, we investigated a role of BAFF and IL-6 as mediators in the stimulation of B cells by monocytes.

Methods: Peripheral monocytes were co-cultured with B cells in the presence of soluble BAFF (sBAFF) and an anti-human sIL-6R antibody. B cells and monocytes were either directly mixed or separated with a transwell insert in the well. The amounts of IL-6 and IgG in the culture supernatants were measured by ELISA. The expression of BR3 and CD14 on monocytes was analyzed by FACS.

Results: Stimulation of a co-culture of pSS B cells and monocytes with sBAFF drastically enhanced IgG production *in vitro*. It should be noted that separation of these cells by a transwell insert in a well did not suppress the IgG production, suggesting an involvement of humoral factor(s) in the IgG production. Moreover, addition of an anti-human sIL-6R antibody to the co-culture significantly inhibited the IgG production.

Conclusions: Based on these findings, we presume that IL-6 produced by BAFF stimulated monocytes plays a pivotal role in IgG production by pSS B cells.

4528

Methylation transferase EZH2 was involved in the effect of let-7e on TNF- α expression in DENV2-infected THP-1 cells

Zhang, Y., Zhang, Q., Gui, L., Cai, Y., Guo, Q., Huang, J., Junqi

Huang's Lab

The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China

In a small proportion of cases, dengue fever (DF), a mosquito-borne tropical disease caused by the dengue virus (DENV), develops into the life-threatening dengue hemorrhagic fever (DHF), or into dengue shock syndrome (DSS). Patients with DHF/DSS present a ‘cytokine storm’, with high levels of circulating cytokines and chemokines, including tumor necrosis factor α (TNF α). Our previous study revealed that the increased level of miRNA let-7e was associated with downregulation of TNF α in DENV-infected PBMCs. In this study we demonstrated that let-7e was able to inhibit TNF α expression and confirmed that not TNF α but methylation transferase enhancer of zeste homolog 2 (EZH2), histone methyltransferase catalyzing histone H3K27me₃, was the direct target of let-7e. We found silence of EZH2 (siEZH2) could inhibit TNF- α expression by downregulation of Arfaptin2 and ARFGAP1, two vesicular transporter that are helpful for

cytokine secretion with the function in ADP-ribosylation factor (Arf)-dependent membrane traffic outside the ER-Golgi. siEZH2 can also inhibit NF kappa B pathway to downregulate TNF- α expression.

3745

ApoE production in monocytes is altered by anti-inflammatory drugs

Beckman, L.¹, Kumagai-Braesch, M.², Lind, K.¹, Braesch-Andersen, S.¹
¹Mabtech AB, Nacka Strand, Sweden, ²Karolinska Institutet, Transplantation Surgery, Stockholm, Sweden

We have previously shown that peripheral blood monocytes produce considerable amounts of apoE when induced by TGF β and that this apoE production is inhibited by inflammatory signals, similarly to tissue macrophages. In apoE deficient mice it has been shown that restoring apoE production in tissue macrophages is crucial for preventing damage to blood vessels. It is well known that patients suffering from atherosclerosis have signs of chronic low grade inflammation in their vessels and it is likely that this will affect apoE production vital for healing. There are a large number of different anti-inflammatory substances on the market and here we want to see if any of these could be useful for restoring apoE production in atherosclerotic tissues. We have used Peripheral Blood Mononuclear Cells (PBMC) and the monocytes present in these preparations to look at the effects of a large number of anti-inflammatory pharmacological substances on apoE production. By addition of TGF β with or without TNF α in the culture, we can alter the basic apoE production of PBMC and then investigate how the production is affected by co-culturing with pharmacological substances at concentrations achieved by recommended doses. We analyzed apoE production by ELISA, ELISpot and Fluorospot. We can show that several anti-inflammatory substances affect the monocyte apoE production. Ibuprofen significantly increases apoE production while Diclofenac, acetyl-salicylic acid and Exendin-4 have no such effects. This indicates that these substances vary in their ability to increase monocyte apoE production and maybe how they modulate the immune system in an anti-atherogenic direction.

338

Zebrafish as a genetic model for leukemia and other hematopoietic disorders

Basheer, F., Liougue, C., Ward, A.C.
 Deakin University, School of Medicine, Waurn Ponds, Australia

Cytokines play a major role in regulating hematopoiesis and immunity. Perturbed cytokine signalling has been implicated in a variety of myeloproliferative disorders, including leukemia. Unravelling the molecular mechanisms involved in the pathogenesis of these hematological malignancies can provide major insights into their mechanism of action and contribute to the development of novel therapeutics. Indeed cytokine receptors and the associated tyrosine kinases are attractive targets for oncogenic therapy, with dysregulation often leading to constitutive activation that can be successfully inhibited

by pharmacological agents. Zebrafish is well suited to model hematopoiesis and leukemogenesis, providing information into underlying genetic causes, as well as providing a platform for therapeutic testing. This project aims to generate mutants of relevant cytokine signalling components by genome editing to create zebrafish models of hematologic malignancies and other disorders. The first of these targets the *jak3* gene, encoding a key tyrosine kinase downstream of the interleukin 2 receptor family.

Autoimmunity 1

632

Anti-myeloperoxidase pathogenic autoimmunity can be induced by a *Staphylococcus aureus* plasmid derived peptide

Ooi, J.D.¹, Jiang, J.-H.², Eggenhuizen, P.J.¹, Holdsworth, S.R.^{1,3}, Peleg, A.Y.^{2,4}, Kitching, A.R.^{1,3,5}

¹Monash University, Centre for Inflammatory Diseases, Clayton, Australia, ²Monash University, Infection and Immunity Program, Monash Biomedicine Discovery Institute, Department of Microbiology, Clayton, Australia, ³Monash Health, Department of Nephrology, Clayton, Australia, ⁴Monash University, Department of Infectious Diseases, The Alfred Hospital and Central Clinical School, Prahran, Australia, ⁵Monash Health, Department of Paediatric Nephrology, Clayton, Australia

Microscopic polyangiitis is a small-vessel vasculitic autoimmune disease whereby autoreactivity to the neutrophil enzyme myeloperoxidase (MPO) causes rapidly progressive glomerulonephritis. It is unknown how tolerance is lost, but there are reports in patients that *S aureus* infections precede anti-MPO autoimmunity, and in other forms of vasculitis, molecular mimicry has been shown to induce cross-reactive pathogenic autoimmunity.

This study tests the hypothesis that molecular mimicry induces cross-reactive pathogenic anti-MPO autoimmunity. A BLAST search was used to identify 5 candidate microbial peptides with homology to the immunodominant MPO T-cell epitope, MPO₄₀₉₋₄₂₈. Anti-MPO T-cell responses were determined by immunizing C57BL/6 mice with candidate peptides then measuring recall responses *ex vivo* to MPO by ³[H]-T proliferation and IFN- γ and IL-17 ELISPOTs. Anti-MPO antibodies were measured by ELISA, immunofluorescence on ethanol-fixed neutrophils and by enumerating neutrophil glomerular recruitment after passive transfer of antibody. Disease was assessed by immunizing mice with peptide or whole bacteria followed by depositing MPO in the glomerulus using low-dose sheep anti-mouse glomerular basement membrane antibodies, then measuring functional and histological endpoints.

Only 1 peptide induced cross-reactive T-cell responses to MPO. This peptide was derived from a *S aureus* plasmid. Antibodies from mice immunized with this peptide cross-reacted with MPO, bound to neutrophils in a perinuclear fashion, and induced neutrophil glomerular recruitment. Furthermore, mice immunized with either this peptide or a clinical strain of *S aureus* containing the plasmid, developed albuminuria and focal necrotizing glomerulonephritis. This shows that molecular

mimicry by a *S aureus* plasmid derived peptide induces anti-MPO pathogenic autoimmunity.

2111

Short chain fatty acids regulate CD1d-restricted natural killer T cells in limiting autoimmune diabetes

Richards, J.L., McLeod, K.H., Yap, Y.A., Mackay, C.R., Mariño, E. Monash University, Biochemistry, Clayton, Australia

Type 1 Diabetes (T1D) is a T cell-mediated autoimmune disease leading to serious complications, with rising incidence in western countries. Protection in non-obese diabetic (NOD) mice deficient in MyD88, and housed in specific pathogen free facilities, have reduced T1D incidence, while germ-free counterparts experience exacerbated disease, implicating gut microbiota in T1D protection. High fiber diet modify commensal gut microbiota to produce short-chain fatty acids (SCFAs) acetate and butyrate, which have anti-inflammatory effects. Acetate is the main ligand for the receptor GPR43, and deficiency of this receptor in NOD mice results in accelerated disease under SPF housing. Here we showed that NOD.Gpr43^{-/-} mice as well as germ free NOD mice presented a reduced frequency of CD1d-restricted iNKT cells compared to NOD mice. iNKT cells are implicated in the regulation of different autoimmune diseases in mice and humans. We found that feeding NOD mice with high acetate-yielding diets increased splenic iNKT numbers coincident with decreased mature CD1d⁺ marginal zone B cell numbers. Interestingly, IL-17A⁺ iNKT cells from the pancreatic lymph nodes were reduced, whereas IL-4⁺ NKT (NKT2) cell numbers were elevated. Thus, SCFA may target iNKT cell subsets differently based on tissue distribution and T1D progression. This is a critically important since poor quality of diets affecting the gut microbiota towards pathogenic bacteria could contribute to the increased T1D in western countries.

2357

Autoimmune response to amyloidogenic transthyretin in childhood arthritis

Moncrieffe, H.¹, Clement, C.C.², Lele, A.¹, Porcelli, S.A.³, Santambrogio, L.^{2,3}

¹Cincinnati Children's Hospital Medical Center, Center for Autoimmune Genomics & Etiology, Cincinnati, United States,

²Albert Einstein College of Medicine, Department of Pathology, New York, United States, ³Albert Einstein College of Medicine, Pediatric Rheumatology, New York, United States

The most common pediatric rheumatologic condition is juvenile idiopathic arthritis (JIA): an estimated 1 child in every 1000 develops inflammation of the joints before the age of 16. The antigens which trigger JIA are unknown and are hypothesized to be autoantigenic. The aim of this study was to identify putative autoantigenic triggers using a cohort of 50 patients with JIA and 24 age-matched healthy controls. Through detailed proteomic profiling, the transporter protein transthyretin (TTR) was revealed as a candidate antigenic target for B and T cell immune responses in JIA. To assess whether TTR was located in the JIA inflamed site, ELISA was performed and

both TTR protein and anti-TTR autoantibodies were present at a significantly higher amount in JIA patients and compared to controls ($p < 0.001$). Profiling of immune complex-associated antigens in JIA synovial fluid revealed transthyretin was bound to IgG at the site of inflammation. TTR peptides were found in peptidome of JIA patient synovial fluid and synthesized TTR peptides were used to test T cell reactivity. HLA-DR1-restricted peptides induced production of the proinflammatory cytokines IFN γ and TNF α as well as CD4⁺ T cell proliferation in a subset of patients. Together, these data indicate a role of TTR as an autoantigen involved in JIA pathology.

2478

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) suppresses joint inflammation and bone erosion by inhibiting T cell activation in experimental arthritis

Chyuan, I.-T.^{1,2}, Tsai, H.-F.^{3,4}, Hsu, P.-N.^{5,6}

¹National Taiwan University College of Medicine, Graduate Institute of Clinical Medicine, Taipei, Taiwan, Republic of China,

²Cathay General Hospital, Department of Internal Medicine, Taipei, Taiwan, Republic of China, ³Taipei Medical University College of Medicine, Institute of Clinical Medicine, Taipei, Taiwan, Republic of China,

⁴Taipei Medical University Shuang Ho Hospital, Department of Internal Medicine, Taipei, Taiwan, Republic of China, ⁵National Taiwan University College of Medicine, Graduate Institute of Immunology, Taipei, Taiwan, Republic of China, ⁶National Taiwan University Hospital, Department of Internal Medicine, Taipei, Taiwan, Republic of China

Rheumatoid arthritis (RA) is a complex, heterogeneous systemic autoimmune disease involving a wide array of joint inflammation and bone erosion, eventually leading to disabling with a significant morbidity and mortality. Targeting to key points of the pathogenesis of RA is the current mainstay of new therapeutics development. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) belongs to TNF superfamily; from recent studies and accumulated evidences, in addition to triggering apoptosis, TRAIL has been implicated its actual biological function in immune-regulation and immune-mediated diseases. However, the role of TRAIL in autoimmune arthritis remains to be elucidated. In this study, we demonstrated that TRAIL significantly inhibited joint inflammation and development of arthritis in collagen-induced arthritis (CIA) rats. The effect of TRAIL-induced suppression of arthritis in CIA was as potent as TNF inhibitor, etanercept. Furthermore, TRAIL profoundly suppressed the joint inflammation, synovium proliferation and restored bone erosion. Interestingly, the inhibition of joint inflammation by TRAIL was not through the effects of inducing apoptosis in T cells or macrophages. By contrast, TRAIL could directly inhibit T cell proliferation and T cell-derived inflammatory cytokines, indicating TRAIL may exert its anti-arthritic effect through T cell inactivation. Our results provided evidence of a novel mechanism of TRAIL in anti-inflammatory arthritis and shed light on the possible therapeutic application in inflammatory arthritis and in developing better strategies for treating RA in the future.

3045**IL-33 modulates IL-10 and Treg responses in a mouse model of drug hapten, immune-mediated hepatitis**

Cottagiri, M.¹, Mallory, B.¹, Chen, G.¹, Thomas, D.¹, Mantila, J.¹, Rose, N.^{2,3}, Njoku, D.⁴

¹Johns Hopkins University, ACCM, Baltimore, United States, ²Johns Hopkins University, Pathology, Baltimore, United States, ³Harvard University, Pathology, Boston, United States, ⁴Johns Hopkins University, ACCM, Pathology, Pediatrics, Baltimore, United States

Hepatitis is a global health problem linked to autoimmunity, fibrosis and cancer. IL-33 modulates hepatitis and fibrosis in animal models; however, in these models IL-33/ST2 signaling is either protective¹ or pathogenic². IL-33 has been detected in sera of hepatitis C patients. We detected significantly elevated IL-33 in sera from patients with immune-mediated hepatitis from anesthetics, as well as in BALB/c mice with drug-hapten hepatitis modeled after this disease, where mice are immunized on days 0 and 7 with cytochrome P450E1 epitopes covalently modified by trifluoroacetyl chloride drug haptens formed during anesthetic metabolism. In our mouse model, we detected innate lymphoid cells expressing the IL-33 receptor ST2 by flow cytometry, as well as IL-4 and IL-6-driven hepatitis by 3 weeks. Intraperitoneal anti-IL-33 diminished hepatitis in BALB/c mice, and IL-33 deficient (-/-) mice produced significantly less IL-4 and IL-6 (ELISA) early in the disease pathogenesis. However, IL-33-/- mice developed significant hepatitis by 3 weeks along with significantly down-regulated hepatic IL-10 (ELISA) and regulatory T cell (Treg)-associated Foxp3 mRNA (NanoString®), suggesting that IL-33 modulates IL-10 and Treg responses to immunizations. A recent study reported that IL-33 promotes Treg function in rodent experimental colitis³. We previously showed that Tregs significantly reduced drug-hapten hepatitis⁴. Our preliminary findings suggest that IL-33 has both proinflammatory and suppressive roles in drug-hapten hepatitis possibly via Treg immunomodulation. Investigating IL-33 immunomodulation in drug-hapten hepatitis could uncover targets that modulate hepatitis from other etiologies.

1 Sakai et al, 2012

2 McHedlidze et al., 2013

3 Schering et al., 2014

4 Cho et al., 2013

564**The role of somatic hypermutation and N-glycosylation in the anti-NET immunoreactivity of RA synovial monoclonal antibodies**

Corsiero, E., Carlotti, E., Prediletto, E., Jagemann, L., Pitzalis, C., Bombardieri, M.

William Harvey Research Institute, Centre for Experimental Medicine & Rheumatology, London, United Kingdom

Anti-citrullinated peptide/protein antibodies targeting citrullinated antigens in neutrophils-extracellular-traps (NET) can be manufactured within ectopic-germinal-center-like structure (GC-LS) in the rheumatoid arthritis (RA) synovium. Here, we aimed to characterise: whether RA-synovial anti-NET antibodies had undergone antigen-driven affinity maturation,

somatic hypermutations (SHM) importance within VH/VL chain for NET binding and for Fab-domains N-glycosylation.

82 recombinant monoclonal antibodies (RA-syn-rmAbs) were generated from single CD19+B-cells FACS-sorted from fresh GC-LS+ synovial cell suspensions following IgVH+VL genes cloning. N-glycosylation was predicted with NetNGlyc1.0-Server and glycans presence was detected by total glycoprotein staining on gel. IgV genes reversion into germ-line (GL), generation of hybrid clones (VH/VL or selected CDRs/FRs reverted into GL, n=5) and N-glycosylation mutants (NtoQ, n=7) was performed by overlap-PCR. Anti-NET immunoreactivity was detected using cell-based immunoassays with activated peripheral blood or RA-synovial fluid neutrophils.

The RA-syn-rmAbs anti-NETs immunoreactivity was dependent on affinity maturation within GC-LS and was completely abrogated in the GL counterpart. Similarly, when only the single IgVH/VL gene was reverted into GL, NETs reactivity was lost suggesting that both VH+VL chains are important in this reactivity. The increased molecular weight observed in selected anti-NET antibodies was dependent on the presence of Fab-linked glycans and was lost in the GL counterpart.

Thus, SHM seems necessary for high-affinity NET-binding antibodies development in synovial GC-LS and for N-glycosylation introduction sites in the Fab-domain which could influence the NET-antigens binding. Defining the contribution of individual CDRs/FRs to the affinity of antigen-binding sites may help to engineer new therapeutic Abs and design of CDRs/FRs-specific peptides for tolerogenic strategy.

3291**A small-molecule preclinical candidate targeting ROR γ t shows a benign safety profile and effectively reduces clinical scoring and biomarker levels in mouse disease models**

Nielsen, S.J., Christiansen, S.J., Bengtsson, M., Andersson, J.L., Nørager, N.G., Ryborg, S., Jensen, K., Vestergaard, M., Stasi, L.P., Gouliarov, A.H., Franch, T., Glad, S.
Nuevolution A/S, Copenhagen, Denmark

Excessive production of the cytokine IL17A is the key driver in multiple autoimmune diseases, including Psoriasis, Psoriatic arthritis, and Ankylosing spondylitis as supported by clinical data using IL17A-directed antibodies.

The nuclear hormone receptor ROR γ t is a ligand-activated transcription factor, exclusively expressed in cells of the immune system, acting as a master regulator of IL17A production. Consequently, a small molecule targeting the ligand binding domain (LBD) of ROR γ t preventing the production of pro-inflammatory IL17A may offer a convenient oral therapy for IL17A-triggered inflammatory diseases.

We identified potent small molecule inhibitors from the screening of 830 million DNA-encoded compounds against ROR γ t-LBD. A lead series preventing SRC-1 co-activator recruitment was selected for further medicinal chemistry optimization. The resulting candidate compound has an IC₅₀ of 8nM in cell-based reporter assays and >100-fold selectivity against other NHRs. It is a low nM inhibitor of IL-17 production in PBMC and T-cell polarization assays with no effect on T_H1/2

cytokines. The candidate has attractive DMPK properties, high oral bioavailability and shows strong efficacy in clinical scoring and on biomarkers in several animal models of arthritis and dermatitis. In 7-day exploratory toxicology studies in mice, daily doses up to 600 mg/kg were well tolerated with no adverse effects, and only minimal findings in lymphoid tissues, indicating a safety window of ≥ 20 fold over a maximally efficacious dose. Our studies validate ROR γ t as a therapeutic target and indicate that our candidate warrants further investigation as a once-daily oral medicine in human autoimmune diseases.

Poster Monday

15:30:00 - 16:30:00

Adoptive Cell Therapy

1

CAR T cells are potent serial killers

Davenport, A.¹, Jenkins, M.R.¹, Cross, R.S.¹, Yong, C.S.¹, Trapani, J.A.¹, Kershaw, M.H.¹, Ritchie, D.S.^{1,2}, Darcy, P.K.¹, Neeson, P.J.¹

¹Peter MacCallum Cancer Centre, Cancer Immunology Research, Melbourne, Australia, ²Royal Melbourne Hospital, ACRF Lab, Department of Medicine, Melbourne, Australia

Adoptive therapy with autologous chimeric antigen receptor (CAR) T cells was rapidly translated into the clinic with success in refractory B-ALL and B cell lymphoma, but not other types of cancer. We postulate unknown features of CAR-T cell biology contribute to this low response rate in non-B cell cancers. We investigated whether the antigen receptor (CAR vs endogenous TCR) affected CAR-T cell immune synapse formation and tumour cell killing kinetics. We developed a novel transgenic mouse (designated CAR.OT-I), in which CD8⁺ T cells co-express the OVA₂₅₇-specific T cell receptor (TCR) and a second generation CAR with an scFv specific for human HER2. Day 7 OVA₂₅₇ activated CAR.OT-I T cells were re-stimulated with OVA₂₅₇-pulsed (TCR) or HER2-expressing tumour cells (CAR) and killing kinetics examined using time-lapse and confocal microscopy.

We show for the first time, irrespective of the mode of recognition, individual CAR.OT-I cells kill multiple tumour cells ('serial killing') and they detach more quickly from dying targets. We provide evidence for a different immune synapse structure between CAR and TCR mediated killing by CAR.OT-I T cells. Despite a faster detachment rate, CAR-T cells do not display enhanced cytotoxicity of tumour target cell populations in the long term (48 hrs), as CAR expression becomes downregulated. This study provides visual evidence that CAR T cells can serially kill multiple tumour targets in quick succession and provides important insights into CAR-T/tumour cell molecular interactions, which may have wider implications for solid tumour therapy using CAR T cells.

2

Combined immune and epigenetic therapy for high risk and relapsed leukaemias and lymphomas

Dolnikov, A.¹, Yang, S.², Shen, S.¹, Xu, N.¹, O'Brien, T.¹

¹Sydney Children's Hospital, Cord and Marrow Transplant Laboratory, Randwick, Australia, ²Children's Cancer Institute for Medical Research, Randwick, Australia

T-cells genetically modified with Chimeric Antigen Receptor (CAR) to target malignant cells is a novel approach with proven success in early phase human trials. A patient's own T-cells genetically modified with CAR can be amplified ex-vivo to numbers suitable for adoptive cell therapy and administered to the patient. Engineered T cells traffic to the tumour site

and kill cancer cells. CART cells then up-regulate immune-inhibitory receptors that suppress their immune function. In addition, CART-cell functionality can be compromised by the immunosuppressive tumour microenvironment. We have proposed the use of immune modulatory agents to maximise the effect of CART-cells. Using xenograft mouse model of chemoresistant paediatric B-cell lymphoblastic leukaemia (B-ALL) we have shown that CART-cell therapy can be potentiated by administration of a demethylating agent azacytidine (AZA) given prior to CART-cell infusion. Pre-treatment with AZA increased the numbers of short-lived effector memory (Tem)-cells with the robust anti-tumour activity. AZA-treated leukaemia cells expressed increased levels of CD80 ligand activating co-stimulatory CD28 signalling in Tem cells and commonly down-regulated in paediatric B-ALL. We hypothesise that AZA up-regulates the immune stimulatory ligands expressed by cancer cells thus converting tumour environment from immunosuppressive to immune-stimulating. B-ALLs demonstrate variable levels of multiple immune stimulatory and inhibitory molecules. Significant differences in CART-cell functions were found to be target B-ALL-dependent and correlate with the expression of co-stimulatory and inhibitory molecules on B-ALL cells. Our data justify the use of combined checkpoint inhibitor and epigenetic treatments to further potentiate CART-cell therapy.

3

Haematopoietic stem cells to generate chimeric antigen receptor (CAR)-modified T cells

Shen, S.¹, Xu, N.², Yang, S.³, O'Brien, T.², Dolnikov, A.²

¹Sydney Children's Hospital, Randwick, Australia, ²Sydney Children's Hospital, Cord and Marrow Transplant Laboratory, Randwick, Australia, ³Children's Cancer Institute for Medical Research, Randwick, Australia

Using T cells modified with a chimeric antigen receptor (CAR) recognising tumour antigen is a novel approach to treat cancer with proven success in clinical trials demonstrating a potent anti-cancer effect of CART-cells. However, the proportion of patients demonstrated only transient *in vivo* persistence of CART-cells was demonstrated, resulting in tumour recurrence. We and others have proposed that T cells with younger phenotype - central memory or naïve T cells and even stem memory T cells and T-cell precursors transduced with CARs can provide better *in vivo* expansion and long-term *in vivo* persistence of mature CART-cells. Here we tested a new approach to improve CART-cell persistence through the transduction of CARs into haematopoietic stem cells (HSC). Retroviral vector was used to transduce cord blood derived CD34⁺HSCs with CAR targeting human CD19 expressed in B-cell malignancies and normal B-cells or GD2 expressed in neuroblastoma and sarcomas.. Transduction with CARs achieved 60-70% and CAR-transduced HSCs engrafted immune-deficient mice with some delay compared to mock-HSCs. T-cells generated from CAR-transduced HSCs developed memory subsets confirming the expectation of development of long-lasting phenotypes. Mice transplanted with CAR-HSCs targeting CD19 had decreased CD19⁺B-cell populations confirming functionality of CAR targeting *in vivo*.

Delayed cancer progression following challenge with CD19⁺ leukaemia Raji cells or GD2⁺ neuroblastoma was observed in CAR-HSC-reconstituted mice compared to untreated controls. Anti-cancer effect was more robust and durable in stem cell reconstituted mice compare to mice received mature CART-cells. In summary, our data support the concept of genetic modification of HSCs with CARs.

4

Scalable human T cell isolation, activation and expansion using EasySep™ and ImmunoCult™

Kokaji, A.L.¹, Sun, C.A.¹, Ng, V.¹, Lam, B.S.¹, Clarke, S.J.¹, Woodside, S.M.¹, Eaves, A.C.^{1,2}, Thomas, T.E.¹

¹STEMCELL Technologies Inc., Vancouver, Canada, ²Terry Fox Laboratory, BC Cancer Agency, Vancouver, Canada

Cancer immunotherapy using chimeric antigen receptor T cells has demonstrated unprecedented success in early clinical trials. To enable researchers advancing this exciting field, we have developed novel cell isolation and activation reagents, and specialized culture medium required for the manufacturing of these cellular products. EasySep™ is a fast and easy immunomagnetic cell isolation platform for purifying T cells directly from leukopheresis samples. To induce T cell activation, we developed the soluble ImmunoCult™ Human T Cell Activators that have been optimized for use with our serum- and xeno-free ImmunoCult™-XF T cell expansion medium.

EasySep™ isolated T cells were cultured using soluble ImmunoCult™ activators or a bead-based CD3/CD28 activator in the presence of IL-2 or IL-7 and IL-15. The expression of activation markers including CD25, CD137, and PD-1 was monitored and the phenotype of the expanded T cells was determined with an antibody panel that included CCR7, CD27, CD28, CD45RA, CD95 and CD62L.

To demonstrate the scalability of our reagents, EasySep™ isolated T cells were expanded using ImmunoCult™ and bead-based CD3/CD28 activators in two large-scale systems: G-REX flasks and the GE Xuri cell expansion system. Both bioreactor systems and activators effectively expand T cells over the course of a 10 day culture period and upon restimulation, the T cells can continue to proliferate and produce effector cytokines, thereby confirming their functionality post-expansion. Taken together, our EasySep™ and ImmunoCult™ reagents can be used for the isolation and large-scale expansion of functional human T cells with equivalent performance to bead-based activation reagents.

5

iPSC based adoptive immunotherapy by using genome editing strategy

Minagawa, A.¹, Hotta, A.¹, Uemura, Y.², Nakatsura, T.², Kawana, K.³, Kaneko, S.¹

¹Kyoto University, Center for iPSC Research and Application, Kyoto, Japan, ²National Cancer Center, Kashiwa, Japan, ³University of Tokyo, Gynecology, Tokyo, Japan

We have reported that differentiating iPS cells established from antigen specific monoclonal CD8 T cells (T-iPSCs)

enables us to produce large amount of young CD8 T cells that are comparable to original CD8 T cells in antigen specificity. This technology enables us to establish iPSC based adoptive cancer immunotherapy. However it turned out that loss of antigen specificity occurs during optimized differentiation method into functional CD8 T cells. As a solution for this, we have developed Recombination Activating gene2 (RAG2) knock-out strategy by using CRISPER Cas9 system.

Method and outcome: We established Glypican-3 (liver cancer expressing antigen) specific TCR having T-iPSCs and knocked out RAG2 gene. By differentiating these cells into CD8 T cells and analyzing TCR $\alpha\beta$ sequence we confirmed the positive effect of RAG2 KO for preserving Glypican-3 specificity. We also confirmed the specific killing function of differentiated RAG2 KO CD8 cells against Glypican-3 expressing tumor in vitro and in vivo.

Discussion: We have succeeded in making safer and more practical cells for clinical application by using genome editing strategy. This strategy would enables us to supply safe and effective cells for adoptive cancer immunotherapy in the future.

6

High throughput flow screening assays to profile cell-mediated killing

Chan, Y.Y., Kedarnath, S., Skiba, B.,

IntelliCyt Corporation, Albuquerque, United States

While enhancing T-cell mediated killing of tumor cells is emerging as a successful therapeutic approach for a variety of cancers, improvements to these therapies are actively being sought. Traditional assays for monitoring cell-mediated killing are only capable of homogenous live/dead readouts for an entire sample. As an alternative platform for cell-mediated killing studies, IntelliCyt's iQue Screener can identify multiple cell types in suspension and report multiple cell killing readouts in streamlined no wash assay formats. We demonstrate two example high throughput assays for cell-mediated killing using NK cells and chimeric antigen receptor (CAR) T-cells. Using the NK92 cell line as an effector cell and fluorescently encoded Jurkat cells as target cells, viability and Caspase 3 activation was determined for both Jurkat and NK92 cells in the same sample, and compounds that were generally cytotoxic to both cells could be identified. Specificity of the cell-mediated killing response was demonstrated using known signal transduction inhibitors including sunitinib, U73122, pp2, and wortmannin that would attenuate the NK cell killing activity, at a fixed target to effector cell ratio. In the CAR T-cell assay, efficacy of different CARs at targeting and killing a B-cell line (NALM-6) was profiled using multiplex readouts for cell health and secreted cytokines. Multiple cytokines including inflammatory markers and Granzyme B were quantified using bead-based ELISA on the same analysis platform. These application examples highlight the robustness and flexibility of the iQue Screener for performing multiplexed screening assays with cells and beads.

7

High potency dendritic cells generated via CD137L reverse signaling for dendritic cell-based immunotherapy

Dharmadhikari, B.¹, Harfuddin, Z.², Schwarz, H.^{1,2}

¹National University of Singapore, Physiology and Immunology Programme, Singapore, Singapore, ²National University of Singapore, 2. NUS Graduate School of Integrative Sciences and Engineering, Singapore, Singapore

Dendritic cell (DC)-based immunotherapy has relied on the use of monocyte-derived DCs, generated in vitro in the presence of GM-CSF and IL-4. Despite reported clinical benefits, the overall response remains low, largely due to the inability of DCs to mount a sufficiently strong T cell response. We have previously shown that CD137L reverse signaling induces differentiation of human monocytes to a novel type of dendritic cell (CD137L-DC) which are more potent than GM-CSF + IL-4 derived DCs. Here we report that CD137L-DCs have an inflammatory phenotype and are potential candidates for immunotherapy of Epstein-Barr Virus (EBV)-associated cancer(s). In our current study, CD137L-DCs generated from peripheral human monocytes (isolated by EasySep, STEMCELL Technologies) were used to test T cell responses against the LMP1 and LMP2 proteins of EBV via ELISPOT, followed by a cytotoxicity assay to test the ability of T cells activated CD137L-DCs to lyse HLA-matched, antigen-pulsed target cells. Our data shows that induction of proliferation and activation of EBV-specific T cells was significantly higher by CD137L-DCs than by GM-CSF + IL-4 derived DCs. Also the killing activity of CD137L-DC activated T cells was about 2-fold higher. The T cell exhaustion level was significantly lower upon activation of T cells by CD137L-DCs. Further characterization of CD137L-DCs showed low expression of PD-L1, an inflammatory phenotype with caspase-dependent IL-1 β secretion and a significant enrichment of inflammatory gene signatures. Thus, CD137L-DCs are a novel and highly potent type of in vitro generated DCs that should be of benefit for cancer immunotherapy.

8

Enhancing adoptive immunotherapy: redirecting immune subsets and metabolic pathways

Yong, C.^{1,2,3,4}, Devaud, C.⁵, Darcy, P.K.^{2,3,6}, Kershaw, M.H.^{2,3,6},

Dardalhon, V.^{1,4}, Taylor, N.^{1,4}

¹IGMM UMR5535 CNRS, Montpellier, France, ²University of Melbourne, Sir Peter MacCallum Department of Oncology, Victoria, Australia, ³Cancer Immunology Research, Program Sir Peter MacCallum Department of Oncology, University of Melbourne, Australia, ⁴University of Montpellier, Montpellier, France, ⁵INSERM U1043 Centre de Physiopathologie Toulouse Purpan (CPTP), Toulouse, France, ⁶Monash University, Prahran, Department of Immunology, Victoria, Australia

Adoptive anti-tumor T cell immunotherapies have demonstrated promising results, notably for the treatment of chemotherapy-resistant cancers. Specifically, CD8⁺ T lymphocytes, genetically modified to express a chimeric antigen receptor (CAR) against the CD19 antigen, have been used to successfully treat refractory B leukemias/ lymphomas. However, recent studies also highlight

the potential role of other immune subsets in improving anti-tumor immunity. In order to fully explore the potential of different T cell subsets as well as other leukocytes armed with an anti-tumor CAR, we generated a preclinical transgenic mouse model expressing a CAR specific for the Her2 (ERB) tumor antigen in all immune subsets. Using this model, wherein the Her-CAR is expressed under the control of the *vav* pan-hematopoietic promoter, we found that Her2-CAR T cells exhibit Her2-specific immune responses, monitored as a function of cytokine secretion as well as cytotoxicity. Furthermore, we are assessing whether the metabolic features of Her2-CAR immune cells regulate their relative persistence, proliferation, differentiation and anti-tumor potential. To this end, the cellular metabolism of Her2-CAR immune cells has been altered by nutrient availability and Her2-CAR cells segregated on the basis of their glycolytic state, identified by surface Glut1 glucose transporter levels. The capacity to modulate the function of anti-tumor immune cells through a metabolism-based approach opens new avenues for optimizing adoptive immunotherapies.

9

Construction of CD47 specific CAR-NK and its anti-tumor activity *in vitro*

Huang, Q., Ren, H., Yang, H., Wang, Y., Li, Q., Shi, J., Shao, D., Zhang, M. Northwestern Polytechnical University, School of Life Sciences, Xi'an, China

T cells engineered with chimeric antigen receptors (CAR) and transferred into oncology patients is a promising approach to eradicate tumor cells. One major obstacle with CAR-T is the need to collect and utilize autologous cells. In contrast to T cells, natural killer (NK) cells are known to mediate anti-cancer effects without the risk of inducing graft-versus-host disease (GvHD). Therefore NK cells can be used as allogeneic effector cells since they do not require HLA matching.

At present, the CAR lentiviral expression vector designed for immuno-therapy CAR-NK was generated by connecting an extracellular antigen-binding domain of CD47 MAb single-chain variable fragment (scFv) to transmembrane domain of CD28 and intracellular signal transduction domain of CD28 and CD3z. A signal peptide of CD8 was introduced at N-terminal. Lentiviral particles were produced by transfecting 293 T cells with the lentiviral expression vector and the packaging vectors. CAR modified primary human NK cells were prepared by infecting the NK cells with lentiviral particles during the NK cells were expanded by co-culture with the irradiated K562 cell line expressing membrane bound IL-15 and 41BBL. For the anti-tumor activity of the CAR-NK *in vitro*, HepG2 and Caco2 cells were used as target cell. The cytotoxicity of CAR modified human primary NK cells showed 15% and 10% higher cytotoxicity of lysing HepG2 and Caco2 cells than that of unmodified human primary NK cells. This results will provide the basic data for the further study of CD47 specific CAR NK *in vivo*.

10

A rapid and easy system for screening of antigen-specific TCRs

Hamana, H., Kishi, H., Shitaoka, K., Xiuhong, P., Ozawa, T., Muraguchi, A. University of Toyama, Toyama, Japan

T cell receptor (TCR) gene therapy is a promising strategy for the treatment of various cancers. We reported an efficient cloning system for TCR cDNA from single antigen-specific human T cells within 10 days (Nature Med, 2013). However, evaluation of antigen specificity of cloned TCRs by using T cell line and retroviral vector is still troublesome and time-consuming. Therefore, we tried to establish a more convenient strategy. In this study, we constructed a 293T-NFAT-Luc cell line that enables us to detect activation of TCR by luciferase reporter assay. To this end, firstly we transduced NFAT-RE-luciferase reporter gene, cDNAs of mouse-CD8 and human-CD3 complex, -CD8, -SYK, -NFAT-C2 into 293T cells. To confirm the cell function, we expressed a 1G4 TCR that was specific to NY-ESO-1 on the cells and co-cultured them with COS-7 cells expressing NY-ESO-1 peptide, HLA-A02 and human beta-2-microglobulin. As a result, we observed the luciferase production in a peptide-dependent manner. Next, we tried to express TCR by using transcriptionally active PCR (TAP) fragments that have CMV promoter and poly-A additional signal. The 293T-NFAT-Luc cells transfected with TAP fragment of OT-I TCR cDNA produced luciferase when the cells were co-cultured with OVA peptide pulsed EL4 cells. These results suggest that a combination of 293T-NFAT-Luc and TAP fragment is useful for rapid and easy analysis of the specificity of cloned TCR from human as well as mouse. Now, we are trying high-throughput screening of melanoma-associated antigen TRP2-specific TCRs in mice by using our strategy.

11

Adoptive transfer of Tc17 CD8 T cells as an approach to elicit a better immune response to vaccination

Yen, H.-R.^{1,2}

¹China Medical University, School of Chinese Medicine, Taichung, Taiwan, Republic of China, ²China Medical University Hospital, Research Center for Traditional Chinese Medicine and Department of Chinese Medicine, Taichung, Taiwan, Republic of China

In our previous study, we found a novel subset of IL-17 producing CD8 Tc17 cells persisted longer and convert to an IFN- γ producing phenotype in a self-antigen autoimmune model; however, such an IL-17 producing Tc17 cells had less expression of cytolytic molecules and activity. In the present study, we adoptively transferred a relatively small numbers (100,000) of IL-17 secreting TCR-transgenic antigen-specific CD8 T cells one day before vaccination with recombinant vaccinia virus encoded with full-length hemagglutinin. Tc 17 cells activated, expanded better than Tc1 cells 9 days after vaccination (Mean = 7.425×10^6 versus 1.350×10^6 , $p < 0.05$). Interestingly, Tc17 can convert to an IFN- γ producing phenotype after vaccination. Our preliminary data fit the niche that is required to improve vaccination and immunotherapy and we suggest that, to treat infectious disease, CD8 Tc17 could be a promising

effector T cell subset to achieve the goal. Additional mechanisms are being explored.

12

Functional signature of CD19 CAR transduced T cells

Magalhaes, I., Mattsson, J., Uhlin, M.

Karolinska Institutet, Stockholm, Sweden

B cell malignancies consist of a heterogeneous group of leukemias and lymphomas and despite improvements in treatment strategies many patients still succumb to the diseases. Several clinical trials of adoptive cell therapy of chimeric antigen receptor (CAR) modified T cells targeting CD19 have shown dramatic results in both pediatric and adult patients.

CD19 CAR transduced T cells were generated from patients with chronic lymphocytic leukemia (CLL), acute lymphoblastic leukemia (ALL) and healthy donors. CD4⁺ and CD8⁺ CD19 CAR transduced T cells displayed an effector memory (CD45RO⁺CCR7⁻) phenotype. We evaluated the cytokine (IL-2, IFN- γ , TNF- α and IL-17) production and degranulation (CD107a) of CD19 CAR transduced T cells after stimulation with either K562-CD19⁺ cells or autologous B cells. We show that when stimulated with K562-CD19⁺ cells the majority of CD4⁺ and CD8⁺ CD19 CAR transduced T cells showed a polyfunctional (3⁺ functions) profile and cytotoxic capacity (CD107a⁺). Furthermore, CD4⁺ CD19 CAR transduced T cells produced IL-17 specifically against CD19⁺ target cells. Interestingly when stimulated with autologous B cells, as compared to cells stimulated with K562-CD19⁺, CD19 CAR transduced T cells were less polyfunctional with the majority of the cells (CD4⁺ and CD8⁺) exhibiting only one function and particularly CD107a⁺. These results suggest that CD19 CAR transduced T cells stimulated with different CD19⁺ target cells have different functional signatures (cytotoxicity vs cytokine secretion). We are investigating the role of CD19 density on CD19⁺ target cells and the signaling profile in CD19 CAR transduced T cells stimulated by different CD19⁺ target cells.

13

Generation of T cell effectors using LNCap-loaded DC vaccine for adoptive T cell therapy

Vavrova, K., Vrabцова, P., Horvath, R., Bartunkova, J.

2nd Faculty of Medicine, Charles University, Dept. of Immunology, Prague, Czech Republic

Adoptive T cell transfer (ACT) is a cell coculture-based approach whereby anti-tumor-specific lymphocytes derived from a patient are expanded *in vitro* and transferred back into the patient. ACT has been shown to be an effective method used to boost tumor-specific immune responses in several types of malignancies. In our study, we set out to optimize the ACT approach for treatment of prostate cancer with an expansion protocol that involves the generation of DC vaccine-primed autologous T cells. The protocol includes a pre-stimulation step where T cells were primed with autologous DCs loaded with high hydrostatic pressure-treated prostate cancer cell line LNCap. Primed T cells were further *in vitro* expanded with

anti-CD3/CD28 Dynabeads and tested for cytotoxicity using a lactatedehydrogenase assay. Our data indicate that Dynabead expansion leads to the expansion of tumor-specific CD4⁺IFN- γ ⁺ T cells while the frequencies of CD8⁺ IFN- γ ⁺ T cells remain largely unchanged. The majority of both CD4⁺ and CD8⁺IFN- γ ⁺ producing cells were CD62L⁻, CCR7⁻ and CD57⁻ but CD28⁺ and CD27⁺ positive, indicating an early/intermediate effector memory phenotype (T_{EM}) in non-terminal differentiation stage. Expanded T cells showed significantly greater cytotoxicity against LNCap cells compared to control SKOV-3 cells, with the highest efficiency of killing at the ratio of 20:1. Our data show that the ACT approach in combination with a DC-based vaccine provides a viable way to generate prostate cancer reactive T cell effectors which could be tested for clinical treatment of this malignancy.

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Identification of tumor-specific T cell receptors of primary tumor-infiltrating lymphocytes (TILs) from B16F10 melanoma-bearing mice at single cell levels

Shitaoka, K., Hamana, H., Kishi, H., Ozawa, T., Muraguchi, A.

University of Toyama, Toyama-shi, Japan

T cell receptor (TCR) gene therapy is a promising cancer therapy and requires tumor-specific TCR gene. In general, tumor-specific antigens are pre-requisite for identifying tumor-specific T cells and cloning their TCR. However, identification of tumor-specific antigen in each patient is not easy and time-consuming. Consequently, the research is skewed to the major HLA haplotypes such as HLA-A24 in Asia and HLA-A2 in Europe and USA. The patients with minor HLA haplotypes do not benefit from those researches. It has been reported that tumor-specific T cells are activated and proliferate in the tumor. In this study, we tried to clone cDNAs of tumor-specific TCR from primary tumor-infiltrating lymphocytes (TIL) without using tumor-specific antigen. To this end, we sorted CD8⁺ or CD8⁺CD137⁺ single TILs from B16F10 melanoma tissues in C57BL/6 mice with FACS and their TCR α and β cDNAs were obtained. TCR repertoire analysis indicated the clonal expansion of CD8⁺ CD137⁺ T Cells in the tumor. We inserted the TCR cDNA into a retroviral vector, transduced the cDNA into splenic T cells, and examined their reactivity against B16F10 melanoma cells. Most of the TCRs obtained from clonally expanded TILs induced cytotoxicity against B16F10 melanoma cells and interferon- γ production. Thus, our protocol enables us to easily obtain tumor-specific TCR from primary TILs without antigenic peptides presented on individual MHC haplotypes.

15

Engineering T cells for cancer therapy by expressing a chimeric antigen receptor (CAR) targeting the tumour endothelial marker ROBO4

Cawkwell, L.¹, Bicknell, R.², Lee, S.¹

¹University of Birmingham, Institute of Immunology and Immunotherapy, Birmingham, United Kingdom, ²University of

Birmingham, Institute of Cardiovascular Science, Birmingham, United Kingdom

Adoptive T cell transfer of tumour-specific engineered T cells has become an attractive area of cancer research. However, few groups have attempted to target the tumour vasculature using T cells. Roundabout Homolog 4 (ROBO4) is an endothelial specific gene that is overexpressed in the vasculature of multiple different solid tumours but is absent or expressed at very low level in normal adult tissue. Chimeric antigen receptors (CARs) are fusion proteins linking an extracellular antigen binding domain derived from an antibody, with an intracellular signalling domain. Here we have attempted to target ROBO4 using T cells engineered to express a CAR. Two monoclonal antibodies that both bind human and mouse forms of ROBO4 were engineered into second generation CARs (named R1 and R2) containing a CD28 signalling domain. R1 and R2 CARs successfully retargeted T cells to recombinant human and mouse ROBO4 protein and ROBO4-expressing cell lines in multiple in vitro assays of T cell function, with R1 displaying greater functional activity. When mouse T cells engineered with these CARs were injected into healthy mice, no signs of toxicity were observed. However, both R1 and R2 CAR

T cells were selectively and rapidly removed from the peripheral blood whereas T cells expressing a non-targeting control CAR persisted. We are currently investigating the fate of these ROBO4-specific CARs, with the possibility that they are accumulating in response to ROBO4 expressed in healthy organs.

16

Ex vivo generation of alloantigen-specific T regulatory cells using selective T-cell co-stimulation blockade

Watanabe, M.¹, Kumagai-Braesch, M.¹, Thunberg, S.², Henrikson, J.¹, Sellberg, F.³, Lundgren, T.¹, Yao, M.H.¹, Jorns, C.¹, Berglund, D.³, Berglund, E.¹, Ericzon, B.-G.¹

¹Karolinska Institutet, Transplantation Surgery, Stockholm, Sweden, ²Karolinska Institutet, Laboratory Medicine, Stockholm, Sweden, ³Uppsala University, Immunology, Genetics and Pathology, Uppsala, Sweden

Purpose: Adoptive transfer of alloantigen-specific T regulatory cells (Tregs) generated ex vivo by co-culture with anti-CD80/CD86 mAbs (2D10.4/IT2.2) has been shown to allow early withdrawal of immunosuppressant and graft acceptance after liver transplantation. Belatacept, a clinically approved CTLA4-Ig, may be an alternative agent for the ex vivo generation of alloantigen-specific Tregs. The efficacies of anti-CD80/CD86 mAbs and Belatacept on ex vivo generation of Tregs were evaluated.

Methods: Human peripheral blood mononuclear cells (PBMCs) (50 x 10⁶ cells) were co-cultured with irradiated donor PBMCs (20 x 10⁶ cells) in the presence of 2D10.4/IT2.2, or Belatacept in eight different pairs. At day 7, irradiated donor PBMCs, culture media, and 2D10.4/IT2.2, or Belatacept, were replenished. Phenotypes and immunomodulatory effects of the generated cells were assessed at day 14.

Results: After 14 days of co-culture CD4⁺CD25⁺CD127^{lo}Foxp3⁺ Tregs increased from 4.1±1.0% to 7.1±2.6% and 7.3±2.6% in

2D10.4/IT2.2 and Belatacept groups, respectively. Concurrently, delta-2 FOXP3 mRNA expression increased significantly. Generated cells from both treatment groups effectively impeded proliferative responses of freshly isolated recipient PBMC against donor-antigen in mixed lymphocyte reactions, in a generated cell-number dependent fashion. While, such effect was minor against third-party antigens. IFN-γ production was downregulated and IL-10 production increased in ELISPOT and ELISA assays from 2D10.4/IT2.2 and Belatacept treated groups, as compared to sham treated cells.

Conclusion: Alloantigen-specific Tregs generated with Belatacept showed comparable immune-modulatory effects as compared to those of 2D10.4/IT2.2. Belatacept seems to be a promising agent for ex-vivo generation of alloantigen-specific Tregs.

17

Generating CD4⁺ and CD8⁺ T cells using BMDCs and BMMΦs improves adoptive cell therapy for B16 melanoma

Shields, N., Young, K., Li, K., Young, S.

University of Otago, Department of Pathology, Dunedin, New Zealand

The adoptive transfer of tumour-specific T cells, known as adoptive cell transfer (ACT), is a promising novel approach for the treatment of cancer. Antigen presenting cells (APCs) can serve as a tool for the *ex vivo* expansion of tumour-specific T cells for use in ACT, with the functional state of these cells during antigen presentation playing a pivotal role in shaping resultant T cell responses. Due to their status as the most potent APC, dendritic cells (DCs) have been the focus of intense investigation in regards to

T cell activation, while macrophages have received little attention. We have previously shown that a combination of CD4⁺ and CD8⁺ T cells generated using DCs as APCs *ex vivo* effectively controls B16 melanoma growth. Here we compared the ability of DCs and macrophages to further enhance ACT through the *ex vivo* expansion of tumour-specific T cells, using ovalbumin (OVA) as a model tumour antigen. In our model, naïve OVA-specific CD4⁺ and CD8⁺ T cells were expanded for 10 days using bone marrow-derived DCs, re-stimulated with either DCs or macrophages and cultured for a further 10 days before adoptive transfer into mice bearing B16-OVA tumours. ACT using a combination of CD4⁺ T cells re-stimulated with macrophages and CD8⁺ T cells re-stimulated with DCs achieved 100% complete tumour-rejection and provided full protection following tumour re-challenge. Future experiments will investigate the differences in antigen uptake and processing by DCs and macrophages and their effects on T cell generation for ACT.

18 Identification of new genes that enhance effector function of chimeric antigen receptor T cells

Li, P., Lai, Y., Qin, L.

Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, China

Chimeric antigen receptor (CAR) T cell immunotherapy has shown unprecedented success in the treatment of leukemia, but not solid tumors. And a large number, usually $> 10^9$ cells, of CAR T cells are needed for clinical trials and a long time (>20 d) is needed for in vitro expansion. To further improve the effector function of CAR T cells in both leukemia and solid tumors, we performed a genetic screening and identified that insertion of the intracellular signaling domain of a gene, N1, to the C end of 2nd generation CARs was able to significantly enhance effector function of CAR T cells. T cells expressing α CD19-28zN1 showed enhanced cytotoxicities against CD19⁺ cell lines NALM6, REH and K562-CD19 compared with T cells expressing α CD19-28z. Similarly, T cells expressing α MSLN-28zN1 exhibited stronger cytotoxicities against MSLN⁺ lung cancer cell lines A549 and H23 compared with T cells expressing α MSLN-28z. Also, in xenograft models, T cells expressing α CD19-28zN1 showed enhanced cytotoxicities against CD19⁺ NALM6 cells; and T cells expressing α MSLN-28zN1 showed stronger cytotoxicities against subcutaneous tumor of A549 cells compared with T cells expressing α MSLN-28z. Importantly, we are conducting a clinical trial with α CD19-28zN1 T cells in which the infusion of 4.5×10^6 CAR T cells achieved CAR T cell expansion and B-ALL remission in the patient. Our results revealed N1 as a contributing component in CAR T cell immunotherapy whose incorporation simplifies the production of CAR T cells by infusing fewer CAR T cells for therapy and potentially reduces the cytokine releasing syndrome in patients.

19 NFkB acts as a central integrator of cell extrinsic signals to regulate CD8⁺ T cell fate and tumor efficacy

Shrikant, P., Myles, A., Vardham-Kaur, T.

Mayo Clinic, Immunology, Scottsdale, United States

Exploiting mechanisms underpinning integration of antigen and cytokine generated signals to produce tumor-antigen specific CD8⁺ T cells with low proinflammatory profile and memory functions can boost efficacy of adoptive cell therapy (ACT) of cancer. Herein, we show that IL-21 via STAT3 induces the atypical IKK family member, Bcl3, to dampen antigen driven NF- κ B (p65/p50) activation. IL-21 decreases antigen induced CD8⁺ activation and proliferation, but enhances clonal expansion due to reduction in apoptosis in a Bcl3 dependent manner. Strikingly, Bcl3 mediated NF κ B dampening skews expression of master transcriptional factors including T-bet, Eomesodermin, Bcl6, Blimp-1 and FoxO1 to facilitate transition of antigen induced polyfunctional CD8⁺ effector cells to memory precursor cells (MPCs) that retain cytotoxicity but produce reduced IFN γ and IL-2. Moreover, ex vivo IL-21 conditioned CD8⁺ T cells are persistent and produce greater tumor efficacy upon adoptive transfer into intact syngeneic recipients. Our results reveal a pivotal role for NF- κ B to integrate antigen and cytokine signals

and control differentiation as well as survival to establish fate of antigen stimulated CD8⁺ T cells. We submit that new strategies to regulate T cell responses for durable tumor immunity should target NF κ B activity.

20 Activity of engineered antigen-specific T cells as a function of the relationship between affinity, avidity and antigen density

Greenman, R., Oren, R., Haus-Cohen, M., Reiter, Y.

Technion, Biology, Haifa, Israel

The use of engineered Ag-specific T cells in adoptive cell transfer therapies has recently gained significant focus due to initial clinical success. Such engineered cells are generated using a chimeric Ag receptor (CAR) based on common formats composed from Ag-recognition elements, such as $\alpha\beta$ -TCR genes or Ab variable domain fragments, fused with T cell-signaling moieties. We recently combined these recognition elements using Abs that recognize peptide-MHC (TCR-like Abs), and compared such a high-affinity TCR-like Ab CAR to a native low-affinity TCR. Unexpectedly, the high-affinity CAR was less effective than the low-affinity TCR, suggesting an upper affinity threshold for TCR-based effective functional outcomes of engineered T cells. That is, exceeding this threshold leads to reduced functionality. To further characterize this affinity threshold and to achieve optimal T cell function, we characterized a series of anti-Tyr single chain fragment binding domains, ranging from 4nM to 1000nM Kd, and constructed Tyr-specific second generation CARs with ranging affinities. The interplay between CAR affinity, Ag density and CAR expression (i.e. avidity) and their effect on T cell functionality will then be studied. Hence, the precise combination of affinities and avidities (termed functional avidity) of T cells that leads to optimized functional outcome can be of crucial importance in adoptive cell transfer immunotherapies that utilize CARs.

21 Blockade of PD-1 enhances the cytotoxic function of ex vivo expanded CD8⁺ T-cells in EpCAM⁺ PDL-1⁺ cancer

Kumar, R.¹, Fang, Y.², Zhen, Y.-H.³, Bo, L.⁴, Yuan, Y.⁴, Ge, H.-X.⁵, Hu, P.¹, Zin, X.¹

¹Affiliated Hospital of Guizhou Medical University, Clinical Research Center/Cancer Immunology and Immunotherapy Center, Guiyang, China, ²Affiliated Hospital of Guizhou Medical University, Clinical Research Center, Guiyang, China, ³Affiliated Hospital of Guizhou Medical University, Department of Colorectal Surgery, Guiyang, China, ⁴Affiliated Hospital of Guizhou Medical University, Cancer Immunology and Immunotherapy Center, Guiyang, China, ⁵Affiliated Hospital of Guizhou Medical University, Department of Surgery, Guiyang, China

Background: In recent years, adoptive cell therapy has proven to be an encouraging modality for treating cancer patients. The increased expression of inhibitory receptors (iRs) on T cells in the tumour microenvironment is a major obstacle in tumour rejection. Programmed cell death protein-1 (PD-1) negatively

regulates the function of CD8+ T cells during *ex vivo* expansion, which is responsible for reduced killing. Here, we investigated whether the blockade of PD-1 on CD8+ T cells can potentiate tumour rejection in cancer patients.

Methods: We investigated the tumour-killing activity of CD8+ T cells after PD-1/PD ligand-1 (PDL-1) blockade. CD8+ T cells were co-cultured with EpCAM+ tumour cells such as Tumour Epithelial Cells (TECs), DLD-1, SW480 and SW620 for cytotoxicity activity, ELISpot was used to detect IFN- γ and GrB, and CD107a mobilisation was quantified by flow cytometry.

Results: We found a dramatic increase in PD-1 expression on the surface of CD8+ T cells during *ex vivo* expansion. PD-1 was downregulated by ~40% after using PD-1 mAb, which induced apoptosis in tumour cells during co-cultured with CTLs. Secreted cytolytic molecules, such as IFN- γ and GrB, and CD107a mobilization also increased upon PD-1/PDL-1 blockade during co-culture, which enhanced CD8+ T cells tumour interactions, as well as cytosolic exchange between effector and target cells.

Conclusion: Our findings suggest that PD-1/PDL-1 blockade increased the killing efficiency of CD8+ T cells and the secretion of functional cytolytic granules and cytokines during tumour invasion. These findings advance the clinical investigation for a more effective approach to cell-based adoptive immunotherapy.

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Using Merkel cell polyomavirus specific TCR gene therapy for treatment of Merkel cell carcinoma

Lyngea, R.¹, Wulff Pedersen, N.¹, Linnemann, C.², Schrama, D.³, Ibrani, D.⁴, Met, Ö.⁵, thor Straten, P.⁵, Nghiem, P.⁴, C. Becker, J.³, Reker Hadrup, S.¹

¹Veterinary Institute, DTU, Section of Immunology and Vaccinology, Fredriksberg C, Denmark, ²Netherlands Cancer Institute, Dept. of Immunology, Amsterdam, Netherlands, ³Medical University of Graz, General Dermatology, Graz, Austria, ⁴University of Washington, Departments of Medicine/Dermatology, Pathology, Seattle, United States, ⁵University Hospital Herlev, Center for Cancer Immune Therapy, CCIT, Department of Hematology, Herlev, Denmark

T cell receptor gene-therapy has entered the clinic and shown potential for successful cancer treatment. However, the clinical evaluation has also highlighted the need for selection of truly cancer-specific targets. Merkel cell carcinoma (MCC) is a highly aggressive skin cancer associated with Merkel cell polyomavirus (MCPyV). Due to the clear viral correlation CD8+ T cells specific for viral epitopes could potentially form cancer-specific targets in MCC patients. We have identified MCPyV specific T cells using a high-throughput platform for T-cell enrichment and combinatorial encoding of fluorescence-labeled major histocompatibility complex (MHC) class I multimers. We identified 35 T cell epitopes among 398 MCPyV derived peptides analyzed. Strikingly, T-cell responses against the two oncogenic MCPyV proteins Large T antigen and small T antigen were exclusively present in blood of MCC patients when compared to healthy donors. We demonstrate both the processing and presentation of oncoprotein-derived epitopes, as well as lytic activity of specific T cells towards MHC-matched MCC cells. Demonstrating the presence of oncoprotein-specific

T cells among tumor infiltrating lymphocytes *ex vivo* further substantiated the relevance of the identified epitopes. The viral epitopes represents specific targets and should be ideal for TCR-gene therapy approaches. We have isolated and sequenced MCPyV oncogenic protein specific

T cell receptors and are currently testing *in vitro* transduction systems with the purpose of introducing the TCRs into human PBMC, injecting them into immune deficient NOG mice carrying HLA matched MCPyV positive tumor to investigate the tumor rejection capacity of these gene-modified T cells.

23

Selection process of the optimal T-iPSC clone from among clones derived from T cells specific to melanoma antigen MART-1

Nagano, S., Maeda, T., Shimazu, Y., Masuda, K., Kawamoto, H. Institute for Frontier Medical Sciences/Kyoto University, Department of Immunology, Kyoto, Japan

We have previously reported that iPSC cells (iPSCs) were established from mature cytotoxic T cells specific to MART-1 antigen from a melanoma patient, and that CD8+ mature T cells were generated from these iPSCs (Cell Stem Cell, 2013). This method represents a novel tool for the cloning and expansion of T cells that can be applied for cell therapy against cancer. Whereas this approach has been based on the idea of autologous transplantation, we are also thinking of applying this method to the allogeneic transplantation settings.

In this study, we tried to established a method to select optimal T-iPSC clone from multiple clones. We firstly expanded MART-1 specific CD8+ T cells from a healthy donor, and then reprogrammed these cells using Yamanaka factors. A total of 8 iPSC clones were established, and regenerated T cells from these clones were found to express different T cell receptor (TCR). We found that the affinity of these TCR varied very widely, and accordingly the difference was observed in the cytotoxic activity of regenerated T cells. We also found that these iPSC clones were intrinsically very heterogeneous in terms of the efficiency in the *in vitro* regeneration of T cells. These findings propose that selection process of the best clone among multiple T-iPSC clones is required for this strategy. We are now testing allo-reactivity of regenerated T cells, which would be a risk in allogeneic transplantation setting. Such information in total will be very important for the development of fundamental technology in this strategy.

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Using agonist anti-4-1BB to enhance CART cell responses against cancer

Mardiana, S.^{1,2}, John, L.B.^{1,2}, Beavis, P.A.^{1,2}, Henderson, M.A.^{1,2}, Giuffrida, L.^{1,2}, Slaney, C.Y.^{1,2}, Cross, R.S.^{1,2}, Haynes, N.M.^{1,2}, Trapani, J.A.^{1,2}, Johnstone, R.W.^{1,2}, Kershaw, M.H.^{1,2}, Darcy, P.K.^{1,2}

¹University of Melbourne, Cancer Immunology Research Program, Sir Peter MacCallum Department of Oncology, Melbourne, Australia, ²University of Melbourne, Sir Peter MacCallum Department of Oncology, Melbourne, Australia

Adoptive immunotherapy involving the genetic modification of T cells with Chimeric Antigen Receptor (CAR) is a promising strategy for targeting tumor-specific T cells to cancer. Clinical trial results have revealed that CART cell therapy is most successfully employed in hematological cancers, but less successful against solid cancers. Therefore new approaches to enhance CAR T cell function are required. Activation of the CD137 (4-1BB) pathway has been shown to provide important co-stimulation to T cells, and the use of an agonistic α -4-1BB antibody has been reported to have potent effects in improving T cell function. We hypothesized that the combination of CAR T cells and agonistic α -4-1BB would act synergistically to increase anti-tumor responses. We found significantly enhanced tumor growth inhibition following the combination therapy compared to either treatment alone against two different Her2⁺ solid tumors. Investigation into the mechanism *in vivo* revealed increased expression of the proliferation marker Ki67 and IFN γ production by CAR T cells following α -4-1BB administration. Interestingly, α -4-1BB also affected host endogenous immune cells favoring CD8⁺ T cell expansion with a concomitant decrease in CD4⁺ Treg cell frequency. This data suggests that the administration of α -4-1BB may have a dual effect of enhancing anti-tumor responses from both Her2-specific CAR T cells and host CD8⁺ T cells. In summary, the addition of exogenous α -4-1BB antibody is a potentially promising strategy for enhancing adoptive T cell immunotherapy for cancer patients.

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Human gingiva-derived mesenchymal stromal cells inhibit Graft-versus-host disease through CD39 and IDO

Wang, J., Olsen, N., Zheng, S.G.

Penn State University, Medicine, Hershey, United States

Mesenchymal stem cells have the capacity to maintain immune homeostasis and prevent autoimmunity. However, it is unclear whether GMSCs can suppress human T cell-mediated diseases. GMSC were isolated from human gingival tissues. GMSC were cultured with allogenic T cells labeled with CFSE, and the T cell proliferation and cytokine production was evaluated by CFSE dilution and cytokine staining in FACS. Mouse xenogenic Graft-versus-host-disease (GVHD) was induced in NOD/SCID mice. GMSC, human fibroblast (negative control) or human bone marrow mesenchymal stem cells (positive control) were co-transferred with human PBMC into NOD/SCID mice. Weight loss and symptoms of xeno-GVHD were assessed. We observed that GMSCs potently suppressed the proliferation and cytokine production by T cells *in vitro*, and that co-transfer of GMSC and human PBMC significantly prolonged mouse survival. We also demonstrated that GMSC is even better than MBSC on treating xeno-GVHD. We further revealed that GMSCs inhibited human PBMCs-initiated xeno-GVHD *via* CD39 and IDO signals. Thus, GMSC can suppress human immune responses and immune system-mediated diseases, offering a potential clinical option to be used for modulating GVHD and other autoimmune diseases.

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CD4⁺CD26⁺ T cells exhibit enhanced persistence and antitumor activity *in vivo*

Bailey, S.^{1,2}, Nelson, M.^{1,2}, Butcher, A.³, Majchrzak, K.^{1,2}, Bowers, J.^{1,2}, Meek, M.^{1,2}, Neal, L.^{1,2}, Paulos, C.^{1,2}

¹Medical University of South Carolina, Microbiology & Immunology, Charleston, United States, ²Medical University of South Carolina, Surgery, Charleston, United States, ³College of Charleston, Charleston, United States

Adoptive T cell transfer has been an impressive therapy for treating cancer patients, but can be inconsequential if the transferred cells are unable to engraft and persist. Our lab has found that human CD4⁺ T cells that express intermediate or high levels of CD26—termed CD26^{intermediate} and CD26^{high}

T cells, respectively—display striking antitumor activity in NSG mice bearing large, established mesothelioma. Importantly, these cells also exhibit increased engraftment and persistence compared to mice treated with bulk CD4⁺ or CD26^{negative} T cells. While the multi-functional nature (IL-17, IFN- γ , IL-2, TNF- α , IL-22) and heightened cytotoxicity (CD107A, Granzyme B) of CD26^{high} T cells can help explain their antitumor activity *in vivo*, their ability to persist long-term despite their differentiated phenotype (CD45RO⁺) is intriguing. After further evaluation, we found that both CD26^{intermediate} and CD26^{high} T cells express elevated β -catenin and BCL-2, as well as decreased Caspase 3 cleavage, compared to CD4⁺ and CD26^{negative} T cells. Gene array analysis revealed that CD26^{high} T cells also express heightened levels of CEBPD and ATF5. Excitingly, both CD26^{intermediate} and CD26^{high} T cells displayed enhanced

T cell persistence and significantly delayed tumor growth when transferred into mice bearing established PANC1 pancreatic tumors. Furthermore, PANC1-bearing mice treated with bulk CD26⁺ T cells also displayed a better antitumor response and persistence than those treated with bulk CD4⁺ or CD26^{negative} T cells. Overall, this data suggests that the presence of CD26 on transferred CD4⁺ T cells could be crucial for an effective antitumor response and can be utilized to enhance future immunotherapy strategies.

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Manipulating PD-1 axis to generate melanoma-specific high avidity CD8 T cells for adoptive cell transfer for

Simon, S.^{1,2,3,4}, Vignard, V.^{1,2,3,5}, Florenceau, L.^{1,2,3,4,5}, Khammari, A.^{1,2,3,5}, Dréno, B.^{1,2,3,5}, Lang, F.^{1,2,3,4}, Labarrière, N.^{1,2,3,4}

¹University of Nantes, Nantes, France, ²INSERM, U 892, Nantes, France, ³CNRS 6299, Nantes, France, ⁴LabEx IGO, Nantes, France, ⁵Nantes Hospital, Nantes, France

Therapeutic strategies using anti-PD-1 blocking antibody reported unparalleled effectiveness for cancer immunotherapy, in terms of clinical response rates. Nonetheless, the search for biomarkers unequivocally associated with clinical efficiency remains a crucial issue to improve patients' management. Despite its negative role in anti-tumor immunity, PD-1 also identifies reactive tumor-infiltrating T cells. Starting from this ambiguous observation, we documented for the first time the existence of melanoma specific T cell clones unable to express

PD-1. This stable feature was due to the persistent methylation of the *PDCD1* promoter. These PD-1^{neg} clones were globally of lower avidity than their PD-1^{pos} counterparts, suggesting that in physiological conditions, high affinity specific T cell clones negative for PD-1 expression are not or rarely present in peripheral blood, as they are probably eliminated by negative selection, due to a too high reactivity. This strongly suggests the implication of PD-1 in shaping an antigen-specific repertoire. We further demonstrated *in vitro* that the blockade of PD-1 signaling during the selection and amplification process of melanoma specific T cells from patients' PBMC, resulted in the proliferation of specific T cells with a biased TCR Vbeta repertoire exhibiting a better functional avidity (Simon *et al.*, *Oncoimmunol*, 2016, *in press*). This phenomenon likely occurs *in vivo* in anti-PD-1 treated patients and may represent a marker of clinical efficiency. These results offer new prospects for monitoring immune responses of cancer patients treated with anti-PD-1 antibody and for the selection of optimal effector T cells for adoptive cell transfer treatments.

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Ex vivo expansion of cytolytic natural killer cells in combined immune therapy for relapsed and refractory neuroblastoma

Shen, S.^{1,2,3}, Dolnikov, A.^{1,2,3}, O'Brien, T.^{1,2,3}

¹Sydney Children's Hospital, Kids Cancer Centre, Sydney, Australia,

²University of New South Wales, Faculty of Medicine, Sydney, Australia,

³Children's Cancer Institute for Medical Research, Sydney, Australia

Neuroblastoma (NB) is an aggressive childhood malignancy with limited treatment options for relapsed and refractory disease. Natural Killer (NK) cells are part of innate immune system; they detect and destroy virally-infected transformed cells and have anti-tumor activity. NB is sensitive to NK-mediated cytotoxicity suggesting the possibility of NK cell infusions as a treatment. Human tumours harbour numerous genetic and epigenetic alterations, and develop multiple resistance mechanisms to evade immune destruction. Thus, combining NK therapy with immune-modulatory agents might be beneficial. In this study, we investigated the cytolytic activity of ex vivo expanded NK cells against NB, either as a mono-agent or in combination with histone deacetylase inhibitor (HDACi) and/or Programmed death 1 (PD-1/PD-L1) blocker. NK cells were readily expanded from peripheral blood mononuclear cells by co-culture with K562-mbIL15-41BBL cells (from Prof. Campana, National University of Singapore), with an average 81.9±28.4 fold-expansion after 10-14 days culture. Expanded NK cells expressed activating receptors NKG2D and NKp30 & 46. Co-culture of NK cells with NB cells SK-N-SH, SK-N-AS and SK-N-Be2 resulted in robust cytotoxicity against NB. Treatment of NB cells with HDACi entinostat up-regulated cell surface expression of NKG2D ligand MIC-A/B and increased NK cell killing. Activation of NK cells up-regulated PD-1 expression; blockade of PD-1/PD-L1 axis via neutralizing mAb against PD-1 increased cytotoxicity of NK cells. We speculate that the use of HDACi and/or PD-1/PD-L1 blockade will act to increase the efficacy of NK cell therapy against NB. Effects of such combined treatments in an *in vivo* model is currently being investigated.

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A rapid and efficient single-cell manipulation method using microwell array chip (ISAAC) technology for screening antigen-specific cytokines-secreting T-cells

Ozawa, T., Kishi, H., Hamana, H., Tajiri, K., Lyu, F., Muraguchi, A.

University of Toyama, Department of Immunology, Toyama, Japan

Identification of antigen-specific T-cells and obtaining their T-cell receptor (TCR) cDNA is becoming important as T-cell adoptive therapy and TCR gene therapy are expected as a promising next generation therapy for tumor or infectious diseases. The interaction of TCRs on T-cells and peptide/MHC on antigen-presenting cells initiates T-cell activation. Using immunospot array assay on a chip (ISAAC) technology with a microwell array chip, we have recently shown that TCRs and peptide/MHC molecules on a single CD8+ T-cells can interact and induce cytokine secretion. ISAAC technology with a microwell array chip enables direct identification of antigen-specific antibody-secreting cells from human peripheral blood lymphocytes, rapid cloning of antibody cDNA, and efficient and rapid production of human monoclonal antibodies. In this study, we applied ISAAC technology to detect antigen-specific T-cells and cloning their TCR cDNA.

To detect antigen-induced cytokine-secretion on the chip, we prepared CD8+ cells from HLA-A24(+) healthy donors, arrayed them on an anti-IFN-gamma antibody-coated chip and stimulated them with Epstein-Barr virus BRLF1-peptide (TYPVLEEMF) that binds to HLA-A*2402 molecules. After 6 h, IFN-gamma immunospots were detected using fluorescence-labeled anti-IFN-gamma antibody. We retrieved the IFN-gamma-secreting cells from the chip and amplified TCR alpha and beta-chain variable region cDNAs using single-cell RT-PCR. We then expressed the cloned TCRs on a T-cell line. Flow cytometry analysis revealed that the cloned TCRs bound to BRLF1-peptide/MHC tetramer. These results demonstrated that ISAAC technology could detect antigen-specific T-cells and yield antigen-specific TCRs. This novel method may provide a powerful tool for the analysis of antigen-specific T-cells.

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Influence of TCR and pMHC structure on the polyfunctional phenotypes of TCR gene-modified T cells

Spear, T.¹, Wang, Y.², Simms, P.³, Foley, K.¹, Murray, D.¹, Scurti, G.¹, Hellman, L.², Rosen, H.⁴, Baker, B.², Nishimura, M.¹

¹Loyola University Chicago, Cardinal Bernardin Cancer Center,

Maywood, United States, ²University of Notre Dame, Department

of Chemistry & Biochemistry, Notre Dame, United States, ³Loyola

University Chicago, Flow Cytometry Core Facility, Maywood,

United States, ⁴University of Colorado Denver, Division of

Gastroenterology & Hepatology, Aurora, United States

As the quality of a T cell's functional response has been most closely attributed to the affinity between the TCR-pMHC interaction, strategies to enhance TCR affinity through random alterations by yeast/phage display develop a presumably "better" TCR for adoptive cell transfer. Yet increasing evidences suggests this relationship may not be so straightforward. We examined TCR-pMHC structure-function relationships using a panel of 9

naturally occurring HCV NS3:1406-1415 pMHC variants, NS3⁺ hepatocellular carcinoma cells, and T cells gene-modified with a cross-reactive A2-restricted TCR. We demonstrated inconsistent correlations between variant TCR-pMHC binding measurements (surface plasmon resonance, tetramer binding curves, and tetramer dissociation rates) with biological responses (target lysis, cytokine secretion, and 7-dimensional intracellular cytokine production). Recognition of multiple peptides were CD8-independent, but lost independence in the context of natural processing by tumor cells. Surprisingly, dependence on CD8 required both affinity enhancement (MHC α 3 binding domain) as well as intracellular signaling contributions (Ick-binding domain). TCR pairing competition also influenced biological responses as TCR-engineered Jurkats lacking an endogenous TCR exhibited broader cross-reactivity and reduced CD8-dependence. Stark differences in polyfunctional responses existed between peptide and tumor stimulations, and hierarchical clustering analysis of polyfunctional populations did not always "relate" responses with affinity. Having solved the crystal structure of the WT TCR-pMHC interaction, we can now rationalize these inconsistencies. We propose that modifications in therapeutic TCRs should be rationally designed rather than randomly affinity-enhanced, and that optimal T cell polyfunctionality relies on a more complex cooperation of affinity, ligand and receptor densities, co-receptor signaling components, and structural integrity.

Immunodeficiency

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The effects of CD247 deficiency on activating NK cell receptors

Blázquez-Moreno, A., Valés-Gómez, M., Reyburn, H.T.
National Center for Biotechnology, Immunology and Oncology Department, Madrid, Spain

Immunoreceptor complexes expressed at the cell surface are composed of single-pass transmembrane subunits, responsible for extracellular ligand-binding, coupled to signal transducing adaptor molecules. One of the most important signalling adaptors, present in both T and Natural Killer (NK) cells is CD247 (CD3 ζ , TCR ζ). We have recently studied NK cells in a new case of inherited CD247 deficiency, where we observed that expression of CD16a (TM form) was reduced in function of the CD247 genotype, with residual expression on CD247-deficient patient cells, presumably due to Fc ϵ R γ adaptor molecule. Expression of the NKp30 (NCR3) receptor was also markedly reduced in heterozygous relatives and essentially absent in patient NK cells, suggesting that NKp30 expression is more dependent on CD247 than Fc ϵ R γ , whereas, surprisingly, NKp46 (NCR1) expression did not vary with CD247 genotype. These observations have led us to analyse in detail the organisation and assembly of complexes containing the different activating NK cell receptors and CD247 or Fc ϵ R γ . Our results show that CD16a surface expression is totally dependent on association with either CD247 or Fc ϵ R γ . Expression of NKp30 isoform b, the most common isoform present in the patient, was also entirely dependent on association with adaptors, particularly CD247. Finally, NKp46

was able to reach the cell membrane by itself, independently of either CD247 or Fc ϵ R γ . A better understanding of the regulation of these receptors, key for NK cell function in tumour clearing and killing of virally infected cells, will aid manipulation of NK recognition for therapeutic benefit.

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Stem cell based strategies for re-establishing T cell immunity

Wong, K., Hun, M., Barsanti, M., Hammett, M., Lepletier, A., Alsharif, A., Boyd, R., Chidgey, A.
Monash University, Anatomy and Developmental Biology, Melbourne, Australia

Thymic epithelial cells (TEC) provide most of the specialist functions in the thymus and are critical for the establishment of a microenvironment competent to induce development of self-tolerant T cells and T regulatory cells. From early in life, the thymus gradually atrophies due to a loss in TEC number, precipitating a progressive decline in T cell specificities and immune system function. Further damage to the thymus from aggressive cytoablative treatments associated with cancer therapies and hematopoietic stem cell transplantation can lead to a protracted period of severe immune deficiency in adults/elderly.

We recently identified and characterized thymic epithelial stem cells (TESC) in young adult mice (Wong et al, 2104) which express low levels of major histocompatibility complex II (MHCII) and Ly51, high levels of α 6 integrin, Sca1 and epithelial stem cell associated genes. We found reduced colony forming efficiency of adult TESC during ageing, which implicates TESC loss-of-function as a significant mechanism behind the profound loss in lineage specific progenitors and mature epithelial cells. To identify candidate molecules and pathways that could potentially modulate TESC regeneration and differentiation, we have undertaken a systematic transcriptome analysis of fetal mouse thymus development and adult TECs, spanning thymic primordium (E9.5; 3rd pharyngeal pouch endoderm), TESC (E10.5 - E11.5), transitioning TESC-TEC (E12.5-13.5) and adult/aged TECs. Candidates included factors such as fibroblasts growth factors (FGFs), insulin like growth factors (IGFs), bone morphogenic proteins (BMPs) and members of the R-spondin family, involved in Wnt signaling, previously associated with Foxn1 expression and TEC lineage commitment.

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NFKB2 mutations in CVID: variability in clinical and immunologic phenotypes

Slade, C.^{1,2,3}, Scerri, T.¹, Bahlo, M.^{1,3}, Douglass, J.^{2,3}, McLean, C.⁴, Hodgkin, P.^{1,3}, Bryant, V.^{1,3}

¹Walter & Eliza Hall Institute of Medical Research, Parkville, Australia, ²Royal Melbourne Hospital, Parkville, Australia, ³University of Melbourne, Parkville, Australia, ⁴The Alfred Hospital, Prahran, Australia

Common variable immune deficiency (CVID) is the most common form of primary immune deficiency, affecting up to 1 in 25,000 individuals worldwide. We, and others have shown defects

of both canonical and non-canonical NF κ B signaling cause common variable immune deficiency in humans. In addition to early-onset hypogammaglobulinaemia and B lymphopaenia, individuals with *NFKB2* mutations have been reported to suffer from central adrenal insufficiency, and autoimmune conditions, especially alopecia areata. Here, we report a family with a novel *NFKB2* mutation that demonstrates significant heterogeneity in their clinical and immunologic phenotypes. The proband was diagnosed with CVID at the age of 9, having suffered from failure to thrive, recurrent pneumonia and severe molluscum contagiosum. Her younger sister was diagnosed with CVID at age 7. Their healthy father was also identified to harbor the mutation. Despite receiving replacement immunoglobulin since diagnosis, at the age of 28 the proband succumbed to enteroviral meningoencephalitis. Although extensive investigations for infectious agents were performed during the illness, the cause was unidentified until post-mortem cerebral tissue was examined. Histologic analysis of secondary lymphoid tissue revealed an absence of B cells and germinal centre formation. In contrast the sister had normal naive, but reduced memory B cell counts, and the father normal naive and memory B cell counts. The varied immunologic and clinical phenotypes observed within this pedigree highlight both the challenges of diagnosis and management of this autosomal dominant form of CVID, and the need to identify the mechanisms by which the variability in phenotype occurs.

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Susceptibility to cryptococcosis due to anti-granulocyte-macrophage colony-stimulating factor autoantibodies in the absence of pulmonary alveolar proteinosis

Yeh, C.-F.¹, Kuo, C.-Y.², Tu, K.-H.³, Shih, H.-P.⁴, Ding, J.-Y.⁴, Lin, C.-H.⁴, Huang, W.-C.⁵, Ho, M.-W.⁶, Chi, C.-Y.⁶, Ku, C.-L.⁴

¹Chang Gung Memorial Hospital, Infectious Disease, Taoyuan, Taiwan, Republic of China, ²Chang Gung Memorial Hospital, Pediatric Infectious Diseases, Taoyuan, Taiwan, Republic of China, ³Chang Gung Memorial Hospital, Nephrology, Taoyuan, Taiwan, Republic of China, ⁴Chang Gung University, Graduate Institute of Clinical Medical Science, Taoyuan, Taiwan, Republic of China, ⁵Chang Gung Memorial Hospital, Infectious Disease, Kaohsiung, Taiwan, Republic of China, ⁶China Medical University Hospital, Infectious Disease, Taichung, Taiwan, Republic of China

Background: Anti-cytokine autoantibodies have emerged as a cause of immunodeficiency in healthy adults without human immunodeficiency virus infection. Granulocyte-macrophage colony-stimulating factor (GM-CSF) regulates the function of phagocytes and pulmonary alveolar macrophages. Anti-GM-CSF autoantibodies have been reported in patients with pulmonary alveolar proteinosis. The patients are concurrent susceptible to cryptococcal infection.

Methods: We reviewed the clinical histories and performed immunologic evaluation and screening of anti-cytokine autoantibodies in patients with cryptococcal infection. The impact of autoantibodies on immune function was assessed by intranuclear staining of GM-CSF-induced STAT5 phosphorylation and inhibit macrophage inflammatory protein 1 alpha (MIP-1 α) production.

Results: A total of eleven patients with cryptococcal infection was examined. We identified neutralizing anti-GM-CSF autoantibodies from two patient with disseminated cryptococcal infection, [one involved central nervous system (CNS), lung and skin; one involved CNS and lung], one patient with cryptococcal infection of eyes, and one patients with cryptococcal infection with meningitis and encephalitis. The existence of anti-GM-CSF autoantibodies could block GM-CSF signaling and further inhibit STAT5 phosphorylation and production of MIP-1 α .

Conclusions: GM-CSF is critical in the host defense mechanism against *Cryptococcus*. Anti-GM-CSF autoantibodies will increase cryptococcal susceptibility in host. In the face of patients with severe cryptococcal infection, it is important to consider the existence of neutralizing anti-GM-CSF autoantibodies even without the concurrent presence of pulmonary alveolar proteinosis.

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Analysis of the NT5E (ecto-5'-nucleotidase) gene in Common Variable Immunodeficiency

Shields, D.^{1,2,3}, Smith, A.², Hissaria, P.⁴, Al-Kindi, M.⁴, Ferrante, A.^{1,2,3}, Quach, A.^{2,3}

¹University of Adelaide, Department of Molecular and Cellular Biology, Adelaide, Australia, ²SA Pathology at the Women's and Children's Hospital, Department of Immunopathology, Adelaide, Australia, ³Robinson Research Institute, Department of Paediatrics, Adelaide, Australia, ⁴Flinders Medical Centre, Immunology and Allergy Unit, Adelaide, Australia

Common variable immunodeficiency (CVID) is a primary immunodeficiency characterised by low serum IgG and IgA levels. Attempts to elucidate the causes of this heterogeneous disorder have identified several genetic mutations that are associated with CVID, but in the vast majority of cases the cause remains unknown. Approximately 10% of CVID cases described in the literature have been associated with variants within the TNFRSF13B gene encoding TACI, a protein involved in B cell activation. Our group has recently discovered that a further 25% of CVID express a specific TACI haplotype associated with a decrease in B cell surface TACI expression. Recently it has been reported that expression of CD73, an ecto-5'-nucleotidase associated with immunoglobulin class switch recombination, is significantly decreased in a proportion of CVID patients. To our knowledge, genetic analysis of NT5E, the gene that encodes this protein, has not been performed in relation to CVID. We have examined the coding regions and identified several single nucleotide polymorphisms and one 3' UTR 5' nucleotide deletion in our CVID patient cohort (n=37). A case control comparison with a healthy population (n=61) of the frequency of these anomalies revealed no significant association with CVID. These data imply that decreased CD73 expression reported previously in CVID patients is unlikely due to mutations in the NT5E gene.

36**Super-resolution imaging reveals the role of WASP in the ultrastructure of LFA-1 and actin cytoskeleton at the lytic synapse**

Guipouy, D.^{1,2,3}, Houmadi, R.^{1,2,3}, Vasconcelos, Z.⁴, Valitutti, S.^{1,2,3}, Allart, S.^{1,2,3}, Dupré, L.^{1,2,3}

¹Centre de Physiopathologie de Toulouse Purpan, Inserm UMR 1043, Toulouse, France, ²Université Toulouse III Paul Sabatier, Toulouse, France, ³CNRS, UMR 5282, Toulouse, France, ⁴Fernandes Figueira Institute, Fiocruz, Rio de Janeiro, Brazil

CD8⁺ cytotoxic T lymphocytes (CTL) require LFA-1 to adhere to target cells and deliver the lethal hit. LFA-1 activation is controlled via conformational changes, which depend on anchorage to the actin cytoskeleton. How these synaptic events are coordinated at a nanoscale remains to be explored. Super-resolution microscopy approaches (dSTORM and SIM) revealed that LFA-1 was distributed into a few thousands of nanoclusters at the interface between primary human CTL and planar surfaces coated with ICAM-1 and anti-CD3 mAbs. Costaining with antibodies specific of different LFA-1 conformations revealed that each cluster contained LFA-1 under a predominant conformation, with active nanoclusters being excluded from the outer ring of the synapse. Rather than being anchored to actin filaments, LFA-1 nanoclusters were preferentially positioned on the side of actin filaments in areas of intermediate meshwork density. To decipher the link between LFA-1 topology and the actin cytoskeleton, we studied CTL from Wiskott-Aldrich syndrome (WAS) patients, which lack WASP, a key actin regulator. WAS CTL displayed a global reduction of LFA-1 activation, an unstable lytic synapse and a reduced killing activity. In WAS CTL, the outer lamellipodia was replaced by arrays of microspikes, while the synapse center was sustained by an actin meshwork of reduced density. Active LFA-1 nanoclusters were abnormally localized to the edge of the synapse. In conclusion, our work reveals the nano-architecture of LFA-1 at the CTL synapse and its association to the actin cytoskeleton meshwork. The WASP-deficiency model highlights how spatial control of LFA-1 activation tunes synapse stability and cytotoxic activity.

37**Subcutaneous gammaglobulin replacement therapy in chronic lymphocytic leukemia - 5 years of experience**

Grywalska, E., Rolinski, J.

Medical University of Lublin, Department of Clinical Immunology and Immunotherapy, Lublin, Poland

Introduction and aim: In patients with chronic lymphocytic leukemia (CLL) who are at high risk of infection due to secondary hypogammaglobulinemia, subcutaneous immunoglobulin (SCIg) may be administered every week to reduce the risk of serious infection. Weekly SCiG therapy is an alternative to intravenous immunoglobulin (IVIg) in the treatment of patients with secondary antibody deficiencies. The objective of this study was to investigate for the first time the efficacy, safety, quality of life and cost effectiveness of SCiG in patients with CLL. Materials and methods: The study included 20 patients with CLL receiving SCiG. Median age was 64.84 years. Obtained data were

compared with those from the last year with IVIg.

Results: The median trough serum IgG level was 526 mg/dl with IVIg. In patients in whom the SCiG dose was maintained or reduced compared to IVIg, the median trough serum IgG level was 674 mg/dl ($p < 0.001$). Annual rate of infection was lower in comparison to IVIg (32 vs. 69, $p = 0.004$). In 5-year observation, there were 8 treatment-related local adverse events (AE) reported with SCiG. IgG4 levels, undetectable in 11 patients, increased substantially during SCiG treatment ($p < 0.001$). Switching to SCiG treatment led to significant improvements in quality of life and substantial cost savings.

Conclusions: We conclude that subcutaneous administration of 1.65% SCiG is a safe and cost-effective alternative to IVIg for replacement therapy of CLL-related antibody deficiency. Median trough serum IgG levels were higher with SCiG. Local AE were rare and mild. Quality of life is significantly improved.

38**The efficacy of different programs of the interferon- and immunotherapy in children with combined secondary immunodeficiencies associated with recurrent respiratory infections**

Nesterova, I.¹, Malinovskaj, V.², Kovaleva, S.³, Chudilova, G.³, Lomtatidze, L.³

¹People' Friendship University of Russia, Department of Allergology and Immunology, Moscow, Russian Federation, ²Federal Research Center of Epidemiology and Microbiology. N.F. Gamalei, Department of Ontogenesis of Interferon System, Moscow, Russian Federation, ³Kuban State Medical University, Department of Clinical and Experimental Immunology, Krasnodar, Russian Federation

The restoration of defects of immune and interferon systems in immunocompromised children suffering from recurrent viral and viral-bacterial infections is the actual problem. We studied 3 groups of immunocompromised children 5-8 years suffering from recurrent respiratory infections. Group I included 13 children with recurrent acute respiratory viral infections (ARVI), group II - 14 children with recurrent ARVI, group III - 20 children with recurrent ARVI and chronic bacterial infections of upper respiratory tract (URT). Mono- or mixed herpes viral infections were detected in all groups. Immune and interferon (IFN) systems were investigated: T-, B-chains, NK, neutrophilic granulocytes (NG), serum IFN α and IFN γ . Immunodeficiencies (ID) with the predominant defects of NG and IFNs were identified. 3 different programs of IFN- and immunotherapy were created: **in group I** local and system therapy recombinant IFN α 2 (viferon) was used;

in group II IFN therapy and for correction of defects of NG - glyukozoaminilmuramildipeptid (likopid) were used; **in group III** - IFN therapy and for the correction of humoral immunity - IRS-19 were used; in children of all 3 groups inosine pranobex was used for elimination of herpesviruses.

The study demonstrated: reducing the frequency of ARVI in 3,2 (group I), in 5,8 times (group II), exacerbations of chronic diseases of URT in 4 times, increasing the period of "days free from diseases" from 7-10 to 100-150 days. IFN restored levels of IFN α in all groups. Likopid corrected defects of NG in group

II, IRS-19 restored the humoral immunity in group III. Created programs shown high clinical and immunological efficiencies.

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Impact of a gain-of-function mutation in CXCR4 on haematopoiesis in mice

Nguyen, J.¹, Freitas, C.¹, Wittner, M.², Biajoux, V.¹, Gaudin, F.¹, Bachelerie, F.¹, Dalloul, A.¹, Donadieu, J.³, Louache, F.², Balabanian, K.¹

¹Université Paris-Saclay, Laboratoire 'Cytokines, Chimiokines et Immunopathologie', UMR_S996, Inserm, Clamart, France,

²Université Paris-Saclay, Laboratoire 'Hématopoïèse Normale et Pathologique', U 1009, Inserm, Villejuif, France, ³Service d'Hématologie Pédiatrique, Hôpital Trousseau, AP-HP, Registre Français des Neutropénies Chroniques Sévères, Paris, France

The Warts, Hypogammaglobulinemia, Infections and Myelokathexis Syndrome (WS) is a rare immunohaematological disorder characterized by a chronic lymphopenia. It is mostly caused by inherited heterozygous autosomal gain-of-function mutations in CXCR4, which engender a distal truncation in the C-tail domain and lead to a desensitization-resistant receptor. We hypothesized that WS-associated circulating lymphopenia arises from altered CXCR4-mediated signaling that skews tissue distribution and differentiation of lymphocytes. For this purpose, we generated a knock-in mouse strain harboring a WS-linked heterozygous *Cxcr4* mutation. Mutant mice displayed lymphocytes with enhanced migration to *Cxcl12* and phenocopied severe lymphopenia. To determine the origin of the lymphopenia, we explored the biological impact of the gain-of-*Cxcr4*-function on haematopoiesis in the bone marrow (BM). In mutant mice, the number of haematopoietic stem cells (HSCs) was not altered in the BM, while those of multipotent (MPP) and lymphoid-biased (LMPP and CLP) progenitors were decreased in an allele dose-dependent manner. In contrast, the myeloid lineage was preserved. Long-term BM reconstitution assays revealed that these alterations involve both intrinsic (HSC) and extrinsic (stroma) defects. In line with this, deregulated expression of *Cxcl12* was observed in the BM of mutant mice. Together, these findings identify the MPP stage as defective in mutant mice and this could account for the lymphopenia. As the BM microenvironment is also altered in mutant mice, we currently investigate the impact of a gain-of-*Cxcr4*-function on the maintenance of BM niches and determine their capacity to support lymphoid-biased MPP localization and differentiation.

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Woodsmoke inhalation injury causes pulmonary recruitment of neutrophils and alters cytokine production late after injury

Kartchner, L.¹, Dunn, J.¹, Stepp, W.², Sjeklocha, L.², Glenn, L.³, Maile, R.^{1,3}, Cairns, B.A.^{1,3}

¹University of North Carolina at Chapel Hill, Microbiology and Immunology, Chapel Hill, United States, ²University of North Carolina at Chapel Hill, School of Medicine, Chapel Hill, United States, ³University of North Carolina at Chapel Hill, Surgery, Chapel Hill, United States

Patients who suffer inhalation injury following severe burn trauma experience up to a 20% increase in mortality, increased comorbidities, and severe immune dysregulation. Currently, there are few models that recapitulate the immune dysfunction following burn plus acute inhalation injury and test potential therapeutic targets. Our lab created a clinically relevant murine model in which C57BL/6 mice (female, 8-12 weeks, >18g) were intubated and subjected to a woodsmoke inhalation injury, burn injury (20% total body surface area), or combined injury (burn+inhalation). We observed 50% increased mortality following combined injury when compared to sham treated mice. Immune cells were isolated from digested whole lung and bronchioalveolar lavages (BAL) collected 14 days post-injury and stimulated using PMA and ionomycin. Inhalation and combined injury caused neutrophil recruitment to airways, observed by neutrophil infiltration into the BAL and digested lung. Cells isolated from inhalation, burn and combined injury treated animals and stained for intracellular flow cytometric analysis revealed increased levels of IL-10 (macrophages) and IL-12 (neutrophils) upon stimulation. In our model, inhalation injury and combined injury cause cellular recruitment to the lungs and stimulation causes increased production of both pro- and anti-inflammatory cytokines as is often found in patient populations who suffer similar injuries. We propose to use this model as a means to determine if changes in neutrophil and macrophage populations contribute to increased susceptibility to bacterial infections and poor outcomes following inhalation or combined injury.

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Effect of BAY 41-2272, a soluble guanylate cyclase agonist, in lymphocytes

Uchoa Wall Barbosa de Carvalho, M.¹, Vendramini Ferreira Rosa, P.¹, Soeiro Pereira, P.V.², Antunes, E.³, Condino Neto, A.⁴

¹University of Sao Paulo, Department of Immunology, Sao Paulo, Brazil, ²University of Maranhao, Sao Luis, Brazil, ³University of Campinas / Faculty of Medical Sciences, Campinas, Brazil, ⁴University of Sao Paulo, Sao Paulo, Brazil

Our group has studying new therapies with potential therapeutic chemical components for Primary immunodeficiencies, which are disorders that predispose individuals to recurrent infections and other immune manifestations. Recently, our studies has been shown that BAY 41-2272, a soluble guanylate cyclase agonist (sGC), has proven to exert an effect in modulation of monocytes, leading to infection control. In T cells, activation of sGC by NO, increasing the levels of cyclic guanosine monophosphate (cGMP), selectively induces expression of IL-12 β 2 receptor or induced calcium influx and IL-4 production. Thereby, BAY 41-2272, and its pathway, has a potential for activation of T cells. Thus we evaluate the potential of BAY 41-2272 and its pathway as a tool for modulating of lymphocytes. To this end, pharmacological treatments were performed with BAY 41-2272 to evaluate cytokine production by ELISA, expression of CD69, FOXP3 and ROR γ T. It was observed that BAY 41-2271, as direct activator did not induce the production of IFN γ , IL-4 and IL-10 and expression of CD69, FOXP3 e ROR γ T in lymphocytes ($p < 0,001$, ANOVA with Tukey post-test). However, pretreatment

for 24 hours with BAY 41-2272, with subsequent activation with 90 nM of phorbol myristate acetate (PMA), has showed inhibitory effect on IFN γ production and expression of CD69 in lymphocytes ($p < 0,05$), but did not interfere on expression of transcription factors FOXP3 and ROR γ T. These results suggests a immunomodulator effect of BAY 41-2272 in patients with PIDs, which have autoimmunity and phagocytes deficiency, since it shows an improvement of microbicidal response of monocytes and inhibits lymphocytes.

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A nation-wide study of clinical phenotypes and *TACI* mutations in patients with common variable immunodeficiency (CVID) and IgA deficiency (IgAD) in Greece

Kapoussouzi, A.¹, Sevdali, E.¹, Farmaki, E.², Taparkou, A.², Tsinti, G.¹, Kakkas, I.³, Paterakis, G.⁴, Germenis, A.E.¹, Speletas, M.¹, on behalf of the Study Group of Primary Immunodeficiencies of the Hellenic Society of Haematology

¹University of Thessaly, School of Health Sciences, Faculty of Medicine, Larissa, Greece, ²Aristotle University of Thessaloniki, Hippokraton General Hospital, Thessaloniki, Greece,

³Evangelismos General Hospital, Athens, Greece, ⁴Gennimatas General Hospital, Athens, Greece

CVID and IgAD represent the most common immunodeficiencies in humans. This study is an updated record of the clinical presentation and *TACI* mutations of both diseases in Greece. Medical records of 93 patients (85 families), 47 with CVID (male/female:24/23, mean age of analysis: 34.2y, range: 4-70), and 46 with IgAD (male/female:26/20, mean age: 21.2y, range: 5-79), were evaluated. *TACI* was amplified and sequenced by standard protocols. In CVID patients, a remarkable delay of diagnosis (mean: 9.2y, range: 0-34) was observed. Recurrent infections of the upper and lower respiratory track, complicated also by bronchiectasis, were the most common clinical manifestations (78.7%, 65.9% and 29.6% of patients, respectively). Lymphoproliferation was observed in 26 patients (55.3%), and autoimmunity in 22 (46.8%), with thrombocytopenia and thyroid disease representing the most common manifestations (27.7% and 17.0%, respectively). Enteropathy was observed in 25.5% and granulomatous disease in 8.5% of patients, while neoplasia in 4 patients (8.5%; 3 lymphoma, 1 NSCL). Autoimmunity was the most common manifestation in IgAD patients (23, 50.0%), followed by recurrent infections (18, 39.1%) and allergy (6, 13.1%). *TACI* mutations were detected in 7 CVID patients (14.9%; all except one in heterozygous state); p.C104R was the most common defect, associated with splenomegaly ($p=0.022$) and neoplasia ($p < 0.001$). Only 2 asymptomatic IgAD patients displayed *TACI* defects (p.C104R & p.P151L). Our study was in accordance with previous ones in Caucasian patients providing further evidence that *TACI* mutations are associated with a more aggressive CVID phenotype, representing a marker for a higher awareness in patients' management.

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Conserved IL-2R signalling in zebrafish

Sertori, R., Liongue, C., Ward, A.

Deakin University, School of Medicine, Geelong, Australia

The interleukin 2 receptor (IL-2R) family of class I cytokine receptors plays important roles in lymphocyte development and function. The mammalian IL-2R family utilize the shared signalling component interleukin 2 receptor gamma common (IL-2R γ c) that signals via JAK3. Mutations of this receptor chain in humans or mice leads to severe combined immunodeficiency (SCID). The aim of this research project is to investigate the evolution of the il-2r family and analyse the role of key components in zebrafish lymphopoiesis. Using morpholino-mediated knockdown, pharmacological inhibition and genome editing with Transcription Activator-Like Effector Nucleases (TALENs), we demonstrate that the zebrafish *il-2ryc.a* paralogue has a conserved role in T-lymphopoiesis mediated via jak3 and other downstream pathways, consistent with human T-B+ SCID observed in humans with defective IL-2R γ c.

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Recurrent Infected lymphangioma in hyper-IgE syndromes

Sari, N.I.N.

Harapan Kita Women and Children Hospital, Pediatric, Jakarta, Indonesia

Introduction: Hyper-immunoglobulin E Syndrome (HIES) is a rare primary immunodeficiency disease which is characterized by high serum of IgE levels, eczema, and recurrent bacterial infections. We present the case of a patient with HIES from our hospital.

Method: Case Presentation.

Results: A 5 year-old girl was admitted to our hospital due to recurrent infected lymphangioma in cervical area. She was born full term without perinatal problems. She had a history of recurrent skin and respiratory tract infections such as skin abscess, otitis media, sinusitis and pneumonia since 6 months of age. There is no genetic relationship between her parents. Her father has history of recurrent abscesses. Her brother died with severe pneumonia in the first year of age with the IgE serum was 16.764 IU/ml. On physical examination, we found chronic eczematous skin rashes on the whole body and infected lymphangioma caused by *S. aureus*. Laboratory tests revealed a WBC count of 19,660 /uL, elevated eosinophil count 860/uL and high level of Ig E (16,452 IU/ml). Chest radiography revealed interstitial pneumonia. The patient's National Institutes of Health (NIH) Score for HIES was 61 means suggestive of autosomal dominant-HIES. The genetic examination should be done to verified. Antimicrobial prophylaxis to prevent *S. aureus* was started (2.5 mg/kg of the trimethoprim-sulfamethoxazole twice daily), and had a good response.

Conclusion: HIES should be considered in patients with recurrent bacterial infections. NIH-HIES scoring system help to distinguish HIES patients from patients with suspected primary immunodeficiency disease.

Keywords: Lymphangioma, Hyper IgE Syndrome (HIES), Immunodeficiency

45**Delayed puberty and gonadal failure in patients with HAX 1 mutation**Cekic, S.¹, Saglam, H.², Tarim, O.², Kilic, S.S.¹¹Uludag University Medical Faculty, Pediatric Immunology, Bursa, Turkey, ²Uludag University Medical Faculty, Pediatric Endocrinology, Bursa, Turkey

Purpose: Severe congenital neutropenia (SCN) comprises a heterogeneous group of disorders characterized by early childhood onset of profound neutropenia and recurrent life-threatening infections. Hypergonadotropic hypogonadism has been reported in two female patients with SCN due to P.Glu190X mutation in Hax-1 gene which is known to play a role in apoptosis and cell migration. In this investigation, gonadal development and function of patients with Hax-1 mutation in our clinic are evaluated.

Method: Detailed history, pubertal development, physical, and laboratory findings of 8 patients with SCN due to P.Trp44X mutation in HAX1 gene are evaluated retrospectively.

Results: The pubertal development of the only male patient was normal and complete. Four of the remaining 7 female patients were diagnosed with hypergonadotropic hypogonadism and hormone replacement therapy was prescribed. The rest of the three patients had small ovaries. One patient had small ovaries and menstrual irregularity, but gonadotropin levels were not elevated. One patient had small ovaries, but hypergonadotropic hypogonadism was not diagnosed, because she had normal menarche and gonadotropin concentrations. Although ovaries could not be visualized in one patient, puberty had just started and hypergonadotropic hypogonadism was not established. They are being followed up with gonadal dysfunction.

Conclusion: These results demonstrate that female patients with HAX1 mutation may develop primary ovarian failure. It is crucial to follow and evaluate the gonadal development of functions of female patients with HAX 1 mutation.

46**Mutations underlying autosomal dominant hyper-IgE syndrome impair distinct stages of STAT3 signaling**Pelham, S.J.^{1,2}, Lenthall, H.¹, Deenick, E.K.^{1,2}, Tangye, S.G.^{1,2}¹Garvan Institute of Medical Research, Immunology Division, Sydney, Australia, ²University of New South Wales, St Vincent's Clinical School, Sydney, Australia

Autosomal dominant hyper IgE syndrome (AD-HIES) is a primary immunodeficiency caused by heterozygous dominant negative mutations in the gene encoding Signaling transducer and activator of protein 3 (STAT3). These mutations cause a multisystem disorder, characterized immunologically by susceptibility to infection with pathogens such as *C. albicans* and *S. aureus* due to defective Th17 responses, and a failure to generate effective humoral immune responses. Interestingly, disease-causing mutations have been identified throughout all functional domains of STAT3 including the DNA binding, SH2 and transactivation domains. However, no phenotype-genotype correlations have been observed. To better understand how different mutations all result in the same

clinical and functional outcome we sought to characterize which stage(s) of STAT3 signaling was impaired by each of these mutations and if this was consistent for mutations in different domains. We used B-cell lines from AD-HIES patients or cell lines transfected with mutant STAT3 to determine the abilities of the mutant STAT3 proteins to undergo phosphorylation, cytokine receptor docking, dimerization, nuclear translocation, bind DNA and induce gene transcription. This revealed that while all mutations resulted in a distal impairment in STAT3 binding to DNA the block occurred at different proximal stages in signaling: transactivation domain mutant had reduced phosphorylation, SH2 domain mutants either were not phosphorylated or had reduced dimerization, and DNA binding domain mutants had defective nuclear translocation or DNA binding. Thus, this study reveals the mechanism underlying dysfunctional STAT3 signaling in AD-HIES and also identifies residues and regions of domains that are critical for normal STAT3 function.

47**Alteration of T cell responses in adult-onset immunodeficiency with acquired anti-interferon-γ autoantibodies**Pata, S.¹, Sirikul, C.², Chruewkamlow, N.¹, Mahasongkram, K.¹, Chaiwarith, R.³, Salee, P.³, Supparatpinyo, K.^{3,4}, Kasinrerak, W.¹¹Biomedical Technology Research Center, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency at the Faculty of Associated Medical Sciences, Chiang Mai University, Department of Medical Technology, Chiangmai, Thailand, ²Biomedical Technology Research Center, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency at the Faculty of Associated Medical Sciences, Chiang Mai University, Chiangmai, Thailand, ³Faculty of Medicine, Chiang Mai University, Department of Internal Medicine, Chiangmai, Thailand, ⁴Research Institute for Health Sciences, Chiang Mai University, Chiangmai, Thailand

Autoantibodies to IFN-γ can cause immunodeficiency syndrome in adults and are associated with various opportunistic infections. We speculate that, besides the presence of the anti-IFN-γ antibodies, T cell dysfunction may also be befallen in these patients. Upon TCR activation, T cell proliferation and expression of interleukin 2 receptor (CD25) of the patients remained intact. In comparison with the healthy subjects, the percentage of degranulating CD8 T cells was unchanged. However, the percentage of degranulating cytotoxic CD4 T cells was up-regulated in patients with anti-IFN-γ autoantibodies. Furthermore, the enhancement of Th1 cytokine (IFN-γ and TNF-α) production was observed, the production of Th2 (IL4) and Th17 (IL-17) cytokines were at the same level as healthy controls. We suggest that, in addition to the presence of anti-IFN-γ autoantibodies, alterations in T cell functions may also contribute to this adult-onset immunodeficiency.

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Memory B cells and plasmablasts in patients after splenectomy

Kral, V.¹, Pohorska, J.¹, Jilkova, E.², Zizkova, J.¹, Jilek, D.¹, Richter, J.¹

¹Institute of Public Health, Centre of Immunology and Microbiology, Ústí nad Labem, Czech Republic, ²Institute of Public Health, Centre of Health Services, Ústí nad Labem, Czech Republic

The spleen is crucial in regulating immune homeostasis through its ability to link innate and adaptive immunity and in protecting against infections. Patients after splenectomy are lifelong susceptible to the risk of bacterial infections mainly with encapsulated bacteria. Existing studies have reported the reduction of memory B cells in these patients.

Aim of the study was to perform more detailed analysis of B cells population subsets and monitoring their representation depending on vaccination.

B cells immunophenotyping was done by flow cytometry with CD19, CD27, CD38, IgM and IgD in the splenectomized patients suffering with hereditary spherocytosis (HS, n=15), immune thrombocytopenic purpura (ITP, n=10), injury (I, n=57) and with healthy control (HC, n=12).

Reduction of CD27⁺ memory B cells and more profoundly CD27⁺IgM⁺ immunophenotype was found in all splenectomized groups in comparison to HC group (p < 0,01). These results correlate with significantly increased levels of naive B cells in all patients groups (p < 0,01). Higher proportions of plasmablasts (IgM⁺CD38^{hi}) and transitional B cells (IgM⁺CD38^{hi}) were found in all groups of splenectomized patients, significantly in HS group (p < 0,01 when compared to HC group), despite the fact that last vaccination was done more than one year ago.

Our data affirm that splenectomy is associated with diminished numbers of peripheral memory B cells, mainly IgM⁺CD27⁺ subset. There has been also shown increased proportion of transient B cells and plasmablasts in periphery without any relation to previous vaccination history.

Our results could support the view of the fundamental importance of the spleen marginal zone in IgM memory B cells development.

Innate Lymphoid Cells

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Innate lymphoid cells contribute to allergic airway disease exacerbation by obesity

Everaere, L.¹, Ait Yahia, S.¹, Molendi-Coste, O.², Vorng, H.¹, Quemener, S.², Le Vu, P.², Fleury, S.², Bouchaert, E.², Fan, Y.¹, Duez, C.¹, de Nadai, P.¹, Staels, B.², Dombrowicz, D.², Tsigopoulos, A.¹

¹Inserm U 1019, Institut Pasteur de Lille, Pulmonary Immunity, Lille, France, ²Inserm U 1011, Institut Pasteur de Lille, EGID, Lille, France

Background: Epidemiological and clinical observations identify obesity as an important risk factor for asthma exacerbation, but the underlying mechanisms remain poorly understood. Type 2 and 3 Innate Lymphoid Cells (ILC2 and ILC3) have been implicated respectively in asthma and adipose tissue homeostasis, and in obesity-associated airway hyperresponsiveness (AHR).

Objective: To determine the potential involvement of ILC in

allergic airway disease exacerbation by high fat diet- (HFD) induced obesity.

Methods: Obesity was induced by HFD feeding and allergic airway inflammation was subsequently induced by intranasal administrations of house dust mite (HDM) extract. AHR, lung and visceral adipose tissue (VAT) inflammation, humoral response, cytokine and innate and adaptive lymphoid populations were analysed in the presence or absence of ILC.

Results: HFD feeding exacerbated allergic airway disease features including humoral response, airway and tissue eosinophilia, AHR, and Th2 and Th17 pulmonary profiles. Notably, non-sensitized obese mice already exhibited increased lung ILC and tissue eosinophil infiltration compared with lean mice in the absence of AHR. The number of total and cytokine-expressing lung ILC2 and ILC3 further increased in HDM-challenged obese mice compared to HDM-challenged lean mice and was accompanied by high levels of IL-33 and IL-1 β and decreased ILC markers in VAT. Furthermore, depletion of ILC with an anti-CD90 antibody, followed by T cell reconstitution, led to a profound decrease of allergic airway inflammatory features in obese mice, including Th2 and Th17 infiltration.

Conclusion: These results indicate that HFD-induced obesity may exacerbate allergic airway inflammation by mechanisms involving ILC2 and ILC3.

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T-bet regulates IL-33-induced airway inflammation by suppressing IL-9 production from innate lymphoid cells

Matsuki, A., Takatori, H., Makita, S., Yokota, M., Tamachi, T., Suto, A., Suzuki, K., Hirose, K., Nakajima, H.

Graduate School of Medicine, Chiba University, Allergy and Clinical Immunology, Chiba, Japan

Thy1⁺ CD25⁺ Lineage⁻ group 2 innate lymphoid cells (ILC2s) are a subset of immune cells that produce large amounts of Th2 cytokines such as IL-5 and IL-13 in T-cell-independent allergic inflammation and helminth infection. Recent studies have shown that T-box expressed in T cells (T-bet), which is well known as a master regulator of Th1 cells, plays pivotal roles in the development of ILC3s and ILC1s. However, the role of T-bet in lung ILC2s remains unknown. In this study, we examined the roles of T-bet in the development and the function of lung ILCs and airway inflammation induced by IL-33 administration. At steady-state conditions, the development and cytokine production of lung ILCs were normal in T-bet^{-/-} mice. On the other hand, IL-33-induced accumulation of lung ILC2s and eosinophilic airway inflammation were exacerbated in T-bet^{-/-} mice and T-bet^{-/-} RAG2^{-/-} mice as compared with those in wild-type (WT) mice and RAG2^{-/-} mice, respectively, suggesting that T-bet expressed in non-T/non-B cells regulates the development of eosinophilic inflammation. Transcriptome analysis revealed that IL-9 expression was up-regulated in IL-33-stimulated T-bet^{-/-} ILCs as compared with that in IL-33-stimulated WT ILCs. Moreover, enforced expression of T-bet suppressed IL-9 production in lung ILC2s. Furthermore, anti-IL-9 antibody attenuated IL-33-induced lung inflammation in T-bet^{-/-} mice. Taken together, these results suggest that T-bet suppresses IL-9 expression in lung ILC2s and thereby inhibits IL-33-induced eosinophilic inflammation.

51**Systemic and airway derived Th2 cells and ILC2s in asthmatics susceptible to virus triggered exacerbations***Malmhäll, C., Johansson, K., Lötvall, J., Rådinger, M.**University of Gothenburg, Sahlgrenska Academy, Krefting Research Centre, Gothenburg, Sweden*

Background: Asthma is a chronic inflammatory disease, in which Th2 cells and type 2 innate lymphoid cells (ILC2) play roles. These cells express the interleukin-33 receptor (IL-33R), and/or the prostaglandin D2 (PGD2) receptor CRTH2. IL-33 serves as a barrier guardian, and PGD2 induces cellular migration. The expression of these receptors on Th2 cells and ILC2s in subgroups of asthmatics has not been studied.

Aim: Our aim was to determine the expression of IL-33R/CRTH2 on blood versus airways derived Th2 cells and ILC2s obtained from asthmatics reporting recurrent viral exacerbations or not, and healthy volunteers.

Method: Bronchial lavage and blood were obtained from participants selected from an epidemiological cohort (West Sweden Asthma Study). Th2 cells (Lin+CD45+CD4+and IL-33R+ and/or CRTH2+), ILC2s (Lin-CD45+CD127+and IL-33R+ and/or CRTH2+) were analyzed by flow cytometry.

Results: Th2 cells express IL-33R and CRTH2 similarly in blood and airways. IL-33R expression was seen on a majority of airway ILC2s, but only half of blood ILC2s expressed IL-33R. CRTH2+ILC2s demonstrated an opposing pattern with more CRTH2 positive ILC2s in blood vs airways. Th2 cells were found in higher quantities in blood vs bronchial lavage. However, the number of ILC2s were comparable in blood and bronchial lavage. Asthmatics and healthy volunteers demonstrated similar distribution of Th2 cells and ILC2s in blood and airways at baseline.

Conclusion: ILC2s display highly distinct compartment-specific phenotypes, suggesting a more IL-33-responsive but less migration-prone phenotype in airways compared to blood. In contrast, our data suggest a shared Th2 phenotype in circulating blood and in airway lumen.

52**Soluble fibrinogen-like protein 2 regulates differentiation and enhances immunosuppressive function of myeloid-derived suppressor cells in allograft immunity***Yang, C.**Shanghai Key Laboratory of Organ Transplantation, Shanghai, China*

Soluble fibrinogen-like protein 2 (sFGL2) is a novel immunoregulatory molecule, secreted mainly by regulatory T cells. CD11b⁺ Gr1⁺ myeloid-derived suppressor cells (MDSCs) are an important regulatory innate cell population and have significant inhibitory effect on T cell-mediated responses. Here, we synthesized murine full length sFGL2 by eukaryotic expression system, and investigated the impact on differentiation and function of MDSCs. Bone marrow cells from BABL/c mice were cultured with or without 10 µg/ml sFGL2 for 3 days and 5 days under 10 ng/ml GM-CSF stimulation. Compared with PBS, sFGL2 significantly induced CD11b⁺Ly6G⁺Ly6C^{high} MDSC (MO-MDSC) differentiation but inhibited CD11b⁺Ly6G⁺Ly6C^{low}

MDSC (PMN-MDSC) differentiation. The sFGL2-induced MO-MDSCs significantly inhibited T cells proliferation compared with those induced by PBS. Besides, sFGL2-induced MO-MDSCs demonstrated higher expression of arginase-1 and iNOS at both mRNA and protein level. Furthermore, adoptive transfer sFGL2-induced MO-MDSCs prolonged the skin allograft survival in mice. In the sFGL2-induced MO-MDSCs infusion group, the transplanted skin allograft showed mild inflammatory immune cell infiltration, less apoptosis and necrosis, and lower pro-inflammatory cytokines expression. T cells in the recipient mouse displayed a lower autoimmune phenotype (lower TCR⁺ CD44^{high} CD62^{low} cells). Taken together, our results indicate sFGL2 prompts MO-MDSCs differentiation and enhances their immunosuppressive function.

53**A novel effect of extracellular histones released by innate immune cells on erythrocyte fragility***Kordbacheh, F.¹, O'Meara, C.H.^{1,2}, Coupland, L.A.¹, Lelliott, P.M.³, Parish, C.R.¹**¹Australian National University, John Curtin School of Medical Research, Cancer Biology and Therapeutics, Canberra, Australia,**²The Canberra Hospital, ENT Unit, Canberra, Australia, ³Australian National University, John Curtin School of Medical Research, Immunology and Infectious Disease, Canberra, Australia*

Extracellular histones are released from activated immune cells by extracellular traps. Extracellular traps are first observed in activated neutrophils (NETs) in response to pathogens and tissue injury and exhibit very effective anti-microbial activity. Uncontrolled formation of NETs can, however, become pathogenic particularly via their associated histones that can be cytotoxic in the vasculature causing endothelial cell death, systemic vascular obstruction and multiple organ failure. They can also initiate coagulation by both activating platelets and damaging erythrocytes such that they become pro-thrombotic. Recently we discovered an additional effect of histones on erythrocyte, which is to enhance their fragility when they are subjected to sheer stress associated with blood flow through the vascular system and spleen in particular. The spleen is the largest blood filter and is composed of branching arterial vessels that prevent old, damaged or abnormal erythrocytes from returning to the bloodstream. We have used a novel mechanically-induced sheer stress method as well as an *in vitro* spleen filtration model to investigate the effects of NETS and mammalian histones, over a wide concentration range, on RBC fragility under both isotonic and hypotonic conditions. Our results revealed that free histones bind to erythrocytes with high affinity and render erythrocytes susceptible to lysis by sheer stress in a dose-dependent manner. Based on these data we propose that erythrocytes can capture histones in the circulation and transport them to the spleen for disposal. Overload of this disposal system could cause the unexplained anaemia associated with sepsis, cancer and other pathological conditions.

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Human and murine fibroblastic reticular cells play specialized roles in innate immune cellular crosstalk

D'Rozario, J.^{1,2,3}, Knoblich, K.³, Roberts, D.³, Dias De Campos, J.⁴, Astarita, J.⁵, Mansell, A.⁶, Denton, A.⁷, Fearon, D.^{8,9}, Withers, D.³, Barone, F.⁴, Turley, S.⁵, Boyd, R.L.¹, Heng, T.^{1,10}, Fletcher, A.L.^{3,10}

¹Monash University, Department of Anatomy and Developmental Biology, Clayton, Australia, ²Monash Biomedicine Discovery Institute, Infection and Immunity Program, Clayton, Australia, ³University of Birmingham, Institute of Immunology and Immunotherapy, Birmingham, United Kingdom, ⁴University of Birmingham, Institute of Inflammation and Ageing, Birmingham, United Kingdom, ⁵Genentech, 1 DNA Way, Department of Cancer Immunology, San Francisco, United States, ⁶Hudson Institute of Medical Research, Centre for Innate Immunity and Infectious Diseases, Clayton, Australia, ⁷University of Cambridge, Department of Medicine and Cancer Research UK Cambridge Institute, Cambridge, United Kingdom, ⁸Cold Spring Harbor Laboratory, New York, United States, ⁹Weill Cornell Medical School, Department of Microbiology and Immunology, Joan and Sanford I. Weill Department of Medicine, New York, United States, ¹⁰These authors contributed equally to this work, Clayton, Australia

Fibroblastic reticular cells (FRCs) are a subset of lymph node stromal cells which have evolved to play important structural and immunoregulatory roles within the lymph node. While structurally they form the mesenchymal backbone of the lymph node, and surround the reticular network, FRCs have also been found to facilitate leukocyte migration and priming via the secretion of chemokines, cytokines and growth factors to regulate immune responses within the periphery. Furthermore, new studies have demonstrated additional capabilities of FRCs including deletional tolerance, antigen presentation and T cell suppression mediated via cellular crosstalk mechanisms.

Here we focus on the immunological crosstalk that occurs between FRCs and the innate immune system. Human and mouse transcriptomic expression profiles indicate that FRCs constitutively produce factors associated with the innate immune response and innate cell chemotaxis. FRCs express CCL2 and CXCL12, providing stimuli for the chemoattraction of macrophages and monocytes. FRCs also express functional TLR3 and TLR4, as stimulation with LPS or Poly I:C led to p38 phosphorylation and upregulation of chemotactic and growth factors by FRCs, inducing the maturation of monocytes to macrophages. Furthermore, ablation of FRCs in FAP-/- DTR mice led to the depletion of innate immune cell types within the lymph node and impairment of the immune response. Our data suggest that FRCs have the capacity to respond to bacteria and viruses and support a novel role for FRCs in the regulation of the innate immune system.

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Regulation in the production of natural antibody by innate lymphoid cells

Flores-Vázquez, J.C., Jiménez-Zamudio, L.A., Carnalla-Cortés, M., García-Latorre, E.A., Domínguez-López, M.L.
Instituto Politécnico Nacional, Escuela Nacional De Ciencias Biológicas, Ciudad de México, Mexico

Introduction: B1 lymphocytes are responsible of the production of natural antibody, essential in the defense against infectious agents but maybe dangerous in large quantities because of their autoreactivity. Moro et al. described the type II innate lymphoid cells (ILC2s) as lymphocyte stimulator of B1 cells to parasitic infections; so we asked if they would be responsible not only of stimulation in infection, but also to have a role in the spontaneous function of B1 cells.

Materials and methods: BALB/c healthy mice were used, and to develop the murine plasmacytoma at 9 months after induction with pristane. They were divided in two groups: the control group and a group that was treated with monoclonal antibody anti c-kit to deplete the ILC2s. Serum and peritoneal fluid were obtained to analyze antibody titers of IgM and IgA by ELISA.

Results: An increased of IgM and IgA production was observed in both serum and peritoneal fluid from mice treated with anti c-kit compared to healthy mice control group; however in the plasmacytoma group no significant difference was observed.

Conclusions: The increase in the production of IgM and IgA in the c-kit treated groups suggested a regulatory role by ILC2s. These cells not only are responsible for detecting danger signals to increase production of natural antibody, but are also responsible for regulation; however in the pristane induced animals, the depletion was not seen, probably because of the increase of ILC2s during the inflammatory process.

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Peyer's patch innate lymphoid cells regulate commensal bacteria expansion

Hashiguchi, M., Kobayashi, A., Kashiwakura, Y., Kojima, H., Kanno, Y., Kobata, T.
Dokkyo Medical University School of Medicine, Department of Immunology, Tochigi, Japan

The mammalian intestinal tract is colonized by trillions of bacteria comprising thousands of different species and anatomical containment of commensal bacteria in the intestinal mucosa is promoted by innate lymphoid cells (ILCs). However, the mechanism by which ILCs regulate bacterial localization to specific regions remains unknown.

Flow cytometric analyses revealed distinct cytokine profiles of ILCs from various tissue. Especially, Peyer's patch (PP) ILCs robustly produce IL-22 and IFN- γ , even in the absence of exogenous stimuli. The IL-22-producing ILCs showed typical phenotypes of group 3 ILCs; the IFN- γ -producing ILCs showed phenotypes of group 1 ILCs. Antibiotic treatment of mice decreased both IL-22⁺ and IFN- γ ⁺ cells in PPs. Blockade of both IL-2 and IL-23 signaling *in vitro* lowered IL-22 and IFN- γ production. Combination of IL-2 and IL-23, and IL-12 alone partly restored IL-22, and IFN- γ production of PP ILCs from Antibiotic-treated PP ILCs, respectively. PP ILCs induced mRNA expression of the antibacterial proteins RegIII β and RegIII γ in intestinal epithelial cells. PP contains large numbers of Proteobacteria and small parts of Bacteroidetes and Firmicutes by 16s rRNA analyses. *In vivo* depletion of ILCs rather than T cells altered bacterial composition from Proteobacteria-dominant to Bacteroidetes-dominant. qPCR revealed that ILC depletion also allowed Bacteroidetes and Firmicutes expansion in PPs. Collectively, our results show that ILCs regulate the expansion of commensal bacteria in PPs.

57**Dysregulation of ERK signaling in CD4-expressing cells induces osteochondromas**

Wehenkel, M.¹, Corr, M.², Guy, C.¹, Edwards, B.¹, Calabrese, C.³, Pages, G.⁴, Pouyssegur, J.⁴, Vogel, P.⁵, McGargill, M.¹

¹St. Jude Children's Research Hospital, Immunology, Memphis, United States, ²UCSD, Division of Rheumatology, Allergy and Immunology, La Jolla, United States, ³St. Jude Children's Research Hospital, Small Animal Imaging, Memphis, United States,

⁴University of Nice Sophia-Antipolis, Institute for Research of Cancer & Aging (IRCAN), Nice, France, ⁵St. Jude Children's Research Hospital, Pathology, Memphis, United States

ERK1 and ERK2 are serine/threonine kinases critical for the proliferation and development of many cell types. Mice with a conditional deletion of *Erk1* and *Erk2* mediated by *CD4cre* (DKO^{CD4} mice) exhibit a profound block in T cell development. Surprisingly, the DKO^{CD4} mice spontaneously developed osteochondromas by 28 weeks of age. Histological analysis of these lesions revealed excessive accumulation of hypertrophic chondrocytes originating from the growth plates in the bone, but no mononuclear infiltrate indicative of inflammation. As the majority of CD4⁺ cells are T cells, we investigated whether these lesions were caused by deletion of *Erk* in T cells. Unexpectedly, osteochondromas still appeared in DKO^{CD4} mice in the absence of T cells. In fact, osteochondromas developed faster and were more severe in DKO^{CD4} mice that lacked T cells, indicating that T cells play an important role in regulating cartilage homeostasis, and deletion of *Erk* in other cell types mediates excess cartilage accumulation. Furthermore, the development of the osteochondromas was influenced by changes in the microbiota, as DKO^{CD4} mice treated with an antibiotic cocktail had a delay in osteochondroma development, while mice housed in non-specific pathogen free conditions had accelerated onset of tumors. Together these data suggest that *Erk* plays a critical role in a CD4⁺ cell type other than T cells, possibly an innate lymphoid cell, to alter cartilage growth. In addition, we present a novel role for T cells in regulating cartilage homeostasis.

58**The role of dietary antigens on homeostasis and functional activity of innate lymphoid cells (ILCs) in the small intestine**

Lee, M.J., Kim, K.S., Hong, S.-W., Ko, H.-J., Jung, J., Lee, J.-Y., Surh, C.D. Academy of Immunology and Microbiology (AIM), Institute for Basic Science (IBS), Pohang, Korea, Republic of

ILCs are found in increased numbers in the mucosal tissues and mediate local tissue homeostasis. ILCs lack an Ag-specific receptor, but closely resemble effector CD4⁺ T cell subsets in terms of cytokine profiles and transcription factors. The precise role of commensal microbiota and dietary Ags in the intestinal immune homeostasis of ILCs is poorly understood. In order to address this issue we have examined small intestinal ILCs in B6 mice raised under germ-free (GF) conditions and also in antigen-free (AF) conditions whereby GF mice were raised on an elemental diet deprived of dietary macromolecules. We found that a fraction of Type3 ILCs was depleted in GF mice, and more strikingly, AF mice have a dramatically elevated number of Type2

ILCs that produced considerably higher amounts of TH2 effector cytokines than Type2 ILCs in SPF and GF mice. Moreover, Type3 ILCs in AF mice were severely depleted compared to that in SPF and GF mice. Accordingly, while the basal proliferation rate of Type3 ILCs was similar in SPF and GF mice, it was minimal in AF mice. Various experiments confirmed the role of dietary Ags in modulating Type2 and Type3 ILCs. Notably, we found markedly increased mRNA expression levels of TSLP and IL-25, but not IL-33, in the intestinal epithelial cells from AF mice compared to SPF and GF mice. We are currently investigating how dietary Ags from normal chow diet block strong expansion of ILC2s and promote normal ILC3 homeostasis in the steady state.

59**Mucosal BCG vaccination promotes function and accumulation of innate lymphoid cells in murine lungs**

Steigler, P., McCulloch, T., Kirman, J.R.

University of Otago, Department of Microbiology and Immunology, Dunedin, New Zealand

Tuberculosis (TB) has surpassed human immunodeficiency virus as the deadliest infectious disease. The intradermally applied TB vaccine, Bacille Calmette-Guérin (BCG), prevents disseminated childhood TB but fails to protect against pulmonary TB, the most prevalent form. TB vaccines currently in clinical trials target conventional T cells; innate cells are neglected in these vaccine approaches. New evidence suggests that some innate cell subsets exhibit features of immunological memory. In keeping with this concept, epidemiological studies show that BCG can protect against unrelated diseases. Located at mucosal entry sites and able to secrete cytokines rapidly, innate lymphoid cells (ILCs) are crucial in defense against many pathogens; however their role in protection against TB and ability to exhibit features of memory are as yet unexplored. Several studies have shown that mucosal BCG delivery enhances immunity against pulmonary TB infection compared to parenteral administration. We hypothesised that intranasal BCG vaccination would enhance the number and function of ILCs within lungs. Using a new multicolour flow cytometry panel, we measured ILC phenotype and function in murine lung and secondary lymphoid tissues post intradermal, intranasal or subcutaneous BCG vaccination or in unvaccinated controls. We found increased numbers of all ILC subsets in lungs and increased interferon-gamma production by natural killer cells, ILC1s and ILC3s compared to other delivery routes; implying that intranasal delivery leads to enhanced accumulation and effector function of ILCs in lungs. Mucosal BCG delivery might be the required vaccination route for effective induction of ILC immunity in lungs to mediate early protection against TB.

60**STAT4 modulates innate lymphoid cell effector function and early responses to intestinal infection**

Dulson, S., Harrington, L.

University of Alabama at Birmingham, Cellular Developmental and Integrative Biology, Birmingham, United States

Innate Lymphoid Cells (ILCs) are a newly discovered subset of cells primarily found at barrier surfaces that serve both protective and pathogenic roles in intestinal health. ILCs are subdivided into distinct groups and perform effector functions similar to CD4 T cells, but lack a specific receptor and are activated early during an immune response. Since the initial events following intestinal infection dictate the course of the immune response, it is critical to know what molecules regulate ILC function both during homeostasis and in disease. Signal transducer and activator of transcription 4 (STAT4) is important for driving many pro-inflammatory responses and phenotypes in T cells, but its effects on ILC development and function remain unclear. Contrary to its known role in Th1 cell biology, we find STAT4 to be dispensable in Group 1 ILC development. However, we observe that STAT4 is critical for IFN- γ production by Group 1 ILCs, thus influencing the capacity of this subgroup to mount an effector response. Whereas STAT4 deficiency did not impact IL-22 or IL-17 production by Group 3 ILCs, this transcription factor specifically modulates the secretion of IFN- γ by plastic Tbet⁺ ILC3s, a subgroup that is greatly expanded during inflammation. Importantly, STAT4 signaling is critical early for survival against gastrointestinal infection with a genetically modified strain of *Listeria monocytogenes* that is capable of binding mouse E-cadherin on the intestinal epithelium. These results indicate that STAT4 has the potential to directly influence responses by ILCs, thereby contributing to host protection against intestinal infection.

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Interferon- γ negatively regulates cytokine production of group 2 innate lymphoid cells

Kudo, F., Takaki, S.

National Center for Global Health and Medicine, Research Institute, Department of Immune Regulation, Ichikawa, Japan

Group 2 innate lymphoid cells (ILC2s) contribute to type 2 immune responses in response to allergic inflammation and tissue repair by producing high amounts of IL-5 and IL-13. IL-5 supports eosinophil responses in various tissues. IL-13 induces mucus production and contributes to tissue repair or fibrosis. ILC2s are activated by alarmins, such as IL-33 released from epithelia, macrophages and NKT cells in response to infection and allergen exposure, leading to epithelial injury.

To get insight for regulations operating in ILC2s, we conducted SAGE analysis and found that ILC2s expressed *Ifngr1*, the receptor for Th1 cytokine. IFN- γ severely inhibited IL-5 and IL-13 production by lung and kidney ILC2s in culture. In order to evaluate the effects *in vivo*, we used α -galactosylceramide (α -GalCer) to induce NKT cells to produce IL-33 and IFN- γ . Intraperitoneal injection of α -GalCer in mice induced NKT cell activation resulting in IL-5 and IL-13 production by ILC2s. Administration of anti-IFN- γ together with α -GalCer significantly enhanced the production of IL-5 and IL-13 by ILC2s in lung and kidney. Conversely, cytokine production from ILC2s was markedly suppressed after injection of exogenous IL-33 in *Il33*^{-/-} mice pre-treated with α -GalCer. Our findings demonstrate that IFN- γ directly inhibits IL-5 and IL-13 production by ILC2s, and that blocking IFN- γ restored the ability of activated ILC2s to produce IL-5 and IL-13. IFN- γ antagonizes ILC2 function

similar to the way IFN- γ impacts Th2 cells and constrains type 2 immune responses.

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Characterising the role of group 1 innate lymphoid cells during malaria

Ng, S.S.^{1,2}, Guimaraes, F.^{3,4}, de Labastida Rivera, F.¹, Amante, F.H.¹, Smyth, M.³, Engwerda, C.R.¹

¹QIMR Berghofer Medical Research Institute, Immunology and Infection, Herston, Australia, ²Griffith University, School of Natural Sciences, Nathan, Australia, ³QIMR Berghofer Medical Research Institute, Immunology of Cancer and Infection, Herston, Australia, ⁴Walter & Eliza Hall Institute, Molecular Immunology Division, Parkville, Australia

Innate lymphoid cells (ILCs) resemble T helper (Th) cells in terms of transcription factor requirements and cytokine profiles, but do not express antigen-specific receptors. Group 1 ILCs consist of natural killer (NK) cells and ILC1s. Together, they share many characteristics with Th1 cells such as a requirement for T-bet and the ability to produce IFN γ . However, the developmental relationship between NK cells and ILC1s is still a matter of debate. An ILC1 subset that is liver-resident has recently been described. Given the resemblance between ILC1s and Th1 cells, and the importance of Th1 cells in the immune response during *Plasmodium* infection, we aimed to characterise the role of ILCs during malaria. Initial findings suggested that ILC1s and NK cells have a limited role in conferring protection or causing pathology during *P. chabaudi chabaudi* AS (PcAS) infection. However, in a controlled human malaria infection study, we showed that the frequencies of circulating ILC1s and NKs were decreased at the peak of infection, compared to frequencies before infection. A similar observation was made for liver and splenic ILC1s in PcAS-infected mice. The decrease in liver ILC1 frequencies in mice was associated with increased apoptosis but did not directly relate to blood parasitaemia. Together, our results show that ILC1s are lost early during rodent and human malaria, and this observation may explain the limited role for ILC1s and NK cells in controlling blood stage malaria.

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The role of c-Kit in the activation of innate lymphoid cell and respiratory viral infection-induced acute asthma

Lai, A.¹, Wu, H.-C.¹, Chuang, Y.-T.², Wu, Y.-H.¹, Baumgarth, N.³, Umetsu, D.², Chang, Y.-J.¹

¹Academia Sinica, Institute of Biomedical Sciences, Taipei, Taiwan, Republic of China, ²Children's Hospital, Harvard Medical School, Division of Immunology and Allergy, Boston, United States, ³University of California, Center for Comparative Medicine, Davis, United States

Asthma, which is characterized by airway inflammation and airway hyperreactivity (AHR), is thought to be mediated by allergen-specific Th2 cells, adaptive immunity and allergic inflammation. Current treatments for asthma generally focus on minimizing the Th2-driven eosinophilic inflammation with the use of anti-inflammatory therapies such as corticosteroids.

However, this approach, targeting the adaptive Th2 cell immunity with corticosteroids, has not reduced the high rates of hospitalizations for acute asthma, most often caused by acute viral infection, including influenza. Recently, type 2 innate lymphoid (ILC2) cells expressing c-Kit have been shown to expand during influenza infection and to directly cause airway hyperreactivity (AHR), a cardinal feature of asthma. We now show that the treatment of *influenza*-infected mice with c-Kit kinase inhibitors blocked the development of AHR and airway inflammation and greatly reduced the number and function of ILC2 cells, whereas the treatment with corticosteroids, commonly used to treat acute asthma, failed to prevent the *influenza*-induced AHR. We also demonstrated that c-Kit function is required for normal ILC2 cell function and for *influenza*-induced AHR, and that c-Kit kinase inhibitor inhibits murine lung ILC2 cell proliferation and cytokine productions *in vitro* and *in vivo*. Furthermore, the treatment of c-Kit inhibitor did not affect *in vivo* viral clearance. Our study provides mechanistic insights into the crucial role of c-Kit in modulating the ILC2 activation in the lungs, particularly in respiratory viral infection induced acute asthma.

Invariant & $\gamma\delta$ T Cells

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Comparison of IFN- γ production of peripheral blood $\gamma\delta$ T cells between latent tuberculosis infections and patients with active pulmonary tuberculosis

Li, B.¹, Sheng, L.-L.^{1,2}, Chen, C.¹, Jin, H.², Wang, Z.³, Tang, J.¹, Xia, H.¹
¹Bengbu Medical College, Department of Immunology, and Anhui Key Laboratory of Infection and Immunity, Bengbu, China, ²The First Hospital of Taizhou, Taizhou, China, ³The Infectious Disease Hospital of Bengbu City, Department of Lung Disease, Bengbu, China

Aim: The Interferon- γ (IFN- γ) release assays (T-SPOT.TB), which based on the re-activation of *M. tuberculosis* (*M. tb*) antigen specific memory CD4 T cells, are specific for diagnosis of *M. tb* infection, but not sufficient for discrimination of active tuberculosis (TB) patients from latent TB infection (LTBI). This study was to investigate the difference of IFN- γ producing $\gamma\delta$ T cells between the LTBI and active pulmonary TB patients.

Methods: Eighteen healthy donors without *M.tb* infection (HD), 17 subjects with LTBI, and 22 active pulmonary TB patients were enrolled in this study. Peripheral mononuclear cells were separated from whole blood, and cultured with phosphoantigens (DMAPP, or IPP) and *M.tb* heat resistant antigen (Mtb-Hag) for 20 hours and added monesin during last 6 hours. The antigen stimulated cells were stained with anti cell surface molecule fluorescence monoantibodies (mAb), followed with anti IFN- γ fluorescence mAb. The proportions of IFN- γ producing $\gamma\delta$ T cells, and $\alpha\beta$ T cells were measured by flow cytometry.

Result: The proportions of IFN- γ producing $\alpha\beta$ T cells stimulated with phosphoantigen, or Mtb-HAg, among HD, LTBL and TB groups were from 0.13% to 0.25%, there were no differences among groups. The proportions of IFN- γ producing $\gamma\delta$ T cells that stimulated with phosphoantigen and Mtb-HAg among

LTBI group (7.53%, and 5.82%, respectively), were significantly higher than that among active TB patients (3.36%, and 2.32%, respectively) (both $p < 0.05$).

Conclusion: IFN- γ release assays by $\gamma\delta$ T cell specific antigens are potential means to differentiate LTBI from active TB patients.

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MAIT cells in *Helicobacter* induced gastritis

Dsouza, C.^{1,2}, Chen, Z.¹, Every, A.², Scheerlinck, J.P.², Corbett, A.J.¹, McCluskey, J.¹

¹University of Melbourne, Department of Microbiology and Immunology, Melbourne, Australia, ²University of Melbourne, Centre for Animal Biotechnology, Melbourne, Australia

Mucosal Associated Innate T (MAIT) cells are type of innate like T lymphocytes that express a semi-invariant TCR and are restricted by the non-classical MHC class I-related molecule, MR1. These cells can recognize a novel class of antigens, intermediates of the riboflavin synthesis pathway that are only produced by certain class of bacteria and yeasts.

Helicobacter pylori is a bacterium that infects the human stomach and is associated with causing gastritis, duodenal ulcers and gastric cancer. We have examined whether MAIT cells play a role in the pathology of chronic *H. pylori* infection. We have recently developed highly specific MR1 tetramers that have been used to detect and characterize MAIT cells in mice. Our studies show that MAIT cells play a key role in the regulation of gastric inflammation in *H. pylori* infection. Using a mouse model that first enriches MAIT cells in the lung, we show repopulation of MAIT cells to other mucosal sites including the stomach. On challenge with *H. pylori*, these mice develop an accelerated inflammatory response leading to atrophic gastritis. In our model, MAIT cells have a pathogenic rather than protective effect. We are working to understand their role with a view to therapeutic intervention.

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CAR $\gamma\delta$ -T cell for solid tumor immunotherapy based on tumor specific CDR3 δ

Teng, D., Chen, H., Zhang, J., He, W.

Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China

The adoptive transfer of engineered T cells that express artificial chimeric antigen receptor (CAR) which target CD19 has shown impressive antitumor efficacy in B cell malignancies. But current CAR-T cell therapy has less therapeutic effects in solid tumors, mainly because the limited antigen specificity of single-chain antibody (scFv) expressed on CAR-T cell, is not suitable for highly heterogeneous solid tumors. T cell receptor (TCR) $\gamma\delta$ can recognize stress-induced antigens highly expressed on several tumor cells and the antigen specificity of TCR $\gamma\delta$ solely depends on CDR3 δ . Previously, we found tumor-specific CDR3 δ sequence named OT3 derived from tumor infiltrated lymphocytes. TCR $\gamma\delta$ 2 (OT3)-Fc fusion protein triggered an ADCC response against series of solid tumors including ovarian epithelial carcinoma, hepatic carcinoma, lung carcinoma, etc. Moreover, TCR $\gamma\delta$ 2 (OT3) engineered $\alpha\beta$ T cells also revealed significant

antitumor reactivity both *in vitro* and *in vivo*. In this study, we found several tumor-specific CDR3 δ sequences by immune repertoire technology. Then designed series of third generation CAR $\gamma\delta$ molecules, extracellular domain of which were composed of single chain of V γ and V δ (tumor-specific CDR3 δ grafted), instead of scFv. These molecules were then transferred to CD8⁺T cell by lentivirus and detected the cytotoxicity of CAR $\gamma\delta$ -T cells to tumor cells. Considering the characteristics of solid tumor, we combined heparanase, PD-1 antibody, IL-12, soluble IL-6 receptor with CAR $\gamma\delta$ -T cells, which might enhance the anti-tumor activity and reduce adverse reactions of CAR $\gamma\delta$ -T cells *in vivo*. Based on TCR $\gamma\delta$ broad-spectrum recognition, CAR $\gamma\delta$ -T cells is expected to provide perspective on the development of adoptive immunotherapy against solid tumor.

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TCR-ligand interactions are required for murine epidermal V γ 3V δ 1 T cell development

Witherden, D.A., Garijo, O., Kelly, R., Komori, H.K., Havran, W.L. Scripps Research Institute, Department of Immunology and Microbial Science, La Jolla, United States

Dendritic epidermal T cells (DETC), which bear the V γ 3V δ 1 T cell receptor (TCR), are the exclusive T cell population resident in the murine epidermis where they act as sentinels for neighboring keratinocytes. $\gamma\delta$ T cells differentiate from lymphoid precursors largely within the thymic microenvironment in a highly organized manner. Cellular selection processes are also believed to be involved and indeed several lines of evidence suggest that proper development and homing of the V γ 3V δ 1 subset does require positive selection events in the fetal thymus. To evaluate the role of TCR interactions with cognate ligand in V γ 3V δ 1 T cell development and maturation, we used a soluble DETC TCR tetramer to characterize ligand expression in the fetal thymus and skin. We show that temporal expression of ligand in the thymus closely mimics the window of DETC intrathymic development. Ligand expression is independent of Skint1, a molecule shown to be important for thymic maturation of DETC and their subsequent residence in the epidermis. Furthermore, using the soluble DETC TCR tetramer as a ligand blocking reagent, we show that in fetal thymic reaggregation cultures, TCR-ligand interactions are essential for DETC development and maturation. Current experiments are investigating the nature of the ligand-expressing thymic epithelial cell population. Understanding TCR-ligand interactions involved in DETC development may provide important insight into identifying the enigmatic DETC antigen.

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Dynamic expression of CCR6 and CCR2 balances homeostatic and inflammatory trafficking of $\gamma\delta$ T17 cells

McKenzie, D.¹, Kara, E.¹, Tyllis, T.¹, Bastow, C.¹, Fenix, K.¹, Gregor, C.¹, Kallies, A.^{2,3}, Nutt, S.^{2,3}, Comerford, I.¹, McColl, S.¹

¹University of Adelaide, Department of Molecular & Cellular Biology, Adelaide, Australia, ²Walter & Eliza Hall Institute of Medical Research, Parkville, Australia, ³University of Melbourne, Department of Medical Biology, Parkville, Australia

Successful detection and eradication of invading pathogens requires collaboration between appropriately placed immune sentinels in barrier tissue and infiltrating inflammatory cells. Fulfilling both roles are interleukin 17-producing $\gamma\delta$ T cells ($\gamma\delta$ T17), which exhibit unique migratory characteristics. While most $\gamma\delta$ T17 cells reside in mucocutaneous tissues at homeostasis, these cells also expand in lymphoid organs during inflammation and then infiltrate inflammatory sites. While crucial for various rapid protective responses, $\gamma\delta$ T17 cells have been shown to be detrimental in models of autoimmunity and cancer. Therefore, investigating the poorly understood migratory cues regulating their homeostatic positioning and inflammatory trafficking is of clinical significance. We show that $\gamma\delta$ T17 cells constitutively express the chemokine receptors CCR6 and CCR2. $\gamma\delta$ T17 cell positioning in the dermis at homeostasis is CCR6-dependent, while CCR2 drives recruitment of $\gamma\delta$ T17 cells to the central nervous system in experimental autoimmune encephalomyelitis and into B16 melanomas. We find no requirement for CCR6 in $\gamma\delta$ T17 cell homing to inflammatory lesions despite its established inflammatory role in other cell types. Accordingly, upon activation $\gamma\delta$ T17 cells quickly extinguish CCR6 expression in an IRF4- and BATF-dependent manner, which inhibits their recruitment to uninfamed skin. These findings suggest a novel paradigm of cell migration, whereby $\gamma\delta$ T17 cells constitutively express chemokine receptors that drive recruitment into non-inflamed (CCR6) and inflamed (CCR2) tissues, but rapidly downregulate the homeostatic regulator CCR6 upon activation to avoid recruitment to uninfamed skin. This allows CCR2 to efficiently drive $\gamma\delta$ T17 cells to sites of inflammation and orchestrate early immunity.

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Identification of CD1b-autoreactive T cells using tetramers

Ross, F.J.¹, Souter, M.¹, Pellicci, D.G.¹, Uldrich, A.P.¹, Rossjohn, J.², Godfrey, D.I.¹

¹The Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Microbiology and Immunology, Melbourne, Australia, ²School of Biomedical Sciences, Monash University, Department of Biochemistry and Molecular Biology, Melbourne, Australia

The ability of conventional T cells to recognize peptide antigens presented by MHC has been well characterized. However, there also exist T cell populations that interact with lipid antigens presented by CD1. Four CD1 isoforms are expressed in humans, and include CD1a-d. Of the lipid-reactive T cell populations, CD1d-reactive Natural Killer T cells have been the most thoroughly described, largely due to the fact that CD1d is the only isoform present in mice and that the antigen, α -GalCer, loaded into CD1d tetramers permits clear identification of these cells.

One difficulty in investigating T cells specific to CD1a-c is a lack of reagents to identify them. In spite of this, it has been demonstrated that at least a subset of these cells can be identified by *in vitro* stimulation assays due to their ability to recognize both self-lipids and bacterial lipids. We utilized endogenous antigen-loaded tetramers to probe for CD1b-autoreactive cells within healthy human blood samples. Following *in vitro* expansion of

rare tetramer+ T cells, a defined population could be identified and further examined using single cell abTCR sequencing. Five distinct clones were identified across two donors, each of which was CD4⁺ CD8⁻. The TCR chains expressed by these cells were TRAV12-3/TRBV28, TRAV13-2/TRBV19, TRAV27/TRBV5-8, TRAV27/TRBV5-4, and TRAV41/TRBV6-2, suggesting diverse TCR usage. Generation of retrovirally transduced TCR+ cell lines confirmed CD1b-reactivity, and allows us to investigate their reactivity to a large panel of lipid-based antigens. This work is helping to shed light on the poorly understood autoreactive CD1b-restricted T cell population.

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Visualising MAIT and NKT cells in colorectal tumours

Kelly, J.^{1,2}, Toohey, B.², Gray, D.³, Kannourakis, G.², Berzins, S.P.^{1,2}

¹Federation University Australia, Ballarat, Australia, ²Fiona Elsey Cancer Research Institute, Ballarat, Australia, ³The Walter and Eliza Hall Institute of Medical Research (WEHI), Molecular Genetics of Cancer, Immunology, Melbourne, Australia

Unlike conventional T cells, which have a diverse T cell receptor repertoire and recognize antigen presented on MHC, several unconventional T cell subsets have a semi-invariant TCR α -chain and are restricted by non-MHC antigen presenting molecules, such as MR1 (MAIT cells) and CD1d (NKT cells). The role of these cells is not yet fully defined, but their responsiveness to microbes suggests they may have important roles in immune regulation within mucosal tissues. As colorectal cancers involve a breach of the mucosal barrier and an interaction between colorectal tissue and the gut microbiota, we investigated where, if any, MAIT and NKT cells were located in relation to these lesions, and whether their activity in these locations contribute to cancer evolution and progression. We have previously identified NKT and MAIT cells in colorectal tissue using flow cytometry and molecular analysis and, utilizing convention immunohistochemistry and immunofluorescence staining of formalin-fixed paraffin-embedded archived tissue blocks, we have now identified the location of these cells in both normal and cancerous regions of patients with colorectal cancers.

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Circulating mucosal-associated invariant T (MAIT) cell levels and their functional capacity in healthy individuals and patients with chronic lymphocytic leukemia (CLL)

Alcantara, M.^{1,2}, Wallace, M.¹, Kannourakis, G.^{1,2}, Berzins, S.^{1,2,3}

¹Federation University, Biomedical Science, Ballarat, Australia,

²Fiona Elsey Cancer Research Institute, Ballarat, Australia,

³Melbourne University, Melbourne, Australia

Chronic Lymphocytic Leukemia (CLL) is a malignancy characterized by the accumulation of mature B cells and is the most common form of adult leukemia in the western world. The progression of CLL is unpredictable; patients can remain stable for variable periods (1- 20 years), before rapidly progressing to aggressive and symptomatic disease.

Immune regulation may play an important role in the progression of CLL, with increased numbers of Foxp3+ T regulatory cells

correlated with disease progression. Other regulatory subsets such as NKT cells, $\gamma\delta$ T cells and MAIT cells may also be important but are not as well characterized in patients. MAIT cells are a regulatory T cell population with a semi-invariant TCR repertoire and an anti-microbial role. Their significance in cancer has not been well studied but they interact with B cells and may therefore influence immune activity in CLL patients.

Our preliminary characterization of MAIT cell subsets in CLL patients at different stages of the disease, including enumeration and analysis of cytokines and cell surface molecules have identified a deficiency of MR1 restricted MAIT cells in patients with CLL, within the well-studied CD161+CD8⁺ subset, as well as the CD161-CD8⁺ and CD161+CD8⁻ subsets. In addition, our results also indicate a correlation between patient group characteristics and deficiencies in these cells. We are looking forward to providing important insights on the role of MAIT cells in the immune regulation of CLL patients and potentially how this may be used to improve current treatments or provide new targets for immune therapy.

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The role of the CD8 co-receptor on MAIT cells

Souter, M.N.T., Chen, Z., Pellicci, D.G., Godfrey, D.I., Eckle, S.B.G.,

McCluskey, J.

University of Melbourne, Peter Doherty Institute for Infection and Immunity, Microbiology and Immunology, Melbourne, Australia

T cells have historically been characterized by their ability to recognize peptides presented by the major histocompatibility complex (MHC) molecules.

These 'conventional' T cells express either CD4 or CD8 $\alpha\beta$ co-receptors and can be reliant on these molecules for the recognition of MHC-II and MHC-I respectively. In the last two decades, T cells bearing receptors reactive to lipid and small molecule antigens have been discovered. These include Mucosal-Associated Invariant T (MAIT) cells, which are restricted by the monomorphic MHC molecule MR1.

Using MR1-tetramers we showed that MAIT cell co-receptor expression is discordant with conventional T cells. MAIT cells predominantly express CD8 $\alpha\alpha$ or to a lesser extent can be deficient of co-receptor molecules. The high frequency of CD8⁺ MAIT cells suggests that the CD8 co-receptor may influence immune outcomes in this population of cells. Using a CD8 α transduced cell line, we have determined that CD8 $\alpha\alpha$ is capable of binding to MR1-tetramers in the absence of a T cell receptor (TCR) and fails to bind to MR1-tetramers mutated at residues shown to abrogate the CD8 interaction with MHC-I. Our observations suggest that MAIT cells use the CD8 co-receptor in a similar manner as conventional T cells to bind MHC-I, whereby CD8 can bind MR1 to enhance the overall avidity of the MAIT TCR interaction with MR1. We aim to characterize the CD8 interaction with MR1 quantitatively and determine its importance in MAIT cell biology.

73 TCR $\gamma\delta^+$ CD8 $\alpha\beta^+$ T cell in health and disease: a novel and functionally active subpopulation of T cells enriched within the gut

Kadivar, M.¹, Petersson, J.¹, Bekiaris, V.², Marsal, J.³, Svensson, L.¹

¹Lund University, Medical Faculty, Immunology, Lund, Sweden,

²Technical University of Denmark, DTU Vet., Copenhagen,

Denmark, ³Lund University, Skane University Hospital, Gastroenterology, Lund, Sweden

$\gamma\delta$ T-cells have been implicated in the pathogenesis of immune-mediated diseases such as inflammatory bowel disease (IBD). However, a potential role of different immune cell subsets in IBD and also in the process of mucosal healing upon treatment is unknown. $\gamma\delta$ T-cells have been divided into CD8 $\alpha\alpha^+$ and CD8 $\alpha\beta^+$ T-cells. By using flow cytometry and RT-PCR, we described for the first time a novel subset of human $\gamma\delta$ T-cells expressing CD8 $\alpha\beta$ heterodimers on their surface. We found that these TCR $\gamma\delta^+$ CD8 $\alpha\beta^+$ T-cell subset exist in both human peripheral blood as well as in the gut, however they were differentially enriched within the gut. TCR $\gamma\delta^+$ CD8 $\alpha\beta^+$ T-cells displayed high cytotoxic activity by expressing Fas Ligand on their surface and also producing Granzyme B and Perforin. We showed that these cells can produce INF γ and TNF α but they did not show the ability to produce IL-17 in healthy individuals. In patients with IBD, we found a decrease in the percentage of intestinal CD8 $\alpha\beta^+$ $\gamma\delta$ T-cells compared to healthy controls. Moreover, the percentage of TCR $\gamma\delta^+$ CD8 $\alpha\beta^+$ T-cells out of $\gamma\delta$ T-cells showed a negative correlation with Crohn's disease activity. Three months of anti-TNF α (adalimumab) therapy increased the percentage of TCR $\gamma\delta^+$ CD8 $\alpha\beta^+$ T-cells close to the level of healthy controls. These results suggest that TCR $\gamma\delta^+$ CD8 $\alpha\beta^+$ T-cells might possibly play a role in gut inflammation and also intestinal wound healing after anti-TNF α treatment. These results are likely to have implications for the development of novel therapies to treat mucosal inflammatory diseases.

74 A broad family of MR1-restricted T cells

Gherardin, N.A.^{1,2,3}, Keller, A.N.^{4,5}, Woolley, R.E.^{4,5}, Le Nours, J.^{4,5}, Ritchie, D.S.^{2,6}, Neeson, P.J.², Birkinshaw, R.W.^{4,5}, Eckle, S.B.¹, Waddington, J.N.¹, Liu, L.^{7,8}, Fairlie, D.P.^{7,8}, McCluskey, J.¹, Pellicci, D.G.^{1,3}, Uldrich, A.P.^{1,3}, Rossjohn, J.^{4,5,9}, Godfrey, D.I.^{1,3}

¹Peter Doherty Institute for Infection and Immunity at The University of Melbourne, Department of Microbiology and Immunology, Melbourne, Australia, ²The Peter MacCallum Cancer Centre, Cancer Immunology Research, East Melbourne, Australia, ³ARC Centre of Excellence in Advanced Molecular Imaging, The University of Melbourne, Melbourne, Australia, ⁴Monash University, Department of Biochemistry and Molecular Biology, School of Biomedical Sciences, Clayton, Australia, ⁵ARC Centre of Excellence in Advanced Molecular Imaging, Monash University, Clayton, Australia, ⁶University of Melbourne, Department of Medicine, Melbourne, Australia, ⁷University of Queensland, Division of Chemistry and Structural Biology, Institute for Molecular Bioscience, Brisbane, Australia, ⁸ARC Centre of Excellence in Advanced Molecular Imaging, University of Queensland, Brisbane, Australia, ⁹Institute for Infection and

Immunity, Cardiff University School of Medicine, Heath Park, Cardiff, United Kingdom

MR1 is an MHC class Ib molecule that presents a series of small metabolite antigens derived from vitamin B metabolism. Conserved microbial ligands derived from microbial riboflavin (Vitamin B2) biosynthesis have been shown to be T cell agonists, activating a unique subset of T cells called mucosal-associated invariant T (MAIT) cells. MAIT cells are highly abundant in humans, accounting for up to 10% of circulating T cells. They are defined by expression of a semi-invariant T cell receptor (TCR) that imbues pattern-recognition-like detection of MR1-presented microbial riboflavin metabolites. Thus MAIT cells are emerging as key players in antimicrobial immunity. Beyond MAIT cells however, little is known about MR1-mediated immunity, including the breadth of antigens presented by MR1 and the repertoire of T cells that recognise them. Very recently, we showed that human MAIT cells can recognise non-microbial antigens derived from folate (Vitamin B9), and furthermore that a second population of 'atypical' MR1-restricted T cells exists, characterised by a surface phenotype and transcription factor profile distinct to that of MAIT cells, and a diverse TCR repertoire¹. Thus, MAIT cells represent only a subset of a broader population of MR1-restricted T cells.

We subsequently extend upon these findings and provide further detail on the phenotypic features and functional capacity of these atypical MR1-restricted T cells. We provide novel insight into the TCR and antigenic repertoires of these cells and show that they use a variety of docking modes atop MR1 to mediate MR1-Ag recognition.

¹Gherardin & Keller *et al.*, *Immunity*, 2016

75 Differentiation and expansion of $\gamma\delta$ T-cells by Zoledronic acid efficiently activates cytotoxicity *in vitro*

Thunberg, S.^{1,2}, Uhlin, M.², Önfelt, B.¹

¹Science for Life Laboratory and Royal Institute of Technology, Applied physics, Stockholm, Sweden, ²Karolinska Institute, Oncology and Pathology, Stockholm, Sweden

Between 1-10 % of circulating T-cells express the $\gamma\delta$ T-cell receptor. Dissimilar to conventional $\alpha\beta$ -T-cells recognizing peptide antigens, the $\gamma\delta$ -T-cells recognize phosphorylated antigens expressed by various pathogens and tumours. Metabolites of zoledronic acid (ZOL), a bisphosphonate, can stimulate proliferation and expansion of $\gamma\delta$ -T-cells. The aim of the study was to expand $\gamma\delta$ -T-cells using ZOL and evaluate their cytotoxic function.

Peripheral mononuclear cells (PBMCs) (n=10) was stimulated without or with 2.5 μ M ZOL and 200 U/mL of IL-2 for up to 18 days. T-cell differentiation and expansion was followed with flow cytometry. Cytotoxic function of $\gamma\delta$ - and $\alpha\beta$ -T-cells were evaluated using 4 h co-cultures in 1:1 and 5:1 ratios with the cell lines K562, HL-60 and KU812, all lacking MHC-expression, as targets.

The median frequency of $\gamma\delta$ -T-cells at day 0 was 3.6, increasing with ZOL to 37, 79 and 89 % at day 3, 9 and 13 respectively and absolute number increased between 2 to 4 times per week.

The majority of $\gamma\delta$ -T-cells were NKG2A positive and displayed a mature and activated T-cell phenotype, defined by CCR7-CD45RA-HLA DR+. Both $\gamma\delta$ - and $\alpha\beta$ -T-cells effectively killed K562 and HL-60 cells, while KU812 was predominately killed by $\gamma\delta$ -T-cells. Moreover, the cytotoxicity by $\gamma\delta$ -T-cells was not due to degranulation, since only an average of 0.69 % expressed CD107a, compared to 8.35 % of the $\alpha\beta$ -T-cells.

ZOL are able to expand and activate $\gamma\delta$ -T-cells to become effective killers for potential use in immunotherapy. However, the mechanism of their cytotoxic function remains to be elucidated.

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Immunological role of alternatively spliced isoforms of MR1

Li, S.^{1,2}, McWilliam, H.E.G.^{1,3}, Smith, J.M.¹, Meehan, B.¹, Herold, M.^{4,5}, Villadangos, J.^{1,3}, Rossjohn, J.^{6,7,8}, Eckle, S.B.G.¹, Uldrich, A.P.^{1,2}, McCluskey, J.¹, Godfrey, D.I.^{1,2}

¹University of Melbourne, Peter Doherty Institute for Infection and Immunity, Department of Immunology and Microbiology, Melbourne, Australia, ²University of Melbourne, Department of Microbiology and Immunology, Australian Research Council Centre of Excellence in Advanced Molecular Imaging, Melbourne, Australia, ³University of Melbourne, Bio21 Molecular Science and Biotechnology Institute, Department of Biochemistry and Molecular Biology, Melbourne, Australia, ⁴Walter & Eliza Hall Institute of Medical Research, Melbourne, Australia, ⁵University of Melbourne, Department of Medical Biology, Melbourne, Australia, ⁶Monash University, Department of Biochemistry and Molecular Biology, School of Biomedical Sciences, Melbourne, Australia, ⁷Monash University, Department of Biochemistry and Molecular Biology, Australian Research Council Centre of Excellence in Advanced Molecular Imaging, Melbourne, Australia, ⁸Cardiff University, Institute of Infection and Immunity, School of Medicine, Cardiff, United Kingdom

Mucosal-associated invariant T (MAIT) cells are a subset of T-cells restricted by the MHC-related protein 1 (MR1). While the full-length transcript of MR1, (MR1A), is conserved across all mammalian species, MR1 is also alternatively spliced into species-specific isoform variants. In humans, three isoforms; MR1A, MR1B/D and MR1C are ubiquitously transcribed. MR1A, the most well characterised isoform, encodes for a heavy chain with three extracellular domains (α 1- α 3), a transmembrane domain and a cytoplasmic domain. In contrast, MR1B/D encodes for a truncated membrane bound protein lacking the α 3-domain, and MR1C for a soluble MR1B/D variant. It has recently been shown that MR1A has the capacity to present riboflavin (vitamin B2) and folate (vitamin B9) derivatives as antigens. In contrast, less is known about the function of the other isoforms. Like MR1A, MR1B/D has been suggested to express on the cell surface and activate MAIT cells. Thus, MR1B/D may act as an antigen-presenting molecule although presented antigens and antigen presentation pathways may be distinct.

To address the physiological role of alternative splice forms of MR1 we embark on verifying the existence of and quantifying alternative splice forms in various human cell types and tissues at the transcriptional and translational levels. Initial transcriptional analysis has suggested that MR1 isoforms are

differentially expressed in a tissue and cell type specific manner. Furthermore, in contrast to MR1B/D, MR1A was virtually undetectable in blood cells from all donors. More strikingly, it appears MR1B/D is the dominant MR1 transcript in both human thymus and blood.

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Role of TCR γ enhancer 'Ey4' for the development and function of $\gamma\delta$ T cells

Tani-ichi, S., Ikuta, K.

Institute for Virus Research, Kyoto University, Kyoto, Japan

Development of lymphocytes requires functional receptor gene rearrangements which generate the diversity of lymphocyte repertoire. Whereas the enhancers in the TCR α , β , and δ loci are critical for their V(D)J recombination, a previous study showed that simultaneous deletion of two TCR γ enhancers, Ey1 and HsA, only marginally blocks some V-J rearrangements in the TCR γ locus. We previously reported that the transcription factors Stat5 and Runx bind to not only Ey1 and HsA but also Ey4 and increase its activity *in vitro*. However, the function of Ey4 *in vivo* remains unknown. In this study, we generated mice lacking Ey4 (Ey4^{-/-}), the most 3' located TCR γ enhancer. In Ey4^{-/-} thymus, V γ 1.1⁺ $\gamma\delta$ T cells were almost completely disappeared, and V γ 1.2⁺ and V γ 5⁺ $\gamma\delta$ T cells were also reduced. Analysis of Ey4^{-/-} thymus revealed that Ey4 is essential for V γ 1.1-J γ 4 rearrangement and, to a lesser extent, for V γ 1.2-J γ 2, but not for other V γ -J γ rearrangements. Ey4 was also required for transcription of entire TCR γ genes in the thymus and specific V γ -J γ transcription in peripheral tissues. In addition, effector function of these $\gamma\delta$ T cells in peripheral tissue was changed in Ey4^{-/-} mice. This study suggests that Ey4 has a non-redundant role in the development and function of $\gamma\delta$ T cell subsets.

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Universal immunity against influenza: human $\gamma\delta$ T cells

Sant, S.¹, Crowe, J.², Thomas, P.³, Lappas, M.⁴, Loh, L.¹, Kedzierska, K.¹

¹Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Melbourne, Australia, ²Deeplene Surgery, Melbourne, Australia, ³St Jude Children's Research Hospital, Memphis, Department of Immunology, Memphis, United States, ⁴The University of Melbourne, Department of Obstetrics and Gynaecology, Melbourne, Australia

$\gamma\delta$ T cells link innate and adaptive immunity, exhibiting myriad of effector functions, thus implicating they might provide universal immunity to influenza across throughout life. Studies with *in vitro* expanded human $\gamma\delta$ 2 T cells have shown their protective role against influenza infection, however the potential role of peripheral blood $\gamma\delta$ T cells directly *ex vivo* and their TCR dynamics are understudied. To investigate this, we utilised a single-cell multiplex-nested-RT-PCR to study the TCR γ/δ repertoire in healthy individuals across three major age groups (neonates, adults and elderly donors). Neonatal $\gamma\delta$ T cells displayed diverse TCR γ/δ repertoires, while healthy adults showed an enrichment of $\gamma\delta$ 2 TCR pair with diverse CDR3 region. Furthermore, the elderly donors displayed different

dynamics with clonal expansion of CDR3 regions. To investigate the role of $\gamma\delta$ T cells on the early onset of influenza virus infection, we established an *in vitro* co-culture model of virus infection, in which peripheral blood mononuclear cells were co-cultured with influenza-infected (H1N1) human lung epithelial cells. Following infection, IFN- γ producing cells were isolated via IFN- γ secretion assay for paired CDR3 γ/δ analysis. Healthy adult PBMCs showed enrichment of $\gamma9\delta2$ subset T cells. Preliminary results show that lack of monocytes in A549-PBMC culture abrogated IFN- γ by $\gamma\delta$ T cells. Our study provides insights into the composition and diversity of TCR γ/δ repertoires responding to human influenza viruses. Future investigations are required to understand the level of $\gamma\delta$ T cell cross-reactivity across different influenza viruses and accessory function of monocytes in amplifying $\gamma\delta$ T immune responses.

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Triple costimulation via CD80, 4-1BB and CD83 ligand elicits the long-term growth of V γ 9V δ 2 T-cells in low levels of IL-2

Cho, H.-W.¹, Kim, S.-Y.², Sohn, D.-H.¹, Park, M.-Y.², Sohn, H.-J.², Cho, H.-I.^{2,3}

¹The Catholic University of Korea, Department of Microbiology, Seoul, Korea, Republic of, ²The Catholic University of Korea, Hematopoietic Stem Cell Bank, Seoul, Korea, Republic of, ³The Catholic University of Korea, Cancer Research Institute, Seoul, Korea, Republic of

Human $\gamma\delta$ T-cells play important roles in the regulation of infection and cancer. To understand the roles of costimulatory signals in activation and expansion *ex vivo*, V γ 9V δ 2 T-cells were grown with artificial antigen presenting cells that express CD83, 4-1BBL, and/or CD32, which allowed a loading of α CD3 and α CD28 antibodies. The costimulatory signals through CD80, 4-1BB, CD83L in low levels of IL-2 triggered an explosive *ex vivo* proliferation of V γ 9V δ 2 T-cells capable of secreting high levels of IL-2, IFN- γ , and TNF- α . Moreover, the triple costimulatory signals cause augmented cell viabilities for long-term growth of V γ 9V δ 2 T-cells, resulting in phenotypic changes to CD27⁺CD45RA⁺ effector memory-like cells. Notably, we observed that CD83L signaling is crucial to promote *ex vivo* expansion, survival, and cytolytic effector functions of V γ 9V δ 2 T-cells. In contrast, 4-1BB signaling is moderately important in up-regulating surface molecules on V γ 9V δ 2 T-cells. Consequently, $\gamma\delta$ T-cells stimulated in the presence of triple costimulatory signals have diverse cytolytic effector molecules including perforin, granzyme A, granzyme B, and Fas ligand eliciting potent cytolytic activities against tumor cells. Overall, our results provide insights the roles of costimulatory signals in manufacturing long-lived and fully functional V γ 9V δ 2 T-cells, which could be useful against cancers.

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Mucosal associated invariant T cells are deficient in human airways diseases

Hinks, T.^{1,2,3}, Wallington, J.², Williams, A.^{2,3}, Djukanovic, R.^{2,3}, Staples, K.^{2,3}, Wilkinson, T.²

¹University of Melbourne, Microbiology and Immunology, Melbourne, Australia, ²University of Southampton, Clinical and

Experimental Sciences, Faculty of Medicine, Southampton, United Kingdom, ³University of Hospital Southampton, NIHR Respiratory Biomedical Research Unit, Southampton, United Kingdom

Mucosal associated invariant (MAIT) cells have not been studied in the human airways. Infection with non-typeable haemophilus influenzae (NTHi) is linked to recurrent exacerbations and disease progression in COPD.

We hypothesised airway MAIT-cells may be dysregulated in asthma and COPD and contribute to susceptibility to infection.

Methods: Using bronchoscopy we analysed airway MAIT-cell frequencies by flow-cytometry in 93 adults with asthma, COPD and health. Subgroups underwent trials of inhaled and oral corticosteroids. *In vitro* monocyte derived macrophages (MDM) were infected with NTHi.

Results: CD3+TCR-V α 7.2+CD161+ MAIT cells comprise up to 10% of airway T cells. There was a striking deficiency of MAIT-cells in asthma in blood (P=.005), sputum (P=.002) and biopsies (P=0.02). This deficiency was related to disease severity and chronic treatment with inhaled corticosteroids. Blood MAIT cell frequencies were markedly reduced by 7 days of treatment with oral prednisolone (P=.03). There is significant seasonal variation in MAIT-cell frequencies (R²=0.16, P< 0.0001) which is specific to MAIT cells. 28% of the variability in MAIT cell frequencies was attributable to variation in serum vitamin-D3 concentrations. MAIT cells are also deficient in blood and biopsies (P< .05) in steroid-treated COPD.

In vitro, lung macrophages express MR1. Infection with live NTHi induced expression of MR1 on MDM and MAIT-cell IFN- γ expression. Corticosteroids reduce bacterially-induced MR1 surface expression and IFN- γ production.

Conclusions: Airway MAIT-cells are numerically and functionally deficient in severe asthma and COPD, likely due to corticosteroids. This may contribute to susceptibility to bacterial infection, and to changes in the airway microbiome.

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Innate memory formation of dermal IL-17-producing $\gamma\delta$ T cells in a mouse model of psoriasis

Hartwig, T.¹, Pantelyushin, S.², Croxford, A.¹, Kulig, P.¹, Becher, B.¹

¹University of Zurich, Zurich, Switzerland, ²University of Geneva, Geneva, Switzerland

Subsets of dermal $\gamma\delta$ T cells rapidly produce pro-inflammatory cytokines after challenge with Aldara, an immunostimulatory substance inducing psoriasis-like lesions in murine skin. The ability of 'experienced' innate-like $\gamma\delta$ T cells to form a long-lasting immunological memory is an emerging concept. Using fate mapping studies, we observed long term persistence of V γ 4⁺V δ 4⁺IL-23R⁺IL-7R⁺ T cells after Aldara-induced inflammation. These cells exhibit enhanced cytokine production compared to unchallenged controls, and elicit an exacerbated skin inflammation upon re-challenge. We also describe that longevity of V γ 4⁺V δ 4⁺ T cells in the skin is dependent on IL-7 signaling. In addition to identifying a unique feature of $\gamma\delta$ T cells during inflammation, our results have direct relevance to the human disease as this quasi-innate memory provides a mechanistic insight into relapses and chronification of psoriasis.

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Herpes simplex virus (HSV) attracts resident $\gamma\delta$ T cells to the epidermal and dermal junction of human neonatal skin

Yu, U.^{1,2}, Fernandez, M.A.^{1,2}, Kim, M.³, Harman, A.N.³, Holland, A.J.^{1,2,4}, Li, Z.⁵, Maitz, P.⁵, Cunningham, A.L.³, Alexander, S.I.^{1,2}, Jones, C.A.^{1,2}

¹The Children's Hospital at Westmead, NSW, Australia, ²The University of Sydney, Discipline of Paediatrics and Child Health / Marie Bashir Institute for Infectious Diseases and Biosecurity, NSW, Australia, ³Westmead Institute for Medical Research, NSW, Australia, ⁴Westmead Private Hospital, NSW, Australia, ⁵Concord Repatriation General Hospital, NSW, Australia

$\gamma\delta$ T cells are a small population of innate effectors predominantly located in the skin and mucosa, the main sites of HSV entry. Our aim here was to determine the effects of HSV on human neonatal skin $\gamma\delta$ T cells. We observed that neonatal skin $\gamma\delta$ T cells accumulated to the site of HSV infection 6 hours post infection, peaking at 24 hours post infection, compared to mock controls. Localizations of $\gamma\delta$ T cells changed from the epidermal-dermal junction to the deep dermis from 6 hours to 48 hours after HSV infection, associated with increased expressions of inflammatory chemokines CXCL8, CXCL9, CCL2, and CXCL10 in infected neonatal skin. We observed that HSV infected neonatal skin explants showed clusters of cells formed by inflammatory cells including $\gamma\delta$ T cells, Langerhans cells (LCs), neutrophils, and IL-17A-expressing non- $\gamma\delta$ T cells. Our results suggest that $\gamma\delta$ T cells contribute to the cellular response in neonatal skin after HSV infection but their roles is to be determined.

Neuroimmunology

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STING modulates neuro-inflammation in acute neural injury

Abdullah, A.D.I., Zhang, M., Taylor, J.M., Crack, P.J.
University of Melbourne, Pharmacology and Therapeutics,
Melbourne, Australia

Traumatic brain injury (TBI) represents a major cause of disability and death worldwide with sustained neuro-inflammation a key driver of cellular damage. STING-induced type-1 interferon (IFN) signaling is known to modulate the innate immune response in the periphery, however its role in the CNS remains unclear. We previously identified the type-1 IFN pathway as a key mediator of neuro-inflammation and neuronal cell death in TBI (Karve *et al.*, 2016). This study investigated the role of STING in modulating the type-1 IFN mediated neuroinflammatory response following TBI.

WT and STING^{-/-} mice (male, n=6) were subjected to controlled cortical impact (CCI) injury and brains removed 2h or 24h for QPCR, western and immunohistochemical analysis. QPCR identified increased STING expression (4.45 ± 0.93 fold) in WT mice at 24h that was confirmed by western blot and immunohistochemistry. Elevated TNF- α , IL-6, i-NOS and IFN- β levels were also detected in the WT mice. Significantly, pro-inflammatory gene expression was suppressed in the STING^{-/-} mice with a smaller infarct volume identified at 24h (WT; 4.16 ± 0.27mm³, STING^{-/-} ; 3.20 ± 0.17 mm³; p < 0.05) as assessed by

triphenyl tetrazolium chloride (TTC) staining. Supporting a role for STING in human TBI, a significant upregulation in STING expression (2.25 ± 0.50 fold; p < 0.0001) was detected in late trauma human brain samples as compared to the control group. These studies have identified STING as a novel mediator of neuroinflammation in TBI and therefore a potential new therapeutic target.

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Transgenic expression of H-2Db class I molecules influences the development of brain atrophy during picornavirus infection

Huseby Kelcher, A.¹, Atanga, P.², Gamez, J.², Cumba-Garcia, L.¹, Willenbring, R.³, LaFrance, S.¹, Macura, S.⁴, Johnson, A.J.¹

¹Mayo Clinic, Immunology, Rochester, United States, ²Mayo Clinic, Neurology, Rochester, United States, ³Mayo Clinic, Virology, Rochester, United States, ⁴Mayo Clinic, NMR Core Facility, Rochester, United States

Brain atrophy is a common feature of numerous neurological diseases in which the role of neuroinflammation remains poorly defined. We have previously demonstrated the development of brain atrophy in the Theiler's murine encephalomyelitis virus (TMEV) model of multiple sclerosis. However, cellular and molecular mechanisms of brain atrophy remain poorly understood. We therefore evaluated the contribution of major histocompatibility (MHC) class I molecules in atrophy development during TMEV infection. To accomplish this, we created a novel transgenic FVB/NJ mouse by introducing the H-2Db (Db) class I molecule. Expression of Db class I molecule confers resistance to persistent TMEV infection and demyelination in the normally susceptible FVB/NJ strain (H2-Dq class I haplotype). Next, we compared the development of brain atrophy, assessed by volumetric analysis of T2-weighted MRIs, in FVB/Db to FVB/NJ wild-type mice following viral infection. Significant brain atrophy was observed in the FVB/Db mice over a four-month period. FVB/Db mice also developed a significant increase in the overall number of CNS infiltrating CD8 T cells and a strong CD8 T cell response towards an immunodominant Db TMEV epitope, VP2121-130. Several CD8 T cells were also observed in close proximity to virus infected neurons. We therefore propose a hypothesis that class I restricted CD8 T cell responses promote the development of brain atrophy. This model provides a unique opportunity to analyze the contribution of immune cells to neuronal loss and brain atrophy in a system where persistent virus infection and demyelination are not confounding variables.

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Characterising B cells in multiple sclerosis

Rathbone, E.¹, Durant, L.¹, Douglas, M.^{1,2,3}, Curnow, S.J.¹

¹University of Birmingham, Institute of Inflammation and Ageing, Birmingham, United Kingdom, ²Dudley Group NHS Foundation Trust, Department of Neurology, Dudley, United Kingdom, ³Aston University, School of Life and Health Sciences, Birmingham, United Kingdom

Multiple Sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS). In addition to the presence of intrathecal immunoglobulin, clonally expanded, hypermutated B cells are found in many compartments of the MS brain including the cerebrospinal fluid (CSF). The presence of ectopic meningeal B cell follicles in late disease suggests a sustained immunopathological B cell response, however the relative contributions of recently recruited peripheral B cells versus long-lived CNS-expanded B cells remains unclear.

We investigated the phenotype of B cells and antibody-secreting cells in the blood and CSF of MS patients using flow cytometry, recruiting patients with a clinically isolated syndrome (the first clinical event suggestive of MS), relapsing-remitting MS, MS patients ceasing chronic natalizumab (anti- $\alpha 4$ integrin monoclonal antibody) treatment and control patients with other neurological diseases.

We demonstrated that both B cells and antibody-secreting cells display a more activated phenotype in the CSF versus those in the blood and are more prevalent in MS patients than controls. Antibody-secreting cells in the CSF almost exclusively expressed IgG, compared to the predominant IgA expression seen in the blood, whereas B cells displayed similar immunoglobulin expression profiles in both compartments. Furthermore, antibody-secreting cells in the CSF of MS patients demonstrated a higher degree of kappa light chain restriction compared to B cells.

This suggests that in the CNS of MS patients, B cells arise predominantly from non-specific recruitment from the periphery, whereas antibody-secreting cells are involved in a persistent antigen-driven immune response and are likely involved in disease maintenance.

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Effects of clioquinol on the aggregation of beta-amyloid peptides in the presence and absence of metal ions and astrocyte-mediated inflammation

Tahmasebinia, F., Emadi, S.

Institute for Advanced Studies in Basic Sciences, Biochemistry, Zanjan, Iran, Islamic Republic of

Amyloid β (A β) fibrils, amorphous aggregates and oligomers are found in the brain of patients with Alzheimer's disease (AD). Amyloid plaques in the brains are mainly composed of Amyloid β -(1-40) and β -(1-42). These plaques are surrounded by activated astrocytes and microglia which participate in the inflammatory process in the brain. Metal imbalance is also the leading cause for AD, owing to the fact that A β aggregation takes place in the synaptic cleft where A β , Cu(II) and Fe(III) can be found together in abnormally high concentration. Recent studies show that metal dyshomeostasis is involved in aggregation and inflammation. In addition, at the early stage of AD, small diffusible oligomers (or protofibrils) activate microglia leads to inflammation, particularly early inflammatory responses. On the other hand, fibrillar A β showed less increase of pro-inflammatory molecules and sustain the chronic inflammation associated with AD. In this work, first, we examined the formation of all types of A β 40 and A β 42 aggregates (oligomers, fibrils, and amorphous aggregates) in the presence of the added metal ions, Fe(III) and

Cu(II), by using fluorescence spectroscopy and atomic force microscopy (AFM). Second, we studied the ability of Clioquinol, to bind these metal ions in order to prevent A β accumulation. We found that A β 42 may be more oligomerogenic than A β 40 and may have higher effects in inflammation at early stage of AD and Cu(II) induces oligomeric formation. Fe(III) were found to be involved in fibril formation. So it may be more involved in sustaining the chronic inflammation associated with the AD.

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Protective role of interferon-gamma during the chronic phase of experimental autoimmune encephalomyelitis

Arellano, G., Tichauer, J.E., Acuña, E., Castillo, C., Naves, R.

University of Chile, Faculty of Medicine, Program of immunology, Biomedical Sciences Institute, Santiago, Chile

Several studies have described opposing effects of Interferon (IFN)- γ in the pathogenesis of multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE). We have shown that IFN- γ has a stage-specific role in EAE: pathogenic during the inductive phase but protective in the effector acute phase. In this study, we analyzed the protective effects of IFN- γ in the chronic phase of EAE. Clinical symptoms of EAE were significantly ameliorated in mice treated with IFN- γ compared with PBS-treated control mice. Histological analysis revealed that EAE suppression was associated with less CNS inflammation and demyelination. Furthermore, IFN- γ -treated mice showed a lower frequency of CD4⁺ T cells but a significantly higher frequency of regulatory T cells infiltrating the CNS, compared to control mice. Spinal cords and CNS infiltrating mononuclear cells obtained from mice treated with IFN- γ produced higher IL-10 than those from PBS-treated mice. Overall, these results indicate that IFN- γ plays a protective role in the chronic phase of EAE suppressing CNS inflammation by inhibiting CD4⁺ T cell infiltration and inducing regulatory T cell activity.

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Differentially expressed genes in iron-induced prion protein conversion

Kim, M.-S.¹, Choi, B.-R.², Kim, E.-H.¹, Woo, H.-J.¹

¹Seoul National University, Immunology, College of Vet Medicine, Seoul, Korea, Republic of, ²Johns Hopkins University, Neuroscience, Baltimore, United States

The conversion of the cellular prion protein (PrP^C) to protease-resistant isoform is the key event in prion diseases. Increase of iron in prion disease has been observed with the prion protein-ferritin complex. The accumulation and conversion of recombinant PrP (rPrP) is specifically derived by Fe(III) but not by Fe(II). Fe(III)-mediated PK-resistant PrP (PrP^{res}) conversion is a more complex cellular environment than a direct contact between rPrP and Fe(III). We identified the differentially expressed genes that correlate with the prion degeneration depending on Fe(II) and Fe(III) using Affymetrix microarrays. In

Fe(III)-treated environment, we detected 97 genes that were differentially expressed (≥ 1.5 -fold change in expression). The 85 were upregulated and 12 were downregulated as compared with negative controls. However, Fe(II)-treated environment produced moderately altered gene expression level and did not induce a profound alteration of gene expression profile. Moreover, Gene Set Enrichment Analysis indicated that the differentially regulated genes were highly associated with cell growth and maintenance, immune response and cell-cell interaction system. These findings show that Fe(III) might specifically influence the expression of genes involved in many cellular processes. The identification of genes with altered expression patterns in neural cells may provide an insight to the PrP conversion mechanisms in prion disease development and progressing.

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Immunological markers as risk factors in clinically isolated syndrome (MS)

Posova, H.¹, Horakova, D.², Hrušková, Z.¹, Čapek, V.¹, Havrdová, E.¹
¹1st Medical Faculty, Charles University in Prague, Institute of Immunology and Microbiology, Prague, Czech Republic, ²1st Medical Faculty, Charles University in Prague, Department of Neurology and Centre of Clinical Neuroscience, Prague, Czech Republic

Clinically isolated syndrome (CIS) represents first neurological symptoms suggestive of demyelinating lesion in CNS according to MRI with the risk of multiple sclerosis (MS) development. So far there are no sufficient immunological markers predicting disease activity and potential disability or efficiency of registered CIS treatment - interferon beta. The aim of our study was to evaluate immunological predictors of CIS conversion into MS.

Method: 212 patients with CIS treated with interferon beta have been followed for 4 years. Lymphocyte subsets (CD3+, CD4+, CD8+, CD19+, CD3+CD16+56+ and chemokine receptors CXCR3+ and CCR5) were analyzed by flow cytometry at the time of first relaps, after 1-2 month before therapy and after 6, 12, 24, 36 and 48 months. 88 patients converted to MS and switched to another therapy. Multivariate Cox proportional hazard regression were used to compare this group with patients who did not develop clinically defined MS.

Results: Higher levels of B lymphocytes predicted relapse-free status. On the other hand a decrease of NK cells was associated with an increased risk of relapse activity. Decreases of the naïve subset of cells (CD45RA+ in CD4+) after 12, 24, and 36 months of follow-up were associated with increased risk of a new relapse activity and increase expression of chemokine receptors on CD4+ T lymphocytes increase signals a clinical deterioration.

Conclusion: Our data point to the importance of regular examination of B lymphocytes, NK cells and naïve T helper cells; a decrease of these subpopulations increases the risk of relapse or clinical worsening.

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Distinct cytokine profiles differentially induce white and grey matter inflammation in the experimental autoimmune encephalomyelitis model of multiple sclerosis

Orian, J.M., Dang, P.T., D'Souza, C.

La Trobe Institute for Molecular Science, Biochemistry and Genetics, Melbourne, Australia

Progress in the elucidation of mechanisms underlying neurodegeneration in the central nervous system (CNS) autoimmune disorder multiple sclerosis (MS) has demonstrated distinct pathological processes in grey matter (GM) and white matter (WM). To identify WM and GM-specific mechanisms multiple approaches were applied to WM and GM separately, in the experimental autoimmune encephalomyelitis MS model and resident CNS cell responses and axonal/neuronal survival, in relation to lesion development evaluated in each compartment, over the disease course. These include

- (a) laser microdissection of defined WM and GM regions, followed by expression profiling of pro-inflammatory and apoptosis/anti-apoptosis molecules,
- (b) quantitative immunochemistry and
- (c) in-situ hybridization.

Results showed that in the spinal cord, distinct pro-inflammatory profiles evolve in WM and GM from pre-clinical times, where WM disease is driven by IFN-gamma, but GM disease by TNF-alpha. Secondly, upregulation of the extrinsic apoptosis pathway is observed in neuronal cell bodies; this is in opposition to evidence of mitochondrial dysfunction as the cause of axonal degeneration. However, concurrent upregulation of anti-apoptosis molecules is observed in neuronal cell bodies, without significant neuronal loss. We conclude

- (1) that the microenvironment generated within WM or GM in response to circulating inflammatory mediators is determined by resident CNS cells,
- (2) each microenvironment drives a unique apoptosis mechanism and
- (3) neurons exhibit innate survival mechanisms in early disease. These data are in agreement with evidence of different cytokine/chemokine profiles along the neuraxis during neuroinflammation, but also highlight differences between WM and GM responses which may underlie the divergence in pathophysiological pathways.

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To investigate the immunological role of chemokine decoy receptor ACKR2 in experimental autoimmune encephalomyelitis

Chen, J.W.¹, Lin, G.-J.², Chen, S.-J.^{3,4,5}

¹National Defense Medical Center, Graduate Institute of Life Science, Taipei, Taiwan, Republic of China, ²National Defense Medical Center, Graduate Institute of Biology and Anatomy, Taipei, Taiwan, Republic of China, ³National Defense Medical Center, Graduate Institute of Medical Sciences, Taipei, Taiwan, Republic of China, ⁴National Defense Medical Center, Department of Pediatrics, Tri-Service General Hospital, Taipei, Taiwan, Republic of China, ⁵National Defense Medical Center, Department of Microbiology and Immunology, Taipei, Taiwan, Republic of China

Experimental autoimmune encephalomyelitis (EAE) is an animal model of multiple sclerosis, which is inflammatory demyelinating disease of central nervous system (CNS). Astrocytes and microglial cells were stimulated by damaging stress, they secrete inflammatory chemokines, like the CCL2 and CCL5. These inflammatory chemokines stimulate leukocytes activation, and the activating leukocytes transmigrate to brain parenchyma. Atypical chemokine receptor 2 (ACKR2) is a chemokine decoy and scavenger receptor, which has been proven express on lymphatic endothelial cells and some immune cells. ACKR2 through binding inflammatory chemokine, internalization and degradation regulate chemokine-driven inflammatory response. Lack ACKR2 has been proven to increase the cutaneous inflammation, colitis and colon cancer. In addition, high expression level of ACKR2 had also been shown to slow down skin tumor and type 1 diabetes. Despite the role of ACKR2 in the peripheral system has clearly understood, the role of the CNS is not clear. In this study, we investigate the immunological functions of ACKR2 in EAE. The expression level of ACKR2 on immune cells was detected through flow cytometry. In the PBMC from patients, there was high expression on the CD4+ T cell. In addition, splenic B220+ B cells, from the EAE, expressed higher ACKR2 than wild type on the early phase of EAE. The mRNA expression of ACKR2 will be measured by real-time PCR. Furthermore, the protein expression of ACKR2 will be detected by western blot and immunohistochemistry.

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Adrenoreceptor signalling influences T cell responses during viral infection

Loi, J.K.^{1,2}, Devi, S.^{1,2}, Sloan, E.³, Mueller, S.N.^{1,2}

¹The University of Melbourne and The Peter Doherty Institute for Infection and Immunity, Department of Microbiology and Immunology, Parkville, Australia, ²ARC Centre of Excellence in Advanced Molecular Imaging, The University of Melbourne, Melbourne, Australia, ³Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Australia

T cells express adrenergic receptors (AR) that enable them to respond to neurotransmitters including those of the sympathetic nervous system (SNS), noradrenaline (NA) and adrenaline. These neurotransmitters bind to α and β AR inducing downstream signalling and modulating cell functions, although whether this is stimulatory or inhibitory in T cells during the context of immune responses to virus infection remains unclear. We confirmed that naive CD4 and CD8 T cells expressed predominantly b2AR that induced cyclic AMP production upon stimulation *in vitro*. We examined the role of adrenoceptor signals via treatment of mice with the bAR agonist isoprenaline early during acute and chronic lymphocytic choriomeningitis virus (LCMV) infection. While the magnitude of antigen-specific CD4 and CD8 T cell responses to LCMV clone 13 infection was unchanged by 5 days of isoprenaline treatment, viral clearance in the spleen and blood was impaired and mice displayed increased morbidity and mortality. Using 2-photon microscopy we found that T cell migration was altered by indirect bAR signals, suggesting that adrenoceptor signals may influence T cell responses to infection by influencing behaviour in tissues.

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Tolerogenic activity of IFN- γ on macrophages/microglia in experimental autoimmune encephalomyelitis

Tichauer, J., Arellano, G., Acuña, E., Naves, R.

Universidad de Chile, Program of Immunology, Biomedical Sciences Institute, School of Medicine, Santiago, Chile

During experimental autoimmune encephalomyelitis (EAE) development, leukocytes are recruited into the CNS mediating a destructive inflammatory response. Resident microglia and infiltrating macrophages present myelin peptides to reactive T cells inducing activation or tolerance. This process is regulated by inflammatory cytokines, such as IFN- γ . Our previous results have shown that IFN- γ has a stage-specific dual activity in EAE: pathogenic during the inductive phase but protective in the effector phase. We hypothesized that as part of its protective role, IFN- γ might exert a tolerogenic activity on innate immune cells in the CNS. Here, we evaluated the effect of IFN- γ on the activation of macrophages/microglia during the chronic phase of EAE. Flow cytometry analysis of CNS cells revealed that IFN- γ -treated EAE mice had a significantly lower frequency of CD11b⁺ cells than PBS-treated control mice. However, CD11b⁺ cells from IFN- γ -treated EAE mice showed significantly higher expression of IL-10 than those from controls. Remarkably, IFN- γ -treated EAE mice had a significantly lower frequency of CD11b⁺CD45^{hi} (activated) cells but a significantly higher frequency of CD11b⁺CD45^{low} (resting) cells associated with higher expression of IL-10, compared with controls. Moreover, CD11b⁺CD45^{hi} cells obtained from CNS of PBS-treated EAE mice were induced to downregulate CD45 expression and to increase IL-10 expression upon *ex vivo* IFN- γ treatment. In addition, macrophages/microglia from IFN- γ -treated EAE mice exhibited lower expression of CD80 costimulatory molecule without affecting MHC-II and CD86 expression, compared to control mice. Overall, our results indicate that IFN- γ induces a tolerogenic phenotype in macrophages/microglia during EAE effector phase.

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Functional effects of antibodies specific for myelin proteolipid protein in multiple sclerosis

Greer, J.M.¹, Muller, D.M.¹, Beasley, S.J.¹, Trifilieff, E.², Pender, M.P.³

¹The University of Queensland, UQ Centre for Clinical Research, Brisbane, Australia, ²Université de Strasbourg, Institut de Physique Biologique, Strasbourg, France, ³The University of Queensland, School of Medicine, Brisbane, Australia

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS). Autoimmune T cells are critical for the pathogenesis of MS, but it is also likely that autoantibodies play a role, although this remains to be proven. We have found that a high proportion of MS patients have elevated levels and titres (compared to healthy controls and patients with other neurological diseases (OND)) of autoantibodies specific for the second extracellular loop of myelin proteolipid protein (PLP), the most abundant CNS

myelin protein. The aim of the current study was to investigate these antibodies further, in order to determine if they could play a functional role in MS. Most PLP-specific antibodies from MS patients showed evidence of isotype-switching to IgG and thus of an ongoing active response; in contrast, anti-PLP antibodies detected in healthy controls and OND patients were almost all IgM. Serum from MS patients opsonized human myelin for uptake by macrophages, whereas antibodies from healthy individuals or OND patients did not; and for 65% of MS sera, pre-adsorption of sera with PLP peptides could remove the opsonizing activity. Anti-PLP antibodies could also label and kill oligodendrocytes in culture. In an animal model of MS, anti-PLP antibody was able to shift the location of lesions within the CNS in a complement-dependent manner. These findings show that antibodies directed against extracellular epitopes of PLP have the potential to induce pathogenic effects in patients with MS, and suggest that specifically removing anti-PLP antibodies might be clinically indicated.

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Trehalose administration inhibits inflammatory responses in the brain of Lewy body disease model mice

Tanji, K., Maruyama, A., Matsumiya, T., Miki, Y., Mimura, J., Mori, F., Itoh, K., Wakabayashi, K.

Hirosaki University, School of Medicine, Hirosaki, Japan

Lewy body disease (LBD) such as Parkinson's disease and dementia with Lewy bodies is a neurodegenerative disorder accompanied by abnormal protein aggregation and inflammatory responses. Actually increased levels of pro-inflammatory cytokines such as TNF, IL-1 β , and IL-6 are observed in postmortem brain and cerebral spinal fluid from patients with LBD. Trehalose is a natural disaccharide composed of two glucose units, and found in a wide range of eukaryotic microorganisms, plants, fungi and insects. Interestingly, trehalose serves as a scaffold molecule that inhibits protein aggregation by direct interaction. Giving this property of trehalose, it can modulate inflammatory responses in the brain. We therefore investigated the effect of trehalose on abnormal aggregation and inflammatory responses in the brains of LBD model mice. Regarding abnormal aggregation of α -synuclein, immunohistochemical studies showed that any differences were not observed between mice with orally trehalose- and maltose-intake. Whereas, GFAP and Iba1 immunoreactivity was weaker in trehalose mice compared with maltose one. Further analyses using real time PCR revealed that the levels of several cytokines were inhibited in the brain with trehalose compared with maltose. In addition, trehalose increased the levels of several chaperone molecules, such as HSP90 and SigmaR1, in the brain of LBD model mice. These findings suggest that trehalose together with chaperone molecules suppresses inflammatory responses in the brain of LBD model mice. Oral administration of trehalose could be used as a safe therapeutic approach for sustained inflammatory responses.

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Role of T cells accumulated in the brain at the late onset of stroke

Ito, M., Shichita, T., Yoshimura, A.

Keio University School of Medicine, Microbiology and Immunology, Tokyo, Japan

Stroke causes ischemic brain injury, which is a leading cause of neurological disability and death worldwide. Post-ischemic inflammation is a hallmark of ischemic stroke pathology. Although intensive efforts have been devoted to understanding innate immunity after stroke, pathogenic significance of adaptive immunity and its long-term effects on the post-ischemic brain remain unclear. In this study, we demonstrated the roles of CD4⁺T cells in adoptive immune response to ischemic brain injury using a transient middle cerebral artery occlusion (MCAO) model induced by means of an intraluminal suture. The infiltration of antigen-specific CD4⁺T cells into the infarct region in the brain was drastically increased on day 7 to 21 after stroke onset. These T cells were mainly composed of IFN- γ ⁺ Th1 and Foxp3⁺ Tregs. By using Rag-deficient mice and transfer experiments, we found that T cells played a crucial role for the formation of astrocytic gliosis which recruits inflammatory cells from uninjured brain cells. We also observed that such delayed T cell accumulation in the infarct area ameliorated neurological dysfunction. Moreover, the T cells from ischemic mice had suppressive effects on infarct volume growth by suppressing inflammatory response and by promoting the activation of astrocytes. These results suggest that the delayed accumulation of CD4⁺T cells is important for the neurological recovery after ischemic brain injury and induction of brain-specific Tregs could be a useful therapy for alleviation of neurological symptoms.

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Blimp-1 deficiency exacerbates experimental autoimmune encephalomyelitis in mice by impairing the suppressive function of Treg cells and enhancing the encephalogenicity of Th1 cells

Lin, M.-H.¹, Sytwu, H.-K.^{2,3}

¹Kaohsiung Medical University, College of Medicine, Institute of Medicine, Department of Microbiology and Immunology, Kaohsiung, Taiwan, Republic of China, ²National Defense Medical Center, Department and Graduate Institute of Microbiology and Immunology, Taipei, Taiwan, Republic of China, ³National Defense Medical Center, Graduate Institute of Life Sciences, Taipei, Taiwan, Republic of China

Recently, we demonstrated that B lymphocyte-induced maturation protein 1 (Blimp-1) has a role in regulating the differentiation and effector function of Th1 and Th17 cells. Since these cells play critical roles in the induction and pathogenesis of experimental autoimmune encephalomyelitis (EAE), we further investigate whether the potential role of T cell Blimp-1 in modulating the pathogenesis of MOG₃₅₋₅₅-induced EAE. We currently established T cell-specific Blimp-1 conditional knockout (CKO) non-obese diabetic (NOD) mice to further dissect the roles of Blimp-1 in EAE using loss-of-function mouse model. Our results indicated that EAE severity is dramatically

exacerbated in Blimp-1 CKO mice. The numbers of CNS-infiltrating Th1, Th17, IFN- γ ⁺IL-17A⁺, and IL-21⁺IL-17A⁺ CD4⁺ T cells are remarkably increased in brain and spinal cord of CKO mice. Moreover, the ratio of regulatory T cells (Tregs)/effectors and IL-10 production of Tregs are significantly downregulated in CNS of CKO mice. The frequency and expression of GM-CSF (granulocyte-macrophage colony-stimulating factor) in Th1 cells are massively upregulated in CNS of Blimp-1 CKO mice with EAE induction. In summary, we concluded that Blimp-1 suppresses autoimmune encephalomyelitis, at least, via downregulating the pathogenicity of Th1 and Th17 cells and enhancing the effector function of Treg cells.

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A choroid-forebrain-lymphatic pathway for the maintenance of CNS immune tolerance

Mohammad, M.G.¹, Tsai, V.², Li, H.², Hassanpour, M.^{2,3}, Hart, P.⁴, Breit, S.², Sawchenko, P.⁵, Brown, D.^{2,6}

¹University of Sharjah, Department of Laboratory Sciences, Faculty of Health Sciences, Sharjah, United Arab Emirates, ²St Vincent's Hospital Center for Medical Research, Inflammation and Cytokine Research Group, Sydney, Australia, ³University of New South Wales, St. Vincent's Clinical School, Darlinghurst, Australia, ⁴University of Western Australia, Telethon Institute for Child Health Research, Centre for Child Health Research, Perth, Australia, ⁵Salk Institute for Biological Studies, Laboratory of Neuronal Structure and Function, La Jolla, United States, ⁶Westmead Hospital, Department of Immunology/Immunopathology, Sydney, Australia

The ability of the central nervous system (CNS) to maintain immune privilege is well known, but poorly understood. However, it appears that in multiple sclerosis and its animal model, experimental autoimmune encephalomyelitis (EAE), the cervical lymph nodes (CxLNs) may participate in this process. We therefore wished to establish whether there was immune cellular traffic from the CNS to the CxLNs, as well as its functional significance. Using IHC, dendritic cells (DCs) were more prominent throughout the rostral migratory stream (RMS), a pathway of neural stem cells. Further, DCs migrated from the RMS to the CxLNs, a process interrupted by Fingolimod treatment. This treatment led to the accumulation of DCs along the RMS and to severer actively induced EAE. The same treatment of 2D2 mice, a T-cell receptor transgenic mice, induced spontaneous with accumulation of antigen specific T-cells in the spinal cords of these EAE-resistant mice. In these mice, spinal cord T-cell infiltration was proportional to the ratio of anti-inflammatory T-regulatory cells (Tregs) to pro-inflammatory T-effector cells in their CxLNs. Investigating this phenomenon showed that RMS-Fingolimod treatment compromised CxLN Treg function. Using a delayed type hypersensitivity model, we demonstrate that the same treatment leads to fewer regulatory DCs in CxLNs. Combined; these data suggest that DCs migrate from the CNS to the CxLNs and regulate anti-CNS immunity representing a novel target for the treatment of CNS autoinflammatory disease.

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TREM-2 promotes amyloid plaque formation and modulates microglia oxidative stress adaption in an experimental Alzheimer's disease mouse model

Yang, F.-C., Chou, N.-Y., Lin, Y.-H., Chen, N.-J.

National Yang-Ming University, Institute of Microbiology and Immunology, Taipei, Taiwan, Republic of China

Alzheimer's disease (AD) is a common neurodegenerative disease characterized with abnormal deposition of amyloid A β plaques in brain. A phagocytic receptor TREM-2 expressed on microglia was recently found interacts with oligo A β and plays roles in AD pathogenesis. We examined the role of TREM-2 in AD by intercrossing J20 A β -transgenic mouse model and *Trem-2* deficient (KO) mouse model. Interestingly, less amyloid A β plaques and fewer infiltrating Iba-1 (+) microglia cells were observed in the brain samples of 12 month-old J20/*Trem-2* KO mice in comparison with those from J20 mice. Further investigation on the inflammatory changes upon AD revealed a significant reduction of iNOS activity, accompanied with a drastic decrease of A β nitration in the brain sections of J20/*Trem-2* KO. Accordingly, the iNOS expression, NO induction and the NO-mediated A β nitration were defective in primary TREM-2 deficient microglia cells and TREM-2 knockdown BV2 cells. Moreover, depleting TREM-2 in BV-2 significantly exacerbates the stimuli-induced intracellular oxidative stress consequently leads to enhanced cell death. In conclusion, in an experimental AD mouse model, TREM-2 plays a motive role on enhancing the amyloid plaque formation via regulating A β nitration. TREM-2 may also contribute to the adaption of microglia on overflowed oxidative stress in response to inflammatory stimuli upon AD.

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Behavioural and cellular effects of immunomodulatory compounds in the cuprizone model

Templeton, N.¹, La Flamme, A.², Kivell, B.², Webster, G.³, Connor, B.⁴

¹Victoria University of Wellington, Biology, Wellington, New Zealand, ²Victoria University of Wellington, Wellington, New Zealand, ³Innate Immunotherapeutics, Auckland, New Zealand, ⁴University of Auckland, Auckland, New Zealand

Multiple Sclerosis (MS) is a disorder of the central nervous system that affects approximately 2.5 million people worldwide. Disease pathology is characterised by the formation of demyelinating lesions in the central nervous system (CNS) which lead to cognitive and motor impairments associated with disease. These CNS lesions can be classified as those with immune cell involvement or those without immune cell infiltrate. To investigate the effect of immune-modulating therapies on lesions without immune cell infiltrate, the cuprizone model of non-immune demyelination was used to determine the effect of the immunomodulatory compounds, MIS416 and clozapine, both of which have been shown to reduce disease burden in an immune-driven animal model of MS. We found that treatment with MIS416 and clozapine led to improved performance on behavioural assays over control, with the cellular changes underlying this difference still being characterised. Protective effects provided by either of these compounds could aid in the

development of unique combination therapies to target both the immune component and the cellular components seen at different stages of MS.

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Mast cell-nerve interactions in the colonic mucosal of *Trypanosoma cruzi*-infected individuals with chagasic megacolon

Martins, P.^{1,2}, Martinelli, P.¹, Oliveira, E.³, d'Avila Reis, D.¹

¹Universidade Federal de Minas Gerais, Morphology Department, Belo Horizonte, Brazil, ²University of Melbourne, Anatomy and Neuroscience, Melbourne, Australia, ³Universidade Federal de Goias, Goiania, Brazil

Chagas disease is an endemic infection in Latin America caused by the parasite *Trypanosoma cruzi*. Nowadays, it affects millions of people worldwide including thousands in Australia and New Zealand. Chagasic megacolon is the most frequent complication of the digestive chronic form. It is characterized by a dilation of the colon, due to lesions of enteric nervous system, followed by motility disorders. The mechanism to induce neuronal lesions is still unknown; nevertheless, several evidences support a role for the immune system. Mast cells (MCs), in particular, participate in the bidirectional communication between the immune and nervous systems. Tryptase released by MCs is a pro-inflammatory protease that through linkage to its protease activated receptor 2 (PAR2) on neurons can induce hyperexcitation or neuronal death, which could contribute to denervation of the megacolon. Accordingly, we investigated the relationship between MCs and enteric nerves in infected individuals with and without megacolon and controls, by immunohistochemistry and ultrastructural studies in the colonic mucosal. The number of tryptase-MCs increased in patients with megacolon. The percentage of PAR2-neurons and the area of PGP 9.5 nerve fibers in individuals with megacolon were lower when compared to controls. Ultrastructural analysis showed that in controls, 59% of the MCs were without signals of degranulation, while 93% of MCs were degranulated and 75% of MCs were in close proximity or contact, located within 5 µm of nerve fibers in infected individuals with megacolon. These data provide evidences about the communication between the immune and nervous systems in the chagasic megacolon.

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Neuromyelitis optica spectrum disorder: a patient with a symptomatic third ventricular floor lesion, anti-aquaporin-4 antibodies and remission with methotrexate

Berry, R.^{1,2}, Panegyres, P.K.^{1,2}

¹Joondalup Health Campus, Neurology Service, Perth, Australia, ²Neurodegenerative Disorders Research Pty Ltd, Perth, Australia

Neuromyelitis optica is a rare severe inflammatory demyelinating disease of the central nervous system previously described to affect only the optic nerves and spinal cord. Since the detection of the highly specific autoantibody to Aquaporin-4 (anti-AQP4), lesions are now recognised outside of these regions. We present a patient with severe debilitating

symptoms resulting from a lesion within the diencephalon, which manifested with abnormal circadian rhythms, autonomic dysfunction, behavioural disturbance and unusual visual phenomenon. Centrally driven sick sinus syndrome resulted in permanent cardiac pacemaker insertion. Disease activity has been suppressed with methotrexate. This experience teaches us of the clinical spectrum of Neuromyelitis Optica Spectrum Disorder and provides evidence that methotrexate might be a useful immunomodulating substance and steroid sparing agent for anti-AQP4 related pathology.

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Immunophenotypic studies in transgenic reporter mice reveal that the uveal tract, but not the neural retina, contains populations of resident dendritic cells

Dando, S., McMenamin, P.

Monash University, Department of Anatomy and Developmental Biology, Clayton, Australia

The question of whether dendritic cells (DCs) reside in the normal mouse retina has been hotly debated for many years. Early studies in rodents failed to identify MHC class II⁺ cells in the quiescent retina, suggesting an element of 'immune-privilege'. Nonetheless, the retina is affected by numerous diseases with an inflammatory or autoimmune nature, such as uveoretinitis, suggesting that resident antigen presenting cells (APCs) may interact with autoreactive T cells to initiate disease. We hypothesised that candidate APCs reside within the uveal tract of the eye (choroid, iris and ciliary body), but not the neural retina. Using transgenic reporter mice (CD11c-eYFP *Crb1^{wf/wf}*, CD11c-DTR-GFP and CX3cr1^{GFP/GFP}) we examined the distribution and phenotype of putative DCs within the uveal tract and retina. *In vivo* imaging revealed an extensive network of CX3cr1^{GFP/GFP+} and CD11c-eYFP⁺ cells within the retina, but no CD11c-DTR-GFP⁺ cells were observed. Retinal CX3cr1^{GFP/GFP+} and CD11c-eYFP⁺ cells were identical in both morphology and phenotype to resting Iba-1⁺ microglia (CD45^{int}, F4/80⁺, CD11b⁺, CD115⁺), but did not express APC or DC markers including I-A/I-E, CD11c, CD103, CD8α, CD80, CD86, CD135, DEC205, 33D1 or Zbtb46. In contrast, CX3cr1^{GFP/GFP+} and CD11c-eYFP⁺ cells within the iris and ciliary body were stout in appearance with few, short processes and expressed APC, DC and macrophage markers. These findings demonstrate that the retina, unlike the iris and ciliary body, is devoid of DCs. Therefore, putative APCs in the uveal tract are most likely involved in the initiation of autoimmune responses of the uvea and retina.

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Immune privilege in the central nervous system revisited: are there dendritic cells in the brain?

Dando, S.¹, Chinnery, H.², Ruitenber, M.³, McMenamin, P.¹

¹Monash University, Department of Anatomy and Developmental Biology, Clayton, Australia, ²University of Melbourne, Department of Optometry and Vision Sciences, Parkville, Australia, ³University of Queensland, School of Biomedical Sciences, St Lucia, Australia

The central nervous system (CNS) was considered to be 'immune

privileged' due to a lack of antigen presenting cells (APCs) and the absence of traditional lymphatics. However, these concepts were recently challenged due to the discovery: (i) of putative dendritic cells (DCs) in the brain parenchyma of CD11c-eYFP mice and (ii) that brain interstitial fluid may be partly absorbed by lymphatic vessels in the dura mater. These findings suggest that afferent communication may exist within the CNS, which has implications for our understanding of immune regulation at this site. However, recent data from our laboratory questioned whether the putative 'brain DCs' described within the brain parenchyma of CD11c-eYFP mice do indeed fit the profile of DCs. We microdissected brains from CD11c-eYFP *Crb1^{wt/wt}* mice and determined the phenotype of CD11c-eYFP⁺ cells within the brain parenchyma, meninges and choroid plexus. CD11c-eYFP⁺ cells within the brain parenchyma (CD45^{int}, F4/80⁺, CD11b⁺, I-A/I-E, CD11c, CD115⁺, CD103⁻, CD8α⁻, CD80⁻, CD86⁻, CD135⁻, DEC205⁻, 33D1⁻, Zbtb46⁻) did not display the immunophenotype of DCs or their precursors and were phenotypically indistinguishable from microglia. Thus we challenge the notion that CD11c-eYFP⁺ cells within the brain parenchyma are 'brain DCs'. In contrast, CD11c-eYFP⁺ cells within the pia mater and choroid plexus (non-parenchymal, supportive tissues of the brain) contained rich populations of macrophages and DCs. These data confirm that the brain parenchyma is devoid of DCs and suggest that autoimmune responses may be initiated in the meninges and/or choroid plexus. Studies are currently underway to determine the functional capacity of these cells.

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Infection driven inflammation severely attenuates influenza specific immunity in a chronic model of spinal cord injury

Andreansky, S., Valerie Bracchi-Ricard, Ji Zha, Annalise S. Barnette, Darlah Rodriguez-Lopez, John R. Bethea
University of Miami, Pediatrics (D-820), Miami, United States

Crosstalk between the nervous system and the immune system plays a pivotal role in maintaining homeostasis of the host. This coordination is achieved via the sympathetic nervous system (SNS) and the hypothalamic-pituitary-axis (HPA), which regulates a variety of cytokines, hormones and neurotransmitters. Thus direct injury to the neural pathways innervating the spinal cord contributes to functional deficit in the peripheral immune response and renders patients with spinal cord injury (SCI) more susceptible to viral and bacterial infections and account for 50% mortality. We therefore hypothesized that chronic SCI causes 'immune paralysis' and impairs virus specific host immunity. A low-level (T9) thoracic contusion injury model was utilized as it minimally disrupts the innervations from preganglionic sympathetic neurons to lymphoid organs. Comprehensive analysis of virus specific immunity was performed at various time points post-infection in comparison to uninjured controls. We demonstrate that infection of chronically injured mice with influenza A virus causes severe morbidity and mortality, which was driven by virus induced inflammation at the site of infection. Analysis of adaptive immunity demonstrated severe deficits in virus specific antibody and CD8T cell responses. Furthermore we provide evidence that chronic injury also regulates the formation of immunological memory in a secondary virus infection model.

To our knowledge, this is the first comprehensive analysis of virus specific immunity against a clinically relevant respiratory pathogen in a chronic SCI preclinical model. Our goal is to develop more effective therapies and reduce mortality due to complications from infections in chronic SCI patients.

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Brain mast cells evoke central nervous system inflammation by inducing microglial activation

Zhang, S., Dong, H., Zhang, X., Wang, Y., Zhou, X., Qian, Y.
The First Affiliated Hospital of Nanjing Medical University, Nanjing, China

Brain inflammation has a critical role in the pathophysiology of brain diseases. Microglia, the resident immune cells in the brain, plays an important role in brain inflammation. While brain mast cells are the "first responder" in the injury rather than microglia. Functional aspects of mast cell-microglia interactions remain poorly understood. Our results demonstrated that site-directed injection of the "mast cell degranulator" compound 48/80 (C48/80) in the hypothalamus induced mast cell degranulation, microglial activation and inflammatory factors production, which initiated the acute brain inflammatory response. "Mast cell stabilizer" disodium cromoglycate (cromolyn) inhibited this effect, including decrease of inflammatory cytokines, reduced microglial activation. We also demonstrated that C48/80 had no effect on microglial activation in mast cell-deficient KitW-sh/W-sh mice. In vitro study, we found that the conditioned medium from activated HMC-1 could stimulate microglial activation and subsequent production of pro-inflammatory factors TNF-α and IL-6. The antagonists of H1R, H4R, PAR2 or TollR4 reduced HMC-1-induced pro-inflammatory factors production and MAPK and PI3K/AKT pathway activation. These results implicate that activated mast cells could trigger microglial activation and CNS inflammation, and interactions between mast cells and microglia could constitute a new and unique therapeutic target for CNS immune inflammation-related diseases.

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Attenuation of pro-inflammatory parameters and memory impairment by erythropoietin in experimental pneumococcal meningitis

Barichello, T.¹, Simões, L.R.², Generoso, J.S.², Sangiogo, G.², Danielski, L.G.², Florentino, D.³, Domingui, D.², Comim, C.M.⁴, Petronilho, F.³, Quevedo, J.¹

¹University of Texas Health Science Center at Houston, Houston, United States, ²Universidade do Extremo Sul Catarinense, Criciúma, Brazil, ³Universidade do Sul de Santa Catarina, Tubarão, Brazil, ⁴Universidade do Sul de Santa Catarina, Palhoça, Brazil

Pneumococcal meningitis is characterized by a severe inflammatory reaction in the subarachnoid and ventricular space of the brain, disruption of the blood-brain barrier, hearing loss, and neurologic sequelae in as many as 27% of surviving patients. Several experimental studies have shown that erythropoietin (EPO) and its receptor are expressed in the central nervous system and have neuroprotective properties through the inhibition of apoptosis, as well as anti-inflammatory,

antioxidant, angiogenic, and neurotrophic effects. In the current study, we demonstrated the effect of erythropoietin (EPO) on lipid peroxidation, protein carbonylation, superoxide dismutase (SOD), catalase (CAT), myeloperoxidase (MPO), and behavioral parameters in rats with pneumococcal meningitis. EPO decreased lipid peroxidation and protein carbonylation, and it prevented protein degradation in the hippocampus and frontal cortex. MPO activity was decreased, and both SOD and CAT activity were increased in the first 6 hours after pneumococcal meningitis induction. Novel object recognition memory was impaired in the meningitis group; however, adjuvant treatment with EPO prevented memory impairment during both the short- and long-term retention tests. The meningitis group showed no difference in motor and exploratory activity between training and test sessions in the open-field task, which indicates that habituation memory was impaired; however, adjuvant treatment with EPO prevented habituation memory impairment. Although there are some limitations with respect to the animal model of pneumococcal meningitis, this study suggests that adjuvant treatment with EPO contributed to decreased oxidative stress and prevented cognitive impairment.

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Pain is an inducer for relapse in multiple sclerosis models via the sensory-sympathetic nerve activation

Arima, Y., Higuchi, K., Nishikawa, N., Stofkova, A., Ohki, T., Kamimura, D., Murakami, M.

Hokkaido University, Division of Molecular Neuroimmunology, Institute of Genetic Medicine, Graduate School of Medicine, Sapporo, Japan

Pain is a common symptom in many diseases, but is considered as one of their by-products. Because a large proportion of multiple sclerosis patients experiences remission and relapse during the course of disease progression with the frequent association of pain, we assessed whether pain induces the relapse of experimental autoimmune encephalomyelitis (EAE), which is an animal model of multiple sclerosis. EAE symptoms relapsed in mice that had recovered from the initial episode of EAE after pain induction. The pain-induced excitation of sensory neurons activated the anterior cingulate cortex (ACC) in the brain, followed by the activation of sympathetic neurons that specifically control the ventral vessels of the whole spinal cord. The activation of sympathetic neurons caused the release of norepinephrine around the ventral vessels, which led to the expression of chemokine CX3CL1 from activated monocytes and further accumulation of activated monocytes at the ventral vessels. Because the numbers of activated monocytes increased in L5 cord, pain-mediated EAE relapse was induced from L5 cord. Importantly, the activated monocytes had an ability to present myelin antigens to autoreactive T cells for the induction of IL-17A and IL-6, which are important cytokines for the inflammation amplifier, a chemokine inducing mechanism in endothelial cells. Indeed, their neutralizing antibody treatments inhibited the pain-induced relapse. Thus, we concluded that pain-induced sensory-sympathetic neural activation triggers EAE relapse via the inflammation amplifier activation. These results provide the possibility that suppressing pain-mediated

regional neural signaling may prevent the relapse of CNS diseases, including MS.

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Intrahepatic development of liver-resident NK cells

Tian, Z., Peng, H., Tang, L., Sun, R.

University of Science and Technology of China, Hefei, China

Natural killer (NK) cells are an important component of the innate immune system and play critical roles in host defense against infections and tumors. Accumulating studies have shown that the NK cell pool is highly heterogeneous, consisting of different cell subsets with distinct phenotypic and functional features. Recently, in collaboration with Professor Wayne Yokoyama, we showed that a novel NK cell subset expressing CD49a and lacking DX5 rarely recirculates and selectively resides in the liver, that is, liver-resident NK cells. While bone marrow (BM) cells are efficient in generating conventional NK (cNK) cells, the frequency of liver-resident NK cells is significantly reduced after BM transplantation. Conversely, lethally irradiated mice adoptively transferred with fetal liver cells exhibit normal frequency of liver-resident NK cells. Further analyses reveal that the adult liver retains hematopoietic potential and contains a few hematopoietic progenitor cells (HPCs), which are phenotypically distinct from BM lineage (Lin)-negative progenitor cells but resemble fetal liver counterparts. Moreover, adoptive transfer of these adult liver HPCs have the capacity to generate liver-resident NK cells, but not cNK cells. Finally, Liver HPCs express higher levels of the transcription factors that are essential for liver-resident NK cell development than their BM counterparts. Thus, our studies suggest an alternative development pathway for liver-resident NK cells.

NK Cells

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Expansion of adaptive NK cells in HIV disease persists following combination antiretroviral therapy

Hearps, A.¹, Zhou, J.¹, Brunt, S.², Agius, P.³, Price, P.⁴, Elliott, J.⁵, Jaworowski, A.¹

¹Burnet Institute, Centre for Biomedical Research, Melbourne, Australia, ²University of Western Australia, Pathology and Laboratory Medicine, Perth, Australia, ³Burnet Institute, Center for Population Health, Melbourne, Australia, ⁴Curtin University, Biomedical Science, Bentley, Australia, ⁵The Alfred Hospital and Monash University, Infectious Diseases Unit, Melbourne, Australia

Introduction: Combination antiretroviral therapy (cART) does not effectively target inflammation or innate immune activation in HIV+ individuals, leading to increased risk of inflammatory non-AIDS co-morbidities. Understanding how cART impacts innate immune activation is required for better long-term management of HIV patients.

Methods: Blood was analysed from 20 HIV+ men who have sex with men (MSM) pre-cART and 6, 12 and 24 months post-cART, and 15 HIV- MSM controls. NK, T and monocyte populations and

activation were measured using whole-blood flow cytometry. Antibodies to HCMV glycoprotein B (gB) and lysates from infected human foreskin fibroblasts (HFF) were measured in plasma. Changes of individual parameters over time were modeled using latent growth-curve modeling.

Results: CD56dimFcyR γ - adaptive NK cells were elevated at baseline in HIV+ compared to HIV- MSM ($p=0.003$) and our modelling revealed that their proportion did not change after at least 14 months of cART ($p=0.128$). Expansion of adaptive NK cells was associated with persistently elevated CMV Ab although there was no correlation between the two in HIV+ MSM ($p=0.858$ for HFF; $p=0.477$ for gB). NK activation assessed with the early activation marker CD69 decreased ($p=0.003$) whereas HLA DR+CD38+ NK cells did not ($p=0.103$). In contrast, T cell activation (HLA DR+CD38+ CD4 and CD8 both $p < 0.001$) and expansion of the proportions of intermediate ($p < 0.001$) and non-classical ($p=0.001$) monocytes significantly decreased.

Conclusions: cART reduces NK, CD4 and CD8 T cell activation at different rates. The expansion of the adaptive NK cell population following HIV infection was not altered by cART.

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Effects of resveratrol on cytotoxicity of human NK cells in vitro

Shao, D., Di, Y., Li, Q., Wang, Y., Ren, H., Shi, J., Huang, Q.

Northwestern Polytechnical University, School of Life Sciences, Xi'an, China

Resveratrol (RES), a natural compound in plants, possesses obvious immune modulation activities, such as anti-tumor. Natural killer (NK) cells are important in host defense against tumors and Res may influence the cytotoxicity of NK cells through affecting the NKG2D receptor on NK cells or NKG2D-ligands on tumor cells. The effects of RES on NK cytotoxicity through its influence on NK cells and tumor cells were assessed in this study, respectively.

RES with different concentrations (3 μ M, 6 μ M, 12 μ M, 25 μ M and 50 μ M) were used in the present study. Results showed that when directly treated to NK cells, RES exhibited concentration dependent biphasic effects on the cytotoxicity of NK cells to K562 cells. At high concentration (50 μ M), RES lowered the NK cells cytotoxicity by down-regulating the expression of NKG2D. However, contrary results were obtained when at low concentration (3 μ M). On the other hand, after K562, HepG2, Caco2 and Hela cells were pre-treated with RES, there were no significant change on the sensitivity of K562, Caco2 and Hela cells to NK cells. However, 50 μ M RES treated HepG2 cells were resistant to NK cells with 15% decreased cytotoxicity than that of control. It was found to be because of the decrease in the gene expression of some NKG2D ligands (MICA, MICB, MICC, ULBP1 and ULBP2) in the HepG2 cells with 50 μ M RES treatment. The results demonstrated it was selective for RES to tumor cells. Our results may provide a new perspective of sight into RES function on immuno-cells and tumor cells.

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Ascites containing ovarian cancer cells impair NK cells functionality and hinder their activation after IL-2 stimulation

da Silva, R.E.¹, Yoshida, A.¹, Cardoso, D.M.², Jales, R.M.¹, Derchain, S.¹, Guimarães, F.³

¹University of Campinas / Facult of Medical Sciences, Tocogynecology, Campinas, Brazil, ²University of Campinas / Facult of Medical Sciences, Campinas, Brazil, ³University of Campinas / CAISM, LCE, Campinas, Brazil

Peritoneal ascites is a unique feature of patients with advanced ovarian cancer that displays soluble factors and cells consistent with the tumor microenvironment. The goal of this study was to evaluate the NK cells functionality and their possible suppressive mediators in ascites from ovarian cancer patients. Healthy donor's ($n=12$) and patient's blood ($n=21$), and ascites without (Asc, $n=11$) and with cancer cells (AscCA, $n=6$) were collected after signed consent. Functionality of NK cells resting and IL-2 stimulated (overnight) were evaluated against K562 (1:1 ratio) by the expression of CD107a, together with the expression of DNAM-1, NKp30, NKG2D and CD16, and soluble cytokines IL-2, IL-4, IL-5, IL-10, TNF, IFN- γ and TGF- β in the serum and ascites were also quantified. The functional activation of NK cells from Asc increased significantly ($p < 0.05$) after IL-2 stimulation, (median/range, resting=17.60/6.29-29.55 to IL-2=37.07/11.20-49.50), however, the increase of the functional activation of NK cells from AscCA was not significant (resting=9.81/6.04-18.40 to IL-2=15.45/11.75-18.50). All activating receptors observed in AscCA samples were significantly ($p < 0.05$) down-regulated in respect to healthy donor's blood samples. TNF was the only cytokine in which increased significantly from Asc to AscCA. Furthermore, the population of T-regs in AscCA was significantly higher than in Asc, within the CD4⁺ population (Asc T-regs=3.36/1.38-9.58, AscCA T-regs=6.05/3.58-14.95). NK cells functionality and their response to IL-2 is impaired in ascites containing ovarian cancer cells. This effect is associated to the down-regulation of NK cells activating receptors and a higher percentage of T-regs.

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Natural killer activating receptors in Behcet disease

Sallakci, N.¹, Aktas Cetin, E.², Tahrali, I.², Kucuksezer, U.C.², Yilmaz, A.², Gul, A.³, Deniz, G.²

¹Akdeniz University, Medical Faculty, Hematology Laboratory, Antalya, Turkey, ²Istanbul University, Institute of Experimental Medicine, Immunology, Istanbul, Turkey, ³Istanbul University, Istanbul Medical Faculty, Department of Internal Medicine, Division of Rheumatology, Istanbul, Turkey

Behcet's disease (BD) is a systemic inflammatory disorder of unknown etiology. Besides genetic predisposition, immune dysregulation in Natural Killer (NK) cells are reported to be involved in the pathogenesis of BD. NK cells have activating receptors that can be modulate adaptive immune system. NK activating receptors can shape immune responses, but very limited studies have reported about NK cells and their receptors.

In this study, expression of NKp30, NKp46, NKG2D receptors and proliferative capacity of NK cells in BD were investigated.

Peripheral blood mononuclear cells (PBMCs) were isolated from Behcet's patients (n=8) and healthy subjects (n=4). PBMCs were incubated with various human recombinant cytokine combinations including IL-12, IL-15, IL-18 and IL-23. After culturing for 40 hours, cells were stained with fluorochrome labeled antibodies and analyzed by 8-color flow cytometer. Proliferative activity were evaluated by CFSE staining.

Compare to healthy donors, CD16⁺ cells in lymphocyte population were significantly diminished in BD patients when stimulated with IL-12+IL-15 and IL-15+IL-23. Expression of CD16⁺NKp30⁺, CD16⁺NKp46⁺ and CD16⁺NKG2D⁺ cells were also lower when stimulated with IL-12+IL-13 in Behcet's patients. But on unstimulated conditions, there was borderline differences both CD16⁺NKp46⁺ and CD16⁺NKp30⁺ expression. When the stimulation with cytokine combinations including IL-12+IL-15, IL-18+IL-15 and IL-23+IL-15, proliferative capacity of CD3⁺CD56⁺ cells of Behcet's patients were significantly increased compared to healthy subjects.

Combination of the cytokines could be playing a role immune dysregulation of BD. Expression of low activating receptors in Behcet's patients might be contributed disease pathology. Excessive proliferative capacity of Behcet's patients might be a factor IL-15.

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Tumor therapeutics work as stress inducers to enhance tumor sensitivity to NK cell cytotoxicity by upregulating NKp30 ligand B7-H6

Cao, G., Wang, J., Zheng, X., Wei, H., Tian, Z., Sun, R.

University of Science and Technology of China, Hefei, China

Immune cells are believed to participate in initiating anti-tumor effects during regular tumor therapy such as chemotherapy, radiation, hyperthermia and cytokine injection. One of the mechanisms underlying this process is the expression of so-called stress-inducible immunostimulating ligands. Although the activating receptor NKG2D has been proven to play roles in tumor therapy through targeting its ligands, the role of NKp30, another key activating receptor, is seldom addressed. In this study, we found that the NKp30 ligand B7-H6 was widely expressed in tumor cells and closely correlated to their susceptibility to NK cell lysis. Further studies showed that treatment of tumor cells with almost all standard tumor therapeutics, including chemotherapy (cisplatin, 5-fluorouracil), radiation therapy, non-lethal heat shock, and cytokine therapy (TNF- α), could upregulate the expression of B7-H6 in tumor cells and enhance tumor sensitivity to NK cell cytotoxicity. B7-H6 shRNA treatment effectively dampened sensitization of tumor cells to NK-mediated lysis. Our study not only reveals the possibility that tumor therapeutics work as stress inducers to enhance tumor sensitivity to NK cell cytotoxicity but also suggests that B7-H6 could be a potential target for tumor therapy in the future.

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HLA haplotype homozygous iPSC-derived cells can be rejected by haploidentical recipient in NK cell dependent manner

Ichise, H., Kawamoto, H.

Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan

iPSC stock project are planned to be produced from donors with homozygous HLA haplotype (HLA-homo) in Japan. It is expected that HLA-homo iPSC-derived cells can be transplanted to recipients who have identical haplotype in one of alleles (HLA-hetero). In homo-to-hetero transplantation, rejection by allo-reactive T cells may not occur. However, recipient NK cells may attack the graft, by the mechanism called missing-self. In recent years, NK cell-mediated rejection has been suggested to occur in some organ transplantation cases in human. The aim of this study is to examine whether NK cell mediated rejection occurs against human iPSC-derived regenerated tissue in homo-to-hetero transplantation setting. NK cells express inhibitory receptors called killer cell immunoglobulin-like receptors (KIRs), and HLA-C serve as major ligands for KIRs. HLA-C are divided into two groups (C1 and C2), each of them binds to different KIRs (2DL3, 2DL1). We established iPSCs from HLA-homo and hetero donors, and those iPSCs were differentiated into T cells and endothelial cells. NK cells from a HLA-hetero donor (C1/C2) were cultured with regenerated T cells or endothelial cells from a HLA-homo donor (C1/C1), and production of inflammatory cytokine and cytotoxic activity were measured. We found that NK cells expressing C2 specific KIRs (2DL1) were selectively activated and produced IFN- γ . More importantly, regenerated HLA-homo T cells were found to be killed by HLA-hetero NK cells. We further found that such cytotoxic activity was suppressed by transducing HLA-homo iPSCs with HLA-C2 gene.

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MiR-146a negatively regulates NK cell functions via STAT1 signaling

Xu, D.¹, Han, Q.¹, Zhang, C.¹, Zhang, J.²

¹Shandong University, School of Pharmaceutical Sciences, Jinan, China, ²Shandong University, School of Pharmaceutical Sciences, Jinan, China

It is known that NK cell function is down-regulated in chronic hepatitis B virus infection (CHB) and hepatic carcinoma (HCC). However, the mechanisms underlying this functional down-regulation are largely unclarified. In this study, microRNA (miR)-146a expression increased in NK cells from CHB and HCC patients compared to NK cells from healthy donors, while miR-146a levels were negatively correlated to NK cell functions. Overexpression of miR-146a diminished NK cell mediated cytotoxicity and production of IFN γ and TNF α , observations which were reversed upon inhibition of miR-146a. MiR-146a in NK cells was induced by cytokines IL-10 and TGF- β , but reduced after treatment with IL12, IFN α , and IFN β . We further revealed that miR146a targeted STAT1 and thereby regulated NK cell functions. Taken together, the up-regulated miR146a expression attributes to, at least partly, the NK cell dysfunction in CHB and HCC patients. So, miR-

146a may become a therapeutic point with great potential to ameliorate NK cell functions in liver disease.

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Differential phenotypic and functional properties of liver-resident NK cells and mucosal ILC1s

Tang, L.¹, Peng, H.¹, Zhou, J.¹, Chen, Y.¹, Wei, H.¹, Sun, R.¹, Yokoyama, W.M.², Tian, Z.¹

¹University of Science and Technology of China, Institute of Immunology and The CAS Key Laboratory of Innate Immunity and Chronic Disease, Hefei, China, ²Washington University School of Medicine, Department of Medicine, St. Louis, American Samoa

Group 1 innate lymphoid cells (ILCs) consist of conventional natural killer (cNK) cells, tissue-resident NK cells and mucosal ILC1s. Recently identified liver-resident NK cells, which can mount contact hyper-sensitivity responses, and mucosal ILC1s that are involved in pathogenesis of colitis are distinct from cNK cells in several aspects, but the issue of how they are related to each other has not been clearly clarified. Here, we show that liver-resident NK cells and mucosal ILC1s have different phenotypes, as evidenced by distinct expression patterns of homing-associated molecules. Moreover, mucosal ILC1s exhibit tissue residency akin to liver-resident NK cells. Importantly, liver-resident NK cells express relative high levels of cytotoxic effector molecules, which are poorly expressed by mucosal ILC1s, and exhibit stronger cytotoxic activity compared with mucosal ILC1s. These results demonstrate differential phenotypic and functional characteristics of liver-resident NK cells and mucosal ILC1s, shedding new light on the diversity of ILC family.

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The role of natural killer cells in graft versus leukaemia following T cell deplete allogeneic stem transplant

Maggs, L., Kinsella, F., Zuo, J., Moss, P.

University of Birmingham, Birmingham, United Kingdom

Leukaemic relapse is the major cause of treatment failure after reduced intensity T cell deplete haematopoietic stem cell transplant (HSCT). A graft versus leukaemia (GvL) effect derived from cells co-transferred with a donor's stem cell population eliminates residual tumour. Natural killer (NK) cells are purported to contribute to this effect but their precise function remains to be properly identified. Here we assessed whether 18 patients who relapsed following T cell deplete HSCT received different NK cell populations compared to 56 patients who did not relapse by immunophenotyping donor apheresis product samples and comparing these to patient outcomes. Apheresis product NK cell CD56^{dim} proportions as well as activation receptors NKp46, NKG2D and DNAM expression were decreased compared to healthy individuals, but not between the different outcome groups. Patients who relapsed received a lower percentage, as well as a lower total dose, of NK cells in their apheresis product. Further functional analysis of apheresis product NK cell cytotoxic potential, via killing of 721.221 cells, found no difference between patients who relapsed and those who did not. The phenotype and function of NK cells in

the apheresis products of allogeneic stem cell transplants did not differ between patients who go on to relapse and those who did not. However the overall number of NK cells in the transplant may be important for the success of the GvL effect in a reconstituted haematopoietic environment.

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Heat killed *Helicobacter pylori* increases NK cell cytotoxicity through induction of NKG2DL surface expression on gastric adenocarcinoma cells

Hernández, C.J.¹, Garrido-Tapia, M.², Kramm, K.², Ribeiro, C.H.², Molina, M.C.²

¹Universidad de Chile, Escuela de Tecnología Médica, Facultad de Medicina, Santiago, Chile, ²Universidad de Chile, Programa disciplinario de Inmunología, Facultad de Medicina, Santiago, Chile

The Natural Killer Group 2, member D (NKG2D) is an activating receptor present on the surface of cytotoxic cells. Its ligands (NKG2DLs) are expressed on the cell surface in response to cellular stress. *Helicobacter pylori* can induce cellular damage on gastric cells through HSP60 and lipopolysaccharide (LPS), which are recognized by Toll like receptors (TLR). Our aim was to evaluate NKG2DLs expression on gastric adenocarcinoma cell lines in response to *H. pylori* through TLRs activation and its effect on NK cell cytotoxicity activation. NKG2DL expression was assessed at the protein and mRNA levels in MKN-45 cells upon heat killed *H. pylori* (HKHP) treatment by flow cytometry and real time PCR, respectively. NK cells degranulation and cytotoxicity on target cells was evaluated, respectively, by CD107a surface expression, by flow cytometry, and lactate dehydrogenase release assay after co-culture with MKN-45 cells previously treated with HKHP. HKHP induced a selective modulatory effect on MICA, ULBP2/5/6, ULBP4 and TLR4 cell surface expression and mRNA levels on MKN-45 cells. Both, LPS from *Rhodobacter sphaeroides*, a TLR4 antagonist, and a peptide that inhibits the dimerization of MyD88, reversed the effect of HKHP. Accordingly, increased MICA, ULBP2/5/6 and ULBP4 expression on target cells upon HKHP stimulus resulted in increased NK cell cytotoxicity, mainly through NKG2D receptor activation. Our results suggest that an inflammatory microenvironment could be associated to induction of NKG2DL expression through TLR stimulation on gastric epithelial cells, which may contribute to the maintenance of epithelial tissue damage. Financial support: FONDECYT Grant 1130330

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Characterising natural killer cell subset responses to a herpes simplex virus (HSV) lipopeptide

Truong, N.^{1,2}, Kim, M.^{1,2}, Zeng, W.³, Esparon, S.⁴, Wines, B.⁴, Hogarth, M.⁴, Jackson, D.³, Cunningham, A.^{1,2}

¹The Westmead Institute for Medical Research, Sydney, Australia, ²University of Sydney, Sydney Medical School, Sydney, Australia, ³University of Melbourne, Department of Microbiology and Immunology, Parkville, Australia, ⁴Burnet Institute, Melbourne, Australia

There are two subsets of human natural killer (NK) cells. In

circulation, >95% are CD56^{dim}CD16⁺, which are highly cytolytic against infected target cells. < 5% are CD56^{bright}CD16^{-/dim}, which have decreased cytolytic capacity, but increased production of cytokines. We are investigating HSV vaccine development using a Toll-like receptor-2 (TLR-2) stimulating lipid-conjugated HSV peptide, "Pam2Cys-30-5". Previously, we observed that this lipid conjugation enhanced CD4 T lymphocyte responses to Pam2Cys-30-5 in coculture with NK cells and dendritic cells. Furthermore, NK cells were directly activated by Pam2Cys-30-5 and could activate CD4 T lymphocytes in the absence of other antigen presenting cells, so their responses to Pam2Cys-30-5 were further investigated. Both cultured NK cell subsets were activated by Pam2Cys-30-5 as shown by increased CD69 expression, and CD16 expression on CD56^{dim} NK cells decreased over 18 hours. The two stimulated subsets showed different patterns of cytokine and chemokine production. Furthermore, this emerging CD56^{dim}CD16^{-/dim} population showed highest expression of IFN- γ . CD16 involvement in NK cell TLR-2 responses has not been explored. Hence, we began investigating the mechanism of CD16 downregulation induced by Pam2Cys-30-5. CD16 can be cleaved, so shedding into the supernatant was measured by Cytometric Bead Array. Direct binding between Pam2Cys and CD16 was not observed by ELISA, and CD16 only infrequently interacted with TLR-2 by proximity ligation assay. Finally, CD16⁺ cytolytic NK cells selectively infiltrated the HSV infected epidermis of initial herpetic lesion biopsies. Therefore they may play a role in innate control of initial genital HSV infection and should be considered in vaccine development.

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Flow cytometric multicolor NK cell phenotyping as a potential tool for donor selection and NK cell reconstitution analysis in different hematopoietic stem cell transplantation setups

Pflitsch, S., Huppert, V., Richter, A., Siewert, C., Dose, C. Miltenyi Biotec GmbH, Research & Development, Bergisch Gladbach, Germany

Natural killer (NK) cells are lymphocytes of the innate immune system and show the capacity to kill cancer cells. Their function is regulated by the balance of inhibitory and activating signals transmitted mainly by killer-cell immunoglobulin-like (KIR), killer-cell lectin-like (KLR) and natural cytotoxicity (NCR) receptors. Genotyping, phenotyping and haplotyping of NK cell receptors are discussed in different haploidentical and allogeneic hematopoietic stem cell transplantation settings to predict the incidence of Graft-versus-Host-Disease (GvHD), Graft-versus-Leukemia effects (GvL), graft rejection and relapse, and therefore to improve donor selection. Furthermore NK cell receptor phenotyping is a potent tool to analyze NK cell reconstitution after haploidentical and allogeneic hematopoietic stem cell transplantation to further improve the understanding of the complex NK cell biology. Here we present four robust multicolor flow cytometry NK cell receptor phenotyping panels (up to 8 colors) with the focus on a) inhibitory KIRs recognizing HLA-C2 (CD158a - KIR2DL1), HLA-C1 (CD158b - KIR2DL2/3) and HLA-Bw4 (CD158e - KIRDL1/DS2) alleles of the HLA class I molecules,

b) other inhibitory and activating KIRs, c) KLRs and NK cell maturation markers and d) NCRs. Panels were compiled by including recombinant antibodies (REAFinity™ Antibodies), which provide the advantages of high purity, lot-to-lot consistency for greater reproducibility and high specificity, so that FcR blocking is no more required. Additionally, the brighter VioBright™ FITC fluorophore is used to substitute the conventional FITC fluorochrome, where feasible.

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Natural killer cytotoxicity and single nucleotide polymorphisms in transient receptor potential ion channels and acetylcholine receptors of isolated natural killer cells in chronic fatigue syndrome

Marshall-Gradisnik, S.^{1,2}, Huth, T.^{1,2}, Chacko, A.^{1,2}, Johnston, S.¹, Smith, P.¹, Staines, D.¹

¹Menzies Health Institute Queensland, National Centre for Neuroimmunology and Emerging Diseases, Parklands, Australia, ²Griffith University, School of Medical Science, Parklands, Australia

Nucleotide polymorphisms (SNPs) and genotypes in transient receptor potential (TRP) ion channels and Acetylcholine receptors (AChRs) in isolated NK cells from CFS patients.

Methods: Thirty-nine Fukuda defined CFS patients (age = 51.69±2.00 years) and 30 non-fatigued controls (age = 47.60±2.39 years) were included. Flow cytometric protocols were used to examine NK cytotoxic activity. A total of 678 SNPs from isolated NK cells were examined for 21 TRP ion channel genes and for 9 AChR genes using Agena Biosciences iPLEX Gold assay. SNP association and genotypes were determined using ANOVA and PLINK analysis.

Results: CFS patients had a significant reduction in NK% lysis (17±4.68) compared to non-fatigued controls (31±6.78). Eleven SNPs for TRP ion channel genes were identified in the CFS group. Five of these were associated with TRPM3 and the remainder were associated with TRPM8, TRPC2 and TRPC4. Fourteen SNPs were associated with nicotinic and muscarinic AChRs: six were nAChR α 3, while the remainder were nAChR α 2, nAChR β 4, nAChR α 5 and nAChR epsilon. Sixteen genotypes were identified from SNPs in TRP ion channel and AChR for TRPM3, TRPM, TRPC4, TRPC2, nAChR epsilon, nAChR α 2, nAChR α 3 and nAChR β 4.

Conclusions: We identified SNPs and genotypes for TRP ion channels and AChRs from isolated NK cells in patients with CFS, which may be involved in changes in NK cell function and development of CFS pathology.

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Generation, characterization and functional analysis of HLA-DR-positive NK cells

Erokhina, S., Streltsova, M., Kanevskiy, L., Kovalenko, E. M.M. Shemyakin and Yu.A. Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences, Moscow, Russian Federation

Activated NK cells are able to express HLA-DR. However, HLA-DR-positive subpopulation and functional significance of HLA-

DR on NK cells are still not fully characterized. In this work, we investigated HLA-DR-positive NK cells generated in the in vitro activation system using IL-2 and K562 cells with membrane-bound IL-21 (K562-mbIL-21). This cytokine combination is very promising for NK cells expansion for adoptive immunotherapy. Stimulation of NK cells for 6 days significantly increased the proportion of HLA-DR⁺ subpopulation, with combination of IL-2 and K562-mbIL-21 causing the highest rates (93% of HLA-DR⁺ NK cells). IL-2 only, unmodified K562, soluble IL-21, and their various combinations also led to a certain increase in HLA-DR⁺ cells proportion, but to a lesser extent (less than 45% of NK cells). This phenomenon resulted from the induction of HLA-DR expression on NK cells, rather than propagation of HLA-DR-positive subpopulation: proportion of HLA-DR⁺ cells increased in the pre-sorted HLA-DR-negative subpopulation too.

HLA-DR⁺ cells, obtained after 6 days of stimulation with IL-2 and K562-mbIL-21, demonstrated higher IFN γ production and cytotoxicity than HLA-DR⁻ cells, along with higher expression of NKG2C, NKG2D, CD86 and degranulation marker CD107a. At the same time, we found out a significant increase in proportion of HLA-DR-positive NK cells in the peripheral blood of tuberculosis-infected patients comparing to healthy donors. This might be the clue to elucidating the physiological role of HLA-DR-positive NK cells, but further investigation is needed, including the potential to use these cells with elevated effector functions in adoptive immunotherapy.

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Killer cell immunoglobulin-like receptor genes and allelic polymorphism from natural killer cells in chronic fatigue syndrome/myalgic encephalomyelitis patients

Huth, T.^{1,2}, Brenu, E.^{1,2}, Staines, D.^{1,2}, Marshall-Gradisnik, S.^{1,2}

¹National Centre for Neuroimmunology and Emerging Diseases, Menzies Health Institute Queensland, Gold Coast, Australia, ²School of Medical Sciences, Griffith University, Gold Coast, Australia

Variations in *Killer cell immunoglobulin-like receptor (KIR)* gene content and allelic polymorphism have been identified to influence KIR surface expression and receptor ligation required to initiate NK cell cytotoxic activity. NK cell cytotoxic activity has consistently been reported to be reduced in patients with Chronic Fatigue Syndrome/ Myalgic Encephalomyelitis (CFS/ME). The aim of this study was to investigate *KIR* gene content and allelic polymorphism to determine if genetic variations may contribute to reduced NK cell activity in an Australian population of CFS/ME patients. NK cells were isolated by magnetic separation and deoxyribonucleic acid was extracted from CFS/ME patients and non-fatigued controls (NFC). NK cell DNA was sequenced on an Illumina MiSeq platform and the data generated was aligned the Immuno Polymorphism-KIR Database to determine *KIR* content and allelic polymorphism for each participant. A total of 20 CFS/ME patients and 20 non-fatigued controls (NFC) were included in this study. The frequency of individual activating and inhibitory *KIRs* were compared between CFS/ME patients and NFC and no significant differences were observed. *KIRs* alleles were also compared between CFS/ME patients and NFC and no significant differences

were reported. As the frequencies of *KIR* genes and allelic polymorphisms were not significantly associated with NK cells from CFS/ME patients, this suggests that individual *KIR* genes and allelic polymorphism may not contribute to reduced NK cell cytotoxic activity. Further investigations in a larger sample size are required to ensure there is enough statistical power to identify differences between CFS/ME patients and NFC.

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ERK1/2 significantly reduced in CD56^{dim}CD16⁺ NK cells from chronic fatigue syndrome/myalgic encephalomyelitis patients

Huth, T.^{1,2}, Brenu, E.^{1,2}, Staines, D.^{1,2}, Marshall-Gradisnik, S.^{1,2}

¹National Centre for Neuroimmunology and Emerging Diseases, Menzies Health Institute Queensland, Gold Coast, Australia, ²School of Medical Sciences, Griffith University, Gold Coast, Australia

Intracellular signals for Natural Killer (NK) cell effector function are propagated through a phosphorylation cascade by a family of mitogen-activated protein kinases (MAPKs). Extracellular signal-regulated kinase (ERK)1/2 has been identified as a critical signalling molecule required for the secretory granule in cytotoxic NK cells to polarise towards and release lytic proteins into the target cell. Reduced cytotoxic function of NK cells has consistently been reported in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) patients, and the MAPK signalling pathway remains to be investigated. Therefore, the purpose of this study was to examine phosphorylated ERK1/2 in NK cells from CFS/ME patients as a potential mechanism for reducing NK cell cytotoxic function. Peripheral blood mononuclear cells (PBMCs) were isolated from each participant by density gradient centrifugation and incubated with K562 cells at an effector to target ratio of 25:1 for 15 minutes to induce phosphorylation. The PBMCs were subsequently fixed, permeabilised, stained for ERK1/2 with a monoclonal antibody and analysed using flow cytometric methods on CD56^{dim}CD16⁺ NK cells. 14 CFS/ME patients and 11 non-fatigued controls were included in the study. In CFS/ME patients, ERK1/2 was significantly reduced in CD56^{dim}CD16⁺ NK cells following stimulation with K562 cells compared to the non-fatigued controls. The MAPK pathway may contribute to reduced NK cell cytotoxic activity in CFS/ME patients. The significant reduction of ERK1/2 in CD56^{dim}CD16⁺ NK cells from CFS/ME patients may disrupt intracellular signals required for polarisation of the secretory granule towards the target cell.

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MAPK signalling and NK cell cytokine production in chronic fatigue syndrome/myalgic encephalomyelitis patients

Huth, T.^{1,2}, Brenu, E.^{1,2}, Staines, D.^{1,2}, Marshall-Gradisnik, S.^{1,2}

¹National Centre for Neuroimmunology and Emerging Diseases, Menzies Health Institute Queensland, Gold Coast, Australia, ²School of Medical Sciences, Griffith University, Gold Coast, Australia

Natural Killer (NK) cells elicit cytotoxic activity and cytokine

production to remove target cells infected with viruses, bacteria or cells which have been malignantly transformed. Intracellular signalling through the mitogen activated protein kinase (MAPK) pathway induces cytokine production. Interferon gamma (IFN- γ) from NK cells in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) patients has previously been reported to be increased and the purpose of this study was to investigate if signalling molecules in the MAPK pathway may contribute to increased cytokine production in CFS/ME patients. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation. To induce phosphorylation, the PBMCs were stimulated with K562 cells at an effector to target ratio of 25:1 for 15 minutes. The PBMCs were subsequently fixed, permeabilised, stained with monoclonal antibodies for MAPK Kinase1/2 (MEK1/2), p38 and c-Jun N-terminal kinase (JNK) and analysed on CD56^{bright}CD16^{-dim} NK cells using flow cytometric methods. 14 CFS/ME patients and 11 non-fatigued controls were included in the study. CD56^{bright}CD16^{-dim} NK cells from CFS/ME patients expressed significantly increased amounts of p38 and MEK1/2 following stimulation with K562 cells compared to the non-fatigued controls. Phosphorylation of p38 and MEK1/2 activates tyrosine kinase molecules which translocate into the nucleus to regulate cytokine production at the transcriptional level. Therefore, increased levels of phosphorylated p38 and MEK1/2 in CD56^{bright}CD16^{-dim} NK cells may contribute to increased production of IFN- γ in CFS/ME patients.

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SNPs rs4656317 and rs12071048 located within an enhancer in *FCGR3A* are in strong linkage disequilibrium with SNP rs396991 and influence ADCC by transcriptional regulation

Oboshi, W., Yukimasa, N.

Kagawa Prefectural University of Health Sciences, Department of Medical Technology, Takamatsu, Japan

Responses to NK cell-mediated ADCC have been shown to depend on a SNP at CD16 (Fc γ R11a) receptor amino acid position 158. However, a consensus has not yet been reached regarding differences in the structure and expression levels of the CD16 molecule among Fc γ R11a-F158V genotypes. The aim of the present study was to further investigate the relationship between ADCC activity and functional SNPs in the *FCGR3A* gene. SNP analyses were performed on 101 healthy Japanese volunteers (46 males and 55 females). Direct sequencing using *FCGR3A* gene sequence-specific primers were conducted for SNP rs396991 (T>G; F158V) and other SNPs located within an enhancer, i.e., rs10917571 (G>T), rs4656317 (C>G), and rs12071048 (G>A). Twenty-four volunteers participated in measurements of NK cell-mediated ADCC activity, the binding affinity of CD16 to the Fc region, *FCGR3A* transcripts, and cell-surface CD16 expression. The results obtained showed that SNP rs396991 was in strong linkage disequilibrium with SNPs rs4656317 and rs12071048. NK cell-mediated ADCC activity was significantly different in the genotypes of their SNPs. Binding affinity for SNP rs396991 was significantly greater in G/G subjects than in G/T or T/T subjects. *FCGR3A* transcript levels and surface CD16 expression levels indicated a significant

difference in the genotypes of SNPs rs4656317 and rs12071048, respectively. We revealed a detailed relationship between ADCC activity and functional SNPs in NK cells obtained from Japanese subjects. In conclusion, two regulatory SNPs are in strong linkage disequilibrium with SNP rs396991 in *FCGR3A* and influence NK cell-mediated ADCC by transcriptional regulation.

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Deficiency of autophagy resulted in high cytotoxicity activity and strong anti-tumor effect in NK (natural killer cells)

Yang, J., Wang, X., Zhang, Y., Chen, W.

Zhejiang University, Institute of Immunology, School of Medicine, Hangzhou, China

Macroautophagy (hereafter referred to autophagy) is an evolutionary conserved self-homeostasis mechanism. It has been demonstrated that autophagy played an important role in the development, survival and function of immune cells. However, the role of autophagy in the function of NK cells remained unknown. Here, we utilized Atg3 and Atg7 conditional knock-out mice with ER-Cre, which could induce Atg gene knockdown in mature NK cells without affecting cell development. We found that the NK cells from both of Atg3-deficient and Atg7-deficient mice showed higher expression levels of perforin and granzyme B compared with NK cells from control mice, and therefore possessed increased cytotoxicity activity in vitro. We also found that these autophagy-deficient mice exhibited more obvious anti-tumor effect on the NK-sensitive RAM-S tumor in vivo. Furthermore, the expression of killer activatory receptors (KAR) and killer inhibitory receptors (KIR) on surface of NK cells did not change after Atg7 gene knockdown. In addition, the release of cytolytic granules detected by CD107a staining was not impaired in NK cells from Atg7 knockout mice. These data indicated that autophagy controlled the cytotoxicity activity of NK cells by regulating the expression levels of perforin and granzyme B, and KAR/KIR on surface of NK cells as well as the release of lytic granule remained unchanged.

NKT Cells

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Increase in NKT cell numbers protects mice from central nervous system autoimmunity

Gemiarto, A., Xuyen Dinh, T., Jordan, M., Baxter, A.

James Cook University, Molecular and Cell Biology, Townsville, Australia

Natural killer T (NKT) cells are a distinct subset of T lymphocytes that expresses both TCR and natural killer cell receptors. NKT cells require the presentation of glycolipid antigen by CD1d and they express an invariant TCR- α chain Va14-Ja18. They rapidly produce an array of cytokines upon stimulation, including IFN- γ , IL-4, and IL-10. In this study, we examined the role of NKT cells in an animal model of central nervous system (CNS) autoimmunity, Experimental Autoimmune Encephalomyelitis (EAE).

We compared the progression of EAE in wild-type C57BL/6, NKT cell-deficient C57BL/6.CD1d^{-/-} (CD1dKO) and NKT cell-abundant C57BL/6.Va14 transgenic (Va14tg) mice. Mice were immunised with myelin oligodendrocyte glycoprotein 35-55 in complete Freund's adjuvant and supplemented with pertussis toxin. Mice were scored for signs of CNS damage for 30 days, after which the CNS was harvested for histological analysis. The proportions of NKT cells in the lymphoid organs were determined using flow cytometry.

There was no significant difference in disease severity of CD1dKO mice compared to the wild-type strain. Conversely, Va14tg mice developed a significantly milder form of EAE. Histological analysis confirmed a lower degree of inflammation in the CNS of Va14tg mice compared to wild-type and CD1dKO mice. We also observed significantly milder symptoms in wild-type mice following transfer of NKT cells from Va14tg mice, compared to PBS-injected control mice.

These findings support a role for NKT cells in suppressing autoimmune disease and may lead to the development of a novel MS therapy.

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Role of killer-associated protein NKG7 in NK and NKT cells

Baxter, A.G.¹, Gemiaro, A.¹, Oliaro, J.², Dinh, T.X.¹, Smith, L.¹, Godfrey, D.I.³, Jordan, M.A.¹

¹James Cook University, Comparative Genomics Centre, Townsville, Australia, ²Peter MacCallum Cancer Centre, Cancer Immunology, Melbourne, Australia, ³University of Melbourne, Microbiology and Immunology, Melbourne, Australia

NKG7 is a poorly characterised integral membrane protein expressed on cytotoxic granules of quiescent NK and CD8 T cells and is translocated to the plasma membrane of cytotoxic cells following target engagement. It is expressed on expanded populations of cytotoxic CD4 T cells in patients with effective antiviral responses to HIV and CMV. In a transcriptional analysis of NKT cell development, we found that *Nkg7* is one of the most strongly up-regulated transcripts (35-fold) in NKT cells at the point of lineage commitment, suggesting it plays an important role in NKT cell activities. We have produced B6.*Nkg7*^{-/-} mice and found a decrease in NK cell-mediated cytotoxicity on a per cell basis *in vitro*, but not an effect on numbers of peripheral NK cells. B6.*Nkg7*^{-/-} mice had fewer thymic resident NK1.1⁺ NKT cells but increased numbers in spleen and liver. The expression of the NK1.1 marker in NKT cells was associated with a "killer transcriptional program," including strong expression of *Nkg7*, *Gzma*, *Gzmb*, *Prf1*, *Fasl* and a wide range of NK cell markers that were not expressed on other subsets.

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Phenyl glycolipids with different glycosyl groups exhibit marked differences in murine and human iNKT cell activation

Huang, J.-R.¹, Wu, T.-N.^{2,3}, Hung, J.-T.¹, Lin, K.-H.^{2,4}, Wu, Y.-T.², Wu, J.-C.¹, Wong, C.-H.^{2,3,4}, Yu, A.L.^{1,2}

¹Chang Gung Memorial Hospital, Institute of Stem Cell and Translational Cancer Research Center, Taoyuan, Taiwan, Republic

of China, ²Academia Sinica, Genomics Research Center, Taipei, Taiwan, Republic of China, ³National Taiwan University, Institute of Biochemical Sciences, Taipei, Taiwan, Republic of China, ⁴National Taiwan University, Department of Chemistry, Taipei, Taiwan, Republic of China

Phenyl glycosphingolipids (GSLs) bearing α -galactosyl head group (α Gal) and acyl-chain terminated with a phenyl derivative were found to be more potent than α -galactosylceramide (α GalCer) to stimulate both murine and human invariant natural killer T (iNKT) cells. Their activities have a strong correlation with the binding avidities and stability of the ternary interaction between iNKT T-cell receptor (TCR) and CD1d-GSL complex. In this study, we replaced the galactosyl group with glucose (α Glc) or mannose (α Man), and showed that GSLs with α Glc were stronger than those with α Gal in human but weaker in mice in inducing cytokines and expansion/activation of immune cells. However, addition of fluoride on the 4' or 6'-glucose-head phenyl-glycolipids could rescue the IFN- γ secretion in mice to comparable level as induced by GSL with α -galactosyl head. Using Ja18 knockout (KO) mice, we showed that most cytokines/chemokines induced by GSLs with either α Glc or α Gal were iNKT-dependent. On the other hand, it was the binding of GSLs to iNKT-TCR rather than CD1d that dictated the species-specific responses, as demonstrated by mCD1d vs. hCD1d swapping assay. Computer modeling of the ternary complex suggested that a H-bond interaction between Glc-GSL and human iNKT-TCR is lacking in murine TCR, which may attribute to the species differences in their iNKT responses to GSLs. Thus, our studies have not only uncovered a species specific immune response to GSLs with different glycosyl groups, due to differences in the TCR-glycolipid interactions but also provided a new insight for designing new generation of GSL for human use.

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Induction of adipocyte resident NKT cells can be instructed by non ligand-binding motifs of the TCR β chain

Vieih, J., Das, J., Ranaivoson, F., Comoletti, D., Denzin, L., Sant'Angelo, D.

Robert Wood Johnson Medical School, Rutgers University, The Child Health Institute of NJ, New Brunswick, United States

Invariant Natural Killer T (NKT) cells, a CD1d-restricted subset of ab T cells, have been increasingly recognized for their role in a wide variety of functions associated with autoimmunity, cancer, infection, tolerance, and obesity. The semi-invariant TCR expressed by NKT cells is unique in its recognition of glycolipids presented by CD1d, heterodimer rigidity, and limited binding footprint compared to conventional TCR:MHC interactions. This distinctive interaction has been studied extensively *in vitro*, though the role of the rigid $\alpha\beta$ heterodimer in the selection and development of NKT cells remains elusive. We investigated a hydrophobic patch on the b chain of the NKT TCR, suggested to play a role in heterodimer stability. Partial disruption of this patch, while permissive of ligand binding, resulted in decreased populations of NKT cells, diminished development, and effector function. Complete disruption of the patch resulted in the ablation of the NKT cell compartment entirely, while still

accommodating of conventional T cell development. NKT cells expressing the mutant NKT TCR acquired an adipose-resident NKT phenotype while in the thymus, and accumulated in the adipose tissue in mice. Hence, we show that *in vivo*, non-ligand binding regions of the NKT TCR are critical for the development of these important immune cells, and mediate the selection of adipose-resident NKT cells.

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A novel epigenetic regulator of early NKT cell development

Inoue, M.¹, Okamoto, K.¹, Negishi-Koga, T.², Nakashima, T.³, Takayanagi, H.¹

¹University of Tokyo, Tokyo, Japan, ²Showa University School of Dentistry, Tokyo, Japan, ³Tokyo Medical and Dental University, Tokyo, Japan

Natural killer T (NKT) cells are differentiated from CD4⁺CD8⁺ double positive (DP) cells that recognize CD1d-glycolipid complexes. After commitment to the NKT cell lineage, NKT cells undergo differentiation from stage 0 to stage 3 cells in thymus, in which several transcription factors such as PLZF and T-bet are involved. However, no epigenetic regulators that contribute to NKT cell development were reported. Here we identified a histone modifying enzyme (termed Himez) as an essential regulator of NKT cell development. Himez epigenetically regulates the expression of various genes related to cell cycle and survival in many tissues. Since Himez is highly expressed in T cells, we newly generated Himez-floxed mice and then crossed Himez-floxed mice with *Cd4-Cre* mice to generate T cell-specific Himez knockout mice. These mice possessed normal thymic cellularity. The Treg cell number was mildly decreased in thymus and spleen of Himez^{flox} *Cd4-Cre* mice. On the other hand, NKT cell number was markedly reduced in the thymus, spleen and liver of Himez^{flox} *Cd4-Cre* mice, due to an impaired differentiation in stage 1. Mixed bone marrow chimera experiments demonstrated that Himez regulates NKT cell development in a cell intrinsic manner. The transcriptome analysis using NKT stage 1 cells indicated that Himez is necessary for expression of several genes associated with NKT cell development. Collectively, Himez plays an essential role in early NKT cell development by regulating gene transcriptions. We would like to discuss the epigenetic modifications by Himez that influence NKT cell generation.

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Characterization of sulfatide-reactive type II NKT cells in mouse lungs

Kato, S.¹, Pasquet, L.¹, Adams, T.¹, Sharrow, S.¹, Davies-Hill, T.¹, Jaffe, E.¹, Xia, Z.¹, Suzuki, M.², Kovalovsky, D.¹, Berzofsky, J.¹, Terabe, M.¹

¹National Cancer Institute, National Institute of Health, Bethesda, United States, ²National Heart, Lung and Blood Institute, National Institute of Health, Bethesda, United States

Type II NKT cells are CD1d-restricted T cells that do not express an invariant TCR α expressed on all type I NKT cells. Similar to type I NKT cells, type II NKT cells have been reported to play a critical role in many disease models including autoimmune diseases and cancer. However, in contrast to type I NKT cells

much less is understood about type II NKT cells because of lack of widely available tools to identify them. Although there is no known antigen that can be recognized all type II NKT cells, sulfatide is a relatively well-defined self-antigen recognized by a fraction of type II NKT cells. By creating sulfatide-loaded CD1d-tetramers, we analyzed distribution of sulfatide-reactive type II NKT cells among different organs of mice and found a significant number in lungs. Characterization of lung sulfatide-reactive type II NKT cells revealed a strong contrast with type I NKT cells. They consisted of CD4⁺ and CD8⁺ populations while type I NKT cells are either CD4⁺ or double-negative. Lung sulfatide-reactive type II NKT cells do not express PLZF, which is a master regulator of type I NKT cell development. This result was confirmed with the observation that sulfatide-reactive type II NKT cells could develop in PLZF^{-/-} mice. Their V β repertoire was diverse like conventional T cells. Unexpectedly we also found that a significant fraction of sulfatide-reactive type II NKT cells express CD11b and Ly6C although they had lymphocytic morphology. Altogether, lung type II NKT cells have very unique characteristics.

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TAC1 is required for maintaining the numbers and functions of invariant NKT (iNKT) cells

Quah, P.S.¹, Fairfax, K.², Andrews, D.¹, Mackay, F.^{1,3}

¹Monash University Central Clinical School, Department of Immunology and Pathology, Melbourne, Australia, ²Walter and Eliza Hall Institute of Medical Research (WEHI), Parkville, Australia, ³University of Melbourne, Peter Doherty Institute for Infection and Immunity, Department of Microbiology and Immunology, Melbourne, Australia

Invariant NKT (iNKT) cells are innate-like T lymphocytes which co-express the invariant Ja18 T cell receptor and NK cell markers. Murine iNKT cells recognize lipid antigens presented on CD1d. The primary function of NKT cells is cytokine production where CD4⁻ iNKT cells produce Th1 cytokines while CD4⁺ iNKT cells produce both Th1 and Th2 cytokines. Among all antigen presenting cells that express CD1d, marginal zone (MZ) B cells express the highest levels. MZ B cells also express high levels of B cell activating factor (BAFF) receptor (BAFF-R) and transmembrane activator and calcium modulator and cyclophilin ligand interactor (TAC1). BAFF-R binds only to BAFF whereas TAC1 binds to both BAFF and a proliferation inducing ligand (APRIL). The purpose of this study is to investigate the role of BAFF, APRIL and their receptors in regulating iNKT cell numbers and functions. Comparing the numbers of iNKT cells in BAFF^{-/-}, BAFF Tg (overexpress BAFF), BAFF-R^{-/-} and TAC1^{-/-} mice to wildtype mice, we observed a significant reduction of iNKT cell numbers in TAC1^{-/-} mice. In addition, the level of IL-4 was significantly decreased in the sera of TAC1^{-/-} mice injected intraperitoneally with α -galactosylceramide. Our results show that TAC1 is important for maintaining the number and function of iNKT cells. iNKT cell defects have been observed in human patients with common variable immunodeficiency (CVID) and autoimmune disorders. Some of these patients are also TAC1 deficient. Therefore, studying the link between TAC1 and iNKT cells might provide a better clinical outcome for these patients.

136**c-Maf positively regulates IL-17A production in invariant NKT cells**

Yu, J.-S.¹, Hamada, M.², Ohtsuka, S.³, Yoh, K.⁴, Takahashi, S.², Miaw, S.-C.¹

¹College of Medicine, National Taiwan University, Graduate Institute of Immunology, Taipei, Taiwan, Republic of China, ²Faculty of Medicine, University of Tsukuba, Department of Anatomy and Embryology, Tsukuba, Japan, ³Graduate School of Comprehensive Human Sciences, University of Tsukuba, Department of Respiratory Medicine, Tsukuba, Japan, ⁴Faculty of Medicine, University of Tsukuba, Department of Nephrology, Tsukuba, Japan

c-Maf, a member of the large Maf family transcription factors, plays an important role in regulation of cytokine production and differentiation in Th2, Th17, Tfh and Tr1 cells. Many studies have shown that invariant natural killer T (iNKT) cells can produce Th-related cytokines such as IFN- γ , IL-4 and IL-17A. However, the role of c-Maf in iNKT cells and iNKT cells-mediated diseases is poorly understood. In this study, we demonstrate that α -galactosylceramide (α -GalCer)-stimulated iNKT cells express c-Maf transcript and protein. We further show that c-Maf-deficient iNKT cells produce less IL-17A, but not IFN- γ and IL-4, than their WT counterparts after α -GalCer stimulation by using c-Maf-deficient fetal liver cells-reconstituted mice both *in vitro* and *in vivo*. c-Maf deficiency does not affect the development and activation state of iNKT cells. In addition, induction of IL-17-producing iNKT (iNKT17) cells by IL-6, TGF- β and IL-1 β is also positively regulated by c-Maf *in vitro*. Furthermore, c-Maf-deficient iNKT17 cells lose the ability to recruit neutrophils to the lungs. Taken together, c-Maf is a positive regulator for IL-17A production in iNKT cells and potential therapeutic target in iNKT17 cells-mediated inflammatory diseases.

137**Repeated activation of lung invariant NKT cells results in chronic obstructive pulmonary disease-like symptoms**

Chuang, Y.-H.

National Taiwan University, Department of Clinical Laboratory Sciences and Medical Biotechnology, Taipei, Taiwan, Republic of China

Chronic obstructive pulmonary disease (COPD) is characterized by chronic airway inflammation, mucus hypersecretion, and emphysema, which lead to reduced lung function and breathlessness. The pathologies of COPD are due to an abnormal immune response. Invariant natural killer T (iNKT) cells are important population of innate lymphocytes and have been implicated in the regulation of immune responses associated with a broad range of diseases including COPD. We have here analyzed the role of iNKT cells in a model of COPD induced by repeated intranasal administration of iNKT cell agonist α -galactosylceramide (α -GalCer). Our results demonstrated that mice received repeated intranasal administration of α -GalCer had molecular and inflammatory features of COPD including airway inflammation with significant increases in infiltration of macrophages and lymphocytes, CD8⁺ T cells, as well as proinflammatory cytokines IL-6 and TNF- α . In particular, these mice also showed the presence of pulmonary emphysema,

mucus production, and pulmonary fibrosis. Furthermore, neutralization of IL-4 reduced α -GalCer induced emphysema. This study indicates the importance of iNKT cells in the pathogenesis of COPD by an IL-4 dependent mechanism.

138**CD1d-dependent lipid antagonist DPPE-PEG attenuates atherosclerosis**

Li, Y.^{1,2}, Kanellakis, P.¹, Hosseini, H.¹, Cao, A.¹, Deswaerte, V.¹, Tipping, P.², Toh, B.-H.², Bobik, A.^{1,3}, Kyaw, T.^{1,2}

¹Baker IDI Heart and Diabetes Institute, Melbourne, Australia, ²Southern Clinical School, Faculty of Medicine, Nursing and Health Sciences, Monash University, Centre for Inflammatory Diseases, Department of Medicine, Melbourne, Australia, ³Central Clinical School, Faculty of Medicine, Nursing and Health Sciences, Monash University, Department of Immunology, Melbourne, Australia

Atherosclerosis is responsible for heart attacks and stroke-leading causes of global mortality. As current lipid-lowering statins reduces risk of cardiovascular events by only one-third in patients with atherosclerosis. Thus, additional therapies to reduce atherosclerosis-related deaths are urgently needed. In this study, we investigate pharmacological effect of DPPE-PEG₃₅₀ that can prevent NKT atherogenicity in development and progression of atherosclerosis.

To test its effect in the development of atherosclerosis, DPPE-PEG350 was administered to ApoE^{-/-} mice at the beginning of 8 week high fat diet (HFD). We found that DPPE-PEG350 reduced aortic root atherosclerosis as well as lesion lipid content and macrophage accumulation without affecting NKT cells or other lymphocyte numbers. CD4/CD8 T cells and B cells in atherosclerotic lesions and lesion contents of MCP-1 and VCAM-1 proteins were decreased in DPPE-PEG350-treated mice. Necrotic core size, not lesion smooth muscle and collagen content were also reduced in DPPE-PEG350-treated mice. In progression experiment, 6 week HFD-fed ApoE^{-/-} mice were treated with DPPE-PEG350 for 6 weeks. DPPE-PEG350-treatment also showed reduced atherosclerosis in similar fashion. To confirm if DPPE-PEG350 reduces atherosclerosis by preventing CD1d-dependent lipid antigen presentation to NKT cells, we treated mice with DPPE-PEG₃₅₀ followed by NKT agonist, α -GalCer. We found that α -GalCer failed to aggravate atherosclerosis in DPPE-PEG₃₅₀-treated mice. In conclusion, our data suggest that DPPE-PEG₃₅₀ treatment attenuates both development and progression of atherosclerosis by preventing CD1d-dependent activation of NKT cells. DPPE-PEG₃₅₀ in combination with current lipid lowering statins has a therapeutic potential to reduce atherosclerosis-related mortality.

Tumour Immunology

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Characterization of the antitumor and immunomodulatory properties of new HDAC inhibitors as targeted anti-cancer drugs

Veloso Caixeta, A.¹, Soares Romeiro, L.A.², Grace Magalhães, K.¹

¹University of Brasilia, Laboratory of Immunology and Inflammation, Brasilia, Brazil, ²University of Brasilia, Laboratory of Development and Therapeutic Innovation, Brasilia, Brazil

The chemotherapy still remains the treatment of choice in the clinic against cancer. However its toxicity and side effects are limiting factors for its use. Immunotherapy is also often used in combination with other anticancer drugs. Currently there is a difficulty in obtaining drugs that have both antitumor and immunomodulatory effects. Inhibitors of histone deacetylases (HDACi) represent a new drug target class in which the histone acetylation associated with chromatin is changed, thereby modulating a gene transcription and factors involved in proliferation, migration, inflammation and cell death. HDACs appear to have a role in innate immunity and adaptive immunity. Thus, new drugs that target HDAC (LD 536, LDT537, and LDT80) were synthesized in order to be verified its antitumor and immunomodulatory properties. Murine macrophages, human monocytes (THP-1), peripheral blood mononuclear cell (PBMC), human breast cancer (MCF-7) and murine (4T1), and leukemia (K562) cell lines were treated in vitro with different HDAC inhibitors (LDT536, LDT537 and LDT80) as targeted anti-cancer drugs at different concentrations. After 24 hours cell viability was analyzed by MTT test. LDT536 and LDT537 compounds induced a significant selective decrease in cell viability non-invasive human breast cancer (MCF-7). The LDT536 decreased the cell viability of THP-1 only in the higher concentration 50 μ M. However, LDT537 showed to be selectively cytotoxic only over leukemia K562 cells. The data show that the drugs LDT536 and LDT537 could be potential drugs to be used in anti-breast and anti-leukemia cancers.

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Tumour resection in patients with non-small cell lung cancer does not modulate the adaptive immune system in a systemic manner

Cook, A.^{1,2}, Khong, A.^{1,2}, Salmons, J.^{1,2}, Millward, M.^{2,3}, Lake, R.^{1,2}, Nowak, A.K.^{1,2,3}, Robinson, B.W.S.^{1,2}

¹National Centre for Asbestos Related Diseases, Perth, Australia, ²University of Western Australia, School of Medicine & Pharmacology, Perth, Australia, ³Sir Charles Gairdner Hospital, Medical Oncology, Perth, Australia

Tumour resection is widely used to treat patients with early non-small cell lung cancer (NSCLC); however it is often not curative due to subsequent local recurrence or occult distant metastases. We have previously shown in preclinical models that survival outcomes of 75% tumour debulking surgery can be improved by combination with immunotherapy - enabling the adaptive immune system to delay outgrowth of any remaining tumour and having a curative effect in some animals. In the work described

here, our aim was to identify any relevant systemic changes in T cell-mediated immunity resulting from tumour resection in the clinical setting. Pre-surgery (< 1 week) and 1 month post-surgery peripheral blood samples were prospectively collected from 24 consenting adult patients with stage 1a, 1b or 2a operable NSCLC, including adenocarcinoma (15/24) or squamous cell carcinoma (8/24). Peripheral blood mononuclear cells (PBMC) were isolated, cryostored and phenotypically analysed by flow cytometry. Initial results show no significant alterations in the proportion of activated CD8+ T cells or CD4+ regulatory T cells, nor in the expression of activation (ICOS+) or proliferative (Ki67+) markers, after surgical resection. Lymphocyte expression of PD-1 and PD-L1 is currently under investigation and will be reported. We conclude that tumour resection in lung cancer does not modulate systemic adaptive cell-mediated immunity phenotypes, although functional implications remain unknown. This supports the potential for surgery to offer a partnership with immunotherapies such as checkpoint blockade with minimal additional considerations when compared to checkpoint blockade alone.

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Killed *Klebsiella pneumoniae* activates an organ-specific innate immune response to reduce tumor burden

Costa, A.¹, Perekslis, T.¹, Alexander, M.¹, Sanders, K.¹, Gunn, H.², Fiering, S.¹, Mullins, D.¹

¹Dartmouth College, Microbiology and Immunology, Lebanon, United States, ²Qu Biologics, Vancouver, Canada

Acute infection in a cancer-bearing organ has been associated with improved prognosis and enhanced survival, although the anti-cancer immune mechanisms remain undefined. We hypothesized that stimulating pathogen-specific immunity, using bacteria that have previously infected specific organs, may induce protective anti-cancer immune responses in those target organs. In collaboration with Qu Biologics, we evaluated *Klebsiella*-mediated anti-cancer efficacy in metastatic-like B16 melanoma using heat-killed *Klebsiella pneumoniae* (Qu Biologics' drug product QBKPN Site Specific Immunomodulator [SSI]). Subcutaneous injection of QBKPN SSI significantly reduced tumor burden in mice unexposed to *K. pneumoniae*, and pre-exposure significantly enhanced QBKPN SSI-induced anti-tumor immunity and control of metastatic-like B16 melanoma in the lungs. The anti-tumor efficacy in exposed mice correlated with an influx of monocytes and neutrophils, and is effective in RAG-knockout (lymphocyte deficient) mice, suggesting innate memory may be responsible. Furthermore, subcutaneous treatment with heat-killed *E. coli* (QBECO SSI) reduced lung tumor burden but not as substantially as with QBKPN, suggesting a species-specific response. Collectively, these data suggest pre-exposure to live *K. pneumoniae* may induce an innate memory response that is re-activated upon injection of subcutaneous heat-killed *K. pneumoniae* and induces a potent anti-tumor response in the lungs.

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Immune escape in primary melanoma*Behren, A.^{1,2}, Paessler, S.¹, Deb, S.¹, Cebon, J.^{1,2,3}, Klein, O.^{1,2}**¹Olivia Newton-John Cancer Research Institute, Cancer Immunobiology, Heidelberg, Australia, ²La Trobe University, School of Cancer Medicine, Heidelberg, Australia, ³Melbourne University, Department of Medicine, Melbourne, Australia*

Tumour infiltrating lymphocytes are prognostic in all stages of melanoma and may also predict as part of an inflamed tumour microenvironment for a response to immunotherapeutic interventions. Melanoma cells can escape the anti-tumour immune response either by immunoselection or immunosuppression, and it is currently not well understood if both processes are mutually exclusive or not. In immune selection, melanoma cells hide from the recognition of CD8⁺ T lymphocytes via downregulation and loss of melanoma specific antigens or components of the HLA class I complex. Alternatively, melanoma cells can escape anti-tumour immune responses by the expression of molecules or immunosuppressive cytokines that inhibit the cytotoxic potential of melanoma-specific T cells by engaging co-inhibitory receptors on their cell surface. One of these molecules, the programmed cell death ligand-L1, PD-L1 has been recognized as an important therapeutic target with monoclonal antibodies blocking the PD-1/PD-L1 interaction demonstrating significant clinical activity in patients with advanced melanoma.

The immune escape mechanisms of melanoma in early stages of the tumour development are currently not well defined. It can be predicted that anti-melanoma immunity is shaped at the initial encounter of the tumour and the host immune system and that this interaction may influence the further disease course and potentially the responsiveness to immunotherapy. Here, we defined the immune microenvironment in early stages of melanoma with a particular focus to discriminate immunoselection and immunoevasion by investigating the expression of PD-L1, HLA-class I and lymphocyte subset infiltration using multi-colour immunofluorescence in a large cohort of primary melanomas (n>50).

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Regulatory T cells in experimental colon carcinoma: effect of differential ratios of fish oil and corn oil*Malik, R., Agnihotri, N., Bhatnagar, A.**Panjab University, Biochemistry, Chandigarh, India*

The present study highlights effect of different ratios of fish oil (FO) and corn oil (CO) on T regulatory cell populations in rodent model of colon carcinoma.

Male Wistar rats were divided into six groups: Ethylenediamine-tetra acetic-acid (EDTA) treated group, dimethylhydrazine-dihydrochloride (DMH) treated (DMH/week for four weeks), FO:CO(1:1)+EDTA, FO:CO(1:1)+DMH, FO:CO(2.5:1)+EDTA, FO:CO(2.5:1)+DMH. The animals were maintained for 16 weeks after last treatment and sacrificed by cervical dislocation. CD4⁺/CD25⁺/Foxp3 (nTregs), CD4⁺/Foxp3 (iTregs) and intracellular cytokines (IL-6 and IL-10) were analyzed in colonic intraepithelial lymphocytes (IELs).

A significant decrease was observed in nTregs (1.38±0.42%) on DMH treatment whereas iTregs (9.22±2.93%) increased significantly (p<0.001) in this group as compared to control group (8.87±1.96%, 5.35±1.61% respectively). In FO+CO(1:1)+DMH and FO+CO(2.5:1)+DMH both, the Tregs increased significantly as compared to DMH group. In FO+CO(1:1)+DMH group, percentage of nTregs and iTregs was 14.4±2.96 and 13.67±3.15. In FO+CO(2.5:1)+DMH the percentage of nTregs was 16.86±2.58 and iTregs was 16.4±4.15. A significant (p<0.001) increase was observed in IL-10 and IL-6 expression in CD4 cells (13.76±1.96 and 40.37±7.5) on DMH treatment as compared to control group (6.88±0.11 and 9.15±2.3). Treatment with both the ratios of fish oil and corn oil led to a considerable decrease (p<0.001) in IL-10 expression (4.86±0.56 and 1.3±0.31 respectively) and IL-6 expression (25.14±4.8 and 20.05±2.6) with respect to DMH treated group.

The regulatory T cells and their cytokine expression was found to be affected by fish oil & corn oil in isolated IELs from rat colon cancer model in a dose and time dependent manner.

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Immunophenotyping breast tissue for the identification of new immune-therapeutic targets for breast cancer*Unsworth, A.¹, Vieusseux, J.¹, Dall, G.^{1,2}, Haynes, N.¹, Anderson, R.¹, Britt, K.¹**¹Peter MacCallum Cancer Centre, East Melbourne, Australia,**²Monash University, Anatomy and Developmental Biology, Clayton, Australia*

Background: Tumor infiltrating lymphocytes have been shown to predict clinical outcome in breast cancer (BCa) patients, leading to the application of several antibody-based immunotherapies for treatment of late stage BCa. The specific immune cell subtypes within the breast tissue responsible for predicting outcome, and their role in the early stages of breast cancer initiation however, has not been addressed. Investigating this area will determine whether currently available immunotherapies are applicable to BCa patients, and may also identify new immunotherapeutic targets for BCa.

Materials and methods: Using the transgenic MMTV-neuT spontaneous model of HER2+ BCa, we assessed the timing of breast cancer progression from hyperplasia through to DCIS and metastatic disease. We developed a mammary specific immunophenotyping FACS panel and used this to assess the immune cell compartment present in the mammary gland during hyperplasia and DCIS.

Results: Our immune profiling revealed an increase in the number of macrophages present in MMTV-neuT mice throughout hyperplasia and DCIS compared to WT mice. These changes are also evident prior to the histological appearance of hyperplasia. Furthermore, dendritic cells are also increased in MMTV-neuT mice during DCIS. We are currently treating these MMTV-neuT mice with anti-CSF-1R monoclonal antibody to deplete macrophages, and block the early stages of breast cancer development. This will allow us to determine the role of macrophages in the initiation and progression of BCa.

Conclusions: This research has identified macrophages and dendritic cells, as new potential targets for the use of immune-based therapeutics to treat early stage HER2+ breast cancer.

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IFN- γ exposure augments immunoinhibition caused by ganglioside enrichment of dendritic cells*Dillinger, B.¹, Ahmadi, S.¹, Erhart, F.¹, Lau, M.¹, Hölzl, M.A.¹, Jürgens, B.¹, Fuchs, D.², Heitger, A.¹, Ladisch, S.³, Dohnal, A.M.¹**¹Children's Cancer Research Institute, Tumor Immunology, Vienna, Austria, ²Innsbruck Medical University, Division of Biological Chemistry, Innsbruck, Austria, ³Children's National Medical Center, Center for Cancer and Immunology Children's Research, Washington, United States*

Gangliosides, known as biologically active molecules, are shed by tumor cells into their microenvironment (TME). There they can bind and inhibit the function of infiltrating dendritic cells (DCs), central for effective anti-tumor cellular immune responses. We posed the question, whether interferon-gamma (IFN- γ), capable both of priming and boosting DC function, is able to counteract ganglioside inhibitory effects on DCs. Dissecting the effects, we found:

- (i) DC ganglioside exposure reduced LPS/p38 signaling, explaining the previously observed strong down-modulation of factors that drive cellular immune responses including CD80, TNF- α and IL-12.
- (ii) IFN- γ , while strongly increasing LPS-dependent DC responses, did not overcome the inhibitory effect of GD1a.
- (iii) Instead, IRF-1 expression and LPS-independent induction of immunosuppressive indoleamine 2,3-dioxygenase (IDO) indicated that the immunoinhibitory IFN- γ /STAT1-signaling axis was active and unaffected by GD1a ganglioside enrichment.
- (iv) IFN- γ exposure reinforced the strong immune inhibition of LPS/GD1a-treated DCs, resulting in markedly impaired DC potential to stimulate CD8⁺ T-cells.

This combination--potent ganglioside inhibition of IFN- γ -boosted LPS immune stimulation of DCs and retained integrity of IFN- γ -induced immunosuppressive IDO activity--may contribute in creating a tolerogenic microenvironment *in vivo*. These factors, both released by tumors such as glioma, may play a keyrole in triggering clinically relevant immunosuppression. As future work we will validate the interplay of IFN- γ with gangliosides in clinical samples.

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Transgenic CAR mice for cancer immunotherapy*von Scheidt, B.^{1,2}, Yong, C.S.M.^{1,2}, Davenport, A.J.^{1,2}, Slaney, C.Y.^{1,2}, Darcy, P.K.^{1,2,3}, Kershaw, M.H.^{1,2,3}**¹Peter MacCallum Cancer Centre, Cancer Immunology Program, East Melbourne, Australia, ²University of Melbourne, Sir Peter MacCallum Department of Oncology, Parkville, Australia, ³Monash University, Department of Immunology, Clayton, Australia*

Adoptive immunotherapy using genetically modified lymphocytes has shown great promise in the treatment of blood cancers in the clinic. However, the challenge of eradicating solid tumours still remains. We developed a transgenic mouse model using a pan-haematopoietic promoter (*vav*) to drive the expression of a chimeric antigen receptor (CAR) specific for the tumour antigen human epidermal growth factor receptor 2 (Her2). This mouse model allows us to study the ability of

different subsets of CAR⁺ cells to mediate anti-tumour activity and their potential role in enhancing immunotherapy against cancer. We also describe two transgenic mouse models which generate dual-specific CAR T cells. T cells in these mice express a Her2-specific CAR as well as either a T cell receptor (TCR) specific for the pre-melanocyte antigen (pMEL, gp100) (CARaMEL) or alternately an OT-I TCR, recognising the ovalbumin peptide SIINFEKL (CAR.OT-I). We found that CAR⁺ cells in all three mouse models were functional and mediated robust antigen-specific responses through proliferation, cytokine release and cytotoxicity, *in vitro* and *in vivo* against Her2-expressing tumours. Our data show that expression of an antigen-specific TCR by dual-specific CAR T cells can induce the expansion of CAR⁺ T cells in the tumour in response to vaccination, thereby enhancing the anti-tumour response. These new CAR mouse models provide a ready source of CAR expressing immune cells for further studies to improve the adoptive immunotherapy against solid cancers.

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System-level effects of ectopic galectin-7 reconstitution in cervical cancer and their microenvironment*Rincon-Orozco, B.¹, Higareda, J.², Rabinovich, G.³**¹Universidad Industrial de Santander, School of Microbiology, Bucaramanga, Colombia, ²Helmholtz Zentrum München Deutsches Forschungszentrum für Gesundheit und Umwelt, Institute for Diabetes and Cancer, Neuherberg, Germany, ³Instituto de Biología y Medicina Experimental (IBYME), Laboratorio de Inmunopatología, Buenos Aires, Argentina*

Cancer can be conceived as a complex cross talk of molecular networks that are shaped by a particular microenvironment in a spatio-temporal fashion. However, our understanding of how these networks adapt in response to a specific gene expression remains largely unexplored. Such knowledge would be critical to comprehend the role of galectin-7, which is negatively regulated in cervical cancer, and appears to be a link between the apoptotic response triggered by cancer and the surveillance of the immune system to annihilate the malignant cell.

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Immune cell composition in human non-small cell lung cancer*Stankovic, B.¹, Korsmo, H.A.¹, Müller, E.^{1,2}, Aamodt, H.^{3,4}, Beraki, K.¹, Bækkevold, E.⁵, Woldbæk, P.R.³, Jahnsen, F.³, Øynebråten, I.¹, Corthay, A.^{1,2}**¹Oslo University Hospital-Rikshospitalet, Department of Pathology, Tumor Immunology Group, Oslo, Norway, ²University of Oslo, Department of Biosciences, Oslo, Norway, ³Oslo University Hospital Ullevål, Department of Cardiothoracic Surgery, Oslo, Norway, ⁴Oslo University Hospital-Rikshospitalet, Oslo, Norway, ⁵Oslo University Hospital, Rikshospitalet, Liipat, Department of Pathology, Oslo, Norway*

Lung cancer is the most common cause of cancer related death worldwide. Non-Small Cell Lung Cancer (NSCLC) is the most frequent type, representing about 85% of all lung cancer patients. Presently, the TNM staging, which is based on tumor size and localization, is used for diagnosis and prognosis in

NSCLC. However, previous reports suggest that the analysis of tumor-infiltrating immune cells may represent a more accurate prognostic tool. The aim of this project was to perform comprehensive analysis of tumor-infiltrating immune cells in NSCLC using flow cytometry, as a first step to understanding the relationship between tumor-infiltrating immune cells and clinicopathological parameters. We used 10-color flow cytometry to investigate immune cells in tumors, distant lung tissue, lymph node, and peripheral blood, from 67 patients with primary NSCLC. The following populations of tumor-infiltrating immune cells were identified: CD4⁺ T cells, CD8⁺ T cells, each with memory and naive phenotypes; CD19⁺ B cells, with naive, memory, germinal center and plasma cell subsets; CD14⁺ macrophages, CD123⁺ plasmacytoid dendritic cells, CD11c⁺CD1c⁺ dendritic cells (DCs), and CD11c⁺CD141⁺ DCs, CD3⁺CD56⁺ natural killer T cells (NKT), CD56⁺ natural killer (NK) cells, with CD16⁺ and CD16⁻ subsets, and all four granulocyte populations. Statistical analysis revealed increased percentage of CD45⁺ leukocytes within tumors compared to distant lung tissue. Among leukocytes, B cells showed increased frequency in tumor compared to the distant lung ($p=0.0001$). This suggests that the tumor microenvironment of NSCLC recruits immune cells and has a different immunological structure compared to normal lung tissue.

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Distribution of the potential target for immunotherapy BTN2A1 in cancer and normal tissues

Tutuka, C.¹, Hudson, C.¹, Woods, K.¹, Prato, S.², Panousis, K.², Maraskovsky, E.², Cebon, J.¹, Behren, A.¹

¹Olivia Newton-John Cancer Research Institute, Cancer Immunology, Heidelberg, Australia, ²CSL Limited, Parkville, Australia

Cancer immunotherapy targets immune-regulatory signal molecules such as the programmed cell death receptor PD-1 or its ligand PD-L1 and cytotoxic T lymphocyte antigen CTLA4. Clinical trials have shown encouraging outcomes including durable responses first seen in melanoma. However, a subset of patients does not respond.

With the success of these therapies and the deeper understanding of the immune-system and its regulation in the context of cancer, more and more potential immune-regulatory molecules are being discovered and examined. We recently discovered one of these molecules following a high-throughput screen for novel tumor antigens in melanoma in collaboration with CSL Limited. The screening outcome led to the discovery of a protein belonging to the Butyrophilin (BTN) family on the surface of melanoma cells called BTN2A1. BTN2A1 shares sequence and structure similarity to the B7 family of immune-regulatory molecules, which include B7-1 and B7-2 in mice. The purpose of this project was to evaluate BTN2A1 expression in human cancers. We have found that BTN2A1 was generally more highly expressed in cancer cells compared to their normal tissue counterparts. BTN2A1 had high levels of membrane expression in 8/10 melanomas tested and was over expressed in 9/9 pancreatic adenocarcinomas, 8/15 hepatocellular carcinomas, 14/24 breast and 6/14 prostate cancers.

Multiplex fluorescent immunohistochemical staining with

the VECTRA (Perkin Elmer®) system also enables staining of multiple immune markers. In ongoing studies we are evaluating the expression patterns and spatial relationships of different immune-regulatory molecules such as BTN2A1 and PD-L1 within the same tumour samples.

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Reduction of gastric cancer proliferation and invasion by miR-15a mediated suppression of Bmi-1 translation

Wu, C.¹, Zheng, X.², Li, X.³, Fesler, A.⁴, Hu, W.¹, Chen, L.², Xu, B.², Wang, Q.², Tong, A.⁵, Burke, S.⁴, Ju, J.⁴

¹The Third Affiliated Hospital of Soochow University, Department of Oncology, Changzhou, China, ²The Third Affiliated Hospital of Soochow University, Department of Biological Treatment, Changzhou, China, ³The Third affiliated Hospital of Soochow University, Department of Oncology, Changzhou, China, ⁴Stony Brook University, Translational Research Laboratory, Department of Pathology, New York, United States, ⁵BioGenex Inc, Fremont, United States

B-cell specific moloney leukemia virus insertion site 1 (Bmi-1) gene plays important roles in gastric cancer, but the epigenetic regulatory mechanism by microRNA (miRNA) and the functional significance of Bmi-1 inhibition in gastric cancer remains elusive. In this study, we systematically investigated the functional roles of miRNA mediated Bmi-1 suppression in gastric cancer. Our results show that the expression of miR-15a is significantly reduced in gastric cancer and the protein expression levels of Bmi-1 are inversely correlated with miR-15a ($P=0.034$) in gastric cancer patientsamples. Functional studies revealed that ectopic expression of miR-15a decreased Bmi-1 in gastric cancer cell lines with reduced proliferation and tumor invasion. High levels of Bmi-1 in gastric cancer patients are significantly associated with better overall survival ($P=0.02$) based on the Kaplan-Meier survival analysis.

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High-dose vitamin C modulates CXCR4/CXCR7/CXCL12 chemokine receptor/ligand axis in MDA-MB-231 breast cancer cells

Akekawatchai, C.¹, Changsri, K.¹, Soymanee, T.¹, Fungkrajay, M.¹, Tuntipopipat, S.²

¹Faculty of Allied Health Sciences, Thammasat University, Department of Medical Technology, Pathumthani, Thailand, ²Institute of Nutrition, Mahidol University, Nakornpathom, Thailand

High-dose ascorbic acid as a treatment for cancer has recently been reexamined and mechanisms underlying its anti-tumor activity are still unclear. CXCR4/CXCR7/CXCL12 chemokine system has been demonstrated to participate in natural selection of breast cancer cells in invasion and metastasis. Functional CXCL12 expression appears to promote invasiveness and apoptosis in the cells. Therefore, this study aimed to examine an effect of high-dose ascorbic acid, potentially achieved by clinical intravascular injection, on expression and function of CXCR4/CXCR7/CXCL12 system in MDA-MB-231 breast cancer cells. The cells were characterized for their response to ascorbic acid in

clinical relevant conditions and expression of CXCR4, CXCR7 receptors and CXCL12 ligand by real-time PCR, immunostaining and flow cytometry, and ELISA. The data showed that there was up to approximately 50% reduction of viability of the treated-MDA-MB-231 cells. Analysis by annexin V-PI stain and flow cytometry, and activated caspase detection assay indicated that the decrease of cell survival was due to apoptosis. In this cell line, a strong constitutive expression of CXCR4 was observed whereas CXCR7 and CXCL12 were weakly expressed. Ascorbic acid treatment caused a significant increase of CXCL12 secretion and induction of CXCR7 expression, while CXCR4 remained highly expressed in the treated cells. Blocking CXCL12 ligands by the selective inhibitor chalcone 4 decreased ascorbic acid-induced apoptosis of the cells. Our findings indicated that high-dose ascorbic acid modulates CXCR4, CXCR7 and CXCL12 expression, potentially affecting the selection processes in metastasis of breast cancer cells, and are useful for therapeutic application of high-dose vitamin C in cancer.

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The role of NADPH oxidase 2 on M2 tumor-associated macrophages differentiation in pancreatic cancer progression

Chen, C.-J.¹, Hou, Y.-C.¹, Wang, H.-C.¹, Shieh, C.-C.^{1,2}, Shan, Y.-S.^{1,3}
¹National Cheng Kung University, Tainan, Taiwan, Republic of China, ²National Cheng Kung University Hospital, Pediatrics, Tainan, Taiwan, Republic of China, ³National Cheng Kung University Hospital, Surgery, Tainan, Taiwan, Republic of China

M2 Tumor-associated macrophages (M2-TAMs) are a dominant component of inflammatory cells in the tumor microenvironment and have been shown to promote tumor development and progression. Reactive oxygen species (ROS) have been reported essential for differentiation of TAMs. In this study, we aimed to explore the role of NADPH oxidase 2 (NOX2) in M2-TAMs differentiation. After orthotopically inoculated cancer cells (KPC) from Pdx-Cre Kras^{G12D/+}p53^{-/-} mice into the pancreas of *Cybb*^{-/-} and wild type mice, the tumor size was significantly smaller in *Cybb*^{-/-} mice than in wild type mice. Interestingly, the expression of mouse M2-TAM markers CD206 and YM1 was also reduced in pancreatic tumors of *Cybb*^{-/-} mice, suggesting that knockout of *Cybb* gene to downregulate NOX2 function could impede M2-TAMs formation and inhibit tumor growth. Then bone marrow cells isolated from the *Cybb*^{-/-} mice were stimulated using GM-CSF to differentiate into macrophages. After co-cultured with KPC cells, the *Cybb*^{-/-} mice-derived macrophages failed to skew M2-TAMs. In conclusion, our study demonstrates that CYBB gene function is required for M2-TAMs differentiation and may be a potential therapeutic target for pancreatic cancer treatment.

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Modulating T-cell cholesterol metabolism to treat cancer

Yang, W., Bai, Y., Xu, C.

Institute of Biochemistry and Cell Biology Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China

CD8+ T cells play a central role in antitumour immunity, but their activity is suppressed in the tumour microenvironment. Reactivating the cytotoxicity of CD8+ T cells is of great clinical interest in cancer immunotherapy. Here we report a new mechanism that the antitumour response of CD8+ T cells can be potentiated by modulating cholesterol metabolism. Upon activation, T cells undergo a reprogramming of cholesterol metabolism to support acquisition of effector function and fast proliferation. Inhibiting cholesterol esterification leads to potentiated effector function and enhanced proliferation of CD8+ T cells. This is attributed to the elevation of the plasma membrane cholesterol level of CD8+ T cells that causes enhanced T-cell receptor clustering and signalling as well as more efficient formation of immunological synapse. CD8+ T cells with impaired cholesterol esterification showed better effect in controlling melanoma growth and metastasis in mice than wild-type CD8+ T cells. We further show that a small molecule inhibitor of cholesterol esterification has good antitumour effect in preclinical models. A combined therapy of the inhibitor and α -PD-1 antibody showed better efficacy than monotherapies in controlling tumour progression. Modulating T-cell cholesterol metabolism is therefore a promising new strategy to treat cancer.

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Immunotherapy of solid tumours using complete Freund's adjuvant

Carroll, C.S.E.¹, Andrew, E.R.¹, Neeman, T.², Fahrer, A.M.¹

¹Australian National University, Research School of Biology, Acton, Australia, ²Australian National University, ANU Statistical Consulting Unit, Acton, Australia

The field of cancer immunotherapy aims to modulate immune responses to enhance tumour destruction. The aim of this project is to investigate the efficacy of an immunotherapeutic cancer vaccine. The hypothesis is that injecting a strong immunostimulant directly into tumours can induce an anti-tumour immune response (Fahrer, 2012). Mice bearing subcutaneously injected tumours were treated with intratumoural CFA. Tumours were measured regularly, and immune infiltrates in tumours were collected at pre-determined time points by fine needle aspiration and analysed by flow cytometry. Fine-needle aspiration allowed us to investigate the time course of immune cell infiltration and then correlate it to survival data. CFA treatment of P815 mastocytomas resulted in a statistically significant survival increase and a reduction in tumour growth rate (p-value < 0.0001, < 0.0001 respectively). CFA treatment also resulted in a reduced incidence of mice with observable liver metastases at necropsy (p-value 0.0121). In the P815 model, increased survival was correlated to increased infiltration of neutrophils into tumours on days 1-5 post-treatment. Neutrophil depletion abrogated CFA treatment efficacy.

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Tim-3, a negative player in liver tumor microenvironment*Ma, C.**Shandong University School of Medicine, Jinan, China*

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide that accounts for half a million deaths per year. Accumulated data highlighted the important role of tumor microenvironments in the progression of HCC. Liver is rich of innate immunes such as NK cells and macrophages which constitute the important stromal components of tumor microenvironments. T cell immunoglobulin and mucin-domain containing protein-3 (Tim-3) has been recognized as a key negative regulator of

T cell-mediated responses, and abundant Tim-3 expression has also been demonstrated in both NK cells and macrophages. In the present study, we investigated the expression pattern and the roles of Tim-3 in tumor infiltrated macrophages and NK cells in HCC. Augmented Tim-3 expression was detected on tumor associated macrophages (TAMs) and tumor infiltrated NK cells (TINK) from HCC patients and orthotopic transplanted mice model of liver tumor. The increased Tim-3 expression on TAM negatively correlated with survival of HCC patients. Interference of Tim-3 expression in NK cells or macrophages suppressed HCC growth. In vitro study showed that Tim-3 expression was promoted by TGF- β . Tim-3 blocking greatly enhanced NK cells cytotoxicity and IFN- γ /TNF- α production. Moreover, Tim-3 enhanced the TGF- β mediated alternative activation of macrophages, which might be due to the reduced production of IL-6 in macrophages. These data suggest Tim-3 as the potential target in HCC immunotherapy which might both booster NK cells-mediated antitumor immunity and revise macrophages-induced immunosuppression in tumor microenvironments.

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The impact of microbiota on the efficacy of immune checkpoint blockade*Duong, C.^{1,2}, Vétizou, M.^{1,2}, Zitvogel, L.^{1,2,3,4}*

¹Gustave Roussy Cancer Campus, Villejuif, France, ²INSERM Unit U 1015, Villejuif, France, ³Université Paris-Saclay, Faculté de Médecine, Le Kremlin-Bicêtre, France, ⁴Center of Clinical Investigations in Biotherapies of Cancer, Villejuif, France

The curative potential of immunotherapy has been demonstrated through the combined targeting of the immune checkpoints CTLA4 and PD1. Accordingly, ipilimumab and nivolumab have recently been approved in metastatic melanoma following reported clinical response rates of 60%. However, reports of immune-related adverse events are concerning, these often occurring at sites exposed to commensal microbiota. Recent studies have demonstrated the importance of gut microbiota to the efficacy and toxicity of CTLA4 and PDL1 blockade. Therefore, we hypothesise that the efficacy and toxicity of combined CTLA4 and PD1 blockade is also dependent on microbiota. To test this, mice were treated with or without a cocktail of antibiotics (ATBx),

inoculated with either MCA205 sarcomas or the melanoma cell line RET, and treated with α -CTLA4, α -PD1, or a combination of both Abs. Interestingly, we also observed a decrease in overall survival (MCA205) and tumour growth (RET) in ATBx-treated mice receiving the combination therapy. We will next determine whether specific bacteria identified to improve the efficacy of CTLA4 and PDL1 blockade in mice are able to compensate for the lack of microbiota in our combination. Various organs, including lymph nodes and spleen will be analysed by flow cytometry to assess changes in the immune status and gut pathology will be assessed for toxicity by immunohistochemistry. This project will evaluate which species of microbiota are involved in the efficacy and toxicity of immune checkpoint inhibitors and will enhance our understanding of their mechanism of action, enabling better prediction of patients susceptible to treatment-related toxicity.

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Transmissible cancers and the immunogenic role of *Trypanosoma cruzi* calreticulin*Cruz, P.¹, Torres, C.², Ferreira, A.¹*¹University of Chile, Faculty of Medicine, Immunology, Santiago, Chile,²University of Chile, Faculty of Veterinary Medicine, Santiago, Chile

Transmissible cancers are important due to both their capacity to be easily transmitted among allogenic individuals, and their significant potential of endangering wildlife. Canine transmissible venereal tumor (CTVT) and the devil facial tumor disease (DFTD) are transmissible cancers naturally occurring in dogs (*Canis lupus familiaris*) and Tasmanian devils (*Sarcophilus harrisi*), respectively. CTVT cells evade the host immune system by secreting TGF- β 1 and downregulating the expression of MHC class I molecules in tumor cells. Furthermore, tumor-secreted TGF- β 1 downregulates cell-mediated cytotoxicity by impairing IFN- γ secretion by T and natural killer cells. Treatments of CTVT and DFTD are complex (e.g. vincristine treatment is effective only in CTVT and recurrences are frequent). Therefore, in order to promote tumor rejection, new therapeutic or prophylactic alternatives are needed, especially in Tasmanian devils, which are under severe conservation threat due to DFTD. We have determined that *Trypanosoma cruzi* (the agent of American trypanosomiasis) calreticulin (TcCRT), an endoplasmic reticulum (ER) resident chaperone, is a potent anti-angiogenic and anti-tumor agent. (Indeed, TcCRT can be translocated from the ER to the parasite exterior). We have also cloned and expressed recombinant TcCRT (rTcCRT). Currently we investigate whether TcCRT promotes tumor rejection by increasing the immunogenicity of CTVT cells. We now assess the rTcCRT binding to the surface of cultured CTVT cells, with subsequent and consequent binding of complement component C1q, which opsonizes cancer cells for engulfment by dendritic cells (DCs). In the regional lymph nodes these DCs will promote cytotoxic

T cell responses will resulting significant tumor growth inhibition.

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The critical role of macroautophagy for MHC class II presentation of tumor antigens by dendritic cells

Oh, D.S., Lee, H.K.

KAIST, Daejeon, Korea, Republic of

Autophagy has been implicated in the presentation of cytoplasmic and viral antigens on MHC class II molecules. However, the role of the autophagic process in the presentation of phagocytized tumor associated antigens (TAAs) *in vivo* remains unclear. Following administration of apoptotic tumor antigen, mice with a dendritic cellspecific deletion of Atg5, which is a key autophagy gene, exhibited reduced CD4+ cell priming, but not CD8+ cytotoxic T cell priming. Atg5 deficient dendritic cells have a defect in the MHC class II processing and presentation of phagocytized tumor antigens. In contrast, Atg5 is not essential for crosspresentation of peptides on MHC class I molecules in dendritic cells or macrophages. Further, Atg5 deficient dendritic cells and macrophages had elevated mRNA expression of class A scavenger receptors and showed increased phagocytosis of apoptotic tumor cells. Thus, our study demonstrated that dendritic cells utilize autophagic machinery to process and present apoptotic tumor antigens for MHC class II presentation.

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Autologous bone marrow Th cells can support multiple myeloma cell proliferation *in vitro* and *in vivo*

Wang, D.^{1,2}, Fløisand, Y.³, Tveita, A.⁴, Bürgler, S.⁵, Parente-Ribes, A.⁴, Hofgaard, P.⁴, Schjesvold, F.⁴, Szodoray, P.⁴, Nakken, B.⁴, Bogen, B.^{2,4,6}, Tjønnfjord, G.^{2,3}, Dalgaard, J.^{3,7}, Munthe, L.^{2,4}

¹Oslo University Hospital-Rikshospitalet, Institute of Immunology/Centre for Immune Regulation, Oslo, Norway, ²Institute of Clinical Medicine, University of Oslo, Oslo, Norway, ³Oslo University Hospital, Dept. of Hematology, Oslo, Norway, ⁴Centre for Immune Regulation, Oslo University Hospital, Dept. of Immunology, Oslo, Norway, ⁵Swiss Experimental Infectious Diseases and Cancer Research, University Children's Hospital, Zurich, Switzerland, ⁶KG Jebsen Centre for Influenza Vaccine Research, Institute of Clinical Medicine, University of Oslo, Oslo, Norway, ⁷Vestre Viken Trust, Drammen Hospital, Dept. of Medicine, Oslo, Norway

Multiple myeloma (MM) is a cancer of end-differentiated B cells (plasma cells). MM cell growth is supported by the microenvironment by poorly defined cellular and molecular mechanisms. MM cells express CD40, a receptor known to activate autocrine secretion of cytokines and proliferation. However, the source of CD40 ligand has been unclear. Here we show that activated autologous T helper (Th) cells can support MM cell growth *in vitro* and *in vivo*. CD40-activated MM cells secreted cytokines that attracted Th cells, suggesting that MM cells actively recruit Th cell partners. MM cells had retained sufficient antigen presentation function to activate Th cells and engage in a MM cell - Th cell collaboration. The results suggest that Th cells may support the expansion of MM cells in patients and that such a mechanism may be a caveat in terms of treatment of multiple MM patients with check-point inhibitors.

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Inhibitory activity and major effective compounds of the products converted from glucose by *Phomopsis sp.* XP-8 resting cells

Shi, J.¹, Li, Q.¹, Zhang, Y.², Wang, Y.¹, Huang, Q.¹, Shao, D.¹

¹Northwestern Polytechnical University, School of Life Sciences, Xi'an, China, ²Northwest A&F University, College of Food Science and Engineering, Yangling, China

Resting cells of *Phomopsis sp.* XP-8, an endophytic fungus isolated from *Eucommia ulmoides* Oliver, were previously found to have capability to convert glucose into pinoresinol (Pin), a plant-derived lignin that has multiple functions including potential of anti-tumor. Inhibition effects on HepG-2 and K562 cells of the culture broth of *Phomopsis sp.* XP-8 resting cells in glucose solution were assessed and the major effective compounds were identified in this study. As results, the culture broth significantly inhibited HepG-2 and K562 cells by decreasing their viability and inducing apoptosis via up-regulated the expression of apoptosis related genes. It also significantly inhibited HepG-2 cells adhere to the plate, and inhibited them migrate by blocking the expression of MMP-9. After separately evaluating the inhibitory activity of each compound detected in the culture broth, Pin and pinoresinol monoglucoside were identified as the major effective components. Methoxyquercetin also found in a high amount but did not show inhibitory effects on tumor cells. The results indicated the potential application of *Phomopsis sp.* XP-8 in producing anti-tumor products by bioconversion of glucose.

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Tumor-infiltrating T-cells of ovarian cancer express markers associated with functional impairment

Rådestad, E.¹, Klynning, C.², Mattsson, J.^{1,3}, Norström, M.M.⁴, Sundberg, B.¹, Levitsky, V.⁵, Uhlin, M.^{1,3,6}

¹Karolinska Institutet, Department of Oncology-Pathology, Stockholm, Sweden, ²Karolinska University Hospital, Department of Gynaecology, Stockholm, Sweden, ³Karolinska University Hospital, Centre for Allogeneic Stem Cell Transplantation, Stockholm, Sweden, ⁴Pharmaceutical Sciences (PS), Roche Pharmaceutical Research and Early Development (pRED), Roche Innovation Center, Basel, Switzerland, ⁵Molecular Partners AG, Schlieren-Zurich, Switzerland, ⁶Royal Institute of Technology, Department of Applied Physics, Stockholm, Sweden

Suppression of immune reactivity by increased expression of co-inhibitory receptors has been discussed as a major reason as to why the immune system fails to control tumor development. Elucidating the co-inhibitory pattern of tumor-infiltrating lymphocytes in different cancer types is of great importance for development of future treatment strategies.

We performed an extracellular characterization with primary focus on coinhibitory and co-stimulatory receptors on T-cells freshly isolated from blood, tumor-associated ascites, and metastatic tumor tissue from ovarian cancer patients (n=24) at late-stage disease (III-IV).

The results showed significantly higher frequencies of T-cells expressing co-inhibitory receptors LAG-3 (median 9.4%), PD-1 (62.6%), TIM-3 (7.5%), and CTLA-4 (4.0%) in tumor tissue

compared to T-cells isolated from blood (0.8%, 13.4%, 0.1%, and 0.7% respectively). The frequency of T-cells lacking expression of LAG-3, PD-1, and TIM-3 were decreased in tumor tissue (35.4%) and ascites (59.5%) compared to blood (87.0%). A significantly higher frequency of potential regulatory T-cells (CD4⁺/CD25^{high}CD127^{-low}) was observed in both tumor tissue (24.1%) and ascites (12.6%) compared to blood (5.7%). Analysis of soluble factors by Luminex revealed higher concentrations of G-CSF, IL-5, IL-6, IL-8, IL-10, IP-10, MCP-1, MIP1a, MIP1b, and TNF- α in ascites compared to blood.

Our data confirms that the late stage metastatic ovarian tumor environment exhibits an inflammatory milieu with abundant presence of infiltrating immune cells expressing co-inhibitory receptors. Clinical data and functional studies are necessary to obtain insight into the anti-tumor reactivity of these cells, elucidate the observed heterogeneity between patients, and evaluate possible novel treatment strategies such as checkpoint inhibitors.

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Assessment of checkpoint blockade combined with NKT cell-dependent glycolipid-peptide vaccines

Petley, E.V.^{1,2}, Osmond, T.L.¹, Farrand, K.J.¹, Tang, C.¹, Ancelet, L.R.¹, Anderson, R.J.³, Painter, G.F.³, Hermans, I.F.¹

¹Malaghan Institute of Medical Research, Wellington, New Zealand, ²Victoria University of Wellington, School of Biological Science, Wellington, New Zealand, ³Ferrier Research Institute, Wellington, New Zealand

While inhibition of immune checkpoints treatments have validated the concept of enhancing T cell-mediated immune responses for cancer treatment, it is clear that not all patients respond. Therefore, there is considerable interest in whether the use of vaccines can improve responses to checkpoint blockade, particularly in patients that do not have pre-existing anti-tumour immunity. Here we generated a new synthetic cancer vaccine conjugating the tumour-associated antigen survivin to an isomer of α -Galactosylceramide (α -GalCer), a potent invariant natural killer T (iNKT) cell agonist with documented adjuvant activity. Upon antigen encounter, activated iNKT cells are capable of licensing dendritic cells (DCs) through CD40-CD40L interaction to stimulate potent anti-tumour CD8⁺ T cell responses. Conjugation of α -GalCer to the survivin-derived peptide was achieved via an enzymatically cleavable linker sensitive to cathepsin B activity. This strategy thereby favours the co-presentation of both active components on the same DC. We combined our vaccine with the immune-checkpoint blockade inhibitors α -PD-1, α -CTLA-4, α -LAG-3 and α -CD40, in an implantable mouse model of glioma, to fully reveal the immunogenicity of our vaccine. Our preliminary data demonstrates that combining the conjugate vaccine with α -CTLA-4 improved tumour clearance compared to α -CTLA-4 monotherapy in an implantable mouse model of glioma, suggesting that this combination may be a useful therapy for patients with glioma.

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Immune infiltrates in murine solid tumour models treated with bacterial based cancer immunotherapies

Andrew, E.¹, Carroll, C.¹, Schulte, K.-M.², Neeman, T.³, Fahrer, A.¹

¹The Australian National University, Research School of Biology, Acton, Australia, ²The Australian National University, The John Curtin School of Medical Research, Acton, Australia, ³The Australian National University, Statistical Consulting Unit, Acton, Australia

Our lab is testing a novel, simple cancer immunotherapy: intra-tumoural injection of Complete Freund Adjuvant (CFA). CFA contains heat-killed mycobacteria in oil which is emulsified into a water-in-oil emulsion. Our hypothesis is that, due to the Pathogen-associated Molecular Patterns (PAMPs) present on the mycobacterium, CFA will stimulate a strong anti-tumour immune response when injected directly into the solid tumour. Coley's toxins, heat-killed preparations of *Streptococcus pyogenes* and *Serratia Marcescens* used by Dr William Coley in the early 19th Century, may also work by this mechanism. Previous work in the Fahrer lab analysing the anti-tumour effect of CFA on a murine mastocytoma model revealed that CFA increases survival of treated mice. Using the novel technique of repeated fine needle aspiration (FNA) combined with flow cytometry we are able to monitor tumour infiltrates in individual mice over time. Currently we can analyse the distribution of 7 immune cell subtypes for 8 time points per mouse, thus linking changes of immune cell subtypes with the survival of our mice. This technique allows us to distinguish the immune infiltrates resulting from bacterial based immunotherapies in multiple solid tumour types. We have associated survival with high levels of neutrophils between 1-5 days after CFA injection in mastocytoma. The aim of this study is to analyse the immune infiltrates and compare the efficacy of CFA and Coley's toxins in multiple murine tumour models. The results of this study are presented.

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Simple and effective tumour immunotherapy using intratumoural complete Freund's adjuvant

Fahrer, A.¹, Carroll, C.¹, Andrew, E.¹, Orange, M.², Allavena, R.³

¹The Australian National University, Research School of Biology, Acton, Australia, ²Arlesheim Klinik, Onkologie, Arlesheim, Switzerland, ³University of Queensland, School of Veterinary Science, Gatton, Australia

We demonstrate that intra-tumoural injection of Complete Freund's Adjuvant can result in a potent anti-tumour immune response.

In order to test the efficacy of this treatment, we have initiated pre-clinical trials in three species: mice, dogs and horses. Efficacy has been demonstrated in a range of solid tumours, including mastocytoma, mammary tumours and melanoma. Complete tumour regressions have been observed in all three species. Evidence of systemic immune responses (regression of non-injected metastases) have also been observed. We characterise the immune cells infiltrating mouse tumours after treatment. Finally we provide case reports on the treatment of four human cancer patients; suffering from lung cancer, metastatic

osteosarcoma, or breast cancer, with one patient having a partial response to treatment with intra-tumoural Complete Freund's Adjuvant.

Taken together, our data demonstrate that our treatment has major anti-tumour effects in a proportion of treated animals, and is safe for use in human cancer patients. Further trials in human cancer patients are therefore strongly warranted. Our novel treatment provides a simple and inexpensive cancer immunotherapy, applicable to a wide range of solid tumours, and of potential benefit to cancer patients around the world, including patients from developing countries.

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Cancer vaccine design based on tumor cell lysates and adjuvants

Salazar-Onfray, F.

Universidad de Chile, Facultad de Medicina, Santiago, Chile

Dendritic cells (DCs) loaded with tumor-associated antigens (TAAs) have emerged as promising immunological tools for cancer therapy due to their abilities to stimulate the antitumor response and trigger immunological memory in cancer patients. Nevertheless, an important percentage of patients treated with DCs remain refractory. The optimal delivery of antigens (Ags) to DCs is a relevant aspect for vaccine success. Indeed, antigen peptides, tumor-associated proteins, tumor cells, autologous tumor lysates, and tumor-derived mRNA have been tested as antigen sources. We have previously shown that DCs loaded with allogeneic tumor cell lysates can induce a potent immunological response in melanoma and prostate cancer patients. This strategy provides a reproducible pool of almost all potential Ags, suitable to be used in a broad number of patients independently of their MHC haplotypes or the availability of autologous tumor tissue. However, the development of an optimal autologous tumor cell lysate preparation is crucial to enhance the efficacy of these approaches. Additionally, Ag-presenting cells, used as DC-vaccines, possess innate-related receptors that sense molecular signatures derived from pathological microenvironments as well as stressed cancer cells, thus helping in triggering anti-cancer immune responses. Calreticulin cell surface mobilization, and HGBM1 release during stress are close related to DCs activation through TLR4. Combination of stressed tumor cell lysates together with different adjuvants is strongly immunogenic, as we show in a melanoma model using immunological competent mice. This approach may constitute a useful strategy for melanoma, prostate and gallbladder cancer vaccines that can be associated with prolonged survival of cancer patients.

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Effect of new oncogene-targeted therapies on NK cell function

Manzini, C.¹, Venè, R.², Cossu, I.¹, Queirolo, P.², Moretta, L.³, Mingari, M.C.⁴, Pietra, G.⁴

¹IRCCS Istituto G. Gaslini, Genova, Italy, ²IRCCS San Martino/IST, Genova, Italy, ³IRCCS Bambin Gesù Hospital, Roma, Italy, ⁴University of Genova-IRCCS AOU San Martino-IST, DIMES, Genova, Italy

Oncogene-targeted therapies based on mutant BRAF-specific inhibitors and/or MEK inhibitors have recently been developed for melanoma treatment. Although these targeted drugs induce tumor regression in a high percentage of patients, clinical responses are frequently narrow in time. Understanding the impact of these targeted therapies on immune cell function is an important area of research as the oncology field moves to combinations of targeted drugs with immunotherapies. Natural killer (NK) cells are suitable effector cells in tumor immunotherapy. Thus in this study, we investigated whether Vemurafenib (a BRAF^{V600} inhibitor) and PD0325901 (a MEK inhibitor) could interfere with NK cell function.

Vemurafenib has no effect on the phenotypic and functional properties of peripheral blood NK cells cultured in the presence of IL-2 or IL-15. In contrast, the PD0325901-induced MEK inhibition reduces NK cell proliferation and down-regulates the main activating NK receptors resulting in inhibition of NK cell function. However, interestingly, the inhibitory effect of PD0325901 can be overridden if NK cell are activated in the presence of IL-15+IL-18, or pre-activated with IL-2 or IL-15. Finally, given that BRAF/MEK co-inhibition is a possible clinical practice, the combination of BRAF and MEK inhibition was also tested in this study.

Our data may offer a rationale for future trials that combine IL-15/IL-18 cytokine administration with MAPK inhibitors. In addition, they suggest that both these drugs may represent synergistic tools to improve the efficacy of NK cell-based adoptive therapy.

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Andosan™ extract of the immunomodulating and antitumor mushroom *Agaricus blazei*, protects against development of intestinal cancer in the APC Min^{+/-} mouse model for colorectal cancer

Hetland, G.¹, Eide, D.M.², Tangen, J.M.³, Haugen, M.H.⁴, Mirlashari, M.R.³, Paulsen, J.E.⁵

¹Oslo University Hospital, Immunology, Oslo, Norway, ²Norwegian Institute of Public Health, Oslo, Norway, ³Oslo University Hospital, Oslo, Norway, ⁴Oslo University Hospital -The Norwegian Radium Hospital, Tumor Biology, Oslo, Norway, ⁵Norwegian University of Life Sciences, Food Safety and Infection Biology, Oslo, Norway

Similar to human Familial Adenomatous Polyposis, APC^{Min/+} mice have a deletion in the *apc* gene, giving spontaneous rise to multiple intestinal polyps that develop into adenocarcinomas. *Agaricus blazei* Murill (AbM) is an edible *Basidiomycetes* mushroom used in traditional medicine against cancer and other diseases. The mushroom is rich in immunomodulating β-glucans and has antitumor effects in murine models for fibrosarcoma, ovarian-, lung - and prostate cancer. Andosan™ is a water extract based on AbM (82%), also containing medicinal *Basidiomycetes* mushrooms, *Hericeum erinaceus* and *Grifola frondosa*. Tap water with 10% Andosan was provided as drinking water for 4.5 months to APC^{Min/+} mice and wild-type mice, which then were exsanguinated, their intestines preserved in formaldehyde and the serum frozen. The intestines were examined blindly by microscopy and also stained for the pro-inflammatory and metastasis-promoting protease, legumain. Serum cytokines (pro- and anti-inflammatory, Th1-, Th2 -and

Th17 type) were measured by Luminex analysis. Andosan treated APC^{Min+/-} mice had significantly fewer adenocarcinomas in both the small intestines and colon, and a 60% significantly reduced tumor load (# tumors x size). There was also reduction in legumain content in intestines from Andosan-treated animals. Moreover, Andosan had a significant cytotoxic effect in vitro on the human cancer colon cell line, CaCo2. However, there were no differences in serum cytokine levels between treated and untreated APC^{Min+/-} mice, and no correlations between cytokine level and tumor load. Interestingly, there was an increase in pro-inflammatory cytokine levels in Andosan-treated wild type mice that was not observed for the APC^{Min+/-} mice.

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Pediatric B- and T-ALL minimal residual disease levels in Turkey

Cinar, S., Yilmaz, A., Gelmez, M.Y., Tahrali, I., Ozcit, G., Deniz, G. Institute of Experimental Medicine, Istanbul University, Immunology, Istanbul, Turkey

In patients with acute lymphoblastic leukemia (ALL), treatment response is increasingly evaluated with minimal residual disease (MRD) assays. Assessment of MRD by flow cytometry is faster and cheaper when compared to molecular methods and it is the main reason for utilization in many centers.

In this study, paired bone marrow samples from diagnosis and early follow up from 454 consecutive B-cell precursors ALL and 71 consecutive T-ALL cases were analyzed. Blasts were detected in bone marrow samples of ALL patients on the 15th day of treatment and MRD ratio and relapse risk were evaluated. For B-ALL MRD detection, CD10, CD11a, CD19, CD20, CD34, CD38, CD45 and CD58 expression levels were determined by 4-color flow cytometry. For T-ALL MRD detection CD45, CD3, CD4, CD5, CD7, CD8, CD99, cytoplasmic CD3 and cytoplasmic TdT antigen expressions in samples were detected by 8-color flow cytometry. According to MRD risk in B-ALL diagnosis of 153 cases was FLR (33.70%), 232 cases were FMR (51.10%) and 69 cases were FHR (15.20%); In T-ALL 24 cases were FLR (33.80%), 27 cases were FMR (38.03%) and 20 cases were FHR (28.17%).

MRD is now used in several clinical trials for risk assignment and to guide clinical management overall. Further studies, to investigate relationship between prognosis and survival or correlation of PCR and MRD findings in more detailed studies with contribution of clinics will be more supportive to apply sensitive and accuracy therapy in either B-ALL or T-ALL patients.

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CTLA-4 positive breast cancer cells inhibit dendritic cells function and the reverse effect of anti-CTLA-4 antibody therapy

Chen, X., Hao, S., Shao, Q., Zhao, Z., He, Y., Gao, W., Mao, H. Qi Lu Hospital, Shandong University, Institute of Basic Medical Sciences, Jinan, China

Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) expressed by T lymphocytes is traditionally known as a potent immunoregulatory molecule that down-regulates T-cell activation and thus inhibits the anti-tumor immune response.

Antibodies that specifically block the binding of CTLA-4 with its ligands can enhance immune response. In the present study, we have discovered that cytoplasmic and surface expression of CTLA-4 in human breast cancer cells (BCCs) is also detectable by flow cytometry and immunohistochemical staining. Moreover, CTLA-4⁺ breast cancer cells could inhibit dendritic cells (DCs) maturation and function. LPS-stimulated human DCs decreased the expression of major histocompatibility complex class II (HLA-DR) and co-stimulatory molecules (CD40, CD80, CD83 and CD86) following coculture with human CTLA-4⁺ BCCs. Also, the suppressed DCs further inhibited the proliferation of CD4⁺ T lymphocytes and promoted the secretion of Th2 cytokines, such as IL-4, IL-6. However, the addition of functional anti-CTLA-4 antibody in coculture system could recover LPS-stimulated DC maturation and DC-elicited allogeneic T-cell proliferation. In addition, anti-CTLA-4 antibody therapy also significantly inhibited the proliferation of CTLA-4⁺ BCCs. Collectively, our findings that CTLA-4 molecule is expressed and functional on human breast cancer cells suggest a new sight into the role of CTLA-4 as a negative regulator to influence the maturation and function of DCs in tumor milieu, and anti-CTLA-4 antibody therapy not only recovers the antigen-presenting function of DCs and T lymphocytes activation but also suppresses breast cancer cells proliferation. This study highlights the clinical application of anti-CTLA-4 antibody therapy in breast cancer.

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Characterization of immune cell phenotypes in response to Ras activation in the mouse intestinal epithelium

Henn, S.J.¹, Gutting, T.¹, Janssen, K.-P.², Ebert, M.¹, Burgermeister, E.¹ ¹University of Heidelberg, Department of Medicine II, Universitätsklinikum Mannheim, Mannheim, Germany, ²Technical University Munich, Department of Surgery, Munich, Germany

Evidence for the importance of the role of immune cells in the tumor microenvironment has increased over recent years. The priming of the immune system by oncogenic stimuli and the transition of inflammation to malignancy has not yet been fully elucidated and is of significant importance regarding disease prevention and therapy in patients with inflammatory bowel disease. In the intestine and other organs, constitutively active mutant Ras stimulates multiple interconnecting pathways resulting in tumor initiation and progression. These pathways act not only directly on epithelial cells, but also stimulate the release of chemotactic cytokines and chemokines, known to result in immune cell infiltration. However, specific immune cells targeted by oncogenic *ras*-activated epithelial cells and the subsequent change in immune cell phenotypes during *ras*-specific tumor initiation and progression have not been previously characterized. Using a mouse model with an activating *ras* mutation specific to intestinal cells, we characterized the subsets of T cells and macrophage phenotypes in the intestine involved in the initiation and progression of cancer compared to wildtype mice and *ras*-inhibitor treated mice. Techniques included flow cytometry, immunohistochemistry and RTqPCR. Our data indicate that transgenic overexpression of the mutated *ras* oncogene in the intestinal epithelium promotes a chronic inflammatory state, a precondition to malignant transformation.

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Significance of INHBA expression in EBV-infected nasopharyngeal carcinoma*Fong, H.L., Lee, H.F.**University of Hong Kong, Hong Kong, China*

Nasopharyngeal carcinoma is common in Asia and Africa, especially in southern China. The cause of NPC is a combination of factors which can be environmental influences, genetic susceptibility, and including the viral influence associated with the infection of Epstein-Barr virus (EBV). The relationship between NPC and EBV has not been fully understood yet, meanwhile it is also suggested to be associated with human papillomavirus (HPV).

Inhibin beta A, INHBA as a member of the transforming growth factor β superfamily, it is known to have association with several human cancer. However, the significance of INHBA in NPC has not been evaluated. There were clinical data showed that a poorer overall survival rate was resulted in the group of patients with high INHBA expression comparing to the group of patients with low INHBA expression

($P < 0.05$). Moreover, we found that INHBA was able to enhance the ability of growth, metastatic, migratory and invasive capabilities of NPC cell lines in vitro.

This study can give us a new insight in the viral oncogenesis of INHBA in NPC and in order to contribute to its early diagnosis and effective treatment.

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Chitosan hydrogel vaccine generates CD8⁺ T cell memory protective in a mouse tumour model of colorectal cancer*Highton, A., Niemi, V., Hook, S., Kemp, R.**University of Otago, Dunedin, New Zealand*

Vaccination generating a robust memory population of CD8⁺ T cells may provide protection against cancer. We investigated the generation of murine memory CD8⁺ T cells using a sustained antigen release vaccine vehicle (chitosan gel; Gel+OVA) containing a model antigen, ovalbumin, or conventional dendritic cell vaccination (DC+OVA) using the same protein antigen. The aims of this work were to evaluate the efficacy of Gel+OVA in generating memory CD8⁺ T cells at peripheral and gut associated lymphoid sites; and to determine whether this vaccination provided protection in murine tumour challenge models.

Mice were euthanised at memory time points following subcutaneous vaccination and cell populations were phenotypically assessed in peripheral and gut-associated lymphoid tissues using flow cytometry. Following vaccination with Gel+OVA, CD8⁺ T cell memory populations specific for ovalbumin protein were detected in both peripheral and gut-associated lymphoid organs. A subcutaneous melanoma model and an orthotopic colorectal cancer model were used to assess the protective capacity of Gel+OVA vaccination at peripheral and gut sites. Vaccination with Gel+OVA or DC+OVA increased survival of mice following subcutaneous tumour challenge compared to unvaccinated controls. Only vaccination with Gel+OVA gave decreased tumour burden compared to unvaccinated or DC+OVA-

vaccinated mice in the colorectal cancer challenge model. This protection was associated with IL-2 production by antigen-specific cells.

These results indicate that subcutaneous vaccination with Gel+OVA generates a population of functional CD8⁺ memory T cells in peripheral and gut-associated lymphoid tissue able to protect against tumour challenge.

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Myeloid STAT3 promotes hepatocarcinogenesis by inactivating natural killer T cells*Wang, H.¹, Yin, S.², Li, J.³, Gao, B.⁴**¹Department of Oncology, The First Affiliated Hospital of Anhui Medical University, Hefei, China, ²Department of Geriatrics,**Affiliated Provincial Hospital of Anhui Medical University, Hefei,**China, ³Institute for Liver Diseases, Anhui Medical University, Hefei,**China, ⁴NIAAA, National Institutes of Health, Rockville, United States*

Background/objectives: Activation of signal transducer and activator of transcription 3 (STAT3) occurs virtually in most liver diseases, but how STAT3 links liver inflammation and cancer remains elusive.

Methods and results: Using cell-specific STAT3 deletions mouse strains, we have investigated the interplay of myeloid and hepatic STAT3 in controlling hepatocarcinogenesis induced by diethylnitrosamine (DEN). We show that in DEN model hepatic STAT3 plays an important role in mediating a cytoprotective action and enhancing tumor growth, while myeloid STAT3 also significantly affects tumor growth via its anti-inflammatory effect. We further revealed that higher natural killer T cells expansion in liver tumor microenvironment of DEN-treated myeloid cells specific STAT3 knockout mice when compared with that in the wild-type mice.

Conclusions: Orchestral crosstalk between hepatic and myeloid STAT3 enables survival of mutagenized cells and subsequent tumor promotion in DEN-induced hepatocarcinogenesis. Myeloid STAT3 promotes hepatocarcinogenesis by inactivating natural killer T cells.

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Zoledronic acid induces dose-dependent increase of antigen-specific CD8 T-cell responses in combination with peptide/poly-IC vaccine*Cho, H.-I.¹, Park, H.-M.², Shin, C.-A.³, Shon, H.-J.², Kim, T.-G.⁴**¹The Catholic University of Korea, Catholic Hematopoietic Stem Cell Bank, Cancer Research Institute, Seoul, Korea, Republic of,**²The Catholic University of Korea, Catholic Hematopoietic Stem Cell Bank, Seoul, Korea, Republic of, ³The Catholic University of**Korea, Department of Microbiology, Seoul, Korea, Republic of,**⁴The Catholic University of Korea, Department of Microbiology, Catholic Hematopoietic Stem Cell Bank, Seoul, Korea, Republic of*

Zoledronic acid (ZA) is used for treating osteoporosis and for preventing skeletal fractures in cancer patients suffering from myeloma and prostate cancer. It is also reported to directly

induce cancer cell apoptosis and indirectly modulate T-cell immune response as an antitumor agent. In this study, the effect of ZA following peptide/ polyinosinic-polycytidylic acid (poly-IC) vaccination was investigated in a murine tumor model. The combination of ZA with peptide/poly-IC vaccine showed a synergistic effect on the induction of antigen-specific CD8 T-cell response. Three consecutive intravenous administrations of ZA was defined to induce the highest CD8 T-cell response. Further, total splenocyte counts and antigen-specific CD8 T-cell response gradually increased depending on the dose of ZA. In tumor-bearing mice, ZA showed a dose-dependent decrease of growth and prolonged survival. Treatment with ZA only decreased the number of CD11b⁺Gr1⁺ myeloid cells in blood. Our results demonstrate that the use of ZA could improve antitumor immune responses induced by the peptide/poly-IC vaccine.

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Activin-A potentiates anti-tumor immunity and protects against lung cancer progression in vivo

Morianos, I., Semitekoulou, M., Stellas, D., Xanthou, G.

Biomedical Research Foundation of the Academy of Athens, Athens, Greece

Activin-A is a pleiotropic cytokine that shapes T cell-mediated responses in allergic and autoimmune disorders. Pertinent to cancer, activin-A exerts both anti-tumorigenic and pro-tumorigenic functions, depending on the cell type and activation status. Activin-A is overexpressed at the tumor site in patients with lung cancer; still, its *in vivo* role in tumor progression remains elusive. Our aim was to decipher whether activin-A can enhance T cell-mediated anti-tumor responses and inhibit lung cancer progression. To address this, we administered recombinant activin-A, in mice which were intravenously inoculated with either Lewis Lung Carcinoma or B16F10 melanoma cells and studied its effect on lung tumor development and induction of anti-tumor immune responses. Our data demonstrated a significant increase in activin-A serum levels in lung tumor-bearing mice. Immunohistochemical studies revealed that activin-A was increased in bronchial epithelial cells at the tumor site. Notably, *in vivo* administration of activin-A in preventive and therapeutic protocols induced a marked regression in lung tumor development, evidenced by macroscopic, PET/CT imaging studies and leukocytic infiltration observations of lung tissue sections, followed by an extended overall survival. Activin-A anti-tumor effects were associated with a striking increase in effector T cell populations in the lung and decreased expression of the immune checkpoint inhibitors, PD-1 and CTLA-4. Administration of activin-A also enhanced the immunostimulatory potential of lung-infiltrating CD11c⁺DCs, reflected by increased MHCII, CD80 and CD86. Collectively, our studies uncover activin-A as a novel anti-cancer agent that may be utilized for enhancing anti-tumor immune responses leading to effective lung tumor regression.

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Role of intestinal CD103⁺CD11b⁻ dendritic cells in colorectal cancer

Soncini, L., Sheng, J., Ruedl, C.

Nanyang Technological University (NTU), SBS, Singapore, Singapore

The tumor microenvironment is comprised of proliferating neoplastic cells, tumor stroma, blood vessels and infiltrating immune cells such as T lymphocytes, occasional B and NK cells as well as effectors of innate immunity, including dendritic cells (DC), macrophages and granulocytes.

We have recently characterized at least nine distinct colon intra-tumoral myeloid cells including dendritic cells, resident macrophages, monocyte-derived macrophages, neutrophils/MDSCs and eosinophils.

Since the intestinal DCs represent a heterogeneous cell family that mediates diverse immune functions, we studied which DC subset regulates the integrity of the epithelial barrier and contributes to the development of colitis-associated malignancies. To unravel the function of the CD103⁺CD11b⁻ DC subset in promoting or repressing intestinal tumor development, Clec9A-DTR mice were backcrossed with the APC^{Min/+} mouse strain, widely used as mouse model for colon cancer. The conditional *in vivo* ablation of CD103⁺CD11b⁻ DCs in Clec9A-DTR APC^{Min/+} mice resulted in a higher adenomas formation when compared to the wild-type APC^{Min/+} mice, suggesting an anti-tumoral function of this DC subset. A higher infiltration of myeloid cells such as neutrophils and monocytes was observed, which could contribute to the enhanced tumor progression. In order to untangle the role of the intra-tumoral CD103⁺CD11b⁻ DCs, other parameters such as T cell activation state, were evaluated.

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Type I interferon agonists as potent anti-metastatic cancer therapeutics

Rautela, J.^{1,2,3}, Anderson, R.⁴, Hertzog, P.⁵, Parker, B.⁶

¹The Walter and Eliza Hall Institute, Molecular Immunology,

Parkville, Australia, ²The University of Melbourne, Sir Peter

MacCallum Cancer Institute, East Melbourne, Australia, ³La Trobe

Institute for Molecular Science, Biochemistry, Bundoora, Australia,

⁴Sir Peter MacCallum Cancer Institute, East Melbourne, Australia,

⁵Hudson Institute of Medical Research, Clayton, Australia, ⁶La Trobe

Institute for Molecular Science, Bundoora, Australia

Metastatic disease accounts for almost all breast cancer related deaths. The mechanisms that dictate this spread to key sites such as the lung and bone are only now emerging. Our recent work supports a role for the host immune system in restricting breast cancer metastasis, and that the balance between pro- and anti-metastatic immune responses is a critical determinant of metastatic progression. Previous studies in our laboratory have shown that breast tumour cells suppress their intrinsic type-I interferon (IFN) pathway, and hence type-I IFN secretion, in order to evade host immunosurveillance and colonise distant organs. These findings are supported by our recent

work showing that breast cancer patients who present with tumours expressing low levels of key IFN pathway members had a much greater risk of developing metastatic disease. These data argued for the re-examination of type-I IFN therapies in the prevention of disseminated disease using our unique models of spontaneous breast cancer metastasis. We show that the induction of endogenous type-I IFN (through the administration of TLR agonists) provided superior anti-metastatic protection than the use of recombinant type-I IFN alone. We also show that the efficacy of such therapies depends on their administration early in tumour dissemination, and, through the use of models of innate immunodeficiency, uncover the importance of NK cells in this anti-metastatic immune response. These data, together with our recent and comprehensive review of IFN clinical trials in breast cancer, form a compelling basis for re-visiting this cytokine pathway in the prevention of metastatic breast cancer.

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Antibodies pay Toll to improve their therapeutic efficacy: improving antibody-mediated anti-cancer treatment via co-activation of Fc-receptors and Toll-like receptors

Ganzevles, S.¹, Stigter-van Walsum, M.¹, Schilham, M.², Tuk, K.³, van Egmond, M.³, Leemans, R.¹, Brakenhoff, R.¹, Bakema, J.¹

¹VUMC, Department of Otolaryngology/Head-Neck Surgery, Amsterdam, Netherlands, ²LUMC, Department of Pediatrics, Leiden, Netherlands, ³VUMC, Molecular Cell Biology and Immunology, Amsterdam, Netherlands

The development of immunotherapies aiming to employ the patient's own immune system to fight cancer has increased dramatically in the last decades. Unfortunately, malignant cells have evolved various immune escape mechanisms to suppress anti-tumor immune responses, which hampers clinical success of anti-cancer therapeutic antibodies in most patients. Many of these harmful suppressive mechanisms of the tumor are, in fact, based on natural regulators that resolve inflammatory immune responses once pathogens have been cleared.

We now aim to re-activate the inflammatory immune response during antibody mediated immunotherapy to overcome the immune suppressive tumor microenvironment. We recently demonstrated that simultaneous activation of antibody binding Fc-receptors (FcRs) and pathogen recognizing Toll-like receptors (TLRs) on various immune cells induces unique and robust pro-inflammatory immune responses. We therefore explored the synergistic properties of a combined treatment with cetuximab, an antibody targeting the epidermal growth factor receptor (EGFR), and synthetic ligands for TLRs to target EGFR positive tumors.

We demonstrated that simultaneous activation of TLRs during cetuximab treatment improved the tumor killing capacity of natural killer (NK) cells of healthy donors as well as NK cells from cancer patients. Furthermore, analyzing various cytokines and chemokines we could demonstrate that a profound pro-inflammatory immune response was evoked when cetuximab treatment was combined with TLR agonists. These results show that combined activation of TLRs and FcRs during cetuximab treatment can overcome the immunosuppressive tumor environment and as such improve the tumoricidal capacity

of immune effector cells. This strategy may improve various immunotherapies targeting patient's own immune system.

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Lactococcus lactis as a new vehicle in delivering tumoricidal recombinant human protein tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)

Wieckiewicz, J., Ciacma, K., Baran, J.

Jagiellonian University Medical College, Clinical Immunology, Krakow, Poland

Introduction: TRAIL (Tumour Necrosis Factor-Related Apoptosis-Inducing Ligand) has a unique ability to induce apoptosis of tumour cells in vitro and in vivo without cytotoxic effect on normal cells. Recombinant TRAIL developed for clinical application (Dulanermin®) was found to be well tolerated, however without therapeutic effect, indirectly caused by the form of its administration. *Lactococcus lactis* is a non-pathogenic and endotoxin-free bacteria, which might serve a new vehicle for TRAIL administration and expression.

Objective: To assess if our genetically engineered *Lactococcus lactis* bacteria, harbouring constructed recombinant plasmid pUSP45TRAIL3, can efficiently express bioactive hsTRAIL.

Methods: Recombinant hsTRAIL-cDNA was synthesized using RT-PCR, inserted to plasmid vector pTrc99A and transformed into the *E.coli* DH5α host strain. The screening of hsTRAIL antitumour activity in vitro and its effect in combination with chemotherapeutics (puromycin, 5-Fluorouracil) were performed by incubation of broth supernatant from *E.coli* DH5α culture with human pancreatic adenocarcinoma HPC-4 cell line. The final hsTRAIL-secretable vector (pUSP45TRAIL3) was obtained by insertion of hsTRAIL-cDNA (cut from constructed *E.coli* plasmid) and cDNA of *L.lactis* usp45 gene coding signal peptide, into plasmid vector for *L.lactis* pNZ8148 and was transformed into the *L.lactis* NZ9000 host strain. The presence of the protein in lysates from *L.lactis* was examined by Western blot.

Results: Supernatants from culture of *E.coli* DH5α expressing hsTRAIL exhibited strong antitumour activity onto HPC-4 cells in a dose dependent manner. The addition of chemotherapeutics enhanced hsTRAIL-induced apoptosis of cancer cells. Identification of hsTRAIL protein in the lysates of *L.lactis* NZ9000 proved production of this protein by transformed strain.

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The role of T cells in the immune regulation of Langerhans cell histiocytosis (LCH)

Mitchell, J.^{1,2}, Pellicci, D.G.³, Berzins, S.^{1,2}, Kannourakis, G.^{1,2}

¹Federation University Australia, Ballarat, Australia, ²Fiona Elsey Cancer Research Institute, Ballarat, Australia, ³Peter Doherty Institute, University of Melbourne, Dept. Microbiology and Immunology, Parkville, Australia

LCH is characterised by lesions containing CD1a⁺ and/or CD207⁺ histiocytes as well as inflammatory cells, including T and B cells. New treatment options are required for the large proportion of patients who remain unresponsive to current standards. FOXP3⁺ regulatory T cells (Tregs) are present in high

numbers in LCH lesions (suggestive of an immune-suppressive environment), but the frequency and importance of other T cells with regulatory functions, such as mucosal associated invariant T (MAIT) cells and type I natural killer T (NKT) cells is not known. We have characterised blood and LCH lesions using 13-colour flow cytometry, and *in vitro* assays of T cell function. We report that MAIT cells and type I NKT cells are altered in LCH patients compared with healthy donors and we have established a method to isolate FOXP3⁺ Tregs from LCH patients to conduct *in vitro* functional studies. Immune regulation may be defective in LCH and changes in these and other regulatory T cell subsets could be important factors in LCH onset and progression. Targeting regulatory T cell subsets could therefore be a promising avenue of investigation in the development of new immune based therapies.

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Novel regulators of CD8⁺ T-cell functions in the skin

Bridge, J.A., Overgaard, N.H., Cruz, J.L.G., Veitch, M., Frazer, I.H., Steptoe, R.J., Wells, J.W.

The University of Queensland Diamantina Institute, Woolloongabba, Australia

Cancer Council statistics show that Australia has the highest rate of skin cancer in the world (twice that of the USA and UK), and predicts 2 in 3 Australians will be diagnosed with skin cancer before the age of 70. Tumour-specific CD8⁺ T-cells are well-recognised for their importance in eliciting tumour-rejection, however, in many cases tumour-specific CD8⁺ T-cells within the tumour microenvironment are dysfunctional. The regulation of CD8⁺ T-cell activity in the tumour microenvironment is poorly understood. This study aimed to explore the mechanisms involved in the regulation of CD8⁺ T-cells in the skin as a prelude to tumour studies. We have generated a new experimental mouse model in which activated CD8β⁺ T-cells from donor mice were introduced into RAG1KO mice in order to assess CD8⁺ T-cell deregulation in the absence of conventional-regulatory T-cells (Treg). When RAG1KO mice subsequently received CD4-depleting antibody, CD8⁺ T-cell-mediated destruction of the ear skin occurred. However, this did not occur in mice administered control-antibody. Analysis of lymph nodes 30 days post CD8β⁺ T-cell transfer showed no evidence of classical CD4⁺FoxP3⁺ Treg indicating regulation is mediated by a separate, distinct cell type. Using the model, we have identified CD4⁺ cells, which are distinct from classical-Treg, and we are subsequently defining the mechanism by which these cells exert control of CD8⁺ T-cell function in the skin. Uncovering novel pathways of CD8⁺ T-cell regulation will shed new light onto regulatory influences of CD8⁺ T-cell function within tumours and yield opportunities to develop better treatment options for cancer patients.

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Eliciting regression by combination of therapeutic vaccine STDENVANT and PD-L1 blockade in a glioma mouse model

Zhu, S.¹, Zhang, X.¹, Lv, X.¹, Zang, G.¹, Chen, W.², Liu, Y.-J.³, Chen, J.¹

¹The First Hospital of Jilin University, Institute of Translational Medicine, Chuangchun, China, ²University of Minnesota

Medical School, Department of Hematology-Oncology and BMT Department of Pediatrics, Minneapolis, United States, ³MedImmune, Gaithersburg, United States

Glioblastoma multiforme (GBM) is the most malignant primary type of brain tumor in adults. Glioma stem cells (GSCs) are resistant to radiotherapy and chemotherapy, and are responsible for glioblastoma recurrence. In order to develop an immunotherapy for GBM treatments, we explored a cancer vaccine called STDENVANT, which combined with lysate of glioma stem-like cells, dendritic cells (DCs), and Toll-like receptor 9 agonist CpG ODN and studied its anti-tumor effects in an intracranial glioma mouse model. The mouse model was generated with luciferase-expressing GL261 or GL261-derived GSCs in C57BL/6 mice. STDENVANT vaccine was injected every seven days for three times after tumor inoculation. Mice were monitored for immune responses. Our data showed that STDENVANT vaccine inhibited tumor growth, prolonged survival and increased lymphocyte infiltration in mouse model. The STDENVANT vaccine performed anti-tumor effects in both GL261 and GL261-derived GSCs groups. Furthermore, we found that PD-L1 expression was upregulated in GL261-derived GSCs. Administration of a PD-L1 blocking antibody with STDENVANT elicited complete regression of established tumors. Taken together, the current study demonstrated that PD-L1 blockade could promote STDENVANT to kill the GL261-derived GSCs in mouse glioma model, and the combination of PD-L1 blockade and STDENVANT could provide a feasible immune therapeutic approach against GBM.

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PD-1 blockade boosts radiofrequency ablation-elicited adaptive immune responses against tumor

Jiang, J.¹, Shi, L.¹, Chen, L.¹, Zhu, Y.¹, Wu, C.¹, Lu, B.²

¹Third Affiliated Hospital of Soochow University, Department of Tumor Biological Treatment, Changzhou, China, ²University of Pittsburgh, Department of Immunology, Pittsburgh, United States

Purpose: Radiofrequency ablation (RFA) has been shown to elicit tumor-specific T cell immune responses but is not sufficient to prevent cancer progression. Here we investigated immune suppressive mechanisms limiting the efficacy of RFA. Experimental design: We performed a retrospective case-controlled study on patients with synchronous colorectal cancer liver metastases who had received primary tumor resection with or without pre-operative RFA for liver metastases. Tumor infiltrating T cells and tumoral PD-L1 expression in human colorectal cancer tissues were analyzed by immunohistochemistry. T cell immune responses and PD-1/PD-L1 expression were also characterized in a RFA mouse model. In addition, the combined effect of RFA and PD-1 blockade was evaluated in the mouse RFA model.

Results: We found that RFA treatment of liver metastases increased not only T cell infiltration but also PD-L1 expression in primary human colorectal tumors. Using mouse tumor models, we demonstrated that RFA treatment of one tumor initially enhanced a strong T cell-mediated immune response in tumor. Nevertheless, tumor quickly overcame the immune

responses by inhibiting the function of CD8+ and CD4+T cells, driving a shift to higher Treg to Teff ratio, and up-regulating of PD-L1/PD-1 expression. Furthermore, we established that the combined therapy of RFA and anti-PD-1 antibodies significantly enhanced T cell immune responses, resulting in stronger antitumor immunity and prolonged survival.

Conclusions: The PD-L1/PD-1 axis plays a critical role in dampening RFA-induced antitumor immune responses. And this study provides a strong rationale for combining RFA and the PD-L1/PD-1 blockade in the clinical setting.

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Heterogeneity of immune response within single melanoma metastases

Mathy, J.E.^{1,2}, Dunbar, P.R.^{1,2}

¹University of Auckland, School of Biological Sciences, Auckland, New Zealand, ²Maurice Wilkins Centre for Molecular Biodiscovery, Auckland, New Zealand

Numerous cellular and molecular mechanisms can block tumour-directed immune responses within the tumour microenvironment. Determining optimal immune therapy for each cancer patient will ultimately depend on reading out which mechanisms are active in available tumour samples, using histological or molecular diagnostic techniques. However, the potential heterogeneity of immune responses within metastases remains under-studied -despite the growing recognition of cancer cell heterogeneity.

We are using four-colour immunofluorescence microscopy on tissue sections spanning entire human melanoma metastases to determine the activation status of tumour-infiltrating leukocytes, and their distribution relative to both the melanoma cells and the other immune and stromal cells within each microenvironment. Preliminary results have revealed remarkable heterogeneity within single tumour sections, with distinct microenvironments bearing contrasting markers for T cell activity, e.g. cytotoxic granule markers in one zone, and high FoxP3 expression in another. Heterogeneity of macrophage populations in these microenvironments is also striking. In follow up studies, contrasting microenvironments from single tumours are being sampled using laser capture microdissection for differential gene expression analysis.

Our preliminary data are consistent with the concept that immune status within tumours is highly dynamic, resulting in microenvironments in close proximity showing sharp contrasts in T cell activity and in the cellular and molecular mechanisms that influence that activity. Our results also suggest that highly localised approaches for patient biopsy, such as single point fine-needle aspiration, may be insufficient for comprehensive assessment of the immune response status of a patient's tumours in order to select or modify therapy.

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T cell phenotype and receptor expression in pleural effusions associated with malignant mesothelioma

McDonnell, A.¹, Chee, J.¹, Nowak, A.^{1,2}, Robinson, B.^{1,3}, Lake, R.¹

¹University of Western Australia, National Centre of Asbestos

Related Diseases, School of Medicine and Pharmacology, Nedlands, Australia, ²Sir Charles Gairdner Hospital, Department of Medical Oncology, Nedlands, Australia, ³Sir Charles Gairdner Hospital, Department of Respiratory Medicine, Nedlands, Australia

Cancer of mesothelial cells that line the pleural cavity is known as malignant pleural mesothelioma. A common feature of the disease is abnormal build-up of fluid in the cavity as a pleural effusion. The effusion contains erythrocytes, leukocytes and malignant cells. It is often drained for palliation and diagnostic tests. However, the importance of cells in the pleural effusion is still relatively unknown. We postulated that the pleural effusion is a site that reflects the immunological status of the tumour, i.e. that T cells detected in the effusion would represent those that infiltrate the tumour. If so, the pleural effusion will be an excellent source of immune cells for further analysis of tumour immunity.

We characterised T cells from pleural effusions of mesothelioma patients by multi-parameter flow cytometry, and compared them with concurrently drawn T cells from blood. Notably, we observed increased expression of T cell inhibitory receptors (PD1, LAG3), similar to the phenotype of tumour-infiltrating lymphocytes (TILs). We performed TCR sequencing on T cells isolated from a pleural effusion and compared receptor usage with the relevant blood sample. There was no clear bias in the usage of any Vb gene families in either CD4+ or CD8+ compartments, but we noted several clonotypes differentially expressed in the antigen-experienced (PD1+) compartment between the sites. Our initial studies reveal that T cells in the effusion have a similar phenotype to TILs. The specificity of T cells and the importance of TCR repertoire diversity in the effusion still need to be further tested.

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Inhibition of osteoclast differentiation by NDRG2 expression in breast cancer cells

Kim, B.¹, Nam, S.¹, Lee, H.G.², Lim, J.-S.¹

¹Sookmyung Women's University, Department of Biological Science, Seoul, Korea, Republic of, ²Korea Research Institute of Bioscience and Biotechnology, Medical Genomics Research Center, Daejeon, Korea, Republic of

Bone matrix is properly maintained by osteoclasts and osteoblasts. In the tumor microenvironment, osteoclasts are increasingly differentiated by the various ligands and cytokines secreted from the metastasized cancer cells at the bone metastasis niche. The activated osteoclasts generate osteolytic lesions. For this reason, studies focusing on the differentiation of osteoclasts are important to reduce bone destruction by tumor metastasis. The N-myc downstream-regulated gene 2 (NDRG2) has been known to contribute to the suppression of tumor growth and metastasis, but the precise role of NDRG2 in osteoclast differentiation induced by cancer cells has not been elucidated. In this study, we demonstrate that NDRG2 expression in breast cancer cells has an inhibitory effect on osteoclast differentiation. RAW 264.7 cells, which are monocytic preosteoclast cells, treated with the conditioned media (CM) of murine breast cancer cells (4T1) expressing NDRG2 are less

differentiated into the multinucleated osteoclast-like cells than those treated with the CM of 4T1-WT or 4T1-mock cells. Interestingly, 4T1 cells stably expressing NDRG2 showed a decreased mRNA and protein level of intercellular adhesion molecule 1 (ICAM1), which is known to enhance osteoclast maturation. Osteoclast differentiation was also reduced by ICAM1 knockdown in 4T1 cells. In addition, blocking the interaction between soluble ICAM1 and ICAM1 receptors significantly decreased osteoclastogenesis of RAW 264.7 cells in the tumor environment. Collectively, these results suggest that the reduction of ICAM1 expression by NDRG2 in breast cancer cells decreases osteoclast differentiation, and demonstrate that excessive bone resorption could be inhibited via ICAM1 down-regulation by NDRG2 expression.

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Expression of OCT4 and EMT-associated factors in invasive breast cancer and its relationship with clinicopathological features and prognosis

Ai, J.

The Fourth Hospital of Hebei Medical University, Research Center, Shijiazhuang, China

Objective: In order to investigate the correlation between EMT and cancer stem cells, this experiment is about the expression of EMT related factors E-cadherin, vimentin and stem cells factor OCT4 in breast cancer.

Method: Quantitative real-time PCR and immunohistochemical staining were used to detect the mRNA and protein expression of OCT4, E-cadherin and vimentin in invasive breast cancer tissue and the corresponding adjacent tissues. Furthermore, the correlation between OCT4 and EMT-related proteins and the survival of breast cancer patients were analyzed.

Results: In invasive breast cancer tissues, the expression rate of OCT4, E-cadherin and vimentin were 30% (12/40), 55% (22/40) and 65% (26/40) respectively. OCT4 gene expression levels was correlated with the age, histological grade, lymph node metastasis and the expression in Her-2 of breast cancer patients. The expression of OCT4 mRNA and protein negatively correlated with the expression of E-cadherin mRNA and protein. And the expression of E-cadherin mRNA and protein correlated negatively with the expression of vimentin mRNA and protein. The survival rate of OCT4 positive expression breast cancer patients was lower than patients with OCT4 negative expression significantly.

Conclusion: Stem cells factor OCT4 may promote the metastasis of breast cancer cells via EMT and is an independent prognostic factor for breast cancer.

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Emergence of a neutrophil precursor population that serves as granulocytic myeloid-derived suppressor cells in cancer

Kim, M.-H.¹, Yang, D.², Kim, M.³, Kim, S.-Y.³, Kim, D.², Kang, S.-J.¹

¹Korea Advanced Institute of Science and Technology (KAIST), Biological Sciences, Daejeon, Korea, Republic of, ²Korea Advanced Institute of Science and Technology (KAIST), Bio and Brain Engineering, Daejeon, Korea, Republic of, ³Korea Research Institute

of Bioscience and Biotechnology (KRIBB), Medical Genomics Research Center, Daejeon, Korea, Republic of

Hematopoietic alteration is one of the major features of cancer. In particular, cancer progression is often accompanied by dysregulated granulopoiesis. Studies of granulopoiesis dynamics in clinical conditions like cancer have been hampered by technical limitations in defining neutrophil precursors, thus preventing a clearer understanding of the underlying biological process. In cancer, myeloid-derived suppressor cells (MDSCs) arise and contribute to tumor promotion by suppressing anti-tumor immunity. MDSCs are a heterogeneous cell population that is composed of granulocytic and monocytic cells. Immature neutrophil like granulocytic-MDSCs are major T cell suppressive population contributing pro-tumor immunity. However, the maturation stage and differentiation process of MDSCs remains undetermined. Here, we define a population of neutrophil precursor cells with unprecedented purity, which we call NeuPs (Neutrophil Precursors). We demonstrated that NeuPs have the potential to proliferate and differentiate into mature neutrophils both *in vitro* and *in vivo*. By analyzing the gene expression profiles of NeuPs, we identified stage-specific genes and characterized patterns of gene regulation throughout granulopoiesis. Importantly, NeuPs displayed shared but unique modes of granulopoiesis dynamics in different hematopoietic stress settings, indicating that NeuPs are poised at a critical step to regulate granulopoiesis. Significantly, we demonstrate that NeuPs acquire T cell suppressive activity in cancer condition and can serve as granulocytic MDSCs. Our study reveals that MDSC differentiation branches out at the NeuP stage of neutrophil development in cancer.

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Immunization with glycosylated-MUC1 induces effective humoral responses and suppression of MUC1-expressing carcinoma growth and metastasis

Denda-Nagai, K.¹, Namba, A.², Noji, M.¹, Irimura, T.¹

¹Juntendo University School of Medicine, Tokyo, Japan, ²University of Tokyo, Graduate School of Pharmaceutical Sciences, Tokyo, Japan

Mucin 1 (MUC1) has been considered as a promising target of cancer immunotherapy through the vaccination, and aberrant glycosylation was considered as the basis of immunological distinction between normal and carcinoma-associated MUC1. However, the precise nature of the immune response to the glycoforms of MUC1 was previously unknown. In the present study, we aimed to evaluate the efficacy of MUC1-IgG fusion protein with Tn (GalNAc) or T (Gal-GalNAc) epitopes in the induction of immune response and in the suppression of MUC1-expressing carcinoma growth and metastasis in C57/BL6 mice. Mice immunized with glycosylated MUC1-IgG produced significantly higher levels of anti-MUC1 IgG1 antibodies, especially anti-Tn-MUC1 antibodies, than those with MUC1-IgG without carbohydrate chains. The antibodies after immunization with glycosylated MUC1-IgG also efficiently bound to the surfaces of MUC1-expressing carcinoma cells. Macrophage galactose-type C-type lectins (MGL1 or MGL2 in mice) expressed on dendritic cells were involved in the effective

humoral response at 1 week after immunization of glycosylated MUC1-IgG. Growths at the site of injection (spleen) and liver metastasis of MUC1-transfected mouse colon carcinoma cells were suppressed after immunization with glycosylated MUC1-IgG but those by mock-transfected cells were not. These results strongly suggest that MUC1 with truncated *O*-glycans (Tn and/or T epitopes) induces effective humoral responses and the anti-Tn-MUC1 antibodies contribute to the suppression of carcinoma growth and metastasis.

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Gamma-interferon-inducible lysosomal thiol reductase is upregulated in human melanoma and halo nevi

In, K.¹, Menon, H.¹, Nguyen, J.¹, Sebastiao, N.², Kang, P.¹, Hu, C.¹, Bernert, R.³, DiCauda, D.⁴, Hastings, K.¹

¹University of Arizona College of Medicine, Phoenix, United States, ²Ventana Medical Systems, Tucson, United States, ³Arizona Dermatopathology, Scottsdale, United States, ⁴Mayo Clinic, Scottsdale, United States

T cell-mediated immunity has the ability to produce durable anti-melanoma responses resulting in improved survival of patients with advanced melanoma. Gamma-interferon-inducible lysosomal thiol reductase (GILT) is critical for MHC class II-restricted presentation of multiple melanoma antigens to CD4⁺ T cells. However, GILT expression in human melanocytic lesions has not been defined. We evaluated GILT and MHC class II expression in human primary and metastatic melanomas and nevi using immunohistochemistry. Both GILT and MHC class II expression were increased in melanocytes of primary and metastatic melanomas compared with nevi ($p < 0.01$ for GILT; $p < 0.001$ for class II). GILT expression was increased in APCs of primary and metastatic melanomas compared to nevi ($p < 0.001$), whereas MHC class II had equivalent high expression in APCs of all melanocytic lesions. GILT, but not MHC class II, expression was increased in keratinocytes of primary melanomas compared to nevi and metastases ($p < 0.01$). To evaluate whether GILT expression was related to inflammation, GILT expression in halo nevi was compared to nevi without lymphocytic infiltrates. A halo nevus is a benign nevus with a lymphocytic infiltrate which results in a zone of depigmentation surrounding the nevus. GILT, but not MHC class II, expression was increased in melanocytes and keratinocytes of halo nevi ($p < 0.05$) compared to nevi without lymphocytic infiltrates, suggesting that GILT expression is induced by the inflammatory environment. GILT expression is anticipated to result in improved presentation of melanocyte antigens and more effective immune-mediated destruction.

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Mechanisms of Response and Resistance to checkpoint blockade in models of murine colorectal carcinoma

Wiehagen, K., Pizutti, D., DeAngelis, N., Ferrante, C., Yamada, D., Verona, R.

Janssen Research and Development, LCC, Spring House, United States

PD-1 and TIM-3 are inhibitory cell surface molecules that function to limit T cell responses during infection, autoimmunity, or cancer. In tumors, co-expression of PD-1 and TIM-3 promote

an exhausted or dysfunctional state on T cells. This state of exhaustion is reversible, however. Inhibition of PD-1 with function-blocking antibodies can control tumor growth in syngeneic transplantable murine tumor models. Combination of PD-1 and TIM-3 blockade exhibits greater efficacy than monotherapy, although mechanisms underlying this response remain unclear. We profiled the composition and functionality of infiltrating immune cells in CT26 and MC38 tumors treated with anti-PD-1, anti-TIM-3 or combination. Intratumoral CD8⁺ T cells were detected in higher frequencies after PD-1 blockade. More CD8⁺ T cells expressed patterns of cell surface markers associated with effector lymphocytes, including CD62L, LFA-1, and costimulatory receptors CD137 and GITR. Furthermore, expression of inhibitory receptors also increased with PD-1 blockade, suggesting the emergence of mechanisms for acquired resistance to PD-1 immunotherapy. CD8⁺ T cells from anti-PD-1 and combination treated mice expressed higher levels of T-bet and lower Eomesodermin, two transcription factors associated with balancing effector function and exhaustion in T cells. TIM-3 blockade elicited its own effects, extending from T cells to other immune populations. Combination of PD-1 and TIM-3 induced changes consistent with both monotherapies. Analysis of the CD8 T cell transcriptome of infiltrating lymphocytes revealed gene expression changes in response to anti-PD-1 treatment that correlated with the flow cytometry data. Ongoing studies are comparing PD-1 monotherapy to anti-PD-1 combination with other immunotherapies in multiple models.

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Extracellular pH and hypoxia affect on the function and development of antigen-specific cytotoxic T lymphocytes

Ishii, K., Nakagawa, Y., Negishi, Y., Shimizu, M., Takahashi, M., Takaku, S., Takahashi, H.

Nippon Medical School, Microbiology and Immunology, Tokyo, Japan

CD8⁺ cytotoxic T lymphocytes (CTLs) are the major effector cells in cellular adaptive immunity, which can recognize and kill virus-infected cells or tumor cells. Although CTLs exhibit strong cytolytic ability against target cells in vitro, a number of studies have shown that CTLs function is often impaired within tumors. Nevertheless, CTLs regain their cytotoxic activity after escaping from the tumor environment. These results suggest that the milieu created by growing tumors influence the cytotoxicity of CD8⁺ CTLs. The patho-physiological situation present in vivo has been shown to differ from in vitro regarding the tumor environment. In particular, low pH and hypoxia are the most important microenvironmental factors within growing tumors. To determine the effect of these factors on CTL function in vivo, we examined the cytolytic activity of CTLs against their targets using murine CTL lines and the induction of these cells from memory cells under low pH or hypoxic conditions using antigen-primed spleen cells. The results indicated that both cytotoxic activity and the induction of functional CTLs were markedly inhibited under low pH. On the other, in hypoxic conditions, although cytotoxic activity was almost unchanged, the induction of CTLs in vitro showed a slight enhancement, which was completely abrogated in low pH conditions. Therefore, antigen-specific CTL functions may be more

vulnerable to low pH than to the oxygen concentration in vivo. These findings provide new therapeutic approaches for controlling tumor growth by retaining CTL cytotoxicity through the maintenance of higher pH conditions.

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Neutrophils as therapeutic targets in metastatic breast cancer

Mouchemore, K.^{1,2}, Swierczak, A.^{1,3}, Haynes, N.¹, Hamilton, J.³, Anderson, R.^{1,4}

¹Peter MacCallum Cancer Centre, Melbourne, Australia, ²Monash University, Department of Biochemistry and Molecular Biology, Melbourne, Australia, ³Arthritis and Inflammation Research Centre, Department of Medicine, Melbourne, Australia, ⁴The University of Melbourne, Sir Peter MacCallum Department of Oncology, Melbourne, Australia

We have demonstrated that targeting Tumour Associated Macrophages (TAMs) via their growth factor Colony Stimulating Factor-1 (CSF-1) unexpectedly increased metastasis to lung and bone in two mouse models of breast cancer, accompanied by an increase in circulating and Tumour Associated Neutrophils (TANs) and an increase in plasma levels of the neutrophil growth factor Granulocyte Colony Stimulating Factor (G-CSF). Targeting the G-CSF receptor (G-CSFR) overcame this increase in metastasis and neutrophil accumulation. This project now aims to characterize the role of neutrophils and G-CSF signalling in enhancing breast cancer metastasis, and to identifying appropriate chemotherapy regimes suitable for combination with G-CSFR antibody treatment potentially for patients.

Syngeneic mouse models of breast cancer were established by 4T1.2 tumour cell injection into the mammary gland and mice were treated alone or in combination with cytotoxic chemotherapy and G-CSFR antibody. Treatment efficacy was assessed by flow cytometry for changes in immune populations in blood, tumours and lungs. Therapeutic benefit was assessed by survival and level of metastatic burden in lung.

Low dose Doxorubicin alone depletes myeloid (CD11b⁺) cell populations, including neutrophils, that suppress T-cell activity in the 4T1.2 model, resulting in enhanced anti-tumour responses. However, combination with G-CSFR antibody treatment had little therapeutic benefit. The exact mechanism of action by which G-CSFR antibody alone reduces metastasis is still being characterised. In conclusion, G-CSFR antibody treatment may benefit more from combination with radiotherapy or chemotherapy that directly stimulates T-cell infiltration/activity, rather than therapies also affecting neutrophils themselves.

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The association of lentinan with PD-1/ PD-L1 axis in gastric cancer

Ina, K.¹, Kataoka, T.¹, Ina, H.², Yoneda, M.²

¹Nagoya Memorial Hospital, Nagoya, Japan, ²School of Nursing and Health Aichi Prefectural University, Nagoya, Japan

Background: The outcomes of anticancer therapy for gastric cancer remain unsatisfactory. Aiming at improving the survival, lentinan, beta- (1, 3)-glucan with beta- (1, 6) branches, has been

used in combination with oral fluoropyrimidines such as S-1. We examined the association of lentinan with programmed death (PD)-1/ PD receptor-ligand 1 (PD-L1) axis which may elude immune surveillance.

Methods: The expression of PD-1 and PD-L1 was examined using biopsy samples from chemo-sensitive cases (n=5). Three human gastric cancer cell lines (MKN1, MKN45, Kato III) were used in vitro study. The drug solutions of 5-FU and CDDP and lentinan powder were purchased from commercial sources. All cell lines were cultured in RPMI-1640 supplemented with 10 % fetal bovine serum in the presence of various concentrations of agents. Total RNA was extracted and cDNA was synthesized. Real-time PCR analysis for PD-L1 and b-actin was performed.

Results: PD-L1 expression was demonstrated in gastric cancer cells of chemo-sensitive cases. The exposure to CDDP (0, 0.25, 0.5, 1, 2 microgram/ ml), but not 5-FU, up-regulated PD-L1 expression on gastric cancer cell in a dose-dependent manner, and co-incubation with lentinan down-regulated its expression in vitro.

Discussion: Up-regulated PD-L1 expression should be related to immune escape and chemo-resistance. Lentinan decreased platinum-induced overexpression of PD-L1 on tumor cells in vitro. This effect of lentinan on PD-1/ PD-L1 axis might be related with restoration of chemo-sensitivity, leading to enhance tumor clearance.

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The novel function of immunoregulatory molecule TIM-4 in non-small-cell lung cancer development

Zhang, Q., Wang, H., Liang, X., Ma, C., Gao, L.

Shandong University School of Medicine, Jinan, China

TIM-4 is a novel cell-surface glycoprotein belonging to TIM family. TIM-4 is exclusively expressed on antigen presenting cells. However, the role of TIM-4 in tumor development and progression remains unclear. This study aims to investigate the role of TIM-4 in lung cancer development and further explore related mechanisms. We found that the expression of TIM-4 in lung cancer tissues was significantly higher than that of adjacent tissues and closely related with lung carcinoma histological differentiation and life span of patients. Although TIM-4 expression was relatively low in lung cancer cell lines, LPS and cytokines increased its expression. Moreover, TIM-4 overexpression promoted lung cancer cell growth and proliferation, accompanied by accumulation of lung cancer cells in S phase. Consistently, TIM-4 overexpression upregulated the expression of PCNA, cyclinA, cyclinB1 and cyclinD1. More importantly, we found that TIM-4 interacted with $\alpha\beta 3$ integrin. Interestingly, Arg-Gly-Asp (RGD) motif mutation of TIM-4 abolished the effect of wild type TIM-4 on growth, proliferation and cell cycle progression of A549 cells. Moreover, TIM-4 mediated promotion of lung cancer growth and proliferation by its RGD motif was further verified by tumor xenografts in mice. These data suggest that TIM-4 might be a potential diagnostic marker and therapeutic target of lung carcinoma.

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The role of vasculogenic mimicry vessels in mediating leukocyte recruitment to melanomas

Tan, L.^{1,2}, Sachi, P.¹, Brown, M.^{1,3}, Bonder, C.¹, Ebert, L.¹

¹Centre for Cancer Biology, University of South Australia and SA Pathology, Adelaide, Australia, ²University of South Australia, School of Pharmacy and Medical Sciences, Adelaide, Australia, ³Royal Adelaide Hospital, Department of Medical Oncology, Adelaide, Australia

The endothelial cells of the vasculature act as a gatekeeper between the leukocytes and underlying tissues, including solid tumours such as melanoma. By regulating adhesion molecule expression on the endothelium, tumours can selectively recruit pro-tumourigenic leukocytes to enhance tumour growth. Interestingly, melanoma cells themselves can also form vessel-like structures, a process known as vasculogenic mimicry (VM). The occurrence of VM closely correlates with disease progression in melanoma and other cancers such as breast cancer. While VM vessels appear to function like the conventional endothelium in conducting blood, as indicated by the presence of blood cells within the VM vessels and their connection with the endothelium, whether or not they can recruit leukocytes remains unknown. We and others have shown that melanoma cells can express various adhesion molecules and chemokines, thereby hinting at the potential of these cells to interact with leukocytes. Indeed, preliminary results obtained from parallel plate flow chamber assays and transwell migration assays have revealed that leukocytes, in particular monocytes, could roll, adhere and transmigrate across a melanoma cell monolayer. Interestingly, the capability of the melanoma cells in recruiting leukocytes appeared to correlate with their capability of forming vessel-like structures when subjected to a Matrigel tube formation assay, which reflects their VM capability *in vitro*. Current work focuses on further defining the adhesion molecules and chemokines involved in this process, as well as the detailed phenotype of the recruited leukocytes. Taken together, VM vessels may play an important role in leukocyte recruitment and ultimately dictate melanoma disease outcome.

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Loss of AIM2 expression promotes hepatocarcinoma progression through activation of mTOR-S6K1 pathway

Ma, X.¹, Guo, P.¹, Qiu, Y.¹, Li, T.², Han, L.¹

¹Shandong University School of Medicine, Department of Immunology, Jinan, China, ²Provincial Hospital Affiliated with Shandong University, Department of Gastroenterology, Jinan, China

Absent in melanoma (AIM2) is recognized as a cytoplasmic DNA sensor playing important role in both innate immunity and tumor pathology. However, the role of AIM2 in the development of hepatocellular carcinoma (HCC) remains to be clarified. We here investigate the mRNA and protein levels of AIM2 expression in liver cancer tissues and corresponding non-cancerous liver tissues from a total of 113 HCC patients. Our data showed that expression of AIM2 was significantly decreased in liver cancer tissues, and this decreased expression was significantly

correlated with more advanced tumor progression. Our *in vitro* and *in vivo* assay showed that loss of AIM2 expression significantly promoted the proliferation, colony formation and invasive capability of these HCC cells. Further analysis showed that mammalian target of rapamycin (mTOR)-S6K1 pathway was significantly activated in the AIM2-absent HCC cells, and treatment with mTOR pathway inhibitor rapamycin verified that this mTOR pathway activation contributed to HCC progression. Thus, these data suggested that AIM2 played a critical role as a tumor suppressor and might serve as a potential therapeutic target for future development of AIM2-based gene therapy for human liver cancer. This study also paves a new avenue to treat AIM2-deficient cancer by suppression of mTOR.

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Metabolic reprogramming of myeloid cells during tumor progression

Huang, L.-R., Jian, S.-L., Zhou, Y.-J., Chen, W.-W.

National Health Research Institutes, Institute of Molecular and Genomic Medicine, Miaoli County, Taiwan, Republic of China

Myeloid-derived suppressor cells (MDSCs) are heterogeneous populations of immature myeloid cells, which expand and differentiate in responses to growth factors, cytokines and chemokines derived from cancer cells and then are released from bone marrow to peripheral tissues including tumor sites. MDSCs play important roles not only in T-cell suppression but also in angiogenesis, lymphangiogenesis and metastasis during tumor progression. The influence of tumor cells on the activation and proliferation of myeloid cells has been extensively studied. However little is known about the metabolic status of MDSCs during tumor progression. We therefore try to elucidate the metabolic changes during differentiation of myeloid cells into MDSCs.

We have found that myeloid cells up-regulated their glycolytic pathway and immunosuppressive features when encountering cancer cells *in vitro*. Results from DNA microarray and real-time PCR also revealed that monocytic and granulocytic MDSCs from primary tumor sites of 4T1 tumor-bearing mice promoted their glycolysis in comparison to their healthy counterparts, monocytes and neutrophils, from bone marrow of healthy mice. We further proved that glycolytic pathway contributed to the survival and immunosuppression of MDSCs *in vitro* and *in vivo* using a hexokinase inhibitor, 2-DG, to block glycolysis. We are currently examining the association of metabolites from glycolysis and differentiation of myeloid cells during tumor progression and expect to identify specific metabolite responsible for driving differentiation of MDSCs.

199 miR-200c regulates migration of breast cancer cell BT549 by targeting slug

*Jia, L., Tian, Y., Shi, Y., Zhang, L., Rong, S., Zhang, Y., Li, J.
Third Affiliated Hospital of Zhengzhou University, Clinical
Laboratory, Zhengzhou, China*

This study regarded breast cancer BT549 cell as the research object and transferred miR-200c into the BT549 to explore the relationship of miR-200c and the expression of Slug and the influence of miR-200c to the migration of BT549 cell in order to provide laboratory basis for clinical diagnosis and treatment.

Method: miR-200c mimic was transfected into a highly metastatic breast cancer cell line BT549 which was cultured through RPMI-1640 medium within 10% fetal bovine serum. The experiment was divided into 3 groups: miR-200c group, miR-control group and blank control group. 3 wells were prepared in each group.

Transwell migration assay, Wound healing assay, Western blot assay and Real time PCR were used in the study process.

Result:

1. Compared with blank control groups and miR-control group, the migratory ability of the BT549 cell in miR-200c group was reduced and the difference had statistical significance ($P < 0.05$).
2. Compared with blank control group and miR-control group, the expression of Slug mRNA in the BT549 cell of miR-200c group was decreased significantly ($P < 0.05$), but the expression of E-cadherin mRNA was increased significantly ($P < 0.05$).
3. Compared with blank control group and miR-control group, the expression of Slug in the BT549 cell of miR-200c group was declined significantly ($P < 0.05$).

Conclusion: The miR-200c in BT549 could reduce the expression of correspondent Slug protein by suppressing the translation of Slug mRNA and subsequently promote the expression of E-cadherin, inhibit EMT (Epithelial-Mesenchymal Transition) and block the migration of BT549 cell.

200 Up-regulated S100A9 in stroma functions as an early recurrence marker for early-stage oral cancer patients through increased tumor invasiveness, angiogenesis, macrophage recruitment and IL-6 expression

*Wu, L.-W.
National Cheng Kung University, Institute of Molecular Medicine,
Tainan, Taiwan, Republic of China*

S100A9 is a calcium-binding protein with two EF-hands and frequently deregulated in several cancer types, however, with no clear role in oral cancer. In this report, the expression of S100A9 in cancer and adjacent tissues from 79 early-stage oral cancer patients was detected by immunohistochemical staining. Although S100A9 protein was present in both tumor and stromal cells, only the early-stage oral cancer patients with high stromal expression had reduced recurrence-free survival. High stromal S100A9 expression was also significantly associated with non-well differentiation and recurrence. In addition

to increasing cell migration and invasion, ectopic S100A9 expression in tumor cells promoted xenograft tumorigenesis as well as the dominant expression of myeloid cell markers and pro-inflammatory IL-6. The expression of S100A9 in one stromal component, monocytes, stimulated the aggressiveness of co-cultured oral cancer cells. We also detected the elevation of serum S100A9 levels in early-stage oral cancer patients of a separate cohort of 73 oral cancer patients. The release of S100A9 protein into extracellular milieu enhanced tumor cell invasion, transendothelial monocyte migration and angiogenic activity. S100A9-mediated release of IL-6 requires the crosstalk of tumor cells with monocytes through the activation of NF- κ B and STAT-3. Early-stage oral cancer patients with both high S100A9 expression and high CD68+ immune infiltrates in stroma had shortest recurrence-free survival, suggesting the use of both S100A9 and CD68 as poor prognostic markers for oral cancer. Together, both intracellular and extracellular S100A9 exerts a tumor-promoting action through the activation of oral cancer cells and their associated stroma in oral carcinogenesis.

201 Mesenchymal stromal cells derived from cervical cancer tumors induce an increase of CD73 expression in cervical cancer cells

*Avila Ibarra, L.R.¹, Mora Garcia, M.D.L.², Hernández Montes, J.²,
Garcia Rocha, R.¹, Weiss-Steider, B.², Monroy Garcia, A.¹
¹IMSS, CMN SXXI, UIMEO, Mexico City, Mexico, ²UNAM, FES
Zaragoza, Mexico City, Mexico*

Mesenchymal stromal cells (MSCs) are important components in the tumor microenvironment and participate together tumor cells in the suppression of antitumoral immune response, via production of immunosuppressive factors such as TGF- β . Since the expression of the ectonucleotidase CD73 in malignant cells is also associated with immunosuppression via adenosine production, and that its expression is dependent of TGF- β production, the aim of this work was to analyze whether MSCs derived from cervical tumors (CeCa-MSCs) increase the expression of CD73 on cervical cancer cells through the production of TGF- β . For this purpose CeCa-MSCs and MSCs obtained from normal cervix (NCx-MSCs), were co-cultured with CaSki and Hela cervical cancer (CeCa) cell lines. After 96hrs, CD73 expression was determined by flow cytometry on tumor cells, as well as, the content of TGF- β in the supernatants by ELISA. Interestingly, we observed that CeCa-MSCs as well as NCx-MSCs, induced an important increase of the expression of CD73 on tumor cells, which was dependent of the amount of MSCs added to the cell co-culture, as well as of the presence of TGF- β in the supernatants, since the addition of anti-CD73 mAb strongly blocked the increased expression. These results provide evidence that MSCs collaborate with CeCa cells to increase their expression of CD73 through the production of TGF- β , providing a potential mechanism for immunosuppression in CeCa. This work was supported by DGAPA-PAPIIT (IN226516), FIS/IMSS/PROT (1314 and 1383) and CONACYT (240635) grants.

202**HBV infection promotes proliferation, migration and invasion of hepatocellular carcinoma via the regulation of miR-181a/362 by targeting HSPA5**

Ma, S., Qin, K., Ouyang, H., Jiang, Q., Li, H., Wu, M., Zhu, H., Lei, P., Shen, G.

Huazhong University of Science and Technology, Immunology, Wuhan, China

HSPA5, which belongs to the heat shock protein 70 family, is a stress-inducible endoplasmic reticulum (ER) chaperone and a major regulator of the unfolded protein response (UPR). Tumors are subjected to ER stress triggered by both intrinsic and extrinsic factors, such as hypoxia, acidosis, viral infection and genetic lesions leading to UPR activation and HSPA5 induction. The up-regulation of HSPA5 in a variety of tumors and its induction after drug therapies has been considered as a main contributor to oncogenesis and therapeutic resistance. Moreover, HSPA5 participates in the process of immunoregulation and tumor escape. Notwithstanding these studies, the molecular mechanism underlying the increased HSPA5 expression in HCC remains poorly understood. Given that microRNAs (miRNAs) are implicated in HCC progression, we explored the potential role of miRNAs in HCC by performing miRNA expression profiling between HepG2 and HepG2.215. We discovered miR-181a/362 as two of the most markedly overexpressed miRNAs and highly associated with HBV-positive HCC. We found the overexpression of miR-181a/362 displayed significantly stimulative effects on the proliferation, migration and invasion potentialities of HCC cell lines. Using an integrated approach, we uncovered that HSPA5, whose expression was obviously increased in HCC specimens in comparison with the adjacent noncancerous hepatic specimens and liver hemangioma specimens, was a novel target of miR-362. Furthermore, we showed an inverse influence on the expression levels of miR-181a/362 and HSPA5 exerted by transfection of HBV related plasmids. Altogether, our findings indicate miR-181a/362 may serve as potential prognostic biomarkers and novel targets in the therapeutic strategies of HCC.

203**Role of immune cells in prostate cancer development and clearance**

Mishra, M.¹, Kumar, S.¹, Singh, U.², Scissum-Gunn, K.¹, Manne, U.³, Ponnazhagan, S.³

¹Alabama State University, Montgomery, United States, ²University of South Carolina School of Medicine, Columbia, United States,

³University of Alabama at Birmingham, Birmingham, United States

Prostate Cancer (PCa) is the most common non-skin malignancy and the most commonly diagnosed cancer in men in the United States. The immunotherapeutic role of immune cells in regulation of PCa has been studied but still the specific interplay between these cells to regulate cancer needs further investigation. In this study, we analyzed the role of immune cells in tumor development and progression using a prostate cancer model. We used TRAMP cells (TRAMP C1, C2 and C3 derived from transgenic adenocarcinoma of mouse prostate) to induce tumor

in C57/B6 mouse. Interestingly, TRAMP-C1 and TRAMP-C2 cells are tumorigenic while TRAMP-C3 cells fail to form tumor. Briefly, tumor induction studies were performed on different groups of mice. Mice were inoculated with these three cell lines, tumors were analyzed at different time points, and the percentage and absolute number of different immune cells such as CD4, CD8, NK, NKT, Macrophages, regulatory T cells, and Dendritic cells were analyzed. Our data demonstrated that the capacity of TRAMP-C1 and TRAMP-C2 cells to form tumors and the inability of TRAMP-C3 cells to induce tumors is mediated by number of different immune cells such as NK, NKT, macrophages and regulatory T cells in the tumor microenvironment. Therefore, the data suggest an understanding of function and effect of immune cells during tumor progression and clearance is needed to successfully develop a targeted therapy to modulate the number of immune cells in the tumor microenvironment.

204**The production of cytokines induced by breast tumor antigens in dendritic cells is modulated by cholinergic activation**

Lombardi, M.G.¹, Oroño, M.², Giambalvo Gómez, D.², Martínez Pulido, P.², Sales, M.E.²

¹Centro de Estudios Farmacológicos y Botánicos, Facultad de Medicina, UBA, Segunda Cátedra de Farmacología, FMED, UBA, CABA, Argentina, ²Centro de Estudios Farmacológicos y Botánicos, Laboratorio de Inmunofarmacología Tumoral, Segunda Cátedra de Farmacología, CABA, Argentina

We have previously described the presence of a functional non neuronal cholinergic system in human dendritic cells (DC). The addition of cholinergic agonists, carbachol and acetylcholine modulates the function of DC. Taking into account that a deficiency in the activity of DC was reported in tumor bearers, we investigated the effect of cholinergic receptors (ChR) activation in human DC cells regarding their cytokine profile. Experiments were performed in the absence or presence of tumor antigens obtained from the human luminal breast adenocarcinoma, MCF-7.

Methods: DC were obtained from human monocytes and were treated during 24h with different concentrations of MCF-7 tumor extract (MTE). Afterwards, DC were stimulated with carbachol during 1 h in the absence or presence of cholinergic antagonists (atropine or mecamylamine). Finally, DC were cultured without or with lipopolysaccharide during 24 h to obtain immature or mature DC (iDC or mDC). The production of cytokines in culture supernatants was quantified by ELISA.

Results: In iDC, the addition of increasing doses of MTE triggered a potent up-regulation of interleukin (IL)-10 production while the effect on IL-12 or tumor necrosis factor alpha (TNF- α) levels was less potent ($495 \pm 19\%^{**}$; $295 \pm 28\%^{*}$; $269 \pm 20\%^{*}$, respectively; $^{**}p < 0.0001$; $^{*}p < 0.01$ vs. control). Carbachol prevented the effect of MTE on IL-10 production by iDC ($p < 0.0001$ vs. MTE). MTE did not modify IL-10 or TNF- α levels in mDC, while the addition of carbachol strongly increased TNF- α production ($125 \pm 18\%$; $p < 0.0001$ vs. MTE).

Conclusions: These results show that the activation of ChR promotes an inflammatory response that could modulate the

tolerogenic/immunosuppressive profile induced during DC-tumor interaction.

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Time-scale analysis of interplay between occult immunogenic tumor and immune response

Mojic, M.¹, Sato-Matsushita, M.², Tahara, H.², Hayakawa, Y.¹

¹University of Toyama, Institute of Natural Medicine, Division of Pathogenic Biochemistry, Department of Bioscience, Toyama, Japan, ²The University of Tokyo, The Institute of Medical Science, Department of Surgery and Bioengineering, Tokyo, Japan

The implication of controlling cancer growth *in vivo* by immune response has been mostly based on the results obtained by monitoring palpable tumors. Consequently, the time-scale position of specific immune response to control occult tumor remained unclear. To tackle this question, we established a bioluminescence imaging model to monitor the interplay between occult immunogenic tumor and various components of innate and adaptive immunity. By using mouse B16 melanoma cells co-expressing ovalbumin (OVA) and luciferase (B16OVA-Luc cells), we monitored the exact behavior of B16OVA-Luc cells under the different immunological conditions. In mice immunized with tumor antigen ovalbumin (OVA-mice), we observed time-limited immune control of tumor growth that was far more dynamic than detected with caliper measurement. Anti-tumor immune response in OVA-mice was critically dependent on IFN- γ and CD8⁺ T cells, which inhibition/depletion as late as a 5 days after tumor inoculation resulted in abolition of tumor growth inhibition. In the absence of NK cells, tumor growth suppression in OVA-mice was still conceivable, yet time of immune control over tumor growth was significantly shorter. Compared to tumor antigen-naïve group, we observed significantly higher expression of PD-1 and PD-L1 in OVA-specific CD8⁺ T cells and B16OVA-Luc cells, respectively, even during the period of time where immune control over tumor growth was evident. We are now further exploring the mechanism how IFN- γ plays specific role in the maintenance and/or the effector phase of anti-tumor immune response to control occult tumor in the tumor antigen experienced mice.

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Immunological markers of persistence & regression in cervical intraepithelial neoplasia grade 2 in women under 25

Saito, M.¹, Budhwani, M.¹, Coffey, K.², Sykes, P.³, Hibma, M.¹

¹University of Otago, Department of Pathology, Dunedin School of Medicine, Dunedin, New Zealand, ²University of Otago, Department of Women's and Children's Health, Dunedin School of Medicine, Dunedin, New Zealand, ³University of Otago, Department of Obstetrics and Gynaecology, Christchurch, New Zealand

Cervical cancer is the second most common cause of death from cancer in women. High-risk human papillomavirus (hr-HPV) infection has been recognized as the major risk factor for cervical cancer. The development of cervical cancer, and the

preceding high grade cervical lesions (cervical intraepithelial neoplasia [CIN] 2 and CIN3) is likely to be associated with a lack of an effective immune response against hr-HPV. However, there is a relatively high spontaneous regression rate for CIN2, which is considered to be immune mediated. The aim of this study is to identify immune markers of regression and persistent of CIN2 to identify novel predictive markers of disease outcome.

A range of immunological markers, including CD4, CD8, FoxP3, granzyme, CD1a and Langerin, were used to identify T cell subsets and antigen presenting cell populations in infected lesions from women under 25 with persistent or regressing CIN2 lesions. We found a significant increase in the number of CD8⁺, granzyme positive T cells in regressing lesions when compared with persistent lesions, but no significant difference in the number of Tregs between the two groups.

From these experiments we conclude that granzyme positive CD8⁺ T cells are more likely to be identified in lesions that will regress, even in lesions that contain populations of Tregs. This suggests that the Tregs may not exert a strong suppressive effect on the CD8 T cytotoxic T cells in CIN2 lesions.

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Prebiotic supplement effect on the response to a mutated PAP-derived vaccine in old mice

McArdle, S.E.¹, Rees, R.C.¹, Pockley, A.G.¹, Gibson, G.R.²

¹Nottingham Trent University, The John van Geest Cancer Research Centre, Nottingham, United Kingdom, ²Reading, Food and Nutritional Sciences, Reading, United Kingdom

Vaccine-based immunotherapy can increase the overall survival of patients with advanced prostate cancer. However, the efficacy of vaccine-elicited anticancer immune responses is heavily influenced by the physical, nutritional, and psychological status of the patient. Given their importance, these parameters should be carefully considered for the design of future clinical trials testing this immunotherapeutic paradigm in prostate cancer patients.

We have recently demonstrated the ability of a prostatic acid phosphatase-derived 15mer peptide (PAP-114) to reduce the growth of established TRAMP C1 tumours in a pre-clinical murine model. These results are encouraging and show the efficacy potential of vaccine based therapy, however these were obtained using relatively young animals (maximum 12 weeks) and therefore might not represent the responsiveness/effectiveness which would be generated in older mice or in prostate patients, the average of which is 65 years. We have since elongated, mutated and patented this PAP-derived sequence and demonstrated its superiority in generating PAP-peptides specific T-cells compared to the wild-type, short or long, PAP-sequences. Moreover, due to its length this sequence contains several peptides with high binding affinity to multiple HLA-haplotypes thereby avoiding the need, in the future, to HLA type patients prior to vaccination.

People over 60 typically exhibit a marked reduction in gut bacteria, however supplementation with pre-biotics (e.g. GOS) can restore it, Here we show that older mice benefited from prebiotic supplementation in their diet two weeks prior to vaccination.

These results have profound consequences for the management of any vaccination trial involving older patients, especially cancer patients.

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Can IL-25 remove the malignant cells spontaneously? An in vitro evaluation of IL-25 versus IL-17B on breast cancer cell lines

Shokrollahi Barough, M.^{1,2}, Pak, F.¹, Kokhaei, P.^{1,3}, Barati, M.¹, Barabadi, M.⁴

¹Semnan University of Medical Sciences, Department of Immunology and Cancer Research Center, Semnan, Iran, Islamic Republic of, ²Tehran University of Medical Sciences, Immunology, Asthma and Allergy Research Institute, Tehran, Iran, Islamic Republic of, ³Karolinska Institute and Karolinska University Hospital Solna, Immune Gene Therapy Lab, CCK, Stockholm, Sweden, ⁴Tehran University of Medical Sciences, Department of Immunology, Teheran, Iran, Islamic Republic of

Introduction: IL-25 (IL-17E) and IL-17B are two members of IL-17 family, which bind to a unique receptor (IL-25R). IL-25 can induce apoptosis and IL-17B can enhance the survival of tumor cells in breast cancer. IL-25R as a poor diagnostic marker is widely expressed on breast cancer tissue the exact role of these cytokines is unknown.

Materials and methods: MCF-7 (Estrogen receptor (ER) positive), MDA-MB231 (ER-) like malignant cells and MCF-10A as a non-malignant breast cell line (ER-) was treated with recombinant human cytokines (rhIL-25/IL-17E and rhIL-17B). Apoptosis by flow cytometry and the proliferation by MTT assay was evaluated. Gene expression analysis was performed for IL-25R, IL-17B and MMP-1 by Real Time PCR before and after treatment.

Results: IL-25 treatment induced apoptosis up to 20% and IL-17B promoted cell viability in malignant cell lines without effect on MCF-10A cells. In combinational cytokine treatment the apoptosis assay showed antagonistic effect of IL-17B on IL-25. Both cytokines increased expression level of IL-25R only in ER+ cells and IL-17B expression level in both malignant cells. Although IL-17B expression wasn't observed at non-treated MCF-10A cells, IL-17B treatment caused to 105 fold increment of IL-17B expression. Both cytokines induced elevated level of MMP-1 expression in MDA-MB231.

Conclusion: ER status can affect the IL-25R expression in response to IL-25 and IL-17B treatments without effect on MMP-1. IL-25 may promote the apoptosis in malignant cells, however overexpression of IL-17B in response to IL-25 switch on the IL-17B expression in non-malignant cells which may enhance the proliferation in ER+ cells and promote the metastasis in ER- cells.

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The relationship between tumor associated neutrophils and tumor progression and the mechanism

Han, M., Liu, H., Bai, R.

Ningxia Medical University, Department of Immunology, Yinchuan, China

Objective: To study the relationship of tumor associated neutrophils (TANs) with the occurrence and progression of

gastric cancer, and its molecular mechanism.

Methods: Collecting gastric tumor tissues and adjacent noncancerous tissues from 48 patients with final diagnosis of gastric cancer. Immunohistochemistry method was used to detect the number of TANs and the levels of NE and CXCL-1. The correlations between TANs and CXCL-1, NE, MMP-9, LOX, angiogenesis in gastric cancer tissues were evaluated with Pearson correlation analysis. Peripheral blood neutrophils of normal person were separated, and induced through IFN- β antibody with concentration of 5ug/ml (IFN- β antibody group) and 50% tumor cultivation mediums (tumor cultivation mediums group). The RNA and proteins of LOX MMP-2 MMP-9 generating from neutrophils were detected using real-time PCR and ELISA. And enzymatic activity of MMP-2 and MMP-9 were analyzed with gelatin zymography assay.

Results: The number of TANs was more in gastric cancer tissue than that in adjacent noncancerous tissues; the number of TANs were positively correlated with the expression levels of NE, CXCL-1, LOX, VEGF and tumor angiogenesis; when the neutrophils from normal person were induced to N2 type, the amounts of MMP-9, MMP-2 and LOX secreting by neutrophils were increased significantly.

Conclusions: The most of tumor associated neutrophils in gastric cancer were N2 type, they adjust the angiogenesis and occurrence of gastric cancer through NE, CXCL-1, LOX, MMP-2, MMP-9 and VEGF.

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Immune biomarkers of response to chemoradiotherapy in locally advanced rectal cancer

McCoy, M.J.^{1,2}, Hemmings, C.^{3,4}, Anyaegbu, C.C.¹, Lee-Pullen, T.F.^{1,4}, Austin, S.J.¹, Miller, T.J.^{1,4}, Bulsara, M.K.⁵, Zeps, N.^{1,4}, Nowak, A.K.^{2,6}, Lake, R.A.², Platell, C.F.^{1,4}

¹St John of God Subiaco Hospital, Perth, Australia, ²The University of Western Australia, School of Medicine and Pharmacology, Perth, Australia, ³St John of God Pathology, Perth, Australia, ⁴The University of Western Australia, School of Surgery, Perth, Australia, ⁵The University of Notre Dame, Institute for Health Research, Fremantle, Australia, ⁶Sir Charles Gairdner Hospital, Medical Oncology, Perth, Australia

Background: Chemoradiotherapy prior to surgery (neoadjuvant CRT) is recommended for locally advanced rectal cancer. Individual response to CRT varies considerably with ~20% of patients achieving a pathologic complete response (pCR), which is associated with improved long-term outcome. Factors associated with response are poorly understood. This study aims to identify immunological markers of response to CRT.

Methods: Foxp3+, CD3+, CD8+, CD4+, and IL-17+ cell density was assessed by standard immunohistochemistry in post-CRT surgical samples from 128 patients with advanced rectal cancer. Pre-CRT biopsy samples were obtained from 114 (89%) of cases and infiltrating Foxp3+, CD3+ and CD8+ cells identified using the same methods. Cell densities were quantified using digital image analysis software and correlated with clinical outcome (tumour regression grade and survival).

Results: Low stromal Foxp3+ Treg density post-CRT was strongly associated with pCR (OR: 5.27 (95% CI 1.62 - 17.16), P

= 0.006, after adjustment for pre-surgery clinical factors) and improved recurrence-free survival (HR: 0.46 (95% CI: 0.23 - 0.92), P = 0.03). However, density of Treg, total T cells, or CD8+ T cells in pre-CRT biopsy samples was not predictive of subsequent response to CRT or long-term outcome. Peripheral blood T cell subsets pre- and post-CRT are currently being assessed in a second patient cohort for correlation with CRT response and tumour-infiltrating T cell subset densities.

Conclusions: Pretreatment density of T cell subsets in the local tumour environment does not predict response to neoadjuvant CRT in rectal cancer. However, depletion of Treg during CRT may contribute to treatment efficacy.

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***P. gingivalis*-induced inflammation and immunoregulation promote the progression of pancreatic cancer**

Jan, M.-S.^{1,2,3}, Lin, C.-W.¹, Lin, Y.-C.¹, Chen, W.-T.¹, Chen, Y.-J.¹, Tsai, C.-H.⁴, Lee, H.-L.⁵, Hsu, L.-J.⁶

¹Chung-Shan Medical University, Institute of Biochemistry, Microbiology and Immunology, Taichung, Taiwan, Republic of China, ²Chung Shan Medical University Hospital, Division of Allergy, Immunology, and Rheumatology, Taichung, Taiwan, Republic of China, ³Chung-Shan Medical University, Immunology Research Center, Taichung, Taiwan, Republic of China, ⁴Chung-Shan Medical University, Institute of Medicine, Medical College, Taichung, Taiwan, Republic of China, ⁵Chung Shan Medical University Hospital, Division of Surgery, Taichung, Taiwan, Republic of China, ⁶National Cheng Kung University, Department of Medical Laboratory Science and Biotechnology, Tainan, Taiwan, Republic of China

Pancreatic cancer is one of the most malignant cancers, with a very low five-year relative survival rate. It is noteworthy that recently a few epidemiological research studies have suggested an association between periodontitis and pancreatic cancer. Our studies demonstrated that inoculation of periodontal pathogen *Porphyromonas gingivalis* (*P. gingivalis*) could promote pancreatic cancer (PC) progression in *LSL-Kras^{G12D/w}Pdx-1-Cre^{+/-}* transgenic mice. Significant rapid pancreatic intraepithelial neoplasias (PanINs) progression, which was not found among the *vehicle group mice*, was discovered in *pancreata* of six-week-old *LSL-Kras^{G12D/w}Pdx-1-Cre^{+/-}* mice one month following *P. gingivalis* inoculation episodes. The levels of epithelial-mesenchymal-transition increased in *P. gingivalis*-treated group as well. On the other hand, in vitro treatments of *P. gingivalis* or *P. gingivalis*-cultured broth could induce inflammatory responses including IL-1b, TNF-a, and COX-2 expression of PC and macrophage cell lines. These findings significantly support that periodontitis can accelerate pancreatic cancer progression through induction of inflammation, which, in turn can provide guidance for future research into prophylactic strategy for pancreatic cancer development.

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Anti-proliferation effects of *Scrophularia oxypepala* medicinal plant extract on the prostate cancer cell line (PC3)

Baradaran Gavvani, M.¹, Nasirzonouzi, M.², Nasirzonouzi, P.³, Baradaran, M.⁴

¹Tabriz University of Medical Sciences, Tabriz, Iran, Islamic Republic of, ²Azad Islamic University of Tabriz, Biomedical Engineering, Tabriz, Iran, Islamic Republic of, ³Azad Islamic University of Tabriz, Tabriz, Iran, Islamic Republic of, ⁴Technical University of Sahand, Tabriz, Iran, Islamic Republic of

Introduction: Medical plants have been intensively studied as a source of antitumor compounds and immunomodulatory effect. The antitumor effects of the *Scrophularia oxypepala* medicinal plant extract is not studied on the PC3 prostate cancer cell lines. In the present study, cytotoxic effects of the *Scrophularia oxypepala* extract were investigated on viability of PC3 prostate cancer cell lines.

Material & methods: The cytotoxic effects of *Scrophularia oxypepala* on PC3 prostate cancer cell lines were studied using MTT assay, Trypan blue staining, and DNA fragmentation assay were done at selected concentrations of the plant extract.

Results: According to the findings, the *Scrophularia oxypepala* medicinal plant extract (stems and leaves) altered cells morphology. The *Scrophularia oxypepala* extract inhibited cell growth albeit in a time and dose dependent manner and resulted in degradation of chromosomal DNA.

Conclusion: Our data well established the anti-proliferative effect of *Scrophularia oxypepala* extract, and clearly showed that the plant extract can induce apoptosis in vitro, but the mechanism of its activities remained unclear.

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Pre-clinical development of a first-in-class heat shock protein-based personalized cancer vaccine

Khattar, M., Morin, B., Uduman, M., Zelin, J., Stein, R., Exley, M., Castle, J., Levey, D.

Agenus Inc., Lexington, United States

Tumors accumulate mutations which can give rise to neo-epitopes recognizable as non-self by T-cells. Advances in next-generation sequencing and bioinformatics make it possible to analyze individual patients' tumor genomes and predict immunogenic mutations, which can be synthesized as short peptides, RNA or DNA for use in personalized cancer vaccines. However, these approaches need optimization for safe and effective induction of anti-tumor T-cell responses. We addressed this challenge by complexing synthetic long peptides comprising predicted tumor neo-epitopes to recombinant Hsc70, creating personalized vaccine candidates, AutoSynVax™ (ASV™), administered with QS-21 Stimulon® adjuvant. The same platform comprising Hsc70 plus synthetic long viral peptides was previously validated in a Phase-2 trial, demonstrating effective T-cell responses and reduced viral shedding in HSV-2+ subjects. ASV immunization is designed to facilitate antigen-processing and presentation of neo-epitopes to T-cells, resulting in robust immune responses. Therapy with ASV comprising two previously validated neo-epitopes in B16.F10 melanomas

resulted in significantly lower tumor burden and prolonged survival in mice in a dose-dependent manner. The relationship between tumor protection conferred by ASV and T-cell response to neo-epitopes is under investigation. In addition, novel B16.F10 neo-epitopes have been identified using our proprietary algorithm, AIM™ v1.1, which analyzes whole-exome and RNA-sequencing data to identify immunogenic mutations. We continue to optimize ASV to facilitate complexing of larger pools of peptides to Hsc70. With a positive record of Hsc70 and QS-21 Stimulon exposure in humans and demonstrated preclinical anti-tumor activity of ASV, we are poised to begin a first-in-human clinical trial in second half of 2016.

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Cytokine/chemokine pattern in cutaneous melanoma

Neagu, M.^{1,2}, Constantin, C.¹, Surcel, M.¹, Zurac, S.^{3,4}

¹Victor Babes National Institute of Pathology, Immunology, Bucharest, Romania, ²University of Bucharest, Bucharest, Romania, ³Colentina University Hospital, Pathology, Bucharest, Romania, ⁴University of Medicine and Pharmacy, Pathology, Bucharest, Romania

Circulatory cyto/chemokines pinpoint inflammation processes enabling melanoma cells metastasis through blood/lymphatic systems to other tissues. Patterns of these immune-related molecules and their secretory immune cells can be useful in immune-therapy monitoring of cutaneous melanoma patients. Hence, the aim of our study was the identification in melanoma patients' peripheral blood the cyto/chemokines and the percentage of immune cells that can secrete them, as an outline of the disease stage and clinical outcome.

50 patients (AJCC stages) were included and followed for 36 months. Pre and post-surgery/therapy immune parameters evaluation was performed for all patients including peripheral immune cell populations (*Intracellular Cytokine Staining Kit - Human (BD Pharmingen)*) and circulating cytokines / chemokines using xMAP technology; 170 normal subjects (matching gender and age) represented the control group. All enrolled subjects gave their written consent and the study was approved by the Ethical Committees.

We have found different circulatory cyto/chemokines patterns matching disease stages. Out of the studied cyto/chemokines, IL-6, TNF-alpha, IL-10 and IL-8 were found statistically increased with advanced stages. In the follow-up study, IL-6 correlated with survival, while IL-8, IL-10 and IL-12 did not correlate with overall survival or relapse free survival. Regarding circulatory immune cells capable of secreting the studied cyto/chemokines we found that there is a significant proportion of lymphocytes that can be induced to secrete IFN-γ or TNF-α, while no differences compared to controls for IL-2 secreting cells.

Circulatory immune markers can complete the immune status of the patient.

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Roles of CD11c⁺ T cells in anti-tumor immune responses

Chiba, M., Nakano, N.

Tokyo University of Science, Research Institute for Biomedical Sciences, Noda, Japan

Immune responses against tumor cells are suppressed as tumor microenvironment changes during tumor progression. T cells that attack tumor cells are energized by various mechanisms including signals induced through PD-1 and CTLA-4. In order to find out the molecules that control T cell activation and suppression in tumor microenvironment, we analyzed infiltrating cells in the grafted tumors from early time point (day 10~15) to later stages (day 20~30). Tumor microenvironment was clearly shifted from the M1 macrophage-dominant stage (early phase) to the M2 macrophage stage. Among T cells infiltrating in an early phase, there were T cells expressing CD11c, however, these T cells were rare in the later stage. CD11c⁺ T cells in tumor tissues were CD44^{high}, CCR2⁺, Foxp3⁻, expressing lower levels of PD-1 than CD11c⁻ T cells. In normal mice, T cells with CD11c expression were also found in the spleen, lymph nodes and the thymus although the frequency was low. CD11c⁺ T cells were frequently found within the intestinal epithelial lymphocytes, most of which were expressing CD8aa.

In vitro activation of T cells with anti-CD3+CD28, PMA+ionomycin failed to induce CD11c expression while T cells stimulated with Concanavalin A (Con A) became CD11c⁺. Interestingly, these CD11c⁺ T cells were always PD-1 negative. These results suggested that Con A could activate a distinct signaling pathway, which induced CD11c expression and suppressed PD-1 induction.

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Chemokine receptor patterns in lymphocytes mirror metastatic spreading in melanoma

Jacquelot, N.^{1,2}, Enot, D.^{3,4}, Flament, C.¹, Vimond, N.⁵, Blattner, C.^{6,7}, Pitt, J.¹, Yamazaki, T.¹, Roberti, M.P.¹, Daillère, R.¹, Vétizou, M.¹, Poirier-Colame, V.¹, Semeraro, M.^{1,8}, Caignard, A.⁹, Slingluff, C.L.¹⁰, Sallusto, F.¹¹, Rusakiewicz, S.¹, Weide, B.^{12,13}, Marabelle, A.¹, Kohrt, H.¹⁴, Dalle, S.¹⁵, Cavalcanti, A.⁵, Kroemer, G.^{3,4,16,17,18}, Di Giacomo, A.M.¹⁹, Maio, M.²⁰, Wong, P.²¹, Yuan, J.²¹, Wolchok, J.²¹, Umansky, V.^{6,7}, Eggermont, A.⁵, Zitvogel, L.^{1,2,22}

¹Gustave Roussy Cancer Campus, INSERM U 1015, Villejuif, France, ²University Paris-Saclay, Kremlin Bicêtre, France, ³Gustave Roussy Cancer Campus, Metabolomics and Cell Biology Platforms, Villejuif, France, ⁴Centre de Recherches des Cordeliers, INSERM U 1138, Paris, France, ⁵Gustave Roussy Cancer Campus, Villejuif, France, ⁶German Cancer Research Center, Skin Cancer Unit, Heidelberg, Germany, ⁷University Medical Center Mannheim, Department of Dermatology, Mannheim, Germany, ⁸Center of Clinical Investigation Hôpital Necker Enfants Malades, Paris, France, ⁹Cochin Institute, INSERM U 1016, CNRS UMR 8104, Paris, France, ¹⁰University of Virginia, Division of Surgical Oncology, Department of Surgery, Charlottesville, United States, ¹¹Università della Svizzera Italiana (USI), Cellular Immunology Laboratory, Institute for Research in Biomedicine, Bellinzona, Switzerland, ¹²Eberhard Karls Universität Tübingen, Department of Immunology, Tübingen, Germany, ¹³University Medical Center Tübingen, Department of Dermatology, Tübingen, Germany, ¹⁴Stanford University, Division

of Oncology, Department of Medicine, Stanford, United States, ¹⁵Centre Hospitalier Lyon-Sud, Cancer Research Center of Lyon, Lyon, France, ¹⁶Université Paris Descartes, Paris, France, ¹⁷Université Pierre et Marie Curie, Paris, France, ¹⁸Hôpital Européen Georges Pompidou, Pôle de Biologie, Paris, France, ¹⁹University Hospital of Siena, Medical Oncology and Immunotherapy Division, Siena, Italy, ²⁰University Hospital of Siena, Medical Oncology and Immunotherapy, Department of Oncology, Siena, Italy, ²¹Ludwig Center for Cancer Immunotherapy, Memorial Sloan-Kettering Cancer Center, Department of Immunology and Department of Medicine, New York, United States, ²²Gustave Roussy Cancer Campus, CIC Biotherapie IGR Curie, CIC 1428, Villejuif, France

The prognosis of a variety of human malignancies including melanoma is dictated by the abundance, spatial trafficking and quality of intratumoral T cell infiltrates (Erdag G, Cancer Res, 2012). Salerno *and al.* recently studied chemokine receptors (CC/CXCR) expression on Tumor-Infiltrating T cells (TILs) from several metastasis localizations and suggest a limited tissue-specific homing to human melanoma (Salerno EP, Int J Cancer, 2014). We studied the expression of these CC/CXCR on peripheral T cells from 57 metastatic melanoma (MM) patients, during Ipilimumab therapy (47 patients) and healthy volunteers (HV). Firstly, correlation matrices indicated similar chemokine receptor expression on CD4 and CD8 T cells, except for CXCR4 and CD103, and concordant patterns in naïve, effector and memory T cells. After classification of patients based on their metastasis localizations with skin and lymph node, lung or other distant organs with or without lung involvement, 62 parameters were found to differ significantly (FDR < 0.1) according to distinct localizations metastasis. Expression level normalized with respect to HV indicated that specific signatures could identify preferential metastasis localizations. Loss of CCR6 or CXCR3 (but not CLA) on circulating T cell subsets was associated with skin or lymph node metastases, loss of CXCR4, CXCR5 and CCR9 with lung involvement, and a raise in CCR10 or CD103 with widespread dissemination. High frequencies of CD8⁺CCR9⁺ TN correlated with prolonged OS, while neutralizing the CCR9/CCL25 axis in mice stimulated tumor progression. Effector memory CLA⁺CD8⁺TEM cells increased upon CTLA4 blockade, predicting disease control at 3 months of therapy.

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Panobinostat can enhance the curative activity of trastuzumab in anti-HER2 refractory breast tumors by immune mediated mechanisms

Medon, M.¹, Jenkins, M.R.^{2,3}, Ramsbottom, K.M.², Smyth, M.J.⁴, Atadja, P.W.⁵, Henderson, M.A.⁶, Johnstone, R.W.^{1,3}, Haynes, N.M.^{1,3}

¹Peter MacCallum Cancer Centre, Cancer Therapeutics Program, East Melbourne, Australia, ²Peter MacCallum Cancer Centre, Cancer Immunology Research, East Melbourne, Australia, ³The University of Melbourne, Sir Peter MacCallum Department of Oncology, Melbourne, Australia, ⁴QIMR Berghofer Medical Research Institute, Immunology in Cancer and Infection Laboratory, Herston, Australia, ⁵China Novartis, Institute for Biomedical Research, Shanghai, China, ⁶Peter MacCallum Cancer Centre, Division of Surgical Oncology, East Melbourne, Australia

Trastuzumab is a humanized antibody to the human epidermal growth factor receptor-2 (HER2/neu) and mainstay therapy for HER-2-positive breast cancer. However, many patients on Trastuzumab develop resistance. Here we demonstrate the unique and potent ability of the histone deacetylase inhibitor, panobinostat to augment the therapeutic efficacy of anti-HER2 therapy in models of trastuzumab sensitive and resistant HER2⁺ cancer. Against human BT474 tumors that are intrinsically sensitive to the cytostatic effects of trastuzumab, the combination therapy caused tumor regression in both NOD SCID IL-2 receptor gamma^{-/-} (NSG) and SCID mice. Rejection of the BT474 tumors in response to trastuzumab and panobinostat co-treatment was dependent on therapy-induced inactivation of AKT in the BT474 tumor cells. Most striking and clinically important however, was the combined ability of panobinostat and trastuzumab to eradicate trastuzumab-resistant HER2⁺ tumors. An intact host innate immune system was critical to the curative activity of the combination therapy in trastuzumab resistant AU565 xenografts and BT474 tumors, expressing constitutively active myristoylated AKT (MyrATK). Against these tumors, therapy-induced recruitment of NK cells and antibody dependent cell mediated cytotoxicity underpinned the combined curative effects of panobinostat and trastuzumab. Importantly these findings highlight the novel immune enhancing effects of panobinostat. They also provide compelling evidence that this HDACi can effectively cooperate with the tumor-extrinsic actions of trastuzumab to evoke durable therapeutic responses in settings of relapsed refractory HER2⁺ breast cancer.

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Blockade of interleukin-6 signaling reduces regulatory T cells in ascites of advanced epithelial ovarian cancer

Kampan, N.C.^{1,2}, Madondo, M.T.¹, Quinn, M.A.^{3,4}, McNally, O.M.^{3,4}, Plebanski, M.¹

¹Monash University, Immunology, Melbourne, Australia, ²University Kebangsaan Malaysia, Obstetrics & Gynaecology, Kuala Lumpur, Malaysia, ³Royal Women Hospital, Gynae-oncology Unit, Melbourne, Australia, ⁴Melbourne University, Obstetrics & Gynaecology, Melbourne, Australia

Epithelial ovarian cancer (EOC) has the highest gynaecologic mortality rate globally, as up to two-thirds are diagnosed at an advanced-stage. Ascites, an accumulation of peritoneal fluid is linked to poor prognosis and chemo-resistance. Malignant ascites may aid tumour persistence by harboring immunosuppressive regulatory T cells (Tregs) that render effector T cells (Teff) dysfunctional, as well as soluble factors such as interleukin 6 (IL-6) that promote an inflammatory niche. Upregulation of Tregs and IL-6 have been correlated separately with tumour progression and reduced survival in advanced EOC, however their relationship is not well understood. We studied the immunomodulatory effect of IL-6 on Tregs within malignant ascites *in-vitro*. Peripheral blood mononuclear cells (PBMC) from healthy donors were incubated in two conditions with controls- media with exogenous IL-6 or cell-free malignant ascites from advanced EOC patients for 48 hours. Exogenous IL-6 was used at 50ng/ml to mimic the natural level in ascites.

Bioactive IL-6 within the ascites was neutralized with human monoclonal antibody to IL-6. The frequency and phenotype of Tregs as well as Teff were then evaluated. The results from this study show for the first time that a blockade of IL-6 activity in ascites decreases the frequency of Treg, otherwise induced by exogenous IL-6 in media and ascites, and increases the ratio of Teff/Tregs. Antibody blockade of IL-6 specifically within ascites may offer a novel, clinically translatable strategy to increase immunocompetence in the local tumour microenvironment, and combined with other treatments, may offer more effective modalities of management for advanced EOC.

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Adenosine receptor A_{2A} blockade increases the efficacy of anti-PD-1 through enhanced anti-tumor T cell responses

Beavis, P.¹, Milenkovski, N.¹, Henderson, M.¹, John, L.¹, Allard, B.², Loi, S.¹, Kershaw, M.¹, Stagg, J.², Darcy, P.¹

¹University of Melbourne, Peter MacCallum Cancer Centre, Melbourne, Australia, ²Centre Hospitalier de l'Université de Montréal, Montreal, Canada

Immunotherapy is rapidly emerging as a cancer treatment with high potential. Recent clinical trials with anti-CTLA-4 and anti-PD-1/PD-L1 antibodies (mAbs) suggest that targeting multiple immunosuppressive pathways may significantly improve patient survival. The generation of adenosine by CD73 also suppresses anti-tumor immune responses through the activation of A_{2A} receptors on T cells and natural killer (NK) cells. We sought to determine whether blockade of A_{2A} receptors could enhance the efficacy of anti-PD-1 mAb. The expression of CD73 by tumor cells limited the efficacy of anti-PD-1 mAb in two tumor models, and this was alleviated with concomitant treatment with an A_{2A} adenosine receptor antagonist. The blockade of PD-1 enhanced A_{2A} receptor expression on tumor-infiltrating CD8(+) T cells, making them more susceptible to A_{2A} -mediated suppression. Thus, dual blockade of PD-1 and A_{2A} significantly enhanced the expression of IFN γ and Granzyme B by tumor-infiltrating CD8(+) T cells and, accordingly, increased growth inhibition of CD73(+) tumors and survival of mice.

The results of our study (1) indicate that CD73 expression may constitute a potential biomarker for the efficacy of anti-PD-1 mAb in patients with cancer and that the efficacy of anti-PD-1 mAb can be significantly enhanced by A_{2A} antagonists. We have therefore revealed a potentially novel biomarker for the efficacy of anti-PD-1 that warrants further investigation in patients. Because our studies used SYN-115, a drug that has already undergone phase IIb testing in Parkinson disease, our findings have immediate translational relevance for patients with cancer.

1. Beavis PA et al. 2015 Cancer Immunol Res.

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Comparison of the distribution of ERAP1 single nucleotide polymorphisms in non-small cell lung cancer patients and healthy controls in Poland and China

Yao, Y.¹, Wiśniewski, A.², Ma, Q.³, Kowal, A.⁴, Porębska, I.⁴, Pawełczyk, K.⁵, Yu, J.¹, Dubis, J.⁶, Żuk, N.⁶, Li, Y.⁷, Shi, L.¹, Kuśnierczyk, P.⁸

¹Institute of Medical Biology, Chinese Academy of Medical Sciences

& Peking Union Medical College, Kunming, China, ²Ludwik Hirsfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Laboratory of Immunogenetics and Tissue Immunology, Wrocław, Poland, ³No.3 Ailiated Hospital of Kunming Medical University, Thoracic Surgery, Kunming, China, ⁴Wrocław Medical University, Pulmonology and Lung Cancer, Wrocław, Poland, ⁵Wrocław Medical University, Thoracic Surgery, Wrocław, Poland, ⁶Regional Specialist Hospital in Wrocław, Research and Development Centre, Wrocław, Poland, ⁷No.1 Ailiated Hospital of Kunming Medical University, Geriatrics, Kunming, China, ⁸Ludwik Hirsfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Laboratory of Immunogenetics and Tissue Immunology, Wrocław, Poland

HLA-I-bound peptide antigen presentation depends on antigen-processing machinery molecules. Aminopeptidase residing in endoplasmic reticulum 1 (ERAP1) trims peptides to optimal length for HLA-I binding. Single nucleotide polymorphisms (SNPs) in the ERAP1 gene result in changes in aminopeptidase activity and specificity, which may affect susceptibility to cancer. However, non-small cell lung carcinoma (NSCLC), the most frequent type of lung cancer, has not been studied in this respect yet. Here, we present results of our study of genotype and haplotype frequencies of four coding and nonsynonymous ERAP1 SNPs, rs26653G>C [R127P], rs26618T>C [I276M], rs30187C>T [K528R], rs27044C>G [Q730E], in NSCLC in two genetically distant populations, Chinese Han and Poles. We tested 420 cases and 385 healthy control individuals in China as well as 317 cases and 506 controls in Poland. We found associations of all four SNPs with NSCLC in Chinese but not in Poles. No differences in SNP frequencies between squamous cell carcinoma and adenocarcinoma were found, although a weak trend in Poles for two SNPs (rs26653 and rs30187) was observed. The differences between Chinese and Poles might be explained by highly significant differences in SNP genotype frequencies between Chinese and Poles (except for rs26618). In accord with this, most frequent ERAP1 haplotypes were distributed differently in cases versus controls in Chinese, but not in Poles. Our findings add to differences between Orientals and Caucasians in genetics of disease susceptibility.

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p16 overexpression and high levels of Treg cells in smokers young patients with oropharyngeal cancer

Faxas, M.E.¹, Hernández, D.¹, Carballoso, E.¹, Jiménez, J.¹, González, N.², Arango, M.¹

¹Institute of Oncology and Radiobiology, Immunology, Havana, Cuba, ²Institute of Oncology and Radiobiology, Havana, Cuba

Introduction: Head and neck cancers are malignancies virally mediated and tobacco and alcohol associated. In these diseases, tumor development is close related to the host immune system. Material and methods: Twenty-two young patients (Median age: 30 years, range, 20-37 years), heavily smokers, with locally advanced oropharyngeal carcinoma were studied. Pretreatment peripheral blood lymphocytes were analyzed by flow cytometry (CD3, CD4, CD8, CD16, CD20, and CD4+CD25+Foxp3+ Treg cells). All patients were subjected to immunochemistry assays

for p16 and lymphocyte subsets.

Results: Seventeen tumors (80%) were p16-positive. The tissue samples from p16 positive patients showed an infiltration by intratumoral CD20 B cells and FoxP3 Treg cells. In peripheral blood, high levels of Tregs, NK and of CD8 cells were detected. On the contrary, low values of CD3 and CD4 cells were detected. In assessing conventional prognostic factors, there were no correlations of lymphocyte subsets with tumor grade or nodal status.

Conclusions: In young patients with oropharyngeal carcinoma an immunosuppressive status could favor the carcinogenic effects of tobacco and virus.

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Blockade of inhibitory receptor TIGIT prevents tumor-induced NK cell exhaustion

Zhang, Q., Bi, J., Zheng, X., Sun, R., Tian, Z.

University of Science and Technology of China, Hefei, China

Checkpoint immunotherapy against tumor has drawn extensive attentions. Emerging studies showed that TIGIT might be a potential checkpoint supplementary for existing ones inducing CTL exhaustion. Since TIGIT expressed on both CTL and NK cells, we want know whether TIGIT is involved in NK cell exhaustion and its blockade improves NK cell-based immunotherapy. Here, we found that TIGIT expression is strongly associated with exhaustion of NK cells during tumor progression while NK cells barely expressed CTLA-4 or PD-1, two well-recognized checkpoints on CTL. TIGIT blockade reversed exhaustion of tumor-infiltrating NK cells, independent of adaptive immune system, and improved adaptive recall response against tumor reloading. Absence of NK cells more significantly diminished the therapeutic effects from TIGIT blockade if compared with CTL. Thus, our findings indicate that TIGIT might be critical checkpoint for reversing exhaustion of not only CTL but also NK cells, implying more promising application.

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The molecular and immunological mechanism of hepatoma development promoted by HBV-SALL4

Han, Q.

Shan Dong University, Jinan, China

HBV infection and liver cancer are closely related, but the mechanism of HBV-induced liver cancer has not been fully elucidated. Recent research demonstrated that the SALL4 is highly expressed in patients with hepatoma, but the relationship between HBV infection and SALL4 is not clear, also the effect of SALL4 on hepatic immune tolerance remains unclear. In our study, DEN-induced liver cancer was established in HBV transgenic mice. Dynamic SALL4 up-regulation expression was observed during HCC development. Using the bioinformatics and molecular biology methods, we confirmed the molecular mechanism that HBV regulate of SALL4 expression by STAT3 signal pathway. And luciferase reporter assay validated several target genes of SALL4. When SALL4 is overexpressed or silenced, liver immune tolerance state was assayed, and we found MDSC

accumulation when SALL4 is high expressed in liver. This study revealed the relationship between HBV infection and SALL4 expression, and reveal the molecular and immunological mechanism how SALL4 promotes hepatoma. Thus, our study will finally provide more precise evidence for diagnosis and treatment of liver cancer.

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PEG10 promotes human breast cancer cells proliferation, migration and invasion

Zhang, Q., Li, X.

School of Basic Medical Sciences, Wuhan University, Department of Immunology, Wuhan, China

Paternally expressed imprinted gene 10 (PEG10), derived from the Ty3/Gypsy family of retrotransposons, has been implicated as a genetic imprinted gene. Accumulating evidence suggests that PEG10 plays an important role in tumor growth in several cancers, including hepatocellular carcinoma, lung cancer and prostate cancer. However, the correlation between PEG10 and breast cancer remains unclear. In the present study, we evaluated and characterized the role of PEG10 in human breast cancer proliferation, cell cycle, clone formation migration and invasion. The expression level of PEG10 was significantly elevated in breast cancer tissues and associated with distant metastasis and poor clinical outcome. Gene set enrichment analysis indicated that high expression of PEG10 could enrich cell-cycle related processes in breast cancer tissues. Ectopic overexpression of PEG10 in breast cancer cells enhanced cell proliferation, cell cycle, clone formation along with migration and invasion. Cell-to-cell junction molecule E-cadherin was downregulated and matrix degradation proteases MMP-1, MMP-2, MMP-9 were upregulated after PEG10 overexpressed. Our results demonstrated that PEG10 is a crucial oncogene and has prognostic value for breast cancer, which could be applied in breast cancer diagnosis and targeting therapy in future.

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HER-2⁺ breast cancer cells expressing GPI-anchored cytokines induce long lasting anti-tumor memory response

Bommireddy, R., Margaroli, C., Shafizadeh, Y., Bozeman, E., Munoz, L., Menon, A., Selvaraj, P.

Emory University School of Medicine, Department of Pathology, Atlanta, United States

Breast cancer is the second leading cause of death among female cancer patients. Human epidermal growth factor receptor 2 (HER-2) overexpression is associated with poor prognosis in 15-20% of breast cancer patients. Anti-HER-2 antibody (Herceptin) prolongs the survival of the HER-2⁺ breast cancer patients. However, resistance to Herceptin is a cause for relapse of aggressive, metastatic cancer in these patients. Our earlier studies have demonstrated that PD-L1 blockade enhances the efficacy of HER-2⁺ breast cancer whole cell vaccine by increasing the infiltration of T cells into the tumor. The goal of the present study is to determine the duration of protective anti-tumor memory responses to HER-2⁺ breast cancer. In this

study, we demonstrate the beneficial effect of GPI-anchored cytokine molecules as adjuvants for generating long lasting memory response against HER-2⁺ as well as HER-2 negative breast cancer in syngeneic tumor models. Female BALB/c mice were challenged with D2F2/E2 (HER-2 positive) cells or D2F2/E2 transfected with GPI-IL-12 or GPI-GM-CSF. While the wild-type challenged mice developed tumors, the mice challenged with GPI-cytokine expressing D2F2/E2 cells were protected. Protected mice were re-challenged with D2F2/E2 cells 3 months later and D2F2 cells 4 months later. All of the mice challenged with D2F2 (HER-2 negative) or D2F2/E2 were protected. We have observed strong antibody responses against HER-2 and D2F2 tumor specific antigens in these mice. Our results show that long lasting protective anti-tumor memory response against D2F2 and D2F2/E2 is generated by vaccination with D2F2/E2 cells expressing GPI-anchored cytokine molecules.

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Galectin-3 in prostate cancer proliferation and metastasis

Aalinkel, R., Abou-Jaoude, E., Parikh, N., Reynolds, J., Sykes, D., Mammen, M., Mahajan, S., Schwartz, S.
University at Buffalo, Medicine, Buffalo, United States

Bone metastasis occurs in >90% of patients in advanced, metastatic prostate cancer (CaP). Development of therapies to prevent bone metastases are a high priority in CaP research. In CaP, factors released from infiltrating CaP cells can alter the equilibrium between the host's bone-degrading cells, osteoclasts and osteoblasts, facilitating the process of bone metastases. Galectin-3 (Gal-3) is implicated in apoptosis, angiogenesis, and tumor progression. We hypothesized that silencing of Gal-3 expression using RNAi technology would suppress the progression of CaP. Our goal was to investigate how Gal-3 suppression would affect osteoclast and osteoblasts within the microenvironment of metastasis and identify the molecular mechanisms underlying the role of Gal-3 in CaP metastasis. Gal-3 may regulate the expression of osteopontin (OPN), which regulates osteoclast motility and osteoclast-mediated bone resorption. We evaluated if Gal-3 knockdown in PC-3 CaP cells was associated with reduced proliferation, invasion and colony formation, and if Gal-3 potentiates the transcription of OPN in ST2 human osteoblasts. Our results show a significant decrease in cell proliferation (62% decrease; $p < 0.01$), colony formation (77% decrease; $p < 0.01$), and decreased expression of VEGF (41% decrease; $p < 0.05$), IL-8 (56% decrease; $p < 0.05$) and MMP-9 (69% decrease; $p < 0.01$) in PC-3 CaP cells treated with Gal-3 siRNA, additionally, we observed a significant decrease in OPN expression (73% decrease; $p < 0.01$), indicating that Gal-3 plays a role in progression of CaP metastasis. Our results suggest that Gal-3 may be a therapeutic target to prevent bone metastases in advanced CaP.

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IL-23, an inflammatory cytokine, decreased by shark cartilage and vitamin A oral treatment in patient with gastric cancer

Zarei, R.

Azad Islamic University, Faculty of Medical Science-Immunology Department, Sari, Iran, Islamic Republic of

Introduction: IL-23 is responsible for the differentiation and expansion of Th17/ThIL-17 cells from naive CD4⁺ T cells. TGF- β is also share for the differentiation Th17 producing IL-17 and CD4⁺CD25⁺Foxp3^{hi}T regulatory cells from naive CD4⁺ T cells which are involved in the regulation of immune response,..... Vitamin A and it's derivatives inhibit or reverse the carcinogenic process in some types of cancers . Shark cartilage has antitumor peptides to prevent angiogenesis, in vitro. Our purpose is whether simultaneous oral treatment vitamin A and shark cartilage can modulate IL-23/IL-17 and CD4⁺CD25⁺Foxp3 T regulatory cell/TGF- β pathways and Th1/Th2 immunity in patients with gastric cancer or not.

Materials and methods: The first investigated an imbalanced supernatant of cytokines in patients with gastric cancer existing was measured by ELISA. Associated with cytokines measuring such as IL-23,IL-17,TGF- β ,IL-4 and γ -IFN, then flow cytometry was employed to determine whether the peripheral blood mononuclear cells such as CD4⁺CD25⁺Foxp3^{high}T regulatory cells in patients with gastric cancer were changed correspondingly.

Results: An imbalance between IL-17 secretion and TGF- β /Foxp3 t regulatory cell pathway and so, Th1 immunity (γ -IFN production) and TH2 immunity (IL-4 secretion) was not seen in patients with gastric cancer treated by vitamin A and shark cartilage. But, the simultaneous oral treatment of shark and vitamin A indicated a down-regulation of IL-23, at least cytokine level.

Conclusion: IL-23, as a pro-angiogenesis cytokine, probably, helps with tumor growth. Hence, it is suggested that down-regulation of IL-23, at least cytokine level, is useful for anti-tumor immune responses in patients with gastric cancer.

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MPL adjuvant encapsulated in liposome coated with P5 HER2/neu peptide: a potent prophylactic vaccine for breast cancer

Shariat, S.¹, Jalali, S.A.², Jafari, M.R.³, Alimohammadi, R.²

¹School of Pharmacy, Shahid Beheshti University of Medical Sciences, Department of Pharmaceutics, Tehran, Iran, Islamic Republic of, ²Medical School, Shahid Beheshti University of Medical Sciences, Department of Immunology, Tehran, Iran, Islamic Republic of, ³Mashhad University of Medical Sciences, Biotechnology Research Center, Nanotechnology Research Center, Mashhad, Iran, Islamic Republic of

Vaccines containing synthetic peptides derived from tumor-associated antigens (TAA) can elicit potent cytotoxic T lymphocyte (CTL) response if they are formulated in an optimal vaccine delivery system. The aim of this study was to develop a simple and effective lipid-based vaccine delivery system

using P5 HER2/ neu-derived peptide conjugated to Maleimide-PEG2000-DSPE. The conjugated lipid was then incorporated into liposomes composed of DMPC:DMPG:Chol:DOPE containing Monophosphoryl lipid A (MPL) (LipDOPE-P5-MPL). Different liposome formulations were prepared and characterized for their physicochemical properties. To evaluate anti-tumoral efficacy, BALB/c mice were immunized subcutaneously 3 times in two-week intervals and the generated immune response was studied. The results demonstrated that LipDOPE-P5-MPL induced a significantly higher IFN- γ production by CD8+ T cells intracellularly which represents higher CTL response in comparison with other control formulations. CTL response induced by this formulation caused the lowest tumor size and the longest survival time in a mice model of TUBO tumor. The encouraging results achieved by Lip-DOPE-P5-MPL formulation could make it a promising candidate in developing effective vaccines against Her2 positive breast cancers.

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Targeting tumor hypoxia improves Immunotherapy based on peptide vaccination and immune-checkpoint blockade

Noman, M.Z., Chouaib, S.

INSERM U1186, Equipe Labellisée Ligue Contre le Cancer, Gustave Roussy Campus, Villejuif, France

Immune checkpoint blockade has shown impressive results in treating previously untreatable cancers, however a significant proportion of patients still do not respond to treatment. Understanding the influence of microenvironmental hypoxia on the peptide/ immune-checkpoint blockade combination induced tumor regression could provide insight into how to improve cancer immunotherapy. We have previously shown that hypoxia is an essential driver of tumor resistance and immune suppression. In this study, our aim was to investigate whether targeting tumor hypoxia will improve the outcome of immunotherapy based on peptide-based vaccination and immune-checkpoint blockade. Using B16-F10 xenograft tumor model, we examined the effect of simultaneous *in vivo* inhibition of hypoxia and peptide-based vaccination and immune-checkpoint blockade. We observed that targeting tumor hypoxia along with peptide-based vaccination and immune-checkpoint blockade significantly decreased tumor growth as compared to targeting tumor hypoxia alone or peptide-based vaccination or immune-checkpoint blockade. More interestingly, triple combination treatment (targeting tumor hypoxia along with peptide-based vaccination and immune-checkpoint blockade) improved significantly the double combination treatment (targeting tumor hypoxia and peptide-based vaccination or peptide-based vaccination and immune-checkpoint blockade). The impact of triple combination treatment (targeting tumor hypoxia along with peptide-based vaccination and immune-checkpoint blockade) as compared to double combination treatment (targeting tumor hypoxia and peptide-based vaccination or peptide-based vaccination and immune-checkpoint blockade) on tumor immune infiltration will be discussed.

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The induction of autophagy in tumor cells by hypoxic stress and the acquisition to CTL resistance involves the stemness transcription factor NANOG

Hasmim, M.¹, Noman, M.Z.¹, Janji, B.², Chouaib, S.¹

¹INSERM U1186, Equipe Labellisée Ligue Contre le Cancer, Gustave Roussy Campus, Villejuif, France, ²Luxembourg Institute of Health, Luxembourg, Luxembourg

Both experimental and clinical studies have shown that hypoxia plays an important role in tumor progression, affecting both metastatic spread and selection of cells with more resistant phenotypes. Nanog is an essential transcription factor maintaining the self-renewal and pluripotency of embryonic stem cells. Our laboratory reported that, under hypoxic stress, NANOG was upregulated in non small cell lung carcinoma cells and involved in hypoxia-induced tumor cell resistance to autologous specific cytotoxic T lymphocytes. We are now investigating the molecular mechanisms underlying NANOG-mediated tumor cell resistance to specific lysis. We have obtained evidence of a link between upregulation of NANOG and autophagy activation under hypoxic stress. Autophagy is a mechanism of tumor cell resistance to CTL-mediated killing. The inhibition of NANOG expression by siRNA under hypoxic stress led to a significant reduction of the number of autophagosomes formed inside tumor cells and a decrease in the expression of genes regulating autophagy. Furthermore, overexpression of NANOG in tumor cells significantly increased the number of autophagosomes per cell and the autophagic flux. Several NANOG-binding sites were found in the promoter region of genes regulating autophagy, and we are performing chromatin immunoprecipitation (ChIP) and luciferase assays to confirm the functional link between NANOG and autophagy activation under hypoxia. The relationship between the expression of this stemness transcription factor and the activation of autophagy under hypoxia will be discussed.

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Specific targeting of the RAS/MAPK pathway in combination with immunotherapy promotes anti-tumor immune responses against triple negative breast cancers

Dushyanthen, S.¹, Henderson, M.¹, Virassamy, B.¹, Mintoff, C.¹, Zhou, C.¹, Savas, P.¹, Caramia, F.¹, Teo, Z.L.¹, Beavis, P.¹, Darcy, P.^{1,2}, Loi, S.^{1,2}

¹Peter MacCallum Cancer Centre, Melbourne, Australia, ²University of Melbourne, Sir Peter MacCallum Department of Oncology, Melbourne, Australia

Tumor infiltrating lymphocytes (TILs) are an important prognostic factor in triple negative breast cancer (TNBC); where their presence is associated with improved survival. Research involving a genomically and clinically characterized cohort of TNBCs suggests an association between activation of the RAS/MAPK pathway with lower levels of TILs and poorer outcomes in patients. However, insight into the cell intrinsic molecular pathways behind the host anti-tumor immune response is lacking. Our studies have demonstrated that the MEK targeted inhibitor (trametinib) increased immunogenicity (MHCII/II₁) of tumors and reduced tumor growth. However, whilst MEK

inhibition effectively inhibited tumor growth, blockade of this pathway triggered adverse inhibitory effects on crucial ERK signaling in immune cells, thus reducing their functional activity and overall efficacy of this treatment. We have previously shown that combining anti PD-1 with trametinib can enhance anti-tumor effects [1]. In the current study immune agonists anti-4-1BB or anti-OX-40 were combined with trametinib in order to recover immune cell function independently of the MEK1/2 pathway. These agonists restored T cell proliferation (Ki67), cytokine production (IFN γ) and redirected signaling through p38 activation following trametinib treatment, leading to enhanced TIL function and more effective anti-tumor immune responses in vivo. Accordingly, this data suggests that immune agonists with anti-PD-1 checkpoint blockade may be an effective strategy, as the combination can directly overcome T cell suppression induced by MEK inhibition. As such, we propose that combining trametinib with agonist/checkpoint immunotherapy could be a promising strategy in the clinic for TNBC patients.

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Combination immune checkpoint blockade as an effective therapy for mesothelioma

Fisher, S.^{1,2}, Solin, J.^{1,2}, Casey, T.^{1,2}, Lesterhuis, W.J.^{1,2}, Lansley, S.³, Robinson, B.W.S.^{1,2}, Lake, R.^{1,2}

¹National Centre for Asbestos Related Diseases, Perth, Australia, ²University of Western Australia, School of Medicine & Pharmacology, Perth, Australia, ³Institute for Respiratory Health, Perth, Australia

Immune checkpoint blockade (ICPB) offers great promise for treating cancer, including mesothelioma. Most ICPB therapies are focused on CTLA4 and PD1 with dual blockade appearing superior. However, at least 22 other checkpoint molecules have been identified and the best combinations for each cancer have yet to be defined. We used an established preclinical mesothelioma tumour models to characterise the expression profile of checkpoint molecules on tumour resident immune cells to identify likely combinations for effective checkpoint blockade therapy.

Flow cytometry was used to characterise expression of immune checkpoint molecules CTLA-4, OX40, TIM-3, GITR and PD-1 on tumour resident immune cells. Tumour bearing mice were then treated with ICPB antibodies to identify the best combinations for inducing anti-mesothelioma immunity.

OX40 and CTLA-4 are highly expressed on tumour resident regulatory T cells compared to tumour infiltrating lymphocytes (CD4 and CD8 TILs). PD-1 expression was observed on non-Treg CD4 and CD8 TILs, while broad TIM-3 expression was observed on both effector and regulatory T cells.

Treatment with either α CTLA-4 or α GITR, but not α TIM-3, α PD-1 or α OX40, significantly delayed tumour growth and improved overall survival. Combining α CTLA4 with α OX40 provided no additional benefit in tumour growth delay over CTLA-4 alone. Conversely, a small delay was observed when PD-1 and TIM-3 were combined. These combinations are currently being tested

in an orthotopic (intrapleural) mesothelioma mouse model. These data suggest that immune checkpoint molecules are highly expressed on tumour infiltrating immune cells and that immunotherapies targeting these molecules can delay mesothelioma development and improve survival.

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Targeted depletion of regulatory T cells in solid cancers

Fisher, S.^{1,2}, Solin, J.^{1,2}, Aston, W.^{1,2}, Cleaver, A.^{1,2}, Lesterhuis, W.J.^{1,2}, Khong, A.^{1,3}, Robinson, B.W.S.^{1,2}, Lake, R.^{1,2}

¹National Centre for Asbestos Related Diseases, Perth, Australia, ²University of Western Australia, School of Medicine & Pharmacology, Perth, Australia, ³University of Western Australia, Perth, Australia

Regulatory T cells (Treg) play an important role in suppressing anti-tumour immunity and their depletion has been linked to improved outcomes. To better understand the role of Treg in limiting the efficacy of anti-cancer immunity, we used diphtheria toxin (DTX) to specifically deplete Treg from tumour bearing BALB/c FoxP3.dtr transgenic mice.

Mice bearing solid tumours were subject to different treatment protocols, with or without Treg depletion and tumour growth and survival monitored. Flow cytometry was used to confirm Treg depletion, immune cell phenotyping and assess immune correlates with treatment outcomes.

DTX administration specifically depleted Treg in a transient, dose dependent manner. Treg depletion correlated with delayed tumour growth, increased effector T cell activation and enhanced survival. Tumour regression was dependent on effector T cells as the depletion of both CD4 and CD8 T cells completely abrogated any survival benefit. Severe morbidity following Treg depletion was only observed when consecutive doses of DTX were given during peak CD8 T cell activation, demonstrating that Treg can be depleted on multiple occasions, but only when CD8 T cell activation has returned to base line levels.

Clinical translation of targeted Treg depletion as a cancer immunotherapy is currently limited by the lack of Tregs specific reagents. However, Treg express high levels of the immune checkpoint molecules CTLA-4, GITR and OX40, while tumour infiltrating effector cells express PD-1 and TIM-3, suggesting that specific combinations of checkpoint immunotherapies could be designed to specifically target Treg and simultaneously enhance effector T cell function to generate effective cancer immunity.

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Impaired signalling in T cells from patients with colorectal cancer in response to IL-2 and IL-7

Shen, S.¹, Taylor, E.¹, Ward-Hartstonge, K.¹, Mccall, J.², Kemp, R.¹

¹University of Otago, Department of Microbiology and Immunology, Dunedin, New Zealand, ²University of Otago, Department of Surgical Sciences, Dunedin, New Zealand

T cells are key components of the antitumour response, particularly in mediating an effector function against colorectal cancer (CRC) to prevent tumour progression. The survival and

proliferation of T cells are dependent on signals from IL-2 and IL-7; these cytokines are therefore important for initiating and maintaining an immune response against colorectal tumours. The aim of this study was to measure the phosphorylation of signalling molecules downstream of IL-2 and IL-7 receptor binding in T cells isolated from patients with CRC. This was achieved by using phosphoflow cytometry to measure STAT5 phosphorylation in T cells after IL-2 or IL-7 stimulation. We hypothesised that patients with CRC would have a dysregulation in T cell signalling in response to IL-2 and IL-7 when compared to healthy subjects.

Preliminary results indicate an increase in STAT5 phosphorylation in T cells from CRC patients after IL-7 stimulation when compared to healthy controls; however this increase was not due to an increased expression of IL-7Ra. Conversely, no aberrant phosphorylation of STAT5 was observed in T cells from CRC patients that were stimulated with IL-2. Results from this study will contribute to understanding the functionality of tumour-infiltrating T cells in patients with CRC.

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Anti-tumor CTL effect targeting the ASB4 gene-derived natural peptide presented by HLA-A24 of human colon cancer stem cells

Miyamoto, S., Kanaseki, T., Takahashi, A., Kochin, V., Hongo, A., Hirohashi, Y., Torigoe, T., Sato, N.
Sapporo Medical University School of Medicine, Department of Pathology, Sapporo, Japan

Cancer stem cells (CSC) are the small cell population that is responsible for cancer growth in vivo as well as resistant to chemo/radiotherapies. We enriched a highly tumorigenic CSC population from the human colon cancer SW480 line based on their elevated transporter activities of ATP-binding cassette, followed by screening peptides that were naturally presented by HLA-A24 of the cells. Large-scale MS/MS sequencing following affinity purification with an HLA-A24-specific antibody allowed us to identify the natural peptide, which source gene, ASB4, was specifically expressed in the CSC population. We found the gene was expressed in a variety of cancer cell lines as well as primary colon cancer tissues, but not in a panel of normal tissues. Moreover, the identified peptide was immunogenic to elicit CTL responses from healthy donor and cancer patient PBMC. In immunodeficient mice, intravenous injection of the induced CTL clone successfully prevented growth of the original SW480 cells that consist of CSC and non-CSC populations. Thus, the CTL induced by the ASB4 gene-derived peptide is specific to CSC and is effectively able to inhibit cancer growth in vivo.

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Prognostic relevance of tumor infiltrating immune populations and of circulating miRNAs and cytokines/chemokines in Breast Cancer (BC)

Fortis, S.¹, Mahaira, L.¹, Sotiriadou, N.², Sofopoulos, M.², Haritos, C.¹, Anastasopoulos, N.¹, Arnogiannaki, N.², Ardavanis, A.³, Perez, S.¹, Baxevas, C.¹

¹Saint Savas Cancer Hospital, Cancer Immunology and

Immunotherapy Center, Athens, Greece, ²Saint Savas Cancer Hospital, Pathology Department, Athens, Greece, ³Saint Savas Cancer Hospital, Oncology Department, Athens, Greece

There is accumulating evidence supporting the pivotal role of miRNAs and cytokines/chemokines for cancer progression and therefore it is reasonable to suggest that they could be potential biomarkers predicting cancer progress. Moreover, tumor infiltrating lymphocytes (TILs) shape an efficient immune microenvironment with favorable clinical outcome. Here, we profile the composition of TILs, from a retrospective BC patient cohort (n=100) and correlate with clinical outcome, depending on TILs spatial distribution (Tumor Invasive Margin, TIM vs Tumor Center, TC). We also, investigate possible correlations between TILs at TIM vs TC and circulating biomarkers in a prospective cohort of BC patients (n=50).

Peripheral blood from women diagnosed with invasive breast carcinoma (IBC) (prospective cohort), without neoadjuvant therapy, was obtained one day before surgery. Selected circulating miRNAs (e.g. miR-21, miR-23a, miR-146a, miR-155, miR-181a), were measured in the serum. The levels of cytokines/chemokines (e.g. TGF- β 1, Rantes, IL-9, IL-10, IL-1Ra), were also evaluated. The infiltration analysis of immune subpopulations (CD4, CD8, FoxP3 and CD163) was performed by IHC in FFPE tissues of patients with histologically-confirmed IBC in both cohorts.

We have identified strong correlations between systemic parameters (circulating miRNAs & cytokines/chemokines) and tumor microenvironment (density & localization of TILs) in the prospective cohort. Additionally, we observed favorable outcome (longer DFS/OS) with certain TILs patterns in the retrospective cohort. We report for the first time that (i) the spatial distribution of TILs plays a significant role in clinical outcome in IBC and (ii) peripheral immune-signatures may predict density and the localization of TILs in IBC. Thus, the combination of peripheral with intratumoral immune-signatures may more precisely predict clinical outcome.

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Altered expression and function of NK receptors in pediatric acute lymphoblastic leukemia (ALL)

Mathew, S.¹, Powers, S.¹, Bowman, W.P.^{2,3}, Aldy, K.¹, Mathew, P.¹
¹UNT Health Science Center, Cell Biology & Immunology, Fort Worth, United States, ²UNT Health Science Center, Pediatrics, Fort Worth, United States, ³Cook Children's Medical Center, Oncology & Hematology, Fort Worth, United States

Acute lymphoblastic leukemia (ALL) is the most common pediatric cancer in the developed world with peak prevalence between the ages of 2 and 5 years. Despite current treatment protocols achieving significant improvement in survival of ALL patients, serious acute and late complications are frequent and resistance to chemotherapy often develops. Although NK cells have long been known to mediate antigen independent tumor killing, the therapeutic potential of NK cell-based immunotherapy has yet to be realized. NK cells have the capacity to kill virus-infected or tumor-transformed cells and they express several surface molecules that regulate NK cell

function both positively and negatively. We have previously identified 2B4 (CD244) and CS1 (CRACC) playing a major role in NK activation. In this study, we evaluated the expression and function of NK cell receptors in 12 ALL subjects and 11 healthy subjects both male and female in the age range of 3 - 18 yrs. The peripheral blood samples were collected and analyzed by flow cytometry and real-time PCR for expression and cytotoxicity assays were done for functional analysis. A significant increase in the expression of CS1 and NKp30 in B cells and 2B4 and CS1 in the CD14+ monocytes was observed in ALL subjects as compared to healthy controls. NK cells from 3 ALL subjects also showed impaired cytotoxic function as they demonstrated lower killing of K562 cells compared to a healthy control subject. These results implicate that 2B4, CS1 and NKp30 may play a role in NK-mediated surveillance of childhood ALL.

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Manipulation of tumor microenvironment by cytokine gene transfection enhances dendritic cell-based immunotherapy

Wijesekera, D.P.H.¹, Sugiura, K.¹, Ichida, C.¹, Yuba, E.², Kono, K.², Yamate, J.³, Kanegi, R.¹, Nishimura, T.¹, De Silva, N.H.¹, Hatoya, S.¹, Inaba, T.¹

¹Osaka Prefecture University, Department of Advanced Pathobiology, Osaka, Japan, ²Osaka Prefecture University, Department of Applied Chemistry, Sakai, Japan, ³Osaka Prefecture University, Department of Integrated Structural Biosciences, Osaka, Japan

The tumor microenvironment strongly influences the clinical outcome of immunotherapy. By using intratumoral (i.t.) injection of interferon (IFN) γ , a cytokine which induces Th1 polarization and dendritic cell (DC) -maturation, we obtained satisfactory clinical results in dog tumor therapy. This manipulation is expected to be more effective and persistent by transfecting genes of the relevant cytokine into tumor cells *in vivo*. To verify this hypothesis, we transfected genes of the cytokine into tumor cells growing in mice, and examined the effect on DC therapy. For the transfection we used a novel synthetic vector composed of a cationic lipid bound with pH-sensitive liposomes, which effuses enclosed DNAs from endosomes into the cytoplasm. The efficiency of transfection into the LM8 osteosarcoma growing in syngeneic C3H mice was approximately 10% by i.t. even with intravenous (i.v.) injection of the vector enclosing the plasmid containing GFP-cDNA (GFP-vector). Therapeutic experiments were performed to inhibit LM8 growth by i.t. or i.v. injection of the IFN γ -vector or the CD40L-vector, followed by i.t. injection of the DCs presenting the LM8 antigens. This treatment was performed four times at 7-day intervals. Growth of the tumor was significantly suppressed by the i.v. treatment of the IFN γ -vector and the i.t. and i.v. treatment of the CD40L-vector, relative to untreated mice. The CD40L-vector treatment led to significantly improved survival. These results indicate that gene transfection of the cytokine genes manipulate the tumor microenvironment effectively and significantly improve DC-based tumor immunotherapy.

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Targeting endosomal receptor of immune cells by multivalent polymer nanocomplex for enhanced immunostimulation and anti-tumor response

Kim, S.-Y., Phuengkham, H., Lim, Y.T.

Sungkyunkwan University, Suwon-Si, Korea, Republic of

In this work, we designed and developed a multivalent polymer nanocomplex for the effective intracellular delivery and subsequent multivalent display of stimulating epitope through the combination of multivalent immune adjuvants with CpG ODNs (as a TLR9 ligands) and cationic PLL (for the enhancement of cellular uptake) in antigen presenting cells such as dendritic cell and macrophage cells.

Cytosine-Guanine (CpG) oligodeoxynucleotides can be recognized by Toll-like receptor 9 (TLR9) and induce powerful immune response via cell signaling pathways including NF κ B. Hyaluronic acid is a biocompatible, biodegradable, non-toxic polysaccharide, which has been applied for various drug delivery systems.

Finally, the multivalent polymer nanocomplex caused the enhanced cellular uptake efficiency and modulation of the continuous stimulation of endosomal TLR9. We show that multivalent polymer nanocomplex exhibit a potent immunostimulatory activity *in vitro*. Inspired by the *ex vivo* immunostimulating capacity of the nanocomplex, we investigated the *in vivo* tumor therapeutic effect. Mice vaccinated with the nanocomplex exhibited tumor growth inhibition as well as a strong anti-tumor memory response.

We demonstrated that multivalent polymer nanocomplex exhibited potent immunostimulatory activity both *in vitro* and *in vivo*. The future, these studies could be used to develop programmed immune-cells for effective cancer immunotherapy.

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Adjuvanticity and chemo-therapeutic potential of novel Phytol-derived immunoadjuvants

Ghosh, S.¹, Johnson, D.¹, Steding, C.¹, Fitch, R.²

¹Indiana State University, Biology, Terre Haute, United States,

²Indiana State University, Chemistry, Terre Haute, United States

This study was initiated on the premise that surgery and radiotherapy often fail to eliminate cancer. The residual cancer portends danger, but also provides antigenic stimuli exploitable for development of immunotherapy using immuno-adjuvants. If the latter also exhibits chemotherapeutic properties, the treatment would boost immunity and destroy cancer. This led us to test lipophilic terpenoid adjuvants, PHIS01 (phytanol) and squalene, and an amphiphilic adjuvant phytanyl sulphate. We have been systematically investigating adjuvanticity of long-chain acyclic diterpenoid phytol of photosynthetic green pigment chlorophyll and several of its derivatives (Ghosh: USA Patent 8088395). We observed that some derivatives, e.g. PHIS01 and PHIS03 (phytanyl mannose) are excellent immunoadjuvants for different cancer cells (Front Immunol. 2012; 3:49). Moreover, there are reports highlighting cancer-killing properties of some terpenoids. In this study, we addressed:

(a) Could an oil-in-water micro-emulsion of PHIS01 be an

effective immune-stimulator comparable to squalene as in commercial MF59?

(b) Could phytol-based adjuvants be chemotherapeutic causing tumor regression?

Our results demonstrated effective adjuvanticity of PHIS01 formulated as in MF59 even at low doses. Also, its direct administration into murine lymphomas A20, and 2C3 caused tumor regression. Studies *in vitro* with mammary tumor 4T1 demonstrated cytotoxic effects with both PHIS01 and phytanyl sulphate. The latter, being more surfactant-like, was effective at lower doses than PHIS-01. Based on ongoing studies, we speculate that necrosis induced by isoprenoid micro-emulsion help recruit and activate dendritic cells and induce anti-tumor immunity. Supported by the Fraternal Order of Eagles, and the University Research Committee (URC319).

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Diagnostic and prognostic immunobiological markers of bladder cancer

Avdonkina, N.¹, Slavyanskaya, T.¹, Salnikova, S.²

¹People's Friendship University of Russia, Moscow, Russian Federation, ²Institute of Immunophysiology, Moscow, Russian Federation

Bladder cancer (BC) is the second most common urinary tract malignancy. Early diagnosis and prognosis monitoring are very important for the survival of patients with bladder cancer. To find current immunological and biological markers (IBM) for BC. Search results revealed, that the expression levels of proteins p53, p63 и p16; EGFR, VEGF, HER2/neu; Ki 67, PCNA; bcl-2, bcl-x, CD 95; cytokeratin profile had no prognostic significance and connection with disease state. At the same time it is determined, that the levels of expression of Cyclin D1, antiadhesive glycoprotein tenascin, matrix metalloproteinases and their tissue inhibitors; the reduced expression of adhesion molecules EpCAM E-cadherin and b-catenin were correlated with disease prognosis. The importance of determining the levels of inflammatory markers was shown: IL-6, IL-8, CRP; thrombomodulin, expression of chemokine receptor CXCR7 for assessment of the disease state and prediction of its course. A promising marker of tumor progression survivin was installed, which may be a potential target with immunotherapy. The possibility of using overexpression of cancer-testicular antigen MAGEA and oncofetal protein IMP3 is considered as independent predictors of progression and recurring of urothelial carcinoma. Worth noticing is the definition of circulating tumor cells in the blood and microRNAs involved in transcriptional and posttranscriptional regulation of gene expression. The heterogeneity of the IBM, moderate level of evidence of their clinical and diagnostic significance, and also insufficient quantity of works demands further experimentations for their selection and accurate significance in cases of BC.

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Polyporus alveolaris extract (PAE) induces anti-tumor activity of RAW264.7 macrophage

Kanai, H.¹, Matsuzaki, T.¹, Sasajima, Y.¹, Kawamura, M.¹, Aradate, T.², Katagiri, T.¹

¹University of Toyama, Dept. Biology (Faculty of Pharmaceutical Sciences), Toyama-shi, Japan, ²University of Toyama, Dept. Biology (Faculty of Medical Sciences), Toyama-shi, Japan

From the screening results of a number of hot-water extract, finally we revealed the extract from Hachinosutake (*Polyporus alveolaris*), PAE, is the good activator for macrophages to anti-tumor. PAE stimulated growth inhibitory activity against tumor cells by RAW264.7 was approximately equal to high concentration of LPS. However, unlike LPS, anti-tumor effect of PAE induced in RAW264.7 was not affected under Polymyxin B coexist. We examined the effects of PAE to the production of anti-tumor effector molecules, TNF- α and NO production, from RAW264.7. As a result, it was confirmed to induce TNF- α production and NO production in a concentration dependent manner in RAW267.4. Moreover, it was not affected to these activity under Polymyxin B coexist. To examine these production mechanism, it analyzes the intracellular signaling were examined for phosphorylation of tyrosine phosphorylation and MAP kinase ERK, JNK, p38. As a result, the phosphorylation time course pattern with PAE was quite unique which compared from LPS pattern. From the result of the analysis of the receptor necessary for effect of PAE, with neutralizing antibodies, at least a portion of the anti-tumor effect induction revealed that from Dectin-1 is a receptor for β -glucan. Moreover, fractionated molecular weight with cutting filters by molecular size contained in PAE using molecular weight, were studied active ingredient. As a result, components of molecular weight of 100~1,000kDa has induced the highest activity to the macrophages. These results are characteristic β -glucan contained in PAE are believed to indicate that the beneficial anti-tumor activity induction of macrophages.

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Understanding the anti-tumour immune response to melanoma in the brain

George, T.^{1,2}, Endersby, R.¹, Brizard, G.¹, Kuchibhotla, M.¹, Waithman, J.¹

¹Telethon Kids Institute, The University of Western Australia, Subiaco, Australia, ²Curtin University, Bentley, Australia

Up to 75% of patients suffering from malignant melanoma develop brain metastases. Treatment options are currently limited for these patients. While immunotherapy has shown success in treating melanoma, patients with brain involvement have traditionally not been considered for immunotherapies. This is due to the long-standing concept that the brain is immune privileged. Recently, however, it was reported that the brain is not immune privileged, and possesses lymphatic-like vessels that provide a connection between the immune system and the central nervous system (CNS).

To understand the coordination of brain tumour immunity, B16 melanoma cells expressing ovalbumin (OVA) were intracranially implanted into C57BL/6 mice. OVA-specific T cells

were transferred into tumour-bearing mice to assess antigen presentation. CD69 upregulation, used as a marker of T cell activation, was observed on OVA-specific T cells in both deep and superficial cervical lymph nodes (LNs). This indicated that antigen presentation occurred in these LNs. To further assess whether migratory antigen presenting cells (APCs) coordinate this CD8 T cell response, T cell activation was investigated in CCR7^{-/-} mice. In these mice, migration of APCs is compromised. CD8 T cell activation in deep and superficial cervical LNs was impaired in tumour-bearing CCR7^{-/-} mice, suggesting that activation of brain tumour-specific CD8 T cells is dependent on CCR7⁺ migratory APCs.

Future studies will aim to elucidate which APCs drive this immune response to brain tumours, which may provide insight for the design of better treatment strategies against this disease and potentially other cancers involving the CNS.

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Difference in proinflammatory response induced by Newcastle disease virus in tumor and normal cells contributes to tumor-specific cytotoxicity

Ginting, T., Suryatenggara, J., Christian, S., Mathew, G. Mochtar Riady Institute for Nanotechnology - Universitas Pelita Harapan, Immunology, Tangerang, Indonesia

Newcastle disease virus (NDV) has been clinically proven to activate immune responses that are harmful to tumor cell but not to normal cell. In order to investigate the actual role of immune responses induced by lentogenic NDV for its antitumor effect, we infected NDV LaSota strain into human cells i.e. small lung carcinoma (A549), glioblastoma (U87MG), lung fibroblast (HF19), skin fibroblast (NB1RGB), transformed embryonic kidney (HEK293) and monocyte cells at low (0.0001) and high (0.01) MOI (multiplicity of infection); and observed the cytotoxicity and expression of proinflammatory cytokines. Both A549 and U87MG cell lines showed viability of less than 20%, while normal cell lines NB1RGB, HEK293, and monocyte showed more than 50% viability 72 hours post infection. Proinflammatory cytokines profiling showed that NDV mainly induced the secretion of interferon alpha (IFN- α), beta (β) and lambda (λ) in tumor cells and only IFN- λ in normal cells.

We found that NDV infection stimulated apoptosis in both tumor and normal cells and this effect is reduced with the addition of type I IFN-blocker in tumor cells but not in normal cells. The cumulative effect of IFN- α , β , and λ expression in tumor cells enhances tumor cells cytotoxicity when compared to that observed in normal cells after NDV LaSota infection. This finding introduces a new perspective on the role of IFN- λ in NDV-mediated oncolysis.

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The regulatory effects of NKT and MAIT cells in cancer

Meredith, T.^{1,2}, Minoda, Y.^{1,2}, Toohey, B.², Kannourakis, G.², Berzins, S.^{1,2,3}

¹Federation University Australia, Ballarat, Australia, ²Fiona Eley Cancer Research Institute, Ballarat, Australia, ³Melbourne University, Melbourne, Australia

Natural killer T (NKT) cells and mucosal associated invariant T (MAIT) cells contribute to immune regulation in several settings, including cancer. NKT cells in particular have a strong association with cancer in mice, but less is known about their role in humans. We seek to understand whether NKT and MAIT cells have a regulatory effect on cells such as natural killer (NK) cells, dendritic cells and conventional T cells that are known to have important anti-tumour effects.

Our approach is to measure the effector functions of these cells in the presence or absence of NKT and MAIT cells, and their ligands. Our preliminary data suggests that altering NKT/MAIT cell concentrations can cause a decrease in T and NK cell cytokine production, as well as T cell proliferation.

We are also investigating the impact of NKT and MAIT cells on the tumour lysing abilities of NK cells using primary and established tumour cell lines as targets or as sources of stimulation. Preliminary data suggests that NK cells lysing ability is unaffected by MAIT cell levels.

These experiments will seek to determine the importance of NKT and MAIT cells in anti-cancer immunity and if these cells could be useful targets in new immune therapies.

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TCR beta sequencing to determine clonal T-cell populations in melanoma patients undergoing immunotherapy

Witkowski, T.¹, Behren, A.², Woods, K.², Fritz, B.³, Wong, S.Q.⁴, Colebatch, A.⁴, McArthur, G.⁴, Cebon, J.², Dobrovic, A.¹

¹Olivia Newton-John Cancer Research Institute, Translational Genomics & Epigenomics, Heidelberg, Australia, ²Olivia Newton-John Cancer Research Institute, CIBL, Heidelberg, Australia, ³Adaptive Biotechnologies Corp, Seattle, United States, ⁴Peter MacCallum Cancer Centre, Department of Cancer Research, Melbourne, Australia

The adaptive immune system elicits clonal expansion of tumour-infiltrating lymphocytes (TILs) in melanoma¹. Immunotherapeutics targeting immune suppressive receptors such as CTLA-4 and PD-1 have been shown to influence prognosis with a correlated increase in TILs^{2,3}. However, immune-checkpoint inhibition can also prompt immune-related adverse events such as rare instances of acute inflammation including complications like cytomegalovirus (CMV) pneumonia⁴. Assessing clonality and TIL numbers is therefore of interest in solid tumours. Next Generation Sequencing (NGS) assays designed across the V, D and J regions of the *TCRB* gene can be used to infer T-cell clonality. The immunoSEQ hsTCRB kit from Adaptive Biotechnologies utilizes an initial VDJ gene-specific amplification with a proprietary set of primers, followed by adapter ligation and sequencing on an Illumina platform. The sensitivity of the assay was assessed by spiking in a single

clone population, isolated using NY-ESO-1 derived peptides in proportions with healthy donor peripheral blood mononuclear cell (PBMC) DNA. It was shown that genomes derived from healthy donor PBMCs contain 85% unique clones of TCR, and we were able to detect our clone spiked at 0.01%. This ability to detect specific clones of lymphocytic DNA indicates that the immunoSEQ NGS assay is readily adapted to the study of clonal T-cell populations and will be sensitive enough to target limited lymphocytic DNA in FFPE from tumour tissues including serial studies of patients undergoing immunotherapy. Following sequencing individual clones can also be monitored using uniquely designed qPCR assays utilising rearrangement specific primers.

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Vascular endothelial growth factor-A and placental growth factor regulate tumor progression via increased vasculature in cutaneous T cell lymphoma

Miyagaki, T., Sugaya, M., Oka, T., Takahashi, N., Kawaguchi, M., Suga, H., Fujita, H., Yoshizaki, A., Asano, Y., Sato, S.
Faculty of Medicine, University of Tokyo, Department of Dermatology, Tokyo, Japan

Angiogenesis and lymphangiogenesis are regarded as essential steps to support tumor growth and metastasis. Also in hematological malignancies including cutaneous T cell lymphoma (CTCL), angiogenesis and lymphangiogenesis are increased and serum levels of some proangiogenic and prolymphangiogenic markers are elevated. In this study, we investigated expression levels of vascular endothelial growth factor (VEGF) family members in lesional skin and sera in CTCL patients and assessed their association with CTCL development. We found that mRNA and protein expression of VEGF-A and placental growth factor (PIGF) but not VEGF-C was significantly elevated in lesional skin of CTCL. Tumor cells expressed VEGF-A in most cases and PIGF in some cases. Moreover, serum VEGF-A and PIGF levels were elevated in patients with advanced CTCL and both of them decreased after treatment. Increased serum VEGF-A levels were associated with pruritus, while serum PIGF levels reflected disease severity. Remarkably, local administration of PIGF enhanced tumor growth of lymphoma cells through increasing vasculature in vivo. These findings suggest that VEGF-A and PIGF play important roles in angiogenesis during progression of CTCL. In addition, considering the correlations of expression of VEGF-A and PIGF with pruritus and disease severity, inhibitors of those proangiogenic factors, such as bevacizumab, or VEGF receptor inhibitors could be promising therapies in CTCL.

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High expression of Ecto-nucleotidases CD39-CD73, A2A-A2B adenosine receptors and IL-10 is associated with HLA class I downregulation in advanced cervical cancer

Gutierrez, V.^{1,2}, Mora, M.D.L.¹, García, R.², Weiss, B.¹, Monroy, A.²
¹UNAM, FES Zaragoza, Laboratory of Immunobiology, UIDCC, UMIÉZ, Ciudad de México, Mexico, ²IMSS, CMN SXXI, Laboratory of Immunology and Cancer, UIMEO, H. Oncology, Ciudad de México, Mexico

Development of Cervical cancer (CC) is strongly associated with high-risk HPV infection and several factors involved in the tumor microenvironment, including immunosuppressive cytokines, such as IL-10, that inhibit cellular immune response and favor HLA class I downregulation. On the other hand, the co-expression of the ectonucleotidases CD39 and CD73 in malignant tumors is also associated with immunosuppression, via adenosine production and high expression of IL-10. The aim of this study was to analyze the co-expression of molecules involved in the adenosinergic pathway such as the CD39 and CD73 ecto-nucleotidases, and A2A-A2B adenosine receptors, as well as the expression of IL-10 and HLA-Class I molecules in tissue samples derived from different stages of the development of CC. The expression of these cell markers was analyzed in immunohistochemical samples from normal cervix (20), LSIL (20), HSIL(20) and CC(20) by using the Image-pro Plus 6.0 program. We found that the expression of CD39 and CD73, as well as, A2A and A2B was strongly associated with an increase of the IL-10 expression and HLA-Class I downregulation in relation to the degree of disease progression. These results suggest that the expression of molecules participating in the adenosinergic pathway, can be a favorable condition to generate IL-10, providing a possible mechanism for immunosuppression and immune evasion during the development of CC. This work was supported by DGAPA-PAPIIT (IN226516), FIS/IMSS/PROT (1314 and 1383) and CONACYT (240635) grants.

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Cancer chemo immunotherapy - exploiting the immunogenic potential of cytotoxic chemotherapy

Aston, W.J.^{1,2}, Rinaldi, C.^{1,2}, Fisher, S.A.^{1,2}, Nowak, A.K.^{1,2,3}, Lake, R.A.^{1,2}, Lesterhuis, W.J.^{1,2}

¹National Centre for Asbestos Related Diseases, Perth, Australia, ²University of Western Australia, School of Medicine & Pharmacology, Perth, Australia, ³Sir Charles Gairdner Hospital, Medical Oncology, Perth, Australia

Background: Cytotoxic chemotherapy is the primary treatment for many cancers however is often not curative. Recently, a number of novel immunotherapeutic treatments have shown promising clinical results including the checkpoint blocking antibodies. It has long been believed that these could not be combined with chemotherapy due to the latter largely exhibiting myelosuppressive effects. However, it has recently been shown that selected chemotherapeutics may be immunestimulating and so this research aimed to investigate the antitumour immune responses of cyclophosphamide specifically focusing on lymphoid and myeloid subsets.

Methods: BALB/c mice were inoculated subcutaneously with 5x10⁵ AB1 mesothelioma cells. On day 12 they were given 250 mg/kg cyclophosphamide intraperitoneally. Tumours were harvested at various times after chemotherapy and analysed using H&E histology and flow cytometry to characterise immune cell infiltrates.

Results: AB1 tumours treated with cyclophosphamide regressed, appeared to regrow then regressed completely over a 20 day period. Based on this four time points were selected for histology and flow cytometry analysis: growth before treatment,

growth after treatment, regrowth phase and final regression phase. The most notable finding was in the final regression phase in which the resected tumour section showed large leukocytic infiltrates. Gross changes to immune cell populations included a decrease in FoxP3+ Tregs and a significant increase in both CD4+ and CD8+ T lymphocytes. Functional studies showed that CD8+ T cells were necessary for the curative effect of cyclophosphamide.

Conclusion: Cyclophosphamide treatment of mesothelioma *in vivo* results in leukocytic infiltrates that mediate regression, particularly CD8+ T cells.

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IL-15 derived from gastric cancer mesenchymal stem cell involved in the progression of gastric cancer

Li, S.¹, Chen, B.¹, Sun, X.¹, Xu, R.¹, Han, Y.¹, Zhao, Y.¹, Wang, M.², Zhu, W.²
¹Jiangsu University, Zhenjiang, China, ²Jiangsu University, School of Medicine, Zhenjiang, China

Objective: In previous study has shown that interleukin 15 (IL-15) involved in the progression of leukemia, but the role of IL-15 in solid tumor was unclear. In the present study we aims to investigate the role of IL-15 derived from gastric cancer-derived MSCs (GC-MSCs) in the progression of gastric cancer (GC).

Methods: The expression of IL-15 was assessed using ELISA and immunohistochemical (IHC). The GC cell lines were treated with culture media of GC-MSCs (GC-MSC-CM) and their proliferation and migration ability was detected using colony formation assay and transwell migration assay, respectively. The stemness and epithelial-mesenchymal transition (EMT) of GC cell lines were detected by western blot.

Results: We found that the level of IL-15 was higher in GC patients compared with that in healthy individuals, which were associated with lymph node metastasis. The results of IHC showed that there was no IL-15 expression in gastric epithelial cells in healthy individuals. However, the tumor tissues of GC patients expressed different intensities of IL-15 and were also associated with lymph node metastasis. We also discovered that IL-15 derived from GC-MSC enhance proliferation, migration and stemness and promote EMT of GC cell lines. We found that IL-15 derived from GC-MSC promote proliferation of regulatory T cells and inhibit proliferation of regulatory Th17 cells.

Conclusion: IL-15 is involved in the occurrence and progression of gastric cancer. IL-15 may be a novel marker for GC diagnosis and treatment.

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Molecular mechanisms of DUSP22 ablation in enhancing EGFR-mediated lung tumor development: potential involvement of immunosuppression

Lin, H.-P., Ho, H.-M., Chang, C.-W., Tan, T.-H., Lin, W.-J.
 National Health Research Institutes, Immunology Research Center, Miaoli County, Taiwan, Republic of China

DUSP22, a member of the dual-specificity phosphatases (DUSPs) family, is a key phosphatase controlling the activity of protein kinases and transcription factors through the dephosphorylation

(serine/threonine or tyrosine) process, such as MAPKs, FAK, Lck, and STAT3. Many members of DUSP22-targeted proteins are important for tumorigenesis, and DUSP22 downregulation has been reported in some cancers. Importantly, DUSP22^{-/-} mice manifest multi-organ inflammation, supporting a role for DUSP22 in maintaining immune homeostasis. However, little is known about the *in vivo* role of DUSP22 during tumor development. Here, utilizing a murine lung tumor model driven by the combination of mutated EGFR amplification (exon 19 deletion) and the loss of DUSP22, we found genetic ablation of DUSP22 promoted aggressive EGFR-mediated lung tumor development at early age, and identified MDSCs and exhausted T cells as the two main tumor-infiltrating immune cell types. Our cytokine array profiling found a significant increase of IL-1 and CXCL1 in EGFR/DUSP22 KO lung tumors, suggesting these two cytokines may potentially serve as EGFR/DUSP22 KO tumor-derived factors to attract immunosuppressive cells and promote tumor development. Consistently, GEO data analysis shows that lung adenocarcinoma patients with low DUSP22 expression have poor survival. Collectively, our current data indicate that DUSP22 underexpression may facilitate the recruitment of MDSCs and dysfunctional T cells and establish an immunosuppressive tumor microenvironment for tumor development. Currently, we are using pharmacological and immune approach to elucidate molecular mechanisms of DUSP22 ablation for enhancing lung tumor development.

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T-cells in the anal mucosa of men with high-grade squamous intraepithelial lesions

Tong, W.W.Y.^{1,2}, Turville, S.³, Roberts, J.⁴, Zaunders, J.^{1,3}, Jin, F.³, Poynten, I.M.³, Hillman, R.^{1,5,6}, Grulich, A.³, Kelleher, A.^{1,3}, Carr, A.^{1,2}, for the SPANC Study Team

¹St Vincent's Centre for Applied Medical Research, HIV, Immunology & Infectious Diseases, Darlinghurst, Australia, ²University of New South Wales, Sydney, Australia, ³The Kirby Institute for Infection and Immunity in Society, University of New South Wales, Sydney, Australia, ⁴Douglas Hanly Moir Pathology, Sydney, Australia, ⁵Western Sydney Sexual Health Centre, Sydney, Australia, ⁶University of Sydney, Sydney, Australia

Introduction: Anal mucosal cellular immune responses and their role in controlling human papilloma virus (HPV)-induced precancerous lesions have not been previously studied.

Methods: The Study of the Prevention of Anal Cancer (SPANC) is a longitudinal natural history study of anal HPV infection in men ≥35 years who have sex with men. 26 participants with anal high-grade squamous intraepithelial lesions (HSIL) had 44 anal biopsies. Lymphoid aggregates were detected by inspection of haematoxylin & eosin-stained sections. Additional sections were immunofluorescently stained to enumerate stromal and intra-epithelial CD4+ and CD8+ T-cell counts. Whole slide imaging of full tissue architecture (x600) was used. Student's t-test of log10-transformed T-cell density was used to compare means; a generalized, linear model was used to determine factors associated with total T-cell density (biopsy-based analysis with intra-subject adjustment).

Results: Of 26 men (mean age 53 years, SD 10.5), 7 (27%) were HIV-infected. Seventeen (68%) had concurrent anal HPV16 detected. Of 44 biopsies, 26 (59%) revealed HSIL. Twenty-four (55%) had lymphoid aggregates localised in the stroma adjacent to the epithelium. Lymphoid aggregate presence was associated with higher CD4+ T-cell density (mean 192 vs. 69 cells/mm², $P < 0.001$), but not higher CD8+ T-cell density (106 vs. 62 cells/mm², $P = 0.077$). A biopsy with HSIL was significantly associated with higher total T-cell density (OR 11.80, 95%CI 1.51 - 92.08, $P = 0.02$), as was having anal HPV16 detected (OR 14.08, 95%CI 1.15 - 172.71, $P = 0.04$).

Conclusion: CD4+ T-cell enriched lymphoid aggregates in the anal mucosa were associated with anal HSIL and HPV16.

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Effects of upregulation of Id3 in human lung adenocarcinoma Q1 cells on proliferation, apoptosis, mobility and tumorigenicity

Li, X.-J.^{1,2}, Chen, F.-F.¹, Liu, Y.¹, Wang, F.¹, Pang, X.-J.¹, Zhu, C.-D.¹, Xu, M.¹, Yu, W.¹

¹Center of Clinical Laboratory Science, Jinling Hospital, School of Medicine, Nanjing University, Nanjing, China, Nanjing, China,

²State Key Laboratory of Analytical Chemistry for Life Science, Nanjing University, Department of Chemistry, Nanjing, China

The inhibitor of DNA-binding/differentiation 3 (Id3) protein is a helix-loop-helix transcription factor and may have an important role in cell proliferation and differentiation. This study was to evaluate the effects of upregulation of Id3 in human lung adenocarcinoma cells on proliferation, apoptosis, mobility and tumorigenicity. Short interference RNA suppression of Id3 (miRId3) in A549 cells was used to investigate the functional role(s) of Id3. Next, we used in vitro wound-healing assay and trans-well assay to study the effects of overexpressed Id3 on migration and invasion of A549 cells. Furthermore, to explore the influence of overexpressed Id3 on in vivo tumorigenesis, adenoviruses containing Id3 gene (Ad-Id3) and empty vector (Ad-LacZ) were generated. Co-transfection of pcDNA/miRId3 and pEGFP/Id3 into A549 cells reversed the Id3-induced cell proliferation inhibition and apoptosis. Upon Id3 transfection, A549 cells displayed decreased migratory and invasive capabilities, however, co-transfection of miRId3 and Id3 into A549 cells reversed the Id3-induced inhibitions of migratory and invasive capabilities. Three groups of nude mice were inoculated with Ad-LacZ, Ad-Id3 transfectants and untransfected A549 cells, respectively. Twenty-eight days after inoculation, tumors induced by Ad-Id3 transfectants grew much more slowly compared with Ad-LacZ transfectants and control group. This study provides for the first time both in vitro and in vivo proofs that forced expression of Id3 in lung adenocarcinoma cells reduces tumor growth rate and may be a potential target for tumor suppression.

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Hepatocyte growth factor activates stromal fibroblasts to promote tumorigenesis in gastric cancer

Chen, X.

Shanghai Jiaotong University, Ruijin Hospital, Shanghai, China

Cancer-associated fibroblasts (CAF), as the activated fibroblasts in the tumor stroma are important modifiers of tumor progression. However, the mechanisms underlying stromal fibroblast activation and their promotion of tumor growth remain largely unknown in gastric cancer. In this study, we show that normal fibroblasts (NFs) from non-cancerous regions of gastric cancer exhibit the traits of CAFs when grown together with gastric cancer cells in vivo. Activation of NFs can be induced by co-culture with gastric cancer cells. while deprivation of hepatocyte growth factor (HGF) using a neutralizing antibody inhibits the activation of NFs. Moreover, we identify HGF as an important factor from CAFs that acts in a paracrine manner to promote tumorigenesis in vitro and in vivo

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Preclinical modeling of EGFR-specific mAb and immune pathways that impact immunotherapeutics for squamous cell carcinoma

He, X.^{1,2}, Gonzalez Cruz, J.¹, Simpson, F.¹, Wells, J.¹

¹The University of Queensland Diamantina Institute, Brisbane, Australia, ²The University of Queensland, Brisbane, Australia

Non-melanoma skin cancers (NMSCs) are the most common skin cancer within the caucasian population. Basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) are the most frequent NMSCs, and it has been shown that UV irradiation and immunosuppression are two of the main triggers of these pathologies. While BCCs typically react positively to topical treatments, SCC does not and is especially prone to local recurrence. This problem highlights the need for new and more effective treatments. However, in order to develop new treatments we first need to develop a reliable pre-clinical model. It has been previously reported that 95% of UV-induced SCC over-express the epidermal growth factor receptor (EGFR) on their surface, making EGFR an attractive target for anti-SCC therapies. In fact, the use of anti-EGFR antibody therapy has been demonstrated to mediate tumour regression by interrupting oncogenic signals and by inducing Fc-mediated innate mechanisms.

In our laboratory we have recently developed a UV-induced SCC mouse model with arising tumours that over-express EGFR. In this project we will use this mouse model to investigate the efficacy of monoclonal antibody therapies that target the EGFR using one of the two anti-murine EGFR antibodies (7A7) available worldwide, in order to investigate the impact that these therapies have on immune system activation.

The insights derived from our study will benefit the development of successful combination therapies for the treatment of EGFR-positive cancers, such as SCC and head and neck cancer.

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Overview of T cells populations in human colorectal cancer by single cells analysis*Simoni, Y.¹, Koo, S.-L.², Tan, I.², Newell, E.²**¹A*STAR, SigN, Singapore, Singapore, ²A*STAR, Singapore, Singapore*

T lymphocytes are one of main cell subsets infiltrating colorectal tumor (CRC), however technical and inherent experimental limitations have hampered a clear profiling of these cells in humans tumors. By using mass spectrometric analysis (CyTOF), we optimized two panels of 38 markers each, that allowed us to simultaneously assess all T cells subsets (CD4, CD8, T γ δ , MAIT cells) and their phenotypes (exhaustion/activation), transcription factor expression profiles (FoxP3, T-bet, EOMES, Ki67) and tumors antigen specificity of CD8 T cells using combinatorial peptide-MHC tetramer staining. With t-Distributed Stochastic Neighbor Embedding (t-SNE) to display phenotypic diversity, our data showed much greater complexity in the CD4⁺ and CD8⁺ tumor-infiltrating T cell population than previously appreciated, including distinct niches occupied by tumor specific CD8 T cells. We also observed a non-uniform pattern of variation across patients tested, highlighting diversity as previously described. In addition, we observed striking variation in T cell composition between different sectors of the same tumor. Overall, the large degree of diversity we observe, and particularly with respect to markers of T cells exhaustion, may help to explain heterogeneity in clinical outcome during various forms of immunotherapy.

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Caspase-1 is involved in drug resistance to triterpenoid anticancer drug in adult T-cell leukemia*Nakanishi, T., He, C., Wang, D., Song, Y., Morita, K., Kanazawa, T., Yoshida, Y.**University of Occupational and Environmental Health, Kitakyushu, Japan*

We previously reported that the inflammasome inhibitor cucurbitacin D (CuD) induces apoptosis in human leukemia cell lines. Here, we investigated the effects of CuD and a B cell lymphoma extra large (Bcl-xL) inhibitor on autophagy in peripheral blood lymphocytes (PBL) isolated from adult T cell leukemia (ATL) patients. CuD induced PBL death in patients but not in healthy donors. This effect was not significantly inhibited by treatment with rapamycin or 3-methyladenine (3-MA). The Bcl-xL inhibitor Z36 induced death in primary cells from ATL patients including that induced by CuD treatment, effects that were partly inhibited by 3-MA. Similarly, cell death induced by the steroid prednisone was enhanced in the presence of Z36. A western blot analysis revealed that Z36 also promoted CuD-induced poly ADP ribose polymerase cleavage. Interestingly, the effects of CuD and Z36 were attenuated in primary ATL patient cells obtained upon recurrence after umbilical cord blood transplantation, as compare to those obtained before chemotherapy. Furthermore, cells from this patient expressed high level of caspase-1, and treatment with caspase-1 inhibitor enhanced CuD-induced cell death. Taken together, these

results suggest that rescue from resistance to steroid drugs can enhance chemotherapy, and that caspase-1 can serve as a marker of drug resistance in ATL patients.

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Identification and quantification of innate like T cells in patients with cancer*Minoda, Y.¹, Godfrey, D.², Kannourakis, G.¹, Toohey, B.¹, Berzins, S.¹**¹Fiona Elsey Cancer Research Institute, Ballarat, Australia, ²The University of Melbourne, Melbourne, Australia*

Innate-like T cells, including invariant natural killer T (iNKT) cells, mucosal associated invariant T (MAIT) cells and gamma delta T (γ δ T) cells, are associated with regulation of anti-tumour responses in humans and mice, although their precise role, particularly within the tumour microenvironment is poorly defined. We have studied innate-like immune cells isolated from tumours, blood and bone marrow of patients undergoing treatment for cancer and compared their phenotypic and functional characteristics with cells from healthy donors. Our results indicate that the frequency of NKT cells and MAIT cells in tissue samples from patients with solid or blood cancer was usually significantly lower than in healthy individuals, and the distribution of their subsets was often abnormal. Interestingly, cytokine productivity by these innate like T cells was relatively similar to healthy individuals, however, MAIT cell proliferation in blood and tumour from patients with solid cancer was significantly reduced compared to healthy individuals. Collectively, our results indicate that innate like T cells in patients with cancer are often abnormal and targeting them in new therapies could a viable option for new treatments.

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Epac1 knockdown inhibits the proliferation of serous ovarian carcinoma cells by inactivating AKT/CyclinD1/CDK4 pathway in vitro and in vivo*Wang, X.¹, Gao, M.¹, Ma, Y.¹, Bast Jr., R.C.², Wei, Z.³**¹Shandong University School of Medicine, Department of Immunology, Jinan, China, ²Division of Cancer Medicine, University of Texas MD Anderson Cancer Center, Department of Experimental Therapeutics, Houston, United States, ³Shandong University School of Medicine, Department of Gynecology and Obstetrics, Jinan, China*

Ovarian cancer is the leading cause of death among gynecological malignancies, and high grade serous ovarian carcinoma is the most common and most aggressive subtype. Recently, it was demonstrated that cAMP mediates protein kinase A-independent effects through Epac (exchange protein directly activated by cAMP) proteins. Epac proteins, including Epac1 and Epac2, are implicated in several diverse cellular responses, such as insulin secretion. Several reports document that Epac1 could play vital roles in promoting proliferation, invasion and migration of some cancer cells. However, the expression levels and roles of Epac1 in ovarian cancer have not been investigated. In the present study, we detected the expression levels of Epac1 protein in serous ovarian carcinomas and normal ovarian tissues by immunohistochemistry. Furthermore, the effect of Epac1

knockdown on the proliferation and apoptosis of serous ovarian carcinoma cell lines SKOV3 and OVCAR3 were evaluated in vitro and in vivo. The results showed that the expression of Epac1 was significantly upregulated in serous ovarian carcinoma tissues and cells lines. Epac1 knockdown inhibited the proliferation of serous ovarian carcinoma cells in vitro and in vivo. Decreased proliferation may be due to Epac1 downregulation-induced G1 phase arrest by inactivating the AKT/CyclinD1/CDK4 pathway, but not to alterations in the MAPK pathway or to apoptosis of ovarian carcinoma cells. Taken together, our data provide new insight into the essential role of Epac1 in regulating growth of serous ovarian carcinoma cells and suggest that Epac1 might represent an attractive therapeutic target for treatment of serous ovarian carcinomas.

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N-glycosylation HMGB1 preferentially promoted M-MDSCs expansion via driving differentiation from bone marrow, contributing conversion of monocytes into MDSC-like cells and facilitated breast cancer progression

Su, Z.¹, Ni, P.², Liu, Y.², Lu, H.², Su, X.², Xu, H.²

¹Jiangsu University, Immunology, Zhenjiang, China, ²Jiangsu University, Zhenjiang, China

Myeloid-derived suppressor cells (MDSCs) are major cell populations that regulating immune responses. It is accumulated in tumor progression, chronic inflammatory and autoimmunity. Previous data indicated that high mobility group box 1 (HMGB1) facilitated MDSCs differentiation from bone marrow, and suppressed antigen-driven activation of CD4⁺ and CD8⁺ T cells and involved in cancer development. whereas the potential mechanisms of HMGB1 facilitating MDSCs differentiation from bone marrow are unclear. In the present work, we clearly demonstrated that:

- (i) HMGB1 secreted by MCF-7 cells was glycosylation at Asn37
- (ii) N-glycosylation HMGB1 not normal HMGB1 preferentially driven M-MDSCs differentiation from bone marrow progenitor cells STAT3/Erk1/2/NFκB pathway;
- (iii) N-glycosylation HMGB1 contributed conversion of monocytes into MDSC-like cells;
- (iv) In vivo, HMGB1 blockade by ethyl pyruvate (EP) significantly reduced the accumulation of MDSCs in tumor-bearing mice, thus leading to the delayed tumor growth. Therefore, our results shown that N-glycosylation HMGB1 facilitated MDSCs expansion and contributed cancer progression; which indicated that HMGB1 might be a potential tumor immunotherapy targets.

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Opposing effects of the adenosine/PGE2/cAMP vs. STAT3 pathways on CD73 expression on human tumour-associated monocytes

Al-Taei, S.¹, Spary, L.¹, Salimu, J.¹, Clayton, A.¹, Lester, J.², Tabi, Z.¹

¹Cardiff University, Division of Cancer and Genetics, Cardiff, United Kingdom, ²Velindre NHS Trust, Velindre Cancer Centre, Cardiff, United Kingdom

ATP, released by damaged or dying cells, represents a danger signal that contributes to the initiation of immune responses. On the other hand, the ATP-metabolite adenosine has extensive immunosuppressive effects and also directly supports tumour growth, differentiation and metastasis. ATP conversion into ADP-5'-AMP-adenosine is catalysed by a highly regulated stepwise activity of ectonucleotidase enzymes CD39 and CD73. Most malignant cells express CD73, while tumour stroma, including immune cells, express CD39. The aim of this study was to reveal if there is a unique expression pattern of CD73 on tumour-associated immune cells in malignant pleural mesothelioma.

We found CD73-expressing CD14⁺ cells in tumour-associated pleural effusion (PF) but not in the peripheral blood of mesothelioma patients. The co-expression of CD39 and CD73 makes tumour-associated myeloid cells uniquely capable of metabolising ATP to adenosine. CD73 was induced on normal CD14⁺ cells, but not on CD3 cells, with PF, adenosine and cAMP-activators forskolin and prostaglandin E₂ (PGE₂). Inhibition of the adenosine A_{2a} receptor or PGE₂ receptors (EP2/EP4) abolished CD73 induction by PF. Monocyte treatment with PF also induced STAT3 phosphorylation; however, STAT3 blocking resulted in enhanced CD73 expression, pointing towards a negative control of the adenosine pathway by STAT3.

Here we demonstrate a link between prostaglandin, cAMP and adenosine pathways under the negative control of STAT3 in human monocytes. The complex control of CD73 expression on myeloid cells points towards the importance and potential therapeutic targeting of the ATP:adenosine balance in the tumour microenvironment.

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Human mesothelioma induces defects in dendritic cell numbers and antigen processing function which predict survival outcomes

Cornwall, S.^{1,2}, Wikstrom, M.³, Musk, A.⁴, Alvarez, J.⁵, Nowak, A.^{6,7}, Nelson, D.^{1,2}

¹Curtin University, School of Biomedical Sciences, Immunology and Cancer Group, Bentley, Australia, ²Curtin University, CHIRI Biosciences Research Precinct, Bentley, Australia, ³University of Western Australia, Lions Eye Institute, Nedlands, Australia, ⁴Sir Charles Gairdner Hospital, Department of Respiratory Medicine, Nedlands, Australia, ⁵The Mount Hospital, Perth, Australia, ⁶University of Western Australia, School of Medicine and Pharmacology, Nedlands, Australia, ⁷Sir Charles Gairdner Hospital, Department of Medical Oncology, Nedlands, Australia

Mesothelioma is an almost invariably fatal tumor with chemotherapy extending survival by a few months. One immunotherapeutic strategy is to target dendritic cells (DCs), key antigen presenting cells involved in antigen presentation, to induce antigen-specific T cell responses. However, DC-targeting will only be effective if DCs are fit-for-purpose, and the functional status of DCs in mesothelioma patients was not clear. We found that mesothelioma patients have significantly decreased numbers of circulating myeloid (m)DC1 cells, mDC2 cells and plasmacytoid (p)DCs relative to healthy age and gender-matched controls. Blood monocytes from patients could not

differentiate into immature monocyte-derived DCs (MoDCs), indicated by a significantly reduced ability to process antigen and reduced expression of costimulatory (CD40, CD80 and CD86) and MHC (HLA-DR) molecules, relative to controls. Activation of mesothelioma-derived MoDCs with LPS+/-IFN γ generated partially mature MoDCs, evident by limited up-regulation of the maturation marker, CD83, and the costimulatory markers. Attempts to rescue mesothelioma-derived DC function using CD40Ligand(L) also failed, indicated by maintenance of antigen processing capacity and limited up-regulation of CD40, CD83, CD86 and HLA-DR. These data suggest that mesothelioma patients have significant numerical and functional DC defects and that their reduced capacity to process antigen and reduced expression of costimulatory molecules could induce anergized/tolerized T cells. Nonetheless, survival analyses revealed that individuals with mesothelioma and higher than median levels of mDC1s and/or whose MoDCs matured in response to LPS, IFN γ or CD40L lived longer, implying their selection for DC-targeting therapy could be promising especially if combined with another treatment modality.

45 Minute Oral

16:45:00 - 17:30:00

Lymphocyte Signalling

3815

Cytokine-driven loss of plasmacytoid dendritic cell function in chronic lymphocytic leukemia

Saulep-Easton, D.¹, Vincent, F.B.¹, Le Page, M.¹, Wei, A.², Ting, S.B.², Croce, C.M.³, Tam, C.⁴, Mackay, F.⁵

¹Monash University Central Clinical School, Department of Immunology, Melbourne, Australia, ²The Alfred Hospital and Monash University, Department of Haematology, Melbourne, Australia, ³The Ohio State University, Department of Molecular Virology, Immunology and Medical Genetics, Columbus, United States, ⁴Peter MacCallum Cancer Institute, Department of Haematology, Melbourne, Australia, ⁵University of Melbourne, School of Biomedical Sciences - Department of Microbiology and Immunology, University of Melbourne, Australia

Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of CD5+CD19+ B cells in the peripheral blood, and in primary and secondary lymphoid organs. A major complication associated with CLL is severe recurrent infections, which are often fatal. Vulnerability to infection is due to a wide variety of immunological defects, yet the initiating events of immunodeficiency in CLL are unclear. Using CLL patient samples and a mouse model of CLL, we have discovered that plasmacytoid dendritic cells (pDCs), which underpin the activity of effector immune cells critical for anti-viral immunity and anti-tumor responses, are reduced in number and functionally impaired in progressive CLL. As a result, the levels of interferon alpha (IFN α) production, a cytokine critical for immunity, are markedly reduced. Lower pDC numbers with impaired IFN α production was due to the decreased expression of FMS-like tyrosine kinase 3 receptor (Flt3) and Toll-like receptor 9 (TLR9), respectively. Reduced Flt3 expression was reversed using inhibitors of TGF- β and TNF, an effect correlating with a reduction in tumor load. Defects in pDC numbers and function offer new insight into mechanisms underpinning the profound immunodeficiency affecting CLL patients and provide a potentially novel avenue for restoring immunocompetency in CLL.

Tumour Immunology

Novel mediators of immune suppression within the tumor microenvironment

Restifo, N.P.

National Cancer Institute, NIH, Bethesda, MD, USA

Tumours progress despite being infiltrated by effector T cells. Tumour necrosis is associated with poor survival in a variety of cancers. Here, we report that that necrosis causes release of an intracellular ion, potassium, into the extracellular fluid of human and mouse tumours. Surprisingly, elevated extracellular potassium [K⁺] was sufficient to profoundly suppress mouse and human T cell anti-tumor function. Elevations in extracellular [K⁺] acted to acutely impair T cell receptor (TCR) dependent Akt-mTOR phosphorylation and effector function. Potassium mediated suppression of Akt-mTOR signaling and T cell effector function required intact activity of PP2A, a serine/threonine phosphatase. The suppressive effect mediated by elevated extracellular [K⁺] required a T cell-intrinsic increase in intracellular potassium [K⁺] and was independent of changes in plasma membrane potential (V_m). Finally, ionic reprogramming of tumour-specific T cells via over-expression of the voltage-gated potassium channel Kv1.3 lowered intracellular [K⁺] and improved effector functions in vitro and in vivo, with this gain of function being dependent on intact channel function. Consequently, Kv1.3 T cell expression enhanced tumour clearance and the survival of melanoma-bearing mice. These results uncover a previously undescribed ionic checkpoint against T cell function within tumors and identify new strategies for cancer immunotherapy.

T Cell Memory

Transcriptional regulation of Memory T cell Formation

Goldrath, A.

University of California San Diego

Long-lived memory T cells mediate protection from reinfection with previously encountered pathogens; keep chronic, opportunistic and latent pathogens at bay; and can serve as endogenous defenders against tumor growth and metastases. The memory T cell population is heterogeneous, typically categorized into central memory cells found in the blood and lymphoid tissues or effector memory cells predominantly located in the blood and non-lymphoid tissues. The recent recognition of a third subset of memory lymphocytes, termed tissue-resident memory cells, that reside strictly within tissues and do not recirculate requires a revision of our understanding of memory T cell differentiation. The goal of our research is to understand how each population of CD8⁺ memory T cells is generated and maintained by identifying the transcriptional, epigenetic and signaling events that regulate the survival and differentiation of T cells as they navigate the immune response and become long-lived memory T cells.

Tuesday, 23 August 2016

30 Minute Oral

08:30:00 - 10:15:00

Golden anniversary of IgE: WAO –IUIS session

Hierarchy and molecular properties of house dust mite allergens

Thomas, W.

Telethon Kids Institute

Antiserum produced to the purified major allergen of ragweed was central to the discovery of IgE but largely due to variable allergen extracts the use of IgE to define allergens was slow to develop. Molecular cloning and production of recombinant allergens reshaped the field by providing defined and pure allergen components required for reproducible assays and take confounding reactivities to degradation products, cross-reactivities and isoforms into account. House dust mite allergy can now be clinically and scientifically investigated within the framework that most of the response is caused by sensitization to a small number of immunodominant and mid-tier allergens that show a characteristic hierarchy in their ability to induce IgE antibody making proportionate contributions to the overall response of all responding subjects. The allergen components bound by IgE by children susceptible to recurrent and persistent asthma are the same as those with manageable disease although sensitised subjects without disease have both lower overall IgE titres and detectable antibody to fewer components. Both the likelihood of children developing allergic disease and the likelihood of mite allergens being responsible for extant disease are proportional to the titres of their anti-mite IgE but the probabilities are not significant at the low titres required for positive skin tests and for mite about 40% of subjects with high titre do not develop disease. Anti-mite IgG antibodies that reach titres the same as those of anti-microbial responses are largely confined to sensitised subjects and to the allergens but are in low subjects susceptible to di.

Rational of blockade of IgE in Allergic Airway Disease

Pawankar, R., MD, Ph.D., FRCP, FAAAAI

Department of Pediatrics, Nippon Medical School, Tokyo, Japan

The strategy for the treatment of allergic diseases like allergic rhinitis and asthma lies in its capacity to reduce the severity of symptoms either by interfering with or modulating the allergic inflammatory cascade. IgE plays a crucial role in the pathophysiology of allergic diseases. In view of this pivotal role that IgE has in triggering immediate-type hypersensitivity responses, its capacity to bind to its receptors, IgE represents a potentially important therapeutic target. In addition, the identification of both FcεR1 and FcεR2 on

monocytes, dendritic cells and epithelial cells, suggests important roles for IgE in the regulation of allergic inflammation, including enhanced allergen presentation. Moreover, increase in FcεR1 expressing cells and increased FcεR1 expression in mast cells, basophils, dendritic cells, epithelial cells, its upregulation by IL-4 or IgE is reported in patients with AR and asthma. Targeting IgE is therefore an important strategy in the treatment of allergic diseases. Omalizumab is a humanized anti-IgE monoclonal antibody that binds to free IgE and prevents its binding to specific receptors on the surface of cells, thus preventing the release of immune mediators. Treatment with omalizumab in asthmatics results in a rapid and pronounced decrease in serum IgE levels correlated with improvement in the severity of symptoms and in the quality of life and reduction in asthma exacerbation and emergency visit rates, down-regulation of IgE receptors on basophils and dendritic cells, reductions in tissue eosinophils and IgE+ cells, as well as T cells and B cells. Long term treatment showed disease modifying effects.

Infection

Immune control of chronic malaria

Langhorne, J., Perez-Mazliah, D, Sodenkamp, JC, McLaughlin, S.

Francis Crick Institute

CD4+ T-cells play a central role in immune control of erythrocytic stages of the malaria parasite *Plasmodium*. Classical studies have shown that IFN-γ-producing Th1 cells are important for controlling acute infections, however they also contribute to acute blood-stage pathology. CD4+ T-cell help and antibodies are required for elimination of chronic infection. However, in humans in endemic areas, antibody responses can be slow to develop, and are short lived, and exhausted B and T cells are expanded suggesting some impairment of host responses. We have investigated the requirements for activating a protective B cell response in malaria using a model of *P. chabaudi* in C57bl/6 mice. We show that IL-21 produced by T follicular helper cells, co-expressed with IFN-γ and acting on the IL-21 receptor on B cells is required to resolve chronic infection, and for immunity to re-infection. The absence of IL-21, IL-21R and bcl/6 in T cells resulted in abrogation *P. chabaudi*-specific IgG responses, including memory B cells. The SLAM-associated protein, SAP, which has been shown to regulate T:B cell interactions particularly for long-term immunity was not required in this infection for Tfh activation, IgG responses or control of infection. Our data show that CD4+ T cells and B cells can control parasitemia during chronic blood-stage malaria through a single cytokine produced by Tfh cells. Knowledge of how T-cell help and antibodies are regulated during blood-stage malaria will help us understand how humoral responses can be impaired, and how this impacts on protective immunity.

Oral Abstract Sessions

10:30:00 - 12:10:00

Granulocytes

527

Neutrophil necroptosis triggers NET release via RIPK3 and MLKL to combat *Staphylococcus aureus* infection

D'Cruz, A.¹, Bliss-Moreau, M.¹, Al-Obeidi, A.¹, Gavillet, M.¹, Williams, D.¹, Ericsson, M.², Croker, B.^{1,2}

¹Boston Children's Hospital, Boston, United States, ²Harvard Medical School, Boston, United States

Neutrophil extracellular traps (NETs) are networks of chromatin and microbicidal proteins released by neutrophils in response to infection and tissue damage. Although classically viewed as a discrete cellular process in neutrophils, the genetic factors controlling NETosis are poorly defined. To investigate the involvement of regulated cell death pathways in NETosis, we examined the consequences of activating positive regulators of necroptosis including receptor-interacting protein kinase-3 (RIPK3) and mixed lineage kinase domain-like (MLKL). The release of hyper-citrullinated histone H3 and double-stranded DNA (dsDNA) from neutrophils was dependent on reactive oxygen species (ROS), RIPK3 and MLKL. Phosphorylated MLKL was identified at the plasma membrane of necroptotic neutrophils, and MLKL colocalized to regions of the membrane releasing DNA. Necroptotic mouse and human neutrophils restricted the growth of *Staphylococcus aureus* (*S.aureus*) but not in the presence of DNaseI. Consistently, mice deficient in RIPK3 or MLKL displayed increased numbers of *S.aureus* in the blood and kidney. Our studies place NET formation in the context of non-apoptotic cell death pathways to prevent bacteremia via regulated DNA release.

1007

Redefining myeloid Cell subsets in murine spleen

Hey, Y.Y., Tan, J.K.H., O'Neill, H.C.

Bond University, Gold Coast, Australia

Spleen is known to contain multiple dendritic and myeloid cell subsets, distinguishable on the basis of phenotype, function and anatomical location. As a result of recent intensive flow cytometric analyses, splenic dendritic cell (DC) subsets are now better characterised than other myeloid subsets. In order to identify and fully characterise a novel splenic subset termed 'L-DC' in relation to other myeloid cells, it was necessary to investigate myeloid subsets in more detail. In terms of cell surface phenotype, L-DC were initially characterised as a CD11b^{hi}CD11c^{lo}MHCII⁻Ly6C⁻Ly6G⁻ subset in murine spleen. Their expression of CD43, lack of MHCII, and a low level of CD11c was shown to best differentiate L-DC by phenotype from conventional DC subsets. A complete analysis of all subsets in spleen led to the classification of CD11b^{hi}CD11c^{lo}MHCII⁻Ly6C^{lo}Ly6G⁻ cells as monocytes expressing CX₃CR1, CD43 and

CD115. Siglec-F expression was used to identify a specific eosinophil population, distinguishable from both Ly6C^{lo} and Ly6C^{hi} monocytes, and other DC subsets. L-DC were characterised as a clear subset of CD11b^{hi}CD11c^{lo}MHCII⁻Ly6C⁻Ly6G⁻ cells, which are CD43⁺, Siglec-F⁻ and CD115⁻. Changes in the prevalence of L-DC compared to other subsets in spleens of mutant mice confirmed the phenotypic distinction between L-DC, cDC and monocyte subsets. L-DC development *in vivo* was shown to occur independently of the BATF3 transcription factor that regulates cDC development, and also independently of the FLT3L and GM-CSF growth factors which drive cDC and monocyte development, so distinguishing L-DC from these commonly defined cell types.

1286

Sugar modification of carrier protein in allergen determines the magnitude of IgE- and basophil-mediated allergic inflammation

Nagao, T., Takahashi, S., Kawawa, M., Miyake, K., Yoshikawa, S., Sato, S., Yamaniishi, Y., Karasuyama, H.

Tokyo Medical and Dental University, Department of Immune Regulation, Tokyo, Japan

In the field of immunological research, ovalbumin (OVA) is commonly used as allergen, but it remains why OVA has strong allergenicity in animals. Recently we have established a basophil-dependent allergy model "IgE-mediated chronic allergic inflammation (IgE-CAI)", in which mice sensitized with hapten-specific IgE are challenged intradermally with hapten-conjugated OVA. In this model, mice show delayed-onset ear swelling with accumulation of inflammatory cells including eosinophils and macrophages. Intriguingly, when challenged with the same hapten-conjugated bovine serum albumin (BSA) instead of OVA, mice showed poorer IgE-CAI response with reduced ear swelling and cellular infiltration, suggesting that the nature of carrier protein is important for the induction of IgE-CAI. Notably, BSA and OVA, when conjugated with the same number of hapten, showed comparable capacity to activate IgE-sensitized basophils *in vitro*. To address the molecular mechanism underlying the functional difference *in vivo* between OVA and BSA, we focused on their post-transcriptional modifications. OVA but not BSA has one sugar chain, giving the hypothesis that the sugar modification in carrier proteins may determine the magnitude of the IgE-CAI response. Consistent with this hypothesis, the addition of sugar moiety conferred the capacity of eliciting the efficient IgE-CAI response on BSA. Collectively, our results indicate that post-transcriptional sugar modification of carrier protein plays a significant role in the induction of IgE-CAI, and this finding may account for the reason why OVA, but not BSA, is preferentially used as allergen to induce allergy.

1961**Engagement of the ITIM receptor CD31 regulates neutrophil adhesion and rolling on activated endothelial cells in vitro and in vivo**

Andreata, F., Ollivier, V., Syvannarath, V., Rasser, C., Loste, A., Procopio, E., Nicoletti, A., Le Borgne, M., Caligiuri, G.
INSERM U 1148, Paris, France

CD31 is an ITIM-bearing transhomophilic receptor highly expressed by both endothelial cells and neutrophils but, intriguingly, nothing is known about the dynamic of CD31 expression and the role of its engagement during neutrophils rolling and adhesion onto activated endothelial cells.

By using flow cytometry and fluorescence microscopy, we found most of the CD31 extracellular portion is rapidly lost, leaving a membrane-proximal extracellular fragment expressed by activated neutrophils. Western Blot analysis revealed that the use of a synthetic peptide able to engage the lingering CD31 extracellular fragment sustains, in a dose-dependent manner, the phosphorylation of the intracellular CD31 ITIMs and the consequent SHP2 activation.

Such CD31 agonist peptide prevents neutrophil rolling and adhesion onto activated endothelial cells in vivo in C57BL/6 mice as detected by intravital videomicroscopy after application of ionomycin onto a mesenteric venule. The administration of the CD31 agonist peptide resulted in a durable 60% inhibition of leukocytes adhesion onto the activated endothelium as compared to control mice (n=4/group). In separate *in vitro* experiments we used a flow system in which purified human neutrophils were let stream with a post-capillary flow rate (5 dynes/cm²) on human coronary artery endothelial cell (HCAEC) previously cultured until confluence under continuous flow on cellix VENAFLUOR® chambers and activated overnight with 10ng/ml of TNF α . As compared to control cells, neutrophils pre-treated with the CD31 agonist peptide rolled weakly and virtually did not arrest onto the activated HCAEC.

We conclude that CD31 signalling modulates neutrophils interactions with activated endothelial cells and effectively controls neutrophil extravasation.

588**Lfc regulates the formation of neutrophil extracellular traps**

Weng, C.-M., Chiang, H.-S.

National Taiwan University, Life Science, Taipei, Taiwan, Republic of China

Neutrophils are the most abundant type of white blood cells in most mammals, and play a critical role in innate immunity. Previous studies have shown that neutrophils trap and kill a variety of pathogens by neutrophil extracellular traps (NETs), whose formation depends on dynamic microtubule networks. The guanine nucleotide exchange factor Lfc is crucial in coupling microtubule dynamics to Rho GTPase activation in a variety of normal biological situations. It is also a newly defined component of cellular defenses for the detection of microbial effectors during cell invasion by pathogens. However, it remains unknown whether Lfc regulates NET formation in response to pathogen infection. Here we show that upon activation by

phorbol 12-myristate 13-acetate (PMA), the rate of NET release was reduced in Lfc-deficient neutrophils compared to wild-type neutrophils. The reduced NET formation in Lfc-deficient neutrophils was not due to the impaired granulopoiesis for neutrophils in the bone marrow. We further found that Lfc deficiency lead to impaired NET formation in response to *shigella flexneri* infection. Overall, our results suggested a potential role for Lfc in the regulation of NET formation.

1462**mMCP-8, a basophil specific protease, triggers an inflammatory response by stimulating fibroblasts to produce chemokines**

Tsutsui, H., Yamanishi, Y., Yoshikawa, S., Sato, S., Karasuyama, H.
Tokyo Medical and Dental University (TMDU), Department of Immune Regulation, Tokyo, Japan

Basophils store a variety of bioactive molecules including proteases in the secretory granules and rapidly release them upon degranulation that contributes to allergic response or host defense against pathogens. Mouse mast cell protease-8 (mMCP-8) is specifically expressed in murine basophils and widely accepted as a basophil-specific marker in mice. However, the physiological function of mMCP-8 has not been elucidated. In the present study, we show evidence that basophil-derived mMCP-8 provokes an inflammatory response in the skin lesion. Notably, mMCP-8 was localized to the secretory granules in basophils and released promptly after IgE/antigen-induced degranulation. Subcutaneous injection of recombinant mMCP-8 (rmMCP-8) caused transient skin swelling with local accumulation of neutrophils and macrophages in a dose-dependent manner. Consistent with the leukocyte accumulation, increased expression of chemokines such as CXCL1 and CCL2 was observed in the rmMCP-8-injected skin lesions. To distinguish what cell types are a main source of CXCL1 and CCL2 in rmMCP-8-injected skin lesion, we sorted the skin cells into hematopoietic cells, fibroblasts, and others. Importantly, the chemokine expression was exclusively induced in dermal fibroblasts, suggesting that fibroblasts were a possible target for mMCP-8. To address this possibility, primary-cultured fibroblasts were stimulated with rmMCP-8 *in vitro*. Of note, significant amount of CXCL1 and CCL2 was produced by fibroblasts in response to rmMCP-8 stimulation. Collectively, our results suggest that basophil-specific protease mMCP-8 activates dermal fibroblasts that produce chemokines and attract inflammatory cells at the site, leading to skin inflammation.

310**Functional and metabolic reprogramming drives the development of a pathogenic subset of neutrophils in inflammatory airway diseases**

Forrest, O.¹, Ingersoll, S.¹, Preininger, M.², Laval, J.², Limoli, D.¹, Brown, M.¹, Lee, F.³, Bedi, B.³, Sadikot, R.³, Goldberg, J.¹, Tangpricha, V.³, Tirouvanziam, R.¹

¹Emory University, Pediatrics, Atlanta, United States, ²Emory University, Atlanta, United States, ³Emory University, Medicine, Atlanta, United States

Background: Airway inflammation in cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), and severe asthma (SA) is characterized by the massive recruitment of polymorphonuclear neutrophils (PMNs) to the lumen. Neutrophil elastase (NE) released from primary granules is a strong predictor of lung function in CF and COPD. However, it remains unclear how NE and other PMN granule mediators are released into the lumen.

Methods: Blood and sputum were collected from CF, COPD, SA, and healthy control subjects, and PMNs were analyzed for viability, degranulation, and other changes. In a transepithelial migration assay, airway fluid from the same four cohorts of subjects was used to induce blood PMN recruitment into the airway lumen. In vitro migrated PMNs were analyzed for survival, degranulation, and metabolic and bacterial killing activities.

Results: CF, COPD, and SA airway fluid induced rapid transepithelial migration and survival of PMNs in vitro; (ii) CF, COPD and SA, but not HC, airway PMNs release their primary granules actively, both in vivo and in vitro; and (iii) migration into CF airway fluid reprograms PMNs to upregulate glycolysis while paradoxically dampening their ability to kill the CF pathogen *P. aeruginosa*, which is consistent with prior in vivo observations.

Conclusions: Together, these data suggest a primary role for the inflammatory airway milieu in promoting survival, functional and metabolic reprogramming of recruited PMNs.

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2875

Pathological manifestations in lymphatic filariasis correlate with lack of inhibitory properties of IgG4 molecules on IgE-armed granulocytes

Prodjinotho, U.F., von Horn, C., Hoerauf, A., Adjobimey, T. Institute for Medical Microbiology, Immunology and Parasitology (IMMIP), University Hospital Bonn, Germany, Bonn, Germany

Background: Helminth parasites are renowned for their capacity to dampen inflammation to support their own survival, thus generating a modified Th2 immune response characterized by the presence of regulatory cytokines and high plasma levels of the non-cytolytic antibody IgG4. This particular isotype is described in both helminth and allergy models to inhibit diverse effector cells. How IgG4 molecules affect granulocyte activation and functions is still not well characterized.

Methods: Using isolated granulocytes and affinity purified IgG and IgG4 fractions from plasma of endemic normals (EN), lymphatic filariasis pathology patients (CP), asymptomatic microfilaraemic (MF+) and amicrofilaraemic (MF-) infected individuals, we analyzed the impact of bulk plasma and IgG positive or negative fractions on IgE/IL-3 stimulated granulocytes by flow-cytometric analysis of CD66b/CD63/HLADR expression and ELISA assessment of histamine, eosinophil cationic protein and neutrophil elastase in culture supernatants. In addition, the granulocyte modulation pathways were investigated by FcγRs blocking, immunofluorescence and western blot.

Results: Granulocytes activation and granules content release were significantly inhibited by plasma of EN and MF+ individuals. This inhibitory capacity was abrogated upon depletion of IgGs

from the plasma of MF+ individuals but persisted in EN plasma. Interestingly, affinity-purified IgG4 molecules from EN, MF+ and MF-, but not those of CP, interact with FcγRI and FcγRII while significantly inhibiting granulocytes activation in a Src, AKT and MEK dependent mechanism.

Conclusion: Our data indicate that, during helminth infections, MF+ individuals display IgG4 antibodies with potent inhibitory activities on granulocytes. In addition, we have identified possible functional differences between IgG4 molecules.

912

Advances in practical clinical blood analysis for eosinophilic disorders using high throughput table top imaging flow cytometry

Piasecka, J.¹, Summers, H.D.², Thornton, C.A.¹

¹Institute of Life Science, Swansea University Medical School, Swansea, United Kingdom, ²Swansea University, College of Engineering, Swansea, United Kingdom

Autofluorescence that localizes to cytoplasmic granules can be used during flow cytometry to identify eosinophils within mixed leukocyte preparations such as blood. A wider range of cellular features can be captured using imaging flow cytometry to provide more detail about cell phenotype including, potentially, the activation status of the cells. We have developed a simple, sensitive and rapid imaging cytometry technique for identifying activated and non-activated eosinophils in clinical whole blood samples.

Whole human peripheral blood and isolated blood eosinophils from healthy volunteers were exposed to various stimuli responsible for eosinophil recruitment, growth, survival, and for inducing selective protein release from eosinophilic granules (IL-3, IL-5, GM-CSF, Eotaxin, and RANTES) for different time points. Results from imaging flow cytometry were compared by treatment, and for isolated eosinophils, with images from electron microscopy.

Eosinophils were readily identified in whole peripheral blood by imaging cytometry in a label-free manner and populations analysed from spatial metrics (size, shape, granularity) obtained from light scatter (dark field), light transmission (bright field) or autofluorescence images. Texture information relating to intra-cellular granules can be extracted from bright field images and this reports on eosinophil activation status. Thus we demonstrate a label-free method, allowing continuous and harmless monitoring of both eosinophil number and cellular state.

This project provides early validation that eosinophil images captured through imaging cytometry have potential as a clinical tool for monitoring of various eosinophilic diseases.

Invariant and $\gamma\delta$ T cells

2823

The protective role of $\gamma\delta$ T cells in neonatal influenza

Guo, X.^{1,2}, Dash, P.¹, Thomas, P.^{1,2}

¹St. Jude Children's Research Hospital, Immunology, Memphis, United States, ²University of Tennessee Health Science Center, Microbiology, Immunology and Biochemistry, Memphis, United States

Influenza virus infection is a continuing worldwide health threat, especially in infants and the elderly. Neonates are highly susceptible to influenza infection. $\gamma\delta$ T cells are the first T cells to develop during embryogenesis and are a significant component of the neonatal immune system. In the neonatal period, conventional $\alpha\beta$ T cell responses are functionally less prominent. Hence, $\gamma\delta$ T cells are poised to play a critical role in neonatal infections, however, the specific role of $\gamma\delta$ T cells in neonatal influenza infection remains to be elucidated. Using a neonatal mouse model of intranasal influenza infection, we observed the activation and proliferation of $\gamma\delta$ T cells within two days after infection. By utilizing *Nur77*-GFP reporter mice, we demonstrated the activation of these $\gamma\delta$ T cells was via the TCR and its downstream signaling. IL-17-producing $\gamma\delta$ T cells, rather than IFN- γ -producing cells, dominated in this response. A comparison between wild-type and *TCR δ* -deficient neonates identified a protective function of $\gamma\delta$ T cells in neonates, as an increased survival rate were observed in the wild-type compared to *TCR δ* -deficient animals. In infected lungs, wild-type neonates also had increased IFN- β and inflammatory cytokine production, especially among the Th2-relevant cytokines. Consistent with this observation, more eosinophils infiltrated into the lungs of wild-type neonates. Our findings demonstrate a vital role for $\gamma\delta$ T cells in mediating protection during neonatal influenza virus infection via a mechanism which appears to include promotion of a Type 2 immune environment that may provide a new therapeutic target for pediatric influenza treatment.

3683

Identification of phenotypically and functionally heterogeneous mouse Mucosal Associated Invariant T cells using MR1 tetramers

Koay, H.-F.^{1,2}, Rahimpour, A.¹, Enders, A.^{3,4}, Clanchy, R.¹, Eckle, S.¹, Meehan, B.¹, Chen, Z.¹, Whittle, B.⁴, Liu, L.^{5,6}, Fairlie, D.^{5,6}, Goodnow, C.³, McCluskey, J.¹, Rossjohn, J.^{7,8,9}, Uldrich, A.^{1,2}, Pellicci, D.^{1,2}, Godfrey, D.^{1,2}

¹University of Melbourne, Peter Doherty Institute for Infection and Immunity, Microbiology and Immunology, Parkville, Australia, ²Australian Research Council Centre of Excellence in Advanced Molecular Imaging, The University of Melbourne, Parkville, Australia, ³John Curtin School of Medical Research, Australian National University, Department of Immunology and Infectious Disease, Canberra, Australia, ⁴John Curtin School of Medical Research, Australian National University, Australian Phenomics Facility, Canberra, Australia, ⁵University of Queensland, Institute for Molecular Bioscience, Brisbane, Australia, ⁶Australian Research Council Centre of Excellence in Advanced Molecular Imaging, University of Queensland, Brisbane, Australia, ⁷Monash University, Department of Biochemistry and Molecular Biology, Clayton,

Australia, ⁸Australian Research Council Centre of Excellence in Advanced Molecular Imaging, Monash University, Clayton, Australia, ⁹Institute of Infection and Immunity, Cardiff University, School of Medicine, Heath Park, United Kingdom

Studies on the biology of Mucosal-Associated Invariant T (MAIT) cells in mice have been hampered by a lack of specific reagents. Using MR1-antigen (Ag) tetramers that specifically bind to the MR1-restricted MAIT cell receptors (TCRs), we demonstrate that MAIT cells are detectable in a broad range of tissues in C57BL/6 and BALB/c mice. These cells include CD4-CD8-, CD4-CD8+, and CD4+CD8- subsets and their frequency varies in a tissue- and strain-specific manner. MAIT cells are relatively rare in spleen and thymus, but more abundant in liver, lymph nodes and lungs, where they represent up to 3% of T cells. Mouse MAIT cells have a CD44^{hi}CD62L^{lo} memory phenotype and produced high levels of IL-17A, and other cytokines including IFN- γ , IL-4, IL-10, IL-13, GM-CSF at moderate levels. Consistent with high IL-17A production, most MAIT cells expressed high levels of retinoic-acid-related orphan receptor (ROR) γ t, whereas (ROR) γ t^{lo} MAIT cells predominantly expressed T-bet and produced IFN- γ . Most MAIT cells expressed the promyelocytic leukaemia zinc finger (PLZF) transcription factor and their development was largely PLZF-dependent. Residual MAIT cells in PLZF deficient mice had an immature, CD4+ phenotype and failed to produce cytokines upon stimulation, indicating a key role for this transcription factor in MAIT cell biology. Thus, MAIT cells can be readily identified in mice using MR1 tetramers, as soon as they emerge in the thymus in a PLZF dependent manner, functionally diverse subpopulations can be detected. These studies represent a key component in understanding the function of MAIT cells in the immune system.

4122

New pathways regulating immunosurveillance by tissue-resident gd T cells

Iannitto, M.L.^{1,2}, Nussbaumer, O.¹, Woolf, R.¹, Hayday, A.^{1,2}

¹King's College London, London, United Kingdom, ²Francis Crick Institute, London, United Kingdom

gd-T cells are unique unconventional lymphocytes, mostly tissue-resident, and able to mount rapid "lymphoid stress-surveillance responses" to sentinels of tissue dysregulation. We ignore the molecules regulating these potentially potent responses. The importance of the PVR-TIGIT-DNAM1 axis is emerging in conventional T cell activation and tumour immunology, but its relevance to tissue-resident T cell populations, particularly gd-T cells, is unknown. PVR, expressed in cell-cell junctions and over-expressed in tumours, can switch from a DNAM1-dependent activator to a TIGIT-dependent suppressor.

We found that human peripheral blood Vd2⁺ cells and human skin-resident Vd1⁺ cells both express DNAM1, but Vd1⁺ cells constitutively express TIGIT whereas Vd2⁺ cells express TIGIT only transiently post-activation. These data suggest that human tissue-resident gd-T cells are pre-activated cells regulated by TIGIT that relays inhibitory signals in response to PVR engagement. Indeed, rhPVR inhibited TCR-induced cytokine production by Vd1⁺ cells, whereas Vd2⁺ cells were unaffected.

Constitutive TIGIT expression was likewise displayed by murine, tissue-resident, epidermal Vg5⁺Vd1⁺ cells. TIGIT expression commenced during their *Skint1*-dependent developmental checkpoint, suggesting that TIGIT is switched on to regulate the cells' innate-like immune-surveillance potentials. Under physiological conditions, PVR is expressed primarily by Langerhans Cells, the other large epidermal immune population, and only by a fraction of the CD45⁺ compartment. This argues that the PVR-TIGIT-DNAM1 axis is utilised by local DCs to suppress local T cell activation, until a threshold of immunogenicity is exceeded. These findings have profound implications and clinical relevance to tissue-mediated regulation of anti-tumour responses and of local T cell regulation in inflammatory diseases.

4133

HEB plays a critical role in the installation of IL-17 program in $\gamma\delta$ T cells

In, T.^{1,2}, Trotman-Grant, A.¹, Anderson, M.K.^{1,2}

¹Sunnybrook Research Institute, Biological Sciences, Toronto, Canada, ²University of Toronto, Immunology, Toronto, Canada

IL-17 producing $\gamma\delta$ T cells that arise during fetal development form an integral part of the immune system of various lymphoid and mucosal tissues in mice. Here, we aim to better understand the transcriptional network that drives functional programming of the IL-17 producing $\gamma\delta$ T cells. Specifically, we characterized the role of HEB in this process by utilizing fetal thymic organ culture (FTOC) from mouse embryos at E14. First, we observed that the HEB-deficient fetal $\gamma\delta$ T cells lacked the ability to upregulate SOX13, a hallmark transcription factor of IL-17 producing $\gamma\delta$ T cells, at the immature stage, leading to an inability to express IL-17 upon maturation, marked by downregulation of CD24 and upregulation of CD73. These differences in the gene expression profile of developing fetal thymic $\gamma\delta$ T cells in the absence of HEB were also met by a significant reduction in the frequency of ROR γ t⁺ $\gamma\delta$ T cells and a profound deficiency in their ability to produce IL-17 in response to stimulation with IL-1 β , IL-23 and IL-21 as determined by intracellular flow cytometry analysis and ELISA. Reflecting the deficiency in IL-17 producing $\gamma\delta$ T cells in HEB-deficient fetal thymus, HEB conditional knockout mice on a *vav*-Cre background had significantly lower frequencies of ROR γ t⁺ IL-17 producing $\gamma\delta$ T cells in the spleen, lymph nodes and lungs. Collectively, our work shows for the first time that HEB plays an indispensable role in the transcriptional network that drives the installation of IL-17 program in $\gamma\delta$ T cells.

742

$\gamma\delta$ T cells and the regulation of immune responses at epithelial surfaces

Havran, W.¹, Johnson, M.¹, Borkowski, A.¹, Tekkam, S.², Crooke, S.², Finn, M.G.², Witherden, D.¹

¹Scripps Research Institute, Immunology and Microbial Science, La Jolla, United States, ²Georgia Institute of Technology, Chemistry and Biochemistry, Atlanta, United States

Intraepithelial $\gamma\delta$ T cells play unique roles in homeostasis, tissue repair, inflammation and protection from malignancy in mice

and man. Human epidermal T cells contribute to wound healing and are defective in patients with chronic wounds. Increasing numbers of elderly and diabetic patients have defects in tissue repair leading to chronic, non-healing wounds. We have identified key regulators of intraepithelial $\gamma\delta$ T cell recognition of damaged epithelial cells leading to activation and participation in local immune responses, including the JAML and CAR costimulatory molecules. Expression of the CAR costimulatory ligand is upregulated on epithelial cells around human healing wounds but not in chronic wounds. Surgical debridement to remove non-healing tissue can create a new acute wound, lead to upregulated CAR expression, and result in improved healing. Since expression of CAR correlates with effective wound healing, we hypothesize that defective costimulation due to lack of CAR expression is responsible for the T cell dysfunction in chronic wounds. We are utilizing biodegradable hydrogels to deliver costimulatory ligands directly into non-healing wounds as a new therapeutic strategy targeting the dysfunctional T cells. Further characterization of the molecules and mechanisms that regulate interactions between tissue-resident T lymphocytes and neighboring epithelial cells may allow for the development of this and other new therapeutic strategies to treat chronic wounds and other epithelial disorders.

1151

Hybrid $\alpha\beta$ - $\gamma\delta$ T cells, novel T cell populations with a critical pathogenic role in CNS autoimmunity

Edwards, S.C.¹, Sutton, C.E.¹, McLaren, J.E.², Ladell, K.², Ribot, J.C.³, Baik, S.⁴, Moran, B.¹, Anderson, G.⁴, Silva-Santos, B.³, Price, D.A.², Mills, K.H.G.¹

¹School of Biochemistry and Immunology, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin, Ireland, ²Institute of Infection and Immunity, Cardiff University, School of Medicine, Cardiff, United Kingdom, ³Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Lisboa, Portugal, ⁴MRC Centre for Immune Regulation, Institute for Biomedical Research, Medical School, University of Birmingham, Birmingham, United Kingdom

$\gamma\delta$ T cells and CD4 T cells are the main sources of IL-17 during inflammation and host immunity. V γ 4⁺ $\gamma\delta$ T cells are found at high frequency in the central nervous system (CNS) of mice with experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis. Here, we describe novel populations of T cells, hybrid $\alpha\beta$ - $\gamma\delta$ T cells distinct from conventional $\alpha\beta$ or $\gamma\delta$ T cells which play a critical pathogenic role in CNS autoimmunity. Hybrid $\alpha\beta$ - $\gamma\delta$ T cells which express V γ 4⁺ cells arise in the foetal thymus on day 16 of ontogeny, where they constitute around 10% of V γ 4⁺ cells in the peripheral lymphoid organs. Hybrid $\alpha\beta$ - $\gamma\delta$ T cells are present in WT but absent in TCR α ^{-/-} and TCR β ^{-/-} mice, however hybrid $\alpha\beta$ - γ T cells were identified in TCR δ ^{-/-} mice. Co-expression of α , β , γ and δ chains was confirmed at the molecular level, revealing a restricted V γ repertoire with more heterogeneous V α and V β usage. Hybrid $\alpha\beta$ - $\gamma\delta$ T cells express the master transcription factor, ROR γ t, and IL-1R, IL-23R, CD44, CCR6 and α 4 β 1. Furthermore, these cells secrete IL-17A, IFN- γ and IL-22, following stimulation with IL-1 β and IL-23 and respond to autoantigens when purified from mice

with EAE. Depletion of V γ 4⁺ cells from either WT or TCR δ ^{-/-} mice dramatically attenuated EAE, and this effect was associated with a significant reduction in CNS-infiltrating Th17 cells. These data identify novel populations of T cells, hybrid $\alpha\beta$ - $\gamma\delta$ T cells which play a critical role in autoimmunity via the activation of Th17 cells.

3871

Thymic precursors to the mucosal-associated invariant T cell lineage

Godfrey, D.^{1,2}, *Koay, H.-F.*¹, *Gherardin, N.*¹, *Enders, A.*³, *Loh, L.*¹, *Chen, Z.*¹, *Corbett, A.*¹, *Eckle, S.*¹, *Meehan, B.*¹, *d'Udekem, Y.*⁴, *Konstantinov, I.*⁴, *Lappas, M.*^{5,6}, *Liu, L.*^{7,8}, *Goodnow, C.*³, *Fairlie, D.*^{7,8}, *Rossjohn, J.*^{9,10}, *Kedzierska, K.*¹, *Berzins, S.*¹¹, *McCluskey, J.*¹, *Uldrich, A.*^{1,2}, *Pellicci, D.*^{1,2}
¹University of Melbourne, Melbourne, Australia, ²Australian Research Council Centre of Excellence in Advanced Molecular Imaging, The University of Melbourne, Melbourne, Australia, ³John Curtin School of Medical Research, Australian National University, Canberra, Australia, ⁴Royal Children's Hospital, Parkville, Australia, ⁵University of Melbourne, Heidelberg, Australia, ⁶Mercy Hospital for Women, Melbourne, Australia, ⁷University of Queensland, Brisbane, Australia, ⁸Australian Research Council Centre of Excellence in Advanced Molecular Imaging, The University of Queensland, Brisbane, Australia, ⁹Monash University, Clayton, Australia, ¹⁰Australian Research Council Centre of Excellence in Advanced Molecular Imaging, Monash University, Clayton, Australia, ¹¹Federation University, Ballarat, Australia

Mucosal-associated invariant T (MAIT) cells, are activated by microbial vitamin B2 (riboflavin) derivatives presented by the major histocompatibility complex (MHC) class I related protein, MR1. In humans, MAIT cells represent between 5 and 50% of T cells in different human tissues. The number of MAIT cells varies widely between individuals and the factors that govern the development of these cells are unclear. Using MR1 tetramers to detect MAIT cells in mice and humans, we have now identified thymic precursors to the MAIT cell lineage and delineated three distinct stages in MAIT cell development. In mice, the least mature, stage 1 (CD24⁺CD44⁻) cells progress to an intermediate stage 2 (CD24⁻CD44⁻) before maturing into stage 3 (CD24⁻CD44⁺) MAIT cells. Progression through each of these checkpoints is MR1 dependent, while the final maturation checkpoint that gives rise to functional MAIT cells requires the transcription factor, promyelocytic leukemia zinc finger (PLZF). In humans, stage 1 (CD161⁻CD27⁻) and stage 2 MAIT cells (CD161⁻CD27⁺) predominate in the thymus, while stage 3 cells (CD161⁺) progressively increase in percentage in umbilical cord blood, young peripheral blood and adult peripheral blood. MAIT cell maturation can also occur after thymic emigration of immature MAIT cells in both humans and mice. Accordingly, this study maps the intrathymic developmental pathway and identifies key checkpoints that control the maturation of functional MAIT cells in mice and humans.

2592

Common drugs modulate mucosal-associated invariant T cell function

Keller, A.N.^{1,2}, *Eckle, S.B.G.*³, *Xu, W.*^{4,5}, *Liu, L.*^{4,5}, *Hughes, V.A.*^{1,2}, *Mak, J.*^{4,5}, *Meehan, B.*³, *Pedionco, T.*³, *Birkinshaw, R.W.*¹, *Chen, Z.*³, *Wang, H.*³, *d'Souza, C.*³, *Kostenko, L.*³, *Corbett, A.J.*³, *Purcell, A.W.*¹, *Fairlie, D.P.*^{4,5}, *McCluskey, J.*³, *Rossjohn, J.*^{1,2,6}

¹Monash University, Infection and Immunity Program, Monash Biomedicine Discovery Institute, Biochemistry and Molecular Biology, Clayton, Australia, ²ARC Centre of Excellence in Advanced Molecular Imaging, Monash University, Clayton, Australia, ³University of Melbourne, Peter Doherty Institute for Infection and Immunity, Department of Microbiology and Immunology, Melbourne, Australia, ⁴Institute for Molecular Bioscience, The University of Queensland, Brisbane, Australia, ⁵ARC Centre of Excellence in Advanced Molecular Imaging, University of Queensland, Brisbane, Australia, ⁶Institute of Infection and Immunity, Cardiff University School of Medicine, Cardiff, United Kingdom

Mucosal associated invariant T lymphocytes (MAIT) are a class of innate like T cells, which use their semi-invariant T-cell receptors (TCRs) to detect small molecules presented by the monomorphic MHC Class-I related protein (MR1). Ubiquitously expressed, MR1 captures compounds associated with folic acid degradation and riboflavin biosynthesis - the latter being indicative of bacterial infection, stimulating MAIT cells into producing an array of cytokines in an innate-like response.

Considering the monomorphic nature of MR1, its ability to bind small molecules and the relatively high abundance of MAIT cells in humans; two fundamental questions are whether MR1 will capture drugs or other naturally derived compounds, and importantly, do these molecules then modulate MAIT cell activity. We have addressed these fundamental questions. Using an *in silico* approach followed by *in vitro* screening, we identified a panel of small molecules that are captured by MR1. These include; the degradation products of methotrexate and sirtinol, formyl-salicylates, as well as the non-steroidal anti-inflammatory drug diclofenac, and its metabolite 5-OH-diclofenac. Moreover, we have shown that they can inhibit or activate MAIT cell activity *in vitro* and *ex vivo*. Finally, we solved the X-ray crystal structures of MR1-Ag-TCR of six of these compounds, revealing the mechanisms used by these diverse chemical scaffolds to modulate MAIT cell activity.

These findings show how heterogeneous chemical scaffolds, including FDA-approved drugs, are sequestered within MR1 and can modulate MAIT cell activity. Another crucial chapter in understanding the immunological repercussions of the current pharmacological age is beginning to be realised.

3241

Obesity triggers changes in skin IL-17-producing $\gamma\delta$ T cells

*Stolp, J.*¹, *Corpuz, T.*¹, *Pinget, G.*¹, *Sprent, J.*^{1,2}, *Webster, K.*^{1,2}

¹Garvan Institute of Medical Research, Sydney, Australia, ²St Vincent's Clinical School, Sydney, Australia

Obesity is associated with a state of chronic, low-grade inflammation in adipose tissue. It has been found that

altered ratios of Tregs to activated T cells augment the enhanced cytokine and chemokine output of macrophages and adipocytes to generate this inflammatory environment. However, whether obesity alters the balance of immune cells in lymphoid tissues has remained largely unexplored. We placed mice on a high fat diet (HFD) for 15 weeks, after which time we examined T cell populations in the spleen and lymph nodes (LN). We did not observe any significant changes in proportions of Tregs or cytokine-producing CD4⁺ or CD8⁺ T cells. However, we did observe an increase in IL-17-producing cells in the peripheral, but not mesenteric, LN. These cells were identified as ROR γ t⁺ γ δ T cells, and we subsequently found an increased proportion of dermal IL-17-producing γ δ T (γ δ T-17) cells in the skin. Surprisingly, we not only found these cells in the dermis, but also found a significant proportion had migrated into the epidermis. Upon further analysis of HFD mice we found that cells in the epidermis, most likely keratinocytes, had upregulated chemokines and cytokines key to γ δ T-17 cells. Furthermore, we found that this transepidermal migration of γ δ T-17 cells coincided with an increased disease severity in the IMQ model of psoriasis. These data suggest that excess adiposity has distinct consequences for immune cells in the skin.

T Cell Development

3743

The critical roles of IRF1 and BATF during differentiation of Tr1 cells

Karwacz, K.¹, Miraldi, E.^{2,3}, Pokrovskii, M.³, Asaf, M.⁴, Bonneau, R.^{2,5,6}, Dan, L.^{7,8}, Kuchroo, V.⁹

¹University College London, London, United Kingdom, ²Simons Center for Data Analysis, Simons Foundation, New York, United States, ³New York University School of Medicine, New York, United States, ⁴Evergrande Center for Immunologic Diseases, Brigham and Women's Hospital and Harvard Medical School, Boston, United States, ⁵Center for Genomics and Systems Biology, Department of Biology, New York University, New York, United States, ⁶Courant Institute of Mathematical Sciences, Computer Science Department, New York University, New York, United States, ⁷Molecular Pathogenesis Program, The Kimmel Center for Biology and Medicine of the Skirball Institute, New York University School of Medicine, New York, United States, ⁸The Howard Hughes Medical Institute, New York University School of Medicine, New York, United States, ⁹Evergrande Center for Immunologic Diseases, Brigham and Women's Hospital and Harvard Medical School, Boston, United States

Tr1 cells are IL-27-induced IL-10 producing cells crucial for the control of autoimmunity. We show that IL-27 induces IRF1 and BATF transcription factors (TFs), which are critical for Tr1 differentiation and their suppressive function *in vitro*.

In vivo, *Irf1*^{-/-} and *Batf*^{-/-} mice fail to induce Tr1 cells when treated with an anti-CD3 monoclonal antibody and during autoimmune disease (EAE). Furthermore, transfer of *in vitro* IL-27-treated *Irf1*^{-/-} and *Batf*^{-/-} CD4⁺ T cells into MOG₃₅₋₅₅/CFA immunized mice fails to suppress EAE progression, while transfer of WT control cells does suppress it. *Irf1*^{-/-} cells develop more severe disease, characterized by impaired recovery and increased numbers of

IL17A⁺IFN γ ⁺ double producing cells as well as decreased IL10 in the central nervous system.

We further show that both IRF1 and BATF bind to the IL-10 promoter in overlapping sites and require each other for this binding. Moreover, cMaf and AhR, which also bind to and induce IL10, cannot bind to IL10 in the absence of IRF1 or BATF. While IRF1 can directly transactivate IL10, while BATF cannot.

We have analyzed chromatin changes in *Irf1*^{-/-} and *Batf*^{-/-} IL-27 primed cells using ATAC-seq and found that both TFs influence chromatin accessibility. IRF1-induced chromatin changes are local, while BATF-dependent changes are global and drastically alter the transcriptional state of the knockout cells, suggesting a role for BATF as a Tr1 pioneer factor. Finally, by coupling chromatin accessibility data with RNA sequencing of *Irf1*^{-/-} and *Batf*^{-/-} cells, we constructed a network of IRF1 and BATF interactions during Tr1 differentiation.

2480

Novel role for heparan sulfate in intrathymic T cell development

Simon Davis, D.A., Parish, C.R.

John Curtin School of Medical Research, The Australian National University, Cancer Biology & Therapeutics, Canberra, Australia

The thymic stromal microenvironment is crucial for MHC-restricted positive and negative selection of T cells in the thymus to generate functional and self-tolerant α β T cells. Our recent studies have provided new insights into this important process. We found that CD8 β on CD4⁺CD8⁺ double positive (DP) thymocytes strongly interacts with highly sulfated heparan sulfate (HS) carrying 6-O sulfate groups. Furthermore, we discovered that a HS mimetic (dextran sulfate 500-kDa) can trigger a sustained Ca²⁺ flux in DP thymocytes that is CD8 β and Slp76, but not Zap70 dependent, and results in a lowering of the TCR activation threshold in DP thymocytes. In contrast, α 2-3 sialylation of CD8 β on single positive thymocytes and peripheral CD8⁺ T cells blocks HS binding and prevents the HS-induced Ca²⁺ flux, an inhibitory effect that is reversed by enzymatic desialylation. Moreover, cortical thymic epithelial cells (cTEC) express high levels of highly sulfated HS and readily form 'rosettes' with thymocytes, rosettes formation being accompanied by a sustained Ca²⁺ flux in TEC-bound thymocytes, that is substantially blocked by HS mimetics and is markedly reduced in CD8-deficient thymocytes. Additionally, MHC on cTEC synergises with HS to enhance thymocyte rosetting capacity and the extent of the rosette-induced Ca²⁺ flux in cTEC-bound thymocytes. Collectively, the data imply that the CD8-HS interaction

- (1) enhances the interaction of DP thymocytes with cTEC expressing self-peptide/MHC complexes and
- (2) triggers unique CD8-dependent accessory signals, additional to TCR signals, that lower the threshold required for positive selection of TCR clones for self-peptide/MHC complexes.

4114**Notch/RBPJ signaling in a conditional inducible state in vivo**

Zuniga-Pflucker, J.

University of Toronto, Department of Immunology, Toronto, Canada

T-lymphopoiesis depends on temporally- and spatially-regulated settling of the thymic niches by bone marrow-derived progenitors. Thymocyte differentiation and progressive restriction to the T-lineage is contingent on Notch signaling together with other transcriptional networks. However, how exactly Notch modulates these activities during T cell development is still largely unresolved. A clear understanding of these processes has been limited by the unavailability of appropriate assays, which would permit regulation of Notch responsiveness within the same cellular microenvironment, thus making precise assessments regarding the role of Notch at various stages of T-lymphopoiesis possible. In order to more precisely and extensively address the role of Notch signaling throughout T cell development, we have developed an inducible mouse model system, in which Notch responsiveness can be temporally regulated in bone marrow stem/progenitor cells, developing lymphocytes. This mouse model will allow us to address the many Notch signaling requirements at various stages of lymphocyte development, such as thymic settling by progenitors, T-lineage commitment, b-selection checkpoint, ab versus gd or CD4 versus CD8 lineage decisions.

236**The linear ubiquitin chain assembly complex: a new function in thymic T cell differentiation and regulatory T cell homeostasis**

Teh, C.^{1,2}, Lalaoui, N.^{1,2}, Jain, R.^{1,2}, Policheni, A.^{1,2}, Heinlein, M.^{1,2}, Alvarez-Diaz, S.^{1,2}, Rieser, E.³, Deuser, S.³, Koay, H.-F.^{4,5}, Hu, Y.^{1,2}, Kupresanin, F.¹, O'Reilly, L.^{1,2}, Godfrey, D.^{4,5}, Smyth, G.^{1,2}, Bouillet, P.^{1,2}, Strasser, A.^{1,2}, Walczak, H.³, Silke, J.^{1,2}, Gray, D.^{1,2}

¹The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia, ²The University of Melbourne, Department of Medical Biology, Melbourne, Australia, ³Centre for Cell Death, Cancer and Inflammation, University College London, London, United Kingdom, ⁴The Peter Doherty Institute for Infection and Immunity, Melbourne, Australia, ⁵The University of Melbourne, The Australian Research Council Centre of Excellence for Advanced Molecular Imaging, Melbourne, Australia

T cell differentiation involves the integration of diverse inputs, including signals from the TCR, cytokine and TNF receptors. The linear ubiquitin assembly complex (LUBAC) is a signalling hub composed of three proteins, HOIL-1, HOIP and SHARPIN, that post-translationally modifies substrates via the addition of linear ubiquitin chains. Although LUBAC has essential roles in regulating innate immunity, its role in adaptive immunity is less clear. T cell deficiency observed in patients with loss-of-function mutations in LUBAC proteins prompted us to examine a role for this complex in T cell differentiation.

We show that mice lacking the LUBAC proteins, HOIL-1 or HOIP, in the T cell lineage were almost devoid of peripheral T cells, Foxp3+ regulatory T cells (Treg) and non-conventional T cell

subsets, due to a block late in thymic differentiation. By contrast, mice deficient in a third LUBAC component, SHARPIN, had normal conventional T cell development, but selective loss of thymic Foxp3+ regulatory (Treg) cells. Treg cell-specific ablation of HOIP led to severe Treg cell deficiency in the periphery and lethal immune pathology, revealing a requirement for ongoing LUBAC activity for Treg cell homeostasis. Genetic or pharmacologic blockade of cell death pathways did not rescue these defects. SHARPIN, HOIL-1 or HOIP were required in thymocytes to propagate NFκB and MAP kinase signals, but not ERK phosphorylation, downstream of TCR or TNFR ligation. These data reveal a novel differential, stage-specific requirement for LUBAC components in coordinating the signals required for T cell differentiation.

1631**Specific requirement of C-terminal Zn finger motifs in Bcl11b transcription factor for lineage dissection upon receiving positive selection signals in the thymus**

Kojima, S., Seo, W., Muroi, S., Taniuchi, I.

RIKEN Center for Integrative Medical Sciences, Laboratory for Transcriptional Regulation, Yokohama, Japan

Several T cell subsets with distinct functionality arise from CD4⁺CD8⁺ DP precursors according to TCR signals through induction lineage specifying transcription factor, ThPOK, Runx3 and FoxP3 for helper, cytotoxic and regulatory T cells, respectively. However, it remains elusive how differences in TCR signals are linked with transcriptional regulation that control expression of above key molecules. In order to address this question, we have used a *Thpok* as a model locus and identified a transcriptional silencer as a key cis-regulatory element that control helper-lineage specific expression of *Thpok* gene. Our biochemical approach identified Bcl11b transcription factor, known as a commitment factor for T-lineage, as relevant regulator for silencer function. In addition, Bcl11b turned out to play essential role to regulate lineage specificity of *Runx3* gene. Another Bcl11b mutation that get rid of the last Zn-finger motif at C-terminal-end of Bcl11b protein also failed to control expression of *Thpok*, *Runx3* and *FoxP3* genes, while it could support commitment to T-lineage. Analyses of promoter and enhancers usage in *Thpok* locus in the absence of Bcl11b suggested that *Thpok* locus behaves like as it was in non-T cells. Our results thus provide a novel insight that pre-priming of lineage specifying genes by Bcl11b through its C-terminal Zn finger motifs upon T-lineage is essential to couple TCR signals with lineage dissection in the thymus.

3632**The TCR signalosome specifies thymic selection outcome by a quantitative network mechanism that sums activity across a single protein-interaction signature**Schrum, A.¹, Neier, S.¹, Smith, S.¹, Ferrer, A.¹, Wilton, K.¹, Chen, Z.², McCluskey, J.², Burrows, S.³, Rossjohn, J.⁴, Gil, D.¹

¹Mayo Clinic College of Medicine, Immunology, Rochester, United States, ²University of Melbourne, Microbiology & Immunology, Parkville, Australia, ³University of Queensland, QIMR Berghofer

Medical Research Institute, Brisbane, Australia, ⁴Monash University, Biochemistry & Molecular Biology, Clayton, Australia

During conventional alpha-beta T cell selection, TCR engagement initiates biochemical signals based on protein-protein interactions (PPI). Still unclear is the network mechanism by which the TCR signalosome transmits signal specificity to program the cellular response for positive- versus negative-selection. This could occur by signatures differing *qualitatively* (customized, unique PPI combinations for each signal), *quantitatively* (graded amounts of a single PPI combination), or *kinetically* (speed of PPI pathway progression). To gain insight, we mounted an adaptable multiplex matrix system applicable to physiologic network signaling protein complexes, with >200 pairwise measurements of proteins in shared complexes detected by exposed surface epitopes (PiSCES). Upon stimulation with peptide/MHC ligands on APCs, we found that pre-selection OT1.b2m-/-RAG2-/- thymocytes produced PiSCES network signatures that differed quantitatively between positive versus negative selection conditions, but did not differ qualitatively or kinetically. This was also true when overall signaling activity was amplified by using hyper-responsive genetically engineered Jurkat cells expressing either murine OT1 or human LC13 TCRs. To test whether this quantitative difference in the network was deterministic for signaling outcome, we found peptide doses of classic negative-selection ligands that produced network signatures with intensity equal to that of positive-selection ligands, and discovered that when used in FTOC these specially-low-dosed negative-selection ligands now produced positive selection of conventional T cells. We confirmed this concept a second way, observing that in reduced-TCR-expressing thymocytes from OT1.b2m-/-RAG2-/-CD3d+/- mice, some classic negative-selection peptides induced positive selection. These data indicate that a quantitative network signaling mechanism through the TCR signalosome specifies thymic selection outcome.

3641

The unique features of pTa mediate pre-TCR down-regulation rather than constitutive signalling

Call, M., Krshnan, L., Call, M.

The Walter and Eliza Hall Institute, Parkville, Australia

The T cell receptor (TCR) is formed by the step-wise rearrangement of the TCR loci. To ensure rearrangement leads to a functional TCR β chain prior to TCR α rearrangement, TCR β assembles with pTa and CD3 signalling dimers to form the constitutively active pre-TCR, which initiates β -selection. Knockout of pTa stalls thymocyte development at this checkpoint resulting in the accumulation of DN3a cells (CD4⁺CD8⁺CD44⁺CD25⁺CD28⁻). We hypothesised that a structural understanding of how pre-TCR maintains constitutive activity would lead to mechanistic insights into ligand-induced TCR triggering, an unresolved problem in T cell biology. We developed a retrogenic approach where pTa or TCR α chains could be introduced into pTa knockout foetal liver cells to reconstitute lethally irradiated mice. Our results showed TCR α and pTa could both mediate transition from DN3a to DN3b (CD4⁺CD8⁺CD44⁺CD25⁺CD28⁺)

but OT-I TCR α was inefficient at transitioning past DN4 (CD4⁺CD8⁺CD44⁺CD25⁺CD28⁺). A second TCR α chain raised in a different MHC background however, could match wild-type pTa function suggesting that DN4 cells are highly sensitive to MHC-induced signalling. Like TCR α , pTa has two basic residues in the transmembrane domain that mediate stable recruitment of CD3. Mutation of both these residues resulted in pTa chains that were unable to pass β -selection, however pTa chains carrying single mutations were better than wild-type at reconstituting host thymi. These data support a model where pre-TCR signalling is not a special property of the pre-TCR, but instead suggests a role for the unique features of pTa in destabilising receptor after β -selection initiation to allow efficient transition through DN4.

2673

Cooperative TCR-pMHC-CD8 catch bond distinguishes thymocyte positive versus negative selection

Hong, J., Zhu, C.

Georgia Institute of Technology, Atlanta, United States

For a developing thymocyte to mature into a functional peripheral T cell, the T cell receptor (TCR) must recognize self-peptide-major histocompatibility complex (pMHC) well enough to signal survival while remaining below the activation threshold that would induce clonal deletion. Thus, the binding propensity for self pMHC provides the selection criteria to separate thymocyte positive from negative selection. Although this process is well known, the biophysical measurements to date cannot easily explain thymocyte fate. Using mechanical-based assays, we show that the quality, not quantity, of binding determines thymocyte fates. Force regulates selection outcomes through the induction of cooperative binding to convert two TCR-pMHC and MHC-CD8 slip bonds into a TCR-pMHC-CD8 trimolecular catch bond for negative but not positive selecting ligands. Thymocytes exert more sustained tension on TCR and CD8 that form higher quality catch bonds with negative selecting ligands compared to lower quality slip bonds with positive selecting ligands, resulting in their differential engagement times. Our results reveal a novel mechanism driving thymocyte selection, highlighting the roles of force and CD8 coreceptor.

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Search for substrates of protein kinase D during thymocyte development

Ishikawa, E.¹, Kosako, H.², Yasuda, T.³, Kurosaki, T.^{3,4}, Saito, T.^{5,6}, Yamasaki, S.¹

¹Division of Molecular Immunology, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan, ²Division of Cell Signaling, Fujii Memorial Institute of Medical Sciences, Tokushima University, Tokushima, Japan, ³Laboratory for Lymphocyte Differentiation, RIKEN Center for Integrative Medical Sciences, Yokohama, Japan, ⁴Laboratory for Lymphocyte Differentiation, WPI Immunology Frontier Research Center (iFReC), Osaka University, Suita, Japan, ⁵Laboratory for Cell Signaling, RIKEN Center for Integrative Medical Sciences, Yokohama, Japan, ⁶Laboratory for Cell Signaling, WPI Immunology Frontier Research Center (iFReC), Osaka University, Suita, Japan

Thymic selection shapes an appropriate T cell antigen receptor (TCR) repertoire during T cell development. In contrast to tyrosine phosphorylation events through protein tyrosine kinases, the role of serine/threonine kinases in this process is not fully understood.

We found that a serine/threonine kinase PKD (protein kinase D) is phosphorylated upon stimulation with antigen peptides in preselection CD4⁺CD8⁺ double positive (DP) thymocytes. We then established T cell-specific PKD-deficient mice (PKD^{ΔT} mice) to investigate a role of PKD in T cell development. In PKD^{ΔT} mice, the generation of CD4 single positive (SP) thymocytes was abrogated. By crossing with OT-II TCR Tg mice, we found that positive selection of CD4 SP thymocytes was severely impaired in PKD^{ΔT} mice. The generation of CD4⁺CD8^{int} population was also significantly impaired and the CD5 expression was low, suggesting that TCR signaling during positive selection is attenuated in PKD^{ΔT} mice.

TCR-induced proximal tyrosine phosphorylation events and Ca²⁺ influx were not affected in the absence of PKD. We thus searched for cellular substrates for PKD using phosphoproteomic analyses. Two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) followed by LC-MS/MS identified several proteins that are selectively phosphorylated in wild-type but not PKD-deficient thymocytes upon TCR engagement. *In vitro* kinase assay revealed that these proteins are direct substrates for PKD. These results suggest that PKD enhances TCR signaling to promote CD4⁺ T cell development through a novel signaling pathway.

HIV 2

948

Public TCRs confer high-avidity CD4 responses to HIV controllers

Benati, D.¹, Galperin, M.¹, Lambotte, O.², Gras, S.³, Lim, A.⁴, Mukhopadhyay, M.¹, Nouël, A.¹, Campbell, K.-A.³, Lemercier, B.⁴, Claireaux, M.¹, Hendou, S.⁵, Lechat, P.⁶, De Truchis, P.⁷, Boufassa, F.⁵, Rossjohn, J.³, Delfraissy, J.-F.², Arenzana-Seisdedos, F.¹, Chakrabarti, L.¹

¹Institut Pasteur, Viral Pathogenesis Unit, Department of Virology, Paris, France, ²AP-HP, Department of Internal Medicine and Clinical Immunology, University Hospital Paris Sud, Le Kremlin Bicêtre, France, ³Monash University, Department of Biochemistry and Molecular Biology, School of Biomedical Sciences, Melbourne, Australia, ⁴Institut Pasteur, Department of Immunology, Paris, France, ⁵INSERM U 1018, Centre de Recherche en Epidémiologie et Santé des Populations, Le Kremlin Bicêtre, France, ⁶Institut Pasteur, Genomic Bioanalysis Group, Paris, France, ⁷Raymond Poincaré University Hospital, AP-HP, Infectious and Tropical Diseases Department, Garches, France

Rare patients termed HIV Controllers are able to spontaneously control HIV replication to undetectable levels and maintain normal CD4⁺ T cell counts in the absence of anti-retroviral therapy. Accumulating evidence suggests that control of viral replication in these patients is mediated by a particularly efficient cellular immune response. To identify the molecular determinants underlying this response, we characterized the

TCR repertoire directed at the most immunoprevalent CD4 epitope in HIV-1 capsid, Gag293. HIV Controllers from the ANRS CODEX cohort showed a highly skewed TCR repertoire characterized by a predominance of TRAV24 and TRBV2 variable gene families, the presence of conserved motifs in both CDR3 regions, and a high frequency of public clonotypes (n=18 for each TCR chain). The most prevalent public clonotypes generated TCRs with affinities in the micromolar range, at the higher end of values reported for naturally occurring TCRs. These high-affinity Gag293-specific TCRs were cross-restricted by up to 5 distinct HLA-DR alleles, accounting for their expression in HIV Controllers of diverse genetic backgrounds. Transfer of these TCRs to healthy donor CD4⁺ T cells conferred high antigen sensitivity and polyfunctional cytokine responses, thus recapitulating key features of the Controller CD4 response. Transfer of a high-affinity Gag293-specific TCR could also redirect

CD8⁺ T cells to target HIV-1 capsid via nonconventional MHC II restriction. These findings indicate that TCR clonotypes with superior functions are associated with HIV control. Amplifying or transferring such clonotypes may contribute to immunotherapeutic approaches that aim at a functional HIV cure.

2032

Association of the G801A polymorphism in chemokine cxc ligand 12 (CXCL12) gene with liver complication in HIV-infected Thais

Chiraunyanann, T.¹, Changsri, K.², Sretapanya, W.¹, Yuenyongchaiwat, K.³, Akekawatchai, C.²

¹Graduate Program in Medical Technology, Faculty of Allied Health Sciences, Thammasat University, Pathumthani, Thailand, ²Faculty of Allied Health Sciences, Thammasat University, Department of Medical Technology, Pathumthani, Thailand, ³Faculty of Allied Health Sciences, Thammasat University, Department of Physical Therapy, Pathumthani, Thailand

Our previous study has reported high prevalence of elevated liver enzymes in Thai HIV patients receiving combined anti-retroviral therapy (cART) and risk of liver disease in the patients requires more investigation. CXCL12 participates in immune cell homing to the liver and hepatic inflammation. This study aimed to examine an impact of 3'UTR G801A polymorphism in CXCL12 gene on liver complication in the patients ongoing cART. A cross-sectional study was conducted in 164 HIV-infected Thais. cART was received by 71.3% of the patients with median ART duration of 39 (16-55) months. Prevalence of coinfection with hepatitis B (HBV), hepatitis C virus (HCV) and HBV/HCV were 9.1%, 8.3% and 0.8% respectively. The rates of transaminitis, defined as increased levels of aspartate aminotransferase (AST) and/or alanine aminotransferase (ALT), was 28% while there were 14% of the patients with higher baseline levels of the hepatic fibrosis marker AST to platelet ratio index (APRI). The G801A polymorphism was studied by tetra-primer PCR. In this study group, frequencies of AA/GA and GG genotypes were 25.6% and 74.4% consequently. Analysis demonstrated a significant association of the genetic polymorphism with transaminitis ($p=0.014$) and APRI ($p=0.045$). Univariate and

multivariate analyses indicated that the AA/GA genotypes were significant predictive factors for the higher baseline APRI levels (OR: 4.1, 95% CI: 1.1-14.5, $p=0.030$), together with being male, CD4⁺ cell count less than 350 cells/ul and HCV coinfection. Our data suggested a contribution of the CXCL12 G801A polymorphism in progression of chronic liver disease in Thai HIV patients ongoing cART.

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Fc effector polyfunctionality in individuals that develop HIV-specific broadly neutralizing antibodies

Richardson, S.^{1,2}, Chung, A.³, Lassauniere, R.¹, Lambson, B.¹, Abdool Karim, S.⁴, Tiemessen, C.^{1,2}, Moore, P.^{1,2,4}, Mkhize, N.^{1,2}, Alter, G.³, Morris, L.^{1,2,4}

¹Centre for HIV and STIs, National Institute for Communicable Diseases, Johannesburg, South Africa, ²Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa, ³Ragon Institute of MGH, MIT and Harvard, Boston, United States, ⁴Centre for the AIDS Programme of Research in South Africa (CAPRISA), University of KwaZulu-Natal, Durban, South Africa

The finding that antibody-dependent cell-mediated cytotoxicity (ADCC) was a correlate of protection in the RV144 HIV-1 vaccine trial, has suggested that a vaccine should elicit antibodies with both neutralizing and effector functions. However, it is unknown if individuals who develop broadly cross-neutralizing antibodies (BCN) also have antibodies with increased Fc effector polyfunctionality. We therefore assessed the gp41, gp120 and gp140-targeted ADCC, antibody-dependent complement deposition (ADCD), antibody-dependent cellular phagocytosis (ADCP), NK degranulation and measured HIV-specific IgG1-4 levels of polyclonal IgG isolated from 14 BCN and 11 non-BCN individuals at 6, 12 and 36 post-infection (p.i.) from the CAPRISA cohort. Gp120 and gp140 but not gp41- specific ADCP and ADCD responses were significantly higher ($p = 0.01$) in the BCN group at 6 months p.i. indicating that HIV epitopes may be targeted differently by antibodies bound to Fc-receptors. Gp120-specific IFN- γ secretion following NK degranulation was significantly higher in the BCN group ($p = 0.03$) at all three time points. Since IFN- γ enhances IgG3 (the most polyfunctional of all isotypes) class-switching, we also noted that IgG3 levels were higher in the BCN group ($p = 0.04$). Overall our study shows that there are differences in Fc-mediated effector function between BCN and non-BCN individuals in early infection and that this is likely to be antigen specific. Regardless, while elicitation of broadly neutralizing antibodies remains a focus, vaccination strategies should also aim to enhance Fc effector function of the overall HIV immune response which may be easier to achieve than neutralization.

3804

Accumulation of follicular T Helper (Tfh) cells, despite preferential infection, allows establishment of a large and unexpected viral reservoir during HIV infection

Xu, Y.¹, Bailey, M.¹, Phetsouphanh, C.¹, Harvey, R.², Turville, S.¹, Aggarwal, A.¹, Wong, A.¹, Suzuki, K.^{1,2}, Graff-Dubois, S.³, Autran, B.³, Moris, A.³, Munier, M.L.¹, Koelsch, K.¹, Kent, S.⁴, Zaunders, J.^{1,2}, Kelleher, A.^{1,2}

¹Kirby Institute, UNSW, Immunovirology and Pathogenesis Program, Sydney, Australia, ²St Vincent's Hospital, St Vincent's Centre for Applied Medical Research, Sydney, Australia, ³Sorbonne Universités, Center for Immunology and Microbial Infections, Paris, France, ⁴Melbourne University, Doherty Institute, Melbourne, Australia

HIV-1 infection depletes CD4⁺ T-cells, but induces chronic follicular hyperplasia. HIV infection in humans and SIV infection in macaques is characterized by accumulation of CXCR5⁺PD-1^{high}Bcl-6⁺ Tfh cells in lymphoid tissues (LT). In both infections Tfh are infected at similar or higher rates to other memory CD4⁺ T-cells. Further, despite Tfh being characterised as CCR5⁻, the envelopes of viruses found in these cells are phylogenetically indistinguishable from viruses infecting other memory CD4⁺ T-cells and are CCR5 tropic. We confirm that in both infected and uninfected macaques, Tfh cells are CCR5⁻, but a subset of their precursors are either CCR5⁺ or transiently become CCR5⁺ as they differentiate into Tfh. We show that in macaques, SIV infection of Tfh is indirect, occurring at this precursor stage. In humans the same route of infection of Tfh is available, but we have also found a small subset of Tfh, isolated directly from tissue, that expresses CCR5. This population exists regardless of HIV status. Fusion assays show these cells are susceptible to infection by CCR5 tropic virus, with viral entry blocked by CCR5 inhibitors. CXCR5, PD-1 and Bcl-6 expression in these cells is indistinguishable from the CCR5⁻ Tfh. Intriguingly, fine needle biopsies of LT, show that increases in Tfh in HIV infection are not normalised by antiretroviral therapy, suggesting ongoing follicular hyperplasia, and a long lasting reservoir of infected Tfh despite aviremia. Although we have uncovered mechanisms for the establishment of this viral reservoir, the mechanisms of survival and expansion of infected Tfh require further investigation.

3928

Plasmacytoid DCs control HIV latency in resting T-cells by type I IFN α

van der Sluis, R.M.¹, Kumar, N.A.¹, Evans, V.A.¹, Harman, A.N.², Mota, T.¹, Tennakoon, S.¹, Hertzog, P.J.³, Lewin, S.R.^{1,4}, Cameron, P.U.^{1,4}

¹Doherty Institute for Infection and Immunity, University of Melbourne, Parkville, Australia, ²Centre for Virus Research, Westmead Millennium Institute, Westmead, Sydney, Australia, ³Hudson Institute of Medical Research and Monash University, Melbourne, Australia, ⁴Department of Infectious Diseases, Alfred Health and Monash University, Melbourne, Australia

Introduction: We have previously demonstrated that myeloid DC (mDC), but not plasmacytoid DC (pDC), induce HIV latency in T-cells in vitro. pDC produce high levels of type-I IFN so we asked whether different IFNs have an effect on the establishment, maintenance and reversal of HIV latency.

Methods: Resting CD4⁺ T-cells from blood were labelled with the proliferation dye eFluor670, cultured with SEB and autologous mDC or pDC for 24h and infected with an eGFP-expressing-CCR5-tropic virus. At day 5 post-infection, non-productively-infected, non-proliferating (eGFP-eFluor670hi) and proliferating (eGFP-eFluor670lo) T-cells were sorted and inducible latent

infection quantified by measuring eGFP expression in sorted subsets after activation with anti-CD3/CD28+IL-7+IL-2 in the presence of Raltegravir.

Results: Using qPCR, type-I and type-III IFN mRNA could be detected in T+pDC co-cultures. When IFN α was added to T+mDC prior to infection, there was a reduction in productive infection, with a percentage mean inhibition (MI) of 60% at 1000U/ml, compared to untreated controls. Similar results were obtained with IFN β and IFN ω but not with IFN λ 1 and IFN λ 3. Latent infection was significantly reduced in non-proliferating T-cells in the presence of 100U/ml IFN α (MI=62%, p=0.014, n=4). When IFN α was added after latency was established, eGFP expression significantly increased compared to unstimulated controls in non-proliferating cells (mean fold change (MFC) eGFP=1.3, p=0.015, n=8).

Conclusion: Multiple type-I interferons can inhibit productive infection and the establishment of HIV latency in vitro, however, once latency is established IFN α can reverse latency in non-proliferating T-cells. IFN α should be explored as a potential therapeutic intervention to perturb latency.

311

Variability in HIV-1 envelope glycan shield vulnerability: implications for immune escape from anti-glycan neutralising antibodies

Moyo, T.^{1,2}, Ferreira, R.³, Davids, R.^{1,2}, Sondag, Z.¹, Moore, P.L.^{4,5}, Travers, S.³, Wood, N.⁶, Dorfman, J.R.^{1,2}

¹International Centre for Genetic Engineering and Biotechnology, Cape Town, South Africa, ²University of Cape Town, Pathology, Cape Town, South Africa, ³South African National Bioinformatics Institute, South African Medical Research Council Bioinformatics Unit, University of the Western Cape, Cape Town, South Africa, ⁴University of the Witwatersrand, Johannesburg, South Africa, ⁵National Institute for Communicable Disease, Johannesburg, South Africa, ⁶University of Cape Town, Integrative Biomedical Sciences, Cape Town, South Africa

The HIV-1 envelope protein is heavily glycosylated. These glycans serve multiple functions including shielding epitopes from antibodies. This is particularly important for the gp120 V3 loop because it is targeted by very common, narrowly-neutralizing antibodies. However, some broadly neutralizing antibodies directly target the V3/glycans, i.e those at amino acid positions 301, 332 and 334, making these glycans important in vaccine immunogens. We show that removal of the potential N-linked glycosylation site (PNG) at position 301 has substantially different effects in two subtype C viruses with similar sequences. In virus Du156.12, removal of the 301 PNG reveals epitopes recognized by 18/64 sera from chronically HIV-1-infected individuals. In contrast, removal of the 301 PNG in CAP45.2 only revealed epitopes recognized by 3/64 sera. This suggests that removing PNG 301 had less of an effect on the integrity of the glycan shield in CAP45.2 than Du156.12. This difference was not substantially affected by whether the nearby V3/glycan was at position 332 or 334. Molecular modeling of fully glycosylated Envelope trimers predicted that the absence of PNG 301 would likely result in greater exposure of portions of the V3 and C4 regions in Du156.12 compared to CAP45.2.

Our data indicate that the contribution of PNG 301 to resistance to common neutralizing antibodies is variable. Thus, certain viruses may be subject to less antibody-mediated pressure to maintain the 301 PNG, allowing them to more easily evade broadly neutralizing anti-V3/glycan antibodies. This may impair the efficacy of passively-infused anti-V3/glycan antibodies or those induced by a vaccine.

1463

Comparison of the antigen sensitivity of Gag-specific CD4+ T cell responses in controlled HIV infection and HIV vaccination

Mukhopadhyay, M.¹, Galperin, M.¹, Nouël, A.¹, Vasan, S.², Ho, D.D.³, Lambotte, O.⁴, Benati, D.¹, Chakrabarti, L.¹

¹Institut Pasteur, Department of Virology, Viral Pathogenesis Unit, Paris, France, ²Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand, ³Aaron Diamond AIDS Research Center, New York, United States, ⁴Hôpital de Bicêtre, AP-HP, Médecine Interne et Maladies Infectieuses, Le Kremlin-Bicêtre, France

Spontaneous control of HIV infection is characterized by a highly efficient cellular immune response. We showed in particular that HIV Controllers from the ANRS CO21 CODEX cohort harbor a population of specific CD4⁺ T cells that detect the immunodominant CD4 epitope Gag293 with high antigen sensitivity. To determine whether candidate vaccines can induce the high sensitivity responses seen in Controllers, we analyzed Gag293-specific responses in healthy volunteers who received the ADVAX DNA vaccine administered by electroporation. Comparison of Gag293-specific responses in primary CD4⁺ T cell lines via IFN- γ ELISpot revealed that the median antigen sensitivity in vaccinees was similar to that observed for Controllers (5×10^{-8} M) but higher than that in treated patients (5×10^{-7} M). However, antigen sensitivity remained higher in a subset of Controllers compared to vaccinees. TCR repertoire analysis of Gag293-specific CD4⁺ T cells from vaccinees revealed a preferential amplification of TCR β family chain TRBV2, which also predominates in Controllers. However, TRAV family gene usage appeared more diverse in vaccinees compared to Controllers. Sequence analysis of the TCR chains amplified in 4 vaccinees revealed a biased TCR repertoire with the presence of public clonotypes (3 TRAV24 and 2 TRBV2) shared with HIV Controllers. In conclusion, DNA vaccination administered by electroporation has the potential to induce Gag-specific CD4⁺ T cells responses with a high antigen sensitivity and partial TCR repertoire overlap with that of Controllers. Monitoring the amplification of public TCR clonotypes could provide a novel approach to evaluate the quality of HIV vaccine responses.

3104

Molecular characteristics and use of bovine IgG with broad HIV-1 neutralizing activity from colostrum of cows vaccinated with Env-gp140 trimers

Heydarchi, B.¹, Center, R.¹, Cuthbertson, J.¹, Bebbington, J.¹, Mackenzie, C.¹, Muller, B.^{1,2}, Kramski, M.¹, Chai, Z.Q.¹, Rawlin, G.², MacInnes, D.², Khoury, G.¹, Gonelli, C.¹, Purcell, D.¹

¹University of Melbourne, Peter Doherty Institute for Infection and

Immunity, Microbiology and Immunology, Melbourne, Australia,
²*Reef Pharmaceuticals Pty Ltd, Melbourne, Australia*

HIV-patient serum with elite virus-neutralizing breadth has led to preparation of monoclonal antibodies (mAb) with long and highly mutated CDRH3 domains that can neutralize a broad array of viral strains and prevent transmission in animal models. Primate vaccination has not matched these broad neutralizing antibody responses, but vaccination of cows before then during pregnancy with four 100µg doses of purified HIV-1_{AD8} Env-gp140 trimers in Montanide adjuvant yields vast quantities, up to 1kg, of polyclonal IgG in colostrum that binds HIV Env-gp140 with titres of 1x10⁵ and neutralise all 27 Env (24-strongly, 3-moderately) from clade A, B and C reference panels. We assessed this potent bovine IgG by preparing mAbs with matched chimeric bovine-VH and -VL with human C-regions from HIV gp140-binding IgG+ CD21+ circulating memory B-cells present at 0.66%. The 33 mAbs had long CDRH3 sizes ranging from 12 - 64 aa's with a high Cys and aromatic-aa frequency. Two mAbs, 6A and 8C, displayed strong binding to HIV-1_{AD8} Env-gp140 uncleaved trimers, but not monomer, and bound cleaved covalently-stabilized HIV-1_{AD8} SOSIP gp140 trimers. The VH somatic mutation rate for 6A and 8C was 27% and 25% respectively and their 21 and 14aa CDRH3 domains were 57% and 93% mutated from germline. Mutation of CDRH3 Cys or Trp eliminated the HIV-specific binding characteristics. Despite strong trimer-specific binding, these mAbs didn't match the broad neutralizing activity of the polyclonal IgG. We have now produced large quantities of HIV-1 hyperimmune colostrum polyclonal antibodies for further evaluation as a microbicide to block HIV transmission.

3729

Characterization of gut tissue resident memory CD8⁺ T-cells in HIV infection

Kiniry, B.¹, Ganesh, A.¹, Hunt, P.², Somsouk, M.², Deeks, S.², Shacklett, B.¹

¹*University of California, Davis, Davis, United States,* ²*University of California, San Francisco (UCSF), San Francisco, United States*

The gastrointestinal tract (GIT) is an important site of HIV transmission and pathogenesis. Combating HIV will likely require maintenance of cells capable of protective anti-HIV immune responses in the GIT. Tissue resident memory T-cells (T_{RM}) are long-lived, non-recirculating memory T-cells localized in tissues like the GIT. Positioned near sites of infection, T_{RM} are a first line of defense against pathogens. A knowledge gap exists regarding CD8⁺ T_{RM} in the human GIT and their role in HIV infection. We used flow cytometry to characterize T_{RM} in blood and rectal biopsies. CD8⁺ T-cells from HIV+ and HIV- adults were phenotyped using CD45RO, CD69, CD103, S1PR1, T-bet, and Eomesodermin (Eomes). Functionality was assessed in a 6-hour *ex vivo* stimulation assay. Cells were stimulated with DMSO, Gag or CMV-EBV-Flu (CEF) peptides, or Staphylococcal Enterotoxin B, and stained for CD107a, IFNγ, Mip-1β, TNFα, IL-2, and Granzymes A and B. Most rectal CD8⁺ T-cells were CD103⁺CD69⁺S1PR1⁺, indicative of tissue residency. This subset was significantly more abundant in gut than blood (P=0.005). CD45RO⁺ cells in this subset were classified as T_{RM}⁺. Consistent with murine studies, CD8⁺ T_{RM} were mainly T-bet^{Low}Eomes^{Neg}. The rectal mucosa has

an expanded population of GrzA^{High} CD8⁺ T-cells compared to blood; these were predominantly CD69⁺CD103⁺S1PR1⁺. Participants who control HIV without drug therapy displayed stronger polyfunctional and cytotoxic (CD107a⁺GrzA⁺GrzB⁺) Gag-specific CD8⁺ T_{RM} responses compared to antiretroviral-treated subjects. In summary, a large subset of CD8⁺ T-cells in rectal mucosa are T_{RM} and likely play an important role in HIV containment. *Supported by NIH/NIAD R01 AI057020.*

Cell Trafficking

2754

Implications of Spns2-deficiency on S1P-driven lymphocyte egress, HEV-integrity and immunity

Simmons, S.¹, Sasaki, N.², Umemoto, E.³, Yoshizumi, N.², Fukuhara, S.⁴, Kitazawa, Y.⁵, Okudaira, M.⁶, Inoue, A.⁶, Motooka, D.⁷, Nakamura, S.⁷, Iida, T.⁷, Aoki, J.⁶, Mochizuki, N.⁴, Matsuno, K.⁵, Takeda, K.³, Miyasaka, M.², Ishii, M.¹

¹*Osaka University, WPI Immunology Frontier Research Center and Graduate School of Medicine & Frontier Biosciences, Department of Immunology & Cell Biology, Osaka, Japan,* ²*Osaka University, Graduate School of Medicine, Laboratory of Immunodynamics, Osaka, Japan,* ³*Osaka University, WPI-Immunology Frontier Research Center and Graduate School of Medicine, Laboratory of Mucosal Immunology, Osaka, Japan,* ⁴*National Cerebral and Cardiovascular Center Research Institute, Department of Cell Biology, Osaka, Japan,* ⁵*Dokkyo Medical University, Department of Anatomy (Makro), Tochigi, Japan,* ⁶*Tohoku University, Graduate School of Pharmaceutical Sciences, Laboratory of Molecular and Cellular Biochemistry, Sendai, Japan,* ⁷*Osaka University, Research Institute for Microbial Diseases, Department of Infection Metagenomics, Osaka, Japan*

In order to reveal the role of the S1P-specific transporter Spinster-homologue-2 (Spns2) in controlling S1P-mediated lymphocyte egress from secondary lymphoid organs into the lymphatic system we conditionally deleted Spns2 in Lyve1+ lymphatic endothelial cells (LECs).

We detected a strong reduction of S1P in the lymph of Lyve1-Spns2^{Δ/Δ} (KO) mice, leading to a strong accumulation of lymphocytes in Peyer's patches (PPs) and the development of hypotrophic lymph nodes (LNs). The difference between lymphocyte immigration and egress in pLN and PPs caught our particular attention. We could show that total WT congenic splenocytes transplanted *i.v.* showed reduced immigration into pLNs of KO mice in comparison to Spns2^{fl/fl} (WT) mice, whereas the appearance of B/T-cells in PPs was not affected. Importantly, we detected severe impairment in morphology and size of high-endothelial venules (HEVs) in pLNs of KO mice. We observed that DCs which enter pLNs by afferent lymphatics were absent in close proximity to the HEVs. Interestingly, DCs, which co-localize in WT animals with HEVs in order to provide lymphotoxin-β for proper development and function, are unable to overcome a restricted area around HEVs of KO mice. Impaired micro-anatomical co-localization of DCs and HEVs in LNs was also observed if mice were treated with several S1PR-specific antagonists. Furthermore, S1PR1-signalling was reduced in HEVs of KO mice.

Taken together all these studies reveal the importance of Spns2 to control S1P-driven lymphocyte egress from lymphoid organs and uncovers new insights in the role of LEC-derived S1P in controlling micro-anatomical migration of immune cells in LNs.

4210

Boosting migration and function of tumor-specific T cells using integrin transregulation

Cantor, J.¹, Ginsberg, M.²

¹University of California San Diego, Medicine, La Jolla, United States, ²University of California San Diego, La Jolla, United States

Poor homing and tumor-mediated suppression of T cells are barriers to adoptive ex vivo-modified T-cell immunotherapy for solid tumors. Inhibiting Protein Kinase A(PKA)-mediated phosphorylation of $\alpha 4$ integrin in human T cells results in an increase in $\alpha \text{L}\beta 2$ -mediated migration on mixed ICAM-1-VCAM-1 substrates *in vitro*, a phenomenon termed "integrin trans-regulation." We have created an $\alpha 4$ (S988A)-bearing mouse, a mutation which precludes PKA-mediated $\alpha 4$ phosphorylation, to examine the effect of integrin trans-regulation *in vivo*. The $\alpha 4$ (S988A) mouse exhibits selectively increased migration of lymphocytes but not myeloid cells to an inflammatory site, strikingly reduced growth of B16 melanoma tumors, and increased tumor-infiltrating T cells with lower expression of exhaustion markers. Boosting $\alpha 4$ trans-regulation of $\alpha \text{L}\beta 2$ integrin function thus increases migration and function of tumor-specific T cells. This novel mechanism could enhance adoptive immunotherapy using modified T cells for solid tumor cancers.

1169

Exocrine gland surveillance by tissue resident memory T cells depends on cell contractility and Cdc42-mediated polarity but not integrin and chemokine signaling

Ficht, X.¹, Stolp, B.², Thelen, F.¹, Kreuzfeldt, M.³, Page, N.³, Fukui, Y.⁴, Merkler, D.³, Stein, J.V.¹

¹University Bern, Theodor-Kocher-Institute, Bern, Switzerland, ²University Clinic Heidelberg, Department of Virology, Heidelberg, Germany, ³University of Geneva, Institute for Pathology and Immunology, Geneva, Switzerland, ⁴Kyushu University, Department of Immunobiology and Neuroscience, Fukuoka, Japan

Distinct CD8⁺ memory T cell populations persist after infection to ensure host efficient protection upon pathogen re-encounter and actively migrate in their target tissues for immunosurveillance. T cell motility is presumed to be driven by chemokine-induced F-actin treadmilling while force transmission is mediated by specific binding of integrins to extracellular matrix. This canonical view is largely built on 2D *in vitro* findings, which do not take into account the complex architecture of tissues and organs, nor cell intrinsic properties of memory T cell subsets. Here, we used multiphoton intravital imaging to dissect the cellular requirements for *in vivo* migration of central memory T cells (T_{CM}) in lymph nodes and tissue resident memory T cells (T_{RM}) in salivary and lacrimal glands. We found that inhibition of αE -, αL -, $\alpha 4$ - and $\beta 1$ - integrins or G_{ai} does impair

the migration of T_{CM} but not T_{RM} cells. Rather, T_{RM} migration required ROCK-mediated contractility and pseudopodia formation mediated by the Cdc42 activator DOCK8. In sum, our findings show that T_{RM} cell migration in exocrine glands doesn't rely on specific adhesion of integrins nor on signaling of G_{ai} -coupled chemokine receptors. Instead, we propose a novel model of resident memory T cell migration: Intrinsically active pseudopodia formation and myosin contractility use confinement-induced friction to generate momentum. Our findings imply that memory T cell migration is less dependent on specific molecular interactions than previously anticipated and may instead be influenced to a greater extent by physical tissue properties such as confinement and extracellular matrix architecture.

1417

CXCR4 mediates homeostatic compartmentalization of monocytes and pulmonary inflammatory responses

Chong, S.Z.¹, Evrard, M.¹, Devi, S.¹, Balabanian, K.², Chen, Q.³, Renia, L.¹, Wang, C.-I.¹, Looney, M.R.⁴, Krummel, M.F.⁴, Randolph, G.J.⁵, Biswas, S.K.¹, Ginhoux, F.¹, Hidalgo, A.⁶, Bachelier, F.², Ng, L.G.¹

¹Singapore Immunology Network (SiGN), A*STAR, Singapore, Singapore, ²INSERM UMR-S 996, Université Paris-Sud, Clamart, France, ³Institute of Molecular and Cell Biology (IMCB), A*STAR, Singapore, Singapore, ⁴University of California (UCSF), Department of Medicine and Pathology, San Francisco, United States, ⁵Washington University, Division of Immunobiology, St. Louis, United States, ⁶Fundación Centro Nacional de Investigaciones Cardiovasculares (CNIC), Area of Cell and Developmental Biology, Madrid, Spain

The migration of monocytes between tissue compartments represents a critical aspect of their immune surveillance function. However, mechanisms that regulate their distribution between tissue compartments remain unclear. Using *in vivo* assays in transgenic mice with either loss- or gain-of-function in CXCR4-signaling, we show that CXCR4 is a key regulator in maintaining circulating monocyte numbers by controlling their shuttling between the circulation, bone marrow (BM) and lung marginal pool. We show through BrdU pulse-chase assays and parabiosis that CXCR4-signalling not only regulated the bi-directional trafficking of monocytes between the blood and BM compartments, but fluctuations in its expression levels regulated the circadian rhythmic oscillation of monocyte numbers. In addition, we observed that BM Ly6C^{hi} monocytes that have differentiated from the common monocyte progenitor (cMoP) consists of a heterogenous population with distinct maturity and ability to respond to mobilization cues. Specifically, CXCR4 expression delineates this functional heterogeneity, with "immature" monocytes being CXCR4^{hi} and "mature" monocytes being CXCR4^{lo}. Importantly, our intravital imaging revealed that CXCR4 orchestrates monocyte margination in the pulmonary vasculature and this process was augmented upon exposure to endotoxins. In particular, CXCR4 inhibition reduced monocyte margination, which resulted in decreased occurrence of lung injury and mortality in mouse models of sepsis. Together, our data identify CXCR4 as a key regulator of monocyte compartmentalization and homeostasis in both steady and inflammatory states.

2695

L-selectin controls trafficking of chronic lymphocytic leukemia cells in lymph node high endothelial venules *in vivo*Lafouresse, F.^{1,2,3}, Bellard, E.^{1,2}, Laurent, C.^{4,5,6}, Moussion, C.^{1,2}, Fournié, J.-J.^{2,4,5}, Ysebaert, L.^{4,5}, Girard, J.-P.^{1,2}¹CNRS, IPBS (Institut de Pharmacologie et de Biologie Structurale), Toulouse, France, ²Université de Toulouse, UPS, Toulouse, France, ³Swinburne University of Technology, Melbourne, Hawthorn, Australia, ⁴IUCT (Institut Universitaire du Cancer Toulouse)-Oncopôle, Toulouse, France, ⁵INSERM, U 1037, CRCT (Centre de Recherches en Cancérologie de Toulouse), Toulouse, France, ⁶INSERM, U 563, Centre de Physiopathologie de Toulouse Purpan, Toulouse, France

Chronic Lymphocytic Leukemia (CLL) is characterized by the progressive accumulation of CLL cells in the blood and lymph nodes (LN). Although chemotherapy and immunotherapy are effective at targeting circulating CLL cells, LN resident CLL cells are resistant to treatment and are often source of relapse. We studied the first step of CLL migration to LN, their interaction with high endothelial venules (HEV), specialized blood vessels for lymphocyte recruitment (Moussion & Girard, *Nature* 2011; Girard et al., *Nature Rev Immunol*, 2012).

We observed the frequent presence of CLL cells within HEV pockets in human CLL LN, indicating intense trafficking of CLL cells through HEV. Using *in vivo* imaging, we visualized for the first time, the behavior of human CLL cells within murine LN microcirculation. We found that CLL cells roll, stick and crawl on HEV endothelium. Functional analyses revealed that the lymphocyte homing receptor L-selectin (CD62L) is the key factor controlling the binding of CLL cells to HEV walls *in vivo*. Interestingly, L-selectin expression was decreased on CLL cells from patients treated with idelalisib, a phosphoinositide 3-kinase delta inhibitor recently approved for treatment of CLL patients. Reduced rolling and sticking of CLL cells to HEV was observed after treatment with idelalisib, correlating with L-selectin downregulation.

Together, our findings indicate that interference with L-selectin-mediated trafficking in HEV could be useful to limit the entry of circulating CLL cells into LN in order to increase the efficacy of conventional therapy (Lafouresse et al., *Blood*, 2015, Cover page and Highlighted in commentaries).

3293

Platelets direct leukocytes to their sites of extravasationZuchtriegel, G.^{1,2}, Uhl, B.², Pühr-Westerheide, D.², Pörnbacher, M.², Lauber, K.³, Krombach, F.², Reichel, C.A.^{1,2}¹Ludwig Maximilians University Munich, Department of Otorhinolaryngology, Head and Neck Surgery, Munich, Germany, ²Ludwig Maximilians University Munich, Walter Brendel Centre of Experimental Medicine, Munich, Germany, ³Ludwig Maximilians University Munich, Department of Radiation Oncology, Munich, Germany

Leukocyte recruitment from the microvasculature to the site of injury or infection is a key event in the inflammatory response. Whereas the principles of this highly complex process have been

elucidated in the past decades, it remained poorly understood how these immune cells 'find' their site of extravasation.

Interactions of platelets, neutrophils, inflammatory monocytes, and endothelial cells were analyzed by multi-color *in vivo* microscopy in the cremaster muscle of male CX₃CR-1^{GFP/+} mice (exhibiting fluorescence-labeled monocytes) upon stimulation with CCL2. Expression profiles of adhesion and signaling molecules in the microvasculature were assessed *ex vivo* by confocal microscopy in cremasteric tissue whole mounts. Different *in vitro* assays were used to further characterize the underlying mechanisms.

Upon onset of inflammation, circulating platelets immediately adhered at distinct sites in venular microvessels enabling these cellular blood components to capture neutrophils and, in turn, inflammatory monocytes via CD40-CD40L-dependent interactions. In this cellular crosstalk, ligation of leukocyte PSGL-1 by P-selectin initiates conformational changes in surface-expressed leukocyte integrins via ERK1/2 MAPK which subsequently promote the successive extravasation of neutrophils and inflammatory monocytes to the perivascular tissue. Conversely, blockade of this cellular partnership resulted in misguided, inefficient leukocyte responses.

Here, we report a previously unrecognized role of platelets as pathfinders navigating neutrophils and inflammatory monocytes to their exit points in the inflamed microvasculature. This platelet-directed guidance enhances the efficacy of the leukocyte transmigration process and is essential for effective leukocytes responses.

3163

Real-time intravital longitudinal imaging of osteoclast formation, function and fate during bone remodelingMcDonald, M.^{1,2}, Butt, D.^{1,2}, Terry, R.^{1,2}, Quinn, J.^{1,2}, Rogers, M.^{1,2}, Brink, R.^{1,2}, Croucher, P.^{1,2}, Phan, T.^{1,2}¹Garvan Institute of Medical Research, Sydney, Australia, ²UNSW Australia, Faculty of Medicine, Sydney, Australia

Osteoclasts are large multinucleated cells derived from the recruitment and fusion of cells of the monocyte/macrophage lineage in response to signals provided by bone lining stromal cells and osteoblasts, such as M-CSF and RANKL. Bone resorption by osteoclasts and bone formation by osteoblasts is tightly coupled in the basic multicellular unit (BMU). By resorbing bone and remodeling the endosteal bone niche, osteoclasts also control the mobilisation of hemopoietic stem cells, and the activation of dormant cancer cells that metastasise to bone. Thus, in addition to their role in skeletal homeostasis, osteoclasts also play key roles in hematopoiesis, and in controlling bone metastasis. However, because of the inaccessibility of the bone to real-time interrogation in live animals, little is known about the dynamic cellular and molecular events that regulate osteoclast formation, function and fate during physiological and pathological bone remodeling. To address this, we have developed a novel method for intravital two-photon microscopy that enables longitudinal deep tissue imaging of intact long bones at high resolution. This has allowed us to image these processes using lineage reporters and fluorescent probes in the steady state and following perturbation with osteoclast

inhibitors and activators. Our data reveals a previously unappreciated dynamic equilibrium between osteoclasts and macrophages under homeostatic conditions in the BMU that can be shifted in favour of osteoclasts during bone resorption and against them during bone formation. Thus, manipulating osteoclasts to alter this equilibrium may provide novel strategies to treat metabolic bone disease, control hematopoiesis and regulate cancers in bone.

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MST1 dependent vesicle trafficking regulates neutrophil transmigration through the vascular basement membrane

Kurz, A.R.M.¹, Pruenster, M.¹, Rohwedder, I.¹, Schäfer, K.², Breithaupt, U.³, Gouveia, G.^{1,4}, Nussbaum, C.^{1,5}, Immler, R.¹, Wiessner, J.R.¹, Margraf, A.¹, Lim, D.-S.⁶, Walzog, B.¹, Dietzel, S.¹, Moser, M.¹, Klein, C.⁵, Vestweber, D.², Haas, R.³, Sperandio, M.¹

¹Ludwig Maximilians University, Walter Brendel Center of Experimental Medicine, Munich, Germany, ²Max Planck Institute for Molecular Biomedicine, Department of Vascular Cell Biology, Münster, Germany, ³Ludwig Maximilians University, Max von Pettenkofer-Institute for Hygiene and Medical Microbiology, Munich, Germany, ⁴Ministry of Education of Brazil, CAPES Foundation, Brasilia, Brazil, ⁵Ludwig Maximilians University, Dr. Von Hauner Children's Hospital, Munich, Germany, ⁶Korea Advanced Institute of Science and Technology, Department of Biological Sciences, Biomedical Research Center, Daejeon, Korea, Republic of

Extravasation of neutrophils from postcapillary venules into inflamed tissue is a crucial step during the inflammatory response. Within this process, neutrophils migrate across the endothelium and subsequently need to penetrate the perivascular basement membrane. The precise regulation of both steps is not fully understood, yet. By using multiphoton intravital microscopy and immunofluorescence staining we identified mammalian sterile 20-like kinase 1 (MST1) as a key player for the migration of neutrophils through the perivascular basement membrane. *Mst1* knock out neutrophils (*Mst1*^{-/-}) persist between the endothelium and the basement membrane of inflamed murine cremaster muscle venules and fail to migrate into inflamed tissue. *Mst1*^{-/-} neutrophils also fail to extravasate from gastric submucosal vessels in a murine *Helicobacter pylori* infection model. Mechanistically, impaired extravasation of *Mst1*^{-/-} neutrophils was accompanied by defective translocation of VLA-3, VLA-6, and neutrophil elastase from intracellular vesicles to the neutrophil surface, a requirement for neutrophils to penetrate the basement membrane. Taken together, our findings identify MST1 as a critical regulator of neutrophil transmigration and emphasize the importance of MST1-dependent vesicle trafficking for the recruitment process.

2594

Tc17 cells: a non-cytotoxic pro-inflammatory effector CD8⁺ T cell subset?

McCull, S.¹, Fenix, K.¹, Gregor, C.¹, Kara, E.¹, McKenzie, D.¹, Bastow, C.¹, Sutton, V.², Trapani, J.², Gartlan, K.³, MacDonald, K.³, Hill, G.³, Comerford, I.¹

¹The University of Adelaide, Molecular and Cell Biology, Adelaide, Australia, ²Peter MacCallum Cancer Centre, Cancer Immunology Program, Melbourne, Australia, ³Queensland Institute of Medical Research, Immunology, Brisbane, Australia

Tc17 cells are a subset of effector CD8⁺ T cells that develop similarly to their CD4⁺ T cell counterpart Th17 and have emerging functional significance. Both Tc17 and Th17 cells share developmental characteristics and cytokine expression profiles with Tc17 cells thought to fulfil a pro-inflammatory role in response to MHC class-I restricted antigens. However, their reported function as cytotoxic cells is controversial and the trafficking mechanisms used by these cells are not well understood. We have detected Tc17 cells in a number of model immune responses and shown an important functional role for these cells in experimental graft versus host disease through pro-inflammatory cytokine production. Our investigation of the migratory properties of Tc17 cells reveals that the chemokine receptor CCR6 is important for their recruitment. Regarding their cytotoxic potential, we show that Tc17 cells have only very limited capacity to kill targets and this property derives from their reliance on TGFβ for development, which also inhibits expression of granzymes and perforin. Highly cytotoxic Tc17 cells, dependent on granzymes A and B, develop in conditions where TGFβ signalling is limited, indicating additional complexity within the Tc17 cell subset to encompass both cytotoxic and non-cytotoxic cell types.

Dendritic Cells 1

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Dendritic cell development is dependent on Drosha-mediated cleavage of mRNAs

Johanson, T.^{1,2,3}, Keown, A.³, Cmero, M.³, Yeo, J.^{1,2,3}, Kumar, A.³, Lew, A.^{2,4,5}, Zhan, Y.^{1,2}, Chong, M.^{6,7}

¹The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia, ²The University of Melbourne, Department of Medical Biology, Melbourne, Australia, ³St. Vincent's Institute of Medical Research, Melbourne, Australia, ⁴The Walter and Eliza Hall Institute of Medical Research, Immunology Division, Melbourne, Australia, ⁵The University of Melbourne, Department of Microbiology & Immunology, Melbourne, Australia, ⁶St. Vincent's Institute of Medical Research, Melbourne, Australia, ⁷The University of Melbourne, Department of Medicine (St. Vincent's), Melbourne, Australia

Drosha and Dicer are enzymes required for microRNA biogenesis. By genetic ablation in mice, we found that Drosha is critical for the development of dendritic cells (DCs) and other myeloid lineages. Drosha deficiency resulted in a block early in DC development, leading to an accumulation of early haematopoietic progenitors in the bone marrow, and a near

total absence of mature DCs in the periphery. In contrast, Dicer deficiency only partially affected DC numbers. This suggests that the impact of Drosha deficiency on DC development is largely independent of the microRNA pathway.

In addition to the cleavage of microRNA precursors, our lab and others discovered that Drosha is capable of directly degrading specific messenger RNAs (mRNAs). This occurs via the recognition and cleavage of stem loop structures within the target mRNA. Transcriptional profiling of haematopoietic progenitors revealed a series of transcripts that accumulated upon Drosha, but not Dicer deletion, suggesting microRNA independent regulation. We showed that the derepression of two of these transcripts, Myl9 and the previously unannotated Target Of Drosha 1 (Todr1) are responsible for the block in DC development in the absence of Drosha. Furthermore, knockdown of Myl9 or Todr1 via shRNA in Drosha deficient haematopoietic progenitors restores DC development. We also demonstrated that these two transcripts are specific targets of, and are directly cleaved by, Drosha in haematopoietic progenitors. Thus, Drosha is required for DC development through degrading inhibitors of myelopoiesis. This is the first demonstration of a microRNA independent function for Drosha in the haematopoietic system.

3129

Clec9A-mediated induction of Tfh and B cell responses

Kato, Y.¹, Lahoud, M.², Kaisho, T.³, Shortman, K.^{4,5}, Mueller, S.¹, Caminschi, I.², Heath, W.¹

¹The University of Melbourne at the Peter Doherty Institute for Infection and Immunity, Parkville, Australia, ²Monash University, Department of Biochemistry and Molecular Biology, Clayton, Australia, ³Wakayama Medical University, Department of Immunology, Institute of Advanced Medicine, Wakayama, Japan, ⁴The Walter and Eliza Hall Institute of Medical Research, Parkville, Australia, ⁵The University of Melbourne, Department of Medical Biology, Parkville, Australia

Clec9A is a surface molecule expressed by CD8⁺ DCs. We have previously shown that targeting Ag to this receptor induces a potent humoral immunity without adjuvant. Here we demonstrate that Clec9A targeting promotes antibody responses by two distinct mechanisms. Upon Clec9A-targeted priming, CD8⁺ DCs induced Tfh expressing CXCR5, PD1, Bcl6 and IL-21 that localized within GCs. Furthermore, targeting gD peptide derived from HSV-1 to Clec9A induced memory CXCR5⁺ PD1⁺ CD4⁺ T cells that proliferated extensively upon secondary challenge with the virus and rapidly developed into effector Tfh. This was associated with an enhanced GC B cell response and accelerated antibody production. Strikingly, Clec9A targeted antigens also mediated direct interaction between CD8⁺ DCs and Ag-specific B cells. Ag captured via Clec9A was efficiently retained in the native form on the surface of CD8⁺ DCs. CD8⁺ DCs induced rapid activation of Ag-specific B cells, resulting in their accumulation at the T-B border in the spleen and lymph nodes within 12h. Intravital two-photon imaging showed Ag-specific B cells physically interacted with CD8⁺ DCs prior to their accumulation at the T-B border. The B cells activated by CD8⁺ DCs readily acquired help from cognate T cells and underwent divisions, resulting in generation of strong antibody responses. These findings collectively show that a Clec9A targeted

vaccination strategy harnesses the ability of steady-state CD8⁺ DCs to prime Tfh and to share intact antigens with cognate B cells, facilitating their rapid activation. These findings have important implications for development of novel vaccination strategies.

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Dendritic cells, division of labour during peripheral herpes simplex virus-1

Whitney, P., Tebartz, C., Macleod, B., Bachem, A., Turner, A., Bedoui, S.

University of Melbourne, Microbiology and Immunology, Melbourne, Australia

Dendritic cells (DC) continually patrol peripheral tissues and transport antigen to draining lymph nodes (LN) to drive antigen-specific T cell responses. The ever-expanding knowledge of different DC subsets provides ever-expanding questions; such as what is each DC subsets actual contribution during infections. Skin-draining LN consists of both migratory DCs (Langerhans cells, dermal DC and CD103⁺ DC) and lymph node-resident DCs. In this study, we utilised a localised cutaneous HSV-1 infection model to assess the relative *in vivo* contributions for the different DCs subsets involved in priming HSV-specific CD8⁺ T cells. Using mouse models with complete or conditional depletion of various DC subsets (eg. *irf8*^{-/-} and Langerin-DTR) we could confirm importantly *in vivo*, that CD8α⁺ DC and/or CD103⁺ DCs are the main antigen-presenting subsets priming HSV-1-specific CD8⁺ T cells. These findings were further expanded utilising mice expressing diphtheria toxin under the control of the ROSA26 locus in combination with HSV-1 expressing Cre recombinase. This allowed for the selective killing of HSV-1-infected cells and thereby eliminated direct antigen-presentation by infected DC. This approach showed convincingly that the majority of antigen presentation driving HSV-1-specific CD8⁺ T cells occurs via cross presentation. These findings are consistent with skin-resident DCs, either directly infected or containing virally infected material migrating to the draining lymph nodes and handing over their cargo to LN-resident CD8α⁺ DC for priming of CD8⁺ T cells. This work highlights that not all DCs are contributing equally and that indeed a division of labour exists allowing unique tailoring to fit different situations.

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BCAP regulates DC homeostasis in response to immune stimulus through bi-switch NF-κB and PI3K/Akt pathway

Fang, E.^{1,2}, Jiang, M.^{1,2}, Miao, Y.^{1,2}, Qi, L.^{1,2}, Xiao, W.^{1,2}

¹University of Science and Technology of China, Hefei, China, ²Key Laboratory of Innate Immunity and Chronic Disease, Chinese Academy of Sciences, Hefei, China

B cell adapter protein (BCAP) is a transduction adapter protein expressed only in B cell, NK cell, macrophage and dendritic cell (DC). Notably, BCAP regulates distinct signaling pathways in different cell types, suggesting that it exerts a cell-specific feature. The function of BCAP in DC was still unknown. Our study demonstrated that bone marrow-derived DC (BMDC)

and spleen conventional-DC from BCAP gene knockout mice showed diverse characteristics with increased expression of co-stimulation molecules, cytokine production, specific antigen presentation, but decreased migration compare to the control DC. The activities of NF- κ B and PI3K/Akt showed distinct status, where NF- κ B pathway was prolonged and PI3K/Akt was reduced during LPS-induced maturation in BCAP-KO BMDC compare to the control BMDC. Further study showed that, in the steady state, BCAP associated with MyD88 to inhibit the activation of downstream NF- κ B signaling; when induced with LPS, BCAP dissociated from MyD88 and interacted with p85 α , which freed MyD88 from the MyD88/BCAP complex and activated NF- κ B. At the same time, BCAP bound to p85 α and promoted PI3K/Akt activation. Short after the response, BCAP dissociated with p85 α and interacted with MyD88 again. Thus, the BCAP served as a bi-switch of MyD88 and PI3K pathways in response to LPS stimulation. Additionally, the similar results were observed in DC stimulated with TLR2 agonist Pam3CK, but not TLR3 agonist poly:I:C. These observations clearly suggested that BCAP plays a critical role in maintaining homeostasis through bi-switch of NF- κ B and PI3K/Akt pathways in MyD88-dependent manner in DC response to immune stimulus.

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The dendritic cells that prime Th2 immune responses in vivo are transcriptionally heterogeneous

Connor, L.¹, Cognard, E.¹, Ochiai, S.¹, Tang, S.C.¹, Hilligan, K.¹, Old, S.¹, Pellefigues, C.¹, Smith, A.T.¹, Eccles, D.¹, McConnell, M.², Ronchese, F.¹

¹Malaghan Institute of Medical Research, Wellington, New Zealand, ²Victoria University of Wellington, Wellington, New Zealand

Dendritic cells (DC) acquire the capacity to prime immune responses by undergoing a coordinated programme of transcriptional changes where they upregulate expression of costimulatory signals and soluble mediators to induce CD4⁺ T cells proliferation and differentiation into the appropriate effector cell type. To gain an understanding of the signals involved in the differentiation of IL4-producing Th2 cells in vivo, we used RNAseq to carry out a transcriptional characterization of skin-derived DC subsets in the lymph nodes of mice exposed to two different Th2 stimuli, the TSLP-independent nematode parasite *Nippostrongylus brasiliensis* (Nb), and the TSLP-dependent contact sensitizer DBP-FITC. We find that both stimuli induced extensive but distinct transcriptional changes in the CD11b⁺CD103⁻CD326⁻ (CD11b⁺) and CD11b⁻CD103⁻CD326⁻ (TN) DC subsets. Pathway analysis revealed a clear cytokine signature that was specific to DC subsets from Nb-injected mice. This signature was functionally important as treatment with cytokine receptor blocking antibodies at the time of immunization suppressed the development of IL4-producing T cells in Nb-primed, but not DBP-FITC-primed, mice. Surprisingly, comparison of the DC transcriptional changes in Nb-primed and DBP-FITC-primed mice revealed only a small number of similarly regulated genes, mostly consisting of well-known molecules associated with DC migration, activation, and Th2 immunity. Thus the priming of Th2 immune responses

does not appear to be associated with a unique DC signature, a finding that may be consistent with a model where incomplete or insufficient signals from DC result in a "default" development of Th2 responses.

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Activation of plasmacytoid dendritic cells in colon-draining lymph nodes during *C. rodentium* infection involves pathogen-sensing and inflammatory pathways distinct from CD103⁺ DC

Toivonen, R.¹, Kong, L.², Rasool, O.², Lund, R.², Lahesmaa, R.², Hänninen, A.¹

¹University of Turku, Medical Microbiology and Immunology, Turku, Finland, ²Turku Centre for Biotechnology, Turku, Finland

Dendritic cells (DC) bear the main responsibility for initiation of adaptive immune responses necessary for antimicrobial immunity. In the gut, CD103⁺ migratory DC traffic from small intestinal lamina propria to small intestinal mesenteric lymph nodes (siMLN) and induce regulatory and antimicrobial immunity there. Immune surveillance of the large intestine and the role of CD103⁺DC therewith are less well characterized. We identified three small mesenteric lymph nodes, distinct from small intestinal lymph nodes, which drain lymph specifically from the colon, and studied DC responses to the attaching and effacing pathogen *C. rodentium* in these. Transcriptional profiling of CD103⁺ DC and the lymph-node resident pDC populations during steady-state conditions revealed activity of distinct sets of genes in these two DC subsets, both in small intestinal and colon-draining lymph nodes. *C. rodentium* activated DC especially in colon-draining lymph nodes, and gene expression changed in pDC more profoundly than in CD103⁺DC. Among the genes most upregulated in pDC were C-type receptor CLEC4E, IL-1-receptors (IL1R1-2), proinflammatory cytokines (IL1a, IL-6) and TLR6. The coMLN and siMLN also showed differential lymphocyte response to bacterial signals as seen with increased IFN γ production by CD4⁺ cells in the coMLN, but not in the siMLN.

Our results indicate that colon immune surveillance is distinct from that of the small intestine in terms of draining lymph nodes, and identify pDC as active sentinels of colonic inflammation and/or microbial dysbiosis.

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Human plasmacytoid dendritic cells diversify into specialized innate and adaptive subsets after viral activation

Alculumbre, S.G.¹, Vargas, P.², Di Domizio, J.³, Maiuri, P.², San Roman, M.¹, Terrier, B.⁴, Saadoun, D.⁵, Gilliet, M.³, Soumelis, V.¹

¹Institut Curie, INSERM U932, Paris, France, ²Institut Curie, CNRS UMR144, Paris, France, ³University Hospital CHUV, Department of Dermatology, Lausanne, Switzerland, ⁴Cochin Hospital, APHP, Department of Internal Medicine, Paris, France, ⁵Groupe Hospitalier Pitié Salpêtrière, APHP, Department of Internal Medicine, Paris, France

Microbial activation stimulates plasmacytoid pre-dendritic cells (pDC) to secrete large amounts of type I interferon (IFN) and

differentiate into mature dendritic cells capable of modulating the adaptive immune response. Here, we show that peripheral innate activation of steady state human pDC induced their differentiation into three phenotypically, morphologically, and functionally distinct subsets, in the absence of cell proliferation: P1 (PD-L1⁺CD80⁻), P2 (PD-L1⁺CD80⁺) and P3 (PD-L1⁻CD80⁺). The innate and adaptive functions previously associated to pDC biology were differentially linked to each of the subsets. P1-pDCs display a plasmacytoid morphology and innate immune functions, comprising IFN production, whereas P3-pDCs adopt a dendritic morphology and adaptive immune functions, including T cell activation and proliferation. P2-pDCs had an intermediate functional profile. Different stimuli induced variable proportions of the subsets, suggesting that steady state pDC are multipotent. We found distinct pDC subsets associated with human autoimmune and inflammatory diseases, supporting their pathophysiological relevance. We propose that peripheral innate activation induces pDC subset diversification with reciprocal exclusion of innate and adaptive functions.

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Inflammation induces two types of inflammatory dendritic cell in lymph nodes

Min, J.¹, Yang, D.¹, Kim, M.², Haam, K.², Kim, Y.S.², Kim, D.¹, Kang, S.J.¹

¹Korea Advanced Institute of Science and Technology, Daejeon, Korea, Republic of, ²Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea, Republic of

The spatio-temporal regulation of immune cells in lymph nodes (LNs) is crucial to mount protective T cell responses, which are orchestrated by dendritic cells (DCs). However, it is unclear how the DC subsets are altered by the inflammatory milieu of LNs. Here, we show that the inflamed LNs of *Listeria*-infected mice are characterized by the clustering of neutrophils and monocytes, and IFN- γ production. Significantly, the early inflammatory responses are coupled to the differentiation of not one, but two types of inflammatory DCs. Through assessment of chemokine receptor dependency and gene expression profiles, we herein unveiled a novel inflammatory DC population that arises from conventional DCs (cDCs), contrary to the current consensus on the single monocytic origin of inflammatory DCs (moDCs). The cDC-originated inflammatory DCs had a higher potential to stimulate T cell proliferation and Th1 differentiation than moDCs, whereas the latter displayed more robust expression of inflammatory genes. These findings reveal the role of early inflammatory responses for driving the differentiation of two inflammatory DC subsets with distinct competencies. Further our study delineates unprecedented regulatory mechanisms that are set by the early innate immune responses for orchestrating the following adaptive immunity.

2330

The non-canonical Wnt pathway negatively regulates the differentiation of dendritic cell by inhibiting the expansion of Flt3⁺ lymphocyte-primed multipotent precursors

Xiao, J., Zhou, H., Wu, N., Wu, L.

Tsinghua University School of Medicine, Beijing, China

The differentiation of dendritic cells (DC) altered during the process of aging. However, the molecular mechanisms for the alteration of DC development in aged mice are far from clarified. Recently, Wnt5a has been reported as an important aging related molecule in hematopoietic system. Here, we suggest that the increased expression of Wnt5a in aged hematopoietic precursors led to deficient differentiation of DC in aged mice. Compared with young mice, the percentages and cell numbers of plasmacytoid DC (pDC) and CD172aCD8 α ⁺ conventional DC (cDC) decreased in aged mice. Further analysis indicated that the hematopoietic precursors that can give rise to DC, including Flt3⁺ lymphocyte-primed multipotent precursors (LMPP), common lymphoid progenitors (CLP) and common DC precursors (CDP) all decreased in the bone marrow of aged mice. Overexpression of Wnt5a in hematopoietic precursors strongly affected the differentiation of cDC and pDC in vivo. Treatment of hematopoietic stem cells (HSC) with Wnt5a led to a significant decrease in the differentiation of LMPP, CLP and CDP populations, which was similar to that of the HSC in BM of aged mice. Molecular studies demonstrated that Wnt5a negatively regulated the expression of an array of genes important for DC differentiation, including Flt3, Gfi-1, Ikaros, Bcl11a and IL-7R by activating Wnt5a-Cdc42 pathway. Finally, we rejuvenated DC differentiation from aged precursors by blocking the non-canonical Wnt pathway. Therefore, our study identified the key roles of non-canonical Wnt pathway in DC differentiation and DC aging.

Mucosal Immunology 1

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Profiling immunogenic bacteria within the microbiota of SKG mice associated with spondyloarthritis and ileitis using IgA-SEQ

Rehaume, L.M.¹, Kang, A.¹, Zbarskaya, O.¹, Mullaney, J.A.¹, Kim, M.¹, Ó Cuív, P.¹, Angel, N.², Lachner, N.², Daly, J.², Morrison, M.¹, Hugenholtz, P.², Thomas, R.¹

¹The University of Queensland Diamantina Institute, Brisbane, Australia, ²Australian Centre for Ecogenomics, Brisbane, Australia

IgA is the main barrier mechanism of mucosal surfaces. High affinity IgA binds to pathogenic bacteria, whereas low affinity IgA binds commensal bacteria. Next-generation sequencing of IgA-coated bacteria (IgA-SEQ) identified a consortia of bacteria from human gut microbiota that exacerbates murine colitis, including a *Prevotella* species. *Prevotella copri* was associated with new-onset rheumatoid arthritis. BALB/c ZAP-70^{W163C} (SKG) mutant mice housed in specific pathogen-free (SPF) conditions treated with beta-glucan develop spondyloarthritis, in which ileitis is microbiota-dependent. Our aim was to define bacteria driving ileitis development in a genetically susceptible host.

Fecal samples were collected from germ-free (GF) SKG and GF-BALB/c mice recolonized with altered Shaedler flora (ASF), and the microbiota composition determined by real-time PCR. Fecal samples were collected from naive SPF-SKG mice, and IgA-coated bacteria enriched by magnetic- and fluorescence-activated cell sorting. IgA⁻, IgA⁺, IgA^{dim} and IgA^{bright} bacteria were identified by deep sequencing.

Four bacterial strains were detected in ASF-SKG and ASF-BALB/c mice: *Clostridium* sp., *Lactobacillus murinus*, *Mucispirillum schaedleri*, *Parabacteroides* sp. Whilst the *Clostridium* sp. and *Parabacteroides* sp. were decreased in SKG, the dominant bacteria in both strains of mice was the *Parabacteroides* sp. *Prevotellaceae* were enriched among IgA^{bright} bacteria, while *Lactobacillaceae* were diminished.

Interaction of the microbiota and immune system of SKG mice alters the composition of the bacterial community. The immunogenic IgA^{bright} bacteria within the microbiota of SKG mice are enriched in *Prevotellaceae*, which has been associated with rheumatoid arthritis and inflammatory bowel disease. IgA-SEQ is a powerful tool to identify immunogenic bacteria from a highly diverse microbiota.

3057

Epithelial HVEM regulates intestinal homeostasis of microbiota and immune cells

Takahashi, D.¹, Geo, G.², Lu, H.-H.³, Kronenberg, M.², Shui, J.-W.³

¹Keio University, Division of Biochemistry, Tokyo, Japan, ²La Jolla Institute for Allergy and Immunology (LIAI), La Jolla, United States,

³Academia Sinica, Institute of Biomedical Sciences, Taipei, Taiwan, Republic of China

Blocking TNF has been proven effective in treating inflammatory disorders including inflammatory bowel disease (IBD). HVEM (herpes virus entry mediator), also called TNF receptor superfamily 14, has been recently reported as an IBD risk gene. We found HVEM is highly expressed in intestinal epithelium and HVEM can regulate epithelial innate function by inducing epithelial Stat3 activation. Furthermore, we showed epithelial HVEM provides indispensable host defense against bacterial infection in the gut and lung (*Nature* 488:222, 2012), suggesting epithelial HVEM serves as an important surface receptor for intestinal homeostasis.

To further understand whether epithelial HVEM regulates intestinal immunity at the steady state, we generated epithelium-specific HVEM conditional knockout mouse. Epithelial scrape samples from small intestines of co-housed littermates were prepared and subjected to RNAseq analysis. Significantly, Paneth cell-derived anti-microbial products (defensin, lysozyme and phospholipase A2), commensal bacteria SFB-associated host defense mediators (Reg3g, Mmp7, Saa1, Nos2), and pro-inflammatory products (IL18bp, Ceacam1, Socs3, Dmbt1) were up-regulated in HVEM conditional knockout mice. Correlated to this, co-housed aged (7-month old) HVEM conditional knockout mice had increased SFB, which likely led to over-activation of immune cells (Th17 and ILC3) in the lamina propria of ileum (SFB has tropism to ileum). Furthermore, vancomycin treatment of co-housed littermates wiped out SFB and restored over-activation of immune cells in HVEM knockout mice. Together,

our data indicate that epithelial HVEM is important for intestinal homeostasis of microbiota and mucosal immune cells. Our findings will also provide insights into how HVEM could be targeted for treating intestinal inflammatory conditions.

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TGF- β signaling in ILC3 prevents spontaneous microbiota-induced colorectal cancer

Bauché, D.¹, Lélu, K.¹, Noiret, J.¹, Ferreira, C.², Deceneux, C.¹, Guibert, P.³, Treilleux, I.⁴, Veldhoen, M.², Marie, J.^{1,5}

¹Cancer Research Center of Lyon, Immunology Virology and Inflammation Dpt, Lyon Cedex, France, ²Babraham Institute, Cambridge, United Kingdom, ³Centre Léon Berard, Gastroenterology, Lyon, France, ⁴Centre Léon Berard, Anapathology, Lyon, France, ⁵DKFZ, Tumor Immunology, Heidelberg, Germany

The incidence of intestinal cancer is particularly high in the colorectal region, coinciding with a high microbial load. Whether microbiota contributes to spontaneous induction of colorectal cancers (CRC) and which mechanisms are in place to prevent CRC are unknown. Here, we show that the TIF-1 γ and SMAD4-dependent branches of (transforming growth factor β) TGF- β signaling maintain the expression of AhR (arylhydrocarbon receptor) in type 3 innate lymphoid cells (ILC3), sustain their IL-22 production and thereby the intestinal barrier-integrity. The concomitant deprivation of both SMAD4 and TIF-1 γ results in microbial translocation responsible for excessive TH17 cell-response, and subsequent CRC- induction. Antibiotics, neutralization of IL-17, recombinant IL-22 or the reconstitution of an AhR-sufficient ILC3-compartment are adequate to prevent CRC induction. Furthermore, we confirm that CRC patient-derived colonic ILC3 are unable to sustain high levels of AhR upon TGF- β stimulation. This work reveals an unsuspected role for TGF- β in the maintenance of the host-intestinal barrier integrity, keeping in check the ability of the microbiota to induce an IL-17 environment responsible for spontaneous CRC development.

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Hyper inflammasome signaling in *Nlrp3* mutant mice alters gut microbiota and confers resistance to colitis and colorectal cancer

Yao, X.¹, Xing, Y.¹, Zhang, C.², Zhao, L.³, Meng, G.⁴

¹Institut Pasteur of Shanghai, Chinese Academy of Sciences, Shanghai, China, ²State Key Laboratory of Microbial Metabolism, Shanghai Jiao Tong University, Shanghai, China, ³State Key Laboratory of Microbial Metabolism, Shanghai Jiao Tong University, Department of Biological Sciences, School of Life Sciences and Biotechnology, Shanghai, China, ⁴Institut Pasteur of Shanghai, Chinese Academy of Sciences, Unit of Innate Immunity, Shanghai, China

Inflammatory bowel disease (IBD) is a prominent threat to human health. Moreover, uncontrolled progression of IBD leads to increased risk for colorectal cancer. Dysbiosis in the gut microbiota has long been recognized as a key factor mediating IBD pathogenesis. As an important gene in the innate immune

system, NLRP3 has been linked with IBD and colorectal cancer. But whether and how gut microbiota is involved in NLRP3 function in IBD remains obscure. Here we report that mice carrying the R258W mutation which acquires an autoactivation of the Nlrp3 inflammasome remains gut homeostasis through remodeling their gut microbiota. During DSS colitis induction, this reshaped microbiota responds differently compared to that of wildtype, which is revealed by the internal microbial networks. Strikingly, this special microbiota composition confer Nlrp3^{R258W} mice protection against DSS induced acute colitis as well as AOM plus DSS induced colorectal cancer. Mechanistically, we found the mutated Nlrp3^{R258W} protein mainly acts in lamina propria CD11C+ macrophages. Interestingly, it was IL-1 but not IL-18 mediating the microbiota change and resistance to colitis. In summary, we demonstrate that the Nlrp3^{R258W} mutation led to a shift in the gut microbiota of the mice, which turned out to be a key causative factor to mediate the resistance to colitis and colorectal cancer.

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High-affinity, poly-reactive IgA is required for gut homeostatic maintenance to prevent colitis in mice

Okai, S.¹, Usui, F.¹, Hasegawa, M.², Nakamura, T.³, Yamamoto, K.⁴, Nishiyama, E.⁴, Mori, H.⁴, Yamada, T.⁴, Kurokawa, K.⁵, Matsumoto, S.⁶, Nanno, M.⁶, Naito, T.⁶, Kato, T.⁷, Miyauchi, E.⁷, Ohno, H.^{7,8,9}, Shinkura, R.¹
¹Nagahama Institute of Bio-Science and Technology, Immunology, Shiga, Japan, ²Nagahama Institute of Bio-Science and Technology, Protein Function Analysis, Shiga, Japan, ³Nagahama Institute of Bio-Science and Technology, Epigenetics, Shiga, Japan, ⁴Tokyo Institute of Technology, Graduate School of Bioscience and Biotechnology, Tokyo, Japan, ⁵Tokyo Institute of Technology, Earth-Life Science Institute, Tokyo, Japan, ⁶Yakult Central Institute, Tokyo, Japan, ⁷RIKEN Center for Integrative Medical Sciences, Kanagawa, Japan, ⁸Chiba University, Graduate School of Medicine, Chiba, Japan, ⁹Yokohama City University, Graduate School of Medical Life Science, Kanagawa, Japan

Growing evidence suggests that dysbiosis resulting from compromised immune responses has a role in the pathogenesis of inflammatory bowel disease (IBD). Immunoglobulin A (IgA) is the main antibody isotype secreted into the intestinal lumen. It plays a critical role in the defense against pathogens and in the maintenance of intestinal homeostasis. However, the molecular mechanisms of how secreted IgA (SIgA) regulates intestinal microbiota are not completely understood but a plausible explanation is that it selectively coats disease-associated bacterial taxa. In this study, we isolated a monoclonal IgA antibody (clone W27), which had high-affinities to a variety of commensal bacteria. Activation-induced cytidine deaminase mutant (AID^{G235}; glycine to serine at the 23rd amino acid) mice produce only low-affinity Igs due to somatic hypermutation (SHM) defect and develop lymph proliferative disease associated with of dysbiosis. Oral administration of the W27 IgA modulated gut microbiota composition and prevented lymph proliferative disease in AID^{G235} mice. Identical beneficial effects of W27 were observed in different models of colitis. Thus a high-affinity, poly-reactive IgA oral treatment is a potential remedy for IBD, acting through restoration of the host-microbial symbiosis.

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Microbiota of the small intestine are selectively engulfed by phagocytes of the lamina propria and Peyer's patches

Morikawa, M., Tsujibe, S., Shibata, J., Watanabe, Y., Nagaoka, N., Shida, K., Matsumoto, S.

Yakult Central Institute, Kunitachi, Japan

Phagocytes, such as dendritic cells and macrophages that are distributed in the small intestinal mucosa, play a crucial role in maintaining mucosal homeostasis by sampling the luminal gut microbiota. However, there is limited information regarding the microbial uptake in steady state. We investigated the composition of gut microbiota that is engulfed by the phagocyte subsets in the small intestinal lamina propria (SILP) and Peyer's patches (PP) from C57BL/6 mice.

On the basis of CD11b/CD11c expression patterns, SILP and PP cells were divided into four and three subsets, respectively. Each phagocyte subset exhibited distinct immune properties, such as cytokine gene expression and abilities to induce helper T cell subsets from naïve T cells. Interestingly, phagocytes that had engulfed bacteria were observed in every subset using FISH. Analysis of bacterial 16S rRNA gene amplicon sequencing revealed 1) all the phagocyte subsets in the SILP mostly engulfed *Lactobacillus* (the most abundant microbe in the small intestine), whereas CD11b^{hi} and CD11b^{hi}CD11c^{hi} cell subsets in PP mostly engulfed segmented filamentous bacteria (indigenous gut bacteria in rodents), and 2) among the *Lactobacillus* species engulfed by the SILP cell subsets, *L. murinus* were engulfed more often than *L. taiwanensis*, although both these *Lactobacillus* species were abundant in the small intestine.

These results suggest that small intestinal microbiota is selectively engulfed by phagocytes that localize in the adjacent intestinal mucosa under a steady state. These observations may provide insight into the crucial role of phagocytes in immune surveillance of the small intestinal mucosa.

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Zymosan induces Th17 cell differentiation by promoting IL-6 secretion from small intestinal lamina propria dendritic cells

Park, A.¹, Jin, S.², Yang, B.-G.³, Hong, C.-P.¹, You, D.-J.², Jang, M.H.³, Kim, Y.-M.^{1,4}

¹Pohang University of Science and Technology, Division of Integrative Biosciences and Biotechnology, Pohang, Korea, Republic of, ²Osaka University, World Premier International Research Center Initiative-Immunology Frontier Research Center, Suita, Japan, ³Academy of Immunology and Microbiology, Institute for Basic Science, Pohang, Korea, Republic of, ⁴Pohang University of Science and Technology, Department of Life Sciences, Pohang, Korea, Republic of

The homeostasis of small intestinal immune system is regulated by food antigens and molecules derived from commensal microorganisms. Certain commensal bacteria control CD4+ T cell differentiation and specifically induce Th17 or Treg cells. Commensal microorganisms also include fungi such as *Saccharomyces cerevisiae* and *Candida albicans*, however much less is known on the regulation of intestinal immune system

by these commensal fungi. To understand how fungi affect the intestinal immune system, we first investigated the effects of zymosan, a cell wall derivative of *Saccharomyces cerevisiae*, on lamina propria dendritic cells (LP-DCs). We found that zymosan specifically induced IL-6 in LP-DCs but not in splenic dendritic cells (SP-DCs) whereas both cell types produced IL-12p40 and TNF- α upon zymosan stimulation. Zymosan can signal via TLR-2 and dectin-1. By using TLR-deficient mice and dectin-1-blocking antibodies, we found that IL-6 induction by zymosan in LP-DCs is exclusively mediated by dectin-1. Correlated with production of IL-6, zymosan-activated LP-DCs, but not SP-DCs, induced differentiation of naive CD4⁺ T cells into Th17 cells and concomitantly inhibited Treg differentiation in the presence of TGF- β *in vitro*. We confirmed that these effects by zymosan depends on IL-6 by using dendritic cells from the IL-6-deficient mice. Furthermore, oral delivery of zymosan significantly increased small intestinal Th17 cell population. These findings suggest that zymosan from commensal fungi may provide a Th17-promoting condition in the small intestine via the induction of IL-6 in LP-DCs.

2406

Oral immunization induces an effective mucosal immune response that protects mice against intravaginal HSV-2 challenge

Mulvey, P.¹, Aaskov, J.¹, Aldwell, F.², Beagley, K.¹

¹Queensland University of Technology, Brisbane, Australia,

²University of Otago, Otago, New Zealand

Immunity against genital Herpes Simplex Virus 2 (HSV-2) infection is dependent on local memory immune responses in the genital tract. Oral immunisation can induce mucosal immune responses in the genital tract but has not been tested against viral STIs. Here we describe the mucosal immune responses elicited by an oral vaccine that protects mice against intravaginal challenge from lethal HSV-2 infection. Control of HSV-2 was attributed to HSV-2-specific IgG and IgA in the genital mucosa together with CD4 and CD8 T cells recruited to the genital epithelia. Furthermore, combining this vaccine with vaginal application of non-specific inflammatory or chemoattractants, CXCL9 and CXCL10, led to the recruitment of CD8 tissue resident memory cells in the genital epithelia that controlled new infection with HSV-2. Thus, this represents the first oral vaccine that can protect mice against lethal intravaginal HSV challenge.

3142

Interferon- ϵ -regulated gene expression patterns in protection against female reproductive tract infection

Mayall, J.¹, Mangan, N.², Chevalier, A.¹, Kim, R.¹, Rae, B.¹, Hertzog, P.², Horvat, J.¹, Hansbro, P.¹

¹Hunter Medical Research Institute & The University of Newcastle, New Lambton Heights, Australia, ²Hudson Institute of Medical Research and Monash University, Clayton, Australia

Chlamydia trachomatis is the most common sexually transmitted bacterial infection and frequently causes female reproductive tract (FRT) sequelae such as infertility. However, the processes

involved in clearance and immunopathology of *Chlamydia* infection are not well understood. In previous studies, we showed that interferon (IFN)- ϵ is constitutively expressed in the FRT and plays an important role in protecting against *Chlamydia* infections from the earliest stages of infection. To elucidate the mechanisms of IFN- ϵ -mediated protection, we examined the effects of IFN- ϵ on the expression of genes in the FRT in *Chlamydia muridarum* and sham-infected, wild type and IFN- ϵ deficient ($\epsilon^{-/-}$) C57BL/6 mice using whole-genome microarray-based analyses. IFN- ϵ uniquely regulated the expression of 744 genes at baseline and 802 genes during *Chlamydia* infection, and universally regulated the transcription of 61 genes, regardless of infection status. The majority of these transcripts were down-regulated in IFN- $\epsilon^{-/-}$ mice, particularly during infection, and pathway analysis revealed that many of these genes are involved in immune processes. Transcripts associated with leukocyte haematopoiesis, infiltration, and communication were down-regulated in IFN- $\epsilon^{-/-}$ mice at baseline. In *Chlamydia*-infected IFN- $\epsilon^{-/-}$ mice, pathways associated with innate responses, such as IFN regulatory factor activation and pattern recognition receptor signalling were down-regulated. Interestingly, several metabolic pathways were also dysregulated during IFN- ϵ deficiency, highlighting the intimate relationship between host metabolism and defence. These data demonstrate that IFN- ϵ both regulates baseline expression of a wide variety of genes and primes for the rapid expression of factors involved in the host response to *Chlamydia* infections in the FRT.

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1143

Mitochondrial stress contributes to differential type I interferon induction by *Mycobacterium tuberculosis* and *Mycobacterium africanum*

Wiens, K.¹, Ernst, J.²

¹NYU School of Medicine, Pathology, New York, United States, ²NYU School of Medicine, Medicine, New York, United States

Type I interferon (IFN $\alpha\beta$) induction varies by *Mycobacterium tuberculosis* (Mtb) strain and by patient disease state, and may contribute to Mtb pathogenesis. The mechanism for variation in IFN $\alpha\beta$ induction is thought to lie in the cytosolic signaling pathway upstream of IFN β transcription. In this pathway Mtb gains access to the cytosol and then triggers STING signaling, either directly or through DNA binding to cGAS in the cytosol. In order to examine this mechanism we infected bone marrow derived macrophages with two Mtb strains and a *Mycobacterium africanum* (Maf) strain. We found that Maf induced less IFN β than either Mtb strain induced. Differences in IFN β were not due to differences in bacterial numbers and were entirely dependent on STING and cGAS. We found that Maf accessed the host cytosol to the same extent as Mtb. We also found that Maf shed similar amounts of bacterial DNA as Mtb. Intriguingly, Maf infection was associated with reduced mitochondrial DNA in the cytosol. Correspondingly, Maf infection was associated with reduced mitochondrial stress as measured by ATP and superoxide production. Finally, we found that treating macrophages with a mitochondria-specific antioxidant reduced

the differences in IFN β induction between the strains. Our results indicate that Maf induces less mitochondrial stress, which results in the accumulation of less mitochondrial DNA in the cytosol and lower IFN β induction. This suggests that mitochondrial dynamics contribute to IFN β induction by Mtb and may also contribute to mycobacterial virulence.

3445

Infection of mice by *Trypanosoma brucei* ameliorates host immune response against *Brucella melitensis*

Machelart, A.¹, Potemberg, G.¹, Truyens, C.², Romano, M.³, Pays, E.⁴, De Trez, C.⁵, Magez, S.⁵, Letesson, J.J.¹, Muraille, E.²

¹University of Namur, Namur, Belgium, ²Université libre de Bruxelles, Laboratory of Parasitology, Bruxelles, Belgium, ³Scientific Institute for Public Health, Bruxelles, Belgium, ⁴Université libre de Bruxelles, Laboratory of Molecular Parasitology, Bruxelles, Belgium, ⁵Vrije Universiteit Brussel, Bruxelles, Belgium

Brucella spp. are facultative intracellular bacterial pathogens responsible for brucellosis, a worldwide zoonosis that causes abortion in domestic animals and chronic febrile disease associated with serious complications in humans. There is currently no approved vaccine against human brucellosis, and antibiotic therapy is long and costly.

Identification of key factors regulating host resistance to brucellosis or other pathogens is classically performed in immunologically naive animals. This approach is highly reductionist and ignores that host contract frequently several pathologies at the same time. In order to characterize the impact of parasitic infection on the course of *Brucella* infection, we administrate *Trypanosoma brucei*, an extracellular protozoan responsible for African trypanosomiasis, in *Brucella melitensis* infected mice.

Surprisingly, our results demonstrate that *T. brucei* infection facilitates the control and the elimination of *B. melitensis* by the host immune system. A large proportion of coinfecting mice display no detectable bacteria in spleen at the time where *Brucella* is always detected in control mice. Higher control of *Brucella* infection appears correlated with a boost of IFN- γ producing CD4⁺ T cells induced by *T. brucei* infection. The importance of CD4⁺ T cells in the *T. brucei*-mediated control of infection is confirmed by the absence of *Brucella* clearing in spleen of CD4 genetically deficient mice.

1449

Brahma complex modulates immune signalling in *Drosophila melanogaster*

Valanne, S.¹, Järvelä-Stöltzing, M.¹, Vanha-aho, L.-M.¹, Vesala, L.¹, Myllymäki, H.¹, Harjula, S.-K.¹, Rämetsä, M.^{1,2,3}

¹University of Tampere / BioMediTech, Laboratory of Experimental Immunology, Tampere, Finland, ²University of Oulu, PEDEGO Research Unit, and Medical Research Center Oulu, Oulu, Finland, ³Oulu University Hospital, Department of Children and Adolescents, Oulu, Finland

Drosophila melanogaster is a widely used model in immunology. *Drosophila* defence against yeast and bacteria is mainly

mediated by two evolutionarily conserved NF-kappaB signalling pathways, the Toll and the Immune deficiency (Imd) pathway. Although the core Toll signalling is well-known, the negative regulation of Toll signalling is poorly understood. In our genome-wide RNAi *in vitro* screen in S2 cells, we identified 14 genes that negatively regulate Toll pathway activity. Among these genes there were four components of the so-called Brahma complex that is involved in chromatin remodelling. We studied the *in vivo* significance of the Brahma complex in immune signalling using the UAS-GAL4 method in *D. melanogaster*. The RNAi flies were crossed to the C564-GAL4 driver, to silence the expression of two members of the Brahma complex (*brahma* and *osa*) in the fat body (equivalent to mammalian liver). The progeny flies were infected first with Gram-positive bacteria, *Micrococcus luteus*, to activate the Toll pathway. 24h post infection, the flies were further infected with more virulent Gram-positive bacteria, *Enterococcus faecalis*. The infection experiments demonstrate that silencing of components of the chromatin remodelling Brahma complex increases survival of the flies against *E. faecalis*. Quantitative PCR analysis of antimicrobial peptide gene expression further suggested that the Brahma complex negatively regulates the Toll pathway. These results indicate that DNA modification may play a role in the regulation of *Drosophila* Toll signalling. Further studies will elucidate the more specific function of the Brahma complex in *Drosophila* immune response.

1115

TLR2/1 orchestrate human pDC response to Gram+ bacteria

Rajeli, S., Soumelis, V.

Institute Curie, U 932, Paris, France

Tuberculosis is worldwide life-threatening disease. Moreover, infections by Gram+, multi-drug resistant bacteria are emerging as a cause of mortality. In both cases new studies are highlighting the pathological role of Type I interferon (IFN-I). Plasmacytoid dendritic cells (pDCs) produce high amounts of IFN-I following viral sensing. Recent evidence suggests that human pDCs might sense bacteria. However, human pDCs are reported to express only few pathogen pattern recognition receptors, namely Toll like receptor (TLR)-7 and TLR9. The receptors mediating bacterial sensing in pDCs are not known.

We show here that pDCs respond to Gram+ bacteria *Mycobacterium tuberculosis*, *Staphylococcus aureus* and *Listeria monocytogenes* by upregulating costimulatory molecules and secreting IFN-I. pDCs activation in these settings was blocked by a TLR2/1 competitive antagonist (CU-CPT22). Our work showed that human primary pDCs express TLR1 at the mRNA and protein level. We demonstrated that pDCs respond to TLR2/1 synthetic ligand (Pam3CSK4) with the up-regulation of costimulatory molecules (as MHCII, CD80 and CD86) and cytokines secretion (IL-6, TNF- α , IFN-I). In human primary pDC we found that in response to bacterial lipoproteins up-regulation of costimulatory molecules is TLR1-dependent while IFN-I secretion is TLR2-dependent. TLR2/1 ligand-stimulated pDCs primed naïve CD4+ T-cells, inducing proliferation and differentiation to TH1/TH2 subsets.

Overall, we showed the pDCs sense Gram+ bacteria through

the TLR2/1 pathway, and suggest a differential role for TLR1 and TLR2 in the induction of IFN- λ . This work provides the rationale to explore pDCs in human bacterial infection.

2046

Functional and structural insights into a unique SEIXy staphylococcal superantigen

Langley, R.¹, Ting, Y.T.², Radcliff, F.¹, Clow, F.¹, Young, P.², Choi, J.¹, Baker, H.², Fraser, J.¹

¹The University of Auckland, Faculty of Medical and Health Sciences and The Maurice Wilkins Centre for Molecular Biodiscovery, Auckland, New Zealand, ²The University of Auckland, The School of Biological Sciences and The Maurice Wilkins Centre for Molecular Biodiscovery, Auckland, New Zealand

Staphylococcus aureus is a serious human pathogen. It causes higher rates of disease in New Zealand than other developed countries and is a major cause of skin and soft tissue infections, bacteraemia, and endocarditis. Its success as a pathogen can be attributed to the large number of diverse virulence factors it produces. The superantigen (SAg) family of toxins is a major class of virulence factors with twenty four distinct members identified to date. SAGs are infamously known for causing staphylococcal toxic shock syndrome and have been shown to be important for bacterial persistence and immune evasion during infection. By concurrently binding and activating major histocompatibility complex (MHC) class II molecules on antigen-presenting cells and T-cell receptors (TCRs) on T lymphocytes bearing susceptible V β regions SAGs promote massive activation of immune cells, leading to the release of inflammatory mediators that can ultimately result in hypotension, shock, organ failure, and death. The most recently discovered SAg, Staphylococcal Enterotoxin-Like X (SEIX), is unique in several respects. In contrast to other SAGs, it is chromosomally located and thus present in over 95% of *S. aureus*. SEIX lacks homology with the known MHC class II binding sites of SAGs. It possesses a unique N-terminal domain and its C-terminal domain shows greater homology to the Staphylococcal Superantigen-Like (SSL) family of immune evasion molecules than the SAGs.

Here we present functional and structural evidence that SEIX is an SSL-like SAg with features of both related families and additional features that are unique to SEIX.

3077

PD-1 blockade rescues *Mycobacterium tuberculosis* specific polyfunctional T cell responses

Kamboj, D.¹, Thakral, D.¹, Mitra, D.K.¹, Mohan, A.², Basil, M.V.³

¹All India Institute of Medical Sciences, Department of Transplant Immunology and Immunogenetics, New Delhi, India, ²All India Institute of Medical Sciences, Department of Pulmonary Medicine and Sleep Disorders, New Delhi, India, ³V.P. Chest Institute, Department of Microbiology, New Delhi, India

Tuberculosis remains one of the leading causes of death worldwide. Th1 cytokines, particularly IFN- γ and TNF- α , are known to play a crucial role in protection against tuberculosis. IFN- γ is protective but not sufficient, whereas TNF- α alone can

lead to self tissue damage by causing necrosis. Therefore, the role of polyfunctional T cells is emerging in immunity against *Mycobacterium tuberculosis*. In this study, we found that polyfunctional T cell response was diminished in tuberculosis patients, which could be rejuvenated by blocking PD-1. PBMCs from tuberculosis patients were stimulated with *Mycobacterium tuberculosis* whole cell lysate in presence or absence of PD-1 blocking antibody and cytokine producing antigen specific T cells were determined using polychromatic flow cytometry. We demonstrated reduced frequency of *Mycobacterium tuberculosis* specific polyfunctional (CD4+IFN- γ +TNF- α) T cell responses in tuberculosis patients as compared to healthy controls. Blocking of PD-1 showed increased percentage rescue of polyfunctional response. This was because of higher expression of PD-1 on IFN- γ + TNF- α (dual positive) CD4 T cells as compared to the single cytokine producing T cells.

Further, we wanted to investigate the effect of cytokine milieu in altering the death pattern (apoptosis versus necrosis) of infected macrophages. The effect of cytokines (IFN- γ and TNF- α) either individually or in combination, was tested *in vitro* on MDM (monocyte derived macrophages) infected with *Mycobacterium tuberculosis* H37Rv strain. Our results suggest a critical role of IFN- γ and TNF- α in shifting the death of infected macrophages from necrosis towards apoptosis. This may have implications in the containment of infection.

3867

Understanding antigen specificity of CD4⁺ T cell responses in *Salmonella* infection is key to vaccine design

Wang, N.¹, Schittenhelm, R.B.², Purcell, A.W.², Scott, T.A.³, Kupz, A.⁴, Bedoui, S.¹, Strugnell, R.A.¹

¹Peter Doherty Institute for Infection and Immunity at The University of Melbourne, Department of Microbiology and Immunology, Melbourne, Australia, ²Monash University, Department of Biochemistry and Molecular Biology, Clayton, Australia, ³Institute of Structural and Molecular Biology, University College of London, London, United Kingdom, ⁴Max Planck Institute for Infection Biology, Berlin, Germany

Pathogenic serovars of *Salmonella enterica* is one of the leading causes of gastroenteritis and febrile diarrhoeal diseases including typhoid fever invasive non-typhoidal *Salmonella* (NTS) infections. Despite the disease burden and rising prevalence of antibiotic resistance, a highly efficacious vaccine against *Salmonella* is still lacking. Elucidating immunogenic *Salmonella* antigens is critical to rational vaccine design, but it has not been successfully resolved to date.

We have recently generated a novel live-attenuated vaccine (LAV) strain of *Salmonella*, denoted TAS2010. We observed that vaccination with TAS2010 conferred superior protection against lethal infection with wild-type *Salmonella* compared to vaccination with the benchmark LAV strain, BRD509. In contrast to BRD509, vaccination with TAS2010 elicited significantly increased activation of CD4⁺ T cells, alongside with increased inflammation as marked by increased neutrophil recruitment and elevated production of cytokines such as IFN- γ , TNF- α and IL-6. The magnitude of immune activation is positively correlated with net bacterial load during the first two weeks

post-vaccination, suggesting a fundamental tension between immunogenicity and safety in *Salmonella* LAV design. Ongoing studies utilise the TAS2010 LAV to elucidate *Salmonella* antigens that are key to optimal vaccine efficacy. Our results suggest that the population of antigen-specific CD4⁺ T cells lack apparent structure of immunodominance but is most likely polyclonal, with each specificity contributing incrementally to overall protection, highlighting the importance of antigen diversity in effective immunity mediated by CD4⁺ T cells during *Salmonella* infection. We anticipate this work will provide critical information relevant for future development of efficacious *Salmonella* vaccines.

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The plasmacytoid dendritic cells exert a tolerogenic function during a pulmonary model of fungal infection

Calich, V., Araújo, E., Medeiros, D., Araújo, V., Loures, F.
Institute of Biomedical Science, University of São Paulo,
Department of Immunology, São Paulo, Brazil

Paracoccidioidomycosis (PCM) is a systemic mycosis with the highest incidence in Latin America. A previous study has demonstrated that the resistance and susceptibility to *Paracoccidioides brasiliensis* were associated with diverse dendritic cell (DC) subpopulations. Furthermore, plasmacytoid DCs (pDCs), originally described as the main regulators of viral infections, was shown to promote an effective growth control of *Aspergillus fumigatus* hyphae. The purpose of this study was to evaluate the role of pDC during *P. brasiliensis* infection. pDCs were depleted in 129 mice with 250 µg of the anti-pDC antibody (BX44). The control group received equivalent doses of the GL113 antibody. After 72h, 2 and 8 weeks of infection with one million yeasts, the mice were sacrificed and several aspects of infection evaluated. The depletion resulted in a less severe disease. A higher frequency of activated T CD4⁺ and CD8⁺ lymphocytes was detected in the lungs of pDC-depleted mice when compared with control mice. In addition, the depletion resulted in a prominent expansion of Th1 cells and elevated numbers of activated macrophages and neutrophils to the lungs. The analysis of lung homogenates showed diminished levels of type I IFN as well as reduced levels of anti-inflammatory cytokines such as TGF-β and IL-10. Besides, reduced expression of Foxp3 (Treg) and indoleamine 2,3-dioxygenase (IDO) mRNA was found in the lungs of pDC-depleted mice. Taken together, our results suggest a non-redundant role of pDCs during *P. brasiliensis* infection, and demonstrate a tolerogenic function of these cells associated with Treg cells activation and IDO-mediated mechanisms.

Genetics

3184

Cross-talk between DNA break repair and chromosome conformation in developing lymphocytes

Collins, P.¹, Porter, S.¹, Purman, C.¹, Bednarski, J.¹, Sleckman, B.², Oltz, E.¹
¹Washington University, Pathology and Immunology, Saint Louis, United States, ²Cornell University, Department of Pathology and Laboratory Medicine, New York, United States

To maintain genomic stability, developing lymphocytes must deal with physiologic double strand breaks (DSBs) that are continually generated by the processes of V(D)J recombination. These genomic lesions must be repaired with high fidelity, minimizing oncogenic alterations such as chromosomal translocations. The DSB response leads to extensive revision of flanking chromatin, including phosphorylation of the histone variant H2AX (termed γ-H2AX), which spreads for 100s of kb from a DSB. In non-cycling cells, the γ-H2AX domain serves as a chromatin-based platform to facilitate repair by the non-homologous end joining (NHEJ) machinery. In this regard, mechanistic links between DNA repair and epigenetic landscapes around DSBs are beginning to emerge. A feature that may bridge these processes is the 3D conformation of chromatin flanking a DSB. However, the impact of DSBs on locus conformation and, conversely, the role of its reconfiguration in stabilizing DNA ends for repair, remain unexplored. We have used ChIP followed by sequencing to finely map and characterize γ-H2AX domains following persistent DSBs at antigen receptor (AgR) loci. We have discovered that DSBs in precursor lymphocytes induce compaction of chromatin over 100s of kb flanking DSB sites, paralleling the spread of γ-H2AX. This compaction of chromatin at broken Ig loci in pre-B cells is dependent upon a transducer of NHEJ signaling, 53BP1, which is recruited to the γ-H2AX domain. We provide evidence that conformational mechanisms are important to generate compact platforms for repair complexes and to spatially restrict DSBs from other regions of the genome.

1763

TLR4 plays a critical role in allowing the persistent, asymptomatic infection with a new commensal *Neisseria*

Powell, D.¹, Ma, I.^{1,2}, So, M.^{1,2}, Frelinger, J.^{1,2}
¹University of Arizona College of Medicine, Immunobiology, Tucson, United States, ²University of Arizona, BIO5 Institute, Tucson, United States

Bacteria of the *Neisseria* genus are part of the normal flora of many animals and humans. Two species, *Neisseria gonorrhoeae* and *Neisseria meningitidis*, are important human pathogens. A major limitation of studying *Neisseria* biology is the lack of a mouse model. Our group has isolated a new species of *Neisseria*, *N. musculi*, from a wild mouse that is able to colonize inbred CAST mice and persists in them at high levels for at least one year. Only CAST mice (among the 8 tested) are colonized and maintain high carriage levels indefinitely. B6 and B6-*rag*^{-/-} mice are relatively resistant to colonization. In contrast, B6 MyD88 deficient mice are colonized at high frequency and

high levels.

We compared the sequences of MyD88, CD14, TLR4, and MD2 in the 8 strains tested. The only unique coding polymorphism in CAST was found in the TLR4 gene. We hypothesized CAST mice might not recognize *N. musculi* lipopolysaccharide (LPS).

Splenocytes from CAST and C57BL/6 (B6) mice were stimulated with a variety of TLR ligands and secreted IL-1, IL-6 and TNF α were measured. Both strains of mice responded equally well to TLR9 ligand ODN, which requires functional MyD88; however, the response of CAST mice to purified *E. coli* LPS and intact *N. musculii* was diminished compared to B6. Thus, the diminished cytokine response was likely due to TLR4. To account for other differences in mouse strains we are currently expressing the TLR4s from both mouse strains in the same genetic background.

2028

Neuroinflammatory pathways and risk genes associated with Alzheimer's disease

Hodges, A.¹, Lim, Y.M.¹, Lupton, M.K.², Dumas, A.¹, King, A.³, Troakes, C.³, Murray, C.⁴, Lin, K.¹, Al-Sarraj, S.³, Kazakoff, S.², Waddell, N.², Pearson, J.V.², Sivakumar, L.¹, Papouli, E.⁵, Mirza, G.⁵, Saxena, A.⁵, Lashley, T.⁴, Powell, J.³

¹King's College London, Old Age Psychiatry, London, United Kingdom, ²QIMR Berghofer Medical Research Institute, Royal Brisbane Hospital, Brisbane, Australia, ³King's College London, Basic & Clinical Neuroscience, London, United Kingdom, ⁴University College London, Institute of Neurology, London, United Kingdom, ⁵NIHR Biomedical Research Centre, Genomics Core Facility, Guy's Hospital, London, United Kingdom

Growing evidence suggests inflammation contributes to Alzheimer's disease (AD). Activated microglia cluster around amyloid and correlate with disease symptoms. Inflammatory genes including TREM2 contribute to AD risk. We investigated whether additional rare variants in 112 genes from a TREM2 containing brain co-expression module may also contribute to AD risk. We also investigated the effects of TREM2 variants on microglia activation in post-mortem human brain to establish whether microglia behave similarly in AD patients regardless of their TREM2 genotype. We sequenced the exons of 144 genes (including controls) in 950 DNA samples from AD, MCI and control individuals. After de-multiplexing, alignment and quality control, we tested for variant association with AD. 80% of bases were covered at $\geq 12 \times$ read depth in the majority of samples. A number of rare variants were found to be associated with AD, suggesting multiple linked pathways may lead to AD vulnerability. Validation of these findings in independent cohorts is underway. We investigated microglia activation using the established markers CD68, Iba-1 and HLA in CA1 and CA4 hippocampal regions. AD/TREM2+ cases were found to have significantly fewer HLA and CD68-stained microglia compared to AD and control TREM2- cases, while Iba-1-stained microglia numbers were similar between groups. It appears having a TREM2 risk variant attenuates microglial activation in AD patients and there may be multiple points of vulnerability in microglia which can together contribute to AD pathology. These results have implications for strategies which target neuroinflammation to treat AD.

3768

Immunogenetic traits of a heritable CD56+ cell population dysregulated in Multiple sclerosis: clinical utility and implications for pathogenesis

Booth, D.¹, McKay, F.¹, Gatt, P.¹, Fewings, N.¹, Parnell, G.¹, Schibeci, S.¹, Barnett, M.¹, Slee, M.², Kermode, A.³, McCauley, J.⁴, Stewart, G.¹, Vucic, S.¹

¹University of Sydney, Westmead Institute for Medical Research, Sydney, Australia, ²Flinders University, Neurology, Adelaide, Australia, ³University of Western Australia, Medicine, Perth, Australia, ⁴University of Miami, John P. Hussman Institute for Human Genomics, Miami, Australia

Multiple Sclerosis (MS) is a common neurological disease driven, in part, by an autoimmune response. Genes affecting susceptibility have been identified and indicate many immune cell subsets contribute to pathogenesis. Recent studies have identified that the proportion of some immune cell subsets in peripheral blood is highly heritable, such that environmental factors have limited effect on their relative populations in individuals over time. We recently identified that a population of CD56+ cells are under-represented in MS from studies of multiple cohorts. In whole blood this dysregulation is tagged by expression of the MS genetic risk factors EOMES and TBX21. From transcriptomic studies a module of genes co-regulated with these two has been identified. This module was confirmed in independent cohorts from Sydney, Perth, and Adelaide in Australia; and Miami in USA; and the correlated gene set is present in controls and people with MS. MS risk factor genes are over-represented in the module genes. The downregulated genes in MS control cytolytic activity and immune cell trafficking. Expression of the module genes is tightly associated with Vitamin D activation, suggesting an interaction of these environmental and genetic risk factors which may be tractable to therapeutic intervention. MS therapies fingolimod and tysabri affect this cell population. The clinical utility of this phenotype is discussed.

1812

Diversity of Killer cell immunoglobulin-like receptors (KIRs) and HLA ligands in susceptibility to dengue infections in northeastern Thais

Chaisri, S.^{1,2}, Jumniansong, A.^{2,3}, Romphruk, A.V.^{2,4}, Leelayuwat, C.^{2,3}

¹Chulabhorn International College of Medicine (CICM), Thammasat University, Pathum Thani, Thailand, ²Khon Kaen University, Centre for Research and Development of Medical Diagnostic Laboratories (CMDL), Faculty of Associated Medical Sciences, Khon Kaen, Thailand, ³Khon Kaen University, Department of Clinical Immunology and Transfusion Sciences, Faculty of Associated Medical Sciences, Khon Kaen, Thailand, ⁴Khon Kaen University, Blood Transfusion Center, Faculty of Medicine, Khon Kaen, Thailand

Killer cell immunoglobulin-like receptors (KIRs) are cell surface receptors on natural killer (NK) cells and subsets of T cells. The interactions between KIRs and human leukocyte antigen (HLA) class I molecules on target cells can modulate function of NK cells in responses to infected or transformed cells. In order to study

genetic variations of *KIRs* and *HLA* ligands relevant to dengue infections, a case-control study was investigated in 253 dengue infections and 235 healthy controls in northeastern Thais (NETs) using polymerase chain reaction with sequence specific primer (PCR-SSP) technique. *KIR2DS1* was significantly increased in healthy control (47.2% vs 37.6%, $p = 0.030$), whereas *KIR2DL3* and *2DS4F* were significantly increased in dengue infections with 96.4% vs 90.2%, $p = 0.005$ and 61.7% vs 52.8.2%, $p = 0.028$, respectively. For analysis of *HLA* ligands, the frequencies of *HLA-C1* were significantly higher in the control group (97% vs, 92.1% $p = 0.017$), but *HLA-A11* was significantly lower in dengue infections (60.4% vs 78.7% $p < 0.001$). *KIR+HLA* combinations in dengue infections were analyzed. Consequently, a *KIR2DS4F+A11* combination was positively associated with dengue infections (49% vs 30.2%, $p < 0.001$), whereas a negative association of *KIR2DL2+ C1* was found (24.8% vs 38%, $p = 0.026$). Moreover, B haplotype showed a possible protective factor against dengue infections. This study indicates that the diversity of *KIR* and *HLA* ligands is associated with dengue infections. *KIR-HLA* interactions would play an important role in immune responses and susceptibility to dengue infections.

2473

Identification of differentially methylated genes in purified disease relevant blood cell populations in patients with spondyloarthritis

Miceli-Richard, C.^{1,2}, Bugge Tinggaard, A.³, Wang-Renault, S.-F.³, Busato, F.³, Dougados, M.¹, Tost, J.³

¹Université Paris Descartes (Paris V), Rheumatology - Hôpital Cochin, Paris, France, ²Pasteur Institute of Paris, Immunoregulation, Paris, France, ³Institut de Génomique - Centre National de Génotypage - CEA, Epigenetics, Evry, France

Background: Spondyloarthritis (SpA) is a complex disease involving genetic, epigenetic and environmental contributions to disease risk.

Objective: This study aimed to perform a genome-wide DNA methylation analysis in sorted CD4 and monocytes from SpA patients compared with controls.

Methods: Genome-wide DNA methylation patterns were analyzed in cell-sorted (MACS) monocytes and CD4 T-lymphocyte populations from 24 SpA patients and 16 controls using the Illumina 450K Infinium Human Methylation 450K BeadChip allowing the simultaneous quantitative monitoring of more than 480,000 CpG positions.

Results: In CD4 cells 122 CpGs in 82 promoter regions of genes were found to be differentially methylated using stringent quality thresholds including several genes involved in disease-relevant signaling cascades such as Wnt-signaling and genes in which genetic polymorphisms have previously been associated with susceptibility to SpA. With 158 CpGs located in 86 promoter regions, slightly more genes were found to be differentially methylated in monocytes. Differentially methylated loci included again genes in Wnt signaling and bone metabolism, osteoblast or chondrocyte-specific genes as well as genes that have previously been shown to be implicated in related diseases such as psoriasis. A single gene was found differentially methylated in both monocytes and T-lymphocytes underlining

the importance to perform these epigenetic analyses in purified cell populations.

Conclusions: This study is the first to analyze genome-wide DNA methylation in purified disease-relevant blood cell populations in SpA bringing into evidence a moderate number of promoters whose deregulation might contribute to the pathogenesis of SpA.

4717

Association study involving polymorphisms in IL-6, IL1-RA, and CTLA4 genes and rheumatic heart disease in New Zealand population of Māori and Pacific ancestry

Azevedo, P.¹, Merriman, T.², Topless, R.², Sika-Paotonu, D.³, Crengle, S.⁴, Wilson, N.⁵, Percival, T.⁶, Lennon, D.⁷

¹Hospital Israelita Albert Einstein, Rheumatology, Sao Paulo, Brazil, ²University of Otago, Biochemistry, Dunedin, New Zealand, ³Victoria University of Wellington, GSNMH, Wellington, New Zealand, ⁴University of Auckland, Tomaiora Māori Health Research Centre, Auckland, New Zealand, ⁵Starship Children's Hospital, Paediatric Cardiology, Auckland, New Zealand, ⁶University of Auckland, Pacific Health, Auckland, New Zealand, ⁷University of Auckland, Paediatrics, Auckland, New Zealand

Introduction: Rheumatic Fever (RF) incidence among New Zealand (NZ) individuals of Māori and Pacific ancestry remains among the highest in the world. Polymorphisms in the IL-6, IL1RN, and CTLA4 genes have been associated with RF, and their products are modulated by new medications. Confirmation of these previous associations could help guide clinical approaches.

Objective: To test IL-6, IL1-RA (IL1RN), and CTLA4 functional SNPs in 204 rheumatic heart disease (RHD) patients and 116 controls of Māori /Pacific ancestry.

Method: Self-reported ancestry of the eight great-grandparents defined ancestry of participants. Severity of carditis was classified according to the 2012 World Heart Federation guideline for the echocardiographic diagnosis of RHD. The IL-6 promoter rs1800797, IL1RN rs447713 and CTLA4 rs3087243 SNPs were genotyped by Taqman. Correlations were assessed by logistic regression analysis adjusting for gender and ancestry.

Results: The IL-6 rs1800797 variant was significantly associated with RHD, being the carriers of the GG genotype 6.09 (CI 1.23; 30.23) times more likely to develop RHD than the carriers of the AA genotype ($p = 0.027$). No significant associations with RHD were found for IL1RN rs447713 and CTLA4 rs3087243. Patients carrying the G allele (GG plus AG genotype) for the IL1RN rs447713 SNP had 2.36 times (CI 1.00; 5.56) more severe carditis than those without this allele (the AA genotype) ($p = 0.049$).

Conclusion: The IL-6 promoter rs1800797 (-597G/A) SNP may influence susceptibility to RHD of people of Māori and Pacific ancestry living in NZ, and that the IL1RN rs447713 SNP may influence the severity of carditis in this population.

4193**Regulation of CD4 commitment is conserved between marsupial and placental mammals***Kappes, D.J., Hua, X., Mookerjee-Basu, J.**Fox Chase Cancer Center, Philadelphia, United States*

Lineage- and stage-specific transcriptional silencers play critical roles in control of gene expression and development. The ThPOK transcription factor acts as master regulator of CD4/CD8 lineage choice, which is necessary and sufficient for CD4 commitment. In mice, a silencer element (Sil^{ThPOK}) restricts ThPOK expression to MHC class II-restricted thymocytes. The Sil^{ThPOK} element is highly conserved at the DNA level between placental and marsupial mammals, raising the question of whether its function has also been preserved across this evolutionary span. To test this, we generated knockin mice, in which the murine Sil^{ThPOK} is replaced by the homologous marsupial (*Monodelphis domestica*) element. Remarkably, knockin mice display normal control of ThPOK transcription and lineage choice, indistinguishable from wt mice, as verified by crossing to MHC class I- and II-restricted TCR transgenes. Next we compared inherent functional properties of the ThPOK silencer with the CD4 silencer, another T lymphoid silencer, by carrying out reciprocal inter-chromosomal silencer swaps. Importantly, knockin mouse lines in which the Sil^{ThPOK} was replaced by the Sil^{CD4}, or vice versa, exhibited severe defects in CD4 expression and lineage-specification, indicating important inherent differences in their regulatory capacities. We show that the Sil^{ThPOK} encodes the unique capacity to discriminate between MHC class I- and class II-restricted TCR signals. Our data indicate that the function of the ThPOK silencer has been subject to severe evolutionary constraints, since at least the time of marsupial/placental divergence 165 million years ago, including the ability to respond selectively to MHC class II-restricted TCR signals.

45 Minute Oral

12:30:00 - 13:15:00

Microbiome

4198

Intestinal interleukin-17 receptor signaling mediates reciprocal control of the commensal microbiota and autoimmune inflammation

Kumar, P., Castillo, P., Kolls, J.

University of Pittsburgh, Department of Pediatric, Pittsburgh, United States

Despite recent advances in understanding the role of IL-17 in host immunity, its role in regulating enteric and systemic immune responses as well as its impact on the commensal microbiome has not been well studied. To further understand this, we have generated intestinal epithelial cells specific IL-17R knockout mice.

Our data shows that global (*Il17ra*^{-/-}) and intestinal epithelial cell specific (*Il17ra*^{fl/fl} *x villin* cre+) mice have overgrowth of segmented filamentous bacteria (SFB), suggesting a critical role of IL-17 signaling in SFB colonization. Higher SFB colonization in *Il17ra*^{fl/fl} *x villin* cre+ mice results in expansion of IL-17A and IL-22 producing Th17 cells. 16S microbial analysis demonstrated that the overall microbial community remained largely unchanged, however, a detailed analysis of operational taxonomic units (OTUs) revealed differential abundance of SFB, S24-7 and the *Clostridiales* family in *Il17ra*^{fl/fl} *x villin* cre+ mice. RNA sequencing data from mouse primary intestinal organoid and terminal ileum of SFB-colonized *Il17ra*^{fl/fl} *x villin* cre+ mice revealed a direct role of IL-17 in regulating *Nox1* (an apical NADPH oxidase) and *Pigr* expression. Furthermore, *Nox1*^{-/-} and *IgA*^{-/-} mice showed a higher degree of SFB colonization as compared to cohoused control WT mice. When subjected to experimental autoimmune encephalomyelitis, *Il17ra*^{fl/fl} *x villin* cre+ mice demonstrated earlier disease onset and worsened severity that was associated with increased intestinal *Csf2* expression and elevated systemic GM-CSF cytokine concentrations.

Our study highlights the importance of intestinal IL-17R signaling in the host-microbiome interaction and its impact on intestinal and peripheral autoimmune inflammation.

Oral Abstract Sessions

13:30:00 - 15:10:00

2118

Changes of microbiota, and their metabolites, in the development of T1D

McLeod, K.H.¹, Stanley, D.², Richards, J.L.¹, Yap, Y.A.¹, Moore, R.³, Mackay, C.R.¹, Marino, E.¹

¹Monash University, Biochemistry, Clayton, Australia, ²Central Queensland University, School of Medical and Applied Sciences, Rockhampton, Australia, ³MIT University, School of Applied Sciences, Bundoora, Australia

The rise of autoimmune diseases, such as type 1 diabetes (T1D) in the western world over the last 60 years has been of concern. The leading hypothesis for the increased prevalence of autoimmune illnesses is due to changes in diet. Understanding the microbiota and its metabolites is important to understand the link between diet and the development of T1D. Studies in twins suggested that differences in diabetes progression in identical twins could be due to altered microbiota. Here we use diets rich in resistant starches to target colonic bacteria in the non-obese diabetic (NOD) mice. We found that high acetate-yielding diets induced protection in the NOD mice towards increased abundance of *Bacteroides* genus and increased production of microbial short chain fatty acid (SCFAs) acetate. Similarly, germ free (GF) NOD mice were re-colonized with fecal acetate-modified microbiota showed delayed onset of T1D and higher abundance *Clostridium* and reduced *Parabacteroides*. This is consistent with one study, abundance of bacterial species that either produce acetate, such as *Bifidobacterium adolescentis*, or *Clostridium cluster XIVa* or *IV* species (which produce butyrate from acetate), correlated inversely with a number of B-cell autoantibody specificities. Interestingly, protected GF NOD mice re-colonized with acetate-shaped microbiota showed increased CD4⁺Foxp3⁺ Treg cells in the PLN. This is consistent with bacterially-derived butyrate directly promoting Treg cell differentiation. Our study highlights an alternative approach for the treatment or prevention of T1D in humans- use of specialized diets that operate at many levels to correct defects in gut microbiota and immune homeostasis.

1714

Anticancer probiotics: a novel tool of the oncological armamentarium

Daillère, R.¹, Vétizou, M.¹, Waldschmitt, N.², Chamaillard, M.², Plebanski, M.³, Cattoir, V.⁴, Gomperts Boneca, I.⁵, Zitvogel, L.¹

¹Gustave Roussy Cancer Campus, Villejuif, France, ²Institut Pasteur, Lille, France, ³Monash University, Melbourne, Australia, ⁴CHU de Caen, Caen, France, ⁵Institut Pasteur, Paris, France

The tumor microenvironment is influenced by anticancer therapies, and even more so by those affecting the gut homeostasis. We reported that a deviated repertoire of the intestinal microbiome called « dysbiosis », caused by broad spectrum antibiotics compromised the efficacy of

cyclophosphamide (CTX), an immunomodulatory alkylating agent exerting cytotoxic effects against cancer (Viaud, Science, 2013). Lately, we reported the importance of the gut microbiota in the efficacy of ipilimumab, a human monoclonal antibody targeting CTLA-4 (Vétizou, Science, 2015). Mechanisms underlying this gut-cancer axis in these two therapeutic contexts are different.

CTX is responsible for disrupting the gut barrier integrity as well as the intestinal homeostasis, allowing a NOD1/2-dependent translocation of several Gram-positive bacteria into secondary lymphoid organs. CTX breaks the intestinal tolerance towards the intestinal microbiota and leads to the immunization of the host against some bacterial strains. We identified a Gram positive bacteria, *Enterococcus hirae*, which markedly modulates the intestinal and systemic immunity through the elicitation of bacterial-specific Th1 and pathogenic Th17 cells. Moreover, we have shown that *E. hirae* is capable of enhancing tumor-specific CD4⁺ and CD8⁺ T cell responses against candidate tumor antigens. Finally, *E. hirae* specific-memory Th1 immune responses selectively predicted longer progression-free survival in advanced lung or ovarian cancer patients treated with immunotherapy and chemotherapy (platinum salts- or CTX-based chemotherapy).

Altogether, *E. hirae* represents a valuable probiotic against cancer, an oncobiotic ameliorating the efficacy of the most common alkylating immunomodulatory compound. Other oncobiotics discovered in the context of CTX will be presented at this meeting.

Early life host-microbiota interactions have lifelong consequences for humoral immunity

Poyntz, H.¹, Robinson, M.¹, Pitt, L.¹, Everitt, C.¹, Jones, A.¹, Plunkett, C.¹, van den Elsen, L.¹, Naidoo, K.¹, Jones, J.¹, Geuking, M.², McCoy, K.², Weyrich, L.³, Le Gros, G.¹, Forbes-Blom, E.¹

¹Malaghan Institute of Medical Research, Gut Inflammation, Wellington, New Zealand, ²University of Bern, Bern, Switzerland, ³University of Adelaide, Adelaide, Australia

The infant immune system co-evolves with the developing gut microbiota in a mutualistic relationship, providing signals that imprint immune health for life. Recent studies have demonstrated the immunomodulatory effects the microbiota exert on humoral immune development using germ-free mice and antibiotic treatment. However, examining the impact that naturally divergent gut microbial communities have on the development of humoral immunity remains to be elucidated.

We have developed a model system whereby two lines of BALB/c mice harbour naturally and prominently divergent gut microbiota compositions. These two lines of mice demonstrate disparate levels of immune activation, resulting in markedly different antibody responses. In turn, protective immunity to infectious disease and susceptibility to allergic disease is affected. Microbial transfer has identified a critical window in early life during which microbial imprinting of immune capability occurs and elucidated the key associated microbes. Taken together, we have established a critical window during the ontogeny of the immune system in which gut microbial composition may be therapeutically manipulated to promote optimal humoral immunity.

1478

Depletion of gut microbiota protects against renal ischemia reperfusion injury by reducing bone-marrow derived monocyte and renal resident macrophage maturation status and function

Emal, D.¹, Stroo, I.¹, Rampanelli, E.¹, Butter, L.M.¹, Teske, G.J.¹, Claessen, N.¹, Stokman, G.¹, Florquin, S.^{1,2}, Leemans, J.C.¹, Dessing, M.C.¹

¹Academic Medical Center, University of Amsterdam, Department of Pathology, Amsterdam, Netherlands, ²Radboud University Nijmegen Medical Center, Department of Pathology, Nijmegen, Netherlands

Background: An accumulating body of evidence shows that gut microbiota play an important role in health and disease by modulating local and systemic immunity. The importance of the microbiome in the development of kidney disease is however largely unknown.

Methods: To study this, we depleted gut microbiota with broad-spectrum antibiotics and performed renal ischemia reperfusion injury (IRI) in mice. In addition, we analysed the function and phenotype of bone-marrow derived (BMD)-monocytes and renal resident macrophages from antibiotic-treated mice.

Results: Our results reveal that depletion of microbiota significantly attenuates renal damage, dysfunction, tubular integrity and remote organ injury after renal IRI. We found a significant reduction in expression levels of F480, chemokine receptors CX3CR1 and CCR2 in renal resident macrophages and BMD-monocytes from antibiotic-treated mice. In addition, our data present that BMD-monocytes from gut flora depleted mice are less responsive to TLR2 but not TLR4 ligands and have a decreased migration capacity compared to control mice. To study whether observed phenotype/effects were driven by depletion of microbiota, we performed fecal transplantation in antibiotic-treated mice and show that fecal transplantation restores the expression level of F480/CX3CR1/CCR2 and reverses the protective effect against renal IRI.

Conclusions: In conclusion, our data strongly indicate that signals from the gut microbiota are essential for priming of renal resident macrophages and BMD-monocytes. Therefore, renal resident macrophages from gut flora depleted mice are less responsiveness to ischemic insult, resulting in protection against renal IRI.

568

Molecular characterization of alpha-galactosylceramides from commensal Bacteroidales and their immunomodulatory functions to the host

Oh, S.F.^{1,2}, Zheng, W.¹, Iyer, S.², Gensollen, T.², Blumberg, R.S.², Kasper, D.L.¹

¹Harvard Medical School, Boston, United States, ²Brigham and Women's Hospital, Boston, United States

Education of the immune system by commensal microbiota is recognized as critical to host development. The basic molecular mechanisms and effectors of these such processes are of considerable interests. Recently, we have identified alpha-galactosylceramides (aGCs) from gut commensal *Bacteroides fragilis*, as having an immunomodulatory impact on host natural

killer T (NKT) cells in the early stage of life. We have carried out multi-pronged (microbiological, chemical and immunological) approaches to understand the biological relevance of these symbiotic factors. Untargeted lipidomic profiling of related commensal Bacteroidales and non-Bacteroidales revealed that the aGCs are unique and limited to only two of more than 20 gut commensal species studied, as opposed to the most of commensals in genera *Bacteroides*, *Prevotella* and *Porphyromonas* can synthesize ceramides. Genome-wide metabolomic screening of *B. fragilis* transposon library with high-throughput MALDI-TOF/TOF mass spectrometry has identified multiple responsible target loci for aGC biosynthesis and further genetic characterization is under way, along with the *in vivo* functional analysis with monoclonized mice model.

1273

Targeting gut microbial composition to enhance protective immunity against influenza

van den Elsen, L., Poyntz, H., Jones, A., Plunkett, C., Forbes-Blom, E. Malaghan Institute of Medical Research, Wellington, New Zealand

Influenza remains a substantial public health burden with significant rates of morbidity, mortality and economic loss. The efficacy of influenza vaccination could be improved. Toll like receptors (TLR) play an important role in promoting antibody responses and can also adjuvant vaccine-induced antibody responses. It has recently been demonstrated that TLR-mediated sensing of gut microbiota is required for antibody responses induced by influenza vaccine. We investigated the effect of gut microbial composition on protective immunity in two lines of BALB/c mice with divergent gut microbiota (BALB/c A versus B). BALB/c A showed significantly increased serum concentrations of IFN- γ , MCP-1 and IL-12p70 compared to BALB/c B, especially 6h following systemic LPS or CpG administration. Body weight loss after intranasal influenza infection with HKx31 (H3N2) was lower in BALB/c A. On the contrary, systemic immunization with influenza only protected BALB/c B mice from secondary intranasal influenza infection as a result of higher antibody production in this line of mice. Vaccination with trivalent inactivated influenza vaccine (TIV) resulted in higher primary and secondary (5 days post boost) immune responses in BALB/c B mice, as measured as TIV-specific antibody titers. In conclusion, the innate versus adaptive immune responses to influenza are differentially altered in these two lines of mice with divergent gut microbiota.

3371

The effect of microbiome/Toll-like receptor interactions on susceptibility to type 1 diabetes

Jordan, M.A.¹, Stanley, D.², Moore, R.³, Crowley, B.¹, Baxter, A.G.¹

¹James Cook University, Comparative Genomics Centre, Townsville, Australia, ²Central Queensland University, School of Medical and Applied Sciences, Rockhampton, Australia, ³MIT University, Applied Sciences, Melbourne, Australia

Changes in microbiome, mucosal immunity and intestinal integrity have been associated with the onset of Type 1 Diabetes

(T1D) in children. Toll-like Receptors (TLR) have been associated all three factors. The role of TLR and their effects on microbiome in autoimmunity were studied by crossing TLR1,2,4,6,9 and MyD88 targeted deficiency mutations to the type 1 diabetes (T1D)-prone NOD mouse strain. While NOD.*Tlr9*^{-/-} and NOD.*Tlr6*^{-/-} mice were unaffected, T1D in NOD.*Tlr4*^{-/-} and NOD.*Tlr1*^{-/-} mice was exacerbated and that in NOD.*Myd88*^{-/-} and NOD.*Tlr2*^{-/-} mice ameliorated. Physical parameters of the intestines were compared; ileal weight was reduced in NOD.*Tlr1*^{-/-} mice. Similarly, by histology, these mice had reduced villus length and width. The intestinal microbiomes of NOD wild-type (WT), NOD.*Tlr1*^{-/-}, NOD.*Tlr2*^{-/-} and NOD.*Tlr4*^{-/-} mice were compared by high throughput sequencing of 16S ribosomal DNA (rDNA), in two cohorts, 18 months apart. Analysis of caecal 16S sequences clearly resolved the mouse lines and there were significant differences in beta diversity between the strains, with individual bacterial species contributing greatly to the differences in the microbiota of individual TLR-deficient strains. To test the relationship between microbiome and T1D, all strains were re-derived onto the parental NOD/Lt line and the incidence of T1D re-assessed within two generations. All rederived lines expressed an incidence of disease similar to that of the parental line. TLR deficiencies are associated with changes in microbiome; changes of microbiome are associated with T1D; the effects of TLR deficiencies on T1D appear to be mediated by their effects on gut flora.

3674

An expansion of rare lineage intestinal microbes characterizes rheumatoid arthritis

Taneja, V.

Mayo Clinic, Rochester, United States

Genetic and environmental factors are involved in predisposition to develop rheumatoid arthritis (RA). Although the etiology of RA is unknown, recent studies on the role of gut microbiota in adaptive immune response have led to the concept that interaction between the host microbiome and genetic factors influences autoimmunity. In this study, we characterized intestinal microbiome signatures in patients with RA. 16S ribosomal DNA from fecal samples of RA patients and healthy non-RA comparator subjects were sequenced and analyzed for an association between variables of interest and the overall microbiota composition. The machine learning algorithm 'Random Forest' was used to build a predictive model, and identify the most discriminatory taxa between patients and controls. Patients with RA exhibited decreased gut microbial diversity compared to controls. Increased rheumatoid factor levels and disease duration were associated with decreased species richness and diversity ($P < 0.05$ and $P < 0.1$ respectively). PERMANOVA based on Bray-Curtis distance showed that the structure of the microbiota of patients with RA differed significantly from control subjects. A taxon-level analysis suggested an expansion of the rare taxa, Actinobacteria, with a decrease in abundant taxa in patients with RA compared to controls. Prediction models based on the Random Forests algorithm suggested that 3 genera segregated with RA. These observations suggest dysbiosis in patients with RA resulting

from the abundance of certain rare bacterial lineages. A correlation between the intestinal microbiota and metabolic signatures could determine a predictive profile for disease causation, progression, and drug efficacy.

3208

Association of differential microbiota profile and cytokines expression levels at cervical level in the different stages of cervical cancer

Torres Poveda, K.J.¹, Audirac Chalifour, A.¹, Bahena Román, M.¹, Téllez Sosa, J.¹, Martínez Barnetche, J.¹, Delgado Romero, K.², Burguete García, A.¹, García Carrancá, A.³, Madrid-Marina, V.¹
¹Instituto Nacional de Salud Pública, Chronic Infections and Cancer Division, Cuernavaca, Mexico, ²Health Services of the State of Morelos, Capasam, Cuernavaca, Mexico, ³Instituto Nacional de Cancerología, Division of Basic Research, Cuernavaca, Mexico

The cervical cancer (CC) is given by HPV persistence due to the immunosuppressive tumor microenvironment generated by immunosuppressive cytokines such as IL-10. It is known that the vaginal microbiota determines the presence of certain cytokines locally. We assessed the association between cervical microbiota diversity and the histopathological diagnosis of each stage of CC, and evaluated IL-4, IL-6, IL-10, TGF- β 1, TNF- α and IFN-gmRNA expression levels in cervix across histopathological diagnosis and specific bacterial clusters. We determined cervical microbiota by High-throughput sequencing of 16S rDNA amplicons and classified it in community state types (CST). Mean difference analyses between alpha-diversity and histopathological diagnosis were evaluated. β -diversity analysis within histological diagnosis was carried out. Cytokine mRNA expression at the cervix level was analyzed across CST and histopathological diagnosis. We found a significant difference of microbiota diversity between NCL-HPV negative vs SIL and CC ($p=0.006$, $p=0.036$). When β -diversity was evaluated, the CC samples had the highest variation within groups ($p < 0.0006$) and largest distance compared to NCL-HPV negative ($p < 0.00001$). Predominant bacteria in women with normal cytology are *L. crispatus* and *L. iners*; *Sneathia* spp. in SIL and *Fusobacterium* spp. in CC. We found higher median levels of IL-4 and TGF- β 1 mRNA at the cervix level, in CST dominated by *Fusobacterium* spp. These results show that cervical microbiota may play a role in cervical cancer pathology.

3522

The immune pathogenesis of neonatal infection: relevant to digestive health

Kent, P., Merani, S., Xu, G., Elahi, S.

University of Alberta, Edmonton, Canada

Neonates are highly susceptible to disseminated infections, which are often fatal. The mechanisms underlying the susceptibility of neonates to infections and the molecular basis for the transition of immunologic function from fetal to postnatal life has remained a mystery. While most vaccines do induce protective immunity in older children and adults, their efficacy in the very young often requires further manipulation

and optimization. Recently, we have reported that CD71⁺ erythroid cells are physiologically enriched in neonatal mice and human cord blood with distinctive immune suppressive properties. Our most recent data indicate that CD71⁺ cells impair neonatal adaptive immune responses by suppressing T cell functions *in vitro* and *in vivo*. In addition, CD71⁺ erythroid cells by expression of PD-1 ligands (PDL-1 and PDL-2) and production of TGF- β could play an essential role in immune regulation. More interestingly, CD71⁺ cell mediated susceptibility to infection is counterbalanced by protection against aberrant immune cell activation in the intestine where brisk postnatal colonization with commensal microbes occurs. CD71⁺ cells quench excessive upregulation of TLRs induced by abrupt commensal colonization after parturition. Our data provide new insight into the role of this novel subset of "suppressor cells" in immunopathogenesis of neonatal infections and immune regulation early in life.

Late Breaker Symposium 1

4584

The transcription factor Myb plays an important role in eosinophil development

Fairfax, K., Bolden, J., Lucas, E., Hilton, D.

Walter and Eliza Hall Inst, Parkville, Australia

Eosinophils are important immune modulators, involved in the early innate inflammatory response to many pathogens, the most studied of which is the helminth. They function by engulfing small pathogens and secreting many proteins including peroxidases, RNases and enzymes, that destroy large pathogens (and host tissue). They also secrete chemokines and cytokines that heighten subsequent immune responses. However, the genetic control of the development of eosinophils is poorly understood (in contrast to innate lymphocytes). Myb is an important transcription factor involved in both erythroid and lymphocyte lineage specification, and our expression data shows it is highly expressed in eosinophil precursors, and present in eosinophils. Published data suggests Myb hypomorphs are eosinophil deficient, therefore we set out to examine the role of Myb in eosinophil development. In contrast to published reports, we show that Myb hypomorphic (Myb^{Plt4/Plt4}) mice do have eosinophils, however, these eosinophils have substantially altered side scatter properties (and thus are not detected using differential blood analysis). RNAseq and ELISA data suggests these eosinophils have reduced expression of a number of genes important for eosinophil function, such as eosinophil peroxidase. The eosinophils from Myb^{Plt4/Plt4} mice still resemble eosinophils by cyto-spin and TEM, however, preliminary experiments show a reduction in the number of crystalloid granules. Thus these cells resemble something that may represent an immature eosinophil. *In vitro* differentiation and *in vivo* challenge experiments show Myb^{Plt4/Plt4} eosinophils develop and recruit normally up to a certain stage, but are unable to mature into fully functional mature eosinophils *in vivo*.

4743

The use of *ex vivo* expanded human invariant natural killer T cells as a novel cell therapy to modulate graft versus host disease

Im, J.S.¹, He, H.², Besra, G.³, Shpall, E.J.², Porcelli, S.⁴, Molldrem, J.M.²

¹The University of Texas MD Anderson Cancer Center, Stem Cell Transplantation and Cellular Therapy, Houston, United States,

²The University of Texas MD Anderson Cancer Center, Houston,

United States, ³The University of Birmingham, Birmingham, United Kingdom, ⁴Albert Einstein College of Medicine, Bronx, United States

CD1d-restricted invariant Natural Killer (iNK) T cells are innate cells that can influence adaptive immunity toward inflammation or immune-suppression via production of Th1-type or Th2-type cytokines, respectively. Several studies suggest that iNK T cells may play a role in preventing graft versus host disease (GVHD) in allogeneic stem cell transplantation (ASCT), thus can become novel therapy to prevent GVHD in ASCT. Here, we sought to compare phenotypic and functional differences of expanded iNK T cells from cord blood (CB) and adult PBMC, and evaluated the modulatory property in a murine xenograft GVHD model. We successfully expanded polyclonal iNK T cells from CB or adult PBMC to a greater than 90% purity via co-culturing enriched iNK T cells with dendritic cells in the presence of α -GalCer and IL-2. Expanded CB iNK T cells were mostly CD4⁺CD25⁺Foxp3⁺ and preferentially produced Th2-type cytokines, while adult iNK T cells contained a varying degree of CD4⁺ and preferentially produced Th1 type cytokines. Despite preferential Th1 type responses, adult iNK T cells showed a trend towards decreased GVHD-related mortality in murine xenograft GVHD model (HR 0.3921, $p=0.0712$). In summary, we demonstrated it is feasible to selectively expand iNK T cells from CB and adult PBMC, and that expanded CB iNK T cells maintained regulatory phenotype and function, suggesting that CB might be a preferred source for iNK T cell therapy to modulate GVHD in ASCT. Further investigation is needed to delineate unique regulator-effector function of CD4⁺ vs CD4⁻ iNK T cells in modulating GVHD.

4551

Regulation of T cell receptor signaling by lipid metabolites

Wang, F.¹, Beck-García, K.², Zorzín, C.², Schamel, W.², Davis, M.¹

¹Stanford University, Stanford, United States, ²University of

Freiburg, Freiburg, Germany

Most adaptive immune responses require the activation of specific T cells through the T cell receptor/CD3 complex (TCR). Here we show that cholesterol sulfate (CS), a naturally occurring cholesterol metabolite, inhibits CD3 ITAM phosphorylation, a crucial first step in T cell activation. Biochemical studies show that CS disrupts TCR multimers, apparently by displacing cholesterol, known to bind TCR β . Moreover, CS deficient mice show a heightened sensitivity to a self-antigen, whereas increasing CS levels by intrathymic injection inhibits thymic selection, indicating that this molecule is an intrinsic regulator of thymocyte development. These results show a novel regulatory role for sterol metabolism in TCR signaling and thymic selection, highlighting the importance of the membrane microenvironment in modulating cell surface receptor activation.

4633**Antigen-linked rather than T_{FH} cell-derived signals select high affinity germinal centre B cells for plasma cell differentiation**

Suan, D.¹, Krautler, N.^{1,2}, Butt, D.¹, Bourne, K.¹, Hermes, J.¹, Schofield, P.¹, Jackson, J.¹, Phan, T.G.¹, Christ, D.¹, Brink, R.¹

¹Garvan Institute, Darlinghurst, Australia, ²ETH Zurich, Zurich, Switzerland

Preferential activation of high affinity germinal centre (GC) B cells provides the basis for serological immune protection and vaccine efficacy. Within GCs, B cells undergo somatic hypermutation (SHM) to modify the specificity of their clonally-defined B cell antigen receptor (BCR). Rare clones developing increased antigen affinity are either retained in the GC (positive selection) or differentiate into plasma cells (PCs) that secrete high-affinity antibodies. The key stimuli for GC B cells reside in the light zone (LZ) in the form of intact antigen held on follicular dendritic cells (FDCs) and T follicular helper (T_{FH}) cells that bind to processed antigenic peptides presented on the B cell surface. Whilst preferential provision of T_{FH}-derived help is thought to mediate the selective activation of high-affinity B cells, direct validation of this is lacking. To resolve this issue, we developed the capability to directly identify and compare high and low affinity GC B cells. High affinity LZ B cells (LZhi) showed greater cell cycle activation compared to low affinity cells (LZlo) but did not possess a gene expression signature consistent with preferential reception of T_{FH} cell help. Indeed, initiation of PC differentiation within the LZhi compartment did not require T_{FH}-cell help but was instead dependent on signals derived from direct engagement of intact antigen. T_{FH}-cell derived signals were subsequently required for the progression of PC differentiation and the migration of mature PCs out of the GC.

4450**Identification of broadly conserved cross-species protective Leishmania antigen and its responding CD4⁺ T cells**

Mou, Z., Okwor, I., Uzonna, J.

University of Manitoba, Immunology, Winnipeg, Canada

Recovery from natural or experimental infection with *Leishmania major* induces long-term protection to reinfection collectively known as infection-induced resistance. However, it is not known what antigens induce and maintain this resistance and whether these antigens preferentially favor the development of memory T cells. To identify protective *Leishmania* antigens, we eluted and identified naturally processed *L. major* peptides were from I-A^b MHC II molecules on infected BMDCs and by immunoproteomics approach. One of the peptides activated *Leishmania*-reactive T cells from mice that have healed their primary *L. major* infection *in vitro*. Interestingly, the source protein of this peptide, glycosomal phosphoenolpyruvate carboxykinase (PEPCK), was expressed in both the promastigote and amastigote stages of the parasite. Also, cellular immune responses against PEPCK were detected in *L. major*-infected patients, while antibody responses were detected in infected mice, dogs and human. I-A^b-PEPCK₃₃₅₋₃₅₁ tetramer identified for the first time protective *Leishmania*-specific CD4⁺ T cells at

clonal level, which comprised ~ 20% of all *Leishmania*-reactive CD4⁺ T cells at the peak of infection. PEPCK₃₃₅₋₃₅₁-specific CD4⁺ T cells are oligoclonal in their TCR usage, produce polyfunctional cytokines (IL-2, IFN- γ and TNF) and undergo expansion, effector activities, contraction and stable maintenance following lesion resolution. Vaccination with PEPCK peptide, DNA expressing full length PEPCK or rPEPCK induced strong durable cross-species protection in both resistant and susceptible mice. Given the remarkable effectiveness and durability of protection in vaccinated mice, our study suggests a real possibility for development of a broadly cross-species protective vaccine against different forms leishmaniasis by targeting PEPCK.

4360**LYVE-1 expressing macrophages as gatekeepers of large blood vessel homeostasis**

Angeli, V.¹, Lim, H.Y.¹, Lim, S.Y.¹, Tan, C.K.T.², Goh, C.C.G.³, see, P.³, Tan, N.S.T.², Ng, L.G.³, Ginhoux, F.³

¹National University of Singapore, Singapore, Singapore, ²Nanyang Technological University (NTU), Singapore, Singapore, ³A*STAR, Singapore, Singapore

Although lymphatic vessel endothelial hyaluronic acid receptor-1 (LYVE-1) is a specific marker for lymphatic vessels, it has also been reported on some tissue macrophages. However, the function of these macrophages has been largely overlooked and limited to embryonic development. Unexpectedly, imaging of adult skin, trachea and aorta revealed that LYVE-1 expressing macrophages are lining the outer layer of vessels exhibiting smooth muscle cells (e.g veins, arteries/arterioles) but not those displaying pericytes (capillaries). Parabiosis, bone marrow transplantation and fate-mapping revealed that these tissue-resident macrophages derive from fetal monocyte and mainly sustain in adulthood through self-renewal. Comparative gene-expression analysis showed that LYVE-1+ macrophages exert homeostatic rather than immune functions. Consistent with this, we found that severe depletion of LYVE-1+ macrophage in aorta following colony stimulating factor receptor-1 inhibition leads to arterial wall remodeling and arterial stiffness characterized by changes in collagen and elastin and decreased smooth muscle cell contractility. We have evidence that LYVE-1+ macrophages mediate this homeostatic function by acting in part on smooth muscle cells. Altogether, our data reveals LYVE-1+ macrophages as a previously unrecognized perivascular population of macrophages that are essential for maintaining large blood vessel integrity and function.

4728**Tissue-resident ILC2 and inflammatory ILC2: two distinct ILC populations**

Huang, Y.¹, Mao, K.², Chen, X.¹, Kawabe, T.¹, Zhu, J.¹, Urban Jr., J.F.³, Germain, R.N.², Paul, W.E.¹

¹National Institute of Allergy and Infectious Diseases, National Institutes of Health, Laboratory of Immunology, Bethesda, United States, ²National Institutes of Health, Laboratory of System Biology, National Institute of Allergy and Infectious Diseases, Bethesda, United States, ³Beltsville Human Nutrition Research

Center, Agricultural Research Service, USDA, Diet, Genomics, and Immunology Laboratory, Beltsville, United States

Innate lymphoid cells (ILCs) have important functions not only in immune defense against pathogens but also in allergy, inflammation, tissue remodeling and metabolic homeostasis. ILCs are generally considered as tissue-resident cells in the intestine, lung, skin and other mucosal surfaces, but ILC subsets have been also identified in non-mucosal sites, such as lymphoid organs and adipose tissues. We have reported the existence of a distinct ILC population, which is not present in these tissues under steady state conditions but appears in high numbers in lung, liver, mesenteric lymph nodes and spleen upon systemic administration of the cytokine IL-25 or *Nippostrongylus brasiliensis* infection. This cell population has been termed "inflammatory ILC2", as it only appears in diverse tissues under inflammatory circumstances (*Nat Immunol.* 2015 Feb; 16(2): 161-9). Inflammatory ILC2 cells develop into natural ILC2-like cells *in vitro* and *in vivo* and contribute to the expulsion of *N. brasiliensis*. They can also acquire IL-17-producing ability and provide partial protection against *Candida albicans*, indicating their multipotentiality or plasticity. Very recent experiments reveal that inflammatory ILC2 cells are not generated in peripheral, non-gut tissues *in situ*; they are circulating cells and are well exchanged in parabiosis mice in contrast to natural ILC2 cells that are tissue-resident in the steady state and barely exchange in parabiotic mice. Thus, inflammatory ILC2 cells are a unique circulating ILC population that is distinguishable from tissue-resident ILC subsets and that plays an important role in peripheral tissue host defense.

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4656

Bile acids control inflammation and metabolic disorder through inhibition of NLRP3 inflammasome

Guo, C., Xie, S., Chi, Z., Wang, D.

Institute of Immunology, School of Medicine, Zhejiang University, Hangzhou, China

Reciprocal interactions between the metabolic system and immune cells play pivotal roles in the pathogenesis of a variety of inflammatory diseases, but the underlying mechanisms are still unclear. The activation of bile acid-mediated signaling has been linked to improvement in metabolic syndromes and enhanced control of inflammation. Here, we demonstrate that bile acids inhibit NLRP3 inflammasome activation via the TGR5 signaling. TGR5 signaling induces the phosphorylation of NLRP3 on a single, evolutionarily conserved residue. Importantly, the TGR5-induced phosphorylation leads to the ubiquitination of NLRP3, and this serves as a critical brake on NLRP3 inflammasome activation. Several gain-of-function mutations in *Nlrp3* that cause cryopyrin-associated periodic fever syndromes (CAPS) disrupt the phosphorylation and ubiquitination of NLRP3 *in vitro*, potentially allowing the uncontrolled CAPS-associated activation of the NLRP3 inflammasome. In addition, *in vivo* results indicate that TGR5 activation blocks NLRP3 inflammasome-dependent inflammation, including

lipopolysaccharide-induced systemic inflammation, alum-induced peritoneal inflammation, and type-2 diabetes-related inflammation. Altogether, our study unveils a novel mechanism with combinational post-translational modifications of the NLRP3 protein through which bile acids endogenously constrain NLRP3-related inflammation. Thus, pharmacological targeting of TGR5 may be a potential strategy for the treatment of NLRP3 inflammasome-related diseases.

4444

A novel function of human pumilio proteins in innate immune responses

Narita, R.^{1,2}, Takahashi, K.³, Murakami, E.¹, Hirano, E.¹, Yamamoto, S.P.⁴, Yoneyama, M.⁵, Kato, H.^{1,6}, Fujita, T.^{1,6}

¹Institute for Virus Research, Kyoto University, Kyoto, Japan,

²Research Institute for Sustainable Humanosphere, Kyoto University, Kyoto, Japan, ³Department of Life Science, Gakushuin University, Tokyo, Japan, ⁴Osaka City Institute of Public Health and Environmental Sciences, Osaka, Japan, ⁵Medical Mycology Research Center, Chiba University, Chiba, Japan, ⁶Graduate School of Biostudies, Kyoto University, Kyoto, Japan

RIG-I-like receptor (RLR) plays a pivotal role in the detection of invading pathogens to activate type I interferon (IFN) gene transcription. Since aberrant IFN production is harmful to the host, RLR signaling is strictly regulated. However, the regulatory mechanisms are not fully understood. By expression cloning, we identified Pumilio proteins, PUM1 and PUM2, as candidate positive regulators of type I IFN induction. Overexpression of Pumilio proteins and their knockdown augmented and diminished IFN- β promoter activity induced by Newcastle disease virus (NDV), respectively. Both proteins showed a specific association with LGP2, but not with RIG-I or MDA5. Furthermore, all of these components were recruited to NDV-induced antiviral stress granules. Interestingly, biochemical analyses revealed that Pumilio increased double-stranded (ds) RNA binding affinity of LGP2; however, Pumilio was absent in the dsRNA-LGP2 complex, suggesting that Pumilio facilitates viral RNA recognition by LGP2 through its chaperon-like function. Collectively, our results demonstrate an unknown function of Pumilio in viral recognition by LGP2.

B Cells 2

3182

PU.1 and SPI-B transcription factors mediate Ig light chain accessibility during B cell development

Batista, C.R.¹, Solomon, L.A.¹, Xu, L.S.¹, Li, S.K.H.¹, DeKoter, R.P.^{1,2}

¹Western University, Microbiology & Immunology, London, Canada,

²Centre for Human Immunology, London, Canada

PU.1 and Spi-B are members of the E26 transformation-specific family of transcription factors and regulate the transcription of genes important for B-cell development. Mice carrying a conditional deletion of PU.1 in the absence of Spi-B, under the control of the *Cd79a* gene, show a severe block in B cell development in the bone marrow at the stage at which B

cells undergo rearrangement of immunoglobulin light chain genes (IgL). Therefore, we hypothesized that PU.1 and Spi-B are pioneer transcription factors mediating accessibility and rearrangement of IgL chain genes. In order to test this, we reintroduced PU.1 expression using a doxycycline-inducible system in an IL-7-dependent PU.1 and Spi-B knockout pro-B cell line lacking IgL rearrangements. We performed RNA and chromatin immunoprecipitation (ChIP) sequencing analysis to determine differential gene expression and the PU.1 binding profile in pro-B cells. We found that PU.1 changed the expression of 4528 genes, and bound to approximately 49,000 regions in the mouse genome. PU.1 peaks were identified at several sites in the immunoglobulin kappa (Igκ) and lambda (Igλ) chain loci, including in the Igκ3' and Igλenhancers. PU.1 bound to *Rag1* and *Rag2* genes and induced their transcription. PCR analysis confirmed that PU.1 induced transcription of unarranged Vκ regions, as well as rearrangement of the Igκ locus, indicating a role for PU.1 and Spi-B in controlling IgL accessibility for RAG-mediated rearrangement. Our findings point out a key role for PU.1 and Spi-B as pioneer factors mediating the pre-B cell checkpoint during bone marrow B cell development.

3130

Complete degradation of CD74-invariant chain by the endopeptidase SPPL2A is necessary for B cell survival

Bergmann, H.¹, Vega-Ramos, J.², Yabas, M.¹, Barthel, N.¹, Young, C.¹, Goodnow, C.C.³, Villadangos, J.², Enders, A.¹

¹John Curtin School of Medical Research, Department of Immunology and Infectious Disease, Canberra, Australia, ²The University of Melbourne, Peter Doherty Institute for Infection and Immunity, Melbourne, Australia, ³Garvan Institute of Medical Research, Immunology Division, Sydney, Australia

Mice lacking the intramembrane protease SPPL2A exhibited profound humoral immunodeficiency and specifically lack most mature B cells. Surviving B cells are characterized by low surface expression of the B-Cell Receptor (BCR) and BAFF-Receptor (BAFFR) and do not produce specific antibodies in response to immunizations. Mature B cell accumulation, but not expression of the BCR and BAFFR could be rescued by over-expression of the pro-apoptotic protein BCL2, indicating a survival defect of mature B cells.

On a molecular level we identified SPPL2A as the long sought-after, final enzyme in the sequence of CD74 (MHC II invariant chain) cleaving proteases. In the absence of SPPL2A the ultimate step of CD74 degradation is blocked. This causes a dramatic build-up of short N-terminal CD74 peptide fragments including the p8 product of Cathepsin S processing in the MHCII antigen presenting compartment. Accumulation of CD74 fragments was responsible for the cellular phenotype as deletion of CD74 in SPPL2A deficient animals reconstituted mature B cell numbers to levels seen in mice solely deficient in CD74, and also restored surface BCR and BAFFR expression. Despite a very similar accumulation of CD74 fragments, the observed cellular phenotype in SPPL2A^{-/-} mice strongly contrasts with the phenotype observed in Cathepsin S^{-/-} mice. Furthermore, block of CD74 egress from the ER through loss of MHCII did not prevent accumulation of CD74 N-terminal fragments. Taken together,

these results show a novel mechanism of CD74 degradation in B cells that is essential for B cell survival and function.

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Defining antigen specific plasmablast and memory B cell subsets in blood following viral infection and vaccination of humans

Ellebedy, A.¹, Jackson, K.², Boyd, S.², Ahmed, R.³

¹Emory University, Atlanta, United States, ²Stanford University, Stanford, United States, ³School of Medicine, Emory University, Atlanta, United States

Upon antigen exposure B cells eventually bifurcate into two functionally and phenotypically distinct subsets, plasmablasts and memory B cells. We previously reported that plasmablasts or antibody-secreting cells (ASCs) are transiently detected in blood shortly after infection or vaccination of humans. Here, we define the phenotype and the transcriptional program of a novel human antigen-specific B cell subset, referred to as activated B cells (ABCs). ABCs do not spontaneously secrete antibodies and possess a unique transcriptional profile that distinguishes them from ASCs and resting memory B cells. The clonal lineages of ABCs and ASCs are partially overlapping, and both show elevations in frequency among day 90 memory B cell pool compared to pre-vaccination levels, suggesting that they both contribute to longer-term B cell memory. ABCs and ASCs are clearly distinguishable in blood following influenza and Ebola virus infections in humans. Our data clearly suggest that ABCs could potentially be precursors for long-term memory B cells. Defining and studying ABCs along with ASCs in humans will reveal the intrinsic differences between B cells constituting the two compartments of humoral immune memory, which in turn could enhance our understanding of how to make better vaccines.

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Blimp1 controls plasma cell function through regulation of immunoglobulin secretion and the unfolded protein response

Tellier, J.^{1,2}, Shi, W.^{3,4}, Minnich, M.⁵, Liao, Y.^{2,3}, Crawford, S.⁶, Smith, G.K.^{3,7}, Kallies, A.^{1,2}, Busslinger, M.⁵, Nutt, S.L.^{1,2}

¹Walter & Eliza Hall Institute, Molecular Immunology, Parkville, Australia, ²The University of Melbourne, Medical Biology, Parkville, Australia, ³Walter & Eliza Hall Institute, Parkville, Australia, ⁴The University of Melbourne, Computing and Information Systems, Parkville, Australia, ⁵Research Institute of Molecular Pathology (IMP), Vienna, Austria, ⁶The University of Melbourne, School of BioSciences, Parkville, Australia, ⁷The University of Melbourne, Mathematics and Statistics, Parkville, Australia

The differentiation of plasma cells requires resetting of gene expression to silence B-cell transcription, whilst establishing antibody-secretory function and long-term survival. The transcription factors Blimp1 and Irf4 are essential for the initial differentiation of plasma cells, however their function in mature plasma cells has remained elusive. We have found that while Irf4 was essential for plasma-cell survival, Blimp1 was dispensable.

Blimp1-deficient cells retained the plasma-cell transcriptional signature, but lost the ability to secrete antibody. Blimp1 regulated many components of the unfolded protein response (UPR), including Xbp1 and Atf6. The overlap of Blimp1 and Xbp1 function was restricted to the UPR, with Blimp1 uniquely regulating mTOR activity and plasma cell size. Thus, Blimp1 is required for the unique physiological capacity of plasma cells that enables the secretion of protective antibody.

844 miRNAs are critical for the regulation of RAG expression and secondary Ig rearrangement in peripheral B lymphocytes

Coffre, M.¹, Benhamou, D.², Riess, D.³, Blumenberg, L.¹, Snetkova, V.¹, Chakraborty, T.³, Jensen, K.³, Chong, M.¹, Blleloch, R.⁴, Littman, D.¹, Skok, J.¹, Melamed, D.², Rajewsky, K.⁵, Korolov, S.¹

¹NYU School of Medicine, Dept of Pathology, New York, United States, ²Technion, Haifa, Israel, ³Harvard Medical School, Children's Hospital Boston, Boston, United States, ⁴UCSF, Urology, San Francisco, United States, ⁵Max Delbruck Center, Berlin, Germany

The differential expression of miRNAs throughout B cell development suggests that these ncRNAs contribute to stage-specific regulation of the intricate transcriptional program during B cell differentiation. Conditional ablation of Dicer or deletion of the miR-17~92 cluster in pro-B cells, revealed a critical role of miRNAs in B cell differentiation.

In the present study we analyzed mice in which enzymes critical for miRNA biogenesis, Dicer, Drosha and DGCR8 are conditionally ablated in B lymphocytes. Global ablation of miRNAs in B lymphocytes lead to an early block in B cell development. Rescue of B cell survival by overexpression of the anti-apoptotic factor Bcl2 revealed that in the absence of miRNAs, B cells in the periphery expressed low levels of Ig heavy chain without expressing light chain. We demonstrate that miRNA-deficient B cells fail to regulate recombination machinery in the periphery, resulting in ongoing Ig light chain gene rearrangement. In addition to the upregulation of RAG1/2 in peripheral B cells, we demonstrated ongoing DNA double strand breaks at Ig loci and upregulation of surrogate light chain components. We show that these events occur downstream of deregulated PI3K signaling and we recapitulate many of these defects in wild-type B lymphocytes by targeting individual components of PI3K signaling network. Furthermore, we achieve complete rescue of miRNA deficient B cells when we introduce a pro-survival Bcl2 transgene along with an Ig transgene resistant IgL editing. Our data highlight an important and novel role for miRNAs in the maintenance of a mature phenotype in peripheral B cells.

3644 Differential responsiveness of IgM and IgD B cell receptors regulate early B cell development and the activation of mature B cells

Übelhart, R.¹, Surova, E.², Iype, J.², Reth, M.², Juma, H.¹

¹University Hospital Ulm, Institute of Immunology, Ulm, Germany,

²Albert Ludwigs University Freiburg, Centre for Biological Signaling Studies (BIOSS), Freiburg, Germany

During development, B cells express IgM and IgD variants of their BCR. However, despite possessing similar antigen specificity, it is unclear why B cells shift from IgM to IgD during maturation. We have recently demonstrated that IgM-BCR possess the characteristic property of inducing signaling in response to monovalent antigens, while IgD-BCR required polyvalent antigens for activation.

To further confirm our findings, we addressed the function of IgM and IgD in the context of an autoreactive BCR expressed at the immature B-cell stage. While IgM efficiently supported BCR editing in response to the low-affinity autoantigen, expression of IgD supported editing of the autoreactive idiotype only upon engagement with the high-affinity autoantigen. Retention of the autoreactive IgD-BCR idiotype in the presence of the low-affinity autoantigen is accompanied by the loss of allelic exclusion of the Ig heavy-chain gene. In order to show that these findings also apply for human B cells, we generated a human mature BCR-deficient B-cell line and reconstituted expression of IgM- and IgD-BCRs with defined antigen specificities. We found that human mature B cells behave similar to murine B cells in that monovalent antigen stimulated only IgM-expressing cells, while those expressing IgD required polyvalent antigen for activation.

Taken together, these data demonstrate that the low activation threshold of IgM-BCR is essential for early B cell development and selection, while the increased expression of IgD on mature B cells is important for selective responses towards polyvalent antigens in immune complexes.

22 Effects of neonatal microbial exposure on B cell development and protection against allergic and autoimmune disease

Kearney, J.F.

University of Alabama at Birmingham, Microbiology, Birmingham, United States

Our goals in this project are to identify factors affecting the establishment of the mouse B cell clonal repertoire reactive to polysaccharide (PS)-associated epitopes expressed by a select group of bacteria and also occurring as self neo-epitopes. The antibody response to PS in immunized neonatal mice is delayed and protracted and follows similar kinetics regardless of the age at immunization. These results suggest that the advent of antibody production to PS antigens following neonatal immunization may reflect the maturation of the system more than expansion of antigen specific clones. Neonatal immunization (Day 3 v14) with *Streptococcus pyogenes* (expressing GlcNAc residues on the cell wall PS) results in identical alterations in the repertoire in absence of increased antibody titers convincingly demonstrating that these two effects are decoupled. Detailed examination of the anti-GlcNAc repertoire in single B cells shows a restriction in IGvH usage but includes many different nucleotide CDR3 sequences, however there is a remarkable degree of identical public translated protein sequences indicating a strong role for antigen selection in the development of the anti-GlcNAc B cell repertoire. Our preliminary studies of germ-free mice show that the commensal intestinal flora has a strong influence on

the selection and composition of the peripheral splenic B cell anti-GlcNAc repertoire. Finally we show that neonatal but not adult exposure of mice to bacteria has protective effects against the development of allergic airway disease to multiple allergens and dampens the development of type one diabetes in NOD mice. Supported by NIAID AI100005, AI14782 and JDRF.

2922

Early B cell development requires balanced expression of miR-191

Blume, J., Lyszkiewicz, M., Witzlau, K., Krueger, A.

Hannover Medical School, Institute for Immunology, Hannover, Germany

B cell development is a tightly regulated process yielding cells capable of producing highly diverse antibodies by random rearrangement of the B cell receptor gene loci. MicroRNAs (miRs) have been reported to regulate hematopoiesis by influencing transcription factor networks. However, little is known about individual miRs influencing lineage fate and development in the B cell lineage.

We found that miR-191 is dynamically regulated during B cell development and activation and identified three transcription factors E2A, Foxp1 and Egr-1 as direct targets of miR-191. In consequence, ectopic expression of miR-191 led to an accumulation of preB1 cells at the expense of later developmental stages in bone marrow chimeras. This developmental delay was possibly due to down-regulation of the rearrangement machinery during heavy chain recombination and reduced IL-7R α expression. Furthermore, we detected increased levels of apoptosis during negative selection upon overexpression of miR-191. Forced expression of E2A or FoxP1 was able to rescue the block in B cell development introduced by ectopic expression of miR-191.

Aggressiveness of diffuse large B cell lymphoma (DLBCL) is correlated to high expression of Foxp1. Consequently, overexpression of miR-191 reduced expression of Foxp1 in DLBCL lines and resulted in increased levels of apoptosis in DLBCL and thereby inhibited tumor formation and expansion in immunodeficient mice.

Overall, our results suggest that modulation of miR-191 expression is required to maintain balanced B cell development and differentiation.

3391

AP4 mediates resolution of chronic viral infection through amplification of germinal center B cell responses

Chou, C., Verbaro, D., Tonc, E., Cella, M., Colonna, M., Bhattacharya, D., Egawa, T.

Washington University School of Medicine, Pathology and Immunology, Saint Louis, United States

B cells undergo clonal expansion and somatic hypermutation in the germinal center (GC) in response to survival and proliferation signals provided by follicular helper T (T_{FH}) cells. This interaction with T_{FH} cells induces transient c-MYC expression in non-cycling GC B cells in the light zone (LZ), however, B-cell proliferation

occurs after migration into the dark zone (DZ), paradoxically after their separation from T_{FH} cells in the LZ and the extinction of c-MYC expression. Here, we resolve this paradox by showing that the transcription factor AP4, which functions downstream of c-MYC to control the gene expression programs related to cellular growth and survival, is responsible for expansion of GC B cells in the DZ and for diversification of BCR repertoires required for protective antiviral immunity. AP4 is initially induced by c-MYC in the LZ, but is sustained by IL-21 produced by T_{FH} in a c-MYC-independent manner. These results indicate that AP4 integrates T-cell help signals in response to affinity selection in the LZ to drive GC B cell clonal expansion at a distance in the DZ.

1344

Activation induced cytidine deaminase (AID) expression in human B-cell precursors is essential for central B-cell tolerance

Cantaert, T.¹, Schickel, J.-N.¹, Bannock, J.¹, Ng, Y.-S.¹, Massad, C.¹, Oe, T.¹, Wu, R.¹, Lavoie, A.², Walter, J.³, Notarangelo, L.³, Waleed, A.-H.⁴, Sebnem Kilic, S.⁵, Ochs, H.⁶, Nonoyama, S.⁷, Durandy, A.⁸, Meffre, E.¹

¹Yale University School of Medicine, Immunobiology, New Haven, United States, ²Centre Hospitalier de l'Université Laval, Quebec, Canada, ³Harvard Medical School, Boston, United States, ⁴Kuwait University, Kuwait, Kuwait, ⁵Uludag University Medical Faculty, Bursa, Turkey, ⁶University of Washington, Seattle, United States, ⁷National Defense Medical College, Tokorozawa, Saitama, Japan, ⁸Institut Imagine UMR 1163, Paris, France

Activation-induced cytidine deaminase (AID), the enzyme mediating class switch recombination (CSR) and somatic hypermutation (SHM) of immunoglobulin genes, is essential for the removal of developing autoreactive B cells. How AID mediates central B-cell tolerance remains unknown. By analyzing patients with various types of *AID* mutations, we could show that removal of developing autoreactive B cells in the bone marrow is regulated by gene dosage and AID enzymatic activity. In addition, we report that AID proteins are expressed during early B-cell development in both human fetal liver and adult bone marrow. AID expression was found restricted in to early immature B cells that co-express recombination-activating gene 2 (RAG2), suggesting that they undergo secondary recombination to edit autoreactive antibodies. However, most AID⁺ immature B cells lacked anti-apoptotic MCL-1 and were deleted by apoptosis. Furthermore, we show that AID inhibition by lentiviral-encoded short hairpin (sh) RNA in B cells developing in humanized mice impaired the counterselection of autoreactive clones.

Hence, B-cell intrinsic AID expression mediates central B-cell tolerance potentially through its RAG-coupled genotoxic activity in self-reactive immature B cells.

NK Cells 2

3067

Identification of two MCMV immunoevasins that modulate NK cell recognition via the NKR-P1B:Clr-b axis

Aguilar, O.A.^{1,2}, Rahim, M.M.³, Reichel, J.⁴, Lau, T.^{1,2}, Samaniego, J.^{1,2}, Sampaio, I.S.^{1,2}, Krmpotić, A.⁴, Jonjić, S.⁴, Makrigiannis, A.P.³, Allan, D.S.J.^{1,2}, Carlyle, J.R.^{1,2}

¹University of Toronto, Department of Immunology, Toronto, Canada, ²Sunnybrook Research Institute, Toronto, Canada, ³University of Ottawa, Department of Biochemistry, Microbiology and Immunology, Ottawa, Canada, ⁴University of Rijeka, Department for Histology and Embryology, Rijeka, Croatia

Natural killer (NK) cells are a subset of innate lymphoid cells (ILC) capable of recognizing pathological target cells through germline-encoded receptor-ligand interactions. Murine cytomegalovirus (MCMV) is a betaherpesvirus that has co-evolved with its natural host, and as such, a significant portion of its genome encodes immunoevasins that directly target NK cell receptor-ligand interactions. Here, we identify two putative immunoevasins that modulate host-pathogen interactions via the inhibitory NKR-P1B:Clr-b recognition system. First, we identify an m145 family member, m153, that actively prevents MCMV infection-mediated Clr-b downregulation. Ectopic expression of m153 in mouse fibroblasts increases Clr-b expression at the cell surface. In contrast, infection with a Δ m153-mutant MCMV shows more substantial Clr-b loss at the cell surface compared to wild-type MCMV. Importantly, enhanced Clr-b loss upon infection using Δ m153-mutant MCMV could be reversed upon m153 complementation by overexpression. Secondly, we have identified an m02 family member, m12, that directly interacts with the NKR-P1B inhibitory receptor in reporter cell assays. Notably, the interaction between NKR-P1B and m12 is Clr-b/ β_2 m-independent, and infection of fibroblasts using Δ m12-mutant MCMV abrogates NKR-P1B ligation of reporter cells. This interaction, suggestive of decoy ligand function, is also MCMV strain-dependent and host NKR-P1B allele-specific, in turn suggesting that polymorphisms have evolved at both the pathogen and host levels that affect NK cell recognition of infected target cells.

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Interleukin-1 receptor 8 (IL-1R8) plays a crucial role in NK cell differentiation and function

Molgora, M.¹, Bonavita, E.¹, Ponzetta, A.¹, Barbagallo, M.¹, Polentarutti, N.¹, Jaillon, S.¹, Riva, F.¹, Magrini, E.¹, Benigni, G.², Bernardini, G.², Santoni, A.², Mantovani, A.^{1,3}, Garlanda, C.¹

¹Humanitas Clinical and Research Centre, Rozzano (MI), Italy, ²Istituto Pasteur-Fondazione Cenci Bolognetti, Sapienza University of Rome, Rome, Italy, ³Humanitas University, Rozzano (MI), Italy

IL-1R8 is an Interleukin-1R receptor (ILR) family member and acts as a negative regulator of ILR and Toll-like receptor (TLR) signaling. Although it was demonstrated to be a tumor suppressor able to control cancer-related inflammation, it is also highly expressed by NK cells, which activation contributes to the elimination of tumor cells.

The aim of the study was to characterize its role in modulating ILR and TLR signaling in NK cells. We showed that both murine and human NK cells expressed high levels of IL-1R8 and it was differentially expressed during NK cell maturation. IL-1R8-deficient NK cells displayed a more mature phenotype and enhanced effector functions, in terms of IFN- γ production, FasL expression and cytotoxicity. To investigate the mechanism responsible for this phenotype, we focused on IL-18, which is a key regulator of NK cell activities and may be potentially targeted by IL-1R8. Indeed, IL-1R8 regulated IL-18-MyD88 axis during NK cell differentiation, acting through the inhibition of the mTOR and JNK pathways.

To assess the role of IL-1R8 in NK cells in pathology, we used a model of sarcoma-derived metastasis. The primary tumor similarly grew in IL1R8-competent and deficient mice, whereas the number and dimension of lung metastasis were significantly reduced in Il1r8^{-/-} mice. The depletion of NK cells in this model totally abrogated the protection from lung metastasis in Il1r8^{-/-} mice.

IL-1R8 plays therefore a nonredundant role in the regulation of NK cell biology and it is a crucial regulator of NK cell antitumoral activity.

3688

The IRE1/XBP1 pathway is a critical regulatory hub for human NK-cell mediated lysis and motility that modulates the actin-binding protein XIRP1

Gandhi, M.K.¹, Mujaj, S.¹, Vari, F.¹, Cui, Q.¹, Nourse, J.P.¹, Han, E.¹, Cristino, A.²

¹University of Queensland Diamantina Institute, Blood Cancer Research Laboratory, Brisbane, Australia, ²University of Queensland Diamantina Institute, Systems Biology, Brisbane, Australia

We recently demonstrated NK-cell effector function is impaired in aggressive lymphoma (Keane, Lancet Haem 2015). However, the underlying mechanisms are poorly understood. Using NK-cell-lines and unmanipulated *ex-vivo* primary NK-cells (pNK), and various blood cancer targets, we show the critical role of the inositol-requiring enzyme 1/X-box-binding protein 1 (IRE1/XBP1) signaling pathway, involving non-conventional splicing of XBP1 to its active form XBP1s. Minimal splicing of XBP1 occurred in unstimulated NK-cells, with rapid XBP1s induction following activation. Small molecule blockade resulted in dose-dependent reduced direct cytotoxicity and ADCC whereas activation up-regulated effector function, associated with increased granzymeB/TNFA/FasL/GM-CSF/NKG2D/CD16. XBP1-splicing inhibition reduced pseudopodia, motility and migration implicating altered cytoskeletal function. Transcriptome-array identified candidates associated with the IRE1/XBP1 pathway and its downstream effects on NK-cell cytotoxicity. Overlapping differentially expressed genes (DEG) show significant positive correlation ($r=0.88$ and $r=0.95$; both $P < 0.0001$). One-third of DEG promoter regions were enriched in putative XBP1s-binding sites, indicating a robust/conserved molecular kernel involving both direct lysis and ADCC. The cytoskeletal actin-binding protein gene XIRP1 was a key DEG, and IRE1-inhibitors reduced XIRP1 proteins in a dose-dependent manner. Intriguingly, the

XBP1-target network was enriched with binding sites for the microRNAs which were themselves targeting XBP1 target genes. Highly enriched microRNAs included mir-1234 and mir-4488, for which binding sites have been identified in the 3'UTR of XIRP1 consistent with this gene being under concerted direct/indirect regulation by XBP1s and its targeted microRNAs. These data highlight a hitherto unrecognized and potentially targetable role for IRE1/XBP1s as a master regulator of human NK-cell effector function.

1335

Disarming a killer: novel viral infection of human NK Cells interferes with function and phenotype

Campbell, T.M.¹, McSharry, B.P.¹, Steain, M.¹, Ashhurst, T.M.², Slobedman, B.¹, Abendroth, A.¹

¹University of Sydney, Infectious Diseases & Immunology, Sydney, Australia, ²University of Sydney, Discipline of Pathology, Sydney, Australia

Clinical evidence has revealed that NK cell function is critical to controlling infection with varicella zoster virus (VZV), the alphaherpesvirus responsible for varicella and herpes zoster. Despite the crucial role of NK cells, understanding their interaction with VZV has remained surprisingly overlooked. VZV is an established lymphotropic virus, however our findings are the first to demonstrate VZV infection of human peripheral blood NK cells, with remarkably 40% of cells antigen positive after 48 hours, as detected by flow cytometry and immunofluorescence. Importantly, NK cells were able to pass on infection to surrounding cells, suggesting a possible role for these circulating innate lymphocytes in the dissemination of virus during host infection. Additionally it was found that a marked increase in VZV antigen positive NK cells occurs in the presence of interleukin-2, which is typically elevated during infection. To assess the effect of infection on NK cell function a calcein-release cytotoxicity assay was employed. Analysis revealed that infection dramatically disabled cytolytic function of NK cells. In exploring this effect by multi-parameter flow cytometry, human NK cells were assessed for changes in phenotype following VZV infection, through examining expression of 22 receptors. Analysis revealed that VZV-cultured NK cells showed elevated activation, as detected by CD69 expression, and potent downregulation of the Fc receptor CD16, which regulates antibody-dependent cell-mediated cytotoxicity. Our findings identify a novel infection of NK cells and begin to reveal the complex manipulation and evasion of NK cell anti-viral function by VZV.

2121

Role of the non-classical MHC H2-Q10 in NK cell biology

Goodall, K.¹, Sullivan, L.², Andrews, D.¹

¹Monash University, Immunology, Melbourne, Australia, ²Peter Doherty Institute, University of Melbourne, Melbourne, Australia

As a member of the frontline immune response, natural killer (NK) cells can respond rapidly to pathogens by killing infected cells. NK cells in the mouse express Ly49 receptors that recognise both

classical and non-classical MHC-I receptors. Non classical MHC-I receptors typically display tissue-restricted expression, and a restricted peptide repertoire. H2-Q10, however, is a secreted non-classical MHC-I receptor that is expressed primarily in the liver but binds a classical repertoire of peptides, and therefore has both classical and non-classical properties. Recent work has shown that H2-Q10 interacts with the NK cell via Ly49C^{B6}. However, the 129 mouse does not display Ly49C on NK cells, and has impaired function in several infection models. Here we show that H2-Q10 also recognises Ly49I¹²⁹, however unlike Ly49C^{B6}, H2-Q10 shows peptide specificity. We also showed that H2-Q10 binding is significantly impaired when Ly49I is bound in *cis* with H2-K^b/D^b. Alanine substitution has revealed the binding sites of H2-Q10 to Ly49I. This data aims to determine the nature of H2-Q10 recognition of Ly49 receptors on NK cells in mice, and its potential role in infection.

2832

Orientation of CD56^{dim} NK cells toward IFN- γ production directed by NKG2A/KIR expression upon Fc γ RIIIa engagement

Lajoie, L., Congy-Jolivet, N., Bolzec, A., Sung, H.C., Thibault, G. Université François Rabelais, GICC CNRS UMR 7292, Tours Cedex, France

It is not known which antibody-dependent NK cell function, *i.e.* killing or cytokine production, mediates clinical responses to therapeutic monoclonal antibodies (mAbs). Fc γ RIIIa/CD16a is the sole activating receptor of CD56^{dim} NK cells whose engagement induces substantial degranulation and cytokine synthesis. However, it is currently unclear how NK cells are directed toward these effector functions upon Fc γ RIIIa/CD16a engagement. Degranulation and/or IFN- γ -production and the expression of the inhibitory receptors NKG2A, CD158a and CD158b were simultaneously evaluated after stimulation by plate-bound anti-CD16 3G8 mAb, rituximab or trastuzumab. After 4h of 3G8 stimulation, CD107⁺IFN- γ ⁺, CD107⁺IFN- γ ⁻ and CD107⁻IFN- γ ⁺ cells represented $27.1 \pm 14.2\%$, $61.1 \pm 13.9\%$ and $11.8 \pm 5.9\%$ of responding CD56^{dim} NK cells, respectively. This partial functional segregation persisted for up to 20h of stimulation. No correlation was detected between the percentages of degranulating and IFN- γ -producing NK cells from 26 donors. Importantly, IFN- γ production was positively associated with CD158a and CD158b expression and negatively associated with NKG2A expression after Fc γ RIIIa engagement by 3G8, rituximab or trastuzumab. Conversely, degranulation was positively although weakly associated with NKG2A expression. It was concluded that the individual inhibitory receptors expression of CD56^{dim} NK cells, influences their orientation toward degranulation and IFN- γ production in response to the engagement of Fc γ RIIIa by therapeutic mAbs.

4100**Antigen-specific memory responses of human liver- and skin-derived NK cells**

Stary, V.¹, Strobl, J.², Haegele, S.¹, Pereyra, D.¹, Starlinger, P.¹, Stary, G.²

¹Medical University of Vienna, General Hospital, Department of Surgery, Vienna, Austria, ²Medical University of Vienna, General Hospital, Department of Dermatology, Division of Immunology, Allergy and Infectious Diseases, Vienna, Austria

Until now, vaccine strategies have been exclusively focused on promoting effector activity by the two classical arms of the adaptive immune system, namely B and T cells. Mounting evidence points to an important role for a third lymphocyte subset, natural killer (NK) cells, in host resistance to infections. Murine liver-resident NK cells were discovered with long-lived memory of haptens and viral antigens. Despite phenotypic analysis of human liver NK cells, the existence and consequences of antigen-specific NK cell memory still needs to be proven.

We isolated human liver NK cells from individuals vaccinated against hepatitis A / B and characterized them phenotypically and functionally in killing assays against these viral antigens. Furthermore, we evaluated the distribution of NK cells in epicutaneous patch test reactions, a model for delayed-type hypersensitivity reactions.

In contrast to the peripheral blood, two distinct NK cell populations were found in the liver based on their expression of CD16 and CD49a. CD49a⁺CD16⁻ NK cells (54.6% ± 4.2 of total NK cells) performed antigen-specific killing comparable to CD8 T cells. Blood-derived and CD49a⁺CD16⁺ liver NK cells did not exert antigen-specific cytotoxicity, but recognized MHC-I^{low} target cells. Although absent in healthy human skin, 57,8 ± 5.1 % of total NK cells in hapten-induced epicutaneous patch tests were found to belong to the CD49a⁺CD16^{low} NK cell subset.

These results suggest that memory NK cells in humans can be found in the liver and inflamed skin, which might lead to novel strategies of vaccination by harnessing this NK cell subset.

3003**Adenosine impairs the proliferative capacity of terminally mature NK cells**

Young, A.^{1,2}, Souza-Fonseca-Guimaraes, F.^{1,2,3}, Ngiew, S.F.^{1,2}, Gao, Y.^{1,2}, Linden, J.⁴, Huntington, N.D.³, Smyth, M.J.^{1,2}

¹QIMR Berghofer Medical Research Institute, Herston, Australia, ²The University of Queensland, Herston, Australia, ³The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia, ⁴La Jolla Institute for Allergy & Immunology, San Diego, United States

Adenosine is a critical immunosuppressive metabolite, necessary for protecting against overzealous immune responses. Group 1 innate lymphoid cells (ILCs), both natural killer (NK) and ILC 1 cells, express high levels of the A2A adenosine receptor (A2AR), but not alternate adenosine receptors. Therefore, we interrogated the role of adenosine signaling via the A2AR in these cellular subsets. At baseline, complete and conditional Nkp46^{cre} deletion of the A2AR significantly enhanced accumulation of terminally mature (CD11b⁺CD27⁻ and CD11b⁺KLRG1⁺) NK cells in lymphoid organs. As terminally mature wild type NK

cells display reduced proliferative potential, we performed competitive 1 to 1 transfers of wild type (CD45.1⁺) and A2AR-deficient (CD45.2⁺) bone marrow or sorted splenic NK cells into sublethally irradiated or lymphocyte-deficient Rag^{-/-}γc^{-/-} mice, respectively, to assess their proliferative ability. Surprisingly, significantly greater expansion of A2AR-deficient NK cells was observed compared to wild type. In addition, following bone marrow reconstitution, A2AR-deficient NK cells maintained an altered maturation profile. By sorting individual NK cell subsets, based on CD11b and CD27, we found that terminally mature A2AR-deficient NK cells, but not wild type, proliferated *in vitro* following IL-15 stimulation. Finally, we assessed whether conditional Nkp46^{cre} deletion of the A2AR modified tumour development. Using the SM1WT1 melanoma and MCA *de novo* tumorigenesis models we identified a significant reduction in primary tumour growth and delayed tumour initiation, respectively. These results indicate that A2AR adenosine signaling limits proliferation and anti-tumour responses of NK cells, which may have therapeutic implications for improved NK cell activity as A2AR antagonists reach clinical utility.

384**TGF-beta inhibits NK cell activation and cytotoxicity through repression of the mTOR pathway**

Souza-Fonseca-Guimaraes, F.^{1,2}, Viel, S.³, Marçais, A.³, Huntington, N.¹, Bartholin, L.⁴, Walzer, T.³, Smyth, M.²

¹Walter & Eliza Hall Institute, Melbourne, Australia, ²QIMR Berghofer Medical Research Institute, Herston, Australia, ³CIRI, Centre International de Recherche en Infectiologie - International Center for Infectiology Research, Lyon, France, ⁴INSERM U 1052, CNRS UMR 5286, Centre de Recherche en Cancérologie de Lyon (CRCL), Centre Léon Bérard, Lyon, France

Deciphering the regulation of natural killer (NK) cell maturation is essential to understanding how NK cells are mobilised to combat cancer and infection. TGF-β is a major immunosuppressive cytokine maintaining immune homeostasis and preventing autoimmunity. TGF-β has anti-proliferative and anti-inflammatory properties in various cell types that remain to be characterized at the molecular level. Here, we provide genetic, pharmacologic and biochemical evidences that a major target of TGF-β in mouse and human Natural Killer (NK) cells is the serine/threonine kinase mTOR. *In vitro* treatment with TGF-β or with rapamycin leads to a rapid blockade of IL-15-induced mTOR activation in mouse or human NK cells. TGF-β and rapamycin also had identical effects on NK cell metabolic activity, proliferation and cytotoxic activity. Constitutive TGF-β signaling or mTOR deletion results in similar developmental arrests in NK cells while reciprocally TGF-βRII deletion unleashes mTOR activity and cytotoxic potential upon IL-15 treatment. Suppression of TGF-β signaling in NK cells does not impact on NK cell development and homeostasis. However, it endows NK cells with a better ability to control metastases in two different tumor models. Altogether, these results establish the mTOR kinase as a crucial signaling integrator of pro and anti-inflammatory cytokines in NK cells. Moreover, they suggest that improving metabolic activity of anti-tumor lymphocytes could be a valid strategy to promote tumor suppression.

Stem Cells & Immunity

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High salt driven Th17 response induces stem cell mobilization and accelerates atherosclerosis

Lee, M.K.S.^{1,2}, Dragoljevic, D.^{1,2}, Kraakman, M.J.¹, Shihata, W.^{1,2}, Al-sharea, A.^{1,2}, Whillas, A.^{1,2}, Khan, S.^{1,2}, Nguyen, T.¹, Heywood, S.¹, Masters, S.L.³, Chin-Dusting, J.^{1,2}, Murphy, A.J.^{1,2}

¹Baker IDI Heart and Diabetes Institute, Melbourne, Australia, ²Monash University, Melbourne, Australia, ³Walter & Eliza Hall Institute of Medical Research, Melbourne, Australia

Introduction: A high-salt diet (HSD) is linked to increased atherosclerosis. Recently, a HSD has been shown to alter the immune response in both mice and humans, by promoting inflammatory and suppressing anti-inflammatory immune cells. We hypothesized that a HSD would drive Th17 cells to promote hematopoietic stem progenitor cells (HSPCs) mobilization, monocytosis and increased atherosclerosis.

Methods: Atherosclerotic-prone Apoe^{-/-} mice were fed a normal salt (1%) or HSD (8%) for 12wks. Levels of HSPCs and myeloid progenitors, myeloid cells and Th17 cells were measured in the blood, spleen and bone marrow using flow cytometry. CD68 and Oil Red O staining were used to quantify sinus plaques that identify macrophage and lipid abundance.

Results: A HSD significantly promoted rupture-prone atherosclerotic lesion formation with increased macrophage and lipid content in the sinus. These changes were associated with monocytosis and neutrophilia. We found HSPC mobilization from the bone marrow to the spleen where more monocytes were produced, suggesting extramedullary hematopoiesis. HSD-fed mice also had increased levels of circulating Th17s, likely driven by the increased abundance of CD24⁺ (IL-23 producing) dendritic cells in the spleen. Using an IL-1RA (Anakinra) and neutralizing antibodies to IL-17 and IL-23R, we found that this signaling axis was responsible for HSPC mobilization and ultimately unstable lesion formation.

Conclusions: A HSD promotes rupture-prone atherosclerotic lesions by increasing Th17 cells to influence HSPC mobilization to the spleen. This results in extramedullary hematopoiesis, expanding the monocyte population, which enter the atherosclerotic lesion. The IL-1/IL-23/IL-17 signaling axis appeared to be responsible for this process.

2133

Desmoglein 2 (DSG2) is a novel surface marker of endothelial and haematopoietic progenitor cells, and plays a critical role in neoangiogenesis

Ebert, L.^{1,2}, Tan, L.Y.^{1,3}, Min, K.K.M.^{1,3}, Cockshell, M.¹, Johan, M.Z.¹, Parham, K.^{1,3}, Betterman, K.^{1,3}, Ruszkiewicz, A.^{1,2}, Zannettino, A.^{4,5}, Gronthos, S.^{4,5}, Koblar, S.^{4,5}, Harvey, N.^{1,3}, Lopez, A.^{1,2}, Shackleton, M.⁶, Bonder, C.^{1,3}

¹Centre for Cancer Biology, Adelaide, Australia, ²SA Pathology, Adelaide, Australia, ³University of South Australia, Adelaide, Australia, ⁴South Australian Health and Medical Research Institute, Adelaide, Australia, ⁵University of Adelaide, Adelaide, Australia, ⁶Peter MacCallum Cancer Centre, Melbourne, Australia

Endothelial progenitor cells (EPC) are rare but clinically important cells present in blood and bone marrow (BM) which contribute to blood vessel growth and repair. We recently undertook a genome-wide search for new surface markers of EPCs, for the purpose of improving EPC detection and isolation. The top candidate was desmoglein 2 (DSG2), an adhesion molecule belonging to the cadherin family. Flow cytometry revealed that DSG2 is expressed on short-term cultured EPCs derived from umbilical cord blood, and on circulating EPCs (CD34⁺ VEGFR2⁺) in peripheral blood. Interestingly, DSG2 was also expressed on the closely related CD34⁺ CD45^{dim} haematopoietic progenitor cell (HPC) population in peripheral and cord blood. Detailed characterisation of BM subsets revealed uniformly high DSG2 expression on the most primitive HPCs (CD34⁺ CD38⁻ CD90⁺) with progressively lower expression on more committed progenitors, while mature leukocytes lacked DSG2. In contrast, DSG2 was maintained on a subset of mature endothelial cells (EC), suggesting DSG2 may play an important role in this lineage. To address this possibility, a 'knock-out first' approach was used to generate a *Dsg2* loss-of-function strain of mice (*Dsg2*^{0/0}). In response to reduced levels of *Dsg2*, EPCs displayed proliferation and differentiation defects, neoangiogenesis was significantly impaired and ECs in some tissues displayed morphological aberrations. Together, our results define DSG2 as a novel, and potentially useful, surface marker for the identification and isolation of EPCs and HPCs, and show that, independent from its classical function as a component of desmosomes, this cadherin also plays a critical role in the vasculature.

1351

The parasympathetic nervous system is involved in the regulation of hematopoietic stem cell activity

Al-Sharea, A.^{1,2}, Whillas, A.^{1,2}, Shihata, W.^{1,2}, Andrews, K.¹, Purton, L.³, Chin-Dusting, J.¹, Murphy, A.¹

¹Baker IDI Heart and Diabetes Institute, Melbourne, Australia, ²Monash University, Melbourne, Australia, ³St Vincent's Institute, Melbourne, Australia

Objective: The sympathetic arm of the autonomic nervous system regulates retention/release of hematopoietic stem cells (HSCs). We studied if the parasympathetic nervous system (PNS) also has a regulatory role.

Methods: Wild-type (C57BL/6) mice were injected with the anticholinergic atropine (2mg/kg, bi-daily for 3days), to inhibit the activity of the PNS under normal conditions. Using flow cytometry, levels of HSCs and their downstream myeloid progenitors were quantified in the blood and bone marrow (BM).

Results: Inhibition of the PNS promoted HSC mobilization, associated with changes in the expression of the key genes involved in HSC mobilization including stromal cell-derived factor 1, matrix metalloproteinase 9 (MMP9) and stem cell factor. PNS inhibition appeared to alter the HSC niche, causing a decrease in endothelial cells and down-regulation of ICAM-1. To determine whether stimulating the PNS prevents HSC mobilization, mice were injected with the mobilizing agent G-CSF (250µg/kg bi-daily x 3days) and treated with pyridostigmine (1mg/kg daily), to increase endogenous PNS ligands. G-CSF caused a robust

mobilization of HSCs, marked neutrophilia and splenomegaly, which was reversed with pyridostigmine. We identified alpha7-nicotinic acetylcholine receptor (alpha7nAChR) subunit to be highly expressed in the BM. This appeared to be the dominant receptor, as the alpha7nAChR specific agonist GTS-21 (4mg/kg bi-daily), also prevented G-CSF-induced HSC mobilization.

Conclusion: The PNS appears to play a role in retaining HSCs in the BM under homeostatic conditions. Stimulating the PNS can inhibit G-CSF-induced HSC mobilization. This occurs via acting on the alpha7nAChR to modulate the HSC niche.

1795

BCAP inhibits myeloid cell development from hematopoietic progenitors

Duggan, J.^{1,2}, Hamerman, J.^{1,2}

¹Benaroya Research Institute, Immunology, Seattle, United States,

²University of Washington, Immunology, Seattle, United States

B cell adaptor for PI3-kinase (BCAP) is a signaling adaptor expressed in hematopoietic cells including macrophages, monocytes, and neutrophils. Here we asked if BCAP plays a role in development of these myeloid cells. We found that BCAP was expressed in bone marrow (BM) hematopoietic progenitors, including LSK (Lin⁻Sca1⁺cKit⁺), CMP (Common Myeloid Progenitor) and GMP (Granulocyte/Macrophage Progenitor) cells, suggesting that BCAP may impact myelopoiesis. BCAP^{-/-} mice had more BM monocytes than WT mice, and in mixed chimeras generated with a 1:1 ratio of WT and BCAP^{-/-} BM, monocytes and neutrophils in the BM, blood and spleen exhibited skewing towards BCAP^{-/-} origin, showing a competitive advantage for BCAP^{-/-} myeloid cells. Thus we hypothesized that BCAP inhibits myeloid development. Consistent with this hypothesis, BCAP-deficient BM LSK, CMP and GMP cells out-competed WT progenitors in mixed chimeras. In an in vitro myeloid colony-forming-unit assay, sorted BCAP^{-/-} progenitors produced more myeloid cells than WT progenitors, supporting a cell-intrinsic role of BCAP in inhibiting myeloid differentiation. Furthermore, BCAP^{-/-} progenitors were more mature than WT progenitors, indicating that BCAP^{-/-} progenitors display accelerated myeloid development. During cyclophosphamide-induced myeloablation or specific monocyte depletion, BCAP^{-/-} mice replenish circulating monocytes earlier than WT mice. Lastly, BCAP^{-/-} progenitors showed increased expression of the myeloid-differentiating transcription factors IRF8 and C/EBPα compared to WT progenitors. Together, these data identify BCAP as an inhibitor of myeloid development from BM hematopoietic progenitors in the steady state and during demand conditions.

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Mesenchymal cells expressing Tlx1 serve as an extramedullary niche in the spleen

Oda, A., Kasahara, T., Goitsuka, R.

Tokyo University of Science, Division of Development and Aging, Chiba, Japan

Extramedullary hematopoiesis (EMH) is initiated by the pathological stress responses, through an alteration in a

specific hematopoietic microenvironment (niche) in the bone marrow that support steady-state hematopoiesis from the hematopoietic stem and progenitor cells (HSPCs). The pathological stresses, such as chronic hemolytic anemia and severe bacterial infection, induce to mobilize HSPCs from the bone marrow to the periphery, leading to EMH mainly at the spleen and liver, sites that support embryonic hematopoiesis. However the precise mechanisms and nature including the niche leading to EMH and the source of hematopoietic factors remain poorly understood. Here we show that mesenchymal stromal cells expressing Tlx1 (Tlx1⁺ cells), a transcription factor essential for spleen organogenesis, potentially serve as the HSPCs niche during EMH. We generated the *Tlx1^{creER-Venus}; Rosa26-iDTA* mouse that enables both visualization of Tlx1⁺ cells by Venus and depletion of Tlx1⁺ cells by tamoxifen administration. A majority of Tlx1⁺ cells are localized in the red pulp, particularly a perifollicular region, and display immature mesenchymal stem/progenitor phenotypes on their cell surface. When EMH was induced by phenylhydrazine- or LPS-treatment, depletion of Tlx1⁺ cells by tamoxifen administration drastically reduced splenomegaly as well as EMH. Since CXCL12 and SCF, the key niche factors, is highly expressed in Tlx1⁺ cells among other cells in the spleen, these findings suggest that Tlx1⁺ mesenchymal cells in the splenic red pulp serve as a HSPC niche that supports EMH.

3806

Production of BMP4 by endothelial cells is crucial for endogenous thymic regeneration

Wertheimer, T.¹, Velardi, E.¹, Xiao, S.², Palikuqi, B.³, Ottmuller, K.⁴, Beilhack, A.⁴, Butler, J.³, Manley, N.², Rafii, S.³, van den Brink, M.¹, Dudakov, J.^{1,5}

¹Memorial Sloan Kettering Cancer Center, Immunology Program,

New York, United States, ²University of Georgia, Department of

Genetics, Athens, United States, ³Weill Cornell Medical College,

Ansary Stem Cell Institute, New York, United States, ⁴Wurzburg

University Hospital, Department of Medicine, Wurzburg, Germany,

⁵Fred Hutchinson Cancer Research Center, Program in Immunology,

Seattle, United States

Although the thymus has a remarkable capacity for repair following damage, the mechanisms underlying this regeneration remain poorly understood. Here we reveal that endothelial cells (ECs), which are highly damage-resistant, represent a critical pathway of endogenous thymic regeneration. Analysis revealed that ECs significantly increase their production of BMP4 after thymic damage; and abrogating BMP4 signalling or production leads to worse thymic repair. Recovery of thymic function could be significantly boosted with therapeutic administration of *ex vivo* propagated and expanded ECs (exEC), but intriguingly only if they were derived from thymus, not kidney or heart. Treatment with exECs led to upregulation by thymic epithelial cells (TECs) of *Foxn1*, a key TEC transcription factor, as well as the FOXN1 target gene *Dll4*. *In vitro* co-culture studies revealed that conditioned media from exEC derived from the thymus, but not heart or kidney, induced expression of *Foxn1* by thymic epithelial cells (TECs) and that this was abrogated by Noggin, an inhibitor of BMP signaling. Consistent with these studies, exEC^(Thy) produced

significantly more BMP4 compared to exEC derived from the heart or kidney; and silencing *Bmp4* expression by shRNA within exEC^(Thy) limited their capacity to mediate exogenous thymic regeneration, and failed to induce the expression of *Foxn1* and *Dll4*. These studies not only detail a novel pathway underlying endogenous thymic regeneration, but offer an innovative clinical approach to enhance T cell immunity in individuals with T cell deficiencies due to aging, infectious disease, and common cancer treatments such as chemo- and radiation-therapy.

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Menstrual blood stromal stem cells modulate the properties of dendritic cells

Bozorgmehr, M.^{1,2}, Zarnani, A.H.^{2,3}, Sheikhan, A.⁴, Salehnia, M.⁵, Nikoo, S.³, Moazzeni, S.M.¹

¹Faculty of Medical Sciences, Tarbiat Modares University, Immunology Department, Tehran, Iran, Islamic Republic of,

²Nanobiotechnology Research Center, Avicenna Research Institute, ACECR, Tehran, Iran, Islamic Republic of, ³Reproductive Immunology Research Center, Avicenna Research Institute, ACECR, Tehran, Iran, Islamic Republic of, ⁴Faculty of Medicine, Lorestan University of Medical Sciences, Immunology Department, Khorramabad, Iran, Islamic Republic of, ⁵Faculty of Medical Sciences, Tarbiat Modares University, Anatomy Department, Tehran, Iran, Islamic Republic of

Menstrual blood stromal stem cells (MenSCs) share several characteristics with mesenchymal stem cells. Nevertheless, data regarding the potential impacts of MenSC on different arms of the immune system is still scarce. Hence, we sought to explore, whether MenSCs affect the generation and function of dendritic cells (DCs) from human peripheral blood monocytes.

MenSCs were separated from menstrual blood of normal women. Monocytes from unrelated donors were differentiated towards immature DCs (iDCs) and mature DCs (mDCs) using appropriate cytokines. Generation of DCs was performed in the presence or absence of MenSCs. The effect of MenSCs on phenotypic and functional characteristics of DCs was evaluated through immunophenotyping, measuring cytokine concentration and regulatory T cell (Treg) induction.

DCs generated in the presence of MenSCs acquired regulatory properties, as judged by the expression level of surface markers (CD14, CD1a, CD40, CD80, CD86), the amount of typical several immunostimulatory and immunoinhibitory cytokines (IL-6, IL-10, IL-12, and TNF- α), and the ability to induce Tregs. Moreover, our data showed that these impacts were exerted on the first step of DC generation (i.e. monocyte to DC differentiation) rather than the second step (i.e. iDC to mDC differentiation); this impact was shown to be in part related to the presence of IL-6 and IL-10 in the cocultures.

This is the first work addressing the modulatory impacts of MenSCs on generation and function of DCs. Accordingly, since DCs play key roles in regulation of immune responses, MenSCs could be regarded as potential tools to be used in future clinical settings.

2345

Differential TLR activation of murine mesenchymal stem cells generates distinct immunomodulatory effects in EAE

Vega-Letter, A.M., Kurte, M., Fernández-O'Ryan, C., Gauthier-Abeliuk, M., Moya, I., Carrión, F.

Universidad de los Andes, Santiago, Chile

Mesenchymal stem cells (MSCs) are multipotent, nonhematopoietic progenitor cells that exhibit potent anti-inflammatory and immunomodulatory properties. Recently, it has been observed that MSCs can modulate their immunomodulatory capacities depending on the specific *in vitro* activation of different Toll-like receptors (TLR), such as TLR3 and TLR4. In the present study, we evaluated the effect of poly(I:C) and LPS pretreatment on the immunological capacity of murine MSCs *in vitro* and in murine experimental autoimmune encephalomyelitis (EAE). In comparison with untreated MSCs, pretreatment of MSCs with poly(I:C) significantly reduced the proliferation of splenocytes as well as that of Th1 and Th17 cells and increased the levels of the soluble immunosuppressive factor nitric oxide. In contrast, MSCs pretreated with LPS increased splenocytes proliferation, and that of Th1 and Th17 cells, as well as the levels of proinflammatory cytokine IL-6. Finally, we showed that intraperitoneal administration of MSCs pretreated with poly(I:C) significantly reduced the severity of EAE as well as the percentages of Th1 and Th17 proinflammatory subsets, while the pretreatment of MSCs with LPS completely reversed the therapeutic immunosuppressive effect of MSCs. Taken together, these data show that pretreatment of MSCs with poly(I:C) improved their immunosuppressive abilities. This may provide an opportunity to better define strategies for cell-based therapies for autoimmune diseases.

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Characterisation of mesenchymal cell subsets in human lymph nodes

Park, S.M.^{1,2}, Eom, J.^{1,2}, Brooks, A.^{1,2}, Feisst, V.^{1,2}, McIntosh, J.¹, Dunbar, P.R.^{1,2}

¹The University of Auckland, School of Biological Sciences, Auckland, New Zealand, ²Maurice Wilkins Centre for Molecular Biodiscovery, Auckland, New Zealand

Lymph nodes (LN) depend on several subsets of mesenchymal stromal cells to construct and maintain their elaborate architecture, and support immune function. Major stromal cell subsets in LNs include fibroblastic reticular cells (FRCs), follicular dendritic cells (FDCs), and marginal reticular cells (MRCs). All these cell subsets need to participate in LN remodeling during immune responses, yet the relationship between these subsets and any potential precursor populations has remained unclear. A lack of specific markers for mesenchymal cell subsets and their precursors in humans has also prevented full exploration of their potential roles in health and disease.

We have recently characterised mesenchymal subsets in human LNs. We discovered that CD141 uniquely labelled MRCs amongst the stromal cell populations in human LNs, revealing that MRCs line all boundaries between sinuses and the parenchyma. Migration of both T cells and Antigen-Presenting Cells (APCs) between MRCs could be observed in human LNs, and enzymes

that generate the sphingosine-1-phosphate (S1P) gradient within LNs showed striking spatial relationships with MRCs. We also identified Mesenchymal Precursor Cells in a distinctive niche within human LNs, with both phenotypic and functional characteristics similar to those we have previously characterised in human adipose tissue and dermis. We hypothesise that these cells may represent precursors of the other mesenchymal cell subsets present in human LNs, and may help increase the populations of these subsets during the substantial LN expansion that occurs during the early phases of an immune response.

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Elucidating genetic pathways in SLE and stratifying patients via whole genome sequencing

Jerjen, R.¹, Kreft, L.¹, McEwan, E.¹, Silva, A.¹, Field, M.^{1,2}, Athanasopoulos, V.^{1,2}, Jiang, S.^{1,2,3}, Andrews, D.^{1,2,4}, Pascual, V.⁵, Liston, A.⁶, Peterson, P.⁷, Fulcher, D.^{1,2}, Alexander, S.^{2,8}, Cook, M.^{1,2,9}, Vinuesa, C.^{1,2}, Ellyard, J.^{1,2}

¹JCMSR, ANU, Immunology and Infectious Disease, Canberra, Australia, ²Centre for Personalised Immunology, ANU, Canberra, Australia, ³Canberra Hospital, Department of Renal Medicine, Canberra, Australia, ⁴National Computational Infrastructure, ANU, Canberra, Australia, ⁵Baylor Institute for Immunology Research, Dallas, United States, ⁶VIB, KU Leuven, Leuven, Belgium, ⁷University of Tartu, Department of Biomedicine, Tartu, Estonia, ⁸Centre for Kidney Research, Children's Hospital at Westmead, Sydney, Australia, ⁹The Canberra Hospital, Department of Immunology, Canberra, Australia

Systemic lupus erythematosus (SLE) is a heterogeneous autoimmune disease. Twin studies indicate a strong genetic contribution to lupus, yet often the pathogenic variant remains unknown. Using next generation sequencing technologies (WES/WGS) it is now possible to identify rare/novel gene variants that cause disease. We previously used WES to identify a genetic variant in TREX1 as a cause of cerebral SLE; providing proof of principle that rare genetic variants do contribute to complex autoimmunity. It also revealed the patient to be a prime candidate for tailored therapies targeting type-I interferons. Using our validated bioinformatics pipeline and methodology, we have now identified two other cohorts of patients with genetic variants that impair thymic tolerance and toll-like receptor (TLR) 2 signaling, respectively. Biochemical assays on patient PBMCs or overexpression in cell lines confirmed the variants impair protein function. Furthermore, flow cytometry identified endophenotypes in the patients' PBMCs that may explain disease pathogenesis. These endophenotypes and the mechanisms by which they drive SLE pathogenesis are being evaluated in mice with CRISPR/Cas9-engineered patient-specific alleles. Our data suggests the first cohort have defects in thymic epithelial cells and developing thymocytes that combine to affect central tolerance, characterized by impaired regulatory T cells. In contrast, in the second cohort, we identified a pathway that primarily affects myeloid cells and neutrophils to cause SLE through production of type-I interferon. Thus by understanding

the precise genetic mechanisms that contribute to SLE pathogenesis, our data is able to stratify patients and through a personalized approach, identify tailored therapeutic options.

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Antigen-specific maturation of autoantibodies in systemic lupus erythematosus

Sakakibara, S.^{1,2}, Arimori, T.³, Yamashita, K.², Jinzai, H.¹, Motooka, D.⁴, Nakamura, S.⁴, Takeda, K.¹, Yasui, T.^{1,2}, Narazaki, M.^{2,5}, Tanaka, T.⁶, Standley, D.M.^{2,7}, Takagi, J.³, Kikutani, H.^{1,2}

¹Research Institute for Microbial Diseases, Osaka University, Suita, Japan, ²WPI Immunology Frontier Research Center, Osaka University, Suita, Japan, ³Institute for Protein Research, Suita, Japan, ⁴Genome Information Research Center, Research Institute for Microbial Diseases, Osaka University, Suita, Japan, ⁵Osaka University Graduate School of Medicine, Department of Respiratory Medicine, Allergy and Rheumatic Diseases, Suita, Japan, ⁶Osaka University Graduate School of Medicine, Department of Clinical Application of Biologics, Suita, Japan, ⁷Institute for Virus Research, Kyoto University, Kyoto, Japan

The developing pathway of self-reactive B cells in systemic lupus erythematosus (SLE) has not been firmly defined. To understand how self-reactive B cells are generated in SLE, we investigate antigen specificity, clone size and diversity of disease-associated self-reactive B cells. Among monoclonal antibodies generated from blood plasmablasts by a single cell cloning, we isolated distinct anti-nuclear antibody (ANA) clones that show high reactivity with nuclei of Hep2 cells in an indirect fluorescent assay. We obtained the ANAs only from untreated acute patients, but not from patients in remission or healthy donors. Moreover, their staining patterns resemble those of sera from respective donors, suggesting that the ANA clones we obtained are disease-associated autoantibodies. Most of the ANA clones have a relatively small number of mutations, which are, however, critical for the self-reactivity. For further analyses, we chose the anti-dsDNA ANA clone, 71F12, which exhibits a nano-molar equilibrium dissociation constant (K_D) to both ds and ssDNA. Next-generation sequencing (NGS) analysis revealed that the blood lymphocyte-derived immunoglobulin library contained a substantial frequency of 71F12 lineage sequences, most of which shared three somatic mutations. The crystal structure of the ligand-bound Fab revealed that through the mutated residue 71F12 interacted with nucleobases, suggesting that this antibody may unwind dsDNA to bind to nucleobases unlike a previously proposed model where an anti-dsDNA antibody interacts with the backbone of duplex DNA. Collectively, our results demonstrate that self-reactive B cells diverged from less or non-reactive precursors through rapid affinity maturation in SLE.

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Harnessing homeostatic mechanisms to resolve innate-directed neuroinflammation

La Flamme, A.C.^{1,2}, Webster, G.³, Mayo, N.⁴, White, M.¹, Sim, D.⁵

¹Victoria University of Wellington, Wellington, New Zealand, ²Malaghan Institute of Medical Research, Wellington, New Zealand

Zealand, ³Innate Immunotherapeutics, Auckland, New Zealand, ⁴McGill University, Montreal, Canada, ⁵University of Otago, Wellington, New Zealand

In recent years, a key role for the innate immune system in regulating neuroinflammation during secondary progressive multiple sclerosis (SPMS) has been revealed and is supported by the inability of therapeutics that target the peripheral adaptive immune response to reduce disease during SPMS. MIS416 is a novel microparticle that targets innate cells by activating TLR9 and NOD2, and this innate-directed therapy is currently undergoing a phase 2b efficacy trial for use during SPMS. Our preliminary clinical and preclinical studies have indicated that MIS416 is activating an IFN- γ -driven homeostatic mechanism that regulates both the peripheral and CNS-resident innate immune environment. A multidimensional scaling analysis of SPMS patient responses to MIS416 therapy as well as analysis of patient outcomes highlighted concordance between a regulated induction of the IFN- γ axis and improvements in SPMS disease parameters post treatment. Together these studies have begun to dissect the mechanism by which MIS416, a first-ever innate-mediated SPMS therapeutic, may regulate innate-driven neuroinflammatory processes during SPMS.

1244

Type 1 diabetes susceptibility genes impair gut physiology and lead to perturbations in the microbiota

Mullaney, J., Stephens, J., Hamilton-Williams, E.
University of Queensland, UQ Diamantina Institute,
Woolloongabba, Australia

Alterations in the gut microbiota have been associated with many autoimmune and inflammatory diseases including type 1 diabetes (T1D). It is postulated that microbial dysbiosis can perturb normal immune development and increase autoimmune responses. It is not known whether T1D associated microbial alterations are caused by altered environmental exposures or whether genetically driven host factors may also contribute. We have used the T1D susceptible NOD mouse model to test whether the introduction of genetic variants that protect from T1D also lead to changes in the microbiota and whether this may be due to reduced intestinal inflammation. We have observed that NOD mice have a significantly different microbiota and signs of inflammation and perturbations in both the ileum and colon compared with protected C57BL/6 mice. We then tested whether specific genetic loci that are highly associated with T1D susceptibility contribute to these changes. NOD.H-2^b mice have the T1D associated MHC region replaced with the C57BL/10 derived H-2^b locus also had a distinct microbiota to wild-type NOD mice. NOD.H-2^b mice had significantly restored Paneth cell morphology along with increased Paneth cell derived cryptdin expression. T1D protected, NOD congenic *Idd3/5* mice also had a distinct microbiota to NOD mice along with increased goblet cell mucous area in the colon. These findings support the conclusion that T1D associated genetic variants lead to changes in the inflammatory state of the intestine, leading to alterations in the gut microbiota.

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Targeting the survival mechanism of plasmacytoid dendritic cells for immune intervention in lupus

Zhan, Y.¹, Carrington, E.¹, Brady, J.¹, Oon, S.¹, Morand, E.², Lew, A.¹
¹The Walter and Eliza Hall Institute of Medical Research, Parkville, Australia, ²Monash University, The Monash Centre for Inflammatory Diseases, Clayton, Australia

Plasmacytoid dendritic cells (pDCs) and their products (eg. IFN- α and BAFF) have a strong association with the pathogenesis of autoimmune B cell-mediated diseases like lupus. We recently showed that pDCs of lupus-prone mice have prolonged survival, leading to increase in IFN- α production. Thus, we suggest that altered lifespan of pDCs might be a pathogenic factor for certain autoimmune diseases. We also showed recently that pDCs but not lymphoid-resident conventional DCs (cDCs) rely chiefly on BCL-2 for survival. Accordingly, BCL-2 antagonists selectively kill mouse and human pDCs. In addition, we found that BCL-2 antagonists have strong synergy with steroids, one of main drugs for lupus treatment, to kill pDCs. Interestingly, we also demonstrated that pDC subsets had differential sensitivity to BCL-2 antagonists. Together, we advocate that BCL-2 antagonists have potential as a treatment for lupus and could lower steroid usage and reduce steroid associated side effects. These strategies are currently being investigated in mouse lupus models and humanized mouse models of lupus.

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IL-1 receptor antagonist-deficient mice develop autoimmune arthritis due to intrinsic activation of IL-17-producing CCR2⁺V γ 6⁺ γ δ T cells

Akitsu, A., Iwakura, Y.
Tokyo University of Science, Research Institute for Biomedical Sciences, Chiba, Japan

IL-17-producing γ δ T cells (γ δ 17) cells are implicated in autoimmune diseases, but the pathogenic mechanisms, especially the determination of tissue-specific inflammation by these cells, remain unclear. We found that a high proportion of γ δ 17 cells were detected in joints of IL-1 receptor antagonist (IL-1Ra) KO mice, one of a model for rheumatoid arthritis, which depends on IL-17 and T cells. Furthermore, We demonstrated that only a mixture of γ δ T and CD4⁺ T cells induces both γ δ 17 cell localization in joints and arthritis development by using adoptive transfer experiments, suggesting that activated CD4⁺ T cells are required for the tissue distribution of γ δ T cells. To analyze further detailed mechanism, we generated IL-17-reporter mice. We also found that activated CD4⁺ T cells directed γ δ T cell infiltration, in a CCL2-dependent manner. These mice showed that among the CCR2-expressing γ δ T cells, the cells specifically expressing V γ 6 TCR have a dominant role in IL-17 production in the inflamed joints. IL-1Ra suppressed IL-1R expression on γ δ T cells *in vitro* experiments, and thus IL-1Ra KO mice enhance IL-1R expression on specific V γ 6 subset, which plays a pivotal role in activation of them to produce IL-17. Our findings demonstrate a pathogenic mechanism in which adaptive and innate immunity induce an autoimmune disease in a coordinated manner.

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N-terminal additions to the WE14 peptide of Chromogranin A creates strong autoantigen agonists in type I diabetes*Jin, N.^{1,2,3}, Wang, Y.³, Crawford, F.^{2,3}, White, J.^{2,3}, Marrack, P.^{2,3,4}, Dai, S.^{3,4,5}, Kappler, J.W.^{1,2,3,4,5}**¹University of Colorado, Barbara Davis Center for Childhood Diabetes, Aurora, United States, ²Howard Hughes Medical Institute, Denver, United States, ³National Jewish Health, Department of Biomedical Research, Denver, United States, ⁴University of Colorado School of Medicine, Department of Immunology and Microbiology, Aurora, United States, ⁵University of Colorado School of Medicine, Program in Structural Biology and Biochemistry, Aurora, United States*

Chromogranin A (ChgA) is an autoantigen for CD4⁺ T cells in the NOD mouse model of type-1 diabetes (T1D). The natural ChgA processed peptide, WE14, is a weak agonist for the prototypical T cell, BDC-2.5, and other ChgA specific T cell clones. Mimotope peptides with much higher activity share a C-terminal motif, WXR(M/D/E), that is predicted to lie in the p5 to p9 position in the IA^{g7} binding groove. This motif is also present in WE14 (WSRMD), but at its N-terminus. Therefore, to place the WE14 motif into the same position as seen in the mimotopes, we added the amino acids RLGL to its N-terminus. Like the other mimotopes, RLGL-WE14, is much more potent than WE14 in T cell stimulation and activates a diverse population of CD4⁺ T cells, which also respond to WE14 as well as islets from wild type, but not ChgA^{-/-} mice. The crystal structure of the IA^{g7}-RLGL-WE14 complex confirmed the predicted placement of the peptide within the IA^{g7} groove. Florescent IA^{g7}-RLGL-WE14 tetramers bind to ChgA specific T cells clones and easily detect ChgA specific T cells in the pancreas and pancreatic lymph nodes of NOD mice. The prediction that many different N-terminal amino acid extensions to the WXR(M/D/E) motif are sufficient to greatly improve T cells stimulation leads us to propose that such a post-translational modification may occur uniquely in the pancreas or pancreatic lymph nodes, perhaps via the mechanism of transpeptidation. This could account for the escape of these T cells from thymic negative selection.

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A role for circulating AIM protein in the pathogenesis of autoimmune type 1 diabetes*Matsumoto, A., Arai, S., Miyazaki, T.**University of Tokyo, Faculty of Medicine, Tokyo, Japan*

Type 1 diabetes (T1D) is an autoimmune disease characterized by the destruction of the insulin producing beta cells in the pancreatic islet, which is caused by self-reactive T cells infiltrating to the islets. The non-obese diabetic (NOD) mouse, the most commonly used animal model for T1D, reveals inefficient macrophage differentiation, resulting in a reduction in various macrophage functions including phagocytosis, which is believed to be one of the causes of disease acceleration. The apoptosis inhibitor of macrophage (AIM, encoded by *cd51* gene; ref. 1) is a circulating protein, produced by tissue macrophages. We found that NOD mice possessed significantly reduced

number of F4/80^{high+} liver Kupffer cells, the major AIM producing macrophage, and thus, the blood AIM level was lower in NOD mice than in non-diabetic C57BL/6 mice. Parallel results were obtained in T1D patients, whose AIM levels were lower than those in healthy individuals. We recently demonstrated that AIM accumulates at dead cells and promotes their removal by phagocytes (ref. 2). This fact suggests that lower AIM levels in NOD mice and T1D patients might contribute to the disease acceleration due to inefficient dead pancreatic beta cell clearance by macrophages. To further assess this hypothesis, we successfully generated AIM-deficient NOD mice using CRISPR/Cas9 system. We will present some results from analysis of the mice. Refs.

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1442

Nanoengineered myelin oligodendrocyte glycoprotein peptides suppressed experimental autoimmune encephalomyelitis: Implications in Multiple Sclerosis therapy*Prashant, S.¹, Payne L., N.², Sun, G.², Anusha, A.¹, Wilbin, X.¹, Gowd G., S.¹, Nair V., S.¹, Menon N., K.¹, Bernard, C.C.A.², Koyakutty, M.¹**¹Amrita Vishwa Vidyapeetham University, Amrita Centre for Nanosciences & Molecular Medicine, Kochi, India, ²Australian Regenerative Medicine Institute (ARMI), Monash University, Clayton, Australia*

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) and is characterized by the destruction of myelin and axons leading to progressive disability. Peptide epitopes from CNS proteins, such as the myelin oligodendrocyte glycoprotein (MOG), possess promising immunoregulatory potential for treating MS; however their instability and poor bioavailability is a major impediment for their use clinically. To overcome this problem, we have prepared different sized silica nanoparticles (100-500nm in diameter) and conjugated selected MOG peptides onto them in order to test the ability of such nanoengineered particles to prevent and/or treat a model of MS, experimental autoimmune encephalomyelitis (EAE) in mice. Using this approach, we showed that the intravenous injections of nanoparticles coated with MOG peptides could prevent and/or significantly delay the development of the chronic form of EAE in C57BL/6 mice when administered during the priming phase of disease. Decreased clinical scores were associated with reduced T-cell proliferative responses, pro-inflammatory cytokine secretion as well as a reduction in inflammation, demyelination and axonal damage. Significant suppression of the relapsing-remitting form of EAE was also observed in NOD/Lt mice following injections of MOG conjugated nanoparticles. Collectively these data demonstrate the therapeutic effects of nanoengineered myelin peptides in two models of MS and suggest that such an approach may have potential therapeutic value for MS.

3919

Molecular basis for the increased susceptibility of Indigenous North American tribes to seropositive rheumatoid arthritis

Sally, S.¹, Law, S.-C.², van Heemst, J.³, Wieles, D.³, Moustakis, A.⁴, Papadopoulos, G.⁵, van der Woude, D.³, Hitchon, C.⁶, Robinson, D.⁶, Huizinga, T.³, Reid, H.¹, Toes, R.³, El-Gabalawy, H.⁶, Rossjohn, J.¹, Thomas, R.²

¹Monash University, Department of Biochemistry and Molecular Biology, Clayton, Australia, ²University of Queensland, Diamantina Institute, Brisbane, Australia, ³Leiden University Medical Center, Leiden, Netherlands, ⁴Technological Educational Institute of Ioanian Islands, Argostoli, Greece, ⁵Epirus Institute of Technology, Arta, Greece, ⁶University of Manitoba, Arthritis Centre, Winnipeg, Canada

TCR recognize foreign peptides to trigger T cell activation and expansion for pathogen control; however TCR are both degenerate and cross-reactive. Polymorphisms in the HLA-DRbeta chain arose out of selection pressure imposed by lethal infections in exposed populations, enriching particular HLA-DRB1 alleles within certain ethnic groups. Rheumatoid arthritis (RA) is linked to specific HLA-DRB1 alleles. In Caucasians, alleles associated with anti-citrullinated peptide antibody (ACPA)-positive RA share a conserved motif at positions 11, 13, 71 and 74 of DRbeta. Indigenous North American Native (INA) tribes have a high risk of early-onset ACPA+ RA, associated with the rare allele HLA-DRB1*1402 association, which unexpectedly has a His13betaSer polymorphism. We determined the molecular mechanism of HLA-DRB1*1402 with ACPA+ RA. Unlike HLA-DRB1*04:01/04 where citrulline, but not positively-charged arginine, was accommodated within the electro-positive P4-pocket, both citrulline and arginine were accommodated in the P4-pocket of HLA-DRB1*14:02. When vimentin-64Cit(59-71) was bound to HLA-DRB1*04:01/04 or HLA-DRB1*14:02, citrulline was upright, solvent-exposed, and potentially accessible to TCR. When bound to HLA-DRB1*14:02, arginine-64 of vimentin(59-71) was buried in P4. The TCR repertoire of peripheral blood vimentin(59-71) and vimentin-64Cit(59-71)-specific CD4+ T cells in HLA-DRB1*14:02+ INA RA patients and at-risk first-degree relatives revealed oligoclonality among TCRalpha and TCRbeta, including a public TRBV/CDR3beta/TRBJ sequence. These data indicate broader presentation of native and citrullinated autoantigen by HLA-DRB1*14:02 than HLA-DRB1*04:01/04, and limited TCR diversity among the HLA-DRB1*14:02-restricted autoreactive CD4+ TCR repertoire. Engagement of a "best-fit" TCR for persistent pathogen control may provide a survival advantage for carriage of HLA-DRB1*1402 at the expense of self-epitope cross-reactivity.

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Bypassing STAT3-mediated Id2 inhibition improves the anti-tumor efficacy of dendritic cells

Li, H.¹, Liu, C.², Xiao, Y.¹, Chu, F.³, Liang, X.¹, Peng, W.², Neelapu, S.³, Sun, S.-C.¹, Hwu, P.², Watowich, S.¹

¹MD Anderson Cancer Center, Immunology, Houston, United States, ²MD Anderson Cancer Center, Melanoma Medical Oncology, Houston, United States, ³MD Anderson Cancer Center, Lymphoma and Myeloma, Houston, United States

STAT3, a signal transducer for various cytokines and growth factors, is constitutively activated in numerous cell types in the tumor microenvironment, including tumor-infiltrating immune populations. STAT3 has broad immunosuppressive function in cancer, as evidenced by studies using pan-hematopoietic *Stat3*-deficient animals. Nonetheless, it remains unclear whether and how STAT3 regulates the immunogenic properties of antigen-presenting dendritic cells (DCs) within tumors. Our current work suggests a previously unrecognized STAT3-regulated immunosuppressive mechanism in DCs. Here, we show that introduction of GM-CSF-derived DCs (GM-DCs) to murine melanoma tumors in vivo, or GM-DC exposure to melanoma-secreted cytokines, inhibits DC-intrinsic expression of the transcriptional regulator Id2. Melanoma-infiltrating CD103⁺ DCs also demonstrate reduced Id2 expression relative to their skin-resident counterparts. We found melanoma-associated cytokines inhibit *Id2* transcription via STAT3 signaling in DCs. Using DC-restricted *Stat3*-deficient mice and a tumor vaccination strategy with GM-DCs, we show that blockade of STAT3-mediated Id2 repression significantly improves the outcome with melanoma. Furthermore, we found Id2 expression influences GM-DC production of the pro-inflammatory cytokine TNF- α , GM-DC-mediated naive CD4⁺ T cell polarization, and the proportion of tumor-infiltrating immunoregulatory and immunostimulatory T cell populations upon GM-DC vaccination. In addition, we show the efficacy of the Id2-GM-DC vaccine is improved by combinatorial treatment with anti-PD-1 antibody. Collectively, our data indicate Id2 regulates DC-mediated tumor immunity by modulating tumor-associated CD4⁺ T cell responses. Since DCs have shown relatively modest success in cancer immunotherapy, this information may be useful to enhance the efficiency of DC vaccines in cancer.

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The mechanism of pathogenicity of IL-17A in CNS autoimmunity

Mc Ginley, A., Mills, K.H.G.

Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin, Ireland

IL-17 and IL-17-producing Th17 cells and $\gamma\delta$ T cells are emerging as central players in the pathogenesis of many autoimmune diseases. MS is an inflammatory disorder of the CNS, involving autoreactive T cell responses to myelin antigens. In this study we used a murine model of MS, EAE, to examine the role of IL-17A in CNS inflammation. Compared to WT control mice, IL-17A^{-/-}

mice had significantly attenuated EAE with a delayed onset, indicating a requirement for IL-17A in the initiation of neuronal inflammation. Consistent with this, there were significantly fewer CD4 and $\gamma\delta$ T cells secreting GM-CSF, IL-17F, or IFN γ in the lymph nodes of IL-17A^{-/-} mice during the induction phase of EAE, and in the CNS during the acute phase of disease. Furthermore, WT mice treated with anti-IL-17A antibody only at induction of EAE showed similar attenuation of EAE and reduction in Th17 responses as IL-17A^{-/-} mice, indicating a key role for IL-17A in the priming of autoreactive Th17 cells. Surprisingly, T cells from MOG-immunized IL-17A^{-/-} mice were capable of inducing disease following transfer into WT mice, demonstrating that IL-17A production by autoreactive T cells is not essential for development of EAE. Finally, we found decreased expression of Th17-promoting cytokines IL-1 β , IL-23 and IL-6 in the lymph nodes of IL-17A^{-/-} compared with WT mice early in EAE, providing further evidence that IL-17A may function not only as an effector cytokine in host defence, but also as a pathogenic cytokine in autoimmunity through the induction or the expansion of Th17 cells.

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A20 as a master regulator of islet inflammation and diabetes

Zammit, N.¹, Horikawa, K.², Langley, D.¹, Walters, S.¹, Goodnow, C.¹, Grey, S.¹

¹Garvan Institute, Darlinghurst, Australia, ²The Australian National University, The John Curtin School of Medical Research, Canberra, Australia

Aims: Single-nucleotide polymorphisms (SNPs) within tnfaip3 (A20) associate with susceptibility to autoimmune and inflammatory diseases including type-1-diabetes. How polymorphisms within the A20 locus contribute to disease at the tissue level is not understood.

Methods: In a genome-wide ENU-mutagenesis screen of C57BL/6 mice we identified a mouse line harboring a non-synonymous change of an evolutionary conserved isoleucine at amino-acid position 325 within the A20-OTU domain (A20I325N). We tested its effect on islet homeostasis.

Results: When challenged with mild inflammatory stress by syngeneic transplantation, A20I325N/I325N islet grafts show abnormal production of inflammatory factors and immune infiltration with severe glucose intolerance due to impaired beta-cell function. When directly challenged with an immunological insult by allogeneic transplantation, A20I325N/I325N islet grafts were hyper-inflammatory and more rapidly destroyed compared to WT allografts. When stimulated with TNF α , A20I325N cells exhibited exaggerated poly-ubiquitination of RIP1 at TNFR1 with enhanced NF- κ B and JNK/AP1. Conversely, ectopic expression of WT-A20 rescued the hyperinflammatory phenotype of A20I325N islets; suggesting that A20 may be used therapeutically to suppress tissue inflammation. Indeed, forced expression of A20 allowed permanent survival of ~50% of islet grafts across a full MHC-mismatch where control grafts are destroyed. The A20-expressing grafts at >100 days showed normal morphology, high frequencies of intra-graft antigen-specific regulatory T-cells with high expression of IL-10 & TGF-beta.

Conclusion: Human GWAS associate polymorphisms within the A20-OTU domain to inflammatory disease. Our data show how SNP's within this domain uncouple A20's protective effect, and provide a model for understanding how subtle A20 polymorphisms contribute to human disease.

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Regulation of IL-10 expression in human T-cells in response to TNF blockade

Roberts, C.A.¹, Durham, L.¹, Fleskens, V.¹, Rajasekhar, M.¹, Frederiksen, K.S.², Evans, H.G.¹, Taams, L.S.¹

¹King's College London, Division of Immunology, Infection and Inflammatory Diseases, Centre for Molecular and Cellular Biology of Inflammation, London, United Kingdom, ²Novo Nordisk A/S, Biopharmaceuticals Research Unit, Inflammation Biology, Måløv, Denmark

It is well established that CD4⁺ effector T-cell subpopulations expressing pro-inflammatory cytokines such as interleukin-17 (IL-17) and interferon- γ (IFN γ) can acquire regulatory potential characterised by IL-10 expression. However, the underlying cellular and molecular mechanisms in these subpopulations are not yet fully elucidated. We recently showed that TNF blocking drugs increase IL-10 expression in human IL-17⁺ CD4⁺ T-cells. Here we further characterised the regulation of IL-10 expression via blockade of TNF α signalling, or other cytokine/co-stimulatory pathways, in human T-cell subpopulations.

Addition of the anti-TNF drug adalimumab to anti-CD3-stimulated human CD4⁺ T-cell/monocyte co-cultures promoted IL-10 expression in pro-inflammatory IL-17⁺, IFN γ ⁺, TNF α ⁺ and GM-CSF⁺ CD4⁺ T-cell subpopulations. Conversely, exogenous TNF α suppressed IL-10 expression. TNF α signalling similarly regulated IL-10 expression in CD8⁺ T-cells in anti-CD3-stimulated PBMC. IL-1R1 blockade, but not blockade of IL-17 or IFN γ (using neutralising antibodies), IL-6R (using tocilizumab) or co-stimulation (using abatacept), significantly increased IL-10 expression in CD4⁺ T-cell subpopulations. We found that TNF blockade directly promoted IL-10 in effector CD4⁺ T-cells activated by anti-CD3/CD28, in the absence of monocytes or regulatory T cells and independently of IL-27.

Gene expression profiling revealed 220 genes commonly regulated by TNF blockade in both IFN γ ⁺ and IL-17⁺ T-cells; these genes were enriched for cell cycle-associated functional annotations. Addition of TNF blocking drugs impaired CD4⁺ T-cell proliferative responses; however, elevated IL-10 expression and reduced CD4⁺ T-cell proliferation were not interdependent. Current experiments seek to characterise phenotypic changes induced by TNF blockade in CD4⁺ T-cell subpopulations, which may identify further mechanistic pathways relevant to IL-10 regulation.

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Co-expression of receptors type 1 and 2 to TNF-alpha on immunocompetent cells

Alshevskaya, A., Lopatnikova, J., Belomestnova, I., Sennikov, S. Federal State Budgetary Scientific Institution 'Research Institute of Fundamental and Clinical Immunology', Novosibirsk, Russian Federation

Background: Changes in number of membrane-bound receptors type 1 and 2 to pleiotropic cytokine TNF-alpha were shown in pathological conditions, and ratio of different type membrane receptors could be critical to switch type of cell response to mediator.

Aims: To estimate ratio of cells with different combinations of membrane-bound receptors to TNF-alpha in immunocompetent cell subsets (ICS) and assess the stability of their presence on cell membrane.

Methods: Optimization of flow cytometry sorting protocol of ICS with different expression level of TNFR1/2 allowed to allocate among T-lymphocytes, B-lymphocytes and monocytes in four subsets: TNFR1+TNFR2-, TNFR1+TNFR2+, TNFR1-TNFR2+, TNFR1-TNFR2-. Cultivation of sorted fractions over 72 hours was conducted to assess the spontaneous change in the receptor representation on cell membrane.

Results: The average percentages of allocated subpopulations among intact cells were, respectively: 20.8%:4.95%:9.35%:64.9% (for T-cells), 10.8%:11.9%:22.5%:54.75% (for B-cells), 16.3%:47.1%:17.5%:19.2% (for monocytes). Sorted T-lymphocytes with initially high level of TNFR2 expression retained it after 72h cultivation. Among TNFR2- T-lymphocyte fraction not more than 2% of the cells began to express TNFR2 after cultivation.

Conclusion: Immune cells differ in the co-expression of TNFR1 and TNFR2. For T-lymphocyte sorted fractions the expression level of TNFR2 remained stable during 72 hours. Further research of apoptosis level and proliferation activity of cells with different expression levels and different combinations of membrane-bound receptors to TNF-alpha are needed to enhance understanding of the relationship between the number of receptors and functional activity of cells.

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Augmented vascular T-cell levels promote vascular dysfunction in the stroke prone spontaneously hypertensive rat via prostanoid pathways

Khan, S.L.¹, Andrews, K.L.¹, Vinh, A.², Jefferis, A.-M.¹, Memon, B.¹, Jennings, G.L.¹, Sampson, A.K.¹, Murphy, A.J.¹, Chin-Dusting, J.P.F.¹
¹Baker IDI Heart and Diabetes Institute, Melbourne, Australia,
²Monash University, Department of Pharmacology, Melbourne, Australia

Vascular dysfunction is a hallmark of hypertension and is associated with vascular T-cell infiltration. The direct effect of T-cells on vascular function is undefined. We aimed to determine how aortic T-cells effect vascular function in the stroke-prone spontaneously hypertensive rat (SHRSP) compared to

normotensive Wistar Kyoto (WKY) rats. We found impaired endothelium-dependent relaxation in the SHRSP aorta, due to a combination of reduced basal nitric oxide (NO) levels, enhanced constrictor prostanoid activity and vasodilatory prostacyclin receptor dysfunction. This was accompanied by increased aortic T-cell levels in the SHRSP compared with the WKY. Isolated T-cells from the aorta, displayed a T-helper-1 cytokine skewing in the SHRSP compared with the WKY, indicated by a higher ratio of IFN γ to IL-4 production. Interestingly, *in situ* stimulation of the aortic T-cells with anti-CD3⁺ and anti-CD28⁺ antibodies worsened endothelial function only in the SHRSP aorta. Importantly, we found that basal NO levels were compromised in both strains after stimulating the T-cells, suggesting that this was not the mechanism responsible for T-cell-mediated vascular dysfunction. Instead, we found that T-cell-mediated vascular dysfunction in the SHRSP was associated with enhanced constrictor prostanoid production that was reversed with cyclo-oxygenase (COX) inhibition. Scavenging of reactive oxygen species (ROS) also abrogated vascular dysfunction and prevented elevated constrictor prostanoid production in T-cell-stimulated SHRSP aortae, highlighting a close interaction between ROS and COX activity. In conclusion, perivascular T-cells appear to contribute to vascular dysfunction in the SHRSP through the elevated production of ROS which in turn stimulates COX to increase constrictor prostanoids.

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IL-13-dependent biomarkers with liver inflammatory differentiation after pneumococcal sepsis during rapid inflammatory phase

Yamamoto, N.^{1,2}, Nakamura, K.¹, Arai, K.³, Dela Cruz, C.², Machida, T.⁴, Suzuki, Y.⁵, Ogura, Y.⁶, Abe, Y.⁷, Sekine, H.⁴, Iseki, K.⁷, Askenase, P.², Kanemitsu, K.¹

¹Fukushima Medical University, Department of Infectin Control, Fukushima, Japan, ²Yale University School of Medicine, Section of Allergy and Clinical Immunology, New Haven, United States, ³Kitasato University School of Medicine, Laboratory of Infectious Diseases Science, Tokyo, Japan, ⁴Fukushima Medical University, Department of Immunology, Fukushima, Japan, ⁵Fukushima Medical University, Department of Blood Transfusion and Transplantation Immunology, Fukushima, Japan, ⁶Nara Women's University, Division of Human Life and Environmental Sciences, Nara, Japan, ⁷Fukushima Medical University, Department of Emergency and Critical Care Medicine, Fukushima, Japan

Recently, we demonstrated that single IL-13-gene-dysrupted rodents (BALB/c mice) had a clear defective immune response as highly sustained lung colony-forming units (CFU) to *S. pneumoniae* infection via trachea (*Immunology*, doi:10.1111/imm.12544). The IL-13^{-/-} hosts exhibited a more distinct defect as a shortened survival duration compared to control BALB/c hosts when using an intravenously-injected sepsis model of the *S. pneumoniae*. To elucidate the responsible reason of the severe mortality, serum IgM, and several biomarkers via mRNA, that include CD14, TNF α , IL-6, HMGB1, CXCL2, NLRP3, and 4, IL-1 β , IL-18, caspase1, were investigated for the peripheral mononuclear cells (PBMC), the liver tissue, and blimp-1 for splenocytes in short time of 2.5 hours up to 48 hours after induction of intravenous

infection with *S. pneumoniae*.

The significant and distinct differences were observed with a decreased TNF α and an increased NLRP3 mRNA level within 10 hours post sepsis in the liver tissue in *IL-13*^{-/-} hosts compared with control BALB/c hosts. An averaged serum anti-polysaccharide IgM level, which was measured by ELISA, was lower in *IL-13* mice compared to that of control BALB/c before sepsis, but this was inverted within 48 hours post infection.

We hypothesized that *IL-13*^{-/-} hosts bear yet unknown immunological defects in liver inflammatory differentiation and splenic natural IgM-secretory systems, and both systems affect to the poor prognosis in an acute septic condition with *S. pneumoniae*.

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Regulation of immune cell function by short chain fatty acids

*Xu, Y.*¹, *Naselli, G.*¹, *Bandala-Sanchez, E.*¹, *Thomas, T.*¹, *Harrison, L.C.*¹, *Zhang, Y.*^{1,2}

¹Walter & Eliza Hall Institute of Medical Research, Melbourne, Australia, ²Guangzhou Institute of Pediatrics, Guangzhou Women and Children's Medical Center, Guangzhou, China

Short chain fatty acids (SCFAs) including acetate, propionate and butyrate are produced by bacterial fermentation in the colon of dietary fibre and undigested carbohydrate. They have been shown to modulate immune cell function by inhibiting histone deacetylases and activating G-protein coupled receptors. We studied the contribution of SCFAs to cytokine expression in adult human T cells. In the presence of IL-2, SCFAs promoted IFN- γ expression in both CD4⁺ and CD8⁺ T cells upon T cell activation. This was abrogated by either IL-2 neutralization or inhibition of glycolysis or fatty acid synthesis (FAS). SCFAs also promoted the expression of IL-4, most strikingly in CD8⁺T cells activated in the absence of IL-2, or when FAS was inhibited following CD4⁺T cell activation. This correlated with increased expression of carnitine palmitoyltransferase I (CPT1), which is critical in mitochondrial fatty acid oxidation (FAO), and decreased phosphorylation of ribosome protein S6, a target molecule of mTOR pathway in CD8⁺T cells. Thus, SCFAs may promote both IFN- γ and IL-4 expression, either by promoting glycolysis and FAS in the presence of IL-2 or by promoting FAO in the absence of IL-2. Our findings highlight the complexity of immune modulation by SCFAs and caution in their clinical application.

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Regulation of myeloid cell differentiation by S100A8 and S100A9

Defrene, J., Laouedj, M., Cesaro, A., Pagé, N., Barabé, F., Tessier, P.
Université Laval, Quebec, Canada

S100A8 and S100A9 are calcium-binding proteins highly expressed by myeloid cells. These damage-associated molecular patterns are found at high concentrations in the serum of patients suffering from inflammatory diseases and myeloid leukemia. S100A8 and S100A9 are secreted by a mechanism presumed to be linked to inflammasome activation,

and once secreted stimulate cytokine secretion by neutrophils and monocytes by binding to TLR receptors. Recent reports suggest that S100A8 and S100A9 modulate dendritic cell maturation. In this study, we examined the roles of S100A8 and S100A9 on myeloid cell differentiation. Deletion of the *S100a8* gene led to an increase of myeloid (CD11b⁺) cells in the blood and bone marrow, including granulocyte (Ly6C⁺Ly6G^{high}) and monocyte/macrophage (Ly6C⁺Ly6G⁻CD115⁻) precursors. Similarly, expression of myeloid markers (CD11c, Gr1, and 7/4) was increased in the tissues of *S100a8*^{-/-} mice. Blocking S100A8 using antibodies induced the differentiation of acute myeloid leukemia cells in granulocytes and monocyte/macrophages, indicating that S100A8 was acting as an extracellular factor. While anti-S100A9 had no effect on myeloid cell differentiation, S100A9 protein increased expression of the neutrophil marker 7/4 in bone marrow cells, and induced the differentiation of AML cells in granulocytes and monocyte/macrophages. We conclude that S100A8 and S100A9 are differential regulators of myeloid cell differentiation. While S100A8 inhibits differentiation, S100A9 promotes differentiation of myeloid precursors/progenitors in granulocytes and monocyte/macrophages.

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Interleukin-13 predisposes to more severe influenza infection in mice and human epithelial cells by suppressing interferon responses and activating the microRNA-21/PI3K signaling pathway

*Starkey, M.R.*¹, *Dua, K.*¹, *Hsu, A.C.*¹, *Nair, P.M.*¹, *Haw, T.J.*¹, *Nguyen, D.H.*¹, *Kim, R.Y.*¹, *Horvat, J.C.*¹, *Godfrey, D.I.*², *McKenzie, A.N.*³, *Lukacs, N.W.*⁴, *Wark, P.A.*⁵, *Foster, P.S.*¹, *Hansbro, P.M.*¹

¹The University of Newcastle and Hunter Medical Research Institute, School of Biomedical Sciences and Pharmacy, New Lambton Heights, Australia, ²University of Melbourne, Peter Doherty Institute for Infection and Immunity, Department of Microbiology and Immunology, Parkville, Australia, ³Medical Research Council (MRC) Laboratory of Molecular Biology, Cambridge, United Kingdom, ⁴University of Michigan Medical School, Department of Pathology, Ann Arbor, United States, ⁵John Hunter Hospital, Department of Respiratory and Sleep Medicine, New Lambton Heights, Australia

Introduction: People with asthma and COPD are more susceptible to viral infections, which in turn are major causes of exacerbations. However, the immunological mechanisms underpinning these associations are poorly understood.

Methods: Mice were subjected to Ovalbumin (Ova)-, house dust mite (HDM)- or recombinant IL-13 (rIL-13)-induced models of allergic airway disease (AAD), or cigarette smoke-induced experimental COPD and infected with influenza (A/PR/8/34). Some groups were treated with anti-IL-13 neutralizing antibody, miRNA-21-specific antagomirs, PI3K inhibitors, steroids or relevant vehicle controls during influenza infection. Complimentary in vitro studies using bronchial epithelial cells (pBECs) from asthmatics and COPD patients were performed. The cellular source of IL-13 was determined using IL-13-reporter mice.

Results: Influenza infection enhanced the severity of Ova-, HDM- and rIL-13-induced AAD by increasing lung eosinophils, mucus

and airway hyperresponsiveness (AHR). Importantly, anti-IL-13 treatment during AAD suppressed eosinophils, mucus and AHR. Influenza virus infection also exacerbated experimental COPD. All models resulted in increased viral load, which correlated with suppressed interferon's and increased miR-21 and PI3K levels in the lung. Anti-IL-13 treatment also improved anti-viral responses and reduced viral load. IL-13 increased both miR-21 and PI3K, and inhibition of these molecules protected against infection in both mice and human pBECs. Steroids were ineffective and actually increased viral load. Influenza infection increased IL-13-production by NKT cells, ILC2 and Th2 cells.

Conclusion: IL-13 responses during asthma and COPD lead to impaired anti-viral immune responses resulting in more severe influenza infection that exacerbates the underlying disease. These studies identify anti-IL-13 as a potential therapy in influenza infections.

Vaccines 2

2055

Off-target effects of a live attenuated pertussis vaccine

Locht, C.

Inserm U 1019, Institut Pasteur de Lille, Center for Infection and Immunity of Lille, Lille, France

Pertussis, caused by *Bordetella pertussis*, is a severe respiratory disease that can be fatal, especially in young infants. Despite high vaccination coverage, pertussis is still not under control in any country and its incidence is even increasing in several industrialized countries. Although protective against pertussis disease, none of the current vaccines provide protection against infection by *B. pertussis*, which may be the main reason for its resurgence. We have developed a live attenuated pertussis vaccine, named BPZE1 that can be administered nasally. BPZE1 was constructed by the genetic alteration or removal of three toxins. It was found to be safe and efficacious in animal models and has now successfully completed a first-in-man phase I safety trial. During its pre-clinical evaluation, BPZE1 was found to possess interesting off-target properties. A single nasal administration provided protection against experimental asthma in mice. BZE1 also protected against Influenza virus-induced inflammatory pathology in mice, without significantly affecting the viral load. No T- or B-cell cross-reactivity between influenza virus and *B. pertussis* was detected, suggesting that the protection involves innate immune mechanisms. Interestingly, two administrations of BPZE1 increased the protective effect, suggesting innate immune memory. A comparison of the transcriptomic profiles of mice infected with anti-inflammatory BPZE1 compared to pro-inflammatory virulent *B. pertussis* revealed that most genes specifically activated by BPZE1 within the first days after administration code for several cytochrome P450 polypeptides, glutathione S-transferases and enzymes involved in lipid metabolism. These findings establish a link between lipid metabolism, detoxification mechanisms and boostable anti-inflammatory effects of BPZE1.

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Vaccine-induced, antigen-specific T_H9 immune response blocks tumor cell engraftment

Garipey, J., Abdul-Wahid, A., Cydzik, M., Prodeus, A., Alwash, M., Stanojic, M., Huang, E., Thompson, M.

Sunnybrook Research Institute, Toronto, Canada

Ninety percent of all cancer-related deaths are associated with the occurrence of metastases. The development of a vaccine that would prevent the implantation of circulating cancer cells or the expansion of micrometastases would represent an ideal strategy for averting the recurrence of disease (relapse) in cancer patients. We now report that a vaccination strategy yielding an antigen-specific T_H9 response can induce long term host immune surveillance that prevents the engraftment of circulating cancer cells. Specifically, the vaccination of immunocompetent CEA transgenic mice with an altered-self form of the CEA IgV-like N domain, formulated with the TLR3 agonist poly I:C, elicits a CEA N domain-specific T_H9 response, wherein IL-9 secreting T_H cells act in concert with CEA N domain-specific antibodies as well as activated mast cells in preventing tumor cell engraftment. In contrast, vaccine-imparted protection was reversed upon depleting CD3⁺ T cells, neutralizing serum IL-9, pharmacologically stabilizing or by depleting of CD117⁺ mast cells, *in vivo*. The induced immune response was specific for the N domain of CEA (CEACAM5) with no autoimmune-related pathologies observed in CEA transgenic mice that express a complement of related human CEACAMs. Collectively, these results highlight the first instance of a vaccine developing a tumor Ag-specific T_H9 immune response and points to the existence of an alternate tumor eradication mechanism that can be exploited for developing metastasis-preventing immunotherapies. (Supported by OCIR-IPDC, CBCF and CIHR).

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RNA- and protein-based influenza vaccines induce antigen-specific CD8⁺ T-cells identified by MHC class I pentamers with different gene expression profiles

Meldgaard, T.^{1,2,3}, Blengio, F.^{1,3,4}, Sammiceli, C.^{1,3}, Tavarini, S.^{1,3}, Mangiavacchi, S.^{1,3}, De Gregorio, E.^{1,3}, Rappuoli, R.^{1,3}, Geall, A.^{3,5}, Siena, E.^{1,3}, Nuti, S.^{1,3}, Kratzer, R.^{3,6}, Bertholet, S.^{1,3}

¹GSK Vaccines, Siena, Italy, ²University of Siena, Siena, Italy, ³Formerly Novartis Vaccines & Diagnostics, Siena, Italy, ⁴University of Turin, Siena, Italy, ⁵Avidity NanoMedicines, La Jolla, United States, ⁶Transgene, Lyon, France

Priming of antigen-specific CD8⁺ T cells by vaccination is key for subsequent viral clearance. To characterize the heterogeneity of vaccine-induced CD8⁺ T-cells, we performed gene expression profiling at the single-cell level, after delivery of RNA-based (SAM[®](H1) (A/California/2009/H1)) or MF59[®]-adjuvanted Monovalent Influenza Vaccine (aMIV (A/California/2009/H1N1)). CD8⁺ T cells were isolated from mouse splenocytes, stained with MHC class-I HA533-554 pentamer, single-cell sorted, lysed, and processed for multi-parametric RT-qPCR analysis of 96 individual genes.

SAM(H1) induced higher frequencies of pentamer+ CD8⁺ T cells compared to aMIV at all timepoints tested. Ten days after the

second immunization, Principal Component Analysis (PCA) revealed a transcriptional difference of CD8⁺ T cells induced by each vaccine, with upregulation of cytotoxic (GrzA+Grzb+) and inflammatory homing (Cxcr3+Cxcr6+) markers for SAM(H1), and survival markers for aMIV (CD27+). Refined analysis of effector(CD62L⁻CD127⁻CD44⁺), effector-memory(CD62L⁻CD127⁺CD44⁺) and central-memory(CD62L⁺CD127⁺CD44⁺), highlighted significant differences in gene expression profiles between and within the vaccines. SAM(H1) induced mostly effector-memory cells, while aMIV induced equal number of central-memory and effector-memory cells. Both vaccines induced few effector cells, and only CD8⁺ T cells from SAM(H1) showed terminally differentiated short-lived effector cells subpopulation (klrg1⁺IL7ra⁺CD62L⁻CXCR3⁻Tbet⁺Blimp-1⁺). Effector-memory, unlike central-memory, showed a transcriptional difference between the vaccines. SAM(H1) and aMIV upregulated members of the cytolytic/FasL and Tumor Necrosis Factor Superfamily pathways, respectively. In conclusion, this approach identified distinct pentamer⁺ CD8⁺ T-cell subsets elicited by different vaccines. Data is being analyzed for other timepoints and confirmed at the protein level using the CyTOF platform. Such results might unravel new vaccine-specific immune signatures possibly correlating with protection.

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Cross-reactive neutralizing antibody responses of high-growth enterovirus 71 genotype B5 vaccine candidate in rabbits

Chia, M.-Y.¹, Chung, W.-Y.¹, Wang, C.-H.², Chang, W.-H.², Lee, M.-S.¹

¹National Health Research Institutes, NIIDV, Zhunan, Taiwan, Republic of China, ²Academia Sinica, Institute of Chemistry, Taipei, Taiwan, Republic of China

Enterovirus 71 (EV71) is a non-enveloped RNA virus of the family *Picornaviridae*. Based on phylogenetic analysis of the most variable VP1 gene, EV71 could be classified into 3 major genogroups (A, B and C) including 11 genotypes (A, B1~B5, and C1~C5). Since 1997, different EV71 genotypes have caused life-threatening epidemics with severe neurologic complications in Asia, including Malaysia, Taiwan, Singapore, Brunei, Vietnam, Cambodia and China. Therefore, development of EV71 vaccines is a national priority in these countries. Currently, five vaccine candidates have been evaluated in clinical trials in China (3 genotype C4 candidates), Singapore (1 genotype B2 candidate), and Taiwan (1 genotype B4 candidate). Overall, these 5 vaccine candidates could not grow very well (~10⁷ PFU/ml) in cells qualified for vaccine production. In addition, genotypes of these 5 candidates are different from the current predominant genotype B5 in Taiwan and South-Eastern Asia. Therefore, we generated high-growth EV71 genotype B5 virus (HG-B5) which could grow to high titers (~10⁸ PFU/ml) in Vero cells. Infectious HG-B5 particles were purified using sucrose gradient ultracentrifugation and collected to immunize rabbits. Rabbits (two rabbits for each group) were intramuscularly immunized with two doses of purified HG-B5 at 0 and 14 day post-immunization (DPI) using two dosages (0.05 and 0.25 ug of total protein) adjuvanted with alum. The purified HG-B5

could induce cross-reactive neutralizing antibody responses against three EV71 major genogroups. The results indicate that Vero cell-derived HG-B5 vaccine candidate is immunogenic in rabbits and has commercial potential for development of new-generation EV71 vaccines.

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Multi-epitope chimaeric VLP as a therapeutic vaccine for colorectal cancer

Donaldson, B.¹, Ward, V.², Young, S.¹

¹University of Otago, Pathology, Dunedin, New Zealand, ²University of Otago, Microbiology and Immunology, Dunedin, New Zealand

The incidence of colorectal cancer (CRC) in New Zealand is more than double the world average, estimated to be responsible for almost 700,000 deaths worldwide annually. Treatment options for CRC are limited, and therapeutic vaccination is a promising alternative or supplement to conventional therapy. Therapeutic vaccines for cancer are designed to invigorate the immune system, triggering a cytotoxic immune response targeting tumour-associated antigens (TAAs). Our laboratory produces chimaeric virus-like particles (VLP) formed from the Rabbit hemorrhagic disease virus (RHDV) capsid protein VP60 as a vector for therapeutic cancer vaccines. RHDV VLP are an effective cancer vaccine vector, because they promote TAA epitope cross-presentation.

In this study, we recombinantly inserted epitopes derived from murine CRC TAAs into VLP. These VLP induced a potent cytotoxic immune response specific for these epitopes in *in vivo* cytotoxicity assays. Vaccination with VLP containing a single CRC epitope had efficacy comparable to a model antigen construct in a subcutaneous murine CRC trial, delaying tumour growth with 50% overall survival. Tumour growth rate was further delayed with a vaccine containing two different CRC epitopes, with overall survival boosted to 60%. 100% of mice cured of their primary tumour with either of these CRC vaccines were protected from rechallenge in their opposing flank, indicating the presence of persistent systemic immunity. The enhancement of vaccine efficacy by incorporating a second TAA epitope may be indicative that further improvement is possible by including more targets in a multi-epitope therapeutic vaccine.

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Induction of mucosal and systemic immune responses following intranasal vaccination with liposomes containing surface-expressed peptide, encapsulated carrier protein: protection from group A streptococcus following challenge

Zaman, M., Ozberk, V., Reiman, J., Langshaw, E., Pandey, M.,

Batzloff, M., Good, M.

Institute for Glycomics, Griffith University, Southport, Australia

Group A streptococcus (GAS) infections are extremely important clinical problems due to their global health burden. Rheumatic fever and rheumatic heart disease are responsible for the majority of morbidity and mortality (estimated at 12 million cases annually with 380,000 fatalities) (1). Current leading GAS vaccine candidates are challenged by their limited efficacy

against primary GAS infections of the upper respiratory tract (URT) due to lack of mucosal antibody responses (2). This is due in large part to the lack of human approved mucosal adjuvants. We describe an innovative vaccine strategy to induce mucosal and serum antibodies against GAS in animal models based on an important neutralizing antibody determinant from the cell surface GAS M protein. Incorporation of carrier protein and M protein-based B cell epitopes onto liposomal vesicles induce potent serum and mucosal antibodies without the need for an additional adjuvant and which was capable of preventing GAS infection post challenge. The liposomal construct was capable of activating human dendritic cells, highlighting the promise of the self-adjuvanting construct to translate from murine studies to application in humans as a vaccine candidate. Our findings are an important step toward overcoming current obstacles in the development of a GAS vaccine to prevent infection at mucosal sites and community dissemination. The study provides important mechanistic insights into how liposomal particulate delivery systems can collectively induce the desired mucosal immune responses to combat GAS infection. The strategy reported here is relevant to the development of subunit mucosal vaccines against other pathogenic organisms.

1023

A novel TB vaccine based on *Mycobacterium tuberculosis* sulphur metabolism enzymes formulated with Advax™ polysaccharide adjuvant

Counoupas, C.^{1,2}, *Pinto, R.*^{1,2}, *Ngalingam, G.*^{1,2}, *Britton, W.*^{1,2}, *Petrovsky, N.*^{3,4}, *Triccas, J.*^{1,2}

¹University of Sydney, Infectious Diseases and Immunology, Camperdown, Australia, ²Centenary Institute, TB Program, Camperdown, Australia, ³Flinders Medical Centre, Vaxine Pty Ltd, Adelaide, Australia, ⁴Flinders University, Department of Endocrinology, Adelaide, Australia

There is an ongoing need for the rational design of novel safe and effective vaccines against *Mycobacterium tuberculosis* infection. We have previously identified members of the *M. tuberculosis* sulphate assimilation pathway (SAP) as protective antigens that are expressed during multiple stages of tuberculosis disease in animal models (e.g. active and chronic phases). Fusion proteins based on CysD, the major SAP protective component, were highly immunogenic in mice, resulting in generation of a high frequency of multi-functional CD4⁺ T cells, both pre- and post-aerosol *M. tuberculosis* challenge. To further enhance vaccine potency, the lead candidate vaccine, termed CysVac2, was tested in combination with a range of adjuvants including Advax, a novel polysaccharide adjuvant derived from delta inulin. The combination of CysVac2 and Advax provided significantly reduced bacterial burden in the lungs of *M. tuberculosis*-challenged mice, with no safety or reactogenicity issues identified. Following further optimization of the CysVac2-Advax formulation, studies were undertaken into the nature of the observed protective effect. Vaccine administration resulted in a rapid influx of monocytes/macrophages and neutrophils to the site of vaccination, as well as pronounced priming of *M. tuberculosis*-specific CD4⁺ T cells, resulting in increased IFN- γ ⁺TNF⁺IL-2⁺ effector CD4⁺ T cells. As delta inulin adjuvants have

shown a good safety profile when previously trialed in humans, this particular CysVac2 vaccine formulation appears a strong candidate for further preclinical evaluation for progression to human trials.

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A model to study the impact of polymorphism in vaccine design, using altered peptide ligands of an immunodominant CD8 T cell epitope

Wilson, K.L., Xiang, S.D., Plebanski, M.

Monash University, Melbourne, Australia

Vaccines to complex pathogens containing polymorphic antigens, such as pre-erythrocytic malaria parasites, need to be highly immunogenic as well as broadly cross-reactive to account for antigen variability. Little is known about how adjuvant selection for inclusion in vaccines affects the breadth of the immune response being induced. Variant T cell epitope sequences with single or multiple amino acid substitutions are termed altered peptide ligands (APLs). Herein, we present a model investigating APL reactivity to the immunodominant CD8 T cell epitope of the murine malaria strain *Plasmodium berghei*, SYIPSAEKI (pb9, or KI for short). Different adjuvants, representing natural adjuvants (*ex-vivo* peptide pulsed dendritic cells), conventional inflammatory adjuvants (Montanide and poly I:C), as well as non-inflammatory nanoparticle carriers in the viral size range (40-50nm polystyrene nanoparticles), were compared for their ability to induce high homologous and heterologous (cross-reactive) immune responses. Interestingly, whereas the overall magnitude of homologous immune responses was affected by the adjuvant used, changing adjuvants did not alter the pattern of cross-reactivity. By contrast, including APLs in the vaccine formulation was able to increase the breadth of cross-reactive responses. These results indicate that it may be pertinent to consider natural or re-engineered APLs of variant epitopes as vaccine candidates in future studies.

3617

Vaccine antigen design to maximize anti-HIV CD4+ T cell responses

*Cunha-Neto, E.*¹, *Ribeiro, S.*¹, *Santoro Rosa, D.*², *Almeida, R.*¹, *Kalil, J.*¹

¹University of São Paulo, São Paulo, Brazil, ²Federal University of São Paulo, São Paulo, Brazil

Eliciting CD4⁺ T cell responses has been essentially unexplored in the HIV vaccine field, despite the increasing importance of the CD4⁺ T cell response in protection against HIV. We used rational vaccine design to develop a DNA vaccine encoding HIV-1 B subtype or M-type conserved, multiple HLA-DR-binding CD4⁺ T cell epitopes, found to be recognized by multiple HIV-1-infected patients. Vaccines elicited broad, polyfunctional, and long-lived CD4⁺ T cell responses in BALB/c and HLA class II transgenic mice, eliciting extensive cross-clade immunity. Immunization of BALB/c mice increased CD8⁺ T cell responses against subsequent whole HIV protein immunization, and reduced viral titers after challenge with a recombinant vaccinia virus encoding HIV proteins. Immunization prior to recombinant

gp140 HIV envelope protein drastically increased the IgG2a/IgG1 ratio of elicited anti-gp140 antibodies. Immunization of Rhesus macaques induced broad IFN- γ ELISPOT responses 10-fold higher than those found in mice; we found a predominantly CD4⁺ T cell intracellular cytokine response basically consisting of IFN γ , TNF α IL-2, Granzyme B. By virtue of inducing broad responses against multiple conserved CD4⁺ T cell epitopes that can be recognized across diverse HLA class II alleles, this vaccine concept may induce T cell responses against multiple peptides in large proportion of the population. By increasing the chance of matching the responses with multiple epitopes in the infecting HIV isolate, the vaccine concept may also cope with HIV genetic variability. It may be a candidate for standalone use or in association with conventional immunogens, to increase the amplitude and coverage of the induced response.

Tumour Immunology 2

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The immunoreceptor TIGIT regulates anti-tumor and anti-viral T cell effector function

Grogan, J.

Genentech, Cancer Immunology, South San Francisco, United States

Strategies to reactivate exhausted antitumor immune responses with antibody blockade of key T cell coinhibitory receptors such as PD1/PDL1 or CTLA-4 have demonstrated transformational potential in the clinic. We recently identified T cell immunoreceptor with immunoglobulin (Ig) and ITIM domains (TIGIT), a co-inhibitory receptor on T and NK cells, which critically limits anti-tumor and other CD8⁺ T cell-dependent chronic immune responses. TIGIT is an Ig super family member expressed on the surface of activated T cell and natural killer (NK) cell subsets. TIGIT interacts with high affinity with CD155 (poliovirus receptor [PVR]). Activation of TIGIT on T and NK cells limits proliferation, effector cytokine production, and killing of target tumor cells. TIGIT is elevated in the tumor microenvironment in many human tumors, is coordinately expressed with other checkpoint immune receptors such as PD1. In models of both cancer and chronic viral infection antibody co-blockade of TIGIT and PD-L1 enhanced CD8⁺ T cell effector function, resulting in significant tumor and viral clearance respectively. This effect was abrogated by blockade of TIGIT's complementary co-stimulatory receptor, CD226, whose dimerization is disrupted upon direct interaction with TIGIT in cis. These results define a key role for TIGIT in inhibiting chronic CD8⁺ T cell-dependent responses. Therapeutic blockade of TIGIT may result in improved eradication of malignancies when used in conjunction with other anticancer therapies including those that modulate anti-tumor immune responses.

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Monoclonal antibodies against GARP/TGF- β 1 complexes inhibit the immunosuppressive activity of human regulatory T cells *in vivo*

Liénart, S., Coulie, P., Lucas, S.

Université Catholique de Louvain / de Duve Institute, Brussels, Belgium

Regulatory T lymphocytes (Tregs) are essential to prevent autoimmunity, but excessive Treg function contributes to cancer progression by inhibiting anti-tumor immune responses. Tregs exert contact-dependent inhibition of immune cells through the production of active TGF- β 1. On the Treg cell surface, TGF- β 1 is in an inactive form bound to membrane protein GARP and then activated by an unknown mechanism. We demonstrated that GARP is involved in this activation mechanism. Two anti-GARP monoclonal antibodies were generated that block the production of active TGF- β 1 by human Tregs. These antibodies recognize a conformational epitope that requires amino-acids GARP¹³⁷⁻¹³⁹ within GARP/TGF- β 1 complexes. A variety of antibodies recognizing other GARP epitopes did not block active TGF- β 1 production by Tregs. In a model of xenogeneic graft-versus-host disease in NSG mice, the blocking antibodies inhibited the immunosuppressive activity of human Tregs. These antibodies may serve as therapeutic tools to boost immune responses to infection or cancer, via a mechanism of action distinct from that of currently available immunomodulatory antibodies. Used alone or in combination with tumor vaccines or antibodies targeting the CTLA4 or PD1/PDL1 pathways, blocking anti-GARP antibodies may improve the efficiency of cancer immunotherapy. We recently obtained the crystal structure of GARP/TGF- β 1 complexes interacting with the Fab of a blocking anti-GARP antibody. We will also explain how these data help us understand the mechanism of action of such antibodies.

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Interactions between ILC3 and tumor microenvironmental cells in lung cancer: role in the development of intratumoral tertiary lymphoid structures

Carrega, P.¹, Campana, S.², Mingari, M.³, Moretta, L.⁴, Ferlazzo, G.⁵

¹Istituto G. Gaslini, Genova, Italy, ²University of Messina, Messina, Italy, ³University of Genova, Genova, Italy, ⁴IRCCS Bambin Gesù Hospital, Rome, Italy, ⁵University of Messina, Human Pathology, Messina, Italy

Tertiary lymphoid structures (TLS) are a common finding in non-small-cell lung cancer (NSCLC) and are predictors of favorable clinical outcome. Here we show that NCR⁺ Innate Lymphoid Cells (ILC)3 are present in the lymphoid infiltrate of human non-small-cell lung cancer (NSCLC) and are mainly localized at the edge of tumor-associated TLS. This intra-tumoral lymphocyte subset is endowed with lymphoid tissue-inducing properties and, upon activation, produces IL-22, TNF- α , IL-8, IL-2 and activates endothelial cells. Tumor-NCR⁺ILC3 may interact with both lung tumor cells and tumor-associated fibroblasts, resulting in the release of cytokines primarily upon engagement of the NKp44 activating receptor. On the other hand, the interaction between

ILC3 and BDCA1⁺ dendritic cells (DCs), but not BDCA3⁺ DCs, results in ILC3 activation, proliferation and production of a large amount of relevant cytokines, including lymphotoxins, but DC-induced activation of ILC3 functions are NKp44 independent and mediated by DC-derived soluble factors.

In patients, NCR⁺ILC3 are present in significantly higher amounts in stage I/II NSCLC than in more advanced tumor stages and their presence correlate with the density of intratumoral TLS. Our results indicate that NCR⁺ILC3 accumulate in human NSCLC tissue, where they interact with different cells of the tumor microenvironment, possibly contributing to the formation of protective tumor-associated TLS.

2899

High dimensional analysis of tumor mutant antigen-specific CD8⁺ T cells shows that checkpoint blockade immunotherapy targets T cells in tumors but not the periphery

Fehlings, M.¹, Penny, H.L.¹, Gubin, M.M.², Ward, J.P.², Simoni, Y.J.¹, Wong, S.C.¹, Schreiber, R.D.², Newell, E.W.¹

¹A*STAR, Singapore Immunology Network, Singapore, Singapore,

²Washington University School of Medicine, Department of Pathology and Immunology, St. Louis, United States

Recent advances in the field of cancer immunotherapy provide evidence that checkpoint blockade mediated tumor rejection can depend on mutant tumor antigen-specific CD8⁺ T cells. However, analysis of such T cells in tumor bearing individuals is challenging due to the need to detect rare antigen-specific T cell populations in patient samples that are limited in size. Here, we evaluate CD8⁺ T cells from mice bearing progressively growing methylcholanthrene (MCA)-induced sarcomas that are susceptible to checkpoint blockade immunotherapy. By coupling mass cytometry with multiplex combinatorial tetramer staining we screened for CD8⁺ T cells targeting 82 potential mutant peptide-MHC complexes, while retaining the ability to further characterize these cells by several functional and phenotypic marker molecules. We identified significant numbers of CD8⁺ T cells restricted to two major mutant epitopes, mutant Lama4 (mLama4) and mutant Alg8 (mAlg8), simultaneously in tumors, spleens, draining and non-draining lymph nodes in untreated tumor bearing mice. Notably, antigen-specific CD8⁺ T cells from tumors were higher in frequencies and displayed higher levels of PD-1, Tim-3, and Lag-3 expression than their peripheral counterparts. Following anti-CTLA-4 therapy, we detected an increase in frequencies of mutant tumor antigen-specific CD8⁺ T cells isolated from tumors but not the periphery. In addition, whereas tumor infiltrating T cells showed a significantly reduced expression of markers associated with exhaustion, peripheral T cells were not affected. In conclusion, our approach facilitates mapping of a wide variety of tumor epitope candidates for the comprehensive analysis of rare mutant tumor antigen-specific T cells within different tissues.

3980

T cell acute leukaemia development and chemo-resistance are mediated by dynamic interactions with heterogeneous bone marrow microenvironments

Hawkins, E.^{1,2}, Duarte, D.², Akinduro, F.², Khorshed, R.², Passaro, D.³, Nowicka, M.⁴, Strazkowski, L.⁵, Ruivo, N.², Scott, M.², Rothery, S.², Foster, K.³, Waibel, M.⁶, Johnstone, R.⁶, Robinson, M.⁴, Purton, L.⁵, Bonnet, D.³, Lo Celso, C.²

¹WEHI, Melbourne, Australia, ²Imperial College London, London, UK, London, United Kingdom, ³Francis Crick Institute, London, United Kingdom, ⁴University of Zurich, Zurich, Switzerland, ⁵St Vincent's Hospital, Melbourne, Australia, ⁶Peter MacCallum Cancer Centre, Melbourne, Australia

It is widely accepted that complex interactions between immune cells and their surrounding microenvironments contribute to normal cell function but also disease development. In light of this observed interdependency, novel interventions that target specific stromal cell lineages and their interactions with malignant lymphocytes are being sought. To this end, we studied both a mouse and human xenograft model of T cell acute lymphoblastic leukaemia (T-ALL) and developed novel intravital microscopy methods that allowed us to monitor the same cells and microenvironments in the bone marrow (BM) for up to 14 hours and repeated imaging sessions over multiple days. We observed highly dynamic interactions and promiscuous distribution of cells that migrated across the BM, without any preferential association with microenvironments. Unexpectedly, this environment-agnostic behaviour was maintained throughout disease including development of resistance to chemotherapy. Our results reveal that malignant lymphocytes do not depend on specific microenvironments for propagation of disease, nor the selection of chemo-resistant clones, suggesting a stochastic mechanism underlies these processes. Yet, while infiltration and progression are independent of the stroma, accumulation of malignant lymphocytes leads to rapid, selective remodelling of the endosteal space, resulting in a complete loss of mature osteoblastic cells whilst perivascular cells are maintained. This outcome leads to a shift in the balance of endogenous BM stroma, towards a composition associated with less efficient haematopoietic cell function. This novel, dynamic analysis of T-ALL highlights that future therapeutic interventions should target cell-intrinsic mechanisms, in order to combat the invasion by, and survival of, therapy-resistant lymphocytes.

1694

Fibroblast activation protein induces inflammatory fibroblasts via STAT3-CCL2 signaling to promote tumor immunosuppression

Lin, Y.¹, Yang, X.¹, Shi, Y.², Li, B.¹, Dang, Y.³, Chu, Y.¹, Fan, J.², He, R.¹

¹Fudan University Shanghai Medical College, Shanghai, China,

²Fudan University Zhongshan Hospital, Shanghai, China, ³Fudan University Shanghai Medical College, Department of Biochemistry and Molecular Biology, Shanghai, China

Cancer associated fibroblasts (CAFs) recently are demonstrated to be inflammatory and mediate tumor-promoting inflammation,

however, the key molecules that determine inflammatory CAFs are poorly understood. Here we demonstrate that fibroblast activation protein-a (FAP) is required for the induction of a subset of inflammatory CAFs, as primary FAP⁺CAF, but not FAP⁻CAF, exhibited greatly increased STAT3 activation and inflammatory gene expression, particularly CCL2, and forced FAP expression endowed the normal fibroblasts with similar inflammatory phenotype. We further identified FAP as a persistent activator of fibroblastic STAT3 through FAK-c-Src-JAK2 signaling, which is dependent on its interaction with urokinase-type plasminogen activator receptor (uPAR). Moreover, FAP⁺CAF are major source of CCL2 within a transplanted murine liver tumor, and STAT3-CCL2 signaling is responsible for the tumor-promoting capability of FAP⁺CAF by enhancing tumoral recruitment of myeloid-derived suppressive cells (MDSCs) and immunosuppression, which was abrogated in *Ccr2*^{-/-} mice. Lastly, the positive correlation among fibroblastic expression of FAP, p-STAT3 and CCL2 was confirmed in a highly desmoplastic human intrahepatic cholangiocarcinoma (ICC), and increased stromal FAP levels predicted its poor outcome. Together our study reveals the critical role of fibroblastic FAP-STAT3-CCL2 axis in tumor-promoting inflammatory CAFs, and provides potential targets for specifically normalizing inflammatory CAFs to treat some inflammation and desmoplasia-associated cancers.

3694

Neutrophils are protective in cancerogenesis by altering tumor microenvironment and controlling intestinal microbiota

Ponzetta, A.¹, Galdiero, M.R.¹, Barbagallo, M.¹, Molgora, M.¹, Bonavita, E.¹, Magrini, E.¹, Polentarutti, N.¹, Garlanda, C.^{1,2}, Mantovani, A.^{1,2}, Jaillon, S.^{1,2}

¹Humanitas Clinical and Research Center, Lab of Experimental Immunopathology, Rozzano, Italy, ²Hunimed - Humanitas University, Rozzano, Italy

The view of neutrophil as a cell involved only in the early phases of inflammation has recently been challenged and neutrophils are now considered key players in the orchestration of the immune response. Though several studies relied on antibody-based neutrophil depletion to determine their contribution to tumor development, rigorous in vivo genetic evidence explaining the neutrophil role in cancerogenesis is missing.

We investigated this issue using key preclinical models of chemically-induced cancer (3-MCA induced sarcoma and AOM/DSS induced colorectal cancer, CRC) and taking advantage of a genetic model of neutrophil deficiency (i.e. *csf3r*^{-/-} mice).

Neutrophil deficiency was associated with increased susceptibility to sarcoma and CRC, and tumor microenvironment displayed protumoral features (e.g. increased frequency of M2 macrophages and Tregs, higher levels of IL-10 and reduced IFN γ concentration). In addition, in WT mice increased neutrophil infiltrate significantly correlated with reduced proliferation rate of tumor cells and adoptive transfer of naïve neutrophils reduced tumor growth in *csf3r*^{-/-} mice. Finally, the increased susceptibility to CRC in *csf3r*^{-/-} mice was dependent on intestinal microflora, and was abolished in cohousing experiments.

Collectively, our data support that genetic deficiency of

neutrophils affects the anti-tumor response and is associated with increased susceptibility to chemically-induced cancerogenesis.

Until recently neutrophil function was mostly related to acute inflammation and defense against pathogens. We (and others) have challenged this dogma and demonstrated that neutrophils represent an essential component in the control of tumor onset and development.

1897

Tissue resident CD103⁺CD69⁺ T cells develop rapidly and do not require cognate antigen within the epidermis of a mouse model of precancerous skin

Leggatt, G., Veitch, M., Sheehan, B., McKee, S., Linedale, R., Jazayeri, S.D., Mattarollo, S.

University of Queensland, Diamantina Institute, Brisbane, Australia

Skin resident memory CD8 T cells (Trm), characterized by expression of CD103 and CD69 surface markers, are compartmentalised in the skin epithelium and play an important role in recall responses against infectious pathogens such as herpes simplex virus. Less understood is the development of Trm cells in cancer settings particularly in the early stages of non-melanoma skin cancer. Our study examines the development of CD103⁺CD69⁺ skin CD8 T cells in a mouse model of early NMSC where the HPVE7 oncoprotein expressed in keratin 14⁺ cells drives epithelial hyperplasia (K14E7 transgenic mice). The hyperplastic skin leads to a prominent accumulation of Trm cells which are resistant to male antigen mediated deletion in female mice. Upon transfer of activated, ovalbumin-specific OT-1 cells to the K14E7 transgenic mice, expression of CD103 and CD69 was upregulated on transferred OT-1 cells within the skin, but not lymph node, within one week. This suggests that skin resident cells develop rapidly in a process that does not require cognate antigen expression within the skin. The skin-resident OT-1 cells also showed reduced function while the inhibitory molecule, PD-1, was shown to be increased on endogenous CD8 T cells within K14E7 mice. Consequently, the data suggest that development of Trm-like cells can occur early in skin cancer development although the suppressive microenvironment might reduce their ability to impact on tumour progression.

2889

STK4 regulates TLR pathways and protects against chronic inflammation-related hepatocellular carcinoma

Li, W.¹, Xiao, J.¹, Wang, H.¹, Wei, B.^{1,2}

¹Institute of Biochemistry and Cell Biology Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China, ²Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China

Hepatocellular carcinoma (HCC) is frequently associated with pathogen infection-induced chronic inflammation. Large numbers of innate immune cells are present in HCCs and can influence disease outcome. Here, we demonstrated that the tumor suppressor serine/threonine-protein kinase 4 (STK4) differentially regulates TLR3/4/9-mediated inflammatory

responses in macrophages and thereby is protective against chronic inflammation-associated HCC. STK4 dampened TLR4/9-induced proinflammatory cytokine secretion but enhanced TLR3/4-triggered IFN- β production via binding to and phosphorylating IL-1 receptor-associated kinase 1 (IRAK1), leading to IRAK1 degradation. Notably, macrophage-specific Stk4 deletion resulted in chronic inflammation, liver fibrosis, and HCC in mice treated with a combination of diethylnitrosamine (DEN) and CCl₄, along with either LPS or *E. coli* infection. STK4 expression was markedly reduced in macrophages isolated from human HCC patients and was inversely associated with the levels of IRAK1, IL-6, and phospho-p65 or phospho-STAT3. Moreover, serum STK4 levels were specifically decreased in HCC patients with high levels of IL-6. In STK4-deficient mice, treatment with an IRAK1/4 inhibitor after DEN administration reduced serum IL-6 levels and liver tumor numbers to levels similar to those observed in the control mice. Together, our results suggest that STK4 has potential as a diagnostic biomarker and therapeutic target for inflammation-induced HCC.

These proposed observations were published in latest *J Clin Invest.* 2015 Nov 2;125(11):4239-54.

3636

The implications of vaccination-induced IFN-gamma signalling on the control of B-cell lymphoma and the acquisition of immune resistance

Rearden, R., Sah, A., Doff, B., Kobayashi, T., McKee, S., Leggatt, G., Mattarollo, S.

University of Queensland Diamantina Institute, Woolloongabba, Australia

Immunomodulatory therapies can effectively control haematological malignancies by promoting anti-tumour immunity. However, underdeveloped mechanistic insights and frequent tumour relapse limits the translation of many promising strategies. We previously developed a therapeutic natural killer T (NKT) cell-targeting vaccine that achieves transient control of poorly immunogenic murine non-Hodgkin B-cell lymphomas (B-NHL) and almost total control when combined with the agonistic monoclonal antibody anti-4-1BB (CD137). Therapeutic efficacy is highly dependent on the vaccine's ability to provoke rapid interferon-gamma (IFN γ) production from NKT and NK cells. By manipulating the capacity of either host or lymphoma cells to signal through IFN γ receptor (IFN γ R), we investigated whether the therapeutic effect conferred by vaccine-induced IFN γ results from immune cell activation, lymphoma IFN γ sensitivity or a combination of both. We demonstrated that successful therapeutic vaccination requires intact IFN γ signalling within host cells but not tumour cells. IFN γ R-deficient mice failed to mount an effective anti-tumour immune response following vaccination despite elevated IFN γ levels. With consecutive exposures to vaccination, lymphomas acquired increasingly therapy-resistant phenotypes and displayed a reduction in MHC-I and CD1d surface expression, independent of tumour intrinsic IFN γ signalling. Our results suggest that immunotherapy-induced IFN γ production exerts its therapeutic effect via signalling through host cells, rather than directly upon tumour cells in lymphoma. Consequently, intact IFN γ signalling within patients' immune compartment rather than tumour cell

sensitivity to IFN γ may be more critical for successful treatment. Finally, tumour IFN γ signalling alone does not drive acquired tumour resistance to vaccination, implying that additional immunoediting pathways are responsible for tumour immune escape.

Mini Oral Sessions

15:30:00 - 16:30:00

Dendritic Cells 1

2170

Blood dendritic cell immunotherapy as consolidation therapy for acute myeloid leukaemia

Hsu, J.¹, Fromm, P.¹, Papadimitriou, M.¹, Bryant, C.^{1,2}, Orellana, D.², Gasiorowski, R.^{1,3}, Brown, R.², Ho, J.², Iland, H.², Gibson, J.², Joshua, D.², Clark, G.¹, Hart, D.¹

¹ANZAC Research Institute, Dendritic Cell Research, Sydney, Australia, ²Royal Prince Alfred Hospital, Department of Haematology, Sydney, Australia, ³Concord Repatriation General Hospital, Department of Haematology, Sydney, Australia

Acute myeloid leukaemia (AML) relapse after chemotherapy is common. Allogeneic haematopoietic cell transplantation enables immune-mediated removal of residual leukaemic blasts but graft versus host disease (GVHD) is a major risk. Dendritic cell (DC) vaccination may amplify or induce anti-leukaemic T cell responses with no risk of GVHD. Monocyte-derived DC (Mo-DC) vaccines loaded with Wilms Tumour 1 (WT1) mRNA have generated encouraging clinical results in AML but improved DC preparations are essential.

We identified the window of opportunity for blood DC (BDC) vaccination in AML after induction chemotherapy by documenting BDC subset recovery 6-12 weeks post chemotherapy. Using a human chimeric CMRF56 antibody (hCMRF56), we purified BDC from AML patients and showed that these BDC stimulated autologous viral and tumour specific T cell responses, confirming the feasibility of the strategy. Importantly, we showed that hCMRF56 does not bind to AML blasts and that it enriched both CD1c and CD141 myeloid DC. Loading BDC with *in vitro* transcribed mRNA encoded antigen results in superior, prolonged MHC I antigen presentation compared to Mo-DC, which is transitory. Stimulation of hCMRF56⁺ BDC with GM-CSF and loading with WT1 mRNA expanded functional cytotoxic antigen specific T cells.

The use of BDC has the potential to improve therapeutic efficacy by harnessing a superior antigen-presenting cell that has fundamentally different properties to *in vitro* manufactured Mo-DC. This new generation of BDC vaccine may consolidate AML remission with minimal risk and prevent the otherwise predictable relapse.

1437

EpCAM⁺ dermal dendritic cells drive FITC/DBP-induced Th2 differentiation

Cho, Y., Kang, S.J.

Korea Advanced Institute of Science and Technology (KAIST), Daejeon, Korea, Republic of

Despite the reputation of dendritic cells as being the most potent antigen presenting cell, the involvement of dendritic cells

in T helper 2 cell immunity is not well established yet compared to other types of T helper responses. By using Fluorescein isothiocyanate (FITC) dissolved in acetone:dibutyl phthalate (DBP), a conventional method to induce Th2-mediated contact hypersensitivity, we sought to identify immune cells that are responsible for inducing Th2 responses. We focused on the early events by applying FITC/DBP once without inducing contact hypersensitivity. A single application of FITC/DBP was sufficient to drive Th2 responses as we observed early production of IL-4 from T cells and basophils in cutaneous lymph nodes. Although basophils produced IL-4 earlier than CD4 T cells, they were not essential for Th2 differentiation. A previous study has emphasized on the role of PDL2-positive DCs in Th2 differentiation. However, they are unable to differentiate naive CD4 T cells to Th2 cells *in vitro*. We examined the kinetics of migratory DC subsets in the setting of FITC/DBP Th2 differentiation and found the differential expression of PDL2 on each migratory DC subset. We found that EpCAM⁺ dermal DCs are required in Th2 differentiation after FITC/DBP as transient depletion of EpCAM⁺ dermal DCs failed to induce Th2 response. Surprisingly, we found that the cooperation of PDL2-negative EpCAM⁺ dermal DCs and CD11b⁺ dermal DCs are important for Th2 immune response in FITC/DBP Th2 differentiation.

3932

Abnormalities of antigen presenting cells in type 1 diabetes

Boks, M.¹, Buchanan, K.¹, Nagl, L.¹, Harris, M.^{1,2}, Thomas, R.¹

¹University of Queensland Diamantina Institute, Translational Research Institute, Brisbane, Australia, ²Mater Children's Hospital, Brisbane, Australia

In type 1 diabetes (T1D) islet beta cell-specific autoimmunity promotes their inflammatory destruction by islet-reactive T-cells and progressive decline in capacity to produce insulin. Autoreactive T-cell activation is driven by antigen presenting cells, such as dendritic cells (DC), presenting islet self-antigens in draining pancreatic lymph nodes. We showed that the proportion of CD14^{lo}CD16⁺ monocytes was significantly increased in children with T1D and first degree relatives (FDR) compared to unrelated healthy control (HC) children. While the lack of CD14 and CD16 expression identifies peripheral blood (PB) DC precursors, these markers fail to distinguish the different functional DC subsets in circulation. Therefore, we further analysed the CD1c⁺ and CD141⁺ myeloid, and the CD123⁺ plasmacytoid DC subsets in PB of 11 children aged 9-13y presenting with T1D within the previous 3 months, 15 age and sex-matched FDR and 10 unrelated adult HC. The proportion of CD123⁺ pDC was significantly increased in children with T1D and FDR compared to HC. Presence of islet autoantibodies did not influence the proportion of pDC in FDR. The expression of HLA-DR by pDC of diabetic subjects was significantly higher than that of FDR or HC, indicating increased pDC activation and antigen presenting capacity. These data suggest that genetic or environmental factors increase pDC in children at risk, but a specific increase in pDC activity characterizes children who progressed to diabetes. Since pDC normally tolerate CD4⁺ T cells, this activation has important implications for the erosion of immune tolerance in this group.

1670**Effect of TNF- α on the process of Langerhans cell induction from human peripheral mononuclear cells***Otsuka, Y.^{1,2}, Koike, E.¹, Azuma, H.¹, Mayumi, N.^{1,2}, Saeki, H.², Takahashi, H.¹*¹*Nippon Medical School, Department of Microbiology and Immunology, Tokyo, Japan, ²Nippon Medical School, Department of Dermatology, Tokyo, Japan*

It has become widely known that anti-tumor necrosis factor (TNF)- α (TNF- α) agents (infliximab, etanercept, and adalimumab) are promising drugs to treat various inflammatory autoimmune diseases. However, we have to consider new-onset of various skin lesions such as lupus, eczema, alopecia-areata or psoriasis as an adverse side effect that can be triggered by the anti-TNF- α therapy. Epidermal Langerhans cells (LCs) originally derived from yolk sac or fetal liver in the pregnant state, while, after birth, they move to reside predominantly in the stratum spinosum of epidermis as sentinels and renew themselves from specialized local precursor cells in a steady state by interacting with keratinocytes through their E-cadherin as we have reported recently (*Eur. J Immunol.*, 43:270-280, 2013). However, when LCs in the epidermis were in the inflammatory phase, they may be renewed from peripheral mononuclear cells with the assistance of various cytokines, such as IL-6, TNF- α , or IL-1 β . Among them, TNF- α is essential for innate LCs to migrate from epidermal region to the T-cell rich paracortical area to generate Ag-specific primary acquired T-cell responses and thus, anti-TNF- α therapy may inhibit LC function and develop various cutaneous lesions. In the present study, we examined the effect of TNF- α on the process of induction or renewal of LCs when peripheral blood monocytes (PBMo) were stimulated with GM-CSF, IL-4, and TGF- β 1. We found up-regulation of Langerin as well as down-regulation of DC-SIGN. Thus, anti-TNF- α reagents may deteriorate renewal and function of LCs and inhibit a repair of various skin lesions.

4467**Immunomodulatory Drugs (IMiDs), lenalidomide and pomalidomide, have regulatory functions for human myeloid dendritic cells***Phan Thi, X.V., Ito, T., Inaba, M., Kibata, K., Iwata, R., Tanaka, A., Katashiba-Inagaki, N., Nomura, S.**Kansai Medical University, First Department of Internal Medicine, Hirakata City, Japan*

Dendritic cells (DCs) are pivotal in orchestrating both innate and acquired immunity. Series of recent analyses have revealed that DCs also play a pathogenic role in inflammatory and autoimmune diseases by their dysregulated function and cytokine production. According to the function of novel anti-tumor drugs, so-called Immunomodulatory Drugs (IMiDs) that have the potential to enhance T and NK cells function and to induce selective reduction of regulatory T cells, we hypothesized that these agents could modulate the functions of DCs as the center of the immunoregulatory system. Here we investigated the effect of two new analogs of thalidomide, lenalidomide (LEN) and pomalidomide (POM) on the function of human

myeloid DCs (mDCs). We found that both reagents at clinical concentration of 0.1 μ M to 1 μ M did not affect cell survival and maturation in response to TLRs (LPS, pIC, or R848) and TSLP. Either LEN or POM inhibited dose-dependently the production of Th1-type cytokine (IL-12, IFN- λ , and TNF α) and enhanced Th2-type cytokine (CCL17). Both agents significantly enhanced the production of IL-10 from mDCs, and this capacity of POM was stronger than that of LEN. As in myeloma cells, we also found that both LEN and POM downregulated IRF4 but upregulated IRF8 mRNA of mDCs. Our data suggest that IMiDs, although enhance the effector cell function, lead to regulatory action at DC phase. To further unveil the intracellular mechanism underlying the functions of IMiDs, we are now proceeding the analyses of STAT-family and PU.1 expression in this stream of action.

2073**Circulating tolerogenic monocytes are a hallmark of active human tuberculosis***Mascart, E., Dirix, V., Corbière, V., Bosse, J., Wyndham-Thomas, C., Sélis, E., Van Praet, A., Libin, M.**Université Libre de Bruxelles, Laboratory of Vaccinology and Mucosal Immunity, Brussels, Belgium*

Tuberculosis (TB) still represents a major global health problem, and the development of improved preventive and therapeutic approaches is hindered by an incomplete understanding of its pathogenesis. Effective cellular immune responses are key for protection against TB, and they are depressed in patients with active TB (aTB). The role of antigen-induced regulatory T cells (Treg) is recognized, but their mechanism of induction remains unknown. In view of the major role of antigen-presenting cells (APC) in their induction, we analyzed by flow cytometry the expression of different receptors on circulating monocytes (Mo) and dendritic cells (DC) in 15 patients with aTB, 15 latently-TB-infected (LTBI) subjects and 15 uninfected controls. The proportions of CD14⁺CD16⁻ and CD14⁺CD16⁺ Mo were higher in aTB compared to LTBI and controls ($p < 0.01$), as was that of mDC1 (CD11c⁺CD123⁻CD141⁻) ($p < 0.05$). The percentages of PDL-1⁺CD14⁺CD16⁻ Mo and mDC were higher among aTB patients than LTBI and controls ($p < 0.01$), with higher Mo PDL-1 MFI ($p < 0.01$), suggesting a role of T cell apoptosis in the down-regulated T cell responses. Analysis of ILT-3 expression, known to be expressed by tolerogenic APC and to mediate Treg cell generation, indicated higher proportions of ILT-3⁺CD14⁺CD16⁻ Mo, with higher MFI of this marker in aTB compared to LTBI subjects and controls ($p < 0.05$). We therefore not only found abnormal proportions of circulating Mo and mDC with abnormal phenotypes in aTB, but we identified high proportions of tolerogenic monocytes that could play a major role in the induction of Treg cells during aTB.

4154**Protein kinase C dependent differentiation of dendritic cells***Chavel, C., Lee, K.**Roswell Park Cancer Institute, Immunology, Buffalo, United States*

Dendritic cells (DC) are professional antigen presenting cells that are important activators of adaptive immune responses and play a role in the activation of CD8+ T cells that target tumor cells. Our lab has also previously shown that protein kinase C (PKC) β II is essential for DC differentiation and that tumor derived factors, particularly IL-6, inhibit DC differentiation by repressing the expression of PKC β II through a Stat3 dependent mechanism. However, the pathways activated downstream of PKC β II during DC differentiation have yet to be fully characterized. To study this, we used the K562 cell line, which differentiates into DCs with addition of PMA, a known chemical activator of conventional PKC isoenzymes. We have also shown previously that PMA specifically activates PKC β II in K562 cells. Addition of PMA to K562 cells showed the activation of the ERK1/2 and NF κ B pathways, including both the canonical and non-canonical NF κ B pathways. Chemical inhibition of either the ERK1/2 or NF κ B pathway yielded immature DCs with a reduced capacity to stimulate T-cell proliferation when incubated with PMA. Inhibition of ERK1/2 also showed a change in levels of RelB, a NF κ B transcription factor, with PMA treatment and during PMA induced DC differentiation. Inhibition of either the ERK1/2 or NF κ B pathways showed an increase in Foxo3a levels, indicating a role for the ERK1/2 and NF κ B pathways in Foxo3a regulation during differentiation into mature DCs. Taken together, these results show an integral role for both the ERK and NF κ B pathways downstream of PKC β II activation during DC differentiation.

T Cell Development**2246****Spatial interplay between Polycomb and trithorax complexes controls transcriptional activity in T lymphocytes***Onodera, A.¹, Tumes, D.², Kiuchi, M.¹, Kokubo, K.¹, Watanabe, Y.¹, Hirahara, K.¹, Kaneda, A.¹, Sugiyama, F.³, Suzuki, Y.⁴, Nakayama, T.¹**¹Chiba University, Chiba, Japan, ²South Australian Health and Medical Research Institute, Adelaide, Australia, ³University of Tsukuba, Tsukuba, Japan, ⁴University of Tokyo, Kashiwa, Japan*

Polycomb group (PcG) and Trithorax group (TrxG) complexes exert opposing effects on the maintenance of transcriptional status, and play a critical role in the expression of developmentally regulated transcription factors through methylation at histone H3-K27 (H3K27me₃; a repressive mark) and H3-K4 (H3K4me₃; a permissive mark), respectively. However, how they together mediate transcriptional counterregulation remains unknown. Genome-wide analysis revealed that binding of Ezh2 and Menin, central members of the PcG and TrxG complexes, respectively, were reciprocally correlated. Moreover, we identified a developmental change in the fine scale positioning of Ezh2 and Menin in differentiated

T lymphocytes compared to embryonic stem cells (ESCs). Ezh2 and Menin co-occupancy was most frequently observed in ES

cells and often lost during development into T lymphocytes. Ezh2-binding upstream and Menin-binding downstream of the transcription start site (TSS) was frequently found at genes with higher transcriptional levels, and Ezh2-binding downstream and Menin-binding upstream was found at genes with lower expression in T lymphocytes. Interestingly, of the Ezh2 and Menin co-occupied genes, those exhibiting occupancy at an identical position displayed greatly enhanced sensitivity to loss of Ezh2. Finally, we also found that different combinations of Ezh2 and Menin occupancy were associated with expression of specific functional gene groups important for T cell development. Therefore, spatial cooperative gene regulation by the PcG and TrxG complexes may represent a novel mechanism regulating the transcriptional identity of differentiated cells.

1787**Thymic precursors of CD8aa+ intestinal epithelial lymphocytes (IEL) divide into an emigrating and a retained population***Ruscher, R., Kummer, R., Lee, Y.J., Huggenberger, S., Hogquist, K.A. University of Minnesota, Minneapolis, United States*

Intestinal TCR β +CD4-CD8b-CD8a+ (CD8aa) IELs alleviate T cell induced colitis and have been suggested to play a role in virus infection and cancer. Their thymic development has been elucidated to some extent, as IEL precursors (IELp) are known to be CD4-CD8-CD5+TCR β +, but is not yet fully understood. Within the thymus, mature IELp were identified based on their expression of CD122 and MHC class I. Two major phenotypic subsets exist within this mature thymic IELp population: a PD1+Tbet- population that preferentially expresses a4b7, and a PD1-Tbet+ population with preferential CD103 expression. These two populations were also distinct in their Valpha repertoire. The PD1+a4b7+ population contains clones that are strongly self-reactive as judged by Nur77GFP and their dramatic increase in Bim deficient mice, while the PD1-Tbet+ population did not show these characteristics. Both gave rise to CD8aa IELs upon adoptive transfer into RAG-/- recipients. However intrathymic labeling revealed that PD1+a4b7+ IELp were the major thymic emigrating population, and emigration was S1P1-dependent. In contrast, PD1-Tbet+ IELp expressed CXCR3, were retained, and accumulated in the thymus with age. Preliminary immunofluorescence data furthermore indicate differential thymic cortico-medullary localization of the IELp subtypes. These experiments more precisely define the behavior of IEL precursors.

3064**Peripheral CD8 T cell receptor revision contributes to the plasticity of the immune repertoire***Dash, P.¹, Wang, G.¹, Bajracharya, R.¹, Contento, R.², McClaren, J.¹, Morris, M.¹, Love, J.C.², Doherty, P.¹, Thomas, P.¹**¹St Jude Children's Research Hospital, Immunology, Memphis, United States, ²Koch Institute for Integrative Cancer Research at MIT, Chemical Engineering, Cambridge, United States*

Using a technique for the paired amplification of TCR α and β chains from single cells, we have discovered a surprising

plasticity in the peripheral TCR repertoire, particularly under conditions of inflammation. This plasticity includes multiple instances of TCR revision in the periphery, where changes are made in the TCR coding sequence by *de novo* rearrangement. Here we describe this process in murine CD8+ T cells derived from influenza-infected lungs and human T cells derived from CMV-infected individuals *ex vivo* or in naïve single-cell cultures stimulated *in vitro*. Taking advantage of the co-expression of productive and non-productive *Tcra* alleles, we demonstrate secondary rearrangement in the TCR β locus *ex vivo*, while the *in vitro* cultures demonstrated a remarkably high rate of *Tcra* revision following strong stimulation. Not surprisingly, revision was found to be RAG-dependent. Utilizing a model of peripheral deletion of RAG2, we showed that mice deficient in revision showed delayed onset and severity of disease in an experimental autoimmune encephalomyelitis (EAE) model, and also produced a less functional T cell response following challenge with influenza virus. Additionally, in the absence of revision, the dominant NP₃₆₆ response was compromised in the recall phase while the responses to the minor epitopes were increased. Thus, peripheral TCR revision appears to be a robust process that contributes to optimal T cell functionality and repertoire diversity during infection but may also contribute to the development of autoimmunity.

3152

Tolerance by deletion and regulatory T cell differentiation arise from different TCR repertoires during two waves of thymic selection

Hu, D.Y.¹, Singh, M.², Yap, J.Y.¹, Wirasinha, R.C.³, Howard, D.¹, Archer, S.³, Goodnow, C.C.², Daley, S.R.³

¹The John Curtin School of Medical Research, The Australian National University, Canberra, Australia, ²Garvan Institute of Medical Research, Sydney, Australia, ³Monash Biomedicine Discovery Institute, Infection and Immunity, Melbourne, Australia

Thymocytes that receive a strong T cell receptor (TCR) signal may be deleted by apoptosis or diverted into the Foxp3+ T-regulatory (Treg) cell lineage. We are investigating whether this divergence is governed by the developmental stage at which thymocytes register a strong TCR signal. Analysis of apoptosis-defective mice suggests that ~50% of TCR-signalled thymocytes are deleted < 3 days after the cells proliferate as TCR^{hi} precursors. We characterise these short-lived Helios+ CCR7- PD-1^{high} thymocytes as wave 1 of thymic selection. Another subset of Helios+ thymocytes, defined by high CCR7 expression, forms ~5 days after proliferation and contains cells that upregulate Foxp3 protein 6-8 days after proliferation. The Helios+ CCR7+ thymocytes represent wave 2 of thymic selection. To compare TCRs within wave 1 or wave 2 of thymic selection with TCRs expressed by naïve CD4+ T cells, we purified thymocyte populations from TCRbeta-transgenic mice and deep-sequenced TCR-alpha transcripts. The data reveal that ~40% of TCRs were expressed selectively in wave 1 cells and these TCRs were rare or absent in CD4+ and CD8+ splenocyte samples, consistent with deletion of these TCRs. The thymic naïve CD4+ and wave 2 TCR repertoires were partially distinct from each other and were most similar to non-Treg and Treg

subsets of CD4+ splenocytes, respectively. We interpret these data as evidence that strong TCR-signalling triggers deletion of CCR7- thymocytes whereas it primes CCR7+ thymocytes for Treg differentiation. Moreover, the self-antigens that induce wave 1 and wave 2 of thymic selection would appear to be distinct.

3687

How many TCR clonotypes does a body maintain?

Lythe, G.¹, Molina Paris, C.¹, Callard, R.², Hoare, R.²

¹University of Leeds, Leeds, United Kingdom, ²University College London, London, United Kingdom

The number of T cells of one clonotype is an integer that increases or decreases by one cell at a time, starting when the clonotype is released from the thymus to the periphery, and ending with extinction of the clonotype. We present a stochastic model of naïve T-cell homeostasis based on competition between large numbers of TCR clonotypes, each with a unique recognition profile in the environment of self-pMHC stimuli. Thymic activity is included by the release of new clonotypes that compete in the pool of pre-established ones. Our point of view is to consider the stochastic dynamics of the number of T cells of one clonotype as a function of time, which increases or decreases according to the rates of cell division and death. By considering distributions of times to clonal extinction, we give estimates of clonal sizes, and hence diversity, in adult mice and humans. With the estimate that the ratio of thymic production to peripheral division of naïve CD4+ T cells is four percent, our model predicts that the number of distinct clonotypes in the human body is nine percent of the total number of naïve CD4+ T cells.

1339

Competitive signaling between STAT5 and PI3K of the IL-7R modulates T cell development and homeostasis in vivo

Cui, G., Shimba, A., Tani-ichi, S., Hara, T., Ikuta, K.

Laboratory of Biological Protection, Institute for Virus Research, Kyoto University, Kyoto, Japan

IL-7 is an important cytokine for T cell development and homeostasis. STAT5 and PI3K are two major signal molecules of the IL-7R involved in these processes. The tyrosine residue Y⁴⁴⁹ of IL-7Ra is essential for interaction and activation of both STAT5 and PI3K, while the methionine residue M⁴⁵² is additionally required for PI3K recruitment. Nevertheless, how STAT5 and PI3K signals are precisely controlled and contributed under the IL-7R has not been well understood. To characterize the differential roles of these two signals in vivo, we established two lines of IL-7Ra mutant mice, IL7R-Y449F and IL7R-M452L. Interestingly, the levels of phosphorylated STAT5 were downregulated in Y449F mice but significantly upregulated in M452L mice, whereas the levels of phosphorylated Akt were reduced in both Y449F and M452L mice. The Y449F mice showed markedly decreased T cells in thymus and periphery. In contrast, only the thymocytes at the ETP and DN2 stages were reduced in the M452L mice. In addition, the M452L mice also showed slightly increased T cells in periphery. Finally, memory precursor effector cells were

decreased in M452L mice during memory T cell development after *Listeria monocytogenes* infection. Thus, this study suggests a competition between STAT5 and PI3K for the Y⁴⁴⁹ of IL-7Ra in vivo and indicates that the balance between STAT5 and PI3K signals is important for development of early thymocytes and memory T cells.

Immunity to Viruses 2

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IL-17 promotes pulmonary B-1a cell differentiation via induction of Blimp-1 expression during influenza virus infection

Lu, L., Wang, X.

University of Hong Kong, Pathology, Hong Kong, Hong Kong

B-1 cells play a key role in early protection during influenza infections by production of natural antibodies. However, the molecular mechanisms involved in regulating this process are largely unclear. In this study, we found that during influenza infection pleural cavity B-1a cells rapidly infiltrated lungs, where they underwent plasmacytic differentiation with enhanced IgM production. This process was promoted by IL-17A signaling via induction of Blimp-1 expression and NF- κ B activation in B-1a cells. Deficiency of IL-17A led to severely impaired B-1a-derived antibody production in the respiratory tract and is associated with a deficiency in viral clearance. Transfer of B-1a-derived natural antibodies rescued IL17a^{-/-} mice from otherwise lethal infections. Together, we identify a critical role of IL-17A in regulating pulmonary B-1a responses during influenza infection. Our findings provide new insights in understanding the natural antibody response by B-1a cells and its regulatory mechanisms.

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1490

The subcellular localization of viral antigen after infection shapes the antigenic profile and protective capacity of poxvirus-mediated T cell immunity

Tao, S.¹, Tao, R.¹, Patra, M.², Drexler, L.¹

¹*Heinrich-Heine-University Düsseldorf, University Hospital Düsseldorf - Institute for Virology, Düsseldorf, Germany,*

²*Helmholtz-Zentrum München and Technical University Munich, Institute of Virology, Munich, Germany*

Poxviruses engineered to express foreign genes are established tools for target protein synthesis and vaccine development in biomedical research. Modified vaccinia virus Ankara (MVA) is an attenuated vaccinia virus (VACV) strain which is currently evaluated in clinical studies for immunotherapy of infectious diseases and cancer.

Cytotoxic CD8⁺ T cell (CTL) responses play an essential role in antiviral immunity. We demonstrated the requirement of

cross-presentation of MVA-encoded antigens to efficiently prime naïve T cells, which allows for the efficient induction of early and late antigen-specific CTL in the primary response. However, there is a dramatical rearrangement of the immunodominance hierarchy in the recall response. Here, the preferential expansion of CTL specific for early viral epitopes is shaped by cross-competition, while presentation of epitopes derived from VACV late gene products is substantially delayed or completely absent. We identified the cytoplasmic poxviral factories to serve as compartments for sequestration of viral and recombinant antigens which are produced late in infection. The transient trapping in viral factories resulted in deprivation and preservation of late antigens for further processing and presentation on MHC class I. In addition, proteasomes which represent the major source of peptide ligands for MHC class I presentation, were inactive in viral factories. Subsequently, viral CD8⁺ T cell activation was significantly reduced and the protective capacity of MVA vaccines based on late antigens in mouse challenge experiments was absent. We conclude that the prolonged antigen sequestration of late antigens in this immunological impaired environment prevents efficient antigen processing and presentation to CTL.

1553

Mast cell activation in severe dengue

Jeewandara, K.C.^{1,2}, Udari, S.¹, Gomes, L.¹, Paranavitane, S.A.¹, Shyamali, A.³, Ogg, G.S.^{4,5}, Malavige, G.N.^{1,4}

¹*Center for Dengue Research, Faculty of Medical Sciences, University of Sri Jayewardenapura, Nugegoda, Sri Lanka,*

²*Faculty of Medical Sciences, University of Sri Jayewardenapura, Department of Family Medicine, Nugegoda, Sri Lanka,*

³*Faculty of Medical Sciences, University of Sri Jayawardanapura, Department of Medicine, Nugegoda, Sri Lanka,*

⁴*MRC Human Immunology Unit, Weatherall Institute of Molecular Medicine, Oxford NIHR Biomedical Research Centre and University of Oxford, Oxford,*

⁵*Churchill Hospital, Department of Dermatology, Oxford, United Kingdom*

Background: Mast cells have been shown to play a role in the pathogenesis of dengue, in mouse models. In our previous studies, we found that platelet activating factor (PAF), which is a mast cell product, played an important role in vascular leak. Therefore, we set to investigate the potential role of mast cell activation in the pathogenesis of dengue virus infection.

Methods: Serial twice daily blood samples were obtained from 38 adult patients with acute dengue from the time of admission to discharge. Mast cell tryptase level; viral loads, secretory phospholipase (sPLA2) activity and platelet activating factor (PAF) levels were assessed. All clinical and laboratory features were serially recorded until discharge of the patients. Disease severity was classified based on the WHO 2011 dengue guidelines.

Results: sPLA2 activity, mast cell tryptase level, and PAF levels were significantly elevated during the critical period in patients with severe dengue (SD) infection compared to non severe dengue infection (NSD). The highest sPLA2 activity, tryptase and PAF levels were seen on day 6 of illness, which co-occurred with the critical phase. Mast cell tryptase (p=0.006), PAF levels

($p=0.01$) and sPLA2 activity ($p=0.01$) were significantly higher in those with SD when compared to NSD during the critical phase.

Conclusion: mast cell tryptase level, secretory phospholipase (sPLA2) activity, and platelet activating factor (PAF) levels were significantly higher in SD indicating mast cell activation which is likely to play a role in the pathogenesis of vascular leak in severe dengue.

1857

Transcriptional regulation in immune and epithelial cell types during influenza A infection

Ma, J.¹, Wijburg, O.¹, Brooks, A.¹, Reading, P.^{1,2}

¹University of Melbourne, Peter Doherty Institute for Infection and Immunity, Melbourne, Australia, ²WHO Collaborating Centre for Reference and Research on Influenza, Melbourne, Australia

In humans, infection with seasonal influenza A virus (IAV) is generally restricted to the respiratory tract. The lower airways are comprised of parenchymal cells such as airway epithelial cells (AEC) and haematopoietic cell populations such as airway macrophages (M Φ) and dendritic cells (DC). Infection of AECs by seasonal IAV results in productive virus replication whereas the infection of M Φ and DC results in abortive virus replication. Recent studies have identified antiviral host cell factors that limit intracellular replication of a range of viruses, including IAV, in epithelial cells. We previously showed that certain antiviral factors were differentially expressed in mouse M Φ and AEC following *in vitro* infection with IAV, consistent with the hypothesis that one or more differentially expressed genes may restrict productive IAV replication in M Φ /DC. However, they do not give a complete picture of the particular genes which show elevated expression at basal levels or after IAV infection in M Φ /DC.

To address this, we examined the cell intrinsic responses systemically in AEC, M Φ and DC *ex vivo*, as they form the initial epithelial-immune barrier and are the first cells to encounter and respond during IAV infection. Here we show that the differential transcription networks in epithelial and immune cell types correlate with different viral outcomes.

2197

Severe influenza disease is associated with prolonged immune activation

Wong, S.-S.¹, Oshansky, C.^{2,3}, Xizhi, G.², Ralston, J.⁴, Wood, T.⁴, Reynolds, G.⁵, Huang, S.⁴, Thomas, P.², Webby, R.¹, SHIVERS Study Group

¹St. Jude Children's Research Hospital, Infectious Diseases, Memphis, United States, ²St. Jude Children's Research Hospital, Immunology, Memphis, United States, ³Biomedical Advancement Research and Development Authority, Office of the Assistant Secretary for Preparedness and Response (ASPR), US Dept of Health and Human Services, Washington D.C, United States, ⁴Institute for Environmental Science and Research, NCBID, Wellington, New Zealand, ⁵University of Auckland, Auckland, New Zealand

The immunological basis underlying severe influenza disease is poorly understood. We compared the immunological profile

of patients who received ambulatory care for acute respiratory illness (ARI) (N=24) vs. patients hospitalized with severe ARI (SARI, N=25) associated with PCR-confirmed influenza virus infection. Peripheral blood lymphocytes collected during acute and recovery phases were analyzed for differences in levels of myeloid dendritic cells, monocyte subpopulations, and T-cell responses. Serum cytokine levels were also assessed. Continuous variable data were log-transformed and differences between ARI and SARI were analyzed by T-test while cytokines associated with SARI were analyzed using ordinal logistic regression. We found that during the acute phase, SARI patients had significantly lower levels of CD192+ monocytes and myeloid dendritic cells compared to ARI patients ($p < 0.05$). Further, number of influenza-specific CD4+ and CD8+ T cells were generally lower in SARI during acute infection, especially the IFN γ +CD8+ T-cells ($p < 0.05$), compared to ARI individuals. However, these responses tended to be reversed during convalescence, where levels of CD16+ monocytes, circulating effector and CD8 T+ cells remained higher in the SARI patients ($p < 0.05$). Lower levels of serum cytokines MDC and IL12p40 were found to be significantly associated with SARI during the acute phase ($p < 0.05$). Conversely, higher levels of growth-related chemokines. GRO and FGF2 were associated with SARI during convalescence ($p < 0.05$). Our results suggests that patients hospitalized with severe influenza illness could experience a delay in induction of inflammatory immune responses, but a prolonged activation of cellular immune responses compared to individuals with milder infections.

1216

Nuclear domain 10 components upregulated via interferon during human cytomegalovirus infection potentially regulate viral infection

Ashley, C., McSharry, B., Slobedman, B.
University of Sydney, Sydney, Australia

Human Cytomegalovirus (HCMV) is a ubiquitous beta-herpesvirus that causes life-threatening disease in immunocompromised and immunonaive individuals. Type I interferons (IFNs) are crucial molecules in the innate immune response to HCMV. Type I IFNs upregulate several components of the interchromosomal multiprotein aggregates collectively referred to as Nuclear Domain 10 (ND10). ND10 components also are also known to restrict herpesvirus gene expression. This raises the question, could key ND10 components (PML, Sp100 and hDaxx) act as antiviral interferon stimulated genes (ISGs) during HCMV infection? Analysis of ND10 component transcription following HCMV infection illustrated that PML and Sp100 were upregulated whilst hDaxx was not. This upregulation was independent of *de novo* viral gene expression and was also detected following treatment with infected cell supernatant suggesting that a soluble factor(s) is responsible. In cells engineered to block the production or response to type I IFNs, upregulation of PML and Sp100 was not detected following HCMV infection. IFN- β neutralising antibody pre-treatment inhibited upregulation of PML and Sp100 following both infection and treatment with infected cell supernatant. The significance of ND10 components functioning as antiviral ISGs during HCMV infection was

determined by infecting cells engineered using shRNA to have simultaneous knockdown of PML, Sp100 and hDaxx. ND10 knockdown cells were significantly more permissive to HCMV infection and, in contrast to control cells, could be infected by HCMV even with IFN- β pre-treatment. Together these data provide evidence that ND10 component upregulation is a key mediator of the antiviral activity of IFN- β .

1054

ECM remodelling by ADAMTS5 regulates influenza-specific immunity

McMahon, M.¹, Ye, S.¹, Izzard, L.¹, Tripp, R.², McCulloch, D.¹, Stambas, J.¹

¹Deakin University, Waurn Ponds, Australia, ²University of Georgia, Athens, United States

Members of the ADAMTS (A disintegrin-like and metalloproteinase with thrombospondin-1 motifs) metalloproteinase family remodel the extracellular matrix (ECM) to facilitate immune cell migration and cell adhesion. In this current study, we infected *Adamts5*^{-/-} mice with X31 influenza virus (10⁴ pfu/mouse) and measured weight loss, viraemia and T cell immunity following acute infection. *Adamts5*^{-/-} mice showed greater weight loss and increased viraemia when compared to WT counterparts. Furthermore, we observed reduced NP₃₆₆ and PA₂₂₄ influenza specific CD8⁺ T cells in the spleen and lungs of *Adamts5*^{-/-} mice with a corresponding accumulation of these T cell specificities in the mediastinal lymph node (MLN) 10 days post infection. Critically, these responses correlated with increased expression of a key ADAMTS5 substrate; versican, in the MLNs of *Adamts5*^{-/-} mice. We therefore hypothesize that versican accumulation in the MLNs of *Adamts5*^{-/-} mice results in T cell accumulation and that ADAMTS5 is a key enzyme involved in facilitating T cell migration from the MLN to the periphery following acute influenza virus infection.

Allergy 2

3115

A20 levels in dendritic cells control Th17-mediated severe neutrophilic asthma

Vroman, H.¹, Bergen, I.M.¹, Lukkes, M.¹, Schuijs, M.², van Loo, G.², Lambrecht, B.N.^{1,2,3}, Hendriks, R.W.¹, Kool, M.¹

¹Erasmus MC, Pulmonary Medicine, Rotterdam, Netherlands, ²VIB-UGent, Zwijnaarde, Belgium, ³University Hospital, Ghent, Belgium

Asthma is a chronic Th2-cell-mediated inflammatory disease of the airways in response to inhaled antigens such as house dust mite (HDM). However, in severe asthma patients, Th17-cells have been implicated. Dendritic cells (DCs) drive Th-cell differentiation and are essential during sensitization and challenge in experimental allergic asthma models. Activation of DCs is controlled by A20 (*Tnfrsf3*), a negative regulator of the proinflammatory NF- κ B pathway. We hypothesize that A20 in DCs critically controls Th-cell differentiation and asthma severity in a chronic HDM-driven asthma model. *Tnfrsf3*^{fl/fl}*LysMcre*⁺ mice (DC-A20 mice) were treated 25 μ g

HDM or PBS (3xpw) for 5 weeks. Additionally, HDM-specific Th-cell differentiation was determined in DC-A20 mice after intravenously injection of Derp1-specific TCR Tg (1-der) T cells and HDM exposure. HDM-treated DC-A20^{WT} and DC-A20^{HZ} mice showed massive eosinophilic infiltration in the airways, increased mucus-producing goblet cells, and increased numbers of IL-5 and IL-13 producing CD4⁺ T cells. In contrast, DC-A20^{KO} mice developed a mixed eosinophilic/neutrophilic inflammation, and showed increased ROR γ ^t Th17-cell and AHR⁺ Th22-cell numbers in the airways. Moreover, HDM-treated DC-A20^{KO} mice exhibited even higher airway hyper-responsiveness than HDM-treated DC-A20^{WT} mice. 1-der Th-cells in DC-A20^{KO} mice showed increased differentiation into Th17-cells, whereas in DC-A20^{WT} mice they developed into Th2-cells. In conclusion, we showed that lack of A20 in DCs causes a Th17-cell response rather than a Th2-cell response to HDM. This implicates that increased DC activation is a crucial driver of Th17-cell-mediated severe neutrophilic asthma, and could be a novel therapeutic target for treatment of severe asthma patients.

1639

Repeated exposures to common cosmetic preservative biocide methylisothiazolinone produces sensitivity to touch and pressure in sensitized ND4 female mice

Chatterjea, D., Mengistu, H., Chai, N., Landry, J.

Macalester College, Saint Paul, United States

Recent attention to the allergenic effects of the synthetic biocide methylisothiazolinone (MI) has revealed widespread sensitization to this common cosmetic preservative in populations tested. Allergic reactions to the chemical include swelling, redness, itchiness and in some cases neurologic damage and scarring. While epidemiological studies support the increased risk of developing allergic contact dermatitis and neurological complications from repeated exposure to MI in leave-on cosmetics, little is known about the mechanisms underlying tissue damage from chronic or repeated exposure to this biocide preservative. Here we show that in a model of cutaneous contact hypersensitivity, repeated exposure to MI produces not only tissue edema and up-regulation of inflammatory cytokines, but also sensitivity to touch and pressure lasting for up to 3 weeks after exposures have ceased in female ND4 Swiss mice previously sensitized with topical application of this chemical. Given the emerging evidence that allergic exposures and history of atopy are significant risk factors for the development or exacerbation of chronic pain conditions such as migraine headaches, vulvodynia and inflammatory bowel pain, it is important that we establish pre-clinical models of interaction between allergic and hypernociceptive pathophysiologies where novel therapeutic targets may be identified and validated. To our knowledge, this is the first pre-clinical model of contact hypersensitivity to MI; our current studies are focused on characterizing the cellular and molecular changes in the tissue produced by repeated MI exposure.

2920**Post transcriptional regulation of I κ B- ζ expression controls inflammatory responses***Maruyama, T.^{1,2}*¹Gifu University, Gifu, Japan, ²Tohoku University, Sendai, Japan

A nuclear I κ B family protein, I κ B- ζ can be up-regulated in T cells in response to TGF- β +IL-6 and positively regulate IL-17 gene expression cooperate with ROR γ t. However, we show that T cells specific I κ B- ζ deficient mice occurred massive inflammation with aging and produce large amount of IFN- γ . Thus, I κ B- ζ is necessary for both maintaining immune homeostasis and inflammatory responses. Previously study have reported that IL-1 β stimulation enhanced protein level of I κ B- ζ in T cells and promote pathogenic Th17 cells differentiation. We have shown that post transcriptional mechanisms were more important for stimulation specific I κ B- ζ expression (especially IL-1 β /LPS stimulation), but the mechanisms are ill define. Therefore, we hypothesis that control of stimulation specific I κ B- ζ expression plays an important role for regulation of inflammatory responses without breaking immune homeostasis.

We focus on 3'-UTR region of I κ B- ζ mRNA (which plays an important role for post transcription) and found that three putative miRNA binding elements and AU-rich element. However, these elements were dispensable for stability of mRNA expression. Next, we performed reporter assay using by I κ B- ζ 3'-UTR reporter and 9600 kinds of cDNA libraries. We identified that MyD88, a down stream molecule of IL-1 β / LPS stimulation, are key to stabilize I κ B- ζ mRNA expression. In addition, MyD88 deficiency shows less half life of I κ B- ζ mRNA expression. Thus, post transcriptional regulation by MyD88 is a key to regulate I κ B- ζ expression and pathogenic Th17 differentiation.

2577**Interaction of the probiotic strain *E. coli* O83 with the host immune system and its impact on allergic airway inflammation in mice***Zwicker, C., Drinic, M., Sarate, P., Wiedermann, U., Schabussova, I. Institute for Specific Prophylaxis and Tropical Medicine, Center for Pathophysiology, Immunology and Infectiology, Medical University of Vienna, Vienna, Austria*

For primary prevention of allergic diseases by probiotic bacteria, several different treatment approaches, like peri- or postnatal application, have been shown to be very effective. Clinical studies have suggested that the postnatal application of the probiotic *E. coli* strain O83 to newborns of allergic mothers is associated with reduced prevalence of allergies in later life.

In the present study we investigated whether maternal exposure to *E. coli* O83 during gestation and lactation can affect the development of airway inflammation in the offspring in a mouse model of type I allergy.

Surprisingly, the offspring derived from *E. coli* O83-treated dams showed significantly increased airway hyperresponsiveness (AHR) after exposure to ovalbumin compared to mice from PBS-treated mothers. Increased AHR in mice born to bacteria-treated mothers was not associated with enhanced systemic IgE levels

or elevated numbers of eosinophils in bronchoalveolar lavage fluid. However, we detected significantly increased IL-4 levels in the lungs of mice derived from *E. coli* O83-treated mothers but decreased levels of the typically AHR-associated cytokines IL-5 and IL-13 compared to control mice.

Here, we found that maternal supplementation with *E. coli* O83 during pregnancy and lactation leads to increased AHR and elevated IL-4 levels in lungs of their offspring. The role of IL-4 in this phenotype and its cellular source still need to be determined. However, this study emphasizes that the time point of application (peri- vs postnatal) of probiotic bacteria seems to be crucial for its effect on allergic diseases.

3027***Clethra barbinervis* leaf extract inhibits allergic reaction of basophilic leukemia cell line RBL 2H3***Hachiya, M.¹, Ooba, K.¹, Aradate, T.², Ogawa, R.³, Murata, T.³, Katagiri, T.¹*¹University of Toyama, Dept. Biology (Faculty of Pharmaceutical Sciences), Toyama-shi, Japan, ²University of Toyama, Dept. Biology (Faculty of Medical Sciences), Toyama-shi, Japan, ³Tohoku Medical and Pharmaceutical University, Dept. Pharmacognosy, Sendai, Japan

We examined the series of samples from the plants products affect the activation of Fc epsilon RI mediated signals in basophiles in order to find new anti-allergic reagent. As a result, we found a fact that *Clethra Barbinervis* Leaf Extract (CBLE) had strong anti-allergic effects. CBLE inhibited degranulation and TNF- α production in a concentration without cytotoxicity. Based on these facts, we had an attempt to find a mechanism of CBLE's inhibiting the activation pathway. Using RBL-2H3 cells (Rat basophilic leukemia), we analyzed the effects of CBLE to Fc epsilon RI mediated signals. As a result, CBLE was not affect to Fc epsilon expression on RBL 2H3. On that condition, we found that CBLE inhibited calcium influx induced by antigen stimulation. In other word, it means that CBLE contains some compounds regulate an early stage of this signaling. While analyzing the effects of CBLE, we also identified effective components of CBLE. CBLE has quercetin analogues, caffeic acid and epicatechin. These compounds, however, had no effects on degranulation by themselves alone respectively. These results may suggest a possibility of being of some unknown anti-allergic components in CBLE. Or, there is a possibility that shows the combined effect is effective allergy suppression of low effective compounds alone. We have a result that caffeic acid inhibits TNF- α production induced by calcium ionophore and PMA. As a conclusion, we have much possibility of developing new allergic therapeutic drugs with the combination of caffeic acid and unknown components of CLBE.

301***Toxoplasma gondii* serine-protease inhibitor-1: a novel vaccine candidate for asthma treatment***Soto, A.¹, Fenoy, I.², Sánchez, V.¹, Aldirico, M.D.I.A.¹, Perrone Sibilia, M.¹, Picchio, M.¹, Arcón, N.¹, Acosta, P.³, Martín, V.¹, Goldman, A.¹*¹Universidad Nacional de General San Martín, Buenos Aires, Argentina, ²Instituto de Investigaciones Biotecnológicas (IIB-

NTECH), Chascomús, Argentina, ³Fundación Infant, Consejo Nacional de Investigaciones Científicas y Técnicas, Cuidad Autónoma de Buenos Aires, Argentina

Serine proteases are important players in the pathogenesis of asthma, promoting inflammation and tissue remodeling. In addition, some allergens have serine protease activity. It's known that many serine protease inhibitors display immunomodulatory properties. TgPI-1 is a *Toxoplasma gondii* protein that exhibits broad spectrum inhibitory activity against serine proteases. We sought to investigate whether rTgPI-1 administration could suppress experimental asthma. BALB/c mice were intraperitoneally sensitized with ovalbumin/alum and airway-challenged. Once the asthmatic phenotype was achieved they were intranasally treated with different doses of rTgPI-1 alone (PI) or co-administrated with ovalbumin (OPI). Treatment with PI and OPI reduced 13.8% and 50.3% the airway hyperresponsiveness, respectively. This effect was accompanied by a diminished bronchoalveolar eosinophilia, mucus production and peribronchial lung infiltration. Moreover, bronchoalveolar lavage fluid from treated mice showed a reduction in IL-4 and IFN- γ levels. In agreement with these results, both treatments resulted in lower levels of IL-4, IL-5 and IFN- γ in thoracic lymph node cells stimulated with ovalbumin. Regulatory IL-10 was also diminished. Furthermore, OPI induced a reduction in ovalbumin specific T cell proliferation. Treatment did not alter ovalbumin-specific humoral response. These results show that both rTgPI-1 treatments reduced asthma hallmarks; however, OPI was more effective denoting an adjuvant capacity. The effect of rTgPI-1 in modulating allergic lung inflammation could be the consequence of a decrease in the overall local response to the allergen. Although further studies should be undertaken to completely elucidate the mechanisms involved, TgPI-1 is a promising therapeutic vaccine candidate for intervention in patients with asthma.

Mucosal Immunology 1

2081

Microbial metabolites: a critical element in the fight against obesity and inflammatory diseases

Yap, Y.A.¹, Richards, J.L.¹, McLeod, K.H.¹, Watt, M.J.², Mackay, C.R.¹, Marino, E.¹

¹Monash University, Biochemistry and Molecular Biology, Clayton, Australia, ²Monash University, Physiology, Clayton, Australia

There has been an alarming increase in global obesity rates within the past decade. Obesity poses a serious risk for many other diseases, including type 2 diabetes mellitus (T2DM), cardiovascular disease, kidney disease and even some forms of cancer. In recent years, the spotlight has been on the role of gut microbiota, in particular the production of short-chain fatty acids (SCFA), in health and disease. SCFA (acetate, propionate and butyrate), produced through colonic microbial fermentation of indigestible dietary fibre, mediate beneficial effects through G-protein coupled receptors (GPCR), not only in the gut but also in peripheral tissues. Here we are uncovering the anti-inflammatory effects of SCFA-enriched diets in the

treatment of obesity and related metabolic dysfunctions. In the diet-induced obesity (DIO) mouse model, high fat/butyrate-yielding diets reduced body weight gain, significantly improved glucose tolerance and insulin sensitivity, and remarkably also reduced kidney damage evidenced by less PASS staining and reduced proteinuria. Additionally, we found increased lipolysis in white adipose tissue (WAT) and decreased hepatic lipogenesis in mice fed SCFA-enriched diets. Flow cytometry and histological analyses revealed interestingly, that these changes occurred concomitantly with increased Foxp3⁺ regulatory T cells in the gut, kidney and other peripheral tissues. In contrast, GPR109-deficient mice showed reduced glucose tolerance. Our findings suggest that dietary SCFAs modulate the gut microbiota, through GPCR-associated mechanisms, ameliorating inflammation in the DIO model. Therefore, we strongly believe that our results are extremely important in forming the basis for future human studies.

3354

Lachnospiraceae protect from colitis by regulating colonic group 3 innate lymphoid cells

Surana, N.^{1,2}, Kasper, D.²

¹Boston Children's Hospital, Boston, United States, ²Harvard Medical School, Department of Microbiology and Immunobiology, Boston, United States

In previous work, we demonstrated that host-specific bacteria are required for proper maturation of the small-intestinal immune system. In sharp contrast to gnotobiotic mice colonized with normal mouse microbiota (MMb), which have a well-developed small-intestinal immune system, we demonstrated that gnotobiotic mice colonized with normal human microbiota (HMb) have an immature small-intestinal immune system indistinguishable from that of germ-free (GF) mice. Using the dextran sodium sulfate (DSS)-induced colitis model that relies heavily on the innate immune system, we have now found that—similar to GF mice—MMb mice are exquisitely sensitive to DSS colitis with a 100% mortality rate; in contrast, HMb and specific pathogen-free (SPF) mice are protected. Based on flow cytometric analysis of the colonic immune systems, we identified group 3 innate lymphoid cells (ILC3s) as the immunologic correlate of disease. Co-housing MMb and HMb mice facilitated a bi-directional transmission of microbes that led to an intermediate disease phenotype in both groups. Bioinformatic analysis of the fecal microbiota of SPF, MMb, HMb, and co-housed mice identified the bacterial family Lachnospiraceae as a microbiological correlate for protection from disease. Using semi-selective media, we cultured a Lachnospiraceae-enriched pool of bacteria from HMb mice that—when gavaged to MMb mice—led to decreased numbers of colonic ILC3s and protection from DSS-induced mortality. This work provides an immunologic explanation for why multiple cohorts of patients with inflammatory bowel disease have demonstrated that Lachnospiraceae is associated with reduced risk of disease and provides a mechanistic foundation for the potential therapeutic use of these organisms.

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Aberrant accumulation of HLA-DR and reduced mucin production in colonic epithelial cells of HLA-DR4 transgenic mice and ulcerative colitis patients

Irie, A.¹, Imamura, T.², Michibata, Y.¹, Kubo, T.², Takeda, N.³, Shibuya, I.⁴, Sogo, S.⁴, Araki, K.³, Nishimura, Y.¹

¹Kumamoto University, Graduate School of Medical Sciences, Immunogenetics, Kumamoto, Japan, ²Kumamoto University, Graduate School of Medical Sciences, Molecular Pathology, Kumamoto, Japan, ³Institute of Resource Development and Analysis, Kumamoto University, Developmental Genetics, Kumamoto, Japan, ⁴Microbiological Research Institute, Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan

Objective: Homozygotes of HLA-DR4/I-Ed-chimeric transgenic mice (tgm) spontaneously develop colitis similar to human ulcerative colitis (UC). We explored whether the pathogenesis of this colitis is due to the disruption of the genome structure by the transgenes or the HLA-DR4/I-Ed over-expression.

Design: The transgenes were identified to be inserted in chromosome 3 with 39 kb deletion. Using CRISPR/Cas9 system, mice with equivalent genomic deletion were established to see whether they develop colitis or not. In addition, MHC class II transactivator (CIITA) knock out background homozygous tgm were established to see HLA-DR4/I-Ed expression was involved in the pathogenesis of the colitis. Histological and cellular analyses of the colons of the tgm were performed and effect of antibiotic administration was examined.

Result: Lack of CIITA and antibiotic administration were effectively suppressed the development of the colitis, while lack of the 39 kb had no effect. Aberrantly accumulated HLA-DR4/I-Ed in the colonic epithelial cells was observed with up-regulated endoplasmic reticulum (ER) stress marker BiP (Grp78) and significant reduction of mucin-producing goblet cells. Importantly, in 3 out of 12 colonic tissues of UC patients, the association of high HLA-DR expression, reduced number and mucin production of goblet cells and BiP expression was observed.

Conclusion: The pathogenesis of the colitis observed in the homozygous tgm was likely due to the ER stress that resulted in goblet cell loss and significant reduction of mucin in the colonic epithelial cells in which HLA-DR4/I-Ed was aberrantly accumulated. Similar mechanisms could be a fundamental component of pathogenesis of some human UC cases.

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Th17 cell production of amphiregulin contributes to intestinal homeostasis to microbiota

Cao, A., Yao, S., Chen, F., Cong, Y.

University of Texas Medical Branch, Microbiology and Immunology, Galveston, United States

Th17 cells produce the signature cytokines interleukin (IL)-17A (IL-17), IL-17F, IL-21, and IL-22, and have been implicated in a variety of autoimmune disorders. Despite their highly inflammatory nature, Th17 cells are highly enriched in the intestines under homeostatic conditions and primarily respond to commensal bacteria. However, it remains unclear as to their

exact role during intestinal homeostasis. We demonstrated in this report that CBir1 TCR transgenic (Tg) mice, which are specific for a commensal bacterial flagellin, were more resistant to dextran sulfate (DSS)-induced colitis than wild-type mice. CBir1 Tg mice demonstrated a higher level of intestinal Th17, and expressed higher levels of amphiregulin (Areg), which has been recently shown to be highly protective along the epithelial surfaces of the airway and intestine, than wild-type mice. Administration of Areg to wild-type mice led to decreased intestinal injury and higher level of tight-junction protein expression upon DSS insult. We further demonstrated that Th17 cells produced high amounts of Areg, and Areg inhibited T cell proliferation. Collectively, these findings reveal that intestinal Th17 cells contribute to epithelial homeostasis and immune regulation by Areg production.

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Early-life respiratory bacterial infection-induced chronic lung disease is driven by a novel TLR2/IL-13/miR-21/PI3K signaling pathway

Starkey, M.R., Nguyen, D.H., Kim, R.Y., Haw, T.J., Nair, P.M., Essilfie, A.T., Horvat, J.C., Foster, P.S., Hansbro, P.M.

The University of Newcastle and Hunter Medical Research Institute, School of Biomedical Sciences and Pharmacy, New Lambton Heights, Australia

Introduction: There is a critical window in early-life where the lung and immune system are still maturing and this increases susceptibility to infection. Indeed, severe respiratory bacterial infections in early-life are a risk factor for the development of chronic lung diseases.

Methods: Neonatal wild-type (WT), TLR2 deficient (-/-), IL-13-/-, MyD88-/- and STAT6-/- mice were infected with the natural mouse pathogen *Chlamydia muridarum*, as a model of a moderate-to-severe respiratory bacterial infection in early-life. In some experiments WT mice were treated with miR-21-specific antagomirs, the pan-PI3K inhibitor LY294002, or relevant vehicle controls during early-life infection. The impact of these specific immune molecules on early-life bacterial infection-induced chronic lung disease were assessed.

Results: Neonatal respiratory infection increased TLR2, IL-13-receptor, miR-21 and PI3K expression and/or activity in the lung. TLR2 signaling induced IL-13-receptor expression, IL-13 signaling induced miR-21 expression and miR-21 increased PI3K activity. TLR2 signaling also increased the number of IL-13+ type-2 innate lymphoid cells in the neonatal lung. TLR2-/- and IL-13-/- mice were protected against deleterious infection-induced lung function changes in later-life. This TLR2/IL-13 mediated phenotype was independent of MyD88, but dependent on STAT6 signaling. Specific targeting of miR-21 and PI3K during early-life infection also prevented infection-induced chronic lung disease.

Conclusion: Early-life respiratory bacterial infections increase TLR2, IL-13, miR-21 and PI3K responses that promote the development of chronic lung disease in later-life. This study identifies a novel signaling network that may be targeted for the prevention of the long-term deleterious effects of early-life infection on lung function.

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Maternal antibodies are present at the newborn's respiratory tract in the absence of a structured polymeric immunoglobulin secretory immune system (PISIS)

Vega-López, M.A., Guzmán-Bautista, E., Ramírez-Estudillo, C., Rojas, O.

CINVESTAV-IPN, Infectómica y Patogénesis Molecular, México, D.F., Mexico

We have shown that PISIS development occurs during the first 4 weeks of age in the porcine experimental translational model (Dev. Comp. Immunol. 2015. 53:271-282). However, mucosal immune protection in the newborn is still not well understood. We demonstrated that newborn piglets lack of immunoglobulin (Ig) producing cells in the respiratory tract. However polymeric immunoglobulin receptor (pIgR) positive cells are present in the trachea and bronchi submucosae. Using weaned at birth (n=6) and 2 day-old colostrum fed (n=8), SPF Vietnamese minipigs, we measured the amounts of IgG, IgM and total and secretory IgA at serum, saliva, nasal mucus, tracheal and bronchial lavages, by quantitative ELISA. Our results showed low levels of self produced IgM and IgA at birth in serum, and no or very low levels at respiratory surfaces. After 2 days of suckling, important amounts of these Igs and IgG were detected at the respiratory tract, presumably from maternal origin. Furthermore, IgA lacking the secretory component (monomeric and polymeric) were more abundant than SIgA at mucosal samples up to: 10000X in nasal secretion, 200X in saliva, 50X in tracheal lavage and 20X in bronchi lavage. Therefore, non-secretory Igs, including IgG, also reached mucosal surfaces by a non yet described mechanism (newborn's pIgR?) and these Igs may play an important role in protecting the newborn. These results suggest a compensatory role of maternal immunity in mucosal protection of the newborn while the PISIS is fully mature.

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The authors declare no conflict of interests.

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Oral administration of lactic acid bacteria prevents inflammation and steatosis in a murine model of non-alcoholic steatohepatitis (NASH) and hepatocellular carcinoma (HCC)

Tsuji, N.¹, Suabjakyong, P.¹, Watanabe, Y.¹, Kamiya, T.¹, Kimoto-Nira, H.², Kanto, T.³

¹National Institute of Advanced Industrial Science and Technology (AIST), Biomedical Research Institute, Tsukuba, Japan, ²NARO institute of Livestock and Grassland Science (NILGS), Animal Products Research Division, Tsukuba, Japan, ³National Center for Global Health and Medicine, The Research Center for Hepatitis and Immunology, Ichikawa, Japan

The number of Non-alcoholic fatty liver disease (NAFLD) patients has rapidly increased during the past couple of decades. NAFLD is a progressive form of non-alcoholic steatohepatitis (NASH), a hepatic manifestation of metabolic syndrome and highly associated with Hepatocellular carcinoma (HCC). Therefore establishment of diet-solutions, in addition to symptomatic

treatments, are keenly required. We previously reported that a majority of lactic acid bacteria are able to induce high level of anti-inflammatory interferon- β (IFN- β) from dendritic cells by stimulating endosomal Toll-like receptors. One such strain, *Lactococcus lactis* subsp. *cremoris* C60, induces high level of IL-10 also, and stabilizes oral tolerance. In the present study, we show that oral administration of C60 to NASH model mice up-regulated the level of IL-10 in the intestine and significantly improved NAFLD including steatosis, lobular inflammation, and hepatocellular ballooning. We are currently examining the role of these anti-inflammatory mediators (IL-10/IFN- β) and resultant immune regulatory cells in suppressing chronic inflammation to reveal a novel interaction between intestinal immunity and NASH. (This work was partially supported by Grant-in-Aid for Scientific Research by Yakult Bio-Science Foundation and AMED.)

Immunity to Bacteria & Fungi 2

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An inhibitor of Wnt protein production improves host control of *Listeria monocytogenes*

Tran, T.T.¹, Andrades, M.G.¹, Nguyen, T.T.K.¹, Wyer, O.¹, Kling, J.C.¹, Begun, J.^{2,3}, Blumenthal, A.^{1,4}

¹The University of Queensland Diamantina Institute, Translational Research Institute, Brisbane, Australia, ²Mater Research Institute, The University of Queensland, Brisbane, Australia, ³School of Medicine, The University of Queensland, Brisbane, Australia, ⁴Australian Infectious Diseases Research Centre, The University of Queensland, Brisbane, Australia

Bacterial infections remain an important clinical challenge despite our extensive arsenal of antibiotics. This is exemplified by lengthy treatments of chronic infections, high mortality rates due to excessive inflammation, and an alarming increase in antibiotic resistance. One attractive strategy for improved treatments for challenging infections is to enhance endogenous anti-microbial defence. We and others have associated the Wnt pathway with bacterial infections in patients and model systems, implicating novel roles for this well-known developmental signalling pathway in immune responses to infection. However, the nature of immune-related Wnt functions remains to be clearly defined. Here we demonstrate differential expression of multiple Wnt family members in macrophages and intestinal epithelial cells in response to infection with the intracellular bacterial pathogen *Listeria monocytogenes*. *Listeria* burden was increased in Wnt-overexpressing cells, whereas treatment with small molecule inhibitors of Wnt production resulted in restriction of *Listeria* replication by infected host cells. This was associated with significant alterations in the early *Listeria*-containing intracellular compartments, suggesting that Wnt proteins guide the intracellular fate of ingested bacterial pathogens. In an *in vivo* model of *Listeria* infection, inhibition of Wnt production significantly lowered bacterial burden of infected mice underpinning the relevance of our findings to host control pathogens. These observations affirm the functions of Wnt proteins in the immune defence against infection and warrant the exploration of Wnt pathway modulation to enhance endogenous host control mechanisms against pathogenic bacteria.

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Enhancement of IL-22/IL-22RA1 signaling to decrease bacterial burden and extrapulmonary dissemination during pneumococcal pneumonia

Trejejo-Nunez, G., Chen, K., Kolls, J.

University of Pittsburgh School of Medicine/Richard King Mellon Foundation for Pediatric Research/Children's Hospital of Pittsburgh, Pittsburgh, United States

Community acquired pneumonia is caused primarily by *Streptococcus pneumoniae*, which is responsible for 4 million illness episodes, 445,000 hospitalizations and 22,000 deaths annually in the US. Interleukin 22 (IL-22) is a secreted cytokine produced mainly by cells of the lymphoid lineage and innate lymphoid cells. Ligation of IL-22 to IL-22RA1 and IL-10R2 results in downstream phosphorylation of STAT3. IL-22RA1 is characteristically expressed in epithelial cells, and its activation by IL-22 is involved in the pathogenesis of extracellular organisms. We hypothesized that enhancement of IL-22/IL-22RA1 through recombinant IL-22 translates into decrease pneumococcal burden and dissemination. Wild type mice had endogenous IL-22 production upon *S. pneumoniae* lung infection as early as 5 hours post infection and IL-22KO mice had higher pneumococcal burden in lungs and extrapulmonary organs compared to wild type controls. To assess the importance of IL-22RA1 signaling in the liver, IL-22RA1 conditional knockouts were infected with *S. pneumoniae* and had higher bacterial burdens in the lung, liver and spleen compared to littermate controls. Furthermore, pretreatment with IL-22:Fc in wild type mice decreased pneumococcal burden at 24 hours post infection. Taken together, we have evidence that IL-22 has a protective role against pneumococcal burden and dissemination and enhancement of this signaling pathway may be beneficial during pneumonic process. Our data also suggests that during pneumococcal pneumonia, IL-22 signaling to hepatic IL-22RA1 controls bacterial dissemination. We propose that therapeutic use of IL-22 may decrease bacterial burden by sustaining STAT3 phosphorylation in lung and liver tissues compared to other cytokines induced during pneumonia.

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Decreased micro RNAs (miRNAs) and soluble interleukin-7 receptor (sIL-7R) plasma levels in tuberculosis patients in Kumasi, Ghana

Afum-Adjei Awuah, A.^{1,2}, Owusu-Dabo, E.¹, Rimpler, J.³, Kohns, M.³, Heesch, K.⁴, Frempong, M.⁵, Jacobsen, M.³

¹Kwame Nkrumah University of Science and Technology (KNUST), Kumasi Centre for Collaborative Research in Tropical Medicine (KCCR), Kumasi, Ghana, ²GSK Vaccines s.r.l., In vitro Research, Siena, Italy, ³University Children's Hospital, General Pediatrics, Neonatology and Pediatric Cardiology, Duesseldorf, Germany, ⁴Bernhard Notch Institute for Tropical Medicine, Immunology, Hamburg, Germany, ⁵Kwame Nkrumah University of Science and Technology (KNUST), School of Medical Sciences, Kumasi, Ghana

Although a little over two billion people are infected with *Mycobacterium tuberculosis* resulting in significant morbidity and mortality especially in Africa, little is understood about the

dynamics of immune response and host dependent immune modulation. In this prospective cohort study, we investigated the dynamics of interferon-gamma (IFN- γ) responses and the possible role of miRNAs and sIL-7R in 61 tuberculosis cases and 150 exposed but healthy household controls (LTBI).

Antigen-specific IFN- γ responses, sIL7R plasma levels and 29 candidate miRNAs were quantified in active tuberculosis patients before, during and after TB chemotherapy and in controls.

We show from our findings that IFN- γ responses were significantly higher in TB patients than LTBI

($p = 0.0183$) but we did not observe any significant differences in IFN- γ responses during chemotherapy. 7 of the candidate miRNAs (miR-25, miR-26a, miR-29a, miR-99b, miR-101, miR-146a and miR-223) were significantly dysregulated during chemotherapy ($p < 0.05$). We detected lower sIL-7R concentrations in tuberculosis patients as compared to healthy contacts ($p < 0.001$). In addition, during therapy (about 2 months) and after recovery (> 6 months) sIL-7R concentrations were higher than prior to therapy ($p = 0.04$ and $p = 0.003$, respectively). Successful treatment and recovery restored sIL-7R levels especially in tuberculosis patients with low sIL-7R concentrations prior to therapy ($R = 0.59$, $p < 0.001$).

We concluded that differential miRNA expression of 7 miRNAs, IFN- γ and plasma sIL-7R levels are diminished in tuberculosis patients possibly affecting T-cell immunity during acute disease.

4049

Reduced neutrophil differentiation drives aberrant neutrophil function in beta-hemoglobin disorders: understanding the molecular and cellular mechanism

Siwaponanan, P.¹, Siegers, J.², Ng, T.^{2,3}, Svasti, S.¹, Fucharoen, S.¹, Wijburg, O.⁴, Vadolas, J.²

¹Mahidol University, Thalassemia Research Centre, Institute of Molecular Bioscience, Nakornpathom, Thailand, ²Murdoch Childrens Research Institute, Cell and Gene Therapy Group, Melbourne, Australia, ³The University of Melbourne at the Peter Doherty Institute for Infection and Immunity, Department of Microbiology and Immunology, Melbourne, Australia, ⁴The University of Melbourne at the Peter Doherty Institute for Infection and Immunity, Microbiology and Immunology, Melbourne, Australia

β -Thalassemia is an important β -haemoglobin disorder resulting in iron overload, and is associated with several abnormalities in the immune system, including defective neutrophil functions, predisposing patients to infections with bacterial pathogens. The molecular mechanisms involved in impaired neutrophil function during bacterial infection in thalassemia patients are not well understood. We used the Hbb^{th3/+} β -thalassemia mouse model and patient blood samples to investigate neutrophil function and susceptibility to infection. Hbb^{th3/+} β -thalassemia mice were highly susceptible to infection with *Streptococcus pneumoniae* (Spn). Blood and splenic neutrophils from Hbb^{th3/+} β -thalassemia mice, but not bone marrow derived neutrophils, showed defective chemotaxis, reduced opsonophagocytosis and decreased reactive oxygen species production compared with neutrophils from normal mice. Quantitative RT-PCR

analysis on mRNA from purified neutrophils demonstrated that CXCR2, CD11b, p22phox, p40phox, p67phox and p91phox gene expression levels were significantly repressed during systemic infection with *Spn*. Morphological analysis of neutrophils from uninfected animals showed a striking expansion of a subset of immature neutrophils, possibly granulocytic myeloid-derived suppressor cells (G-MDSCs), in Hbb^{th3/+} β -thalassemia mice. Comparison of expression of genes associated with neutrophil maturation showed that expression of the master transcriptional regulator for terminal myeloid differentiation and neutrophil function, PU.1, was significantly decreased in neutrophils of thalassaemic mice. Similar changes in cellular morphology and PU.1 expression in neutrophils from HbE/ β -thalassaemia patients were subsequently shown. The outcome of this study provides evidence for direct molecular mechanisms resulting in aberrant neutrophil functions in the context β -haemoglobinopathies and iron overload, which should be further explored to develop novel treatments.

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Phenol-soluble modulins alpha induce G2/M phase transition delay and impair immune response of eukaryotic cells

Deplanche, M.^{1,2}, Filho, R.A.E.-A.^{1,3}, Semenovskaya, K.^{1,2}, Alekseeva, L.^{1,4}, Jardin, J.^{1,2}, Henry, G.^{1,2}, Azevedo, V.³, Germon, P.⁵, Rainard, P.⁵, Dessauge, F.⁶, Finot, L.⁶, Laurent, F.⁷, Lina, G.⁷, Vandenesch, F.⁷, Le Loir, Y.^{1,2}, Smith, D.⁸, Otto, M.⁹, Götz, F.¹⁰, Berkova, N.N.^{1,2}

¹INRA, UMR 1253, STLO, Rennes, France, ²Agrocampus Ouest, UMR 1253 STLO, Rennes, France, ³Federal University of Minas Gerais, Belo Horizonte, Brazil, ⁴Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Moscow, Russian Federation, ⁵François Rabelais University of Tours, Nouzilly, France, ⁶INRA, Saint Gilles, France, ⁷University of Lyon, INSERM U 1111, Lyon, France, ⁸University of Glasgow, Glasgow, United Kingdom, ⁹US National Institute of Health, Bethesda, United States, ¹⁰University of Tübingen, Tübingen, Germany

Staphylococcus aureus is responsible for a wide range of infections in human and animals. We found that *S aureus* slowed down host cell proliferation and induced a cytopathic effect. We demonstrated that *S aureus* induced a G2/M phase transition delay in host cells, which was associated with accumulation of the cyclin-dependent kinase Cdk1/cdc2 and unphosphorylated histone H3. We found that a G2 phase delay was preferential for bacterial internalization and intracellular proliferation. Using size exclusion chromatography and mass spectroscopy analysis, we identified phenol-soluble modulin alpha (PSMa) peptides as the candidates for this effect. The implication of PSMa in cell cycle alteration was confirmed by testing of synthetic PSMa and by comparison of LACwt with the isogenic mutant LAC Δ psm, which lacks the operon encoding PSMa. The delay was associated with a decrease of defensins expression in a G2 phase, suggesting that PSMa-induced G2/M phase transition delay deteriorates antibacterial state of the epithelial surface. Investigation of the response to *Escherichia coli* and *S. aureus* showed a higher expression of key cytokines IL-6, IL-8, as well as IL-32 (which is involved in dendritic cell maturation) in *E. coli*-infected host cells. Comparison of cytokines expression in

response to LACwt with isogenic mutants, which lack the operon encoding PSMs, show that PSMs inhibit interleukins production, thus impair the innate and adaptive immune response during *S. aureus* infection. Therefore we show, that PSMs alter the host cell cycle, resulting in a reduction of defense response of host' cells, that reveal a newly-identified mechanism for promoting infection.

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The role of regulatory T cells in nontuberculous mycobacterial lung disease

Jung, M.¹, Kang, S.M.², Shin, E.-C.¹

¹KAIST, Daejeon, Korea, Republic of, ²Gachon University Gil Hospital, Incheon, Korea, Republic of

Although nontuberculous mycobacterial lung disease (NTMLD) are becoming prevalent worldwide, immunopathogenesis is poorly understood. NTMLD occurs in individuals with distinct predisposing conditions, suggesting specific immune dysregulation may exist. Regulatory T cells (Tregs) suppresses effector T cell functions. However, little is known about Tregs with NTMLD. To investigate immunological characters of the patients with NTMLD, especially focused on the association of Tregs and their clinical relevance in patients with NTMLD. Treg frequency and CD39 expression on Tregs were enhanced, especially subpopulation II and subpopulation III, in patients with NTMLD compared with healthy controls. Treg frequency, subpopulation II, and subpopulation III showed a tendency toward inverse correlation with total CT scores. The frequency and CD39 expression of Treg subpopulation II were positively correlated with FEV₁ in patients with NTMLD. In conclusions Tregs and Treg subpopulations are dysregulated quantitatively and qualitatively in patients with NTMLD. Enhancing frequency or activity of Tregs may be a novel therapeutic strategy to reduce inflammatory burden and improve lung function in patients with NTMLD.

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Profile of events leading to innate resistance to mycobacterial infection

ADAM, L., Poux, C., Spetz, A.-L., Fernandez, C.

Stockholm University, Molecular Biosciences the Wenner-Gren Institute, Stockholm, Sweden

Pulmonary tuberculosis is a highly contagious infectious disease causing around 1 million deaths each year. Remarkably, many individuals in contact with Mycobacterium do not get infected. Increasing evidence suggests that host genetic heterogeneity affects the immune response to intracellular pathogens, such as Mycobacterium. While BALB/c and C57BL/6 mice are relatively resistant differences exist in their susceptibility towards Mycobacterium infection.

Here, we have used BALB/c and C57BL/6 BCG infection models, to highlight these differences.

We compared CD45⁺ lung populations and observed that C57BL/6 mice present higher frequencies of eosinophils, alveolar macrophages, monocytes and CD103⁺DC compared to BALB/c at steady state. Mice were then infected intranasal with

a live, either low or high BCG dose. We found that after a low BCG exposure dose, C57BL/6 were more efficient than BALB/c in the early control of bacterial growth in the lungs. However, at a higher BCG dose, both strains displayed similar CFU counts. BALB/c mice developed early inflammatory innate responses with significant increases in neutrophils, eosinophils, alveolar macrophages, CD11b⁺DC and CD103⁺DC. Whereas in C57BL/6 mice, most of the CD45⁺ subsets remained stable compared to baseline also after inoculation of a high dose, with the exception of neutrophils and CD103⁺DC that increased. Interestingly, our results suggest that the early strong inflammatory innate response observed in BALB/c is not sufficient to control mycobacterial growth. In contrast, other innate signatures and cellular parameters observed in C57BL/6 mice as well as the increase of CD103⁺DC and neutrophils seem to correlate with the early control of Mycobacterium.

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Tumor cell intrinsic TLR4 signaling promotes tumor growth and metastasis

Rogava, M.¹, Bald, T.¹, Shridhar, N.¹, van den Boorn-Konijnenberg, D.², Schmid-Burgk, J.³, Hornung, V.³, Hölzel, M.², Tüting, T.¹

¹University Hospital Bonn, Experimental Dermatology, Bonn, Germany, ²University Hospital Bonn, Institute for Clinical Chemistry and Clinical Pharmacology, Bonn, Germany, ³University Hospital Bonn, Institute of Molecular Medicine, Bonn, Germany

Metastatic melanoma is the most deadly type of skin cancer as it spreads quickly. We and others have shown that Toll-like receptor 4 (TLR4) signaling in the tumor microenvironment is important for tumor cell survival and metastasis. However, the role of TLR4 signaling in melanoma cells is not entirely clear. Therefore, we hypothesized that tumor cell intrinsic TLR4 signaling contributes to melanoma cell survival and metastasis. To address this hypothesis, we utilized a mouse melanoma cell line established in our laboratory. We generated TLR4 deficient melanoma cell variants using the CRISPR/CAS9 genome editing technology. TLR4 deficient clones were validated by Next Generation Sequencing and in functional assays. As a following step, TLR4 deficient tumor cells were transplanted in immunocompetent syngeneic C57BL/6 mice and monitored for tumor growth and metastasis. Here we find that melanoma cells also express functional TLR4. Activation of TLR4 signaling in tumor cells increases proliferation and migration in vitro. Furthermore, TLR4 deficient cells demonstrate delayed growth kinetics upon transplantation into immunocompetent mice. Additionally, TLR4 deficient melanoma cells show decreased numbers of metastases in the lungs. Genetic reconstitution of TLR4 in melanoma cells reversed the observed impairment in tumor growth and metastasis. Taken together, our results provide experimental evidence that TLR4 signaling has melanoma cell-intrinsic function and promotes cell survival and metastasis. This further supports strategies to inhibit TLR4 signaling as an adjuvant treatment option for melanoma patients with a high risk for metastatic dissemination.

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Exploring the mechanism for cancer associated fibroblasts recruitment and the potential therapeutic value in esophageal squamous cell carcinoma

Qi, J.¹, Zhang, L.¹, Guan, X.²

¹University of Hong Kong, Clinical Oncology, Hong Kong, China,

²University of Hong Kong, Hong Kong, China

Nowadays, a growing body of evidence suggests that cancer is the wound that never heals¹. Similar to normal wound healing, cancer development and progression requires the participation of many different cell types^{2,3}. Host cells such as fibroblasts, endothelial cells and immune cells are recruited into cancer beds to regulate survival, proliferation, invasion, metastasis, angiogenesis, cancer stem cell maintenance and even the outcome of therapy. This research focuses on cancer associated fibroblasts (CAFs), which is the major cellular component of tumor stroma. It is found that tumors frequently recruit CAFs to enhance tumor growth, invasion and angiogenesis by secreting growth factors, matrix degrading enzymes and angiogenic factors. CAFs are also able to prepare metastatic niche for tumors, even affect tumor metabolism. FGFR2 was identified to show greatest expression difference between CAFs and NFs based on microarray results. *In vivo* experiments have shown that FGFR2 (+) fibrocytes can be recruited by ESCC tumors. IF and flow cytometry data show that FGFR2(+) fibrocytes are recruited from bone marrow, circulating in peripheral blood and finally reached site of tumor. RNA sequencing data indicate that after being trained by the tumor, fibrocytes differentiate into CAFs and facilitate tumor progression. Thus blocking the tumor-CAF interaction will strongly impede the tumor growth therapeutic targeting of FGF-2 and FGFR2 may impaired cancer-stroma interaction by inhibit accumulation of CAFs in tumor tissues may serve as an effective treatment approach to hundreds of thousands of ESCC patients.

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IL-17B/IL-17RB axis potentiates tumor growth and migration via activating AKT/ β -catenin pathway in gastric cancer, which accompanied by upregulation of stemness markers

Bie, Q., Ma, J., Su, Z., Wang, S., Xu, H.

Jiangsu University, Immunology, Zhenjiang, China

Background: Inflammation is a critical component involved in tumor progression. IL-17 cytokine family, has recently emerged as important players in diseases. However, the role of IL-17B/IL-17RB signal in tumor growth and migration remains unknown.

Methods: 25 patients diagnosed newly with gastric cancer(GC) were included in the study, Western blotting and immunofluorescence staining were used to determine the expression of IL-17RB, and serum level of IL-17B was measured by ELISA. The expression levels of IL-17RB, IL-17B, IL-25, Oct4, Nanog and Lgr5 mRNA were detected by qRT-PCR. In addition, MGC-803 cell line was chosen to verify the effect of IL-17RB on tumor cells via administrating IL-17RB-shRNA and AKT inhibitor, the ability of cell proliferation, migration was analyzed by colony-formation, cell scratch and transwell assay respectively,

and the expression levels of E-cadherin, N-cadherin, AKT, β -catenin and some stemness markers were detected by qRT-PCR and Western blotting.

Results: The expression of IL-17B and IL-17RB were significantly increased in cancer tissues, there were positive correlations between IL-17RB and some stemness markers. The expression of phosphorylated AKT, GSK-3 β and β -catenin were significantly increased in MGC-803 cell line which expressed higher IL-17RB. In IL-17RB-shRNA transduced MGC-803 cells, the ability of cell-proliferation and migration or expression of E-cadherin, N-cadherin, Nanog, Sox 2 and Oct 4 protein were obviously influenced. In addition, the AKT inhibitor, LY294002 could reverse IL-17B-induced upregulation of β -catenin and some stemness markers.

Conclusions: The IL-17B/IL-17RB axis promoted the growth and migration of tumor cells through up-regulating the cells stemness and activating AKT/ β -catenin pathway in GC.

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Low dose Irradiation retunes Immunosuppressive tumor associated macrophages (TAM), and induces immune mediated rejection of established Tumors of Pancreas

Prakash, H.

University of Hyderabad, Hyderabad, India

Inefficient migration of immune effector cells in the tumor is a major limitation of effective therapy against solid tumors in general, but more specifically in pancreatic tumours. This is believed to be due to impermeable tumor endothelium and accumulation tumor-associated macrophages (TAM) in the majority of tumors. These cells represents major barrier in therapies and have been associated with the poor prognosis in the majority of cancer patients. Therefore it is anticipated that targeted activation of the tumor microenvironment may overcome this barrier dysfunction. Among various tumor directed therapies, Radiotherapy has been recently introduced as a potential strategy to improve cancer immunotherapy and tumor immune rejection. This is the only clinically advanced approach for noninvasive, site-specific intervention in cancer patients. Majority of cancer patients are routinely irradiated with therapeutic doses of γ -radiations which range between 45-60Gy. Such high doses of γ -radiations during radiotherapy procedures frequently manifests moderate to severe local / systemic toxicities. Low dose radiation (LDR) on the other hand provides good alternatives of HDR for avoiding such toxicities. In this line, our pioneer studies (Klug and Prakash et, 2013, Prakash et al, 2016) demonstrated that low dose irradiation (2Gy) when applied either locally or systemically, effectively modified tumor microenvironment and facilitated infiltration of peripheral immune effectors cells (T-cells) in neuro endocrine, solid, highly invasive and non-resectable insulinoma in RIP1-Tag5 (RT5) mice, primary human pancreatic carcinoma as well as in xenotransplanted human melanoma. Such tumor infiltration of T cells remained strictly dependent on iNOS+ peritumoral macrophages.

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Natural Killer cells display increased efficiency towards Vemurafenib resistant melanoma cell lines

Frazao, A.¹, Colombo, M.¹, Bouquet, F.², Toubert, A.¹, Caignard, A.¹, Savina, A.²

¹INSERM U 1160, IUH - Hopital Saint Louis, Paris, France, ²Roche Scientific Partner, Boulogne Billancourt, France

Melanoma is an aggressive cutaneous cancer with high metastatic potential. Presence of a BRAFV600 mutation in 60% of melanoma showed the interest of BRAF specific inhibitors such as Vemurafenib. This treatment induces high rates of clinical responses in patients but most of them relapse after several months of treatment. Lately, focus has been given to immunotherapy for melanoma tumors. Melanoma cells express stress molecules and are susceptible to lysis by Natural Killer (NK) cells, suggesting interest for combined NK-based therapies. From BRAF mutated melanoma cell lines, we generated Vemurafenib resistant cell lines by culturing the cells with increasing doses of the drug. These resistant variants were characterized and compared to the corresponding parental cell lines, susceptible to Vemurafenib. We showed that drug resistant variants are more susceptible to NK-cell mediated lysis compared to parental cell lines. Resistant cell lines induced stronger activation of NK cells. NK cells showed higher degranulation potential and production of IFN-gamma in response to stimulation with resistant variants. Comparing parental and resistant variants, we showed an increase of NKG2D ligands on resistant variants. Interestingly, we also observed increased death domain receptors (Fas and Trail-R) expression by resistant cell lines compared to their parental counterparts. Thus, certain Vemurafenib resistant variants display increased visibility to NK cells and higher susceptibility to death domain receptors apoptosis.

Altogether, our observations indicate the interest of combining BRAF inhibitors with NK based therapies.

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Ovarian cancer ascites-activated neutrophils suppress T cell proliferation in a contact-dependent mechanism

Singel, K.L.¹, Khan, A.N.H.², Emmons, T.R.¹, Mayor, P.C.³, Moysich, K.B.⁴, Odunsi, K.^{3,5}, Segal, B.H.^{1,2,6}

¹Roswell Park Cancer Institute, Immunology, Buffalo, United States,

²Roswell Park Cancer Institute, Medicine, Buffalo, United States,

³Roswell Park Cancer Institute, Gynecologic Oncology, Buffalo, United States,

⁴Roswell Park Cancer Institute, Cancer Prevention and Control, Buffalo, United States,

⁵Roswell Park Cancer Institute, Center for Immunotherapy, Buffalo, United States,

⁶University at Buffalo, Medicine, Buffalo, United States

Ovarian cancer (OC) is often diagnosed at advanced stages. Necrosis is a hallmark of advanced cancer and releases DAMPs that activate innate immune responses. CD8⁺ T cells mediate antitumor immunity while TAMs and MDSCs can impair T cell responses. Knowledge is limited on the role of activated neutrophils in the tumor microenvironment. In ascites from patients with newly diagnosed OC, ~90% of cells were inflammatory (CD45⁺) with varying proportions of neutrophils,

monocytes and lymphocytes. Neutrophils comprised ~15% of CD45⁺ cells and the neutrophil:CD8⁺ T cell ratio was 1.5:1. In *ex vivo* studies, cell-free ascites (CFA) attracted normal donor neutrophils (NDN). Neutrophil extracellular traps (NETs) are a distinct mode of death, and we found that CFA induced NDN to generate NETs *ex vivo*. In co-culture studies, normal donor T cell proliferation was measured by [³H] thymidine incorporation. While CFA or NDN alone did not impair CD3/CD28-stimulated T cell proliferation, CFA-activated NDN completely suppressed proliferation. Suppression did not affect T cell viability or induce apoptosis, and was not reversed by the addition of arginine, N-acetyl-L-cysteine or IL-2. Suppression required T cell contact with NDN. We also found that PD-1 expression on the suppressed T cells phenocopied the unstimulated controls, suggesting that T cell-NDN contact may block activation and subsequent checkpoint upregulation. These results support a model in which neutrophils in the ascites of OC patients may suppress T cell responses, thereby abrogating anti-tumor immunity. Further studies will identify ascites constituents that activate neutrophils and contact mechanisms for neutrophil-mediated T cell suppression.

Cytokines, Interferons & Inflammation 2

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High fat diets induce colonic epithelial cell stress and inflammation that is reversed by IL-22

*Gulhane, M.*¹, *Murray, L.*², *Lourie, R.*², *Tong, H.*², *Kang, A.*³, *Schreiber, V.*², *Magor, G.*⁴, *Denman, S.*⁵, *Begun, J.*², *Florin, T.*², *Perkins, A.*⁴, *O Cuiv, P.*³, *McGuckin, M.*², *Hasnain, S.Z.*²

¹Mater Research Institute-University of Queensland, Brisbane, Australia, ²Mater Research Institute-University of Queensland, Immunity, Infection and Inflammation Group, Brisbane, Australia, ³University of Queensland Diamantina Institute, Translational Research Institute, Brisbane, Australia, ⁴Mater Research Institute-University of Queensland, Blood and Bone Diseases Program, Brisbane, Australia, ⁵The Commonwealth Scientific and Industrial Research Organization, Brisbane, Australia

Prolonged high fat diets (HFD) induce low-grade chronic intestinal inflammation in mice, and diets high in saturated fat are a risk factor for the development of human inflammatory bowel diseases. We hypothesized that HFD-induced obesity, oxidative stress, protein misfolding and endoplasmic reticulum (ER) stress occur in intestinal secretory goblet cells, triggering inflammatory signaling and reducing synthesis and secretion of proteins that form the protective mucus barrier. A prolonged HFD of 22 weeks elevated the intestinal inflammatory cytokine signature, alongside compromised mucosal barrier integrity with a decrease in goblet cell differentiation factor Kruppel-like factor-4 and Muc2, along with a loss in the tight junction protein, claudin and increased serum endotoxin levels. In a spontaneous model of colitis (*Winnie* mice), HFD increased ER stress, further compromised the mucosal barrier and increased the severity of pathology. We discovered using primary intestinal cells that non-esterified fatty acids directly increased oxidative and ER stress leading to protein misfolding which was suppressed by the oxidative stress suppressing cytokine IL-22. IL-22 therapy

reduced ER/oxidative stress and improved the integrity of the mucosal barrier, and reversed microbial changes associated with obesity. Consistent with epidemiological studies, our experiments suggest that high fat intake and obesity is likely to impair intestinal barrier function, particularly in early life, which at least partially involves direct effects of free fatty acids on intestinal secretory cells, and we demonstrate this can be reversed by IL-22 therapy.

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Antimicrobial peptide derived from insulin-like growth factor-binding protein 5, AMP-IBP5, activates human mast cells

Niyonsaba, F.^{1,2}, *Okumura, K.*¹, *Ogawa, H.*¹

¹Juntendo University Graduate School of Medicine, Atopy (Allergy) Research Center, Bunkyo-ku, Japan, ²Juntendo University, Faculty of International Liberal Arts, Bunkyo-ku, Japan

Apart from exhibiting killing activities against invading pathogens, antimicrobial peptides (also known as host defense peptides, HDPs) broadly modulate the immune response through activation of different cell types. Recently, a novel antimicrobial peptide derived from insulin-like growth factor-binding protein 5 (AMP-IBP5) was discovered and shown to display strong antimicrobial activities at concentrations comparable to those of well-characterized HDPs such as human β -defensins and cathelicidins. Given that human β -defensins and cathelicidins are known to activate various cell types including mast cells, we hypothesized that AMP-IBP5 could also stimulate mast cells.

In the current study, we demonstrated that AMP-IBP5 activated human mast cells (LAD2 cells) to degranulate and secrete various eicosanoids such as prostaglandin D₂, E₂ and leukotriene C₄. Moreover, AMP-IBP5 enhanced intracellular calcium flux, cell migration and production of various cytokines/chemokines by mast cells. We observed that AMP-IBP5 activated human mast cells through a Mas-related G protein-coupled receptor member MrgX2, but not other MrgX receptors. Moreover, AMP-IBP5-induced mast cell activation was controlled by the phospholipase C, MAPK and NF- κ B pathways, as evidenced by their specific inhibition.

In conclusion, the present study provides novel evidence that, in addition to its microbicidal properties, host defense peptide AMP-IBP5 may also contribute to the regulation of innate immunity through activation of various functions of mast cells.

539

Anti-inflammatory properties of the hookworm protein Na-AIP-1

Buitrago, G., *Pickering, D.*, *Giacomin, P.*, *Shepherd, C.*, *Jones, L.*, *Loukas, A.*

Centre for Biodiscovery and Molecular Development of Therapeutics, Australian Institute of Tropical Health and Medicine, James Cook University, Cairns, Australia

Inflammatory bowel disease (IBD) is an umbrella term for a group of immune-mediated conditions, which are characterised

by idiopathic chronic inflammation of the gastrointestinal tract. Current treatment protocols for IBD are often poorly tolerated or ineffective. The decline in prevalence of human parasitic infections and concurrent increases in the occurrence of various inflammatory conditions has been recognised, provoking the hypothesis that some of these parasites may possess anti-inflammatory capabilities.

Multiple experimental studies have documented the efficacy of live helminth infection in the alleviation of symptoms of intestinal inflammation, using both human and animal models of disease. Consequently, there is now interest in the potential immunomodulatory proteins derived from helminths, which may be safer and more acceptable alternatives to live worm therapy. We have isolated and expressed a novel candidate anti-inflammatory molecule from the secretions of the hookworm *Necator americanus* (*Na*-anti-inflammatory protein (AIP)-1).

Recombinant *Na*-AIP-1 was cloned and expressed using *Pichia pastoris* and purified via immobilised metal ion affinity chromatography. Intra-peritoneal treatment of mice with recombinant *Na*-AIP-1 ameliorated clinical, immunological and histological features of disease in murine models of TNBS-induced colitis and CD4⁺ T-cell transfer-induced colitis. Thus, *Na*-AIP-1 represents an excellent candidate for further development as a novel treatment of IBD and other immune-mediated inflammatory disorders.

276

The impact of the pro-inflammatory cytokine TNF- α in prostate cancer development

Banzola, I.¹, Mengus, C.², Wyler, S.³, Sais, G.¹, Keller, E.X.⁴, Poyet, C.⁴, Eberli, D.⁴, Sulser, T.⁴, Spagnoli, G.C.², Provenzano, M.¹

¹University Hospital Zurich, Schlieren, Switzerland, ²University Hospital Basel, Basel, Switzerland, ³Cantonal Hospital Aarau, Aarau, Switzerland, ⁴University Hospital Zurich, Zurich, Switzerland

Prostate cancer (PCa) constitutes a major health problem due to its high prevalence in the population and the current lack of effective treatment for its metastatic, castration-resistant form. A great deal of literature suggests that inflammation might play an important role in the onset and progression of PCa, although evidence remains scant. Inflammation of the prostate is almost ubiquitous in mature men and histological analysis of prostatic samples has revealed that areas surrounding adenocarcinomas often display inflammation, specifically in lesions designated as early stages of the disease.

In this study we addressed the role of the inflammatory cytokine TNF- α in the development of PCa. We particularly focused on PCa-specific tumor-derived soluble factors (PCa-TDSFs) that have already been reported as possible mediators of PCa morbidity and on how the TNF- α -driven release of those microenvironmental modifiers might affect cancer progression. Our results showed a clear picture in which the inflammatory stimulus modulates the release and functions of TDSFs primarily involved in specific steps of PCa progression. Indeed, both cancer immune evasion and cancer aggressiveness were permanently strengthened by the TNF- α -driven modulation of factors, such as IL-6, IL-1 β and MMP9, which are involved in the mechanisms of cell proliferation, migration and invasion.

Our results argue for a role of pro-inflammatory cytokines in the transformation of localized PCa into a more aggressive and invasive form. This opens a discussion on the possibility of administering inflammatory cytokine-based cancer treatments, such as immunotherapy, and on possible modulations of their effects.

1797

Impact of the timing of morphine administration on lipopolysaccharide-mediated lethal endotoxic shock in mice

Kato, H.¹, Fukada, T.¹, Imanishi, K.², Ozaki, M.¹, Yagi, J.¹

¹Tokyo Women's Medical University, Tokyo, Japan, ²Japan Health Sciences University, Saitama, Japan

Sepsis is a serious condition related to systemic inflammation, organ dysfunction, and organ failure. It is a subset of the cytokine storm caused by dysregulation of cytokine production. Morphine influences the severity of infection in vivo and in vitro because it regulates cytokine production. We investigated the immunological function of morphine using a mouse model of septic shock. We treated mice with α -galactosylceramide (α -GalCer, 2 μ g/mouse) to induce lethal endotoxic shock following a challenge with lipopolysaccharide (LPS, 1.5 μ g/mouse). This model represents acute lung injury and respiratory failure and reflects the clinical features of severe septic shock. We evaluated the effect of the timing of morphine (0.8 mg/mouse) administration on the survival rate, cytokine production in vivo, and histological changes of mice with LPS-mediated lethal endotoxic shock. Morphine treatment before LPS challenge suppressed lethal endotoxic shock. In contrast, when we administered after LPS, morphine exacerbated lethal endotoxic shock; hematoxylin and eosin staining revealed a marked increase in the accumulation of infiltrates comprising polymorphonuclear leukocytes and mononuclear cells in the lung, and Elastica van Gieson staining revealed the destruction of alveoli. The plasma levels of TNF- α , IFN- γ , MCP-1, and IL-12 in the group treated with morphine after LPS challenge were higher than those treated with morphine before LPS challenge. In conclusion, one of the factors that determine whether morphine exacerbates or inhibits infection is the timing of its administration. Morphine treatment before shock improved the survival rate, and morphine treatment after shock decreased the rate of survival.

3810

Aspirin-triggered Resolvin D1, ATRvD1, reverts chronic pulmonary fibrosis in a murine model

Guilherme, R.¹, Nogueira, T.¹, Neves, J.², Canetti, C.¹, Benjamim, C.¹

¹Federal University of Rio de Janeiro, Program of Immunology, Rio de Janeiro, Brazil, ²Federal University of Rio de Janeiro, Program of Pharmacology and Inflammation, Rio de Janeiro, Brazil

Pulmonary fibrosis is characterized by diffuse chronic interstitial inflammation, increased fibroblast proliferation, and enhanced extracellular matrix synthesis and deposition. This disease is a major cause of morbidity and mortality with a prognostic of 5

years of life, and there are few treatments strategies available. The resolvin D1 (RvD1) is a lipids derivative of omega-3 PUFAs (polyunsaturated fatty acids) and has anti-inflammatory and pro-resolution effects, however their anti-fibrotic effect has not been demonstrated yet. Our aim is to evaluate the mechanisms underlying the anti-fibrotic effect of ATRvD1 on bleomycin-induced pulmonary fibrosis in mice. Pulmonary fibrosis was induced in C57BL/6 mice by administration of bleomycin (BLM - 0.06U i.t.). ATRvD1 treatment was performed at days 7 (25 µg/ Kg iv) and 10 (5 µg/ Kg) after BLM administration, and the analysis was done 14 days after. Cell population was enhanced in the BAL of BLM group and the ATvD1 treatment reduced total cells, macrophages, and neutrophils infiltration in the BAL at day 14. ATRvD1 administration reversed BLM-induced collagen and fibronectin deposition, OH-proline content, collagen I and III secretion, α-sma expression and TNF-α, MCP-1, IL-1β, TGF-β production in the lungs. A marker of fibroblast activation, FSP1, was highly expressed after BLM and ATRvD1 was able to reduce this expression. ATRvD1 has an anti-fibrotic effect by controlling inflammation and reversing fibrotic phenotypes, however further experiments are necessary to characterize the mechanism of action of ATRvD1 in the mice fibrosis.

2300

Proinflammatory milieu and excitotoxic glutamate on glioblastoma cell infiltration

Fathima K, H., Dalavaikodihalli Nanjaiah, N.

NIMHANS, National Institute of Mental Health and Neurosciences, Neurochemistry, Bangalore, India

Background: Tumor cell migration and diffuse infiltration into brain parenchyma are the major hallmarks of Glioblastoma (GBM). GBM cells release extensive amounts of glutamate leading to excitotoxic cell death and necrosis of surrounding brain parenchyma, which triggers inflammation and release of the major proinflammatory cytokine IL-1β. Also, humans GBM cells themselves are found to overexpress IL-1β. We have previously shown that IL-1β plays a role in glioma invasion.

Objective: The objective of this study was to examine the effect of IL-1β and the activation of AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) and NMDA (N-methyl-D-aspartate) receptors of glutamate on glioma cell migration and matrix metalloproteinase-2 (MMP-2) activity and the possible effects of their interaction.

Materials: Cultured U87MG cells were treated with IL-1β, AMPA or NMDA and in vitro wound healing scratch assay was carried out to analyze their effect on migration of these cells. The effect on MMP-2 was analyzed using gelatin zymography for MMP-2 activity and qPCR for its mRNA expression.

Results: IL-1β showed increase in cell migration and MMP-2 activity in U87MG glioma cells. Treatment with either AMPA or NMDA increased the cell migration, while MMP-2 activity was found to increase only on NMDA treatment. IL-β induced cell migration and MMP-2 activity was enhanced on the pretreatment of cells with NMDA, but no such effect was observed on pretreatment with AMPA.

Conclusion: These results suggest that increase in migration of glioma cells in IL-β proinflammatory microenvironment

is enhanced by glutamate excitotoxicity, mainly through interaction with NMDA receptors.

Autoimmunity 2

1243

DNase 1 is protective in murine experimental myeloperoxidase anti neutrophil cytoplasmic antibody associated glomerulonephritis

O'Sullivan, K.M.¹, Gan, P.-Y.¹, Lo, C.Y.², Kitching, A.R.^{1,3,4}, Holdsworth, S.R.^{1,3}

¹Monash University, Medicine, Melbourne, Australia, ²Monash University, Monash Micro Imaging, Melbourne, Australia, ³Monash Health, Nephrology, Melbourne, Australia, ⁴Monash Health, Paediatrics, Melbourne, Australia

Myeloperoxidase (MPO) anti-neutrophil cytoplasmic antibody (ANCA) associated glomerulonephritis (MPO-ANCA GN) is an important cause of renal failure. With current standard treatment 30% of patients die or become dialysis dependent within 3 years. Studies in murine models have shown that the disease is neutrophil dependent. We have shown recently in patients that glomerular neutrophil extracellular traps (NETS) are a prominent feature. Human recombinant deoxyribonuclease 1 (DNase 1) is already used safely in children with Cystic Fibrosis. DNase 1 in addition to its other anti-inflammatory properties can inhibit net formation *in vitro*. We used DNase 1 intravenously twice daily to inhibit net formation in a murine model of experimental MPO-ANCA glomerulonephritis. DNase 1 treatment significantly decreased the percentage of glomeruli with NETs as defined by co-localisation of NET components: citrullinated histone 3 (H3Cit), protein arginine deiminase 4 (PAD4), MPO and chromatin (2.5% ± SEM 1.5, versus 38% ± SEM 5.6 in saline treated, P< 0.0001) and significantly reduced the amount of extracellular MPO (P< 0.05). Functional injury as assessed by proteinuria was significantly reduced (albuminuria/creatinine ratio reduced from 0.3± 0.09 in controls versus 0.07±0.03) as was histological injury (segmental glomerular necrosis, 38.5% ± 4.2% in controls versus 13.2% ± 1.9%). Glomerular recruitment of CD4

T cells, CD8 T cells, macrophages and neutrophils, were reduced with DNase 1 treatment (all, P< 0.05). This data suggests that NET formation is a critical component of inflammatory injury in this disease and that DNase 1 is effective in inhibiting NET formation, and reducing inflammation.

2219

CCR6 intrinsic signaling promotes Th17 cell differentiation during autoimmunity

Kulkarni, N., Lal, G.

National Centre for Cell Science, Infection and Immunity, Pune, India

Background: Chemokine receptor CCR6 is a G protein-coupled receptor (GPCR) expressed on various immune cells, including Treg and Th17, and its intrinsic signaling is well characterized

in the migration of cells. Does CCR6 signaling also control the differentiation of the effector Th17 cells?

Methods: We induced colitis by giving dextran sodium sulfate (DSS) in drinking water to C57BL/6 mice or adoptively transferred naïve CD4 T cells (CD4+Foxp3rfp-CD44-CD45RBhi cells) into RAG1^{-/-} mice.

Results: CCR6+CD4⁺ T cells showed significantly increased expression of ROR γ t and IL-17 in DSS induced colitis compared to controls, and CCR6^{-/-} mice showed reduced colitis. To test the contribution of CCR6 on ROR γ t expression, we injected CCR6^{-/-} CD4 T cells (CD45.2⁺) in the CD45.1⁺ congenic mice, and mice were given DSS. Compared to the CD45.1+(CCR6+/+) CD4 T cells, CD45.2+(CCR6^{-/-}) cells did not increase ROR γ t expression. To faithfully monitor the effect of CCR6 on ROR γ t expression in Foxp3⁺ Tregs, we adoptively transferred naïve CD4 T cells from CCR6gfp^{+/-}::Foxp3rfp^{+/+} mice to RAG1^{-/-} mice. During colitis, CCR6gfp⁺Foxp3rfp⁺ Tregs showed significant increased ROR γ t expression compared to the CCR6gfp⁻ Tregs. Addition of CCL20 (CCR6 ligand) in Treg and Th17 lineages differentiation culture conditions significantly increased ROR γ t and IL-17 expression. Binding of CCL20 with CCR6 induced phosphorylation of Akt, mTOR and STAT3, and which in turn promoted the binding of ROR γ t specifically on IL-17A promoter and CNS5, and increased IL-17 reporter activity.

Conclusions: CCL20-CCR6 signaling promotes differentiation of Th17 cells, and designing strategies to block CCR6 signaling will help in controlling gut inflammation and autoimmunity.

2595

Proinsulin specific CD4⁺ T cells isolated from peripheral blood of people with type 1 diabetes have similar specificities to islet-infiltrating CD4⁺ T cells

So, M., Pathiraja, V., Tresoldi, E., Elso, C., Kay, T., Mannering, S. St Vincent's Institute of Medical Research, Melbourne, Australia

Type 1 diabetes (T1D) is an autoimmune disease caused by T-cell mediated destruction of the insulin-producing beta cells. Genetic risk for T1D is most strongly associated with HLA-DQ2 and HLA-DQ8, and the insulin gene promoter. Recently we isolated CD4⁺ T-cell clones from the residual pancreatic islets of a deceased organ donor who had T1D. Many of these clones recognized epitopes from C-peptide, a natural product of the conversion of proinsulin to insulin. All C-peptide specific clones were restricted by HLA-DQ8, or HLA-DQ8 transdimer that only forms in HLA-DQ2/DQ8 antigen presenting cells. Here we ask if similar CD4⁺ T-cell responses can be detected in peripheral blood mononuclear cells (PBMC) of individuals with recent onset T1D when stimulated with full length (31 aa) C-peptide. C-peptide responsive CD4⁺ T cells were cloned from PBMC of people with recent-onset T1D. CD4⁺ T-cell responses to C-peptide were detected in PBMC from 9/21 individuals with recent-onset T1D but 0/7 individuals with long-standing T1D. C-peptide specific clones were isolated from two recent-onset subjects. Currently, we have three C-peptide responsive clones, one from Donor 1 and two from Donor 2. All three clones recognize proinsulin 47-62, which overlaps the epitopes recognized by 4/14 islet-infiltrating CD4⁺ T cells. Two clones, one from each donor, are HLA-DQ8 restricted and one is HLA-DR restricted. This work

highlights the importance of CD4⁺ T-cell responses to C-peptide in T1D. Furthermore, it supports the notion that C-peptide specific CD4⁺ T-cells detected in PBMC have similar properties to those that infiltrate human islets in T1D.

2870

Lupus-associated complement classical pathway proteases degrade apoptotic nuclear proteins and dampen cell activation by subclinical endotoxin

Cai, Y.¹, Yeo, J.G.^{1,2}, Leong, J.Y.³, Lu, J.¹

¹Yong Loo Lin School of Medicine, National University of Singapore, Department of Microbiology and Immunology, Singapore, Singapore, ²KK Women's and Children's Hospital, Division of Medicine, Singapore, Singapore, ³Singapore Translational Immunology and Inflammation Centre, Singapore Health Services, Singapore, Singapore

The complement system has long been investigated in host defense against microbial infections but genetic deficiency of the different complement components revealed involvements of complement in broader physiological processes. Deficiency of early (C1q, C1r/C1s and C4) but not late components of the classical pathway are strong causal risk factors for lupus-like conditions characterized by antinuclear autoimmunity. In search for a connection between these complement elements to antinuclear autoimmunity, we identified C1q recognition of the nucleolus late in apoptotic cells and we also found that this caused degradation of multiple nucleolar proteins including nucleolin and nucleophosmin-1. These C1 proteases also cleave high mobility protein box-1 (HMGB1), a well-known nuclear alarmin released by apoptotic cells which causes lupus-like conditions in mice. The cleavage inactivated HMGB1 in its ability to synergize with subclinical levels of endotoxin in the activation of monocytes, macrophages and dendritic cells. Apoptotic cells also release many autoantigens and some of these autoantigens are also cleaved by C1 proteases. In summary, our data stresses on novel substrate specificities of C1 proteases among intracellular proteins and help explain their deficiency being among the strongest risk factors for autoimmunity.

3795

Preclinical characterization of JAK1/JAK2 inhibitors for treatment of type 1 diabetes

Scott, N.A.^{1,2}, Trivedi, P.M.^{1,2}, Graham, K.L.^{1,2}, Fynch, S.¹, Jenkins, M.R.³, Kay, T.W.^{1,2}, Thomas, H.E.^{1,2}

¹St Vincent's Institute, Immunology and Diabetes Unit, Fitzroy, Australia, ²University of Melbourne, Department of Medicine, St Vincent's Hospital, Fitzroy, Australia, ³Peter MacCallum Cancer Centre, Cancer Immunology Research, East Melbourne, Australia

Type 1 diabetes is an organ specific autoimmune disease in which insulin-producing beta cells are destroyed by autoreactive T cells. Cytotoxic CD8⁺ T lymphocytes (CTL) and helper CD4⁺ T cells mediate beta cell destruction. Our aim is to inhibit the interaction between beta cells and CD8⁺ T cells and curb effector CD4⁺ T cell-dependent inflammation in the islets to prevent beta cell death. MHC class-I is upregulated on beta cells in

response to cytokines secreted by immune cells infiltrating into islets. Blocking cytokine signaling by overexpressing suppressor of cytokine signaling (SOCS)-1 in beta cells inhibits MHC class I upregulation and protects beta cells from CTL-mediated killing. We hypothesize that drugs that selectively inhibit JAK1 and JAK2 kinases downstream of cytokine signaling will have the same effect as SOCS-1 overexpression. JAK1/JAK2 inhibitors successfully blocked IFN γ -induced phosphorylation of STAT1 and IFN γ -induced MHC class-I upregulation in mouse and human islets. Mouse islets were protected from CTL-mediated killing in vitro by JAK1/JAK2 inhibitors. Time-lapse microscopy shows the inhibitor prevented the direct interaction between CTLs and beta cells, reducing calcium flux in the CTL and synapse duration. JAK1/JAK2 inhibitors blocked the effect of cytokines on beta cells in vivo by inhibiting MHC class I upregulation, and reduced infiltration of immune cells into islets in CD8 and CD4 mediated models of type 1 diabetes. Furthermore, JAK1/JAK2 inhibitors protected NOD mice from cyclophosphamide-induced diabetes. This preclinical study provides a platform for designing proof of concept clinical trials for blocking JAK/JAK2 signaling in human type 1 diabetes.

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CCDC134 suppresses the development of experimental autoimmune encephalomyelitis in C57BL/6 mice

Huang, J., Xia, P., Gong, X., Xiao, L., Yu, B., Qiu, X.

School of Basic Medical Sciences, Peking University Health Science Center, Department of Immunology, Beijing, China

CCDC134, a cytokine-like molecule served as a potential member of the γ_c cytokine family, was previously reported to have potent antitumor effects by augmenting CD8⁺ T cell-mediated immunity. However, whether CCDC134 is actively released in central nervous system (CNS) and involved in experimental autoimmune encephalomyelitis (EAE) remains unclear. In this study, we found that CCDC134 level in mouse sera was increased in the onset and peak stage of EAE. Moreover, we demonstrated that CCDC134 was efficacious in a model of EAE that mirrors chronic progressive multiple sclerosis. A short-term systemic treatment with recombinant CCDC134 reduced clinical severity and incidence of EAE. In addition, we used CCDC134-transgenic mice model and found that the onset and severity of EAE, the appearance of inflammatory infiltrates in spinal cord and the subsequent demyelination and axonal damage were significantly alleviated in CCDC134-transgenic mice compared with control mice. We further sought to elucidate the basis of CCDC134 protective effect on EAE by characterizing the T effector/regulatory responses. And the presence/activation of encephalitogenic Th17 cells and several inflammatory mediators in peripheral lymphoid organs and CNS were down-regulated in CCDC134-transgenic mice, while a remarkable upregulation of IL-10 and TGF- β in the spinal cord of CCDC134-transgenic EAE mice was detected. These findings indicate that CCDC134 plays a pivotal role in the pathogenesis of EAE and provide insights into the role of recombinant CCDC134 as a unique therapeutic agent for the treatment of autoimmune diseases.

1757

Molecular targets for inhibiting progression of myocarditis to inflammatory cardiomyopathy

Forsthuber, T.¹, Casabar, J.¹, Maldonado, D.¹, Forsthuber, I.¹, Al-Abed, Y.², Nalawade, S.¹

¹University of Texas at San Antonio, Biology, San Antonio, United States, ²Feinstein Institute for Medical Research, New York City, United States

Inflammatory dilated cardiomyopathy (DCMI) is a leading cause of heart failure and heart transplantation, yet there are currently no specific treatments available. Substantial evidence supports a key role for inflammation and chronic myocarditis in progression to DCMI and an autoimmune origin is supported by autoantibodies against heart tissue in myocarditis patients and induction of experimental autoimmune myocarditis (EAM) with cardiac autoantigens in animal models. Surprisingly, immunosuppressive treatments such as corticosteroid (CS) drugs are not very effective and cannot prevent DCMI. We hypothesized that macrophage migration inhibitory factor (MIF) may play a role in treatment resistance since MIF is induced by CS and counter-regulates CS-mediated immunosuppression. Here, we will present our results showing that MIF knockout mice treated with Dexamethasone (Dex) were resistant to EAM and did not progress to DCM. Furthermore, small molecule inhibitors of MIF combined with Dex were similarly effective in preventing DCMI in wild-type mice. Studies investigating the underlying mechanism implicated key chemokines and adhesion molecules in the progression of EAM to DCMI. Our results suggest that therapeutic inhibition of MIF in combination with CSs may be a novel treatment approach to prevent DCMI. Last, our studies may provide new insights into the mechanisms driving DCMI.

Poster Tuesday

15:30:00 - 16:30:00

Complement

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Complement receptor immunoglobulin (CRIg) and adaptive immunity: regulation of expression in human dendritic cells by cytokines

Small, A.^{1,2}, Munawara, U.^{1,3}, Quach, A.^{1,2}, Hii, C.^{1,2}, Abbott, C.³, Ferrante, A.^{1,2}

¹University of Adelaide, Adelaide, Australia, ²SA Pathology, Department of Immunology, Adelaide, Australia, ³Flinders University, Bedford Park, Australia

The B7 family-related protein, V-set and Ig domain (VSIg4)/Complement Receptor (CRIg) is expressed on dendritic cells (DC) and promotes T lymphocyte suppression. Thus CRIg is likely to be a control point in immune responsiveness through which cytokines exert their influence on adaptive immunity. However little is known on the interplay between cytokines which regulate immune responsiveness and expression of CRIg on DC. CRIg expression was examined at the mRNA (qRT-PCR) and protein level (western blot) in DC derived from human peripheral blood monocytes. Our data demonstrated that DC express three isoforms of CRIg. The cytokines modulated the expression of all three isoforms of CRIg in a similar manner. A key result was the finding that those cytokines (IL-10, TGF- β 1) and agents (dexamethasone, PKC inhibitors) upregulated CRIg expression also promote the development of tolerogenic DC and those which decrease expression (TNF, IFN- γ) break the tolerance function of DC. The results emphasise ways of modulating CRIg expression on DC to optimise their tolerogenic function and this may provide avenues for tackling diseases with an autoimmune origin.

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The role of complement in antibody-mediated immunity against malaria in pregnancy

Opi, D.H.^{1,2}, Boyle, M.¹, Reiling, L.¹, McLean, A.¹, Zhou, J.¹, Stanicic, D.^{3,4}, Baiwog, F.³, Rogerson, S.⁵, Jawarowski, A.^{1,6,7}, Fowkes, F.^{1,8}, Beeson, J.^{1,5,9}

¹The Burnet Institute of Medical Research and Public Health, Centre for Biomedical Research, Melbourne, Australia, ²Kenya Medical Research Institute-Wellcome Trust Research Programme, Kilifi, Kenya, ³Papua New Guinea Institute of Medical Research, Goroka, Papua New Guinea, ⁴Institute for Glycomics, Griffith University, Gold Coast, Australia, ⁵University of Melbourne, Department of Medicine, Melbourne, Australia, ⁶University of Melbourne, Department of Chemical and Biomolecular Engineering, Melbourne, Australia, ⁷Monash University, Department of Infectious Diseases, Melbourne, Australia, ⁸University of Melbourne, Centre for Epidemiology and Biostatistics, Melbourne, Australia, ⁹Monash University, Department of Microbiology, Clayton, Australia

Malaria in pregnancy is a leading cause globally of poor maternal health, and low birth weight and premature delivery of neonates leading to greatly increased mortality and morbidity. Malaria in pregnancy (MiP) is characterised by the accumulation of *Plasmodium falciparum*-infected red blood cells (pRBCs) in the placenta. Infection is less prevalent with successive pregnancies partly due to the acquisition of antibodies directed against parasite antigens that mediate pRBC sequestration. However, effector mechanisms of antibodies are incompletely understood. Antibody responses are predominantly IgG1 and IgG3 subtypes, supporting a potential role for complement, but this has not been defined. Using sera collected from a longitudinal cohort of 350 pregnant women from a malaria-endemic province in Papua New Guinea we have established that acquired antibodies among pregnant women promote the deposition of complement on the surface of placental-binding pRBCs through binding C1q, and activate complement leading to fixation of C3b. Interestingly, complement fixation does not lead to cell lysis or parasite killing. Instead, antibodies with complement inhibit binding of pRBCs to placental vascular receptors and enhance phagocytosis by monocytes, mechanisms likely to provide protection against MiP. Using genetically-engineered *P. falciparum*, we identified the parasite antigen PfEMP1 as the major target of complement-fixing antibodies. Analysis of longitudinal data among pregnant women supports a role for complement fixation by acquired antibodies in protection against placental infection and adverse pregnancy outcomes. These findings provide new insights into the mechanisms mediating immunity to malaria in pregnancy to advance vaccine development.

265

A sjogren's syndrome-like condition in Gln137Gly mutant RP S19 gene knocked-in C57BL/6J female mice

Nishiura, H., Yamanegi, K., Yamada, N., Nakasho, K.
Hyogo College of Medicine, Pathology, Nishinomiya, Japan

We have demonstrated that apoptotic cells express C5a receptor (C5aR) and produce its antagonist/agonist ligand cross-linked S19 ribosomal proteins (RP S19s) between Lys122 and Gln137 by tissue transglutaminases. We recently found that RP S19 polymer additionally interacts not only with an apoptosis inducing transcription factor delta lactoferine in apoptotic cells but also with an extracellular calcium inducer annexin A3 in macrophages. Therefore, we suggested that RP S19 polymer participates in the phagocytic clearance of apoptotic cells by macrophages via C5aR.

To validate the roles of RP S19 polymer, we prepared Gln137Glu mutant RP S19 gene knocked-in C57BL/6J mice. In comparison with wild-type female mice, in knocked-in female mice (1) numbers of CD4-/CD8- double minus cells were increased and vice versa those of CD4+/CD8+ double positive cells in thymus, (2) numbers of peripheral blood lymphocytes were decreased, (3) CD4+ single positive cell ratio to the CD8+ was increased in spleen, (4) B cells expressed an activation marker programmed cell death receptor-1 (PD-1) in spleen,

(5) anti-submandibular gland antibodies were present in plasma and
 (6) the PD-1 positive lymphocytes infiltrated into submandibular glands.

We suggested that the loss of C5aR-mediated phagocytic clearance of apoptotic cells by macrophages functions to initiate a Sjogren's syndrome-like condition in C57BL/6J female mice.

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Long-term effects of gestational chronodisruption on the complement system: a novel determinant of allergy in the adulthood?

Sarmiento, J., Carmona, P., Perez, B., Espinoza, G., Henriquez, C., Folch, H.

Universidad Austral de Chile, Valdivia, Chile

Substantial evidences strongly suggest that conditions during the intrauterine period of life play a critical role on the homeostasis of immune system, and the environment of mother is an important factor that influences the susceptibility to allergic disease in the adulthood. The allergic pathology is characterized by bad-adapted T helper cells (Th) drive immune response. Different data strongly suggest that complement system factors are expressed by Th cells and involved in the regulation of Th cells polarization. We have analyzed the effects of chronodisruption on the expression of complement system factors in a well characterized animal model of gestational chronodisruption in Sprague-Dawley rats. In this model, the exposure of pregnant dams to constant light during the second half of gestation suppressed the maternal melatonin rhythm, reduced intrauterine growth and impair the fetal adrenal function. By microarrays analysis, we found that several components of the complement system were significantly deregulated in the fetal liver; which was confirmed by quantitative PCR for the up-regulation of C1qbp and the down-regulation of C3 and C9. Notably, C3 and C9 transcripts remained significantly down-regulated in the adult offspring, which was accompanied by significantly decreased levels of plasma C3 protein. Because C3 is a key factor of complement system activation that could affect the overall efficiency of the pathway, and that play important roles in the homeostasis of Th cells polarization we postulated that downregulation of C3 by gestational chronodisruption as a new mechanism of bad-adapted pathological immune response in adulthood
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The complement C5a receptor, C5aR2 contributes to motor deficits in mouse models of Huntington's and Parkinson's disease

Li, R., Levin, S., Lee, J., Gordon, R., Woodruff, T.

The University of Queensland, School of Biomedical Sciences, Brisbane, Australia

Huntington's disease (HD) and Parkinson's disease (PD) are chronic neurodegenerative diseases characterised by progressive motor and cognitive dysfunction. Recent studies

have shown widespread up-regulation of innate immune complement factors by neurons and glia in the brains of HD and PD patients, which suggests that complement system may be a key contributor to disease progression of HD and PD. C5a is the most potent inflammatory effector of the complement system which functions by binding to its two receptors termed C5aR1 and C5aR2. The aim of this study was to determine the functional role of C5aR2 on motor functions in mouse models of HD and PD. We used an established model of HD, the R6/1 transgenic mouse, and a dopaminergic neurotoxin (6-OHDA) intra-striatally injected mouse model for PD. We bred the R6/1 transgenic mice with C5aR2 knockout (KO) mice and 6-OHDA injected C5aR2 KO to identify any potential protective or pathogenic function for C5aR2 in these disease models. Several behavioural tests were conducted including rota-rod, balance beam, grip strength and amphetamine-induced rotational tests. We found that 6-OHDA injected C5aR2 KO mice had better performances in both balance beam and amphetamine-induced rotational tests when compared to wild-type mice. Similarly, knocking out C5aR2 in R6/1 Huntington's mice resulted in improved motor function in rota-rod, balance beam and grip strength tests. These studies demonstrate a pathogenic role for C5aR2 in HD and PD, and identify a new potential therapeutic target to mitigate motor deficits in these diseases.

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Factor B and factor H dysregulation during dengue virus infection: *in vitro* and *in vivo* evidence

Cabezas, S.¹, Rudd, P.², Mahalingam, S.², Gordon, D.¹, Carr, J.M.¹

¹School of Medicine, Flinders University, Microbiology and Infectious Diseases, Adelaide, Australia, ²Griffith University, Emerging Viruses and Inflammation Group, Southport, Australia

Dengue virus (DV) is responsible for one of the most important viral diseases in terms of geographical distribution and human morbidity. The pathogenesis of severe DV is not fully understood; however hyper-activity of the complement alternative pathway (AP) has been associated with severe disease. Our study aimed to determine the mechanisms underlying the hyper-activity of the complement AP during DV infection.

Factor B (FB) and factor H (FH) are the main regulatory factors of the AP, with FB an activator and FH a negative regulator, keeping the AP activity tightly controlled. Using Real-Time RT-PCR we quantitated FH and FB mRNA in cell lines (mouse embryonic fibroblasts [MEF], primary human umbilical vein endothelial cells [HUVEC], human monocyte-derived macrophages [MDM]) and in the AG129 mouse infected with DV. Our results show that both FB and FH mRNA levels significantly increase during DV infection, in both mouse and human cell infection models. Moreover, incubation of HUVEC with a neutralizing IFN- β antibody blocked DV-induction of FH and FB suggesting an IFN-dependent mechanism. In contrast, induction of FH and FB mRNA was observed in AG129-DV-infected mouse liver, but not kidney during the viremic phase of infection. Since the AG129 mice are IFN-receptor deficient, this implies IFN-independent induction of FH and FB also occurs in response to DV infection. Together these results support the notion that the AP is dysregulated during DV infection which may be associated with

complement-mediated effects on the vasculature and could drive development of the severe forms of DV disease.

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The lysosomal cysteine protease legumain is required for normal Th1 induction in vitro

Freeley, S.¹, Cardone, J.¹, Gunther, S.², Reinheckel, T.², Watts, C.³, Kemper, C.¹

¹King's College London, Department of Transplant Immunology and Mucosal Biology, London, United Kingdom, ²University of Freiburg Medical Center, Institute for Molecular Medicine and Cell Research, Freiburg, Germany, ³University of Dundee, Division of Cell Signalling & Immunology, Dundee, United Kingdom

Introduction: Complement is now emerging as a critical checkpoint regulator in Th1 biology: intracellular cleavage of complement C3 into C3a and C3b by cathepsin L (CTSL) drives the autocrine activation of the C3aR and the C3b receptor CD46 on human CD4+ T cells, which is critically required for IFN γ induction upon TCR activation. Importantly, increased intracellular processing of C3 by CTSL contributes to Th1 hyperactivity in rheumatoid arthritis and can be targeted pharmacologically by CTSL inhibition. CTSL may therefore be a potential therapeutic target in autoimmune disease.

Objectives: We noticed that the lysosomal asparagine endopeptidase Legumain (AEP) is also expressed in Th1 cells. As AEP can drive proteolytic maturation/functional modulation of cathepsins B, H and L in myeloid cells and is connected with dysregulation of cellular responses, we assessed for a potential role of AEP in Th1 responses.

Methods: Purified CD4+ T cells were activated in the presence or absence of a specific Legumain inhibitor (MV026630) and assessed for intracellular CTSL maturation and C3 activation as well cell survival/proliferation and Th1 induction.

Results: Legumain inhibition caused changes in CTSL localisation and a significant and 'dose-dependent' reduction in intracellular C3a generation ($P=0.0276$) and reduced the production of both IFN γ ($P=0.0004$) and IL-17 ($P=0.0007$) without affecting cell viability or IL-4 production.

Conclusion: These data suggest that upstream regulation of CTSL activity by Legumain may control intracellular C3a generation and normal human Th1 induction. We are currently assessing the biological significance of this novel Legumain/CTSL/C3 axis in appropriate animal models.

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Role of factor H and effects of C-terminal mutations on control of human platelet/granulocyte aggregate formation

Blatt, A.¹, Saggu, G.¹, Cortes, C.², Herbert, A.³, Kavanagh, D.⁴, Ricklin, D.⁵, Lambris, J.⁵, Valenzuela, J.⁶, Ferreira, V.¹

¹University of Toledo College of Medicine, Department of Medical Microbiology and Immunology, Toledo, United States, ²Oakland University William Beaumont School of Medicine, Department of Biomedical Sciences, Rochester, United States, ³University of Edinburgh School of Chemistry, Edinburgh, United Kingdom, ⁴Newcastle University, Institute of Genetic Medicine, Newcastle upon Tyne, United Kingdom, ⁵Perelman School of Medicine, University of Pennsylvania, Department of Pathology and

Laboratory Medicine, Philadelphia, United States, ⁶National Institute of Allergy and Infectious Diseases, NIH, Vector Molecular Biology Section, Rockville, United States

Atypical hemolytic uremic syndrome (aHUS) is characterized by renal damage and thrombosis, as a result of dysregulated complement alternative pathway (AP) activity on cell surfaces. Platelets activated during thrombosis can bind to granulocytes to form stable platelet/granulocyte aggregates (PGA), which can enhance thromboinflammation in the vasculature. Mutations in the C-terminal domains, 19 and 20, of the AP negative regulator factor H are a common cause of aHUS. We have recently shown that domains 19-20 are also critical for control of PGA formation in human whole blood stimulated with thrombin receptor-activating peptide (TRAP), yet there are no known associations between aHUS and PGA formation. Here, we determined that (a) domains 19-20, were the most critical regions of factor H for controlling the AP on human platelets and neutrophils, and in TRAP-stimulated human whole blood,

(b) factor H limits PGA formation primarily by preventing AP-mediated C5a generation, (c) aHUS-related mutations in domains 19-20 have differential effects on control of TRAP-mediated PGA formation and AP activity, as measured by C3b deposition and C5a generation, and

(d) the effects of mutations in 19-20 have similar differential effects on AP activity on isolated platelets and neutrophils.

Our results indicate a key role for factor H C-terminal domains in controlling AP activity on cells involved in PGA formation, and suggest that mutations associated with aHUS result in varying degrees of impairment of complement regulation on cell surfaces. These data have important implications for understanding pathophysiologic mechanisms of PGA formation and potential treatment options for aHUS.

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Pathogenic role for C5aR1 in chronic inflammation and renal fibrosis in a murine model of chronic pyelonephritis

Zhou, W.¹, Choudhry, N.¹, Li, K.², Zhang, T.², Wu, K.-Y.², Song, Y.², Farrar, C.A.¹, Wang, N.², Liu, C.-F.², Peng, Q.¹, Wu, W.¹, Sacks, S.H.¹

¹King's College London, Department of Innate Immunity, Medical Research Council (MRC) Centre for Transplantation, London, United Kingdom, ²Xi'an Jiaotong University, Core Research Laboratory, the Second Affiliated Hospital, School of Medicine, Xi'an, China

C5a/C5aR1 interaction participates in the pathogenesis of a number of inflammatory diseases, including acute kidney injury. However, its role in chronic inflammation, particularly in pathogen-associated disorders, is largely unknown. Here we show that development of chronic inflammation and renal fibrosis is dependent on C5aR1 in a murine model of chronic pyelonephritis, induced by human uropathogenic *Escherichia coli* (UPEC) strain IH11128. C5aR1-deficient (*C5aR1*^{-/-}) mice showed a significant reduction in bacterial load, tubule injury and tubulointerstitial fibrosis in the kidneys following the infection, compared with C5aR1-sufficient mice. This was associated with: i) reduced leukocyte infiltration of the kidney,

specifically for the population of Ly6C^{hi} pro-inflammatory monocytes/macrophages (MO/MΦ); ii) reduced intrarenal gene expression of key pro-inflammatory factors (e.g. TNF- α , KC, MCP-1) and pro-fibrogenic factors (e.g. TGF- β , PDGF) in C5aR1^{-/-} mice following infection. Antagonizing C5aR1 reduced renal bacterial load, tissue inflammation and tubulointerstitial fibrosis. *Ex vivo* and *in vitro* studies showed that under infection conditions, C5a/C5aR1 interaction up-regulated the production of pro-inflammatory and pro-fibrogenic factors by renal tubular epithelial cells (RTEC) and MO/MΦ, whereas the phagocytic function of MO/MΦ was down-regulated. Our data demonstrate a pathogenic role for C5aR1 in chronic inflammation and renal fibrosis in an experimental model of chronic pyelonephritis. The observations suggest that C5a/C5aR1-mediated up-regulation of local inflammatory responses to UPEC and impairment of phagocytic function of phagocytes contribute to persistent bacterial colonisation of the kidney, chronic renal inflammation and subsequent tubulointerstitial fibrosis. C5aR1 may be a new therapeutic target for this disorder.

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Transfusion-related immunomodulation: Importance of cell-to-cell interactions in studying underlying mechanisms

Perros, A.^{1,2,3}, Flower, R.^{1,3}, Christensen, A.-M.^{1,3}, Dean, M.^{1,3}

¹Australian Red Cross Blood Service, Research and Development, Brisbane, Australia, ²University of Queensland, School of Medicine, Brisbane, Australia, ³Queensland University of Technology, Faculty of Health, School of Biomedical Sciences, Brisbane, Australia

Background and aims: Immune-modulation and adverse patient outcomes associated with transfusion are collectively referred to as transfusion-related immunomodulation (TRIM). Platelet concentrates (PC) are stored for 5 or 7 days, depending on the jurisdiction. During storage, platelets undergo significant biophysical changes and release soluble mediators (sCD40L, RANTES, IL-8). Laboratory models are a valuable tool to define mechanisms associated with TRIM. This study investigated the modulation of monocyte inflammatory responses by PC in an in-vitro model.

Methods: "Recipient" whole blood was cultured with PC-supernatants (PC-SN) stored for 2, 5, or 7 days at doses representing a 1, or 2-3 unit PC-transfusion. Lipopolysaccharide (LPS) was added in parallel wells to model TRIM during infection. Inflammatory responses were measured using flow cytometry. Responses from the whole blood model were also compared with responses from isolated monocyte cultures.

Results: Exposure to PC-SN alone had no impact on monocyte inflammatory responses in the whole blood model however, in the presence of LPS, monocyte production of MIP-1 β , IL-12, IL-10 and IL-6 were significantly suppressed. Dose, more than storage was associated with immunomodulation. Of note, in the isolated monocyte model, effects of PC-SN were less apparent with only IL-1 α and MIP-1 β modulated.

Conclusions: These findings suggest that the transfusion recipient's underlying condition and the number of PC transfused both contribute to a modulation of the monocyte inflammatory profile that may precipitate development of TRIM.

Further, this study highlights the importance of cell-to-cell interactions and demonstrates the limitation of using isolated cell models in the context of transfusion outcomes.

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Serum lipid and cytokines profile in the initial and advanced insulin resistance

Marcelino-Rodríguez, I.^{1,2}, Alemán-Sánchez, J.J.³, Almeida-González, D.³, Gannar, F.^{3,4}, Brito Díaz, B.³, Rodríguez-Pérez, M.D.C.³, Cabrera de León, A.^{3,5}

¹La Laguna, Santa Cruz de Tenerife, Spain, ²University Hospital NS La Candelaria, Santa Cruz de Tenerife, Spain, ³University Hospital NS La Candelaria, Research Unit, Santa Cruz de Tenerife, Spain, ⁴Carthage University, Bizerte, Tunisia, ⁵University of La Laguna, Preventive Medicine, La Laguna, Spain

Objective: To describe the serum lipid and cytokine profile of the non-diabetic general population that suffers early insulin resistance (IR).

Methods: Cross-sectional study of 5943 non-diabetic adults from the general population. They were stratified into: group 1 (*non-IR*: C-peptide < third tertile and glycaemia < 100 mg/dL), group 2 (*early IR*: C-peptide \geq third tertile but glycaemia < 100 mg/dL), and group 3 (*advanced IR*: glycaemia \geq 100 mg/dL). Bivariate, ordinal regression and multinomial regression analysis were performed.

Results: At least 20% of the non-diabetic general adult population was part of the early IR group. They presented significant bivariate differences with the non-IR group in the serum concentration of insulin (p < 0.001), triglycerides (p < 0.001), HDL-cholesterol (p < 0.001), LDL-cholesterol (p < 0.001), non HDL-cholesterol (p < 0.001), sCD40L (p < 0.001), CRP (p < 0.004), leptin (p < 0.001) and adiponectin (p < 0.001). These associations were corroborated in the ordinal and multinomial multivariate regression models for all biomarkers but sCD40L. The *advanced IR* group presented the same biomarkers significant differences with *non-IR* group and, in addition, it also showed a significant difference for the resistin serum concentration (p < 0.001).

Conclusion: Early IR is not detected in the clinical practice because this population group shows normal glycaemia. However it presents a hazardous serum lipid and cytokine profile.

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Serum resistin and physical activity in the general population

Marcelino-Rodríguez, I.^{1,2}, Almeida-González, D.¹, Gannar, F.^{1,3}, Rodríguez-Pérez, M.D.C.¹, Brito Díaz, B.⁴, Alemán-Sánchez, J.J.⁴, Cabrera de León, A.^{4,5}

¹University Hospital NS La Candelaria, Research Unit, Santa Cruz de Tenerife, Spain, ²University of La Laguna, La Laguna, Spain, ³Carthage University, Bizerte, Tunisia, ⁴University Hospital NS La Candelaria, Santa Cruz de Tenerife, Spain, ⁵University of La Laguna, El Sauzal, Spain

Objective: Resistin is mainly a cytokine in human being. It's been related with inflammation and ischaemic heart disease. Physical activity (PA) improves the obesity-related chronic inflammation, and prevents ischaemic cardiopathy. We explored the relationship between serum resistin and PA in general population.

Methods: Cross-sectional study of 6636 adults, randomly recruited from the general population. In addition to serum resistin and PA (in MET; 1 MET = 4,184 KJ/Kg*h), we measured: age, gender, smoking, alcohol consumption and HDL-cholesterol (HDL-c). The bivariate associations of resistin and HDL-c with PA, were adjusted for age, alcohol and smoking in multivariate regression models.

Results: Serum resistin concentration was higher in women (6,06±2,41 ng/mL) than men (5,63±2,18 ng/mL). Resistin was inversely correlated with HDL-c ($p < 0.001$). Most of PA measures were inversely correlated with resistin, but with some differences between men and woman: Time of PA ($p < 0.001$), MET spent in leisure time ($p < 0.001$), moderate leisure PA ($p < 0.001$), etc. On the contrary, most of PA measures were directly correlated with HDL-c, also with some differences between men and woman: Daily total MET ($p < 0.001$), daily active MET ($p < 0.001$), and moderate leisure PA ($p < 0.01$), etc. Men (OR=0.78 [$p < 0.05$]) and women (OR=0.75 [$p < 0.01$]) in the highest quintile of PA time, showed a decreased risk of elevated serum resistin.

Conclusion: In the general population, resistin is inversely associated to PA and HDL-c. Resistin shows a stronger association with PA than HDL-c, and it may be a good biomarker of PA.

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Aberrant TDP-43 expression is related to obesity

Park, A., Lee, S., Lee, T.A., Park, B.

Yonsei University, Seoul, Korea, Republic of

Tat-activating regulatory DNA-binding protein-43 (TDP-43) is involved in several major neurodegenerative diseases. Cellular TDP-43 expression is tightly controlled through a negative feedback loop involving its mRNA processing. Recently, We reported that TDP-43-containing sub-nuclear bodies are specific cytokine-splicing compartments important for efficient RNA production during activation and modulation of the immune response. Here, We demonstrate that TDP-43 expression is highly elevated in the liver and adipose tissue in concert with increased IL-6 levels in high-fat diet-fed mice. IL-6 expression and RNA processing are dependent on the expression of TDP-43 in pre-adipose and adipose cells. Our findings provide valuable insight into the molecular pathology of obesity and may contribute to the development of improved therapies for treating obesity-related diseases.

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Vorinostat ameliorates repeated corticosterone-induced depressive-like behavior in mice: Implication of stress paradigm on neuroinflammation

Athira, K.V.¹, Rajaram, M.M.¹, Mangala, L.^{1,2}, Swapnil, S.³

¹National Institute of Pharmaceutical Education and Research (NIPER)-Guwahati, Department of Pharmacology and Toxicology,

Guwahati, India, ²Gauhati Medical College, Department of Pharmacology, Guwahati, India, ³National Institute of Pharmaceutical Education and Research (NIPER)-Guwahati, DST WOS-A Scientist, Department of Biotechnology, Guwahati, India

Depression is a chronic illness projected to be the most debilitating to society by 2030. Exogenous corticosterone (CORT) administration in rodents mimics the chronic stress associated hypothalamic-pituitary-adrenal (HPA) axis dysregulation, a well-established feature found in depressive patients. Our study aimed to examine the antidepressant potential and the possible mechanism of vorinostat, a histone deacetylase inhibitor against repeated corticosterone-induced mice model of depression. Depressive-like state was induced in male Swiss albino mice, aged 6-7 weeks, weighing 20-25 g by daily injections of CORT (40 mg/kg; subcutaneous) for 21 days. Vorinostat (25 mg/kg; intraperitoneal) and fluoxetine (15 mg/kg; oral) were administered 30 min prior to the CORT injection from day 8 to 21. After 21 days, neurobehavioral tests were conducted; followed by estimation of molecular parameters in serum and hippocampus through ELISA, biochemical assays and RTPCR analysis. CORT injected mice exhibited depressive-like behavior, as indicated by the significant decrease in sucrose consumption and increase in immobility time in the forced swim test and tail suspension test. Depressive-mice showed concomitant significant increase in the levels of serum CORT, ACTH, pro-inflammatory cytokines (IL-1 β , IL-6, TNF- α), hippocampal malondialdehyde, nitric oxide, mRNA expression of GRP-78, NF κ B and C-reactive protein along with significant decrease in hippocampal BDNF as compared to normal mice. Vorinostat treatment, in a similar manner to the classical antidepressant fluoxetine, significantly ameliorated all the behavioral and molecular changes induced by corticosterone. Thus vorinostat mitigated depression via amelioration of the hippocampal neuroinflammation inflicted by the oxidative-nitrosative-endoplasmic reticulum stress associated with hypercortisolemia, thereby promoting neurogenesis.

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Hypoxia mediated changes in human mast cell profile of genetic expression

Walczak-Drzewiecka, A., Sałkowska, A., Ratajewski, M., Dastych, J.
Institute of Medical Biology Polish Academy of Sciences, Lodz, Poland

We investigated the effect of prolonged hypoxia on profile of genetic expression of human mast cells using de novo sequencing of RNA. Culture of LAD2 mast cells were maintained for 5 days under standard conditions or under hypoxic conditions (1% O₂). Cells were harvested and lysed to obtain RNA that was next used for de novo sequencing of transcripts. Numbers of copies of each detected gene transcript were analyzed and genes showing statistically significant differences in expression under hypoxic conditions were identified.

We have observed changes in expression of 179 among analyzed 23 616 genes. Hypoxia upregulated expression of multiple genes, which products are involved in process of cell adhesion. Upregulated genes involved gene coding for proinflammatory

peptide adrenomedullin known to increase cell adhesion, genes regulating focal adhesion (TNS1) and formation of tight intercellular junctions (JAM3), several genes (ITGA3, ITGA5, ITGA7, ITGAL) coding for subunits of integrins adhesion receptors mediating mast cell adhesion to extracellular matrix and to other immune cells including lymphocytes.

Interestingly hypoxia also upregulated cytokine genes supporting Th17 differentiation (IL18 and TGF β) and receptors responding to IL17 family of cytokines (IL17RA, IL17RB). Finally, hypoxia has increased expression of genes coding for elements of epigenetic machinery such as HDAC5 and MBD1.

Pattern of changes in human mast cell gene expression suggest upregulation of mast cell adhesiveness and secretion of cytokines that might stimulate direct interaction with Th17 lymphocytes. Hypoxia may lead to prolonged changes in mast cell phenotypes as it regulated expression of genes engaged in epigenetic processes.

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Inhibition of autophagy reduces irradiation-induced inflammation in cerebellum in the immature mouse brain

Zhu, C.^{1,2}, Wang, Y.^{1,3}, Xie, C.¹, Sun, Y.^{1,2}, Xu, Y.^{1,2}, Li, T.^{1,3}, Zhang, Y.^{1,3}

¹Gothenburg University, Gothenburg, Sweden, ²Zhengzhou University, Zhengzhou, China, ³Zhengzhou Children's Hospital, Zhengzhou, China

Objective: To investigate the effects of autophagy inhibition on irradiation-induced cell death and inflammation in the cerebellum of immature mouse brain.

Methods: Ten-day-old Atg7 neuronal knockout and wild type littermate pups were subjected to a single 6 Gy dose of whole-brain irradiation. The animals were euthanized at 6 hours or 5 days after irradiation. Neuronal cell death-related markers were evaluated at 6 h, and brain injury was evaluated at 5 days, inflammation was evaluated at both 6h and 5 days after irradiation.

Results: Cell death was evaluated at 6 h after irradiation in the cerebellum by hematoxylin and eosin staining. There was a greater number of pyknotic cells in the wild-type mice compared to the Atg7 knockout mice ($p < 0.05$). Caspase-3 activity was measured in the cerebellum at 6 h after irradiation, and the level of activity was lower in the Atg7 knockout mice compared to their wild-type litter mates ($p = 0.038$). The volumes of cerebellum were reduced at 5 days after irradiation compared to the non-irradiation controls. However, there was no difference between Atg7 knockout and wild-type mice in either irradiated or non-irradiated mice. IL-6 and KC were increased significantly in wild type mice but not Atg7 knockout mice at 6h after irradiation. There was no significant difference at 5 days after irradiation.

Conclusion: The inhibition of autophagy prevents irradiation-induced proliferating cell death and inflammation in cerebellum, and autophagy could be considered as a potential target for preventing irradiation-induced cell death and inflammation.

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Toll-like receptor 2 promotes vascular calcification via the chondrogenic transdifferentiation of vascular smooth muscle cells

Lee, G.-L., Kuo, C.-C.

National Health Research Institutes, Miaoli County, Taiwan, Republic of China

Objective: vascular smooth muscle cell (VSMC) transformation to osteoblast/ chondrocyte Phenotype is an initiative event for arterial calcification, which is highly correlated with cardiovascular disease morbidity and mortality. Toll-like receptors (TLRs) play key roles in the development of vascular diseases, but their regulation in vascular calcification remains unclear.

Methods and results: Calcification assays by Alizarin red S staining revealed that TLR2 agonists promoted VSMC calcification. TLR2 deficiency or inhibition of TLR2 signaling with anti-TLR2 antibody suppressed TLR2 agonist-induced VSMC calcification and IL-6 production. Neutralizing anti-IL-6 antibodies impaired TLR2-mediated VSMC calcification, while addition of IL-6 recombinant protein promoted VSMC calcification. Additionally, ApoE^{-/-} mice fed high-fat diet (HFD) exhibited vascular calcium accumulation and serum IL-6 elevation, which were ameliorated in Tlr2^{-/-}ApoE^{-/-} mice. Further studies demonstrated that TLR2 agonist time-dependently increased chondrogenic transdifferentiation transcription factors SOX9 and osterix but suppressed osteo/ chondrogenic transdifferentiation transcription factor Runx2 and osteoprotegerin (OPG) in wild-type but not in Tlr2^{-/-} VSMCs. Furthermore, TLR2-induced transdifferentiation of wild-type VSMCs into chondrogenic cells was suppressed by both of neutralizing anti-IL6 antibodies and OPG recombinant protein.

Conclusions: Our results suggest that upon ligand binding, TLR2 promotes chondrogenic transdifferentiation of VSMC via modulating expression of IL-6 and OPG production, SOX9 and osterix. The chondrogenic VSMCs in turn contributes to vascular calcification during the pathogenesis of atherosclerosis.

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Lfc attenuates tumorigenesis during colitis-associated cancer

Lin, C.¹, Chang, H.W.², Tsai, P.S.², Reinecker, H.C.³, Chiang, H.S.¹

¹National Taiwan University, Life Science, Taipei, Taiwan, Republic of China, ²National Taiwan University, School of Veterinary Medicine, Taipei, Taiwan, Republic of China, ³Gastrointestinal Unit and Center for the Study of Inflammatory Bowel Disease, Massachusetts General Hospital, Harvard Medical School, Boston, United States

Colitis-associated cancer (CAC) is a colorectal cancer subtype that is associated with inflammatory bowel disease. The recruitment and activation of immune cells enhance the production of proinflammatory cytokines and play a major role in CAC. Lfc, a guanine nucleotide exchange factor that promotes the activation of Rho GTPase by exchanging of GDP for GTP and mediates NF κ B-dependent induction of proinflammatory cytokines. Although the role of Lfc in expression of

proinflammatory cytokine is well studied, its role in the development of CAC has not been characterized. To evaluate the role of Lfc in the initiation and progression of CAC, *Lfc*^{-/-} mice were subjected to the azoxymethane/dextran sodium sulfate (AOM/DSS) recurring inflammation-driven CAC model. Lfc-deficient mice demonstrated significant weight loss compared with the wild-type animals. Surprisingly, Lfc deficiency led to enhanced expression of *Il6*, *Il10*, *Il1b* and *Tnfa* in the colons of *Lfc*^{-/-} mice. Colon histopathology also showed greatly increased crypt hyperplasia in Lfc-deficient mice compared to wild-type controls. These findings suggest Lfc functions as an attenuator of CAC through modulating proinflammatory cytokine levels in the colon.

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Proinflammatory cytokines induced by gastric fluid aspiration in GERD (Gastro Esophageal Reflux Disease) and pulmonary fibroblast differentiation

Su, H.-H.¹, Chiang, C.-C.², Shih, W.-A.², Cheng, C.-M.²

¹*Kaohsiung Medical University, Graduate Institute of Medicine, College of Medicine, Kaohsiung, Taiwan, Republic of China,*

²*Kaohsiung Medical University, Department of Biomedical Science and Environmental Biology, Kaohsiung, Taiwan, Republic of China*

GERD (gastro esophageal reflux disease) is a reflux disease that gastric content regurgitate into esophagus. Epidemic studies indicated that micro aspiration of gastric fluid in GERD patients is associated with the prevalence of chronic respiratory diseases. Our previous studies have demonstrated that gastric fluid acts as an inflammatory mediator in macrophages and airway smooth muscle cells. We further hypothesized that, in the context of pulmonary residential cells, micro aspiration of gastric fluid initiates inflammatory microenvironment and lead to subsequent fibrotic progression. Gastric fluid treated conditioned mediums collected from NL-20 and Raw264.7 were used for the assessment of factors involved inflammatory mediators and fibroblast differentiation. The pulmonary fibroblast cells (HFL-1) was used to evaluate the fibrotic progression induced by gastric fluid. Our data shows gastric fluid significantly induced eotaxin, IGFBP-4 and IL-6 expression in pulmonary epithelial cells by cytokine array analysis. Similarly, induction of GCSF, GM-CSF, IL-6, and MCP-1 by gastric fluid was observed in macrophages treated with gastric fluid. Both conditioned mediums from NL-20 and Raw264.7 induced cell migration and α -SMA protein expression as well as actin polymerization in pulmonary fibroblast cells. By using RT-PCR and ELISA assay, we observed that IL-6, IL-8 and transforming growth factor-beta (TGF- β) were enhanced in pulmonary fibroblast cells by gastric fluid and conditioned medium treatment. In summary, our current data indicated that, the poor mixture of proinflammatory mediators induced by chronic gastric fluid aspiration in the venue of pulmonary context may cause the pulmonary fibroblast cell differentiation and is causal for subsequent pulmonary fibrosis.

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The possible mechanism by which MicroRNA-122 controls inflammation in mice

Leu, C.-M.¹, Wei, C.-W.¹, Su, Y.-R.¹, Hsu, K.-H.¹, Chou, T.¹, Yang, F.-C.¹, Tsou, A.-P.^{2,3}, Chen, N.-J.¹, Hsu, C.-L.¹

¹*National Yang-Ming University, Institute of Microbiology and Immunology, Taipei, Taiwan, Republic of China,* ²*National Yang-Ming University, Department of Biotechnology and Laboratory Science in Medicine, Taipei, Taiwan, Republic of China,* ³*National Yang-Ming University, VYM Genome Research Center, Taipei, Taiwan, Republic of China*

MicroRNA-122 (miR-122) is the most abundant miRNA in the liver. An increase in Kupffer cells, neutrophils, IL-6, and TNF- α in the livers of *Mir122a* knockout mice indicates that miR-122 possesses an anti-inflammatory function; however, the mechanism was unclear. Here, we report moderate splenomegaly and augmentation of myeloid cells in the spleens of *Mir122a*^{-/-} mice. Intriguingly, serum IL-6 was significantly increased after LPS injection in the *Mir122a*^{-/-} mice. A low level of miR-122a was detected in peritoneal macrophages, indicating the potential of miR-122 to modulate macrophage activity. *In vitro* assay demonstrated that LPS-induced IL-6 secretion was concomitantly higher in the *Mir122a*^{-/-} macrophages. We further discovered several proteins participating in the activation of IL-6 production as miR-122a targets using a reporter assay. The expression of these miR-122a targets were significantly increased in the *Mir122a*^{-/-} macrophages. Taken together, our findings imply that miR-122 may control signal pathway regulating IL-6 production by macrophages. Because myeloid cells is critical for the development of hepatocellular carcinoma, we speculate that miR-122 may inhibit tumor formation at least in part by reducing inflammation.

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Salivary expression of dysregulated immune/inflammatory genes provides a useful set of biomarkers in detection of oral cavity squamous cell carcinoma

Kiss, C.¹, Horváth, J.², Lábicsák, P.³, Tar, I.⁴, Szabó, A.⁵, Csósz, É.³, Scholtz, B.², Márton, I.J.⁶

¹*University of Debrecen, Department of Pediatrics, Debrecen, Hungary,* ²*University of Debrecen, Department of Biochemistry and Molecular Biology, Genomic Medicine and Bioinformatic Core Facility, Debrecen, Hungary,* ³*University of Debrecen, Department of Biochemistry and Molecular Biology, Debrecen, Hungary,* ⁴*University of Debrecen, Department of Periodontics, Debrecen, Hungary,* ⁵*University of Debrecen, Department of Maxillofacial Surgery, Debrecen, Hungary,* ⁶*University of Debrecen, Department of Restorative Dentistry, Debrecen, Hungary*

Background: Detection of reliable and sensitive salivary biomarkers in oral cavity squamous cell carcinoma (OSCC) may improve clinical outcome. RNA and protein samples transcribed and translated from immune/inflammatory genes were repeatedly detected in salivary samples of these patients. We evaluated the performance of mRNA and protein samples expressed in saliva of OSCC patients from Hungary, a country characterized by an embarrassing fourfold elevation of OSCC-

related mortality between 1970 and 2000.

Methods: Unstimulated whole saliva was obtained from 31 patients with newly diagnosed OSCC, 30 age- and gender-matched subjects and 29 young healthy subjects. Expression of putative mRNA biomarkers (DUSP1, OAZ1, H3F3A, IL1B, IL6, IL8, SAT, S100P and TNF α) and 8 normalizing genes was quantified using real-time quantitative PCR. Concentrations of IL-1 α , IL-1 β , IL-6, IL-8, TNF- α and VEGF were determined by a Luminex-based multiplex kit. To detect catalase, profilin-1, S100A9/MRP14, CD59, galectin-3-binding protein/M2BP, CD44 and keratin-19, selected reaction monitoring-based targeted proteomic method was developed.

Results: IL6 mRNA was consistently expressed in OSCC patients but not in control subjects. The best mRNA biomarker combination (DUSP1/IL6/IL8/OAZ1/H3F3A) distinguished OSCC vs. young control patients, but not vs. age-matched control patients. S100-A9, thioredoxin, IL-6 and TNF- α molecules proved to be useful protein OSCC biomarkers in the Hungarian population.

Conclusion: We developed a noninvasive method of detecting OSCC biomarkers in order to improve clinical outcome. Useful biomarker combinations included products of dysregulated immune/inflammatory genes. Differences between our results and previous analyses suggest that immune/inflammatory processes in the oral cavity may affect the performance of putative salivary biomarkers.

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Functional interactions of IL6 and *CHRFAM7A* genes in schizophrenia pathogenesis

Venugopal, D.^{1,2}, Agrawal, R.², Kalmady, S.², Venkataram, S.², Subbanna, M.^{1,2}, Rajasekaran, A.^{1,2}, Amaresha, A.C.², Narayanaswamy, J.C.², Debnath, M.¹, Ganesan, V.²

¹National Institute of Mental Health and Neuro Sciences, Department of Human Genetics, Bangalore, India, ²National Institute of Mental Health and Neuro Sciences, Translational Psychiatry Laboratory, Department of Psychiatry, Bangalore, India

Background: CHRNA7 gene coding for $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR) and its partially duplicated chimeric gene CHRFAM7A have been implicated in schizophrenia. Anti-inflammatory cholinergic pathways mediated by these genes play crucial role in mitigating the effects of inflammation acted upon by cytokine such as interleukin-6 (IL6). Disruption of such regulatory feedback mechanism might be involved in immune-mediated pathogenesis of schizophrenia. However, the interaction between these two pathways at the level of gene expression (i.e. IL6 & CHRFAM7A) is yet to be evaluated.

Aim: To quantify the gene expression of IL-6 and CHRFAM7A for assessing their potential functional interactions in schizophrenia patients.

Methods: Antipsychotic-naïve schizophrenia patients (DSM-IV) [N=13] were examined in this study in comparison with healthy controls [N=29]. RNA was extracted from peripheral blood lymphocytes. IL6 and CHRFAM7A gene expression was quantified by reverse transcription-PCR using TaqMan gene expression assay.

Results: There was a significant positive correlation between IL6

and CHRFAM7A gene expression in healthy controls ($r = 0.52$; $p = 0.004$) but not in patients ($r = -0.05$; $p = 0.883$).

Conclusion: Our preliminary observations indicate possible functional interactions between IL6 & CHRFAM7A signalling pathways. Lack of this correlation in schizophrenia patients might have pathogenic significance. This needs replication and further extension in large samples.

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Modulation of immune system by Korean red ginseng via potentiation of cell survival and inhibition of inflammation in pneumococcal infection

Lee, S.Y., Pyo, S., Rhee, D.-K.

Sungkyunkwan University, Pharmacy, Su-Won, Korea, Republic of

More than 50% of sepsis cases are caused by *Streptococcus pneumoniae* (pneumococcus), and hospital mortality related to sepsis comprises 52% of all hospital deaths. Therefore, sepsis is a medical emergency, and any treatment against the agent that produces it, is welcome. Here, the protective effect of Korean red ginseng (KRG) extract against pneumococcal infection and sepsis was elucidated. KRG-pre-treated mice (100 mg/kg of KRG) had significantly higher survival rates and body weights than those of the non-treated controls; KRG-pre-treated mice had lower bacterial number and morbidity than those of the non-treated controls. 100 mg/kg of KRG administration decreased cytokine levels including TNF- α and IL-1 β , nitric oxide level, and neutrophil infiltration 48 h post-infection, *in vivo*. In pneumococcal infection, KRG pre-treatment downregulated TLR4 and TNF- expressions in RAW 264.7 macrophage cells and increased cell survival by activating PI3K/AKT signaling. Taken together, 100 mg/kg of KRG appeared to protect host cells from lethal pneumococcal sepsis by inhibiting inflammation as well as by enhancing bacterial clearance thereby reinforcing cell survival against pneumococcal infection.

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Characterising the effects of interferon lambda on dendritic cells

Pang, E.S.¹, Macri, C.¹, Pooley, J.¹, Fancke, B.¹, Lundie, R.¹, Hochrein, H.², Radford, K.³, O'Keefe, M.¹

¹Biomedicine Discovery Institute, Monash University, Clayton, Australia, ²Bavarian Nordic GmbH, Research Immunology, Martinsried, Germany, ³Mater Research Institute-University of Queensland, Brisbane, Australia

Interferon lambda (IFN- λ) is the most recent addition to the class II cytokine family. It has roles in antiviral and anti-tumour responses, as well as autoimmune disease pathogenesis. Single nucleotide polymorphisms in *IFNL* have been associated with the spontaneous clearance and improved therapeutic outcomes of hepatitis C infection. However, its mechanism of action and impacts on immunity are still unclear. Dendritic cells (DCs) are major producers of IFN- λ . As with IFN- α , plasmacytoid DCs secrete large quantities of IFN- λ in response to toll-like receptor (TLR) 7 and 9 stimulation. CD8+ and BDCA3+ conventional DCs in mice and humans respectively also produce IFN- λ when

stimulated with TLR3, which is dependent on IFN- α receptor (IFN- α R) signalling. Although IFN- α and IFN- λ bind different receptors, they both signal via JAK/STAT pathway and induce the expression of similar IFN-stimulated genes (ISGs). Recently, more reports are elucidating unique regulatory, signalling and functional mechanisms of IFN- λ compared to IFN- α , but these mechanisms in DC biology are still largely unidentified. Using IFN- λ R knockout mice, we have found that DC activation and cross-priming abilities are impaired in response to TLR3 stimulation *in vivo* and serum levels of IFN- α are reduced. Furthermore, IFN- α R expression on splenic DCs *ex vivo* is lower and stimulation with IFN- α is able to rescue DC maturation in these mice. These data suggest that distinct functions and cross-talk exist between IFN- λ and IFN- α in DCs and the identification of these specific effects could potentiate more tailored and/or synergistic therapies in the future.

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Novel anti-inflammatory peptides based on chemokines

McNaughton, E.¹, Sessions, R.², Kungl, A.³, Farris, M.⁴, Broadbridge, R.⁴, Middleton, J.¹

¹Bristol University, Inflammation and Immunology, Bristol, United Kingdom, ²Bristol University, Biochemistry, Bristol, United Kingdom, ³University of Graz, Pharmaceutical Chemistry, Graz, Austria, ⁴Peptide Protein Research Ltd, Bishops Waltham, United Kingdom

Inflammation is an underlying feature of numerous chronic diseases which as yet have no effective treatment. We have identified an interest in the way leukocytes are rapidly recruited from the blood to the site of inflammation in response to chemokine molecules. Chemokines are anchored to the endothelium by glycosaminoglycans (GAGs) such as heparan sulphate (HS) and act to direct leukocytes towards areas of inflammation. We have developed peptides based on chemokines that interfere with chemokine - GAG interactions which we hypothesise will reduce leukocyte migration.

Peptides based on the chemokines CXCL8, CXCL12 and CCL5 were synthesised at PPR Ltd, Southampton employing a solid phase peptide synthesis strategy using conventional Fmoc chemistry. Peptides were purified using HPLC and analysed by mass spectrometry to give a final purity of >95%.

Compared to wildtype chemokine, the peptides exhibited a higher affinity for HS as shown by isothermal fluorescent titration. This corresponded with a reduced leukocyte migration across an endothelial monolayer using a range of peptide concentrations in response to 100ng/ml of chemokine. The optimum peptide concentration was in the picomolar range. Altering the positively charged residues in the peptide removed the inhibitory effects of the peptides. HS was shown to have an important functional role in the observed leukocyte migration as cleavage of HS from the endothelial cells significantly reduced the amount of migration ($p < 0.0001$).

Our studies suggest that peptides based on chemokines that interact with HS can reduce leukocyte migration and may act as a potential novel anti-inflammatory therapeutic.

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ZNF19 acts as a transcription factor to direct the expression of sustained inflammatory genes

Lee, E.¹, Lee, T.A.¹, Kim, J.H.², Ra, E.A.¹, Lee, S.¹, Lee, J.E.², Park, B.¹

¹Yonsei University, Seoul, Korea, Republic of, ²Sungkyunkwan University, Seoul, Korea, Republic of

The transcription of inflammatory genes is an essential step for activating the effective host defense against microbial invaders. Here, we show that zinc finger protein 19 (ZNF19) acts as a transcription factor that is required for activating the innate immune response. In particular, *Znf19* expression was induced by infections through an autoregulatory feedback mechanism and nuclear factor- κ B (NF- κ B) activity, after which it detected persistent inflammatory signals and worked with NF- κ B to robustly produce sustained cytokines. Under lipopolysaccharide (LPS) stimulation, ZNF19 was re-translocated to the nucleus by phosphorylation-mediated dimerization. Consequently, knockdown of ZNF19 in zebrafish showed reduced *il-6* mRNA levels and a greatly decreased number of infiltrating neutrophils into an infected region, thereby leading to severe *Shigella flexneri* infection-induced mortality *in vivo*. Collectively, these observations identified ZNF19 as a transcription factor and a key inducer of the stable activation of immune cells during perpetual infections.

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Characterization of interferon- ϵ expression in mouse mucosal and non-mucosal tissues

De Geus, E.D., Brooks, G.D., Hertzog, P.J., Mangan, N.E.

Hudson Institute of Medical Research, Centre for Innate Immunity and Infectious Diseases, Clayton, Australia

Type I Interferons (IFNs) play an important role in the early control of many pathogens, because of their direct anti-pathogen effects and their effects on both innate and adaptive immune cells. IFN ϵ was previously described by the Hertzog laboratory. In contrast to other type I IFNs, IFN ϵ is not upregulated by PRRs or virus infection. IFN ϵ is highly and constitutively expressed by epithelial cells of the female reproductive tract (FRT), where it is involved in protection against pathogens, as IFN $\epsilon^{-/-}$ mice showed increased susceptibility to infection of the FRT with Herpes Simplex Virus-2 and *Chlamidia muridarum*.

While the expression of IFN ϵ is most abundant in the FRT in mice, expression has also been shown in epithelial cells of other tissues as demonstrated in rhesus macaques, IFN ϵ protein expression was detected in lung epithelial cells lining the bronchioles, but not in alveoli. Furthermore, expression was detected in the epithelium of the jejunum and rectum.

We are currently screening male and female mouse mucosal (e.g. lung and gastro intestinal tract) and non-mucosal tissues (e.g. liver and brain) for IFN ϵ expression using immunohistochemistry, as a basis for the characterisation of this novel IFN in the immune response in mucosal tissues.

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The role of G-CSF in chronic obstructive pulmonary disease*Tsantikos, E.¹, Lau, M.^{1,2}, Maxwell, M.¹, Anderson, G.², Hibbs, M.¹*¹Monash University, Melbourne, Australia, ²University of Melbourne, Melbourne, Australia

Chronic Obstructive Pulmonary Disease (COPD) is a major global health problem affecting tens of millions of people. It is a chronic irreversible lung disease characterized by abnormal enlargement of airspaces (emphysema), obstruction of the airways and chronic bronchitis, and persistent macrophage-rich inflammation. Progressive and permanent destruction of the lung parenchyma and airway thickening leads to unremitting breathlessness and disability. The presence of disease comorbidities, including infection susceptibility, metabolic syndrome, muscle wasting and osteoporosis, further reduce quality of life. Alveolar macrophages are believed to play a key role by secreting factors that exacerbate inflammation and cause irreversible tissue damage. Treatment of COPD involves the use of bronchodilators and potent immunosuppressive drugs, which are only partially effective at managing symptoms and is not curative, thus there is a significant unmet clinical need to develop novel treatments. Cytokine inhibition is an attractive target in COPD, as a multitude of cytokines have been linked to disease severity. Studies by our laboratory have shown that granulocyte-colony stimulating factor (G-CSF) is involved in several pathogenic mechanisms pertinent to the COPD phenotype in a well-characterized animal model of the disease. Deletion of G-CSF was able to attenuate alveolar macrophage activation, limit airway inflammation and tissue destruction and significantly reduce lung pathology. Furthermore, these animals did not develop systemic inflammation and co-morbid phenotypes compared to their G-CSF-sufficient counterparts, yet are immunocompetent upon exposure to pathogenic material. These studies show that G-CSF plays a pathogenic role in inflammatory lung disease and is a potential therapeutic target in COPD.

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Galectin 8 present in conditioned media from breast cancer cells induces increased microvascular permeability via nitric oxide and S-nitrosylation of p120*Zamorano, P.¹, Rebolledo, L.¹, Guequén, A.¹, Ehrenfeld, P.², González, A.³, Soza, A.³, Sánchez, F.¹*¹Universidad Austral de Chile/ Instituto de Inmunología, Valdivia, Chile, ²Universidad Austral de Chile/ Instituto de Histología y Patología, Valdivia, Chile, ³Pontificia Universidad Católica de Chile/ Instituto de Reumatología, Santiago, Chile

Galectins have been involved in many essential functions including differentiation, cell-cell adhesion, cell-matrix interaction, growth regulation and apoptosis. Galectin-8 (Gal-8) is widely expressed in tumoral tissues and seems to be involved in the promoting endothelial cell migration and angiogenesis. Nitric oxide (NO) produced by NO synthase (eNOS) mediates increase in vascular permeability by S-nitrosylation of proteins from the adherens junctions. In cancer, the increase in endothelial permeability promotes the vascularization of

the tumor and dissemination of tumoral cells. Therefore we wanted to study whether or not Gal-8 have a role in endothelial permeability. We tested the hypothesis that Gal-8 present in conditioned medium from breast cancer cell lines acts on the endothelium increasing microvascular permeability via nitric oxide and S-nitrosylation of adherens junction proteins such as p120.

As a model system we use EA.hy926 cells treated with Gal-8 and conditioned medium from MCF-7 breast tumoral cells (MCF-7 CM). Permeability was measured through flux of dextran-FITC. eNOS activation was evaluated through Western-blot and S-nitrosylation of p120 was measured by biotin-switch assay. Gal-8 increases permeability, induces eNOS phosphorylation, S-nitrosylation of p120, and disruption of the adherens junction complex. These effects were inhibited in the presence of L-NMA and lactose. eNOS activation was also evaluated in vivo. Both, Gal-8 and MCF-7 CM, increase eNOS phosphorylation in the mouse cremaster. Furthermore GAL-8 activates FAK and Akt in EA.hy926 cells.

These results provide a new mechanism of action of Gal-8 through eNOS activation on the endothelial cells that can help to the progress of the tumor.

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Modulation of pro- and anti-inflammatory cytokines contribute to the healing effect of the triterpene lupeol in cutaneous wounds in rats*Beserra, F.P.¹, Bérnago, D.A.¹, Bérnago, P.L.¹, Vieira, A.J.¹, Hussni, C.A.², Maia, G.L.d.A.³, Rozza, A.L.¹, Pellizzon, C.H.¹*¹State University of São Paulo, Department of Morphology, Botucatu, Brazil, ²Faculty of Veterinary Medicine and Animal Science, State University of São Paulo, Department of Surgery and Anaesthesiology of Large Animals, Botucatu, Brazil, ³Federal University of São Francisco Valley, Department of Pharmacy, Petrolina, Brazil

Skin wound healing is a dynamic biological process divided into three phases: inflammation, proliferation and remodeling that involve cytokines as important mediators, and the comprehension of their action may lead to new and effective therapeutic strategies. Same natural products change the cytokines levels. The aim of this study was to determine the involvement of lupeol, a triterpene derived from *Bowdichia virgilioides* in the healing process of skin wounds by the levels of cytokines in rats. We used Male Wistar rats (n=8) randomly divided into 5 experimental groups: base ointment, collagenase ointment and lupeol ointment (0.1%, 0.2% or 0.4% w/w). After anesthesia, the animals were submitted to induction of lesion by 2 cm diameter punch in the dorsal region. All animals were submitted to topical treatment, during 3, 7 or 14 days and the quantification of Tumor Necrosis Factor alpha (TNF- α), Interleukin-1 beta (IL-1 β), Interleukin-6 (IL-6) and Interleukin-10 (IL-10) levels were performed on skin samples by ELISA method removed after euthanasia. Topical treatment with lupeol ointment was shown to decrease the levels of TNF- α , IL-1 β , IL-6 in 7 and 14 days of treatment and a significant increase in IL-10 levels in 14 days. No alterations of cytokines levels were observed in the groups after 3 days of treatment. These data

indicate a reduction in the inflammatory response and clearly showed anti-inflammatory effect of lupeol topical treatment by modulating pro- and anti-inflammatory cytokines, important in the wound healing process.

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Antimicrobial peptide derived from IGFBP-5 (AMP-IBP5) stimulates normal human keratinocytes beyond direct antimicrobial properties

Chieosilapatham, P.^{1,2,3}, Niyonsaba, F.^{1,4}, Kiatsurayanon, C.¹, Okumura, K.¹, Ikeda, S.^{1,2}, Ogawa, H.¹

¹Juntendo University Graduate School of Medicine, Atopy (Allergy) Research Center, Bunkyo-ku, Japan, ²Juntendo University Graduate School of Medicine, Department of Dermatology and Allergology, Bunkyo-ku, Japan, ³Chiang Mai Provincial Public Health Office, Ministry of Public Health, Department of Dermatology, Chiang Mai, Thailand, ⁴Juntendo University, Faculty of International Liberal Arts, Bunkyo-ku, Japan

The insulin-like growth factor (IGF) system plays an important role in skin growth and repair, through its ability to modulate cell proliferation, attachment, and migration. Seven IGF-binding proteins (IGFBP-1-7) have been discovered from a variety of vertebrate species, including humans. Among IGFBPs, IGFBP-5 has been reported to enhance the protein synthesis and cell migration in many cell types, including keratinocytes. Recently, it was demonstrated that IGFBP-5 undergoes proteolytic processing to produce a functional antimicrobial peptide named AMP-IBP5 (antimicrobial peptide derived from IGFBP-5). This peptide has been shown to display microbicidal activities against bacteria and fungi, even more potent than those of cathelicidin LL-37 and human β -defensin-2, the major skin-derived antimicrobial peptides (AMPs). Because AMPs activate human keratinocytes and the effects of AMP-IBP5 on these cells remain unknown, we investigated whether AMP-IBP5 would trigger keratinocyte activation. Among several chemokines/cytokines tested, AMP-IBP5 stimulated keratinocytes to increase only the production of IL-8. The molecular mechanism investigation suggested that AMP-IBP5-mediated IL-8 production was controlled by both MAPK and NF- κ B pathways, as evidenced by specific inhibitors for MAPKs and NF- κ B. We also confirmed that AMP-IBP5 upregulated ERK, but not JNK and p38, and also enhanced NF- κ B activation. In addition, AMP-IBP5 markedly stimulated keratinocyte migration and proliferation in a dose- and time- dependent manner. Taken together, the ability of AMP-IBP5 to stimulate the production of IL-8 and mediate keratinocyte migration and proliferation provides a novel evidence that this peptide may contribute to the skin immunity beyond its microbicidal ability.

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An angiogenic peptide AG-30/5C, stimulates various functions of human mast cells

Kanazawa, K.¹, Okumura, K.¹, Ogawa, H.¹, Niyonsaba, F.^{1,2}

¹Juntendo University Graduate School of Medicine, Atopy (Allergy) Research Center, Bunkyo-ku, Japan, ²Juntendo University, Faculty

of International Liberal Arts, Bunkyo-ku, Japan

In addition to their direct antimicrobial activities against invading pathogenic microorganisms, antimicrobial peptides are also involved in various immunomodulatory functions, which have led to antimicrobial peptides being named host defense peptides (HDPs). In fact, HDPs induce the production of cytokines/chemokines, promote chemotaxis, cell proliferation and angiogenesis, and accelerate wound healing. Recently, a novel angiogenic HDP named AG-30/5C has been shown to activate numerous functions of fibroblasts and endothelial cells, in addition to its antimicrobial activity. Because mast cells reside in the vicinity of vessels and are involved in the wound healing process, we hypothesized that angiogenic AG-30/5C may activate mast cells.

In the present study, we observed that AG-30/5C caused degranulation of human mast cells and induced the production of eicosanoids such as leukotriene C₄, prostaglandin D₂ and E₂. Furthermore, we demonstrated that AG-30/5C promoted mast cell chemotaxis and stimulated the production of various cytokines and chemokines by mast cells. Investigation of the molecular mechanism by which AG-30/5C activates mast cells revealed that this peptide stimulates mast cells through the G protein, phospholipase C, MAPK and NF- κ B signaling pathways. Taken together, the current study suggests that a novel angiogenic and antimicrobial peptide AG-30/5C plays a crucial role in the recruitment and activation of mast cells, leading to the control of inflammation and wound healing.

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Investigating the role of IL-33 in the pathogenesis of Behçet's disease

Keskin, G.¹, Inal, A.², Tutluer, S.¹, Çerçi, P.¹, Köse, K.¹, Ölmez, Ü.¹

¹Ankara University School of Medicine, Immunology, Ankara, Turkey, ²Medical School of Başkent University, Immunology, Istanbul, Turkey

Background: Behçet's disease (BD) is a multisystem inflammatory disease, characterized by oral aphthous lesions, recurrent uveitis, skin lesions, genital ulceration, and joints involvement. It's thought that immune system abnormalities play role in the pathogenesis of the disease. In BD, increased release of several cytokines may play a role in inflammatory stages of the disease. IL-33 is a member of the IL-1 cytokine superfamily. It has been reported that IL-33 plays an important role in inflammation and homeostasis. Here, we analyzed serum IL-33 concentration in patients with BD to assess its possible role in the pathophysiology of this disease.

Results: The mean serum IL-33 levels were $4,84 \pm 2,81$ pg/ml in patients with BD and $2,88 \pm 0,42$ pg/ml in the healthy controls. The mean levels of serum IL-33 were $6,16 \pm 2,94$ pg/ml in active stage and $2,86 \pm 0,54$ pg/ml in inactive stage. According to these results; serum IL-33 levels were significantly higher in patients with BD compared with healthy controls ($p < 0.01$). In addition, Serum IL-33 levels were significantly higher in the active patients compared to in the inactive patients and controls ($p < 0.001$ and $p < 0.001$). In the inactive patients with BD, serum IL-33 concentrations were found to be similar compared to healthy controls. In active Behçet's patients, the mean serum IL-33 level

was correlated with arthritis ($r=0.674$, $p < 0.05$).

Conclusion: The high levels of serum IL-33, in active patients with BD suggest that IL-33 may play a significant role in the pathogenesis of BD.

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Pro-inflammatory cytokine and RANKL profile and osteoclast induction by osteoarthritic TMJs synovial fluid

Monasterio Ocares, G.¹, Castillo, F.¹, Garcia, K.¹, Alvarez, C.¹, Rojas, L.¹, Núñez, C.², Flores, G.³, Diaz, W.³, Vernal, R.¹

¹University of Chile, Faculty of Dentistry, Periodontal Biology Laboratory, Santiago, Chile, ²University of Chile, Faculty of Dentistry, Department of Surgery, Santiago, Chile, ³University of Chile, Faculty of Dentistry, Prosthetic Department, Santiago, Chile

Aims: The temporomandibular joint osteoarthritis (TMJ-OA) is a degenerative disorder characterized by synovitis, chronic pain, and progressive degradation and resorption of articular cartilage and subchondral bone. These degenerative changes are mainly associated with osteoclastogenesis; however, the molecular processes associated to these changes remains still unclear. This study aimed to analyze the presence and activity of osteo-destructive factors in synovial fluid (SF) of TMJ-OAs, quantifying the pro-inflammatory and osteoclastogenic cytokine levels and osteoclast activation and function.

Methodology: Five TMJ-OA and two disk-displacement without articular bone-resorption subjects were enrolled for this study. From them, 4 mL of SF were obtained by arthrocentesis and the RANKL, IL-17, and IL-22 levels were measured by ELISA. In addition, the mRNA levels for RANKL, IL-1 β , IL-4, IL-9, IL-17, IL-22, and TGF- β 1 were quantified by qRT-PCR from SF-isolated cells. Finally, 50,000 RAW264.7 cells/mL were seeded in osteo-assay[®] plates, differentiated into pre-osteoclasts with 35 ng/mL rhRANKL and then exposed to serial dilutions of the SF samples. Osteoclast activation was detected by TRAP staining and resorption areas were digitally quantified using a light-microscope.

Results: Higher levels of pro-inflammatory cytokines and RANKL were detected in samples of TMJ-OAs patients compared with disk-displacement controls. In addition, a dose-dependent osteoclast activity was detected in cells induced with TMJ-OAs samples compared with controls.

Conclusions: The higher levels of pro-inflammatory cytokines and RANKL can, at least in part, explain the degradation of articular cartilage and resorption of subchondral bone described during the TMJ-OA pathogenesis.

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Keywords: Osteoarthritis, Temporomandibular Joint, Bone resorption, Cytokines, RANKL.

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Luminex assay manufacturer-based disparity in biomarker concentrations reported in the EQAPOL proficiency program

Lynch, H., Rountree, W., Walker, L., Sempowski, G.

Duke University, Duke Human Vaccine Institute, Durham, United States

Multiplex Luminex assays are widely used for biomarker quantification in research and clinical studies due to their sample sparing capability. These assays use paired antibodies specific to an analyte to determine a concentration using a kit manufactured "standard" protein dilution curve. An international Luminex proficiency program was established within the External Quality Assurance Program Oversight Laboratory (EQAPOL) at Duke University to identify and reduce variables that negatively affect Luminex-based human assay performance through semi-annual proficiency assessments consisting of a common 5-plex kit and cytokine-spiked samples. Accuracy to the consensus mean and precision of site-reported concentrations are components of this proficiency assessment. Recently, we established a second track to assess accuracy and precision using data from each lab's choice of Luminex assay kit manufacturers. We found increased manufacturer dependent variability in reported concentrations impedes assessment of accuracy. Therefore, we tested two ways to mitigate this issue: a blinded pre-diluted internal standard to determine absolute concentration or normalization as a ratio to a calibrator sample to determine relative concentration. A common internal standard, surprisingly, did not reduce manufacturer dependent variability, suggesting there are other contributors (e.g., antibody clones). Normalizing data as a ratio to a calibrator sample, however, reduced variability between manufacturer kits. These studies suggest the need to standardize results across Luminex assay manufacturers and the importance of using a consistent manufacturer for long-term or multi-site studies.

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A study of the role of autophagy and hypoxia in human inflammatory periapical lesions

Huang, H.Y.^{1,2}, Wang, W.C.^{3,4}, Huang, C.P.¹, Lin, P.Y.¹, Chen, C.Y.^{3,4}, Lin, L.M.^{3,4}, Chen, Y.K.^{3,4}

¹Ditanson Medical Foundation Chia-Yi Christian Hospital, Chia-Yi, Taiwan, Department of Dentistry, Chiayi City, Taiwan, Republic of China, ²School of Dentistry, College of Dental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan, Republic of China, ³School of Dentistry, College of Dental Medicine, and Oral & Maxillofacial Imaging Center, Kaohsiung Medical University, Kaohsiung, Taiwan, Republic of China, ⁴Division of Oral Pathology & Maxillofacial Radiology, Kaohsiung Medical University Hospital, Department of Dentistry, Kaohsiung, Taiwan, Republic of China

This study aimed to determine the expression of hypoxia-related (HIF-1 α , BNIP3, and pAMPK) and autophagy-related (LC3, beclin-1, Atg5, Atg12, and p62) proteins in human inflammatory periapical lesions (radicular cyst and periapical granuloma) to explore the role of autophagy in the pathogenesis of these lesions under hypoxia circumstance. Fifteen radicular cysts and 21 periapical granulomas were included in the experimental group; 17 human healthy dental pulp tissues in the control group. ELISA was used to detect IL-1 β cytokine, whilst immunohistochemical and western blot analyses were used to examine autophagy- and hypoxia-related proteins. Kruskal-Wallis and Mann-Whitney U test were employed for statistical analyses (significant different level, $p < 0.05$). Upon ELISA, there was a significant higher IL-1 β expression in periapical lesions than normal pulp tissues.

Immunoscores of immunohistochemical expression for HIF-1 α , beclin-1, Atg5, Atg12, and BNIP3 proteins in periapical lesions (predominantly located in inflammatory, endothelial cells, and epithelial cystic linings) were higher than that of normal pulp tissues. By contrast, lower immunoscores of LC3 and p62 proteins were noted in periapical lesions as compared with normal pulp tissues. Results of immunohistochemical studies were largely compatible to those of western blot analyses where significant higher expressions of hypoxia-related and autophagy-related proteins (except p62 and LC3I) in periapical lesions were noted as compared with normal pulp tissues. In conclusion, our data indicated that autophagy could play a potential causative role in developing and maintaining of the inflamed periapical lesions under hypoxia environments offering an additional concept of the pathogenesis of human inflammatory periapical pathoses.

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Gamma-Aminobutyric acid alleviates the chronic kidney disease progression and renal inflammation

Huang, H.Y., Lin, B.F.

College of Life Science, National Taiwan University, Department of Biochemical Science and Technology, Taipei, Taiwan, Republic of China

Introduction: The disease process of chronic kidney disease (CKD) is irreversible. Previous study showed that Gamma-Aminobutyric acid (GABA) have a beneficial effect on renal function in nephrectomized rats by inhibiting fibrosis and atrophy in tubuli. However, the beneficial effect of GABA on CKD and inflammatory progress are still unknown. Therefore, we further investigated the effect of GABA on renal inflammation and injury.

Materials and methods: C57BL/6 mice were fed aristolochic acid (AA; 6 mg/Kg) to establish the animal model of chronic kidney disease. The mice treatment group were given with GABA rice containing diet for 4-weeks. The urine protein, urine creatinine and serum creatinine levels were measured. HE staining and PAS were also assayed. Furthermore, MES-13 cell or mouse tubular epithelial cell (mTEC) were pre-treated with or without GABA for 1 hr, and stimulated with AA and LPS for 24 hr. The cytokine levels of MCP-1, IL-8, TGF- β , IL-1 β , ROS and HIF-1 protein were measured.

Results: B6 mice fed with AA containing diet significantly increased urine protein earlier than AA and GABA containing diet. Body weight and food intake of mice were no change. In addition, AA directly increased TGF- β and MCP-1 secretion and ROS generation in MES-13 cell. Therefore, GABA improved renal inflammation process might through decreasing inflammatory cytokines secretion and ROS generation.

Conclusions: These data showed that GABA might alleviate the inflammation during early stage of CKD. Furthermore, this study could clarify the mechanisms of GABA for improving the chronic inflammation of renal disease.

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Glucocorticoid-induced leucine zipper regulates inflammation and immunosuppression through its acetylation

*Fan, H.¹, Wang, D.², Cheng, Q.¹, Jones, S.¹, Harris, J.¹, Morand, E.¹
¹Lupus Research Laboratory, Centre for Inflammatory Diseases, School of Clinical Sciences at Monash Health, Monash University, Clayton, Australia, ²Monash University/Hudson Institute Of Medical Research, Clayton, Australia*

Glucocorticoids (GC) have been widely used to inhibit inflammation and immune responses for more than six decades. Unfortunately, the broad spectrum of therapeutic effects of GC is accompanied by a range of adverse effects. Recent studies have shown that glucocorticoid-induced leucine zipper (GILZ), a glucocorticoid-induced protein, mediates many of the anti-inflammatory effects of GC and may do so without GC-triggered adverse effects. GILZ inhibits activation of macrophages, B cells, Th1 and Th17 cells. While GILZ is known to interact directly with NF- κ B, the biochemical pathways and processes involved are incompletely understood. Here we demonstrate that GILZ has a number of putative acetylation sites and TSA, a histone deacetylase inhibitor, increases acetylation of the protein. Moreover, a site mutation (Lys to Arg) at one site, K77, results in abrogation of the anti-inflammatory actions of GILZ in human microvascular endothelial cells. FLIM/FRET analysis demonstrated that acetylation at K77 is critical the interaction between GILZ and NF- κ B. This study provides new insights into how GILZ, a glucocorticoid-induced molecule, regulates anti-inflammatory and immunosuppression functions. Moreover, this information could aid in the development of novel compounds to target this important pathway.

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NNAT plays an anti-inflammatory function in adipose tissue

*Ka, H.L., Yong, H.J., Joo, H.J., Boldbaatar, A., Yang, Y.
Sookmyung Women's University, Department of Life Systems, Seoul, Korea, Republic of*

NNAT (Neuronatin) is known to regulate ion channels during brain development and play a role in maintaining the structure of the nervous system. In the present study, the function of NNAT in adipose tissue was investigated because we found that it is overexpressed in adipose tissue of obesity patients from in silico study. NNAT was increased in white adipose tissue of *db/db* mouse compared with wild type. Since obesity is known to induce low grade inflammation systemically, NNAT expression level was examined in adipose tissue obtained from C57BL/6 mice administered with LPS. However, NNAT expression was decreased in white adipose tissue. To find out a role of NNAT in inflammation, NNAT overexpressing cells were treated with LPS. The level of NF- κ B p65 subunit was decreased and the nuclear localization of NF- κ B p65 subunit was inhibited compared with control cells. It implies that NNAT plays an anti-inflammatory function in adipose tissue.

302**Elevated high mobility group B1 levels in active adult-onset Still's disease associated with systemic score and skin rash***Kim, H.-A., Ye, Y.-M., Jung, J.-Y., Suh, C.-H., Nam, J.-Y.**Ajou University School of Medicine, Suwon, Korea, Republic of*

Objective: High mobility group box-1 (HMGB1) is a nuclear protein and such prototypical damage-associated molecular patterns mediate the immune response in the noninfectious inflammatory response. Adult-onset Still's disease (AOSD) is a systemic inflammatory disorder involved in the dysregulation of innate immunity. We investigated the serum HMGB1 level in patients with AOSD and evaluated its clinical significance.

Methods: Blood samples were collected from 40 patients with active AOSD and 40 healthy controls (HC). Of the patients with AOSD, follow-up samples were collected from 16 patients after a resolution of AOSD disease activity.

Results: Serum HMGB1 levels in patients with AOSD were higher than those of the HC (10.0 ± 5.85 vs. 5.15 ± 1.79 ng/ml, $p < 0.001$). Serum HMGB1 levels were found to be correlated with C-reactive protein (CRP) and the systemic score. The AOSD patient who had a sore throat showed a higher serum HMGB1 level than those patients who did not, and the patient with a skin rash had higher levels than the patients without. In addition, the serum HMGB1 levels were decreased after the resolution of disease activity in the AOSD patients who were followed-up.

Conclusion: The serum HMGB1 levels were elevated in AOSD patients compared to the HC, and were correlated with both CRP and the systemic score. The HMGB1 levels were associated with skin rash and a sore throat in AOSD patients. After the resolution of disease activity, serum HMGB1 levels were found to have decreased.

303**The role of IL-33 in the molecular biology of rosacea in keratinocytes***Cho, H.¹, Byun, J.Y.²**¹Dongguk University, Seoul, Korea, Republic of, ²Ewha Womans University School of Medicine, Department of Dermatology, Seoul, Korea, Republic of*

Interleukin-33 (IL-33) is a member of the IL-1 family that potently drives release of T helper-2 (Th2)-associated cytokines. IL-33 is expressed on a wide variety of cell types, including skin cells, immune cells, endothelial cells, and epithelial cells. IL-33 mediates its biological effects by interacting with the receptors ST2 (also known as IL1RL1), activating intracellular molecules in the NF- κ B and MAP kinase signaling pathways. The ST2 was an orphan receptor that was related in inflammatory process and immune disease. IL-33 as a functional ligand for ST2, it is clearly a potential mediator of diverse inflammatory diseases. Rosacea is chronic inflammatory skin disease, caused an altered innate immune response is involved in inflammatory disease. In this study, we investigated the role of IL-33 in the molecular mechanism of rosacea in keratinocytes. For induction of rosacea, Cathelicidin (LL-37 peptide) and UVB were used. After 2 days treated with LL-37 only in HaCaT keratinocytes cell lines, the growth of these cells were decreased compared to untreated

cells and the mRNA of TNF- α , IL-1 α and IL-33 were expressed. When UVB was exposed to HaCaT cells, IL-33 was also expressed. In rosacea UVB is the source of TLR2 (Toll like receptor 2) activation in skin, and downstream signaling molecules like I κ B and NF- κ B were activated for pro-inflammatory processes. We checked the activation of NF- κ B, p38, ERK using western blot analysis. We suggest that IL-33 is released in rosacea, and it is component involved in the molecular pathology of rosacea.

304**Neutrophil extracellular traps (NETs) formation mediated by oxidized LDL (oxLDL) initiates lipid accumulation in early atherosclerotic plaque***Seo, J.-W., Yang, E.-J., Choi, I.-H.**Institute for Immunology and Immunological Disease, Brain Korea 21 PLUS Project for Medical Science, Yonsei University College of Medicine, Department of Microbiology and Immunology, Seoul, Korea, Republic of*

NETs are an important host defense mechanism to eliminate extracellular pathogens. Also, it is implicated in chronic inflammatory disease, such as atherosclerosis. Recent studies show that NETs components are observed in advanced lesion of atherosclerosis. However, their role in early atherosclerosis is not well examined. In this study, we investigated formation of NETs in early atherosclerosis and sought activating factor that promotes NETs formation. Wild-type and apoE-deficient mice were fed an atherogenic-diet for 4 weeks and NETs formation was evaluated using T2*-weighted MR imaging. NETs were detected in apoE-deficient mice after 2 weeks and it was more clearly detected after 3 weeks. But NETs were not detected in WT mice even after 4 weeks. Next, we isolated neutrophils from WT and apoE-deficient mice and IL-8 induced NETs formation was observed using confocal microscopy. Neutrophils isolated from apoE-deficient mice induced NETs, but the cells from WT mice do not formed NETs. Together with NETs formation in apoE-deficient mice, lipid accumulation in arterial wall was also increased in apoE-deficient mice compared to WT mice. Recent study shows that oxLDL can induce NETs in human neutrophils. So, we assessed levels of oxLDL in WT and apoE-deficient mice. Concentration of oxLDL in plasma was increased in apoE-deficient mice but not detected in WT mice and oxLDL induced NETs in *ex vivo* experiment. Collectively, our results suggest that NETs were induced in early atherogenesis and subsequently lead to lipid accumulation in arterial walls. And oxLDL is a crucial component to promote formation of NETs.

305**The proinflammatory role of matrix metalloproteinase-2 in chronic obstructive pulmonary disease***Nishihama, K.¹, D'Alessandro-Gabazza, C.N.¹, Toda, M.¹, Yasuma, T.¹, Hinneh, J.¹, Etsuko, H.¹, Kentaro, F.², Kobayashi, T.², Gabazza, E.²**¹Mie University School of Medicine, Department of Immunology, Tsu, Japan, ²Mie University School of Medicine, Department Pulmonary and Critical Care Medicine, Tsu, Japan*

Matrix metalloproteinases (MMPs) play crucial roles in the mechanism of alveolar destruction of the lungs in patients with

chronic obstructive pulmonary disease. We have reported that the overexpression of MMP-2 enhances the destructive process in emphysema induced by cigarette smoke using human MMP-2 transgenic mice (TG) exposed to inhaled cigarette smoke extract (CSE).

MMP-2-TG mice showed no phenotype in unstressed conditions, but CSE-exposed mice revealed significant emphysematous changes in the lungs compared to wild type mice within 2 weeks.

In the present study we evaluated the effect of MMP-2 on inflammation *in vivo*.

The results showed that the lungs of MMP-2 transgenic mice have significantly increased infiltration of inflammatory cells and increased level of inflammatory cytokines and chemokines compared to wild type control mice. MMP-2 transgenic mice showed increased emphysematous changes as compared WT mice and also increased the activity of proteases such as MMP-9. Overall, these results suggest that MMP-2 is a key mediator of lung tissue remodeling and inflammation in cigarette smoke induced emphysema.

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Potential involvement of MICA (Major Histocompatibility Class I-related Chain A) in the pathogenesis of endometriosis

Carnevale Marin, M.L.^{1,2}, Coelho, V.^{1,2,3}, Rached Rached, M.⁴, Kalil, J.^{1,3,4}, Simões Abrão, M.⁵

¹Heart Institute (InCor), School of Medicine, University of Sao Paulo, São Paulo, Brazil, ²Histocompatibility and Cellular Immunology Laboratory, LIM-19, School of Medicine, University of São Paulo, São Paulo, Brazil, ³Institute for Investigation in Immunology, National Institute of Science and Technology (iii-INCT), University of São Paulo, São Paulo, Brazil, ⁴Clinical Immunology and Allergy Division, School of Medicine, University of Sao Paulo, São Paulo, Brazil, ⁵Department of Obstetrics and Gynecology School of Medicine, São Paulo University School of Medicine, São Paulo University, São Paulo, Brazil

Endometriosis (EDT) is an inflammatory disease characterized by ectopic implantation/growth of endometrial tissue. Reduced NK and T cell cytotoxicity have been implicated in the pathogenesis of endometriosis. MICA is a stress inducible molecule and its soluble form (sMICA) down-modulates the NKG2D receptor expression on CD8+T and NK cells, reducing the cytotoxic response. We evaluate sMICA levels and MICA expression in women with endometriosis and healthy controls. Soluble MICA levels were determined by ELISA in the serum and the peritoneal fluid (PF) of 153 women with endometriosis and 71 healthy controls. MICA protein *in situ* expression was analyzed in endometriotic lesions and eutopic endometrium of EDT patients (n=22), and in eutopic endometrium of controls (n=17), by immunohistochemistry. We found higher sMICA levels in the EDT (in serum and PF, $p < 0.0001$, Mann-Whitney test), especially in the PF (serum vs PF, $p < 0.0001$), both associated to severe stages of the disease ($p < 0.0001$). MICA expression was positive in 20/22 (91%) of endometriotic lesions and in the correspondent eutopic endometrium, similarly to controls (17/17). Nevertheless, the frequency of MICA high expression

was higher in EDT (11/20, 55%) than in controls (3/17, 18%) ($p=0.0397$, Fisher's exact test). The high protein expression is likely to be related to the inflammatory microenvironment and the increased levels of MICA may be involved in the reduction of the NK and CD8+ T cells cytotoxic, favoring the implantation and growth of endometrial tissue.

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Leptin and resistin are not useful markers to predict the risk of vascular complications in type 2 diabetes mellitus

Zorena, K.¹, Jachimowicz-Duda, O.², Raczyńska, D.³, Lipowski, P.³, Wąż, P.⁴, Bartoszewicz, M.¹, Michalska, M.¹, Raczyńska, K.³

¹Medical University of Gdańsk, Department of Immunobiology and Environment Microbiology, Gdańsk, Poland, ²Diabetic Outpatient Clinic in Gdynia, Gdynia, Poland, ³Medical University of Gdańsk, Department and Clinic of Ophthalmology, Gdańsk, Poland, ⁴Medical University of Gdańsk, Department of Nuclear Medicine, Gdańsk, Poland

The onset of type 2 diabetes mellitus (T2DM) is often insidious and at diagnosis 50% of patients already have diabetes-related organ damage. Therefore there is ongoing search for risk markers of chronic vascular complications in diabetes mellitus. The aim of our study was to investigate leptin and resistin serum levels as potential markers to predict the risk of chronic vascular complications in T2DM patients.

Materials and methods: The study involved 79 subjects including 47 T2DM patients and 32 subjects at risk of diabetes. All study subjects had their clinical parameters recorded and serum leptin and resistin levels measured by ELISA method.

Results: The group of T2DM patients was older ($p=0.001$) and had significantly higher BMI ($p=0.001$), WHR ($p=0.009$), urinary albumin excretion ($p=0.003$) serum creatinin ($p=0.034$), HbA1c ($p=0.001$), fasting glucose ($p=0.001$) and systolic blood pressure ($p=0.002$) values versus the risk group. No significant between-group differences were seen in sex ($p=0.731$), diastolic blood pressure ($p=0.240$), smoking status ($p=0.70$), leptin ($p=0.92$), and resistin ($p=0.45$) levels. Prognostic value for predicting the risk of chronic diabetic complications has been found for HbA1c, total cholesterol and BMI, as assessed on their area under the curve discriminative powers. The Receiver Operating Characteristics statistical analysis has shown no discriminative values for leptin and resistin ($p=0.968$).

Conclusions: In our study sample neither leptin nor resistin occurred to be good markers for predicting the risk of chronic vascular complications in T2DM patients. Further studies on larger samples of patients are needed to better investigate the potential of leptin and resistin.

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Regulation of Jak-Stat signaling pathway by an EF-hand motif protein

Okamoto, K., Inoue, M., Okuhama, A., Takayanagi, H.
Graduate School of Medicine and Faculty of Medicine, The University of Tokyo, Department of Immunology, Tokyo, Japan

Calcium ion (Ca^{2+}) acts as a major second messenger responsible

for a variety of intracellular signaling pathways. Proteins with the EF hand motif, a common Ca^{2+} -binding motif, are important for the intracellular Ca^{2+} signal. A number of proteins with EF hand motif that mediate the biological effects of Ca^{2+} have been identified so far, but the physiological roles of some of them remain largely unknown. Here we identified an EF-hand motif protein (hereafter called Efp) as a new regulator for Jak-STAT signaling pathway. Since Efp is expressed at high level in hematopoietic cell lineage, we newly generated the Efp floxed mice and analyzed the hematopoietic lineage-specific Efp-deficient mice by mating them with Vav-iCre transgenic mice. Efp^{fllox/-} Vav-iCre mice were born with a pale skin at the expected Mendelian ratio and die within 1 day after birth because of severe anemia. Blood test using the peripheral blood from the P0 mice revealed a decrease in the number of red blood cells and low hemoglobin in the Efp^{fllox/-} Vav-iCre mice. On the other hand, we found normal number of CD11b⁺ myeloid cells, CD3⁺ T cells and B220⁺ B cells. Notably, activation of Jak-Stat pathway by erythropoietin was inhibited in the Efp-deficient fetal liver cells, whereas IL-3 or GM-CSF-induced Jak-Stat pathway was normally activated. These results indicate that Efp regulates the Jak-Stat signaling pathway downstream of certain types of cytokines. We will also discuss roles of Efp in lymphocyte development by analyzing T- and B-cell specific Efp knockout mice.

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In vitro comparison of 18 α -glycyrrhizin, silymarin and silybin A on activated human hepatic stellate cells

Kalantar, K.¹, Hosseini, S.Y.^{2,3}, Shahin, K.³, Ghayour, M.⁴, Rajabibazl, M.⁵, Fattahi, M.-R.³, Moini, M.³, Amirghofran, Z.⁶

¹Shiraz University of Medical Sciences, Immunology Department, Shiraz, Iran, Islamic Republic of, ²Shiraz University of Medical Sciences, Department of Bacteriology and Virology, Shiraz, Iran, Islamic Republic of, ³Shiraz University of Medical Sciences, Gastroenterohepatology Research Center (GEHRC), Shiraz, Iran, Islamic Republic of, ⁴Shahid Beheshti University of Medical Sciences, Department of Clinical Biochemistry, Tehran, Iran, Islamic Republic of, ⁵Shahid Beheshti University of Medical Sciences, Department of Clinical Biochemistry, Shiraz, Iran, Islamic Republic of, ⁶Shiraz University of Medical Sciences, Shiraz, Iran, Islamic Republic of

Hepatic stellate cells (HSCs) activation and proliferation is the main cause of liver fibrosis, thus it seems that this cell could be the major target of current fibrosis therapy. In this study the anti-fibrotic role of three medicinal plants-derived components, including 18 α -glycyrrhizin, silymarin and silybin A was compared on activated LX-2 stellate cell, in prophylactic and therapeutic manner. The possible cytotoxic/proliferative effect of components on LX-2 cells evaluated to find the optimal dose of treatment. The HSCs then treated with herbal compounds before (in prophylactic) and after (in therapeutic manner) activation by TGF- β 1. The anti-proliferative effects of components on normal and activated LX-2 cell were measured by MTT assay. The harvested cells were introduced into real-time PCR to determine the gene expression levels of TIMP-1, COL1A1 and MMP-2 as fibrotic markers. Furthermore, TGF- β 1 production

was measured by ELISA assay on culture supernatants. MTT assay results indicated that nearly all components have significant anti-proliferative effect on activated LX-2 cells when applied in a therapeutic manner ($P < 0.01$). In prophylactic manner, only silybin A showed a significant anti-proliferative effect ($P < 0.001$). Real-time PCR was used to detect TIMP-1, COL1A1 and MMP-2 mRNA expression. The results showed that the components decrease these three genes in comparison to the control condition in therapeutic and prophylactic manner. TGF- β production from LX2 cell in presences of extracts, was significantly decrease the level TGF- β in therapeutic and prophylactic manner. Our herbal extracts exhibited some extent of inhibitory effects on TGF- β 1 activated LX-2 cells both prophylactic and therapeutic manner.

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B-cell abnormalities in HIV disease are associated with increased interferon- α activity

Cha, L.¹, Abudulai, L.¹, French, M.^{1,2}, Fernandez, S.¹

¹University of Western Australia, School of Pathology and Laboratory Medicine, Perth, Australia, ²Royal Perth Hospital, Department of Clinical Immunology, Perth, Australia

B-cell dysfunction, such as increased frequencies of exhausted CD21^{lo/-} B-cells and decreased isotype diversification of IgG antibody responses, is observed in HIV-1 infection. Understanding more about these defects may lead to novel therapies for enhancing antibody responses, including those induced by therapeutic HIV vaccines. Here, we have investigated the role of interferon-alpha (IFN- α). In antiretroviral therapy (ART)-naïve (n=19) and ART-treated (n=30) HIV patients, levels of plasma IP-10 and soluble (s) IFNAR2 were examined by ELISA as indicators of IFN- α activity and B-cell activation and exhaustion was assessed by enumeration of CD21^{lo/-} B-cells and serum levels of kappa and lambda free light chains (FLCs), IgG1 and IgG2. In ART-naïve patients, plasma IP-10 and sIFNAR2 levels correlated with frequencies of CD21^{lo/-} B-cells, FLCs and serum IgG1 levels ($r \geq 0.5$, $p \leq 0.03$ and $r \geq 0.6$, $p \leq 0.01$, respectively). In contrast, IP-10 and sIFNAR2 were inversely correlated with serum IgG2 ($r = -0.4$, $p = 0.09$ and $r = -0.6$, $p = 0.007$, respectively). To assess the effect of IFN- α on B cell differentiation in vitro, B cells from non-HIV donors (n=25) were stimulated for 5 days with interleukin-21 and CD40 ligand in the absence or presence of IFN- α 2b. Addition of IFN- α 2b to cultures did not alter the induction of plasmablasts (CD27^{hi}CD38^{hi}) but decreased the production of all IgG subclasses ($p \leq 0.002$) in culture supernatants, particularly IgG2 ($p < 0.0001$). Thus, increased IFN- α activity is associated with B cell activation and exhaustion in HIV patients and IFN- α exerts an inhibitory effect on IgG production which may limit isotype diversification of IgG antibody responses.

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The indicators of systemic inflammation in patients with end-stage renal disease (ESRD), receiving different types of renal replacement therapy

Solomatina, L.^{1,2}, Kuznetsova, Y.³, Groznykh, E.⁴

¹Ural Federal University named after the First President of

Russia B.N.Yeltsin, Ekaterinburg, Russian Federation, ²Institute of Immunology and Physiology, Ural Branch of RAS, Ekaterinburg, Russian Federation, ³Ural Scientific Research Institute of Dermatovenerology and Immunopathology, Ekaterinburg, Russian Federation, ⁴Sverdlovsk Regional Hospital №1, Ekaterinburg, Russian Federation

Objective: To assess the degree of chronic systemic inflammation (CSI) in patients with ESRD receiving different types of renal replacement therapy.

The study included the following groups: patients with ESRD receiving program hemodialysis (12 hours per week, blood samples taken prior to dialysis session (n=42), renal transplant patients with normal allograft function (n=24), patients with chronic allograft dysfunction (CAD, n=23).

Chronic glomerulonephritis was the condition that resulted in the development of ESRD in all the patients.

Blood plasma was tested for the following SI indicators: CRP, IL-6, IL-8, IL-10, TNF α , D-dimers, cortisol, myoglobin, troponin I. On the basis of these markers using an original method we calculated the integral index of intensity of chronic SI (from 0 to 8 points) -CSI Score.

Results: CSI was diagnosed only in hemodialysis patients (90.9%) and in the CAD group (43.5%).

CRP level increase was found in 27.3% (hemodialysis group), 17.4% (CAD group) and 16.7% (normal allograft function group).

Mortality / graft loss were reported in 26.1% of patients (CAD group), of which CSI was found in 4 patients (66.7%) and elevated CRP levels in only 2 patients (33.3%).

Mortality / loss of kidney transplant was 16.7% in the group with normal functioning transplant, and only one patient had an increased level of CRP.

Conclusion: CSI is a severe ESRD complication in hemodialysis patients. Following the renal transplantation CSI is found only in patients with CAD. CSI Score can be used as a predictor of mortality / allograft loss in patients with CAD.

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Superoxide dismutase 1 protects hepatocytes from type I interferon-driven oxidative damage

Bhattacharya, A.¹, Hegazy, A.², Kosack, L.¹, Deigendesch, N.³, Cupovic, J.⁴, Kandasamy, R.¹, Hildebrandt, A.¹, Merkle, D.⁵, Kuehl, A.⁶, Vilagos, B.¹, Schliehe, C.¹, Panse, I.², Khamina, K.¹, Baazim, H.¹, Arnold, I.⁷, Flatz, L.⁴, Xu, H.⁸, Lang, P.⁸, Aderem, A.⁹, Takaoka, A.¹⁰, Superti-Furga, G.¹, Colinge, J.¹, Ludewig, B.⁴, Löhning, M.², Bergthaler, A.¹

¹CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria, ²Experimental Immunology, Charité - Universitätsmedizin Berlin, Department of Rheumatology and Clinical Immunology, Berlin, Germany, ³Max Planck Institute for Infection Biology, Berlin, Germany, ⁴Institute of Immunobiology, Cantonal Hospital St. Gallen, St Gallen, Switzerland, ⁵University of Geneva, Department of Pathology and Immunology, Geneva, Switzerland, ⁶Campus Benjamin Franklin, Charité - Universitätsmedizin Berlin, Department of Medicine I for Gastroenterology Infectious Disease and Rheumatology, Berlin, Germany, ⁷Translational Gastroenterology

Unit, Experimental Medicine Division Nuffield Department of Clinical Medicine, University of Oxford, John Radcliffe Hospital, Oxford, United Kingdom, ⁸Heinrich-Heine-University, Department of Gastroenterology, Düsseldorf, Germany, ⁹Seattle Biomedical Research Institute, Seattle, United States, ¹⁰Division of Signaling in Cancer and Immunology, Institute for Genetic Medicine, Hokkaido University, Sapporo, Japan

Tissue damage caused by viral hepatitis is a major cause of morbidity and mortality worldwide. Using a mouse model of viral hepatitis, we identified virus-induced early transcriptional changes in the redox pathways in the liver, including downregulation of superoxide dismutase 1 (Sod1). Sod1^{-/-} mice exhibited increased inflammation and aggravated liver damage upon viral infection, which was independent of T and NK cells and could be ameliorated by antioxidant treatment. Type I interferon (IFN-I) led to a downregulation of Sod1 and caused oxidative liver damage in Sod1^{-/-} and wild-type mice. Genetic and pharmacological ablation of the IFN-I signaling pathway protected against virus-induced liver damage. These results delineate IFN-I mediated oxidative stress as a key mediator of virus-induced liver damage and describe a mechanism of innate-immunity-driven pathology, linking IFN-I signaling with antioxidant host defense and infection-associated tissue damage.

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Therapeutic effect of *Oleaceae* spp. ethanol extract on the IBD model

Li-Shian, S., Po-Chen, L., Yi-Ling, Y.

National Formosa University, Department of Biotechnology, Huwei Township, Taiwan, Republic of China

Inflammatory bowel disease (IBD) symptoms characterized by chronic inflammation of the gastrointestinal tract. The exact etiology and pathogenesis of these disorders remain unclear. The clinical features include diarrhea, pain, narrowing of the gut lumen leading to strictures and bowel obstruction, severe diarrhea, blood loss, and progressive loss of peristaltic function. The medical therapeutic effect of the *Oleaceae* flower has been recorded in "Ben Cao Gang Mu" traditional Chinese medical literature. It describes the usefulness of these flowers for arrest of dysentery with blood in the bowel. The scientific evidence of this effect has not yet been proved.

In this study, acute colitis was induced and ethanol extract of *Oleaceae* was administered via intragastric administration. The therapeutic effect was measured by body weight loss, colon length. The severity was calculated using disease activity index. Furthermore, MIP-2 secretion, myeloperoxidase activity and histologic examination were also been evaluated. Oral administration of ethanol extract of *Oleaceae* (1000mg/kg) has inhibitory activity on body weight loss and shortening of the colon. It also alleviate severity of colon inflammation according to the histological examination by reducing inflammatory cell infiltration to colonic mucosa, as well as the degree and extent of epithelial damage.

In summary, oral administration of ethanol extract of *Oleaceae* has inhibitory activity on the IBD model.

314**Role of IL-5 in IL-33-induced inflammation***De Vera Mudry, M.C., Bessa, J., Iglesias, A.**F. Hoffmann-La Roche Ltd., Roche Pharma Research and Early Development, Basel, Switzerland*

Introduction: IL-33 knock-in mice (ST2+/+IL33tm1/+) show a characteristic TH2 response with increased serum IL-33 and IL-5, eosinophilia, lethal non-resolving multi-organ inflammation, and altered subcellular localization of IL-33. IL33tm1/+ mice crossed with mice lacking ST2 receptor (ST2-/-IL33tm1/+) have normal serum IL-5 levels and are protected from inflammation, despite increased IL-33.

Methods: ST2-/-IL33tm1/+, ST2-/+IL33tm1/+, ST2-/-wtwt, and ST2-/+wtwt mice were sacrificed at ages 3-24 months, and tissues collected for histopathological evaluation.

Results: ST2-/-IL33tm1/+ showed no significant findings in mesenteric lymph node, small intestine, lung, heart, and bone marrow, similar to ST2-/-wtwt mice. In contrast, ST2-/+IL33tm1/+ mice showed inflammation of various tissues, similar to ST2+/+IL33tm1/+ mice. The mesenteric lymph node showed hypercellularity due to marked infiltration of plasma cells, mononuclear cells, and eosinophils that extended to the capsule and mesenteric fat. The spleen was hypercellular due to marked extramedullary hematopoiesis (myeloid and erythroid) and infiltration of plasma cells and eosinophils. The small intestine showed marked villous thickening, mononuclear and eosinophilic infiltration, and hypertrophy/hyperplasia of smooth muscle layers. ST2-/+IL33tm1/+ mice showed eosinophilic myeloid hyperplasia of bone marrow, minimal goblet cell hyperplasia of pulmonary bronchioles, and fibrosis of the cardiac pulmonary-arterial trunk.

Conclusion: ST2-sufficient ST2-/+IL33tm1/+ mice succumb to IL-33-induced inflammation due to increased IL-5, resulting in eosinophilia, eosinophilic infiltration, fibrosis, and remodeling in the gut and lung. Despite high serum IL-33, ST2-insufficient ST2-/-IL33tm1/+ mice are protected due to normal levels of IL-5. Impact statement: The IL-33 knock-in mouse model of inflammation is a tool for developing agents targeting IL-33 or its receptor ST2.

315**The role of mTORC1 in skin irritant croton oil-induced cytokine expression by mouse keratinocytes***Ohtani, M., Manabe, A., Watanabe, N.**Toho Univ., Fac. of Sci., Dept. of Biomol. Sci., Funabashi, Chiba, Japan*

The mammalian target of rapamycin complex 1 (mTORC1) regulates a diverse array of cellular responses including cell growth, metabolism, survival, and immunity. We and other groups previously demonstrated that mTORC1 regulates cytokine production in myeloid cells, but roles in non-myeloid cells such as keratinocytes remain unclear. In this study, we investigated the role of mTORC1 in skin irritant croton oil-induced cytokine expression in mouse keratinocyte cell line PAM212 cells. The expression of cytokines/chemokines such as GM-CSF, TSLP, IL-1 and MIP-2 mRNA was induced in PAM212 cells stimulated with croton oil. Western blot analysis revealed

that the phosphorylation p70S6K and 4EBP1, downstream targets of mTORC1, was decreased upon croton oil stimulation and was completely blocked by the treatment with mTORC1 inhibitor rapamycin. Rapamycin also increased the expression of GM-CSF, TSLP and IL-1 α mRNA induced by croton oil. In addition, intracellular cytokine staining and ELISA analysis revealed the increase in GM-CSF protein by rapamycin in PAM212 cells, suggesting that mTORC1 negatively regulates GM-CSF expression. To address the mechanism(s) of regulation of GM-CSF gene expression by mTORC1, we examined the promoter activity of *Csf2* (GM-CSF). Luciferase reporter assay demonstrated that the sequence between -688 to +290 is necessary in response to croton oil and of the sequence between -34 to +49 is sensitive to rapamycin. We are currently investigating the transcription factor associated with mTORC1.

316**Activated protein C inhibits rheumatoid synovial fibroblast invasion and prevents inflammatory arthritis in mouse models***Xue, M.¹, Dervish, S.^{1,2}, Little, C.³, March, L.⁴, Jackson, C.¹**¹University of Sydney, Sutton Arthritis Research Laboratory, Kolling Institute of Medical Research, St Leonards, Australia, ²University of Sydney, Westmead Institute, Westmead, Australia, ³University of Sydney, Raymond Purves Research Laboratory, Kolling Institute of Medical Research, St Leonards, Australia, ⁴University of Sydney, Rheumatology, Royal North Shore Hospital, St Leonards, Australia*

Aim: To investigate whether activated protein C (APC), a physiological anticoagulant with anti-inflammatory properties, can inhibit aggressive properties of rheumatoid synovial fibroblasts (RASf) and prevent inflammatory arthritis.

Methods: RASf isolated from patients with rheumatoid arthritis (RA) after surgery were treated with APC, TNF- α or IL-17. Cell viability/proliferation was measured by MTT assay, cytokines and cell signaling molecules by ELISA and western blot, matrix metalloproteinase (MMP)-2 and MMP-9 by zymography, and cartilage degradation by 1,9-dimethylmethylene blue (DMMB) assay. The in vivo effect of APC was evaluated in mouse antigen- and collagen -induced arthritis (AIA and CIA) models.

Results: In vitro, APC reduced IL-1 β , IL-17 and TNF- α production RASf and inhibited IL-17-stimulated cell proliferation and NF- κ B activation in RASf. When co-incubated with RASf and cartilage tissues, APC reduced RASf-induced cartilage degradation and inhibited MMP-9 production in control and TNF-stimulated conditions. In vivo, APC and endothelial protein C receptor (EPCR) were expressed by the synovial joint. APC decreased the incidence and the severity of CIA, inhibited pannus formation, and cartilage and bone destruction within the joint in DBA/J1 mice, 20 days after the second immunization. The serum levels of IL-1 β , IL-6, IL-17 and TNF- α were significantly lower in response to APC treatment in CIA model. In AIA model, APC significantly reduced total histological score and cartilage degradation at day 4 and day 28, after intra-articular injection of methylated bovine serum albumin in C57BL6 mice.

Conclusion: APC suppresses inflammation and invasion of RASf and inhibits the development and severity of inflammatory arthritis.

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Type-I Interferon dependent degradation of microRNA isoforms in mouse macrophages*Nejad, C.¹, Pépin, G.¹, McCoy, C.E.², Gantier, M.¹*¹Hudson Institute of Medical Research, Centre for Innate Immunity and Infectious Diseases, Clayton, Australia, ²Hudson Institute of Medical Research, Centre for Cancer Research, Clayton, Australia

Innate immune activation by pathogens promotes global transcriptional changes in infected cells, quickly affecting the levels of messenger RNAs and non-coding RNAs such as microRNAs (miRNAs). Previous reports indicate that the intracellular levels of select miRNAs can be rapidly down-regulated following innate activation. Given the extreme stability of miRNAs, these reports suggest that select miRNAs could be actively degraded upon innate immune activation. Here we show that the intracellular levels of abundant miRNAs are not decreased upon TLR activation of mouse bone marrow derived macrophages (BMDMs). miR-221 was the exception, with a >80% decrease following TLR4 and TLR3 stimulation, but not when stimulated by other TLRs. The miR-221 decrease was independent on a transcriptional event given that pri-miR-221 levels were increased upon stimulation. miR-221 levels were strongly reduced following IFN- β treatment of BMDMs, consistent with an involvement of TRIF in the TLR3/4-induced miR-221 decrease. Accordingly, miR-221 levels were not impacted on by TLR3/4 in IFN- α/β receptor 1 deficient BMDMs. Studies of miR-221 levels following *Drosha* deletion suggested that this miRNA was not intrinsically unstable following biogenesis, and was rather actively degraded by IFN- β treatment. Indeed, we discovered that miR-221 decrease by TLR signalling was restricted to a long variant of this microRNA, presumably more sensitive to 3'-5' exonucleases. Similar results were found for the closely related miR-222 isoforms. Collectively, our results demonstrate an isoform-specific degradation of abundant miRNAs upon IFN- β treatment of BMDMs, likely impacting the innate immune program in these cells.

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Impact of a nonsteroidal anti-inflammatory drug (NSAID) on serum MCP1 & clinical observations during lipopolysaccharide (LPS) induced inflammation in swine*Myers, M.¹, Smith, E.², Messenheimer, J.², Lewandowski, A.², Groesbeck, C.², Zhang, W.², Swain, T.², Chiesa, O.A.², Deaver, C.M.²*¹US Food and Drug Administration, Center for Veterinary Medicine, Laurel, United States, ²U.S. Food and Drug Administration, CVM/Office of Research, Laurel, United States

The capacity of the veterinary NSAID flunixin meglumine (flunixin) to affect the outcome of an acute LPS induced systemic inflammatory response in swine was evaluated. Flunixin was given either 1 h before or 1 h after a single i.v. administration of lipopolysaccharide (LPS; 2 μ g/kg). Blood samples for MCP1 assessment were collected at 0, 1, 3, 6, 8, & 24 h after LPS administration using an in-dwelling catheter inserted prior to LPS administration. MCP1 concentrations were determined using a swine-specific ELISA. Clinical observations included rectal temperature, heart rate (HR), respiration rate

(RR), skin redness (SR), lethargy, and respiratory character (RC). RR, HR, and clinical scores for RC, SR, and lethargy were elevated from 1 h through 24 h post-LPS administration. Flunixin administration reduced the LPS-induced increase in RR and RC. Flunixin administration had no impact on the LPS-induced increase in the clinical scores for SR or lethargy, regardless of when it was administered. Plasma concentrations of MCP-1 were induced within 1 h of LPS administration, and remained elevated through 8 h, returning to baseline values by 24 h post-LPS. Flunixin had no impact on serum MCP1 levels regardless of time of administration. The severity of the clinical results suggests that the administered LPS challenge dose resulted in toxicity beyond the expected systemic inflammatory response. While these results suggest that RR and RC are potential clinical endpoints useful in assessing the anti-inflammatory action of NSAIDs, the severity of morbidity further suggests the model needs to be refined.

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Absence of CCR5 axis exaggerates thrombus formation through reduced uPA and tPA expression in murine DVT model*Nosaka, M.¹, Ishida, Y.¹, Kimura, A.¹, Kuninaka, Y.¹, Mukaida, N.², Kondo, T.¹*¹Wakayama Medical University, Forensic Medicine, Wakayama, Japan, ²Kanazawa University, Division of Molecular Bioregulation, Cancer Research Institute, Kanazawa, Japan

Deep vein thrombosis (DVT) is multifactorial and often results from a combination of risk factors such as genetic conditions, obesity, drugs, pregnancy, aging, trauma, and malignancy. And DVT is a complex biological event, with endothelial injury, venous stasis and blood hypercoagulability. In this study, we examined the pathophysiological roles of CCL3/CCL5-CCR5 axis in the resolution of DVT in *Ccr5* KO mice. Upon the ligation of the inferior vena cava (IVC) of the mice, venous thrombi formed and grew progressively until 5 days, and, thereafter, the thrombus weight decreased. We observed the thrombi until 21 days after IVC ligation. Immunohistochemically, in WT mice, intrathrombotic CCR5-positive cells were detected in the whole course of the observation period and increased gradually with time after IVC ligation. Concomitantly, *Ccr5* expression was detected in the thrombi. When *Ccr5* KO mice were treated in the same manner, thrombus size was much larger in *Ccr5* KO mice than in WT ones. Moreover, the blood flow of the IVC was less recovered in *Ccr5* KO mice than in WT ones. And intrathrombotic *Plat* and *Plau* mRNA expression was significantly reduced in *Ccr5* KO mice than WT mice. In WT-derived macrophages but not *Ccr5* KO-derived ones, recombinant CCL3 and CCL5 treatment enhanced gene expression of *Plau* and *Plat*, respectively. These observations indicated that CCR5-deficiency impaired fibrinolytic activity and collagen production, both indispensable steps for thrombus resolution. Collectively, the absence of CCR5 can have a detrimental role in the thrombus resolution by suppressing uPA and tPA expression respectively.

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Mevalonate kinase deficiency leads to decreased prenylation of Rab GTPases

Munoz, M.A.¹, Jarczyluk, J.¹, Skinner, O.P.¹, Preston, A.¹, Ali, N.¹, Palendira, U.², Quinn, J.M.W.¹, Argent, E.³, Tangye, S.G.⁴, Brown, A.J.⁵, Ziegler, J.B.⁶, Mehr, S.⁷, Rogers, M.¹

¹Garvan Institute of Medical Research, Bone Biology, Darlinghurst, Australia, ²Centenary Institute, University of Sydney, NSW, Camperdown, Australia, ³Sydney Children's Hospital, Dept. of General Paediatrics, Randwick, Australia, ⁴Garvan Institute of Medical Research, Immunology, Darlinghurst, Australia, ⁵University of New South Wales, School of Biotechnology and Biomolecular Sciences, Darlinghurst, Australia, ⁶Sydney Children's Hospital, Dept. of Immunology and Infectious Diseases, Randwick, Australia, ⁷Children's Hospital at Westmead, Dept. of Immunology and Allergy, Sydney, Australia

Mevalonate kinase deficiency (MKD) is caused by mutations in a key enzyme of the mevalonate-cholesterol biosynthesis pathway. A recurrent autoinflammatory disorder characterised by inflammasome-mediated processing of IL-1b. It is currently believed that the inflammatory phenotype of MKD is triggered by temperature-sensitive loss of mevalonate kinase activity and reduced biosynthesis of isoprenoid lipids required for the prenylation of small GTPase proteins. However, previous studies have not clearly shown any change in protein prenylation in patient cells under normal conditions. With lymphoblast cell lines from 2 compound heterozygous MKD patients, we used a highly sensitive *in vitro* prenylation assay, together with quantitative mass spectrometry, to reveal a subtle accumulation of unprenylated Rab GTPases in cells cultured for 3 days or more at 40°C compared to 37°C. This included a 3-fold increase in unprenylated Rab7A, Rab14 and Rab1A. Inhibition of SREBP activation by fatostatin led to more pronounced accumulation of unprenylated Rab proteins in MKD cells but not parent cells, suggesting that cultured MKD cells may partially overcome the loss of isoprenoid lipids by SREBP-mediated upregulation of enzymes required for isoprenoid biosynthesis. Furthermore, while inhibition of Rho/Rac/Rap prenylation promoted the release of IL-1b, specific inhibition of Rab prenylation by NE10790 had no effect in human PBMC or human THP-1 monocytic cells. These studies demonstrate for the first time that mutations in mevalonate kinase can lead to a mild, temperature-induced defect in the prenylation of small GTPases, but that loss of prenylated Rab GTPases is not the cause of enhanced IL-1b release in MKD.

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Epigenetic-immunological axis of resveratrol-mediated amelioration of acute lung injury

Algheta, H.F.K.^{1,2}, Mohammed, A.K.^{1,2}, Zumbun, E.¹, Elliot, D.¹, Nagarkatti, P.¹, Nagarkatti, M.¹

¹University of South Carolina, School of Medicine, Department of Pathology, Microbiology and Immunology, Columbia, South Carolina, United States, ²University of Baghdad, College of Veterinary Medicine, Baghdad, Iraq

Resveratrol (RES) is a plant-derived phenol that has antioxidant and anti-inflammatory properties, and has been explored as a

possible treatment against inflammatory diseases. In this study, we explored the role of RES in suppressing inflammation induced by an inhaled super-antigen, staphylococcal enterotoxin B (SEB). To this end, mice were pretreated with RES or vehicle and the acute lung injury (ALI) was induced by dual-dose administration of SEB. All SEB exposed mice pretreated with vehicle succumbed, while all mice pretreated with RES survived. Total RNA was extracted from lung infiltrate for microRNA (miRNA) microarray analysis and data showed several miRNAs were altered upon RES treatment. Downregulation of miR-193a-3p, miR-148a-3p, and upregulation of miR-3103-5p which target TGF- β , SOCS3 and VEGF- α , respectively were confirmed by real-time PCR. Importantly, TGF- β upregulation is known to result in increased Treg cells and reduced activated CD4⁺ cells, as seen with RES treatment where the lymphocyte subsets in the lungs were significantly lower than vehicle-treated mice. We conclude that RES treatment protects against super-antigen induced inflammation by immobilizing T-cells and thereby decreasing vascular endothelial dysfunction at the site of exposure, possibly by acting through miRNAs (Supported by NIH grants P01AT003961, R01AT006888, R01ES019313, R01MH094755, P20RR032684 and VA Merit Award BX001357).

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TNF- α -MEK-ERK1/2 signaling contributes to invasiveness of U87MG glioblastoma cells

Dalavaikodihalli Nanjaiah, N., Ramaswamy, P.

National Institute of Mental Health and Neuro Sciences (NIMHANS), Department of Neurochemistry, Bengaluru, India

Glioblastoma the most aggressive and deadliest form of primary brain tumor accounts for 60% of all glial tumors. These tumors remain incurable, and patients have a median survival time of less than 15 months despite substantial progress in early diagnosis and combined modality therapy including surgical therapy, radiotherapy and chemotherapy. Glioblastoma cells are capable of exploiting tumor microenvironment for their survival and progression. We hypothesized that inflammatory cytokine tumor necrosis factor (TNF)- α milieu, contributes to growth and spread of glioblastoma and probably through mitogen-activated/extracellular signal-regulated kinase kinase (MEK) - extracellular signal-regulated kinase (ERK) 1/2 signaling axis. We investigated the effect of TNF- α on proliferation of U87MG cells by MTT assay, viability by trypan blue dye exclusion assay, migration by scratch wound-healing assay, invasion by *in vitro* trans-well Matrigel invasion assay and expression and activity of matrix metalloproteinases (MMP)-2 and MMP-9 by reverse transcriptase-PCR and gelatin zymography respectively. Migration and invasion of U87MG cells were significantly increased in the microenvironment of TNF- α , without any effect on proliferation or glioma cell viability. Inhibition of MEK kinase and ERK1/2 attenuated the TNF- α induced migration and invasion of U87MG cells. These findings point to the role of inflammatory microenvironment in the migration and invasion of glioma and demonstrate MEK-ERK1/2 pathway in TNF- α mediated growth and spread of glioblastoma. Targeting TNF- α -MEK-ERK1/2 signaling is worth considering in the management of invasion of glioblastoma.

323**Suppression of cutaneous inflammatory reactions by oral administration of whole dihomo- γ -linolenic acid-producing *Saccharomyces cerevisiae****Watanabe, N., Ohtani, M., Uemura, H.**Toho University, Fac. of Sci., Dept. of Biomol. Sci., Funabashi, Chiba, Japan*

Polyunsaturated fatty acids have many biological activities and play important roles in human health and nutrition. Dihomo- γ -linolenic acid (DGLA, C20:3n-6) is known to have an anti-inflammatory activity, but its range of effects was not well studied because of its limited natural sources. Taking advantage of genetic tractability of *Saccharomyces cerevisiae*, we have previously constructed DGLA-producing yeast strain by introducing two types of desaturase and one elongase genes to convert endogenous oleic acid (C18:1n-9) to DGLA. In this study we investigated the efficacy of oral intake of heat-killed whole DGLA-producing yeast cells on skin inflammation such as irritant contact dermatitis (ICD) and allergic contact dermatitis (ACD).

In a murine ICD, topical application of croton oil induced ear swelling along with the production of chemokines and accumulation of infiltrating cells into the skin sites. These inflammatory reactions were significantly suppressed in a dose-dependent manner by oral intake of the DGLA-producing yeast for only 7 days. This suppression was not observed by the intake of the γ -linolenic acid (C18:3n-6, an immediate precursor of DGLA) producing yeast, indicating DGLA itself suppressed the inflammation. Further analysis demonstrated that DGLA exerted an anti-inflammatory effect via prostaglandin E1 formation. In addition to ICD, ACD-induced ear swelling and cellular infiltration to the inflamed site induced was also suppressed by the same treatment. Oral administration of the DGLA-producing yeast is considered to be a simple but efficient method to suppress inflammatory responses because purification of fatty acids was not required and a small amount of DGLA was effective.

324**Type I interferons in the regulation of mucosal immune responses in the female reproductive tract***Mangan, N.¹, Mielke, L.², De Geus, E.¹, Gould, J.¹, Cumming, H.¹, Woodhouse, I.¹, Gearing, J.¹, Hansbro, P.³, Hertzog, P.¹**¹Hudson Institute of Medical Research, Centre for Innate Immunity and Infectious Disease, Clayton, Australia, ²Walter & Eliza Hall Institute, Department of Medical Biology, Melbourne, Australia,**³Hunter Medical Research Institute & The University of Newcastle, Newcastle, Australia*

In the female reproductive tract (FRT), homeostasis is maintained to enable embryo implantation and development in parallel with priming of the immune system, to protect against localised infection from mucosal pathogens including sexually transmitted infections (STIs). Type I Interferons (IFNs) are important cytokines and regulators of the host innate immune response to infections, including STIs. IFN *epsilon* (IFN ϵ) is a novel type I IFN that was discovered in our laboratory and its expression is most abundant in the epithelial cells of the uterus,

where there is constitutive expression unlike other type I IFNs. Importantly, using IFN $\epsilon^{-/-}$ mice we have demonstrated that this novel cytokine protects mice from experimental models of STIs - *Chlamydia muridarum* and HSV-2, and regulates levels of FRT-NK cells, a subset of innate lymphoid cells (ILCs). We now extend our mechanistic studies to assess the role of type I IFNs including IFN ϵ in the induction and regulation of resident mucosal ILC subsets in the FRT. ILCs have been demonstrated as key regulators/effectors of mucosal immune responses in the lung and the intestine. However, the role of these cells in the physiology and infection of the FRT has not been fully elucidated. We now characterise the composition and function of ILC subsets in the hormone regulated FRT. Importantly, we demonstrate type I IFN regulation of FRT-ILC subsets. These studies may identify new therapeutic strategies for targeting and manipulating the innate immune response in disease, through regulation of the endogenous control mechanisms.

325**The role of the P2X7 receptor in an imiquimod-induced psoriasis-like mouse model***Geraghty, N.^{1,2}, Watson, D.^{1,2}, Fuller, S.³, Sluyter, V.^{1,2}, Sluyter, R.^{1,2}**¹University of Wollongong, Wollongong, Australia, ²Illawarra Health and Medical Research Institute, Wollongong, Australia,**³University of Sydney, Medical School Nepean, Sydney, Australia*

Psoriasis is a chronic inflammatory skin disorder that manifests as plaque- or pustular-like lesions due to epidermal hyperplasia and leukocyte infiltration. The damage-associated molecular pattern receptor P2X7 is up-regulated in lesional psoriatic skin of humans and promotes inflammatory immune responses characteristic of psoriasis in human skin explants. This study investigated the role of P2X7 in the development of imiquimod-induced psoriasis-like inflammation in mice. AldaraTM cream (containing 5% imiquimod) was applied daily to the ears of mice for 6 days (days 0-5) to induce psoriasis-like inflammation. Ear swelling was measured daily; from days 0 to 6. On day 6, epidermal hyperplasia and cutaneous inflammation were assessed by histology, and leukocyte percentages (T cells, macrophages and neutrophils) determined by flow cytometry. Imiquimod treatment induced ear swelling in both BALB/c and C57BL/6 mice. However, compared to BALB/c mice, ear swelling was significantly reduced in C57BL/6 mice, which encode a partial loss-of-function (P451L) mutation in the *P2RX7* gene. Epidermal hyperplasia and cutaneous inflammation in imiquimod-treated skin was similar between BALB/c and C57BL/6 mice. Imiquimod-induced ear swelling, epidermal hyperplasia, and cutaneous inflammation were similar between C57BL/6 (wild-type) and P2X7 knockout mice. Further, imiquimod-treated skin demonstrated increased percentages of T cells (CD45⁺CD3⁺), macrophages (CD45⁺Ly6G⁺CD11b⁺), and neutrophils (CD45⁺Ly6G⁺CD11b⁺), which were similar between C57BL/6 (wild-type) and P2X7 knockout mice. Collectively, this study demonstrates that although genetic deletion of the *P2RX7* gene does not prevent imiquimod-induced psoriasis-like disease in this mouse model, investigation into the role of P2X7 in human psoriasis pathogenesis is warranted.

326**Characterising the novel regulation and function of interferon epsilon in the female reproductive tract**

Bourke, N.¹, U-Shane, S.¹, Lim, S.², DeWeerd, N.², Marks, Z.², White, M.², Mesiano, S.², Gargett, C.³, Mangan, N.², Hertzog, P.^{2,4}

¹Hudson Institute of Medical Research, Centre for Innate Immunity and Infectious Diseases, Clayton, Australia, ²Hudson Institute of Medical Research, Centre for Innate Immunity and Infectious Diseases, Clayton, Australia, ³Hudson Institute of Medical Research, The Ritchie Centre, Clayton, Australia, ⁴Monash University, Melbourne, Australia

We identified a novel type I interferon (IFN), IFN ϵ , which differs from other family members as it is constitutively expressed in the female reproductive tract (FRT) and not pathogen regulated in mice, where it is a critical regulator of FRT immunity. IFN $\epsilon^{-/-}$ mice are more susceptible to sexually transmitted infections (STIs) and IFN ϵ regulates FRT innate and adaptive immunity (Fung et al, *Science* 2013). However its expression, regulation and function in the human FRT has not previously been characterised. We have developed novel reagents (recombinant protein, monoclonal antibodies) to fully characterise the regulation and function of IFN ϵ in humans. We have generated a panel of monoclonal antibodies and found IFN ϵ expressed throughout the human FRT, however expression was in different epithelial layers between the upper FRT (endometrium, fallopian tube) and lower FRT (ectocervix, vagina), indicating that IFN ϵ may have different functions at these sites. We also found that the sex hormone progesterone (P4) inhibits expression of IFN ϵ *in vitro* and that *in vivo*, IFN ϵ levels are lower in P4 dominated secretory phase of menstrual cycle, when the FRT is known to be most susceptible to STIs. As P4 based contraceptives have previously been linked with increased susceptibility to STIs (including HIV), we are now examining IFN ϵ levels in women on hormone based contraceptives. We are also exploring the local immune responses directly activated by IFN ϵ in the FRT. In summary, IFN ϵ is highly expressed in the human FRT and emerging as a potential key modulator of FRT mucosal immune responses.

327**Identifying the role of fruit phytochemicals in modulating allergen-induced lung inflammation**

Shaw, O.M.^{1,2}, Sawyer, G.¹, Harper, J.L.², Hurst, R.D.¹

¹The New Zealand Institute for Plant & Food Research Ltd, Food & Wellness, Palmerston North, New Zealand, ²The Malaghan Institute for Medical Research, Inflammation and Arthritis, Wellington, New Zealand

Epidemiological evidence indicates that increased fruit and vegetable consumption can improve lung function and reduce airway inflammation. Research also indicates that these benefits are associated with the modulation of the immune system rather than with reduced free-radical activity. Using a mouse model of allergen-induced lung inflammation, we sought to determine the effects of a number of fruits on acute lung inflammation and to identify potential mechanisms of action. We found that New Zealand-grown blackcurrants high in delphinidin anthocyanin

glycosides, significantly ($P < 0.05$) inhibited ovalbumin-induced epithelial production of the eosinophil chemokine, CCL11, and a 38% decrease in lung eosinophilia. Boysenberry, with a high cyanidin:delphinidin glycoside ratio, inhibited allergen-induced eosinophil infiltration by 63% in conjunction with reduced inducible nitric oxide synthase (iNOS) and increased arginase, but did not inhibit production of the allergy-related mediators CCL11, IL-4, IL-13, or IgE. In contrast, a Boysenberry preparation containing low cyanidin glycosides effectively inhibited CCL11 and reduced eosinophil infiltration by 44% into the lung. Interestingly, a combined preparation of blackcurrant and low cyanidin Boysenberry administered at sub-therapeutic concentrations still reduced allergen-induced cell infiltration by 38%, but had no effect on CCL11 production. This study shows that different individual fruits can protect against lung inflammation via different mechanisms of action which may be related to their phytochemical compositions. Combining these fruits may provide synergistic therapeutic benefits in protection from airway inflammation.

328**MicroRNAs mediate the crosstalk between the cytokines prostaglandin E2/IL-6 in the inflammatory tumor microenvironment**

Li, P., Zhu, Z.

Shanghai Jiaotong University, Ruijin Hospital, Shanghai, China

The inflammation in the tumor microenvironment contributes to the progress of tumor. The inflammatory cytokines are key messengers. Prostaglandin E2 (PGE2) and interleukin (IL)-6 signal pathways are involved in the crosstalk between tumor and stromal cells. However, how PGE2-mediated signaling modulates this crosstalk remains unclear. Here, we show that microRNA (miR)-149 links PGE2 and IL6 signaling mediating the crosstalk between tumor cells and CAFs in gastric cancer (GC). miR-149 inhibited fibroblast activation by targeting IL6 and miR-149 expression was substantially suppressed in the CAFs of GC. miR-149 negatively regulated CAFs and their effect on GC development both *in vitro* and *in vivo*. CAFs enhanced epithelial to mesenchymal transition (EMT) and the stem-like properties of GC cells in a miR-149-IL6-dependent manner. In addition to IL6, PGE2 receptor 2 (PTGER2/EP2) was revealed as another potential target of miR-149 in fibroblasts. Furthermore, *H. pylori* infection, a leading cause of human gastric cancer, was able to induce cyclooxygenase-2 (COX-2)/PGE2 signaling and to enhance PGE2 production, resulting in the hypermethylation of miR-149 in CAFs and increased IL6 secretion. Our findings indicate that microRNA mediates the crosstalk between the cytokines PGE2 and IL-6 in GC and highlight the potential of interfering miRNAs in stromal cells to improve cancer therapy. These findings shed light on the mechanism of action of the PGE2-IL-6 activation loop and further highlight the significance of this loop in tumorigenesis.

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Siglec1 feedback suppresses antiviral innate immune response by inducing TBK1 degradation via the ubiquitin ligase TRIM27

Zheng, Q.¹, Hou, J.², Zhou, Y.², Yang, Y.¹, Xie, B.³, Cao, X.^{1,2,3}

¹National Key Laboratory of Medical Molecular Biology & Department of Immunology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Beijing, China, ²National Key Laboratory of Medical Immunology & Institute of Immunology, Second Military Medical University, Shanghai, China, ³Institute of Immunology, Zhejiang University School of Medicine, Hangzhou, China

Type I interferon (IFN) production plays pivotal roles in host antiviral innate immune response, but excessive production of type I IFN leads to the development of immunopathological conditions. The mechanisms for tightly regulating host type I IFN production are currently of great interest. Here, we found that lectin family member Siglec1 expression was upregulated by viral infection in macrophages, which was dependent on IFNAR/JAK/STAT1 signaling pathway. Siglec1 was then identified to negatively regulate viral infection-triggered type I IFN production. Mechanistically, Siglec1 associates with DAP12 to bind and activate the scaffolding function of SHP2, and then SHP2 recruits E3 ubiquitin ligase TRIM27, which induces TBK1 degradation via K48-linked ubiquitination at Lys251 and Lys372. Therefore, viral infection-induced upregulation of Siglec1 feedback inhibits type I IFN production in antiviral innate response. Our study outlines a new way of negative regulation of type I IFN production, which may help virus to escape immune elimination.

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Exploring the immunosuppressive potential of venom-derived molecules

Ryan, R.^{1,2}, Ikonomopoulou, M.^{1,3}, Haigh, O.¹, Lutzky, V.¹, Jimenez Martinez, R.^{1,2}, Ratnatunga, C.^{1,3}, Wong, Y.^{1,3}, Leal Rojas, I.⁴, Lopez, A.^{1,2}, Fry, B.³, Herzig, V.³, King, G.³, Miles, J.^{1,3}

¹QIMR Berghofer, Brisbane, Australia, ²Griffith University, Brisbane, Australia, ³University of Queensland, Brisbane, Australia, ⁴Translational Research Institute Australia, Brisbane, Australia

The life threatening danger posed by the bite or sting of a venomous animal is due to the unique combinations of potent, specific, and fast-acting molecules contained within venom. A single drop of venom may contain thousands of unique molecules, each with pre-programmed mechanism of action. These molecules rapidly disrupt vital biological processes in predators and/or prey. Ironically, the same characteristics that make venom deadly offer an ideal platform for the exploration of novel therapeutics and biological pathways. As such, venom represents a vast, largely unexplored source of novel drug discovery. There are currently six FDA approved venom-derived drugs. Venom-derived compounds could be used to control immune responses through high affinity targeting of surface receptors and immune pathways. This study involved mapping potent new immunomodulating compounds via the systematic scanning of a large venom BioBank across peripheral blood

mononuclear cells. The anti-inflammatory effects of venoms, crude venoms, and synthetic venom peptides were quantified using multiplex CBA and LEGENDplex. The results showed that certain venom components could potentially neutralise the effects of mitogen-induced production of various pro-inflammatory cytokines. Apoptosis detection assays showed that cytokine modulation was not due to cytotoxicity and multiparametric flow cytometry show that the compounds acted on defined immune cell lineages. Studies examining the mode of action are ongoing. Indeed, synthetic production of these bioactive molecules could catalyse the development of venom peptides as simple, fast-acting drugs for controlling or fine-tuning the function of the human immune system.

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IL-6 deficiency results in specific cytokine responses in a mouse model of irritant contact dermatitis

Gallucci, R., Lockett-Chastain, J., Calhoun, K., Fremph, B.

University of Oklahoma, Pharmaceutical Sciences, Oklahoma City, United States

Irritant contact dermatitis (ICD) is an acute inflammatory response in skin to chemical irritants that is ranked the 2nd most prevalent occupational injury associated with workman's compensation. Interleukin-6 (IL-6) is a pro-inflammatory cytokine that is closely associated with healing and decreased levels are associated with more severe ICD. Inflammatory cytokines can effect the course and severity of ICD by mediating cell type, number, and activity of infiltrate following irritant exposure. To examine the role of IL-6 in the cytokine response during ICD, IL-6KO and C57 mice were exposed to the irritant benzoalkonium chloride (BKC) for up to 7 days. Skin protein was extracted and evaluated via multiplex (36 plex) assay. IL-6KO mouse skin displayed expression profiles and dynamics quite varied from WT. Many proinflammatory cytokines such TNF-alpha, IFN-gamma, and IL-1beta were not significantly different between strains. However, several anti-inflammatory or pro-healing cytokines/chemokines were decreased in IL-6KO skin including, IL-10, IL-13, IL-22, CXCL5 and CXCL10. Additionally, mRNA expression of alternatively activated macrophage cytokine/chemokine associated receptors such as IL-4R and CCR1 were significantly decreased in IL-6KO mouse skin. Overall, these results further confirm a role for IL-6 in determining the cytokine milieu that may determine the severity and affect the resolution of ICD.

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Investigating venom-derived molecules that augment human immune function

Jimenez-Martinez, R.^{1,2}, Ikonomopoulou, M.^{1,3}, Haigh, O.¹, Lutzky, V.¹, Leal-Rojas, I.⁴, Ryan, R.^{1,2}, Ratnatunga, C.^{1,3}, Wong, Y.^{1,3}, Lopez, A.^{1,2}, Fry, B.³, Herzig, V.³, King, G.³, Miles, J.J.^{1,3}

¹QIMR Berghofer Medical Research Institute, Brisbane, Australia, ²Griffith University, Brisbane, Australia, ³University of Queensland, Brisbane, Australia, ⁴Translational Research Institute Australia, Brisbane, Australia

The millions of bioactive compounds found within animal venoms are a veritable war chest of novel immunotherapeutics. Recent discoveries have resulted in six venom-derived drugs currently approved by the FDA for diseases such as blood disorders and diabetes. Venom-derived compounds are known to be highly potent and highly selective for ion channels as well as other key biological pathways. To date only an infinitesimally small fraction of venom-derived compounds have been investigated for medical applications. The aim of this project is the systemic scan of a vast venom biorepository to map novel venom-derived peptides with immunomodulatory function. Using resting and stimulated human leucocytes, we performed high-throughput, multiplex scanning of venom activity using Cytometric Bead Array readout. We found 20% of crude venoms activated or enhanced cytokine production between 2-1,500 fold. From the top crude venom hits, we found candidate compounds that enhanced T cell proliferation to greater extent than classical adjuvants. We performed reverse-phase high-performance liquid chromatography (RP-HPLC) and identified the active venom fractions. Using Matrix-assisted laser desorption/ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) we mapped the purity and molecular weights of the bioactive venom compounds. Current research aims to determine the sequence of these immunomodulatory peptides using Edman degradation and produce them synthetically for mode-of-action studies and further *in vitro* and *in vivo* characterization. We anticipate this research will help catalyze development of new classes of peptide-based immunostimulatory compounds to help augment vaccine effectiveness and enhance effects of current and future immunotherapeutic strategies.

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Cigarette smoke induces mtROS-dependent senescence of CD4+ Th17 lymphocytes

Kerbrat, S.^{1,2}, Baskara, I.¹, Dagouassat, M.^{1,2}, Delost, M.^{3,4}, Henry, A.^{1,2}, Baillou, C.^{3,4}, Decrouy, X.^{1,2}, Lemoine, F.^{3,4}, Boczkowski, J.^{1,2}, Le Gouvello, S.^{1,2}

¹Université Paris Est Créteil, Créteil, France, ²Inserm U 955, Créteil, France, ³UPMC, Paris, France, ⁴Laboratoire de Biothérapies de l'Hôpital Pitié-Salpêtrière, Paris, France

Introduction: The pathogenic role of CD4+ T lymphocytes (LTCD4+) producing IL-17A (Th17) is described in inflammatory diseases, such as MS, PR, COPD, and cancer. Smoking, which is a pejorative parameter for these diseases, is associated with systemic and pulmonary Th17 expansion. We evaluated the potential role of cigarette smoke (CS)-induced oxidative stress in the modulation of senescence and inflammatory potential of Th17.

Methods: CCR6+ Th17 and other "non-Th17" LTCD4+ of healthy donors are exposed to cigarette smoke extract (CSE). ROS production is measured by flow cytometry analysis of H2DCF-DA oxidation. Immunofluorescence analysis of p16 expression evaluated senescence. Inflammatory response is evaluated by expression of relevant genes (RT-qPCR).

Results: CSE induces higher production of ROS and expression of p16 in Th17 compared to other LTCD4+ subsets. Expression of TNF α is more increased in CSE-exposed Th17. Treatment with

the antioxidant NAC or with the mitochondrial uncoupling drug, FCCP, reduced CSE-induced ROS in all LTCD4+ cell subsets, but the higher production of ROS in Th17 compared to other LTCD4+ is maintained. Conversely, FCCP treatment reduced p16 expression to the same level in Th17 as in other CD4+ T cell subsets, whereas NAC diminished p16 expression without changing the differences between LTCD4+ subsets.

Conclusion: Our results show that Th17 present a higher susceptibility to CS-induced mtROS-dependent senescence compared to other LTCD4+. We demonstrate for the first time that mitochondrial uncoupling is effective to dampen Th17 senescent phenotype in oxidative stress conditions, and as such, identify a potential new therapeutic anti-inflammatory strategy for Th17-associated chronic diseases.

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Macrophage migration inhibitory factor could be involved in early inflammatory phase of gouty arthritis

Kim, H.-R., Lee, S.-H.

Konkuk University School of Medicine, Rheumatology, Seoul, Korea, Republic of

Objectives: This study aimed to determine monosodium urate (MSU)-induced MIF production and the role of MIF in gouty arthritis.

Methods: Peripheral blood and clinical data were obtained from 98 patients (31 patients with acute gouty arthritis and 67 patients with intercritical gout). Synovial fluid (SF) was obtained from patients with acute gouty arthritis and SF white blood cells and neutrophils were counted. SF and serum levels of MIF, interleukin (IL)-1, IL-8 and leukotriene B₄, were measured using enzyme-linked immunosorbent assay (ELISA) and their relationship was analyzed. Peripheral blood mononuclear cells (PBMC) were isolated and cultured with MSU crystal, the production of MIF was determined.

Results: SF MIF level was higher in acute gouty arthritis than osteoarthritis. Serum MIF was higher in patients with intercritical gout than patients with acute gouty arthritis or healthy volunteers. SF MIF level was positively correlated with SF WBC and neutrophil counts, IL-1 β and IL-8 levels in acute gouty arthritis. Serum MIF level was correlated with IL-1 β and IL-8 levels in patients with intercritical gout. MSU crystal induced MIF production in PBMC with maximal effect at 100mg/ml.

Conclusions: MIF was highly produced in gouty arthritis and MSU induced MIF production. MIF could be involved in early inflammatory phase in acute gouty arthritis.

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Functional interplay between type I and II interferons is essential to limit influenza A virus-induced tissue inflammation

Stifter, S.^{1,2}, Bhattacharyya, N.^{1,2}, Feng, C.^{1,2}

¹The University of Sydney, Discipline of Infectious Diseases and Immunology, Sydney, Australia, ²The Centenary Institute, Tuberculosis Research Program, Sydney, Australia

Influenza A virus infection is characterised by exuberant

pulmonary inflammation due to the influx of monocytes and neutrophils. It is unclear however, which components of the host immune response regulate this process. Our recent work shows that in addition to its known anti-viral activity, STAT1 signaling coordinates host inflammation during IAV infection in mice. This regulatory mechanism is dependent on both type I IFN and IFN- γ receptor signaling and, importantly, requires functional interplay between the two pathways. As such, type I IFNs act to promote monocyte recruitment to the lungs of infected mice as well as to prevent excessive activation of monocytes by IFN γ . Interestingly, we show that type I IFNs preferentially regulate IFN γ signalling in Ly6C^{lo} compared to Ly6C^{hi} mononuclear cell populations. In the absence of type I IFN signaling, Ly6C^{lo} monocytes/macrophages become phenotypically and functionally more proinflammatory than Ly6C^{hi} cells, revealing an unanticipated function of the Ly6C^{lo} mononuclear cell subset in tissue inflammation. In addition to their effects on mononuclear cells, type I IFNs also suppress the recruitment of neutrophils into IAV-infected tissues, however the full neutrophil-suppressive effect requires the co-operation of both type I and II IFNs. Our study demonstrates that IFN interplay links innate and adaptive anti-viral immunity to orchestrate tissue inflammation and reveals an additional level of complexity for IFN-dependent regulatory mechanisms that function to prevent excessive immunopathology while preserving anti-microbial functions.

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Disruption of TNF receptor assembly elicits an IL-27 response to suppress T_H17-mediated experimental colitis in an IL-10-dependent manner

Fu, S.-H.¹, Lin, M.-H.², Wang, Y.-L.³, Yeh, L.-T.¹, Sytwu, H.-K.¹

¹National Defense Medical Center, Department and Graduate Institute of Microbiology and Immunology, Taipei, Taiwan, Republic of China, ²Kaohsiung Medical University, Department of Microbiology and Immunology, Institute of Medicine, College of Medicine, Kaohsiung, Taiwan, Republic of China, ³Chang Gung Memorial Hospital, Center for Vascularized Composite Allotransplantation, Taoyuan, Taiwan, Republic of China

The soluble pre-ligand assembly domain (PLAD) of tumor necrosis factor receptor 1 (TNFR1) interferes with receptor trimerization to block downstream signaling and mediates Th17 suppression. We explored therapeutic effect of recombinant PLAD.Fc protein on a spontaneous experimental colitis model. A T cell-specific BLIMP-1 knockout mouse model was established and characterized as a Th1/Th17-mediated colitis, resembling Crohn's disease. Mice at 9 weeks of age were treated with PLAD.Fc protein at 5 mg/kg of body weight twice per week for 16 weeks, and the presence of colitis was monitored by the appearance of diarrhea, weight loss and histological scoring of colon pathology. Activation status, cytokine profiles, and transcription factors in T cells were further analyzed. In PLAD.Fc-treated group, the weight lost was recovered and mild disease score revealed by a low level of cellular infiltrate, moderate crypt elongation and reduced thickening of the bowel wall. PLAD.Fc downregulates T cell pathogenicity by suppressing the levels of IFN- γ , IL-17, IL-21, IL-22, IL-23R, GM-CSF and TNF- α in CD4⁺ T cell.

Moreover, IL-27 expression level was induced at an extremely high level along with dramatically increased production of IL-10 in sera from PLAD.Fc-treated mice. The proportions and expression levels of IL-10 and IL-10R in Th1 and Th17 cells were significantly higher in the lymphoid organs of PLAD.Fc-treated mice. Furthermore, PLAD.Fc-restraining pathogenic phenotype of Th1 and Th17 cells is retracted when IL-10 is neutralized. PLAD.Fc protects BLIMP-1-deficiency mice from colitis by eliciting an IL-27 response and promoting IL-10 production by effector T cells as a self-regulatory mechanism.

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Peripheral kappa-opioid receptor (KOR) stimulation exerts anti-inflammatory effects in lipopolysaccharide (LPS)-activated macrophages

Fazalul Rahiman, S.S.^{1,2}, Morgan, M.³, Gray, P.^{4,5}, Shaw, P.¹, Cabot, P.¹

¹The University of Queensland, School of Pharmacy, Brisbane, Australia, ²Universiti Sains Malaysia, School of Pharmaceutical Sciences, Penang, Malaysia, ³The University of Queensland, Institute for Molecular Bioscience, Brisbane, Australia, ⁴The University of Queensland, School of Medicine, Brisbane, Australia, ⁵Princess Alexandra Hospital, Department of Anaesthesia, Brisbane, Australia

Under inflammatory conditions, kappa opioid receptors (KOR) are upregulated at peripheral sensory neurons accompanied with production of opioid peptides from leukocytes. Activation of KOR by kappa (κ)-agonists has been shown to regulate immune responses in addition to producing analgesia. This study aimed to examine the effects of KOR stimulation by selected κ -agonists in LPS-activated macrophages. Phorbol 12-myristate 13-acetate (PMA)-differentiated THP-1 cells were established as an *in vitro* model for macrophages in inflammation. LPS (1 μ g/mL) was used to induce the activation of nuclear factor-kappa B/p65 (NF- κ B/p65) translocation and the release of interleukin-1 β (IL-1 β) and tumor necrosis factor-alpha (TNF- α) followed by co-incubation with dynorphin 1-17, dynorphin 1-7 and U50,488H at specified concentrations and duration. Modulation of the above inflammatory mediators was also examined in the presence of ML-190, a selective KOR antagonist. High-content cellular imaging was used to quantify the nuclear localisation of immunolabelled NF- κ B/p65. The levels of cytokine release were determined using a commercial AlphaLISA kit for IL-1 β and TNF- α . Results showed that dynorphins (1-17 and 1-7) and U50,488H attenuated NF- κ B/p65 nuclear translocation. The release of IL-1 β and TNF- α was inhibited by dynorphin 1-7 and U50,488H whereas DYN 1-17 only reduced IL-1 β without any effect on TNF- α release. Inhibition of NF- κ B/p65 and cytokine release by κ -agonists was significantly antagonised by ML-190. These findings indicate that peripheral activation of KOR exerts anti-inflammatory properties in LPS-initiated signal transduction in macrophages through inhibition of NF- κ B/p65 nuclear translocation and pro-inflammatory cytokine production, thereby limiting the exacerbation of inflammation.

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PEP-1-SIRT2 causes COX-2 expression via the MAPK pathways in rabbit articular chondrocytes

Kim, S.-J.

Kongju National University, Gongju, Korea, Republic of

SIRT2 is a member of the mammalian sirtuin protein family, primarily found in the cytoplasm. It regulates numerous cellular processes including aging, DNA repair, cell cycle, and survival under stress conditions. However, the biological function and mechanism of the SIRT2 protein is not well understood in normal cells such as primary chondrocytes. In this study, we examined the effects of SIRT2 on inflammation in rabbit articular chondrocytes by using a cell-permeative PEP-1-SIRT2 protein. Our results indicate that PEP-1-SIRT2 caused an inflammatory response by inducing the expression of cyclooxygenase-2 (COX-2) and prostaglandin E₂ (PGE₂). In addition, after treatment with PEP-1-SIRT2, phosphorylation of both p38 and ERK was observed. Inhibition of ERK with PD98059 (PD) suppressed PEP-1-SIRT2-induced COX-2 expression. Reduction in PEP-1-SIRT2-induced inflammatory response was observed upon inhibition of p38 by SB203580 (SB). The same pattern was demonstrated in PEP-1-SIRT2-induced inflammatory response during culture with serial passages. During expansion to four passages, levels of COX-2 and SIRT2 increased and activated ERK and p38. Furthermore, PEP-1-SIRT2 enhances and inflammatory response through the ERK and p38 pathways in rabbit chondrocytes in vitro. These findings suggest that PEP-1-SIRT2 induces inflammation through the p38 and ERK pathways in rabbit articular chondrocytes.

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Proinflammatory cytokines production and metal hypersensitivity in patients with failed orthopedic implants

Podzimek, S., Prochazkova, J., Janatova, T., Vinsu, A., Bartova, J., Duskova, J.

School of Dental Medicine, First Faculty of Medicine and General University Hospital, Charles University, Prague, Czech Republic

In most of patients orthopedic implants heal well, nevertheless, there are some individuals, in whom orthopedic implants fail for reasons, which remain unclear.

The aim of our study was to determine metal intolerance and to monitor production of selected pro-inflammatory cytokines in patients with failed orthopedic implants and in patients with allergy to metals and with need of orthopedic implant surgery. The study groups included 35 patients with failed orthopedic implant and 15 patients with suspected allergy to metals and with need of orthopedic implant surgery. Cytokine production after lymphocyte cultivation with chromium, cobalt, titanium and zirconium antigens was determined.

Direct correlation between implant failure and intolerance to its composition in approximately 40 % of patients with failed implants has been determined. In these patients, production of pro-inflammatory cytokines was influenced the most by stimulation by titanium in patients with diagnosed titanium intolerance. Titanium exposure in patients with titanium intolerance will likely lead to titanium implant failure.

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Virus infections impact structural bone cell populations

Maltby, S.^{1,2}, Lochrin, A.^{1,2}, Donges, B.^{1,2}, Tay, H.^{1,2}, Plank, M.^{1,2}, Stewart, J.^{1,2}, Foster, P.^{1,2}

¹Priority Research Centre for Healthy Lungs, Newcastle, Australia,

²Hunter Medical Research Institute, Department of Microbiology and Immunology, School of Pharmacy and Biomedical Sciences, Faculty of Health, Newcastle, Australia

Virus infections cause a variety of symptoms and negatively impact health. We have a detailed understanding of how the immune system responds at sites of infection to destroy viruses. However, growing evidence suggests that viruses also have long-term effects throughout the body, including changes in the bone marrow. The bone marrow serves as the site of immune cell production and also houses cells that maintain bone structure. Regulation of the bone marrow must be delicately balanced to deal with infection, while maintaining long-term bone health. Our previous studies identified increased myeloid cell production as a key feature of infection. This occurs following acute lung infection with pneumonia virus of mice (PVM) and in early stages of systemic lymphochoriomeningitic virus (LCMV) infection. Intriguingly, we also noted decreased structural bone cell populations (including osteoblasts) following infection. Osteoblast numbers decrease during stages of increased systemic inflammation. During chronic LCMV infection, osteoblast numbers remain decreased well after systemic inflammation resolves. Our current studies are addressing the mechanisms underlying these changes and the functional consequences on bone integrity. While bone structural cells are obviously important for the long-term maintenance of bone integrity, they also play key roles regulating hematopoiesis. The regulation of these cells is important for the development of appropriate immune responses to clear infection and directing pathology induced by infection.

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Contact: Steven Maltby - steven.maltby@newcastle.edu.au

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Role of IL-21 in the development of autoimmune myositis

Kageyama, T., Iwamoto, T., Tanaka, S., Suto, A., Nakajima, H.

Graduate School of Medicine, Chiba University, Allergy and Clinical Immunology, Chiba, Japan

Objective: IL-21 has been shown to be involved in the development of various autoimmune diseases; however, the role of IL-21 in autoimmune myositis remains unknown. In this study, we investigated the role of IL-21 in the development of autoimmune myositis.

Methods: We examined serum levels of IL-21 in patients

with inflammatory myopathies and healthy donors. We also investigated the roles of IL-21 in the development of experimental autoimmune myositis (EAM) by using IL-21-deficient mice.

Results: Serum levels of IL-21 were significantly increased in patients with inflammatory myopathies as compared to those in healthy donors ($n = 17$, each, $p < 0.05$). Upon the induction of experimental autoimmune myositis, muscle weakness was less obvious in IL-21-deficient mice as compared to that in wild-type (WT) mice. While the numbers of CD4⁺ T cells, CD8⁺ T cells, and B cells in the affected muscle were similar between EAM-induced IL-21-deficient mice and WT mice, the number of CD11b⁺ cells was significantly decreased in the muscle of EAM-induced IL-21-deficient mice. The numbers of germinal center B cells and GM-CSF-producing gd T cells in the draining lymph nodes were significantly decreased in EAM-induced IL-21-deficient mice as compared to those in EAM-induced WT mice. GM-CSF production was significantly impaired in IL-21-deficient CD27- gd T cells.

Conclusion: IL-21 is involved in the development of autoimmune myositis presumably by inducing the production of GM-CSF in gd T cells.

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Opuntiol/Opuntioside-I: a novel suppressor of cytokine, chemokine and lipid mediators in in-vivo and in-vitro inflammatory models

Roome, T.¹, Ali, P.¹, Aziz, S.¹, Razzak, A.¹, Faizi, S.², Abidi, L.², Lubna, L.²
¹Dow Diagnostics Reference and Research Laboratory, Dow International Medical College, Dow University of Health & Sciences, Molecular Pathology, Department of Pathology, Karachi, Pakistan,
²H.E.J Research Institute of Chemistry, Dr. Panjwani Center of Molecular Medicine and Drug Research, International Centre for Chemical and Biological Science, University of Karachi, Karachi, Pakistan

Cytokines are signaling proteins including chemokines, interleukins, interferons, lymphokines and tumor necrosis factor that induce immune-based inflammatory responses. Elevated levels of cytokines provoke a broad range of cellular and physiological response and inflammation. *Opuntia dillenii* belongs to genus *Opuntia* have shown remarkable effects as an anti-inflammatory agent. In the present investigation we have evaluated novel compounds Opuntiol and Opuntioside-I derived from *Opuntia dillenii* have shown significant decrease in various cytokines (interleukins) and chemokines in a dose dependent manner. Cytokines i.e. Tumor necrosis factor- α (TNF- α), Interleukin 1 β (IL-1 β), interleukin 6 (IL-6), interleukin 10 (IL-10) and chemokine i.e. keratinocyte chemottractant (KC), JE, and monocyte chemottractant factor-1 (MCP-1) were evaluated in the absence and presence of opuntiol and opuntioside-I administered orally 60 minutes prior to induction of peritonitis via carrageenan and zymosan, respectively using ELISA. At highest dose 20mg/kg Opuntiol/Opuntioside-I inhibited IL-1 β , IL-6, TNF- α , JE, KC and MCP-1 by 50-90%, whereas IL-10 levels were unchanged in all doses. Additionally, marked decline in mRNA expressions of pro-inflammatory cytokines (TNF, IL-1 β , IL-6, IL-10) and chemokines (KC and MCP-1) were observed after i.p. treatment with 20mg/kg of Opuntiol/Opuntioside-I during

zymosan induced peritonitis using RT-PCR. IL-1 β , IL-6 and TNF- α activating NF-KB signaling pathway induced by JAK/STAT and I κ B pathways which proceed with upregulation of various inflammatory gene such as COX2 and iNOS, growth factors, cytokines etc. Hence, Opuntiol/Opuntioside-I inhibiting the levels and reducing the expression of aforementioned cytokines and chemokine can be a potential immunomodulatory agent against Asthma, Rheumatoid arthritis, Atherosclerosis, Chronic bronchitis, Psoriasis etc.

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The effect of glomerulus specific over-expression of TGF- β 1 in streptozotocin-induced diabetic mouse model

Yasuma, T.¹, Nishihama, K.², Takeshita, A.², Hinneh, J.¹, Harada, E.¹, D'Alessandro-Gabazza, C.¹, Toda, M.¹, Yano, Y.², Gabazza, E.¹
¹Mie University Graduate School of Medicine, Immunology, Tsu, Japan, ²Mie University Graduate School of Medicine, Diabetes and Endocrinology, Tsu, Japan

Objective: Diabetic nephropathy (DN) is one of the major microvascular complications of diabetes and one of the leading causes of death among patients with diabetes, but the efficacy of available strategies for the prevention of DN remains poor. TGF- β (transforming growth factor- β) is well identified as a central mediator in fibrosis. We prepared glomerulus specific TGF- β 1 transgenic mice and evaluated the effect of TGF- β 1 in streptozotocin (STZ)-induced diabetic mouse model.

Methods: We injected saline (SAL) or STZ to podocin-hTGF- β 1 transgenic mice (hTGF- β 1Tg) and control mice (WT). Mice were divided into 4 groups (WT/SAL, WT/STZ, hTGF- β 1Tg/SAL, hTGF- β 1Tg/STZ). Blood glucose and insulin were measured. Intraperitoneal glucose tolerance test (IPGTT) was performed. Pathological evaluations of kidneys were done after sacrifice.

Results: Compared with WT/STZ group, blood glucose levels of hTGF- β 1Tg/STZ group were significantly higher. Aggravation of glucose tolerance and higher HOMA-IR were observed in hTGF- β 1Tg/STZ mice compared with WT/STZ mice. Interstitial fibrosis and glomerulosclerosis were histologically more advanced in hTGF- β 1Tg/STZ mice compared with WT/STZ mice.

Conclusion: Glomerulus specific over-expression of TGF- β 1 causes nephropathy more rapidly in streptozotocin-induced diabetic mouse model. This TG mouse can be used to develop diabetic nephropathy mouse model in a short time.

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Discriminating between IDA and ACD using TfR as indicators of iron status an oxydative balance

Zaharov, T.
 Odense University Hospital, H. C. A. Children's Hospital, Odense C, Denmark

The concentrations of the TfR (soluble receptor for transferrin) positively correlated with total mass of tissue receptors, increased in response to a deficiency of iron and, conversely, negatively correlated with the content of cellular iron.

Methods: Blood was collected from children with iron/nutritive deficient anemia, ($n=20$), IDA group, from children with

inflammations and/ or recurrent infections (n=53) with anemia syndrome, ACD group, and from healthy controls (n=19). Serum concentrations of soluble transferrin receptor were determined using the Quantikine[®] Human immunoassay kit (R&D Systems) in nmol/l. Plasma nitric oxide (NO) concentration was measured by Cayman Chemical's photometric test ($\mu\text{mol/L}$).

Results: The TfR concentration was increased (mean \pm SD), $52,48 \pm 21,36$ in IDA patients, and was normal ($29,67 \pm 3,81$) in controls and for more than 55% of the ACD patients ($20,4 \pm 8$). The TfR/log ferritin was significantly low ($p < 0.001$) in the ACD group, $11,82 \pm 5,68$ compared to IDA ($55,46 \pm 33,16$) and controls ($24,40 \pm 3,49$), while NO value were extremely high in the ACD group: 123 ± 41 vs IDA 70 ± 49 ; Control 49 ± 12 .

Conclusion: The determination of sTfR is necessary in the differential diagnosis of these two anemias. One explanation proposed for this inadequate sTfR secretion is that the anemia is adaptive; to reflects a reduced rate of oxygen use by tissues in response to increased oxidative stress induced by pro-inflammatory state of the organism. One colorful description of this process is that the "hematologic thermostat has been turned down a bit".

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Interplay between the pro-oxidant and antioxidant systems (AOS) in relation to iron homeostasis (IH)

Zaharov, T.

Odense University Hospital, H. C. A. Children's Hospital, Odense C, Denmark

As there is strong evidence for inflammation and oxidative stress (OS) in anemia of chronic syndrome (ACS), the aim of this study was to elucidate the relationship between oxidative imbalance and cellular immune response and to ask whether these processes are linked with IH. Blood was collected from 53 children with inflammations and/ or recurrent infections with ACS, from 19 healthy, controls (C), in comparing to 20 children with IDA (iron deficient anemia). Erythrocyte catalase (CAT), erythrocyte superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were assayed spectrophotometrically with Synchron CX5 analyzer, Beckman. Plasma nitric oxide (NO) concentration was measured by Cayman Chemical's photometric test.

Results: (mean \pm SD): GSH-Px (U/gHb): ACS $39,55 \pm 16,55$; IDA $41,09 \pm 11,90$; C $59,66 \pm 11,78$; SOD (U/gHb): ACS: $1046,41 \pm 153,03$; IDA $1200,22 \pm 185,26$; C $1262,8 \pm 187,81$; CAT (U/g Hb): ACS $10,95 \pm 4,63$; IDA $15,05 \pm 4,05$; C $14,85 \pm 5,77$. NO ($\mu\text{mol/L}$) ACS 123 ± 41 ; IDA 70 ± 49 ; C 49 ± 12 . We observed a significant decrease in the level of all three enzyme in the first, ACS group with significance $p < 0,001$ compared to the controls, as well as the activities of CAT and SOD in relation to IDA group, but there was no significant difference in in the level of GSH-Px between IDA and ACS groups.

Conclusions: A strong association between antioxidant defense, OS, and IH was revealed and these findings show that ACS is associated with increased OS. We could indirectly conclude increased RBC liability to lipid peroxidation in ACS. Decreased AOS correlates with an increased cellular inflammatory response, whereas both concur with erythron and iron status.

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Changes in cellular iron metabolism (CIM) during chronic inflammatory states (CIS) leading to iron sequestration manifesting as the anemia of chronic disease (ACD)

Zaharov, T.

Odense University Hospital, H. C. A. Children's Hospital, Odense C, Denmark

ACD is result of a complex network of events, acting at the transcriptional and translational levels to alter the expression of proteins involved in the uptake, storage and ironutilization. This paper focuses on the role of nitric oxide (NO) of CIM during CIS. To determine effects of NO on the expression of sTfR (soluble transferrin receptor) we examined also capacity of RBC antioxydative systems (AOS) in 53 children with CIS (I group) compare with 19 healthy children. STfR value was determined using the Quantikine[®] Human immunoassay kit (R&D Systems), NO easured by Cayman Chemical's photometric test, Erythrocyte catalase (CAT, U/gHb), superoxide dismutase (SOD, U/gHb) and glutathione peroxidase (GSH-Px, U/gHb) were assayed spectrophotometrically with Synchron CX5 analyzer, Beckman.

Results: GSH-Px: $39,55 \pm 16,55$ vs. $59,66 \pm 11,78$; SOD $1046,41 \pm 153,03$ vs. $1262,8 \pm 187,81$; CAT $10,95 \pm 4,63$ vs. $14,85 \pm 5,77$; NO ($\mu\text{mol/L}$): 123 ± 41 vs. 49 ± 12 in strongly negatively correlation with sTfR ($p < 0.001$) $20,4 \pm 8$ vs. $29,67 \pm 3,81$, TfR/log ferritin $11,82 \pm 5,68$ vs $24,40 \pm 3,49$, respectively.

Conclusions: sTfR expression was reduced in relation to high NO and low AOS wich proves that NO is one of the factors launching the complicated network of regulatory signals constituting an attempt to establish an equilibrium that might have role to protect from the potential damages during CIS, by removing "the hammer above the anvil" in the oxidative system. Maybe that we call moderate disease is the attempt of the organism to solve the conflict with the least possible damages. Isn't the interaction between iron homeostasis, NO activity and oxygenation exactly the first regulation attempt?

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The role of gut-derived flagellin following burn injury

Eaves-Pyles, T.^{1,2}, Grimes, L.³, Szabo, C.^{2,4}

¹University of Texas Medical Branch, Microbiology and Immunology, Galveston, United States, ²Shriner's Hospital for Children, Galveston, United States, ³University of Texas Medical Branch, Galveston, United States, ⁴Univeristy of Texas Medical Branch, Anesthesiology, Galveston, United States

Burn injury is associated with a loss of gut barrier function consequentially resulting in systemic dissemination of gut-derived bacteria and their products, namely flagellin. Because we detected flagellin in the serum of burn patients, we investigated whether gut-derived flagellin was a primary or secondary contributor to intestinal dysfunction and systemic inflammation following burn injury. As such, human intestinal epithelial cells (IECs), were exposed to flagellin (50 or 500 ng) and *E. coli* (EC; 1×10^5) that is a gut isolate as follows: 1) flagellin added 30 min prior to EC, 2) flagellin and EC added simultaneously, or 3) EC added 30 min prior to flagellin. Our results showed that luminal

flagellin and EC modulated one another, which influenced cytokine secretion and translocation of EC and flagellin. EC added with or pre-flagellin affected the ability of flagellin to stimulate TLR5 and NF- κ B activation. Additionally, we found marked differences in the gut microbiome of mice gavaged with flagellin then immediately given a 30% burn or burned 2 hours pre- or post-burn. In these mice, gut-derived flagellin induced MCP-1 secretion in the serum, mesenteric lymph nodes and lungs 24 and 48h post-burn. The timing of flagellin gavage and burn altered the gut microbiome changes and induced systemic secretion of cytokines. Together our results demonstrate that gut-derived flagellin can function as a luminal immunomodulator, in addition to being a primary or secondary contributor to systemic inflammation depending on the dose and timing of flagellin's presence in conjunction with normal intestinal flora.

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Macrophage migration inhibitory factor-induced autophagy is involved in thrombin mediated endothelial cell hyperpermeability

Yeh, T.M.¹, Chao, C.-H.²

¹National Cheng Kung University, Medical Laboratory Science and Biotechnology, Tainan, Taiwan, Republic of China, ²National Cheng Kung University, Institute of Basic Medicine, Tainan, Taiwan, Republic of China

Sepsis is a disease in which systemic inflammation-induced vascular leakage and disseminated intravascular coagulation lead to multiple organ failure. Despite a high mortality and morbidity, there is currently no specific drug for sepsis. Thrombin, a serine protease that mediates key sepsis-related pathologies, both activates coagulation cascades and acts as a mediator to induce vascular hyperpermeability and inflammation. Previously, it has been shown that thrombin can induce endothelial cells to secrete macrophage migration inhibitor factor (MIF). MIF is a pro-inflammatory cytokine that is increased in sepsis and can cause endothelial hyperpermeability via autophagy. However, it is unclear whether MIF-induced autophagy is involved in thrombin-mediated vascular hyperpermeability. Herein, we show that thrombin treatment induced hyperpermeability, which was accompanied by VE-cadherin translocation in the human microvascular endothelial cell line (HMEC-1). Additionally, thrombin could induce MIF secretion and autophagy in HMEC-1 cells. Inhibition of MIF by an antibody or inhibitors could reduce thrombin-induced endothelial hyperpermeability and autophagy. However, inhibition of autophagy prevented only endothelial hyperpermeability, not MIF secretion. Blockade of autophagy using Atg5 shRNA further confirmed these findings. Finally, both MIF and autophagy inhibitors reduced thrombin- and lipopolysaccharide-induced vascular leakage *in vitro* and in mice, respectively. Together, our data suggest that autophagy in endothelial cells may represent a common pathway that mediates both thrombin and cytokine-induced vascular leakage in sepsis.

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The effect of Parvoviridae VP1 unique region in H9c2 cardiomyocytes

Tzang, B.-S.^{1,2}, Lin, D.-W.², Shi, Y.-F.², Lin, C.-Y.², Hsu, T.-C.²

¹Chung Shan Medical University, Department of Biochemistry, School of Medicine, Taichung, Taiwan, Republic of China, ²Chung Shan Medical University, Institute of Biochemistry, Microbiology and Immunology, Taichung, Taiwan, Republic of China

Human parvovirus B19 (B19) and Human Bocavirus (HBoV) belong to Parvoviridae, are the only two parvoviruses known to be pathogenic in humans. Both VP1 unique region (VP1u) exhibit the secreted phospholipase A2 (sPLA2)-like enzymatic activity. Previous studies showed that B19 infection may trigger heart disease, such as myocarditis, cardiomyopathy, and pericarditis. Moreover, B19-VP1u may play an important role in the pathogenesis of myocarditis. However, the effect of B19-VP1u and HBoV-VP1u in H9c2 cardiomyocytes is not clear.

Methods: We have constructed the B19-VP1u/B19-VP1uD175A/HBoV-VP1u/ HBoV-VP1uV12A gene in a cytomegalovirus episomal vector, pEGFP-C1 and transfected it into H9c2 cardiomyocytes. Both VP1u and mutant expression in transfected cells were monitored and assessed by fluorescence microscopy, RT-PCR and Western blot.

Results: The expressions of TNF- α , IL-1 β , IL-6, NF- κ B (p-p65), and IKK- α protein were significantly increased in the B19-VP1u-transfected cells than that of the control. Additionally, the expression of IL-1 β and IL-6 proteins was significantly increased in the HBoV-VP1u-transfected cells than that of the control. Notably, the expression of TNF- α and NF- κ B (p-p65) was significantly inhibited by the siRNA in the B19-VP1u-transfected cells than that of the control. No significant variation of TNF- α , IL-1 β , IL-6, NF- κ B (p-p65), and IKK- α protein was observed in cells transfected with pEGFP-B19-VP1uD175A and pEGFP-HBoV-VP1uV12A than that of the control.

Conclusions: Altogether, these findings revealed the different effects of B19-VP1u and HBoV-VP1u on the stimulation of inflammatory signaling in cardiac H9c2 cells. Moreover, B19-VP1u rather than HBoV-VP1u likely plays more important roles in the pathogenesis of B19-related myocarditis.

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Clinicopathologic evaluation of chickens infected with a recombinant virulent clone of Newcastle disease virus containing an insert for IL-4 expression

Marcano, V.^{1,2}, Cardenas Garcia, S.^{1,2}, Susta, L.³, Diel, D.⁴, Miller, P.², Afonso, C.², Brown, C.¹

¹University of Georgia, Veterinary Pathology, Athens, United States, ²Southeast Poultry Research Laboratory, Exotic & Emerging Avian Viral Diseases Research, Athens, United States, ³Ontario Veterinary College, University of Guelph, Department of Pathobiology, Guelph, Canada, ⁴College of Veterinary Medicine, University of Illinois, Department of Pathobiology, Urbana, United States

Newcastle disease (ND) is the most economically important disease of poultry, with velogenic strains causing 100% mortality in less than 5 days in unvaccinated poultry flocks.

It is the high consequence animal pathogen most reported to the OIE, with 61 countries reporting severe outbreaks in 2014. This stresses the need of better understand mechanism of pathogenesis, specifically the role of the innate immune response during infection. Mammalian interleukin-4 (IL-4) is an immunoregulatory cytokine that triggers antigen-specific Th2 responses. IL4 has a wide array of functions most of which ultimately stimulate the humoral immunity. Little is known regarding the role of chicken IL4 (chIL4) during infection, however, there is evidence that chIL4 behaves in a manner similar to its mammalian equivalent.

At the Southeast Poultry Research Laboratory (SEPR) of the USDA we have created a virulent recombinant NDV expressing chIL4 (rZJ1-IL4) during replication to determine if IL4 expressed during challenge in naïve animals is capable of inducing protective immune responses. In this experiment birds were infected with rZJ1-IL4 a control virus, rZJ1-GFP, or a sham vaccine. Clinicopathologic disease was compared between groups. Insertion of the IL4 gene into an infectious NDV clone resulted in successful replication of the virus and generation of IL4 protein along with viral transcription and translation. rZJ1-IL4 infected birds had a slightly less severe but more prolonged form of the disease. Pathologic changes were compared between groups, with notably decreased damage in multiple organs in the rZJ1-IL4 birds, suggesting a decreased innate response in the IL4 group.

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EphA2KO mice survive longer in a mouse model of sepsis

Kumar, V.¹, Woodruff, T.², Ting, M.³, Boyd, A.³, Coulthard, M.¹, Souza-Fonseca-Guimaraes, F.⁴, Smyth, M.⁴

¹University of Queensland, Paediatrics and Child Health, Brisbane, Australia, ²University of Queensland, School of Biomedical Sciences, Brisbane, Australia, ³QIMR Berghofer Medical Research Institute, Leukaemia Foundation Laboratory, Brisbane, Australia, ⁴QIMR Berghofer Medical Research Institute, Immunology in Cancer and Infection Laboratory, Brisbane, Australia

Acute inflammation is characterised by accumulation of tissue fluid, neutrophil infiltration and tissue damage and is mediated by loss of endothelial barrier function resulting in vascular leak. The Eph/ephrin receptor ligand system may play a role in the pathogenesis of inflammation by modifying endothelial barrier function mediated through Rho-GTPase signalling. We cultured pulmonary vascular endothelial cells (PVEC) and exposed them to lipopolysaccharide (LPS) stimulation (10 ng/mL) and measured Eph/ephrins and inflammatory cytokines (TNF- α) by quantitative PCR. Our results show that EphA1, EphA2 and ephrinA1 were upregulated in PVECs in response to LPS stimulation. We also confirmed upregulation of TNF- α gene. Wild type (WT) and EphA2 knockout mice (EphA2 KO) were then subjected to caecal ligation and puncture (CLP) induced sepsis and monitored for survival. All WT mice (n=12) were dead by day 6; however, 25% (n=5) of the EphA2KO (n=19) mice survived to 10 days. Serum pro-inflammatory cytokine levels (i.e. TNF- α) were significantly higher in WT mice compared to EphA2 KO mice at all intervals (i.e. 3 and 12 hours post CLP). However, levels of MIP1 α and MIP1 β were significantly higher at 3 hours post

CLP in WT mice compared to EphA2 KO mice but no significant difference at 12 hours post CLP. We conclude that Eph/ephrin receptor signalling plays a role in mediating inflammation in the CLP infectious model of systemic inflammation (i.e. sepsis), whereby EphA2KO mice have decreased serum TNF- α levels and increased survival. Targeting Eph/ephrin signalling pathways may therefore be a future therapeutic approach in severe sepsis.

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Monocyte inflammatory profile differs between individuals, and altered lipid levels may partly contribute to these differences

Patel, V.¹, Williams, H.¹, Li, S.², Fletcher, J.¹, Medbury, H.¹

¹Vascular Biology Research Centre, Department of Surgery, Westmead Hospital, University of Sydney, Westmead, Australia, ²Institute of Clinical Pathology and Medical Research, Westmead Hospital, University of Sydney, Westmead, Australia

In various diseases, such as atherosclerosis, intermediate monocyte levels are elevated. Considered 'inflammatory', they are a possible treatment target. However, as they remain a minor subset (5-10%), the function of the other subsets in disease is important to discern.

To compare the inflammatory profile of monocyte subsets in individuals and relate this to their lipid levels.

Blood was collected from controls and lipid analysis performed. Monocyte subset expression of M1 and M2 markers and production of cytokines (post LPS or PMA stimulation) was determined by whole blood flow cytometry.

No individual subset was distinctively inflammatory. Upon LPS stimulation, intermediates produced more TNF α , IL-1b and IL-6 than classical and non-classical (all P < 0.001), but with PMA, classicals produced more IL-1b than the other subsets (P < 0.001) -TNF α and IL-6 were not induced. Classicals expressed higher levels of M2 (anti-inflammatory), and lower levels of M1 (inflammatory) markers than the other subsets, suggesting they were less inflammatory. Importantly, a greater variation in cytokine production was seen between participants, than between subsets. Indeed, the level of cytokine production by one subset, correlated with that of the next (all P < 0.01). Differences between individuals were related to their lipid levels. LPS-stimulated IL-1b production (by all subsets) correlated positively with Chol/HDL (P < 0.025), ApoB/A1 (P < 0.005) and inversely with HDL (P < 0.025) and ApoA1 (P < 0.025). Upon PMA stimulation, classical IL-1b correlated with cholesterol, LDL (both P < 0.01) and ApoB (P < 0.025).

Perturbation of lipid levels is associated with inflammatory changes in all monocyte subsets.

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Prolyl endopeptidase (PE) activity in the immune system of smoking mice

Abdalla, T.¹, Abdul Roda, M.², Li, J.-D.¹, Blalock, J.E.^{1,3}, Gaggar, A.^{1,3,4}, Xu, X.^{1,3}

¹University of Alabama at Birmingham, Medicine, Birmingham, United States, ²Utrecht University, Pharmaceutical Sciences, Utrecht, Netherlands, ³UAB Program in Protease and Matrix

Biology, Birmingham, United States, ⁴Birmingham VA Medical Center, Birmingham, United States

Rationale: Prolyl endopeptidase (PE) is expressed in various organs and cleaves collagen into the matrikine proline-glycine-proline (PGP), leading to the ongoing process of neutrophil recruitment. Using cigarette smoke (CS), an environmental stimulus causing elevated PGP levels, airway inflammation, and chronic lung disease, we explored PE levels within the immune system of smoke-exposed mice.

Methods: Balb/c mice pretreated either with saline or N-benzyloxycarbonyl-prolyl-proline (ZPP), a selective inhibitor of PE, were exposed to air or CS for 6 weeks followed by bronchoalveolar lavage analyses. PE activity assays were conducted for spleen and thymus lysate while T and B cell purification was completed via nylon column isolation, allowing for PE activity analysis in specific immune cell types.

Results: Neutrophilic airway inflammation was induced by CS exposure and inhibited in the mice with ZPP treatment. PE activity was elevated in the spleens of CS-exposed mice compared with air-exposed mice and smoking mice treated with ZPP showed complete inhibition of PE activity. The PE activity of isolated splenic T and B cell were measured and T cells showed increased PE activity levels compared to B cells. Thymus lysates were unchanged between treatment groups.

Conclusion: PE activity is strongly modulated in CS-exposed mice in both lung and spleen, highlighting the inhibitory effects of ZPP on smoke-induced inflammation. Further research will continue on the study of the effect of smoke-induced PE activity as a new regulator of both innate and adaptive immune responses in the lung.

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Efficacy of activated vitamin D analogues paricalcitol for acute dextran sodium sulfate-induced colitis in mice

Chen, T.¹, Liu, Y.²

¹Hebei General Hospital, Department of Gastroenterology, Shijiazhuang, China, ²Huashan Hospital Fudan University, Department of Gastroenterology, Shanghai, China

Objective: To investigate the effects of Paricalcitol on intestinal inflammation and its mechanism.

Methods: 30 BALB/c mice were divided into five groups: normal control(A), colitis control(B), treatment with low dose(C), treatment with medium dose(D), treatment with high dose(E). Acute colitis was induced by DSS according to standard procedures. Mice were treated by intragastric administration with Paricalcitol or placebo on day 1, 4, 7. DAI score, Serum calcium, histopathological score, the mRNA expression of TNF- α , IL-6, IL-10, IL-17, VDR, P38 MAPK and MKP- 1 and the protein expression of P38, P-P38 and MKP- 1 were detected.

Results: DAI score, histopathological score, TNF- α , IL-6, IL-17, P38 MAPK were high in group B and low in A,C,D,E. ($P < 0.05$) The length of the colons, IL-10, VDR, and MKP- 1 were low in group B and high in A,C,D,E. ($P < 0.05$).

Conclusion: Paricalcitol demonstrated significant anti-inflammatory properties in experimental colitis by inducing the expression of MKP-1 which blocked P38 MAPK pathway.

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The anti-pulmonary fibrotic effect of FMI-1, one phenylethanoid compound from *Osmanthus spp.*

Shi, L.-S., Chuang, Y.-M., Tseng, Y.-F., Ye, Y.-L.

National Formosa University, Department of Biotechnology, Yunlin County, Taiwan, Republic of China

FMI-1 is one phenylethanoid compound derived from *Osmanthus spp.* It has been reported to possess anti-oxidant, anti-inflammatory, antitumor invasion and migration, photoprotective, and neuroprotective effects. Pulmonary fibrosis is a chronic inflammation, progressive and fatal interstitial lung diseases. It is revealed that transforming growth factor- β (TGF- β) is a master switch cytokine during pulmonary fibrosis. The cause of pulmonary fibrosis is characterized by the excessive deposition of extracellular matrix proteins within the pulmonary interstitium. In this study, we used mouse lung fibroblast cell line, primary lung fibroblast cell culture and bleomycin-induced pulmonary fibrosis model to investigate the anti-fibrotic effect of FMI-1. FMI-1 can suppress lung fibroblast cell proliferation and collagen expression through pSMAD 2/3 pathway. In the animal model, FMI-1 also has therapeutic effect. Lung function index such as Rrs (respiratory resistance), Ers (respiratory elastance), G (tissue damping), and H (tissue elastance) by FMI-1 treated mice are all decreased. Transforming growth factor- β 1 (TGF- β 1) and interleukin-6 (IL-6) also decreased after treat FMI-1 in bronchoalveolar lavage fluid by FMI-1 treated mice. In the future, we hope this study can provide the possibilities of FMI-1 for prevention or treatment of pulmonary fibrosis.

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Interleukin-17-induced microRNA-873 contributes to the pathogenesis of experimental autoimmune encephalomyelitis by targeting A20 ubiquitin-editing enzyme

Liu, X., He, F., Pang, R., Zhao, D., Qiu, W., Shan, K., Zhang, J., Lu, Y., Li, Y., Wang, Y.

Nanjing Medical University, Immunology, Nanjing, China

Interleukin 17 (IL-17), produced mainly by T helper 17 (Th17) cells, is increasingly recognized as a key regulator in various autoimmune diseases, including human multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE). Although the aberrant expression of microRNAs (miRNAs) has been shown to contribute to the pathogenesis of MS and EAE, the mechanisms underlying the regulation of abnormal miRNA expression in the astrocytes upon IL-17 stimulation remain unclear. In the present study, we detected the changes of miRNA expression profiles both in the brain tissue of EAE mice and in the cultured mouse primary astrocytes stimulated with IL-17. We first identified microRNA-873 (miR-873) as one of the co-up-regulated miRNAs *in vivo* and *in vitro*. Further studies showed that miR-873 overexpression, demonstrated by targeting the mRNA of A20 (TNF α -induced protein 3, TNFAIP3), remarkably reduced A20 expression and promoted NF- κ B activation *in vivo* and *in vitro* as well as increasing the production of inflammatory

cytokines and chemokines (i.e. IL-6, TNF- α , MIP-2 and MCP-1/5). More importantly, silencing the endogenous miR-873 or A20 gene with lentiviral vector of miR-873 sponge or A20 short hairpin RNA *in vivo* significantly lessened or aggravated inflammation and demyelination in the central nervous system (CNS) of EAE mice, respectively. Taken together, these findings indicate that miR-873 up-regulation in the astrocytes induced by IL-17 promotes the production of inflammatory cytokines and aggravates the pathological process of EAE mice through regulating the A20/NF- κ B pathway, which provides a new insight into the mechanism of inflammatory damage in MS.

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Prevention of colitis and colitis-associated colorectal cancer by a novel small molecule

Lin, Y.-T.¹, Wei, T.-T.¹, Tseng, R.-Y.¹, Shun, C.-T.^{2,3}, Lin, Y.-C.^{1,4,5}, Fang, J.-M.⁶, Chen, C.-C.¹

¹College of Medicine, National Taiwan University, Department of Pharmacology, Taipei, Taiwan, Republic of China, ²Graduate Institute of Forensic Medicine, National Taiwan University College of Medicine, Taipei, Taiwan, Republic of China, ³National Taiwan University Hospital, Department of Pathology, Taipei, Taiwan, Republic of China, ⁴National Taiwan University Hospital, Department of Oncology, Taipei, Taiwan, Republic of China, ⁵Far-Eastern Memorial Hospital, Department of Internal Medicine, New Taipei City, Taiwan, Republic of China, ⁶National Taiwan University, Department of Chemistry, Taipei, Taiwan, Republic of China

Chronic intestinal inflammation is a well-known risk factor for colorectal cancer (CRC) development. Metabolic inflammation (metaflammation) and alteration of gut microbiota (dysbiosis) have been found to elicit CRC with rising annual incidence. Chemoprevention is an important strategy to reduce cancer-related mortality. In this study, we designed and synthesized a small molecule that simultaneously inhibited 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) and histone deacetylases (HDACs).

This small molecule showed both preventive and therapeutic effect in DSS acute colitis mouse models. It ameliorated intestinal inflammatory symptoms, shortening of colon length, loss of crypts, destruction of epithelial integrity, infiltration of inflammatory cells and release of pro-inflammatory cytokines. In addition, in azoxymethane/dextran sodium sulfate (AOM/DSS)-induced CRC mouse model, this small molecule also protected against mucosa dysplasia and adenocarcinoma formation. mRNA of pro-inflammatory cytokines and chemokines, expression of COX-II and cyclin D1 in inflammation and tumor tissues as well as the penetration of macrophages and neutrophils in tumor surrounding zones were all reduced by it. Stemness of CRC and release of endotoxin were also attenuated by this small molecule in AOM/DSS model.

Our results demonstrated that this small molecule showed promising effect on the chemoprevention of CRC, and serum endotoxin level might serve as a potential biomarker for its chemoprevention.

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Interleukin-17A promotes the growth and tumorigenicity of CT26 colon cancer cells

Kim, Y.S.

Chungnam National University, College of Natural Sciences, Department of Biochemistry, Daejeon, Korea, Republic of

Interleukin-17A is a member of the IL-17 family, and is known as CTLA8 in the mouse. It is produced by T lymphocytes and NK cells and has proinflammatory roles, inducing cytokine and chemokine production. However, its role in tumor biology remains controversial. We investigated the effects of locally produced IL-17A by transferring the gene encoding it into CT26 colon cancer cells, either in a secretory or a membrane-bound form. Expression of the membrane-bound form on CT26 cells dramatically enhanced their proliferation *in vitro*. The enhanced growth was shown to be due to an increased rate of cell cycle progression: after synchronizing cells by adding and withdrawing colcemid, the rate of cell cycle progression in the cells expressing the membrane-bound form of IL-17A was much faster than that of the parental cells. Both secretory and membrane-bound IL-17A induced the expression of Sca-1 on the cancer cells. When tumor clones were grafted into syngeneic BALB/c mice, the tumor clones expressing the membrane-bound form IL-17A grew rapidly; those expressing the secretory form also grew faster than the wild type CT26 cells, but slower than the clones expressing the membrane-bound form. These results indicate that IL-17A promotes tumorigenicity by enhancing cell cycle progression. This finding should be considered in treating tumors and immunerelated diseases.

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A novel regulator of NF κ B signaling enhances I κ B α ubiquitination and promotes inflammatory disease development

Okuyama, Y.^{1,2}, Atsumi, T.^{1,3}, Jiang, J.-J.^{1,3}, Nakamura, A.³, Ogura, H.^{1,3}, Meng, J.^{1,3}, Kamimura, D.^{1,3}, Shii, N.², Hirano, T.^{1,3}, Murakami, M.^{1,3}

¹Institute for Genetic Medicine and Graduate School of Medicine, Hokkaido University, Division of Molecular Neuroimmunology, Sapporo, Japan, ²Tohoku University Graduate School of Medicine, Department of Microbiology and Immunology, Sendai, Japan, ³Graduate School of Frontier Biosciences, Graduate School of Medicine, and WPI Immunology Frontier Research Center, Osaka University, Laboratory of Developmental Immunology, Suita, Japan

The inflammation-amplifier is a regulator of chronic inflammation in non-immune cells such as endothelial cells and fibroblasts, and is critical for the development of various diseases and disorders. The inflammation amplifier is activated by the simultaneous activation of NF κ B and STAT3, and enhances the expression of NF κ B targets critical for inflammation development. A functional genome wide screen found over 1,000 candidate genes as positive regulators of the inflammation amplifier. We selected a regulator candidate gene, SAG1. SAG1-deficient cells suppressed the expression of several NF κ B targets, including IL-6, CCL20 and LCN2 after IL-17 and IL-6

stimulation, but not of STAT3 targets such as SOCS3, suggesting that SAG1 regulates the NF κ B pathway but not the STAT3 one. A deficiency of SAG1 by using lentiviruses carrying SAG1-shRNA in the joints suppressed the development of a model NF κ B-mediated disease, the cytokine-induced arthritis. In the NF κ B signaling pathway, I κ B α degradation and p65 nuclear localization were suppressed in SAG1-deficient cells after TNF α stimulation, but upstream events, such as the phosphorylation of IKK α / β , NF κ B, and I κ B α , were unaffected. TNF α -mediated reporter activation was also inhibited in SAG1-deficient cells. These results suggest that SAG1 regulates I κ B α degradation in the cytoplasm. Mechanistic analysis showed that SAG1 directly associated with β TrCP, an E3-ligase, to increase the integrity of an ubiquitination complex, SCF leading to the ubiquitination of I κ B α . These effects on NF κ B-related inflammation suggest SAG1 is a potential therapeutic target for various diseases and disorders.

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Potential roles and mechanisms of CXCR3 and TLR4 in response to CXCL10 in rheumatoid arthritis

Kim, B., Jin, W.J., Lee, Z.H.

Seoul National University School of Dentistry, Cell and Developmental Biology, Seoul, Korea, Republic of

Rheumatoid arthritis (RA) is a chronic autoimmune disease which is characterized by constant joint inflammation and destruction of bone and cartilage. We previously reported that CXCL10 is crucially implicated in joint inflammation and bone destruction in arthritis. However, the specific mechanism by which the recruitment of inflammatory cells and osteoclastogenic cytokine production are regulated by CXCL10 in RA is not fully understood. Here, we found that CXCL10 induced migration of inflammatory cells through CXCR3-mediated, but not TLR4-mediated, ERK activation. Interestingly, both receptors CXCR3 and TLR4 were required for CXCL10-stimulated production of osteoclastogenic cytokines in CD4⁺ T cells, where NFATc1 expression was essential for CXCL10-induced RANKL expression. Based on the *in vitro* results, infiltration of F4/80⁺ macrophages into synovium, serum concentrations of osteoclastogenic cytokines, and bone destruction were all reduced, leading to decreased progression of arthritis in CXCL10^{-/-} and CXCR3^{-/-} mice compared to WT mice in collagen-antibody induced arthritis model. Taken together, these findings highlight the importance of CXCL10 in the pathogenesis of RA and provide the previously unidentified insights into the detailed mechanisms underlying the infiltration of inflammatory cells and the production of osteoclastogenic cytokine in CXCL10-induced development of arthritis.

Granulocytes

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Human neutrophils a new therapeutic target of Tamoxifen

Espinoza, G.¹, Perez, B.², Morales, N.¹, Borlone, C.¹, Folch, H.¹, Moran, G.¹, Sarmiento, J.¹

¹Universidad Austral de Chile, Valdivia, Chile, ²Universidad Austral de Chile, Escuela de Graduados Facultad de Ciencias Veterinarias, Valdivia, Chile

Tamoxifen is a selective estrogen receptor modulator used as treatment of estrogen-positive human breast cancer; its efficacy has been attributed to cell growth arrest and induction of apoptosis in neoplastic cells. Recent studies have demonstrated that tamoxifen produced a reduction of neutrophilic inflammation in acute lung inflammation model in mice and horses. Tamoxifen is metabolized by hepatic cytochrome P450. The antagonist activity for the binding of 17- β -Estradiol nuclear receptors of estrogen α and β (nER α / β) is mediated by the metabolites 4-hidroxytamoxifeno (4-OH) and 4-hidroxy-N-desmethyltamoxifen, but not by tamoxifen. Recently, a membrane receptor for G protein coupled estrogen called GPR30 has been described, for which tamoxifen has an agonist activity. We assessed cell viability by Annexin V/propidium iodide apoptosis assay, phagocytosis capacity performing phagocytosis assay with latex particles opsonized IgG-FTC, measuring both by flow cytometry. Additionally, a respiratory burst assay was carried out using opsonized zymosan in human neutrophils obtained from peripheral blood. Finally, nuclear estrogen receptors (17- β -Estradiol) and membrane GPR30 agonists and antagonists (ICI 182,780 and G15) were employed for assessing whether these receptors are involved in the effects produced by tamoxifen in this study. We detected that tamoxifen does not affect cell viability, neither phagocytosis. On the other side, tamoxifen inhibited the respiratory burst in a dose-dependent way. Interestingly, agonists and antagonist estrogen receptors did not block neither reproduce this effect in the respiratory burst. This study gives preliminary information of the potential of tamoxifen on new therapies for controlling neutrophilic inflammatory disorders in human.

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Evidence for a key role for neutrophils in rheumatoid arthritis

Goldberg, G.¹, Chatfield, S.^{1,2}, Murphy, J.¹, D'Silva, D.¹, Pang, E.S.¹, O'Neill, C.², Tadros, S.², Grebe, K.¹, Chen, Y.¹, Smyth, G.¹, Ng, M.³, Inouye, M.⁴, Busfield, S.³, Wilson, M.³, Andrews, A.³, Wilson, N.³, Wicks, I.^{1,2,4}

¹Walter and Eliza Hall Institute of Medical Research, Parkville, Australia, ²Royal Melbourne Hospital, Parkville, Australia, ³CSL Limited, Parkville, Australia, ⁴University of Melbourne, Parkville, Australia

Rheumatoid arthritis (RA) is characterised by a persistent, but poorly understood interplay between innate and adaptive immunity. Neutrophils are the predominant cell type in inflamed RA synovial fluid (SF). In this study we evaluated the role of

neutrophils in RA. Neutrophil growth factors, chemokines and neutrophil-extracellular traps (NETs) were determined by immunoassay of serum and synovial fluid from RA patients and neutrophil phenotype in peripheral blood (PB) was analysed by flow cytometry. Transcriptional differences in SF and PB neutrophils from RA donors and in white blood cells (WBCs) from both healthy donor and RA patients were determined by RNAseq. Circulating neutrophil proportions and numbers were increased in RA patients (n=73) compared to healthy donors. Neutrophil proportion and number correlated with disease activity in RA patients. G-CSF, GM-CSF and neutrophil chemoattractants, including IL-8, were elevated in SF of RA patients when compared with paired serum samples. NETs were markedly increased in SF of RA patients when compared with both osteoarthritis SF and RA PB. Pathway analysis of WBC transcription profiles identified G-CSF as an upstream regulator of differential gene expression in peripheral RA WBCs (n=50) when compared with healthy donor WBCs (n=50). Analysis of transcription profiles from blood and SF neutrophils from RA patients revealed a decrease in degranulation and cell death-related genes in SF neutrophils. These data together provide evidence that neutrophils contribute to the pathogenesis of RA, provide potential biomarkers for monitoring disease activity and suggest that G-CSF may be a new therapeutic target in RA.

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The different transformatin of the phenotype CD16⁺CD11b⁺ neutrophilic granulocytes in acute viral and acute bacterial infections

Nesterova, L.¹, Kolesnikova, N.², Chudilova, G.², Lomtadidze, L.², Kovaleva, S.², Avdeeva, M.³, Rusinova, T.²

¹Peoples' Friendship University of Russia, Department of Allergology and Immunology, Moscow, Russian Federation, ²Kuban State Medical University, Department of Clinical and Experimental Immunology, Krasnodar, Russian Federation, ³Kuban State Medical University, Department of Disease of Infection, Krasnodar, Russian Federation

Modern data demonstrate: neutrophilic granulocytes (NG) can very quickly realize the antibacterial and antiviral defense. Membrane molecules CD16 and CD11b of NG take part in the implementation of the phagocytosis and/or ADCC in infectious processes of the different nature. We had studied the population CD16⁺CD11b⁺NG in two groups of patients both sexes in age 18-28 years with acute viral and bacterial infections:

group I - 25 patients with acute bacterial tonsillitis (ABT),
group II - 28 patients with acute viral tonsillitis - EBV infection (AEBVI).

Control group included 25 healthy adults.

Comparative analysis of the redistribution of the intensity of the equipment functionally important receptors CD16 and CD11b have detected using the flow cytometry method. Three different subpopulations CD16^{bright}CD11b^{bright}, CD16^{bright}CD11b^{dim}, CD16^{dim}CD11b^{bright} were found in normal and pathological conditions. The subpopulation CD16^{bright}CD11b^{dim}NG dominated in 91,02±5,23% in healthy individuals; in patients with the acute viral infection the subpopulation CD16^{bright}CD11b^{bright}NG dominated in 75,8±4,5% of cases and in patients with the acute

bacterial infections the subpopulation CD16^{dim}CD11b^{bright}NG dominated in 88,6±5,9% of cases. Thus, the membrane equipment of receptors of CD16⁺ and CD11b⁺ was changed in early stage of the acute viral and acute bacterial infections. We propose that very high levels of the expression of CD16 and CD11b molecules in the subpopulation CD16^{bright}CD11b^{bright}NG in patients with the acute viral infection are necessary for realization ADCC in the antiviral defense. From the other side, in the subpopulation CD16^{dim}CD11b^{bright}NG the leading role have CD11b molecules that trigger phagocytosis in early stage of the acute bacterial process.

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NE, CD80 and CD 86 colocalized in NETs

Rodriguez, F.M.¹, Novak, I.T.C.²

¹National University of Cordoba. Faculty of Medicine, Cordoba, Argentina, ²National University of Cordoba. Faculty of Medicine, Institute of Cell Biology, Cordoba, Argentina

Polymorphonuclear neutrophils (PMNs) activated in vitro may express molecules normally associated with antigen presenting cells (APCs). In inflammatory conditions, PMN can release extracellular traps (NETs) composed of chromatin, histone and granule proteins, which trap and kill microbes. NETs have also been implicated in autoimmunity, thrombosis, tissue injury and in cancer immunoediting. Neutrophil elastase (NE) has been shown to play an important role in the degradation of the extracellular matrix, is stored in azurophilic granules and contributes to antimicrobial activity in the phagosome. In previous work we described CD80 and CD86 colocalized in NETs at 30 min of challenge. NETs decreasing activation threshold of T cells. During the formation of NETs NE translocates from the granules to the nucleus via an unknown mechanism. It is described that NE colocalized with chromatin at 120 min after neutrophil activation. We perform the generation of NETs in autologous leukocytes cultures, challenged with lipopolysaccharide (LPS) and perform marking B7-1(CD80) and B7-2(CD86) costimulatory molecules and NE. In LPS assay at 120 min CD80 and CD86 are detected on the cell surface colocalized with NE in NETs, but not in the control paired blood samples. Here, the expression of costimulatory molecules in NETs, would allow PMNs to play function as APCs and modulatory functions of various subpopulations of T cells, could influence the cell environment through the B7-1/B7-2:CD28/CTLA-4 pathway. The NE in NETs may be one adverse effect on host tissues. These findings could have relevance for a break in immune tolerance mediated by NETs.

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Basophils gain the capacity of antigen presentation through the import of MHC class II molecules from dendritic cells

Miyake, K., Shiozawa, N., Nagao, T., Yoshikawa, S., Yamanishi, Y., Karasuyama, H.

Tokyo Medical and Dental University (TMDU), Department of Immune Regulation, Tokyo, Japan

Recent studies demonstrated that basophils play significant roles in chronic allergic inflammation and protective immunity against parasites. Moreover, it has been reported that basophils express MHC class II (MHCII) and can present antigens to naïve CD4 T cells as well as they provide IL-4, leading to Th2 differentiation. However, the mechanisms that regulate MHCII expression on basophils remain elusive.

First, to determine what induces the expression of MHCII on basophils, we generated bone marrow-derived basophils in the presence of various cytokines. We discovered that basophils elicited by GM-CSF plus IL-3 expressed significantly higher levels of MHCII compared to those elicited by IL-3 alone. In contrast, the expression at mRNA level in GM-CSF-elicited basophils is comparable to IL-3-elicited basophils, and is almost negligible compared to expression in dendritic cells (DCs). These data suggest that basophils acquire MHCII molecules from other cells in the culture, rather than they transcribe MHCII by themselves. Indeed, when DCs were depleted from the culture, the MHCII expression on basophils was significantly reduced. Conversely, when purified basophils were co-cultured with DCs, the MHCII expression on basophils was enhanced. Finally, we found that basophils expressing acquired MHCII could present antigenic peptides to naïve T cells and promote their proliferation.

Taken together, our data suggested that basophils acquire MHCII molecules from DCs and these basophils can present antigens to naïve T cells. These data will provide new basophil-DC axis which may be required for antigen presentation by basophils.

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Tpl2 is required for neutrophil antimicrobial functions and bacterial killing

Acuff, N., Elmore, J., Li, X., Rada, B., Watford, W.
University of Georgia, Athens, United States

Tumor progression locus 2 (Tpl2) is a serine/threonine kinase that promotes inflammatory cytokine production by activating the MEK/ERK pathway. Tpl2 has been shown to be important for eliciting the inflammatory properties of macrophages, however, there is relatively little known about Tpl2's contribution to neutrophil effector functions. This is an important consideration, since neutrophils provide the first line of defense against infection in the innate immune system. We found that Tpl2 is expressed in both human and murine neutrophils, suggesting a potential function for Tpl2 within this lineage. Despite significantly higher proportions of bone marrow neutrophils in *Tpl2*^{-/-} mice compared to wild type mice, *Tpl2*^{-/-} mice have significantly reduced proportions of circulating neutrophils. In response to infection, neutrophils secrete inflammatory cytokines and produce reactive oxygen species (ROS), which promote bacterial killing. Tpl2 ablation impaired neutrophil TNF- α production in response to LPS stimulation, superoxide generation in response to the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (fMLP), and killing of the extracellular bacterium, *Citrobacter rodentium*. These results implicate Tpl2 in the regulation of multiple neutrophil antimicrobial pathways, including inflammatory cytokine secretion and oxidative burst. Furthermore, they suggest that Tpl2 functions early during infection to bolster neutrophil-

mediated innate immunity against extracellular bacteria.

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IL-4 signals hamper neutrophil expansion and migration during infection and inflammation

Woytschak, J., Boyman, O.

University of Zurich, University Hospital Zurich, Department of Immunology, Zurich, Switzerland

Upon tissue damage or infection, circulating neutrophil granulocytes (neutrophils) are the first immune cells entering the affected site where they contain invading pathogens and contribute to leukocyte recruitment and restoration of tissue integrity. Interestingly, patients suffering from atopic dermatitis, an allergic skin disease driven by type 2 cytokines including interleukin-4 (IL-4), show a lack of skin neutrophils and a propensity to cutaneous bacterial infections. We hypothesized that IL-4 might interfere with neutrophil recruitment and function. We found that both homeostatic and increased levels of IL-4 dominantly inhibited neutrophil recruitment from the bone marrow (BM) to the circulation and skin during bacterial infection. These effects were a result of IL-4 acting directly on neutrophils to restrict their expansion in the BM and their chemotaxis towards C-X-C chemokine receptor 2 (CXCR2)-binding chemokines via two mechanisms: firstly, IL-4 signals in neutrophils enhanced p38 mitogen-activated protein kinase levels, which in turn suppressed phosphoinositide 3-kinase (PI3K), thus inhibiting CXCR2-mediated PI3K activation and chemotaxis; and secondly, IL-4 interfered with CXCR2-CXCR4-mediated control of neutrophil retention in the BM. These data suggest a novel role of IL-4 in inhibiting neutrophil expansion and migration during infection and inflammation.

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Myocardial ischemia/reperfusion injury is mediated by polymorphonuclear neutrophils through direct activation of phospholipase D (PLD) and mTOR

Ganesan, R.¹, Henkels, K.¹, Pavlov, V.², Stahl, G.², Gomez-Cambroneo, J.^{1,2}

¹Wright State University, Biochemistry and Molecular Biology, Dayton, United States, ²Brigham and Women's Hospital, Harvard Medical School, Center for Experimental Therapeutics and Reperfusion Injury, Boston, United States

During ischemia/reperfusion injury (I/RI), leukocytes release cytokines and exacerbate inflammation causing cardiac dysfunction, while neutrophils produce oxygen radicals that worsen the initial myocardial infarction. Phospholipase D is a cell membrane remodeling and signaling protein implicated in the pathology of I/RI. We analyzed I/RI by ejection fraction, infarct size and serum Troponin levels in wild-type, PLD1^{-/-} and PLD2^{-/-} mice. PLD-deficient mice or inhibition of PLD in WT mice protected against I/RI. Removing PLD2 yielded a slightly better outcome, suggesting PLD2 contributed more to the injury than PLD1, particularly at longer (>4 h) reperfusion times. Three cell signaling mechanisms were investigated: mTOR pathway, Aurora Kinase A and Cyclin-D3. Gene expression of the mTOR

pathway was increased in response to I/RI in WT mice and abrogated in PLD-KO mice suggesting PLD augmented mTOR pathway during IR/I. PLD1^{-/-} mice have lower expression of CCND3/Aurora Kinase A, while PLD2^{-/-} mice expressed more phospho-CyclinD3. We detected neutrophil-associated Ly6G after adoptive transfer of neutrophils during I/RI. Myocardial PMN accumulation was increased following I/R and PLD inhibitors abrogated PMN accumulation. Less myocardial PMN accumulation was observed in PLD-KO mice following IR/I. Adoptive transfer of fluorescently labeled PLD2^{-/-} or PLD1^{-/-} PMN into PLD1^{-/-} or PLD2^{-/-} mice, respectively, demonstrated that PLD1 containing (PLD2^{-/-}) PMNs accumulated more significantly in the myocardium following I/RI. This study demonstrates for the first time the specific mechanistic contribution of mammalian PLD1 and PLD2 to MI/R injury in a murine model and their role in neutrophil infiltration.

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Identification of molecules critical for neutrophil extracellular trap formation

Aihara, R., Fukui, Y.

Kyushu University, Division of Immunogenetics, Medical Institute of Bioregulation, Fukuoka, Japan

Neutrophils are highly motile leukocytes that play important roles in the innate immune response to invading pathogens. Neutrophils rapidly migrate to the site of infections and kill pathogens by producing reactive oxygen species (ROS) and forming neutrophil extracellular traps (NETs). Because NETs are involved in vascular inflammation and autoimmune response, identification of the molecules critical for NETs formation is important to develop effective therapeutic strategy for these diseases. We found that DOCK2 and DOCK5, atypical guanine nucleotide exchange factors (GEFs), critically regulate ROS production in mouse neutrophils through Rac activation. Consistent with this, NETs formation was severely impaired in mouse neutrophils lacking both DOCK2 and DOCK5. Similar results were obtained when human neutrophils were treated with CPYPP, a small-molecule inhibitor of these DOCK GEFs. In addition, we recently identified a molecule that regulates ROS production and NETs formation, independently of Rac activation. Based on these findings, we will discuss the molecular mechanism controlling NETs formation.

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Novel characterisation of mast cell phenotypes from peripheral blood mononuclear cells and identification of progenitor and activated mast cell phenotypes in chronic fatigue syndrome

Nguyen, T.^{1,2}, Johnston, S.^{1,2}, Chacko, A.^{1,2}, Gibson, D.², Cepen, J.², Smith, P.¹, Staines, D.¹

¹*Menzies Health Institute Queensland, National Centre for Neuroimmunology and Emerging Diseases, Parklands, Australia,*

²*Griffith University, School of Medical Science, Parklands, Australia*

Background: Mast cells (MCs) mediate inflammation through neuropeptides and cytokines, along with histamine and reactive oxygen species (ROS). The aim of this study was to determine

mast cell phenotypes in isolated PBMCs, in non-fatigued controls and CFS patients. The study further determined receptor expression of RAGE and its ligand high mobility group box 1 protein (HMGB1).

Methods: Twelve moderate CFS (aged 39.25±3.52 years), severe CFS patients (aged 43.00±4.02 years) and healthy controls (n=13, mean age 42.69 ± SD3.87 years) were included in this study. CFS patients were classified according to the 2011 International Consensus Criteria. LSRFortessa X-20 Flow cytometry was used for identification of phenotypic peripheral mast cells in PBMCs using exclusion marker Lin2 cocktail and inclusion markers following comparative investigation. HMGB1 and soluble RAGE (sRAGE) expression in plasma was measured by sandwich ELISA assay.

Results: There was a significant increase in CD117+CD34+FCeRI-chymase- mast cell populations in moderate and severe CFS compared with healthy controls. There was a significant increase in CD40 ligand and MHC-II receptors on differentiated mast cell populations in the severe CFS compared with healthy controls and moderate CFS. There were no significant differences for HMGB1 and sRAGE.

Conclusions: We report for the first time, significant increase of naïve MCs in moderate and severe CFS patients compared with healthy controls. Moreover, a significant increase was found in CD40 ligand and MHC-II receptors on differentiated mast cells in severe CFS patients. Peripheral MCs may be present in CFS pathology, however further investigation to determine their role is required.

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Control of mast cell differentiation by neuropeptide galanin

Yamaguchi, T., Ikeda, Y., Kawabata, K.

National Institutes of Biomedical Innovation, Health and Nutrition, Laboratory of Stem Cell Regulation, Osaka, Japan

Mast cells play an important role in the pathogenesis of allergic diseases. Activated mast cells can secrete wide variety of mediators, such as histamine. They are generally classified into two phenotypically distinct populations: connective tissue-type mast cells (CTMCs) and mucosal-type mast cells (MMCs). However, the molecular basis determining the different characteristics of these mast cell subclasses remains unclear. At first, we studied the generation and characterization of CTMCs and MMCs derived from mouse bone marrow mast cells (BMMCs). The number of Safranin O-positive cells and the expression levels of MCP-4 mRNA were increased in the BMMC-derived CTMCs (BM-CTMCs) as compared with those in BMMCs. In addition, the expression of MCP-1 and MCP-2 was elevated in the BMMC-derived MMCs (BM-MMCs). These results showed that the BM-CTMCs and BM-MMCs had characteristics similar to CTMCs and MMCs. We also found that the expression of galanin receptor 2 was elevated in BM-MMCs as compared with that in BM-CTMCs. Next, we compared the expression of MCP mRNA levels in BMMCs by quantitative RT-PCR analysis, and found that galanin could induce the expression of MCP-1 and MCP2 mRNA and could reduce the expression of MCP-4 mRNA, showing that galanin could promote the differentiation of BM-MMCs and inhibit the differentiation of BM-CTMCs. Moreover, administration of galanin antagonist into mice decreased the

number of MMCs and prevented the DSS-induced colitis. These results suggest that galanin promotes the *in vivo* differentiation of MMCs. Our results provide important insights into the molecular mechanisms underlying the differentiation of mast cells.

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Particulate matters activated inflammation-induced neutrophils

Miyake, T., He, C., Song, Y., Morita, K., Kanazawa, T., Yoshida, Y.
University of Occupational and Environmental Health,
Immunology and Parasitology, Kitakyushu, Japan

Epidemiological studies have shown the respiratory and cardiovascular effects of particulate matters (PMs). We previously demonstrated that PMs caused neutrophilic alveolitis and bronchitis; however, the effects of PMs on recruited neutrophils are not well understood. To characterize the response to PMs, the effects of different size particles on thioglycolate broth-induced neutrophils were investigated. Peritoneal exudate neutrophils from BALB/c mice, which were harvested 4 hour after the i.p. injection of 2ml of 4% thioglycolate broth were used for following experiments. Flow cytometric analysis shows most of collecting cells are CD11b+Gr1+ cells. It was observed that neutrophils phagocytosed FITC-labeled commercial particles (1 μ m, 3 μ m, and 10 μ m) with different sensitivity by fluorescence microscopy. ATP-based cell viability assay showed that PMs did not changed cellular ATP levels. Inflammatory cytokines such as tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) production from LPS-activated neutrophils were elevated by PMs treatment. These results suggested that phagocytosed PMs activate inflammatory neutrophils.

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Expression and function of IL-1R8 (TIR8/SIGIRR), a regulatory member of the IL-1 receptor family in platelets

Anselmo, A.¹, Riva, F.¹, Gentile, S.¹, Soldani, C.¹, Barbagallo, M.¹, Mazzon, C.¹, Feruglio, F.¹, Polentarutti, N.¹, Carullo, P.¹, Angelini, C.¹, Bacci, M.¹, Mendolicchio, G.L.¹, Voza, A.¹, Nebuloni, M.², Mantovani, A.¹, Garlanda, C.¹

¹Humanitas Clinical and Research Centre, Rozzano, Italy,

²University of Milan, Milan, Italy

IL-1R8, also known as single Ig IL-1-Related Receptor (SIGIRR) or TIR8, is a member of the IL-1R family that negatively regulates responses to IL-1R family members and TLRs. Here we report that IL-1R8 is expressed on human and murine platelets and megakaryocytes. Despite normal levels of circulating platelets IL1R8-deficient (*Il1r8*^{-/-}) mice showed increased homotypic and heterotypic (platelet-neutrophil) aggregation triggered by ADP and IL-1 or LPS. Moreover IL-1R8-deficient mice showed increased soluble P-selectin levels and increased platelet-neutrophil aggregates after systemic LPS administration. Commensal flora depletion and IL-1R1 deficiency abated platelet hyperactivity and the increased platelet/neutrophil aggregation observed in

Il1r8^{-/-} mice *in vitro* and *in vivo*, suggesting a key role of IL-1R8 in regulating platelet TLR and IL-1R1 function. In a mouse model of platelet-dependent pulmonary thromboembolism, IL-1R8-deficient mice showed an increased frequency of blood vessel complete obstruction.

Finally, in a small cohort of patients with severe systemic inflammatory conditions, the expression of platelet IL-1R8 was dramatically abated and it was associated to platelet-derived microparticle release.

Altogether our results highlight a new and efficient mechanism through which platelets, which have a large repertoire of TLRs and IL-1 receptors, regulate thrombocyte activity and identify IL-1R8 as a novel non-redundant player in tuning platelet activation during immunity and inflammation.

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Contribution of MyD88 and protein kinase D1 to the pathogenesis of polyarthritis spontaneously occurring in IL-1R antagonist-deficient mice

Yi, A.-K.¹, Yoon, T.W.¹, Cho, H.², Park, J.-E.³, Hasty, K.^{2,4}, Stuart, J.^{4,5}

¹University of Tennessee Health Science Center, Microbiology, Immunology and Biochemistry, Memphis, United States,

²University of Tennessee Health Science Center, Orthopedics, Memphis, United States, ³University of Tennessee Health Science Center, Pediatrics, Memphis, United States, ⁴Veterans Affairs Medical Center, Research Services, Memphis, United States,

⁵University of Tennessee Health Science Center, Medicine, Memphis, United States

It is thought that rheumatoid arthritis (RA) may be initiated in part by signaling through Toll-like receptors (TLRs) that contribute to the development of a self-perpetuating reaction leading to chronic inflammation and pathogenic auto-reactive Th1/Th17 development. IL-1R antagonist-deficient (*IL-1rn*^{-/-}) mice develop polyarthritis that mimics human RA. We investigated whether a TLR signaling adaptor MyD88 and its downstream protein kinase D1 (PKD1) play a role in the pathogenesis of arthritis in *IL-1rn*^{-/-} mice. Onset of arthritis was significantly delayed in *MyD88*^{-/-}*IL-1rn*^{-/-} mice compared to *MyD88*^{+/+}*IL-1rn*^{-/-} mice. Serum cytokine levels, and macrophage accumulation, matrix metalloproteinase activities and cartilage damage in joints were significantly lowered in *MyD88*^{-/-}*IL-1rn*^{-/-} mice, indicating a critical role for MyD88 in the development and progression of arthritis in *IL-1rn*^{-/-} mice. Because PKD1 is essential for proinflammatory responses mediated by MyD88-dependent TLRs and is hyperactivated in synoviocytes of RA patients, we further investigated the contribution of PKD1 to the development of arthritis in *IL-1rn*^{-/-} mice. Suppression of PKD1 expression *in vitro* resulted in inhibition of TLR-mediated cytokine production in *IL-1rn*^{-/-} macrophages. Daily treatment with a PKD inhibitor substantially reduced the incidence and severity of arthritis in *IL-1rn*^{-/-} mice. In addition, deletion of the PKD1 gene significantly delayed development of arthritis and reduced the severity of arthritis in *IL-1rn*^{-/-} mice. Our findings demonstrated that MyD88 and PKD1 are essential for development of arthritis in *IL-1rn*^{-/-} mice. This also implies that PKD1 might be one of the key factors that modulate proinflammatory responses in RA, and may serve as a therapeutic target.

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Major role for NLRP3 inflammasome in colitis progression*Perera, A.P., Ranson, N., Sohal, S., Eri, R.**University of Tasmania, School of Health Sciences, Launceston, Australia*

Inflammatory bowel disease is a group of intestinal disorders characterised by inflammation of the small intestine and colon. IBD pathogenesis is very elusive and to investigate the main cause of IBD, a number of mouse models have been developed. Inflammasome complex formation involving the innate NLR family receptors, mainly NLRP3, has been extensively studied in mouse models. Inflammasome results in secretion of mature IL-1 β , a major pro-inflammatory cytokine involved in IBD. Unfortunately, the precise role of NLRP3 hasn't been defined due to contrasting results in mouse models. To address the controversial findings we have studied the role of NLRP3 in a well suited spontaneous model of colitis, namely Winnie. Winnie mice have a missense mutation in the MUC2 gene which results in a reduction of intestinal mucus production which is similar to that seen in human ulcerative colitis patients. We have generated Winnie mice that are deficient for NLRP3 which are not embryonically lethal. These mice will be assessed for clinical, histological and immunological parameters to understand the phenotype at 4, 8 and 16 weeks of age. We expect the differential phenotypical and functional expression triggered by the lack of NLRP3 in Winnie/NLRP3^{-/-} against Wild type and NLRP3^{-/-} controlled groups will show that NLRP3 ameliorates colitis. This study is the first to address the inflammatory role of NLRP3 in the Winnie/NLRP3^{-/-} mouse model. The findings will be important because they could lead to a potential target for the development of novel therapeutics for patients with colitis and colitis associated cancer.

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Immunophenotyping of monocytes during human sepsis shows impairment of antigen-presentation, a shift towards non-classical differentiation and up-regulation of Fc-immunoglobulin receptor*Mota, N.V.F.¹, Brunialti, M.K.C.¹, Machado, F.R.², Assunção, M.³, Azevedo, L.C.P.⁴, Salomao, R.¹**¹Disciplina de Infectologia, Escola Paulista de Medicina, Hospital São Paulo, Universidade Federal de São Paulo - UNIFESP, Medicine, Sao Paulo, Brazil, ²Disciplina de Anestesiologia, Escola Paulista de Medicina, Hospital São Paulo, Universidade Federal de São Paulo - UNIFESP, Medicine, Sao Paulo, Brazil, ³Hospital Israelita Albert Einstein, Sao Paulo, Brazil, ⁴Hospital Sírio Libanês, Sao Paulo, Brazil*

Objective: We aimed to characterize the phenotype of monocytes from septic patients and their association with clinical outcomes.

Methods: Sixty one septic patients and thirty-one healthy volunteers (HV) were enrolled in the study. Samples were obtained from patients at admission (n=61), after 7 (n=36) and 14 days (n=22) of therapy. Cells were stained with the following antibodies: CD14-PerCP, CD163-PE, CD206-FITC, CD16-APC-Cy7, CD64-PE-Cy7, CD86-BV421, CD200R-FITC, PD1-BV510, PDL1-PE, CCR1-Alexa-Fluor-647, CCR2-Alexa-Fluor-647, CXCR2-PE-Cy5,

HLA-DR-PE-CF594. Isotypes or FMO controls were used. Results are expressed as percentage of positive cells or as geometric mean fluorescence intensity (GMFI).

Results: Monocytes from septic patients showed decreased expression of HLA-DR, CD86, CD200R, CXCR2 and CD163, compared to HV. CD206, CD64, PD-1 e PDL-1 expression was up-regulated in patients. Among the patients, expression of HLA-DR, CD86, CD64, PD-1 and PDL-1 was lower in non-survivors than in survivors. In follow-up samples, increased expression of CD86, HLA-DR and CXCR2 was observed after 7 and 14 days; CD16 GMFI decreased over time, resulting in an increase of CD16lo expression on day 14 samples; CD206 and CD163 were higher in admission samples than in follow-up ones.

Conclusion: Monocytes from septic patients shown impairment of antigen presentation as characterized by low HLA-DR, increased inhibitory PD-1 and PDL-1, and decreased co-stimulatory CD86. We found conflicting results regarding differentiation towards M2, with increased expression of CD206 and decreased CD163 on monocytes from septic patients, while the subset of non-classical monocytes was demonstrated by increased CD16hi. Co-stimulatory (CD86) and inhibitory receptors (PD1 and PDL1) were down-regulated in non-survivors.

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Inflammasome-dependent IL-1 β release depends upon membrane permeabilisation*Diamond, C.E.^{1,2}, Bagnall, J.², Paszek, P.², Daniels, M.J.², Mortellaro, A.¹, Brough, D.²**¹SigN A*STAR, Singapore, Singapore, ²University of Manchester, Manchester, United Kingdom*

Interleukin-1 β (IL-1) is an essential mediator of inflammation after infection and injury. This proinflammatory cytokine is produced as an inactive precursor, which must be cleaved to its active form via a molecular complex called the inflammasome. IL-1 β is released from the cell via an unknown process, as it lacks the signal peptide required to be conventionally secreted. Several release processes have been proposed, including the use of secretory lysosomes, exosomes and the shedding of microvesicles from the plasma membrane. Until recently, research into this process has been limited by conventional biochemical methods only measuring population dynamics, yet evidence shows much heterogeneity among an immune cell population.

Using lentiviral transfection of a newly developed vector that codes for a fluorescently labeled version of IL-1 β (Venus-IL-1 β) into a bone marrow derived macrophage cell line, we have visualized IL-1 β release on a single cell level in real-time, with confocal microscopy. To simultaneously monitor membrane integrity we included the cell-impermeant nucleic acid stain propidium iodide (PI) in the cell culture medium. The addition of the inflammasome activator ATP caused a simultaneous drop in Venus-IL-1 β fluorescence and increase in PI fluorescence, revealing an intriguing insight into IL-1 β secretion; membrane permeability is required for release of IL-1 β . This process occurs alongside the death of the cell, but its regulation is separate from the non-specific release of proteins during cell death.

Unravelling the secrets of IL-1 β secretion offers key insights into the initiation of the inflammatory cascade and unconventional protein release.

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Salt (sodium chloride) preferentially down-regulates IL-1 β production by gestation-associated tissues

Bryant, A., Scott, L.M., Radley, G., Chong, Y.Y., Jones, R.H., Thornton, C.

Institute of Life Science, Swansea University Medical School, Swansea, United Kingdom

Widespread westernization of diet has caused a rise in average salt (sodium chloride; NaCl) intake to levels well in excess of recommended physiological requirements. Due to the positive association of salt intake with blood pressure, pregnant women have been advised to follow a low salt diet to reduce the risk of developing pre-eclampsia. However, the evidence for this from clinical studies is inconclusive.

Here we examine the impact of altered NaCl levels on lipopolysaccharide (LPS) induced cytokine production by the placenta and choriodecidua. Term placenta and choriodecidua from elective sections were cultured in media supplemented with increasing concentrations of NaCl (10 - 50mM) \pm LPS (10ng/ml) and cytokine production examined. Additional media supplements used were sucrose, sodium gluconate (NaGlu), potassium chloride (KCl) and sodium bromide (NaBr) to determine if any effect of NaCl was mediated through changes in osmolarity, sodium or chloride levels.

A dose dependent decrease in LPS induced IL-1 β by both the placenta and choriodecidua was observed; there was no effect on TNF α and IL-10. The inhibitory effect of NaCl was replicated in both KCl and NaBr supplemented media, suggesting it was dependent on the increase in negative ions and not mediated by changes in sodium level or osmolarity.

The presence of elevated chloride/negative ions down-regulated the secretion of IL-1 β by gestational associated tissues suggesting possible effects of chloride and other anions on inflammasome activity by these tissues.

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Different TLR ligands exhibit contrasting influences on the adhesive properties of human endothelial cells

du Mez, E.¹, Johnson, R.^{2,3}, Kho, D.^{2,3}, Moller-Olsen, C.¹, Feisst, V.^{1,4}, Locke, M.⁵, O'Carroll, S.^{2,6}, Graham, S.^{2,3}, Angel, C.^{1,4}

¹University of Auckland, School of Biological Sciences, Auckland, New Zealand, ²University of Auckland, Centre for Brain Research, School of Medical Sciences, Faculty of Medical and Health Sciences, Auckland, New Zealand, ³University of Auckland, Department of Pharmacology and Clinical Pharmacology, School of Medical Sciences, Faculty of Medical and Health Sciences, Auckland, New Zealand, ⁴University of Auckland, Maurice Wilkins Centre, Auckland, New Zealand, ⁵University of Auckland, Department of Surgery, South Auckland Clinical Campus, Middlemore Hospital, Auckland, New Zealand, ⁶University of Auckland, Department of Anatomy, School of Medical Sciences, Faculty of Medical and Health Sciences, Auckland, New Zealand

Toll-like receptors (TLR) are expressed by antigen presenting cells, which use TLRs to identify foreign pathogens in order to stimulate an appropriate immune response. Earlier studies however, have also detected TLR mRNA expression by human endothelial cell lines. These studies focussed on assessing changes in cytokine secretion in response to TLR ligands but they didn't establish whether TLR ligands influence the adhesive properties of endothelial cells. For the first time we demonstrate that different TLR ligands exhibit contrasting influences on endothelial adhesion.

Using multicolour immunohistochemistry we demonstrate that both blood endothelial cells (BECs) and lymphatic endothelial cells (LECs) express TLR7 and TLR9 protein *in situ* in human skin. Thereafter, we sought to determine whether ligands recognised by TLR7 and TLR9 influenced the adhesive properties of the human dermal microvascular endothelial cell line, HMEC-1. Initially using immunocytochemistry, we confirmed that HMEC-1 cells expressed TLR7 and TLR9 protein. Subsequent experiments using xCELLigence technology demonstrated that different TLR7 (R837, R848 and CLO75) and TLR9 (ODN2006, ODN2216 and ODN2395) ligands displayed contrasting effects on the temporal basolateral adhesive properties of HMEC-1 cells. The trans-endothelial electrical resistance of cell:cell junctions was also measured using ECIS technology following TLR stimulation to assess effects on barrier integrity. These data showed that all three TLR7 ligands increased cell:cell adhesion, in contrast to the decreased adhesion observed with TLR9 ligands.

By establishing how the endothelial barrier is influenced by different TLR ligands we may be able to identify novel therapeutic applications for synthetic TLR ligands.

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IL-1R8 deficiency increases the susceptibility of LPR mice to develop B-cell lymphoma

Riva, F.¹, Ponzone, M.², Polentarutti, N.³, Bertilaccio, S.², Anselmo, A.³, Feruglio, F.⁴, Innocenzi, A.², Veliz-Rodriguez, T.², Simonetti, G.², Muzio, M.², Mantovani, A.³, Garlanda, C.³

¹University of Milan, DIVET, Milan, Italy, ²San Raffaele Hospital, Milan, Italy, ³Istituto Clinico Humanitas, Rozzano (Milano), Italy, ⁴Charles River, Calco, Italy

The association among autoimmunity, chronic inflammation and malignancy has been described and confirmed by epidemiological studies. In particular, patients suffering from autoimmune diseases are prone to develop B-cell Non-Hodgkin's Lymphomas, but the mechanisms triggering the transition from benign B-cell proliferation to malignancy are still poorly understood. We investigated the role of IL-1R8 gene (also known as SIGIRR or TIR8), already known to be associated with autoimmunity, in the development of lymphoma. Indeed, the ability to dampen signalling from IL-1R and TLR family members confers IL-1R8 the ability to act as regulator of inflammation, cancer-related inflammation and autoimmunity. In this study we describe the occurrence of malignant lymphoma in B6lpr/lpr/il-1r8 $^{-/-}$ and B6lpr/lpr mice. Both strains developed a B-cell lymphoma during their late age, but in B6lpr/lpr/il-1r8 $^{-/-}$ mice, it occurred with higher frequency and earlier, and was more aggressive, causing higher mortality. Histopathologic

analysis of spleen and lymph nodes of B6lpr/lpr/il-1r8-/- mice documented clear-cut Diffuse Large B-cell lymphoma (DLBCL) areas arising within a context of atypical lymphoproliferative disorder. These results were corroborated by both molecular analysis and transplantation experiments. Clonal rearrangement was present in both strains, however, only recipients of spleen or lymph node cells collected from B6lpr/lpr/il-1r8-/- mice developed DLBCL. In human, IL-1R8 expression was down-modulated in different lymphoma cell lines, compared to healthy B cells. These observations unveil the involvement of IL-1R8 in the occurrence and development of DLBCL, suggesting its potential role in targeted therapy. In addition, we propose B6lpr/lpr/il-1r8-/- mice as a novel model of autoimmunity-associated B cell lymphomas.

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Phosphorylated tyrosine kinase Syk interacts with ESCRT-0 upon TLR3 activation in murine C8D1A microglial cells

Toka, F.^{1,2}, Mielcarska, M.B.², Bossowska, M.², Gregorczyk, K.P.², Wyzewski, Z.²

¹Ross University School of Veterinary Medicine, Biomedical Sciences, Basseterre, Saint Kitts and Nevis, ²Warsaw University of Life Sciences-SGGW, Preclinical Sciences, Warsaw, Poland

Toll-like receptor 3 (TLR3), found in endosomes, identifies the double-stranded ribonucleic acid (dsRNA), an intermediary during replication of many viruses. TLR3 is expressed in the cells of the central nervous system (CNS), where it serves to control infections of herpesviruses (HSV-1 or HSV-2). Humans or animals with deficiencies in TLR3 signalling are highly susceptible to these infections, hence a justified endeavor to investigate immunological mechanisms behind TLR3 mobility and regulation in the CNS. Our study, conducted on murine C8D1A microglial cell line, shows that following cell stimulation with poly(I:C) (polyinosinic-polycytidylic acid, dsRNA mimetic) tyrosine kinase Syk is immediately phosphorylated. The purpose of activity of Syk kinase is Hrs (*Hepatocyte growth factor*-regulated tyrosine kinase substrate), a component of the ESCRT-0 protein complex (Endosomal sorting complex required for transport-0). ESCRT plays a role in protein trafficking to multi-vesicular bodies and lysosomal compartments. Our findings indicate the existence of interaction between ESCRT-0 and TLR3, after receptor stimulation. Establishment of Syk as an important mediator between TLR3 and ESCRT-0 in the TLR3 signalling pathway in microglial cells after stimulation with poly(I:C) may indicate that TLR3 mobility, en route to the endosomes, is regulated by ESCRT-0. Although recent studies have shown that ESCRT pathway plays an important role in TLR7 and TLR9 sorting and recycling, molecular mechanisms which govern TLR3 allocation and TLR3-mediated elicitation of antiviral innate immune response in cells of the CNS remain weakly understood and require further investigation.

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Cow's milk hydrolysates modulate Toll-like receptor-mediated activation

Kiewiet, M.B.G.¹, Dekkers, R.², Gros, M.², van Neerven, J.², de Vos, P.¹, Faas, M.M.¹

¹University Medical Center Groningen, Pathology and Medical Biology, Groningen, Netherlands, ²FrieslandCampina, Amersfoort, Netherlands

Hydrolyzed cow's milk proteins are thought to elicit effects on the innate and adaptive immune system. Recently, Toll-like receptors (TLRs) have been suggested to be involved in the underlying mechanism of these effects. Therefore, we studied the TLR activation and inhibition capacity of a range of whey and casein hydrolysates.

To screen for immunomodulatory effects, we isolated peripheral blood mononuclear cells (PBMC's) from healthy volunteers and stimulated them with whey, casein and their hydrolysates. Cytokine production was measured by Luminex. Moreover, hydrolysate induced TLR dependent signaling was assessed by incubating the THP-1-XBlue-MD2-CD14 and the THP-1-XBlue-MyD88 deficient reporter cellline (Invitrogen) with the hydrolysates. Individual TLRs were studied using HEK-Blue hTLR reporter cells (Invitrogen). All cells were stimulated with 2 mg/ml hydrolysate for 24 hours.

Stimulation of PBMCs showed that whey and several of the whey hydrolysates induced both IL-10 and TNF α cytokine production, while casein hydrolysates had no effect. Whey hydrolysates with different levels of hydrolysis (between 3.1 and 15%) increased TLR2,3,5 and 9 ($p < 0.05$) activation, while casein hydrolysates did not have an effect on TLR activation. On the other hand, casein and casein hydrolysates inhibited ligand-induced activation of TLRs (mostly TLR 5 and 9) up to 69% ($p < 0.05$), while whey hydrolysates did not.

Overall, we found that generally whey hydrolysates induced cytokine production and TLR activation, while casein hydrolysates did not induce cytokine production and generally showed TLR inhibition. This knowledge could contribute to a better understanding of the immunomodulatory effects of hydrolysates.

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The role of dysregulated TLR signaling in lung tumorigenesis

Yap, G.^{1,2,3}, Lim, L.^{1,3}

¹National University of Singapore, Physiology, Singapore, Singapore, ²NUS Graduate School for Integrative Sciences and Engineering, Singapore, Singapore, ³National University of Singapore, Inflammation and Cancer Laboratory, Immunology Programme, Singapore, Singapore

Toll-like receptors (TLRs) form a part of the larger family of pattern recognition receptors (PRRs), and they play an important protective role in innate immunity. Found on immune cells, TLRs recognize and bind to a wide array of conserved microbial structures called pathogen associated molecular patterns (PAMPs) that will result in the triggering of anti-microbial and anti-viral responses. Recently however, TLRs have been found

on various cancer cells and are furthermore discovered to be functional. In this study, TLR signaling in lung cancer cells and its role in viral-induced tumorigenesis were investigated. Basal expression levels of TLRs were determined using real time PCR and different lung cancer cell lines showed varying levels of TLRs. Activation of certain TLRs signaling pathway via specific TLR agonists showed a possible role of dysregulated TLRs signaling in lung tumorigenesis. As expected, the level of TLRs present in the cell is able to influence the extent of which the cell responds to TLR agonists' treatment. Only H460 cell line which has highest expression of TLR3, showed a decrease in cell viability upon treatment with transfected poly (I:C). Other TLR agonists showed no significant effect on cell viability upon treatment. TLR3 activation in H460 led to a decrease in NfκB promoter activity, thus suggesting a possible signaling pathway. This study deepens our understanding of the role of TLRs in lung tumorigenesis. The role of ANXA1 in dysregulated TLR signaling was also investigated.

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The miR-20a-5p regulates M.tb-induced cell apoptosis through targeting JNK2 in human macrophage

Zhang, G.^{1,2}, Liu, X.³, Wang, W.², Zhang, M.², Zeng, G.⁴, Zhou, B.², Feng, C.G.¹, Chen, X.²

¹The University of Sydney, Infectious Diseases and Immunology, Sydney, Australia, ²Shenzhen Third People's Hospital, Guangdong Key Lab of Emerging Infectious Diseases, Shenzhen, China, ³Sun Yat-sen University, The Fifth Affiliated Hospital, Zhuhai, China, ⁴Sun Yat-sen University, Zhongshan School of Medicine, Guangzhou, China

Depends on the cell death pathway, macrophage could be either beneficial or detrimental to Mycobacterium tuberculosis (Mtb) inside the cell. To facilitate its survival and dissemination, Mtb has developed strategies to promote cell death via necrosis, which avoid through apoptosis. However, the mechanisms underlying these strategies remain to be elucidated. MicroRNAs (miRNAs) are small non-coding RNAs that function in various cellular processes such as differentiation, proliferation, and apoptosis through post-transcriptional regulating target gene expression. In this study, we focused on the potential role of miR-20a, which has been reported to promote gastric cancer cell proliferation and inhibit cell apoptosis, in regulating the cell death pathway of human macrophage. Our findings have demonstrated that Mtb infection significantly inhibited miR-20a-5p expression in monocytes and macrophage. In consistence, miR-20a-5p expression was significantly decreased in the plasma, the whole blood, and CD14 monocytes of active pulmonary TB patients ($P < 0.01$). Functional assay demonstrated that inhibition of miR-20a-5p expression significantly increased apoptosis of Mtb infected macrophage, which is corroborated with that over-expression of miR-20a-5p significantly reduced apoptosis. Mechanism study indicated that miR-20a-5p modulates the expression of BCL2 and BIM expression through directly targeting JNK pathway. Thus, our results uncover a novel regulation of innate immune response during mycobacterial infection, dependent on the down-regulation of miR-20a-5p to promote macrophage apoptosis in attempt to facilitate Mtb clearance.

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NLRX1 attenuates damage following traumatic brain injury through negatively regulating NF-κB signaling

Allen, L.¹, Meza, A.¹, Brickler, T.¹, Coutermarsh-Ott, S.¹, Ives, A.¹, Bertke, A.², Theus, M.¹

¹Virginia Tech, Department of Biomedical Sciences and Pathobiology, Blacksburg, United States, ²Virginia Tech, Department of Population Health Sciences, Blacksburg, United States

Mechanical trauma to the CNS results in the disruption of the cellular microenvironment leading to massive necrotic and apoptotic loss of neuronal and glia populations. The progressive cascade of secondary events, including ischemia, inflammation, excitotoxicity and free radical release contribute to neural tissue damage. Members of the NLR family of pattern recognition receptors are essential mediators of the host immune response. Recently, our lab and others identified a novel sub-group of NLRs that function as negative regulators of inflammation. One of the members of this sub-group, NLRX1, is a potent regulator of interferon, NF-κB and autophagy signaling. Thus, we hypothesized that NLRX1 attenuates Traumatic Brain Injury (TBI) through the negative regulation of overzealous innate immune system signaling. To evaluate this hypothesis, we utilized *Nlr1^{-/-}* mice in a controlled cortical impact (CCI) injury model. The *Nlr1^{-/-}* mice exhibited significantly larger brain lesions and increased motor deficits following CCI injury. We also observed significant proliferation of microglia within the *Nlr1^{-/-}* lesions compared to wild type animals. Mechanistically, our data indicates that NLRX1 attenuates TBI progression through the microglia compartment via negative regulation of NF-κB signaling and IL-6 production. Together, our data extends the function of NLRX1 beyond its currently characterized role in host-pathogen defense and identifies this highly novel NLR as a significant modulator of TBI progression.

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Mal D96N provides new insights into TLR induced signal transduction and inflammation independent of MyD88

Dowling, J., Tate, M.E., Bourke, N.M., Lauterbach, M.A., Golenbock, D.E., Mansell, A.

Hudson Institute of Medical Research, Center for Innate Immunity and Infectious Disease, Melbourne, Australia

Mal is a critical adaptor in TLR2 and TLR4 induced inflammation, facilitating recruitment of MyD88 to these TLRs and is often regarded as a 'bridging' adaptor. However, recent studies have identified roles for Mal in IFNγR and TLR9 signaling. It is the most polymorphic of TLR adaptors, where one single nucleotide polymorphism (SNP), D96N is protective by reducing inflammatory responses demonstrating the critical role Mal plays in modulating the strength of immune responses. We have generated mice expressing this SNP, (MalD96N). BMDMs from D96N mice display reduced inflammatory responses to lipopolysaccharide, LPS (TLR4), producing significantly less IL-6, TNFα, delayed MAP kinase activation and transactivation of NF-κBp65. Critically, MalD96N mice are protected from LPS-induced lethality. Reduced levels of IL-6, TNFα, IL-1β and MCP-1

were also detected in serum following challenge. Latest work describes a role for Mal in maintaining integrity of the intestinal barrier in the non-hematopoietic compartment during infection to *S. typhimurium*, suggesting functions of Mal independent of its 'bridging' role in TLR signaling. While functional studies have been conducted in innate cells there is increasing awareness of the importance between the epithelial barrier and innate immunity during infection. We found that MalD96N gastric epithelial cells, but not BMDMs, display enhanced *S. typhimurium* uptake and MalD96N intestines express substantially reduced Claudin-3, ZO-1 and occludin mRNA compared to WT. Future work aims to clarify results by examining *S. typhimurium* infection in WT, MalD96N and Mal-/- mice. These studies will help elucidate roles for Mal in TLR-signaling and inflammation independent of MyD88.

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Membrane-bound lactoferrin modulates the function of human polymorphonuclear leukocytes partially through TLR-4

Hu, X.M., Xu, Y.R., Dong, H.L., Wang, J., Gao, X.M.
Institutes of Biology and Medical Sciences, Soochow University, Suzhou, China

Lactoferrin (LTF) is a soluble iron-binding glycoprotein of the transferrin family mainly present in mammalian body fluids. Soluble LTF is a versatile molecule that not only regulates the iron homeostasis, but also harbors direct microbicidal and immune-modulating abilities. In contrast, little is known about the function of membrane-bound LTF (mLTF), although its expression on human polymorphonuclear leukocytes (huPMNs) has been reported for decades. Given that LTF/anti-LTF antibodies represent a potential diagnostic/prognostic biomarker and a therapeutic target in patients with various immune disorders, we wished, in the present study, to generate a novel human LTF (huLTF)-specific monoclonal antibody (mAb) suitable for detailed analyses on the expression and function of mLTF. By using the traditional hybridoma cell fusion technology, we obtained a murine IgG1(kappa) mAb, M-860, against huLTF. M-860 is highly efficient in binding to natural huLTF in ELISA, but exhibits no recognition of natural bovine/murine LTF and denatured huLTF in ELISA & Western blot. Moreover, M-860 detects mLTF by FACS and captures endogenous huLTF in total cell lysates of huPMNs. Functionally, M-860 is capable of inducing the activation of huPMNs, which is independent of phagocytosis but signals partially through TLR-4. These data indicate that mLTF serves as a surface receptor on huPMNs, and, upon binding to its ligand like M-860, is capable of modulating the function of huPMNs partially through TLR-4. Thus, detailed analyses on the expression and function of mLTF will further our understandings on the role of LTF in health and disease.

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ATP stimulated P2X7R enhances IL-1 β secretion during *Neisseria gonorrhoeae* infection through a NLRP-3 inflammasome independent pathway

Garcia, K.¹, Ortiz, C.¹, Escobar, G.¹, Mendoza, P.¹, Beltran, C.², Vernal, R.¹, Escobar, A.¹

¹University of Chile, Faculty of Dentistry, Santiago, Chile, ²University of Chile, Faculty of Medicine, Santiago, Chile

Background: *Neisseria gonorrhoeae* (Ngo) is the etiological agent of the sexually transmitted disease gonorrhea. This Gram-negative bacterium has developed multiple immune evasion mechanisms involving the innate and adaptive immune responses. Recent findings have reported that P9-17 Ngo strain can reduce the IL-1 β levels present in culture supernatants of induced human monocyte-derived-macrophages (MDMs). In addition, current data strongly suggest that P2X7 Receptor (P2X7R) is a major component in NLRP3 inflammasome stimulation and subsequent IL-1 β maturation.

Objectives: To analyze the role of ATP-stimulated P2X7R in the IL-1 β maturation pathway in Ngo-infected MDMs.

Methods: Human MDMs were infected with P9-17 Ngo strain (MOI=100) and then stimulated with ATP 5 mM. The secreted levels of IL-1 β were quantified by ELISA. To determine if any NLRP3 inflammasome component was affected by stimulation with ATP, the expression of NLRP3, ASC, and Caspase-1 were assessed by qRT-PCR. In addition, the mature form of IL-1 β was intracellularly detected by immunofluorescence and active Caspase-1 levels were quantified by flow cytometry.

Results: ATP stimulation of P2X7R enhances the secretion of IL-1 β in Ngo-infected MDMs and modifies the NLRP3 expression; however, it does not affect Caspase-1 expression and activity. In addition, in Ngo-infected MDMs, an increment in the cytoplasmic levels of mature form of IL-1 β was detected; however, it was not detected culture supernatants.

Conclusions: ATP and P2X7R have a role in the enhanced secretion of IL-1 β in Ngo-infected MDMs by a Caspase-1 independent pathway. Therefore, the P2X7/IL-1 β secretion pathway could play a role in the pathogenesis of gonorrhea.

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Effect of damps in inflammasome generation in monocytes from pregnant women with preeclampsia

Romao-Veiga, M., Matias, M.L., Rocha Ribeiro, V., Rezeck Nunes, P., Medeiros Borges, V.T., Araujo Jr, J.P., Peracoli, J.C., Serrao Peracoli, M.T.
Sao Paulo State University, Botucatu, Brazil

Preeclampsia (PE) is considered as a major cause of maternal and fetal morbidity and mortality. Plasma of preeclamptic women has elevated levels of molecular structures associated with stress and cell death which can bind to receptors of innate immunity cells and activate an intracellular complex called inflammasome. This study investigated the state of activation both endogenous and induced by Hyaluronan (HA) and monosodium urate (MSU) in monocytes of these patients. Peripheral blood was obtained from 20 preeclamptic (PE), 20 normotensive (NT) pregnant women and 20 non-pregnant (NP) women. Plasma was employed to determine the concentration of HA, uric acid and HMGB1.

Peripheral blood monocytes were separated and cultured with or without HA or MSU. Inflammasome activation was evaluated by RT-qPCR and determined by ELISA. Plasma levels of HA, uric acid and HMGB1 were increased in PE group than in the other groups. In PE group HA stimulation led to an increase in mRNA of NLRP1, NLRP3, caspase-1, IL-1beta, TNF-alpha and HMGB1. All groups studied had IL-1beta increased protein levels detected by ELISA. MSU stimulation increased NLRP3, caspase-1, IL-18, TNF-alpha and HMGB1 mRNA expression in PE and NP groups, and higher protein levels of IL-18 and TNF-alpha in PE group. The endogenous up-regulation of NLRP1, NLRP3, caspase-1, IL-1beta and TNF-alpha mRNA expression in monocytes confirms the activated profile of these cells in PE. NLRP3, caspase-1 and HMGB1 activation by HA and MSU suggest the participation of these DAMPs in the inflammatory process in the pathogenesis of preeclampsia.

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Inflammasomes and intestinal inflammation

Ranson, N.¹, Mitchell, B.², Fanning, S.², Kunde, D.¹, Eri, R.¹

¹University of Tasmania, Launceston, Australia, ²Launceston General Hospital, Launceston, Australia

Background and aims: Inappropriate innate immune responses to invading bacteria and cellular stress contribute to disease development in inflammatory bowel disease (IBD). A key initiating event in the host's immune response is thought to be the activation of the inflammasome complex which enhances the maturation of interleukin (IL)-1 β and IL-18 by providing a signalling platform for the activation of caspase-1. Mice studies have contributed to the overall understanding of inflammasomes, however many have been overshadowed by controversy and others lack consistency. Given the limited number of human inflammasome studies the overall aim of this study was to investigate the expression of different inflammasome complexes in IBD.

Methods: qRT-PCR was performed on inflamed and non-inflamed colon biopsies from 44 ulcerative colitis (UC) patients, 21 Crohn's disease (CD) patients and non-IBD controls from a Tasmanian cohort. The cellular localisation of inflammasomes was determined using immunohistochemistry, immunofluorescence and confocal imagery performed on paraffin embedded sections from inflamed tissue. Targeted RNA sequencing of inflammatory genes was used to identify potential candidate SNPs.

Results: This study describes the gene expression and cellular localisation of inflammasomes in human IBD. Results indicate different expression profiles and localisation of key inflammasome complexes in UC and CD. This study also highlights the possibility of an inflammasome biomarker for distinguishing disease phenotypes.

Significance: Despite conventional treatment may IBD patients still experience active disease therefore targeting specific inflammasomes to reduce IL-1 β levels may represent a more effective treatment option.

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Early endosome autoantigen 1 controls unconventional caspase-1 release after NLRP3 inflammasome activation

Baroja-Mazo, A.¹, Compan, V.², Pelegrín, P.¹

¹Murcia's Biomedical Research Institute (IMIB-Arrixaca), Murcia, Spain, ²Université de Montpellier, Montpellier, France

Caspase-1 is a protease that controls the release of different leaderless proteins, including its own release. Caspase-1 secretome comprises the well studied pro-inflammatory cytokines IL-1b and IL-18, and the alarmin HMGB1. However, little is known about the unconventional release pathway for caspase-1. The release of IL-1b includes microvesicle shedding, lysosomal release and exosome budding. Although endosomal compartments have been suggested to play a role in unconventional protein secretion, IL-1b failed to co-localize with endosome markers, such as early endosome antigen 1 (EEA1), and pro-IL-1b processing by caspase-1 in macrophages has been found to be a cytosolic event that precedes its release. Here we found that activation of the NLRP3 inflammasome in macrophages, mature caspase-1 traffics close to endosomes where it cleaves the endosomal docking protein EEA1. Activation of NLRP3 inflammasome by different means resulted in the processing and release of EEA1. Pharmacological or genetic inhibition of caspase-1 activation abolished the processing and release of EEA1. EEA1 knock-down resulted in a decrease of caspase-1 and IL-1b release after NLRP3 inflammasome activation, but do not affect the release of NLRP3 and ASC particles from macrophages. Our data suggest that the cleavage of the endosome docking protein EEA1 by caspase-1 could affect endosome fusion to form giant endosomes and allow endosomes to fuse with the plasma membrane. This could be a protection mechanism to maintain the integrity of the plasma membrane and preserve cell viability after inflammasome-activation. This caspase-1 dependent mechanism could be used as a release pathway for different cytosolic proteins.

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NLRP3-independent caspase-8 activation is involved in IL-1 β release induced by melittin

Martín-Sánchez, F.¹, Martínez-García, J.J.¹, Rivas, L.², Pelegrín, P.¹

¹Murcia's Biomedical Research Institute (IMIB-Arrixaca), Murcia, Spain, ²Centro de Investigaciones Biológicas CSIC, Madrid, Spain

Melittin, the main component of bee venom, is a cytolytic molecule reported to be able to induce NLRP3 inflammasome activation in macrophages. In addition, previous studies have shown that bee venom induces apoptosis through caspase-8/caspase-3 pathway activation. However, the molecular mechanisms for this inflammasome activation remain poorly understood. Recently, caspase-8 has been suggested to participate in processing and release of IL-1 β , meanwhile caspase-3 has been involved in IL-18 degradation. The aim of this study was to understand the molecular cascades by which melittin induce pro-inflammatory cytokine release. We found that melittin induces classical NLRP3 inflammasome activation by potassium depletion, leading to the formation of ASC specks, caspase-1 activation and IL-1 β maturation and secretion, but

interestingly not IL-18. However, the use of NLRP3, ASC or caspase-1 knock-out BMDMs reduced, but not completely abolished IL-1 β release by melittin, being approximately 30% of IL-1 β released independent of inflammasome and caspase-1. In addition, as expected, caspase-8/caspase-3 pathway was induced by melittin in macrophages, this activation was independent on caspase-1. Inhibition of caspase-8 was able to reduce approximately 30% of the IL-1 β secretion induced by melittin. In addition, caspase-8 inhibition blocked caspase-3 activity leading to an increase in IL-18 secretion. These results suggest that melittin induces IL-1 β processing and secretion mainly via the classical NLRP3/caspase-1 inflammasome pathway, but also by a caspase-8-dependent but NLRP3-independent pathway. In addition, caspase-8 activating caspase-3 appears implicated in degradation of IL-18 and could explain the lack of IL-18 release induced by melittin.

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Engagement of functional cGAS-STING signalling in immortalized SV40 mouse embryonic fibroblasts

Pépin, G.¹, Ferrand, J.¹, Nejad, C.¹, Hornung, V.², Gantier, M.¹

¹Hudson Institute of Medical Research, Centre for Innate Immunity and Infectious Diseases, Clayton, Australia, ²Ludwig-Maximilians University, Gene Center and Department of Biochemistry, Munich, Germany

Cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS) is a key detector of cytoplasmic DNA, which can activate the adaptor protein STING to induce a broad antiviral program in mammalian cells. Recently, several viral oncogenes such as the large T antigen of simian virus 40 (SV40), have been proposed to antagonise the cGAS-STING pathway in response to transfected foreign DNA. However, it is not clear if this potential effect of viral oncogenes is applicable to other cGAS substrates. In this report, we show that endogenous DNA damage by-products can activate a functional cGAS-STING response in SV40-immortalized mouse embryonic fibroblasts (MEFs). This activation leads to increased expression of many interferon-stimulated genes, resulting in a strong antiviral protection against Semliki Forest Virus infection in a STING-dependent manner. Collectively, these results establish that SV40 immortalized MEFs retain a functional cGAS-STING pathway and that they could be used as a useful tool in immunological studies investigating this pathway.

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Deubiquitinating enzyme USP50 is a critical regulator for NLRP3 inflammasome activation

Lee, J.Y., Park, S.H.

Sungkyunkwan University, Biological Sciences, Suwon, Korea, Republic of

NLRP3 is an important component of the inflammasome-mediated immune response that promotes Caspase-1-dependent production of interleukin 1beta (IL-1beta). Its signaling pathway requires the recruitment of adaptor protein ASC after recognition of danger signals. Although NLRP3-mediated inflammasome

signaling pathway is extensively studied in a number of groups, the post-translational modifications regarding the components of NLRP3 signaling pathway such as NLRP3 and ASC remains unclear. In this study, we performed RNAi-based screening in human THP1 cells by using human deubiquitinating enzyme (DUB) siRNA library to identify specific DUBs involved in the regulation of NLRP3 inflammasome activation. Through these systematic approaches, we finally identified USP50 promoting the deubiquitination of the adaptor ASC protein. USP50 specifically bound to ASC and deubiquitinated the K63-linked polyubiquitination of ASC. USP50-knockdown THP1 cells showed the decreased activation of Caspase-1 and subsequently the reduced expression of IL-1beta after stimulation with alum crystals or nigericin. Therefore, our results for the first time demonstrate the underlying molecular mechanism that USP50 is a crucial regulator for NLRP3-mediated inflammasome signaling pathway through deubiquitinating the adaptor ASC protein.

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TLR10 as an innate receptor

Lee, S.M., Yan, S., Yip, T., Li, S., Ip, K., Peiris, J.

The University of Hong Kong, HKU-Pasteur Research Pole and Centre of Influenza Research, Hong Kong, Hong Kong

Among the ten members of toll-like receptors (TLRs) in human, TLR10 remains an orphan receptor without known agonist and function. Since its discovery, multiple attempts have been tried to investigate its function, mechanism of activation or the signaling pathway. However, despite the correlation observed between the induction of TLR10 and viral or bacterial infection, its exact function in innate immune aspect remains unclear. In this study, we aim to identify the potential TLR10 ligand and characterize its associated adaptor protein(s) or signaling molecule(s) upon activation. We revealed that TLR10 physically bound dsRNA in vitro, whilst such binding was favorable at a pH condition similar to that within endosomes. Myeloid differentiation primary response gene 88 (MyD88) was recruited to activate TLR10 for signal transduction which led to interferon regulatory factor (IRF)-7 regulated interferon (IFN) expression. Our result demonstrates that TLR10 is a novel innate receptor which could sense dsRNA within endosomes and subsequently recruit MyD88 to regulate IFN expression mediated via IRF7.

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Modulating innate immune responses to biomaterial-induced inflammation

Christo, S.¹, Diener, K.^{1,2}, Manavis, J.³, Grimbaldston, M.⁴, Bachhuka, A.⁵, Vasilev, K.⁵, Hayball, J.^{1,2,6}

¹University of South Australia, Adelaide, Australia, ²University of Adelaide, Adelaide, Australia, ³SA Pathology, Adelaide, Australia, ⁴Centre for Cancer Biology, University of South Australia and SA Pathology, Adelaide, Australia, ⁵Mawson Institute, Adelaide, Australia, ⁶University of Adelaide, School of Medicine, Adelaide, Australia

Detailing the inflammatory mechanisms of biomaterial-implant induced foreign body responses (FBR) has implications for

revealing targetable pathways that may reduce leukocyte activation and fibrotic encapsulation of the implant. We have adapted a model of poly(methylmethacrylate) (PMMA) bead injection to perform an assessment of the mechanistic role of the ASC-dependent inflammasome in this process. We first demonstrate that ASC^{-/-} mice subjected to PMMA bead injections had reduced cell infiltration and altered collagen deposition, suggesting a role for the inflammasome in the FBR. We next investigated the NLRP3 and AIM2 sensors because of their known contributions in recognising damaged and apoptotic cells. We found that NLRP3 was dispensable for the fibrotic encapsulation; however AIM2 expression influenced leukocyte infiltration and controlled collagen deposition, suggesting a previously unexplored link between AIM2 and biomaterial-induced FBR.

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Linking MIF and NLRP3 in the pathogenesis of IL-1 dependent inflammatory disorders

Lang, T., Lee, J., Fan, H., Cheng, Q., Morand, E., Harris, J. Monash University, Lupus Research Group, Centre for Inflammatory Diseases, Clayton, Australia

Introduction: Macrophage migration inhibitory factor (MIF) is a pluripotent pro-inflammatory factor that acts as a mediator of innate immunity and is implicated in the pathogenesis of a number of autoinflammatory disorders, including Gout. Levels of serum MIF correlate with disease outcomes and clinical studies have shown correlations between elevated levels of IL-1 β and MIF in serum of patients with autoimmune disorders. To date, it is unclear whether MIF specifically regulates the expression and secretion of IL-1 family cytokines.

Methods and results: We inhibited MIF in murine bone marrow-derived macrophages using the small molecule inhibitor, COR123625. We show that depletion of MIF in macrophages significantly reduced IL-1 cytokine family release in response to NLRP3-activating stimuli, but has no effect on the secretion of TNF- α , IL-6 and MCP-1 (CCL2). Moreover, diminished IL-1 responses were independent of NF- κ B function and production of pro-IL-1 β . Instead, MIF depletion specifically inhibits NLRP3-mediated responses as IL-1 cytokine secretion was unaffected following activation of the AIM2 or NLRC4 inflammasomes.

Conclusions: Our findings reveal a novel role for MIF in the modulation of IL-1-dependent inflammatory responses, linking MIF directly to NLRP3 inflammasome activation. This study for the first time implicates a specific role for MIF in the release of IL-1 family cytokines and highlights the potential of targeting MIF in IL-1-dependent pathologies.

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Involvement of NLRP3 inflammasome in *P. gingivalis*-accelerated atherosclerosis and periodontal disease

Kurita-Ochiai, T.¹, Yamaguchi, Y.², Kobayashi, R.¹, Ando, T.²

¹Nihon University School of Dentistry at Matsudo, Matsudo, Japan,

²Tokyo Women's Medical University, Shinjuku, Japan

Periodontal pathogen, *Porphyromonas gingivalis* (*P. g*) has

been shown to accelerate atherosclerotic lesion development in hyperlipidemic animals. We examined a possible association between the inflammasome in atherosclerosis induced by *P. g* infection using Apolipoprotein E deficient spontaneously hyperlipidemic (ApoE^{sh}) mice, and further investigated the effect of NLRP3 deficiency on vascular inflammation and bone loss. Mice were orally infected with *P. g* 33277 (wild type, WT), KDP136 (gingipain-nell mutant), KDP150 (FimA-deficient mutant) 5 times a week for 3 weeks and sacrificed at 18 weeks. Bacteria were detected two days after final infection from each organ. Furthermore, alveolar bone loss, lesion area in the proximal aorta, IL-1 β , IL-18, and TNF- α secretion in peritoneal macrophage, and gene expression of NLRP3, pro-IL-1 β , pro-IL-18, pro-caspase-1 in the gingival tissue and aorta was measured. Oral infection with *P. g* WT significantly increased alveolar bone loss, lesion area of aortic sinus covered with atherosclerotic plaque. IL-1 β , IL-18, and TNF- α production in peritoneal macrophage, gingival or aortic gene expression of NLRP3, pro-IL-1 β , pro-IL-18, and pro-caspase-1 compared with KDP136 or KDP150 challenge. *P. g* genomic DNA was detected in the aorta, gingival tissue, liver, and spleen of WT-challenged-mice not of KDP136- or KDP150-challenged mice. Furthermore, WT-induced expression of pro-IL-1 β and pro-IL-18 mRNA in gingival tissue and aorta were significantly decreased in NLRP3-knockout mice. These results indicate that NLRP3 inflammasome, followed by a response from the IL-1 family, may play a critical role in periodontal disease and atherosclerosis induced by *P. g* challenge through sustained inflammation.

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Dual action of the cytosolic viral sensor MDA-5 in tumor killing and immune modulation triggers type I interferon-dependent antitumor immunity

Wang, X.-Y.(^{1,2}), Yu, X.¹

¹Virginia Commonwealth University School of Medicine, Human and Molecular Genetics, Richmond, United States, ²Massey Cancer Center, Richmond, United States

Melanoma differentiation-associated gene 5 (MDA-5/IFIH1) functions as a major cytosolic innate pattern recognition receptor that recognizes double-stranded RNA (dsRNA). We show that ectopic expression of MDA-5 via replication incompetent adenovirus (*Ad.Mda-5*) causes cytotoxicity and type I interferon (IFN) production in mouse and human prostate cancer cells. This dual activity of MDA-5 requires the adaptor protein IFN- β promoter stimulator 1 (IPS-1/MAVS) and could be functionally uncoupled. Deletion of N-terminal caspase-recruitment domains (CARDs) has no effect on MDA-5-initiated intracellular death signaling in cancer cells, but compromise its capacity to induce IFN- β expression. In contrast to cancer cells that are susceptible to MDA-5-mediated cytotoxicity, normal cells are highly resistant but display a robust type I IFN response. Intratumoral delivery of *Ad.Mda-5* led to regression of established prostate cancers and development of long-lasting antitumor immunity, which was executed through activation of cytotoxic T lymphocytes and/or natural killer cells. Our mechanistic studies using the CARDs-truncated MDA-5 mutant, genetic silencing of IPS-1, and antibody blockade of the IFN-

α/β -receptor reveals that type I IFN signaling is indispensable for protective antitumor immunity induced by in situ MDA-5 therapy. It is the first report that deliberately targeting evolutionarily conserved MDA-5-IPS-1-mediated viral sensing pathway in the tumor can provoke systemic mobilization of both innate and adaptive components of the immune system for tumor destruction. Since the immunosuppressive tumor microenvironment presents a major challenge for successful cancer immunotherapy, our study provides an innovative strategy to reprogram the tumor immune compartment for restoring effective antitumor immunity.

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Activin A regulates neutrophil activation via Smad3 pathway

Qi, Y.¹, Cui, X.², Ge, J.³, Liu, Z.¹

¹College of Basic Medical Sciences, Jilin University, Department of Immunology, Changchun, China, ²College of Basic Medical Sciences, Jilin University, Department of Genetics, Changchun, China, ³College of Basic Medical Sciences, Jilin University, Department of Physiology, Changchun, China

Activin A, a member of transforming growth factor beta (TGF- β) superfamily, acts as a pro-inflammatory factor in acute phase response. Neutrophils are involved in inflammatory response and are the original source of activin A in inflammation. However, whether activin A can directly act on neutrophils is still unclear. In the present study, we found that the type IIA receptor of activin (ActRIIA) was co-expressed with Ly-6G, a marker of neutrophils, on neutrophils of mouse by immunofluorescent staining and flow cytometry, and activin β A, ActRIIA, ActRIIB and Smad3 mRNA were expressed in neutrophils detected by RT-PCR, as well as activin A promoted Smad3 phosphorylation in neutrophils identified by Western blotting. Furthermore, the results showed that the lipopolysaccharide-stimulated peritoneal neutrophils of mouse could release activin A *in vitro*, and activin A stimulated respiratory burst, induced nitric oxide (NO) and interleukin-6 (IL-6) production of the cultured peritoneal neutrophils, while it inhibited migration of neutrophils by transwell method. Additionally, the results revealed that effect of activin A on IL-6 release from neutrophils was significantly attenuated by activin signaling molecule Smad3 knock-down. These above data indicate that activin A can act directly on neutrophils by an autocrine/paracrine manner and may play an important role in regulating activation of neutrophils via Smad3 pathway.

Macrophages

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Dynamic changes in gene regulatory networks control subcapsular sinus macrophage fate and function

Nguyen, A.^{1,2}, Moran, I.^{1,2}, Tanaka, M.³, Tangye, S.^{1,2}, Brink, R.^{1,2}, Phan, T.^{1,2}

¹Garvan Institute of Medical Research, Immunology Division, Sydney, Australia, ²UNSW Australia, Faculty of Medicine, Sydney, Australia, ³Tokyo University of Pharmacy and Life Sciences, Tokyo, Japan

CD169+ subcapsular sinus (SCS) macrophages are strategically positioned at the lymph-parenchyma interface of the lymph node and play a critical role in both the innate and adaptive immune response. SCS macrophages act as “fly paper” and extend processes into the subcapsular space to sample afferent lymph for antigen. Once captured, antigen is then relayed and presented to the B cells in the underlying follicle, a step that is critical for initiation of the antibody response. This intimate contact between SCS macrophages and B cells can be subverted by lymphotropic viruses to gain entry into the follicle and transfect B cells. Inflammation can also disrupt the integrity of the SCS macrophage barrier, possibly by inducing their migration into the follicle and inflammasome-mediated cell death. Recently, we identified lymph-node resident memory T and B cells that occupy a unique niche in the subcapsular region where they scan SCS macrophages. Despite the central role of these cells in immunity, little is known about the underlying molecular networks that govern SCS macrophage function under steady-state conditions and following inflammation, and the niche factors they provide to attract and maintain memory cells in this location. To address this we used a CD169 reporter mouse to track the origin and fate of SCS macrophages by intravital two-photon microscopy, and perform RNAseq analysis of steady-state and inflamed SCS macrophages. Our data defines dynamic changes in gene transcription networks that specify distinct functions for SCS macrophages in infection and immunity.

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Porphyromonas gingivalis-derived RgpA-Kgp complex activates the macrophage urokinase plasminogen activator system

Fleetwood, A.¹, O'Brien-Simpson, N.², Veith, P.², Lam, R.², Achuthan, A.¹, Cook, A.¹, Singleton, W.², Lund, I.³, Reynolds, E.², Hamilton, J.¹

¹The University of Melbourne, Department of Medicine (RMH), Melbourne, Australia, ²The University of Melbourne, Melbourne Dental School, Melbourne, Australia, ³Copenhagen University, Finsen Laboratory, Copenhagen, Denmark

Urokinase plasminogen activator (uPA) converts plasminogen to plasmin, resulting in a proteolytic cascade that has been implicated in tissue destruction during inflammation. Periodontitis is a highly prevalent chronic inflammatory disease characterized by destruction of the tissue and bone that support the teeth. We demonstrate that stimulation of macrophages with the arginine- and lysine-specific cysteine protease complex (RgpA-Kgp complex), produced by the keystone pathogen *Porphyromonas gingivalis*, dramatically increased their ability to degrade matrix in a uPA-dependent manner. We show the RgpA-Kgp complex cleaves the inactive zymogens, pro-uPA (at consensus sites Lys¹⁵⁸-Ile¹⁵⁹ and Lys¹³⁵-Lys¹³⁶) and plasminogen, yielding active uPA and plasmin, respectively. These findings are consistent with activation of the uPA proteolytic cascade by *P.gingivalis* being required for the pathogen to induce alveolar bone loss in a model of periodontitis and reveal a new host-pathogen interaction in which *P.gingivalis* activates a critical host proteolytic pathway to promote tissue destruction and pathogen virulence.

404**Interaction of S100 proteins and the cytoskeleton in phagocytes***Russo, A., Wolf, M., Roth, J., Vogl, T.**Institute of Immunology, Münster, Germany*

Background: Phagocytic functions, such as adherence, migration and phagocytosis are based on the rearrangements of the cytoskeleton. These processes are controlled by intracellular signaling pathways leading to the activation of specific protein kinases and the transient elevation of intracellular calcium concentration. However, the molecular links coordinating the interplay of different cytoskeletal compartments and membrane receptors are not well defined. We previously demonstrated that the major calcium-binding proteins in monocytes, S100A8 and S100A9, can act as cross linker of microtubules dependent on an intracellular calcium concentrations.

Methods: Using Hoxb8 monocytes from wild type and S100A9^{-/-} mice, functional cell assays, immunofluorescence studies and biochemical analysis, we will investigate the role of S100A8/A9 proteins during cellular dynamics of phagocytes.

Results: First immunofluorescence studies confirmed the cross-linking of microtubules and F-actin by S100A8/A9 is dependent on calcium. Furthermore, phosphorylation of S100A9 abrogates cross-linking under the same conditions. In a two chamber filter-based transmigration assay, S100A9^{-/-} monocytes show an decreased number of transmigrated cells after 4 hours of migration.

Conclusion: We provide evidence that S100A8/A9 is associated with microtubules and is strongly involved in crosslinking of actin filaments and MTs with subsequent impact on phagocyte adhesion and transmigration. These findings reveal a novel function of S100A8/A9 in cytoskeletal reorganization as well as transmigration and adhesion of phagocytes.

405**M2-macrophages for stroke treatment***Chernykh, E., Shevela, E., Starostina, N., Morosov, S., Davydova, M., Sachno, L., Ostanin, A.**Institute of Fundamental and Clinical Immunology, Novosibirsk, Russian Federation*

There is a growing body of evidence that monocytes/macrophages may be implicated in the repair of the brain tissue and mediate neurological improvement following stem cell therapy. In the present study we evaluated the safety and clinical efficacy of M2 macrophages in non-acute stroke patients. Thirteen patients (12 males and one female with median 63 years) diagnosed with ischemic (n=10) or hemorrhagic (n=3) stroke have been subjected to M2 cell transplantation (study group). On average 22.0×10^6 of autologous M2 macrophages were injected via intrathecal introduction. Thirteen matched case-control stroke patients with standard therapy formed the control group. There were no any serious adverse effects following M2 cell injection. One patient in the study group and two patients in the control group died during the 6-mo follow-up period due to recurrent stroke. In the study group NIHSS score decreased from 11 to 6 ($p=0.007$) in a 6-mo follow-up

period, whereas the patients in the control group showed a less pronounced neurological improvement (from 11 to 8; $p=0.07$). The improvement of NIHSS score ≥ 3 in the study group was observed in 75% versus 18% in the control group ($p_{\text{FET}}=0.03$). Of note, peripheral blood cells of responder patients differed from that of non-responders by lower spontaneous production of IL-10, FGF- β , PDGF, VEGF and higher stimulation indexes of IL-1 β , TNF- α , IFN- γ and IL-6. These findings suggest the intrathecal administration of M2 cells in stroke patients is safe and leads to a better neurological recovery through the immunomodulatory activity of M2 macrophages.

406**The role of the bone morphogenetic proteins 9 and 10 in endothelial inflammation***Mitrofan, C.G., Appleby, S., Morrell, N.**University of Cambridge, Medicine, Cambridge, United Kingdom*

Atherosclerosis is characterised by chronic inflammation in the vascular wall. Bone morphogenetic proteins (BMPs), BMP2, 4 and 6 signalling via the type-I activin like kinase (ALK) receptors ALK2 have been involved in the inflammatory processes that accelerate atherosclerosis. The endothelial BMP receptor, ALK1, in complex with the bone morphogenetic protein receptor II (BMPRII) signal specifically in response to the circulating BMP9 and 10. Reduced BMPRII endothelial expression has been shown to induce inflammation *in vitro* and in a mouse model of atherosclerosis. We therefore questioned whether BMP9 and 10 signalling via BMPRII confers protection against endothelial inflammation by maintaining endothelial integrity.

Endothelial:monocyte interactions were investigated using an *in vitro* flow-based system. Surface expression of E-selectin, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) were quantified using flow cytometry on human aortic endothelial cells (HAECs).

BMP9 and 10 concentration-dependently increased monocyte recruitment to TNF- α -stimulated HAECs. BMP9 and 10 also increased E-selectin and VCAM-1 surface expression on TNF- α -stimulated endothelial cells. Knockdown of ALK2 reduced the surface expression levels of adhesion molecules.

Pre-treating the endothelium with high concentrations of BMP9/10 followed by TNF- α treatment enhanced the inflammatory response through increased expression of selectins and adhesion molecules and ultimately by upregulating monocyte recruitment. These findings suggest that at high concentrations, BMP9 and 10 signal through the ALK2 receptor, which in turn enhances the surface expression levels of adhesion molecules, ultimately resulting in increased monocyte adherence. These findings are relevant to the potential therapeutic use of BMP9 and 10 in cardiovascular disease.

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The role of zinc in macrophage antimicrobial responses

Stocks, C.J.¹, Kapetanovic, R.¹, Bokil, N.¹, Phan, M.-D.², Lo, A.², Schembri, M.², Sweet, M.J.¹

¹Institute for Molecular Bioscience, The University of Queensland, Brisbane, Australia, ²School of Chemistry and Biological Sciences, The University of Queensland, Brisbane, Australia

Macrophages utilise a host range of strategies to eliminate micro-organisms, however certain pathogens are able to evade these and persist within this innate immune cell. *Salmonella* species including *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) survive and replicate within this intracellular niche, whilst more recently certain strains of *Uropathogenic E. coli* (UPEC) have also been shown survive in macrophages. Regulated zinc trafficking has emerged as a novel antimicrobial mechanism, but specifics regarding its function and role in host defence are not well understood. Recent studies in our laboratory and others, have shown that Toll-like receptor signalling promotes the accumulation of vesicular zinc within human macrophages, with this being harnessed for microbial clearance via metal ion toxicity. These zinc vesicles co-localise with engulfed UPEC strain EC958 and a Δ SPI-1 mutant of *S. Typhimurium*, whilst wild-type *S. Typhimurium* evades zinc-containing vesicles. Intracellular EC958 and *S. Typhimurium* up-regulates the zinc exporter *ZntA*, peaking at 24 hours, indicating exposure of intramacrophage bacteria to zinc stress. For wild-type *S. Typhimurium*, *zntA* expression peaks at 6 hours and declines by 24 hours, suggesting active subversion of zinc-mediated defence. To further investigate this, zinc-stress reporter constructs were designed in EC958 whereby the transcription of *zntA* is coupled to the expression of GFP. The inducible expression of *zntA* and GFP was shown to be highly specific to zinc, with research ongoing to directly visualise the zinc-stress response during bacterial infections. Current research into zinc trafficking is aimed at identifying key molecular players in this response.

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CD11b alveolar macrophage upregulation is a hallmark of acute and chronic inflammatory lung diseases

Duan, M.¹, Steinfort, D.², Smallwood, D.², Hew, M.³, Ernst, M.⁴, Anderson, G.⁵, Irving, L.², Hibbs, M.⁶, Chen, W.¹

¹La Trobe Institute for Molecular Science, Biochemistry and Genetics, Melbourne, Australia, ²Royal Melbourne Hospital, Respiratory Medicine, Melbourne, Australia, ³Alfred Hospital, Allergy, Immunology and Respiratory Medicine, Melbourne, Australia, ⁴Olivia Newton-John Cancer Research Institute, Melbourne, Australia, ⁵University of Melbourne, Pharmacology, Melbourne, Australia, ⁶Monash University, Immunology, Melbourne, Australia

Residential tissue macrophages differentially facilitate organ homeostasis yet also underlie the pathological processes of many acute and chronic diseases. This is particularly true for alveolar macrophages (AMΦs), which predominate within the alveolar airspaces where they are implicated in organ homeostasis, pathogen detection and chronic lung disease pathogenesis.

We have previously shown that AMΦ CD11b expression is an unexpected and translatable marker of lung inflammation in humans and mice. Using an internally standardised flow cytometry approach which compares CD11b expression levels on AMΦs with anchor expression levels in blood neutrophils, we demonstrated that the presence or absence of AMΦ CD11b upregulation and lung eosinophilia could be used to identify distinct inflammatory profiles in mice for the induction and resolution of endotoxin challenge, influenza virus infection and in two genetic models of chronic obstructive pulmonary disease (COPD). Notably, heightened AMΦ CD11b expression was also a novel hallmark of acute lung exacerbations in the SHIP-1^{-/-} model of COPD and the identification of analogous AMΦ profiles in respiratory disease patients highlighted CD11b upregulation as a conserved hallmark of lung inflammation. To elucidate the functional phenotype of AMΦ CD11b upregulation, we have now characterised the proteome of CD11b negative versus CD11b positive alveolar macrophages in response to different acute inflammatory stimuli. Anti-viral as well as classically pro-inflammatory signalling pathways were selectively associated with AMΦ CD11b regulation, and confirms AMΦ CD11b upregulation as a pro-inflammatory pathway in acute and chronic inflammatory lung diseases.

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The comprehensive understanding of the metabolic profile in RAW264.7 murine macrophage during endotoxin tolerance

Ito, Y., Tabata, S., Tomita, M., Fukuda, S.
Keio University, Tsuruoka, Japan

In the intestinal environment, host immune cells interact with commensal microbiota to maintain intestinal homeostasis. Macrophages are one of the most important immune cells in activating the innate immune response by recognition of foreign antigens. The decrease in macrophage response on repetitive antigen stimulation is called endotoxin tolerance. However, molecular mechanisms underlying endotoxin tolerance are still not fully understood. Macrophages change their metabolism drastically in response to the antigen. In this study, we focused on RAW264.7 mouse macrophage cells and comprehensively investigated global changes in metabolism by Capillary Electrophoresis - Time-of Flight Mass Spectrometry (CE-TOFMS, Agilent Technologies) when endotoxin tolerance was induced by lipopolysaccharide (LPS) from *Escherichia coli* serotype O55:B5. RAW264.7 cells were stimulated with LPS for 12 hours and then washed with phosphate buffer saline solution. These cells were then repeatedly stimulated with LPS for 6 hours and then metabolism was analyzed by CE-TOFMS. It was observed that nucleotide sugars such as UDP-N-Acetylglucosamine were accumulated during endotoxin tolerance. In addition, the metabolic pathway switched to the hexosamine biosynthesis pathway which biosynthesized UDP-N-Acetylglucosamine from glycolysis during endotoxin tolerance. These results suggested that repetitive antigen stimulation activated metabolism of nucleotide sugars in macrophage. UDP-N-Acetylglucosamine is known to be related to glycosylation of NF-κB and suppresses the innate immune response. In conclusion, these results

suggest that nucleotide sugar metabolism affects endotoxin tolerance in suppressing NF- κ B. As endotoxin tolerance is known to be related to sepsis and other diseases, these findings may contribute to clinical progress in these aspects.

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Intrinsically disordered *Mycobacterium tuberculosis* protein PPE37 induces proliferation of tolerogenic immune cells and apoptosis by N and C terminal domains, respectively

Ahmad, J.^{1,2}, *Farhana, A.*³, *Babu, M.M.*⁴, *Srinivasan, A.*¹, *Ehtesham, N.Z.*², *Hasnain, S.E.*⁵

¹All India Institute of Medical Sciences, Department of Biophysics, New Delhi, India, ²National Institute of Pathology, New Delhi, India, ³Centre for DNA Fingerprinting and Diagnostics, Hyderabad, India, ⁴MRC Laboratory of Molecular Biology, Cambridge, United Kingdom, ⁵Kusuma School of Biological Sciences, Indian Institute of Technology, New Delhi, *Contributed Equally, India

Mycobacterium tuberculosis (*M.tb*) harbors the highest percentage (14.5%) of intrinsically disordered proteins (IDPs) among bacterial and archaeal genomes. Although their significance vis-à-vis pathogenicity and host pathogen interaction is largely unknown, their abundance may favor pathogen survival both by immune evasion, and possibly facilitating host-pathogen interactions.

The PE/PPE/PGRS protein family of *M.tb*, present nowhere in the living kingdom other than the genus *Mycobacterium*, displays unusually high levels of disordered/partially-disordered stretches. A member of this family, PPE37, is highly expressed under iron stress conditions and is localized to cell membrane. PPE37 is cleaved by *M.tb* protease into an iron-binding N-terminal part and a C-terminal highly disordered region, which harbours the eukaryotic NLS motif that localizes it to the nucleus of infected macrophages. Recombinant N-terminal segment was found to interact and internalize within the monocytic THP-1 cells leading to their proliferation and differentiation into CD11c, DC-SIGN positive semi-mature dendritic cells, known to induce immune tolerance. These cells also demonstrated a high IL-10, negligible IL-12 and low TNF- α secretion, an environment suitable for maintenance of tolerogenic immune cells. The nuclear-targeted C-terminal segment induced apoptosis of the infected host cell through mechanism that involves caspase-3 but without DNA damage.

Our data provide evidence supporting the role of intrinsically disordered stretch within a PPE protein in performing contrasting functions to modulate the host processes possibly through molecular mimicry and cross-talk in two spatially distinct host environments so as to benefit *M.tb* in terms of survival and pathogenesis.

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Macrophages programmed by apoptotic cells inhibit epithelial-mesenchymal transition in lung alveolar epithelial cells via PGE₂, PGD₂, and HGF

Yoon, Y.-S., Choi, Y.-H., Kang, J.L.

Ewha Womans University, Physiology, Seoul, Korea, Republic of

Apoptotic cell clearance results in the release of growth factors and the action of signaling molecules involved in tissue homeostasis maintenance. Here, we investigated whether and how macrophages programmed by apoptotic cells inhibit the TGF- β 1-induced Epithelial-mesenchymal transition (EMT) process in lung alveolar epithelial cells. Treatment with conditioned medium derived from macrophages exposed to apoptotic cells, but not viable or necrotic cells, inhibited TGF- β 1-induced EMT, including loss of E-cadherin, synthesis of N-cadherin and α -smooth muscle actin, and induction of EMT-activating transcription factors, such as Snail1/2, Zeb1/2, and Twist1. Exposure of macrophages to cyclooxygenase (COX-2) inhibitors (NS-398 and COX-2 siRNA) or RhoA/Rho kinase inhibitors (Y-27632 and RhoA siRNA) and LA-4 cells to antagonists of prostaglandin E₂ (PGE₂) receptor (EP4 [AH-23848]), PGD₂ receptors (DP1 [BW-A868C] and DP2 [BAY-u3405]), or the hepatocyte growth factor (HGF) receptor c-Met (PHA-665752), reversed EMT inhibition by the conditioned medium. Additionally, we found that apoptotic cell instillation inhibited bleomycin-mediated EMT in primary mouse alveolar type II epithelial cells *in vivo*. Our data suggest a new model for epithelial cell homeostasis, by which the anti-EMT programming of macrophages by apoptotic cells may control the progressive fibrotic reaction via the production of potent paracrine EMT inhibitors.

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Mer signaling leads to transactivation of anti-inflammatory target gene arginase 2 of liver X receptor via STAT1 transcription factor in macrophages

Kim, S.-Y., Lim, E.-J., Kang, J.L.

Ewha Womans University, Physiology, Seoul, Korea, Republic of

Mer receptor tyrosine kinase (Mer) plays a central role in intrinsic inhibition of the inflammatory response by immune cells. Previously, we demonstrated that the Mer signaling increases the transcriptional liver X receptor (LXR) activity to promote the resolution of acute sterile inflammation. Here we aimed to understand the downstream pathway of Mer signaling after growth arrest-specific protein 6 (Gas6) treatment leading to LXR expression and transcriptional activity in mouse bone-marrow derived macrophages (BMDM). We show the role of signal transducer and activator of transcription1 (STAT1), which acts as an enhancer of LXR expression and LXR-mediated transcription of target genes in BMDM. Gas6-induced STAT1 phosphorylation, and LXR and the target gene expression were inhibited in BMDM from Mer^{-/-} mice or by the specific inhibitor of PI3K, or Akt. Gas6-induced Akt phosphorylation was reduced in BMDM from STAT1^{-/-} mice or BMDM in the presence of STAT1 specific inhibitor, fludarabine. Gas6-induced LXR activity was enhanced through an interaction between LXRA and STAT1 on the DNA promoter of Arg2, which was dependent on STAT1. We show that Gas6 inhibited LPS-induced nitric oxide production through Mer/STAT1/LXR pathway-dependent Arg2 induction in macrophages. Our data suggest that Gas6/Mer signaling leads to increased transcriptional LXR activity and its target genes, including Arg2, in macrophages via STAT1 pathway, consequently exerting the anti-inflammatory response.

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Immunization of recombinant antigen EpC1 from *Echinococcus granulosus* on macrophage regulation*Li, J.¹, Wang, H.¹, Ding, J.², Zhen, X.¹, Wen, H.¹, Zhang, W.¹**¹The First Affiliated Hospital of Xinjiang Medical University, Urumqi, China, ²School of Preclinical Medicine, Xinjiang Medical University, Urumqi, China*

Alternatively activated macrophages (AAMacs) are key to promoting Th2 responses and have been associated with a variety of roles during helminth infection. *Echinococcus granulosus* (E.g) induces a polarized Th2 immune response in its intermediate hosts. Here we investigated the effects of its infection and a recombinant antigen, termed rEpC1, derived from E.g, on murine macrophage *in vivo* and *in vitro*.

Methods: Peritoneal exudate cells (PECs) from E.g and E.m infection mice were harvested by adherence method and then the activation status of macrophages was analyzed by evaluating the expression of genetic markers of alternative activation (Fizzl, Ym1, and Arg1). Then we constructed the rEpC1 and injected hydatid cyst fluid (HCF) or rEpC1 intraperitoneally 6 or 9 times, delivered on alternate days. After 2 days of the final injection, PECs were harvested and analyzed and activation status of macrophages. T-cell cytokine production was assessed by culturing spleen cells with HCF and rEpC1. Supernatants were removed after 72hrs and the concentrations of cytokines were measured by CBA kit. AAMacs were recruited to the peritoneum of mice after E.g and E.m infection. In addition, *in vitro* studies showed that HCF and rEpC1 can directly convert bone marrow macrophages to an alternatively activated phenotype. Our studies suggest that the rEpC1 activates macrophages as an initial step in the induction of Th2 responses by E.g infection to defense against the host immune response.

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Influenza vaccines induce NF- κ B activations and enhancement of TLR expressions in macrophages*Sato, K.¹, Asanuma, H.¹, Ato, M.², Tashiro, M.¹, Odagiri, T.¹, Itamura, S.¹**¹National Institute of Infectious Diseases, Influenza Virus Research Center, Tokyo, Japan, ²National Institute of Infectious Diseases, Department of Immunology, Tokyo, Japan*

Influenza vaccines induce protective immunity via induction of antibody production against subsequent infections, and a variety of different vaccine types are currently available. It has been known that inactivated influenza vaccines induce, through TLR7, type I IFN, which influences vaccine-specific antibody production. Previously, we showed that NF- κ B/AP-1 activations by inactivated whole-virion influenza vaccines were correlated with type I IFN induction. To explore these signaling in detail, the effect of an RIG-I inhibitor, which results in inhibiting TBK1/IKKe, and of an endosomal acidification inhibitor, which results in suppressing endosomal TLR-mediated signaling, was evaluated. PMA-differentiated THP-1 cells were preincubated with either of the inhibitors, and then co-cultured with various concentrations of inactivated whole virion vaccines. After 20 h incubation with vaccines, NF- κ B/AP-1 activities were measured.

Not only the inhibition of TBK1/IKKe but that of endosomal acidification suppressed the NF- κ B activations by vaccines. In addition, both inhibitors also reduced the NF- κ B activity levels in MyD88-deficient THP-1 cells. Upon stimulation with various TLR-specific ligands, TLR7 signaling to activate NF- κ B was not detected in the MyD88-deficient cells. In other experiments, some TLR expressions were enhanced by the vaccine treatment. The enhancement of TLR expressions was not affected by the treatment of both TBK1/IKKe inhibitors and endosomal acidification inhibitors. Taken together, the activations of NF- κ B by vaccines might be mediated through TLR3 as well as TLR7 and RIG-I pathways. Further study will clarify signal transductions of influenza vaccines in macrophages.

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Quercetin suppressed imiquimod-induced activation of alveolar macrophages*Oyabu, S., Matsushima, M., Omura, A., Ogasawara, N., Ochi, H., Kusatsugu, Y., Atsumi, K., Takemura, K., Kawabe, T.**Nagoya University Graduate School of Medicine, Department of Pathophysiological Laboratory Sciences, Nagoya, Japan*

Respiratory viral infections that cause chronic airway and lung disease can result in the activation of the innate immune response. Alveolar macrophages (AMs), one of the first lines of defense in the lung, are abundantly located in alveoli and the respiratory tract. Flavonoids found in fruits and vegetables exhibit cytoprotective effects on various types of cells. In this study, we investigated the effect of quercetin on AMs activated by imiquimod, a ligand of Toll-like receptor (TLR) 7. In both a mouse AM cell line (AMJ2-C11 cells) and mouse bronchoalveolar fluid cells, we demonstrated that quercetin attenuated the TLR7-induced expression of TNF- α and IL-6. In AMJ2-C11 cells, quercetin also attenuated the TLR7-induced CD40 expression; attenuated the translocation of p65; induced translocation of Nrf2 from cytosol to nucleus; and induced heme oxygenase (HO)-1 expression. Notably, tin protoporphyrin IX (SnPP), an inhibitor of HO-1, also attenuated TLR7-induced transcription of the TNF- α and IL-6 genes, suggesting that the effect of quercetin is mediated by HO-1. These results suggest that dietary supplementation with quercetin may have efficacy in the treatment of respiratory viral infection.

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Critical role of selective autophagy adaptor protein p62 in quercetin-induced Nrf2 activation*Kusatsugu, Y., Matsushima, M., Oyabu, S., Ochi, H., Atsumi, K., Ogasawara, N., Takemura, K., Kawabe, T.**Nagoya University Graduate School of Medicine, Department of Pathophysiological Laboratory Sciences, Nagoya, Japan*

Flavonoids are polyphenolic compounds included in vegetables and fruits. We have reported that quercetin, one of the flavonoids, exhibited cytoprotective effects via nuclear factor-E2-related factor 2 (Nrf2)-heme oxygenase (HO)-1 pathway in mast cells, epithelial cells, and fibroblasts. However, the molecular mechanisms how quercetin activates Nrf2 is

still poorly understood. Autophagy, one of the important cytoprotective mechanisms, is an intracellular bulk degradation system for the maintenance of cellular homeostasis in response to cellular stresses. It has been reported that quercetin could induce autophagy. Recently, autophagy has been reported to activate Nrf2 through selective autophagy-adaptor protein p62. In this study, we investigated the involvement of autophagy on quercetin-induced Nrf2 activation, especially focusing on p62. Mouse macrophage cell line, RAW 264.7 cells were used and treated with quercetin for 8-24 hr. We found that quercetin could induce the conversion of LC3-I to LC3-II, a molecular marker for autophagy. Quercetin could also induce selective autophagy by inducing the expression and phosphorylation of p62. Based on these results, we suggested that quercetin-induced Nrf2 activation might be included in p62-mediated pathway.

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Extracellular ATP acting as a danger signal activates the P2X7 receptor in macrophages for the release of a specific secretome

de Torre-Minguela, C., Barberà-Cremades, M., Gomez, A.I., Baroja-Mazo, A., Martín-Sánchez, F., Pelegrin, P., Murcia's Biomedical Research Institute (IMIB-Arrixaca), Murcia, Spain

We aimed to obtain a better characterization of P2X7 receptor (P2X7R) secretome in both M1 and M2 polarized macrophages. The changes in P2X7R secretome were analyzed by LC-MS/MS and antibody array technology. ELISA and western blot were then used to validate P2X7R secretome. The activation of P2X7R-signaling pathways leading to protein release was studied using a pharmacological approach including specific P2X7R antagonists and P2X7R-deficient mice, as well as NLRP3- or Casp1-deficient macrophages. We identified the release of 25 proteins associated with P2X7R and 9 relevant proteins for the inflammatory process were selected for further validation: macrophage mannose receptor, CD14, cathepsin B, cystatin B, thioredoxin, annexin A1, peptidyl-prolyl cis-trans isomerase A, TNF- α and CCL-2. We compared their secretion with the control release of IL-1 β and the p10 subunit of active caspase-1, two well established proteins released upon P2X7R stimulation. We characterized the different P2X7R molecular signaling leading to protein release, which are activated at different times and affecting unconventional and conventional release pathways. P2X7R activation was responsible for the secretion of more proteins than the ones controlled by the inflammasome/caspase-1 pathway. Furthermore, we found that P2X7R induces in M2 polarized macrophages the release of anti-inflammatory proteins that could contribute to the resolution of the inflammation. The characterization of P2X7R-secretome in macrophages contributes to novel conceptual advances into the general biology of macrophages and the release of anti-inflammatory proteins in M2 macrophages, suggesting for the first time a potential role for P2X7R during the resolution of inflammation.

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Interferon lambda drives a pro-inflammatory phenotype in macrophages

Read, S.¹, Ramezani-Moghadam, M.¹, Schibeci, S.², George, J.¹, Ahlenstiel, G.¹

¹Westmead Institute for Medical Research, University of Sydney, Storr Liver Centre, Sydney, Australia, ²Westmead Institute for Medical Research, Centre for Immunology and Allergy Research, Sydney, Australia

Interferon lambdas (IFNLs) are potent antiviral cytokines that are widely implicated in many chronic inflammatory diseases. While IFNLs are best known for their role in clearance of hepatitis C virus infection, there is substantial data supporting their role in acute viral infections such as influenza and viral gastroenteritis, as well as chronic autoimmune and liver disease. Numerous tissues have reported IFNL expression, but there remains controversy over which PBMC subsets are responsive to IFNL and thus contribute to inflammatory activity in chronic diseases.

Upon examining IFNL sensitivity *in vitro*, we demonstrate macrophages but not monocytes are more responsive to IFNL3 than myeloid and plasmacytoid dendritic cells; the only other leukocyte populations with a significant IFNL response. To investigate the role of IFNLs on macrophage maturation and function, human monocytes from healthy subjects were differentiated using either GM-CSF (M1 skewed) or M-CSF (M2 skewed), alone or in combination with IFNL3. M1 macrophages expressed more IFNL receptor (IFNLR1) and were more responsive to IFNL3, supporting their role as pro-inflammatory cytokines. Moreover, IFNL3 increased the expression of numerous inflammatory/chemotactic cytokines and activation markers as measured by qPCR and flow cytometry respectively. Functionally, macrophages differentiated in the presence of IFNL3 were more phagocytic towards apoptotic cells and more chemo-attractant towards CD3, NK and NKT cells.

Lastly, we demonstrated an accumulation of IFNLR1 expressing macrophages in numerous chronic inflammatory conditions, supporting their potential to drive a pro-inflammatory state. Our data suggests that IFNL contributes to chronic inflammation by driving a pro-inflammatory macrophage phenotype.

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A novel alveolar-like macrophage to study influenza A infection *in vitro*

Wood, C.¹, Logue, C.², Silman, N.², Jackson, S.¹, Fejer, G.¹

¹Plymouth University, Plymouth, United Kingdom, ²Public Health England, Porton Down, United Kingdom

Influenza A (IAV) primarily infects and replicates within the respiratory tract, with its infectious and entry mechanisms well characterised in non-immune epithelial cells. However, lung Alveolar Macrophages (AMs) play pivotal roles in the defence against IAV, but yet the mechanisms of viral pathogenesis in these cells are much less understood. Much of our current understanding on IAV induced innate immune responses comes from transformed macrophage cell lines or primary bone marrow derived or peritoneal macrophages, due to the limited

availability of AM's. This limits the understanding we have of IAV pathogenesis within this distinct and specialised branch of macrophages.

The recently described MPI cells (Fejer *et al.*; 2013, PNAS) represent a novel, non-transformed, continuously growing macrophage model that could bridge this gap in our understanding, as they closely resemble AMs. Our aim is to study innate responses in this model, with regards to IAV. It is expected that IAV infection will elicit a distinct innate immune response in MPI cells that closely resembles that of AM's. Moreover, by taking advantage of the unlimited availability of MPI cells and the availability of cells from various gene deficient backgrounds we hope to identify specific innate sensors and mechanisms that lead to the IAV induced innate immune response.

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Tumours from patients with colorectal cancer may dysregulate gut macrophage function and phenotype

Norton, S., Taylor, E., Dunn, E., Munro, F., Black, M., McCall, J., Kemp, R.

University of Otago, Dunedin, New Zealand

In contrast to many cancers, a high infiltration of macrophages in colorectal cancer has been associated with improved prognosis for patients. Cytokines and other stimuli from the tumor microenvironment affect monocyte to macrophage maturation and subsequent phenotype and function. We used a novel flow cytometry panel in both tumor and paired non-tumor bowel from colorectal cancer patients. Heterogeneous macrophage populations, ranging from gut conditioned to a more conventional monocyte-derived macrophage phenotype, were identified in the tissues. The frequency of macrophage subsets with a gut-conditioned phenotype was lower in tumor compared to non-tumor bowel. The frequency of macrophages expressing high CD206 and CD163, both associated with an anti-inflammatory phenotype, was also decreased in the tumour tissue compared to non-tumour bowel. The microenvironment strongly affects macrophage phenotype. Colorectal cancer patient PBMCs were cultured in both tumour and NTB conditioned media, which were assessed for cytokine content using a multiplex assay. Conditioned media that contained high levels of IL-6 and/or TNF α supported an anti-inflammatory macrophage phenotype. This study grants new insight into macrophage function in colorectal cancer and provides methods to examine them *ex vivo* from human tissue.

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Murine macrophage phagocytosis of devil facial tumour disease cells

Li, X.¹, Lyons, A.B.², Korner, H.¹, Woods, G.¹

¹Menzies Institute for Medical Research, University of Tasmania, Hobart, Australia, ²School of Medicine, University of Tasmania, Hobart, Australia

The Tasmanian devil (*Sarcophilus harrisii*) is the world's largest marsupial carnivore. A transmissible cancer known as Devil Facial Tumour Disease (DFTD) threatens its existence. The disease is

fatal as there is no protective immune response. There is no evidence for innate immune responses such as NK cell activity or phagocytosis against the cancer cells to compensate for lack of MHC expression. The aim of this study was to investigate macrophage phagocytosis as a potential defence mechanism against DFTD. As tumour necrosis factor (TNF) is an important cytokine in macrophage activation we also investigated the involvement of TNF in macrophage phagocytosis. Bone marrow derived macrophages (BMDMs) were generated from C57BL/6 wild type (WT) and C57BL/6 TNF^{-/-} (TNF^{-/-}) mice. DFTD cells were labelled with CFSE and co-incubated with CellTrace™ Violet labelled BMDMs. Phagocytosis of DFTD cells was investigated by confocal microscopy and flow cytometry. The results revealed that DFTD cells can be phagocytosed by WT and TNF^{-/-} BMDMs with similar efficiency. It would appear that TNF signalling is not involved in macrophage phagocytosis of DFTD cells before activation. Following activation by exposure to IFN γ /LPS, TNF^{-/-} BMDMs had higher phagocytosis efficiency and lower nitric oxide production compared to WT controls. In addition, using nitric oxide (NO) inhibitor failed to alter phagocytosis efficiency in IFN γ /LPS activated TNF^{-/-} macrophages. Our results indicate that DFTD cells can be phagocytosed and that TNF appears to reduce IFN γ /LPS activated macrophage phagocytosis. NO is unlikely to account for the increased phagocytosis by TNF^{-/-} BMDMs.

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CD169 identifies an anti-tumor macrophage subpopulation in human hepatocellular carcinoma

Wu, Y., Zhang, Y., Zheng, L.

Sun Yat-sen University, Guangzhou, China

Macrophages are a major component of most solid tumors and can exert both anti- and pro-tumorigenic functions. Although the immunosuppressive/pro-tumor roles of macrophages have been widely examined, significantly less is known about macrophage subpopulations that have potential anti-tumor properties in humans. In the present study, a population of CD169⁺ macrophages with relatively high expression levels of HLA-DR and CD86 was identified in human hepatocellular carcinoma tissues. The frequency of CD169-expressing macrophages within cancer nests was significantly lower compared with that found in paired non-tumor areas. *In vitro* experiments revealed that in the presence of anti-CD3 stimulation, CD169⁺ macrophages could significantly enhance the proliferation, cytotoxicity, and cytokine production capacity of autologous CD8⁺ T cells in a CD169 molecule-dependent manner. Autocrine TGF- β produced by tumor-stimulated macrophages was involved in the down-regulation of CD169 expression on these cells. Moreover, CD169⁺ macrophage accumulation in tumor tissues was negatively associated with disease progression and predicted favorable survival in hepatocellular carcinoma patients, which was in contrast to the trend observed for total CD68⁺ macrophages. Therefore, CD169 may act as a costimulatory molecule for cytotoxic T cell activation, and could define a population of tumor-infiltrating macrophages with potential anti-tumor properties in human hepatocellular carcinoma tissues.

All the authors declare no competing financial interests and concur with the submission.

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Roles of macrophages in transplantation of tissue-engineered cartilage in mice

Fujihara, Y.¹, Hikita, A.², Takato, T.¹, Hoshi, K.¹

¹The University of Tokyo Hospital, Department of Oral-maxillofacial Surgery, Dentistry and Orthodontics, Tokyo, Japan, ²The University of Tokyo, Department of Cartilage & Bone Regeneration (Fujisoft), Tokyo, Japan

In regenerative medicine, tissue reactions after transplantation of engineered tissues could affect the regeneration and maturation. In our previous studies, tissue-engineered cartilage consisting of C57BL/6 mice chondrocytes and poly-L-lactic acid (PLLA) scaffolds were transplanted in EGFP transgenic mice, and it was shown that the host-derived EGFP-positive cells were mostly F4/80-positive macrophages. This study was conducted to elucidate the properties and effects of macrophages in transplantation of tissue-engineered cartilage. As to methods, human chondrocytes were cultured and embedded in PLLA scaffold, and then transplanted subcutaneously in athymic mice. After a certain period of time, they were harvested and analyzed using real-time RT-PCR and ELISA. Expression of cytokines related to M1 macrophage was increased from 1 to 2 weeks after transplantation. Meanwhile, arginase I-positive M2 macrophages became distinct around 2 weeks, suggesting that the shift of macrophage from M1 to M2 could occur around 2 weeks. By partially depleting macrophages for initial 7 days with clodronate liposome, more cartilage matrix was produced in tissue-engineered cartilage. It was suggested that suppression of early-stage macrophages, which were considered to be M1-prone, may be advantageous for promoting cartilage maturation.

Metabolism

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Accelerated atherosclerosis in the context of rheumatoid arthritis

Bäcklund, A.¹, Johansson, M.², Ria, M.³, Ketelhuth, D.F.¹, Tsirikla, P.¹, Chernogubova, E.¹, Jin, H.¹, Bäcklund, J.², Eriksson, P.¹, Maegdefessel, L.¹, Holmdahl, R.², Hansson, G.K.¹, Hamsten, A.¹

¹Karolinska Institutet, Medicine Solna, Stockholm, Sweden, ²Karolinska Institutet, Department of Medical Biochemistry and Biophysics, Stockholm, Sweden, ³Karolinska Institutet, Institute Molecular Medicine and Surgery, Karolinska Institute, Stockholm, Sweden

It is well recognized that patients with chronic inflammatory diseases, such as rheumatoid arthritis (RA) have an increased risk for cardiovascular disease (CVD) compared with the general population. It has also been suggested that CVD presented in RA patients is of an altered, more aggressive, phenotype compared to subjects without RA. Thus, there is a need to gain a deeper understanding of how the exacerbated inflammatory

state of arthritis affects the atherosclerosis process when both syndromes are presented in the same individual. We have developed a novel murine model where the human relevant genes of CVD (deficiency of Low Density Lipoprotein (LDL) receptor, thus increased LDL levels) and RA (MHCII, thus susceptible to collagen-induced arthritis) have been crossed into the common C57Bl6/J strain, enabling both arthritis and atherosclerosis being presented simultaneously. This model mirrors the clinical state where the systemic inflammation of arthritis enhances atherosclerosis progression, where mice presenting arthritis have a significant increase in atherosclerotic lesion progression compared with their non-arthritic littermates. Interestingly, there was an inverse correlation with cholesterol levels, but a positive correlation with macrophages, and macrophage associated cytokines but not T cells. This model thus demonstrates that the lipid levels are vital in initiation of lesion development but it is the enhanced innate immune system that is driving the lesion acceleration in a chronic inflammatory state. This novel combined model is now available to be used to investigate altered clinical treatment strategies or novel treatments of atherosclerosis in the context of arthritis.

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Correlation between the metabolome and binder of ST2 receptor in chronic periodontics in elderly

Borges, A.¹, Carvalho, M.², Venturini, G.³, Vieira, C.⁴, Paulino, T.⁵, Botelho Miguel, C.⁶, Oliveira, C.⁷, Pereira, A.³, Binignat, O.⁸, Rodrigues, W.^{4,8,9}

¹Faculdade Mineirense - Fama, Odontology, Mineiros-GO, Brazil, ²Faculdade Mineirense - FAMA, Odontology, Mineiros, Brazil, ³Heart Institute (InCor)/Univ of Sao Paulo Med Sch, São Paulo, Brazil, ⁴Federal University of Uberlandia, Uberlandia, Brazil, ⁵Federal University of GoiasTriangulo Mineiro - CEFORES, Uberaba, Brazil, ⁶Federal University of Triangulo Mineiro, Uberaba, Brazil, ⁷Faculdade Mineirense - Fama, Mineiros-GO, Brazil, ⁸Federal University of GoiasTriangulo Mineiro, Uberaba, Brazil

Introduction: High relationship of senescence with the emergence and/or compromise of disease in oral cavity is a triggering factor of disease in this age group including chronic periodontal disease (CPD). Some markers have been associated with disease prognosis, as well as systemic diseases, such as ST2 binder.

Objectives: Evaluate the correlation between metabolites and ST2 receptor binder in crevicular fluid in the elderly.

Methods: All procedures were approved by the Ethics Committee in Research of the Federal University of Triangulo Mineiro, under number: 017430/2014. Twenty individuals were selected after applying the inclusion and exclusion criteria. They were divided into 2 groups, CPD (N = 10 - Clinical evaluation + probing depth higher or equal to 3 mm, and at least presence of marginal bleeding at a site) without disease - Control (n = 10). To the assessment of metabolites was performed the metabolome (triplicate - GC/MS Agilent -7890B GC; 5977A MS). The ELISA was performed for the detection and quantification of IL-33 (R&D Systems). The Prism software was used for statistical evaluation.

Results: Were identified 969 metabolites, of which 64 were selected to analyze. After analysis 5 metabolic (2,3

dihydroxypropyl icosanoate, glycerol, serine, 5-aminovaleric acid e putrescine) obtained a ratio CPD/Control greater than 2. The elevation was accompanied of increase of the ST2-binder, where was found a positive correlation.

Conclusion/discussion: The data point to a relationship of metabolic activity inducible IL-33/ST2 in CPD in the elderly.

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ATF4 reprograms T cell metabolism and control differentiation of T cell subsets

Du, W.¹, Xia, R.¹, Yang, X.², Lu, B.¹

¹University of Pittsburgh School of Medicine, Pittsburgh, United States, ²Tsinghua University, Beijing, China

T cell metabolism is strongly influenced by the extracellular tissue microenvironment such as the oxidizing status. The underlying molecular mechanisms are not well understood. Here, We demonstrated that the oxidative environment induced ATF4 in activated T cells. ATF4 is a basic leucine-zipper (bZip) transcription factor, which regulates cellular redox state and amino acid metabolism. ATF4 deficient

T cells had a severe aberration in metabolism as illustrated by global gene expression profiling and biochemical analysis. Lack of ATF4 resulted in T cell autonomous defects in the function of Th1 cells. In vivo, ATF4 deficiency resulted in altered antitumor immune responses and exacerbated autoimmune inflammation. Our study establishes that ATF4 plays an important role in T cell metabolism and is important for the Th1 type immune response.

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The impact of intestinal lymph extravasation on adipose tissue function and whole body insulin resistance

Cao, E.¹, Watt, M.², Hu, L.¹, Porter, C.¹, Trevaskis, N.¹

¹Monash University, Monash Institute of Pharmaceutical Sciences, Melbourne, Australia, ²Monash University, Department of Physiology, Melbourne, Australia

Insulin resistance (IR) underpins a spectrum of prevalent and inadequately treated cardiometabolic diseases, including type 2 diabetes (T2D). Excess adipose, particularly visceral adipose tissue (VAT) in the abdomen, increases the risk of IR. The expanded VAT releases pathogenic mediators that promote IR. *db/db* mice showed significantly enhanced lymphatic vessel permeability and in mice with hyperpermeable lymphatics the surrounding adipose is expanded. The content of inflammatory and metabolic mediators in intestinal lymph is also increased in response to high fat diet (HFD). Our aim is to determine whether intestinal lymph access to VAT is increased in HFD thereby promoting VAT expansion and pathogenic changes that promote IR.

Immunofluorescence analysis of mesenteric lymph vessels and Evans blue lymphangiography showed that in mice fed HFD vs chow fat diet (CFD) there were increased initial lymph capillary lymphangiogenesis and the permeability of collecting mesenteric lymph vessels. Metabolomics and flow cytometry analysis revealed increases in lipid species, pro-inflammatory mediators and CD4+ and CD8+ T cells in the HFD vs CFD fed

lymph. Finally, to determine whether increased lymph access to VAT promotes pathogenic changes in adipocytes with the potential to induce IR, co-culture of adipocytes with intestinal lymph enhanced adipocyte adipogenesis, inflammation, lipogenesis, and lipolysis. These effects were greater on treatment with HFD vs CFD fed lymph. Current results provide evidence that intestinal lymph access to VAT is increased in HFD thereby promoting VAT expansion and pathogenic changes that promote whole body IR. These studies advance the fundamental understanding of the pathogenic drivers of IR.

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Tributylin attenuate, but do not revert, the metabolic and inflammatory changes associated with obesity through GPR43-dependent and independent mechanisms

Sato, F.T.^{1,2}, Murata, G.M.³, Ferreira, C.M.⁴, Vieira, A.T.⁵, Marino, E.², Rodrigues, H.G.⁶, Hirabara, S.M.⁷, Crisma, A.R.³, Cruz, M.M.⁴, Alonso-Vale, M.I.C.⁴, Câmara, N.O.S.⁸, dos Santos, M.F.⁹, Curi, R.³, Vinolo, M.A.R.¹

¹University of Campinas / Institute of Biology, Department of Genetics, Evolution and Bioagents, Campinas, Brazil, ²Monash University, Department of Biochemistry & Molecular Biology, Melbourne, Australia, ³University of São Paulo / Institute of Biomedical Sciences, Department of Physiology and Biophysics, São Paulo, Brazil, ⁴Federal University of São Paulo, Department of Biological Science, Diadema, Brazil, ⁵Federal University of Minas Gerais / Institute of Biological Sciences, Department of Biochemistry and Immunology, Belo Horizonte, Brazil, ⁶University of Campinas / Faculty of Applied Sciences, Limeira, Brazil, ⁷Cruzeiro do Sul University / Institute of Physical Activity Sciences and Sports, São Paulo, Brazil, ⁸University of São Paulo, Department of Immunology, São Paulo, Brazil, ⁹University of São Paulo, Department of Cell and Developmental Biology, São Paulo, Brazil

The non-healthy obesity is associated with low degree chronic inflammation involved in dysfunction of several organs and tissues. We investigate the effect of a tributyrin (Tb), pro-drug of a butyrate, a short chain fatty acid produced by bacterial fermentation on a diet induced obesity model (DIO). After 2 months on high fat diet (HFD), C57BL/6 obese mice were treated with 2 g/kg body weight, three times a week or placebo. After 6 weeks of treatment, Tb mice had lower weight gain, better fasting glucose, glucose and insulin tolerance and a decrease in the liver weight and hepatic triglycerides content. Although no difference in white adipose tissues (WAT) weight were observed, a reduction of relative and absolute number of M1-macrophages and IL1- β and MCP-1 mRNA content were present in Tb mice. Tb reverses some but not all the metabolic and inflammatory changes present in obese animals. When we treated obese GPR43 knockout mice with Tb, at the same conditions as the previous experiment, we didn't found alterations of weight gain, insulin tolerance and WAT M1 and M2 macrophages, suggesting the participation of GPR43 on Tb effects. Although this result, we found an improvement in basal glycaemia and glucose tolerance on Tb animals, indicating an independent effect of Tb between the glycemic parameters and weight gain without alteration in insulin tolerance. These results, indicate that the effects of tributyrin on obese animals depends partially of GPR43 receptor.

430**Cell surface Glut1 levels distinguish human CD4 and CD8 T lymphocyte subsets with distinct effector functions**

Cretenet, G., Clerc, I., Matias, M., Mongellaz, C., Dardalhon, V., Taylor, N.

CNRS, Institut de Genetique Moleculaire de Montpellier, Montpellier, France

CD4 and CD8 T lymphocyte activation requires the generation of sufficient energy to support new biosynthetic demands. Following T cell receptor (TCR) engagement, these requirements are met by an increased glycolysis, due, at least in part, to induction of the Glut1 glucose transporter. As Glut1 is upregulated on tumor cells in response to hypoxia, we assessed whether surface Glut1 levels regulate the antigen responsiveness of human T lymphocytes in both hypoxic and atmospheric oxygen conditions. Notably, Glut1 upregulation in response to TCR stimulation was significantly higher in T lymphocytes activated under hypoxic as compared to atmospheric oxygen conditions. Furthermore, TCR-stimulated human T lymphocytes sorted on the basis of Glut1-Low and Glut1-High profiles maintained distinct characteristics, irrespective of the oxygen tension. T cells activated in hypoxia divided less than those activated in atmospheric oxygen. However, in both oxygen conditions, Glut1-High lymphocytes exhibited increased effector phenotype acquisition, augmented proliferation and a skewing towards a cytotoxic fate, resulting in an inverted CD4/CD8 ratio. Consistent with these data, Glut1-High T lymphocytes displayed an enhanced ability to secrete IFN- γ as well as IL-17. Thus, Glut1 surface levels identify human T lymphocytes with distinct effector functions in both hypoxic and atmospheric oxygen tensions. Our data showing that Glut1 acts as a gauge of T lymphocyte function fosters the development of novel therapeutic strategies for manipulating T cell responses, notably in the context of anti-tumoral adoptive T cell therapy.

431**Evidence against a role for NLRP3 - driven inflammation in the *db/db* mouse model of type 2 diabetes**

Kammoun, H.L.¹, Allen, T.L.¹, Henstridge, D.C.¹, Barre, S.¹, Coll, R.C.², Butler, M.S.², Roberston, A.A.B.², O'Neill, L.A.³, Murphy, A.J.¹, Cooper, M.A.², Febbraio, M.A.^{1,4}

¹Baker IDI Heart and Diabetes Institute, Melbourne, Australia,

²Institute for Molecular Bioscience, The University of Queensland,

Brisbane, Australia, ³Trinity Biomedical Sciences Institute, Trinity

College, Dublin, Ireland, ⁴Garvan Institute of Medical Research, Sydney, Australia

Type 2 diabetes (T2D) is considered a chronic, low grade inflammatory disease. Activation of the Nod-like receptor protein 3 (NLRP3) inflammasome, and secretion of its target interleukin 1 β (IL1 β), have been implicated in pancreatic β cell failure in T2D. Specifically targeting the NLRP3 inflammasome to prevent β cell death without compromising all IL1 β -associated immunity could represent a valuable therapeutic option for T2D. We recently demonstrated that MCC950, a compound that specifically inhibits the NLRP3 inflammasome can prevent autoinflammation and autoimmune diseases in

mice *in vivo* (Coll et al. *Nat Med.* 21: 248-255, 2015). Accordingly, we hypothesized that treating a diabetic (*db/db*) mouse with MCC950 would prevent pancreatic β cell death and delay, if not completely prevent, the onset of T2D. We treated *db/db* and littermate controls with MCC950 via their drinking water for 8 weeks. We verified the bioavailability of MCC950 in the blood of all treated animals. We did not detect, however, any differences in body weight, fat or lean mass when comparing the MCC950 treated *db/db* with the control group. Moreover, oral glucose tolerance tests showed similar glucose handling of the MCC950 *db/db* compared with the vehicle *db/db* and both *db/db* groups showed a progressive loss of β cell function when measuring plasma insulin over the course of the treatment. Finally, we did not detect any differences in circulating immune cell profiles between the 2 groups. These data suggest that, in *db/db* mice, NLRP3 driven-pancreatic IL1 β inflammation does not play a key role in the pathogenesis of disease.

432**Glucose and fatty acids in T cell-metabolism and their roles in immune regulation**

Kojima, H., Kashiwakura, Y., Kanno, Y., Hashiguchi, M., Kobata, T.

Dokkyo Medical University, School of Medicine, Department of Immunology, Tochigi, Japan

The glycolytic pathway plays a pivotal role in energy supply for cellular functions. We found that CD4⁺CD25⁺ regulatory T cells (Trs) were less dependent on glycolysis than CD4⁺CD25⁻ conventional

T cells (Tcvs), suggesting that Trs depend on oxidative phosphorylation more than Tcvs. Mitochondria (Mit) are key organelles for energy supply. It was found that the contents of Mit in Trs were similar to those in Tcvs at resting status. On the other hand, it was found that activated Trs contained less Mit than activated Tcvs. In addition, mitochondrial activity in activated Trs was less than that in activated Tcvs. These findings suggested that ATP demand and/or supply in Trs upon activation might be less than those in Tcvs. In fact, ATP production in activated Trs was significantly less than that in activated Tcvs. Though, activated Trs produced ATP significantly higher than resting Trs, suggesting that they may mainly utilize energy source other than glucose. Since fatty acids are well known as one of ATP sources, effects of fatty acids on T cell activation were assessed. To address this issue, palmitic acid (PA) was added into mixed lymphocyte cultures (MLCs). The addition of PA decreased T cell activation in MLCs including Trs more than in Tr-depleted MLCs. It is possible that PA affects Trs rather than other T cells. As the result, Trs energized with PA strongly inhibited Tcv activation. To confirm this hypothesis, assessment of effects of PA on ATP production among T cell-subsets is in progress.

433**A lipid metabolite profile in atherosclerotic plaques associated with increased inflammation and cardiovascular risk**

Tomas, L.¹, Björkbacka, H.¹, Edsfeldt, A.¹, Danielsson, A.¹, Wigren, M.¹, Grufman, H.^{1,2}, Persson, A.^{1,2}, Prehn, C.³, Adamski, J.^{3,4}, Nilsson, J.¹, Gonçalves, I.^{1,2}

¹Lund University, Department of Clinical Sciences Malmö, Malmö, Sweden, ²Malmö University Hospital, Department of Cardiology, Malmö, Sweden, ³Institute of Experimental Genetics, Helmholtz Zentrum München, Neuherberg, Germany, ⁴Institute of Experimental Genetics, Life and Food Science Science Center Weihenstephan, Freising-Weihenstephan, Germany

Atherosclerosis is characterized by the retention of lipids and chronic inflammation in the vasculature. In the present study we asked whether a certain lipid profile in carotid human atherosclerotic plaques is associated with a more inflammatory plaque phenotype and with future cardiovascular events (CE). Carotid plaques (n=159) from patients with and without cerebrovascular symptoms were analyzed for levels of 148 different lipids by electrospray ionization tandem mass spectrometry and phenotyped by measuring inflammatory mediators in homogenates and by immunohistochemical staining of sections.

By using 5 different clustering algorithms the lipid composition of each plaque was classified into two major clusters with different lipid profiles. Symptomatic plaques were significantly overrepresented in cluster 2 (76%, $p < 2.7 \times 10^{-5}$), whereas cluster 1 contained more asymptomatic plaques (60%, $p < 2.7 \times 10^{-5}$). In addition, cluster 2 was found to harbor more plaques with a high vulnerability index (CD68/ORO/Glycophorin A/Masson/ α -Actin; 68%, $p < 1.2 \times 10^{-9}$) as well as more inflammatory plaques (MCP-1/IL-6/IL-1 β ; 73%, $p < 1.3 \times 10^{-10}$), whereas the opposite was true for cluster 1 regarding vulnerability and inflammatory score. A reduced risk for future CE was found for cluster 1 ($p < 0.032$).

After adjusting for possible confounders (age, LDL, HDL, smoking, β -blockers, WBC and eGFR) the hazard ratio for future CE was 0.438 ($p = 0.039$). Adjusting for Framingham risk factors yielded similar results.

These results indicate that the lipid composition of human atherosclerotic plaques is associated with a pro-inflammatory plaque phenotype and increased risk of future CE.

434**Western-type diet induces myelopoiesis and modulates haematopoietic stem cell proliferation**

Karimi Azardaryany, M.¹, Ramezani-Moghadam, M.¹, Bendall, L.², Liddle, C.¹, George, J.¹, Esmaili, S.¹

¹Storr Liver Centre, The University of Sydney and The Westmead Institute for Medical Research, Sydney, Australia, ²Centre for Cancer Research, The Westmead Institute for Medical Research, The University of Sydney, Westmead, Australia

Consumption of a "western-type" diet is associated with obesity and the metabolic syndrome and is linked to widespread meta-inflammation. In obesity, expansion of bone marrow

haematopoietic stem cell and progenitor cells (HSPCs) leads to monocytosis that contributes to the accumulation of macrophages in adipose tissue and to atherosclerosis. Hypercholesterolaemia also induces monocytosis and expansion of HSPCs. We hypothesized that the cholesterol component of a western-type diets plays an important role in HSPC proliferation. We fed C57BL6 mice either a normal chow (NC) diet or a diet rich in sucrose (34%), cholesterol (2%), 0.5% sodium cholate (bile acid) (Atherogenic diet [ATH]) or a high sucrose diet without added cholesterol and cholate (HS) for 8 weeks. HS diet fed mice demonstrated the hallmarks of metabolic syndrome including insulin resistance and obesity. However, ATH diet fed mice were resistant to weight gain. Only the ATH diet induced hepatosplenomegaly with infiltration of immune cells in the liver and spleen. In bone marrow, we detected an increase in the number of HSPCs characterised as Lin⁻ CD45⁺ Sca-1⁺ c-Kit⁺ (LSK) (10-fold, $p = 0.001$) in ATH diet fed mice. The ATH diet also increased the number of Long-Term HSCs (2-fold, $p = 0.001$), characterised as LSK CD48⁻ CD150⁺, compared to the normal chow diet. Despite developing the metabolic syndrome, mice fed a HS diet did not demonstrate an increase in the number of inflammatory and stem cell subsets, indicating that cholesterol in combination with bile acids drives inflammation and stem cell proliferation.

435**Metabolic adaptation of polymorphonuclear neutrophils activated with pro-inflammatory cytokines**

Lachhab, A.^{1,2}, Brisebois, B.¹, Pelletier, M.^{1,2}

¹CHU de Québec-Université Laval Research Center, Infectious Diseases and Immunity Research Division, Quebec, Canada, ²Laval University, Quebec, Canada

Pro-inflammatory cytokines IL-6, TNF and IL-1 β are involved in chronic inflammatory diseases. They can modulate the responses of immune cells like polymorphonuclear neutrophils (PMNs), but their impact on their bioenergetics is not well defined. Using the extracellular flux analyzer, we observed a quick and robust glycolytic response in TNF-activated PMNs compared to IL-1 β - or IL-6-activated cells. TNF also induced rapid oxygen consumption, an effect enhanced with IL-1 β . IL-6 or IL-1 β alone had no effect on PMNs' oxygen consumption. At the molecular level, we observed that TNF- and IL-1 β -activated PMNs had enhanced gene transcripts coding for pro-inflammatory cytokines and for the metabolic adaptor *HIF-1A*. The expression of the glucose transporter *GLUT-3* and the amino acid transporters *CD98* and *ASCT2* transcripts were only modulated by TNF. Pre-incubation of PMNs with a competitive inhibitor of glucose revealed that the cytokine-activated oxidative burst depended on glycolysis. Since carbohydrates regulate this process, we examined the effect of sugars on TNF and IL-1 β -activated PMNs. The cytokine-induced oxidative burst occurred in the presence of sugar-free medium, or in media containing glucose and galactose. Surprisingly, this effect was completely inhibited in the presence of mannose, even when glucose and galactose were added. Moreover, the induction of pro-inflammatory cytokine transcripts in response to TNF and IL-1 β was significantly increased in PMNs when mannose was

used compared to glucose-, galactose- or sugar-free medium. These results indicate that pro-inflammatory cytokines exert different effects on PMNs' energy metabolism and that the carbohydrate nature can influence their cytokine expression and functional responses.

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The ribonuclease Regnase-1 plays a role in iron homeostasis and anemia

Yoshinaga, M., Mino, T., Takeuchi, O.

Institute for Virus Research, Kyoto University, Kyoto, Japan

Iron homeostasis is tightly controlled during inflammatory responses. The coordinate regulation of iron homeostasis is largely dependent on the post-transcriptional control. However, the players and mechanisms of post-transcriptional regulation in the iron homeostasis have not been fully understood. Our group previously found a ribonuclease, named Regnase-1, destabilizes a set of pro-inflammatory cytokine mRNAs, including *Il-6* and *Il-12p40*. In this study, we investigated the role of Regnase-1 in the control of iron homeostasis and anemia through the analysis of Regnase-1-deficient mice. We found that Regnase-1-deficient mice showed severe iron deficiency and anemia, which was partly rescued by the intraperitoneal iron supplementation. Regnase-1-deficient mice did not show the signs of excess blood loss, and iron deficiency observed in Regnase-1-deficient mice was not rescued by the lack of lymphocytes. These findings suggest that Regnase-1 is critical for dietary iron absorption, and this function is largely independent of the control of inflammation. To identify Regnase-1 target mRNAs responsible for the iron uptake, we conducted the transcriptome analysis in duodenum, where iron uptake takes place, and found that several iron-controlling genes, including *Egln3*, were up-regulated under Regnase-1 deficiency. The overexpression of Regnase-1 accelerated the decay of the *Egln3* mRNA via its 3' untranslated region. Consistently, the expression of *Egln3*-regulated HIF-2 α target genes was impaired in Regnase-1-deficient mice compared with iron-deficient control mice. Furthermore, the abrogation of *Egln3* activity rescued the iron deficiency in Regnase-1-deficient mice. Taken together, Regnase-1 not only regulates inflammation but also prevents the development of anemia, by regulating the duodenal iron uptake.

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Hepatic endogenous HMGB1 plays important roles in non-alcoholic fatty liver disease

Deng, M., Loughran, P., Billiar, T., Scott, M.

University of Pittsburgh, Surgery, Pittsburgh, United States

High-mobility group box 1 (HMGB1) is an abundant chromatin-associated nuclear protein and released into the extracellular milieu in non-alcoholic fatty liver disease (NAFLD). However, the function of intracellular HMGB1 in NAFLD is currently unknown. To determine the role of hepatic endogenous HMGB1 in NAFLD, we generated HC-specific HMGB1^{-/-} (HC- HMGB1^{-/-}) mice and induced NAFLD with high fat diet (HFD). At 8 weeks after HFD

administration, specifically deletion of HMGB1 dramatically exacerbated the pathogenesis of hepatosteatosis, evidenced by significantly higher body weight, total fat content, and liver fat content in HC- HMGB1^{-/-} than in control mice. The increase of liver fat content in HC- HMGB1^{-/-} mice with HFD was associated with decreased β -oxidation gene expression (*Lcad*, *Mcad*, and *Vlca*) in liver compared to control mice. Interestingly, at 16 weeks after HFD administration, the difference between HC- HMGB1^{-/-} and control mice in body weight, total fat content, and liver fat content diminished. However, there are more immune cells infiltration and collagen formation in the liver in HC- HMGB1^{-/-} than in control mice. Additionally, liver damage and was correlated to histology changes. We found that these were associated with significantly more reactive oxidative species (ROS) production in the liver in HC- HMGB1^{-/-} mice compared to control. Together, the above data indicate that hepatic endogenous HMGB1 plays important role in hepatosteatosis via regulation of β -oxidation. Lack of hepatic endogenous HMGB1 enhanced liver injury and inflammation at late stage of NAFLD, which may be mediated via increased ROS production in hepatocyte.

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HIF-1 α stabilization through metabolic hypoxia via glutaminolysis in progressive pulmonary tuberculosis

Torres Rojas, M.¹, Abarca Rojano, E.², Huerta Yopez, S.³, Hernández Pando, R.⁴, Rangel Santiago, J.³, Mayoral Márquez, H.⁵, Domínguez López, M.L.⁶

¹Hospital Infantil de México Federico Gómez, Unidad de Investigación en Enfermedades Oncológicas, México, Mexico,

²Instituto Politécnico Nacional, Laboratorio de Respiración Celular, Escuela Superior de Medicina, México, Mexico, ³Hospital Infantil de México Federico Gómez, Unidad de Investigación en Enfermedades Oncológicas, México, Mexico, ⁴Instituto Nacional de Ciencias Médicas y Nutrición "Salvador Zubirán", Departamento de Patología Experimental, México, Mexico, ⁵Instituto Politécnico Nacional, de Respiración Celular, Escuela Superior de Medicina, México, Mexico, ⁶Instituto Politécnico Nacional, Laboratorio de Inmunoquímica I, Escuela Nacional de Ciencias Biológicas, México, Mexico

Tuberculosis is a disease caused by the infection of *Mycobacterium tuberculosis*. According to the last report of the World Health Organization, in 2013 were reported 9 million of new tuberculosis cases and 1.5 million of deaths because of it, becoming the second cause of death caused by an infectious disease. During the infection many processes occur but inflammation is an angular event in the physiopathology of its evolution and can be triggered and promoted by multiple mechanisms. Is of our interest the study of the role played by HIF-1 α in this process and the mechanisms involved on its stabilization. Using tissue microarrays of postmortem lung samples, ones infected with tuberculosis and others none infected, we found out that HIF-1 α is significantly expressed in the infected ones, phenomenon that is also looked with the expression of *Mycobacterium tuberculosis* antigens, suggesting that there is a relation between the progression of the disease

and the presence of HIF-1 α . We also found that glutaminolysis enzymes: glutaminase 1 (enzyme that catalyzes the conversion of glutamine to glutamate) and glutamate dehydrogenase (enzyme that catalyzes the conversion of glutamate to α -ketoglutarate) are statistically increased, also related with the overexpression of succinylated proteins and the succinate receptor GPR91. With these results we suggest the presence of a state of metabolic hypoxia in which glutaminolysis is used as an anaplerotic way of producing succinate and consequently the stabilization of HIF-1 α by a competitive inhibition of the prolyl hydroxylase enzymes, finally promoting inflammation.

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Adipose renin angiotensin system mediates fatty acid-induced endoplasmic reticulum stress in adipose tissue

Ramalingam, L.^{1,2}, Kalupahana, N.³, Moustaid-Moussa, N.^{1,2}

¹Texas Tech University, Nutritional Sciences, Lubbock, United States,

²Texas Tech University, Obesity Research Cluster, Lubbock, United States,

³Faculty of Medicine, University of Peradeniya, Department of Physiology, Peradeniya, Sri Lanka

Angiotensin System (RAS), a key regulator of blood pressure and fluid balance, has been linked to metabolic disorders. RAS components including its precursor angiotensinogen (Agt) and hormone product Angiotensin II are also locally generated in adipose tissue. Accumulation of free fatty acids causes lipotoxicity leading to inflammation and endoplasmic reticulum (ER) stress in adipose. However, the exact mechanisms are not completely understood. Hence, we hypothesize that ER stress in adipose tissue caused by FFA may be mediated through the RAS. To test the hypothesis, mice overexpressing angiotensinogen in the adipose were fed high fat diet supplemented with or without captopril (Angiotensin converting enzyme inhibitor). Markers of ER stress including C/EBP homologous protein (CHOP) and activating transcription factor 4 (ATF4) were significantly decreased in adipose tissue from Agt-Tg mice treated with captopril compared to vehicle-treated Agt-Tg littermate mice. Experiments are currently underway to further dissect these mechanisms in cultured adipocytes treated with fatty acids and/or specific RAS inhibitors. Further, miRNA profiling in adipose tissues from Agt-Tg and Wt mice revealed differential expression of several miRNAs between these 2 genotypes. Specifically, miRNA 99, known to regulate ER stress as was significantly higher in adipose tissue from Agt-Tg compared to Wt mice. Quantitative PCR validation of these findings are underway. In conclusion, RAS system is a potential mediator of fatty acid induction of ER stress that may promote obesity. Identification of novel mechanisms such as miRNAs regulating these pathways may provide novel means to reduce Agt-linked obesity and insulin resistance.

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Glucosamine inhibits CD122 expression through the inhibition of glycolysis pathway in T cells

Kim, E.J.^{1,2}, Yoo, H.S.^{1,2}, Na, K.^{2,3}, Jeon, M.-S.^{1,2,3}

¹Inha University School of Medicine, Molecular Biomedicine, Incheon, Korea, Republic of, ²Inha University Hospital, Translational

Research Center, Incheon, Korea, Republic of, ³Inha University School of Medicine, IRIMS, Incheon, Korea, Republic of

D-glucosamine (GlcN) is known to have a therapeutic role in osteoarthritis (OA) by promoting the formation of joint cartilage. Molecular mechanisms how GlcN influences on immune cells are not fully understood. In this study, we investigated to understand the mechanism how GlcN influences on the T cell activation *in vitro*. We obtained the following results; 1) GlcN inhibited the T-cell proliferation and division. 2) This inhibition was not caused by apoptosis. 3) The initial activated state of the T cells was maintained in the presence of GlcN without cell division. 4) Interleukin-2 (IL-2) production was significantly increased in GlcN-treated T cell culture medium. 5) IL-2 receptor beta chain (CD122) was significantly decreased by GlcN. Thus, the increased IL-2 detection was occurred by the decreased CD122 expression. 6) GlcN markedly reduced the IFN- γ expression. 7) CD122 expression was reduced in T cells by the neutralizing IFN- γ . Thus, the level of IFN- γ production could regulate the CD122 expression. 8) We could detect the high glucose level in T cell culture medium treated with GlcN. This result could suggest that the glucose uptake might be blocked by GlcN. Taken together GlcN suppressed the IFN- γ production through the decreased glucose metabolism which resulted in the inhibition of CD122 expression. Therefore, the decreased CD122 expression impaired the T cells functions.

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STAT5 signaling is a key regulator of glycolysis during immediate activation of naive, but not memory CD4+ T-lymphocytes

Jones, N., Cronin, J., Panetti, S., Thornton, C., Francis, N.

Swansea University, Medical School, Swansea, United Kingdom

Upon stimulation, CD4+ naive (NV) T-lymphocytes undergo rapid proliferation and clonal expansion resulting in a pool of effector (EM) T-lymphocytes that actively target the infection. The majority of this effector pool undergoes apoptosis post-infection, with the surviving cells resulting in a small viable, central memory (CM) population that will remain in the blood to provide a rapid response should reinfection occur. In order to carry out their specific roles, NV, EM and CM T-lymphocytes must obtain energy in the form of ATP.

Here we show that resting EM and CM cohorts have higher baseline metabolism than their NV counterparts, along with higher mitochondrial content. Upon activation with α CD3/28 we found that NV T-lymphocytes rapidly increase their glycolytic rate, with this immediate glycolytic switch being dependent on STAT5 signaling. Inhibition of STAT5 with N-((4-Oxo-4H-chromen-3-yl)methylene)nicotinohydrazide (100 μ M) significantly reduced glycolysis in NV, but not EM or CM T-lymphocyte suggesting divergent pathways of activation in memory populations. This divergence is not observed at the functional level with STAT5 inhibition significantly reducing the production of interferon- γ , interleukin-2 and expression of the activation marker CD69, without altering cell viability in NV, EM and CM populations.

These data suggest a key role for STAT5 during the initial

activation of NV CD4+ T-lymphocytes, but a differential signalling pathway for metabolic reactivation of memory populations.

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UV-irradiation of skin enhances glycolytic flux in bone marrow-differentiated dendritic cells

Hart, P.¹, McGonigle, T.¹, Keane, K.², Newsholme, P.², Ghaly, S.¹, Carter, K.¹, Anderson, D.¹, Scott, N.¹, Goodridge, H.³

¹Teleton Kids Institute, Perth, Australia, ²Curtin University, School of Biomedical Sciences, Perth, Australia, ³Board of Governors Regenerative Medicine Institute, Cedars-Sinai Medical Centre, Los Angeles, United States

Following UV irradiation of skin, dendritic cells (DCs) differentiating from the bone marrow (BM) of mice have a reduced ability to prime new immune responses; their reduced immunogenicity is maintained for at least 16 weeks in UV-chimeric mice. We hypothesized that different metabolic states underpin changes in DC function. Compared with DCs from the BM of non-irradiated mice, DCs from the BM of UV-irradiated mice produced more lactate and utilized greater amounts of glucose, a profile that was supported by greater glycolytic flux when incubated in low-serum-containing medium. Responses to a mitochondrial stress test were similar suggesting that the DCs from the BM of UV-irradiated mice had not switched from a profile of oxidative phosphorylation, but were imprinted for greater glycolytic responses. After microarray profiling, RT-qPCR confirmation and Ingenuity pathway analysis, greater expression of the enzyme, 3-hydroxyanthranilate 3,4-dioxygenase, was identified as a potential contributor to increased glycolysis by BM-differentiated DCs. This enzyme provides the final step of the biosynthetic pathway from tryptophan to quinolinate, the universal de novo precursor to the pyridine ring of nicotinamide adenine dinucleotide (NAD), and may provide a mechanism to ensure sufficient NAD is available to support enhanced glycolysis. Increased lactate production was also measured for DCs from the BM of 16-week engrafted UV-chimeric mice and suggests long-lasting imprinting of progenitor cells for altered immunometabolism in their progeny cells. This study provides evidence of changes to metabolic states that associate with altered DC function.

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Sensitivity to restimulation-induced cell death is linked to glycolytic metabolism in human CD8+ T cells

Larsen, S., Bilenkin, A., Snow, A.

Uniformed Services University of the Health Sciences, Pharmacology, Bethesda, United States

Restimulation-induced cell death (RICD) regulates immune responses by restraining effector T cell expansion and limiting nonspecific damage to the host. RICD is triggered by re-engagement of the T cell receptor (TCR) on a cycling effector T cell, resulting in apoptosis. It remains unclear how RICD sensitivity is calibrated in T cells derived from different individuals or subsets. Here we show that aerobic glycolysis strongly correlates with RICD sensitivity in human CD8+ effector

T cells. Reducing glycolytic activity or glucose availability rendered effector T cells significantly less sensitive to RICD. We found that active glycolysis specifically facilitates the induction of pro-apoptotic Fas ligand upon TCR restimulation, accounting for enhanced RICD sensitivity in highly glycolytic T cells. Collectively, these data indicate that RICD susceptibility is linked to metabolic reprogramming, and that switching back to metabolic quiescence may help shield T cells from RICD as they transition into the memory pool.

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Rebamipide protects against atherosclerosis via regulation of Th17/Treg balance and oxLDL-induced foam cells formation

Kim, S.-Y., Jhun, J.Y., Kim, E.-K., Jeong, J.-H., Cho, M.-L.

Catholic University of Korea, Rheumatism Research Center, Catholic Research Institute of Medical Science, Seoul, Korea, Republic of

Atherosclerosis is a chronic inflammatory disease regulated by T lymphocyte subsets, especially a vascular inflammatory process in the presence of an excess amount of lipid. We reported that rebamipide reduced Th17 cells differentiation and also enhanced regulatory T (Treg) cells. Thereby we have used APoE deficient mice to inquire into whether rebamipide can treat atherosclerosis. We verified that aortas from APoE deficient mice that were treated rebamipide had less atherosclerotic lesions than APoE deficient mice (negative control) and positive control had by oil red O staining. In addition, we stained splenocytes from APoE deficient mice and confirmed that CD4+CD25+Foxp3+ regulatory T(Treg) cells were increased in rebamipide treated mice group compared with negative and positive controls. Moreover, we also confirmed that rebamipide could treat obese arthritis in vivo. Furthermore, we investigated that rebamipide could suppress ox-LDL induced foam cell formation in vitro. In conclusion, rebamipide can make balance between inflammatory T helper cells and Treg cells during processing atherosclerosis in a mouse model of accelerated atherosclerosis. Thus, rebamipide can be a therapeutic agent to suppress the progress of inflammatory diseases.

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Platelet activating factor receptor in adipose tissue macrophages is associated with anti-inflammatory phenotype and metabolic homeostasis

Jancar, S.¹, Ribeiro Filgueiras, L.¹, Mainard Koga, M.¹, Kiyotaka Ishizuka, E.¹, B Montes, M.¹, José Rios, F.²

¹University of São Paulo, Department of Immunology, Institute of Biomedical Science, São Paulo, Brazil, ²University of Glasgow, Institute of Cardiovascular and Medical Sciences, British Heart Foundation Glasgow Cardiovascular Research Centre, Glasgow, United Kingdom

Metabolic dysfunction is associated with adipose tissue inflammation and macrophage infiltration. The Platelet Activating Factor Receptor (PAFR) is expressed in several cell types and binds to PAF and a wide range of oxidized phospholipids. Engagement of PAFR in macrophages drives them towards

the anti-inflammatory phenotype. In the present study, we investigated whether genetic deficiency of PAFR affects the phenotype of adipose tissue macrophages (ATM) and its effect on glucose and insulin metabolism. PAFRKO mice presented increased gene expression of *Ccr7*, *Nos2*, *Il6*, and *Il12*, associated to pro-inflammatory mediators, and reduced expression of the anti-inflammatory *Il10* in the epididymal adipose tissue. Moreover, the adipose tissue of PAFRKO presented higher pro-inflammatory macrophages, characterized by an increased frequency of *F4/80+CD11c+* cells. Blood monocytes of PAFRKO mice also exhibited a pro-inflammatory phenotype (increased frequency of *Lys6C+* cells) and PAFR-ligands were detected in the serum of both PAFRKO and WT. Regarding metabolic parameters, compared to WT, PAFRKO mice had:

- i) higher weight gain and serum glucose concentration levels;
- ii) decreased insulin-stimulated glucose disappearance;
- iii) insulin resistance in the liver;
- iv) increased expression of *Ldlr* in the liver.

When mice were fed high fat diet some of these changes were potentiated, particularly in the liver. Together these results indicate that endogenous ligands of PAFR are responsible for maintaining the anti-inflammatory profile of blood monocytes and adipose tissue macrophages under physiological conditions. In the absence of PAFR signaling, monocytes and macrophages acquire pro-inflammatory phenotype, resulting in adipose tissue inflammation and metabolic dysfunction.

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NK cells link obesity-induced adipose tissue stress to inflammation and insulin resistance

Wensveen, F.M.¹, Valentić, S.¹, Jelenčić, V.¹, Šestan, M.¹, Turk Wensveen, T.², Theurich, S.³, Glasner, A.⁴, Mendrila, D.⁵, Štimac, D.², Wunderlich, F.T.³, Brüning, J.C.³, Mandelboim, O.⁴, Polić, B.¹

¹Faculty of Medicine, Department for Histology and Embryology, Rijeka, Croatia, ²University Hospital Rijeka, Department of Internal Medicine, Rijeka, Croatia, ³Max Planck Institute for Metabolism Research Cologne, Cologne, Germany, ⁴The Lautenberg Center for General and Tumor Immunology, The Hebrew University Hadassah Medical School, Jerusalem, Israel, ⁵University Hospital Rijeka, Department of Surgery, Rijeka, Croatia

Obesity is an increasingly common health issue that predisposes people to metabolic disorders such as insulin resistance (IR), which can progress to diabetes mellitus type 2 (DM2). An important underlying cause of obesity-induced IR is chronic systemic inflammation derived from accumulating pro-inflammatory macrophages in visceral adipose tissue (VAT). Currently, it is unknown which signal initiates adipose tissue macrophage (ATM) activation in VAT.

We find that a phenotypically distinct VAT-resident NK cells provide a crucial link between obesity-induced adipose stress and ATM activation in VAT. Ligands for the NK cell-activating receptor *NKp46/Ncr1* are expressed in human and mouse VAT. Feeding with high-fat diet causes up regulation of *Ncr1*-ligands on adipocytes, leading to localized activation and cellular increase of NK cells. *IFN γ* produced by these cells drives early pro-inflammatory macrophage differentiation and promotes obesity-induced insulin resistance. Lack of NK cells, *Ncr1* or *IFN γ*

prevents macrophage activation in VAT and greatly ameliorates glucose tolerance and insulin sensitivity. Therapeutic blocking of *Ncr1*-signaling forestalls ATM activation. Our study identifies NK cells as key regulators of macrophage polarization and insulin resistance in response to obesity-induced adipose stress. The NK-ATM axis therefore provides an attractive new target for early treatment of patients with metabolic syndrome to prevent progression to DM2.

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Altered nutrient levels caused by B cell lymphoma leads to dysfunctional natural killer cells

Kobayashi, T.¹, Tuong, K.¹, Man, K.², McKee, S.¹, Leggatt, G.¹, Kallies, A.², Mattarollo, S.¹

¹University of Queensland Diamantina Institute/Translational Research Institute, Woolloongabba, Australia, ²Walter & Eliza Hall Institute, Melbourne, Australia

Natural killer (NK) cells are of paramount importance in immune protection against cancers. Optimal anti-tumour immunity requires functional NK cells. Cumulative evidence suggests that efficient cellular metabolism is not only required for maintaining cells, but also is a driving force for specific immune cell functions. In blood cancers, where tumour cells and host immune cells are co-located, tumour cells can impact on immune cell metabolism, which can lead to immune system dysfunction. Here, we found that the metabolic signatures of NK cells are distinct from other lymphocyte populations, having the highest lipid intake and storage capacity compared with that of B cells, T cells, and NKT cells. We also found that, in murine c-myc-driven B cell lymphoma-bearing mice, NK cells showed altered metabolic profiles associated with their developmental, survival, and functional defects. These defects were measured by a decrease in the number, abnormal subset compositions, down-regulated NK activating receptors and suppressed effector molecule production (*IFN- γ* and Granzyme B) respectively. We next hypothesized that the cellular metabolism of NK cells is compromised due to altered availability of nutrients. To this end, we studied metabolite levels in the system and observed that altered amounts of fatty acids and amino acids released by tumour cells led to defects in *AKT/mTORC1* signalling and up-regulation of apoptosis, both of which eventuate in the observed NK cell dysfunction.

This study has demonstrated how blood cancers negatively regulate NK cell metabolism and their functions, and has discovered a potential immunometabolic checkpoint target for cancer immunotherapies.

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The effect of matrix metalloproteinase-2 for the islets of streptozotocin-induced diabetic mouse model

Takeshita, A.¹, Yasuma, T.¹, Nishihama, K.¹, Hinneh, J.², D'Alessandro-Gabazza, C.², Harada, E.², Toda, M.², Yano, Y.¹, Gabazza, E.²

¹Mie University Graduate School of Medicine, Diabetes and Endocrinology, Tsu-City, Japan, ²Mie University Graduate School of Medicine, Immunology, Tsu-City, Japan

Background: Matrix metalloproteinase-2 (MMP-2) is one of the enzymes that decompose extracellular matrix. Although previous reports refer to effect of MMP-2 for islet of pancreas, its detailed role is still unclear.

Objective: To investigate the role of MMP-2 in pancreatic beta cell of streptozotocin (STZ)-induced diabetic mouse model.

Methods: We injected saline (SAL) or STZ to 15-week-old human matrix metalloproteinase-2 over-expression transgenic mice (hMMP-2Tg) and 17-week-old control mice (WT). Mice were divided into 4 groups (WT/SAL, WT/STZ, hMMP-2Tg/SAL, hMMP-2/STZ). Measurement of blood glucose, intraperitoneal glucose tolerance test (IPGTT) and glucose induced insulin secretion test were performed. Mice were killed after 4 weeks of injection. Pathological evaluations of pancreas were done after sacrifice.

Results: Compared with WT/STZ group, blood glucose levels of hMMP-2Tg/STZ group were significantly lower at 14, 21 and 28 days after injection. IPGTT suggest that the changes of blood glucose were milder in hMMP-2Tg/STZ group than WT/STZ group. Glucose induced insulin secretion test revealed the levels of serum insulin of hMMP-2Tg/STZ mice were higher than those of WT/STZ group. Larger islet was significantly preserved in hMMP-2Tg/STZ group against WT/STZ group.

Conclusion: Over-expression of hMMP-2 may be protective against STZ-induced diabetic mellitus.

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Dipeptidyl peptidase 9 enzyme activity alters expression of genes that are important in neonatal immunity, insulin signalling and metabolism

Chen, Y.^{1,2}, Gall, M.^{1,2}, Zhang, H.^{1,2}, McCaughan, G.^{2,3}, Yu, D.^{1,4}, Gorrell, M.^{1,2}

¹Centenary Institute, Molecular Hepatology Laboratory, Newtown, Australia, ²University of Sydney, Sydney Medical School, Sydney, Australia, ³Centenary Institute, Liver Injury and Cancer Program, Newtown, Australia, ⁴Children's Cancer Institute, Kensington, Australia

Dipeptidyl peptidase 9 (DPP9) is a ubiquitous intracellular protease that is up-regulated in activated T and B cells, macrophages and many tumours. DPP9 can influence antigen processing and secretion of pro-inflammatory cytokines. Newly identified DPP9 substrates include two key immune regulators, C-X-C motif chemokine 10 (CXCL10) and interleukin-1 receptor antagonist (IL-1RA), calreticulin and adenylate kinase 2, indicating potential roles of DPP9 in immune function, energy metabolism and homeostasis.

Our homozygous mice lacking DPP9 enzyme activity (*Dpp9* gene knock-in; *Dpp9* gki) display neonatal lethality. Taqman PCR arrays and sequential qPCR assays on neonatal liver and gut revealed differential expression of genes involved in innate immunity, insulin signalling and metabolic pathways, suggesting a less active IL-1 β /TNF α -induced NF- κ B pathway, enhanced insulin sensitivity, dysregulated glucose metabolism including increased hepatic gluconeogenesis and extrahepatic glucose uptake and utilization, disrupted long-chain-fatty-acid uptake, increased VLDL uptake and clearance of circulatory HDL from blood in *Dpp9* gki neonatal mice compared to wild type. *In vitro* mechanistic studies indicated that effects of DPP9 enzymatic activity on neonatal metabolism may occur

via modulating AMPK phosphorylation. The dysregulation of both metabolic genes and the NF- κ B mediated inflammatory pathway in *Dpp9* gki neonatal mice may be linked, and would be expected to exacerbate any other pathogenic process that occurs in the *Dpp9* gki neonates, and so might contribute to the neonatal lethality seen in *Dpp9* gki mice.

These data point to important biological roles for DPP9 in inflammation and metabolism in newborn mice.

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T-cells induced by oral gluten challenge in patients with coeliac disease confirm the in vivo effects of gluten hydrolysed by intestinal bacteria from gnotobiotic mice

Russell, A.¹, Caminero, A.², Galipeau, H.J.², Mccarville, J.L.², Johnston, C.W.³, Bernier, S.P.², Jury, J.², Tye-Din, J.A.¹, Casqueiro, J.⁴, Surette, M.G.^{2,5}, Schuppan, D.⁶, Magarvey, N.A.³, Verdu, E.F.²

¹Walter and Eliza Hall Institute of Medical Research, Immunology, Parkville, Australia, ²Farncombe Family Digestive Health Research Institute, McMaster University, Hamilton, Canada, ³DeGroot Institute for Infectious Disease Research, McMaster University, Biochemistry and Biomedical Sciences, Hamilton, Canada, ⁴Universidad de Leon, Microbiología, Leon, Spain, ⁵DeGroot Institute for Infectious Disease Research, McMaster University, Hamilton, Canada, ⁶Institute for Translational Immunology and Research Centre for Immunotherapy, Johannes Gutenberg University, Mainz, Germany

Coeliac disease (CD) is a gluten-mediated autoimmune condition. At least 30% of the population carry the genetic predisposition for CD, but only 2-4% develop disease, implicating a role for environmental factors in disease onset. Intestinal dysbiosis in CD has been implicated, but the mechanism remains unclear. The ability of intestinal bacteria isolated from CD (*Pseudomonas aeruginosa* (*Psa*)) and non-CD (*Lactobacillus spp*) subjects to hydrolyse wheat gluten/gliadin protein and a 33mer peptide from gliadin containing immunogenic T-cell epitopes was confirmed in a gnotobiotic mouse model. We sought to assess the immunogenic potential of these hydrolysed end-products in human CD volunteers.

HLA-DQ2.5+ CD patients in remission consumed wheat-bread for 3 days. PBMCs were collected before (D0) and after (D6) commencing the challenge. An overnight IFN- γ ELISpot assay was used to enumerate T-cells responding to a range of peptides generated by incubation of bacteria with pepsin/trypsin-digested (PT) gliadin or 33mer.

Gluten-specific T-cells were detected on D6 but not D0. Responses were substantially increased to PT-gliadin (~2x) and 33mer (~3x) after deamidation, and significantly increased by incubation with *Psa* (~5x). In contrast, PT-gliadin incubated with *Lactobacillus spp.* was less immunogenic than PT-gliadin alone. Disease-specific polyclonal T-cells induced by oral feeding challenge were useful to confirm the in vivo relevance of hydrolysed gliadin and 33mer. The findings indicate that human small intestinal bacteria participate in the metabolism of gluten peptides, and these effects differ between bacteria isolated from CD compared to non-CD patients. This describes a potential mechanism through which dysbiosis could modulate CD risk.

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A SAXS based model of a tip pilin protein of *Lactobacillus rhamnosus* GG: insight in its structural properties*Bhattacharjee, A.¹, Douillard, F.P.², Sèle, C.³, Rasinkangas, P.², Klievink, J.¹, Messing, M.¹, de Vos, W.M.^{1,4}*¹University of Helsinki, Research Program Unit, Immunobiology, Helsinki, Finland, ²University of Helsinki, Department of Veterinary Biosciences, Helsinki, Finland, ³University of Helsinki, Institute of Biotechnology, Helsinki, Finland, ⁴Wageningen University, Laboratory of Microbiology, Wageningen, Netherlands

Lactobacillus rhamnosus GG is a well-characterized probiotic strain that displays health-promoting properties for the host. *L. rhamnosus* GG establishes interaction with the intestinal mucosa using mucus-binding pili, protruding protein fibers with a length that equals that of the producing bacterium. These pili have been shown to be an important colonization and persistence factor in the gastrointestinal tract. Specifically, the pilin subunit SpaC, located at the tip of the pili in *L. rhamnosus* GG has mucus-binding properties. It is a large (90 kDa) multi domain protein and has some typical features of gram positive pili proteins. However, the binding domains of SpaC are still largely unknown. Hence, we determined the low resolution structure of purified SpaC by small angle X-ray scattering technique. This gave a clear idea of the envelope of the big protein. Subsequently, we did fit in the pre-modelled individual domains of SpaC in the experimentally determined envelope of SpaC. The resulting structure provided extensive insights into the intra molecular peptide bonds in SpaC and beyond. Preliminary mass spectrometry data also supported the presence of intra- and inter-molecular peptide bonds in the tip pilin of SpaC. Thus, we provided a model of the molecular architecture of the tip pilin of GG, providing a working basis to further explore its role in host interaction and signaling.

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Nutrition-induced changes in microbiome alter inflammatory airways disease outcome*Maddox, D.**Mayo Clinic, Allergic Diseases & Internal Medicine, Rochester, United States*

A recent cohort study suggested that a high intake of fibers in food protects from early death from several causes. Other investigators have reported that intake of grain fibers modulates cytokine levels in blood, and their data actually suggests that certain grain fibers actually reduce pro-inflammatory cytokine levels. The human microbiome contributes many physiologically important moieties to the bloodstream, and some recent studies suggest that grain-related carbohydrates induce changes in the microbiome that are pro-inflammatory on a systemic basis. We here report that patients presenting with chronic non-allergic inflammatory airways disease [chronic rhinosinusitis with nasal polyposis and bronchial asthma] improve clinically when nutritional patterns are modified to exclude grain-related foods. This clinical improvement exceeds the results expected from pharmacologic management alone. This result seems to beg the question of whether the carbohydrate associated with grain-

related foods is more important in modulating microbiome components, or the fiber is the more important element. When grain-related foods are reduced in the nutritional pattern, vegetable-related elements tend to increase, and experiments that isolate storage carbohydrate from structural fiber content will be necessary in order to understand which element predominates in this improved clinical outcome.

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Polymyxin B modulation of maternal gut microbiome during pregnancy and lactation alters neonatal immunity*Nyangahu, D.¹, Jaspan, H.^{1,2}*¹University of Cape Town, Cape Town, South Africa, ²Seattle Children's Research Institute, Seattle, United States

Introduction: The pioneer microbiome in neonates may affect future actions of the immune system. How antibiotic use during pregnancy and lactation may influence neonatal microbiome and immunity is not clear.

Methodology: Pregnant BALB/c mice were treated with polymyxin B 5 days prior to giving birth and while nursing or just while nursing. We killed pups 14 days postpartum and investigated the splenic immunity.

Results: Pups born to mothers treated during either gestation or nursing had significantly increased body weight when compared to controls. However, maternal antibiotic treatment during both gestation and nursing did not associate with a change in neonatal weight. We observed a significant decrease in the proportions of CD4+ T cells in the spleen of pups born to mothers treated with Polymyxin B during gestation ($p=0.005$). Analysis of the activation status of splenic CD4+ T cells in pups showed, pups born to mothers treated with polymyxin B throughout gestation and nursing or in gestation or nursing alone had significantly lower proportions of effector T cells when compared to pups born to untreated mothers. The frequency of T regulatory cells was significantly higher in pups nursed by antibiotic treated mothers ($p=0.011$). Neonatal B cell alterations by maternal antibiotics were less marked. We observed a trend towards decreasing proportions of B cells in infant mice born to mothers treated with polymyxin B during either gestation or lactation.

Conclusions: Maternal microbial perturbations during gestation and nursing are critical for immune development in neonates.

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Therapeutic bacterial transplantation for atopic dermatitis*Myles, I.**National Institutes of Health (NIH), NIAID, Bethesda, United States*

Atopic dermatitis (AD) is characterized by reduced barrier function, reduced innate immune activation, and susceptibility to *Staphylococcus aureus*. Host susceptibility factors are suggested by monogenic disorders associated with AD-like phenotypes, and can be modulated through topical steroids or calcineurin inhibitors. *S. aureus* is another known contributor to AD pathogenesis, and can be mitigated by antibiotics or bleach baths. Recent work has revealed that the skin microbiome

differs significantly between healthy controls and patients with AD. However, little is known about the contribution of dysbiosis to AD symptoms or if microbiome modulation could provide therapeutic benefit. In various cellular and culture-based models, we found that commensal Gram-negative (CGN) bacteria taken from healthy human volunteers but not from patients with AD were associated with enhanced barrier function, innate immunity activation, and control of *S. aureus*. Treatment with CGN from healthy controls improved outcomes in a mouse model of AD. These findings may hold therapeutic promise for AD patients.

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Differences in the breast ductal fluid microbiome from healthy women vs. women with a history of breast cancer

Chan, A.A.¹, Bashir, M.^{2,3}, Duval, K.^{4,5}, Vaishampayan, P.A.², Love, S.⁵, Lee, D.J.¹

¹John Wayne Cancer Institute, Translational Immunology, Santa Monica, United States, ²Jet Propulsion Laboratory, California Institute of Technology, Biotechnology and Planetary Protection Group, Pasadena, United States, ³Medical University of Graz, Division of Endocrinology and Metabolism, Graz, Austria, ⁴University of California Los Angeles, Breast Center, Westwood, United States, ⁵Doctor Susan Love Research Foundation, Encino, United States

Recent studies have reported the presence of bacterial DNA in human breast tissue and milk, leading to a paradigm shift in breast physiology, and a potential role for microbes to stimulate antitumor immunity in breast cancer. Our previous work described *Sphingomonadaceae* family members to be greater in paired normal vs. breast tumor tissue from breast cancer patients. Here we studied the breast ductal microbiome using nipple aspirate fluid (NAF), which is constantly secreted and absorbed by breast ductal epithelium, to investigate the ductal microbiome and its potential association with breast cancer. We used 16S amplicon DNA sequencing to compare nipple skin as well as the NAF microbiome between healthy control women (HC) and women with a history of breast cancer (BC) While the skin microbiome from BC vs. HC showed no differences, we found that the NAF microbiome from BC vs. HC showed statistically significant community-wide differences. The genus *Alistipes* was significantly higher in NAF from BC while an unclassified genus from the *Sphingomonadaceae* family was significantly higher in NAF from HC. The association of *Sphingomonadaceae* with a healthy state in this second independent study suggests a potentially protective role in breast cancer. We detected bacterial DNA in human breast ductal fluid and found that breast ductal microbiome differs based on health status with regard to a woman's history of breast cancer. These findings warrant further investigation of the breast ductal microbiome and the potential role for specific microbes or their genes to promote antitumor immunity in breast cancer.

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Circulating microRNA as a biomarker for diagnosis of tuberculosis

Yuan, L., Cao, S.L., Shen, A.P.

Shihezi University, Shihezi, China

The aim of the study was to explore whether certain microRNA (miRNA) could serve as biomarkers for active pulmonary TB and latent tuberculosis infection diagnosis. Plasma was collected from healthy controls, active pulmonary tuberculosis (PTB) patients and latent tuberculosis infection (LTBI) patients. Expression levels of circulating miR-155-5p, miR-21-5p and miR-29a were validated and compared by RT-qPCR. The expression levels in plasma of miR-155-5p, miR-21-5p, miR-29a in the active pulmonary TB group were increased 12.27, 4.73 and 3.82 times compared to healthy controls. By ROC curve analysis of active pulmonary TB and healthy controls, the area under the curve (AUC) were 0.938, 0.880, 0.808. MiR-155-5p and miR-21-5p levels were increased 3.21 and 2.39 times in LTBI compared with healthy controls. Furthermore active TB patients had higher expression levels of miR-155-5p, miR-21-5p, miR-29a compared with LTBI patients, 3.82, 1.98 and 8.50 times respectively. Thus significant and potentially clinically useful differences of miR-155-5p, miR-21-5p, and miR-29a plasma expression levels were observed among healthy controls, active pulmonary TB and LTBI, suggesting that these miRNA may serve as potential biomarkers for diagnosis, as well as provide new insights into the human response to tuberculosis infection.

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Contribution of gut microbial composition to the development of diet-induced metabolic syndrome in BALB/c mice

van den Elsen, L., Jones, A., Plunkett, C., Forbes-Blom, E.

Malaghan Institute of Medical Research, Wellington, New Zealand

The gut microbiota contributes to the metabolic phenotype. Germ-free mice are protected against diet-induced obesity and transfer of gut microbiota from obese to germ-free mice results in fat mass gain. The development of obesity correlates with low *Bacteroidetes* and high *Firmicutes* in the gut. Generally BALB/c are considered resistant to developing metabolic syndrome. We investigated the susceptibility of BALB/c mice to develop diet-induced metabolic syndrome and the role of the gut microbiota in this process by using 2 lines of BALB/c mice with divergent gut microbial compositions (BALB/c A versus B). Mice were fed a control or high fat/high sugar diet (HFHSD) for 15 weeks. BALB/c A had a higher *Firmicutes/Bacteroidetes* ratio at baseline than BALB/c B. The HFHSD increased the ratio for both BALB/c lines, however their microbial signature remained very different with specific bacterial species present. BALB/c A fed the HFHSD gained more body weight than the control group, whereas BALB/c B did not. Fat pad mass was larger in BALB/c A vs B and increased significantly when fed the HFHSD in BALB/c A only. This is in line with increased serum leptin concentrations. HFHSD-fed BALB/c A also showed increased expression of IL-6 and TNF- α mRNA in adipose tissue. However, BALB/c B but not A developed impaired glucose tolerance and insulin sensitivity

when fed HFHSD. This was associated with higher serum LPS levels. In conclusion, the gut microbiota has a differential effect on hallmarks of the metabolic syndrome, which is associated with the abundance of certain bacterial species.

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Blockade of CB1 cannabinoid receptor attenuates gut microbiota dysbiosis and inflammation in dietary induced obesity

Mehrpouya-Bahrami, P.¹, Ganewatta, M.S.², Tang, C.², Nagarkatti, M.^{1,3}, Nagarkatti, P.¹

¹School of Medicine, University of South Carolina, Pathology Microbiology and Immunology, Columbia, United States,

²University of South Carolina, Department of Chemistry and Biochemistry, Columbia, United States, ³Dorn VA Medical Center, Columbia, United States

Obesity is characterized by chronic low-grade, systemic inflammation, altered gut microbiota and gut barrier disruption. The endocannabinoid system (eCB) plays a major role in the regulation of the inflammation and metabolic disorders associated with obesity. We investigated the effect of treatment of mice with SR141716A, a cannabinoid receptor1 (CB1) antagonist, on Diet Induced Obesity (DIO), specifically addressing whether such a treatment can induce anti-inflammatory state in adipose tissue. We demonstrated that blockade of CB1 receptor reduced plasma LPS level, circulating inflammatory cytokines and the trafficking of M1 macrophages into the adipose tissue. This decreased inflammatory tone was associated with a lower intestinal permeability and improved hyperglycemia and insulin resistance. In order to better understand the beneficial effects of SR141716A in DIO model, we performed 16S rRNA metagenomic sequencing to investigate alterations in the gut microbiome. Analysis of fecal samples revealed that treatment with SR141716A dramatically increased the relative abundance of Akkermansia muciniphila within the Verrucomicrobia phylum. Although an increase in the genus Prevotella also emerged as strong biomarkers of SR141716A treatment, the increase in A. muciniphila was the only strong biomarker consistently detectable across taxonomic levels and sample types. Drastic reduction in immunogenic Lachnospiraceae and Erysipelotrichaceae with SR141716A treatment was beyond its effect on the weight loss and diet intake. High levels of anti-inflammatory propionate and butyrate in cecal content of SR141716A treated group was detected. Our data suggest that blocking of CB1 receptors ameliorates obesity by restoring gut microbiome and consequently their metabolites which modulate macrophage inflammatory mediators.

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Deletion of CD44 leading to amelioration of experimental autoimmune encephalomyelitis results in altered gut microbiome profile and short-chain fatty acid production

Chitralla, K.N.¹, Guan, H.¹, Busbee, B.¹, Gandy, A.¹, Ganewatta, M.S.², Tang, C.², Nagarkatti, P.¹, Nagarkatti, M.^{1,3}

¹University of South Carolina, School of Medicine, Pathology, Microbiology, and Immunology, Columbia, United States,

²University of South Carolina, Chemistry and Biochemistry, Columbia, United States, ³WJB Dorn VA Medical Center, Columbia, United States

Dysbiosis in gut microbiome has been shown to be prevalent in inflammatory and autoimmune diseases. Previous studies from our lab have demonstrated the pivotal role played by CD44 in experimental autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis (MS). Specifically, we showed that CD44KO mice were resistant to induction of EAE when compared to CD44WT mice and this was to be due to inhibition in the pro-inflammatory Th1/Th17 and increased anti-inflammatory Th2/Treg cell activity. In the current study, we determined whether these effects resulted from an alteration in gut microbiota and the short-chain fatty acids (SCFAs) produced in CD44WT and KO mice. For this purpose, we have performed high-throughput bacterial 16S rRNA gene sequencing followed by clustering sequences into operational taxonomic units (OTUs) and biochemical analyses. KO mice showed significant diversity, richness and evenness when compared to WT mice at the phylum level with dominant *Bacteroidetes* (70.37%) and low *Firmicutes* (27.43%). Taxonomic analysis showed a significant change ($P < 0.05$) in the genera *Alistipes*, *Bacteroides*, *Hallella*, *Meniscus*, *Odoribacter*, *Parabacteroides*, *Phocaeicola*, *Flavonifractor*, *Syntrophococcus*, *Daeguia*, *Desulfovibrio*, *Dongia*, *Helicobacter*, *Litorimonas*, *Nitrosococcus*, *Thiobacter*, *Vampirovibrio*, *Wolinella* and *Mucispirillum* of KO mice compared to the WT mice. Further, results showed a significant change ($P < 0.05$) in the SCFAs, butyric acid, propionic acid and valeric acid in KO compared to WT mice. In conclusion, our results showed that alterations in gut microbiota and SCFAs can shape the immunological environment in MS. (Supported by NIH grants P01AT003961, R01AT006888, R01ES019313, R01MH094755, P20RR032684 and VA Merit Award BX001357).

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Natural dietary indole, indole-3-carbinol, prevents gut microbiota dysbiosis during murine TNBS colitis induction, effectively alleviating colitis associated symptoms

Busbee, P.¹, Nagarkatti, M.², Nagarkatti, P.³

¹University of South Carolina School of Medicine, Columbia, United States, ²University of South Carolina School of Medicine, Pathology, Microbiology, and Immunology, Columbia, United States,

³University of South Carolina, Vice President of Research, Columbia, United States

Colitis is an autoimmune disease characterized by acute or chronic inflammation of the large intestine. Currently there is no cure for patients suffering from colitis, and most treatments involve the use of immunosuppressive drugs that can have adverse side-effects or increased toxicity. In the current study, we investigated the effects of indole-3-carbinol (I3C), a component found in a number of cruciferous vegetables, on two murine colitis models (DSS and TNBS). Our data shows that administration of I3C alleviates symptoms associated with colitis in both of these models, which includes reversal of weight loss and colon shortening. In addition, mice treated with I3C showed decreased levels of circulating inflammatory

serum cytokines and cellular infiltration into the colon. In order to better understand the beneficial effects of I3C against colitis, we performed 16S rRNA metagenomic sequencing to investigate alterations in the gut microbiome after induction of colitis by TNBS and treatment with I3C. Analysis of cecal flushes revealed that TNBS administration leads to a decrease in several Firmicute species (e.g. *Sedimentibacter hydroxybenzoicus* and *Clostridium frigidis*), while increasing several species belonging to the Phylum Bacteroidetes (e.g. *Bacteroides acidifaciens*, *Dysgonomonas wimpennyi*, and *Bacteroides xylanisolvens*). However, mice that were treated with I3C showed a remarkable reversal in these gut microbial alterations caused by TNBS colitis induction, having gut microbiome similar to that of vehicle-treated control mice. Collectively, these data suggest that I3C is able to ameliorate colitis by preventing pathogenic gut microbial dysbiosis and restoring gut microbiome composition to a more homeostatic state.

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Effects of folate on gut microbiome and intestinal immune responses in AA-induced kidney inflammation model

Tang, P.Y., Lin, B.-F.

National Taiwan University, Department of Biochemical Science and Technology, Taipei, Taiwan, Republic of China

Introduction: Aristolochic acid is a common ingredient in Chinese medicine for aiding losing weight, but has been found causing kidney inflammation. Folic acid supplementation has been used as a treatment for the patients to reduce adverse effects in chronic kidney diseases. Gut microbiota has been suggested to modulate immune homeostasis and microbial imbalance has been found in many inflammation diseases, including kidney diseases. Besides kidney, AA is also toxic to intestine, folate supplementation can help maintain gut barrier that might prevent intestinal inflammation. So we want to explore the effects of folate supplementation on intestinal immune responses and microbiome in AA-induced kidney inflammation model.

Materials and methods: Feces from C57BL/6 mice fed with AA and supplemented with or without folic acid were collected and fecal genomic DNA were analyzed. Immune cells from payer's patches or mesenteric lymph nodes will be stimulated with ConA and cytokine levels including IL-2, IFN-g, IL-10 and IL-17 will be analyzed.

Results: Due to the results of CNS inflammation model (EAE) we did before, under folate supplementation conditions, the average level of IL-2 in MLN decreased. The gene fold expression of Lactobacilli is lower than normal folate diet. We will further analyze the association between the microbiome and inflammation in murine model of kidney inflammation.

Conclusion: Intestinal immune responses of AA-induced kidney inflammation animal model might be influenced under folate supplementation. Microbial constitution and intestinal immune responses will be correlated to further clarify the effects of folate on gut microbiome in murine model of kidney inflammation.

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High frequency of *Helicobacter pylori* infection in opisthorchiasis and its association with advanced periductal fibrosis in liver fluke endemic areas of Thailand

Deenonpoe, R.¹, Chomvarin, C.², Mairiang, E.³, Rinaldi, G.⁴, Pairojkul, C.⁵, Chamgramol, Y.⁵, Mairiang, P.⁶, Loukas, A.⁷, J. Brindley, P.⁴, Sripa, B.^{5,8}

¹Chulabhorn International College of Medicine Thammasat University, Pathobiology, Pathum Thani, Thailand, ²Khon Kaen University, Microbiology, Khon Kaen, Thailand, ³Khon Kaen University, Radiology, Khon Kaen, Thailand, ⁴George Washington University, Microbiology, Immunology and Tropical Medicine, and Research Center for Neglected Tropical Diseases of Poverty, Washington DC, United States, ⁵Khon Kaen University, Pathology, Khon Kaen, Thailand, ⁶Khon Kaen University, Medicine, Khon Kaen, Thailand, ⁷James Cook University, Australian Institute of Tropical Health & Medicine, Cairns, Australia, ⁸Khon Kaen University, Tropical Disease Research Laboratory, Khon Kaen, Thailand

Objective: To investigate the prevalence of infection with *Helicobacter* spp. and virulence factors in association with advanced periductal fibrosis in people with and without infection with the *Opisthorchis viverrini* in endemic areas of Thailand.

Methods: Five hundred and fifty-four stool samples recruited from case-control Thai people residing in endemic for opisthorchiasis were investigated for infection with species of *Helicobacter*. All *Helicobacter* data were analyzed in association with advanced periductal fibrosis.

Results: Overall prevalence of *H. pylori* infection was significantly higher in *O. viverrini*-infected (69.3%) than uninfected (30.8%) groups (P 0.001). Significantly higher prevalence of infection with *H. bilis*, but not *H. hepaticus*, was also observed in *O. viverrini* infected group (29.3%) compared to non-*O. viverrini* positive participants (5.4%) (P 0.001). Interestingly, participants infected with *O. viverrini* showed significantly higher frequency of *cagA*-positive *H. pylori* than those uninfected (21.1 vs. 5.0%,) (P 0.001) similar to those of *cagE* (10.2 vs. 1.9 % for *O. viverrini*-infected and uninfected groups, respectively)

(P 0.001). Phylogenetic analysis of *CagA* sequences revealed significant higher prevalence of AB type in *O. viverrini*-infected (75.8%) than those uninfected (93.2%) (0.05) whereas the ABC type (typically western *CagA* type) was found only in *O. viverrini*-infected group.

Conclusion: People with opisthorchiasis harbour higher rates of *Helicobacter* than those uninfected in an intensity dependent manner. *H. pylori* and its virulence factors, specifically *CagA* and *CagE* and *H. bilis* may be involved in the pathogenesis of opisthorchiasis-associated hepatobiliary disease and including cholangiocarcinoma.

Keywords: *Helicobacter* spp., *Helicobacter pylori*, *cagA*, *cagE*, opisthorchiasis, periductal fibrosis

463**Altered gut microbiota and resulting immunomodulation is key to alcoholic liver disease pathogenesis: a northeast India based study**

Bose, S.¹, Bose, M.¹, Basumatary, T.K.¹, Begum, R.H.², Baruah, V.¹, Kalita, M.P.², Bujarbaruah, D.¹, Saikia, A.K.³

¹Gauhati University, Biotechnology, Guwahati, India, ²Assam University, Diphu Campus, Lifesciences and Bioinformatics, Diphu, India, ³Central Hospital, NF Railway, Gastroenterology and Medicinal Unit, Guwahati, India

Present study focused on alterations in gut-microbiota and its clinical relevance with alcohol mediated liver disease susceptibility and severity in tribal patients enrolled from northeast India.

Methodology: Cases[ALD=76, alcoholic-without liver disease(AWLD=25)] were enrolled with clinical details and liver stiffness measurement(LSM) score;along with healthy controls(n=180). Decidual aspirations based microbiome load analysis was performed by standard culture methods for ALD and AWLD cases. Serum endotoxin levels were estimated using standard kit (Lonza). Differential sCD14,mCD14,TLR4,TLR2,NK/NKT expression was analyzed by ELISA/flowcytometry. Differential cytokine profile was studied by multiplex ELISA method. Statistical analysis was performed by SPSS software.

Results: Increased total and gram negative bacterial load was significantly associated with ALD compared to AWLD. Serum endotoxin, sCD14 levels increased gradiently: controls< AWLD< CLD< cirrhosis. Monocyte-CD14 expression was significantly higher in cirrhosis cases(73.98±33.46%) compared to controls(44.30±28.84%), AWLD (43.47±17.08%) and alcoholic-CLD(42.28±18.97%). Average TLR4 expression on blood cells was highest in alcoholic-CLD cases(19.04±8.62%) compared to other cohorts which were comparative. Average serum TLR2 expression (in ng/ml) was higher in alcoholic-CLD(6.08±0.763) and cirrhosis cases(6.12±0.78) compared to AWLD(5.39±0.62) and controls(5.43±0.89). TLR4, TLR2 levels significantly positively correlated with higher LSM scores and mCD14 levels; while mCD14 levels also correlated positively with higher LSM score. NK cell expression was higher in cirrhosis. Distinct up-regulation of NFκB, TNF-α combined with significant down-regulation of anti-inflammatory cytokine IL-6, IL-10 at both protein and mRNA level was found to be significantly correlating with ALD pathogenesis, higher LSM score (p< 0.001) and severity.

Conclusion: Higher gut microflora load and resulting altered hyper-immunomodulation is detrimental to ALD susceptibility and severity.

464**A high fibre diet can modulate hallmarks of inflammation and autoimmunity culminating in reduced nephritis in a model of systemic lupus erythematosus**

Gottschalk, T.A., Tsantikos, E., Hibbs, M.L.

Monash University, Department of Immunology and Pathology, Melbourne, Australia

Systemic Lupus Erythematosus (SLE) is a highly complex, heterogeneous autoimmune disease characterized by circulating self-reactive antibodies that deposit in tissues including skin,

kidneys and brain, alongside a chronic inflammatory response that leads to progressive tissue damage and impaired function. Recent evidence suggests that diet and gut microbiota can influence the development of inflammatory and autoimmune diseases through the generation of short chain fatty acids via the fermentation of dietary fibre. Mice deficient in the tyrosine kinase Lyn (Lyn^{-/-}) develop an autoantibody mediated autoimmune disease and age-dependent glomerulonephritis reminiscent of human SLE. To determine whether a high fibre diet could modulate systemic autoimmune and inflammatory pathology, Lyn^{-/-} and C57BL/6/J control mice were weaned and reared on a diet high in both soluble and insoluble fibre (HFD) or a standard control diet until 42 weeks of age. While Lyn^{-/-} mice on a standard diet developed high titer pathogenic IgG anti-dsDNA autoantibodies and plasma cell expansion, as well as splenomegaly and enhanced splenic hematopoiesis, a HFD ameliorated these phenotypes, suggesting that systemic inflammation could be dampened by dietary intervention. Histological analysis of the kidneys revealed that Lyn^{-/-} mice on the HFD had reduced cellular infiltration and glomerular expansion, and indeed, their kidneys resembled those of control mice. These findings indicate a role for diet in modulating autoimmune responses and inhibiting the generation of pathology in an SLE-like environment. Further studies will focus on characterizing the microbiome and the efficacy of high fibre diet as a post-diagnosis lifestyle intervention.

465**Plasmodium berghei ANKA infection induces intestinal dysbiosis**

Taniguchi, T.^{1,2}, Miyauchi, E.³, Nakamura, S.⁴, Hirai, M.⁵, Suzue, K.¹, Imai, T.¹, Nomura, T.⁶, Okada, H.¹, Shimokawa, C.¹, Onishi, R.¹, Ochiai, A.¹, Hirata, J.¹, Tomita, H.⁶, Ohno, H.³, Horii, T.⁷, Hiseada, H.¹

¹Gunma University, Department of Parasitology, Maebashi, Japan, ²Gunma University, Center for Medical Education, Maebashi, Japan, ³RIKEN Center for Integrative Medical Sciences (IMS), Laboratory for Intestinal Ecosystem, Yokohama, Japan, ⁴Research Institute for Microbial Diseases, Osaka University, Department of Genome Informatics, Suita, Japan, ⁵Juntendo University School of Medicine, Department of Molecular and Cellular Parasitology, Bunkyo, Japan, ⁶Gunma University, Department of Bacteriology and Laboratory of Bacterial Drug Resistance, Maebashi, Japan, ⁷Research Institute for Microbial Diseases, Osaka University, Department of Molecular Protozoology, Suita, Japan

Malaria caused by protozoa of the genus *Plasmodium* is the most prevalent infectious disease in tropical and subtropical regions. Gastrointestinal symptoms, such as abdominal pain, vomiting and diarrhea, are frequently observed in patients with *P. falciparum* in addition to the malarial triad of fever, anemia, and splenomegaly. The pathogenesis of malaria which depends on parasite growth and host immunity to malaria parasites is very complicated. However, it remains unknown whether intestinal microbiota are involved in host-parasite interactions during malaria including intestinal pathology. Here we show that infection of C57BL/6 mice with *P. berghei* ANKA, experimental cerebral malaria (ECM), cause intestinal pathological changes such as epithelial detachment and bleeding in the small

intestines, and an apparent dysbiosis characterized by a reduction of Firmicutes and an increase in Proteobacteria. Furthermore, some genera of microbiota correlate with parasite growth and/or ECM development. By contrast, BALB/c mice that are resistant to ECM exhibit milder intestinal pathology and dysbiosis. These results indicate that the severity of cerebral and intestinal pathology coincides with the degree of alteration in microbiota. Further investigations of the relationship between dysbiosis and intestinal pathogenesis during *P. berghei* ANKA will add to our understanding of the mechanisms involved in intestinal pathology in malaria patients.

Mucosal Immunology

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TRAIL regulates egg-allergen induced eosinophilic oesophagitis

Collison, A.¹, Sokulsky, L.A.¹, Starkey, M.R.^{1,2}, Nightingale, S.^{1,3}, Le Fevre, A.³, Percival, E.^{1,3}, Hansbro, P.M.², Foster, P.S.², Mattes, J.^{1,4}

¹University of Newcastle & Hunter Medical Research Institute, Priority Research Centre GrowUpWell, Callaghan, Australia, ²University of Newcastle & Hunter Medical Research Institute, Priority Research Centre Lung Health, Newcastle, Australia, ³John Hunter Children's Hospital, Department of Gastroenterology, Newcastle, Australia, ⁴John Hunter Children's Hospital, Department of Respiratory and Sleep Medicine, Newcastle, Australia

Introduction: Food antigens are common inflammatory triggers in paediatric eosinophilic oesophagitis (EoE), with food elimination often effective in treating the disease. TNF-related apoptosis-inducing ligand (TRAIL) is a key inducer of eosinophilic inflammation in inflammatory disorders such as house dust mite-induced allergic airways disease and Aspergillus-induced experimental EoE through the upregulation of Midline (MID)-1 and subsequent downregulation of Protein Phosphatase 2A (PP2A).

Methods: Oesophageal biopsies collected from paediatric EoE patients and healthy controls were assessed for TRAIL and MID-1 protein and mRNA transcript levels. To investigate the role of TRAIL in vivo, wild type (TRAIL+/+) and TRAIL deficient (TRAIL-/-) mice were administered subcutaneous chicken egg ovalbumin (OVA) followed by an oral challenge of OVA. Oesophageal samples were collected for histological, protein and gene analyses.

Results: TRAIL and MID-1 protein levels were significantly increased in EoE biopsy samples derived from paediatric EoE patients. TRAIL+/+ mice were found to have a significant upregulation of TRAIL and MID-1 in the oesophagus, with eosinophilic inflammation, fibrosis and elevation of EoE associated cytokines TSLP, IL-5, IL-13 and CCL11. This inflammatory phenotype was significantly attenuated in TRAIL-/- mice.

Conclusion: TRAIL and its associated downstream molecules are upregulated in EoE patients and are key inducers of inflammation in OVA-driven EoE. We propose that TRAIL is a key cytokine involved in the perpetuation of EoE and a potential therapeutic target.

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Micro-RNA-125a/b target A20 and MAVS to promote inflammatory and impair antiviral responses in chronic obstructive pulmonary disease

Hsu, A.^{1,2}, Starkey, M.^{1,2}, Hansbro, P.^{1,2}, Wark, P.^{1,2,3}

¹University of Newcastle, Newcastle, Australia, ²Hunter Medical Research Institute, Newcastle, Australia, ³John Hunter Hospital, Newcastle, Australia

Chronic obstructive pulmonary disease (COPD) is characterized by exaggerated airway inflammatory responses and heightened susceptibility to influenza virus infections with increased mortality. The mechanisms of which are unknown.

In this study we infected primary bronchial epithelial cells (pBECs) from COPD and healthy subjects with human influenza H3N2 or H1N1.

Infection led to significantly higher activation of NF-κB and inflammatory cytokines production

(IL-6/-8/-1β), but resulted in impaired induction of antiviral type I interferon (IFN)-β/-I in COPD, leading to higher viral replication.

A20, an important negative regulator of NF-κB, was not induced by infection in COPD. Ectopic expression of A20 decreased NF-κB phosphorylation but minimally affected IFN-β induction.

This reduced level of A20 was the result of increased induction of miR-125a/b. miR-125a/b antagomiRs increased A20 expression and decreased NF-κB phosphorylation, but IFN-β was markedly increased. TargetScan analysis also showed the antiviral adaptor protein MAVS is a potential target of miR-125a/b. By using MAVS-3' UTR-luciferase reporter constructs and miR-125a/b mimetics and immunoprecipitation, enhanced miR-125a/b targeted the putative binding region on 3' UTR, leading to reduced MAVS expression and IFN-β induction in COPD.

In conclusion, influenza virus infection led to enhanced inflammatory but reduced antiviral responses in COPD. miR-125a/b targets A20 and MAVS, leading to enhanced NF-κB activation and impaired IFN response in COPD. miR-125a/b antagomiRs suppressed NF-κB activation and increased IFN-β induction via increased A20 and MAVS, respectively.

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Nasal tissue resident memory CD8+ T cells are highly protective against influenza A infection

Pizzolla, A., Reading, P., Wakim, L.

University of Melbourne, Microbiology and Immunology, Melbourne, Australia

CD8+ tissue resident memory T cells (Trm) develop after viral infection and persist in the tissue after viral clearance without recirculating.

Influenza A virus is a common pathogen infecting mainly the upper respiratory tract and in severe cases the lower respiratory tract (lung). Influenza virus specific CD8+ Trm have been described in the lung following influenza infection. Whether these cells are also deposited in the upper respiratory tract and if they assist in controlling secondary influenza virus infections is currently unknown.

We find influenza specific CD8 Trm persist within the upper respiratory tract (URT) following influenza infection. URT Trm

were capable of producing cytokines upon re-stimulation and were highly effective at reducing virus growth within the nasal tissue upon re-infection. Importantly, we show that following influenza virus infection CD8⁺ Trm in the URT prevented the migration of influenza virus from the upper respiratory tract to the lungs and the development of severe pulmonary influenza infection.

Deeper understanding of acquired memory in the URT, the predominant site of influenza virus infection, will assist in the development of better vaccination regimes.

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CXCR6 deficiency alters the kinetics of acute inflammatory responses to influenza virus and control of chronic *Mycobacterium tuberculosis* infection

Ashhurst, A.¹, Flórido, M.¹, Lin, L.¹, Stambas, J.², Britton, W.J.^{1,3}

¹Centenary Institute, Tuberculosis Research Program, Camperdown, Australia, ²Deakin University, School of Medicine, Geelong, Australia,

³University of Sydney, Medical School, Sydney, Australia

Respiratory infections cause a significant burden on global health. Understanding the inflammatory response to pathogens, such as influenza and *Mycobacterium tuberculosis* (*Mtb*), may assist in the development of more effective vaccines or treatments. Previous studies have suggested a link between CXCR6⁺ CD8⁺ T-cells and protection against *Mtb* at early stages of infection (Lee et al., *Infect Immun.*, 2011). However the role of CXCR6 during pulmonary infection has not been fully elucidated. We assessed vaccine-induced CD4⁺ T-cell responses in CXCR6^{-/-} mice, using a recombinant influenza virus expressing the *Mtb* p25 epitope (rIAV-p25), that is protective against *Mtb* in mice (Flórido et al., *EJI*, 2015, 45:780). Interestingly, CXCR6^{-/-} mice lost less weight and returned to their initial weight earlier after acute rIAV-p25 infection, when compared to WT counterparts. At 3 weeks post infection, CXCR6^{-/-} mice had elevated activated T-cells in the lungs, however there was reduced Th1-cytokine production and increased KLRG-1 expression on parenchymal T-cells. By 6 weeks this was comparable to WT mice. In an aerosol model of *M. tuberculosis* infection, we demonstrated that CXCR6 was redundant for early recruitment of T-cells and control of bacterial growth. Interestingly at late time points, 6 and 12 weeks, CXCR6^{-/-} mice showed a reduction in bacterial counts in the lungs, associated with a decrease in Th1-cytokines and KLRG-1 expression. While CXCR6 deficiency has minimal impact on CD4⁺ T-cell recruitment to the lungs, it may modulate inflammatory kinetics in mice infected with influenza or *Mtb*, which can be beneficial to the clearance of pathogens.

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Immune regulation in the gastrointestinal tract during persistent virus infection

MacLeod, B.L., Elsaesser, H.J., Snell, L.M., Brooks, D.G.

University Health Network, Princess Margaret Cancer Centre, Toronto, Canada

The sustained antigen load during persistent virus infection creates an immunosuppressive environment that results in the

functional alteration of multiple immune subsets, including CD4 and CD8 T cells, B cells, dendritic cells and macrophages. Persistent infection of mice with LCMV Clone-13 results in profound T cell exhaustion in both lymphoid organs and peripheral tissues. However, the extent to which virally exhausted cells are able to persist and function in the intestinal mucosa is poorly understood. Therefore, we compared and contrasted the composition and functionality of mucosal immune cells following infection with the acutely cleared LCMV-Armstrong and the persistent LCMV Clone-13 virus. These studies provide novel insight into the unique gastrointestinal environment in the context of persistent virus infection.

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CCR7 in periodontal lymphoid-like structures formation

Alvarez Rivas, C., Rojas, L., Monasterio, G., García, K., Pujol, M.,

Carvajal, P., Vernal, R.

Universidad de Chile, Periodontal Biology Laboratory, Santiago, Chile

Periodontal lymphoid-like clusters formation has been postulated in chronic periodontitis patients, where dendritic cells may locally present microbial antigens to naïve T-cells and thus promote alveolar bone resorption in a periodontal site-specific manner. CCR7, CCL19, CCL21, and CXCL12 have been widely associated with ectopic lymphoid-like structures formation in other inflammatory diseases. This study aimed to analyze the CCR7, CXCR4, CXCL12, CCL19, and CCL21 production, as well as the CCR7⁺ naïve T-helper lymphocyte detection, in periodontal tissues from healthy or periodontitis-diseased individuals.

Methods: From gingival samples of healthy or moderate-to-severe chronic periodontitis individuals, total cells were obtained using enzymatic digestion and then analyzed by flow cytometry using the following monoclonal-antibodies: anti-CD4, CD25, CD45RA, CD45RO, RORC2, Foxp3, and CD197. In addition, the mRNA expression for CCR7, CXCR4, CXCL12, CCL19, and CCL21 was quantified by qRT-PCR. Correspondingly, CCL19, CCL21, and CXCL12 secretion was quantified from gingival crevicular fluid samples by ELISA. Finally, CCR7 was detected by western-blot and localized by immuno-fluorescence.

Results: Higher levels of CCR7, CXCR4, CCL19, CCL21, and CXCL12 were detected in periodontitis compared with healthy individuals. In addition, the number of CCR7⁺ naïve T-cells and CCR7⁺ RORC2⁺ memory T-cells were greater in periodontitis patients. CCR7 was localized broadly in the gingival connective tissue, and particularly clustered around blood vessels.

Conclusions: Higher levels of CCR7⁺ naïve T-cells were detected in periodontal tissues from periodontitis versus healthy individuals and this increment was associated with local CCL19, CCL21, and CXCL12 production. Therefore, CCR7 could play a role in periodontal lymphoid-like structures formation.

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Investigating the role of Th1 cells in response to infection with the enteric pathogen *Citrobacter rodentium**Kennedy, C.L.¹, Burke, Z.¹, Alison, E.¹, Brown, A.S.^{1,2}, Fung, K.Y.¹, van Driel, I.R.², Hartland, E.L.¹*¹*Peter Doherty Institute for Infection and Immunity, University of Melbourne, Department of Microbiology and Immunology, Melbourne, Australia, ²The Bio21 Molecular Science and Biotechnology Institute, Department of Biochemistry and Molecular Biology, Parkville, Australia*

Citrobacter rodentium is a mouse pathogen that mimics many virulence behaviours of the human pathogen enteropathogenic *E. coli* and is commonly used as a model organism in the investigation of the enteric mucosal immune response. *C. rodentium* is commonly described in the literature as eliciting a Th1-, Th17-, ILC3-mediated immune response where IL-22 is critical for maintaining epithelial integrity and promoting the production of antimicrobial peptides. However a recent paper by Aycheh, et al, (2015) indicates that Th1 cells may, in fact, be detrimental in the host response to *C. rodentium* infection.

This study aims to clarify the role of Th1 cells in the intestinal immune response. The differentiation of naïve T cells into pro-inflammatory Th1 cells is dependent on the heterodimeric cytokine IL-12, which is expressed by dendritic cells and macrophages. IL-12 is made up of two subunits, IL-12p35 which is unique to IL-12, and IL-12p40 which is shared with IL-23. To date, all studies into IL-12 in the *C. rodentium* immune response have utilised IL-12p40^{-/-} mice, which will also be deficient in IL-23. Our study shows that IL-12p35^{-/-} mice, deficient in IL-12 and, consequently, Th1 cells, are able to contain and clear *C. rodentium* infection as well as C57BL/6 mice. Histologically, there was increased inflammation observed in the lamina propria of infected IL-12p35^{-/-} mice compared C57BL/6 mice and increased penetration of the bacteria into the crypts. Overall, however, these data suggest that IL-12 and Th1 cells are largely dispensable in the host response to *C. rodentium* infection.

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Characterisation of CD4⁺ T cells in healthy and diseased koalas (*Phascolarctos cinereus*) using cell-type-specific monoclonal antibodies*Mangar, C.¹, Armitage, C.W.¹, Timms, P.², Corcoran, L.M.³, Beagley, K.W.¹*¹*Queensland University of Technology, Brisbane, Australia, ²University of Sunshine Coast, Sunshine Coast, Australia, ³Walter and Eliza Hall Institute of Medical Research (WEHI), Melbourne, Australia*

The koala (*Phascolarctos cinereus*) is an arboreal herbivorous marsupial that is an Australian icon. *Chlamydia pecorum* and *C. pneumoniae* are obligate intracellular Gram-negative bacteria that infect koalas and can cause, infertility, arthritis, conjunctivitis, respiratory disease, and in some cases death. Due to a lack of koala-specific immune reagents and assays there is currently no way to adequately analyse the immune response in diseased, healthy or vaccinated animals. Using the extracellular domain of koala CD4⁺ this paper reports the production and characterisation of the first anti-koala CD4 monoclonal antibody

(mAb). Fluorochrome-conjugated anti-CD4 mAb was used to investigate the levels of CD4⁺ T helper cells collected from koala spleens, lymph nodes and peripheral blood by flow cytometry. Biotin-conjugated anti-CD4 mAb was also used for Western Blot and immunohistochemistry to identify CD4⁺ T helper cells in the lymph node. The results indicate that CD4⁺ specific T cells could be identified using flow cytometry of cells from both tissues and peripheral blood as well as visualised using immunohistochemistry techniques. Further analysis of the results also showed an increased level of CD4⁺ cells in animals with current signs of chlamydial infection/disease as opposed to those showing no signs of infection or clinical disease. Since CD4⁺ T cells have been shown to play a pivotal role in clearing chlamydial infection in both human and mouse infections, using this novel antibody will help determine the role CD4⁺ T cells play in protection against chlamydial infection in koalas.

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Increased Langerhan cell counts and elevated chemokine gene expression in foreskins from young men with asymptomatic sexually transmitted infections (STIs)*Gray, C.M.¹, Olivier, A.J.¹, Amu, S.², Jaspán, H.B.¹, Harryparsad, R.¹, Wilson, D.³, Dietrich, J.⁴, Martinson, N.⁴, Mukudu, H.⁴, Mkhize, N.⁵, Morris, L.⁵, Cianci, G.C.⁶, Dinh, M.H.⁶, Hope, T.J.⁶, Passmore, J.S.¹, Chiodi, F.²*¹*University of Cape Town, Immunology, Cape Town, South Africa,*²*Karolinska Institutet, Stockholm, Sweden, ³Edendale Hospital, Pietermaritzburg, South Africa, ⁴Perinatal HIV Research Unit, Johannesburg, South Africa, ⁵National Institute for Communicable Disease, Johannesburg, South Africa, ⁶Northwestern University Feinberg School of Medicine, Chicago, United States*

Medical Male Circumcision (MMC) has been shown to reduce HIV acquisition by up to 60%, and additionally decrease the incidence of other sexually transmitted infections (STIs). We hypothesize that the underlying mechanisms for this protection is that MMC results in the removal of potential target cells for HIV infection, and alters levels of keratinisation. In a longitudinal study involving 14-24 year-old HIV-negative men undergoing MMC, we compared the numbers of CD4⁺ T cells, expression of langerin, Ki67, and chemokine genes. Participants were tested for asymptomatic STIs using multiplex PCR (*C. trachomatis*, *N. gonorrhoea*, *M. genitalium*, *T. vaginalis*, HSV-1/2). We found elevated numbers of CD207⁺ Langerhans cells (LCs) in the foreskin of men with asymptomatic STIs (n=28) compared to age-matched STI-negative men (n=28) (median 1 vs 4 cells/mm³, p=0.0005). There were no significant differences in CD4⁺ T cell and Ki67 cell counts as a function of STI status. The outer foreskin had higher keratin thickness compared to the inner foreskin (median difference = 1.712um; p=0.0058), but no difference due to STI status. There was a positive correlation between CD207 and elevated CCL27 gene expression in the inner foreskin (r=0.498; p=0.016). STI-induced inflammation and recruitment of immune cells via chemokines to the foreskin may be elevating the risk of HIV acquisition in uncircumcised men with an asymptomatic STI. MMC may thus reduce HIV acquisition risk in this highly susceptible group of men by removing potential HIV target cells in foreskins. Treatment

of asymptomatic STIs in uncircumcised men would lower HIV acquisition risks.

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Regulation of intestinal immune homeostasis by the retinoic acid-responsive transcriptional factor hypermethylated in cancer 1

Burrows, K.¹, Antignano, F.¹, Chenery, A.¹, Underhill, T.M.¹, Zaph, C.^{1,2}

¹University of British Columbia, The Biomedical Research Centre, Vancouver, Canada, ²Monash University, Department of Biochemistry and Molecular Biology, Clayton, Australia

Retinoic acid (RA) has critical functions in the intestinal immune system. RA licenses CD4 T helper (Th) cells to express gut-tropic homing receptors and is important in determining the balance between regulatory T (Treg) cells and Th17 cells in the intestinal microenvironment. However, the molecular mechanisms linking RA to intestinal immune homeostasis are unknown. Here, we identify the POK/ZBTB family zinc finger transcription factor Hypermethylated in cancer 1 (Hic1) as a critical RA-responsive transcriptional determinant that controls intestinal T cell function. *In vivo*, Hic1 expression within the hematopoietic compartment is restricted to the small intestine. Strikingly, mice fed a vitamin A deficient diet exhibit notably diminished expression of Hic1 in intestinal immune cells. Mice with a specific deletion of Hic1 in T cells (*Hic1^{ΔT}* mice) have reduced frequencies of T cells in the lamina propria of the small intestine at steady state but display increased levels of interleukin (IL)-17A. Following induction of small intestinal inflammation, *Hic1^{ΔT}* mice displayed significantly less signs of pathology, associated with heightened levels of IL-17A and IL-10 at the site of inflammation. *In vitro*, *Hic1*-deficient Th17 cells also displayed increased levels of IL-17A and IL-10. Mechanistically we found that Hic1 and Stat3 interact in Th17 cells, and in the absence of Hic1 there are higher levels of Stat3 binding to both the *Il17a* and *Il10* loci. Taken together, these results demonstrate that Hic1 is a novel intestinally-restricted transcription factor that regulates Th17 cell differentiation and may be a target for therapeutics in treating intestinal inflammation.

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Galectin-9 regulate epithelial innate immunity and is essential for intestinal and inflammation

Lu, D.H.H., Liu, F.T., Shui, J.W.

Academia Sinica, IBMS, Taipei, Taiwan, Republic of China

Galectin-9 (Gal-9), a β -galactosides binding animal lectin, has been recently reported as a risk gene for inflammatory-bowel-disease (IBD) in a genome-wide-association-study (GWAS). However, it remains unclear how Gal-9 is involved in innate immune regulation in gut, particularly mucosal host defense and inflammatory pathogenesis.

To reveal functions of Gal-9 in the mucosa, we first determined Gal-9 expression levels in different segments and compartments of intestine. Interestingly, Gal-9 is predominantly expressed in colonic and ileal crypts, where stem cells and anti-microbial Paneth cells are located. Further analysis indicated that Gal-9^{-/-}

mice had reduced crypt numbers in ileum and colon at steady state. Based on this finding, we hypothesized that Gal-9 might be essential for epithelial cell homeostasis and as such, Gal-9 could be involved in colitis pathogenesis via regulating epithelial innate immunity and barrier integrity. We find that after dextran-sulfate-sodium (DSS)-induced colitis, Gal-9 expression level in epithelial cell were increased and Gal-9^{-/-} mice showed 100% mortality with more severely inflammation and lower IL-22 production (important to epithelial repair and anti-microbial-peptide induction). Also, Gal-9^{-/-} mice were highly susceptible to *Yersinia*, with increased bacterial burden and translocation. Correlated to *in vivo* findings, freshly isolated Gal-9^{-/-} epithelial cells (crypts) showed impaired *Yersinia* killing ability and Gal-9^{-/-} crypts and fragment had defective innate response after *Yersinia* infection.

Our results indicate that galectin-9 not only plays an essential role in mucosal immunity during host defense, but also regulate intestinal inflammation during colitis pathogenesis. Our findings therefore provide evidences that polymorphism of galectin-9 gene might contribute to dysfunction of epithelial barrier, leading to intestinal inflammation.

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The role of host IL-1 α and the microbiome in acute colitis

Nunberg, M.¹, Werbner, N.², Bersudsky, M.¹, Apte, R.N.¹, Koren, O.², Voronov, E.¹

¹Ben Gurion University of the Negev, Beer Sheva, Israel, ²Bar Ilan University, Safed, Israel

Interleukin-1 α (IL1 α) is a pleiotropic cytokine, which acts as an alarmin during inflammation. It is expressed in intestinal epithelial cells (IECs) under homeostatic conditions and is released from damaged IECs and initiates inflammation. The effect of IL-1 α on acute colon inflammation was assessed in DSS-induced colitis model, using complete IL-1 α mice KO mice and mice with a specific deletion of IL-1 α in IECs, we observed a very mild form of colitis with significantly better repair compared to control mice. Thus, various possible mechanisms involved in IL-1 α -induced colon inflammation were studied. Changes in the microbiome could explain how deficiency of IL-1 α leads to reduced DSS-induced damage of intestinal mucosal barrier, as well as high expression of tight junction proteins, preservation of goblet cells or retained epithelial barrier functions. Thus IL-1 α , which induces exacerbated colon inflammation, may be critical in driving the pathologic breakdown of barrier integrity. Co-housing experiments combined with metagenomic analysis confirmed the role of the specific microbiota in IL-1 α KO mice as compared to control mice. After DSS administration, IL-1 α KO co-housed mice developed a disease phenotype similar to DSS-treated control mice rather than to DSS-treated control IL-1 α KO mice. Goblet cells in co-housed mice were also similar to their distribution to control mice. Metagenomic analysis, obtained from fecal samples, revealed a significant shift in β -diversity towards control and co-housed mice. These results confirmed that IL-1 α is a key molecule in acute colon inflammation and affects barrier functions and colon microbiota.

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Dendritic cells from ankylosing spondylitis patients show reduced activation in response to stimulation with gut bacteria

Peyroux, E.¹, Gray, A.², Mc Connell, M.³, Highton, J.⁴, Young, S.¹

¹University of Otago, Pathology, Dunedin, New Zealand, ²University of Otago, Preventative and Social Medicine, Dunedin, New Zealand, ³University of Otago, Microbiology and Immunology, Dunedin, New Zealand, ⁴University of Otago, Medicine, Dunedin School of Medicine, Dunedin, New Zealand

Ankylosing Spondylitis (AS) is a chronic inflammatory arthritis of spine and sacroiliac joints with further peripheral manifestations affecting 0.1- 1.4% of the population. Susceptibility genes identified include HLA-B27 and ERAP1, both essential components in antigen processing for initiation of immune responses. Numerous lines of evidence indicate that bacterial contact at mucosal surfaces is the driving force behind inflammation in AS. We generated monocyte-derived dendritic cells (MoDCs) from the venous blood of AS patients (n= 24) and control group (n= 15) and stimulated them with a range of heat-killed gut bacteria with known associations to Reactive arthritis and inflammation - *Bacteroides fragilis*, *Yersinia enterocolitica*, *Campylobacter jejuni* and *Helicobacter pylori*. We measured expression of MoDC activation markers- MHC-I, MHC-II, CD40, CD80 and CD83 by flow cytometry. Analysis of cytokine secretion in supernatants was assessed by Bioplex immunoassay. Our results indicated the level of CD40 and CD80 costimulatory marker up-regulation was significantly reduced in many of the bacterial treatments in the AS group compared to control group. Reduced MHC-II up-regulation was also observed in two treatments- Polyinosinic-polycytidylic acid and *C. jejuni*. CD83, a MoDC maturation marker, was expressed at similar levels for all treatments. Cytokine analysis showed altered TNF α secretion to *Y. enterocolitica* treatment and IL-23 production to *C. jejuni*. From these results we hypothesize that abnormal signaling through innate pattern recognition receptor signaling pathways induces aberrant activation of MoDCs in AS patients. These MoDCs in turn may direct cells of the immune system toward an inappropriate or misdirected immune response.

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Specification of peripheral Treg cells particular to a commensal bacterium that regulates intestinal homeostasis

O. E.^{1,2}, Hong, S.-W.^{1,2}, Lee, J.^{1,2}, Surh, C.D.^{1,2,3}

¹POSTECH, Department of Integrative Biosciences and Biotechnology, Pohang, Korea, Republic of, ²Institute for Basic Science, Academy of Immunology and Microbiology, Pohang, Korea, Republic of, ³La Jolla Institute for Allergy and Immunology, Division of Developmental Immunology, La Jolla, CA, United States

The establishment and the maintenance of intestinal homeostasis requires generation of peripheral regulatory T (pTreg) cells specific to the commensal microbiota in the colon. However, how microbiota-specific pTreg cells initially develop and maintain is not clearly demonstrated, including the identity of the microbial antigens that induce generation of pTreg cells and how such microbial antigens initiate activation

and differentiation of conventional T cells into pTreg cells. To understand these issues, we have begun to define the specificity of a TCR transgenic (Tg) line, CT6, that was shown to be specific to an uncharacterized *Clostridium* species of commensal bacterium. We recently identify CT6 Tg T cells to react to *Clostridium.symbiosum* and proliferated strongly to *C.symbiosum* when its extract was presented *in vitro* by splenic antigen presenting cells whereas control polyclonal C57BL/6 T cells did not. CT6 Tg T cells also responded to *C.symbiosum in vivo* when CT6 cells were adoptively transferred into specific pathogen-free (SPF) B6 mice that were then i.p injected with live *C.symbiosum*. However, CT6 cells did not respond to *C.symbiosum* when the bacterium was introduced orally in SPF or germ-free (GF) B6 host. These findings suggest that the mucosal immunity in SPF and GF mice prevents unopposed presentation of *C.symbiosum* antigens to peripheral immune system. We are currently in the process of defining factors that form the intestinal barrier to prevent *C.symbiosum* translocation under normal conditions and also determine how this barrier establishes and maintains intestinal homeostasis by generating effector and pTreg cells to *C.symbiosum*.

Synergetic damaging effect of factors decreasing the respiratory epithelial barrier

Waltl, E.E.¹, Selb, R.¹, Eckl-Dorna, J.¹, Valenta, R.², Niederberger, V.¹

¹Medical University of Vienna, Austria, Otorhinolaryngology, Vienna, Austria, ²Medical University of Vienna, Austria, Division of Immunopathology, Department of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, Vienna, Austria

The respiratory epithelial barrier plays an important role in protecting humans against various exogenous factors (e.g. allergens, pathogens, toxic substances). Here we aimed to establish different damaging models in order to enable us to investigate protective factors for the epithelium. Furthermore we performed experiments testing the synergetic effect of barrier-decreasing substances.

A bronchial epithelial cell line (16HBE14o-) was cultured in monolayers in the xCELLigence DP system. This system allows continuous real-time measurement of impedance-based cell responses. The epithelial integrity was impaired by the following damaging conditions: i) Physical damage by scratching the cell layer, ii) infection with human rhinovirus (RV), iii) incubation with standardised cigarette smoke extract (CSE) and iv) exposure to the pro-inflammatory TH1 cytokine interferon-gamma and v) exposure to house dust mite (HDM) extract.

Barrier function decreased in a time- and dose-dependent manner after treatment of cells with all above-mentioned substances. However, the various conditions induced different damaging patterns to the respiratory epithelium. The maximal barrier decreasing effects were observed after 36 hours of RV infection, after 12 hours of exposure to CSE, after 4-5 days of treatment with interferon-gamma and after 3-4 days of exposure to HDM extract. Especially, experiments testing the synergetic effect of HDM and CSE showed a significantly amplified damaging effect of the cells when substances were added in combination, in contrast to application of the substances alone. Our results visualise the synergetic damaging effect of CSE and

HDM extract indicating that smoking may facilitate allergic sensitisation and increase allergic symptoms in patients.

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The polysaccharide maltodextrin exacerbates intestinal inflammation

Laudisi, F.¹, Di Fusco, D.¹, Dinallo, V.¹, Vezza, T.², Marafini, I.¹, Alfredo, C.¹, Ortenzi, A.¹, Monteleone, I.¹, Pallone, F.¹, Monteleone, G.¹

¹University of Rome 'Tor Vergata', Rome, Italy, ²IBS Granada, CIBM, University of Granada, Granada, Spain

In the last decades, there has been an increased incidence of Inflammatory Bowel Diseases (IBD), particularly in previously low incidence areas, mainly due to changes in environmental and lifestyle factors. Food additives commonly used as emulsifiers, stabilizers or bulking agents are frequently present in Western diet. Preliminary evidences indicate that daily consumption of some of these compounds could alter the intestinal homeostasis in mice, by promoting dysbiosis and inflammatory response.

In this study, we evaluated whether a diet rich in Maltodextrin (MDX), a polysaccharide used as food additive, energy supplement and coating agent, can increase the susceptibility of mice to develop intestinal inflammation.

Wild-type mice were exposed to drinking water containing MDX and then challenged with dextran-sulfate sodium (DSS) to induce colitis. Mice were sacrificed and colons harvested for RNA/protein analysis and histopathology.

Mice receiving MDX-enriched diet did not exhibit macroscopic signs of intestinal inflammation. Instead, they showed hyperplasia of mucus-producing cells and altered intestinal microbiota composition with high levels of Dual oxidase-2, an epithelial-specific NADPH oxidase that correlates with mucosal dysbiosis. Once challenged with DSS, MDX-treated mice exhibited significant weight loss, destruction of intestinal epithelium, higher inflammatory cell infiltration and increased expression of pro-inflammatory cytokines compared with mice treated with DSS alone.

In conclusion, a diet rich in MDX may alter the interaction between luminal flora and mucosal cells, thus leading to altered intestinal homeostasis and exacerbated tissue-damaging pathogenic responses.

Altogether, data suggest that daily consumption of MDX may contribute to enhance host's susceptibility to IBD.

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Triggering receptor expressed on myeloid cells-1 plays a crucial role in regulating intestinal barrier in an experimental colitis model

Yang, F.-C., Chen, N.-J.

National Yang-Ming University/Institute of Microbiology and Immunology, School of Life Sciences, Taipei, Taiwan, Republic of China

TREM-1 expression is induced mostly on monocytes, macrophages and neutrophils by various stimuli such as toll-like receptor (TLR) ligands. It contributes to the exacerbation of inflammatory disorders by enhancing the secretion of

pro-inflammatory cytokines. Ulcerative colitis (UC) is one of inflammatory bowel disease (IBD) that majorly causing colonic inflammation associated with type 2 inflammatory mediators induction. It was recently demonstrated that a substantial accumulation of TREM-1 expressing macrophages could be obtained in the inflamed colonic tissue of patients with IBD and of mice with experimental colitis. However, the underlying mechanism contributed by TREM-1 is still unknown. To elucidate the precise role of TREM-1 in intestinal inflammation by using an independent TREM-1 knockout mouse line in DSS-induced mouse colitis model, which can mimic patient with UC. We found TREM-1 is involved in enhanced pro-inflammatory mediators expression, and increased macrophages and neutrophils infiltration in affected colons. Ablation of TREM-1 did not protect mice from DSS-induced colitis. Oppositely, an exacerbated tissue damage resulted from impaired intestinal epithelium integrity was obtained in DSS-treated mice lacking TREM-1, implicating a protective role of TREM-1 in modulating the intestinal barrier integrity during DSS-induced colitis. Furthermore, TREM-1 deficiency associates with a drastic reduction of IL-22 that involves in epithelial repair and regeneration, in line with a significant decrease of group 3 innate lymphoid cell (ILC3s), a well known innate source of IL-22 and IL-17. Thus, our results implicate a unique role of TREM-1-expressing macrophages on attenuating DSS-induced colitis through promoting intestinal barrier reconstitution.

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Investigating the immune composition of the human anorectal tract

Baharlou, H.^{1,2}, Bertram, K.¹, Botting, R.¹, Sandgren, K.¹, Kim, M.¹, Cunningham, A.¹, Harman, A.¹

¹Westmead Institute for Medical Research, Centre for Virus Research, Sydney, Australia, ²Sydney University, School of Medicine, Sydney, Australia

Little is known about the immune cells present in the human anorectal tract; instead information is inferred from genital and intestinal tissues. However, caution should be applied to such inferences as differences in immune cell distribution and function have been observed in other mucosal tissues such as the respiratory tract. This represents a critical gap in our knowledge as the anorectal tract is a key site of transmission for many pathogens, especially HIV.

Therefore, we have gained access to human anorectal tissue from colorectal surgery and defined the immune cell subsets that make up these tissues and compared them to those found in genital and intestinal tissue. We have made several novel observations.

1) In anal and rectal tissue the predominant myeloid populations are macrophages whereas in genital tissue dendritic cells predominate.

2) Nevertheless we have identified 4 separate anal epithelial dendritic cell subsets, two of which are negative for CD1a and differ significantly to those found in genital epithelium.

3) We have observed that rectal tissue contains a high frequency of lymphoid aggregates that are rich in both B and T lymphocytes, occupying discrete zones. Interestingly, the

specific subsets of these lymphocytes differ significantly from those found in the colon and small intestine.

For example the rectum contains fewer numbers of central memory CD4 T cells.

Thus the anorectal tract contains a unique immune environment which may reflect differences in the microbiota. Future studies should lead to preventative treatment strategies to block the transmission of pathogens across this surface.

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Dietary iron and transcription factor Spi-C regulate gut homeostasis through maintenance of CX₃CR1^{high} CD11b⁺ CD11c⁺ cells

Kayama, H.¹, Kohyama, M.², Arase, H.², Takeda, K.¹

¹Osaka University Graduate School of Medicine, Suita, Japan,

²Osaka University, Suita, Japan

Intestinal innate immune cells contribute to maintenance of gut homeostasis. In particular, CX₃CR1^{high} CD11b⁺ CD11c⁺ cells are involved in preventing intestinal inflammation through several mechanisms. Recent studies identified transcription factors and environment factors controlling development of several tissue resident macrophages. However, how CX₃CR1^{high} CD11b⁺ CD11c⁺ cells are maintained in the intestine remains poorly understood. In this study, we show that CX₃CR1^{high} CD11b⁺ CD11c⁺ cells highly expressed the transcription factor Spi-C, which is shown to be induced by heme in red pulp macrophages, and iron metabolism-related genes including *Hmox1*, *Blvrb*, and *Slc40a1*. Iron deficient diet-fed mice showed decreased number of CX₃CR1^{high} CD11b⁺ CD11c⁺ cells in the colon and high sensitivity to dextran sulfate sodium (DSS)-induced colitis. Transfer of CX₃CR1^{high} CD11b⁺ CD11c⁺ cells from control diet-fed mice ameliorated DSS-induced colitis in mice given iron deficient diet. Furthermore, *LysM-cre; Spi-C^{flox/flox}* mice, in which *Spi-C* expression was abrogated in CX₃CR1^{high} CD11b⁺ CD11c⁺ cells, but not in red pulp macrophages, were highly sensitive to DSS colitis. These results indicate that dietary iron and transcription factor Spi-C are required for functional properties and survival of CX₃CR1^{high} CD11b⁺ CD11c⁺ cells and prevention of intestinal inflammation.

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Uncoupling of the pDC-Treg crosstalk during early-life Pneumovirus infection underlies long-lived Treg dysfunction and asthma development

Lynch, J.P.¹, Werder, R.B.¹, Loh, Z.¹, Lane, K.¹, Rogers, M.¹, Diener, K.², Hayball, J.², Hill, G.³, Haque, A.³, Spann, K.⁴, Mazzone, S.¹, Upham, J.W.⁵, Sly, P.⁶, Phipps, S.¹

¹University of Queensland, School of Biomedical Sciences, Brisbane, Australia, ²University of South Australia, School of Pharmacy and Medical Sciences, Adelaide, Australia, ³QIMR Berghofer Medical Research Institute, Brisbane, Australia, ⁴Queensland University of Technology, Brisbane, Australia, ⁵University of Queensland, Lung and Allergy Research Centre, School of Medicine, Brisbane, Australia, ⁶University of Queensland, Queensland Children's Medical Research Institute, Brisbane, Australia

Severe viral bronchiolitis is a major risk factor for asthma, a disease associated regulatory T cell (Treg) dysfunction. Plasmacytoid dendritic cells (pDC) produce type-I IFN and induce Treg cell responses to regulate virus infections. Here we addressed whether inducible depletion of pDC in early-life alone increases the severity of viral bronchiolitis and predisposes toward asthma. Neonatal pDC-diphtheria toxin receptor (pDC-DTR) knock-in mice and wild-type (WT) littermates were infected with low-dose pneumonia virus of mice (PVM, 10pfu/i.n.), and challenged with 100pfu 6 weeks later. DT (3ng/g body weight/i.p.) was administered -1, +1, +3, and +5 days post infection and pathologic features of bronchiolitis or asthma assessed. Temporary pDC depletion reduced antiviral cytokine production, increased viral load and induced severe bronchiolitis (airway epithelial cell sloughing, neutrophilic inflammation). This phenotype was associated with diminished IL-10 production and a failure to expand neuropilin-1+ Tregs. Ligation of neuropilin-1 was necessary for pDC-mediated proliferation of Tregs ex vivo. Viral challenge of pDC-DTR but not WT mice induced the hallmark features of asthma and was associated with lower numbers of antigen-experienced Tregs. Adoptive transfer of antigen-experienced WT Tregs (3x10⁴, i.v. route) during primary infection or prior to viral challenge of pDC-depleted mice prevented the development of both severe bronchiolitis and postviral asthma. In contrast, Tregs from 'asthmatic' pDC-DTR mice were insufficient. pDCs mediate the expansion of Tregs to protect against severe bronchiolitis in early-life. Dysregulation of this crosstalk leads to the generation of aberrant and long-lived Tregs that are insufficient to protect against virus-associated asthma in later-life.

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Absence of interferon-β promoter stimulator-1 (IPS1) induces bronchiolitis and asthma-like pathology in response to pneumoviral infection

Simpson, J.¹, Loh, Z.¹, Zhang, V.¹, Spann, K.², Upham, J.³, Phipps, S.¹

¹University of Queensland, School of Biomedical Science, Brisbane, Australia, ²Queensland University of Technology, School of Biomedical Science, Brisbane, Australia, ³University of Queensland, School of Medicine, Brisbane, Australia

Pneumovirus-induced bronchiolitis causes ~200,000 deaths per year and is a risk factor for subsequent asthma. Impaired antiviral responses may underlie susceptibility to bronchiolitis and subsequent asthma. Interferon (IFN)-β promoter stimulator-1 (IPS1, also known as MAVs) is an adaptor protein involved in IFN and inflammatory cytokine production following cytosolic viral detection. Here, we determined the role of IPS1 in pneumonia virus of mice (PVM)-induced bronchiolitis and asthma. Wild-type (WT) and IPS1-deficient mice were inoculated with PVM (2 pfu), at 7 days of age. Pathologies associated with bronchiolitis were evaluated at 4, 7, 10 and 14 days post infection (dpi). To assess susceptibility to asthma, mice were challenged with PVM (100 pfu) at 42 dpi, and euthanised 7 days later. Infection of WT mice induced IFN production and plasmacytoid dendritic cell (pDC) recruitment. The absence of IPS1 impaired IFN production and decreased pDC numbers in the lungs, accompanied by an elevated viral load in the airway epithelium and the release

of epithelial cell-derived alarmins IL-33 and high-mobility group box 1. Bronchiolitic IPS1-deficient mice also exhibited increased airway smooth muscle growth and mucous secretion. Reinfection of IPS1-deficient mice induced the cardinal features of asthma including airway hyperreactivity, mucous hypersecretion and airway smooth muscle remodelling, but failed to induce a significant type-2 inflammatory response. The absence of IPS1 increased the severity of bronchiolitis and predisposed to the development of asthma following viral challenge in later life. Notably, airway remodelling commenced early in life and occurred in the absence of type 2 inflammation.

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Dietary fiber promotes oral tolerance by modulating vitamin A metabolism in mucosal dendritic cells

Tan, J.¹, Mackay, C.¹, Macia, L.²

¹Monash University, Melbourne, Australia, ²University of Sydney, Sydney, Australia

Food allergy is a potentially fatal disease and its incidence has increased dramatically in recent decades. Diet and the gut microbiota have been implicated in attenuating allergic responses, but the exact mechanisms remain elusive. We show that high-fiber diet feeding in mice establishes an anti-inflammatory mucosal environment, enhances oral tolerance and protected from severe food allergy. Mucosal CD103⁺ dendritic cells (DCs) are critical for establishing oral tolerance by their capacity to induce Treg differentiation as well as imprinting gut homing receptor CCR9 on responding naïve T cells. These effects are dependent on their production of retinoic acid from Vitamin A by the enzyme retinaldehyde dehydrogenase (RALDH). High-fiber feeding promoted oral tolerance by upregulating RALDH activity in CD103⁺ DCs, promoting Treg differentiation *in vitro* and *in vivo* as well as suppressing antigen-specific CD4⁺ T cell proliferation *in vivo*. Inhibition of retinoic acid signaling by retinoic acid receptor antagonist LE540 abrogated the ability of mesenteric lymph node derived CD103⁺ DCs from high-fiber fed mice to promote Treg differentiation as well as induce CCR9 expression. Furthermore, high-fiber feeding under Vitamin A deficient condition abrogated the beneficial effects of dietary fiber on oral tolerance. Thus, dietary elements including fiber and Vitamin A regulate tolerogenic pathways in the gastrointestinal tract upstream of Treg cells, necessary for immune non-responsiveness towards food antigens.

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Dietary macromolecules regulate differentiation of small intestine resident memory T cells in IEL compartment

Jung, J.^{1,2}, Kim, K.S.¹, Surh, C.^{1,2,3}

¹Academy of Immunology and Microbiology (AIM), Institute for Basic Science (IBS), Pohang, Korea, Republic of, ²Pohang University of Science and Technology (POSTECH), Department of Integrative Biosciences and Biotechnology (IBB), Pohang, Korea, Republic of, ³La Jolla Institute for Allergy and Immunology (LIAI), San Diego, United States

Intestinal tissue-resident memory CD8⁺ T (T_{RM}) cells are mostly

located within intraepithelial lymphocyte (IEL) compartment. They are abundantly present in the small intestine and serve as the first line of defense against invading pathogens and to maintain homeostasis of the intestinal epithelial cells. Although small intestine is constitutively exposed to both microbial and dietary antigens (Ags), more attention has been focused on the influences of commensal microbiota. To investigate the role of both microbiota and diet in intestinal T_{RM} cells, we compared IELs in the offspring of germ-free (GF) B6 mice raised with a chemically defined elemental diet. In such "Ag-free" (AF) mice, we found a severe reduction in the development of induced IELs, i.e., TCRαβ⁺CD4⁺, TCRαβ⁺ CD8αβ⁺, TCRαβ⁺ CD4⁺CD8α⁺ IELs, and a partial depletion of natural TCRαβ⁺CD8α⁺ IELs. The TCRγδ⁺CD8α⁺ IEL population was relatively unaffected in AF mice. Furthermore, using our GF and AF mice, we directly demonstrate a larger role of diet than microbiota in inducing development of T_{RM} cells. More importantly, we found that the residual T_{RM} cells in the small intestine of AF mice, unlike those in SPF and GF mice, do not display constitutive effector function. Taken together, our results indicate that consumption of diet is important for normal generation and function of effector memory T_{RM} cells in IEL compartment.

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Aeroallergen sampling in an experimental animal model of respiratory allergy

Leffler, J.¹, Mincham, K.T.¹, Mok, D.¹, Holt, P.G.¹, Stumbles, P.A.^{1,2,3}, Strickland, D.H.¹

¹Telethon Kids Institute, University of Western Australia, Subiaco, Australia, ²School of Veterinary and Life Sciences, Murdoch University, Murdoch, Australia, ³School of Paediatrics and Child Health, University of Western Australia, Crawley, Australia

The prevalence of respiratory allergic sensitisation has increased the last decades yet there is only limited understanding of the immunological processes involved. Our previous studies show that a deficient regulatory T cell response, crucial to prevent sensitisation, is linked to reduced allergen uptake in the respiratory mucosa. To provide insights into the immunological processes, modulating development of tolerance versus sensitization during natural respiratory aeroallergen exposure, we used an experimental animal model employing sensitisation of resistant and susceptible rat strains to mimic clinical observations in humans.

During repeated intra-nasal, adjuvant-free ovalbumin exposures, naïve male rats from the susceptible strain develop antigen-specific IgE sensitisation and airway eosinophil infiltration. This was further associated with skewed T-effector to T-regulatory cell ratio in the airways. In the resistant strain, exposure did not induce IgE but rather IgG and the T-effector:regulatory ratio remained constant. Higher levels of initial allergen uptake by respiratory dendritic cells were observed in the resistant strain, that together with subsequent lymph node delivery, increased following exposure in both strains although to lesser extent in the susceptible strain. To identify if increased antigen uptake could alter the course of sensitisation, we exposed the susceptible strain to an elevated ovalbumin dose. This induced T-regulatory cell proliferation and IL-10 production but did not

alter antigen-specific IgE levels. Current analysis is addressing how antigen dose modulates long-term tolerance in this strain. Given these results, this model may provide a platform for developing future therapeutic regimes based on manipulating allergen exposure to prevent allergic sensitisation.

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Role of regulatory T cells in bacterial induced colitis

Fung, K.Y.¹, Rahman, T.², Kennedy, C.¹, van Driel, I.², Hartland, E.L.¹

¹University of Melbourne, Peter Doherty Institute for Infection and Immunity, Microbiology and Immunology, Parkville, Australia,

²University of Melbourne, Bio21 Molecular Science and Biotechnology Institute, Department of Biochemistry and Molecular Biology, Parkville, Australia

CD4⁺CD25⁺Foxp3⁺ Regulatory T cells (Treg) contribute to the maintenance of immune homeostasis in large intestine and as such, are implicated in the development of inflammatory bowel disease (IBD). To determine the role of Treg cells during colonic inflammation, we examined the course and outcome of *Citrobacter rodentium* infection in mice depleted of Treg cells. We found that mice depleted of Treg cells are more susceptible to mucosal *C.rodentium* infection than non-depleted mice. Treg-depleted mice exhibited exacerbated weight loss, significantly higher bacterial burden in the colon, increased dissemination to systemic tissue, increased epithelial permeability and colonic pathology. Compared to non-depleted mice, at 5 days after infection depleted mice showed altered expression of inflammatory cytokines and antimicrobial peptides in the colon, which have been shown to be protective against *C.rodentium* infection and important for maintaining epithelial integrity. In addition, there is reduced number of B cells infiltrating the colon and less anti-*Citrobacter* IgG in serum of Treg-depleted mice at 10 days post infection. These data suggest that Treg cells are important in supporting the host immune response and maintaining the immune epithelial barrier which is vital for healthy gut homeostasis. More importantly, we have revealed a possible role for Treg cells in regulating or cooperating with B cells to combat bacterial infection or possible involvement of B cells in epithelial barrier maintenance.

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dLac and FOS reverse antibiotic-induced lung defense impairment in a ventilator model through increasing intestinal reactive oxygen species

Chen, L.-W.¹, Chen, P.-H.², Hsu, C.-M.³

¹Kaohsiung Veterans General Hospital, Surgery, Kaohsiung, Taiwan, Republic of China, ²Kaohsiung Veterans General Hospital, Kaohsiung, Taiwan, Republic of China, ³National Sun Yat-Sen University, Department of Biological Sciences, Kaohsiung, Taiwan, Republic of China

Prior antibiotic exposure is associated with increased mortality in gram-negative bacteremia complicated by sepsis or septic shock. This study was to determine effect and mechanisms of antibiotic treatment on lung defense mechanism in a mechanical ventilation (MV) model. C57BL/6 (WT) mice received ventilation

for 3 h at 6 days after intramuscular antibiotic treatment. The effects of dead *L. salivarius* and fructo-oligosaccharides (FOS) feeding on reactive oxygen species (ROS) of intestinal mucosa and *Pseudomonas aeruginosa* (PA) killing activity of alveolar macrophages (AMs) were examined. ROS scavenger, NAC, was used to investigate the involvement of intestinal ROS. Antibiotic treatment decreased ROS production of the intestinal mucosa and PA killing activity of AMs. Dead *L. salivarius* or fructo-oligosaccharides (FOS) feeding reversed the inhibitory effects of antibiotic on PA killing activity of AMs and protein expression of Reg3b as well as TLR4 in intestinal mucosa. Ablation of dead *L. salivarius* or fructo-oligosaccharides (FOS)-induced intestinal ROS with N-Acetylcysteine (NAC) decreased dihydrorhodamine (DHR) production and PA killing activity of AMs. Taken together with the inhibition of the stimulatory effect of FOS on lung defense mechanisms in MyD88 knockout (MyD88^{-/-}) mice, we concluded that antibiotic induces the inhibitory effect of lung defense mechanisms through the decrease of intestinal ROS and DHR production of AMs. Dead *L. salivarius* or fructo-oligosaccharides (FOS) feeding reverses antibiotic-induced lung defense impairment through intestinal TLR4/MyD88 pathway.

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Free neutrophil elastase activity in airways of young children with cystic fibrosis prior to, during and post-Pseudomonas infection

Garratt, L.¹, Kicic, A.^{1,2,3,4}, Ranganathan, S.^{5,6,7}, Stick, S.^{1,2,3,4}, on behalf of AREST CF

¹Telethon Kids Institute, Subiaco, Australia, ²University of Western Australia, School of Paediatric and Child Health, Nedlands, Australia, ³University of Western Australia, Centre for Cell Therapy and Regenerative Medicine, Nedlands, Australia, ⁴Princess Margaret Hospital for Children, Department of Respiratory Medicine, Subiaco, Australia, ⁵Royal Children's Hospital Melbourne, Department of Respiratory Medicine, Parkville, Australia, ⁶Murdoch Childrens Research Institute, The Royal Children's Hospital, Parkville, Australia, ⁷University of Melbourne, Department of Paediatrics, Parkville, Australia

Introduction: Early acquisition of *Pseudomonas aeruginosa* (Psa) by children with cystic fibrosis (CF) is associated with worse prognosis. Neutrophil elastase (NE) activity is also abnormally present in CF airways. We examined NE activity to investigate whether Psa eradication also successfully reduced NE activity and inflammation.

Methods: Bronchoalveolar lavage fluid (BALf) was obtained through the AREST CF longitudinal cohort study for standard clinical microbiology. Children positive for Psa underwent a program-specific eradication regimen. Additional BALf was collected three months later to confirm eradication. All BALf were assessed for inflammation including leukocyte counts, IL-8 and NE activity.

Results: Eighty-one episodes of Psa infection in 74 children (0.27-6.63 years) were assessed. Of those, free NE activity was detected in 46. Mean NE activity (137.80 nM ±385.60) was significantly higher than prior annual BALf (35.31 nM ±84.49, p=0.0444) and peers who never cultured Psa (33.58 nM ±86.93, p< 0.0001). After eradication therapy, Psa was still detected

in 12 cases. Free NE prevalence was lower in 26 patients post-eradication, however mean activity was not significantly different ($124.30 \text{ nM} \pm 614.70$, $p=0.0645$). At following annual assessment ($n=67$), prevalence ($n=23$, 34.33%) and mean activity ($60.60 \text{ nM} \pm 181.10$, $p=0.0334$) remained significantly lower than Psa infection. No significant changes were observed in IL-8 or neutrophil cell count.

Conclusion: Prevalence and activity of NE were associated with Psa infection that was not always resolved following eradication. The treatment of residual protease activity during Psa eradication regimens should also be investigated for potential to prevent additional lung damage.

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NKT cells mediate the recruitment of neutrophils by stimulating epithelial CXCL secretion during colitis

Huang, E.¹, Liu, R.¹, Lu, Z.¹, Liu, X.¹, Chu, Y.^{1,2}

¹Fudan University, Department of Immunology, Shanghai, China,

²Fudan University, Biotherapy Research Center, Shanghai, China

CD1d is a member of the major histocompatibility complex class I (MHC I)-like molecule family and has been proved to present glycolipid or glycerol lipid antigens to NKT cells. NKT cells are cells that express both NK cell markers and semi-invariant CD1d-restricted TCRs, and upon activation, can regulate immune responses via secreting a variety of cytokines. Ulcerative colitis (UC) is a kind of inflammatory bowel diseases characterized by inflammation and ulcer in colon, and its course is usually chronic and recurrent. UC patients have increased risk of getting colorectal cancer. In our research, we adopted the DSS-induced colitis mouse model, which is widely used to simulate UC, to investigate the role of CD1d and NKT cells in the progression of the disease. We found that in CD1d knocked-out (CD1dKO) mice, colitis was relieved. Further investigations revealed that when CD1d is knocked-out, colon epithelium express less chemokines that attract neutrophils (CXCL1, 2 and 3), and this may lead to fewer neutrophil infiltration. Infiltrated neutrophils also produce less reactive oxygen species (ROS) and TNF-alpha, this indicates they may cause less severe epithelial damage in CD1dKO mice. During colitis, NKT cells strongly express TNF-alpha, and TNF-alpha, can stimulate the expression of CXCL 1, 2, 3 by the epithelium. This can partially explain the mechanism of relieved neutrophil infiltration in CD1dKO mice with colitis.

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Regulatory role of IL-22-dependent microRNAs in homeostasis and inflammation in the intestinal epithelium

Sadio, M., Tourneur, E., Bens, M., Vandewalle, A., Chassin, C.
INSERM, U 1149, Paris, France

The intestinal mucosa is constantly exposed to microbes, and the mechanisms implicated in the control of tolerance by intestinal epithelial cells (IECs) to avoid an inappropriate activation of the inflammation are unclear. The stimulation of IECs by Interleukine 22 (IL-22) induces the production of antimicrobial peptides, the reinforcement of the mucus barrier

and of IECs regeneration. Several studies showed the protective role of IL-22 during pathologies such as inflammatory bowel diseases or infections, but the mechanisms implicated in IECs remain not clear. Our group identified a specific panel of small non coding microRNAs (miRs), key regulators of physiological and pathological processes, induced by IL-22 in IECs. The results show that the panel of miRs induced by IL-22 is able to modulate the inflammation by targeting important proteins involved in inflammatory signaling pathway, correlated with a modulation of inflammatory-induced cytokine and of the bacterial load. Results were confirmed in a mouse model of intestinal inflammation. We also showed that the TLR4 expression is modulated by the induction of a miRs specifically targeting TLR4 induced by IL-22 in IECs. This modulation can be reversed with the injection of an antagomiR in our mouse model of intestinal inflammation. This finding could explain the IL-22 induced mechanisms regulating the inflammatory signaling pathway in IECs and highlight the possible use of microRNAs as therapeutic tool to prevent the intestinal inflammation.

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Macrophages regulate steroid resistant airway inflammation in a mouse model of respiratory syncytial virus-induced asthma exacerbation

Nguyen, T.H.¹, Maltby, S.¹, Simpson, J.L.¹, Evers, F.¹, Gibson, P.G.², Foster, P.S.¹, Yang, M.¹

¹Priority Research Centre for Asthma and Respiratory Diseases, Department of Immunology and Microbiology, School of Biomedical Sciences & Pharmacy, Faculty of Health and Medicine and Hunter Medical Research Institute, University of Newcastle, Newcastle, Australia, ²Department of Respiratory and Sleep Medicine, Hunter New England Area Health Service, Newcastle, Australia

Background: About 5-10% of asthma patients with severe disease poorly respond to corticosteroid therapy and account for >50% of asthma-associated healthcare costs. Viral respiratory infections are one of the main factors responsible for exacerbations of asthma. However, the mechanisms how viral-induced asthma exacerbations are poorly understood.

Objectives: The aim of the study was to establish a mouse model of respiratory syncytial virus (RSV)-induced exacerbation of airway hyperresponsiveness (AHR) and airway inflammation to study the underlying mechanisms.

Methods: BALB/c mice were sensitised with OVA (or PBS control) in alum and later exposed to OVA aerosol to establish allergic airways disease. After the final OVA aerosol challenge, some groups were inoculated with either RSV or UV-inactivated RSV and lung function and airway inflammation were assessed.

Results: Infection of OVA-sensitised/challenged mice with RSV significantly increased AHR, macrophage and neutrophil lung infiltration. Exacerbations were accompanied by increased levels of inflammatory cytokines (including TNF α , MCP-1, and KC), compared to OVA-treated mice or OVA/UV-RSV-treated mice. Interestingly, we also observed increased TNF α levels in sputum samples from neutrophilic asthmatic patients. Dexamethasone treatment completely inhibited AHR and eosinophil infiltration in OVA-treated mice. Dexamethasone

treatment in OVA/RSV-treated mice partially suppressed AHR, but failed to dampen macrophage and neutrophil infiltration or inflammatory cytokine production. Furthermore, inhibition of TNF α , MCP-1 or macrophage depletion effectively suppressed features of disease, including AHR and macrophage infiltration.

Conclusions: Our findings highlight critical roles for macrophages and inflammatory cytokines (including TNF α and MCP-1) in viral-induced asthma exacerbations and suggest these as novel therapeutic pathways.

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Structural prediction of receptor binding to IgM and IgA

Agostino, M.^{1,2,3}, Mancera, R.¹, Fernandez-Recio, J.², Ramsland, P.^{3,4,5,6}

¹Curtin University, School of Biomedical Sciences, CHIRI Biosciences and Curtin Institute for Computation, Perth, Australia, ²Barcelona Supercomputing Centre, Life Sciences Department, Barcelona, Spain, ³Burnet Institute, Centre for Biomedical Research, Melbourne, Australia, ⁴RMIT, School of Science, Bundoora, Australia, ⁵University of Melbourne, Department of Surgery Austin Health, Heidelberg, Australia, ⁶Monash University, Department of Immunology, Melbourne, Australia

Receptor binding to the antibody Fc region is a crucial step in the immune response to pathogens, as well as facilitating antibody transport and protection. Although IgM and IgA occupy important niches in host defence, structural knowledge of receptor binding to these immunoglobulins is currently limited. In particular, the large size and flexibility of the IgM pentamer precludes the application of high resolution experimental techniques for structural investigation of protein binding.

In this work, computational molecular docking is applied to predict the structures of pIgR, the pIgR-related receptors Fc α / μ R and Fc γ R, and TRIM21 in complex with IgA and IgM. The docking strategy employed has been specifically optimized for use in predicting Fc-protein interactions. Further analysis of the top 20 ranked poses, using molecular dynamics simulations combined with molecular mechanics generalized Born/surface area calculations, reveals the most likely binding modes of pIgR and TRIM21 in complex with both IgA and IgM. The identified binding modes of pIgR with IgA and IgM are then used to infer the binding modes of Fc α / μ R, based on the fold similarity between the binding domains of these receptors. Structural insight into the selectivity of Fc γ R for IgM over IgA is also revealed.

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Differential cellular expression of the melatonin synthesizing enzyme, Arylalkylamine N-acetyltransferase (AANAT), in the mouse gut E-cadherin-immunolabeled resident enterocytes versus trafficking CD3⁺ and CD117⁺ immune cells

Fazal, N.¹, Al-Ghoul, W.², Royan, A.¹

¹Chicago State University, Pharmaceutical Sciences, Chicago, United States, ²Chicago State University, Biological Sciences, Chicago, United States

Our previous work has demonstrated anti-inflammatory

actions of melatonin in the gut alongside increased *de novo* endogenous melatonin synthesis in the gut in a mouse model of major thermal injury. Here, we investigated the distribution and dynamic changes of AANAT synthesis in the E-cadherin-immunolabeled resident enterocytes versus trafficking CD3⁺ and CD117⁺ immunocytes. Towards this end, AANAT levels were compared in E-cadherin, CD3, and CD117 immunopositive cells as assessed by flow cytometry and/or EasySep magnetic bead purification performed on ileum, colon, spleen, and mesenteric lymph node tissue preparations from control mice versus mice subjected to hot water scald (90°C for 10 seconds) over 20-25% total-body-surface on the back while under anesthesia in the presence or absence of melatonin treatment in accordance with IACUC approved protocols by Chicago State University. Our results suggest differential *de novo* gastrointestinal cell melatonin production and regulation in the resident E-cadherin positive mucosa cells versus and trafficking CD3⁺ and CD117⁺ cells depending on the tissue examined with highest levels being consistently in CD117⁺ cells from unmedicated injury group.

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Lypd8 maintains gut homeostasis by segregating flagellated bacteria and colonic epithelia

Okumura, R., Takeda, K.

Osaka University Graduate School of Medicine, Department of Microbiology and Immunology, Suita, Japan

The intestinal mucosa is protected from commensal and pathogenic microorganisms by several types of barriers. In the large intestine where tremendous numbers of microbiota exist, epithelial cells are covered by thick mucus composed of two layers, the inner firm mucus layer and outer loose mucus layer. Many commensal bacteria are present in the outer mucus layer, whereas none is present in the inner mucus layer. However, the precise mechanisms for the segregation of bacteria and colonic epithelia by the inner mucus layer remain unclear. We found a molecule named Ly6/PLAUR domain containing 8 (Lydp8), which is a highly glycosylated GPI-anchored protein, is highly and selectively expressed in epithelial cells on the uppermost layer of the colonic gland and shed into the intestinal lumen. Bacterial free space in the inner mucus layer disappeared and many flagellated bacteria such as *Escherichia*, *Helicobacter* and *Proteus* invaded colonic epithelia in *Lydp8*^{-/-} mice. In addition, *Lydp8*^{-/-} mice were highly sensitive to dextran sulfate sodium (DSS)-induced colitis. Elimination of intestinal Gram-negative flagellated bacteria by the oral treatment of gentamicin restored colonic bacterial free space and ameliorated DSS colitis in *Lydp8*^{-/-} mice. Lydp8 preferentially bound to flagellated bacteria in the colon. In vitro analysis showed that Lydp8 bound to the flagella of *Proteus mirabilis* and *Escherichia coli*, thereby suppressing their swarming motility. These findings demonstrate that Lydp8 is essential for segregation of flagellated bacteria and colonic epithelial cells through the binding to bacterial flagella and subsequent suppression of their motility.

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Influence of commensal microbiota and dietary antigens on the development and function of antigen presenting cells in intestinal homeostasis

Ko, S.H.-J.^{1,2}, Kim, K.S.^{1,2}, Hong, S.-W.^{1,2}, Jung, J.^{1,2}, Lee, M.J.^{1,2}, Surh, C.D.^{1,2,3}

¹Institute for Basic Science, Academy of Immunology and Microbiology, Pohang-si, Korea, Republic of, ²POSTECH (Pohang University of Science and Technology), Department of Integrative Biosciences and Biotechnology, Pohang-si, Korea, Republic of, ³La Jolla Institute for Allergy & Immunology, Division of Developmental Immunology, La Jolla, United States

Antigen presenting cells (APCs), such as dendritic cells (DCs) and macrophages, are the key immune cells that regulate function of lymphoid populations of immune cells by delivering self or foreign antigens in either steady state or inflammatory state. Intestinal APCs acquire the distinct roles by its constant exposure to commensal microbiota, pathogens, and food antigens. However, it is not elucidated yet how commensal microbiota and dietary antigens influence the development of intestinal APCs and how these cells are involved in establishing and maintaining intestinal homeostasis. To investigate this, we established the unique system that experimental mice are free from commensal microbiota (germ-free (GF mice)) and free from solid food diet (antigen-free (AF)). We examined the phenotypic differences of intestinal APCs from SPF, GF, and AF mice. We found that the number of CD103⁺DCs from AF mice is significantly lower than those from SPF and GF mice while the number of CD103⁺DCs is comparable between SPF and GF mice. When we further distinguished CD103⁺DCs with CD11b, there was more CD103⁺CD11b⁺DCs in AF mice that is similar with those in neonatal GF and SPF mice, indicating that food antigens may be involved in DC differentiation. We speculate that the components in the diet affect immune system without being triggering the Ag-specific receptors of the adaptive immune system. Defining the mechanisms how commensal microbiota and/or dietary antigens influence such a key immune cells in the gut will insight into the understanding of intestinal immunity.

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Can aerosolised bacteriophage preparations against *Pseudomonas aeruginosa* protect against endotoxin-mediated inflammation in the lungs of people with cystic fibrosis?

Trend, S.^{1,2}, Chang, B.³, Kicic, A.^{1,2,4,5}, Stick, S.M.^{1,2,4,5}

¹Telethon Kids Institute, Centre for Health Research, Perth, Australia, ²University of Western Australia, School of Paediatrics and Child Health, Perth, Australia, ³University of Western Australia, School of Pathology and Laboratory Medicine, Perth, Australia, ⁴Princess Margaret Hospital for Children, Department of Respiratory Medicine, Perth, Australia, ⁵University of Western Australia, Centre for Cell Therapy and Regenerative Medicine, School of Medicine and Pharmacology, Perth, Australia

Cystic fibrosis (CF) is a heritable disease caused by mutations to the CFTR gene. Chronic lung infections with bacterial pathogens including *Pseudomonas aeruginosa* are very common in CF,

and cause inflammation and lung destruction that contribute to early mortality. Current therapies for these lung infections include extensive intravenous or inhaled antibiotics, which are burdensome, and do not always eradicate infection. Bacteriophages are viruses that infect bacteria killing their host through replication. Endotoxin-binding bacteriophages are of particular interest to treat infections in CF since they have been shown to exhibit prophylactic anti-inflammatory properties *in vitro* by the reducing the production of reactive oxygen species (Miedzybrodzki *et al.* 2008). We hypothesise that an endotoxin-binding bacteriophage under investigation in our laboratory will have both anti-bacterial and anti-inflammatory properties in CF epithelial cells through hindrance of the interaction of endotoxin with TLR-4. Preliminary results indicate that an endotoxin-binding bacteriophage has broad antibacterial activity against *P. aeruginosa* isolates from children with CF (19 of 21 isolates susceptible). We will use dose-controlled deposition of a bacteriophage preparation on CF primary airway epithelial cells cultured at the air-liquid interface \pm endotoxin to test this hypothesis *in vitro*. RNA will be extracted from stimulated and control cells for analysis of gene expression changes in inflammatory pathways downstream of TLR-4 activation (NF-kappaB, MAPK/AP-1; IL-1beta, IL-6, IL-8, TNF-alpha). This research will explore the potential of bacteriophages to have anti-inflammatory effects in the host human during lung infections in CF using a clinically relevant model, and will inform our future research.

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Dietary N- and O-glycans from cow milk and TLR modulation

Figueroa, S.^{1,2}, Dijkhuizen, L.², Valk, R.², van Leeuwen, S.², de Vos, P.¹

¹University Medical Center Groningen, Pathology and Medical Biology, Groningen, Netherlands, ²University of Groningen, Microbial Physiology, Groningen, Netherlands

Diseases such as type 1 diabetes, food allergies or atopic disorders have been linked with the precocious contact of food components such as glycoproteins from cow milk with the immature intestinal mucosa. The exact mechanism by which food components contribute to food allergy or induce tolerance is still not completely clear. The immune tolerance is lost in MyD88-knockout mice, which made us hypothesize that some food components might signal via TLR-dependent pathways.

In this study cow milk preparations were enzymatically modified to N- and O-glycans. These preparations were exposed to reporter cell lines expressing TLR-receptors. We compared the effect of modified glycans against their intact counterparts and against the original intact glycoprotein. Unlike the original molecule, modified fractions showed an average 3-fold ($p < 0.05$) change in its inhibitory effects on TLR-4. At the same time, the original molecule displayed stimulatory activity on the same receptor. Among modified glycan fractions i.e. glycans with and without sialic acid units, we observed a 2-fold ($p < 0.05$) inhibitory effect on TLR-9.

Our findings suggest a function-structure relationship influence on the inhibition of the signaling cascade for the production of NF-KB mediated by TLR.

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Live but not heat-inactivated *Aquaphilus dolomiae*, a bacteria from aquifer water, inhibit inflammation and ameliorate TNBS-induced colitis in IBD rat's model

Nguyen, T.¹, Helffer, K.¹, Maître, M.¹, Rousset, P.², Vergnole, N.², Libon, C.¹

¹Institut de Recherche Pierre Fabre, Biotechnology, Toulouse, France, ²INSERM U 1220, CHU Purpan, Toulouse, France

Recently, numerous publications have strongly supported the crucial role of Microbiota in immunomodulation of both innate and adaptive mucosal immunity. Many probiotics have showed beneficial effects in immune homeostasis and contributed to human health by complex regulations pathways. *Aquaphilus dolomiae* (ADLM), a non-pathogenic beta-proteobacteria, isolated from a thermal spring water (South of France), was evaluated in inflammatory bowel disease (IBD) experimental model.

Methods: Different doses of live or heat-killed ADLM were daily administered per os for 7 days to Wistar rats prior colitis induction by rectal injection of TNBS, and compared to vehicle controls and to reference drugs (Prednisolone and Remicade™). Development of colitis was assessed daily by checking body weight change, stool consistency and detection of blood in stools. Seven days after TNBS administration, rats were sacrificed. The whole colon was excised to determine macroscopic and microscopic damages and then cut into pieces for myeloperoxidase activity (MPO), proteins and cytokines profile quantification, and histological analysis.

Results: Live ADLM dose-dependently (109 > 108 bacteria/day/rat) protected rats from TNBS colitis. This effect was correlated with reduced inflammation as assessed by MPO activity and inflammatory cytokines (IL-1beta, IL-1RA, MIP-1alpha, IL-6, GRO/KC and TNF-alpha). These effects were at least comparable or better as compared to Prednisolone and Remicade™ treatments. In contrast, heat-inactivated ADLM were less protective, and most of the inflammatory parameters were not modified.

Conclusion: Oral administration of live ADLM is able to prevent colitis development and reduces inflammation. Thus, this probiotic therapy strategy is very promising to alleviate human IBD.

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Long lasting effect of mucosal topical applied microbicides on the subsequent rectal SIV infection and survival by regulating SIV-specific T cell immune responses

Ren, Y., Wan, Y., Wang, J., Xu, J., Zhang, X.

Fudan University, Shanghai Public Health Clinical Center and Institutes of Biomedical Sciences, Shanghai, China

Most previous studies of candidate microbicides focused on the efficacy for the prevention from HIV/SIV infection, few on their potential influences on mucosal immune system and later on the disease progression during subsequent SIV infection. We designed a two-phase study: to mimic microbicide efficacy studies and determine the disease progression in a productive infection model. During the first phase, monkeys were intrarectally pre-treated with tenofovir (TFV), sifuvirtide (SFT) or maroviroc (MVC) formulated microbicides and then

challenged with low-dose SHIV-1157ipd3N4. Secondly, all monkeys were re-challenged with a single high dose SIVmac239 to generate productive infections. The survival rate, viral loads, CD4+ T-cell counts and SIV-specific T-cell responses were determined during the 104-week following up. Repeated rectal challenges resulted in infection in all groups, evidenced by the recurrent viral blips during the first phase of this study. All monkeys were productively infected after the high-dose re-challenge with SIVmac239. Two groups, including MVC- and TFV-treated groups, experienced 100% death during the 104-week following up; In contrast, SFT-treated group survived significantly better ($p=0.0169$ for SFT vs TFV, and $p=0.0067$ for SFT vs MVC), and only 25% (one monkey) dies at week 95. Interestingly, SIV-specific T cell responses were also significantly higher in SFT group comparing to MVC and TFV groups. This study informs a preliminary but important observation on the influence of previously applied microbicides on the disease progression of subsequent SIV infection, and suggests that the long-term immune safety concern for microbicides should be also considered in the effort to develop effective microbicides.

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MAIT cell activation and accumulation after *in vivo* infection depends on microbial riboflavin synthesis and co-stimulatory signals

Chen, Z.¹, Wang, H.¹, D'Souza, C.¹, Sun, S.¹, Kostenko, L.¹, Eckle, S.¹, Meehan, B.¹, Jackson, D.¹, Strugnelli, R.¹, Cao, H.¹, Wang, N.¹, Fairlie, D.², Liu, L.², Godfrey, D.¹, Rossjohn, J.³, McCluskey, J.¹, Corbett, A.¹

¹University of Melbourne, Melbourne, Australia, ²University of Queensland, Brisbane, Australia, ³Monash University, Melbourne, Australia

Despite recent breakthroughs in identifying MAIT cell antigens, the precise requirements for *in vivo* MAIT responses to infection remain unclear. Using MR1 tetramers, the MAIT cell response was investigated in a model of bacterial lung infection employing *rib* gene-competent and deficient bacteria. MAIT cells were rapidly enriched in the lungs of C57BL/6 mice infected with *Salmonella* Typhimurium, comprising up to 50% of ab-T cells after one week. MAIT cell accumulation was MR1-dependent, required antigen derived from the microbial riboflavin synthesis pathway and did not occur in response to synthetic antigen, unless accompanied by a TLR2/6 agonist or by co-infection with riboflavin pathway-deficient *S. Typhimurium*. MAIT cell accumulation involved proliferation in the lungs and regional LN, and was associated with their long-term retention in the lungs. Lung MAIT cells from infected mice were mainly CD4⁺ CD8⁻ double negative (DN) or CD8⁺, displayed an activated/memory phenotype, and most expressed the transcription factor ROR γ t. T-bet expression increased following infection. The majority produced IL-17 while smaller subsets produced IFN γ or TNF, detected directly *ex vivo*. Thus, the activation and expansion of MAIT cells coupled with their pro-inflammatory cytokine production, occurred in response to antigens derived from microbial riboflavin synthesis and was augmented by co-stimulatory signals.

Reproductive Immunology

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Transforming Growth Factor $\beta 1$ (TGF- $\beta 1$) is increased in the sera of postmenopausal osteoporotic women: a case-control study

Samsami-Dehaghani, A.¹, Faraji, A.², Ghaderi, A.³

¹Shiraz University of Medical Sciences, Ob/Gyn, Shiraz, Iran, Islamic Republic of, ²Sadoughi University of Medical Sciences, Ob/Gyn, Shiraz, Iran, Islamic Republic of, ³Shiraz University of Medical Sciences, Shiraz Institute for Cancer Research, Shiraz, Iran, Islamic Republic of

Objectives: TGF- $\beta 1$ and IL-18 play complex roles in normal bone metabolism, and in pathophysiology of postmenopausal osteoporosis. A case-control study was conducted in order to further clarify the role of TGF- $\beta 1$ and IL-18 in osteoporosis of post-menopausal women.

Materials and methods: 65 postmenopausal women with osteoporosis (T score < -2.5 in the lumbar spine or femoral neck) and 69 postmenopausal normal women (T score \geq -1) were enrolled. Serum levels of TGF- $\beta 1$ and IL-18 were measured in both groups.

Results: Serum TGF- $\beta 1$ levels were significantly higher in osteoporotic postmenopausal women than non-osteoporotic individuals (23.80 vs. 15.77 ng/ml; p value=0.009). There was no difference between IL-18 levels in the sera of osteoporotic and non-osteoporotic post menopausal women in this study. There was a positive correlation between body mass index (BMI) and serum level of TGF- $\beta 1$ (p value=0.04).

Conclusion: Aberrant increase in TGF- $\beta 1$ serum levels in postmenopausal ages can result in uncoupled bone resorption and formation, leading to osteoporosis. Screening the levels of TGF- $\beta 1$ in the sera of postmenopausal women can have a predictive value for osteoporosis.

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Immunological modes of pregnancy loss: similarity in hepatitis E infected and non-infected pregnancy cases

Bose, P.D.¹, Tiwari, D.², Bose, M.², Sultana, R.², Begum, A.², Datta, R.³, Das, C.R.⁴, Bose, S.²

¹Cotton College State University, Molecular Biology & Biotechnology, Guwahati, India, ²Gauhati University, Biotechnology, Guwahati, India, ³Gauhati University, Guwahati, India, ⁴Guwahati Medical College, Guwahati, India

We aimed to evaluate the role of alterations in progesterone receptor pathway and altered cytokine profile in deciding the outcome of Hepatitis E virus(HEV) infected pregnancies.

Methods: Blood, placental tissue samples were collected from pregnant cases [term-delivery-cases(TC=30),preterm-cases(TC=50)] and HEV infected PC[HEV-PC,n=22]. Progesterone receptor(PR) polymorphism(PROGINS) was studied by PCR. Differential placental PR expression was studied by IHC. Differential mRNA profile of PR and PIBF expression was analyzed by RT-PCR. Differential cytokine expression at protein and mRNA level was studied by multiplex ELISA (TNF α ,IFN γ ,IL12,IL10,IL6,IL2,IL4,IL8) and RT-PCR, and correlated with PR expression and pregnancy outcome.

Results: Prevalance of PROGINS polymorphism was higher in PC(p=0.216),HEV-PC(p=0.342) compared to TC. PR- expression was down-regulated in both PC and HEV-PC compared to TC;and in IUD/fetal-death PC compared to PC with successful pregnancy outcome. PIBF expression was also down-regulated in both HEV-PC and PC; and PC with negative pregnancy outcome cases compared to TC, and PC with successful outcome respectively. Placental NF κ B expression was significantly higher in PC(0.348 \pm 0.126 μ g/ml)(p=0.032) and HEV-PC (0.329 \pm 0.182 μ g/ml)(p=0.047) compared to term cases(0.289 \pm 0.169 μ g/ml). Cytokine profile clearly indicated significant changes in inflammatory cytokines TNF α ,IL6 and IL8 in both HEV-PC and PC, and correlated with pregnancy outcome; while IFN γ expression was also significantly higher in HEV-PC compared to TC(p< 0.001) and PC(p=0.004). NF κ Bp65,TNF α placental expression was higher in HEV-PC and PC compared to TC,and positively correlated with pregnancy outcome. TNF α mRNA expression significantly negatively correlated with PR-mRNA expression.

Conclusion: Alteration in PR pathway and resulting altered Th1-modulation is detrimental to both HEV-PC and PC; and thus have both prognostic and therapeutic importance.

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Regulatory T cell abundance and phenotype in pregnancy - a novel role for progesterone potentially independent of nuclear progesterone receptor

Green, E.S.¹, Moldenhauer, L.M.¹, Kara, E.E.², Chin, P.Y.¹, Robker, R.L.¹, McColl, S.R.², Robertson, S.A.¹

¹Robinson Research Institute, University of Adelaide, Discipline of Obstetrics & Gynaecology, Adelaide, Australia, ²University of Adelaide, Department of Molecular & Cellular Biology, Adelaide, Australia

Fetal-maternal tolerance is primarily mediated by maternal CD4⁺Foxp3⁺ regulatory T (Treg) cells, with both thymus-derived (tTreg) and peripherally derived (pTreg) cells contributing. Absence or reduced function of Treg cells at embryo implantation causes infertility in mice and is implicated as a cause of reproductive disorders in women. The importance of adequate Treg cell responses during pregnancy is well recognised, however, the factors which control the strength and quality of this response are not defined. Progesterone (P4) has potent immunosuppressive actions. We previously demonstrated that administration of ovarian steroid hormones estrogen and P4 to female ovariectomised mice increases Treg cell numbers in vivo. To further investigate the effects of P4 on Treg cells, mated female mice were administered low doses of the progesterone antagonist RU486 in the peri-implantation period. Flow cytometry analyses showed RU486 treatment resulted in decreased proportions of total Treg cells and increased proportions of IFN γ -producing tTreg and pTreg cells in the uterus-draining para-aortic lymph nodes. In vitro, P4 repressed IFN γ expression in Treg and T effector cells cultured under Th1-, Th17- and non-polarising conditions. Treg cells from mice with a null mutation in the nuclear progesterone receptor (PR) also responded to P4 with attenuated IFN γ production, indicating the P4 effect was not mediated by the nuclear PR and suggesting a non-classical mechanism for progesterone action

on Treg cell phenotype. Collectively, this work demonstrates that P4 can regulate Treg cell abundance and cytokine production, which may be important in the establishment and maintenance of competent maternal tolerance during pregnancy.

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Immune suppression by human semen is mediated by soluble CD52

Harrison, L.C.^{1,2}, Stone, N.L.^{1,2}, Bandala-Sanchez, E.^{1,2}, McLachlan, R.I.³, Merriner, D.J.³, O'Bryan, M.K.³

¹Walter and Eliza Hall Institute of Medical Research, Population Health and Immunity Division, Parkville, Australia, ²The University of Melbourne Victoria 3010, Australia., Medical Biology, Parkville, Australia, ³Monash University, Anatomy and Developmental Biology, Clayton, Australia

Male seminal vesicular fluid or semen not only provides for the carriage and nutritional maintenance of sperm but has immunomodulatory properties that protect against allojection of sperm. Immune modulation by semen has been attributed to several of its components, namely prostaglandins, spermine and transforming growth factor-beta, and to induction of regulatory T cells in the female, but whether these fully account for suppression of lymphocyte activation-proliferation by semen is unclear.

A major 'antigen' in semen, the target of anti-sperm antibodies in some infertile women, is CD52, secreted by the epithelial cells of the distal epididymis and vas deferens, which becomes incorporated into the sperm cell membrane. CD52 is also present as a small GPI-anchored glycoprotein on lymphoid cells. We reported that CD52 is released by activated T cells and suppresses T cells by binding of its glycan to the inhibitory receptor Siglec-10 (1).

Here we show that human semen contains high concentrations of soluble CD52, blockade or depletion of which abrogates suppression of T or B cell function by semen. Blocking by antibodies or by recombinant soluble Siglec-Fc proteins revealed that semen CD52 bound to Siglec-7, which was expressed predominantly by Sertoli cells in the testis. Ligation of Siglec-7 inhibits natural killer cells in the female, which are known to target sperm. In conclusion, the suppression of lymphocyte function by human semen can be accounted for by the Siglec ligand, CD52.

1) Bandala-Sanchez et al (2013). *Nat Immunol* 14: 741-8.

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HLA-G, KIR2DL4 and LILRB1 gene polymorphisms in spontaneous abortion in Poles

Nowak, I.¹, Malinowski, A.², Barcz, E.³, Wilczyński, J.R.⁴, Wagner, M.¹, Majorczyk, E.^{1,5}, Motak-Pochrzęst, H.⁶, Kuśnierczyk, P.⁷

¹Ludwik Hirszfild Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Laboratory of Immunogenetics and Tissue Immunology, Wrocław, Poland,

²Mothers' Memorial Hospital - Research Institute, Surgical, Endoscopic and Oncologic Gynecology, Łódź, Poland, ³Medical University of Warsaw, Poland, First Chair and Clinic of Obstetrics and Gynecology, Warsaw, Poland, ⁴Mothers' Memorial Hospital -

Research Institute, Department of Gynecology and Gynecologic Oncology, Łódź, Poland, ⁵Opole University of Technology, Faculty of Physical Education and Physiotherapy, Opole, Poland, ⁶District Hospital, Strzelce Opolskie, Poland, ⁷Ludwik Hirszfild Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Laboratory of Immunogenetics and Tissue Immunology, Wrocław, Poland

The KIR2DL4 receptor and its ligand HLA-G are considered important for fetal-maternal immune tolerance and successful pregnancy. The absence of a particular variant of KIR2DL4 might be a bad prognostic factor for pregnancy outcome. However, it could be compensated by the presence of the respective LILRB1 allele. Therefore, we investigated the *KIR2DL4*, *LILRB1* and *HLA-G* polymorphisms in 277 couples with spontaneous abortion and 219 control couples by HRM, PCR-SSP and RFLP methods. We found a protective effect of women's heterozygosity in -716 T>G *HLA-G* ($p = 0.0206$) and 5651 G>A *LILRB1* ($p = 0.0131$) against spontaneous abortion. Surprisingly, we observed higher frequency of 9A/10A heterozygotes of *KIR2DL4* gene in the group of male partners from the miscarriage group in comparison to the men from the control group ($p = 0.0288$). Furthermore, there was no association of women's *KIR2DL4* polymorphism with susceptibility to spontaneous abortion. Multivariate analysis indicated that female *HLA-G* -716 T>G and *LILRB1* 5651 G>A and male *KIR2DL4* 9A/10A are important in terms of the protection or susceptibility to miscarriage, respectively ($p = 0.00968$).

In conclusion, a woman's heterozygosity in *HLA-G* and *LILRB1* might be advantageous for a success of reproduction, but the partner's heterozygosity in 9A/10A *KIR2DL4* alleles might be a rather bad predictor.

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Up-regulation of autophagy by sera from women with preeclampsia is associated with pro-inflammatory cytokines

Matias, M.L.¹, Peracoli, M.T.¹, Kanninen, T.², Sisti, G.², Romão-Veiga, M.¹, Peracoli, J.C.¹, Bongiovanni, A.M.², Witkin, S.²

¹UNESP, Botucatu, Brazil, ²Weill Cornell Medical College, New York, United States

Preeclampsia (PE) is a clinical complication of pregnancy associated with elevated levels of inflammatory cytokines and uric acid. Autophagy, an intracellular catabolic process, seems to maintain the cellular homeostasis and can control the inflammasomes activation. Monocytes stimulation with monosodium urate (MSU) demonstrates that uric acid plays a role in inflammasome activation. This study compared the ability of sera from pregnant women with or without PE to induce autophagy in peripheral blood mononuclear cells (PBMC) from healthy non-pregnant women. PBMC from 20 non-pregnant women were incubated with sera from 45 pregnant women, being 23 normotensive (NT) and 22 with PE in the presence or absence of the autophagy inducer, rapamycin. Autophagy induction was evaluated by the expression of p62 by RT-qPCR and ELISA. Higher p62 gene expression was observed in PBMCs

cultured with PE and NT sera or MSU compared to the control cultures. Moreover, gene expression of p62 is increased in PBMCs cultured with PE sera than in culture stimulated with NT sera. Protein levels of p62 in PBMC cultured without rapamycin and treated with sera from NT group were significantly higher when compared to cultures with PE sera. Sera of preeclamptic women showed association between autophagy induction and higher concentrations of IL-1 β and TNF- α . Association between autophagy induction and higher levels of inflammatory cytokines in sera from patients with PE suggests that the enhanced pro-inflammatory state characteristic of PE may result in the up-regulation of autophagy induction in these women.

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Immunoregulation of the decidualization program

Grasso, E.¹, Gallino, L.¹, Soczewski, E.¹, Gori, S.², Salamone, G.², Pérez-Leirós, C.¹, Ramhorst, R.¹

¹University of Buenos Aires, School of Sciences, CONICET, Immunopharmacology Lab. IQUBICEN-CONICET, Buenos Aires, Argentina, ²National Academy of Sciences, IMEX, Buenos Aires, Argentina

Decidualization process involves phenotype and functional changes on endometrial cells and the modulation of different mediators such as cytokines, chemokines and the selective recruitment of immune cells. Particularly, the vasoactive intestinal peptide (VIP) is a neuropeptide produced by endometrial stromal cells that displays multiple target circuits allowing immunetolerance.

To investigate VIP contribution to the decidualization program, we used an in vitro implantation model based in the co-culture of blastocyst-like spheroids (BLS) from trophoblast cells cultured on Human endometrial stromal cell line monolayer (HESC) decidualized with or with medroxyprogesterone and dbcAMP (+control).

Decidualization increased VIP expression and secretion on HESC cells ($p < 0.05$ ANOVA). The decidualization induced by VIP increased differentiation markers as IGFBP1, PRL, KLF13/KLF9 ratio

($p < 0.05$, ANOVA). When BLS from first trimester trophoblast cells (Swan71 cell line) were cultured on HESC monolayer, BLS were able to invade HESC decidualized with VIP or MPA+dbcAMP. In fact, the condition media from developmentally impaired human blastocyst decrease the invasion of on HESC cells decidualized under both treatments. Decidualized HESC CM induced a semi-mature profile on DC preventing CD83 and CD86 induction and increasing IL-10 secretion ($p < 0.05$ Student Ttest), while did not modulate monocytes activation profile. Decidualized cells selective recruit Foxp3+ cells, among monocytes and effector Tcells, associated with and increase CXCL12 expression.

In conclusion, VIP may have an important role during decidualization by allowing BLS invasion contributing to control the immune micro-environment associated with the condition of DC to a tolerogenic profile and to the selective recruitment of maternal Tregs to the uterus.

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The influence of viral infection on the innate immune system during pregnancy

Moore, S.^{1,2}, Garcia Valtanen, P.¹, Robertson, S.², Hayball, J.¹, Diener, K.^{1,2}

¹University of South Australia, Experimental Therapeutics Laboratory, Hanson Institute & Sansom Institute, School of Pharmacy and Medical Science, Adelaide, Australia, ²University of Adelaide, Robinson Research Institute, School of Medicine, Adelaide, Australia

Successful pregnancy is dependent on the establishment of maternal immune tolerance which is predominantly facilitated by the adaptive arm of the immune system. The presence of infection has however the potential to alter pregnancy outcomes, which can be attributed to alterations in tolerance induction. The innate immune system is the first defence against pathogenic invasion and is often considered to educate subsequent adaptive immune responses; however viral-induced changes to the activation and function of innate immune cells during maternal infection and how this may affect reproductive outcomes has largely remained unexplored. Recent evidence suggests that the innate immune system possesses a degree of 'memory' in response to infection, and may therefore be detrimental to pregnancy upon challenge with an unrelated secondary infection. In this study, mated female mice received an infection with murine cytomegalovirus (MCMV) prior to embryo implantation and subsequently challenged with lipopolysaccharide (LPS) later during gestation to assess whether prior innate immune stimulation alters the response to a secondary challenge and what effect this may have on fetal rejection. This was combined with a novel mouse triply-deficient in the innate immune molecules (TLR7, TLR9 and ASC) necessary for MCMV control, which we show results in abrogated natural killer (NK) cell activity. We propose that NK cells alongside monocytes hold the key to differences in pregnancy outcomes between primary and secondary innate immune recognition. These results demonstrate for the first time the innate immune system obtaining memory of infectious challenge and how this may impact on reproductive outcomes.

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Association of IL-17A and IL-17 F gene polymorphisms with recurrent pregnancy loss in Iranian women

Najafi, S.¹, Hadinedoushan, H.², Eslami, G.²

¹Tehran University of Medical Sciences, School of Public Health, Dept. of Pathobiology, Tehran, Iran, Islamic Republic of, ²Yazd University of Medical Sciences, Yazd, Iran, Islamic Republic of

Purpose: Recurrent pregnancy loss (RPL) is defined as the occurrence of two or more miscarriages before the 20th week of pregnancy. T helper17 cells are a novel subset of T cells, which secrete IL (Interleukin)-17 and are known to be involved in inflammation, autoimmunity and rejection of nonself tissues. Herein, we studied the association between IL-17A rs2275913 and IL-17F rs763780 gene polymorphisms with RPL in Iranian women.

Methods: A case-controlled study was performed on two groups

consisting of 85 healthy women with at least one delivery and 85 women with the history of two or more RPLs. The frequency of IL-17A rs2275913 and IL-17 F rs763780 polymorphisms were determined by PCR-RFLP.

Results: In the RPL group, the genotypes frequencies of rs2275913 polymorphism were GG (8.2 %), AG (30.6 %), and AA (61.2 %) and in the control group, were GG (3.5 %), AG (42.4 %) and AA (54.1 %). Statistical analysis showed no significant difference between the genotypes of AA, AG and GG in the two groups ($p=0.1$). The genotypes frequencies of rs763780 polymorphism were TT (43.5 %), TC (49.4 %) and CC (7.1 %) in the RPL group; whereas the frequencies were TT (25.9 %), TC (70.6 %) and CC (3.5 %) in the control group. Statistical analysis revealed a significant difference in the TT, TC, and CC genotypes frequencies between the case and the control groups ($p=0.01$).

Conclusions: Our findings indicate that IL-17F polymorphism, rs763780, might be associated with a high risk of RPL in Iranian women.

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Short and long term effects of pregnancy on memory T cells

Kieffer, T.E.C.¹, Faas, M.M.², Scherjon, S.A.¹, Prins, J.R.¹

¹University Medical Center Groningen, University of Groningen, Department of Obstetrics and Gynecology, Groningen, Netherlands, ²University Medical Center Groningen, University of Groningen, Division of Medical Biology, Department of Pathology and Medical Biology, Groningen, Netherlands

Non-optimal induction of immune tolerance towards paternal antigens during pregnancy is associated with pregnancy complications. Studies have shown that the maternal immune system memorizes paternal antigens and with that possibly reduces the risk of pregnancy complications. This study aims to analyze the short and long term effects of pregnancy on memory T-lymphocytes. Effector memory (EM) and central memory (CM) T-lymphocytes and their activation status were analyzed using flow cytometry in peripheral blood of 14 women that had never been pregnant (nulligravid), 12 women that were pregnant for the first time (primigravid), and 15 women with an uncomplicated pregnancy that were at least 6 months post partum (parous). Populations were compared using one-way ANOVA and Tukey's post hoc test; $p < 0.05$. The short term effects were shown by the significantly higher CD4+ EM cell ($p < 0.05$) and activated CD4+ memory cell ($p < 0.001$) proportions in primigravid women compared to nulligravid women. The long term effects found in this study were the significantly higher proportions of CD4+ EM ($p < 0.05$), CD4+ CM ($p < 0.001$) and activated memory T cells ($p < 0.05$) in parous women compared to nulligravid women. In contrast to CD4+ cells, activation status of CD8+ memory cells did not differ between the groups. Possibly, a shift from CD4+ CM to EM cells occurs during pregnancy followed by an increase of both subsets postpartum. These findings support the hypothesis that memory cells are involved in memorizing paternal antigens.

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The improvement of abnormal pregnancy by IL-10 treatment in *T. gondii*-infected mice

Hu, X.¹, Zhang, H.², Zhao, M.³, Liu, X.², Jiang, Y.²

¹Binzhou Medical University, Department of Immunology, Binzhou, China, ²Binzhou Medical University, Department of Immunology, Yantai, China, ³Binzhou Medical University, Department of Medicine & Pharmacy Research Center, Yantai, China

Aims: To investigate the role of IL-10 on abnormal pregnancy outcomes following *T. gondii* infection.

Methods: C57BL/6 pregnant mice were randomly divided into control group, infected group and rIL-10-treated group. The infected group and rIL-10-treated group were infected with 400 *T. gondii* on gestational day (gd) 7. On gd 6 and 8, rIL-10-treated group was administrated with 1 μ g rIL-10 via a tail vein injection. The mice were sacrificed on gd 12 and their pregnancy outcomes were observed. The expression of NKG2A, an NK cell inhibiting receptor, and NKG2D, an NK cell activating receptor, on NK cells were analyzed by flow cytometry; the level of IFN- γ inside NK cells as well as the total level of IFN- γ in placental tissue extract was measured by flow cytometry and ELISA respectively.

Results: rIL-10-treated group showed significantly alleviated adverse pregnancy outcomes compared with infected group. The expression of NKG2D on NK cells was up-regulated in infected group compared to controls, but was down-regulated in rIL-10-treated group compared to infected group. Conversely, NKG2A was elevated in the infected group, and further increased in rIL-10-treated group. The IFN- γ level inside NK cells as well as in placental tissue extract was up-regulated in infected group compared to controls, but were down-regulated in rIL-10-treated group compared to infected group.

Conclusion: IL-10 may play an immunoprotective role in abnormal pregnancy following *T. gondii* infection through regulating the function of decidual NK cells.

Keywords: IL-10, decidual NK cells, NKG2D, NKG2A, abnormal pregnancy outcomes, *Toxoplasma gondii*.

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Critical differences in the fc receptors of humans and non-human primates with implications for the analysis of antibody effector functions *in vivo*

Chenoweth, A.M.^{1,2}, Trist, H.¹, Tan, P.S.¹, Esparon, S.¹, Anania, J.C.^{1,2}, Munro, J.¹, Wines, B.^{1,2,3}, Hogarth, P.M.^{1,2,3}

¹Burnet Institute, Centre for Biomedical Research, Melbourne, Australia, ²Monash University, Department of Immunology, Melbourne, Australia, ³University of Melbourne, Department of Pathology, Melbourne, Australia

The spectacular success of monoclonal antibodies as therapeutic modalities and of vaccines in preventative medicine, together with the increasing emphasis on translational research, have focused attention on the drive for new therapeutic targets, understanding of the human immune system, and development of appropriate animal models of human immunity, including non-human primates (NHP).

Antibodies, and their Fc receptor (FcR)-based effector functions, are major contributors to the effectiveness of therapeutic antibodies, vaccines, and the regulation of immunity. However, despite the use of NHP as models of human immunity, little is known of their FcR genetics, function, and interaction with immunoglobulins.

We have undertaken a comparative analysis of the interaction of eight high- and low-affinity macaque and human FcγRs with IgG subclasses. We discovered marked differences between macaque FcγR and their human counterparts in their specificity for, and interaction with, human IgG subclasses. Moreover, FcγR sequence analysis revealed surprisingly extensive genetic polymorphisms in macaque, especially in FcγRIIIa, which profoundly affects receptor function as demonstrated by flow cytometry. The molecular basis for these interspecies differences was investigated by engineering 50 IgG subclass mutants, with mutations throughout the IgG Fc region and several "hotspots" of interspecies difference in receptor binding were identified.

Our studies suggest that responses expected in humans may not be faithfully reproduced in NHP due to differences in their FcγRs. This has implications for their use as a model of human adaptive and innate immunity to infection and for their use in preclinical evaluation of human monoclonal antibodies.

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Analysis of toll-like receptor responses in the endangered Tasmanian devil

Patchett, A.¹, Kalodimos, G.¹, Tovar, C.¹, Lyons, A.B.², Woods, G.^{1,2}

¹Menzies Institute for Medical Research, University of Tasmania, Hobart, Australia, ²School of Medicine, University of Tasmania, Hobart, Australia

Devil facial tumour disease (DFTD) is a fatal transmissible cancer threatening Tasmania's largest marsupial carnivore, the Tasmanian devil (*Sarcophilus harrisi*). Development of a vaccine against DFTD is fundamental to the conservation of this unique species. For this vaccine to be effective, a strong adjuvant will likely be required. Toll-like receptors (TLRs) are expressed by immune cells and promote potent immune responses after binding to specific microbial molecules. We have previously shown that TLRs are functional in the Tasmanian devil and, as with human and mouse, we hypothesise that their ligands will act as effective vaccine adjuvants. To analyse TLR responses in the Tasmanian devil, we isolated mononuclear cells (MNCs) from peripheral blood and stimulated these in culture with TLR ligands. Quantitative PCR was performed to measure inflammatory gene expression after TLR stimulation. To assess the response *in vivo*, Tasmanian devils were vaccinated with the model antigen KLH with or without TLR ligands. Blood and serum samples were collected at intervals post-vaccination and the anti-KLH response was measured. Our *in vitro* data showed that a combination of the TLR3 ligand poly(IC) and the TLR7 ligand imiquimod consistently up-regulated inflammatory gene expression in MNCs. These ligands also significantly increased the magnitude of the specific immune response to KLH in Tasmanian devils. Our data provide the first *in vivo* evidence that TLR signaling activates effective immune responses in Tasmanian devils upon vaccination. Furthermore, these findings

could improve the efficacy of candidate DFTD vaccines for enhanced protection against disease transmission.

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Identification and characterization of chicken IFIT5

Rohringer, A.^{1,2}, Lowenthal, B.W.^{1,2}, Butler, J.M.³, Williams, D.T.³, Ward, A.C.², Bean, A.G.D.¹

¹CSIRO AAHL, Immunology, Geelong, Australia, ²Deakin University, School of Medicine, Geelong, Australia, ³CSIRO AAHL, Avian Virology, Geelong, Australia

The comprehensive characterization of antiviral immune pathways is essential for understanding host pathogen mechanisms to underpin improved molecular therapies for both human and livestock use. The interferon pathway impacts the expression of a myriad of interferon stimulated genes (ISGs) that reduce viral replication and infection. One of these, Interferon-Induced protein with Tetratricopeptide repeats 5 (IFIT5), has been shown to be important in the antiviral response in mammals. In this study we identify and characterize chicken IFIT5. This gene shows synteny with the human IFIT genes and encodes a 470 amino acid (AA) protein with strong conservation to mammalian IFITs. IFIT5 expression was induced in chicken splenocytes stimulated with type I interferon or TLR ligand *in vivo*. *In vivo* experiments demonstrated a 400-fold increase in IFIT5 expression in the lungs of chickens following infection with a highly pathogenic avian influenza virus. These data highlight the conserved role of IFIT5 in the response to viral infection and provide information on the chicken immune response to highly pathogenic avian influenza.

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Comparison of immune responses and transmissibility between Korean and Chinese canine influenza viruses in Guinea pigs

Kang, A.¹, Xie, X.², Yuk, H.¹, Yeom, M.¹, Na, W.¹, Song, D.¹

¹Korea University College of Pharmacy, Sejong, Korea, Republic of, ²Nanjing Agricultural University, College of Veterinary Medicine, Nanjing, China

Canine influenza virus (CIV) is a disease that can cause cough, pneumonia even death in dog populations. H3N2 CIV has been circulating in dogs in both Korea and China since 2007. Previous studies showed that guinea pigs have been successfully used as models to evaluate the pathogenicity and transmissibility of different species of influenza in mammalian hosts. But till now, few studies have reported pathogenicity and transmissibility of H3N2 CIV in guinea pigs. Here, we firstly make a comparison about the pathogenicity and transmissibility in same place and time condition of 4 influenza virus, which are Chinese CIV, Korean CIV, CIV/H3N2 mv and pandemic H1N1 virus, respectively. Except for nasal swab collected until days post infection (d.p.i) 10, we collected lung, trachea, nasal terminate, soft palate and rectal from different virus groups for virus load test and histopathology examination. Results showed that four virus strains in infected and contact group (extra guinea pigs put in 4 groups in d.p.i 1) all induce lesions, guinea pigs infected CIV/H3N2 mv and H1N1 showed more severe lesions compared

to CIV strains, lungs in contact group being extracted RNA and amplified to compare with original CIV strains to find mutation point, CIV only replicated in some tissues without production of obvious clinical signs, but was transmissible among guinea pigs. Therefore, the continued evaluation of the pathogenicity and transmissibility of CIVs is critical to the understanding of the evolutionary characteristics of CIVs and emergence of potential pandemic strains.

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Functional and molecular characterization of an invertebrate model immune system reveals evolutionary precursors of vertebrate's hematopoietic lineage

Rosental, B.^{1,2}, *Kowarsky, M.A.*³, *Corey, D.M.*¹, *Ishizuka, K.J.*^{1,2}, *Palmeri, K.J.*^{1,2}, *Chen, S.-Y.*⁴, *Sinha, R.*¹, *Newman, A.M.*¹, *Seita, J.*¹, *Weissman, I.L.*^{1,2}, *Voskoboynik, A.*^{1,5}

¹Stanford University School of Medicine, Institute for Stem Cell Biology and Regenerative Medicine, Palo Alto, United States, ²Stanford University, Department of Pathology, Hopkins Marine Station, Pacific Grove, United States, ³Stanford University, Departments of Applied Physics and Bioengineering, and Howard Hughes Medical Institute, Stanford, United States, ⁴Stanford University School of Medicine, Department of Microbiology and Immunology, Stanford, United States, ⁵Stanford University, Department of Pathology, Hopkins Marine Station. *V.A. and I.L.W. are Equal Contributors, Pacific Grove, United States

The colonial tunicate, *Botryllus schlosseri*, undergoes natural self-nonsel self recognition, resulting in an immune response controlled by a single, highly polymorphic histocompatibility locus we call BHF. Lack of a match in this locus results in a cytotoxic immune rejection that prevents formation of vascular anastomoses between adjacent colonies. Histocompatible colonies form vascular anastomoses that allow circulating germline and somatic stem cells to compete for dominance in the chimeric tissues. Circulating somatic and germ stem cells of one partner can take over the tissues of the chimera. Simultaneously one of the chimeric partners undergoes partial or complete resorption (i.e., morphologically showing death and disappearance of tissue). This resorption may be an immune cell based rejection that operates within BHF histocompatible pairs. Here, using FACS, RNAseq, and diverse functional essays, we have characterized the cells of the *Botryllus* blood and immune system. We have isolated 34 *Botryllus* cell populations, sequenced their transcriptome and analyzed their gene expression. This analysis revealed a cell population resembling hematopoietic stem cells, which upon transplantation, migrated to known stem cell niches and differentiated into other cell lineages. Using functional immunological assays for cytotoxicity and phagocytosis we identified phagocytic cell-type that resemble myeloid cells in vertebrates. Furthermore, we identified a *B. schlosseri* cytotoxic cell population originating from large granular lymphocyte-like cells, which upon allogeneic challenge differentiated into morula cells. Our data suggests that the common ancestor of tunicates and vertebrates had a true hematopoietic myeloid lineage, while the cytotoxic cells may result from a convergent evolutionary mechanism.

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Tamoxifen as a new therapeutic approach for airway inflammation

*Henriquez, C.*¹, *Borlone, C.*², *Morales, N.*¹, *Folch, H.*³, *Uberti, B.*⁴, *Moran, G.*¹
¹Universidad Austral de Chile, Farmacología y Morfofisiología, Valdivia, Chile, ²Universidad Austral de Chile, Graduate Department, Valdivia, Chile, ³Universidad Austral de Chile, Immunología, Valdivia, Chile, ⁴Universidad Austral de Chile, Ciencias Clínicas Veterinarias, Valdivia, Chile

Neutrophils play a central role in innate immunity, acting as the first line of host defense against microorganism. However, this cytotoxic defense machinery has the potential of becoming harmful to surrounding tissues. Dysregulation of apoptosis has been implicated in a range of diseases including allergic asthma and chronic obstructive pulmonary disease in humans and recurrent airway obstruction (RAO) in horses. Tamoxifen (TX) is a non-steroidal estrogen receptor modulator, which has been used for the treatment of breast cancer in women. The efficacy of TX has been attributed to both cell growth arrest and induction of apoptosis. Previous preliminary studies showed that TX improves the clinical status of RAO-affected horses. Therefore, the aim of this study was to evaluate the in vitro effect of TX on chemotaxis, respiratory burst production and phosphatidylserine exposure in equine peripheral blood neutrophils.

We found that IL-8 stimulated cells decrease their chemotactic index when treated with TX (1 and 10µM). Also, respiratory burst production was dampened after treatment with TX. Finally, a significant increase of early apoptotic (Anexin-V positive) cells was observed after treatment with TX (2.5 and 5µM).

These results suggest that tamoxifen has a direct action in equine peripheral blood neutrophils. However, more in vivo and in vitro studies are required to fully understand the mechanisms of action of TX on neutrophils, in order to elucidate if it can be use as treatment in disorders such as allergic asthma in humans and RAO in horses.

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Role of overexpressed *mce4A* gene in pathogenesis of murine tuberculosis

Ahmad, F.^{1,2}, *Gupta, P.*¹, *Owais, M.*², *Bose, M.*³, *Gupta, U.D.*¹
¹National JALMA Institute for Leprosy & OMD, BSL-3 Experimental Animal Facility, Agra, India, ²Aligarh Muslim University, Interdisciplinary Biotechnology Unit, Aligarh, India, ³V.P. Patel Chest Institute, Microbiology, New Delhi, India

The incidence of TB, particularly pulmonary TB is the greatest among those with impaired immunity. The *mce4* is one among the four homologues of mammalian cell entry (*mce*) operons of *M. tuberculosis* involved in steroid transport across the cell. The transport function of the *mce4* system is consistent with proposed role of cholesterol and its metabolism in the pathogenesis of Mtb. Mtb *mce4A* (Rv3499c) deletion mutant shows growth defect and reduced survival in C57BL/6 mice. The *mce4A* gene within this operon is homologous to *mce1A* (Rv0169) that has a role in host cell invasion by Mtb. The

expression of Mce4A protein in non-pathogenic *E. coli* enables it to enter and survive within HeLa cells and the macrophages. As Mce4A protein is expressed during later phase of mycobacterial growth, it is presumed that it may have crucial role in promoting the persistence of the Mtb inside the macrophages. We therefore, decipher the role of *mce4A* gene in pathogenesis of Mtb in Balb/c mice. Mice were infected through aerosol route of infection with overexpressed *mce4A* strain of Mtb and their growth pattern in lungs & spleen were compared with animals infected with standard H₃₇Rv. Animals infected with *mce4A* over expressing strain demonstrated an accelerated growth pattern when compared to animals infected with H₃₇Rv. Log phase of growth was appeared within 10-12 days of infection in mice infected with *mce4A* overexpressed strain while in H₃₇Rv infected animals it was appeared only after standard period of incubation, 21-28 days at 37 °C.

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Milk's microbiota during the periparturient period in Holstein cows: possible implication on animal health and milk quality

Riva, F.¹, Castiglioni, B.², Addis, M.F.³, Soares-Filipe, J.¹, Curone, G.¹, Pollera, C.¹, Bronzo, V.⁴, Moroni, P.⁴, Vigo, D.¹, Cremonesi, P.²

¹University of Milan, DIVET, Milan, Italy, ²Consiglio Nazionale delle Ricerche, Istituto di Biologia e Biotecnologia Agraria, Lodi, Italy,

³Porto Conte Ricerche S.r.l., R&D - Proteomics Lab, Porto Conte, Italy, ⁴University of Milan, VESPA, Milan, Italy

Dairy cattle are exposed to risk for disease during periparturient period with a peak incidence of production problems, metabolic disorders, infectious diseases, metritis and mastitis.

This study aim was to compare the milk's microbiota during the periparturient period to assess the possible implication on animal health and milk quality.

Milk samples were collected from six Holstein cows at T1=dry off, T2=day after calving (dac), T3=7-10 dac, T4=30 dac and T5=60 dac.

The udder health status was monitored by bacteriological analysis and SCC.

The bacterial DNA was extracted from milk and the 16S rRNA gene was analyzed by Miseq.

Milk proteins were evaluated by SDS-PAGE and densitometric analysis for assessment of total protein profiles. The presence of cathelicidin, S100A9 and lysozyme was estimated. The expression of CD45, KRT5, IL-1b and TNFa messengers in isolated milk cells was analyzed. Finally, the expression of PTX3 and IL-1R8 transcripts in milk fat globules was analyzed to evaluate their modulation and check the activation of the mammary epithelial cells.

Bacteriological analysis showed the absence of contagious bacteria. An interesting modification of the leukocyte/exfoliated epithelial cell ratio, a down regulation of cytokine expression and a pick of lysozyme and other inflammatory markers production were observed around T2 and T3. Moreover, this study describes the relative changes in milk protein abundance along lactation and according to composition of the microbial flora.

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DPO system based multiplex PCR for detection of viral and bacterial agents causing canine infectious respiratory diseases

Yuk, H., Na, W., Yeom, M., Kang, A., Song, D.

Korea University, Sejongsi, Korea, Republic of

There are several viral and bacterial pathogens related to canine infectious respiratory diseases ; As relevant virus, there are canine influenza virus (CIV) H3N8, H3N2, novel H1N1, canine distemper virus (CDV) and bacterial agents include *Streptococcus zooepidemicus*, *Bordetella bronchiseptica*. These pathogens recently occurs and are regarded as representative cause of canine respiratory diseases. Especially, influenza virus are epidemiologically important and are needed for continuous surveillance because of their enzootic and contagious properties. Such pathogens are easily transmitted in shelters crowded with dogs and cause secondary and complex infection by multi species. In case of multi infection, it is hard to choose appropriate treatment for specific target. Even, there are some reports identifying some pathogens enhance mutual pathogenicity and cause bad prognosis. Therefore, for proper remedy of canine respiratory disease, fast and exact diagnosis of causal agents is required. However, there are no method detecting those agents at once. Thus, in this study, we firstly designed multiplex PCR for detecting those virus and bacteria simultaneously. This multiplex PCR uses dual-priming-oligonucleotide (DPO) system that consists of stabilizing and determining priming region and polydeoxyinosine linker, which can enhance the sensitivity and specificity by reducing nonspecific amplification. Therefore, this multiplex PCR can be useful diagnostic tool for respiratory diseases of dog population.

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Porphyromonas gulae activates M1 macrophages via TLR2, TLR4 and NOD2

Holden, J.^{1,2}, Lenzo, J.^{1,2}, Orth, R.^{1,2}, O'Brien-Simpson, N.M.^{1,2}, Reynolds, E.C.^{1,2}

¹The University of Melbourne, Melbourne Dental School, Melbourne, Australia, ²Oral Health CRC, Melbourne, Australia

Porphyromonas gulae is an anaerobic, Gram-negative coccobacillus that is frequently isolated from the gingival sulcus of companion animals with periodontal. The aims of this study were to analyse the ligation of pattern recognition receptors by *P. gulae* and to determine how this ligation activated macrophages. Exposure of HEK cells transfected with Toll-like receptors or NOD-like receptors to *P. gulae* resulted in the ligation of TLR2, TLR4 and NOD2 but not NOD1, TLR7, TLR8 or TLR9. The subsequent activation of WT, TLR2^{-/-} or TLR4^{-/-} macrophages was determined by measuring the synthesis of nitric oxide, the expression of CD86 and the production of inflammatory cytokines. The addition of *P. gulae* to naive, unprimed macrophages generated little surface expression of CD86 or nitric oxide production. However, priming macrophages with IFN-γ to generate M1 macrophages resulted in the production of nitric oxide and expression CD86, IL-1β, IL-6 and TNFa in response to *P. gulae*. Nitric oxide and CD86 expression

was abolished in TLR2^{-/-} and reduced in TLR4^{-/-} macrophages, indicating the main TLR ligand of *P. gultae* is lipoprotein and the LPS of *P. gultae* is potentially deacylated and dephosphorylated as in other Porphyromonas spp. These results suggest that inflammatory M1 macrophages responding to *P. gultae* may be important in establishing chronic inflammation in the gingiva of companion animals.

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A new Fc receptor of human and non-human primate granulocytes has altered signalling and cell localisation

Anania, J.C.^{1,2}, Trist, H.¹, Tan, P.S.¹, Wines, B.^{1,2,3}, Hogarth, P.M.^{1,2,3}

¹Burnet Institute, Centre for Biomedical Research, Melbourne, Australia, ²Monash University, Immunology, Melbourne, Australia,

³University of Melbourne, Pathology, Melbourne, Australia

Non-human primates, particularly macaques, are widely used as models of human immunity. Leukocyte Fc receptors (FcR) provide effector cell resistance to infection, however also induce destructive inflammatory processes. Importantly FcR are essential to the success of many therapeutic mAbs. We have defined the function of a newly discovered leukocyte FcR for IgG (designated FcγRIIa3), which is found on circulating leukocytes of humans and macaques. This receptor is abundant on neutrophils¹.

A comparative analysis of genes, proteins and function has revealed remarkable differences between FcγRIIa1 and FcγRIIa3. (i) In both humans and macaques, FcγRIIa3 arises by alternative splicing of the *FCGR2A* gene. The encoded receptor contains a 19 amino acid insertion in the cytoplasmic tail that is absent from the conventional FcγRIIa1.

(ii) Specificity for human IgG was identical for both human and macaque FcγRIIa3 and FcγRIIa1 splice variants, and the expected differences between human and macaque FcR binding of IgG were also observed via flow cytometry.

(iii) Remarkably, confocal microscopy indicates cell localisation of the receptors is distinct, with FcγRIIa3 remaining at the cell surface and FcγRIIa1 being rapidly internalized, potentially altering the receptors signalling potential.

(iv) Indeed ITAM-dependent signalling induced by IgG clustering of FcγRIIa3 is distinctly different in magnitude and kinetics compared to the conventional FcγRIIa1.

Thus the altered functions of FcγRIIa3 may be linked to its increased prevalence in human inflammatory disease¹ and has implications for the analysis of human mAbs in both humans and macaques.

¹ van der Heijden et al. J Allergy Clin Immunol 131:1408(2013)

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Genetic and immunological characterization of Papillon-Lefevre syndrome patients of Sinaloa State, México

Romero-Quintana, J.G.¹, García-Vázquez, R.A.¹, Urquidez-Villanueva, N.J.¹, Frias-Castro, L.O.², Dueñas-Arias, J.E.², Romero-Navarro, J.G.¹, Ramos-Payán, R.¹, Sánchez-Schmitz, G.³, Aguilar-Medina, E.M.¹

¹Autonomous University of Sinaloa, Faculty of Biological and Chemical Sciences, Culiacan, Mexico, ²Sinaloa Pediatrics Hospital,

Culiacan, Mexico, ³Boston Children's Hospital and Harvard Medical School, Division of Infectious Diseases, Boston, United States

Introduction: Papillon-Lefevre Syndrome (PLS) it's a rare autosomic recessive disorder characterized by palmoplantar hyperkeratosis and early onset aggressive periodontitis, it's been associated with Cathepsin C gene mutations witch enzyme its involve in inflammation and phagocytosis. Over 20 cases have been diagnosed in Sinaloa, yielding a high incidence in the region. We previously reported the presence of two loss of function mutations (p.Leu68Arg and p.Thr153Ile). In this study we evaluate PLS patient's mutation founder effect and leukocytes response to infections and in vitro stimuli.

Methodology: STR analysis of CTSC gene region from leukocytes DNA, Flow Cytometry immunophenotyping of leukocytes populations, proliferation, ROS and NOS production of PMN cells infected for 30 and 60 min with *S. aureus*, PMN and Mononuclear cells (Mn) cytokine release stimulated with LPS, ConA and PMA. T-student (p 0.05) comparisons were made for quantitative measures.

Results: Leukocytes subpopulations were among normal range for both patient's and healthy individuals, bactericidal activity was similar in both groups at 30 min (p = 0.270), eliminating 80±9% and 70±11% respectably of the bacterial load. At 60 min both groups eliminate around 50% of the bacteria (p = 0.064). ROS and NOS production had no statistical significance. PMN IL-8 production was higher in patients and IL-1β, IL-10, IL-12p70 and TNF-α was lower in Mn. 50% of patients had a common CTSC haplotype.

Conclusion: Sinaloa's PLS patients with p.Leu68Arg mutation share a common ancestor, such mutation do not affect peripheral leukocytes proportions or response. Further research is needed to better grasp the disease pathogenesis.

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Humoral immune responses against tetanus neurotoxin in human and mouse

Yousefi, M.¹, Shokri, F.²

¹Tabriz University of Medical Sciences, Tabriz, Iran, Islamic Republic of, ²Tehran University of Medical Science, Tehran, Iran, Islamic Republic of

Tetanus is a highly fatal disease caused by tetanus neurotoxin (TeNT) and remains one of the major threats to human and animal health despite preventive strategies.

TeNT composed of heavy and light chains linked by a disulfide bond. The catalytic domain of the toxin resides in the light chain while the non toxic carboxy terminal fragment of the tetanus toxin heavy chain (fragment C) is responsible for neurospecific binding and retrograde transport of TeNT.

In this study, we generated 22 murine and 50 lymphoblastoid cell lines secreting monoclonal antibodies against TeNT. In the next step we determined specificity of these mAbs using different fragments of tetanus toxin. Moreover, we investigate in vitro inhibitory activity neutralizing of these mAbs by ganglioside GT1b assay.

Our results showed that tetanus toxoid immunization in human and mice vigorously induced humoral immune response against

fragment C and antibodies against fragment C potentially neutralize toxin in vitro.

These results propose fragment C as a good candidates for the development of epitope-based vaccines and therapeutic antibodies against tetanus.

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Pathogenicity of attenuated live vaccine candidate base on highly pathogenic porcine reproductive and respiratory syndrome virus in Vietnam

Yeom, M.¹, Na, W.¹, Moon, H.², Kang, B.-K.², Le, V.P.³, Song, D.¹

¹Korea University College of Pharmacy, Sejong-si, Korea, Republic of, ²Green Cross Veterinary Products, Yongin-si, Korea, Republic of, ³Hanoi University of Agriculture, Hanoi, Viet Nam

Porcine reproductive and respiratory syndrome Virus (PRRSV) was first discovered in the United States in 1987. Since its discovery, PRRSV has been spreading worldwide, and nowadays PRRSV is one of the most economically influential infectious diseases in the industry of swine. It is characterized by reproductive failure in pregnant sows and respiratory disorder in growing swine. In northern Vietnam, highly pathogenic PRRS (HP-PRRS) was first recognized in March 2007. The virus isolated from Vietnam showed 99% identity at the genomic level with China. The isolated virus was passaged serially through Marc-145 cells for developing live attenuated vaccine strain candidate. Every 10th passaged samples were collected for identification of nucleotide changes. Three genes (NSP-2, GP5 and GP7) were sequenced and analyzed. Initial passaged virus (5th passage) and attenuated virus (110th passage) were used to compare pathogenicity. A total of 10 healthy 4-week-old PRRSV-free pigs were randomly assigned to 2 challenge groups (4 pigs per group) and one negative group (2 pigs per group). For infection, 2ml of each passaged PRRSV strain (10^{5.5} TCID₅₀/mL) was administered intranasal route. Negative control group were similarly inoculated with the medium. All pigs were monitored daily for clinical signs post-infection until the end of the study. Blood samples were collected from all pigs for serological testing and virus shedding titration. All pigs were euthanized and lung tissue samples were collected at DIP-28 for macroscopic and microscopic test. The pathogenicity was considerably different between wild type and attenuated live vaccine candidate (110th passage).

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Wondering into the Bats' Immune System

Moreno-Altamirano, M.¹, Salinas-Tobón, M.R.², Aguilar-Setien, J.A.³, Rendón-Franco, E.⁴, Santiago-Ojeda, I.P.⁵

¹Instituto Politécnico Nacional, Immunology, Mexico, Mexico, ²Instituto Politécnico Nacional, Immunology, Mexico, Mexico, ³CMNSXXI, Hospital Pediatría, IMSS, Unidad de Investigación Médica en Inmunología, Mexico, Mexico, ⁴UAM, Departamento de Producción Agrícola y Animal, Mexico, Mexico, ⁵Instituto Politécnico Nacional, Inmunología, Mexico, Mexico

Introduction: Bats are a major source of zoonotic viruses worldwide, including those causing lethal infections in humans.

However, they might show not apparent clinical signs, even if they are persistently infected. The only virus causing disease is rabies. To elucidate how bats control a high number of infectious agents, the AIM of this work was to study the bats immune response by analyzing the lymphocytes cell proliferation and cytokines production.

Material and methods: Bats (*Artibeus* sp) were captured and handled under bioethical protocols. Mononuclear cells were obtained by Ficoll-paque density gradient centrifugation of 2-3 mL of peripheral blood, 5X10⁶ mononuclear cells were seeded on cover slips in 8 wells lab teck chambers and activated with Concanavaleine-A or PMA/IONOMYCINE per 5 days and proliferation was assessed with 5uM of carboxyfluorescein (CFSE) by flow cytometry. For cytokines production in cells of human and bats, 2X10⁵ mononuclear cells were cultured in a 96 wells plate in DMEM medium, 100 ug/mL of LPS were added to the cells and supernatant was collected at 6,12,24,48 and 72 Hrs for cytokines detection by using de CBA kit from BD.

Results showed that proliferation of lymphocytes for bats is similar to the humans, and only the IL-8 and IL-6 cytokines is slightly increased at 6 hr post LPS activation for bats, no significant change in the concentration of TNF- α , IL-12P70, IL-1b or IL-10 was observed.

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Evolution of cell death responses to cytosolic DNA

Vitak, N.¹, Johnson, K.N.², Hume, D.A.³, Sester, D.P.¹, Stacey, K.J.¹

¹University of Queensland, School of Chemistry and Molecular Biosciences, Brisbane, Australia, ²University of Queensland, School of Biological Sciences, Brisbane, Australia, ³University of Edinburgh, Roslin Institute and Royal (Dick) School of Veterinary Studies, Easter Bush, United Kingdom

Eukaryotic cells sequester their DNA in the nucleus and organelles. The presence of DNA in the cytosol indicates a danger of either internal or external origin. In mammalian macrophages cytosolic DNA is recognized by AIM2, leading to inflammasome activation and pyroptosis, rapid lytic death. There is no data about such a system for invertebrates, and AIM2 itself is a mammalian-restricted protein. We hypothesized that defence against cytosolic DNA is a feature of all eukaryotic cells, and fundamental to maintenance of genome integrity as well as recognition of infection. To investigate responses to cytosolic DNA in *Drosophila* or non-mammalian vertebrates which lack AIM2, both fruitfly and chicken cells were transfected with DNA via electroporation and viability was assessed at one hour after transfection. Cytosolic DNA but not double-stranded synthetic RNA was toxic for both *Drosophila* and chicken cells, and elicited lytic cell death between 5 and 15 minutes after introduction of DNA. *Drosophila* and chicken cells were sensitive to both single- and double-stranded DNA in contrast to the mammalian cells where the AIM2 response is mediated by only double-stranded DNA. This programmed necrotic death is not pyroptosis or necroptosis, as *Drosophila* does not have the proteins essential to these pathways. Thus we have demonstrated a novel form of rapid necrotic cell death, likely to be an ancient response rendered redundant in mammalian macrophages by the appearance of the AIM2 inflammasome. The retention of

cytosolic DNA-dependent death through evolution suggests a fundamental importance in cellular defence.

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CD44 expression and apoptosis of lymphocytes in *in vitro* stimulation by lipopolysaccharide

Slama, P.¹, Sladek, Z.¹, Kabourkova, E.¹, Havlicek, Z.¹, Kwak, J.Y.²

¹Mendel University in Brno, Brno, Czech Republic, ²Ajou University School of Medicine, Suwon, Korea, Republic of

Lipopolysaccharide (LPS) is a toxin released from the cell wall of Gram-negative bacteria that induces the inflammatory response of bovine mammary gland. CD44 is a proteoglycan that is expressed by most cell types including leukocytes. There is known information about expression of the CD44 on bovine neutrophils and macrophages during the inflammatory response. The virgin, clinically healthy, Holstein × Bohemian Red Pied crossbred heifers were used as mammary gland cell donors for *in vitro* study. Intact lymphocytes from the mammary glands were harvested following the phosphate buffered saline intramammary injection. The samples were incubated at 37 °C in a 5% CO₂ atmosphere for 1, 2 and 5 hours with LPS (0.2, 2 and 20 µg/ml). Apoptosis and CD44-positive lymphocytes were analysed by flow cytometry (FACS Calibur apparatus, Becton Dickinson, CA, USA). Results of this study demonstrate that apoptosis of lymphocytes was modulated with LPS. After 2 and 5 hours of *in vitro* incubation with LPS, there was detected delaying of lymphocyte apoptosis with no significant differences among used concentrations. The proportion of CD44-positive lymphocytes was decreased during *in vitro* cultivation. There was found out the positive correlation between the apoptosis of the lymphocytes and the CD44 expression. The results suggest that the cell surface receptor CD44 may play a role in inflammatory response of mammary gland in connection with lymphocyte apoptosis.

Acknowledgement: The study was supported by the project IGA AF MENDELU TP3/2015.

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Apoptosis of gamma delta T cells during inflammatory response of bovine mammary gland induced by *Staphylococcus aureus*

Slama, P.¹, Sladek, Z.¹, Kabourkova, E.¹, Havlicek, Z.¹, Kwak, J.Y.²

¹Mendel University in Brno, Brno, Czech Republic, ²Ajou University School of Medicine, Suwon, Korea, Republic of

Staphylococcus aureus is one of the most important pathogens causing clinical and subclinical bovine mastitis. This pathogen was used for stimulation of bovine mammary gland inflammatory response. The experiments were carried out on the virgin, clinically healthy, Holstein × Bohemian Red Pied crossbred heifers. Gamma delta T cells and apoptosis of lymphocytes were analysed by flow cytometry (FACS Calibur apparatus, Becton Dickinson, CA, USA) at 24, 48, 72 and 168 hours following the stimulation. The stimulation of mammary gland with *Staphylococcus aureus* resulted in a gradual increase in apoptotic lymphocytes. The portion of gamma delta T cells

was also gradually increased during one week following the stimulation. There was found out the positive correlation between the apoptosis of the lymphocytes and the proportion of gamma delta T cells. The results suggest that gamma delta T cells may play a role in inflammatory response of mammary gland induced by *Staphylococcus aureus* in connection with lymphocyte apoptosis.

Acknowledgement: The study was supported by the project IGA AF MENDELU TP3/2015.

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DDX23, an evolutionary conserved dsRNA sensor, pairs with TRIF or MAVS to trigger the antiviral responses

Yuan, S., Ruan, J., Xu, A.

Sun Yat-Sen University, Department of Biochemistry, Guangzhou, China

DExD/H-box helicases play essential roles in RNA metabolism, and emerging data suggest that they have additional functions in antiviral immunity across species. However, little is known about this evolutionary conserved family in antiviral responses in lower species. Here, by isolation of poly(I:C)-binding proteins in amphioxus, an extant basal chordate, we found that DExD/H-box helicases DHX9, DHX15 and DDX23, rather than RLRs to be responsible for cytoplasmic dsRNA detection in amphioxus. Since the antiviral roles of DDX23 have not been characterized in mammals, we performed further poly(I:C)-pull down assays and found human DDX23 to bind poly(I:C) through its N-terminal region, suggesting that DDX23 is an evolutionary conserved dsRNA sensor. Knockdown of human DDX23 via siRNA enhanced replication of VSV and reduced activation of the NF-κB and IRF3. When stimulated with poly(I:C), human DDX23 translocated from nucleus to cytoplasm and formed complexes with TRIF or MAVS for downstream signaling. This comparative immunological study not only provided a new strategy to identify a novel dsRNA sensor in humans, but also revealed that the essential role of the DExD/H helicase family in viral RNA/DNA sensing extends to the basal chordate, to close a major gap in our understanding of antiviral immunity during evolution.

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Naturally truncated NS gene of H3N8 equine influenza virus attenuates the virulence of the A/Puerto Rico/8/34 virus in mice in mice

Na, W.¹, Yoon, S.-W.², Yeom, M.¹, Song, D.¹

¹Korea University College of Pharmacy, Sejong-si, Korea, Republic of, ²Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea, Republic of

Equine influenza virus (EIV) causes a highly contagious disease of horses and other equid, and also transmits into dogs. Recently, we isolated H3N8 EIV from vaccinated horse that presented symptoms of respiratory disease in South Korea, and the EIV had truncated NS gene segment showing low viral growth kinetics. In order to elucidate correlation between truncation of NS gene and reduced viral virulence, reverse genetics were

applied to generate different NS recombinant virus utilizing an identical [A/Puerto Rico/8/1934(H1N1), PR8] virus. We have analyzed virulence of recombinant viruses in mice and found that naturally truncated NS gene led to incompetent viral pathogenicity and cytokine production compared to other rescued PR8 containing intact NS genes. This study could suggest that the partially deleted NS gene is responsible for the low pathogenicity and inefficient viral replication of the wild type Korean H3N8 EIV.

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The adjuvanticity of seabuckthorn polysaccharide for chicken Newcastle disease vaccine

Zhang, X., Liu, H., Zou, K., Chen, J., Wang, X., Liu, F., Wang, Y.
Inner Mongolia Agricultural University, College of Life Science,
Hohhot, China

The adjuvanticity of Seabuckthorn polysaccharide (SP) was evaluated in vitro and in vivo. In vitro experiment, the effects of SP on chicken peripheral lymphocyte proliferation were measured by Cell Counting Kit-8 assay. The results showed that SP significantly enhanced chicken peripheral lymphocyte proliferation synergistically with ConA. In vivo experiment, 180 one-day-old chickens were randomly assigned into 6 groups. The chickens were vaccinated with 0.2 mL inactivated Newcastle disease (ND) vaccine containing 6.25, 12.50 and 25.00 mg/mL SP as SP low (SP/L), medium (SP/M) and high (SP/H) dose adjuvant group, repeated vaccination on 28th day. Chickens in blank control (BC), non-adjuvant control (NC) and oil-adjuvant control (OC) were injected with equal volume of physiological saline, ND vaccine and ND oil-adjuvant vaccine, respectively. On days 7, 14, 21, 28, 35 and 42 after the first vaccination, the peripheral lymphocytes proliferation, serum ND antibody titers, interferon-gamma (IFN- γ) and interleukin-4 (IL-4) levels were investigated. The results showed that SP could significantly promote chicken peripheral lymphocyte proliferation, enhance serum ND antibody titers and promote the production of IFN- γ and IL-4. Those results indicated that SP act as a novel adjuvant for ND vaccine.

45 Minute Oral

16:45:00 - 17:30:00

Ag Presentation

T cell receptor recognition of non peptide-based antigens

Rosjohn, J.

Infection and Immunity Program and Department of Biochemistry and Molecular Biology, Biomedicine Discovery Institute, Faculty of Medicine, Monash University

T-cells determine the specificity of the immune response via the T-cell Receptor (TCR), of which there is a vast array of different TCRs within an individual, encompassing TCRs that encoded by the α and β TCR loci and the γ and δ TCR loci. Most of the studies in the area of TCR recognition have focused on peptides that are presented by molecules encoded by the Major Histocompatibility Complex (MHC). Great inroads have been made in understanding the TCR-peptide-MHC interaction in the context of key immunological questions. Notably, the three-dimensional fold of the MHC molecule is extremely versatile, and a related group of MHC-I-like molecules play critical roles in immunity by binding to Ags that are non-peptidic in nature. For example, the CD1 family is suited to capture lipid Ags for recognition by lipid-reactive T-cells. Moreover the MHC-I like molecule, MR1, captures vitamin B precursors that activate mucosal-associated invariant T-cells (MAITs). While lipid-specific and vitamin B-specific T-cells play a vital role in the immune system, our understanding of lipid- and metabolite-mediated immunity is in its infancy, and any role in aberrant immune function is unclear. My presentation will detail how the TCR can recognize lipids and metabolites.

Imaging Membranes

The functional importance of T cell receptor clustering

Gaus, K.^{1,2}, Pageonn, S.V.^{1,2}, Tabarin T.^{1,2}, Yamamoto, Y.^{1,2}, Ma, Y.^{1,2}
¹EMBL Australia Node in Single Molecule Science, ²ARC Centre of Excellence in Advanced Molecular Imaging, University of New South Wales, Sydney, Australia

Antigen recognition by the T cell receptor (TCR) is a hallmark of the adaptive immune system. When the TCR engages a peptide bound to the restricting major histocompatibility complex molecule (pMHC), it transmits a signal via the associated CD3 complex. How the extracellular antigen recognition event leads to intracellular phosphorylation remains unclear. Here, we used single-molecule localization microscopy to quantify the organization of TCR-CD3 complexes into nanoscale clusters and to distinguish between triggered and non-triggered TCR-CD3 complexes. We found that only TCR-CD3 complexes in dense clusters were phosphorylated and associated with downstream signaling proteins, demonstrating that the molecular density within clusters dictates signal initiation. Both pMHC dose and

TCR-pMHC affinity determined the density of TCR-CD3 clusters, which scaled with overall phosphorylation levels. Thus, TCR-CD3 clustering translates antigen recognition by the TCR into signal initiation by the CD3 complex and the formation of dense signaling-competent clusters is a process of antigen discrimination.

T-B

How the helper T cell repertoire responds to infection

Jenkins, M.

University of Minnesota, Center for Immunology

CD4⁺ helper T lymphocytes play an important role in adaptive immunity to microbes. My laboratory is trying to understand how the CD4⁺ T cells that are specific for a microbial epitope and are present before the relevant infection generate effector and memory cell types with different immune functions. We found that the secondary lymphoid organs of uninfected mice contained about 100 CD4⁺ T cells expressing T cell receptors capable of high affinity binding to an MHCII-bound peptide from the listeriolysin O protein of *Listeria monocytogenes*. About 90% of the cells in this population had the phenotype of conventional naïve T cells (CD44^{low} Foxp3⁻), while 10% of the cells had the phenotype of thymic regulatory T cells (Foxp3⁺ Helios⁺). Each cell in this population expressed a different T cell receptor and was therefore a unique clone. Following systemic infection with attenuated *L. monocytogenes* bacteria, the small pre-immune population generated a large effector cell population consisting of Th1 and follicular helper T cells but few if any regulatory T cells. As the infection was cleared, about 10% of the effector cells survived in the form of Th1 and follicular helper T cell-like memory cells. My lecture will describe our efforts to identify the factors that influence how single listeriolysin O peptide:MHCII-specific CD4⁺ T cells from a diverse pre-immune repertoire produce different types of effector and memory cells.

Wednesday, 24 August 2016

30 Minute Oral

08:30:00 - 10:15:00

Late Breaker Program Lectures 1

Distinct Gene Regulatory Pathways for Human Innate versus Adaptive Lymphoid Cells

Collins, P., Koues, O.I., Cella, M., Robinette, M.L., Porter, S.I., Pyfrom, S.C., Payton, J.E., Colonna, M., Oltz, E.M.

Department of Pathology and Immunology, Washington University School of Medicine

Upon sensing pathogens or other alterations to their local microenvironments, innate lymphoid cells (ILCs) respond with rapid and robust secretion of cytokines. In turn, these cytokines promote the release of antimicrobial peptides, minimize damage to surrounding cells, and influence the balance of T helper (Th) lymphocytes. While development kinships, functions, and pathologies involving ILCs have been studied extensively in model organisms, little is known about these first responders in humans. Moreover, while ILC and Th subsets rely on common effector genes, distinct signals and kinetics regulate expression programs in innate and cellular counterparts. Using integrated “-omics” analyses of lymphoid cells from a human mucosal environment (tonsils), we show that expression programs for ILC, Th, and NK cells diverge developmentally, but their regulomes devoted to functional polarization are highly convergent. Genes critical for cell-type identity, as well as autoimmune disease-associated polymorphisms, were located in cell-type specific regions of hyperacetylated chromatin known as “super enhancers.” Our results implicate novel pathways for the regulation of genes involved in ILC and Th functional niches, as well as new connections between autoimmune-associated variants and each of the human lymphoid subsets.

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Cancer immunosurveillance by tissue-resident innate lymphoid cells and innate-like T cells

Dadi, S.¹, Chhangawala, S.², Whitlock, B.M.¹, Franklin, R.A.¹, Luo, C.T.¹, Oh, S.A.¹, Toure, A.¹, Pritykin, Y.², Huse, M.¹, Leslie, C.S.², Li, M.O.¹

¹Memorial Sloan Kettering Cancer Center, Immunology, New York, United States, ²Memorial Sloan Kettering Cancer Center, Computational Biology, New York, United States

Malignancy can be suppressed by the immune system in a process termed immunosurveillance. However, to what extent immunosurveillance occurs in spontaneous cancers and the composition of participating cell types remain obscure. Here we show that cell transformation triggers a tissue-resident lymphocyte response in oncogene-induced murine cancer

models. Non-circulating cytotoxic lymphocytes, derived from innate, TCR $\alpha\beta$ and TCR $\gamma\delta$ lineages, expand in early tumors. Characterized by high expression of NK1.1, CD49a and CD103, these cells share a gene expression signature distinct from those of conventional NK cells, T cells and invariant NKT cells. Generation of these lymphocytes is dependent on the cytokine IL-15, but not the transcription factor Nfil3 that is required for the differentiation of tumor-infiltrating NK cells, and IL-15, but not Nfil3, deficiency results in accelerated tumor growth. These findings reveal a tumor-elicited immunosurveillance mechanism that engages unconventional type 1-like innate lymphoid cells and type 1 innate-like T cells.

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Regulation of lung immunity by epithelial derived cytokines

Lloyd, C.

Imperial College London, London, UK, NHLI, London, United Kingdom

Maintaining the balance between immune homeostasis and inappropriate immune activation and associated pathology is particularly complex at mucosal sites that are constantly exposed to potentially antigenic particles. Lung epithelial cells orchestrate pulmonary immune homeostasis and defence against pathogens but also play a critical role in the initiation of allergic responses. Cells within the pulmonary epithelium are a rich source of inflammatory cytokines, chemokines and growth factors. Lung epithelial cells are thought to be particularly important in the development of allergic immune reactions. Indeed, several of the genes identified in asthma GWAS studies are expressed by lung epithelial cells. We have shown that IL-33 contributes to the development of allergic pathology in mouse models and paediatric severe asthma. Moreover, IL-33 was shown to be a steroid resistant cytokine able to promote airway remodelling directly. We have recently identified a relationship between IL33 and epithelial derived TGFbeta in mediating acute pulmonary allergic inflammation. We generated inducible bronchial epithelial cell specific knockouts of TGF-beta and exposed them to inhaled allergen or IL-33. Deletion of TGFbeta solely in the bronchial epithelium resulted in diminished airway hyperactivity (AHR), eosinophilia and pulmonary type2 cytokines. Moreover, accumulation IL-13+ innate lymphoid cells (ILC2s) was significantly reduced. This presentation will highlight a novel interaction between IL33 and epithelial derived TGF-beta to promote ILC2 chemoactivity which facilitates development of allergic inflammation. Our data show that resident epithelial cells instruct immune cells reinforcing the central role of the local environmental niche in defining the nature and magnitude of immune reactions.

Antigen Presentation

Antigen presentation and T cell activation by dendritic cells

Amigorena, S.
Institut Curie

Dendritic cells represent a highly specialized hematopoietic lineage, whose main role is to sense infections in tissues and to activate specific T lymphocytes in lymphoid organs to mount immune responses adapted to the threat. To activate T lymphocytes, dendritic cells need to present peptides derived from infectious antigens on MHC molecules on their plasma membrane. There are two main intracellular sites of peptide loading on MHC molecules: the endocytic pathway for class II and the ER for class I MHC molecules. Because of this localization, the former are mainly (not exclusively) loaded with peptides cleaved by lysosomal proteases from internalized antigens. Peptides to be loaded on class I MHC are mainly derived from proteins that are being translated in the cytosol and are cleaved by the proteasome, before translocation into the ER by dedicated TAP1/2 transporters. In most cells types, the interchange of cargo and membranes between the two compartments is very limited. In dendritic cells, however, ER proteins are quite abundant in phagosomes. We showed recently that Sec22b controls the delivery of a subset of ER resident proteins to phagosomes. In the absence of Sec22b, the abundance of several ER residents in phagosomes is reduced, causing a defect in antigen cross presentation. Intriguingly, we also observed a marked acceleration of phago-lysosome fusion in the Sec22b-silenced dendritic cells, suggesting that the presence of ER in phagosomes delays phagosome maturation. Reduced levels of Sec22b also impaired antigen export from endosomes to the cytosol, suggesting a molecular link between export and the presence of ER-derived proteins in phagosomes. Interestingly, activation of dendritic cells by Toll-like receptor ligands modified both their antigen presentation capacities and their phagocytic functions, especially the fusion of phagosomes with lysosomes. Export to the cytosol, in contrast, seems to be regulated independently through other pathways of stress sensing. The contributions of factors that regulate phagosome functions in dendritic cells, and their relevance to antigen cross presentation will be discussed.

Imaging immunity non-invasively

Ploegh, H.
MIT

We have undertaken a systematic effort to produce single domain antibody fragments, derived from the heavy chain-only antibodies of camelid species to produce a new set of tools. We specifically target proteins of immunological interest, such as the checkpoints PD-L1 and CTLA-4. Antibody fragments composed of the variable region of such heavy chain only antibodies are referred to as VHHs or nanobodies. Their small size accounts for improved tissue penetration compared to full sized antibodies, and they are also cleared from the circulation more rapidly than intact antibodies. These are properties that make VHHs ideal for non-invasive imaging applications, such as positron emission tomography (PET)-based imaging, for example to track CD8 T

cells. These same properties also allow the use of VHHs to deliver payloads such as cytokines to the site of a tumor to improve an immune response against it. Finally, VHHs can survive with in the reducing environment of the eukaryotic cytosol with retention of specificity. This has allowed us to configure screens that select for VHHs that target cytosolic proteins and modulate their function. Examples that will be discussed include VHHs that recognize inflammasome components to interfere with the release of IL-1, and VHHs that impede the replication of influenza virus and vesicular stomatitis virus. An added bonus is the ability of VHHs to serve as crystallization chaperones to identify the functionally relevant epitope.

403

TAPBPR associates with UDP-glucose:glycoprotein glucosyltransferase 1 to provide quality control in the MHC class I pathway

Hermann, C.¹, Neerincx, A.¹, Antrobus, R.², van Hateren, A.³, Trautwein, N.⁴, Stevanović, S.⁴, Elliott, T.³, Deane, J.², Boyle, L.¹
¹University of Cambridge, Department of Pathology, UK, United Kingdom, ²University of Cambridge, Cambridge Institute of Medical Research, Cambridge, United Kingdom, ³University of Southampton, Faculty of Medicine and Institute for Life Science, Southampton, United Kingdom, ⁴Eberhard Karls Universität Tübingen, Department of Immunology, Tübingen, Germany

Our understanding of the antigen presentation pathway has recently been enhanced with the identification that the tapasin-related protein TAPBPR is a second MHC class I-specific chaperone. Recently we revealed TAPBPR influences MHC class I peptide presentation by functioning as a peptide exchange catalyst. Therefore, it is now apparent that tapasin and TAPBPR are both intimately involved in selecting peptides for immune recognition. Here we asked whether any other co-factor associated with TAPBPR in cells. We identify an interaction between TAPBPR and UDP-glucose: glycoprotein glucosyltransferase 1 (UGT1), a folding sensor in the calnexin/calreticulin quality control cycle known to reglucosylate MHC class I. The interaction between TAPBPR and UGT1 is dependent on a conserved cysteine at position 94 in TAPBPR. Our results suggest the formation of a multimeric complex between TAPBPR, UGT1 and peptide-receptive MHC class I. The loss of the association between TAPBPR and UGT1 alters the peptide repertoire expressed on cells and increases the surface expression of peptide-receptive MHC class I. These results suggest TAPBPR and UGT1 can work together to provide an important quality control check-point in the MHC class I antigen presentation pathway.

Innate Molecular

Identification of a novel monocyte subset involved in lung fibrosis

Akira, S.

Laboratory of Host Defense, WPI Immunology Frontier Research Center, Osaka University

Macrophages represent a diverse set of phagocytic cells distributed in the whole body. They play a central role in a variety of biological events including host defense against pathogens, tissue remodelling, chronic inflammation, fibrosis and cancer. Accumulating evidence indicates the existence of multiple and distinct subsets that exert different biological functions. We previously showed that JMJD3, one of H3K27trimethyl demethylases is involved in M2 polarization of macrophages in helminth infection. We also found that Trib-1 knockout mice show severe reduction of tissue-resident M2-like macrophages in various organs, resulting in severe lipodystrophy owing to increases lipolysis. Recently we have identified another monocyte subset critical for development of bleomycin-induced lung fibrosis. We named this novel monocyte subset segregated-nucleus containing atypical monocytes (SatM) based on a unique nuclear shape and a hybrid character of monocyte and granulocyte. C/EBP β deficiency results in a complete lack of SatM. These results demonstrate that C/EBP β is a key transcription factor for differentiation of profibrotic SatM from their committed progenitors.

Pore-forming activity and structural autoinhibition of the gasdermin family

Shao, F.¹, Ding, J.^{1,2}, Wang, K.¹, Liu, W.¹, She, Y.^{1,2}, Sun, Q.¹, Shi, J.¹, Sun, H.¹, Wang, D.C.²

¹National Institute of Biological Sciences (NIBS), Beijing, ²Institute of Biophysics, Chinese Academy of Sciences, Beijing

Inflammatory caspases cleave the gasdermin D (GSDMD) protein to trigger pyroptosis, a lytic form of cell death that is crucial for immune defences and diseases. GSDMD contains a functionally important gasdermin-N domain that is shared in the gasdermin family. The functional mechanism of action of gasdermin proteins is unknown. Here we show that the gasdermin-N domains of the gasdermin proteins GSDMD, GSDMA3 and GSDMA can bind membrane lipids, phosphoinositides and cardiolipin, and exhibit membrane-disrupting cytotoxicity in mammalian cells and artificially transformed bacteria. Gasdermin-N moved to the plasma membrane during pyroptosis. Purified gasdermin-N efficiently lysed phosphoinositide/cardiolipin-containing liposomes and formed pores on membranes made of artificial or natural phospholipid mixtures. Most gasdermin pores had an inner diameter of 10–14 nm and contained 16 symmetric protomers. The crystal structure of GSDMA3 showed an autoinhibited two-domain architecture that is conserved in the gasdermin family. Structure-guided mutagenesis demonstrated that the liposome-leakage and pore-forming activities of the gasdermin-N domain are required for pyroptosis. These findings reveal the mechanism for pyroptosis and provide insights into the roles of the gasdermin family in necrosis, immunity and diseases.

Cytosolic DNA, HIN-200 proteins and inflammasome activation

Stacey, K.J., Vajjhala, P.R., Sagulenko, V., Thygesen, S., Vitak, N., Sester, D.P.

School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane Qld 4072, Australia

Cytosolic DNA can indicate viral or bacterial infection, activity of retroelements, or DNA damage and repair processes. The two major known pathways of response to cytosolic DNA are induction of type I interferon via cGAS/STING, and activation of the AIM2 inflammasome, leading to IL-1 β and IL-18 processing and rapid cell death within 5-10 minutes of DNA introduction. AIM2 is a member of the HIN-200/PYHIN family proteins that contain a DNA-binding HIN domain, and a pyrin domain mediating effector function. AIM2 is the best-studied of this family, and binds to cytosolic DNA eliciting an inflammasome response via recruitment of ASC with subsequent activation of caspase-1. We have also demonstrated the direct recruitment and activation of caspase-8 by inflammasome complexes, leading to apoptotic death. This is mediated by a novel heterotypic interaction between the ASC pyrin domain and caspase-8 death effector domains. This extends the relevance of the inflammasome to cell types not expressing caspase-1. The HIN-200 proteins are restricted to mammals, and have expanded variably and independently in different lineages. Within the family AIM2 is phylogenetically distinct, whereas other family members have no direct orthologues between species, and their function is not well understood. Detection of ASC clustering *in vivo* shows that only AIM2 is competent for direct recruitment of ASC, and the function of the pyrin domain of other family members remains to be determined. Interestingly, some mammalian species have AIM2 pseudogenes and have lost capacity for inflammasome function.

Treg

Regulatory T cells at Barrier Surfaces

Powrie, F.M.

Kennedy Institute of Rheumatology, University of Oxford, UK

The gastrointestinal (GI) tract is home to a large number and vast array of bacteria that play an important role in nutrition, immune system development and host defense. In inflammatory bowel disease (IBD) there is a breakdown in this mutualistic relationship resulting in aberrant inflammatory responses to intestinal bacteria. Studies in model systems indicate that intestinal homeostasis is an active process involving a delicate balance between effector and immune suppressive pathways. In this presentation I will discuss host and bacterial pathways that promote the regulatory T cell response and how these may be harnessed therapeutically.

Oral Abstract Sessions

10:30:00 - 12:10:00

Emerging & Indigenous-Relevant Diseases

2205

Aboriginal Australians with acute rheumatic fever have enhanced Th17 responses to group A streptococcus that can be inhibited *in vitro* by repurposed clinical drugs

Martin, W.J.^{1,2}, Minigo, G.^{3,4}, Kim, M.L.⁵, Keeble, J.¹, Pitman, M.C.⁶, Tipping, P.³, Pacini, G.^{1,7}, Smyth, G.K.^{1,7}, Carapetis, J.^{8,9}, Wicks, I.P.^{1,2,10}

¹Walter and Eliza Hall Institute of Medical Research, Parkville, Australia, ²University of Melbourne, Department of Medical Biology, Parkville, Australia, ³Menzies School of Health Research, Casuarina, Australia, ⁴Charles Darwin University, Casuarina, Australia, ⁵Walter & Eliza Hall Institute of Medical Research, Parkville, Australia, ⁶Royal Darwin Hospital, Casuarina, Australia, ⁷University of Melbourne, Department of Mathematics and Statistics, Parkville, Australia, ⁸Telethon Kids Institute, Subiaco, Australia, ⁹Princess Margaret Hospital for Children, Subiaco, Australia, ¹⁰Royal Melbourne Hospital, Rheumatology Unit, Parkville, Australia

In the Northern Territory of Australia, the rate of rheumatic heart disease (RHD) in Aboriginal and Torres Strait Islanders is 26 times that of the non-Indigenous population. New therapies to reduce RHD and its harbinger, acute rheumatic fever (ARF), have remained out of reach due to a limited understanding of the immunopathogenesis. As ARF/RHD are autoimmune consequences of group A streptococcus (GAS) infection, we evaluated *in vitro* responses of peripheral blood mononuclear cells (PBMC) of ARF patients and healthy individuals to GAS. 19 Aboriginal ARF patients and nine non-ARF controls were recruited to the study. GAS induced Th1 and Th17 CD4 T cell responses in all participants as determined by flow cytometry. ARF patients had elevated Th17 markers in plasma, which rose as CRP levels fell, and also had enhanced Th17 responses to GAS restimulation *in vitro* compared to non-ARF controls. Using an *in vitro* PBMC assay, we assessed a number of drugs used in other autoimmune conditions for the capacity to inhibit T-cell responses to GAS, and to the presumed targets of RHD autoimmunity, streptococcal M protein and cardiac myosin. From a number of positive hits, one drug was of particular interest due to its current cost effectiveness and known safety. This drug was able to inhibit GAS-induced antigen presenting cell function and inhibited IFN γ and IL-23 signalling pathways as assessed by RNAseq analysis. These results demonstrate that ARF patients have elevated Th17 responses to GAS and we propose this drug that could be trialled for RHD prevention.

441

T cell up-regulation of CD127 is associated with reductions in the homeostatic set point of the peripheral T cell pool during malnourishment

Gubbels Bupp, M., Murphy, S., Patrick, K., Thoner, T., Edwards, R.W., Litvin, S.

Randolph-Macon College, Biology, Ashland, United States

The following study was undertaken to better understand the impact of short-term malnutrition on T cell homeostasis, with particular attention focused on the CD127/IL-7 signaling dynamic of peripheral T cells in mice. We report that the total number of peripheral naive and memory CD4⁺ and CD8⁺ T cells notably declined after one week of mild malnourishment a time period too short to be entirely due to malnutrition-induced thymic involution. Peripheral malnourished T cells expressed higher levels of the IL-7 receptor component, CD127, and were less sensitive to death-by-neglect as compared to control T cells. Adoptive transfer studies revealed that CD127 expression did not correlate with increased survival *in vivo* and that all naive CD8⁺ T cells upregulated CD127, regardless of initial expression levels. Corticosterone levels were elevated in malnourished mice and this correlated in time with peripheral T cell up-regulation of CD127 and the diminishment of the peripheral T cell pool. Overall, these data suggest a model in which CD127 levels are up-regulated quickly during malnourishment, thereby increasing the scavenge rate of IL-7, and providing a mechanism to quickly adjust the total number of T cells during malnutrition.

406

IL-33-dependent endothelial activation contributes to apoptosis and renal injury in *Orientia tsutsugamushi*-infected mice

Soong, L.¹, Shelite, T.¹, Liang, Y.¹, Trent, B.², Hu, H.¹, Sun, J.¹

¹University of Texas Medical Branch, Microbiology and Immunology, Galveston, United States, ²University of Texas Medical Branch, Pathology, Galveston, United States

Endothelial cells (EC) are the main target for *Orientia tsutsugamushi* infection and EC dysfunction is a hallmark of severe scrub typhus in patients. However, the molecular basis of EC dysfunction and its impact on infection outcome are poorly understood. We found that C57BL/6 mice receiving a lethal-dose *O. tsutsugamushi* infection had a significant increase in gene expression of IL-33 and its receptor ST2L in the kidneys and liver, but a rapid reduction of IL-33 in the lungs. We also found exacerbated EC stress and activation in infected kidneys, as evidenced by elevated angiopoietin (Ang) 2/Ang1 ratio, increased endothelin 1 and endothelial nitric oxide synthase expression. Such responses were significantly attenuated in IL-33^{-/-} mice. Infected IL-33^{-/-} mice also had markedly attenuated disease due to reduced EC stress and cellular apoptosis. Moreover, we challenged wild-type mice with a sub-lethal dose of *O. tsutsugamushi* and gave mice rIL-33 every 2 days for 10 days. Exogenous IL-33 significantly increased disease severity and lethality, which correlated with increased EC stress and activation, increased CXCL1 and CXCL2 chemokines, but decreased anti-apoptotic gene BCL-2 in the kidneys. To validate EC stress, we infected human umbilical vein endothelial cells

(HUVEC) *in vitro*. We found an infection dose-dependent increase in the expression of IL-33, ST2L soluble ST2 (sST2), and the Ang2/Ang1 ratio at 24 and 48 hours post-infection. This study indicates a pathogenic role of alarmin IL-33 in a murine model of scrub typhus and highlights infection-triggered EC damage and IL-33-mediated pathological changes during the course of *Orientia* infection.

1260

Understanding sepsis-induced lymphopenia

Puthalakath, H.¹, Doerflinger, M.², Pellegrini, M.³, Hotchkiss, R.⁴, Nedeva, C.²

¹La Trobe University, Biochemistry and Genetics, Melbourne, Australia, ²La Trobe University, Melbourne, Australia, ³Walter & Eliza Hall Institute, Melbourne, Australia, ⁴Washington University School of Medicine, St Louis, United States

Sepsis-induced lymphopenia is a major cause of morbidities in intensive care units and in populations with chronic conditions such as renal failure, diabetes, HIV and alcohol abuse. Currently, other than supportive care and antibiotics, there are no treatments for this condition. We developed an *in vitro* assay to understand the role of the ER-stress-mediated apoptosis process in lymphocyte death during polymicrobial sepsis, which was reproducible in *in vivo* mouse models. Modulating ER stress with clinically used chemical chaperones improved survival of mice undergoing polymicrobial sepsis four-fold.

Furthermore, to understand the molecular mechanism of, we biochemically purified the factor released by activated macrophages that leads to lymphocyte apoptosis (Immunoglobulin-binding protein or BiP). Using genome-wide CRISPR knockout, we also identified a new receptor that mediates lymphocyte death known as "Function Unknown Membrane Receptor" or FUMR. Genetic ablation of this receptor results in apoptosis resistance of lymphocyte in mice undergoing polymicrobial sepsis. Administration of a soluble form of the receptor also protected mice from lymphopenia. There have been over fifty clinical trials targeting the inflammatory phase of sepsis without success. It is now thought by many investigators that the immunosuppressive phase (caused by apoptosis) has by far the greatest pathophysiological significance in determining the patient's survival. Our findings open new therapeutic opportunities for treating sepsis.

4088

Development of a MVA-based vaccine against Crimean-Congo Haemorrhagic Fever virus

Graham, V., Dowall, S., Hewson, R.

PHE, Virology and Pathogenesis, Salisbury, United Kingdom

Crimean-Congo Haemorrhagic Fever (CCHF) is a severe tick-borne disease, endemic in many countries in Africa, the Middle East, Eastern Europe and Asia. Between 15-70% of reported cases are fatal. Handling of live virus requires biosafety level 4 facilities, which has restricted work on developing interventions against infection. There is no approved vaccine available, and preclinical protection by an experimental vaccine has not been

demonstrated previously. In the present study, the attenuated poxvirus vector, Modified Vaccinia virus Ankara, was used to develop a recombinant candidate vaccine expressing the CCHF virus glycoproteins. Cellular and humoral immunogenicity was confirmed in two mouse strains, including type I interferon receptor knockout mice, which are susceptible to CCHF disease. The vaccine generated strong humoral and cellular immunity against CCHF viral antigens. This vaccine protected all recipient animals from lethal disease in a challenge model adapted to represent infection via a tick bite. Histopathology and viral load analysis of protected animals confirmed that they had been exposed to challenge virus, even though they did not exhibit clinical signs. Subsequent analysis using passive and adoptive transfer of sera and CD3+ T-cells, respectively, demonstrated that both arms of the immune system were required to exert protective effects against lethal infection. This is the first demonstration of efficacy of a CCHF vaccine that is suitable for development for human use and can meet international regulatory approval.

4522

Andrographolide, a therapeutic agent for chikungunya and other inflammatory diseases

Gupta, S.¹, Mishra, K.P.¹, Ganju, L.¹, Singh, S.B.²

¹Defence Institute of Physiology and Allied Sciences, Immunomodulation, Delhi, India, ²Defence Institute of Physiology and Allied Sciences, Delhi, India

Aim: To study the anti-inflammatory and anti-chikungunya virus (CHIKV) potential of andrographolide (AND).

Method: Anti-inflammatory, anti-arthritic and anti-CHIKV properties of the major bioactive compound of plant *Andrographis paniculata* i.e. AND has been tested both *in vitro* and *in vivo*. Mouse peritoneal macrophages (PM) were used to measure COX-2, iNOS and NF-κB expression by immunoblotting and TNF-α and IL-6 by ELISA. However, *in vivo* studies were carried out in Complete Freund's Adjuvant (CFA) induced arthritis in Sprague Dawley rats. Paw edema in rats was treated by intraperitoneal AND treatment and measured for paw swelling.

In vitro anti-CHIKV studies were performed in THP-1 cell line and intracellular viral load was measured by flow cytometry and immunoblotting. For *in vivo* studies, Balb/c mouse neonates were used as CHIKV animal model. AND treatment was given intraperitoneally to CHIKV infected neonates and tested for CHIKV RNA by qPCR.

Results: *In vitro* studies in PM showed that AND exhibit anti-inflammatory properties by reducing COX2, iNOS and NF-κB expression along with TNF-α and IL-6. A further, *in vivo* study revealed that AND efficiently reduces paw edema formation. The *in vitro* anti-CHIKV study found that AND inhibits CHIKV protein expression in THP-1 cells. Moreover, *in vivo* study suggests that AND reduces CHIKV RNA copy number and percent mortality in Balb/c mouse neonates.

Conclusion: Andrographolide is a potent anti-inflammatory and anti-CHIKV agent and therefore, it can be used as a therapeutic agent for the treatment of virus induced arthritis which sustains for years after CHIKV infection.

464

Cutaneous manifestations of an emerging viral infection: chikungunya fever*Inamadar, A.**BLDE University, Dermatology, Vijaypur, India*

Chikungunya fever is a viral illness caused by an Arbo virus (CHIKV), transmitted by *Aedes sp.* of mosquitoes. The clinical features include high grade fever, arthralgia, myalgia, headache and morbilliform rash. In a recent outbreak of chikungunya fever in India, several new cutaneous features have been documented.

During the earlier epidemics of chikungunya fever in various parts of the world, facial flush, generalized exanthema and purpura were reported as cutaneous features. In the recent outbreak of the disease in south-India, the dermatological manifestations recorded were various patterns of hyperpigmentation (centro-facial freckle like, flagellate and diffuse), vesiculo-bullous (VB) lesions, aphthous-like ulcers in the intertriginous areas, lymphedema, lichenoid eruptions and ecchymosis. The VB lesions were seen among infants and aphthous-like ulcers among adult males. Oral mucosal ulcers, pigmentation of pinna and nails and subungual hemorrhage are the other features noted. Exacerbation of existing dermatoses, such as psoriasis, and unmasking of undiagnosed leprosy were observed.

Skin biopsy from the ulcers showed features suggestive of lymphocytic vasculopathy which might have been viral antigen induced. Evidence of vertical transmission from mothers with acute infection was noted in newborns, which developed diffuse pigmentation present at birth or soon thereafter. Serum IgM antibody for CHIKV was positive in these newborns.

The current outbreak of chikungunya fever in India is due to the African genotype of the virus, in contrast to the Asian genotype on previous occasions. The atypical / newer cutaneous features observed may be attributed to infection due to this different strain of the virus.

1589

Use of the rapid urine lipoarabinomannan antigen-antibody test for bovine tuberculosis diagnosis*Balada-Llasat, J.-M.¹, Sidiki, S.¹, Gebreyes, W.¹, Hunt, W.G.¹, Pan, X.¹, Arcos, J.¹, Hengesbach, J.², Baer, S.³, Smith, R.³, Averill, J.³, Wang, S.-H.¹, Torrelles, J.¹**¹Ohio State University, Columbus, United States, ²Michigan State University, Lansing, United States, ³Michigan Department of Agriculture and Rural Development, Lansing Charter Township, United States*

Bovine-Tuberculosis (BTB) is one of the World Health Organization's seven neglected endemic zoonotic diseases. Currently, there is no rapid-point-of-care, easy-to-use diagnostic test. Therefore, there is an urgent need worldwide for the development and implementation of a BTB point-of-care diagnostic test. The purpose of this study was to assess the accuracy of the lipoarabinomannan antigen-antibody (LAM-Ag-Ab) test to detect BTB. Urine collected from cattle (n=32) suspected of BTB disease in an outbreak in Michigan were tested with the LAM-Ag-Ab test and lipids were removed

from urine to increase test sensitivity. Results were compared to the USDA BTB-standard culture (n=29), gross pathology (n=21), histopathology (n=32) and the IFN- γ blood test (n=21). Ninety percent of the cattle suspected of BTB tested positive by the urine LAM-Ag test. These results were obtained in only 25 minutes, without the requirement of specialized personnel or equipment. Eighty-six percent were positive by gross pathology, 90% positive by histopathology, 57% positive by IFN- γ blood test, and 90% positive for culture. In conclusion, detection of LAM-Ag-Ab in urine offers the potential to diagnose BTB in cattle in a more rapid, accurate, and less expensive fashion than current methods.

2282

Changes in the effector and regulatory functions of CD8⁺ T cells in visceral leishmaniasis patients*Singh, B.¹, Kumar, R.^{2,3}, Engwerda, C.³, Sundar, S.¹**¹Institute of Medical Sciences, Banaras Hindu University, Department of Medicine, Varanasi, India, ²Netaji Subhas Institute of Technology (University of Delhi), New Delhi, India, ³QIMR Berghofer Medical Research Institute, Brisbane, Australia*

Visceral leishmaniasis (VL) is a major neglected tropical disease caused by *Leishmania donovani* in the Indian sub-continent. Following infection, CD8⁺ T cell activation can result in the generation of effector mechanisms allowing killing of target cells. In addition, CD8⁺ T cells can also develop regulatory functions, especially during chronic infection. However, the factors influencing these different fates of CD8⁺ T cells remain poorly understood. We hypothesized that CD8⁺ cell effector functions are compromised in VL patients, and instead CD8⁺ T cells develop immunoregulatory functions that suppress inflammation. To test this hypothesis, we purified CD8⁺ T cells from VL patients on presentation to clinic and after 30 days of drug treatment from blood. CD8⁺ T cells were isolated from the blood of healthy endemic controls and RNA was used to measure the abundance of 130 different mRNA species encoding gene products involved in T cell development, activation, function and regulation using nanostring technology. The results revealed significant differences in mRNA level in VL patients before and after treatment, as well as between cases and controls. CD8⁺ T cells from patients had elevated levels of mRNA encoding genes involved in cytolytic T cell function, including granzymes A, B, H, and perforin. However, these cells also displayed a dominant regulatory phenotype, including high levels of mRNA encoding IL-10, CTLA-4 and CD38 genes. These results will provide new insights into the functions of CD8⁺ T cells during chronic infection diseases and identify potential new strategies to manipulate this

T cell subset for therapeutic advantage.

Late Breaker Symposium 2

4403

Lymph node stromal cells derive from a distinct embryonic FAP+ progenitor

Denton, A.^{1,2}, Magiera, L.², Watts, A.², Fearon, D.^{2,3}

¹*Babraham Institute, Lymphocyte Signalling and Development ISP, Cambridge, United Kingdom,* ²*University of Cambridge, CRUK Cambridge Institute, Cambridge, United Kingdom,* ³*Weill Cornell Medical College and Cold Spring Harbor Laboratories, Cold Spring Harbor, United States*

Lymph node stromal cells (LNSCs) are critical regulators of LN structure and function and are indispensable for adaptive immune responses. The relationship between different LNSCs is poorly defined, as is their developmental origin. In this study, we aimed to understand the origin of stromal cells in the inguinal (i)LN based on expression of fibroblast activation protein- α (FAP). We generated a novel transgenic mouse strain in which the murine FAP transgene drives expression of the Tet-Off tetracycline transactivator. Cross-breeding with tetO-cre and ROSA-lox-stop-lox-tdTomato mice generated a strain in which FAP expression is indelibly marked in a manner that can be inhibited by administration of doxycycline. We show that all gp38+ LNSCs (i.e., fibroblastic reticular cells, FRCs; follicular dendritic cells, FDCs; and marginal reticular cells, MRCs) in adult mice derive from embryonic FAP-expressing cells, whilst endothelial cells - both blood and lymphatic - do not. Doxycycline-regulated fate-mapping revealed that all adult gp38+ LNSCs in the iLN derive from FAP+ cells that arise at e14.5-15.5. 20-25% of gp38+ LNSCs derive from a FAP+ cell present at e14.5, and microscopy analysis demonstrated that FAP+ cells give rise to adult FRCs, FDCs and MRCs in discrete patches that are localised throughout the iLN. Surprisingly, the FAP+ cells that give rise to LNSCs are distinct from the FAP+ progenitors that give rise to bone, muscle and fat tissue. We conclude that a distinct embryonic progenitor, identified by FAP expression, gives rise to adult LNSCs and is thus essential to the generation of adaptive immune responses.

4310

Host genetic polymorphisms affect p24 Gag epitope presentation and T-cell engagement in HIV-1 infection

Josephs, T.M.¹, McLaren, J.E.², Ladell, K.², Geldmacher, C.³, Rossjohn, J.^{1,2}, Price, D.A.², Gras, S.¹

¹*Monash University, Department of Biochemistry and Molecular Biology, Melbourne, Australia,* ²*Cardiff University School of Medicine, Institute of Infection and Immunity, Cardiff, United Kingdom,* ³*Medical Centre of the University of Munich, German Centre for Infection Research, Munich, Germany*

Genetic association studies have consistently linked disease progression rates to particular HLA class I alleles in HIV-1 infection, leading to a consensus in the field that viral epitope presentation and CD8 T-cell targeting patterns are key mechanistic determinants of biological outcome. However, the attractive simplicity of this paradigm is challenged by observations of peptide binding promiscuity, a phenomenon

that enables specific epitope cross-presentation in the context of different HLA class I molecules. The highly conserved p24 Gag-derived epitope TL9 (TPQDLNTML, residues 180-188) elicits protective CD8 T-cell responses restricted by members of the B7 superfamily, most notably HLA-B*4201 and HLA-B*8101. Despite their close molecular homology, the HLA-B*8101 allele is associated with comparatively slower rates of disease progression. To investigate this dichotomy, we examined the functional, biophysical and structural characteristics of TL9 epitope presentation and T-cell receptor (TCR) recognition in HLA-B*4201+ and/or HLA-B*8101+ individuals with chronic HIV-1 clade C infection. The data reveal that clonotype selection from the peripheral TCR repertoire is governed by the presenting HLA class I molecule. This process differentially regulates the mobilization of particular TL9-specific clonotypes defined by a unique mode of antigen engagement, thereby modulating immune escape pathways that influence the course of infection.

4530

Bringing veterinary immunology up to speed using the Babraham pig model of influenza A infection/vaccination

Tungatt, K.¹, Dolton, G.¹, Attaf, M.¹, Hemmink, H.², Morgan, S.B.², Hammond, J.A.², Porter, E.³, Miles, J.J.^{1,4}, Montoya, M.², Bailey, M.³, Tchilian, E.Z.², Charleston, B.², Sewell, A.K.¹

¹Cardiff University, Division of Infection and Immunity, Cardiff, United Kingdom, ²The Pirbright Institute, Pirbright, United Kingdom, ³University of Bristol, School of Veterinary Sciences, Bristol, United Kingdom, ⁴QIMR Berghofer, Brisbane, Australia

Cross-species jumps by viruses such as HIV, ebola and influenza can exploit gaps in existing immune coverage and represent significant threats to both public health and economic stability. Livestock-derived zoonoses represent a particular menace as these animals live in close proximity with humans and can act as mixing vessels for the generation of new and highly pathogenic viral species. Human/avian influenza A viruses typically sit at the apex of such threats because blending of viral strains within their common vector, the pig, fabricates highly dangerous novel fusions. Thus, it is imperative that we develop veterinary vaccines against this peril, however the immunology “toolbox” available for swine lacks routine culture and assay of T-cells, easy definition of dominant T-cell epitopes, robust MHC-binding motifs and effective peptide-MHC tetramers. Here we have studied T-cell responses to influenza A following vaccination of the Babraham inbred pig line and developed novel methods for immunological profiling including:

- i) Defining 3 immunodominant MHC class I restricted peptide epitopes from the pandemic H1N1 nucleoprotein;
- (ii) Large-scale culturing of T-cell clones; and,
- (iii) Routine manufacture and use of porcine-derived peptide-MHC tetramers.

These tools have enabled us to study protective porcine T-cell responses. We are currently in the process of determining Babraham MHC class I crystal structures and will establish peptide-binding motifs through peptide combinatorics and other means. Overall, we expect that these breakthroughs will help establish the Babraham pig as a powerful research platform for both basic and applied infectious disease research that has direct relevance for human health.

2472

Deregulated expression of MiRNAs in purified disease relevant blood cell populations in patients with spondyloarthritis

Miceli-Richard, C.^{1,2}, Bugge Tinggaard, A.³, Wang-Renault, S.-F.³, Busato, F.³, Dougados, M.¹, Tost, J.³

¹Université Paris Descartes (Paris V), Rheumatology - Hôpital Cochin, Paris, France, ²Pasteur Institute of Paris, Immunoregulation, Paris, France, ³Institut de Génomique - Centre National de Génotypage - CEA, Epigenetics, Evry, France

Background: No systematic studies in specific cell populations have been reported so far in Spondyloarthritis (SpA). A recently published computational analysis (Farh et al. Nature 2015) showed a large overlap of active gene regulatory regions with genetic variation associated with SpA for CD4 T cells and CD14

monocytes.

Objective: To analyze the miRNA expression pattern in two disease relevant cell populations of purified CD4 T cells and CD14 monocytes from Spondyloarthritis (SpA) patients and controls.

Methods: We analyzed the expression of 360 MiRNAs in cell-sorted (MACS) monocytes and CD4 T-lymphocyte populations from 24 SpA patients and 16 age and sex-matched controls by qPCR using the Exiqon MicroRNA Ready-to-Use Human Panel I on the miRNA-enriched fraction from both CD4+ and CD14+ cell populations.

Results: In CD4 cells 6 miRNAs were found to be differentially expressed including miR-491-3p, recently reported to be downregulated in ulcerative colitis, and miRNA-26a, a well known inflammation associated miRNA implicated in both allergic inflammation and autoimmune diseases. In monocytes, 25 miRNAs were found to be differentially expressed including the paradigmatic anti-inflammatory miRNA miR-146a and as the most significantly expressed miRNA, a miRNA involved in the degradation of TNF α .

Conclusions: The study is the first to analyze miRNA expression in purified disease-relevant blood cell populations in SpA bringing into evidence a number of interesting miRNAs whose deregulation might contribute to the pathogenesis of the disease.

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4750

Dissecting the IL-21/IL-21R system in human monocytes and macrophages: cell signaling events during phagocytosis adhesion

Vallières, F., Girard, D.

INRS-Institut Armand-Frappier, Laval, Canada

The biological significance of the IL-21/IL-21R system in human myeloid cells, especially in monocytes/macrophages, is not well documented. Previously, we demonstrated that IL-21 enhances the ability of human monocytes and macrophages to phagocytose opsonized sheep erythrocytes by a Syk dependent mechanism. In the present study, we showed that IL-21 enhances FcR-mediated phagocytosis in human monocyte-like THP-1 cells and in GM-CSF monocyte-derived macrophages (HMDM). The ability of IL-21 to enhance FcR-mediated phagocytosis was not associated with an increased expression of CD16 (Fc γ RIII), CD32 (Fc γ RIIA) and CD64 (Fc γ RI) at the cell surface. IL-21 was found to activate ERK-1/2 and p38, Akt and Jak/STAT pathways in THP-1 cells and HMDM, as evidenced by its ability to increase phosphorylation levels of these proteins. Using a pharmacological approach, we demonstrated the importance of ERK-1/2, Akt and Jak/STAT activation in IL-21-induced phagocytosis. Moreover, IL-21 was found to induce phagocytosis of zymosan by a ERK-1/2, Akt and Jak/STAT dependent mechanism. We also investigate the role of IL-21 in regulating other monocyte/macrophage functions, including cellular adhesion and protease secretion and found that it can induce adhesion of THP-1 cells onto the human endothelial hybrid cell line EA.hy 926. The importance of the different cell signaling

pathways are currently under investigation in our laboratory. We conclude that IL-21 possesses important biological effects in mononuclear phagocyte cells. Therefore, future development of therapeutic strategies targeting the IL-21/IL-21R system should consider that monocyte and macrophage cell physiology could be affected by this system.

4393

The complement C3a receptor contributes to melanoma tumorigenesis by regulating neutrophil and CD4⁺ T cell responses

Nabizadeh, J.¹, Manthey, H.¹, Boyle, G.², Steyn, F.^{3,4}, Woodruff, T.³, Rolfe, B.¹

¹The University of Queensland, Australian Institute for Bioengineering and Nanotechnology, Brisbane, Australia, ²QIMR Berghofer Medical Research Institute, Brisbane, Australia, ³The University of Queensland, School of Biomedical Sciences, Brisbane, Australia, ⁴The University of Queensland, The University of Queensland Centre for Clinical Research, Brisbane, Australia

The complement peptide C3a is a key component of the innate immune system, and a major fragment produced following complement activation. We utilised a murine model of melanoma (B16-F0) to identify a hitherto unknown role for C3a-C3a receptor (C3aR) signalling in tumour growth. The results show that the development and growth of B16-F0 melanomas is retarded in mice lacking C3aR, while growth of established melanomas can be arrested by C3aR antagonism. Flow cytometric analysis showed alterations in tumour-infiltrating leukocytes in the absence of C3aR. Specifically, neutrophils and CD4⁺ T lymphocyte sub-populations were increased, whereas macrophages were reduced. The central role of neutrophils was confirmed by depletion experiments which reversed the tumour inhibitory effects observed in C3aR-deficient mice, and returned tumour infiltrating CD4⁺ T cells to control levels. Analysis of the tumour microenvironment showed up-regulation of inflammatory genes which may contribute to the enhanced anti-tumour response observed in C3aR-deficient mice. C3aR deficiency/inhibition was also protective in murine models of BRAF^{V600E} mutant melanoma, colon and breast cancer, suggesting a tumour-promoting role for C3aR signalling in a range of tumour types. We propose that C3aR activation regulates the tumour inflammatory milieu, thereby promoting tumour growth.

Therapeutic inhibition of C3aR may therefore be an effective way to trigger an anti-tumour response in melanoma and other cancers.

4708

Homeostatic monophagocyte signatures regulate anti-tumor immunity

Nirschl, C.¹, Liu, Y.¹, Kim, T.-G.¹, Rezai, M.², Dannenfelser, R.³, Zhu, Q.³, Chau, D.¹, Ducolan Fuentes, J.⁴, Gulati, N.⁴, Song-Zhao, G.¹, Krueger, J.⁴, Sarin, K.², Troyanskaya, O.³, Suarez-Farinas, M.⁵, Anandasabapathy, N.⁶

¹Brigham and Women's Hospital, Harvard Medical School, Dermatology, Boston, United States, ²Stanford University,

Dermatology, Stanford, United States, ³Princeton University, Simmons Foundation, Princeton, United States, ⁴Rockefeller University, Laboratory for Investigative Dermatology, New York, United States, ⁵Icahn School of Medicine at Mount Sinai, New York, United States, ⁶Brigham and Women's Hospital, Harvard Medical School, Dermatology, Dana Farber Cancer Center, Harvard Stem Cell Institute, Boston, United States

An evolutionary balance between immune protection to pathogen challenge and maintenance of self-tolerance may be permissive to autoimmunity and tumor formation, respectively. How tissue immune homeostasis is achieved on a programmatic level is unknown, as are the consequences for tumor immune surveillance and progression. Dendritic cells (DC) are professional antigen-presenting cells, which direct both tolerance and immunity in T cells. DC arriving in lymph node (LN) from peripheral tissue such as skin regulate tolerance to self antigens and have a newly described role in dampening ongoing immunity. Here we show DC and other monophagocytes share a species-conserved homeostatic differentiation program, heightened upon tissue entry and exit. We find this signature associates closely with early melanoma progression, stratifies melanoma survival, and is enriched across multiple human tumors. In particular, loss of the highest species-conserved gene in this signature, expands DC-based priming and adaptive anti-tumor immunity, indicating a critical regulatory role. These findings indicate adaptive immune resistance is closely linked to peripheral immune homeostasis and may be commonly conditioned by cues shared during development and in the tumor microenvironment.

4536

Adaptive NK cells with low TIGIT expression are inherently resistant to myeloid-derived suppressor cell inhibitory activities

Sarhan, D.^{1,2}, Cichocki, F.³, Zhang, B.³, Yingst, A.³, Spellman, S.R.⁴, Cooley, S.³, Verneris, M.R.³, Blazar, B.R.³, Miller, J.S.³

¹University of Minnesota, Masonic Cancer Center, Minneapolis, United States, ²Karolinska Institute, Oncology-Pathology, Stockholm, Sweden, ³University of Minnesota, Adult and Pediatric Blood and Marrow Transplant Program, Minneapolis, United States, ⁴University of Minnesota, Center for International Blood and Marrow Transplant Research, Minneapolis, United States

Human cytomegalovirus (CMV)-induced adaptive natural killer (NK) cells characterized by distinct phenotypic and functional characteristics, including properties of immune memory. We hypothesized that adaptive NK cells are more resistant to suppression mediated by regulatory cell subsets, making them an attractive candidate for cancer therapy. Indeed, we found that relative to conventional NK cells, adaptive NK cells express significantly lower levels of the inhibitory receptor T cell Ig and ITIM domain (TIGIT). Consequently, adaptive NK cells are inherently resistant to suppression mediated by myeloid-derived suppressor cells (MDSCs). In contrast, conventional NK cells were potently suppressed by MDSCs ($p \leq 0.001$), an effect completely abrogated by TIGIT blockade ($p \leq 0.001$). TIGIT signaling in NK cells following co-culture with MDSCs resulted

in a decrease in the phosphorylation of ZAP70/Syk and ERK1/2 ($p \leq 0.003$), which could be reversed by blocking TIGIT on NK cells or by inhibiting reactive oxygen species (ROS) production by MDSCs ($p \leq 0.02$). TIGIT ligand CD155 was highly expressed on MDSCs compared to no/low expression on monocytes. CD155 increased expression was dependent on ROS production in MDSCs or induced by H_2O_2 treatment in monocytes. Thus, NK cells co-cultured with MDSCs in the presence of anti-TIGIT or ROS-inhibitor have normal cytotoxicity against K562 cells. Our results demonstrate that adaptive NK cells arising in response to CMV infection are resistant to MDSC-mediated functional suppression. Furthermore, TIGIT may represent a key checkpoint inhibitor for conventional NK cells that can be neutralized to enhance NK cell-mediated responses against cancer and infection.

Immunity to Bacteria & Fungi 2

2157

The role of CD8 T-cells during anti-PD1 treatment of viral infection-induced secondary bacterial pneumonia

Brown, A.C.¹, Essilfie, A.T.¹, Beckett, E.L.², Thorburn, A.N.¹, Hansbro, N.G.¹, Jarnicki, A.G.³, Yagita, H.⁴, Foster, P.S.¹, Horvat, J.C.¹, Wark, P.A.B.^{1,5}, Hansbro, P.M.¹

¹The University of Newcastle, Centre for Asthma and Respiratory Disease, Callaghan, Australia, ²The University of Newcastle, Callaghan, Australia, ³University of Melbourne, Department of Pharmacology and Therapeutics, Melbourne, Australia, ⁴Juntendo University, Department of Immunology, Tokyo, Japan, ⁵John Hunter Hospital, Department of Respiratory and Sleep Medicine, Newcastle, Australia

Introduction and aim: Secondary bacterial pneumonia frequently occurs following respiratory viral infections with many components of the immune response being dysfunctional. Anti-inflammatory processes such as the PD-1/PD-Ligand (PD-1/PD-L) pathway may be elevated following primary infection and suppress protective immunity to subsequent bacterial exposure. We determined whether primary viral infection increased the PD-1/PD-L pathway. We also assessed the role of this pathway and whether it could be targeted to improve responses to secondary infections.

Methods: We developed a mouse model of respiratory viral infection followed by secondary bacterial infection. Mice were infected with pneumonia virus of mice (PVM), then at viral clearance, they were infected with *Streptococcus pneumoniae*. The levels of PD-1/PD-L on different immune cells were assessed by flow cytometry. The role of PD-1/PD-L was determined by administration of a PD-1 blocking antibody and mediating immune responses determined by flow cytometry. The role of CD8 T-cells during PD-1 blocking treatment was determined by depletion antibodies at the end of viral clearance.

Results: Primary infection with PVM increased bacterial titres and PD-1/PD-L1 expressing cells in secondary *S. pneumoniae* infections compared to controls. Upon blocking PD-1 signalling, bacterial titres were reduced. Respiratory cellular profiles demonstrated increases in activated CD8 T-cells. Depleting CD8 T-cells during this treatment, prevented the suppressive effects

of blocking PD-1 signalling on bacterial titres.

Conclusion: These findings suggest that blocking PD-1 during viral infection-induced secondary bacterial pneumonia improves clearance of bacterial infection, which may be mediated by CD8 T-cells. This may be a potential new therapeutic approach for at risk patients.

1691

Intestine lamina propria CD4⁺T cells promote the bactericidal activity of macrophages via galectin-9 and Tim-3 interaction during *Salmonella typhimurium* infection

Zhang, C., Zhang, H., Zuo, Z., Han, Q., Zhang, J.

Institute of Immunopharmacology & Immunotherapy, School of Pharmaceutical Sciences, Shandong University, Jinan, China

The intestinal immune system is crucial for protection the host from pathogenic infection and maintenance of mucosal homeostasis. The intestine lamina propria macrophages are main effector cells in innate resistance to *intracellular* microbial pathogens. In the present study, we found that *Salmonella typhimurium* infection augmented the Tim-3 expression on intestinal lamina propria CD4⁺T cells and enhanced galectin-9 expression on F4/80⁺CD11c⁺macrophages. Moreover, CD4⁺T cells promote the activation and bactericidal activity of F4/80⁺CD11c⁺macrophages via galectin-9 and Tim-3 interaction during *Salmonella typhimurium* infection. Blockade the interaction of Tim-3 and galectin-9 with α -Lactose significantly attenuated the killing of intracellular bacteria by macrophages. Furthermore, the interaction of Tim-3 and galectin-9 also promoted the formation and activation of inflammasome, which leads to the cleavage of caspase-1 and IL-1 β . The secretion of active IL-1 β further improved bactericidal activity of macrophages and expression of galectin-9 on macrophages. These results demonstrated the critical role of the cross-talk between CD4⁺T cell and macrophage, in particularly the interaction of Tim-3 and galectin-9, in antimicrobial immunity and control intestinal pathogen infection.

There is no conflict of interest.

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Human CD8⁺ T-cells recognizing peptides from *Mycobacterium tuberculosis* presented by HLA-E have an unorthodox Th2-like, multifunctional, *Mtb*-inhibitory phenotype and represent a novel human T-cell subset

Ottenhoff, T.¹, van Meijgaarden, K.E.¹, Haks, M.C.¹, Caccamo, N.², Dieli, F.², Joosten, S.A.¹

¹Leiden University Medical Center, Department of Infectious Diseases, Leiden, Netherlands, ²Central Laboratory for Advanced Diagnostic and Biomedical Research (CLADIBIOR), Dipartimento di Biopatologia e Biotecnologie Mediche e Forensi, Università di Palermo, Palermo, Italy

Mycobacterial antigens are not only presented to T-cells by classical HLA-class Ia and HLA-class II molecules, but also through alternative antigen presentation molecules such as CD1a/b/c, MR1 and HLA-E. We recently described mycobacterial peptides that are presented in HLA-E and recognized by CD8⁺ T-cells.

Using T-cell cloning, phenotyping, microbiological, functional and RNA-expression analyses, we found that these T-cells can exert cytolytic or suppressive functions, inhibit mycobacterial growth, yet express GATA3, produce Th2 cytokines (IL-4,-5,-10,-13) and activate B-cells via IL-4. In TB patients, Mtb specific cells were detectable by peptide-HLA-E tetramers, and IL-4 and IL-13 were produced following peptide stimulation. The frequencies were highest in untreated TB patients and declined following successful treatment. These results identify a novel human T-cell subset with an unorthodox, multifunctional Th2 like phenotype and cytolytic or regulatory capacities, which is involved in the human immune response to mycobacteria and demonstrable in active TB patients' blood. The results challenge the current dogma that only Th1 cells are able to inhibit Mtb growth and clearly show that Th2 like cells can strongly inhibit outgrowth of Mtb from human macrophages. These insights significantly expand our understanding of the immune response in infectious disease.

1501

PKC- δ (delta), a host factor critical for macrophage killing effector functions during *Mycobacterium tuberculosis* in mice

Parihar, S.P.¹, Ozturk, M.¹, Hurdayal, R.¹, Zak, D.E.², Penn-Nicholson, A.³, Scriba, T.³, Guler, R.¹, Brombacher, F.¹

¹University of Cape Town/Institute of Infectious Disease and Molecular Medicine (IDM), Cape Town, South Africa, ²Center for Infectious Disease Research, Seattle, United States, ³South African Tuberculosis Vaccine Initiative (SATVI), Institute of Infectious Disease and Molecular Medicine (IDM) and School of Child and Adolescent Health, University of Cape Town, Cape Town, South Africa

We demonstrated previously that Protein Kinase C delta (PKC- δ), a critical host factor is involved in controlling *Listeria monocytogenes*, *Leishmania major* and *Candida albicans* infection in mice. Here, we aim to demonstrate the role of PKC- δ in *Mycobacterium tuberculosis* (Mtb) infection. Mice deficient for PKC- δ are highly susceptible to tuberculosis infection when compared to wild type mice. Decreased survival of PKC- δ ^{-/-} mice was associated with a rapid weight loss, exaggerated lung pathology and increased mycobacterial burdens in lungs and spleen. Exaggerated lung pathology is reflected by uncontrolled pro-inflammatory cytokine response such as IFN- γ , TNF- α , IL-6, IL-1 β and IFN- β in PKC- δ ^{-/-} mice. Interestingly, PKC- δ ^{-/-} mice exhibited no major differences in the recruiting immune cell populations at the site of infection. However, absence PKC- δ resulted in decreased accumulation of neutral lipid bodies in lungs and in isolated macrophages following Mtb infection. Moreover, the expression of PKC- δ increases following infection in wild-type macrophages however other PKC isoforms remains largely unaffected. *In vitro*, infection of isolated macrophages from PKC- δ mice increased bacterial growth with concomitant decrease in the production of nitric oxide and hydrogen peroxide. Furthermore, PKC- δ is highly expressed in active tuberculosis patients versus healthy adolescent control subjects, which rapidly decreases on the onset of anti-tuberculosis therapy. Thus, we demonstrated

that PKC- δ is required for efficient macrophage killing effector functions and host protection during tuberculosis infection.

1132

Inhibition of IL-1 β expression by anthrax lethal toxin through HDAC8 and enhancer RNAs

Kim, S.O.

Western University, London, Canada

Many pathogenic microbes often release toxins that subvert the host immune responses to render the environment suitable for their survival and proliferation. LeTx is one of the toxins causing immune paralysis through cleaving and inactivating the mitogen-activated protein kinase (MAPK) kinases (MEKs). Here, we show that inhibition of the histone deacetylase 8 (HDAC8) by either the HDAC8-specific inhibitor PCI-34051 or small interference (si)RNAs rendered LeTx-exposed murine macrophages responsive to LPS in pro-IL-1 β production. LeTx induced HDAC8 expression and nuclear localization, in part through inhibiting p38 MAPK, which resulted in a decrease of H3K27Ac levels, and inhibition of recruitment of NF- κ B to IL-1 β enhancer/promoter regions. Inhibition of HDAC8 increased H3K27Ac levels and enhanced NF- κ B-mediated pro-IL-1 β enhancer and messenger RNA production in LeTx-exposed macrophages. Collectively, this study demonstrates a novel role of HDAC8 in LeTx immunotoxicity and regulation of pro-IL-1 β production likely through HDAC8. Targeting HDAC8 could be a strategy for enhancing immune responses in macrophages exposed to LeTx or other toxins that inhibits MAPKs.

2146

Dynamics of phagocytic cells early after mycobacterial lung infection

Ryder, B.M., Manners, K., Kirman, J.R.

University of Otago, Department of Microbiology & Immunology, Dunedin, New Zealand

Dendritic Cells (DC) are essential for instigating adaptive immune responses to mycobacterial infection, however the roles that specific lung DC subsets play in the first two weeks of lung mycobacterial infection are unclear. We used fluorescent *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) in a murine infection model with multi-parametric flow cytometry to track early phagocytic innate immune cell subsets and examine the ability of different lung DC subsets to activate T cells. Our model revealed a rapidly evolving, dynamic range of innate cell association with BCG during the first 14 days of infection, beginning with rapid accumulation of neutrophils in the lung. In contrast with the prevailing dogma, alveolar macrophages remained uninfected during the first two weeks of BCG infection. Although CD103⁺ DC were also uninfected, CD11b⁺ conventional and plasmacytoid DC subsets became associated with BCG as infection progressed. Distinct CD11b^{high} and GR-1^{high} conventional DC subsets and a CD11c^{high} population of plasmacytoid DC were more frequently associated with BCG than CD11b⁺, GR-1^{int} and CD11c^{int} DC populations. Additionally, DC activation states differed between uninfected cells and

those associated with BCG. Collectively, these data demonstrate a highly dynamic early immune response to mycobacterial infection, and the importance of understanding distinct roles played by innate cell subsets to inform development of improved anti-mycobacterial therapies and vaccines against tuberculosis.

3369

Cooperation between monocyte-derived dendritic cells and lymphoid cells in the acute response to a bacterial lung pathogen

Brown, A.S.^{1,2}, Yang, C.^{1,2}, Fung, K.Y.², Bachem, A.², Bourges, D.¹, Bedoui, S.², Hartland, E.L.², van Driel, I.R.¹

¹University of Melbourne, Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, Melbourne, Australia, ²University of Melbourne, Department of Microbiology and Immunology at the Peter Doherty Institute for Infection and Immunity, Melbourne, Australia

Legionella pneumophila is the causative agent of Legionnaires' disease, a potentially fatal lung infection. Alveolar macrophages support intracellular replication of *L. pneumophila*, however the contributions of other immune cell types to bacterial killing during infection are unclear. Here, we observed that during acute respiratory infection of mice, reduced numbers of alveolar macrophages coincided with rapid infiltration of the lung by monocyte-derived dendritic cells (moDC), which, together with neutrophils, became the dominant inflammatory cells associated with the bacteria. moDC were required for bacterial clearance and provided the IL-12 needed to induce IFN γ production by lymphoid cells including NK cells, NKT cells and memory T cells that responded in an antigen-independent fashion. In turn, IFN γ was necessary for bacterial killing by moDC, but not neutrophils. This work has revealed a cooperative innate immune circuit between lymphoid cells and moDC that combats acute *L. pneumophila* infection and defines a specific role for IFN γ .

2418

Galectin-3 negatively regulates CR3-mediated neutrophil anti-*Candida albicans* functions

Wu, S.-Y., Huang, J.-H., Wu-Hsieh, B.

National Taiwan University, Graduate Institute of Immunology, Taipei, Taiwan, Republic of China

Galectin-3 (gal3) a chimera type of galectin that binds to the N-acetyllactosamine motif of glycoproteins through its carbohydrate-recognition domain and polymerizes through its N-terminal domain. Neutrophils are among the immune cells that express gal3. Published studies employing recombinant gal3 demonstrated that gal3 enhances dectin-1 and TLR2-dependent macrophage cytokine response to *Candida albicans*. Here we show that intracellular gal3 suppressed killing of engulfed opsonized *C. albicans* by neutrophils through complement receptor 3 (CR3)-mediated ROS production. Using live cell imaging to track the movement of cytosolic gal3 after phagocytosis of *C. albicans*, we found that cytosolic gal3 moved

towards but did not interact with engulfed *C. albicans* nor membrane CR3. However, both immunofluorescence staining and co-immunoprecipitation assay reveal that cytosolic gal3 physically interacted with Syk after stimulation by *C. albicans* and inhibited the phosphorylation of Syk and PKC β 2 thereby modulated neutrophil ROS production. Moreover, gal3^{-/-} mice were more resistant to systemic *C. albicans* infection than gal3^{+/+} mice with lower mortality. Our results reveal the molecular mechanism of how cell intrinsic gal3 modulates neutrophil anti-*C. albicans* function and demonstrate that neutrophil intrinsic gal3 dampens host defense against candidiasis.

3112

Calcineurin-NFAT signaling control of the innate response

Fric, J.^{1,2}, Zelante, T.^{2,3}, Wong, A.², Mencarelli, A.², Lee, B.², Poidinger, M.², Ricciardi-Castagnoli, P.²

¹International Clinical Research Center (ICRC), Center for Translational Medicine (CTM), Brno, Czech Republic, ²Agency for Science, Technology and Research (A*STAR), Singapore Immunology Network (SIgN), Singapore, Singapore, ³University of Perugia, Department of Experimental Medicine, Perugia, Italy

Treatment of patients with immunosuppressive drugs as Cyclosporine A or Tacrolimus targets calcineurin-NFAT pathway. This treatment often results in different types of immune disorders and higher incidence of different types of infection. Interestingly, although these drugs have an anti-fungal potential, in the clinical setting, the therapeutic usage may also lead to systemic fungal infections as a side effect. The susceptibility to infections cannot be sufficiently explained only through the suppression of T cell function, recent discoveries highlight the importance of the calcineurin-NFAT in innate immune cells and especially in dendritic cells (DCs). Knowing that beta glucan is triggering NFAT translocation in DCs, here we address the question whether NFAT in DCs might have a specific control on innate response to fungal spores, in particular to *Aspergillus fumigatus* conidia. Conditional transgenic mice lacking calcineurin specifically in CD11c expressing cells were screened in order to evaluate calcineurin-NFAT dependent genes induced upon fungal infections. Knockout mice display higher susceptibility to *A. fumigatus* infection. Global expression profiling identified calcineurin-regulated processes in immune response to infection and revealed calcineurin dependent changes in expression of important anti-fungal defense proteins. This result suggests that immunosuppressive drugs based on calcineurin inhibition directly impair important immune defense functions of myeloid cells, this directly resolve in higher susceptibility in models of systemic aspergillosis and invasive pulmonary aspergillosis after allogeneic transplantation of calcineurin knockout bone marrow. These findings are important in context of severity of aspergillus infections in immunosuppressed patients.

Adoptive Cell Therapy

1744

High-affinity PD-1/CD28 chimeric switch receptors improve the potency of CAR-T cells

Salter, A.^{1,2}, Cabanov, A.¹, Balakrishnan, A.¹, Srivastava, S.¹, Riddell, S.^{1,3}

¹Fred Hutchinson Cancer Research Center, Program in Immunology, Seattle, United States, ²University of Washington, Medical Scientist Training Program, Seattle, United States, ³University of Washington, Department of Medicine, Seattle, United States

The adoptive transfer of chimeric antigen receptor-modified T (CAR-T) cells targeting CD19 has produced robust antitumor responses in a subset of patients with B cell malignancies. However, CAR-T cell trials targeting solid epithelial cancers have been less successful. One major obstacle to CAR-T cell therapy in the solid tumor setting is PD-1-mediated immunosuppression. After antigen engagement, CAR-T cells rapidly upregulate PD-1 *in vitro* and *in vivo* and are functionally inhibited by PD-L1, which is commonly expressed on solid tumors. To endow CAR-T cells with an intrinsic ability to resist PD-1-mediated immunosuppression in the tumor microenvironment, we engineered chimeric switch receptors that link the PD-1 ectodomain to the CD28 signaling endodomain and convert negative PD-1 signals into positive costimulatory signals. Previous attempts to develop PD-1/CD28 receptors using the wild type PD-1 ectodomain failed to significantly improve the function of transgenic mouse and human T cells. As interactions between PD-1 and PD-L1/L2 are of low affinity, we hypothesized that these early receptors may have failed to outcompete endogenous PD-1 on the T cell surface or be recruited to the CAR-T cell synapse. We constructed high-affinity PD-1/CD28 chimeric switch receptors using mutated PD-1 ectodomains and show that the high-affinity, but not wild-type, PD-1/CD28 receptors significantly improve activation, cytokine production, and proliferation of ROR1-specific CAR-T cells *in vitro*. These receptors are now being tested in tumor xenograft models. Our results demonstrate that receptor affinity is critical for optimal PD-1/CD28 receptors and provide a strategy for improving CAR-T cell therapy of PD-L1⁺ tumors.

1843

System analysis of the functional fate of tumor infiltrating lymphocytes

Hod Marco, M.¹, Pickman, Y.², Shen-Orr, S.², Besser, M.³, Reiter, Y.⁴

¹Technion, Biology, Haifa, Israel, ²Technion, Immunology, Haifa, Israel, ³Sheba Medical Center, Tel-Hashomer, Ella Institute for Melanoma Research and Treatment, Ramat Gan, Israel, ⁴Technion, Haifa, Israel

Tumor infiltrating lymphocytes (TIL) is a heterogeneous population with potential anti-tumor reactivity. TIL recognition and reactivity with autologous tumor is associated with increased secretion of IFN- γ . Despite the encouraging results obtained from the use of adoptive cell transfer, in stage IV metastatic melanoma patients, and despite extensive research efforts, little is known about the cellular interactions and the mechanisms

determine TIL reactivity. Using multiparametric flow cytometry, computational tools and IFN- γ release assays, we determined the influence of the expansion process on specific subpopulation frequencies, subpopulation signatures, and tumor reactivity of pre- and post-REP Young-TILs. Furthermore, using RNA-seq we defined gene expression signatures for reactive vs. non-reactive post-REP TILs. Analysis of pre-REP Young-TILs discovered distinctive subpopulation signatures which significantly correlate with reactivity and non-reactivity. Importantly, we revealed that pre-REP Young-TIL signatures share similarity, in subpopulation composition and correlation with reactivity, to signatures obtained in previous study for pre-REP Selected-TILs. Further analysis of Pre- vs. post-REP Young-TILs revealed that REP induces alternations in subpopulation composition and thus, alterations in TIL reactivity and TIL subpopulation signatures. Unexpectedly, we obtained that Pre- and post-REP Young-TILs possess similar signature for non-reactivity, but differ in the signatures for reactivity. Interestingly, we found common features of post-REP reactive Young-TILs which are determined in their pre-REP state. The gene expression analysis revealed distinct mechanisms involved in reactivity vs. non-reactivity of TILs. Our studies may shed light on the molecular mechanisms that control TIL reactivity, improve the clinical efficacy of TILs and assist with selecting TILs with potential reactivity.

2814

Dendritic cells electroporated with mRNA encoding the Wilms' tumor protein (WT1) have major effects as adjuvant treatment in cancer patients

Berneman, Z.^{1,2}, Van de Velde, A.¹, Anguille, S.¹, Willemsen, Y.², Huizing, M.^{1,2}, Germonpré, P.², Saevels, K.¹, Cools, N.^{1,2}, Nijs, G.¹, Stein, B.¹, Van Driessche, A.¹, De Reu, H.², Schroyens, W.^{1,2}, Gadsisseur, A.^{1,2}, Verlinden, A.¹, Vermeulen, K.^{1,2}, Maes, M.-B.¹, Goossens, H.^{1,2}, Lammens, M.^{1,2}, Peeters, M.^{1,2}, Van Tendeloo, V.², Smits, E.^{1,2}

¹Antwerp University Hospital, Edegem, Belgium, ²University of Antwerp, Antwerp, Belgium

Previously we reported on vaccination with WT1 mRNA-electroporated dendritic cells (WT1/DC) as a post-remission treatment in 10 patients with acute myeloid leukemia (AML) (Van Tendeloo et al. PNAS 2010). In the present study, we expanded on the initial results and investigated WT1/DC as an adjuvant treatment in 60 high-risk cancer patients.

In AML, there was an antileukemic response in 13/30 patients; 5-yr relative survival was 46.7%, compared to 26.2% in the SEER data. Long-term clinical response was correlated with an increase in poly-epitope WT1-specific tetramer+ CD8 T-cells, long-term survival with WT1-specific IFN- γ + and/or TNF- α + CD8 T-cells from delayed type hypersensitivity sites.

In metastatic breast cancer (n=11), 5 patients had stable disease and 1 partial remission 2 months after starting WT1/DC; and 3 patients had stable disease after 6 months. Median overall survival (OS) was 43.8 months from the time of first documented metastasis, compared to a median OS of 21.7 months in the literature.

In glioblastoma multiforme (n=9), there were 4 patients with stable disease after 2 months; and 1 patient with partial

remission and 2 patients with stable disease after 6 months. Median OS was 30.5 months from time of diagnosis, compared to 14.7 months in the literature.

In non-resectable epithelial-type malignant pleural mesothelioma (n=10), 7 patients showed a stable disease. Median OS was 35.7 months post-start chemotherapy, compared to 22 months in the literature.

These results show that adjuvant WT1/DC-based immunotherapy provides a demonstrable clinical effect in part of these cancer patients and strongly suggest a survival benefit.

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The CARPETS trial: GD2-specific CAR T cell therapy for advanced metastatic melanoma

Gargett, T.^{1,2}, Yu, W.², Dotti, G.³, Yvon, E.⁴, Christo, S.⁵, Hayball, J.⁵, Lewis, I.², Brenner, M.⁶, Brown, M.^{1,2}

¹Royal Adelaide Hospital, Translational Oncology Laboratory, Hanson Institute, Adelaide, Australia, ²University of South Australia, Centre for Cancer Biology, Adelaide, Australia, ³University of North Carolina, Department of Microbiology and Immunology and Lineberger Comprehensive Cancer Center, Chapel Hill, United States, ⁴University of Texas MD Anderson Cancer Center, Houston, United States, ⁵University of South Australia, Experimental Therapeutics Laboratory, Adelaide, Australia, ⁶Baylor College of Medicine, Houston, United States

Chimeric Antigen Receptor (CAR) T cells have shown great promise in the treatment of hematologic malignancies but more variable results in the treatment of solid tumors, and the persistence and expansion of CAR T cells within patients has been identified as a key correlate of anti-tumor efficacy. Lack of immunological 'space', functional exhaustion, suppression due to immune or tumour microenvironments and deletion have all been proposed as mechanisms that hamper CAR T-cell persistence. The world first CARPETS trial (ACTRN12613000198729) investigates third-generation CD3ζ/CD28/OX40 CAR T cells specific for the tumor-associated antigen GD2 in patients with advanced metastatic melanoma. This trial has given us the opportunity to study third-generation CAR signalling and factors effecting CAR T cell persistence. We have performed a detailed analysis of the events following CAR engagement and have identified potent activation followed by significant activation-induced cell death (AICD) *in vitro*, which is in part mediated by PD-1/PD-L1 interactions. PD-1 blockade enhanced both CAR T-cell cytokine production and survival, and promoted killing of PD-L1⁺ tumor lines. We also found PD-L1 expression on CAR T cells was sufficient to ligate PD-1 and cause AICD in the absence of PD-L1⁺ tumor cells. Finally, we have related our *in vitro* findings to CAR T-cell persistence and immune phenotype data from four patients enrolled in the ongoing CARPETS phase 1 clinical trial. Together, these results suggest that CAR T cell activation and deletion also occurs *in vivo* and that combination therapy approaches will be useful to augment CAR T-cell efficacy in patients.

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Expanding the scope of TCR gene therapy for the treatment of AML and other cancers

Perret, R.¹, Valliant-Saunders, K.¹, Cao, J.¹, Greenberg, P.^{1,2}

¹Fred Hutchinson Cancer Research Center, Clinical Research Division, Seattle, United States, ²University of Washington, Seattle, United States

TCR gene therapy hastens the reproducible generation of tumor-specific T cells from patients, creating a promising avenue for treating hematological and solid tumors. Several hurdles remain to making this a broadly effective modality, including variable target antigen expression, risk of tumor antigenic escape, and current therapies limited almost exclusively to HLA-A0201⁺ patients. We previously characterized WT1 and cyclin A1 as favorable T cell immunotherapy targets, based on high expression in malignant versus normal cells and roles in oncogenesis, reducing likelihood of loss variants. We currently have a high-affinity HLA-A0201/WT1-specific TCR in Phase I trials for the treatment of AML and NSCLC, and are now focused on developing WT1- and cyclin A1-specific TCRs for ~7 common HLA types, aiming to cover >90% of patients. T cell lines were generated by stimulating donor cells with overlapping peptide libraries to identify new epitopes, and the highest affinity T-cell clones for each HLA/antigen combination identified by tetramer binding strength. TCRs were then cloned into lentiviral vectors for expression in CD8 T cells. We are currently selecting the best HLA-A0201/cyclin A1-specific TCR to advance to clinical development. We are also optimizing our epitope discovery protocol to improve selection of TCRs for other HLA alleles. Safety and efficacy of selected TCRs are rigorously tested using bioinformatics screens, *in vitro* assays, and humanized MHC-I mice. Our goal is to create a molecular toolbox of off-the-shelf TCR reagents to facilitate rapid treatment of patients, using T cell immunotherapies targeted to each particular tumor antigenic profile and HLA type.

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Of mice and men: HSC transplantation fails to fully regenerate the immune system

Ghosn, E.¹, Waters, J.¹, Phillips, M.¹, Yamamoto, R.^{2,3}, Long, B.⁴, Yang, Y.¹, Gerstein, R.⁵, Stoddart, C.⁴, Nakauchi, H.^{2,3}, Herzenberg, L.¹

¹Stanford University, Genetics and Immunology Program, Stanford, United States, ²Stanford University, Institute for Stem Cell Biology and Regenerative Medicine, Stanford, United States, ³University of Tokyo, Institute of Medical Science, Center for Stem Cell Biology and Regenerative Medicine, Division of Stem Cell Therapy, Tokyo, Japan, ⁴University of California, Department of Medicine, Division of Experimental Medicine, San Francisco, United States, ⁵University of Massachusetts Medical School, Department of Microbiology and Physiological Systems, Worcester, United States

The accepted paradigm has been that a single hematopoietic stem cell (HSC) can regenerate all components of the immune system, including B and T lymphocytes, NK cells, myeloid cells, granulocytes, erythrocytes, and platelets. However, our *in vivo* single-cell transplantation study shows that long-term (LT)-HSC, sorted from adult bone marrow, selectively fails to regenerate

B-1a lymphocytes, a subset of tissue B-cells required for protection against Pneumonia, Influenza, and other infections. Here, continuing with this study, we show that LT-HSCs transplants harvested from mouse fetal liver similarly fail to regenerate B-1a cells in otherwise fully reconstituted host (i.e., fetal liver LT-HSCs fully regenerate follicular B and T lymphocytes, NK cells, myeloid cells, granulocytes, erythrocytes, but do not detectably regenerate B-1a lymphocytes). Thus, our studies establish B-1a as a separate B-cell lineage that develops independently of LT-HSCs and overtly challenge the current paradigm that LT-HSCs regenerate all components of the immune system.

Important from a clinical standpoint, we also show that, in humanized mice, human fetal liver reconstitutes tissue B cells that are phenotypically similar to murine B-1a, raising the question of whether human HSC transplantation, widely used in human regenerative therapies to restore immunity in irradiated or otherwise immune-compromised patients, is sufficient to regenerate human tissue B-1a.

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Optimizing adoptive cell therapy by genetic engineering of T cells for improved homing to tumor site

Idorn, M.¹, Olofsson, G.H.¹, Olsen, M.¹, Larsen, H.L.², van der Berg, J.¹, Met, Ö.¹, Thor Straten, P.^{1,3}

¹Center for Cancer Immune Therapy, Herlev University Hospital, Herlev, Denmark, ²The Danish Stem Cell Center (DanStem), University of Copenhagen, Copenhagen, Denmark, ³University of Copenhagen, Department of Immunology and Microbiology, Copenhagen, Denmark

Adoptive cell therapy (ACT) using *in vitro* expanded tumor infiltrating T lymphocytes (TILs) from biopsy material represents a highly promising treatment of disseminated cancer. A crucial prerequisite for successful ACT is sufficient recruitment of transferred lymphocytes to the tumor site; however, despite transfer of billions of lymphocytes, T-cell infiltration into the tumor post ACT is limited. By PCR and ELISA analyses we found that a majority of malignant melanoma (MM) cell lines expressed chemokines CXCL8/IL-8, CXCL12/SDF-1 and CCL2. Taking advantage of lentiviral transduction, successful transduction of TILs and peripheral blood T cells significantly increased receptor expression of the corresponding chemokine receptors CXCR2, CXCR4 and CCR2. All three chemokine receptors are functional *in vitro* and show ligand specific transwell migration of engineered T cells as well as increased migration towards MM conditioned medium. *In vivo* homing was assessed in a xenograft NOG mouse model. Mice with subcutaneous human melanoma were treated with ACT of tumor specific T cells transduced with either CXCR2 or GFP. Transducing T cells with CXCR2 increased tumor infiltration. In comparison mock transfected T cells appeared to be allocated to other organ-compartments. In conclusion, our CXCR2, CXCR4 and CCR2 transduced T cells are functional *in vitro*, and transduction with CXCR2 improve *in vivo* homing of T cells to tumor site, setting the stage for mixing and matching chemokine-receptor expression to tumor microenvironments. Longitudinal studies assessing cell trafficking and tumor control using *in vivo* imaging are currently ongoing, and results will be presented at the meeting.

1460

Vdelta1 T cells expressing natural cytotoxicity receptors for adoptive cell therapy of leukemia

Silva-Santos, B.¹, Correia, D.V.¹, Almeida, A.², Anjos, D.R.², Silva, M.G.d.³

¹Instituto de Medicina Molecular, Lisbon, Portugal, ²Lymphact SA, Coimbra, Portugal, ³Instituto Português de Oncologia de Lisboa, Lisbon, Portugal

Adoptive transfer of chimeric antigen receptor (CAR)-expressing T-cells constitutes a promising immunotherapy for leukemia. However, antigens targeted by CARs are often expressed by healthy leukocytes or progenitors, thus leading to significant on-target effects. Here we characterize a cellular immunotherapy product composed of genetically unmanipulated gamma-delta T-cells that selectively recognizes malignant (but not normal) leukocytes *in vitro* and *in vivo*. This subset expresses the Vdelta1 TCR and a broad repertoire of natural killer receptors, including NKG2D and the natural cytotoxicity receptors (NCRs), NKp30 and NKp44. Critically, NCR expression is absent in freshly-isolated gamma-delta T-cells but is selectively induced on Vdelta1 T-cells by a cocktail of cytokines and TCR agonists. NCR expression endows these lymphocytes, termed Delta One T (DOT-) cells[®], with enhanced cytotoxicity against lymphoid and myeloid leukemia cells *in vitro*. Importantly, DOT-cells[®] combined TCR and NCR mechanisms to recognize transformation-induced ligands on leukemic (but not healthy) cells. To test DOT-cells[®] *in vivo*, we employed xenograft models of human chronic lymphocytic leukemia and acute myeloid leukemia in NOD-SCID *gc*^{-/-} (NSG) mice. Upon two DOT-cell[®] transfers, we observed a significant reduction in tumor load compared to control animals. DOT-Cells[®] showed an activated and proliferative profile and produced TNF-alpha and IFN-gamma but no interleukins (IL)-17 or IL-10. Most strikingly, DOT-Cells[®] were capable of preventing tumor dissemination to secondary organs and prolonged mouse survival, with no evidence of treatment-associated toxicity (as inferred from blood biochemistry and histology). These data provide the seminal proof-of-concept for pioneering the application of DOT-Cells[®] in leukemia clinical trials.

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Immunotherapy by using rejuvenated antigen-specific T cells

Nishimura, T.¹, Ando, M.², Nakauchi, H.^{1,2}

¹Stanford University School of Medicine, Institute for Stem Cell Biology and Regenerative Medicine, Stanford, United States, ²University of Tokyo, Institute of Medical Science, Tokyo, Japan

Adoptive immunotherapy is a promising approach to fight cancers or chronic viral infections with cytotoxic T cells expressing antigen-specific TCRs. However, its effectiveness is diminished by the exhaustion of antigen-specific T cells during *ex vivo* expansion.

The discovery of induced pluripotent stem cells (iPSCs) has created promising new avenues for therapies in regenerative medicine. Recently, we developed a novel system in which a single antigen-specific T cell can be reprogrammed into an iPSC, expanded and redifferentiated back to the original cell type. Redifferentiated CD8⁺ T cells express the same TCR and exert cytotoxic T cell functions in the same antigen-specific manner

as the original T cell. Rejuvenation via iPSC-reprogramming is highlighted in the redifferentiated CD8⁺ T cells through their characteristic longer telomeres, higher proliferative capacity and younger memory phenotype compared to the original T cells. However, the actual *in vivo* efficacy of rejuvenated T cells has not been tested formally. Here we demonstrate that rejuvenated T cells are effective against EBV-mediated tumors *in vivo* using the tumor-burden mouse model. This unique technique was deployed *in vivo* with inducible Caspase-9 (iC9), a safeguard system which evokes apoptosis via the caspase signaling cascade upon administration of a specific chemical compound. Furthermore, now it becomes possible to obtain abundant CD8αβ⁺ type, CD8αα⁺ type, and CD4⁺ T cells from iPSCs. Although several biological and technical challenges still lie ahead, the application of iPSC-mediated T cell rejuvenation technology could represent a breakthrough in the field of adoptive immunotherapy.

Th Subsets

1440

Single-cell RNA-sequencing resolves Th1-Tfh bifurcation

Lönnberg, T.^{1,2}, *James, K.R.*³, *Svensson, V.*¹, *Fernandez-Ruiz, D.*⁴, *Sebina, I.*³, *Montandon, R.*², *Soon, M.*³, *Stubbington, M.J.T.*¹, *Souza-Fonseca-Guimaraes, F.*³, *Heath, W.R.*⁴, *Billker, O.*², *Haque, A.*³, *Teichmann, S.A.*^{1,2}

¹European Molecular Biology Laboratory, European Bioinformatics Institute, Hinxton, United Kingdom, ²Wellcome Trust Sanger Institute, Hinxton, United Kingdom, ³QIMR Berghofer Medical Research Institute, Herston, Australia, ⁴Peter Doherty Institute, University of Melbourne, Melbourne, Australia

Differentiation of naïve CD4⁺ T cells into functionally distinct T helper subsets is crucial for the orchestration of immune responses. Due to multiple levels of heterogeneity and overlap in differentiating

T cell populations, this process has remained a challenge for systematic dissection *in vivo*. Single-cell RNA-sequencing has provided a powerful tool for identifying such transitional cellular states and elucidating their developmental relationships. Using an antigen-specific transgenic TCR mouse strain, we have dissected the CD4⁺ T cell response to blood-stage *Plasmodium chabaudi* infection, during which both Th1 and Tfh populations emerge and contribute to the successful resolution of the infection. By using a novel computational approach based on overlapping mixtures of Gaussian processes, we have reconstructed the developmental trajectories of Th1 and Tfh cell populations at single-cell resolution in an unbiased way. On a genomic scale, these cell fates emerged from a common, highly proliferative and metabolically active precursor. Moreover, by tracking clonality from T cell receptor sequences, we infer that sibling cells derived from the same naïve CD4⁺ T cell can concurrently populate both Th1 and Tfh subsets. We further found that precursor T cells were coached towards a Th1 but not a Tfh fate by monocytes. Importantly, our integrated genomic and computational methodology is applicable for analysis of any cellular system characterized by differentiation towards multiple fates.

1489

IL-1-induced Bhlhe40 identifies pathogenic T_H cells in a model of autoimmune neuroinflammation

*Lin, C.-C.*¹, *Bradstreet, T.R.*¹, *Schwarzkopf, E.A.*¹, *Jarjour, N.N.*¹, *Chou, C.*¹, *Archambault, A.S.*², *Sim, J.*³, *Zinselmeyer, B.H.*¹, *Carrero, J.A.*¹, *Wu, G.F.*^{1,2,4}, *Taneja, R.*⁵, *Artyomov, M.N.*¹, *Russell, J.H.*³, *Edelson, B.T.*¹
¹Washington University School of Medicine, Dept. of Pathology and Immunology, St. Louis, United States, ²Washington University School of Medicine, Dept. of Neurology, St. Louis, United States, ³Washington University School of Medicine, Dept. of Developmental Biology, St. Louis, United States, ⁴Washington University School of Medicine, Hope Center for Neurological Disorders, St. Louis, United States, ⁵Yong Loo Lin School of Medicine, National University of Singapore, Dept. of Physiology, Singapore, Singapore

The features that define autoreactive T_H cell pathogenicity remain obscure. We have previously shown that T_H cells require the basic helix-loop-helix transcription factor Bhlhe40 to mediate experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. Bhlhe40 regulates the production of specific cytokines by T_H cells, such that *in vitro*-polarized Bhlhe40-deficient T_H1 and T_H17 cells show reduced production of GM-CSF and increased production of IL-10. Here, using novel *Bhlhe40*^{GFP} reporter mice and analyzing both polyclonal and TCR transgenic T_H cells, we found that Bhlhe40 expression by activated T_H cells was heterogeneous after EAE induction. Bhlhe40-expressing T_H cells displayed marked production of IFN-γ, IL-17A, and GM-CSF, while exhibiting reduced expression of both IL-10 and the regulatory T cell transcription factor Foxp3. In adoptive transfer EAE models, Bhlhe40-deficient T_H1 and T_H17 cells were both nonencephalitogenic. Pertussis toxin (PTX), a classical adjuvant for actively induced EAE, promoted IL-1β production by myeloid cells in the draining lymph node and served as a strong stimulus for Bhlhe40 expression in T_H cells. Furthermore, PTX adjuvant activity was Bhlhe40 dependent. IL-1β induced Bhlhe40 expression in polarized T_H17 cells, and Bhlhe40-expressing cells exhibited an encephalitogenic transcriptional signature. *In vivo*, IL-1R signaling was required for full Bhlhe40 expression by T_H cells after immunization. Overall, we demonstrate that Bhlhe40 expression identifies encephalitogenic T_H cells and we define a PTX-IL-1-Bhlhe40 pathway active in EAE.

2835

The stepwise commitment of T follicular helper cells via ICOS signaling pathways

*Pedros, C.*¹, *Zhang, Y.*², *Hu, J.*³, *Choi, Y.S.*^{3,4}, *Canonigo-Balancio, A.*¹, *Yates, J.*², *Altman, A.*¹, *Crotty, S.*^{3,5}, *Kong, K.F.*¹

¹La Jolla Institute for Allergy and Immunology, Division of Cell Biology, La Jolla, United States, ²Scripps Research Institute, Department of Chemical Physiology, La Jolla, United States, ³La Jolla Institute for Allergy and Immunology, Division of Vaccine Discovery, La Jolla, United States, ⁴Seoul National University, Department of Medicine, Seoul, Korea, Republic of, ⁵University of California San Diego, Division of Infectious Diseases, La Jolla, United States

CD4⁺ T cells undergo sequential phases of differentiation and maturation to become mature *bona fide* germinal center (GC) T follicular helper (Tfh) cells, which support the GC reaction and promote the production of high-affinity antibodies. ICOS plays a critical role in Tfh cell development and the only protein so far known to interact with the cytoplasmic tail of ICOS is PI3K. Indeed, PI3K signaling was found to play an important role in Tfh cell differentiation and functions. However, independent studies demonstrated that several events associated with Tfh differentiation are independent of ICOS-mediated PI3K signaling, implying additional, undefined ICOS signaling pathways. Here, we have unraveled a novel signaling pathway emanating from ICOS, which is critical for the full maturation of GC-residing Tfh cells. Specifically, we have:

- i) identified a previously unknown, evolutionarily conserved membrane proximal ICOS motif, IProx, that is required for Tfh development and function;
- ii) identified TBK1 as an activation-induced interacting partner of this motif;
- iii) demonstrated that the recruitment of TBK1 to ICOS is required for the development of fully mature GC Tfh cells while being dispensable for the differentiation of early, nascent Tfh cells; and
- iv) identified the canonical TBK1-binding consensus sequence that is shared between the IProx motif and TRAF2/3 molecules. Therefore, our findings reveal a novel ICOS-TBK1 signaling pathway that specifies Tfh cell commitment to GC reactions. Strategic manipulations of these ICOS-dependent pathways could lead to better vaccine design and treatment of autoimmune diseases.

3088

Peroxisome proliferator activated receptor gamma promotes type 2 immune responses

Coquet, J.

Karolinska, MTC, Stockholm, Sweden

T helper (Th) 2 cells are central to the development of asthma and for immunity to nematode infection. Fully differentiated Th2 cells are marked by their expression of the IL-33 receptor (IL-33R), which promptly induces IL-5 and IL-13 production when ligated. In this study, we report that Th2 cells expressed high levels of the nuclear receptor peroxisome proliferator activated receptor gamma (PPAR-gamma) in models of asthma and following infection with *Heligmosomoides polygyrus*. Mice lacking PPAR-gamma in T cells failed to effectively differentiate into IL-5- and IL-13-secreting cells and as such, did not develop Th2 cell-associated pathologies including goblet cell metaplasia and eosinophilia in response to allergen challenge. Nor did these mice mount protective immune responses to nematode infection. Furthermore, in visceral adipose tissue where PPAR-gamma is important for Treg cell maintenance, we demonstrate that PPAR-gamma also promoted resident Th2 cells and type 2 innate lymphoid cells. Mechanistically, activation of PPAR-gamma was able to induce the expression of surface ST2 *in vivo* and *in vitro* on Th2 cells and ILC2. This study conclusively demonstrates that PPAR-gamma promotes type 2 immune responses and suggests that antagonists of this factor may prevent unwanted allergic immune responses.

3581

Identification of human follicular helper T cells specific miRNAs key to differentiation and function

Lorenzo, M.¹, Ripamonti, A.¹, Provasi, E.¹, De Simone, M.¹, Rossetti, G.¹, Curti, S.¹, Ranzani, V.¹, Arrigoni, A.¹, Bonnal, R.J.¹, Torretta, S.², Pignataro, L.², Pagani, M.³, Abrignani, S.³

¹Fondazione INGM, Milan, Italy, ²Fondazione IRCCS Policlinico di Milano, Milan, Italy, ³Fondazione INGM - University of Milan, Milan, Italy

Follicular helper T cells (T_{FH}) are a CD4⁺T helper subset specialized in helping B cells. T_{FH} cells are critical for humoral immunity, including the generation of long-lived and high affinity plasma cells and memory B cells. Alteration of T_{FH}-specific genes leads to defects in germinal centers (GCs) formation, with consequent autoimmune or immunodeficiency disorders. The molecular mechanisms underlying human T_{FH} cells differentiation are poorly understood. MicroRNAs (miRNAs) are highly conserved non-coding single-stranded small RNA molecules that control gene expression post-transcriptionally by binding the 3' untranslated region of target mRNA. To investigate the role of miRNAs in T_{FH} cells biology we performed RT-qPCR and deep sequencing analysis on T_{FH} and Naive CD4⁺ T cells sorted from secondary lymphoid organs (SLO). We defined a T_{FH}-specific signature of 17 miRNAs that are up or down-regulated in T_{FH} cells compared to other CD4⁺ effector T cells. We showed that miR-31-5p (miR-31) displayed the strongest down-modulation in T_{FH} cells, and found that miR-31 gene promoter is bound by Bcl6, the master transcription factor of T_{FH}, suggesting that that Bcl6 can repress miR-31 expression by binding to regulatory regions in its locus. We combined target prediction with gene-expression analysis and identified CD40L, BTLA and SAP (i.e., key molecules in T_{FH} biology) as predicted miR-31 targets. Modulation of miR-31 expression in T_{FH} cells proved that miR-31 directly regulates the expression of these genes and that increased levels of miR-31 impair B-helper activity of T_{FH} cells suggesting that miR-31 contribute to the modulation of their functional activity.

3021

A dominant function of transcription factor Foxo1 in the development of Th9 and IL-9-producing T cells

Malik, S., Sadhu, S., Awasthi, A.

Translational Health Science and Technology Institute, Faridabad, India

Interleukin 9 (IL-9), a pleiotropic cytokine of common γ -chain cytokine receptor family, plays a crucial role in allergic inflammation, autoimmunity and immunity to extracellular pathogens. Recent identification of anti-tumor function of IL-9 makes it an attractive target for anti-tumor therapy. Although Th2, Th17 cells and Foxp3⁺ Treg cells were shown to produce IL-9, more specialized IL-9-producing Th9 cells were identified as a distinct subset of Th cells that are proinflammatory *in vivo*. The master transcription factors that regulates IL-9 production in Th2, Th9 and Th17 cells has not been identified, although IRF-4, PU.1, Batf and IRF1 were shown to be essential for IL-9 induction in Th9 cells. Here we have identified Foxo1, a forkhead family transcription factor, predominantly required for the induction

of IL-9 in Th9 and Th17 cells. We further identified AKT, an upstream kinase that regulates IL-9 induction in Th1, Th9 and Th17 cells via Foxo1. In addition, c-Jun N-terminal kinase (JNK), a MAPK (Mitogen Activated Protein kinase) enhanced IL-9 induction in Th9 cells via Foxo1. Chromatin Immunoprecipitation identified a direct physical association of Foxo1 with IL-9 and IRF4 promoters. In addition, Foxo1 transactivates both IL-9 and IRF4 genes in Th9 and Tc9 cells, and together with IRF-4 synergistically enhanced IL-9 induction. Furthermore, loss of Foxo1 suppressed IL-9 production in Th9 and Th17 cells. In Th9 mediated asthma model, Foxo1 inhibition substantially ameliorated allergic inflammation and IL-9 induction. Our findings thus identify Foxo1 is the key transcription factor in controlling the development of Th9 and IL-9-producing Th cells.

3542

Tumor suppressor death-associated protein kinase inhibits Th17 by targeting cytoplasmic HIF-1 α for degradation

Chou, T.-F.^{1,2}, Chuang, Y.-T.³, Hsieh, W.-C.¹, Chang, P.-Y.¹, Hsu, T.-S.¹, Miaw, S.-C.⁴, Chen, R.-H.⁵, Kimchi, A.⁶, Lai, M.-Z.¹

¹Academia Sinica, Institute of Molecular Biology, Taipei, Taiwan, Republic of China, ²National Defense Medical College, Institute of Life Sciences, Taipei, Taiwan, Republic of China, ³National Taiwan University Hospital, Department of Medical Research, Taipei, Taiwan, Republic of China, ⁴National Taiwan University, Institute of Immunology, Taipei, Taiwan, Republic of China, ⁵Academia Sinica, Institute of Biological Chemistry, Taipei, Taiwan, Republic of China, ⁶Weizmann Institute of Science, Department of Molecular Genetics, Rehovot, Israel

Death-associated protein kinase (DAPK) is known for its tumor suppressor function, while hypoxia-inducible factor 1 α (HIF-1 α) plays a prominent role in tumorigenesis. Here we report an antagonism between DAPK and HIF-1 α in T helper 17 (Th17) differentiation. HIF-1 α is known to promote Th17 development. We show that DAPK inhibits Th17 and prevents Th17-mediated autoimmune diseases. We identified that DAPK specifically downregulates HIF-1 α in a novel process. In contrast to the predominant nuclear localization of HIF-1 α in many cancer cells, HIF-1 α is located in both cytosol and nucleus in T cells, allowing a cytosolic interaction between DAPK and HIF-1 α . DAPK also binds prolyl hydroxylase domain protein 2 (PHD2) and increases HIF-1 α -PHD2 association. DAPK thereby promotes the proline hydroxylation and proteasome degradation of HIF-1 α . Consequently, DAPK-deficiency led to excess HIF-1 α accumulation, enhanced IL-17 expression, and exacerbated experimental autoimmune encephalomyelitis (EAE). Transgenic DAPK expression suppresses Th17 differentiation and prevents EAE generation. Our results demonstrate a new process involving DAPK-mediated degradation of cytoplasmic HIF-1 α , and suggest that a potential therapy for Th17-associated inflammatory diseases by targeting to DAPK.

1508

Early growth response gene (Egr) 2 and 3 control Th1 differentiation by directly inhibiting T-bet

Miao, T.¹, Li, S.², Symonds, A.¹, Wang, P.¹

¹BICMS, Queen Mary University of London, Immunology, London, United Kingdom, ²Brunel University, Immunology, London, United Kingdom

Egr2 and 3 are inducible transcription factors in T cells that are essential to maintain self-tolerance. In the absence of Egr2 and 3, effector phenotype T cells are hyper-inflammatory resulting in autoimmune diseases. We have now discovered that Egr2 and 3 control Th1 differentiation. Egr2 and 3 deficiency in T cells not only results in excessive Th1 differentiation in unpolarized or Th1 polarized conditions, but a substantial fraction of Egr2 and 3 deficient CD4 cells produced IFN γ under Th2 and Th17 conditions. Significantly, the IFN γ producing cells in Th2 and Th17 conditions did not co-produce IL4 and IL17, indicating Egr2 and 3 inhibit Th1 differentiation in Th2 and Th17 conditions rather than re-directing Th2 or Th17 programmes to Th1. Egr2 and 3 are co-induced with T-bet in Th2 and Th17 conditions. However, in Th1 conditions, T-bet positive cells express low levels of Egr2 and 3. We found that Egr2 and 3 physically interact with T-bet in CD4 T cells, which potently inhibits T-bet mediated IFN γ expression and Th1 differentiation. Egr2 and 3 are induced by antigen stimulation in naive T cells, but this induction is inhibited by Th1 cytokines such as IFN γ and IL12, but not by Th2 and Th17 cytokines. The inhibition of Egr2 and 3 expression by Th1 cytokines is essential for efficient Th1 differentiation. Our findings reveal a novel mechanism of Egr2 and 3 function in controlling Th differentiation.

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PU.1 expressing Th9 cells promote colitis-associated cancer (CAC)

Gerlach, K.¹, Wirtz, S.¹, McKenzie, A.², Neurath, M.F.¹, Weigmann, B.¹

¹Medical Clinic 1, Erlangen, Germany, ²MRC Laboratory of Molecular Biology, Cambridge, United Kingdom

Inflammatory bowel disease (IBD) is linked to an increased risk of developing colitis-associated colorectal cancer (CAC). In IBD especially T cells are critical mediators playing important roles in colorectal cancer. Recently, we have identified Th9 cells as IL-9 producing cells inducing colitis. The pro inflammatory cytokine IL-9 is important in inflammatory disease, but the involvement of IL-9 in CAC is not revealed yet.

IL-9 KO mice were treated with AOM/DSS to induce colon cancer. Miniendoscopic analysis was done to monitor number and size of tumors. For restoration of the IL-9 deficient phenotype an IL-9 expressing minicircle DNA vector was injected into IL-9 deficient mice and the model of AOM/DSS has been investigated. Colonic tumors have been isolated, histological sections were taken out and immunofluorescent analysis was done.

IL-9 deficiency led to significant less inflammation consequently followed with a significant reduction of tumors in the experimental model of AOM/DSS. Furthermore, we explored the effect of IL-9 on tumor growth by overexpression of IL-9 via mini-circle DNA and AOM/DSS treatment, which led to the

development of tumors when IL-9 is absent. Additionally, in cryosections of tumorigenic colon tissue more PU.1+ T-cells were found, pointing out the important relevance for Th9 cells in colon cancer.

Our findings uncover a crucial role of IL-9 in the development of colitis-associated neoplasias as IL-9 led to tumor growth in the model of CAC. The presence of PU.1+ CD4+ -expressing cells in tumorigenic tissue suggests that these cells belong to the recently described Th9 T cell subset.

Immunity to Viruses 2

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Reversed T-cell receptor docking on Major Histocompatibility Class I complexes within the naïve T-cell repertoire limits involvement in the immune response

Gras, S.^{1,2}, Chadderton, J.³, Del Campo, C.M.^{1,2}, Farenc, C.¹, Wiede, F.¹, Josephs, T.M.¹, Mirams, M.³, Sng, X.Y.X.³, Watson, K.³, Tiganis, T.¹, Quinn, K.M.³, Rossjohn, J.^{1,2,4}, La Gruta, N.L.^{1,3}

¹Monash University, Infection and Immunity Program, Monash Biomedicine Discovery Institute, Clayton, Australia, ²ARC Centre of Excellence in Advanced Molecular Imaging, Monash University, Clayton, Australia, ³University of Melbourne, Peter Doherty Institute for Infection and Immunity, Melbourne, Australia, ⁴Institute for Infection and Immunity, Cardiff University School of Medicine, Cardiff, United Kingdom

The anti-viral CD8⁺ T-cell response is drawn from the naïve T-cell repertoire. Selective recruitment and expansion from the available naïve T cell set can significantly shape the characteristics of the responding immune CD8⁺ T cell population. During influenza A virus (IAV) infection, for example, the CD8⁺ T-cell response to an MHC class I H2-D^b-restricted nucleoprotein epitope (NP₃₆₆) is characterized by the preferential expansion of cells that express the T cell receptor beta chain variable region gene (TRBV) 13-1 from the naïve repertoire. In contrast, the TRBV17⁺ set, despite comprising around 25% of all naïve H2-D^bNP₃₆₆-specific cells, is almost completely avoided in the immune response (< 1%). To define the structural basis of this suboptimal recruitment, we examined the docking mode of two TRBV17⁺ TCRs and, strikingly, found that they bound H2-D^bNP₃₆₆ with a 180° reversed polarity in comparison to the canonical TCR-peptide+MHCI (pMHCI) docking mode. The TRBV17⁺ TCR beta chains dominated the interaction, mediating contacts at the C-terminal end of the peptide-binding cleft. Despite this reversed docking mode, the TRBV17⁺ TCRs showed exquisite specificity but a moderate affinity for H-2D^bNP₃₆₆ corresponding to a limited profile of cytokine production after IAV infection. Thus, naturally occurring T-cells in the naïve pool express TCRs with reversed polarity pMHC-I docking modes that confer antigen specificity and functionality, but limit their ability to contribute to the immune response. These data challenge the concept of a germline bias for TCR recognition of pMHC.

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Links between specificity and function in CD4 T cell immunity to influenza virus

Sant, A.¹, Treanor, J.², Nayak, J.³, Richards, K.¹, Knowlden, Z.¹, Dipiazza, A.¹

¹University of Rochester Medical Center, Microbiology and Immunology, Rochester, United States, ²University of Rochester Medical Center, Infectious Diseases, Rochester, United States, ³University of Rochester Medical Center, Pediatrics, Rochester, United States

Memory CD4 T cells contribute a multiplicity of functions and at discreet sites in vivo to protective immunity to influenza virus. Our studies have revealed that in both humans and in animal models of infection and vaccination, the specificity of CD4 T cells is exceptionally broad and includes CD4 T cells specific for virtually all viral proteins, including the most genetically conserved proteins such as NP and M1. However, we have found that some functions of CD4 T cells are selectively conveyed by cells of limited specificity. For example, HA- but not NP-specific CD4 T cells can promote neutralizing antibody responses to influenza and are selectively enriched in expression of CXCR5 in human circulation. This restriction in function is likely in part responsible for the relatively poor neutralizing antibody responses to avian and potentially pandemic influenza viruses and vaccines. Other functions, such as cytotoxicity, are selectively conveyed by CD4 T cells specific for internal virion proteins. Thus, although the circulating memory CD4 repertoire specific for influenza virus is typically abundant and diverse, these complexities in functional potential pose a formidable obstacle to predicting protective immune response to potentially pandemic strains of influenza and in devising strategies to potentiate these responses. Our results suggest that more precise efforts to identify and enumerate both the positive and negative contributors within the CD4 T cell compartment will aid significantly in the achievement of these goals.

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A systems approach to understanding the role of MAVS in Ebola pathogenesis in murine models of infection

Dutta, M.¹, Robertson, S.², Okumura, A.², Rasmussen, A.¹, Chang, J.¹, Weiss, J.M.¹, Katze, M.G.¹, Best, S.²

¹University of Washington, Department of Microbiology, Seattle, United States, ²Rocky Mountain Laboratories, NIAID, NIH, Hamilton, United States

Ebola virus causes lethal hemorrhagic fever in humans and is a threat to biosecurity and public health. Wild-type Ebola virus (WT-EBOV) is not lethal in immunocompetent mice, although virus adaptation to mice is associated with mutations that facilitate replication and increase virulence. In the mouse model, type I interferon is required for resistance to WT-EBOV, but little is known regarding the signaling pathways that coordinate this response in vivo. EBOV infection is detected by RIG-I-like receptors (RLR) that signal through the adapter molecule, mitochondrial antiviral signaling protein (MAVS) to induce innate immunity. To elucidate how MAVS signaling determines cellular

responses to EBOV, we infected MAVS knockout mice (MAVS KO) with WT- or mouse adapted (MA)- EBOV. Compared to C57BL/6 mice, MAVS KO mice showed increased susceptibility to MA-EBOV and succumbed to infection by 5-6 days post-infection (dpi). Analysis of virus replication demonstrated that WT-EBOV replicated in spleen and liver to higher titers than MA-EBOV at 3 dpi in MAVS KO mice. Despite this, replication of WT-EBOV was controlled by 5 dpi whereas MA-EBOV replication increased. Differential expression analysis demonstrated significant differences in host responses when comparing WT- and MA-EBOV, or MAVS KO and C57BL/6 mice. Using a deconvolution approach with digital cell quantification, we demonstrated that pDCs drive MAVS-independent resistance to WT-EBOV in C57BL/6 mice, whereas macrophages have a dominant role in MAVS-dependent resistance to EBOV. These findings suggest that cell-specific responses determine pathogenic outcome. Therapeutic targeting of these cells with RLR agonists may improve clinical outcomes in EBOV-infected humans.

3545

Deep profiling of the murine myelopoietic system: signaling and cell cycle responses to neurotropic viral infection profiled by flow and mass cytometry (CyTOF)

Ashhurst, T.^{1,2}, Niewold, P.¹, Cox, D.¹, Smith, A.^{2,3}, King, N.^{1,2,3}

¹The University of Sydney, Pathology, Sydney, Australia, ²The University of Sydney and the Centenary Institute, The Ramaciotti Facility for Human Systems Biology (RFHSB), Sydney, Australia, ³The University of Sydney and the Centenary Institute, Sydney Cytometry Facility, Sydney, Australia

Viral infection of the central nervous system (CNS) results in a rapid influx of bone marrow (BM)-derived monocytes/macrophages, that ultimately induce fatal pathology in the mouse. Whilst these cells are derived from the BM, little is known about the kinetic and migratory events that mobilise BM monocytes and their progenitors in response to CNS infection. In this study we conducted comprehensive mapping of the murine haemopoietic system in the BM using high-dimensional single cell flow and mass cytometry (CyTOF). Initially, we used this approach to refine the identification of myeloid developmental intermediates involved in monopoiesis and granulopoiesis, during steady state and inflammation. Additionally we examined the expression of the transcription factor IRF8 across the haematopoietic system and its role in instructing myeloid developmental trajectories. During viral encephalitis, we found a reorganisation of cellular outputs to favour monocyte production, resulting in activation and expansion of monocytes and monocyte progenitors, with increased proliferation of mature and progenitor populations. In addition, we observed compensatory downregulation of B-cell lymphopoiesis, and modification of granulopoiesis in the BM, favouring monocyte expansion. The upregulation of the interferon (IFN)-inducible marker SCA-1 on specific lineages suggested a role for IFN signalling, and implicates IFN-gamma (IFN-g) producing NK and T-cells in the BM in this process. As such, antibody blockade of IFN-g resulted in a reduction of monocyte proliferation, associated with improved clinical outcomes. In this study we have used high-dimensional cytometry approaches, and have

characterized modifications to the haematopoietic lineage during viral encephalitis that favour production of pathogenic monocytes.

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Type I interferon preferentially induces GITRL on inflammatory APC compared to classical DC to establish early viral control during chronic LCMV infection

Chang, Y.-H., Clouthier, D.L., Zhou, A., Abdul-Sater, A., Watts, T.H.
University of Toronto, Department of Immunology, Toronto, Canada

Disease outcome in chronic viral infections correlates with early viral set-point, established by the initial T cell response. Previous studies showed that GITR, an NFκB-activating TNFR family member, sustains CD4 T cell accumulation and help for CD8 T cells early during chronic LCMV infection. While the endogenous effect of GITR on CD8 T cells is largely attributed to enhanced early CD4 T cell help, CD8 T cells are directly responsive to exogenous GITR agonist. How endogenous GITR co-stimulation selectively impacts CD4 T cells early during LCMV infection, however, remains elusive. We hypothesize that CD4 and CD8 T cells interact with distinct APC and that the availability of GITRL underscores the regulation of GITR co-stimulation. Here we identified inflammatory monocyte-derived DC and macrophages (infMΦ) as the dominant GITRL-expressing APC with almost 5 times higher GITRL compared to classical DC during LCMV infection. A preliminary experiment showed that deleting exon 2 of GITRL with Lys-M-Cre recapitulates the marked reduction in Th1 response against LCMV observed in the global GITR knockout. During LCMV infection, GITRL expression peaks just after type I IFN expression. Consistently, type I IFN is a potent inducer of GITRL *ex vivo* and blockade of the type I IFN receptor abrogates up-regulation of GITRL on macrophages during LCMV infection. Together, the current study suggests a critical role for infMΦ in GITR-dependent CD4 T cell immunity to LCMV and identifies type I IFN as a key regulator of GITRL on infMΦ *in vivo*. Supported by the Canadian Institutes of Health Research.

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NADPH Oxidase 1 (NOX1) is associated with altered host survival and T cell phenotypes after influenza A virus infection in mice

Hofstetter, A.¹, De La Cruz, J.¹, Cao, W.¹, Patel, J.¹, Belser, J.¹, Liepkalns, J.¹, Amoah, S.¹, McCoy, J.², Cheng, G.², Diebold, B.², Shieh, W.-J.³, Zaki, S.³, Katz, J.¹, Sambhara, S.¹, Lambeth, J.D.², Gangappa, S.¹

¹Centers for Disease Control and Prevention, Influenza Division, Atlanta, United States, ²Emory University, Pathology and Laboratory Medicine, Atlanta, United States, ³Centers for Disease Control and Prevention, Infectious Disease Pathology Branch, Atlanta, United States

The role of the reactive oxygen species-producing NADPH-oxidase (NOX) family of enzymes in the pathology of influenza A virus infection remains enigmatic. Previous reports implicated NOX2 in influenza A virus-induced inflammation. In contrast,

NOX1 was reported to decrease inflammation in mice within 7 days post influenza A virus infection. However, the effect of NOX1 on lethality and adaptive immunity after influenza A virus challenge has not been explored. Here we report improved survival and decreased morbidity in NOX1-deficient mice compared with controls after challenge with A/PR/8/34 influenza A virus. While changes in lung inflammation were not obvious between NOX1-deficient and control mice, we observed alterations in the T cell response to influenza A virus by day 15 post-infection, including increased interleukin-7 receptor-expressing virus-specific CD8⁺ T cells in lungs and draining lymph nodes of NOX1-deficient mice, and increased cytokine-producing T cells in lungs and spleen. Furthermore, a greater percentage of conventional and interstitial dendritic cells from NOX1-deficient draining lymph nodes expressed the co-stimulatory ligand CD40 within 6 days post-infection. Results indicate that NOX1 modulates the innate and adaptive cellular immune response to influenza virus infection, while also playing a role in host survival. Results suggest that NOX1 inhibitors may be beneficial as adjunct therapeutics during acute influenza infection.

2225

Towards identification of immune and genetic correlates of severe influenza disease in Indigenous Australians

Clemens, E.B.¹, Grant, E.J.², Wang, Z.¹, Gras, S.^{3,4}, Mifsud, N.³, Illing, P.³, Tipping, P.⁵, Purcell, A.W.³, Rossjohn, J.^{2,3,4}, Miller, A.⁶, Tong, S.Y.C.⁵, Kedzierska, K.¹

¹The University of Melbourne, at the Peter Doherty Institute for Infection and Immunity, Department of Microbiology and Immunology, Melbourne, Australia, ²Institute of Infection and Immunity, Cardiff University School of Medicine, Cardiff, United Kingdom, ³Infection and Immunity Program, Monash Biomedicine Discovery Institute and Department of Biochemistry and Molecular Biology, Monash University, Clayton, Australia, ⁴Australian Research Council Centre of Excellence for Advanced Molecular Imaging, Monash University, Clayton, Australia, ⁵Menzies School of Health Research, Casuarina, Australia, ⁶Indigenous Research Network, Griffith University, Nathan, Australia

We have investigated immune and genetic factors that could predispose Indigenous Australians to severe influenza disease. We surveyed HLA allelic frequencies in a cohort of Indigenous Australians (n=82), which confirmed previous studies showing predominant usage of HLA-A*02:01, 11:01, 24:02, 34:01 and HLA-B*13:01, 15:21, 40:01/02, 56:01/02. We also identified two novel HLA alleles (HLA-A*02:new and HLA-B*56:new), with homology modelling suggesting that variations within HLA-A*02:new will affect peptide binding. There is a paucity of known influenza epitopes for the majority of these HLAs, with the exception of a universal HLA-A*02:01-M1₅₈ epitope and proposed epitopes presented by HLA-A*11:01/HLA-A*24:02. To dissect universal CD8⁺ T-cell responses, we analysed the magnitude, function and T-cell receptor (TCR) clonality of HLA-A*02:01-M1₅₈⁺CD8⁺ T-cells. We found comparable IFN- γ , TNF and CD107a and TCR α characteristics in Indigenous and non-Indigenous Australians, suggesting that the ~15% of Indigenous people that express HLA-A*02:01 have universal influenza-

specific CD8⁺ T-cell immunity. Furthermore, the frequency of a polymorphic host risk factor, IFITM3-C/C, was comparable between Indigenous Australians and Europeans, suggesting that expression of this allele does not explain increased influenza severity. This suggests that failure of the predominant HLA allomorphs maintained within Indigenous populations to generate a diverse antiviral response may predispose them to more severe influenza disease. Therefore, we undertook peptide presentation studies that identified novel influenza epitopes restricted by HLA-A*24:02, an allele expressed by ~25% of Indigenous Australians. Characterisation of such novel influenza-specific CD8⁺ T-cell epitopes restricted by HLA alleles prevalent in Indigenous populations will inform future rational design of universal T-cell vaccines.

3670

Protective monotherapy against lethal ebola virus infection: structural and molecular basis of potent neutralization

Sullivan, N.J.

NIAID, National Institutes of Health, Vaccine Research Center, Bethesda, United States

Ebola virus disease in humans is highly lethal, with case fatality rates ranging from 25-90%. There is no licensed treatment or vaccine against the virus, underscoring the need for efficacious countermeasures. Here, we demonstrate that a human survivor of the 1995 Kikwit Ebola virus disease outbreak maintained circulating antibodies against the Ebola virus surface glycoprotein for more than a decade after infection. From this survivor we isolated monoclonal antibodies (mAb) that neutralize recent and previous outbreak strains of Ebola virus, and mediate antibody-dependent cell-mediated cytotoxicity *in vitro*. Strikingly, monotherapy with mAb114 protected macaques when given as late as five days after challenge. We defined the mAb binding epitope on Ebola GP and show that blocking a single functional domain in Ebola GP by mAb114 is sufficient for complete protection of primates. These results define a basis of neutralization for protective mAbs, and demonstrate that treatment with a single human mAb as a therapeutic strategy for human Ebola infection may be possible.

2769

Harnessing innate immune responses to inhibit influenza H1N1 infection

Poux, C.¹, Dondalska, A.¹, Järver, P.¹, Contreras, V.², Le Grand, R.², Spetz, A.-L.¹

¹Stockholm University, Department of Molecular Biosciences The Wenner-Gren Institute, Stockholm, Sweden, ²Commissariat à l'Énergie Atomique, UMR 1184, IDMIT Center, Institute for Emerging Diseases and Innovative Therapies, DSV, Fontenay-aux-Roses, France

Influenza viral infection leads to the activation of pattern recognition receptors responding to dsRNA with subsequent pro-inflammatory cytokine and interferon responses. Influenza viruses are often sensitive to interferons but extensive pro-

inflammatory responses contribute to lethal pathology. This is exemplified in TLR3 knock out mice, which have been shown to have increased survival compared with wild-type mice upon influenza infection. To further address the role of dsRNA-induced signaling during influenza infection, we treated influenza H1N1 (IAV) infected human monocyte-derived DCs (MoDCs) with an inhibitor of TLR3 (a single stranded oligonucleotide (ssON)) and/or the synthetic dsRNA analogue, poly I:C. Flow cytometry was used to measure the frequency of IAV nucleoprotein positive cells and the expression of co-stimulatory molecules. The IAV infected cells did not upregulate CD80 and CD86, while the neighboring nucleoprotein negative cells upregulated both co-stimulatory molecules. The impeded MoDC maturation, in IAV infected cells, could not be overcome with polyI:C stimulation. Although the TLR3 inhibitor ssON was able to prevent poly I:C-induced MoDC maturation, it was incapable of blocking MoDC maturation in IAV exposed but non-infected cells. The IAV loads in the cultures were approximately 1×10^5 vp/ml, as measured by real-time PCR, and the combined stimulation of both polyI:C and ssON resulted in a significant reduction in IAV production and/or release (to under 100 vp/ml). Moreover, intradermal injection of macaques with both polyI:C and ssON led to induction of multiple viral restriction factors, but without concomitant pro-inflammatory response. These findings suggest that harnessing innate immunity engaged by oligonucleotides can inhibit IAV infection.

Antigen Processing & Presentation

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MHC class II antigen-processing chaperone H2-O regulates thymic selection in vivo

Sadegh-Nasseri, S., Welsh, R., Song, N.

Johns Hopkins University, Pathology, Baltimore, United States

HLA-DO or H2-O (DO) in mice is a non-classical, non-polymorphic MHC II molecule primarily expressed in the thymic medulla and B cells. While the common belief is that DO inhibits the activity of DM, controversial data opposes that point of view. Physicochemical studies with purified DO, DM and HLA-DR1 demonstrated that DO interacts directly with HLA-DR1 molecules in a peptide-receptive-conformation, augmenting binding of 'DM-resistant' peptides, while inhibiting binding of 'DM-sensitive' peptides to HLA-DR1. We hypothesized that by optimizing presentation of self-epitopes in thymic medulla, thus increasing the density of presented epitopes, DO might ensure full execution of negative selection. We tested this hypothesis in H2-O-Knockout (DO-KO) mice in two ways:

A) a novel 'Prime-Restim' strategy, and

B) by monitoring disease development and severity in a murine model of experimental autoimmune encephalomyelitis (EAE).

In the former, CD4 T cells from DO-WT were immunized and restimulated repeatedly with antigen presenting cells from the DO-KO mice. The results demonstrated that in DO-KO mice, the peptide repertoire of MHC II is different from that of the DO-WT mice. In the latter approach, we found that DO-KO mice develop EAE considerably faster than DO-WT mice. This enhancement in disease onset correlated with

increased numbers of MOG-tetramer-specific-CD4 T cells collected from the CNS of DO-KO mice during the disease, or from periphery in naïve DO-KO mice. Thus, loss of DO correlates with poor deletion of self-reactive T cells leading to larger numbers of MOG-specific T cells, as well as varied epitope selection for presentation by MHCII.

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MR1 is an endoplasmic reticulum-resident sensor of vitamin B metabolites

McWilliam, H.¹, Eckle, S.¹, Theodossis, A.¹, Liu, L.², Chen, Z.¹, Fairlie, D.², Strugnell, R.¹, Mintern, J.³, McCluskey, J.¹, Rossjohn, J.⁴, Villadangos, J.^{1,3}

¹University of Melbourne, Microbiology and Immunology, Melbourne, Australia, ²The University of Queensland, Institute for Molecular Bioscience, Brisbane, Australia, ³Bio21, Biochemistry, Melbourne, Australia, ⁴Monash University, Biochemistry and Molecular Biology, Melbourne, Australia

Antigen presentation by Major Histocompatibility Complex (MHC) and MHC-I like molecules is central to the cellular arm of adaptive immunity. While the cellular pathways of peptide and lipid antigen presentation by MHC and CD1 molecules are well established, our understanding of the presentation of small molecule metabolites is unclear. MR1 is an MHC-I like molecule that presents vitamin B metabolites to MAIT cells, and while diverse cell types ubiquitously express MR1, it only egresses to the cell surface in the presence of antigen(s). We show that, unlike other antigen-presenting molecules, human MR1 does not appear to constitutively present self-ligands, but accumulates in the endoplasmic reticulum (ER) in a ligand-receptive conformation. Here, a lysine residue within MR1 forms a Schiff base with vitamin B metabolites, thereby acting as a "molecular switch" that allows folding and egress of MR1 molecules. Newly formed MR1-antigen complexes follow the secretory pathway, whereupon the half-life of MR1-antigen complexes expressed on the plasma membrane was several hours and not significantly different when ligand was bound. After exposure on the plasma membrane, MR1-antigen complexes underwent endocytosis and degradation, however some recycling back to the cell surface and ligand exchange could be detected. Thus MR1 presentation is characterised by an off-on-off mechanism, where in the steady state the majority of MR1 molecules reside in the ER until they encounter extracellular metabolites. Accordingly the MR1-mediated metabolite presentation is distinct from that of the constitutive presentation of peptides and lipids.

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Immunology by numbers: quantitation of antigen presentation completes the quantitative milieu of systems immunology

Purcell, A.

Monash University, Biochemistry, Clayton, Australia

The classic quantitative tools of immunology have provided critical insights into immune outcome, recent advances have

now taken this to a cell specific and in some circumstances even single cell level of resolution. For example MHC tetramers have allowed the enumeration and characterisation of epitope specific lymphocytes. More recently, the development of mass spectrometry based approaches have provided new avenues to investigate the specificity of the immune response and to address the missing gap of systems wide epitope quantitation on the surface of antigen presenting cells. I will present recent data that highlights the discovery and quantitation of anti-viral epitopes, self epitopes and neoepitopes using next generation mass spectrometric approaches including multiple reaction monitoring and data independent acquisition (SWATH) - mass spectrometry. Within this context we have developed several novel workflows that allow simultaneous measurement of antigen expression and epitope presentation, the identification of post-translationally modified T cell epitopes and differential antigen processing that promotes T cell heterogeneity and immune evasion. Incorporation of this information into system based models facilitates the study of infectious immunity, autoimmunity and allergy at a system wide level.

1953

Highly efficient cross-priming of CD8+ T cells by splenic CD169 macrophages employing a unique endosomal processing pathway

Mauvais, F.-X.¹, Hamel, Y.², Garfa, M.³, Diana, J.¹, van Endert, P.¹
¹INSERM U 1151, Paris, France, ²INSERM U 1163, Paris, France,
³INSERM US 24, Paris, France

Splenic CD169+ marginal zone macrophages (MZM) are strategically located for uptake of soluble and particulate blood-borne antigens. However their role in T cell priming is commonly considered limited to antigen capture and transfer to adjacent dendritic cells (DC). To critically evaluate this concept, we developed a novel protocol allowing for the first time to purify these cells and examine their function in vitro. Purified CD169+ MZM are clearly distinct from dendritic cells by size and morphology and by expression of key genes and of a vast panel of surface receptors. In contrast to current concepts, we demonstrate that purified CD169+ MZM cross-present receptor-targeted antigen as well as CD8+ DCs, and soluble and particulate antigen with greater efficacy. Consistent with this, antigen targeting to CD169+ and DEC205 in vivo induce T cell proliferation with equivalent efficiency. Intravital biphoton live imaging reveals rapid T cell arrest on CD169+ MZM upon antigen targeting.

We also examined the intracellular cross-presentation pathway employed by CD169+ MZM. Although, in the case of DCs, strictly endosomal ("vacuolar") cross-presentation is considered inefficient, we find that, in CD169+ MZM, endosomal processing and presentation of internalized antigen is highly efficient, possibly due to a unique MHC class I recycling pathway. Thus, CD169+ MZM belong together with CD8+ DCs to a small group of professional antigen-presenting cells capable of highly efficient cross-priming. The effector functions primed by CD169+ MZM and the quality of ensuing anti-infectious immune responses are under investigation and will be presented.

2909

In vivo biosensors for pathogen physiological functions reveal distinct effector T cell - APC interaction dynamics depending on intracellular pathogen proliferation

Heyde, S.¹, Formaglio, P.¹, Bousoo, P.², Müller, A.^{1,3}

¹Otto-von-Guericke University, Institute for Molecular and Clinical Immunology, Magdeburg, Germany, ²Institut Pasteur, Immunology Department, Paris, France, ³Helmholtz Centre for Infection Research, Braunschweig, Germany

Pathogen physiology has a profound impact on the outcome of infections: Growth rate and metabolic activity within the pathogen population are important for persistence during chronic infections, but can also have dramatic effects on the deployment of immune responses. How immune cells can distinguish pathogens according to their physiological state, or impact pathogen physiology on a cellular level, is unclear. This is mainly due to difficulties in analyzing at the same time the dynamics of immune cells and pathogen physiology *in vivo*.

To overcome this limitation, we have developed genetically encoded fluorescent reporter systems which permit probing pathogen physiology by intravital 2-photon microscopy. Consequently, we could determine on a single cell level, and longitudinally over the course of an infection, the proliferation and cell death of the intracellular parasite *Leishmania major*. Furthermore, we were able to characterize the cellular niche of high proliferating versus low proliferating pathogens, and could show that pathogen-specific effector

T cells interact preferentially with APCs infected with high proliferating parasites.

Our approach thus provides for the first time an insight into how cells of the immune system recognize and influence distinct pathogen physiological states in the on-going infection.

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The modulation of cellular protease activities by HIV protease inhibitors alters the processing, direct and cross-presentation of pathogens

Kourjian, G., Rucevic, M., Berberich, M.J., Boucau, J., Le Gall, S.
 Ragon Institute of MGH, MIT and Harvard, Cambridge, United States

Immune recognition by T cells relies on the presentation of pathogen-derived peptides by infected cells but the persistence of chronic infections calls for new approaches to modulate immune recognition. Pathogen uptake in phagosomes initiates infection of macrophages and leads to the priming of T cell responses by dendritic cells (DC) whereas viruses like HIV fuses at the plasma membrane of CD4+ cells to access the cytosol. The original degradation of antigens is performed by cytosolic proteases or by pH-dependent endolysosomal cathepsins. We show that HIV protease inhibitors (PIs) prescribed to HIV-infected persons modulate proteasome, aminopeptidases and cathepsin hydrolytic activities in human antigen presenting cells (APCs), DC and macrophages, and CD4 T cells. While they modulate aminopeptidase activities in a residue-specific manner, HIV PIs acted in two complementary ways on cathepsin activities: They directly enhanced procathepsin maturation

and increased cathepsin hydrolytic activities. They indirectly altered regulators of cathepsin functions by inhibiting Akt kinase activities, reducing NADPH oxidase 2 (NOX2) activation, lowering phagolysosomal ROS production and pH, which altogether led to enhanced cathepsin activities. HIV PIs modified endolysosomal degradation and epitope production of proteins from HIV and other pathogens in a sequence-dependent manner. They altered cross-presentation of antigens by DC and epitope-specific T cell-mediated killing. HIV PI-induced modulation of antigen processing partly changed the self MHC-peptidome displayed by primary human cells. This first identification of prescription drugs modifying cellular peptidase activities and the MHC-peptidome may provide an alternate therapeutic approach to modulate immune recognition in immune disease beyond HIV.

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MARCH E3 ligases and ubiquitination events that shape immunity

Mintern, J.D.¹, Liu, H.¹, Ching, A.¹, Huang, P.¹, Ishido, S.², Villadangos, J.A.^{1,3}

¹University of Melbourne, Biochemistry and Molecular Biology, Parkville, Australia, ²Showa Pharmaceutical University, Machida, Japan, ³University of Melbourne, Microbiology and Immunology, Parkville, Australia

Membrane associated RING-CH (MARCH) E3 ligases promote the ubiquitination of membrane associated substrates. Here, we have undertaken a comprehensive analysis of the role of MARCH E3 ligase in the ubiquitination of major histocompatibility complex II (MHC II). Our studies use primary immune cells to monitor ubiquitination in endogenous settings. First, we detect distinct patterns of MHC II ubiquitination in different cell types. In brief, the length of the ubiquitin chain and the ubiquitin chain linkages, as identified by mass spectrometry, associated with MHC II differs in B cells versus dendritic cells, while in both cell types, MARCH 1 is the E3 ligase responsible. This implicates the cell-type specific participation of E2 ligases and/or deubiquitinases that are currently under investigation with CRISPR/cas-9 screening. Second, utilising mice deficient in specific MARCH E3 ligases, we have identified different MARCH family members that are responsible for the ubiquitination of MHC II in haemopoietic versus non haemopoietic cell types. Third, by undertaking proteomic screens of plasma membrane fractions isolated from primary MARCH-deficient dendritic cells and B cells, we have discovered novel substrates for MARCH-mediated ubiquitination. This analysis has identified a surprising role for MARCH 1-mediated ubiquitination in the deposition of complement component 3. In summary, we have undertaken in depth analysis of the role of MARCH E3 ligases in primary immune cells. Our findings uncover cell-type specific ubiquitination events and implicate MARCH and/or ubiquitination in playing unexpected roles in immune pathways.

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GILT-mediated antigen processing in thymic epithelial cells diminishes T cell-mediated protection from melanoma through promoting thymic deletion and regulatory T cells

Rausch, M.¹, Metzger, T.², Waterfield, M.², Cortez, J.², Anderson, M.², Hastings, K.¹

¹University of Arizona College of Medicine, Basic Medical Sciences, Phoenix, United States, ²University of California (UCSF), San Francisco, United States

Central tolerance is critical to prevent autoimmunity, but limits T cell responses to tumor Ags that are self Ags. Gamma-interferon-inducible lysosomal thiol reductase (GILT) is required for thymic deletion of CD4⁺ T cells specific for self and melanoma Ag, tyrosinase-related protein 1 (TRP1). GILT expression was enriched in thymic APCs capable of mediating deletion, medullary thymic epithelial cells (mTECs) and DCs. TRP1 expression was restricted to mTECs. GILT facilitated MHC class II-restricted presentation of endogenous TRP1 by pooled thymic APCs. Bone marrow (BM) chimeras demonstrated that GILT expression in TECs is necessary and sufficient for efficient deletion of TRP1-specific thymocytes. Diminished MHC class II expression on mTECs had no effect on thymic deletion or the percentage or activation of peripheral TRP1-specific T cells, suggesting that TRP1 is processed by GILT in mTECs and transferred to DCs to mediate deletion. In chimeras that express GILT in TECs TRP1-specific thymocytes underwent deletion, and chimeras were not protected from melanoma. Although an intermediate level of TRP1-specific T cells developed in chimeras in which GILT expression was limited to BM-derived cells, only chimeras lacking GILT in both populations were protected from melanoma. Chimeras that expressed GILT in TECs or in which GILT was limited to BM-derived cells had a substantially higher percentage of TRP1-specific Treg cells and lower effector:Treg cell ratio. These findings suggest that GILT operates in mTECs to facilitate the processing of tissue-restricted Ags and promote thymic deletion and development of Treg cells, resulting in diminished T cell-mediated protection from melanoma.

4126

The efficiency of antigen presentation in vitro correlates with the capacity to induce protection in vivo

Gil-Torregrosa, B.¹, Johnstone, C.¹, Rodríguez-Castro, M.¹, Prados, F.¹, Schlicht, H.-J.², Koszinowski, U.H.², Castaño, A.R.¹, Del Val, M.³

¹Instituto de Salud Carlos III, Centro Nacional de Microbiología, Majadahonda, Spain, ²University of Ulm, Virology, Ulm, Germany, ³Centro de Biología Molecular Severo Ochoa (CSIC-UAM), Madrid, Spain

The influence of the flanking sequences of an antigenic epitope on its presentation by MHC class I is established. The efficiency of antigen presentation in vitro has been shown sometimes to parallel the degree of protection conferred in vivo to a lethal challenge infection. However, to be able to predict vaccine potency in vivo these observations had to be extended. To this end, several chimerical proteins encoded in recombinant vaccinia virus (rVACV) and containing the same epitope were produced. All rVACV express the immunogenic nonapeptide

9pp89 (¹⁶⁸YPHFMPNTL¹⁷⁶) of murine CMV inserted in different positions of different carrier proteins. When two different cell lines were infected, all constructions were recognized by specific CTL. The differences in the level of presentation to CTL shown by the different chimeras did not correlate with differences in the total synthesized protein analyzed in the stationary state in the infected cells. Protection induced by 9pp89 is mediated by CD8⁺ T lymphocytes *in vivo*. Therefore, the capacity of the rVACV to protect against a lethal infection by murine CMV was studied. A good correlation was found between *in vitro* antigen presentation and *in vivo* protection capacity induced by the chimerical proteins. In addition, the fact that all the chimeras with the epitope bi-terminally flanked by 5 Ala conferred high presentation and high protection values indicates the beneficial effect of flanking Ala. In conclusion, the differences in the antigenic presentation obtained *in vitro* significantly correlate with the capacity of the different rVACV to induce protection *in vivo*.

Innate Receptors & Inflammasomes 1

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Natural IgG is innate immune responsive

Ding, J.L.¹, Panda, S.²

¹National University of Singapore, Biological Sciences, Singapore, Singapore, ²Skirball Institute of Biomolecular Medicine, NYU School of Medicine, New York, United States

Discovered ~50 years ago, natural IgG (nIgG) has been perceived to be inactive and belong to the adaptive immune system. nIgG exists in neonates and uninfected individuals, but it has been deemed non-functional due to its lack of antigen-specificity and low affinity for pathogens, when studied in isolation. Recently, we discovered that nIgG plays a vital and instant defense role by collaborating with plasma lectins (e.g. ficolin). We demonstrated how nIgG collaborates with ficolin-bound pathogens to kill and clear the invading pathogen through phagocytosis. This nIgG-mediated innate immune response is non-specific to antigens, operates independently of the complement system, and evokes a rapid pro-inflammatory response. Knockout mice lacking nIgG showed increased mortality during *Pseudomonas* infection, but are protected by reconstitution with nIgG. We have defined the contact points of interaction between nIgG:ficolin, revealing that infection-induced local acidosis and hypocalcemia conditions stimulate their interaction. Bioactive peptides derived from nIgG and ficolin could be developed into therapeutic immunomodulators, tunable to pH shifts in the infection-inflammation microenvironment.

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Autocrine NLRP3 inflammasome activity is critical to normal adaptive immunity via regulation of IFN- γ in CD4⁺ T cells

Arbore, G.¹, West, E.², Robertson, A.³, Klos, A.⁴, Rheinheimer, C.⁴, Dutow, P.⁴, Woodruff, T.³, O'Neill, L.⁵, Coll, R.³, Sher, A.⁶, Leonard, W.², Köhl, J.^{7,8}, Monk, P.⁹, Cooper, M.³, Arno, M.¹⁰, Afzali, B.^{1,11}, Lachmann, H.¹², Cope, A.¹³, Mayer-Barber, K.⁶, Kemper, C.¹

¹King's College London, DTIMB, London, United Kingdom, ²National Institutes of Health (NIH), NHLBI, Bethesda, United States,

³University of Queensland, Institute for Molecular Bioscience,

Brisbane, Australia, ⁴Medizinische Hochschule Hannover,

Institute for Medical Microbiology, Hannover, Germany, ⁵Trinity

College Dublin, University of Dublin, School of Biochemistry and

Immunology, Dublin, Ireland, ⁶National Institutes of Health (NIH),

NIAID, Bethesda, United States, ⁷University of Lübeck, Institute for

Systemic Inflammation Research, Lübeck, Germany, ⁸Cincinnati

Children's Hospital Medical Center and University of Cincinnati

College of Medicine, Cincinnati, United States, ⁹University of

Sheffield, Department of Infection and Immunity, Sheffield, United

Kingdom, ¹⁰King's College London, Genomics Centre, Faculty of

Life Sciences and Medicine, London, United Kingdom, ¹¹National

Institutes of Health (NIH), NIAMS, Bethesda, United States,

¹²University College London, UK National Amyloidosis Centre,

London, United Kingdom, ¹³King's College London, DIID, London,

United Kingdom

The NLRP3 inflammasome controls IL-1 β maturation in antigen presenting and other innate immune cells but a direct role in

human adaptive immune cells for canonical NLRP3 activity has not been described. This study demonstrates that NLRP3 inflammasome assembles in human CD4⁺ T cells and initiates caspase-1-dependent IL-1 β secretion, thereby promoting IFN- γ production and Th1 differentiation in an autocrine fashion. Importantly, aside from T cell receptor engagement, NLRP3 activation requires intracellular C5 activation and stimulation of C5a receptor 1 (C5aR1) which leads to subsequent ROS generation. This process is negatively controlled by surface-expressed C5aR2: either C5aR2 agonism or NLRP3 inhibition with MCC950 significantly reduce Th1 response *in vitro*, while inhibition of C5aR2 is accompanied by increased IL-1 β and IFN- γ secretion. Dysregulation of NLRP3 activity in T cells affects inflammatory responses in autoimmune disease or infection: Firstly, CD4⁺ T cells from patients with cryopyrin-associated periodic syndromes (CAPS), who have constitutively-active NLRP3, exhibit overactive Th1 responses that are normalized by treatment with a specific NLRP3 inhibitor. Secondly, IFN- γ production is impaired in T cells from Nlrp3^{-/-} or Il1a/Il1b^{-/-} mice upon viral infection. Our results demonstrate that NLRP3 inflammasome activity is not confined to 'innate immune cells' but occurs in adaptive cells and that its crosstalk with the (intracellular) complement system is an integral component of normal adaptive Th1 responses.

1285

Sid2 transports extracellular dsRNA into the cytoplasm for innate immune recognition

Nguyen, T.¹, Smith, B.¹, Weissman, A.², Elgass, K.³, Belz, G.¹, Masters, S.¹, Hunter, C.², Pang, K.¹

¹Walter and Eliza Hall Institute of Medical Research, Parkville, Australia, ²Harvard University, Cambridge, United States, ³Monash Micro Imaging, Clayton, Australia

During viral infection, extracellular double stranded RNA (dsRNA) acts as a potent trigger of innate immunity via the production of type I interferons (IFNs). The detection of extracellular dsRNA involves different host cell sensors such as Toll-like receptor 3 (TLR3) and the RIG-I-like receptors (RLRs), RIG-I and MDA-5. TLR3 is located within endo-lysosomes, and is therefore well situated to detect dsRNA that has been taken up from the environment. Internalised dsRNA also activates the cytoplasmic RLRs, implying the existence of a mechanism to transport dsRNA across the endosomal membrane. Interestingly, the RLRs are functionally dominant over TLR-3 in the response to extracellular dsRNA and are critical for immunity to multiple viruses, but how dsRNA escapes from the endosome is unknown. Here we report that Sid2, a mammalian orthologue of the *C. elegans* SID-1 dsRNA transporter, is present within the endosomal compartment and co-localises with internalised poly(I:C), a synthetic analogue of dsRNA. Sid2-deficient mice show impaired production of type I IFNs in response to extracellular poly(I:C) *in vivo*. These observations suggest a role for Sid2 in transporting dsRNA from the endosome into the cytoplasm. In support of this, we found that loss of Sid2 does not affect dsRNA internalization, but instead results in the accumulation of poly(I:C) within the endosomal compartment. Sid2-deficient mice produce less type I IFNs and have increased mortality in response to infection

with herpes simplex virus. Our findings demonstrate a key role for Sid2 in the endosomal escape of dsRNA and the subsequent induction of innate immunity.

1289

Inflammatory response sculpting by neutrophil inflammasomes

Chen, K.¹, Gross, C.², Wall, A.¹, Stacey, K.¹, Stow, J.¹, Sweet, M.¹, Schroder, K.¹
¹The University of Queensland, Brisbane, Australia, ²Technische Universität München, Munich, Germany

Macrophage and dendritic cell inflammasomes drive potent innate immune responses against intracellular pathogens, by eliciting rapid caspase-1-dependent pro-inflammatory cytokine production (e.g. interleukins (IL)-1 β and -18 and pyroptotic cell death, as well as caspase-8-directed apoptotic cell death. The contribution of other cell types to inflammasome-mediated host defence had not been examined in detail. Here we demonstrate that neutrophils, typically viewed as cellular targets of IL-1 β themselves activate the NLRC4 inflammasome during acute *Salmonella* infection, and are a major cell compartment for IL-1 β production during acute peritoneal challenge *in vivo*. Importantly, unlike macrophages, neutrophils do not undergo pyroptosis upon *in vitro* or *in vivo* NLRC4, NLRP3 or AIM2 inflammasome activation. Furthermore, neutrophils also resisted inflammasome/caspase-8-directed apoptotic cell death. The ability of neutrophils to resist inflammasome-mediated death is unique amongst inflammasome-signalling cells so far described. Their continued viability allows neutrophils to sustain IL-1 β production at a site of infection, and exert their crucial inflammasome-independent antimicrobial effector functions to clear infection. Additionally, we have found that neutrophils possess a unique specialisation of the NLRP3 pathway that allows these cells to co-ordinate a neutrophil-dominated response only when it is appropriate to do so. This work reveals neutrophils as a surprising new cellular player in shaping inflammasome-mediated inflammatory responses *in vivo*, and highlights the major impact of myeloid cell identity on innate immune signalling pathways.

1600

Differential contribution of the Inflammasome to *L. pneumophila* clearance independently of cell death

Caution, K.¹, Gavrilin, M.², Tazi, M.¹, Kanneganti, A.¹, Layman, D.¹, Hoque, S.¹, Krause, K.¹, Amer, A.¹

¹Ohio State University, Microbial Infection & Immunity, Columbus, United States, ²Ohio State University, Internal Medicine, Columbus, United States

Trafficking and fusion of the phagosome with the lysosome is a key host defense against pathogens. Regulation of the actin cytoskeleton is crucial for proper vesicle trafficking. Inflammasomes are large, multiprotein complexes that promote inflammation and cell death in response to pathogenic microorganisms and sterile stressors. Recently, prominent members of the inflammasome, caspase-1 and caspase-11, were implicated in the fusion of vacuoles harboring intracellular

microbes with lysosomes. However, the molecular mechanisms of caspase-mediated intracellular trafficking are unknown. It is this study's objective to decipher the effects of caspase-1 and -11 on actin dynamics to promote phagolysosomal fusion and restriction. Using single knockout mice lacking caspase-1 or -11, we determined that during *Legionella pneumophila* (*L. pneumophila*) infection, *Casp-1*^{-/-} and *Casp-11*^{-/-} macrophages exhibited diminished F/G-actin ratios compared to WT counterparts. In addition, the absence of caspase-1 or -11 prevented the colocalization of F-actin around the bacterium. We also identified the signaling pathway linking caspase-1 and -11 with actin. We demonstrate that caspase-1 and caspase-11 exert contrasting effects on cofilin phosphorylation state upon intracellular infection. The absence of either caspase maintains actin in the polymerized or depolymerized form, respectively and averts the fusion of pathogen-containing vacuole with lysosomes, allowing bacterial persistence. However, non-pathogenic bacteria were delivered to the lysosome independently of caspase-1 and caspase-11. Notably, caspase-11 targets cofilin via the RhoA GTPase, whereas caspase-1 engages the Slingshot phosphatase. These data establish that inflammasome caspases differentially regulate actin polymerization during infection by modulating F-actin assembly via the activation of cofilin.

32

Rhbdd3 controls innate inflammatory diseases via negatively regulating TLR signaling

Liu, J.¹, Han, C.¹, Liu, S.¹, Xia, M.², Chen, K.³, Cao, X.²

¹National Key Laboratory of Medical Immunology & Institute of Immunology, Second Military Medical University, Shanghai, China, ²National Key Laboratory of Medical Molecular Biology & Department of Immunology, Chinese Academy of Medical Sciences, Beijing, China, ³Institute of Immunology, Zhejiang University School of Medicine, Hangzhou, China

Rhomboid domain-containing protein 3 (Rhbdd3), which belongs to a family of proteins with rhomboid domain, is widely expressed in immune cells; however, the roles of Rhbdd3 in immunity remain largely unknown. Using Rhbdd3-deficient mice, we reveal that Rhbdd3-deficient mice spontaneously developed autoimmune diseases due to increased IL-6 production, and developed more severe inducible autoinflammatory colitis and acute hepatic injury. Rhbdd3 negatively regulates Toll like receptor (TLR)-mediated NK cell and DC activation. Rhbdd3 inhibits TLR3-triggered IFN- γ and granzyme B expression of NK cells in cell-cell contact dependence of accessory cells such as dendritic cells and Kupffer cells. Rhbdd3 interacts with DAP12 and promotes its degradation, inhibiting MAPK activation in TLR3-triggered NK cells. Furthermore, Rhbdd3 directly binds to K27-linked polyubiquitin chains on NEMO via its UBA domain, and recruits the deubiquitinase A20 to induce K63-linked deubiquitination of NEMO and thus inhibits activation of the transcription factor NF- κ B in DCs. Our study identify provide a new mechanistic explanation for the post-translational regulation of TLR triggered activation of MAPK and NF- κ B, indicative of potential intervention strategies for autoimmune or chronic inflammatory diseases.

2029

Anti-phosphorylcholine antibodies decrease the interaction of house dust mite with antigen-presenting cells in the lung through CD36 and platelet activating factor receptor (PAFR)

Patel, P., Kearney, J.

University of Alabama at Birmingham, Microbiology, Birmingham, United States

There is an alarmingly high rate of asthma that occurs among children born in developed countries. Approximately 89% of these asthmatic children demonstrate sensitivity to house dust mite (HDM) allergen. HDM is composed of LPS, chitin, and β -glucan, and studies have shown that blocking the receptors of these PAMPS such as TLR4, Dectin-2, and PAR2 can significantly inhibit both the innate and adaptive immune response to HDM. However, these findings have not lead to the generation of any viable therapeutics. We, and others, have published that HDM contains phosphorylcholine (PC) epitopes. PC receptors such as CD36 and platelet activating factor receptor (PAFR) have been shown to modulate disease states involving oxidized lipids like atherosclerosis, but not much is known about the role of these receptors in allergies and asthma. Our findings demonstrate that mice deficient in CD36, PAFR, or both receptors develop a significantly attenuated form of HDM-induced allergic disease compared to C57BL/6 mice. We further demonstrate that anti-PC IgM antibodies bound to HDM prevent the interaction of the allergen with CD36 and PAFR on APCs in the lung and suppress the development of allergic disease. Additionally, elevated levels of anti-PC IgM antibodies are associated with a decreased incidence of asthma in a local pediatric cohort. Thus, we have indicated that engagement of CD36 and PAFR by HDM plays a critical role in the initiation of allergic disease, and inhibiting the engagement of HDM with these receptors via treatment with anti-PC antibodies can significantly attenuate the development of allergic disease.

1873

The NLRP3 specific inhibitor CP-456,773 potently attenuates crystal-induced kidney fibrosis in vivo

Ludwig-Portugall, I.¹, Bartok, E.², Dhana, E.¹, Evers, B.G.¹, Franklin, B.S.³, Hall, P.⁴, Primiano, M.J.⁴, Hornung, V.⁵, Hartmann, G.², Boor, P.⁶, Latz, E.³, Kurts, C.¹

¹Institute of Experimental Immunology, University Hospital of the Rheinische Friedrich-Wilhelms-Universität, Bonn, Germany, ²Institute of Clinical Chemistry and Clinical Pharmacology, University Hospital of the Rheinische Friedrich-Wilhelms-Universität, Bonn, Germany, ³Institute of Innate Immunity, University Hospital of the Rheinische Friedrich-Wilhelms-Universität, Bonn, Germany, ⁴Inflammation and Immunology Research Unit, Pfizer Inc., Cambridge, United States, ⁵Institute of Molecular Medicine, University Hospital of the Rheinische Friedrich-Wilhelms-Universität, Bonn, Germany, ⁶University of Aachen, Dept. of Nephrology, Aachen, Germany

The Nlrp3 inflammasome in myeloid cells triggers profound inflammatory reaction in response to a variety of stimuli. Mice genetically deficient in inflammasome components have been shown to be protected against inflammation in acute and chronic crystal nephropathy. Here we demonstrate that

a specific inhibitor of the Nlrp3 inflammasome (CP-456,773), potently attenuated the course of a murine model of crystal nephropathy. IL-1 β and IL-18 production, parameters of renal failure, kidney injury and fibrosis were markedly improved after CP-456,773 treatment. In contrast, the crystal burden remained unaltered and the adaptive immune response was not compromised as previously reported for the global inhibition of IL-1 signaling. Using iGLuc, a proteolytic luciferase-based reporter for inflammasome activation, we established an *in vivo* imaging assay making use of iGLuc-transduced bone-marrow cells in order to directly visualize renal inflammasome activation and its inhibition by CP-456,773 *in vivo*. In summary, our study demonstrates the efficacy of NLRP3 inhibition by CP-456,773 for the treatment of crystal nephropathy and introduces a novel imaging technique for *in vivo* inflammasome activation.

1784

E3 ubiquitin ligase TRIM31 negatively regulates NLRP3 inflammasome activation by promoting proteasomal degradation of NLRP3

Huai, W., Song, H., Liu, B., Han, L., Zhang, L., Gao, C., Zhao, W.
Shandong University School of Medicine, Department of Immunology, Jinan, China

NLRP3 inflammasome plays fundamental roles in host defense against microbial pathogens and its deregulation may cause diverse inflammatory diseases. NLRP3 protein expression is considered as a rate-limiting step for inflammasome activation, thus its expression must be tightly controlled to maintain immune homeostasis and avoid detrimental effects. However, how NLRP3 expression is regulated, remains largely unknown. In the present study, we identified E3 ubiquitin ligase TRIM31 as a feedback suppressor of NLRP3 inflammasome. TRIM31 binds to NLRP3, promotes K48-linked polyubiquitination and proteasomal degradation of NLRP3 in both resting and activated macrophages. Our research described a new mechanism by which TRIM31 negatively regulated NLRP3 inflammasome activity and provided an explanation on the limitation of NLRP3 inflammasome activation under physiological conditions.

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Gut microbial metabolites regulate autoimmune T cell responses and protect against type 1 diabetes

Marino, E., Richards, J.L., McLeod, K.H., Yap, Y.A., Stanley, D., Mackay, C.R.

Monash University, Infection and Immunity Program, Monash Biomedicine Discovery Institute, Biochemistry and Molecular Biology, Melbourne, Australia

Diet and gut microbial ecology may underlie the increasing incidence of certain inflammatory diseases. Here, we found that key features of autoimmune diabetes in NOD mice, such as gender differences and protection in *MyD88*^{-/-} NOD mice correlated closely with fecal concentrations of the short chain fatty acids (SCFAs) acetate and butyrate. We then used specialised

diets to deliver high concentrations of acetate and butyrate to the colon and hepatic portal vein of NOD mice, when tolerance to islet antigens has already been broken. High acetate- or butyrate-yielding diets significantly reduced progression to diabetes, through effects on the colonic microbiota, improved gut epithelial integrity and reduced concentrations of pro-inflammatory cytokines. Both acetate and butyrate diets led to dramatically decreased numbers of autoreactive T cells. A high butyrate-yielding diet promoted conversion of naive Foxp3⁻ T cells into Foxp3⁺ Treg cells *in vivo*, through histone modification at the *Foxp3* promoter that led to increased numbers of Treg cells. In contrast, an acetate-yielding diet inhibited histone deacetylase 3 (HDAC3) transcription in B cells, through histone modification at the *CD40*, *b2m* and *Blimp1* promoters, which led to markedly reduced expression of CD86 and MHC1 and reduced their capacity to expand autoreactive CD8⁺ T cells *in vivo*. Control of autoimmune T cell frequencies, and protection from diabetes relied in part on the metabolite-sensor GPR43, a receptor for both acetate and butyrate. Specialised diets that yield high acetate or butyrate may represent an effective non-pharmacologic means to limit autoreactive T cell numbers, and prevent autoimmune disease progression.

4459

cIAP1/2 inhibition downregulates IL-17A and synergizes with TNF- α blockade for sustained suppression of joint inflammation

Kawalkowska, J., Venables, P., Williams, R.

University of Oxford, Kennedy Institute, NDORMS, Oxford, United Kingdom

Cellular inhibitors of apoptosis proteins 1 and 2 (cIAP1/2) are ubiquitin E3 ligases which regulate apoptosis and NF- κ B and MAPK signalling pathways. As cIAP2 may also play a role in regulating TCR signals, by the ubiquitination of BCL10, the aim of this study was to assess the effects of cIAP1/2 inhibition on T cell cytokine secretion, survival, and differentiation using a specific inhibitor (GT13072). Here we show that GT13072 dramatically reduced IL-17A secretion by activated CD4⁺ T cells with little effect on other cytokines, including TNF- α and IFN- γ . Thus, the addition of GT13072 to naive T cell cultures led to a complete abrogation of T_H17 differentiation *in vitro*. We also evaluated the therapeutic potential of GT13072 in collagen-induced arthritis (CIA), a mouse model of rheumatoid arthritis, in which IL-17A plays a pathogenic role. GT13072 profoundly decreased the number of IL-17⁺ cells in joints and lymph nodes resulting in abrogation of joint inflammation. Furthermore, when combined with the TNF- α inhibitor etanercept, GT13072 treatment led to an increase in immunosuppressive Treg numbers and a prolonged therapeutic effect that was sustained after therapy retraction. GT13072 also inhibited the increase in T_H17 cells that has been reported in mice and man after TNF- α blockade. Thus, in summary, cIAP1/2 regulate IL-17A expression by modulating TCR signals and therefore control the balance between Tregs and IL-17A⁺ cells during inflammation. More importantly, we demonstrate that cIAP1/2 antagonism has therapeutic potential and should be further explored for the treatment of inflammatory diseases in which IL-17A plays a role.

1588**Increased frequency of circulating microparticles forming immune complexes, and their putative receptors in monocytes subsets in patients with systemic lupus erythematosus**Burbano, C.^{1,2}, Rojas, M.^{1,2}, Gloria, V.¹, Orejuela, J.¹, Muñoz, C.³, Vanegas, A.³, Castaño, D.¹¹Universidad de Antioquia, Facultad de Medicina, Instituto de Investigaciones Médicas, Grupo de Inmunología Celular e Inmunogenética, Medellín, Colombia, ²Universidad de Antioquia, Unidad de Citometría de Flujo, Sede de Investigación Universitaria, Medellín, Colombia, ³Hospital Universitario de San Vicente Fundación, Sección de Reumatología, Medellín, Colombia

Microparticles (MP) play a critical role in immune responses, due to their ability to transfer and modulate a variety of cellular components. Circulating MP can be recognized by monocytes affecting their functions. Because this interaction could be determinant in Systemic Lupus Erythematosus (SLE) immunopathology, monocytes and MP were studied in the peripheral blood from SLE patients and healthy controls (HC) by multiparametric flow cytometry.

Inactive (i, SLEDAI < 4) and active (a, SLEDAI ≥ 4) SLE patients had elevated percentages of MP IgG+, IgM+, IgM+IgG+, and C1q+; however, iSLE has more MP-IgM+ and aSLE more MP-IgG+. Only aSLE had elevated HMGB1+, and DNA+ MP. We did not observed differences in the MP number, size, neither in the content of RNA and phosphatidylserine compared to HC. To understand the possible pathways by which MP interact with monocytes, we evaluated their putative receptors. The aSLE patients have significantly increased expression of FcγR CD64 in intermediate and non-classical monocytes, and decreased of CD16 in non-classical, respect to HC. There were not differences regarding CD32, complement receptors (CR) 3 and CR4, and FcμR.

These evidences suggest that the interaction of circulating MP with monocytes in iSLE could be through C1qR, FcμR and CD36, favoring their silent clearance. However, in aSLE the recognition of MP could be through FcγR potentially affecting their activation pattern, migration, contact with the endothelium, and induction of tissue damage in SLE patients. The identification of the modulatory effect of MP forming immune complexes in monocytes will provide new immunopathogenic mechanisms in SLE patients.

3078**TCR affinity and bond lifetime for antigen combine to determine avoidance of tolerance and induction of demyelinating disease**

Blanchfield, L., Liu, B., Evavold, B.

Emory University, Microbiology and Immunology, Atlanta, United States

A fundamental question for autoimmunity is how T cells evade tolerance mechanisms in the thymus yet become activated to attack self in the periphery. Ligand discrimination by TCR is often characterized by affinity for peptide:MHC (pMHC), yet T cells also generate dynamic forces during antigen recognition that can limit or enhance TCR-pMHC bond lifetimes. Using an

experimental autoimmune encephalomyelitis (EAE) mouse model, we have analyzed tetramer positive and negative myelin oligodendrocyte glycoprotein (MOG)₃₅₋₅₅-specific TCRs that correspond to high and low affinity TCRs respectively and defined their binding kinetics as they progress through thymocyte development to induction of disease. We report that the bond lifetime of MOG specific T cells under applied force on naïve and activated

T cells are much longer (up to 100 fold) than those on thymocytes, suggesting physiological regulation of TCR kinetics within single T cell clones. Therefore, these data identify unique kinetic properties of autoimmune TCRs where weak interactions in the thymus potentially allow avoidance of negative selection yet in the periphery bind with sufficiently long lifetime to promote autoimmunity. Our work supports a model of T cell activation linking antigen discrimination with actin remodeling and availability of intracellular chemical signaling molecules where with time, high and low affinity T cells are essentially equipotent contributors to autoimmune disease.

3760**Down-regulation of autoreactive T and B lymphocytes by selective therapy in humanized murine SCID model of Systemic lupus erythematosus**Mihaylova, N.¹, Bradyanova, S.¹, Kerekov, N.¹, Chipinski, P.¹, Nikolova-Ganeva, K.¹, Velikova, T.², Ivanova-Todorova, E.², Kyurkchiev, D.², Kalinova, D.³, Herbáth, M.⁴, Prechl, J.⁴, Tchobanov, A.¹¹Institute of Microbiology, Bulgarian Academy of Sciences, Laboratory of Experimental Immunology, Sofia, Bulgaria,²University Hospital 'St. Ivan Rilski'; Laboratory of Clinical³University Hospital 'St. Ivan Rilski'; Clinic of Rheumatology, Sofia, Bulgaria, ⁴Hungarian Academy of Sciences, Immunology Research Group, Budapest, Hungary

Self-specific B and T cells play a main role in pathogenesis of SLE and are a logical target for a selective therapy. The complement receptor type 1 (CR1) on human B-lymphocytes has suppressive activity and the co-crosslinking of this receptor with BCR inhibits B cell activation.

The protein Annexin A1 (ANXA1), is a modulator of the immune system and abnormal expression was found on activated B and T cells in human autoimmunity.

We hypothesize that it may be possible to down-modulate the activity of autoreactive T and B cells from SLE patients in humanized SCID mouse model by treating them with a neutralizing antibody against ANXA1 or by protein engineered molecules, which co-crosslink the BCR and CR1.

The protein chimeric molecules were constructed by coupling an DNA-mimotope peptide to an anti-CR1 antibody. Immunodeficient SCID mice have been used for cell transfer with human PBMC from SLE patients for evaluating the pathogenesis of SLE. The immunomodulatory activities of the therapeutic monoclonal or engineered antibodies were tested *in vitro* and *in vivo*. The levels of anti-dsDNA antibodies and cytokines in the mice sera as well as the apoptosis and the number of dsDNA producing plasma cells were quantified by ELISA, FACS, ELISpot and protein array. Reconstituted SCID mice showed presence of auto-antibodies, as well as immunoglobulin deposition in the

renal glomeruli. Treatment of the transferred SCID mice either with DNA-like chimera and anti-ANXA1 antibody prevented appearance of anti-DNA antibodies and proteinuria, while the PBS-injected animals had high levels after the transfer.

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Self-reactive IgE exacerbates interferon responses associated with autoimmunity

Sanjuan, M.

Medimmune, Gaithersburg, United States

The discovery of Immunoglobulin E (IgE) nearly 40 years ago was a breakthrough in the field of allergy research. IgE directed against parasitic worms and allergens binds mast cells and basophils, triggering an inflammatory response that is characterized by the release of histamine and Th2 cytokines. Here we report that double-stranded DNA (dsDNA)-specific IgE autoantibodies complexed to DNA activate plasmacytoid dendritic cells (pDC), an immune cell type linked to viral defense, leading to the secretion of substantial amounts of IFN- α . Although typical IgE mediated responses are not a hallmark of systemic lupus erythematosus (SLE), IFN- α producing pDCs are a key driver for loss of immune tolerance to host DNA in SLE. We, therefore, investigated this apparent paradox and found that even the small concentrations of circulating dsDNA-specific IgE found in SLE patients greatly potentiated pDC secretion of IFN- α by triggering phagocytosis via the Fc-epsilon receptor 1 (Fc ϵ RI) and TLR9-mediated DNA sensing in the phagolysosome. Consequently, this potent pDC signaling mechanism reduces the threshold of pDC activation, which has the potential to expand anti-DNA responses and greatly exacerbate self-inflicted damage. These findings expand the known pathogenic mechanisms of IgE mediated inflammation beyond those found in allergies and demonstrate that, in addition to recognizing exogenous allergens, IgE can also bind to autoantigens and drive an aberrant self-destructive response. This previously unrecognized link between IgE and the interferon pathway provides additional insights into the pathological mechanisms underlying autoimmunity and may be useful in the rational design of therapies for the treatment of autoimmunity.

1204

Preferential internalization of antioxidant carbon nanoparticles by T lymphocytes to treat autoimmune diseases

Huq, R.¹, Samuel, E.L.G.², Sikkema, W.K.A.², Lee, T.¹, Tanner, M.R.¹, Khan, F.S.¹, Porter, P.C.³, Tajhya, R.B.¹, Patel, R.S.¹, Inoue, T.¹, Pautler, R.G.¹, Corry, D.B.³, Tour, J.M.², Beeton, C.¹

¹Baylor College of Medicine, Molecular Physiology and Biophysics, Houston, United States, ²Rice University, Chemistry, Houston, United States, ³Baylor College of Medicine, Medicine, Houston, United States

The intracellular production of superoxide (O₂⁻) by the mitochondria in T lymphocytes is an integral part of signaling downstream of the T cell receptor as O₂⁻ are required for the translocation of NFAT from the cytosol to the nucleus.

Scavenging O₂⁻ has proven beneficial in reducing the severity animal models of autoimmune diseases. However, dietary antioxidants require the administration of high doses and have not proven effective in the clinic, likely because of their low potency and rapid inactivation. In addition, high doses or long-term use of broad antioxidants, such as Vitamin E, is toxic.

Antioxidant carbon nanoparticles can scavenge reactive oxygen species (ROS) with higher efficacy than dietary and endogenous antioxidants. Furthermore, the affinity of carbon nanoparticles for specific cell types represents an emerging tactic for targeted therapy. Here, we report that nontoxic poly(ethylene glycol)-functionalized hydrophilic carbon clusters (PEG-HCCs), which are selective antioxidants for O₂⁻ and its derivative hydroxyl radical, are preferentially and rapidly internalized by T lymphocytes over other splenic immune cells and localize in T cell mitochondria. We use this selectivity to inhibit T cell function without affecting major functions of macrophages. We also demonstrate the effectiveness of PEG-HCCs in reducing T lymphocyte-mediated inflammation in delayed-type hypersensitivity (DTH) and experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis. Our results suggest that the preferential targeting of PEG-HCCs to T lymphocytes is a new and attractive route for treating

T cell-mediated autoimmune diseases without affecting other immune cells and therefore inducing broad-spectrum immunosuppression.

1531

Synovitis in Juvenile idiopathic arthritis is mediated by TCR-independent CD31-IL17 axis of inflammation

Ferguson, I.^{1,2}, Griffin, P.¹, Yano, H.³, Michel, J.¹, Gaffen, S.^{3,4}, Piganelli, J.^{3,5}, Dvergsten, J.⁶, Rosenkranz, M.^{1,2}, Kietz, D.^{1,2}, Vallejo, A.^{1,2,3}

¹University of Pittsburgh School of Medicine, Department of Pediatrics, Pittsburgh, United States, ²UPMC Children's Hospital of Pittsburgh, Division of Rheumatology, Pittsburgh, United States, ³University of Pittsburgh School of Medicine, Department of Immunology, Pittsburgh, United States, ⁴University of Pittsburgh School of Medicine, Department of Medicine, Pittsburgh, United States, ⁵University of Pittsburgh School of Medicine, Department of Surgery, Pittsburgh, United States, ⁶Duke University Medical Center, Department of Pediatrics, Durham, United States

Juvenile idiopathic arthritis (JIA) is the most prevalent autoimmune, rheumatic disease of childhood. T cells are considered autoimmune effectors, but the autoantigenic basis of JIA pathology remains unknown. Our analyses of joint aspirates from children with oligoarticular and polyarticular JIA showed preponderance of CD28^{null}CD31⁺ $\alpha\beta$ T cells that are deficient in the expression of CD4 and CD8, herein called DN T cells. Along with similar CD28^{null}CD31⁺ CD8 T cells that we reported previously, we hypothesized that CD31 triggering shapes the inflammatory milieu within the inflamed joint. Humoral profiling of synovial fluid (SF) samples showed dominance of IL6, IL10, IL17, IFN γ , and TNF α . Bioassays using primary synovial T cells showed CD31 ligation alone was sufficient to drive production of these cytokines. Moreover, CD31 ligation led to the upregulation ROR γ T transcription factor, and to direct transactivation of *IL17A* gene promoter,

providing a first evidence for TCR-independent production of IL17 by T cells. Cellular analyses of SF also showed high numbers of fibrocyte-like cells (FLC) that expressed procollagen, IL17 receptor A, and CD38, the natural ligand of CD31. FLC exposed to IL17 expressed high levels of IL6, TNF α , and MMPs. Bioassays in the presence of a novel small molecule inhibitor effectively suppressed CD31-driven IL17 production by T cells, as well as the downstream IL17-dependent activation of FLC. Collectively, these data indicate a CD31-IL17A axis of T cell-FLC interaction that likely perpetuates synovitis in JIA. Targeted interruption of CD31-IL17A cell activation cascades may provide new avenue(s) towards alternative therapies for JIA.

3885

Human iPSC-derived neural stem cells modulate adaptive and innate immune responses to protein against autoimmune-mediated demyelination

Payne, N.¹, Shu, R.¹, Sun, G.¹, Sylvain, A.¹, Zhang, Z.¹, Morey, R.², Laurent, L.², Yu, D.³, Bernard, C.¹

¹Monash University, Australian Regenerative Medicine Institute, Clayton, Australia, ²University of California San Diego, Department of Reproductive Medicine, San Diego, United States, ³Monash University, Department of Biochemistry and Molecular Biology, Clayton, Australia

Neural stem cell (NSC) transplantation reduces neuroinflammation and promotes endogenous repair processes in mouse models of multiple sclerosis (MS). We have generated induced pluripotent stem cells (iPSCs) from a set of monozygotic twins discordant for MS, termed patients 6 and 7, using two reprogramming strategies. In the current study, we investigated the immune modulatory activity of iPSC-derived NSCs from patients 6 and 7 using *in vitro* assays and a model of autoimmune-mediated demyelination. Gene expression profiling by RNA-seq and qPCR revealed that genes encoding secreted proteins known to regulate neuroinflammation, such as CX3CL1, LIF, IL-10, and TGF β 2, were upregulated upon neural differentiation of iPSCs. NSC conditioned media reduced the proliferation of human T-cells and regulated the activation state of macrophages *in vitro*, confirming that secreted factors likely play a role in the immune modulatory activity of NSCs. Transplantation of NSCs into the cerebrospinal fluid, but not into the bloodstream, of mice with experimental autoimmune encephalomyelitis (EAE) significantly reduced clinical signs of disease in a preventative treatment protocol. This therapeutic effect was not associated with an expansion of FoxP3+ T regulatory cells, but rather an increase in IL-4+ CD4 T-cells both in the CNS and periphery, as well as a reduction in tissue-invading myeloid cells that mediate demyelination. Our work provides the first evidence that human iPSC-derived NSCs can exert a bystander immune modulatory effect that improves clinical outcomes in a mouse model of MS.

13:30:00 - 15:10:00

Complement

1095

Unraveling a new virulence mechanism: human IgG accentuates experimental *Streptococcus pyogenes* infections in mice

Ermert, D.^{1,2}, *Weckel, A.*¹, *Shaughnessy, J.*², *Kaplan, J.*¹, *Mörgelin, M.*³, *Björck, L.*³, *Rice, P.A.*², *Ram, S.*², *Blom, A.M.*¹

¹Lund University, Translational Medicine, Malmö, Sweden,

²University of Massachusetts Medical School, Medicine, Worcester, United States, ³Lund University, Clinical Sciences, Lund, Sweden

Streptococcus pyogenes, also known as group A streptococcus (GAS) is an important bacterial pathogen that causes disease in humans. This gram-positive bacterium has developed several strategies to evade the human immune system that enables GAS to survive in the host. These include binding of IgG via Fc domains that results in recruitment of soluble complement inhibitors, C4b-binding protein (C4BP) and Factor H (FH) to the surface of GAS, that hinders complement dependent opsonisation and clearance. Protein H is a GAS surface bound virulence factor that, in turn, binds both C4BP and IgG. We characterized IgG's influence on C4BP binding to protein H and the resultant effect of IgG-C4BP interactions on *S. pyogenes* pathogenesis. Human IgG enhanced C4BP binding to GAS protein H over 20-fold. IgG did not augment binding of other protein H ligands such as factor H and did not increase binding of C4BP to streptococcal M protein (another ligand for both C4BP and IgG). Fc fragments derived from human IgG, but not other species were sufficient to increase C4BP binding to protein H. Using a novel humanized C4BP transgenic animal model, we found that mice treated with human Fc and infected with GAS had more severe disease manifestations and higher mortality compared to PBS treated control animals that effectively cleared the infection and survived. Taken together, we have identified a novel virulence mechanism whereby the Fc region of human IgG enhances C4BP binding to GAS and accentuates disease in a novel mouse model.

3818

The ubiquitin ligase MARCH 1 regulates complement C3 deposition on professional antigen presenting cells

Villadangos, J.^{1,2}, *Ching, A.*², *Infusini, G.*³, *Moffat, J.*¹, *Ishido, S.*⁴, *Mintern, J.*²

¹The University of Melbourne, Microbiology and Immunology at the Doherty Institute of Infection and Immunity, Melbourne, Australia, ²The University of Melbourne, Biochemistry and Molecular Biology at the Bio21 Institute, Parkville, Australia, ³The Walter and Eliza Hall Institute of Medical Research, Parkville, Australia, ⁴Showa Pharmaceutical University, Tokyo, Japan

Membrane-Associated Ring-CH1 (MARCH 1) is the best studied of the mammalian MARCHs, a family of E3 ubiquitin ligases specialised in regulation of immunoreceptor expression,

trafficking and turn-over. MARCH 1 ubiquitinates MHC class II and CD86 in professional antigen presenting cells, thereby regulating CD4 T cell antigen recognition and immunity (1). Whether MARCH 1 regulates other immunoregulatory proteins *in vivo* remains unknown.

We have undertaken label-free, comparative proteomic screens of plasma membrane fractions isolated from primary wild-type and MARCH I-deficient dendritic cells (DC) and B cells, and discovered novel substrates for MARCH I-mediated ubiquitination. We have also uncovered an unexpected role for MARCH 1 in deposition and/or removal of complement component 3 (C3) on the plasma membrane of DC and B cells in the steady-state. In the absence of MARCH 1, both cell types accumulated large amounts of C3 on their surface, which was not produced by the cells themselves but captured from the extracellular environment. We will present our studies characterizing how MARCH 1 regulates C3 abundance on professional antigen presenting cells and the functional implications of this novel mechanism of Complement regulation. (1) J. M. Moffat, J. D. Mintern and J. A. Villadangos. 2013. Control of MHC II antigen presentation by ubiquitination. *Curr. Opin. Immunol.* **25**: 109-114.

1985

Collectin-11 recognises stress-induced epithelial cell pattern of L-fucose and triggers the innate immune system

*Zhou, W.*¹, *Farrar, C.A.*¹, *Tran, D.*¹, *Li, K.*², *Wu, W.*¹, *Peng, Q.*¹, *Schwaeble, W.*³, *Sacks, S.H.*¹

¹King's College London, Department of Innate Immunity, Medical Research Council (MRC) Centre for Transplantation, London, United Kingdom, ²Xi'an Jiaotong University, Core Research Laboratory, The Second Affiliated Hospital, School of Medicine, Xi'an, China, ³University of Leicester, Department of Infection, Immunity and Inflammation, Leicester, United Kingdom

The mechanism by which physiochemical stress induces tissue injury involves detection of abnormal molecular patterns by sensory molecules of the innate immune system. In this study, we describe how the recently discovered soluble C-type lectin known as collectin-11 (CL-11) recognises an abnormal pattern of L-fucose on post-ischaemic renal tissue and activates the innate immune response. Rapid local increase in CL-11 expression was associated with post-ischaemic renal damage. Mice with generalised deficiency or specific intrarenal deficiency of CL-11 were strongly protected against loss of renal function and tubule injury due to complement. Detection of the stable complement metabolite (C3d) mapped to proximal tubules, where CL-11 and potential ligand (L-fucose) were co-expressed. *Ex vivo* study of isolated renal tubule cells showed marked capacity for CL-11 binding that was induced by cell culture in hypoxic or hypothermic conditions and prevented after specific removal of L-fucose. Further analysis showed that cell-bound CL-11 required the serine protease MASP-2 to initiate complement deposition on the cell surface. We conclude that lectin complement pathway activation triggered by ligand-CL-11 interaction in post-ischaemic tissue is a potent source of acute kidney injury and is amenable to sugar-specific blockade.

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Scabies mites give mite associated bacterial pathogens a helping handSwe, P.M., Christian, L., Sriprakash, K.S., Fischer, K.
QIMR Berghofer Medical Research Institute, Brisbane, Australia

On a global scale scabies is one of the three commonest dermatological conditions, imposing a considerable economic burden on individuals, communities and health systems. Substantial epidemiological evidence shows that in tropical regions scabies is often linked to pyoderma. Patients can develop serious illness due to invasion by opportunistic bacteria such as cellulitis, heart and kidney diseases, bacteraemia and sepsis. The health burden due to complicated scabies in resource-poor Australian Aboriginal and Torres Strait Islander communities is extreme.

The molecular mechanisms that underpin the link between scabies and bacterial pathogens were unknown. We proposed that scabies mites play a role in the establishment, proliferation and transmission of opportunistic pathogens. We investigate here the impact of a scabies mite complement inhibitors on the growth of the two most recognised mite associated pathogens *Streptococcus pyogenes* and *Staphylococcus aureus*.

Scabies mites secrete several classes of complement inhibiting proteins into the mite gut and excrete them into the epidermal mite burrows. These inhibitors promoted the growth of bacteria *in vitro* in whole blood bactericidal assays. We tested specifically the mite complement inhibitor Serpin SMSB4 on various *S. pyogenes* clinical isolates. Recombinant SMSB4 was produced and purified from *Pichia pastoris*. The complement inhibitory function of SMSB4 was confirmed by haemolytic assays. SMSB4 reduced the opsonisation of the bacteria surface as well as the phagocytosis of bacteria by neutrophils. Investigation of the tripartite interactions between host, parasite and microbial pathogens could serve as a basis to develop novel intervention strategies targeting scabies and associated bacterial infections.

528

Complement C5 regulates TGF- β and response gene to complement -32 expression in experimental autoimmune encephalomyelitisTatomir, A.¹, Boodhoo, D.¹, Tegla, C.¹, Cudrici, C.¹, Weerth, S.², Rus, V.³, Rus, H.^{3,4,5}

¹University of Maryland, School of Medicine, Neurology, Baltimore, United States, ²Albert Einstein College of Medicine, Pathology, New York, United States, ³University of Maryland, School of Medicine, Medicine, Baltimore, United States, ⁴Veterans Administration Maryland Health Care System, Research Service, Baltimore, United States, ⁵Veterans Administration, Multiple Sclerosis Center of Excellence, Baltimore, United States

Complement activation plays a central role in autoimmune demyelination. We have analyzed the role of complement C5 in experimental autoimmune encephalomyelitis (EAE) using C5-deficient (C5-d) and C5-sufficient mice and found that the absence of C5 resulted in fiber loss and extensive scarring. We now show that TGF- β and Response Gene to Complement (RGC)-32 were significantly increased during EAE in C5-d mice.

Since TGF- β profibrotic effects are mediated in part by RGC-32 we investigated its expression during EAE. RGC-32 level was higher in C5-d mice paralleling the pattern seen for TGF- β during acute and chronic EAE. RGC-32 was found to be expressed by CD4⁺ T cells, monocyte/macrophages and astrocytes. To further investigate the role of RGC-32 in mediation of extracellular matrix production we stimulated astrocytes in culture with TGF- β and used siRNA to silence RGC-32 expression and then we determined whether TGF- β was still able to induce collagens I-V and α smooth muscle actin (α SMA). RGC-32 silencing resulted in a significant reduction in TGF- β - induced collagens I, IV and V and α SMA expression. Using astrocytes isolated from RGC-32 knockout (KO) mouse, we found that TGF- β - induced collagen I, IV and α SMA expression was significantly reduced in RGC-32 KO when compared with wild type mouse. These data suggests that TGF- β might be responsible for the gliosis seen in C5-d during chronic EAE and its effects are at least in part mediated by RGC-32. Therefore, RGC-32 may represent a new target for therapeutic intervention in progressive multiple sclerosis.

1516

The complement modulator SALSA in placentaReichhardt, M.P.¹, Jarva, H.^{2,3}, Lokki, A.I.^{2,4}, Laivuori, H.^{4,5,6}, Vuorela, P.^{6,7}, Loimaranta, V.⁸, Siwetz, M.⁹, Huppertz, B.^{9,10}, Meri, S.^{2,3}

¹University of Oxford, Sir William Dunn School of Pathology, Oxford, United Kingdom, ²University of Helsinki, Immunobiology Research Program, Research Programs Unit, and Department of Bacteriology & Immunology, Medical Faculty, Helsinki, Finland, ³Helsinki University Hospital Laboratory (HUSLAB), Helsinki, Finland, ⁴University of Helsinki and Helsinki University Hospital, Medical Genetics, Helsinki, Finland, ⁵University of Helsinki, Institute for Molecular Medicine Finland, Helsinki, Finland, ⁶University of Helsinki and Helsinki University Hospital, Obstetrics and Gynecology, Helsinki, Finland, ⁷Porvoo Hospital, Obstetrics and Gynecology, Porvoo, Finland, ⁸University of Turku, Department of Medical Biochemistry and Genetics, Turku, Finland, ⁹Medical University of Graz, Institute of Cell Biology, Histology and Embryology, Graz, Austria, ¹⁰Medical University of Graz, Biobank Graz, Organizational Unit of Research Infrastructure, Graz, Austria

Background: The salivary scavenger and agglutinin (SALSA) has known functions in the antimicrobial defense at the mucosal surfaces. We have previously shown that SALSA also modulates the activation of the complement system, both in solution and on surfaces. Furthermore, we recently described the presence of SALSA in amniotic fluid. Strict regulation of the maternal immune response to the fetus is essential for a healthy pregnancy, and complications are often accompanied by immune attack towards components of placenta or the fetus itself. In the placenta this involves activation of the complement system.

Methods: In this study we investigated the potential role of SALSA in pregnancy by analyzing its presence in amniotic fluid and placental tissue during healthy and complicated pregnancies. SALSA levels in amniotic fluid were investigated using ELISA, and the expression in placenta was analyzed by fluorescence immunohistochemistry.

Results: SALSA levels in amniotic fluid increased during

pregnancy. Before 20 weeks of gestation the levels were slightly higher in patients who later developed pre-eclampsia than in gestation age-matched controls. In the placenta syncytial damage is often followed by the formation of fibrinoid structures. SALSA was found clustered into these fibrinoid structures in partial co-localization with complement C1q and fibronectin. In vitro analysis showed direct protein binding of SALSA to fibronectin.

Conclusion: In addition to antimicrobial defense, the data presented here suggest that SALSA, together with fibronectin and C1q, play a role in immunological regulation during pregnancy and may be involved in the containment of injured placental structures into fibrinoids.

2077

Susceptibility to leprosy and HBV coinfection is increased by polymorphisms compromising lectin pathway activation and abundance of a complement receptor

Boldt, A.B.W.^{1,2}, Kretzschmar, G.C.¹, Mendes, H.C.W.², Stingham, S.T.², Andrade, F.A.², Ueda, D.M.², Leitão, C.², Braga, A.C.d.M.², Stahlke, E.v.R.S.³, Thiel, S.⁴, Jensenius, J.C.⁴, Messias-Reason, I.J.T.²

¹Universidade Federal do Paraná, Genetics, Curitiba, Brazil,

²Universidade Federal do Paraná, Hospital de Clínicas, Curitiba, Brazil,

³Secretaria da Saúde do Estado do Paraná, Curitiba, Brazil,

⁴Aarhus University, Medical Microbiology and Immunology, Aarhus, Denmark

Thousands of leprosy patients in Brazil and India, not only suffer of physical deformities, but also either have or had HBV (hepatitis B virus) coinfection. Polymorphisms of the complement system modulate susceptibility to leprosy, but genetic susceptibility to coinfection is unknown. We used sequencing and multiplex sequence-specific PCR to genotype 55 functional/tag-SNPs of six genes encoding proteins of the lectin pathway (*MBL2*, *FCN1*, *FCN2*, *FCN3*, *MASP1*, *MASP2*) and two encoding complement receptors (*CR1*, *VSIG4-CR1g*) in up to 190 patients, 74 co-infected (positive for HBsAg and/or HBc) and 116 lepromatous. We also measured serum concentrations of mannan-binding lectin (MBL), MBL-associated proteins (MASP), H-ficolin (FCN-3), soluble complement receptor 1 (sCR1) and MBL mediated C4 activation (MBL-C4) in up to 167 patients. Polymorphisms lowering MBL, FCN-2, FCN-3, MASP-1 and MASP-2 concentrations increased susceptibility: *MBL2*LYQC* (OR=3.5, p=0.03), *FCN2*GGGCAC* (OR=3.6, p=0.01), *FCN3+1637delC* (OR=19.3, p=0.01), *MASP1*ACCA* (OR=4.4, p=0.001), *MASP2*p.126L+p.377A* (OR=4.6, p=0.013), respectively. *VSIG4* genotypes most probably producing a short form of the CR1g receptor, also increased susceptibility (OR=18.8, p=0.01), as well as *MASP2*2B1-i*, causing intermediate MASP-2 levels (OR=6.1, p=0.01) and *CR1*p.1208Arg*, associated with CR1 expression (OR=2.5, p=0.02). Except for *FCN2*, associations were independent of age, gender, ethnicity, institutionalization and lepromatous state, and did not increase susceptibility to leprosy *per se*. Associations for *FCN3*, *MASP1*, *MASP2* and *VSIG4* were also independent of each other. In conclusion, polymorphisms compromising activation of the lectin pathway of complement, as well as modulating the abundance of phagocytic receptors, increase susceptibility to leprosy-HBV coinfection.

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The epidermal growth factor receptor regulates complement activation in the local environment of epithelial tumors

Abu-Humaidan, A., Mohanty, T., Schmidtchen, A., Ekblad, L., Sonesson, A., Sørensen, O.E.

Lund University, Clinical Sciences, Lund, Sweden

The Epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor overexpressed in several epithelial tumors, and has proven to be an effective target for cancer treatment. EGFR controls diverse growth and immune functions, and its inhibition commonly leads to inflammatory skin lesions. Previously, we found that EGFR inhibition significantly enhanced the induction of complement components in keratinocytes and epidermis following stimulation with proinflammatory cytokines. Currently, Using Head and neck squamous cell carcinomas (HNSCC) cell lines, we found that EGFR inhibition decreased the expression of membrane complement regulatory proteins (mCRPs) and promoted activation of the complement system after incubation with serum in a C1q dependent manner in Cetuximab sensitive cell lines but not resistant cell lines. These data demonstrate an important role for EGFR in regulating the expression of complement components and complement activation in human epidermis and epithelial cancers and, to our knowledge, identify for the first time a pathway important for the epidermal regulation of complement activation. We hypothesize that tumors manipulate expression of membrane complement regulatory proteins (mCRPs) and other complement components to make use of complement's growth promoting or immune modulatory potential when faced with growth inhibition.

3579

Association of CR1 gene polymorphisms with severe forms of falciparum malaria

Das, N.^{1,2}, Madhukar, M.^{1,2}, Aggarwal, P.^{1,2}, Samantray, J.^{1,2}

¹Nayati Multi Superspecialty Hospital, Lab Medicine, Mathura,

²All India Institute of Medical Sciences ND, Biochemistry, New Delhi, India

We conducted a case-control study to elucidate the association of two exonic (*exon19*, G3093T, molecular weight and Knop's blood group polymorphisms) and one intronic (*intron27* (A-T), polymorphisms of CR1 with severe *falciparum* malaria. Two hundred healthy subjects, 88 patients with non-severe and 59 patients with severe malaria from the National Capital Region of India were enrolled for the study. Gene analysis was done by PCR-RFLP. frequency distribution of genotypes and alleles of CR1 density and structural polymorphisms were determined by gene counting method and related with disease severity. Regarding *exon19*, G3093T polymorphism, Odd's ratio (OR) of GG genotype was 5.27 (95% CI=2.677-10.78, p< 0.05), 5.139 (95% CI=1.945-15.89, p< 0.05) and 5.243 (95% CI=2.82-9.98, p< 0.05) to develop complicated form of the disease as compared to GT, TT and T allele carriers (GT + TT), respectively. Regarding *intron27* (A-T), *HindIII* density polymorphism, OR for AA genotype was 6.428 (95% CI=3.193-13.61, p< 0.05), 6.779 (95%

CI=2.393-23.81, $p < 0.05$) and 6.525 (95% CI=3.44-12.79, $p < 0.05$) to develop complicated form of the disease as compared to AT', T'T' and T' allele carriers (AT'+T'T'), respectively.

The alleles of these two polymorphisms were in linkage equilibrium, the haplotypes of risk alleles imposed further risk of developing severe complications of the disease.

A population screening for these two risk alleles in the malaria endemic region or epidemic may help identify people at higher risk of malarial complication and save lives. Knops group mutants were absent. Molecular weight polymorphism did not relate to the disease.

2087

Genetic variations of complement factors in schizophrenia

Ghazaryan, H.^{1,2}, Zakharyan, R.¹, Stepanyan, A.¹, Petrek, M.², Arakelyan, A.¹

¹Institute of Molecular Biology NAS RA, Laboratory of Human Genomics and Immunomics, Yerevan, Armenia, ²Palacky University, Laboratory of Immunogenomics, Olomouc, Czech Republic

Alterations in the immune response are involved in pathogenesis of schizophrenia - multifactor complex psychiatric disorder with heterogeneous clinical phenotype. Previous studies, including our own findings, indicated systemic hyperactivation of the alternative complement cascade in schizophrenia. However, molecular pathomechanisms of detected alterations are still unclear.

In the present study, we evaluated the potential association of schizophrenia with single nucleotide polymorphisms (SNPs) in genes encoding the complement factor B (*CFB*), complement factor H (*CFH*) and complement factor I (*CFI*) in Armenian population. Factor B is an essential component of the complement alternative pathway. Factors H and I are negative regulators of alternative pathway. In total, 310 patients with schizophrenia and 310 healthy subjects (controls) were enrolled in this study. Genomic DNA samples of schizophrenia patients and controls were genotyped for SNPs using polymerase chain reaction with sequence specific primers (PCR-SSP). Data were analyzed by Fisher's exact test. We measured also mRNA expression levels of these genes in peripheral blood (n=66 schizophrenia, n=99 controls).

According to the results obtained, *CFH* rs424535, *CFH* rs1061170 and *CFI* rs10033900 SNPs were positively associated with schizophrenia, while *CFB* rs12614 was negatively associated with schizophrenia. *CFH* rs800292, *CFI* rs1000954, *CFI* rs4469075 and *CFB* rs1048709 SNPs were not associated with schizophrenia. No differences in gene expression pattern were found.

In summary, our findings nominated *CFH* rs424535*A, *CFH* rs1061170*C and *CFI* rs10033900*C minor alleles as risk factors for schizophrenia, whereas the *CFB* rs12614 minor allele can be considered as a protective factor against this disease.

Transplantation 1

2744

Understanding the role of Interleukin-6 in human immune system

Ono, R.¹, Tomizawa-Murasawa, M.¹, Sato, K.¹, Matsuda, M.², Hasegawa, T.², Yoshida, H.³, Ohara, O.^{4,5}, Amagai, M.^{6,7}, Koseki, H.², Ishikawa, F.¹

¹RIKEN Center for Integrative Medical Sciences, Laboratory for Human Disease Models, Yokohama, Japan, ²RIKEN Center for Integrative Medical Sciences, Laboratory for Developmental Genetics, Yokohama, Japan, ³RIKEN Center for Integrative Medical Sciences, Laboratory for Immunogenetics, Yokohama, Japan, ⁴RIKEN Center for Integrative Medical Sciences, Laboratory for Integrative Genomics, Yokohama, Japan, ⁵Kazusa DNA Research Institute, Department of Human Genome Research, Kisarazu, Japan, ⁶RIKEN Center for Integrative Medical Sciences, Laboratory for Skin Homeostasis, Yokohama, Japan, ⁷Keio University, Department of Dermatology, Tokyo, Japan

Interleukin-6 (IL-6) is a pleiotropic cytokine and important to the maintenance of immune homeostasis. Over-expression of IL-6 in mice results in anemia, splenomegaly and lymphadenopathy as well as local inflammation in skin and lung. In human, aberrant expression of IL-6 may be related with autoimmune diseases or chronic inflammatory diseases such as rheumatoid arthritis or Castleman's disease.

To study the function of human IL-6 in human immunity, we have developed NOD/SCID/IL2rgKO (NSG) mice expressing human IL-6 (hIL-6 TG NSG mice). By transplanting human hematopoietic stem cells to these mice, we confirmed differentiation of human T cells, B cells, and myeloid cells in the recipient organs. During longer-term observation, hIL-6 TG NSG recipients showed lethargy and skin inflammation. Histological examination demonstrated epidermal thickening and interface dermatitis. In the recipient lung, mice showed perivascularitis and narrowed or obstructed bronchioles. Human CD3+ T cells were detected in the skin and lung of these mice. These observations in hIL-6 TG NSG humanized mice are consistent with chronic GVHD. We found increased human CD4+ T cells producing interferon gamma and Interleukin-17 and decreased regulatory T cells in the recipient spleen.

Our experiments suggest that hIL-6 TG NSG mice may serve as an in vivo model to understand the role of human IL-6 in the pathogenesis of chronic GVHD.

3849

Analysis of sites, kinetics and mechanisms of liver-graft-mediated depletion of alloreactive CD8 T cells reveals a critical role for liver tissue in liver transplant tolerance

Sierro, F.¹, Lu, B.D.², Tay, S.S.¹, Benseler, V.¹, McGuffog, C.¹, Bishop, A.G.³, Cowan, P.J.², Dwyer, K.M.², McCaughan, G.W.⁴, Bowen, D.G.⁴, Bertolino, P.¹

¹Centenary Institute, Liver Immunology, Camperdown, Australia, ²St Vincent's Hospital, Immunology Research Center, Melbourne, Australia, ³University of Sydney, Camperdown, Australia, ⁴Centenary Institute and AW Morrow Gastroenterology and Liver Centre, Camperdown, Australia

Unlike most solid organs, liver transplants are accepted across MHC incompatible barriers and induce donor-specific tolerance. The mechanisms, sites and kinetics of tolerance induction after transplantation remain unclear. Although donor passenger leukocytes (PL) are known to induce abortive activation of alloreactive T cells in recipient lymphoid tissues (RLT), some reports suggest that the liver tissue is a more important contributor to tolerance. We have developed a mouse liver transplantation model in which it is possible to trace the fate of PL, recipient leucocytes and of a reporter liver-reactive transgenic CD8 T cells expressing an H-2K^b-specific TCR. In this model, Ly5.2⁺C57BL/6 livers were transplanted into allogeneic Ly5.1⁺B10.BR recipients adoptively transferred following or prior transplantation with CFSE- or radio-labeled naïve alloreactive Tg T cells. Most liver-reactive CD8 T cells died within RLT within 48hrs following activation by PL. Although rare CD8 T cells surviving deletion, proliferated, recirculated in the blood and accumulated in the transplant, they were unable to reject the liver transplant or donor skin transplanted 100d post-liver transplantation. This tolerance effect was associated with clearance of donor CD8 T cells within the liver tissue following invasion and degradation in hepatocyte lysosomes. Thus, although most liver-reactive CD8 T cells were deleted in RLT within the first 48h post-transplantation, the liver plays a critical role in clearing surviving T cells. These results reveal a novel and unexpected sequence of events in which both RLT and liver tissue contribute to deletion of alloreactive T cells and to the induction of donor-specific tolerance.

3824

Modelling sterile inflammation in syngenic heart transplantation model

Nanayakkara, C.^{1,2}, Perkins, T.¹, Melton, P.^{3,4}, Datta, A.⁵, Larma, I.^{1,6}, Huang, W.H.^{1,7}, Eule, U.¹, Liu, L.¹, Prosser, A.¹, Lucas, A.^{8,9}, Delriviere, L.^{1,7}, Kallies, A.¹⁰, Gaudieri, S.^{2,11}, Lucas, M.^{1,7,11,12,13}

¹University of Western Australia, School of Medicine and Pharmacology, Perth, Australia, ²University of Western Australia, School of Anatomy, Physiology and Human Biology, Perth, Australia, ³University of Western Australia, Centre for Genetic Origins of Health and Disease (GOHaD), Perth, Australia, ⁴Curtin University, Faculty of Health Sciences, Perth, Australia, ⁵University of Western Australia, School of Computer Science and Software Engineering, Perth, Australia, ⁶University of Western Australia, Centre for Microscopy, Characterisation and Analysis (CMCA), Perth, Australia, ⁷Sir Charles Gairdner Hospital, WALK Surgical Transplant Services, Perth, Australia, ⁸Harry Perkins Institute of Medical Research, Institute for Respiratory Health, Perth, Australia, ⁹University of Western Australia, Centre for Cell Therapy and Regenerative Medicine (CTRM), Perth, Australia, ¹⁰Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia, ¹¹Institute for Immunology & Infectious Disease (IID), Murdoch University, Perth, Australia, ¹²PathWest Laboratory Medicine, Queen Elizabeth II Medical Centre, Perth, Australia, ¹³University of Western Australia, School of Pathology and Laboratory Medicine, Perth, Australia

Sterile inflammation is an understudied consequence of surgery which contributes to intra-graft inflammation. In this study, we

took a step towards modelling sterile inflammation using a syngenic murine heart transplantation model where native and grafted hearts were studied at Day 0 (n=5), Day 1 (n=3) and Day 7 (n=3) by RNAseq transcriptomic analysis. Mapping 75Gb of raw data to the reference genome, assembly, quantification and differential expression analysis of transcripts both longitudinally (T₀>T₁>T₇) and conditionally (native vs graft) identified genes with significant p-values which were then entered into Pathways Studio 11.0.5 for identification of significant curated inflammation pathways. Results reveal that pathways involving Anti-Inflammatory Function of Macrophage M2 Lineage, Vascular Endothelial Cell Permeability Activation and CR3-Mediated Phagocytosis in Neutrophils and Macrophages were dominantly up-regulated in both graft and native hearts throughout Days 1-7. Additionally, Eosinophil Activation, CC Chemokine Receptor Signalling and CXC Chemokine Receptor Signalling pathways were also significantly up-regulated but in only the native heart at Day 7. A key finding of transcriptomic analysis was the reversal of the regulation pattern at T₁ for above six pathways in the native heart relative to the graft heart. However, by T₇ three of these pathways had become up-regulated relative to T₁ akin to what was observed in the graft hearts at this later stage. Cellular characterisation of the infiltrates by flow cytometry and the impact of mismatched grafts are now being assessed as an aid to identify the underlying functional nodules at the interface between innate and adaptive immunity governing these pathways.

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IL-28 Is a critical cytoprotectant in transplantation

Henden, A.^{1,2}, Robb, R.¹, Clouston, A.³, Kuns, R.¹, Lane, S.¹, Gartlan, K.¹, Hill, G.¹

¹QIMR Berghofer Medical Research Institute, Bone Marrow Transplantation Laboratory, Herston, Australia, ²University of Queensland, School of Medicine, Herston, Australia, ³Envoi Pathology, Brisbane, Australia

Aims: We have demonstrated a protective role for type I Interferons (IFN) through inhibition of Th1 differentiation invoked by recipient CD8neg dendritic cells after experimental bone marrow transplantation. Recently described Type III IFNs (IFNλ/IL-28) signal through the unique IL-28R primarily expressed in epithelial tissues and implicated in mucosal pathogen defence. Clinical use of type I IFN is associated with adverse neurological, haematological and constitutional symptoms where IL-28 is better tolerated, yet still demonstrates potent anti-viral effects.

Methods: We used IL-28R^{-/-} and IFNαR1^{-/-} donors and recipients in murine models of GVHD and GVL to develop logical therapeutic strategies to improve transplant outcomes.

Results: IL-28R^{-/-} donors invoked similar GVHD and GVL to WT. However IL-28R^{-/-} recipients had accelerated acute GVHD (aGVHD) mortality and disease relative to WT with a phenotype intermediate to that and the hyperacute aGVHD seen in IFNαR1^{-/-} recipients (median survival WT 42 vs. IL28R^{-/-} 26 vs. IFNαR^{-/-} 6.5 days, p< 0.0001). IL28R^{-/-} recipients have augmented colonic GVHD histopathology early (d 7) after BMT (WT 7.111±0.6550 vs. IL28R^{-/-}12.33±0.7993, p=0.0004) and

exaggerated inflammatory cytokine generation (d4 IFN γ WT 331 \pm 41.47pg/mL vs. IL28R $^{-/-}$ 667 \pm 48.79 pg/mL, $p < 0.0001$ and IL-6 WT 61.25 \pm 10.91pg/mL vs. IL28R $^{-/-}$ 91.99 \pm 11.23pg/mL, $p = 0.024$). Re-transplantation of chimeras with WT or IL28R $^{-/-}$ haematopoietic, non-haematopoietic tissue or combinations thereof demonstrated that IL-28 mediated protection required signalling through both compartments, putatively recipient antigen presenting cells and colonic epithelia.

Conclusion: IL-28 represents an attractive therapeutic to mediate cytoprotection within the GI tract and attenuate to GVHD in the peri-transplant period.

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Revealing a critical cross-talk between beta islet cells and the vasculature in diabetes

Bonder, C.¹, Myo Min, K.K.¹, Parham, K.¹, Rojas-Canales, D.², Penko, D.², Drogemuller, C.², Coates, P.T.²

¹Centre for Cancer Biology, University of South Australia & SA Pathology, Adelaide, Australia, ²Centre for Clinical and Experimental Transplantation, Royal Adelaide Hospital & University of Adelaide, Adelaide, Australia

Pancreatic islet transplantation is a promising cellular therapy for Type 1 Diabetes; however, success rates are limited due to suboptimal revascularization of the islet graft once transplanted. Co-culture of endothelial cells with the insulin producing beta islet cells has been shown to improve beta cell survival and function *in vivo*. In light of this novel interaction between endothelial cells and beta islet cells, our study investigates the role of the surface cadherin desmoglein-2 (DSG2) as a novel and important cell adhesion molecule that mediates this cross-talk. We recently discovered that DSG2 is expressed by both endothelial and beta cells, and that beta cells from patients with diabetes have reduced gene expression of DSG2. Therefore, investigation of DSG2's cell-cell adhesion properties will aid in the understanding of the mechanisms of co-dependency between endothelial and beta cells. We generated, and then interrogated, *Dsg2*^{-/-} mice which revealed that *Dsg2* is important for blood vessel formation and that these mice are more susceptible to streptozotocin-induced diabetes. Immunofluorescence has also identified altered morphology of pancreatic vasculature in the *Dsg2*^{-/-} mice, suggesting a previously unrecognized role for this cadherin in the pancreas. Our investigation into the function of *Dsg2* suggests an important role in intercellular communication as well as possibly cell survival and proliferation. These novel findings suggest that DSG2 may be an important target in the fight against a debilitating metabolic disease that affects over 1 million Australians, diabetes.

3915

Understand the role of allo-HLA cross-reactive CD8+ T cells in a transplant setting

Rowntree, L.¹, Van der Heuvel, H.², Claas, F.², Kotsimbos, T.^{1,3}, Purcell, A.⁴, Mifsud, N.⁴

¹Monash University, Department of Medicine, Melbourne, Australia, ²Leiden University Medical Centre, Department of Immunohaematology and Blood Transfusion, Leiden, Netherlands, ³The Alfred Hospital, Department of Allergy, Immunology and Respiratory Medicine, Melbourne, Australia, ⁴Monash University, Department of Biochemistry and Molecular Biology, Melbourne, Australia

Following solid organ transplantation with HLA-mismatched allografts, a proportion of the alloresponse can be attributed to anti-viral T cells which cross-react with the allograft and potentially mediate immunopathology. In addition, infection with or reactivation of latent DNA viruses such as Epstein Barr virus (EBV) have been associated with poor clinical outcomes post-transplantation. We have demonstrated that CD8+ T cells specific for HLA-B*07:02-restricted EBV peptide RPPFIRRL (B7/RPP) cross-recognise HLA-B*40:02, but only minimally recognise HLA-B*40:01. Cross-reactivity was identified when B7/RPP-specific CD8+ T cells were stimulated with allogenic (allo) PBMCs or transfected K562 cell lines (SALs) expressing alternate HLA molecules. Immune reactivity was then measured using proliferation or cytokine production. The T cell receptor (TCR) signature of two cross-reactive B7/RPP T cell clones from the same healthy donor was found to be TRAV14/DV14TRAJ9_TRBV19TRBJ2-7. Subsequent investigations in four HLA-B7+ lung transplant recipients (LTR) who received a HLA-B40 mismatched allograft were screened for cross-reactive B7/RPP-specific CD8+ T cells across a minimum of two time points ranging from pre- to 12 months post-transplant. Cross-reactive T cells were identified in two LTR; present at pre-, 6 and 11 months post-transplant for LTR117 (TCR signature TRAV38-1TRVJ43_TRBV4-1TRBJ2-3) and at 6 and 12 months post-transplant for LTR54 (TCR signature to be determined). Neither patient experienced EBV reactivation during the first 12 months post-transplant, as such LTR117 TCR usage remained static with no adverse clinical events affecting allograft function. This data corroborates our hypothesis that a clinically significant viral reactivation event is necessary to trigger immunopathology driven by cross-reactive T cells.

3992

Characterising the immune response to allogeneic limbal stem cell transplantation

Sagoo, P.¹, Stauss, H.¹, Morris, E.¹, Shortt, A.J.^{1,2}

¹University College London, Institute of Immunity & Transplantation, London, United Kingdom, ²Moorfields Eye Hospital, Biomedical Research Centre for Ophthalmology, London, United Kingdom

Transplantation of *ex vivo* cultivated limbal stem cell (LSC) allografts is a promising approach for the treatment of clinical bilateral corneal disease caused by limbal stem cell deficiency (LSCD). The use of cultivated LSC from donor tissue would provide a bank of tissue-derived stem cell products for the estimated

10 million patients in need worldwide. However, allogeneic transplantation of LSC has a significantly higher rate of clinical graft failure than autologous LSC. Our current understanding of the immunological processes that lead to stem cell graft failure in the eye is limited and further compounded by the lack of model systems of this unique transplantation setting.

To study the immune mechanisms leading to LSC graft rejection, we have established a murine experimental model of LSCD and therapeutic LSC transplantation. LSCD induced through chemical burn injury is established within several days, with characteristic clinical features of corneal opacity, neovascularisation and epithelial defects. In parallel, LSCD induces a profound lymphocytic corneal infiltrate, composed primarily of inflammatory monocytes, NK, neutrophils and T cells. Corneal composite cell layers also respond to injury through the dramatic and sustained upregulation of MHC molecules, not only providing a potential source of donor alloantigen *in vivo*, but also the capacity to stimulate T cells through the direct pathway of allorecognition. Using a combination of traceable stem cell grafts and effector components of the immune response, with real-time imaging techniques, we are performing a detailed longitudinal analysis of the engraftment, survival and fate of LSC allografts in the eye.

2181

Combined blockade of VEGFR- and VEGFR-3 promotes high-risk corneal transplant survival 2

Wen, Y., Zhang, L., Kang, G.J., Chen, L.

University of California at Berkeley, Center for Eye Disease and Development, Program in Vision Science and School of Optometry, Berkeley, United States

Purpose: Though corneal transplantation enjoys a low rejection rate of 10% in uninflamed corneas or "low-risk" setting, the rejection rate in inflamed and vascularized corneas or "high-risk" setting can be as high as 50-90%. This study was designed to investigate the effect of combined blockade of VEGFR-2 and VEGFR-3 on "high-risk" corneal transplant survival.

Methods: High-risk corneal transplantation was performed between normal C57BL/6 (donor) and inflamed BALB/c (recipient) mice. The recipients were randomized to receive intraperitoneal injections of VEGFR-2 and VEGFR-3 neutralizing antibodies (provided by Eli Lilly and Company) or control twice a week for 8 weeks. Corneal grafts were evaluated by ophthalmic slit-lamp biomicroscopy and analyzed by Kaplan-Meier survival curve.

Results: Compared to the control group, the transplants in the treatment group demonstrated a greater degree of transparency and a higher rate of survival as well.

Conclusion: Combined blockade of VEGFR-2 and VEGFR-3 may offer a new and effective strategy to promote high-risk corneal transplant survival.

3807

Sustained mixed chimerism and regulatory t-cell subsets reconstitution in patients with sickle cell disease after allogeneic hematopoietic stem cell transplantation

de Azevedo, J.T.C.^{1,2}, Palma, P.V.B.¹, Marques, A.¹, Simões, B.P.³, Covas, D.T.^{1,3}, Donadi, E.A.^{2,3}, Malmegrim, K.C.R.^{1,4}

¹National Institute of Science and Technology in Stem Cells and Cell Therapy, Regional Hemotherapy Center of the School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, Brazil,

²Department of Biochemistry and Immunology, School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, Brazil,

³Department of Clinical Medicine, School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, Brazil, ⁴Department

of Clinical Analysis, Toxicological and Bromatological, School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Ribeirão Preto, Brazil

Introduction: Currently, allogeneic hematopoietic stem cell transplantation (HSCT) is a curative therapeutic option for patients with sickle cell disease (SCD). Nevertheless, hematologic and immune mechanisms involved with immune tolerance achievement after HSCT in SCD patients are not entirely elucidated. Therefore, to study the immune reconstitution dynamics and role of different cells after transplantation in SCD is of great relevance.

Methods and results: Chimerism analysis after HSCT was carried out by short tandem repeats sequencing of peripheral blood mononuclear cells from fifteen patients. Five patients had full chimerism, nine had mixed chimerism and one patient had graft failure. The average of donor cells was 78.38% at 1 month (N=9), 81.09% at 3 months (N=12), 81.27% at 6 months (N=14) and 80.39% at 12 months after transplantation (N=7). In addition, flow cytometry analyses of peripheral blood cells isolated from SCD patients at pre-transplantation and several periods after HSCT, demonstrated significant increased numbers of regulatory T-cell subsets ($p < 0.05$). CD4+CD25^{hi}GITR+ T-cell counts increased at 12 months (mean±SD: 6.33±6.31) after transplantation when compared to pre-transplantation (1.35±1.54) and CD8+CD28-CD57+ T-cell subset increased at 24 months (247.8±273.7) after transplantation compared to pre-transplantation (29.14±30.67).

Conclusion: Our results demonstrated that most patients did not have full chimerism after transplantation, indicating that sustained mixed engraftment is able to cure SCD patients. Immune reconstitution evaluations showed increased numbers of T-cell subsets with regulatory profile suggesting their involvement with immune tolerance development after HSCT in SCD patients.

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Mucosal Immunology 2

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The IBD-associated autophagy gene *Atg16l1* differentially regulates Treg and Th2 cells to control intestinal inflammation

Kabat, A., Pott, J., Maloy, K.

University of Oxford, Sir William Dunn School of Pathology, Oxford, United Kingdom

Autophagy is a homeostatic cellular degradation pathway with emerging roles in immune regulation. A polymorphism in the essential autophagy gene *Atg16l1* is associated with susceptibility to inflammatory bowel disease, however it remains unclear how autophagy contributes to intestinal immune homeostasis. Using targeted deletion of *Atg16l1* in T cells, we demonstrate a profound requirement for autophagy in the maintenance of balanced adaptive immune responses within the intestinal mucosa. Selective deletion of *Atg16l1* in T cells resulted in spontaneous intestinal inflammation and dysregulated type 2 humoral responses to dietary and microbiota antigens, including IgE responses. Disease correlated with a profound loss of Foxp3⁺ Treg cells, while mucosal Th2 population was selectively enhanced. We demonstrate that, in contrast to other intestinal T helper cells, autophagy intrinsically limits mucosal Th2 cell expansion, revealing a novel aspect of regulation of type 2 responses by autophagy. Furthermore, through selective ablation of *Atg16l1* in Treg cells we show for the first time that disruption of autophagy pathway in Treg cells is sufficient to drive severe systemic and intestinal inflammation, as autophagy is critically important for Foxp3⁺ Treg cells for their survival and metabolic adaptation to the intestinal environment. This study reveals a fundamental role of autophagy in orchestrating mucosal T cell responses, providing a new perspective on how impaired autophagy may predispose to intestinal inflammation and hypersensitivities, suggesting new translational perspectives for their treatment.

3085

TH17 cells express ST2 and are controlled by the alarmin IL-33 in the small intestine

Pascual-Reguant, A.^{1,2}, Bayat-Sarmadi, J.¹, Baumann, C.^{1,3}, Noster, R.⁴, Cirera-Salinas, D.^{1,2}, Caterina, C.^{1,2}, Huber, S.⁵, Zielinski, C.E.⁴, Löhning, M.^{1,3}, Esplugues, E.^{1,2,6}, Hauser, A.E.^{1,2,7}

¹Deutsches Rheumaforschungszentrum, A Leibniz Institute, Berlin, Germany, ²Charité Universitätsmedizin, Cluster of Excellence NeuroCure, Berlin, Germany, ³Charité Universitätsmedizin, Experimental Immunology, Dept. of Rheumatology and Clinical Immunology, Berlin, Germany, ⁴TU München, Medizinische Mikrobiologie, Immunologie und Hygiene, Munich, Germany, ⁵Universitätsklinikum Hamburg-Eppendorf, I. Medizinische Klinik, Hamburg, Germany, ⁶Icahn School of Medicine at Mount Sinai, Immunology Institute, New York, United States, ⁷Charité Universitätsmedizin, Immune Dynamics, Berlin, Germany

Interleukin (IL)-17-producing T helper cells (TH17) are major drivers of inflammation and have been involved in different human autoimmune diseases. Tissue inflammation is a beneficial host response to infection but can also contribute to inflammatory diseases and autoimmunity if it is unregulated. The crosstalk between the inflamed tissue and the immune system during an inflammatory immune response is a key factor for preserving the tissue integrity and to maintain the normal physiological processes. However, how inflamed tissue is able to regulate the magnitude of an immune response by controlling pro-inflammatory immune T cells is not well characterized so far. Here, we show that intestinal epithelial cells (IEC) are the main source of the alarmin interleukin-33 (IL-33) upon T-cell dependent intestinal inflammation. Moreover, we find that TH17 cells express the IL-33 receptor (ST2) *in vivo*. IL-33 promotes a reduced expression of pro-inflammatory genes (*Tbx21*, *Ifng* and *Csf2*) and induces the expression of *Il10* in mouse as well as human TH17 cells. Finally, we show that pro-inflammatory TH17 cells acquire immune-suppressive properties in response to IL-33. Our results give new insights into the control of pro-inflammatory TH17 in both mice and men, and provide a mechanism by which epithelial cells of the small intestine are able to regulate inflammatory responses via the IL-33/ST2 axis in order maintain systemic immune homeostasis.

2783

MR1-dependent activation of intrahepatic MAIT cells by bacterially exposed biliary epithelial cells and liver B cells

Jeffery, H.C.¹, van Wilgenburg, B.², Kurioka, A.², Parekh, K.¹, Stirling, K.¹, Roberts, S.¹, Dutton, E.E.³, Hunter, S.¹, Geh, D.¹, Braitch, M.K.¹, Rajanayagam, J.¹, Iqbal, T.⁴, Pinkney, T.⁴, Brown, R.⁴, Withers, D.R.³, Adams, D.H.^{1,4}, Klenerman, P.², Oo, Y.H.^{1,4}

¹University of Birmingham, Centre for Liver Research & NIHR Biomedical Research Unit, Institute of Immunology and Immunotherapy, Birmingham, United Kingdom, ²Oxford University, Peter Medawar Building for Pathogen Research, Oxford, United Kingdom, ³University of Birmingham, Institute of Immunology and Immunotherapy, Birmingham, United Kingdom, ⁴University Hospital of Birmingham NHS Foundation Trust, Birmingham, United Kingdom

Mucosal-Associated Invariant T-cells (MAITs) are innate-like T-cells characterised by the invariant TCR-chain, Va7.2-Ja33 and high CD161 expression. They are activated by bacterial vitamin B metabolites presented on MR1, and function in antibacterial immunity at mucosal sites. MAITs are found in the human liver but the detailed characteristics of liver-infiltrating MAITs (LI-MAITs) in health and chronic liver diseases and their role in liver immune surveillance remained unexplored and were investigated in this study.

LI-MAITs were present at reduced frequency (% total CD3⁺ T-cells) in diseased compared to normal livers and ratios of CD4⁺/CD8⁺/CD4⁺CD8⁻ subsets altered. In normal and diseased livers LI-MAITs localized predominantly around bile ducts in portal tracts as shown by immunohistochemistry for TCR-Va7.2 and 4-colour confocal microscopy for CD3/CD161/TCR-Va7.2/nucleus. Consistent with this distribution, in flow cytometry analysis they expressed biliary-tropic chemokine receptors CCR6, CXCR6, and integrin α E β 7. LI-MAITs were also present at lower density in the hepatic sinusoids and possessed tissue-homing chemokine receptor CXCR3 and integrins LFA-1 and VLA-4, suggesting their recruitment via hepatic sinusoids. LI-MAITs had an activated, effector memory phenotype, expressed integrin α 4 β 7 and receptors for IL-12, IL-18 and IL-23 and produced cytokines IFN- γ , TNF- α , and IL-17. Importantly, in co-culture assays, in response to *E. coli*-exposed macrophages, liver B-cells and biliary epithelial cells, MAITs up-regulated IFN- γ , TNF- α and CD40-Ligand and degranulated in an MR1-dependent, cytokine-independent manner.

Our findings provide the first evidence of an immune surveillance effector response for MAITs towards biliary epithelial cells and B-cells in human liver that might be targeted therapeutically in the future.

836

Expression of CCR2 on CD4⁺ T-cells is critical for mediating colonic inflammation and modulating gut microbiota to promote intestinal tumorigenesis

Jala, V.¹, Bodduluri, S.¹, Chilton, P.², Chheda, Z.², Sharma, R.², Haribabu, B.¹

¹University of Louisville, Dept. of Microbiology and Immunology, Louisville, United States, ²University of Louisville, Louisville, United States

CCR2/CCL2 axis is critical for recruitment of immune/inflammatory cells including peripheral blood monocytes/macrophages, immature dendritic cells and mast cells. The molecular mechanisms involving CCR2/CCL2 axis in promoting colon cancer are unknown. We investigated CCR2 dependent recruitment of immune cells and modulation of gut microbiota in the context of colon tumorigenesis. Our data demonstrated that lack of CCR2 in the *Apc*^{Min/+} mice (a spontaneous intestinal tumor model) background significantly increased survival and decreased small intestinal and colon tumors both in size and number compared to *Apc*^{Min/+} mice. Analysis of tumors suggested that CCR2^{-/-}*Apc*^{Min/+} displayed decreased tumor infiltrating F4/80⁺ cells, CD8⁺ T cells as well as inflammatory mediators (e.g., IL-6, CXCL1, IL-23 and IL-17) compared to *Apc*^{Min/+} mice. Gut microbiota analysis suggested that significant

reduction in tumor promoting *Bacteroides* genus in CCR2^{-/-}*Apc*^{Min/+} mice compared to *Apc*^{Min/+} mice indicating critical role for CCR2 in maintaining gut homeostasis. To define the role of CCR2 on CD4⁺ T-cell function, we adoptively transferred of splenic CD4⁺ T- cells from CCR2^{-/-}*Apc*^{Min/+} mice into Rag2^{-/-} mice. Interestingly, Rag2^{-/-} mice transferred with CCR2^{-/-}*Apc*^{Min/+} CD4⁺ T- cells did not develop colitis, whereas CD4⁺ *Apc*^{Min/+} T-cell transferred mice developed significantly severe colitis. Next, in CD45Rb^{hi} T-cell induced colitis model, adoptive transfer of CCR2^{-/-} CD4⁺ CD45Rb^{hi} T-cells in to Rag2^{-/-} mice also failed to develop colitis and significantly reduced IL-17 production. In summary, results suggests that expression of CCR2 is critical for recruitment of tumor-associated macrophages (TAMs) as well as activation and recruitment of Th17 cells to promote intestinal inflammation and tumorigenesis.

1058

Aeroallergen-induced IL-33 predisposes to respiratory virus-induced asthma by dampening type I and III interferon production

Werder, R.¹, Lynch, J.¹, Simpson, J.¹, Loh, Z.¹, Zhang, V.¹, Spann, K.², Sly, P.³, Mazzone, S.¹, Upham, J.⁴, Phipps, S.¹

¹University of Queensland, School of Biomedical Science, Brisbane, Australia, ²Queensland University of Technology, Brisbane, Australia, ³University of Queensland, Queensland Children's Medical Research Institute, Brisbane, Australia, ⁴University of Queensland, School of Medicine, Brisbane, Australia

Frequent viral lower respiratory infections (vLRI) and allergic sensitization in early life are independent risk factors for asthma onset, yet together significantly increase the development of persistent asthma. We developed an experimental model of asthma to investigate this synergy. Neonatal BALB/c mice were inoculated with low dose pneumonia virus of mouse (PVM; 1pfu) then exposed to low dose (1 μ g) cockroach antigen or vehicle control at 3 days post infection (dpi). Some mice were re-infected 6 weeks later and exposed to weekly doses of allergen. Virus and allergen co-exposure was critical in both early and later life for disease onset and progression, including airway hyperreactivity, airway remodelling and type 2 inflammation. Allergen exposure during primary vLRI increased IL-33 release and impaired antiviral cytokine production, leading to increased epithelial viral burden, Th2-type inflammation and airway smooth muscle growth. Neutralisation of IL-33 in early life prevented type 2 inflammation, airway remodelling and reversed the dampened interferon response mediated by cockroach antigen. Substitution of allergen with exogenous IL-33 attenuated antiviral cytokines, elevated viral load and promoted airway remodelling. Mechanistically, we found that IL-33 degraded IRAK1 to dampen type I IFN production by plasmacytoid DC. In summary, we identify a novel role for IL-33 in regulating antiviral immunity and as a target to attenuate the synergistic interplay between two important environmental insults in the onset and progression of asthma.

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Notch-STAT5b is central for the development of TCRαβ⁺CD8αα⁺ small intestinal intraepithelial lymphocytes*Ishifune, C.¹, Maekawa, Y.², Hozumi, K.³, Yasutomo, K.¹*¹Graduate School of Medicine, Tokushima University, Department of Immunology and Parasitology, Tokushima, Japan, ²Gifu University Graduate School of Medicine, Department of Parasitology and Infectious Diseases, Gifu, Japan, ³Tokai University School of Medicine, Department of Immunology, Isehara, Japan

Intraepithelial lymphocytes (IELs) in the small intestine are divided into several subsets and TCRαβ⁺CD8αα⁺ IELs and TCRγδ⁺ IELs have suppressive roles in colitis models. However, the molecular mechanisms underlying IELs development and their functions are largely obscure.

We found that TCRαβ⁺CD8αα⁺ IELs and their maturation from Thy1⁺ to Thy1^{neg} Granzyme B⁺ cells in the small intestine were markedly decreased in CD4-Cre dependent Notch1, 2-Rbpj signal deficient mice compared with control mice. We did not find any reduction of IELs precursors in the thymus and their ability to differentiate toward CD8αα⁺ cells in CD4-Cre Rbpj^{fl/fl} mice *in vitro*. Villin1-Cre dependent Jagged1, Dll1 or Dll4 deficient mice had normal number of IELs. Those data indicate that both Notch1 and Notch2 regulate the differentiation of TCRαβ⁺CD8αα⁺Thy1^{neg} IELs but Jagged1, Dll1, Dll4 in the intestinal epithelium is not involved in the differentiation. TCRαβ⁺CD8αα⁺ IELs from CD4-Cre Rbpj^{fl/fl} mice have reduced expression of STAT5b but not STAT5a. CD4-Cre Stat5a/b^{fl/fl} mice had few numbers of TCRαβ⁺CD8αα⁺ IELs, suggesting that STAT5a/b is required for the regulation of TCRαβ⁺CD8αα⁺ IELs. Furthermore, overexpression of constitutive-active STAT5b in CD4-Cre Rbpj^{fl/fl} mice could partially rescue the reduction of TCRαβ⁺CD8αα⁺ IELs but not their maturation.

These data indicate that Notch1 and Notch2 control the differentiation of TCRαβ⁺CD8αα⁺ IELs in the small intestine without affecting its precursor development. Furthermore, Notch-mediated differentiation of TCRαβ⁺CD8αα⁺ IELs would be, at least partly, regulated by the activation of STAT5b signaling.

2535

Probiotics in Crohn disease: selecting strains able to trigger Paneth cells-derived antimicrobial response and downregulate inflammatory responses*Hrdy, J.^{1,2}, Cesaro, A.³, Lapadatescu, C.⁴, Chamailard, M.³, Pot, B.², Grangette, C.²*¹First Faculty of Medicine, Charles University in Prague, Institute of Immunology and Microbiology, Prague, Czech Republic, ²Bactéries Lactiques et Immunité des Muqueuses, Centre d'Infection et d'Immunité de Lille, CNRS UMR 8204, Institut Pasteur de Lille, Université Lille Nord de France, Lille, France, ³Récepteurs Nods-Like dans l'Infection et l'Immunité, Centre d'Infection et d'Immunité de Lille, Inserm U 1019-CNRS UMR 8204, Institut Pasteur de Lille, France, Université Lille Nord de France, Lille, France, ⁴Bioprox Company, Levallois, France

In genetically susceptible individuals, an inappropriate mucosal immune response against intestinal microbiota appears to be the principal mechanism leading to the pathogenesis of IBD. Importantly, Paneth cells showed impaired secretion of

antimicrobial peptides in Crohn disease patients. Our aim was to select probiotic strains able to restore the functionality of Paneth cells and dampen inflammatory immune responses. The capacity of probiotic strains to induce defensin expression was tested *in vitro* using the murine epithelial cell line mCcl2. Induction of activation markers on bone marrow derived dendritic cells (BMDC) after 24 hrs of coculture with bacterial strains was followed by flow cytometry. The potential of probiotic strains to promote either Th17 or Tregs was evaluated by coculture of naive CD4⁺CD25⁻ cells with probiotic-primed BMDC. Mouse model of *Citrobacter rodentium* infection and TNBS acute colitis were used for testing capacity of probiotic strains to decrease inflammation *in vivo*. The best inducers of defensins in mCcl2 were *E. coli*, *L. reuteri* or *L. acidophilus* strains. *L. acidophilus* strains were the most potent inducers of activation markers on BMDC and the best inducer of IL-17, while *L. reuteri* strains were the best inducers of Tregs. In a mouse model of *Citrobacter rodentium* infection, selected strains were able to decrease parameters of inflammation and to increase defensin gene expression in colon of infected mice. In conclusion, we have identified several promising probiotic strains in the context of IBD. Detailed mechanism of action needs to be clarified. This work was supported by ANR project BIOpaneX, AZV15-26877.

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Characterization of the inflammatory response in the upper respiratory tract following influenza infection*Palomino Segura, M., F. Gonzalez, S.**Institute for Research in Biomedicine (IRB), Bellinzona, Switzerland*

Influenza virus is responsible of high morbidity and mortality worldwide and a leading cause of death amongst young children, old people and the immuno-compromised. Despite the fact that the initial phase of the infection occurs in the upper respiratory tract, little is known about the immune reaction that follows infection and how it affects the outcome of the disease. Our research focused on elucidating the role of the inflammatory response in the mucosa of the trachea during early stages following influenza infection. Using 2-photon microscopy we observed a the dense network of dendritic cells (DC) located under the mucosa of the trachea from mice, which increased significantly their number at day 3 post infection (p.i.) with influenza virus. The characterization of the tracheal subgroups of DC according to the expression of different surface markers indicated that at day 3 p.i. the majority of the infiltrated DC correspond to the inflammatory phenotype (CD45⁺, CD11c⁺, MHCII⁺, CD11b⁺, Ly6c^{hi}). The recruitment of DC coincided in time with a prominent infiltration of NK cells and elevated levels of IFNγ at day 3 p.i.. We hypothesized that the latter are responsible of this early secretion of IFNγ, which most likely affected the maturation state of the inflammatory DC. Additionally, we observed that IFNγ production is crucial for the recruitment of neutrophils to the site of infection. In future studies we will evaluate how the manipulation of the early inflammation events will influence the initiation of the adaptive response and the outcome of the disease.

1057

Monoclonal intestinal IgAs are poly-reactive but recognize a specific amino acid sequence of protein expressed by multiple bacteria

Usui, F.¹, Okai, S.¹, Hasegawa, M.², Yamamoto, K.³, Nishiyama, E.³, Mori, H.³, Yamada, T.³, Kurokawa, K.⁴, Shinkura, R.¹

¹Nagahama Institute of Bio-Science and Technology, Immunology, Shiga, Japan, ²Nagahama Institute of Bio-Science and Technology, Protein Function Analysis, Shiga, Japan, ³Tokyo Institute of Technology, Graduate School of Bioscience and Biotechnology, Tokyo, Japan, ⁴Tokyo Institute of Technology, Earth-Life Science Institute, Tokyo, Japan

Immunoglobulin A (IgA) is the main antibody isotype secreted into the intestinal lumen. It plays a critical role in the defense against pathogens and in the maintenance of intestinal homeostasis. However, the molecular mechanisms of how secreted IgA regulates intestinal microbiota are not completely understood. We generated IgA-producing hybridomas derived from the intestinal IgA-secreting cells of wild-type mice. Four monoclonal IgAs (W27, W30, W34, W43) bound to more than 10 different intestinal bacteria with variable affinity. To elucidate their specific antigens, two different bacteria (*Escherichia coli* and *Pseudomonas fulva*) were lysed and used for Western blotting. Unexpectedly, all those four monoclonal IgAs recognized a single protein at the size of about 50 kDa. On the contrary, these four monoclonal IgAs did not recognize any proteins expressed in *Bifidobacterium bifidum*, *Blautia coccoides* and *Lactobacillus casei*. Mass spectrometry analysis revealed that four monoclonal IgAs recognized a same protein, serine hydroxymethyltransferase (SHMT) in *E. coli*. Most bacteria have *glyA* gene encoding SHMT. We further identified the epitope of SHMT in its N terminus. W27 recognized specific four amino acids (EEHI) in their N-terminal motif of *E. coli* and *Pseudomonas fulva*, but W27 did not recognize the different AAs in the corresponding position of *Blautia coccoides* and *Lactobacillus casei*. Our observations strongly suggest that W27 selectively binds to variable colitogenic/pathogenic bacterial species with the strict recognition of SHMT motif, while ignoring the beneficial ones (*Bifidobacterium bifidum*, *Blautia coccoides* and *Lactobacillus casei*). Thus we show a plausible molecular mechanism how intestinal IgA regulates gut microbiota.

Immunity to Parasites 1

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IL-27-producing CD4⁺ T cells regulate protective immune responses during malaria infection

Kimura, D.¹, Doe, H.¹, Miyakoda, M.¹, Kimura, K.¹, Hara, H.^{2,3}, Yoshida, H.², Yui, K.¹

¹Nagasaki University, Molecular Microbiology and Immunology, Nagasaki, Japan, ²Saga University, Biomolecular Sciences, Saga, Japan, ³Kagoshima University, Immunology, Kagoshima, Japan

Malaria infection causes immune modulation or suppression by mechanisms not clearly understood. Here, we report the induction of novel regulatory CD4⁺ T cells that produce IL-27, a heterodimeric regulatory cytokine of IL-12 family consisted of

p28 and EB13 subunits, in mice infected with malaria parasites. IL-27-producing CD4⁺ T cells were induced in mice infected with 4 different strains of malaria parasites, but were undetectable in mice infected with *Listeria monocytogenes*. These cells are foxp3⁻CD11a^{hi}CD49d^{hi} malaria antigen-specific CD4⁺ T cells, express inhibitory receptors, PD-1 and LAG-3, produce IL-27 in response to malaria antigen, and inhibit IL-2 production and clonal expansion of other T cells in an IL-27-dependent manner. Intracellular cytokine staining indicated that these CD4⁺ T cells are distinct from IFN- γ -producing Th1 or IL-10-producing Tr1 cells, and we propose to designate them as Tr27 cells. While innate immune cells have been considered the major IL-27 producers, these T cells were the major source of IL-27 during malaria infection.

In mice lacking IL-27 in T cells, IL-2 production was restored and clonal expansion and IFN- γ production by specific CD4⁺ T cells were improved during malaria infection, culminating in the reduced parasite burden when compared with those in IL-27-sufficient mice. This study highlights novel, IL-27 producing regulatory CD4⁺ T cells (Tr27 cells) and their critical roles in the regulation of the protective immune responses against malaria infection.

1511

Immunomodulation of *Plasmodium* pathogenicity by hepatic gamma-delta T cells in experimental cerebral malaria

Silva-Santos, B., Ribot, J., Neres, R., Mancio-Silva, L., Zuzarte-Luis, V., Gomes, A.Q., Mota, M.M., Pamplona, A.
Instituto de Medicina Molecular, Lisbon, Portugal

Symptomatic malaria infection has been associated with activation and expansion of pro-inflammatory $\gamma\delta$ T cells in the peripheral blood of *Plasmodium falciparum*-infected children. However, previous studies failed to identify a non-redundant role for $\gamma\delta$ T cells in animal models of severe malaria. Here we show that mice lacking $\gamma\delta$ T cells (TCR $\delta^{-/-}$) are strikingly resistant to experimental cerebral malaria (ECM) when infected with *P. berghei* ANKA sporozoites (or mosquito bite), while fully susceptible if challenged with *P. berghei* ANKA-parasitized red blood cells. This reveals an unappreciated pathogenic role of murine $\gamma\delta$ T cells that is strictly dependent on the liver stage of infection. We further show that the presence of $\gamma\delta$ T cells significantly modulated the expression of ~20% of the parasite genome at the end of the liver stage, with a focus on the induction of GPI-anchored proteins associated with pathogenicity. As consequence, TCR $\delta^{-/-}$ mice displayed reduced immune activation and an intact blood-brain barrier, in contrast with TCR $\delta^{+/+}$ controls. Of particular relevance was the impairment in the pro-inflammatory cytokine interferon- γ (IFN γ), which was reduced in the serum, in splenic CD4⁺ and CD8⁺ T cells and in the brain of TCR $\delta^{-/-}$ mice. Importantly, IFN $\gamma^{-/-}$ mice phenocopied the ECM resistance of TCR $\delta^{-/-}$ mice. We thus propose that hepatic $\gamma\delta$ T cells modulate *Plasmodium* pathogenicity and the inflammatory syndrome associated with CM. This unravels the importance of the liver stage, which has been widely neglected in CM pathogenesis, while elucidating the potential of targeting $\gamma\delta$ T cells in future therapeutic approaches.

931

The parasitic worm-derived immunomodulator, ES-62 and its drug-like small molecule analogues are protective in a model of chronic asthma

Harnett, W.¹, Coltherd, J.², Rodgers, D.², Lawrie, R.², Al-Riyami, L.¹, Suckling, C.¹, Harnett, M.²

¹University of Strathclyde, Glasgow, United Kingdom, ²University of Glasgow, Glasgow, United Kingdom

Chronic asthma is associated with persistent lung inflammation and long-term remodelling of the airways. The condition has proved refractory to conventional treatments such as steroids, despite their efficacy in controlling acute airway contraction and bronchial inflammation. As the increase in asthma incidence noted in industrialised countries has not been mirrored in developing regions, it has been suggested that helminth infection may protect humans against developing the disease. Consistent with this, we have previously shown that ES-62, an immunomodulator secreted by the filarial nematode *Acanthocheilonema viteae*, can protect against airway hyper-responsiveness and inflammation in a mouse model of acute asthma. We now report that ES-62 can also prevent cellular infiltration of the lungs, particularly neutrophils and mast cells, and in addition mucus hyper-production and airway thickening in a chronic disease mouse model and this is associated with increased levels of IL-10-producing, putative MZ-like regulatory B cells. Importantly, ES-62 can act even after airway remodelling has been established, arresting pathogenesis and ameliorating the inflammatory flares resulting from repeated exposure to allergen that are a debilitating feature of severe chronic asthma. Moreover, two drug-like synthetic analogues of ES-62, termed 11a and 12b mimic its therapeutic actions in restoring levels of regulatory B cells and suppressing neutrophil and mast cell responses. These studies therefore provide a platform for developing ES-62-based drugs, with compounds 11a and 12b representing the first step in the development of a novel class of drugs to combat the hitherto intractable disorder of chronic asthma.

2366

Low affinity CD8 T cells participate in the T cell response to *Plasmodium* infection

King, T.¹, Neeld, D.², Evavold, B.², Lamb, T.¹

¹Emory University School of Medicine, Pediatric Infectious Diseases, Atlanta, United States, ²Emory University School of Medicine, Microbiology and Immunology, Atlanta, United States

Previous studies have demonstrated expansion and activation of CD8 T cells in response to *Plasmodium*-infected red blood cells (iRBCs). In the case of *Plasmodium berghei* ANKA infections in C57BL/6 mice, activated CD8 T cells mediate the development of symptoms resembling cerebral malaria (experimental cerebral malaria; ECM). Using monomeric MHC-I loaded with immunodominant *Plasmodium* epitopes we measured the frequency and 2 dimensional affinity of the T cell receptor (TCR) of polyclonal CD8 T cells in spleen and brain in *Plasmodium*-infected mice. Here we show that the majority of *Plasmodium*-reactive CD8+T cells that expand in the spleen have low affinity

TCRs; for the glideosome associated protein 50 (GAP50)₄₀₋₄₈ epitope at day 5 post-infection with *P. berghei* ANKA 1.2% of the polyclonal CD8 T cells are tetramer +ve high affinity GAP50₄₀₋₄₈-reactive whereas 30% are tetramer -ve with low affinity TCRs to GAP50₄₀₋₄₈. In *P. berghei* ANKA infection low affinity CD8 T cells migrate to the brain in large numbers indicating that they are important component of the pathogenic response. Adoptive transfer experiments with P14 transgenic T cells reactive to the P14 epitope of lymphocytic choriomeningitis virus (LCMV) show that only *Plasmodium*-reactive cells migrate to the brain during infection providing further evidence that the low affinity CD8 T cells in the brain are *Plasmodium*-reactive. Taken together, these results demonstrate that both low and high affinity CD8 T cells participate in the polyclonal CD8 T cell response to *Plasmodium* infection.

3139

Plasmodium-associated TIM3 expression on $\gamma\delta$ T cells results in functionally impaired cells and decreases the risk of clinical malaria

Schofield, L.^{1,2,3}, Ioannidis, L.^{1,3}, Karl, S.^{1,3}, Robinson, L.^{1,3,4}, Tan, Q.^{1,3}, Poole, D.^{3,5}, Betuela, I.⁴, Hill, D.^{1,3}, Siba, P.^{4,6}, Hansen, D.^{1,3}, Mueller, I.^{1,3}, Eriksson, E.^{1,3}

¹Walter & Eliza Hall Institute of Medical Research, Parkville, Australia, ²Australian Institute for Tropical Health and Medicine, James Cook University, Townsville, Australia, ³Melbourne University, Parkville, Australia, ⁴Papua New Guinea Institute of Medical Research, Goroka, Papua New Guinea, ⁵Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Australia, ⁶School of Veterinary and Biomedical Sciences, James Cook University, Townsville, Australia

Recent studies suggest that dysfunctional $\gamma\delta$ T cells induce tolerance to the *Plasmodium* parasite. However, the immunological basis for this dysregulation is not completely understood. Thus, investigation of the biology and immunoregulation of $\gamma\delta$ T cells will further elucidate host immune factors affecting malaria disease outcomes. This study focused on T-cell immunoglobulin domain and mucin domain 3 (TIM3) expression on $\gamma\delta$ T cells in a malaria mouse model and in children (n=132, 5-10 years) living in malaria endemic areas of Papua New Guinea. We assessed the underlying mechanisms contributing to TIM3 upregulation, effect on functional capacity and association with malaria exposure and outcomes. In mice TIM3 expression was upregulated following malaria infection and was maintained both after clearance of infection and in chronically-infected mice. Similarly, the frequency of TIM3+ $\gamma\delta$ T cells was increased in malaria-exposed individuals compared to healthy controls from non-malaria endemic areas. Of note, children with recent *P. vivax* infection had fewer TIM3+ $\gamma\delta$ T cells compared to children with recent *P. falciparum* infection or co-infections with multiple species. Furthermore, TIM3 upregulation was IL-12/IL18-dependent and associated with exposure to distinct species and clones. Functional assessment of TIM3+ $\gamma\delta$ T cells demonstrated a lack of cytokine production and cytotoxic capacity following stimulation. Notably, presence of these cells was associated with decreased risk of acquiring clinical episodes. Collectively, our findings provide fundamental

insight into the immune-specific processes involved in innate immune regulation and suggest that this is a contributing mechanism to limit clinical symptoms during malaria infection.

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The NLRP3 inflammasome negatively regulates type 2 immunity to gastrointestinal helminth infection

Alhallaf, R.¹, Agha, Z.¹, Miller, C.M.¹, Robertson, A.A.², Dent, L.A.³, Cooper, M.A.², Masters, S.L.⁴, Smith, N.C.¹, Loukas, A.¹, Giacomin, P.R.¹

¹Australian Institute for Tropical Health and Medicine, James Cook University, Cairns, Australia, ²Institute for Molecular Bioscience, The University of Queensland, Brisbane, Brisbane, Australia, ³School of Biological Sciences, University of Adelaide, Adelaide, Australia, ⁴Division of Inflammation, The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia

Inflammasomes have a key role in the immunopathobiology of several chronic inflammatory diseases, as well as controlling immunity to viruses, bacteria, fungi and protozoan parasites, by regulating the function of pro-inflammatory IL-1-family cytokines such as IL-18 and IL-1 β . However, roles for inflammasomes in regulating immunity and inflammation during infections with large, metazoan pathogens such as parasitic helminth remain unclear. Using a murine model of gastrointestinal nematode infection (*Trichuris muris*), we demonstrate that helminth infection is associated with increased IL-18 expression and this is dependent on a component of certain inflammasomes, namely NLRP3. Mice deficient in NLRP3 display reduced parasite antigen-specific Type 1 immune responses and intestinal immunopathology, coincident with amplified Type 2 cytokine responses, goblet cell hyperplasia, eosinophilia and accelerated worm expulsion. Similar results were observed when NLRP3 was targeted therapeutically using a specific chemical inhibitor. Critically, delivery of exogenous rIL-18 to NLRP3-deficient mice restored parasite-induced Th1 responses and chronicity of infection. NLRP3 also negatively regulated protective immunity to infection with the hookworm-like nematode, *Nippostrongylus brasiliensis* and humans experimentally infected with hookworms displayed elevated IL-18 expression, consistent with inflammasome activation. Together, these data highlight a previously unrecognised role for NLRP3 in limiting protective immunity to gastrointestinal helminths, suggesting that targeting the NLRP3-inflammasome axis may be a novel approach for limiting the immunopathology associated with helminth infections.

1558

The IL-12 response to *Toxoplasma gondii* in humans involves a pathogen sensing mechanism and myeloid cell subsets distinct from those utilized by mice

Jankovic, D., Tosh, K., Mittereder, L., Sher, A.
Laboratory of Parasitic Diseases, NIAID, NIH, Bethesda, United States

Toxoplasma gondii is an intracellular protozoan parasite that infects mice as part of its natural transmission cycle and induces

disease in humans, an end-stage host. As one of the natural hosts of *T. gondii*, the mouse has been used extensively for elucidating the cellular and molecular basis of immunity to this pathogen while little is known how human cells detect and respond to *T. gondii*. In addressing this issue, we found that primary monocytes and dendritic cells from peripheral blood of healthy donors produce IL-12 and other proinflammatory cytokines when exposed to toxoplasma tachyzoites. Cell fractionation studies determined that IL-12 and TNF- α secretion is limited to CD16+ monocytes and the CD1c+ subset of dendritic cells. Interestingly, these myeloid subsets represent the opposite counterparts from those triggered by the parasite in mice. Moreover, while the innate cytokine response to *T. gondii* in the mouse depends on the interaction of TLR11 and TLR12 with soluble parasite ligand, profilin, the response in human myeloid cells instead requires phagocytosis of the live pathogen. We speculate that these marked distinctions in the pathways utilized for innate recognition of toxoplasma in mouse and man reflect the differing roles of the two hosts in the biology of this parasite.

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System biology approaches to identify Gene-microRNA pathophysiology related networks in Chagas disease

Ferreira, L.R.P.^{1,2,3,4}, Laugier, L.⁵, Ferreira, F.M.^{1,2,3,4}, Cabantous, S.⁵, Cândido, D.D.S.^{1,2,3}, Rozanski, A.⁶, Lannes-Vieira, J.⁷, Chevillard, C.⁵, Kalil, J.^{1,2,3}, Cunha-Neto, E.^{1,2,3}

¹Heart Institute of São Paulo (InCor), Laboratory of Immunology, Sao Paulo, Brazil, ²University of Sao Paulo - School of Medicine, Division of Clinical Immunology and Allergy, Sao Paulo, Brazil, ³Institute for Investigation in Immunology, iii-INCT, Sao Paulo, Brazil, ⁴Universidade Santo Amaro (UNISA), Sao Paulo, Brazil, ⁵INSERM U906 - AIX Marseille Université AMU, Faculté de Médecine, Marseille, France, ⁶Hospital Sírio-Libanês, Sao Paulo, Brazil, ⁷Laboratory of Biology of Interactions, Oswaldo Cruz Institute (FIOCRUZ), Rio de Janeiro, Brazil

The objective of this study was to integrate gene and miRNA expression profiling data from heart of mice acutely infected with the protozoan parasite, *Trypanosoma cruzi* (*T. cruzi*). The gene expression profiling was obtained using time-series microarray data of hearts from mice uninfected and infected for 15, 30 and 45 days with Colombiana *T. cruzi* strain (n=4 per group), same samples previously used for microRNA profiling. The differentially expressed genes (DEGs) and differentially expressed miRNAs (DEMs) were used to conduct functional enrichment analysis and construct gene-miRNA interaction networks. We found that *T. cruzi* acute infection displays specific gene expression profile involving previously described pathways like: pathogen-associated molecular patterns (PAMPS) recognition, production of Nitric oxide by Macrophages and NF- κ B signaling. We also found pathways, not described yet related to *T. cruzi* infection, like: triggering receptor expressed on myeloid cells (TREM)-1, Tyrosine-protein kinase (Tec), and High mobility group box 1 (HMGB1) signaling. By integrating mRNA and miRNA expression data, we could identify miRNA targets

that are important players in each one of these pathways. We also describe how these identified pathways could lead to the development and establishment of each one of the main clinical parameters of heart Chagas disease: myocarditis, fibrosis, arrhythmia and heart hypertrophy. Our data identified specific molecular features of acute *T. cruzi* infection that may translate in the identification of novel relevant drugable targets.

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Caspase-11 activation is an important effector mechanism against *Leishmania amazonensis* infection

Carvalho, R.V.H.^{1,2}, Lima-Júnior, D.S.¹, Zamboni, D.S.¹

¹University of São Paulo, Department of Cell Biology, Ribeirão Preto Medical School, Ribeirão Preto, Brazil, ²University of São Paulo, Department of Biochemist and Immunology, Ribeirão Preto Medical School, Ribeirão Preto, Brazil

Introduction: Leishmaniasis is a disease that affects millions of people worldwide. Although several studies have been performed in order to better understand its pathogenesis, more effective treatments and vaccines haven't been successfully developed so far. The innate immune response against *Leishmania* spp. has been shown to be coordinated by pattern recognition receptors, such as Toll-like receptors and NLRP3, among others. NLRP3 interacts with an inflammatory caspase called caspase-1, although the functions of another inflammatory caspase, caspase-11, is yet obscure. Thus, the aim of this work is to evaluate the contribution of caspase-11 in the recognition and restriction of *L. amazonensis* infection.

Methods and results: Bone marrow-derived macrophages from C57BL/6 (WT) and caspase-11^{-/-} mice were used to perform in vitro studies with *Leishmania amazonensis*. After 24h of infection, supernatant was collected and used for ELISA. BMDMs from caspase-11^{-/-} mice showed decreased secretion of IL-1 β compared to WT mice, and were less capable of killing *L. amazonensis* parasites, as shown by FACS and Giemsa staining. Mice were infected in vivo in the ear with 10⁶ total parasites and the lesion was followed by eight weeks, then they were euthanized and had their lymph node and ear processed for parasite titer. Caspase-11^{-/-} mice showed larger lesion and higher parasite titers compared to WT mice.

Conclusion: Together, our results suggest that *Leishmania amazonensis* triggers Caspase-11 activation, which could possibly be involved in induction of the non-canonical activation of NLRP3 inflammasome, an important mechanism for the restriction of Leishmaniasis.

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Absence of Bim, a pro-apoptotic member of the BCL-2 family, sensitizes mice to *T. cruzi* infection

Torres, M.H.¹, Nascimento, R.¹, Rodrigues, E.¹, Matteucci, K.², Cabral, P.¹, Gonzales, M.¹, Bortoluci, K.R.^{2,3}, Alvarez, J.M.¹, Amarante-Mendes, G.P.^{1,4}

¹University of São Paulo, Institute of Biomedical Sciences, Department of Immunology, São Paulo, Brazil, ²Federal University of São Paulo, Centro de Terapia Celular e Molecular (CTC-Mol),

São Paulo, Brazil, ³Federal University of São Paulo, Department of Biological Sciences, São Paulo, Brazil, ⁴Instituto de Investigação em Imunologia (INCT-iii), São Paulo, Brazil

Bim is a potent pro-apoptotic member of the BCL-2 family that engages the intrinsic/mitochondrial apoptosis pathway. Bim was shown to participate at different steps of the immune response, such as negative selection of self-reactive thymocytes and elimination of activated T cells. However, little is known about the role of Bim on the control of infections. The overall impression is that Bim has a negative role, since *bim*^{-/-} mice is more resistant to *Leishmania major*, *Mycobacterium tuberculosis* and the lymphocytic choriomeningitis virus (LCMV) infections. Here, we investigated the role of Bim on experimental Chaga's Disease. In contrast to the observations mentioned above, *bim*^{-/-} mice showed an impaired control of the parasitemia, which resulted in overall decreased survival compared to the WT mice. Peritoneal macrophages (PM) from *bim*^{-/-} mice displayed a reduced production of NO, IFN- γ and IL-6, and decreased microbicidal activity, when isolated at the peak of parasitemia. This deficiency seems not to be due to an intrinsic defect of the *bim*^{-/-} macrophages, since PM isolated from naïve WT and *bim*^{-/-} mice behave similarly in response to *in vitro* infection. In fact, the defect seems to represent a differential composition of PM subpopulations in response to *T. cruzi* infection. In addition, we found that the antigen-specific CD8⁺ T response was impaired in *bim*^{-/-} mice also at the peak of parasitemia. Together, our results challenge our understanding of the function of Bim in the control of infections, and suggest that Bim plays a positive role in the control of *T. cruzi*.

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The effects of chronic psychological stress on the immune response to lymphoma

Nissen, M.¹, Sloan, E.², Mattarollo, S.¹

¹University of Queensland Diamantina Institute, Translational Research Institute, Brisbane, Australia, ²Monash University, Institute of Pharmaceutical Sciences, Melbourne, Australia

Immunotherapies have revolutionized treatment for cancer, including lymphomas. However the effectiveness of immunotherapies remain sub-optimal, for reasons that are not well defined.

Chronic stimulation of beta-adrenoceptor signalling pathways, which occurs for example during periods of prolonged stress, has been demonstrated to dysregulate the immune system and may be one factor that adversely impacts immunotherapeutic efficacy. To address this, we investigated the impact of chronic beta-adrenergic signalling on lymphoma progression and response to immunotherapy, by administration of a beta-adrenoceptor agonist isoprenaline.

We injected mice daily with isoprenaline and found that beta-adrenergic stimulation enhances lymphoma growth and leads to increased mortality. It also reduces the effectiveness of an alpha-Gal-Cer based autologous tumor cell vaccine, as demonstrated by increased tumor growth and a corresponding

decrease in survival of treated animals. This reduction in immunotherapy effectiveness is associated with impaired CD8+ T cell function. The proliferation of antigen specific CD8+ T cells in response to autologous tumor cell vaccine is significantly inhibited by beta-adrenergic signalling. Similarly, fewer CD8+ T cells express the activation markers CD44 and KLRG1 in response to anti-PD-1 and anti-4-1BB therapeutic antibody therapy, and the effectiveness of these two therapies are abolished while under the effect of beta-adrenergic signalling.

These findings suggest that chronic beta-adrenergic signalling causes immunosuppression during lymphoma immunotherapy. We predict from this findings that cancer immunotherapies may have limited efficacy in those individuals who are experiencing chronic psychological stress, and that immunotherapies may be improved in this setting by blocking beta-adrenergic signalling pathways.

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Inflammation-dependent upregulation of CCL17 in hippocampal neurons and its influence on neuro-immune interactions

Fülle, L.¹, Offermann, N.¹, Radau, L.¹, Hansen, J.N.², Globisch, T.¹, Brandstätter, O.¹, Abdullah, Z.³, Weighardt, H.¹, Halle, A.², Neumann, H.⁴, Förster, I.¹

¹University of Bonn, Life and Medical Sciences, Bonn, Germany,

²Center of Advanced European Studies and Research,

Neuroimmunology, Bonn, Germany, ³University of Bonn,

Experimental Immunology, Bonn, Germany, ⁴University of Bonn,

Reconstructive Neurobiology, Bonn, Germany

It is well known that systemic inflammation may alter neuronal function via induction of immune responses in the CNS, including expression of chemokines. Here we show that CCL17, a ligand of CCR4, is expressed in a subset of hippocampal neurons. Expression of CCL17 was analyzed in wild-type and CCL17-EGFP (CCL17^{E/+}) reporter mice. Steady-state expression of CCL17 was enhanced by systemic challenge with LPS, but not with CpG or Poly I:C. Gene expression analysis of isolated hippocampi indicated local upregulation of GM-CSF and TNF after LPS treatment. Furthermore, LPS-induced CCL17 expression was partly reduced in GM-CSF^{-/-} mice and nearly absent in TNFR^{-/-} mice. Preliminary data point towards a CCL17-dependent regulation of microglial activity. Consistently, analysis of Iba-1⁺ cells in hippocampal sections revealed an altered morphological activation state of microglia in CCL17^{E/E} mice after systemic LPS challenge. To investigate the functional consequences of inducible ablation of CCL17⁺ cells *in vivo*, CCL17-DTR (CCL17^{DTR/+}) mice were injected with diphtheria toxin (DT). Depletion of CCL17⁺ neurons was confirmed by histology and TUNEL staining, and was associated with an initial rapid loss of body weight and sustained persistent body weight fluctuations. Additionally, increasing numbers of Iba-1⁺ cells in the hippocampal CA1 region were observed. Concurrent with the loss of hippocampal neurons mice showed behavioral abnormalities and sensitivity to stress. In conclusion, CCL17 expression in hippocampal neurons appears to modulate microglial responses to systemic inflammation. Our newly generated CCL17-DTR mice may be a valuable model system

for the analysis of inducible neuroinflammation within the hippocampus.

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IFN- γ signaling at the blood-brain barrier (BBB) endothelial cells has inflammatory function but in parenchyma plays a regulatory role during autoimmunity

Sonar, S., Lal, G.

National Centre for Cell Science, Infection and Immunity, Pune, India

Background: IFN- γ , a Th1 cytokine known to have inflammatory and protective function in neuronal autoimmunity. How does IFN- γ signaling at BBB regulate the transendothelial migration of effector CD4 T cells and also have a regulatory function in experimental autoimmune encephalomyelitis (EAE) is not clearly understood.

Methods: EAE was induced by myelin oligodendrocyte glycoprotein (MOG) peptide injection. MOG specific Th1 and Th17 were adoptively transferred into C57BL/6, T-bet^{-/-}, CCR6^{-/-} or RORc^{-/-} mice. BBB was analyzed by multicolor microscopy.

Results: Passive as well as active EAE induced in the C57BL/6 mice showed a punctate distribution of tight- and adherence-junction molecules ZO-1 and VE-cadherin at the brain and spinal cord endothelial vessels. T-bet^{-/-} mice showed resistance to MOG induced active EAE whereas reconstituted with purified wild-type CD4 T cells resulted in disrupted BBB and caused EAE. Intrathecal injection of IFN- γ in the brain disrupted the BBB and promoted infiltration of leukocytes. ROR γ t^{-/-} Th1 cells were crossed the BBB but did not induce the EAE. IFN- γ induced the nitric oxide in the CNS parenchyma, which in turn induced apoptosis in infiltrating leukocytes and protected from EAE. Furthermore, IFN- γ increased surface expression of ICAM-1, and various chemokines in brain endothelial cells, and promoted apical (luminal side) to basal (abluminal side) transendothelial migration of effector CD4 T cells in STAT-1 and ICAM-1 dependent manner.

Conclusions: IFN- γ signaling at BBB promotes transendothelial migration of encephalitogenic CD4⁺ T cells into neuronal tissues, and plays an active role in the initiation of neuronal autoimmunity.

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The neuroimmunology of encephalitis in a mouse model of Dengue virus infection - interaction between draining cervical lymph nodes and the brain

Vu, L., King, N.J.C.

University of Sydney, Pathology, Sydney, Australia

Currently affecting 3.97 billion people globally, Dengue, the most common arboviral disease, has been increasingly reported as being associated with neurological manifestations. Using a non-lethal mouse encephalitis model, in which wild type C57BL/6 mice were intracranially infected with human DENV-2 isolates, we investigated the neuroimmunology of this disease. Using 18-colour flow cytometry, we found that central

nervous system (CNS) DENV infection of wild type mice results in a strong intracerebral influx of leukocytes, most of which are CD8+ effector T cells. While intracerebral trafficking of these T cells was antigen-independent, correlating with increased CXCL10, CCL5, CCL3, and CCL2 mRNA expression, their antiviral function was MHC-restricted. Using intracranial injection of Ova- or DENV NS4 Ag-loaded splenocyte targets, DENV-specific CD8+ effector T cells exhibited considerable cytolytic activity *in vivo* and were IFN- γ -dependent, suggesting a collaborative role of perforin/granzyme and IFN- γ , which in turn presumably amplifies MHC expression. Monofunctional (IFN- γ + only) DENV-specific CD8+ T cells were detected in the cervical lymph nodes (CLN) by d3 post infection, preceding peak accumulation of multifunctional (IFN- γ +, TNF+ or CD107a+/GrzB+) DENV-specific CD8+ T cells in the CNS on d6. CD8 and/or CD4 depletion or CLN excision resulted in a significant reduction in infiltrating CD8+ T cells into the CNS, slightly increased viral load but did not alter overall clinical outcomes. This suggests that DENV-specific responses in the CNS are initiated within the draining CLN and CNS-lymph node interaction boosts DENV-specific CD8+ effector T cell numbers to limit viral replication in this DENV-2 encephalitis mouse model.

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Anti-inflammatory effects of GABA_A receptor modulators

Neumann, S.^{1,2}, Gowing, E.², Lateef, Z.³, Chebib, M.⁴, Young, S.¹, Clarkson, A.^{2,4}

¹University of Otago, Department of Pathology, Dunedin, New Zealand, ²University of Otago, Department of Anatomy, Brain Health Research Centre, Dunedin, New Zealand, ³University of Otago, Department of Microbiology and Immunology, Dunedin, New Zealand, ⁴The University of Sydney, Faculty of Pharmacy, Sydney, Australia

Inflammatory processes are known to contribute to tissue damage in the central nervous system (CNS) across a broad range of neuro-pathologies, including stroke. The main inhibitory neurotransmitter of the CNS, gamma amino butyric acid (GABA), has been implicated in shaping peripheral immune responses by binding to GABA_A receptors on antigen-presenting cells and lymphocytes. Here, we investigated the effects of several subtype-specific GABA_A receptor modulators on nuclear factor kappa B (NF- κ B)-mediated inflammation *in vitro*. In addition, we tested the most promising of these drug candidates in a mouse model of focal stroke.

We found that GABA_A receptor modulators DS2 and tracazolate reduced NF- κ B activation over a wide concentration range *in vitro*, whereas zolpidem and bumetanide only decreased NF κ B activation at high concentrations. Treatment with DS2 *in vivo* from 1 hour post-stroke significantly reduced infarct sizes when mice were dosed at 0.1 mg/kg but not at higher doses. Motor tasks improved in mice treated with DS2 at all doses; however, the lowest dose of DS2 (0.1 mg/kg) was most beneficial.

Subtype-selectivity of GABA_A modulators influenced their ability to inhibit activation of NF- κ B and thus the production of pro-inflammatory cytokines. It remains to be determined if the improved recovery of stroked animals treated with DS2 depends on modulation of GABA_A receptors in the CNS or on

peripheral immune cells. However, as previous unpublished data shows that DS2 does not cross the blood-brain-barrier, it is likely that DS2 is mediating the degree of infarct volume via the modulation of the peripheral immune response.

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A natural variant of the TCR signaling molecule Vav1 reduces both effector T cell functions and susceptibility to neuroinflammation

Kassem, S.¹, Gaud, G.¹, Colacios, C.¹, Bernard, I.¹, Malissen, B.², Saoudi, A.¹

¹INSERM U 1043, CNRS UMR 5282, Toulouse, France, ²CIML, Marseille, France

The guanine nucleotide exchange factor Vav1 is essential for transducing T cell antigen receptor signals and therefore plays an important role in T cell development and activation. Our previous genetic studies identified a locus on rat chromosome 9 that controls the susceptibility to neuroinflammation and contains a non-synonymous polymorphism in the major candidate gene *Vav1*. To formally demonstrate the causal implication of this polymorphism, we generated a knock-in mouse bearing this polymorphism (Vav1^{R63W}). Using this model, we show that Vav1^{R63W} mice display reduced susceptibility to experimental autoimmune encephalomyelitis (EAE) induced by MOG₃₅₋₅₅ peptide immunization. This is associated with a lower production of effector cytokines (IFN- γ , IL-17 and GM-CSF) by autoreactive CD4 T cells. Despite increased proportion of Foxp3⁺ regulatory T cells in Vav1^{R63W} mice, we show that this lowered cytokine production is intrinsic to effector CD4 T cells and that Treg depletion has no impact on EAE development. Finally, we provide a mechanism for the above phenotype by showing that the Vav1^{R63W} variant has normal GEF activity but reduced adaptor functions. Together, these data highlight the importance of Vav1 adaptor functions in the production of inflammatory cytokines by effector T cells and in the susceptibility to neuroinflammation.

3443**miR-155 controls T helper cell 17 development during autoimmune demyelination via Hsp40 genes expression***Cichalewska, M., Mycko, M., Cwiklinska, H., Selmaj, K.**Medical University of Lodz, Department of Neurology, Laboratory of Neuroimmunology, Lodz, Poland*

MicroRNAs (miRNA) are small, endogenous RNAs that represent a group of non-protein coding gene products that regulate transcription, degradation or activation of mRNA. MicroRNAs have been implicated as regulatory mechanisms in the immune system and miR-155 has been found to play key role in various immune cell functions. We and others have described miR-155 upregulated in T helper (Th) cells during development of the experimental autoimmune encephalomyelitis (EAE), an animal model of MS. Here we address function of miR-155 in Th differentiation during EAE. To manipulate miR-155 activity a set of specific oligonucleotide mimics and antagomir inhibitors were tested. An active myelin antigen immunization EAE model and myelin specific Th transfer as autoimmune demyelination models have been used. We have found that immunization of miR-155 deficient mice with MOG peptide 35-55 led to decreased generation of Th17 response. Searching for molecular mechanisms of miR-155 action in

T cells we have analyzed a gene expression profiles of miR-155 deficient Th cells. Intriguingly, we have found that most upregulated genes in miR-155 deficient were two genes of heat shock protein 40 family, Dnaja2 and Dnajb1. These two genes were also upregulated in Th cells following miR-155 antagomir treatment. Finally, a MOG TCR transgenic Th cells transfected with either Dnaja2 or Dnajb1 were less encephalitogenic in EAE transfer model. Thus our results provide evidence for specific and selective role of miR-155 in the process of myelin antigen adaptive response and autoreactive T cell development via targeting hsp40 genes. Supported: NCN Sonata to MC.

181**The P2X7 receptor in amyotrophic lateral sclerosis***Bartlett, R.^{1,2}, Watson, D.^{1,2}, Sluyter, V.^{1,2}, Yerbury, J.^{1,2}, Sluyter, R.^{1,2}**¹University of Wollongong, Wollongong, Australia, ²Illawarra Health and Medical Research Institute, Wollongong, Australia*

The P2X7 receptor channel is activated by the damage-associated molecular pattern molecule extracellular ATP. P2X7 plays important roles in neuroinflammation and is upregulated in amyotrophic lateral sclerosis (ALS), a rapidly fatal neuromuscular disease characterised by the aggregation of misfolded proteins, microglia activation and motor neuron loss. Cell-to-cell transfer of aggregated proteins, such as mutant superoxide dismutase 1 (mSOD1), is involved in ALS progression. Using G93A mSOD1 cells and mice, this study aimed to identify the roles of P2X7 in ALS. P2X7 was identified in both murine microglia and motor neurons. P2X7 activation resulted in the generation of reactive oxygen species formation and death in microglia. P2X7 activation also caused rapid mSOD1 release from mSOD1-transfected motor neurons independently of cell death. mSOD1 released from motor neurons was predominately in a pelletable fraction indicative of protein aggregation. This

pelletable fraction could be engulfed by microglia and induce tumour necrosis factor release from these cells. Treatment of mSOD1-transgenic ALS mice with the P2X7 antagonist Brilliant Blue G delayed weight loss and prolonged survival in female but not male mice. Treatment had no effect on clinical score, motor coordination, or a number of other disease or systemic features. Combined, these results suggest that P2X7 may be involved in select ALS disease pathways, consistent with the multi-systemic and multi-factorial nature of this disease.

1598**Dynamics of inflammatory immune response in a mouse model of traumatic spinal cord injury, potential therapeutic targets***Hassanpour Golakani, M.¹, Mohammad, M.², Li, H.¹, Saxena, M.¹, Breit, S.¹, Ruitenber, M.³, Brown, D.⁴**¹University of New South Wales/St Vincent's Centre for Applied Medial Research (AMR), Laboratory of Neuroinflammation, Darlinghurst, Australia, ²University of Sharjah, Department of Medical Laboratory Sciences, Faculty of Health Sciences, Sharjah, United Arab Emirates, ³University of Queensland, Queensland Brain Institute, Brisbane, Australia, ⁴Westmead Hospital, Department of Immunology/Immunopathology, Sydney, Australia*

The dynamics of inflammatory responses post spinal cord injury (SCI) are largely unknown. In this study we aimed to characterize the various immune cells in and around the injury site and their relationship with locomotor functional recovery at various time points post SCI.

Severe contusive SCI was induced and locomotor function was assessed at 7, 14, 21, and 28 days post injury (dpi). The entire spinal cord was removed, and immune cells were isolated, characterized, and localized using FACS, immunohistology and immunofluorescence. The frequency of immune cell subsets was correlated with behavioral assessment at various time points post injury.

The number of infiltrating macrophages and CD11c&B220⁺ cells had both beneficial and detrimental effects depending on the time post SCI. While macrophages had adverse effects on locomotion recovery in early stages of 7-14 dpi, they significantly contributed to improved recovery at later stages. CD11c&B220⁺ cells on the other hand were advantageous in early and detrimental in the more chronic stage of SCI.

These findings highlight that the dynamics of inflammatory responses post SCI are a double-edge sword with respect to functional recovery, and that they may be therapeutically manipulated.

4045**Multiple sclerosis patient lymphocytes' crosstalk with microglial cells directs remyelination***El Behi, M.¹, Sanson, C.², Bachelin, C.², Guillot-Noel, L.¹, Fransson, J.², Stankoff, B.³, Maillart, E.³, Sarranzin, N.², Guillemot, V.⁴, Abdi, H.⁵, Rebeix, I.², Fontaine, B.³, Zujovic, V.²**¹Brain and Spine Institute, Neuroimmunology, Paris, France, ²Brain and Spine Institute, Neurobiology, Paris, France, ³AP-HP, Groupement Hospitalier Pitié-Salpêtrière, Neurology, Paris, France,*

⁴Brain and Spine Institute, Biostatistics, Paris, France, ⁵University of Texas, School of Brain and Behavioral Sciences, Texas, United States
Myelin destruction leads to irreversible neurological disabilities in multiple sclerosis (MS), an autoimmune disease of the central nervous system. However, an endogenous remyelination process can be triggered and is highly influenced by the state of activation of microglia cells (MIG) which itself depends on MIG dialogue with T lymphocytes (LT). Therefore, we asked whether LT from healthy donors (HD) and MS patients differentially influence MIG activation state and remyelination.

We adapted experimental designs to study remyelination within a humanized context by developing a new in vivo model combining human LT grafting and lysolecithine-induced demyelination in Nude mice spinal cord. We show that remyelination is significantly decreased in mice grafted with MS LT compared to HD LT. Mechanistically, we demonstrate that MS LT supernatants drove microglial activation towards a pro-inflammatory M1 state, resulting in a decrease in oligodendrocyte precursor cells (OPC) differentiation into myelinating cells.

Finally, we observed heterogeneous remyelination patterns among MS patients. Using multivariate analysis comparing patients with high and low remyelination ability, we uncovered new molecular players with previously unknown roles in OPC differentiation under pathological conditions and validated some of them in vitro as potential targets in MS patients to improve remyelination. Thereby, we propose the concept of using characteristics of MS patients displaying high repair capacities to discover innovative pro-remyelinating molecules.

Innate Receptors & Inflammasomes 2

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Delayed inhibition of the NLRP3 inflammasome during pathogenic influenza A virus infection in mice reduces disease severity and prolongs survival

Tate, M.¹, Pinar, A.¹, Dowling, J.¹, McAuley, J.², Robertson, A.³, Cooper, M.³, Mansell, A.¹

¹Hudson Institute of Medical Research and Monash University, Centre for Innate Immunity and Infectious Diseases, Melbourne, Australia, ²University of Melbourne, Peter Doherty Institute for Infection and Immunity, Department of Microbiology and Immunology, Melbourne, Australia, ³University of Queensland, Institute of Molecular Bioscience, Brisbane, Australia

Introduction: Fatal disease caused by emerging avian influenza A viruses (IAV) is often associated with severe lung pathology, infiltrating leukocytes and an excessive cytokine response termed the 'cytokine storm'. Mortality rates for pathogenic infections such as the current avian H7N9 IAV are >30%, with limited therapeutic strategies available. We recently identified activation of the NLRP3 inflammasome by the PB1-F2 virulence factor found in pathogenic IAV causes hyperinflammation during infection. We have now characterized PB1-F2 derived from H7N9 avian IAV and determined whether targeting of the NLRP3 inflammasome may provide a strategy to protect from fatal IAV.

Results: We have found that a peptide derived from pathogenic

H7N9 PB1-F2 induced IL-1b secretion from human PBMCs and murine macrophages which was Caspase-1-, ASC- and NLRP3-dependent. Furthermore, NLRP3-deficient mice exposed to H7N9 PB1-F2 peptide display reduced neutrophil recruitment to the lung and ablated IL-1b secretion. Importantly, treatment of murine macrophages and human PBMCs with a specific NLRP3 inhibitor termed MCC950 significantly inhibited H7N9-induced IL-1b secretion and mice treated with MCC950 display reduced neutrophil recruitment and IL-1b in the lung.

Critically, temporal targeting of NLRP3 with MCC950 protected mice from pathogenic PR8 IAV-induced mortality, reducing cellular infiltrates and inflammatory cytokines, providing evidence for potentially targeting the inflammasome in pathogenic IAV disease.

Conclusion: This is the first demonstration that reduced IL-1 β and targeted intervention of inflammasome activation therapeutically protects against the detrimental outcomes of pathogenic IAV infection such as that posed by emerging avian influenza.

3257

Familial autoinflammation with neutrophilic dermatosis reveals a novel regulatory mechanism of pyrin activation

Baker, P.J.^{1,2}, Lagou, V.^{3,4,5}, Jéru, I.^{6,7}, Van Eyck, L.^{4,5}, Parry, D.A.⁸, Lawless, D.⁹, De Nardo, D.^{2,10}, Garcia-Perez, J.E.^{4,5}, Dagley, L.F.^{1,2,11}, Holley, C.¹², Dooley, J.^{4,5}, Jeandel, P.-Y.¹³, Scot, R.^{14,15}, Lyras, D.¹⁶, Webb, A.I.^{2,11}, Nicholson, S.E.^{1,2}, De Somer, L.¹⁵, van Niewenhove, E.^{4,5,15}, Ruuth-Praz, J.^{7,17}, Copin, B.⁷, Cochet, E.⁷, Medlej-Hashim, M.¹⁸, Megarbane, A.^{19,20}, Schroder, K.²¹, Savic, S.^{22,23}, Goris, A.³, Amselem, S.^{6,7}, Wouters, C.^{4,15}, Liston, A.^{4,5}, Masters, S.^{1,2}

¹Walter & Eliza Hall Institute of Medical Research, Inflammation Division, Parkville, Australia, ²The University of Melbourne, Department of Medical Biology, Parkville, Australia, ³University of Leuven, Department of Neurosciences, Leuven, Belgium, ⁴University of Leuven, Department of Microbiology and Immunology, Leuven, Belgium, ⁵VIB-KU Leuven, Translational Immunology Laboratory, Leuven, Belgium, ⁶Université Pierre et Marie Curie, INSERM, UMR_S 933, Paris, France, ⁷Assistance Publique - Hôpitaux de Paris, Hôpital Trousseau, Service de Génétique et d'Embryologie Médicales, Paris, France, ⁸University of Edinburgh, Western General Hospital, Centre for Genomic & Experimental Medicine, Institute of Genetics & Molecular Medicine, Edinburgh, United Kingdom, ⁹University of Leeds, Wellcome Trust Brenner Building, St James's University Hospital, Biomedical and Clinical Sciences, Leeds, United Kingdom, ¹⁰Walter & Eliza Hall Institute of Medical Research, Inflammation division, Parkville, Australia, ¹¹Walter & Eliza Hall Institute of Medical Research, Systems Biology and Personalised Medicine Division, Parkville, Australia, ¹²Institute for Molecular Bioscience, The University of Queensland, Cell Biology and Molecular Medicine division, Brisbane, Australia, ¹³Hôpital Archet 1, Université de Nice Sophia-Antipolis, Département de Médecine Interne, Nice, France, ¹⁴University of Leuven, Department of Pathology, Leuven, Belgium, ¹⁵University Hospitals, Leuven, Belgium, ¹⁶Monash University, Department of Microbiology, Clayton, Australia, ¹⁷Université Pierre et Marie Curie, UMR_S 933, Paris, France, ¹⁸Lebanese University, Department of Life and Earth Sciences, Faculty of Sciences II, Hadath, Lebanon, ¹⁹Saint Joseph University, Medical Genetics Unit, Faculty of Medicine, Beirut, Lebanon, ²⁰Institut Jérôme Lejeune,

Paris, France, ²¹Institute for Molecular Bioscience, The University of Queensland, Cell Biology and Molecular Medicine Division, Brisbane, Australia, ²²St James's University Hospital, Department of Clinical Immunology and Allergy, Leeds, United Kingdom, ²³St James's University Hospital, NIHR-LMBRU and LIRMM, Wellcome Trust Brenner Building, Leeds, United Kingdom

Pyrin, encoded by the *MEFV* gene, is implicated in the innate immune response to a number of bacterial pathogens. These bacteria secrete toxins that indirectly stimulate pyrin-dependent IL-1 β release by inhibiting Rho-GTPase signalling and alteration of cytoskeletal dynamics. *MEFV* is so called due to mutations in this gene being responsible for the recessively inherited autoinflammatory disease Familial Mediterranean Fever (FMF). FMF is characterised by short periodic attacks of fever and serositis (often peritoneal or pleural). We have studied several families with a dominantly inherited autoinflammatory disease that is clinically distinct from FMF and is predominantly characterised by neutrophilic dermatosis, prolonged bouts of fever and an absence of any form of serositis. This disease, which we have named Pyrin-Associated Autoinflammation with Neutrophilic Dermatitis (PAAND), is caused by a single amino acid substitution (S242R) in the linker region between the PYRIN and B-box domains of the pyrin protein. Phosphorylation of the highly conserved serine 242 residue creates a non-canonical 14-3-3 binding motif that we show is disrupted by either the S242R mutation or treatment with *Clostridium difficile* Toxin B (TcdB) leading to increased IL-1 β production. This suggests that dephosphorylation of serine 242 is a primary mechanism of pyrin activation in the innate immune response, which is deregulated in individuals presenting with PAAND. Targeting of IL-1 β has proven to be a successful therapy for treating PAAND in at least one patient.

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Human monocytes engage an alternative inflammasome pathway

*Gaidt, M.*¹, *Ebert, T.*¹, *Chauhan, D.*¹, *Schmidt, T.*¹, *Schmid-Burgk, J.*¹, *Rapino, F.*², *Robertson, A.*³, *Cooper, M.*³, *Graf, T.*², *Hornung, V.*¹

¹University of Bonn, Bonn, Germany, ²Universitat Pompeu Fabra and Institutió Catalana de Recerca i Estudis Avançats, Barcelona, Spain, ³University of Queensland, Brisbane, Australia

Interleukin-1 β (IL-1 β) is a cytokine, whose bioactivity is controlled by activation of the inflammasome. However, in response to lipopolysaccharide human monocytes secrete IL-1 β independently of classical inflammasome stimuli. Here, we report that this constituted a species-specific response that is not observed in the murine system. Indeed, in human monocytes, lipopolysaccharide triggered an 'alternative inflammasome' that relied on NLRP3-ASC-caspase-1 signaling, yet was devoid of any classical inflammasome characteristics including pyroptosome formation, pyroptosis induction and K⁺ efflux dependency. Genetic dissection of the underlying signaling pathway in a monocyte trans-differentiation system revealed that alternative inflammasome activation was propagated by TLR4-TRIF-RIPK1-FADD-CASP8 signaling upstream of NLRP3. Importantly, involvement of this signaling cascade was limited to alternative inflammasome activation and did not extend to classical NLRP3

activation. Since alternative inflammasome activation embraces both sensitivity and promiscuity of TLR4, we propose a pivotal role for this signaling cascade in TLR4-driven, IL-1 β -mediated immune responses and immunopathology in humans.

1918

Roles of innate immune regulator TAPE in RIG-I signaling and antiviral defenses

Chen, K.R.^{1,2}, *Yang, C.-Y.*³, *Lin, C.-Y.*², *Lin, W.-Y.*², *Lo, Y.-C.*², *Chuang, H.-C.*³, *Tan, T.-H.*³, *Ling, P.*^{1,2,4}

¹National Cheng Kung University, Institute of Basic Medical Sciences, Tainan, Taiwan, Republic of China, ²National Cheng Kung University, Department of Microbiology and Immunology, Tainan, Taiwan, Republic of China, ³National Health Research Institutes, Zhunan, Taiwan, Republic of China, ⁴National Cheng Kung University, Department of Medical Laboratory Science and Biotechnology, Tainan, Taiwan, Republic of China

Pattern-recognition receptors detect pathogen-associated molecular patterns to trigger downstream pathways leading to type I IFN production to defend pathogen infection. RIG-I-like receptors (RLRs) are key cytosolic sensors for recognizing viral RNA to trigger antiviral immunity. The underlying mechanisms linking RLR-mediated viral recognition to antiviral immunity remain to be further explored. Through our previous work, we uncovered an innate immune regulator termed TAPE (TBK1-Associated Protein in Endolysosomes), also known as CC2D1A, which is implicated in the viral RNA sensor TLR3 and RLR pathways. Yet, the in vivo role of TAPE in antiviral defenses and the mechanistic mechanisms of how TAPE regulates cytosolic RIG-I signaling still remain to be established. TAPE conditional knockout mice were generated for our study. Results from in vivo studies showed that *TAPE*^{eff} CD11c-Cre mice exhibited a more severe mortality than WT mice upon influenza A virus (IAV) infection. Ex vivo studies also showed that TAPE-deficient mouse embryonic fibroblasts and macrophages were defective in type-I interferon induction upon RLR ligand stimulation. In addition, our biochemical analyses showed that the N-terminal region of TAPE was critical for interacting with the CARD domain of RIG-I while the C-terminal region of TAPE contributed most to the interaction with MAVS/IPS-1, a RLR downstream mediator. Together, our results suggest a crucial role for TAPE in linking RIG-I to type I IFN-mediated antiviral responses. Future work will further determine the in vivo role of TAPE in IAV and other RNA virus infection, and explore the mechanistic mechanisms of how TAPE regulates RIG-I signaling.

1230

NLRP3 inflammasome-mediated, IL-1 β -dependent inflammatory responses drive severe, steroid-resistant asthma

*Kim, R.*¹, *Pinkerton, J.*¹, *Essilfie, A.-T.*¹, *Robertson, A.*², *Baines, K.*¹, *Mayall, J.*¹, *Starkey, M.*¹, *Wark, P.*¹, *Gibson, P.*¹, *O'Neill, L.*³, *Cooper, M.*², *Horvat, J.*¹, *Hansbro, P.*¹

¹Hunter Medical Research Institute & University of Newcastle, Priority Research Centre for Healthy Lungs, New Lambton Heights, Australia, ²Institute for Molecular Bioscience, The University

of Queensland, Division of Chemistry and Structural Biology, Brisbane, Australia, ³Trinity Biomedical Sciences Institute, Trinity College Dublin, School of Biochemistry and Immunology, Dublin, Ireland

Excessive NLRP3 inflammasome and concomitant IL-1 β responses are implicated in many inflammatory diseases. However, the direct contributions to pathogenesis, mechanisms involved and potential for therapeutic targeting remain poorly understood. In the lung, NLRP3 inflammasomes are associated with emphysema, infections and severe, steroid-resistant (SSR) asthma, which is the major unmet clinical need in asthma management. It is underpinned by exaggerated innate and T_H1/T_H17 immunity, neutrophilic inflammation and bacterial infections. Here we developed novel mouse models of *Chlamydia*, and *Haemophilus*, respiratory infection and ovalbumin-induced SSR allergic airways disease (SSRAAD). These models share the hallmark features of human disease, including elevated NLRP3 inflammasome and concomitant IL-1 β responses. We also examined the relationships between NLRP3 and IL-1 β levels, and airway neutrophil numbers and disease severity in a population of human asthmatics. The roles and potential for targeting of infection-induced NLRP3 inflammasome, caspase-(CASP)1, and IL-1 β responses in the lung in SSRAAD were examined using a highly-selective NLRP3 inhibitor, MCC950, the specific CASP1 inhibitor, Ac-YVAD-cho, and neutralising anti-IL-1 β antibody, α -IL-1 β , respectively. We show that *Chlamydia* and *Haemophilus* infections increase NLRP3, CASP1, IL-1 β and T_H1/T_H17 responses that drive steroid-resistant neutrophilic inflammation and airways hyper-responsiveness in experimental SSR asthma. Neutrophilic airway inflammation and severity of human SSR asthma correlated with IL-1 β and NLRP3 expression. Treatment with α -IL-1 β , Ac-YVAD-cho, and MCC950 suppressed IL-1 β responses and the important steroid-resistant features of disease. Thus, NLRP3 inflammasome responses may drive SSR asthma and be therapeutically targeted in this and other NLRP3-mediated diseases.

2699

The molecular mechanism of Toll-like receptor 4 signaling in cells

Latty, S.¹, Saki, J.², Klenerman, D.¹, Bryant, C.²

¹University of Cambridge, Chemistry, Cambridge, United Kingdom,

²University of Cambridge, Veterinary Medicine, Cambridge, United Kingdom

The TLR/IL-1 Receptor superfamily are critically required to generate immune responses, yet little is known about the fundamental mechanisms by which they signal. We show that in macrophages Toll-like receptor 4 (TLR4) can exist as monomers or pre-formed dimers. Upon administration of the full agonist lipopolysaccharide (LPS) or other lipid-based ligands, including partial agonists, TLR4 dimers, but not oligomers are present irrespective of ligand-efficacy. To determine how different efficiencies in TLR4 signaling are achieved we first showed in single cell signaling assays that the kinetics of nuclear factor κ B translocation into the nucleus is dose dependently delayed with lower doses of LPS or maximal concentrations of a partial

agonist. LPS stimulation of TLR4 drove the rapid formation of visible, reversible MyD88 oligomeric protein complexes (MyDDosomes) within the cell. In response to a partial agonist there was a reduction in the number of MyDDosomes formed in comparison to LPS suggesting that TLR4 signalling efficiency may be linked to the number of stable MyDDosomes formed over time with, presumably, a critical threshold of MyDDosomes determining when κ B translocation is stimulated.

2619

All-trans retinoic acid enhances extracellular hsp90a dimerization in human monocytes through RAR/RXR- and Ca²⁺-dependent mechanisms

Hoang Thi, X., Jong Hyeok, J., Bon Hyang, N., Bon, H.

Gachon University, Life Science, Seongnam, Korea, Republic of

Heat shock protein 90 (hsp90) is ATP-dependent molecular chaperone and plays a role in maintaining conformational integrity of various cellular proteins. Recent studies have shown that hsp90 can be secreted and communicate with immune system under stress conditions such as inflammation and infection. In this study, we investigated the effects of all-trans retinoic acid (ATRA), an active form of vitamin A, on extracellular hsp90a dimerization of human monocyte THP-1. Cells were treated with ATRA for 24 h, subjected to BS3-cross linking reaction for 30 min, and then analyzed by western blot. Dimer form of hsp90a in ATRA-treated cells increased approximately 3 times in comparison with DMSO-treated cells. Experiments with intracellular cross-linker EGS indicated that ATRA-induced hsp90a dimerization only occurs outside of cells. Experiments with agonists or antagonists for RAR α or RXR revealed that ATRA-induced hsp90a dimerization depends on RAR/RXR pathway. Intracellular Ca²⁺ levels were increased by ATRA and this increase was reversed by the treatment with RAR α or RXR antagonists or calcium channel blockers. ATRA-induced hsp90a dimerization was reversed by the treatment of calcium channel blockers, while addition of exogenous calcium ion enhanced the hsp90a dimerization. Our results suggest that ATRA enhances extracellular hsp90a dimerization of human monocytes through RAR/RXR- and Ca²⁺-dependent mechanisms.

3479

Caspase-4/5 control non-canonical inflammasome activation in human monocytes in response to extracellular LPS

Vigano, E., Diamond, C.E., Mortellaro, A.

Singapore Immunology Network (SigN), Agency for Science, Technology and Research (A*STAR), Singapore, Singapore

Inflammasomes are molecular platforms regulating the release of the pro-inflammatory cytokines IL-1 β and IL-18 in response to microbes and sterile signals. Classical inflammasome activation requires caspase-1, which in turn processes pro-IL-1 β and pro-IL-18 to mature cytokines, as well as inflammatory cell death, known as pyroptosis. In mice a "non-canonical" inflammasome pathway controlled by caspase-11 has recently been identified. Caspase-11 plays a critical role in the control

of IL-1 α secretion and pyroptosis in response to many gram-negative bacteria and cytoplasmic lipopolysaccharide (LPS). Putative human orthologues of caspase-11 are caspase-4 and caspase-5. However, it remains unclear whether the non-canonical inflammasome pathway exists in humans, which roles caspase-4/5 play and the mechanisms regulating their activation. We found that LPS stimulation induces processing of caspase-5, but not that of caspase-4, in human monocytes. Caspase-4 and -5 specifically regulate IL-1 α and IL-1 β release. Caspase-5 processing was dependent on TLR4, activation of the tyrosine kinase Syk and calcium flux, but not on the IFN type I pathway and generation of reactive oxygen species. These mechanisms were unique to monocytes and absent in monocyte-derived macrophages and dendritic cells. We identified caspase-4 and -5 as key immune effectors underpinning inflammasome activation in human monocytes in response to extracellular LPS. This novel view of the molecular mechanisms underlying inflammasome activation in monocytes may open up avenues for new pharmacological interventions aimed at mitigating systemic inflammation in autoinflammatory disorders and more.

3780

RIPK3 promotes cell death and NLRP3 inflammasome activation in the absence of MLKL

Vince, J., Lawlor, K.

The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia

RIPK3 and its substrate MLKL are essential for necroptosis, a lytic cell death proposed to cause inflammation via the release of intracellular molecules. Whether and how RIPK3 might drive inflammation in a manner independent of MLKL and cell lysis remains unclear. Here we show that following LPS treatment, or LPS-induced necroptosis, the TLR adaptor protein TRIF and inhibitor of apoptosis proteins (IAPs: X-linked IAP, cellular IAP1 and IAP2) regulate RIPK3 and MLKL ubiquitylation. Hence, when IAPs are absent, LPS triggers RIPK3 to activate caspase-8, promoting apoptosis and NLRP3-caspase-1 activation, independent of RIPK3 kinase activity and MLKL. In contrast, in the absence of both IAPs and caspase-8, RIPK3 kinase activity and MLKL are essential for TLR-induced NLRP3 activation. Consistent with *in vitro* experiments, interleukin-1 (IL-1)-dependent autoantibody-mediated arthritis is exacerbated in mice lacking IAPs, and is reduced by deletion of RIPK3, but not MLKL. Therefore RIPK3 can promote NLRP3 inflammasome and IL-1 inflammatory responses independent of MLKL and necroptotic cell death.

Immunity to Viruses 3

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Early growth response gene (Egr) 2 and 3 are essential for adaptive immunoresponses of T cells

Lli, S.¹, Miao, T.², Symond, A.², Wang, P.²

¹Brunel University, Bioscience, London, United Kingdom, ²BICMS, Queen Mary University of London, Immunology, London, United Kingdom

Egr2 and 3 are important for maintaining immune homeostasis. Here we defined a fundamental and overlapping function of Egr2 and 3 for controlling the transition between clonal expansion and differentiation of effector T cells. Naive T cells deficient in Egr2 and 3 were severely impaired in clonal expansion and displayed innate like inflammatory differentiation, while forced Egr2 expression in T cells induced hyper-proliferation and impaired effector differentiation in response to viral infection. A novel GFP-Egr2 knockin model demonstrated that two populations of CD44^{high} T cells were induced by viral infection: Egr2 positive highly proliferating cells and Egr2 negative highly differentiated cells. Egr2 bound to and promoted expression of proliferation regulators and differentiation repressors (Myc, Myb, Bcl6, Bcl9, Id3, Lef1, Tcf7 while suppressing expression of transcription factors required for lineage-specific differentiation (T-bet, Runx2, Runx3, Prdm1, Zeb2, ROR α , ROR γ). The function of Egr2 and 3 was tightly regulated in a reciprocal fashion by antigen and effector cytokines, which is essential for optimal coupling of clonal expansion and effector differentiation in response to viral infection. Thus, Egr2 and 3 enforce a checkpoint in early adaptive immunoresponses by promoting clonal expansion and restraining innate like responses of T cells.

4250

Extreme memory inflation of high affinity HLA-C restricted CMV-specific CD8+ T cells specific for immediate-early antigen

Hosie, L.¹, Pachnio, A.¹, Riddell, S.², Moss, P.¹

¹University of Birmingham, Birmingham, United Kingdom, ²Fred Hutchinson Cancer Research Center, Seattle, United States

Human Cytomegalovirus (HCMV) infects the majority of the human population, leading to a uniquely strong cellular immune response that, with age, comes to dominate the peripheral lymphoid pool during chronic infection. This phenomenon is termed 'memory-inflation'. Recently, HCMV infection has been associated with vascular pathology and increased mortality in older individuals, thought due in part to the magnitude of the total HCMV-specific CD8+T-cell response. As such, it is vital to understand the breadth of HCMV immunodominant antigens for inclusion within adoptive immunotherapies.

PBMCs of seropositive individuals were stimulated *ex vivo* with CD8+T-cell HCMV-peptides followed by flow cytometric analysis. Here we report for the first time, to our knowledge, memory-inflation of three HCMV-specific CD8+T-cell populations restricted through HLA-Cw*0702.

We demonstrate that:-

- HLA-C-specific CD8+T-cell clones recognize peptide from **'immediate early'** (IE) HCMV proteins expressed very early during episodes of viral reactivation
- HLA-C-restricted HCMV-specific CD8+T-cells increase dramatically with age to **dominate the CD8+T-cell repertoire** and can individually comprise 32% of the CD8+T-cell pool.
- These CD8+T-cells increase their polyfunctionality, peptide avidity and cytotoxic potential dramatically with age.
- *Ex vivo* CD8+T-cell populations become **increasingly oligoclonal** with age demonstrated by *TCRVB* conservation.

These HCMV-IE-specific, HLA-C-restricted CD8+T-cells, demonstrate both remarkable expansion and selection for strong functional activity *in vivo*, representing one of the **largest CD8+T-cell populations to be recorded**.

These findings are invaluable for understanding the breadth of HCMV-CD8+T-cell memory-inflation. Consequently, it is hoped that the inclusion of these HLA-C specificities will advance current anti-HCMV adoptive therapy to HLA-Cw*0702 positive patients.

2768

The m15 locus of murine cytomegalovirus modulates natural killer cell responses and promotes dissemination to the salivary glands

Chan, B.¹, Arapovic, M.², Masters, L.¹, Jonjic, S.², Shellam, G.¹, Smith, L.¹, Redwood, A.^{1,3}

¹University of Western Australia, Pathology and Laboratory Medicine, Perth, Australia, ²University of Rijeka, Department for Histology and Embryology, Faculty of Medicine, Rijeka, Croatia, ³Murdoch University, Institute for Immunology and Infectious Diseases, Murdoch, Australia

Murine cytomegalovirus (MCMV) expresses in excess of 170 open reading frames (ORFs), many of which have unknown function. In addition, MCMV and other cytomegaloviruses exhibit complex gene regulation, including the expression of alternative splice variants, the use of alternative start sites and the expression of multiple mRNA species from a single locus. Such complex regulation can complicate the study of gene function. We show here that the MCMV locus composed of the m14, m15 and m16 ORFs expresses five overlapping mRNA transcripts that are all co-terminal with the predicted end of the m16 ORF. Functional inactivation of any one of these ORFs has no impact on viral replication. However, disruption of all five transcripts leads to an increase in viral virulence during acute infection. Whilst slight, this elevated virulence is reproducible in multiple mouse strains. Conversely, disruption of this locus also leads to significant viral attenuation, evidenced during chronic infection of the salivary glands. Attenuation at this site is likely due to reduced dissemination. This complex phenotype is associated with heightened natural killer (NK) cell responses, including enhanced proliferation and IFN γ production. Depletion of NK cells, but not T cells, rescues viraemia and salivary gland replication. These data demonstrate that multiple transcripts can modulate, perhaps in a concerted fashion, the function of anti-viral NK cells. In addition, these data highlight

the need to define the transcriptional regulation of a viral locus before assigning gene function.

3663

NF- κ B dependent antiviral immunity in drosophila

Goto, A.¹, Ogado, K.¹, Daeffler, L.¹, Hoffmann, J.¹, Imler, J.-L.²
¹CNRS UPR 9022, IBMC, Strasbourg, France, ²CNRS, UPR 9022, University of Strasbourg, Strasbourg, France

The Toll and IMD pathways of host-defense, which regulate different members of the NF- κ B family of transcription factors, play an important role in the control of fungal and bacterial infections in the model organism *Drosophila melanogaster*. Antiviral immunity involves the cell intrinsic mechanism of RNA interference and ill-characterized inducible responses. We recently showed that the virus-induced cytokine Dieldel suppresses the IMD pathway of host-defense, and that the *dieldel* gene has been hijacked by several insect DNA viruses. These data point to a role of the IMD pathway in the control of viral infections.

Using the picorna-like virus *Drosophila C virus* (DCV) as a model, we show that a restricted number of components of the IMD pathway, including the NF- κ B factor Relish, are required for antiviral immunity *in vivo* and in a macrophage-like cell line. Genome wide profiling of the transcriptome further identified ten genes that are regulated by Relish in flies and this cell line. Their characterization in loss- and gain- of function experiments revealed that two of these ten genes are necessary and sufficient to control viral infection. While one of them is the orthologue of a gene participating in antiviral immunity in mammals, the other one is a recent acquisition of drosophila.

Altogether, our findings point to a new pathway regulating the NF- κ B protein Relish and involved in antiviral immunity. They further highlight how drosophila can be used both to identify evolutionarily conserved defense mechanisms, and to reveal original, host-specific, antiviral factors.

820

Differential recognition of influenza A viruses by M1₅₈₋₆₆ epitope-specific CD8⁺ T cells is determined by extra-epitopic amino acid residues

van de Sandt, C.E.¹, Kreijtz, J.H.C.M.^{1,2}, Geelhoed-Mieras, M.M.¹, Nieuwkoop, N.J.¹, Spronken, M.I.¹, van de Vijver, D.A.M.C.¹, Fouchier, R.A.M.¹, Osterhaus, A.D.M.E.^{1,3,4,5}, Rimmelzwaan, G.F.^{1,3}

¹ErasmusMC, Viroscience, Rotterdam, Netherlands, ²BioNovion, Oss, Netherlands, ³ViroClinics Biosciences BV, Rotterdam, Netherlands, ⁴University of Veterinary Medicine, Research Center for Emerging Infections and Zoonoses (RIZ), Hannover, Germany, ⁵Artemis One Health Research Foundation, Utrecht, Netherlands

Natural influenza A virus (IAV) infections elicit both virus-specific antibody and CD4⁺ and CD8⁺ T cell responses. IAV-specific CD8⁺ cytotoxic T lymphocytes (CTLs) contribute to clearance of influenza virus infections and are generally directed against more conserved epitopes. However, viral CTL epitopes can display variation, allowing IAVs to evade from recognition by epitope-specific CTLs. Due to functional constraints, some epitopes, like

the immunodominant HLA-A*0201 restricted matrix protein 1 (M1)₅₈₋₆₆ epitope, are highly conserved between IAVs regardless of their subtype or host species of origin. We hypothesized that human IAVs evade recognition of this highly conserved epitope by impairing antigen processing and presentation by extra-epitopic amino acid substitutions. Activation of specific T cells was used as read out for antigen presentation. We showed that the M1₅₈₋₆₆ epitope in the M1 protein derived from a human IAV was poorly recognized compared to the M1 protein derived from an avian IAV. Furthermore, we demonstrated that naturally occurring variation at extra-epitopic amino acid residues affect CD8⁺ T cell recognition of the M1₅₈₋₆₆ epitope. These data indicate that human IAVs can impair recognition by M1₅₈₋₆₆-specific CTLs, while retaining the conserved amino acid sequence of the epitope, which may represent a yet unknown immune evasion strategy for IAVs. This difference in recognition may have implications for the viral replication kinetics in HLA-A*0201 individuals and spread of IAVs in the human population. The findings may aid the rational design of universal influenza vaccines that aim at the induction of cross-reactive virus-specific CTL responses.

1346

Control of lymphoid stromal cell expansion during virus infection

Alexandre, Y.¹, Gregory, J.¹, Mueller, S.N.^{1,2}

¹University of Melbourne, Department of Microbiology and Immunology, Parkville, Australia, ²ARC Centre of Excellence in Advanced Molecular Imaging, University of Melbourne, Parkville, Australia

Subsets of lymphoid stromal cells (LSC), including the fibroblastic reticular cells (FRC) create a network on which immune cells migrate in lymphoid organs. FRC also express chemokines to attract naïve T cells and dendritic cells (DC) in the lymph node (LN). Inflammation triggers lymph node swelling and is accompanied by the expansion of the LSC network. It has been suggested that DC trigger FRC stretching while entry of naïve T cells in the LN triggers the expansion of the FRC network. Yet, little is known about the behaviour of LSC during viral infections *in vivo*. We found that infection with herpes simplex virus (HSV) or vaccinia virus induced remodelling of the LSC network in LN, characterised by a strong expansion of FRC. In contrast, LCMV Armstrong infection induced little LN hypertrophy and poor FRC expansion despite expression of Ki67, a marker of proliferation, by FRC. We found that LCMV infected LSC, especially FRC and marginal reticular cells (MRC), after peripheral but not systemic infection. Depletion of CD8 T cells prior to LCMV infection further reduced LSC expansion and increased infection of FRC and MRC, suggesting that this was not due to CTL-mediated killing. Co-infection of mice with HSV and LCMV inhibited LSC expansion without affecting the priming of T cell responses. Our data suggest that viral targeting of LSC by LCMV is influenced by route of infection and limits LN hypertrophy independently of CD8 T cell responses.

3234

Increased severity of influenza in Down syndrome may be due to a rapid inflammatory response rather than virus-induced pathology

Zanin, M.¹, Wong, S.-S.¹, Duan, S.², DeBeauchamp, J.¹, Webby, R.¹

¹St Jude's Children's Research Hospital, Department of Infectious Diseases, Memphis, United States, ²St Jude's Children's Research Hospital, Department of Immunology, Memphis, United States

Down Syndrome (DS), a trisomy of chromosome 21, is associated with increased prevalence and severity of respiratory infections and immunological abnormalities. To study the immune response to influenza virus in DS, we utilized the Ts65Dn mouse strain, a commonly used DS mouse model. Ts65Dn and wild-type mice were inoculated with Influenza A/California/04/2009 (H1N1) and lung, lymph nodes and spleen were collected on day 5 and 8 post inoculation. Ts65Dn mice showed increased morbidity and mortality over wild-type mice, although lung viral replication was less in Ts65Dn mice. Influenza-specific serum antibody titers were reduced in Ts65Dn mice compared to wild-type. By day 5, there were no significant differences in the number of infiltrating innate cells in the lungs. In the spleen, Ts65Dn mice showed significantly fewer CD4⁺ T-cells and a trend of fewer CD8⁺ T-cells. Ts65Dn mice also demonstrated higher serum IL-3 and G-CSF at baseline compared to wild-type, which did not change significantly after infection. Conversely, the pro-inflammatory cytokines IL-6 and IP-10 were significantly elevated in Ts65Dn mice post infection. In the adaptive arm, Ts65Dn mice showed fewer circulating plasma cells (CD138⁺) after infection. Our data suggests that the increased mortality observed in these mice could be due to a rapid hyperinflammatory responses that occurred within 5 days post infection rather than pathology caused by the virus.

3844

A potent clinical stage Smac mimetic antagonizes cellular inhibitors of apoptosis and promotes hepatitis B virus clearance *in vivo*

Ebert, G.^{1,2}, Allison, C.^{1,2}, Preston, S.^{1,2}, Cooney, J.^{1,2}, Toe, J.^{1,2}, Stutz, M.^{1,2}, Ojaimi, S.^{1,2}, Baschuk, N.³, Nachbur, U.^{2,4}, Silke, J.^{2,4}, Pellegrini, M.^{1,2}

¹Walter and Eliza Hall Institute, Infection and Immunity Division, Melbourne, Australia, ²Department of Medical Biology, University of Melbourne, Melbourne, Australia, ³Faculty of Science Technology and Engineering, School of Molecular Science, LaTrobe University, Department of Biochemistry, Melbourne, Australia, ⁴Walter and Eliza Hall Institute, Cell Signalling and Cell Death Division, Melbourne, Australia

Background: Cellular inhibitor of apoptosis proteins (cIAPs) are critically required for the activation of NFκB and the promotion of cell survival downstream of TNF receptor engagement. Drug or genetic targeting of cIAPs results in programmed cell death downstream of death receptor signalling. Smacs/Diablo are mitochondrial derived pro-apoptotic proteins that bind to cIAPs and antagonise their function. Small molecule drug inhibitors of IAPs (Smac mimetics) were developed to promote immune / TNF mediated killing of

cancer cells. We hypothesized that by using Smac mimetics we could promote immune mediated killing of hepatitis B virus (HBV) infected hepatocytes and eliminate infection.

Design: We antagonized cIAPs through gene targeting and by using a clinical stage IAP antagonist in a preclinical mouse model of chronic HBV infection. We examined effects on HBV infection markers in the blood and liver cells.

Results: Loss of cIAPs in gene-targeted animals promoted a rapid control of infection such that serum HBV-DNA was undetectable within 2-3 weeks. Importantly, animals treated with Smac mimetic showed a functional cure of chronic HBV infection with undetectable serum HBV-DNA and HBs antigen and the appearance of anti-HBs antibody within 6 weeks of treatment. The treatment preferentially caused the death of HBV infected hepatocytes *in vivo*. Additionally, Smac mimetics enhanced the efficacy of clinical nucleoside analogues in promoting viral clearance.

Conclusion: cIAPs are central regulators of host responses to HBV, they are critical determinants of infectious outcomes and antagonizing their function by a Smac mimetic can promote the clearance of HBV infection *in vivo*.

1602

High rates of cross-reactivity among apparently unrelated specificities shape antigen-specific T cell receptor repertoires

Thomas, P.¹, Dash, P.¹, Hertz, T.², Gartland, A.³, Bradley, P.³

¹St. Jude Children's Research Hospital, Immunology, Memphis, United States, ²Ben-Gurion University of the Negev, Beersheba, Israel, ³Fred Hutchinson Cancer Research Center, Seattle, United States

We have analyzed several thousand antigen-specific T cell receptor repertoires from multiple animals infected with influenza virus and/or murine cytomegalovirus (mCMV). Insights into the overall structure of antigen-specific repertoire landscapes towards 7 different specificities were generated from single cell paired TCR $\alpha\beta$ sequencing. In addition to characterization of repertoire features such as diversity, clonal structure, convergent recombination, and key determinants features of antigen-specificity, we observed a surprisingly high rate of single or dual chain sharing between otherwise unrelated responses. These shared receptors were often observed in across multiple animals in independent experiments. However, sharing appeared more likely to occur in animals where the two epitopes were presented simultaneously. Cloning and expression of these unique specificities has allowed further receptor characterization. Additional data indicate that some combinations of co-presented epitopes result in broader repertoires recruited to one or both epitopes. In summary, T cell receptors have a greater propensity for cross-reactivity across otherwise unrelated specificities, an effect that appears to be driven by co-presentation and substantially shapes the magnitude and diversity of antigen-specific repertoires.

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3637

Identification of repurposed drugs that increase immune checkpoint blockade efficacy in cancer using network analysis of responding tumours

Zemek, R.^{1,2}, Rinaldi, C.^{1,2}, Millward, M.^{1,3}, Nowak, A.K.^{1,2,3}, Bosco, A.⁴, Lake, R.^{1,2}, Lesterhuis, W.J.^{1,2}

¹University of Western Australia, School of Medicine & Pharmacology, Perth, Australia, ²National Centre for Asbestos Related Diseases, Perth, Australia, ³Sir Charles Gairdner Hospital, Medical Oncology, Perth, Australia, ⁴Telethon Kids Institute, Perth, Australia

Immune checkpoint blockade, such as anti-CTLA4 and anti-PD1 has shown outstanding results in several cancer types, with durable complete regression in a proportion of patients. Conversely, the majority of patients do not display tumour regression. It is unknown what molecular events determine this dichotomous response, nor which co-treatments are likely to combine effectively with checkpoint blockade.

To compare the molecular events in responders versus non-responders, we treated mice with bilateral AB1-HA mesothelioma tumours, which respond symmetrically to anti-CTLA4. We used a weighted gene correlated network analysis (WGCNA) of gene expression profiling data from responding versus non-responding tumours to identify modules associated with response. In addition, we used upstream regulator analysis and interrogated genome-wide drug-perturbation signatures in the connectivity map database to identify repurposed drugs that would phenocopy the response-associated network, and thus increase the response rate to anti-CTLA4.

We found two modules highly significantly differentially expressed; one enriched for cancer-associated genes, one for immune related genes. A central hub in both modules, which was upregulated in responding mice was inducible nitric oxide synthase (iNOS). Cotreatment with competitive iNOS inhibitor L-NNA inhibited the response to anti-CTLA4 while conversely the NO generator isosorbide dinitrate significantly enhanced the cure rate from 10 to 80% in AB1-HA mesothelioma-bearing mice. The drug repurposing approach identified all-trans retinoic acid as a candidate synergistic drug. Indeed, cotreatment with anti-CTLA4 increased the response rate from 10% to 60%.

Thus, we can identify therapeutic targets and drugs that act synergistically with checkpoint blockade in cancer.

3097

A novel approach using a bi-specific CD19xCD47 antibody to harness the phagocytic potential of macrophages to effectively suppress malignant B cells

Chauchet, X., Hatterer, E., Cons, L., Chatel, L., Richard, F., Salgado-Pires, S., Papaioannou, A., Shang, L., Fischer, N., Masternak, K., Johnson, Z., Kosco-Vilbois, M., Buatois, V., Ferlin, W.
Novimmune, Geneva, Switzerland

The up-regulation of the "don't eat me" signal CD47, and the subsequent engagement of SIRP- α , helps malignant cells evade innate immune surveillance and subsequent phagocytosis.

Targeting this axis is therefore an innovative approach to harness local phagocytes for tumor control. Development of monoclonal antibodies (Ab) that neutralize CD47 is hampered by its ubiquitous expression resulting in potential rapid Ab elimination and hematological toxicity including anemia and thrombocytopenia. To overcome such liabilities, a bispecific antibody was generated that relies on a high affinity anti-CD19 and a low affinity anti-CD47 thus focusing blockade of CD47 to B cells. *In vitro*, the bispecific Ab significantly enhances Ab-dependent cellular phagocytosis (ADCP) of B-cell lymphomas as compared to monoclonal Abs engaging either CD47 or CD19. Using xenograft mouse models, the co-blockade of CD19 and CD47 results in tumor growth suppression of several malignant B cell lines. Examined *ex vivo*, macrophages from the tumor microenvironment of bispecific Ab-administered mice show enhanced ADCP of malignant B cells; interestingly, the number of myeloid-derived suppressor cells (MDSCs) is reduced. Mechanistic studies have revealed that the ADCP induced by the bispecific Ab relies on Fc-gamma receptor (FcγR) I and/or FcγRIII engagement but not FcγRIIA ligation on macrophages. The bispecific Ab offers a novel way to safely harness the targeted phagocytic potential of macrophages in the tumor microenvironment.

4235

Somato-germinomics antigens: a new entity of cancer-stem cell-specific functioning antigens

Torigoe, T.¹, Hirohashi, Y.¹, Tsukahara, T.¹, Kanaseki, T.¹, Vitaly, K.¹, Miyamoto, S.², Asano, T.³, Horibe, R.⁴, Morita, R.¹, Sato, N.¹

¹Sapporo Medical University, Dept. of Pathology, Sapporo, Japan,

²Sapporo Medical University, Dept. of Oral Surgery, Sapporo,

Japan, ³Sapporo Medical University, Dept. of Gynecology, Sapporo,

Japan, ⁴Sapporo Medical University, Dept. of Respiratory Medicine, Sapporo, Japan

Cancer stem-like cells (CSCs) are defined as the small population of cancer cells that have stem cell-like phenotypes and high capacity for tumor initiation. These cells may have a huge impact in the field of cancer therapy since they are extremely resistant to standard chemotherapy and therefore likely to be responsible for disease recurrence. We have analyzed the immunopathological properties of CSCs of human solid cancers and identified several genes that were characteristic to CSCs. Remarkably, some of them were expressed exclusively in testicles among normal adult organs and involved in the spermatogenesis and function of spermatozoa. siRNA-mediated knockdown of these genes caused decreased sphere-forming capacity and tumor-initiating capacity, therefore indicating that they might be associated with the maintenance of stem-like phenotype of CSCs. Furthermore, it was indicated that expression of the CSC-specific genes might be regulated by epigenetic mechanisms such as histone modification and DNA methylation. Our data suggest that spermatogenesis-associated genes are aberrantly expressed in CSCs and function as key molecules for tumor initiation. In the field of tumor immunology, the CSC-specific gene products might serve as ideal targets for cancer immunotherapy. We show evidence that CSC-specific cytotoxic T-cells are induced from peripheral blood of cancer

patients. We named these functioning antigens "Somato-Germinomics antigens" and propose an immunotherapeutic strategy targeting to CSCs.

2012

Co-inhibition of adenosine generation and signaling improves anti-tumour immune responses

Young, A.^{1,2}, Ngiow, S.F.^{1,2}, Barkauskas, D.¹, Sult, E.³, Hay, C.³, Sachsenmeier, K.³, Smyth, M.J.^{1,2}

¹QIMR Berghofer Medical Research Institute, Herston, Australia, ²The University of Queensland, Herston, Australia, ³MedImmune, LLC, Gaithersburg, United States

Adenosine is a potent immunosuppressor, which hampers an effective immune reaction towards cancer cells within the tumour microenvironment. In particular, adenosine inhibits tumour cell killing performed by cytotoxic lymphocytes and enhances proliferation of immunosuppressive cell types. Preclinical studies have identified that blockade of CD73 (the ectonucleotidase that generates adenosine) and antagonism of A2A adenosine receptor (A2AR) signaling heightens anti-tumour immune responses. However, it is yet to be established as to whether targeting both stages of the adenosinergic pathway in combination further enhances anti-tumour efficacy. To determine whether co-targeting CD73 and the A2AR would provide improved tumour control or show redundancy we developed double-deficient mice. In response to AT-3 mammary carcinoma and SM1WT1 melanoma, A2AR and CD73 double-knockout mice displayed significantly improved primary tumour control compared to wildtype mice or single gene-deficient controls, indicative of their non-redundant functions. Following, we assessed the therapeutic efficacy of A2AR antagonism alongside anti-CD73 in experimental and spontaneous metastases models. When given concurrently, these therapies reduced metastatic burden and enhanced survival benefit. In particular, anti-CD73 therapeutic activity was mediated by Fc receptor engagement, indicative of the multi-functionality of CD73, alongside adenosine production. Similarly, these *in vivo* findings were paralleled in mixed lymphocyte reactions utilizing human anti-CD73. This study illustrates the importance of targeting the adenosinergic pathway revealing its multi-faceted impact on tumour development and metastatic progression. As we move forward to clinical utility of adenosine-related therapies in oncology, this study provides evidence that targeting different parts of the adenosinergic pathway in combination may potentiate therapeutic response.

827

Treating metastatic disease through manipulation of regulatory T cells

Hughes, E., Bloom, A., Paisey, S., Smart, K., Ager, A., Gallimore, A.
Cardiff University School of Medicine, Infection and Immunity,
Cardiff, United Kingdom

Regulatory T cells (Tregs) play an important role in preventing the development of autoimmunity. Tregs have also been implicated in controlling tumour immunity since depletion of

Tregs slows the growth of primary tumours and, in some cases, causes complete regression. Metastasis is the main cause of fatality in cancer patients but the impact of Tregs in metastatic disease is poorly understood. We hypothesised that depletion of Tregs would prevent the spread of metastases. 4T1 murine mammary carcinomas were grown in DERE mice, which express the diphtheria toxin (DTx) receptor on Tregs. Tumour bearing mice were treated with DTx to deplete Tregs before or after resection of the primary tumour. Metastasis of 4T1 cells in mice was monitored using PET/CT imaging over a period of 3 weeks after removal of the primary tumour. Following Treg depletion, the growth of primary tumours was markedly reduced such that tumours regressed. In marked contrast, the number of metastases was not affected by Treg depletion. Immunohistochemical analysis of primary tumours and lung metastases showed major differences in the organization of tumour cells and immune cell infiltrates. We conclude that there are fundamental differences in the physiology and immune response between the primary tumour and the lung metastases, and depletion of Tregs alone is not enough to control the progression of metastatic disease in breast cancer.

2003

An unusually long 13-mer tumor antigenic peptide "molded" around distinct T-cell receptors when presented by HLA-B*0702 and provided the basis for polyclonal T-cell receptor recognition

Chan, K.F.^{1,2,3,4}, Gras, S.^{3,5}, Beringer, D.^{3,5}, Kjer-Nielsen, L.², Cebon, J.¹, Mccluskey, J.², Rossjohn, J.^{3,5,6}, Chen, W.^{1,4}

¹Ludwig Institute for Cancer Research, Olivia Newton-John Cancer and Wellness Centre, Melbourne, Australia, ²The University of Melbourne, at the Peter Doherty Institute for Infection and Immunity, Department of Microbiology and Immunology, Melbourne, Australia, ³Monash University, Department of Biochemistry and Molecular Biology, School of Biomedical Sciences, Melbourne, Australia, ⁴La Trobe University, Department of Biochemistry & Genetics, La Trobe Institute of Molecular Science, Melbourne, Australia, ⁵Monash University, Australian Research Council Centre of Excellence for Advanced Molecular Imaging, Melbourne, Australia, ⁶Cardiff University School of Medicine, Institute of Infection and Immunity, Cardiff, United Kingdom

Peptides presented by major histocompatibility complex class I (MHC-I) molecules are largely 8 to 10 amino acids in length because of the molecules closed ends. Nonetheless, a growing number of studies have shown that peptide longer than 10 amino acids can be presented for CD8⁺ T-cell (T_{CD8}⁺) recognition and is equally capable of eliciting potent T_{CD8}⁺ responses as peptides of canonical length. Long peptides were frequently observed to form a prominent central bulge when presented by MHC-I molecules. Such bulged peptide/MHC-I (pMHC-I) conformation poses great challenge for T-cell receptor (TCR) binding, thus results in predominantly biased TCR repertoire. Some TCRs were observed to perch on a rigid bulged pMHC-I, but others were shown to deform the bulged pMHC-I and created a flatter conformation. No studies have described that such TCR docking modes could be simultaneously used by distinct TCRs recognizing a common bulged pMHC-I.

We previously reported an immunodominant 13-mer NY-ESO-1₆₀₋₇₂ peptide derived from cancer-testis antigen NY-ESO-1. This peptide is restricted to HLA-B*0702 and is naturally presented by melanoma cells. It adopts a super-bulge in the centre when complexed to HLA-B*0702. Unlike other reported cases, T_{CD8}⁺ responses to the NY-ESO-1₆₀₋₇₂/HLA-B*0702 is not only immunodominant but also involves diverse TCR repertoire, at least 10 different Vβ families.

In this study, we solved the structures of two TCRs, KFJ5 and KFJ37, in complexes with NY-ESO-1₆₀₋₇₂/HLA-B*0702 and showed that the polyclonal TCR recognition is explained by the flexible arrangements of the presented peptide. Our findings elucidated a novel mechanism for polyclonal TCR/pMHC-I interactions.

2955

Systemic inflammation in tumour microenvironment provoked by cytotoxic lymphocyte synapse dwell time

Jenkins, M.R.^{1,2}, Rogers, A.J.¹, Rudd-Schmidt, J.A.¹, Lopez, J.A.¹, Mannering, S.I.^{3,4}, Voskoboinik, I.^{1,5}, Trapani, J.A.^{1,2}

¹Peter MacCallum Cancer Centre, Cancer Immunology Research, East Melbourne, Australia, ²The University of Melbourne, Sir Peter MacCallum Department of Oncology, Melbourne, Australia, ³St Vincent's Hospital, The University of Melbourne, Department of Medicine, Fitzroy, Australia, ⁴St Vincent's Institute of Medical Research, Immunology and Diabetes Unit, Fitzroy, Australia, ⁵The University of Melbourne, The Department of Genetics, Melbourne, Australia

Failure of cytotoxic T lymphocytes (CTL) or natural killer cells (NK) to kill target cells by perforin/granzyme-induced apoptosis causes severe immune dysregulation. Infants deficient in perforin suffer a fatal 'cytokine storm' resulting from macrophage over-activation, but the link to failed target cell death is not understood. We show that prolonged target cell survival amplifies the quanta of inflammatory cytokines secreted by CTL/NK cells and that interferon-γ directly invokes the activation and secondary over-production of pro-inflammatory IL-6 from naïve macrophages. Further, using live cell microscopy to visualise hundreds of synapses formed between WT, perforin-null or granzyme A/B-null CTL/NK and their targets in real time, we show that hyper-secretion of cytokine and chemokines is linked to failed disengagement of perforin- or granzyme-deficient lymphocytes from their targets, with mean synapse time increased five-fold. The signal for detachment arose from the dying target cell and was caspase-dependent, as delaying target cell death with various forms of caspase-blockade also prevented their disengagement from fully competent CTL/NK cells and caused cytokine hyper-secretion. Our findings are supported by recent in vivo data; lymphocytes, which fail to quickly kill tumour cells, cause changes to immune cell recruitment and inflammatory environment. We provide the cellular mechanism through which failed killing by lymphocytes causes systemic inflammation involving recruitment and activation of myeloid cells.

3660**Inflammatory monocytes promote inflammation-induced tumor development in a model of cutaneous squamous cell carcinoma***Lelios, L., Cecconi, V., van den Broek, M., Becher, B., Greter, M.**University of Zurich, Institute of Experimental Immunology, Zurich, Switzerland*

Cutaneous squamous cell carcinoma (SCC) is one of the major non-melanoma skin cancers and derives from transformed epidermal squamous cells. Tumor-associated myeloid cells have been shown to play an important role in tumor development by suppressing anti-tumor immune responses on one hand and supporting tumor growth and metastasis on the other. Langerhans cells (LCs) - the resident mononuclear phagocyte population of the epidermis - have been shown to reside within SCC tumors and to promote epidermal mutagenesis. However, the distribution, phenotype and role of LCs and other skin-resident or infiltrating myeloid cells during SCC development remains unclear. In order to address the functions of these myeloid cell types (monocytes, macrophages, and dendritic cells), we used the *K14-HPV8-E6* transgenic mouse model of ultraviolet light (UV) induced SCC where we blocked the development or recruitment of different myeloid cells to the skin. We demonstrated that neither tumor-associated LCs nor resident macrophages and dendritic cells promoted SCC growth. However, the early recruitment of monocytes to the site of UV exposure was necessary for the development of SCC indicating that chronic inflammation maintained specifically by skin-infiltrating inflammatory monocytes promotes tumorigenesis.

1382**Role of IL17 producing $\gamma\delta$ T cells (T $\gamma\delta$ 17) in gall bladder cancer pathogenesis***Chiplunkar, S.¹, Patil, R.¹, Shah, S.¹, Shrikhande, S.², Goel, M.², Dikshit, R.²**¹Advanced Centre for Treatment, Research & Education in Cancer, Tata Memorial Centre, Chiplunkar Lab, Mumbai, India, ²Tata Memorial Hospital, Mumbai, India*

Gall bladder cancer (GBC) is the most common malignancy of the biliary tract. 70-80% of GBC patients are associated with inflammatory condition of cholelithiasis. The immune microenvironment in GBC is not well investigated. The present study aims to understand the dynamics and functions of pro inflammatory (Th17, T $\gamma\delta$ 17, Tc17) and anti-inflammatory (Regulatory T cells; Tregs) immune cells in GBC. In the current prospective study of GBC patients, we report that the levels of IL17 producing inflammatory subtypes of $\gamma\delta$ (T $\gamma\delta$ 17), CD4 (Th17) and CD8 (Tc17) T cells were increased in peripheral blood and tumor infiltrating lymphocytes (TIL) of GBC patients compared to healthy individuals (HI) as analysed by multicolour flow cytometry. Regulatory T cells (Tregs) were decreased in peripheral blood and increased in TILs of GBC patients but their suppressive potential was comparable to HI. Serum cytokines profile of GBC patients showed elevated levels of cytokines (IL6, IL23 and IL1 β) required for differentiation of IL17 producing cells. We demonstrated that T $\gamma\delta$ 17 cells migrate towards the tumor

bed through CXCL9-CXCR3 axis. IL17 secreted by T $\gamma\delta$ 17 induced VEGF production and other angiogenesis related factors in GBC cells. T $\gamma\delta$ 17, Th17, Treg and serum IL17 were associated with poor survival of GBC patients. Taken together, our data strongly suggests that T $\gamma\delta$ 17 is a pro-tumorigenic subtype of $\gamma\delta$ T cells that contribute to the negative clinical outcome of GBC patients. Our data has unravelled novel pro tumorigenic functions of T $\gamma\delta$ 17 cells in GBC, opening new avenues for targeted therapies.

4758**Targeting cereblon for anti-tumor immunity***Hesterberg, R.^{1,2}, Beatty, M.¹, Epling-Burnette, P.K.¹**¹Moffitt Cancer Center, Tampa, United States, ²University of South Florida, Tampa, United States*

Growing emphasis has been placed on defining novel regulatory circuits to improve anti-tumor immunity. Modulation of intracellular T-cell signaling thresholds could be a valuable approach to extend beyond immune checkpoint blockade. A series of E3 ubiquitin ligases (E3-UbL) are involved in energy and self-tolerance by controlling proteasomal-mediated degradation and molecular trafficking of key signaling intermediates involved in T-cell activation. Recently cereblon (CRBN), a novel E3 ubiquitin ligase substrate receptor for the DDB1/Cul4/Rbx1 complex, has been linked to T-cell signaling regulation. Cereblon is the direct pharmacological target of the immunomodulatory drug (IMiDs[®]) class used in several hematological malignancies. Treatment with IMiDs reduces inflammation and enhances T-cell activation in both optimal and suboptimal stimulatory conditions. Proliferation, IL-2 and IFN γ are enhanced in T-cells from CRBN-deficient (C57B/6 *crbn*^{-/-}) mice with both native polyclonal and fixed T-cell receptor repertoires. Here, we show that CRBN mediates a novel pathway of regulation that is distinct from other E3-UbL proteins associated with tolerance. Compared to wild-type T-cells, *crbn*^{-/-} T-cells display metabolic changes including higher oxygen consumption (OCR) and acidification rates (ECAR) indicating that cereblon-regulated substrates control metabolic activity. It has been previously demonstrated that tumors suppress T-cell function through metabolic competition. B16-melanoma growth was significantly reduced in *crbn*^{-/-} mice consistent with improved anti-tumor T-cell immunity. Adoptive cell transfer of *crbn*^{-/-} T-cells into B16-melanoma bearing mice demonstrated this phenotype to be T-cell intrinsic. These results reveal a novel E3-UbL regulatory circuit involved in metabolic control that may be exploited pharmacologically to improve anti-tumor immunity.

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IL-17 from discovery to targeting

Miossec, P.

University of Lyon, Dpt of Immunology and Rheumatology, Lyon, France

IL-17 now IL-17A, was first identified in 1995 as a T cell derived cytokine with effects on inflammation and neutrophil activation. Using the example of Rheumatoid arthritis (RA), it was shown that its inhibition reduced the production of inflammatory mediators by explants of inflamed synovitis. This effect resulted from synergistic interactions between IL-17 and proinflammatory cytokines such as TNF or IL-1. In addition to its effect on inflammation and related destruction, the role of IL-17 was shown in inflammation chronicity through an effect on reduced apoptosis of mesenchymal cells and induction of anti-apoptotic molecules. At the site of inflammation, increased local production of IL-17 results from local interactions between activated T cells with various mesenchymal cells, either from bone marrow, synovium.

Taken together, these results support the early targeting of IL-17 in chronic inflammation associated with matrix destruction. Reduced effect of IL-17 inhibition can be expected when inflammation had a long term effect on mesenchymal cells. Recent clinical results are in line with these observations. Impressive results with anti-IL-17A antibodies have been observed in psoriasis. This led to the registration of the first anti-IL-17A antibody in January 2015. Interesting results have been obtained in psoriatic, arthritis, spondylarthritis, and rheumatoid arthritis. Other options include the inhibition of TNF and IL-17A with bispecific molecules. As the family members IL-17A and IL-17F share proinflammatory activities, another option is the dual inhibition of IL-17A and IL-17F, even with the added inhibition of TNF with a single tri-specific molecule.

1771

Preclinical efficacy of Periostin short interfering RNA in pulmonary fibrosis

Hinne, J.¹, D'Alessandro-Gabazza, C.N.¹, Toda, M.¹, Yasuma, T.², Kentaro, F.³, Nishihama, K.², Kobayashi, T.³, Etsuko, H.¹, Gabazza, E.¹, Yazunori, Y.⁴

¹Mie University School of Medicine, Department of Immunology, Tsu, Japan, ²Mie University School of Medicine, Department of Diabetes and Metabolism, Tsu, Japan, ³Mie University School of Medicine, Department Pulmonary and Critical Care Medicine, Tsu, Japan, ⁴Aqua Company, Tsu, Japan

Idiopathic Pulmonary Fibrosis (IPF) is a fatal disease with a mortality rate of 3 years after diagnosis. While pathogenetic mechanisms are incompletely understood, the currently accepted paradigm proposes that injury to the alveolar epithelium is followed by a burst of pro-inflammatory and fibroproliferative mediators that invoke responses associated with normal tissue repair. Recent research in this area has focused on treatment regimen which seems to improve lung function but none has so far been found to increase survival.

Periostin which is a recently characterized matricellular protein belonging to the fasciclin 1 family has been shown to be elevated in IPF patients and in bleomycin (BLM) induced lung fibrosis.

In this study we evaluated the effect of periostin siRNA on BLM-induced lung fibrosis.

The results indicate that periostin is significantly increased on day 3 after BLM treatment and reduced on days 7 and 14 but elevated on day 21. There was increased infiltration of inflammatory cells in the scrambled siRNA treated group as compared to periostin siRNA treated group. The levels of periostin, TGF-beta 1 and collagen were significantly reduced in periostin siRNA treated group as compared to scrambled siRNA treated group. Fibrosis was also significantly reduced as measured by the Ashcroft score. The overall survival was also improved in the periostin siRNA treated group as compared to the control groups.

These results show that blockade of periostin with siRNA may be an effective treatment option for the management of pulmonary fibrosis.

3722

The balance between inflammation and immunity in the tumor microenvironment is determined by IL-1 β

Kaplanov, I., Karnetsky, R., Carmi, Y., Voronov, E., Apte, R.N.

Ben Gurion University of the Negev, Beer Sheva, Israel

We used the model of orthotopically injected 4T1 breast cancer cells IL-1 β deficient (IL-1 β KO) and wild-type (WT) mice to probe the role of microenvironment IL-1 β in the balance between inflammation and immunity. Both, WT and IL-1 β KO mice developed tumors, which progress in WT mice and undergo regression in IL-1 β KO mice after 14 days. Progression of the primary tumor in WT mice corresponded with development of lung metastases and poor survival rates, whereas in IL-1 β KO mice regression of primary tumor, with no metastases was observed. Tumor regression in IL-1 β KO mice is CD8⁺ T cell dependent, WT mice, microenvironment IL-1 β promotes, through induction of MCP-1, recruitment of LY6C^{high}CCR2⁺ monocytes to the primary tumor and directs monocyte differentiation to inflammatory and tumor promoting mannose receptor-bearing macrophage lineage cells. In IL-1 β KO mice differentiation pathway of monocytes to macrophages is arrested at the stage of immature macrophages. However, in IL-1 β KO mice, and not in WT mice, LY6C^{low}CCR2 cells were abundant at the tumor site. This cell population might further differentiate into monocyte-derived macrophages or monocyte-derived dendritic cells, possibly with APC function. At the early time intervals after tumor injection into lungs from both WT and IL-1 β KO mice, similar local inflammatory responses were apparent, which progressed in WT mice during development of lung metastases but disappeared in IL-1 β KO mice. Overall, the results suggest that anti-IL-1 β therapy could modify the myeloid cell load and subpopulations in the tumor and modify systemic inflammatory responses, thus reducing tumor invasiveness.

4397

MicroRNAs mediate anti-inflammatory effects of eicosapentaenoic acid in mouse adipose tissue

Moustaid-Moussa, N.¹, Ramalingam, L.¹, Yen, E.¹, Wijayatunga, N.¹, Pahlavani, M.¹, Kottapalli, R.², Gunaratne, P.³, Rajapakshe, K.⁴, Coarfa, C.⁴, Kalupahana, N.S.^{1,5}

¹Texas Tech University, Nutritional Sciences and Obesity Research Cluster, Lubbock, United States, ²Texas Tech University, Center for Biotechnology & Genomics and Obesity Research Cluster, Lubbock, United States, ³University of Houston, Biology & Biochemistry, Houston, United States, ⁴Baylor College of Medicine, Molecular and Cellular Biology, Houston, United States, ⁵University of Peradeniya, Faculty of Medicine, Physiology, Peradeniya, Sri Lanka

Obesity is associated with the expansion of adipose tissue that creates a system in which the body transitions into an inflammatory state. We previously reported that mice fed high fat diets supplemented with the omega-3 fatty acid, eicosapentaenoic acid, (HF-EPA) exhibited reduced adipose and systemic inflammation and macrophage infiltration into adipose tissue, compared to mice fed high fat diets without EPA (HF). Further, microRNAs (miRNAs), a class of small, non-coding RNAs, were identified as key regulators of metabolic genes and processes associated with diseases including obesity. Accordingly, we hypothesized that specific microRNAs are mediating anti-inflammatory effects of EPA in adipose tissue. To test this hypothesis, we tested whether EPA regulates inflammatory genes (RNA-Seq) and microRNAs (miRNA profiling) in visceral white adipose tissue (VAT) of mice. We identified 200 genes and 64 miRNAs that were differentially expressed (95% confidence, $p < 0.05$) in VAT from HF compared to HF-EPA fed mice. Ingenuity® Pathway Analysis (IPA) was used to determine mRNA-miRNA pairs regulated by EPA. Among these, several pro-inflammatory miRNAs such as miR-221 was downregulated by EPA. Other major canonical pathways associated with inflammation were also reduced by EPA. These findings were further validated in mouse fat tissue and confirmed that EPA significantly reduced miR-221 expression ($p < 0.05$). We conclude that global genomic studies are important in identifying new pathways regulated by omega-3 fatty acids that may lead to novel targets to prevent/treat metabolic complications associated with obesity.

1636

mPGES1-dependent PGE₂ orchestrates T cell responses during autoimmunity

Maseda, D., Johnson, E., Crofford, L.J.

Vanderbilt University, Medicine, Nashville, United States

Eicosanoids, particularly prostaglandin E₂ (PGE₂), are critical molecules in the initiation and resolution of inflammation during immune responses. PGE₂ is associated with modulating autoimmunity through altering the IL-23/IL-17 axis and regulatory T cells, whose balance is critical for controlling pathogenic

T cells. The inducible microsomal prostaglandin E synthase 1 (mPGES1) is a membrane enzyme that regulates local PGE₂ levels and is highly expressed at sites of inflammation. Hematopoietic

and stromal cells express mPGES1, but its role in T cell function remains underappreciated. Freshly isolated naïve T cells deficient in mPGES1 depict a significant decrease in *ptger2* and *ptger4* (PGE₂ receptors EP2 and EP4) and *tgfbr1* expression compared with WT naïve T cells. Since TGFβ is key for the generation of both regulatory and Th17 cells we addressed the effect of mPGES1 in Th17 and Treg function. mPGES1^{-/-} naïve T cells produced more IL-17A than their WT counterparts under Th17 polarizing conditions *in vitro*, and exogenous PGE₂ shifted the production from IL-17A to IFNγ. In contrast, during immunization experiments, WT mice demonstrated increased Th17 and Treg responses than mPGES1^{-/-} mice, including enhanced antigen-specific IL-17A, IL-6 and IFNγ recall responses. Furthermore, mPGES1 displayed opposite effects on pathogenic and regulatory T cells in the T-cell transfer colitis model, with mPGES1 deficiency limiting intestinal damage driven by effector T cells, but enhancing *de novo* generation of Tregs and increasing colonic lamina propria homing of CD4⁺FoxP3⁺ cells. Taken together, these data demonstrate the pleiotropic and context-dependent actions of mPGES1 and PGE₂ in inflammation and immunity.

2141

Genetic ablation of CCR6 aggravates colitis in a spontaneous colitis mouse model

Basheer, W.¹, Kunde, D.¹, Korner, H.², Eri, R.¹

¹University of Tasmania, School of Health Sciences, Launceston, Australia, ²Menzies Institute for Medical Research, University of Tasmania, Hobart, Australia

Introduction and Background: Chemokine receptor 6 (CCR6) is expressed by both pro-inflammatory cells and regulatory T cells and its ligand CCL20 is predominantly expressed by intestinal epithelial cells. CCR6/CCL20 axis is implicated to play a major role in the pathogenesis of IBD. The CCR6/CCL20 axis is paradoxical as it is involved in both immune regulation and activation. The contradictory role of CCR6/CCL20 in inflammation/intestinal homeostasis requires further investigation in a relevant spontaneous model of colitis.

Aim: To assess the role of CCR6 in an inflammatory environment of spontaneous colitis model Winnie, with established colitis.

Methods: CCR6 deficient Winnies were generated and assessed with clinical, histological & immunological parameters.

Results: Clinical parameters showed significant increase in the colon weight/body weight in CCR6 deficient Winnie mice compared to sex and age matched controls. Histological examinations revealed the aggravated colitis specifically in mid to proximal colon region. Immuno-phenotyping of colonic lymphocytes indicated reduction in the Fox P3⁺ Tregs and increased Th17 cells.

Discussion and conclusion: Our studies indicate that CCR6 deficiency aggravates colitis in an established chronic colitis setting. Further detailed mechanism of CCR6 function will provide an insight in understanding of CCR6/CCL20 in the pathogenesis of IBD.

3259

IL-33 released from liver sinusoidal endothelial cells induce neutrophil extracellular traps during liver sterile inflammation

Huang, H.^{1,2}, Yazdani, H.¹, Hui-Wei, C.¹, Tohme, S.¹, Loughran, P.¹, Turnquist, H.¹, Tsung, A.¹

¹University of Pittsburgh, Department of Surgery, Pittsburgh, United States, ²Huazhong University of Science and Technology, Department of Surgery, Wuhan, China

Neutrophil extracellular traps (NETs) have been found to exacerbate liver ischemia/reperfusion (I/R) injury which is one of well-known surgical stress. We here sought to determine the role of IL-33 released from Liver sinusoidal endothelial cells (LSECs) in NET formation during liver I/R. There was a significant increase in serum IL-33 level in patients who underwent major liver resection in which liver I/R is inevitable, compared to age/gender-matched healthy volunteers. In a murine non-lethal model of warm liver I/R, NET formation decreased in IL-33 KO mice compared with WT mice, associated with significant less serum level of myeloperoxidase (MPO)-DNA complexes and tissue level of citrullinated-histone H3. Treatment of soluble ST2 (sST2) significantly reduced NET formation whereas recombinant IL-33 (rIL-33)-treatment increased NETs in mice after liver I/R. Significantly less organ damage and lower levels of systemic inflammation were found in IL-33 KO mice or sST2-treated WT mice compared to control mice after liver I/R. rIL-33 administration during I/R exacerbated hepatotoxicity and systemic inflammation. *In vitro*, WT LSECs released IL-33 after hypoxia (1% O₂), promoted WT neutrophils to form NETs. NET formation was blocked by IL-33 neutralizing antibody or sST2. IL-33 KO LSECs failed to induce NET formation in WT neutrophils. ST2 KO neutrophils did not form NETs in response to rIL-33. After neutrophil depletion in mice, the adoptive transfer of ST2 KO neutrophils significantly decreased in NET formation, conferred from liver I/R injury. Our study demonstrates IL-33 from LSECs induces neutrophils to form NETs in excessive inflammation and hepatotoxicity during liver I/R.

353

Promotion of expansion and differentiation of hematopoietic stem cells by IL-27 into myeloid progenitors to control infection in emergency myelopoiesis

Yoshimoto, T.¹, Furusawa, J.-I.¹, Chiba, Y.¹, Xu, M.¹, Hasegawa, H.¹, Nakae, S.², Kobayashi, F.³, Yoshida, H.⁴, Mizoguchi, I.¹

¹Tokyo Medical University, Department of Immunoregulation, Institute of Medical Science, Tokyo, Japan, ²University of Tokyo, Laboratory of Systems Biology, Center for Experimental Medicine and Systems Biology, Institute of Medical Science, Tokyo, Japan, ³Kyorin University of Medicine, Department of Infectious Diseases, Tokyo, Japan, ⁴Saga University, Department of Biomolecular Sciences, Faculty of Medicine, Saga, Japan

Emergency myelopoiesis is inflammation-induced hematopoiesis to replenish myeloid cells in the periphery, which is critical to control the infection with pathogens. Previously, pro-inflammatory cytokines such as IFN- α and IFN- γ were demonstrated to play a critical role in the expansion of

hematopoietic stem cells (HSCs) and myeloid progenitors, leading to production of mature myeloid cells, although their inhibitory effects on hematopoiesis were also reported. Therefore, the molecular mechanism of emergency myelopoiesis remains incompletely understood. Here, we clarify that one of the IL-6/IL-12 family cytokines, IL-27, plays an important role in the emergency myelopoiesis. Among various types of hematopoietic cells in bone marrow, IL-27 predominantly and continuously promoted the expansion of only Lineage-Sca-1⁺c-Kit⁺ (LSK) cells, especially long-term repopulating HSCs and myeloid-restricted progenitor cells with long-term repopulating activity, and the differentiation into myeloid progenitors in synergy with stem cell factor. These progenitors expressed myeloid transcription factors such as *Spi1*, *Gfi1*, and *Cebpa/b* through activation of STAT1 and STAT3, and had enhanced potential to differentiate into migratory DCs, neutrophils, and mast cells. The experiments using mice deficient for one of IL-27 receptor subunits, *WSX-1*, and IFN- γ revealed that the blood stage of *Plasmodium berghei* XAT infection enhanced IL-27 expression through IFN- γ production, and the IL-27 then promoted the expansion of LSK cells, differentiating and mobilizing them into spleen, resulting in enhanced production of neutrophils to control the infection. Thus, IL-27 is one of the limited unique cytokines directly acting on HSCs to promote differentiation into myeloid progenitors downstream of IFN- γ during emergency myelopoiesis.

1239

Bromodomain inhibitors reverse inflammation and disease features in experimental chronic obstructive pulmonary disease

Jones, B.¹, Harrison, C.¹, Dua, K.¹, Hsu, A.¹, Starkey, M.¹, Jarnicki, A.², Smithers, N.³, Knight, D.¹, Wark, P.¹, Adcock, I.⁴, Hansbro, P.¹

¹University of Newcastle, Centre for Asthma and Respiratory Diseases, New Lambton Heights, Australia, ²University of Melbourne, Dept of Pharmacology and Therapeutics, Parkville, Australia, ³GlaxoSmithKline, Epinova DPU, Stevenage, United Kingdom, ⁴Imperial College London, National Heart & Lung Institute, London, United Kingdom

Introduction: Chronic obstructive pulmonary disease (COPD) is a progressive lung disease characterised by chronic airway inflammation that has limited treatment options. Emerging evidence suggests that increased acetylation of histones associated with pro-inflammatory genes drives chronic inflammation in the lungs of patients with COPD. Histone acetylation is governed by histone acetyltransferases (HATs) that drive acetylation, and inhibitory histone deacetylases (HDACs). HAT activity is dependent on a conserved bromodomain, which may be targeted therapeutically.

Methods: We assessed the role(s) and therapeutic potential of targeting HAT bromodomains in our mouse model of cigarette smoke (CS)-induced experimental COPD. We treated mice with a highly-selective, proprietary bromodomain inhibitor for 4 weeks after 8 weeks of CS exposure to induce the development of experimental COPD. Treatment was with either continued CS exposure or smoking cessation. Mechanistic pathways were assessed using RT-qPCR, ELISA and ChIP-Seq.

Results: In mouse and human lung tissues we found aberrant HAT & HDAC expression and activity that led to increased histone H4 acetylation. Treatment with the bromodomain inhibitor abrogated inflammatory cells, particularly neutrophils in the BALF. Furthermore treatment protected against emphysema-like alveolar enlargement and restored lung function parameters including airway resistance and TLC.

Conclusion: COPD is associated with aberrant histone acetylation. Inhibition of bromodomains restricts progression of experimental COPD, by reducing inflammation, alveolar enlargement and restoring lung function parameters. This inhibition suggests that histone acetylation is a potential driving force that leads to the progression and development of COPD. Inhibition could have the potential therapeutic benefit in human COPD.

4606

Protein phosphatase magnesium dependent 1A, the Janus of microenvironment acts as an immunomodulator

Lavi, S.¹, Ben Dov, H.¹, Backal, L.¹, Ben Dov, R.¹, Ben Meir, D.¹, Weingarten, G.², Wertheimer, E.², Barzilai, A.^{2,3}

¹Tel Aviv University, Cell Research and Immunology, Tel Aviv, Israel,

²Tel Aviv University, Pathology, Sackler School of Medicine, Tel Aviv, Israel, ³Sheba Medical Center, Department of Dermatology, Ramat Gan, Israel

Tumor microenvironment has been gradually recognized as a key contributor to cancer progression. Using conditional Protein phosphatase magnesium dependent 1A (PPM1A) knockout mice we identified PPM1A as a major regulator of the microenvironment. In the PPM1A ablated mice the wound healing process goes awry and culminates into uncontrolled inflammation and angiogenesis, features that are two of the hallmarks of cancer. We investigated the role of PPM1A in cancer, using different mouse models. To our surprise, the absence of PPM1A could be either tumor promoting or tumor suppressive depending on the tumor initiating protocol.

Skin fibroblasts play critical roles in normal wound healing and in cancer. While fibroblasts can have a tumor suppressing activity, the phenotype of the fibroblast changes to a tumor promoting state as carcinogenesis progresses. Multipotent skin precursor cells (SKPs) capable to differentiate to fibroblasts were isolated from WT and KO mice and cultivated as 3D spheroids. PPM1A ablation led to major changes in gene expression and in functional characteristics of the SKPs. PPM1A was shown to be a major player in cellular ROS (reactive oxygen species) signaling. The absence of PPM1A led to altered expression of the immunomodulatory genes, genes regulating cell death of immune cells and the recruitment of antigen presenting cells. The role of PPM1A in immunomodulation and tumorigenesis will be discussed.

Our studies might lead to the development of a new strategy in cancer treatment involving immunomodulation of the tumor microenvironment via the manipulation of PPM1A or its targets.

Mini Oral Sessions

15:30:00 - 16:30:00

Antigen Processing & Presentation

1278

Drugs/drug analogues modulate MAIT cell function in an MR1-dependent manner

Eckle, S.B.G.¹, Keller, A.N.^{2,3}, Xu, W.^{4,5}, Meehan, B.¹, Pediongo, T.¹, Liu, L.^{4,5}, Hughes, V.A.^{2,3}, Mak, J.Y.W.^{4,5}, Birkinshaw, R.W.², Chen, Z.¹, Wang, H.¹, D'Souza, C.¹, Kostenko, L.¹, Corbett, A.J.¹, Purcell, A.W.², Fairlie, D.P.^{4,5}, Rossjohn, J.^{2,3,6}, McCluskey, J.¹

¹Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Department of Microbiology and Immunology, Melbourne, Australia, ²Biomedicine Discovery Institute, Monash University, Infection and Immunity Program and Department of Biochemistry and Molecular Biology, Clayton, Australia, ³Monash University, ARC Centre of Excellence in Advanced Molecular Imaging, Clayton, Australia, ⁴Institute for Molecular Bioscience, The University of Queensland, Brisbane, Australia, ⁵University of Queensland, ARC Centre of Excellence in Advanced Molecular Imaging, Brisbane, Australia, ⁶Institute of Infection and Immunity, Cardiff University School of Medicine, Cardiff, United Kingdom

Mucosal Associated Invariant T (MAIT) cells are an abundant subset of T cells, whose T cell receptor is restricted by the monomorphic MHC class I related molecule MR1. Acting as signature biomarkers of microbial infection, stimulating MAIT cell antigens (Ags), such as 5-OP-RU, originate from the microbial biosynthesis of riboflavin. MR1 also binds non-stimulating Ags, which act as competitive inhibitors of stimulating Ags.

Given the small molecule nature of MR1 ligands identified thus far, we reasoned that drugs and drug analogues could represent a source of exogenous MR1 ligands. Indeed when screening a library of drugs/drug analogues we identified novel MR1 ligands. Some of these upregulated MR1 and inhibited human and mouse MAIT cell activity in response to 5-OP-RU, as determined by *in vitro* analyses. Furthermore, these drugs/drug analogues acted in a dose dependent manner as competitive inhibitors of human MAIT cells stimulated with 5-OP-RU to proliferate and to produce IFN γ and TNF α cytokines *ex vivo* and mouse MAIT cell proliferation *in vivo*. Additionally, a drug and metabolites thereof strongly activated a subset of MAIT cells in a dose-dependent and MR1-restricted manner.

Thus MR1 has the potential to accommodate a range of structurally distinct ligands that have the capacity to directly modulate MAIT cell function by either competitively inhibiting MAIT cell activation or activating subsets of MAIT cells. These findings suggest that widely used drugs can inadvertently modulate MAIT cell function. Given the monomorphic and evolutionary conserved nature of the MAIT-MR1 axis this effect has a global impact.

466

CD8 T-cells of *Listeria monocytogenes*-infected mice recognize both linear and spliced proteasome products

Platteel, A.C.M.¹, Mishto, M.^{2,3}, Textoris-Taube, K.², Liepe, J.⁴, Busch, D.H.⁵, Kloetzel, P.M.², Sijts, A.J.A.M.¹

¹Utrecht University, Utrecht, Netherlands, ²Institut für Biochemie, Charité - Universitätsmedizin Berlin, Berlin, Germany,

³Interdepartmental Centre 'Luigi Galvani' for Bioinformatics, Biophysics and Biocomplexity (CIG), Alma Mater Studiorum, University of Bologna, Bologna, Italy, ⁴Centre for Integrative Systems Biology and Bioinformatics, Department of Life Sciences, Imperial College London, London, United Kingdom, ⁵Institute for Medical Microbiology, Immunology and Hygiene, TU Munich, Munich, Germany

CD8 T-cells responding to infection recognize pathogen-derived epitopes presented by MHC class-I molecules. While most of such epitopes are generated by proteasome-mediated antigen cleavage, analysis of tumor antigen processing has revealed that epitopes may also derive from proteasome-catalyzed peptide splicing (PCPS). To determine whether PCPS contributes to epitope processing during infection, we analyzed the fragments produced from a *Listeria monocytogenes* polypeptide by purified proteasomes. Mass spectrometry demonstrated the generation of a known H-2K^b-presented linear epitope (LLO₂₉₆₋₃₀₄) and four spliced peptides, which were trimmed by ERAP into six spliced peptides with *in silico* predicted H-2K^b binding affinity. These spliced peptides, which displayed sequence similarity with LLO₂₉₆₋₃₀₄ bound to H-2K^b molecules in cellular assays and one of them was recognized by CD8 T-cells of infected mice. This spliced epitope differed by one amino acid from LLO₂₉₆₋₃₀₄ and double staining with LLO₂₉₆₋₃₀₄⁻ and spliced peptide-folded MHC multimers showed that LLO₂₉₆₋₃₀₄ and its spliced variant were recognized by the same CD8 T-cells. Thus, PCPS multiplies the variety of peptides that is processed from an antigen and leads to the production of epitope variants that can be recognized by cross-reacting pathogen-specific CD8 T-cells. Such mechanism may reduce the chances for pathogen immune evasion.

635

Counting T cells and peptides: a quantitative view of immunogenicity and antigen presentation during virus infection

Tscharke, D.¹, Croft, N.², Smith, S.¹, Wong, Y.C.¹, Flesch, I.¹, Pickering, J.¹, Keller, E.¹, Purcell, A.²

¹The Australian National University, John Curtin School of Medical Research, Acton, Australia, ²Monash University, Dept. Biochemistry and Molecular Biology, Clayton, Australia

We are using advanced mass spectrometry methods to detect and quantify the presentation of many native vaccinia virus peptides by MHC class I on multiple mouse cell types, and comparing these data with the magnitude of corresponding CD8⁺ T cell responses. We found that presentation of viral peptides on MHC I during virus infection is complex and dynamic. In general, dendritic cells present peptides at much higher levels than infected target cells, with primary fibroblasts being especially poor. Most (~90%) of viral epitopes are generated from their

source protein co-incident with expression, suggesting that few viral proteins reach their fully mature state before entering the antigen presentation pathway. Strikingly, the abundance of peptides being presented is poorly reflected in the anti-viral CD8+ T cell repertoire. We have now identified many new vaccinia virus peptides presented on MHC I during infection and for the first time have been able to directly determine the fraction of presented viral peptide-MHC complexes that are immunogenic. For vaccinia virus we found that around 2/3 of peptide-MHC complexes (>120) are immunogenic, with around a third of these eliciting robust responses. The identification of these was substantially more efficient than has been possible by the use of algorithms or expression cloning approaches. Together these data demonstrate that there are many surprises waiting in viral immunopeptidomes and showcase the advances that can be made with current cutting edge methodologies.

1886

Endoplasmic reticulum aminopeptidases 1 and 2 are part of the peptide loading complex and trim peptides independent of MHC class I molecules

Urban, S., Schmidt, K., Ebstein, F., Kloetzel, P.-M.

Charite Universitätsmedizin Berlin, Institute of Biochemistry, Berlin, Germany

Generation of high-affinity peptide ligands, which are presented to T cells by major histocompatibility complex (MHC) class I molecules on the cell surface involves the sequential action of different proteolytic enzymes. In many cases, the proteasome system generates such peptide ligands as precursor molecules exhibiting the correct C-terminal anchor residue for MHC class I binding, while having an N-terminal extension. In order to increase the affinity and the amounts of antigenic peptides for optimized T cell recognition, these precursor peptides may be trimmed by endoplasmic reticulum aminopeptidases ERAP1 and ERAP2. Up to now, no experimental evidence has been reported to address the outstanding question of whether ERAP1 and ERAP2 interact with the peptide loading complex (PLC), and it is still unclear whether such trimming occurs with or without prior binding of precursor peptides to MHC class I proteins. Here, we observed that both ERAP1 and ERAP2 are intrinsic components of the PLC and furthermore demonstrate that epitope precursor peptides from human cytomegalovirus (HCMV) pp65 and melanoma antigen MART-1 are not trimmed when they are bound to MHC class I molecules.

1337

Peptides dependent degradation of major histocompatibility complex II via ubiquitination

Kozono, Y.¹, Sasaki, Y.², Ishido, S.³, Kanagawa, O.⁴, Kozono, H.¹

¹Tokyo Univ of Science, Res Inst Biomed Sci, Noda, Japan,

²University of Tokyo, Kashiwa, Japan, ³Showa Pharmaceutical

University, Machida, Japan, ⁴Centre International de Recherche en Infectiologie, Lyon, France

MARCH I is an E3 ubiquitin ligase for MHC II that regulate intracellular degradation and transport. We previously

demonstrated differences of structural fluctuation of the MHC II/peptides that can be the basis of variable conformations that often referred as type A and type B. Here, we hypothesized that ubiquitination of MHC II relies on certain structures that formed with bound peptides. With X-ray single molecule analysis that can monitor the real-time motion of proteins, we found that the MSD curves of MHC II/peptide motions were correlated with the SDS sensitivity of the complexes. Pulse-chase experiment revealed that the half-life of type B complexes in BM-DC of wild type mice were much faster than those in BM-DC of MARCH I -/- mice. In contrast, the half-life of type A complexes in BM-DC of wild type mice were similar to those of MARCH I -/- mice. Furthermore, the abilities of T cell stimulation by type B complexes in both immature and mature BM-DC of wild type mice were much weaker than those in BM-DC of MARCH I -/- mice. In contrast, the abilities of T cell stimulation by type A complexes in both immature and mature BM-DC of wild type mice were similar to those of MARCH I -/- mice. Our results suggest that ubiquitination of MHC II/peptides complex occurs bound peptides dependent manner. MARCH I may distinguish conformational differences produced by the motion of MHC II/peptides complexes.

3068

Stability, conformation and peptide binding of HLA-B*27:05 are all influenced by tapasin

Junghans, V.¹, Rasmussen, M.², Buus, S.², Bowness, P.³, Paulsson, K.¹

¹University of Lund, Experimental Medical Science, Immunology, Antigen Presentation, Lund, Sweden, ²University of Copenhagen, Experimental Immunology, Copenhagen, Denmark, ³Oxford University, Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences, Oxford, United Kingdom

Ankylosing spondylitis (AS) is linked to polymorphism in the super family of HLA-B*27 molecules. Up to date the exact role and mechanism of how HLA-B*27 is involved in AS pathogenesis remain unknown. To improve our understanding of HLA-B*27:05 and in particular un-conventional conformations (suggested as a link to disease) of HLA-B*27:05 we used unique conformation specific antibodies against heterodimers (W6/32), free heavy chains (free HC) (HC10) and homodimers (HD6). HLA-B*27:05 were studied at the cell surface of tapasin-deficient and -proficient cells. Tapasin did not affect the cell surface amount of W6/32 reactive conformations of HLA-B*27:05. However, on the tapasin-deficient cells non-conventional conformations of HLA-B*27:05 were present to a higher extent. Interestingly, the stability of not only HD6-reactive conformations but also HC10 was increased. To elaborate on the role of tapasin and also peptides we used recombinant Tpn₁₋₈₇ added exogenously to cells as well as to recombinant HLA-B*27:05. On the cell surface Tpn₁₋₈₇ modulated levels of HD6 reactive epitopes. Moreover, Tpn₁₋₈₇ facilitated folding of HLA-B*27:05 complexes with seven-mers although HLA-B*27:05 formed the most complexes with peptides of 10-11 amino acids in length and stability analysis suggested longer peptides to form complexes of higher stability than those formed with 8-10 amino acid long peptides. In conclusion our data show that HLA-B*27:05 is influenced by tapasin both regarding peptide binding and conformations,

validated biomarker for diagnosing TB in HIV infected patients. Immunoregulatory enzyme, Indoleamine 2,3 dioxygenase (IDO) is capable of modulating cell mediated immunity. IDO catalyses the breakdown of tryptophan (Trp) metabolites collectively known as kynurenines (Kyn). Elevated IDO activity has been proposed as a prognostic biomarker for TB. We investigated whether IDO activity, as measured by Kyn-to-Trp ratio, using UPLC-MS/MS can act as a biomarker for diagnosing TB in HIV infected patients.

We determined IDO activity in the plasma of 32 HIV patients who developed TB during a longitudinal study and compared with 70 control subjects, age and CD4 cell count matched, in the same HIV infected cohort who did not develop TB.

IDO activity was significantly higher in TB cases than controls at the time of TB diagnosis ($P = 0.0001$). At 6 months prior to TB diagnosis, IDO activity was significantly higher in TB cases than controls ($P = 0.0001$). To evaluate diagnostic significance of IDO activity using a receiver operating characteristic curve, we selected 0.70 as the optimal cut-off. At time of TB diagnosis using confirmed TB as gold standard, IDO activity gave a diagnostic sensitivity of 100% and a specificity of 98.5% with Positive and Negative predictive values of 96.9% and 100% for detecting active TB cases.

Our results, demonstrate the plausibility of increased IDO activity as a biomarker of active TB in HIV positive patients.

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HCV induces a strong increase of inflammatory monocytes, more than HIV itself

Schiavone, F.^{1,2}, Lichtner, M.³, Zuccalà, P.³, Mascia, C.³, Tieghi, T.³, Marocco, R.³, Savinelli, S.³, Vita, S.³, Mengoni, F.³, Vullo, V.³, Mastroianni, C.M.³

¹Sapienza University, Molecular Medicine Dep, Rome, Italy, ²Italian Institute of Technology, CLNS, Rome, Italy, ³Sapienza University, Public Health and Infectious Disease Dep, Rome, Italy

Fibrosis progression in HIV-HCV coinfecting patients (pts) is faster compared to HCV mono-infected pts.

Subpopulations of inflammatory monocytes Mo seemed to be implicated in both HCV and HIV infection and were linked to several age related-disease such as atherosclerosis.

In our single-centre study, we enrolled 56 patients (matched for sex, age and degree of liver fibrosis). We stratified them in four groups: 25 HCV pts, 20 HIV/HCV coinfecting pts (between them 10 pts with active HCV infection (AHI) and 10 with HCV viral clearance (VHC)), 17 HIV pts and 10 healthy donors (HD).

We evaluated mDC, pDC, S1anDC and typical, atypical and intermediate Mo by cytofluorimeter. Liver fibrosis was measured using Fibroscan and Fib-4 score. Kruskal-Wallis ANOVA with Dunn's test was used for statistical analysis.

ANOVA shows a statistically difference in all groups for intermediate Mo ($p=0,001$) and atypical Mo ($p=0,0411$). In particular pts with HCV infection presented an increase of intermediate Mo compared to AHI ($p=0,0001$), to VHC ($p=0,0009$) and to HIV+ ($p=0,0001$). Considering atypical Mo we found an increase in pts with HCV infection compared to HD ($p=0,0163$), to VHC ($p=0,0362$) and HIV+ ($p=0,0266$). However considering all the subpopulation of DCs, there were no differences.

Inflammatory Mo are higher in HCV infected pts and can be a constant source for myeloid cells in the liver, but also in other organs suggesting a role of these cells in the higher cardiovascular risk described in HCV infected subjects. A possible immune recovery will be presented in pts undergoing DAA therapy.

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Pathogenesis of HIV persistence: B-cell immune tolerance specific for the conformational antigenic determinants (BIT-CAD) of native trimeric envelope glycoprotein subunits (TEGS)

Vyas, G.^{1,2}, Bozzini, R.², Aiyer, R.²

¹University of California (UCSF), Laboratory Medicine, San Francisco, United States, ²TheraBiol, Inc., San Francisco, United States

Acute HIV infection is marked by rapid destruction of CD4+ T-cells in the gut-associated lymphoid tissues (GALT). We hypothesize that the quaternary structure of membrane-bound trimeric gp41 interacting with gp120 expresses CAD, which are shared by diverse HIV subtypes; furthermore, since B-cells also express CD4, GALT-destroying HIV establishes BIT-CAD to enable persistent infection.

To test this hypothesis, we co-cultured plasma-derived founder-transmitted HIV-R5 (FTHIV) with human lymphocytes. Supernatant virions were concentrated, and inactivated with beta-cyclodextrin and benzonase. TEGS embodying HIV gp41 and gp120 in their native conformation and devoid of p24/polymerase/DNA/RNA were concentrated 100X for immunizing rabbits. TEGS elicited high titer (30-150,000) antibodies assayed by enzyme Immunoassay (EIA) using FTHIV. While FTHIV and TEGS of either R5 or X4 phenotypes, and a cloned gp41 protein blocked the EIA reactivity of limiting antiserum dilutions, monomeric cloned proteins (gp120, gp140, and gp160) did not. These results suggest that TEGS elicited CAD-specific antibodies not reactive with the linear epitopes (LE) of each of the cloned envelope proteins. Isolation of monoclonal anti-CAD from a phage library of the rabbit's splenocytes is pending.

These preliminary data support our hypothesis that BIT-CAD underlies the pathogenesis of persistent HIV infection, and are consistent with other reports that

- (1) HIV-infected sera have high titer antibodies against multiple LE on gp41/gp120;
- (2) clinical trials of HIVIG and IVIG showed no significant difference;
- (3) mAb derived from single B cells of infected patients do not neutralize their own HIV, and
- (4) the cytoplasmic tail of gp41 is key to protective immunity.

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Analysis of monocyte subsets in HIV-1 infected individuals using or not antiretroviral therapy

Silva de Carvalho, A.É.¹, Morais, C.O.B.¹, Barletto, J.¹, Castro, F.O.F.², Ribeiro, C.B.², Tavares, C.², Silva, J.M.², Silva, L.C.S.², Fonseca, S.G.²

¹Pontifical Catholic University of Goiás, Biomedicine, Goiânia, Brazil, ²Universidade Federal de Goiás, Instituto de Patologia Tropical e Saúde Pública, Goiânia, Brazil

Introduction: Monocytes play important role in HIV-1 infection, since it acts as a reservoir and vehicle of spreading the virus in tissues. Based on the levels of CD14 and CD16 expression, monocytes may be divided into 3 subsets: classical (CD14^{high}CD16⁻), intermediate (CD14^{high}CD16⁺) and non-classical (CD14^{low}CD16⁺).

Objective: To evaluate the monocyte subsets in HIV-1 infected individuals in use or not of Antiretroviral Therapy (ART) and non-infected individuals.

Methods: We analyzed four study groups: individuals not infected with HIV-1 (n=11); HIV-1-infected individuals without the use of ART (n=8); HIV-1-infected individuals using two Reverse Transcriptase Inhibitors Nucleoside (T1) (n=6) and HIV-1-infected individuals using two Reverse Transcriptase Nucleoside Inhibitors associated with one Reverse Transcriptase not Nucleoside Inhibitor (T2) (n=10). Monocyte subsets from peripheral blood were determined by flow cytometry and the statistical analyzes performed with the GraphPad Prism 6 and non-parametric test were applied.

Results: The percentages of CD14^{high}CD16⁺ subsets were lower in T1 (p=0.003) and T2 (p=0.0011) groups compared to non-infected individuals. The percentages of CD14^{low}CD16⁺ subsets were reduced in T1 (p=0.01), T2 (p=0.0004) and in the patients who did not use ART (p=0.001) compared to non-infected individuals. CD14^{high}CD16⁻ subsets showed higher percentages in T1 (p=0.0006) and T2 (p< 0.0001) groups compared to non-infected individuals.

Conclusion: Monocytes are also important targets of ART, contributing to the control of HIV-1 infection, and the type of antiretroviral therapy may influence the expansion of monocyte subsets.

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The uncharacteristic role of T memory stem cells in HIV-1 infection

Flynn, J.K.^{1,2,3}, Cashin, K.^{1,2}, Paukovics, G.², Jakobsen, M.R.⁴, Ostergaard, L.⁴, Churchill, M.J.^{2,5}, Gorry, P.R.^{1,2,5}

¹RMIT University, Melbourne, Australia, ²Burnet Institute, Melbourne, Australia, ³Monash University, Melbourne, Australia, ⁴Aarhus University, Aarhus, Denmark, ⁵University of Melbourne, Melbourne, Australia

The long-lifespan, robust proliferative potential and self-renewal capacity of T memory stem cells (TSCM) has generated much clinical interest. However these traits raise particular concerns for the preservation of the HIV-1 reservoir and viral persistence. Defining the susceptibility of CD4⁺ T-cell subsets to HIV-1 infection is important as treatments are currently being investigated to purge latent HIV-1 from CD4⁺ memory T-cell reservoirs.

We quantified the susceptibility of CD4⁺ T subsets cells, including TSCM, to infection by HIV-1 subtype C (C-HIV) strains from treatment-naïve subjects (MUSH cohort, Jakobsen et al 2013). These subjects progressed from chronic to advanced stages of disease whilst maintaining CCR5-using (R5) viruses or experiencing emergence of dominant CXCR4-using (X4) strains.

This is the first study to demonstrate TSCM were susceptible to infection by R5 and X4 C-HIV viruses (higher for R5 strains p< 0.05). Mutagenesis studies established the V3 region of the HIV-1 envelope as the determinant underlying the preferential targeting of naïve CD4⁺ T-cells by emergent X4 C-HIV variants in this study. In contrast, the tropism of R5 C-HIV viruses for memory CD4⁺ T-cell subsets was maintained during chronic disease progression.

This study provides new insights into the natural history of tropism alterations for CD4⁺ T-cell subsets by C-HIV strains. The maintenance of infection of key HIV-1 viral reservoirs, including central memory, transitional memory and TSCM cells, suggest that although not preferentially targeted by X4 viruses, TSCM and other memory T-cells are likely to be viral reservoirs in subjects with either R5 or X4 C-HIV infection.

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VIH-1 induce a deregulation in B-cell populations through a regulatory B-cell-like phenotype *in vitro*

López-Abente, J.¹, Muñoz-Fernández, M.A.², Correa-Rocha, R.¹, Pion, M.¹

¹'Gregorio Marañón' Health Research Institute (IISGM), Laboratory of Immune-Regulation, Madrid, Spain, ²'Gregorio Marañón' Health Research Institute (IISGM), Laboratory of Molecular Immunobiology, Madrid, Spain

HIV-1 patients usually show general immune system deregulation and hyper-activation. At the humoral immunity level, HIV infection induces hyper-gammaglobulinemia and loss of memory B cells. In our group, we have previously reported that HIV-1 particles caused a direct marked effect on the activation, proliferation and phenotype of B cells.

In the present study, we considered the phenotype and functions of HIV-treated B-cell. B cells were extracted from buffy coat and treated with HIV-1 or different stimuli. Using quantitative PCR, we analyzed the expression of different cytokines in B-cell and, by flow cytometry; we analyzed the phenotype of these cells. Moreover, the function of B cells on CD4⁺ or CD8⁺ T cells were determined after co-culture experiments *in vitro*.

Surprisingly, B cells exposed to HIV showed a higher level of mRNA for IL-10, TGF-β, EB13 or IL-12(p35) *in vitro*. In addition, HIV-treated B cells exposed to lymphocytes were able to reduce the proliferative and the TNFα production capacities of CD4⁺ and CD8⁺ T cells, confirming the immunosuppressive profile of these B cells.

These results may explain the general deregulation of the immune system and the high concentration of IL-10 in plasma observed in HIV patients. These preliminary outcomes are highly promising as a means of understanding the hyper-activation of the immunity in HIV patients and further experiments *ex vivo* are needed to confirm these results.

562**Adipose tissue macrophage dynamics in SIV-infected and ART-treated rhesus macaques**

Fahlberg, M.¹, Walker, E.², Penney, T.², Kuroda, M.², Didier, E.³

¹Tulane University National Primate Research Center, Immunology/Microbiology, Covington, United States, ²Tulane University National Primate Research Center, Immunology, Covington, United States, ³Tulane University National Primate Research Center, Microbiology, Covington, United States

Aging-related diseases including cardiovascular disease, lymphoma, and type 2 diabetes occur at younger ages in HIV/HAART chronically-infected people than in the general population. Reasons for this 'accelerated aging' are unclear. Weight gain and lipodystrophy afflict HAART patients, and conversely, untreated HIV infection causes progressive weight loss, indicating that adipose tissue dysregulation may critically contribute to the increased rate of biological aging and pathogenesis of infection. We investigated mesenteric adipose tissue macrophage (ATM) dynamics after a previous study showed high monocyte turnover predicted progression to AIDS in rhesus macaques. We found that CD163+, CD163+CD206+, and CD206+ ATMs represent approximately 12% of total nucleated cells in adipose of uninfected rhesus macaques. We next examined the relative frequency of ATMs in SIVmac239 uninfected (n=4), infected (n=3), and infected + ART (n=4) macaques and found a reduction of CD163+ ATMs and an increase of CD163+206+ ATMs (37.7% ±21.4 and 56.8% ±9.3) in uninfected macaques compared to infected (69.6% ±13.6 and 25.8% ±12.8) and SIV/ART (68.1% ±12.7 and 25.0% ±8.3). We further differentiated ATMs by CD34, a surface marker found on adipose-derived mesenchymal stem cells, and observed novel expression of CD34 on CD163+ ATMs of uninfected macaques (43.3% ±13.0), and this proportion increased significantly after SIV infection (61.7% ±7.2). Furthermore, evidence of recent cell division (via BrdU uptake) was found in CD34+ ATMs at 24 hours post-injection and CD34- ATMs at 48 hours post-injection. Ongoing work is investigating the origins, infiltration, and proliferation of ATMs after infection and antiretroviral treatment.

563**Partial efficacy of broadly neutralizing antibody PGT121 against cell-associated simian-human immunodeficiency virus (SHIV) infection**

Parsons, M.¹, Lloyd, S.¹, Center, R.², Amarasena, T.¹, Swiderek, K.³, Kent, S.¹

¹University of Melbourne, Melbourne, Australia, ²Burnet Institute, Melbourne, Australia, ³Theraclone Sciences, Seattle, United States

Background: Prophylactic vaccines are required to alleviate HIV-1 spread. Broadly neutralizing antibodies (BnAbs), capable of neutralizing arrays of viral isolates, are promising prophylactics. Although BnAbs protect macaques from challenge with cell-free SHIV, their efficacy against cell-associated virus (CAV) remains unclear. While some in vitro studies have raised concerns about BnAbs being less potent against CAV, several BnAbs equally capable of neutralizing cell-free and CAV in vitro have been identified. Amongst these antibodies is PGT121, which

recognizes an epitope including the third variable loop of HIV-1 envelope and surrounding glycans. As HIV-1 preventatives will likely need to eliminate both cell-free virus and CAV, we assessed if passively transferred PGT121 protected macaques from cell-associated SHIV challenge.

Methods: Twelve macaques, six intravenously infused with PGT121 (1mg/kg) and six intravenously infused with isotype control (1mg/kg)/no antibody, were challenged intravenously, one hour following antibody infusion, with 24.5x10⁶ splenocytes from a SHIVSF162P3-infected animal. Animals were followed up for viremia onset.

Results: All six isotype control/no antibody animals developed viremia one week post-challenge. Three of six PGT121-infused animals were completely protected. Two PGT121-infused animals developed peak viremia two weeks post-challenge. Interestingly, these two animals exhibited low plasma PGT121 levels one week post-infusion. The remaining PGT121-infused animal exhibited a seven week delay prior to developing viremia.

Conclusions: These data provide evidence that BnAbs can protect against CAV. The partial nature of the observed protection relates to early suboptimal plasma concentrations of PGT121 and/or long-term persistence of cells harboring virus until waning of therapeutic antibody to suboptimal concentrations.

564**Impact of RNA export TREX-2 component GANP on nuclear transport in viral infection**

Shimoda, M.^{1,2,3}, Maeda, K.^{1,2}, Singh, S.K.², Sakaguchi, N.⁴, Akira, S.^{1,2}

¹Osaka University, Research Institute for Microbial Diseases, Department of Host Defense, Suita, Japan, ²Osaka University, WPI Immunology Frontier Research Center, Department of Host Defense, Suita, Japan, ³Kumamoto University, Department of Immunology, Kumamoto, Japan, ⁴Osaka University, WPI Immunology Frontier Research Center, Suita, Japan

HIV-1 disease is caused by infection with HIV-1 viruses. At the primary step, reverse-transcribed HIV-1 genome is inserted into the host genome by mediating the activity of viral integrase (IN) protein. To encounter the integration sites of host genome, the viral components must be passing through the nuclear pores on nuclear envelope (NE). Nuclear pores are among the largest complex, named as nuclear pore complexes (NPCs). HIV-1 IN forms the preintegration complexes (PICs) with host factors for nuclear import through the NPCs. Mammalian transcription/export-2 (TREX-2) complex is assembled with ribonucleoproteins and involved in RNA transport. We have reported that the TREX-2 component GANP is involved in targeting of AID/APOBEC3 cytidine deaminases in immune cells. Since GANP is localized with NPC at the NE, we examined whether GANP was involved in the maintenance of NE function during the HIV-1 infection. We found that HIV-1 infection dispersed GANP from NE to the cytoplasm and GANP physically interacts with IN through the PIC-independent mechanism during the entry of viral genome into the host chromatin. These results demonstrate that GANP is a unique factor and is interacting with the HIV-1 genome and IN, which might play an important role at the integration process in the viral infection.

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HIV induces interferon stimulated genes in two phases in macrophages while inhibiting interferon induction

Nasr, N.¹, Alshehri, A.A.¹, Wright, T.K.¹, Shahid, M.¹, Heiner, B.M.¹, Harman, A.N.¹, Botting, R.A.¹, Helbig, K.², Kelleher, A.D.³, Suzuki, K.³, Beard, M.², Hertzog, P.⁴, Cunningham, A.L.¹

¹The Westmead Institute for Medical Research, University of Sydney, Centre for Virus Reserach, Westmead, Australia, ²School of Molecular and Biomedical Science, The University of Adelaide, Adelaide, Australia, ³The Kirby Institute, The University of New South Wales, NSW, Australia, ⁴Hudson Institute of Medical Research and Monash University, Clayton, Australia

We have previously shown that HIV induces a subset of Interferon (IFN) stimulated genes (ISG) in monocyte-derived macrophages (MDMs) and dendritic cells while inhibiting type I interferons. Here we show that the induction of the ISGs viperin, IFITs 1-3 occurs in two phases: the first phase is minor and transient occurring early after infection (2-24 hpi) while the second major phase is persistent and induced late (>48hpi). First phase ISG induction was due to the presence of shed extracellular vesicles (EV) in the HIV-1 inocula. Treatment of MDMs with HIV containing EVs induced ISG via IRF1 (as shown by gene knockdown). Similar levels of ISGs were induced upon treatment of MDMs with either isolated EVs or their contained key proteins, Hsp90α, β, or Nef. The second phase was dependent on newly transcribed viral mRNA as shown by knockdown of MAVS, Tat and by transcriptional gene silencing of HIV-1 with shRNA targeting the NF-κB binding site in the HIV-1 promoter. IRF1 and IRF7 bind to motifs in the promotor regions of the ISGs and here we show that their knockdown also significantly decreased second phase ISG induction. Individual knockdown of the IFITs 1, 2 and 3 reduced their antiviral activity against HIV consistent with their activity as a complex. Elucidating the mechanisms of ISG induction and their roles in HIV infection may contribute to immunotherapeutic strategies to limit the size of HIV reservoirs in macrophages.

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Association of FcγRIIIb genotypic variants with markers of disease severity in HIV-1 infected women

Lassauniere, R.^{1,2}, Gray, G.E.³, Louise, K.⁴, Tiemessen, C.T.^{1,2}

¹National Institute for Communicable Diseases, Centre for HIV and STI's, Johannesburg, South Africa, ²University of the Witwatersrand, Faculty of Health Sciences, Johannesburg, South Africa, ³Perinatal HIV Research Unit, Chris Hani Baragwanath Hospital, Soweto, South Africa, ⁴Gertrude H. Sergievsky Centre, College of Physicians and Surgeons, and Department of Epidemiology, Mailman School of Public Health, Columbia University, New York, United States

Cellular receptors for the Fc portion of immunoglobulin G (IgG) - so called Fc gamma receptors (FcγR) - mediate several key immune effector functions that include antibody-dependent cellular cytotoxicity, phagocytosis, respiratory burst, and release of pro-inflammatory mediators. The magnitude of FcγR-mediated immune responses is affected by genotypic variants that alter the receptor's binding affinity for IgG (FcγRIIIa-H131R and FcγRIIIa-F158V), subcellular localization (FcγRIIIb-I232T),

post-translational modification (FcγRIIIb-HNA1a/b/c), and surface density (gene copy number variability). In a cross-sectional study, we indirectly assessed a role for FcγR-mediated effector functions in modulating HIV-1 disease severity using FcγR variants as a proxy for functional capability. We genotyped 282 HIV-1 infected, treatment-naïve South African Black women for all known functional FcγR variants and compared markers of HIV-1 disease severity - plasma viral load and CD4⁺ T cell count - across different genotypes. The FcγRIIIb-HNA1b|1b genotype (confers reduced neutrophil-mediated effector functions) significantly associated with lower viral loads compared to the HNA1a|1a and HNA1b|1c genotypes (P=0.038 and P=0.009, respectively) and higher CD4⁺ T cell counts compared to the HNA1a|1a genotype (P=0.017) and all heterozygous combinations (P< 0.05 for all comparisons). In addition, possession of more than two *FCGR3B* gene copies (confers enhanced neutrophil-mediated effector functions) associated with higher viral loads (P=0.015), while less than two *FCGR3B* gene copies associated with higher CD4⁺ T cell counts (P=0.042). In conclusion, this study describes a novel association for FcγRIIIb allotypes, and by inference a potential role for neutrophils, in modifying HIV-1 disease severity.

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Effect of illicit drug use on inflammation and microbial translocation in HIV infection

Castro, F.O.F.¹, Ribeiro, C.B.¹, Tavares, C.¹, Silva, J.M.¹, Sousa, J.B.², Silva, L.C.S.¹, Pfrimer, I.A.H.², Guilarde, A.¹, Fonseca, S.G.¹

¹Universidade Federal de Goiás, Instituto de Patologia Tropical e Saúde Pública, Goiania, Brazil, ²Departamento de Biomedicina da Pontifícia Universidade Católica de Goiás, Goiania, Brazil

Introduction: The human immunodeficiency virus (HIV) infection causes a rupture in the intestinal mucosa leading to microbial translocation and hyperactivation of the immune system and progression to AIDS. The use of illicit drugs has been shown to alter the immune system function.

Objective: To evaluate the impact of drug use in inflammation and microbial translocation of HIV-infected individuals. The study population consisted of 43 HIV-1-infected individuals including: 18 marijuana-users, 6 cocaine-users and 9 marijuana+cocaine users; and 16 healthy-donors no drug users. All HIV-infected individuals were under antiretroviral therapy.

Methods: TNF-α, IL6, IL10 and soluble CD14 (sCD14) were measured in the plasma samples using Elisa Kit. C-reactive protein was measured in the serum using turbidimetry.

Results: TNF-α plasma levels were higher in HIV-marijuana/cocaine users (p=0,0137) and in HIV-cocaine users (p=0,0029) compared to HIV no-drug-users. IL-6 plasma levels were lower in HIV-marijuana users compared to HIV-no-drug-users (p=0.0087). In respect of IL-10 no differences were found between the groups. CRP levels were higher in HIV-marijuana/cocaine users compared to HIV no-drug-users (p< 0.05). sCD14 levels showed higher levels in HIV-no-drug-users than healthy donors (p< 0,0164), and comparing to HIV-no-drug-users the sCD14 levels were higher in HIV-marijuana users (p< 0,0128), HIV-cocaine users (p< 0,0494), HIV-cocaine/marijuana users (p< 0,0375).

Conclusion: The concomitant use of cocaine and marijuana accelerates the inflammatory responses, increasing the inflammatory cytokine production and microbial translocation in HIV infected individuals and it may lead to faster disease progression.

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Role of complement in progression of HIV-associated neurological disorders (HAND)

Mahajan, S.D., Aalinkeel, R., Abou-Jaoude, E., Parikh, N.U., Reynolds, J.L., Jacob, A., Schwartz, S.A., Quigg, R.J., Alexander, J.J. State University of New York at Buffalo, Medicine, Buffalo, United States

Microglia are believed to be latently infected with HIV-1 and represent a CNS viral reservoir. Microglial activation and latent viral activation contributes to the development of HIV-associated neurological disorders (HAND). The complement system plays diverse roles in the pathogenesis of HIV-1 infection by playing a critical role in clearing HIV-1 and also promoting productive HIV-1 replication. We hypothesize that complement proteins may play a role in the activation of latent virus in the microglia and that complement activation enhances HIV-1 infectivity, contributing to the progression of HAND. We used untransfected (CHME-5) and HIV transfected (CHME-5/HIV) primary human microglial cell lines and evaluated the expression levels of several complement proteins/receptors such as C9, C5aR, C3aR, C5L2 by immunofluorescence and real time QPCR. Our results show significant increase in the expression levels of C9, C5aR and C5L2 in CHME-5/HIV cells as compared to the CHME-5 cells. Additionally, since, complement can be activated through HIV-1 envelope proteins, we treated CHME-5 with HIV-1 Tat (100ng), and observed significant transcriptional upregulation of C5aR (64%; $p < 0.01$), C5L2 (87%; $p < 0.001$) and NF κ B (55%; $p < 0.05$), as compared to the untreated control ($n=3$), indicating that Tat induced complement activation is accompanied by NF- κ B activation. A balance between complement activation and complement regulations may contribute to HIV-1 latency and persistence and the development of HAND, thus, therapeutic approaches targeting the complement system for the treatment of HAND need to be explored.

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Isolation of resting CD4+ T cells from a model system of HIV-infected blood

de Jong, S., MacDonald, N., Morin, P., Kokaji, A., Thomas, T.E. STEMCELL Technologies Inc., Research and Development, Vancouver, Canada

Resting CD4⁺ T cells are of major interest in the study of human immunodeficiency virus type 1 (HIV-1) infection. Although infected cells are largely non-permissive for HIV-1 replication integration of the viral genome into the cell's DNA generates a latent reservoir of potentially infectious cells. Latently infected resting CD4⁺ T cells are rare, can escape the viral immune response and can persist for long periods of time

despite successful antiretroviral therapy. The latent reservoir is recognized as a major barrier to curing HIV-1 infection since given an appropriate stimulus or an interruption of therapy, latently infected cells can reactivate and produce infectious virions.

Currently, complex multi-step immunomagnetic isolations and or flow cytometry-based cell sorting is required to obtain pure resting CD4⁺ T cells. We have recently developed an EasySep™ immunomagnetic column-free method for fast and easy isolation of untouched resting CD4⁺ T cells from human PBMCs. Moreover, the procedure can be automated using RoboSep™, minimizing sample handling and eliminating cross-contamination.

Resting CD4⁺ T cells (defined as CD3⁺CD8⁻CD25⁻CD69⁻HLA-DR⁻) were isolated either from healthy donor PBMCs or from a model system of HIV infection in which in vitro activated CD4⁺ T cells had been spiked into healthy donor PBMCs. Starting with an average resting CD4⁺ T cell content of 21.5±8.6% in healthy donors and 13.5±5.9% in the model system, we obtained average purities of 97.1±1.9% ($n=23$) and 90.0±5.3% ($n=21$), respectively. The purified resting CD4⁺ T cells are suitable for immediate use in downstream assays.

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Investigations on the extra-neural cholinergic system in HIV infection

Ahmad, A.¹, Aldbah, Z.¹, Samarani, S.¹, Allam, O.¹, Routy, J.-P.², Tremblay, C.³

¹CHU Saint Justine, Montreal, Canada, ²McGill University, Montreal, Canada, ³Montreal University, Montreal, Canada

The extra-neural cholinergic system (ENCS) is present in humans as well as in animals. The main effector molecule of the system, Acetylcholine (ACh), is an evolutionarily conserved neurotransmitter. It is also produced abundantly by non-neural cells. Both CD4⁺ T cells and gastro-intestinal tract epithelial cells constitute the main source of this molecule. ACh exerts its effects via Ach receptors (AChR) of nicotinic (n) and adrenergic (a) types, which are expressed abundantly in immune cells in the body. The ENCS modulates differentiation, proliferation and functioning of several types of immune cells. Presently, very little is known about the functioning of the ENCS in HIV infection. We show here for the first time an increase in the concentrations of ACh in the circulation of HIV-infected individuals, whereas anti-retroviral therapy (ART) tends to restore them to their physiological levels. We also found that the expression of the α -7nAChR tended to decrease on CD3⁺CD4⁺ T cells in the virus-infected individuals. Furthermore, the concentrations of SLURP-1 (Secreted Ly6/Urokinase type plasminogen activator Receptor-related Peptide-1), an allosteric ligand for the α -7nAChR, was also increased in the circulation of HIV-infected individuals, and ART also tended to normalize them. Furthermore, a synthetic α -7nAChR agonist decreased and a synthetic antagonist of the receptor increased replication of a T-tropic HIV strain in human IL-2 and PHA-activated blasts. Our results suggest that ACh mimetics and/or α -7nAChR agonists that do not cross blood brain barrier may be beneficial in attenuating aberrantly activated immune system in HIV-infected individuals.

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NKG2D acts as a co-receptor for natural killer cell-mediated anti-HIV-1 antibody-dependent cellular cytotoxicityParsons, M.S.¹, Richard, J.², Lee, W.S.¹, Vandervan, H.¹, Grant, M.³, Finzi, A.², Kent, S.J.¹¹University of Melbourne, Department of Microbiology and Immunology, Melbourne, Australia, ²Centre de Recherche du CHUM, Université de Montréal, Department of Microbiology, Infectiology and Immunology, Montreal, Canada, ³Memorial University of Newfoundland, Division of BioMedical Sciences, St. John's, Canada

There is growing interest in utilising natural killer (NK) cell-mediated antibody-dependent cellular cytotoxicity (ADCC) to protect against HIV-1 acquisition or to eliminate infected cells following latency reversal. While the predominant NK cell receptor responsible for mediating ADCC is CD16 (FcγRIIIa), there are a plethora of other activating and inhibitory receptors that can influence NK cell responses upon encountering antibody-coated target cells. Indeed, it has been shown that ligation of certain activating receptors, including NKG2D, can synergise with CD16 ligation to enhance NK cell calcium flux. Since NKG2D expression levels on NK cells have been linked to HIV-1 disease progression and HIV-1 accessory proteins regulate the expression of NKG2D ligands on HIV-1-infected cells, we examined whether NKG2D signalling was involved in anti-HIV-1 ADCC. We utilised the LDH-release cytotoxicity assay and a flow cytometry-based infected cell elimination assay to assess ADCC against a CD4⁺ T cell line either pulsed with HIV-1 envelope glycoprotein (gp120) or infected with HIV-1. We demonstrate that antibody-mediated blockade of NKG2D on NK cells results in reduced antibody-dependent killing against gp120-pulsed target cells [median: 29.2% (Range: 9.7-55.3%)] as compared to conditions containing an isotype control, [39.7% (22.3-65.1%), $p=0.014$]. The blockade of NKG2D also results in diminished ADCC against cells infected with Nef- and Vpu-deficient HIV-1 [14.1% (9.3-19.8%)] as compared to conditions containing an isotype control [21.3% (20.9-27.1%)]. These observations are highly important for understanding antibody-dependent NK cell responses against HIV-1 and might be helpful in improving the efficacy of antibody-based vaccines and/or therapeutics.

Immunity to Bacteria & Fungi

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Impact of *E. coli* Nissle 1917, *Lactobacillus amylovorus* and *Salmonella* Typhimurium on intestinal IL-8, IL-12/23 p40, TNF alpha, claudin-1 and occludin in gnotobiotic piglets

Splichalova, A., Splichal, I., Slavikova, V.

Institute of Microbiology of the Czech Academy of Sciences, v.v.i., Novy Hradek, Czech Republic

Objectives: A lower part of the gastrointestinal tract (GIT) harbors a majority of body microbiota. Protective effects of *E. coli* Nissle (EcN) and *Lactobacillus amylovorus* (LA) against infection with *Salmonella* Typhimurium (ST) in gnotobiotic piglets were evaluated.

Methods: Colostrum-deprived germ-free piglets (GF) were colonized with EcN or LA four hours after hysterectomy or bred as GF piglets. One week later they were infected with ST for 24 hours. Bacterial colonization of the intestine (CFU counting), secretion of IL-8, IL-12/23 p40 and TNF alpha (xMAP technology) and transcriptions of claudin-1 and occludin (RT-qPCR) and their protein expression (IF) were monitored.

Results: The infection with ST caused bacteremia, somnolence, anorexia, and diarrhea in the piglets and high levels of IL-8, IL-12/23 p40 and TNF alpha. Their levels in the piglets preliminary associated with EcN were almost completely suppressed but not in the case of preliminary associated with LA. The transcription of claudin-1 in GF, EcN and LA piglet groups in colon were comparable but in ST group highly increased. In contrast, the transcription of occludin was depressed in ST group.

Conclusions: The preliminary association of GF piglets with EcN suppressed symptoms of *Salmonella*-induced gastroenteritis and high levels of the inflammatory mediators but LA suppress them partially. Surprisingly the transcriptions of claudin-1 and occludin in *Salmonella*-infected piglets showed quite opposite trends.

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Prevention of actinomycetoma by *Nocardia brasiliensis* in BALB/c mice

Vazquez Marmolejo, A.V., Lopez Ulloa, J.A., Longoria Lozano, O., Zavala Ramirez, J.M., Perez Rivera, L.I., Salinas Carmona, M.C. Universidad Autonoma de Nuevo Leon, Inmunologia, Monterrey, Mexico

Introduction: *Nocardia brasiliensis* is the main etiological agent of actinomycetoma in Mexico. Microabscesses and chronic granuloma are the hallmark of this disease. An animal model of actinomycetoma by *Nocardia brasiliensis* in BALB/c mice has proved helpful for understanding pathogenesis, but it is still not fully understood. IgM but no IgG antibodies protect against infection. Inhibition of nitric oxide synthase (iNOS) function by aminoguanidine hemisulfate (AG) inhibits the capacity of macrophages to eliminate bacteria in vivo and in

vitro, but sustained production of NO can augment the adverse on inflammation of the host.

Objective: To study the effect of pharmacological inhibition of iNOS on the onset and progression of *Nocardia brasiliensis* actinomycetoma.

Methodology: BALB/c mice received a 2% AG solution ad libitum one week before inoculation with either *Nocardia brasiliensis* (1 X106 CFU) or saline in the left footpad. BALB/c mice drinking pathogen-free water were used as controls. The mice were observed for 97 days after infection and sacrificed. Blood, spleen and kidney bacterial burdens were quantitated. Sera were assessed for anti-*Nocardia brasiliensis* IgG antibodies. We determined macrophage phagocytosis in vitro and NO levels were measured in plasma and cell culture supernatants.

Results: AG-treated mice did not develop actinomycetoma. No bacterial burden was found in AG-treated mice. Antibody titers were similar in all infected mice. Macrophages from AG-treated mice cleared less bacterial than those of controls after in vitro infection. NO production was significantly less in AG treated mice.

Conclusions: AG- treatment prevents actinomycetoma by *Nocardia brasiliensis* in BALB/c mice.

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Pre-treatment of in vivo novel ligand molecule generated from a *Staphylococcus aureus* mutant bacterium protected host from MRSA infection

Lee, B., Lee, M.-J., Khan, A.

Pusan National University, College of Pharmacy, Busan, Korea, Republic of

Innate immunity is a first line defense reaction against bacterial infections and links to the activation of host adaptive immunity. To protect hosts from infection of pathogenic bacteria, hosts induce innate immune responses, such as cellular and humoral innate immunity. *Staphylococcus aureus* is a commensal bacterium that induces several diseases in clinic. But, healthy people are almost protected from methicillin-resistant *S. aureus* (MRSA) infection, suggesting that hosts' innate immune cells may produce an unidentified ligand molecule(s) that activates host innate immunity to protect host from bacterial infection. To identify a novel ligand molecule(s) derived from *S. aureus* cells, we screened 10 different *S. aureus* mutants to screen which mutant strain can activate host innate immunity. As results, one *S. aureus* mutant strain produced gamma delta T cell-mediated IL-17A at the early-staged infection, indicating that this mutant is capable of producing unidentified ligand molecule(s) *in vivo* conditions. To purify this molecule(s), large amounts of peritoneal fluids were collected after injection of acetone-treated *S. aureus* mutant bacteria into mice peritoneal cavity. After lyophilization of collected fluid, an active fraction was purified to homogeneity by performing of column chromatographies. When biological activities of the purified fractions were addressed and examined the protection effects from USA300 sMRSA strain infection, purified fraction induced neutrophil-mediated phagocytosis, a typical cellular innate response. Furthermore, pre-treatment of purified fraction protected host from MRSA-infection. These results suggest

that the host defense molecule(s) is invaluable molecule(s) that elicits host's innate immunity, leading to protect host from pathogenic microbe infection.

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A novel proteomic approach for investigating the human antibody response during *Mycobacterium tuberculosis* infection

Hermann, C., Smith, M., Blackburn, J.M.

University of Cape Town, Institute for Infectious Diseases and Molecular Medicine, Department of Integrative Biomedical Sciences, Cape Town, South Africa

Tuberculosis (TB) remains one of the three major killers among the world's infectious diseases. Despite major advances in vaccine research and our understanding of the human immune system, the rules of immune protection against TB remain unclear. Recently, more emphasis has been placed on investigating the antibody response to *Mycobacterium tuberculosis* (M.tb) antigens during infection using mainly microarray technology. However, this technology comes at its own cost and limitations and might therefore be less available to researchers. Here, we report a novel approach for assessing antigens of serum antibodies from TB patients using state-of-the-art proteomics.

In a proof-of-concept study, the human antibody response to M.tb antigens was investigated in patients with active TB and healthy controls. M.tb antigens were isolated from bacterial lysates using purified serum antibodies from patients and healthy controls. The identities of isolated antigens were determined by tryptic digestion, measurement using a Q Exactive Orbitrap mass spectrometer and MaxQuant analysis. Unique antigens were detected in TB patients or healthy controls - TB patients showing more reactivity to Esx proteins and virulence factors. Furthermore, cross-reactivity was observed to M.tb antigens by antibodies in healthy controls. These findings reiterate observations made in recent microarray studies, but also add novel antigens detected in TB patients for the first time.

Thus, this approach might prove an important alternative to microarray technology in the investigation of the antibody response to M.tb infection. Furthermore, this technology might broaden available targets for vaccination and facilitate the future development of an effective TB vaccine.

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Group A streptococcus induced less P65 nuclear translocation and non-classical NF- κ B activation in macrophage, which possibly leads to a weaker inflammatory response

Wu, S.¹, Ma, C.¹, Gao, X.¹, Zhang, L.¹, Miao, Q.¹, Li, W.¹, Song, X.¹, Wang, X.¹, Liu, J.², Wei, L.¹

¹School of Basic Medicine, Hebei Medical University, Department of Immunology, Shijiazhuang, China, ²Saint Louis University School of Medicine, Division of Infectious Diseases, Allergy and Immunology, Departments of Internal Medicine, Molecular Microbiology and Immunology, St. Louis, United States

Objectives: To explore the pathogenic mechanism of Group A streptococci (GAS) and How GAS evades phagocytosis of immune cells?

Methods: We detected the classical inflammatory signal pathway of macrophages infected with GAS by protein microarray, real-time PCR, Western blot, immunoprecipitation, and flow cytometry.

Results: GAS induced a lower level of inflammatory mediators in macrophages than either G+ *Staphylococcus aureus* (SA) or G- *Escherichia coli* (E.coli). Therefore, we checked the conventional inflammatory signal pathway, and found that GAS and SA induced both TLR2 and TLR4 expression, while G- E.coli solely activated TLR4 in RAW264.7 cells. Though MyD88, the main adaptor protein, was activated by the three pathogens, there was no difference in MyD88 expression in macrophages. NF- κ B is the classical transcription factor of inflammatory signals, and our results showed that GAS, similar to E.coli, induced a weaker p65 nuclear translocation compared to SA. Interestingly, GAS activated NF- κ B by inducing p65:p52 heterodimer but not the classical heterodimer of NF- κ B (p65:p50), while E.coli activated NF- κ B by inducing both p65:p50 and p65:p52 heterodimers.

Conclusions: Compared to SA and E.coli infection, GAS induced a weaker nuclear translocation and distinct combination of NF- κ B subunits in macrophages that probably lead to a weak inflammatory response.

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The difference of biological activity of various soluble beta-glucans in cytokine induction

Yamanaka, D., Motoi, M., Ishibashi, K., Adachi, Y., Ohno, N.
Tokyo University of Pharmacy and Life Sciences, Laboratory for Immunopharmacology of Microbial Products, Tokyo, Japan

Beta-glucan is one of the main antigen synthesized by fungi. In the case of 1,3-beta-glucan, dectin-1 plays an important role in mammalian immune systems. On the other hand, several pathogenic fungi such as *Candida albicans* produce not only 1,3-beta-glucan but also long chain of 1,6-beta-glucan. The biological roles of 1,6-beta-glucan in immune systems have not been clarified yet. The aim of this work was to study the reactivity of leukocytes to 1,6-glycosidic chain rich beta-glucans. We first prepared soluble 1,6-/1,3-beta-glucan (AgCAS and ABG) from edible mushroom, *Agaricus brasiliensis*. Soluble fraction of Pustulan (Calbiochem) from *Lasallia pustulata* was also used as a standard for 1,6-beta-glucan. Splenocytes were isolated from C57BL/6 mice and then exposed to various soluble beta-glucans such as SPG (long 1,3-/1,6-beta-glucan), Laminarin (short 1,3-/1,6-beta-glucan), AgCAS and Pustulan in the presence or absence of exogenous GM-CSF. After incubation, the concentrations of IFN- γ were determined by ELISA. Although strong response to SPG and Laminarin was not observed, AgCAS and Pustulan could induce cytokine release in the presence of GM-CSF. We next examined that the influence of direct cell-cell contact or IL-12 on beta-glucan-induced cytokine synthesis. In spite of no change in TNF- α release, IFN- γ production by AgCAS and ABG was significantly suppressed by anti-MHC class II or anti-IL-12p40 antibodies. In conclusion, *Agaricus*-derived beta-glucans and Pustulan has a higher

potential than SPG and Laminarin on cytokine induction in mice and its biological activity was regulated, at least in part, by a IL-12 mediated-pathway and direct cell-cell interactions.

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Improving anti-bacterial immune responses in patients with COPD by blocking inhibitory T-cell receptors

Tan, D.B.A.^{1,2}, Teo, T.-H.¹, Setiawan, A.M.¹, Kirkham, L.-A.³, Moodley, Y.^{1,4,5}
¹University of Western Australia, School of Medicine and Pharmacology, Perth, Australia, ²Institute of Respiratory Health, Perth, Australia, ³University of Western Australia, School of Pediatrics and Child Health, Perth, Australia, ⁴Institute of Respiratory Health, Stem Cell Unit, Perth, Australia, ⁵Fiona Stanley Hospital, Department of Respiratory Medicine, Perth, Australia

Chronic Obstructive Pulmonary Disease (COPD) is the most common respiratory cause of mortality and morbidity worldwide. Notably, acute exacerbations of COPD (AECOPD) accelerates the decline in lung function. AECOPD is usually associated with pulmonary infections but little is known about the mechanisms causing increased susceptibility in COPD. We hypothesize that chronic inflammation in AECOPD patients induces excess T-cell inhibitory molecules (e.g. CTLA-4) that then inhibit anti-bacterial immune responses in COPD patients. Peripheral blood mononuclear cells (PBMC) were isolated from AECOPD patients, stable COPD patients and healthy controls. Production of IFN γ by PBMC was measured by ELISA post-challenge with viable non-typeable *Haemophilus influenza* (NTHI) or anti-CD3. Bacterial killing was assessed by investigating bacterial viability counts. T-cell expression of CTLA-4 was measured by flow cytometry. Plasma levels of inflammatory biomarkers were measured by ELISA. AECOPD patients exhibited higher plasma levels of CRP and IL-6 than stable COPD patients ($p=0.07$ and $p=0.09$ respectively) and healthy controls ($p < 0.01$ for both). PBMC from AECOPD patients have lower production of IFN γ against NTHI compared to stable COPD patients and healthy controls, but increased expression of CTLA-4 on CD4+ T-cells ($p < 0.05$). Blocking of CTLA-4 increased anti-CD3 induced IFN γ by PBMC from COPD and AECOPD patients. However in response to NTHI challenge, IFN γ responses and bacterial killing were only improved in a subset of patients after CTLA-4 blocking. Increased expression of CTLA-4 could account for the increased frequencies of infections in COPD patients. Blocking multiple anti-inflammatory signals could improve anti-bacterial responses to prevent AECOPD.

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Toll-like receptor 8 polymorphisms in *Helicobacter pylori* infection

Ferrand, J.¹, Hold, G.L.², Ferrero, R.L.¹, El-Omar, E.M.², Gantier, M.¹
¹Hudson Institute of Medical Research, Centre for Innate Immunity and Infectious Diseases, Clayton, Australia, ²University of Aberdeen, Division of Applied Medicine School of Medicine and Dentistry, Aberdeen, United Kingdom

Helicobacter pylori-driven chronic inflammation is strongly associated with gastric cancer. Polymorphisms in host

immune sensors have previously been associated with gastric cancer development in patients infected by *H. pylori*, with the example of the DNA sensor Toll-like receptor (TLR) 9. This study aimed to define whether polymorphisms in the RNA sensor TLR8 were also risk factors in *Helicobacter pylori*-driven chronic inflammation and gastric cancer development. Two TLR8 polymorphisms, rs3764879 and rs3764880, previously associated with disease protection against other pathogens, were analysed in a Scottish cohort of Caucasian patients: (i) with *H. pylori* infection, hypochlorhydria and gastric atrophy (n=64); (ii) with *H. pylori* infection but no precancerous lesions (n=46); and (iii) with no evidence of *H. pylori* infection or lesions (n=56). No significant differences were observed between infected patients with or without precancerous abnormalities. However, analyses of infected versus non-infected patients indicated that the A alleles of TLR8 rs3764880 and the C alleles of TLR8 rs3764879 could be associated with increased risk of infection. This hypothesis was refuted in a larger cohort of 253 infected and 187 control German Caucasian patients, where no association between TLR8 rs3764880 and *H. pylori* infection status reached significance.

Collectively these results indicate that the TLR8 gene is not a susceptibility factor in *H. pylori* infection or *H. pylori*-driven cancer development. Nonetheless, it remains a possibility that TLR8 polymorphisms regulate some aspect of the inflammatory response to *H. pylori* infection, which could not be assessed in the cohorts studied.

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The cleaner fish lumpfish (*Cyclopterus lumpus* L.) - immunity, diseases and vaccination

Rønneseth, A.¹, Eggestøl, H.Ø.¹, Lunde, H.S.¹, Colquhoun, D.J.², Wergeland, H.I.¹, Haugland, G.T.¹

¹University of Bergen, Bergen, Norway, ²Norwegian Veterinary Institute, Oslo, Norway

Lumpfish (*Cyclopterus lumpus* L.) are now farmed and increasingly used as cleaner fish in the fight against salmon louse infestation in farmed Atlantic salmon. This biological treatment is done to overcome the increasingly extensive resistance to chemotherapeutics in salmon lice. To improve the health of the farmed lumpfish (10 mill produced in 2015 in Norway) and increase the overall efficiency of the cleaner fish strategy, vaccines against bacterial pathogens of lumpfish have been introduced, but require further development. Salmon vaccines, particularly against bacterial diseases are very successful and development of vaccines for lumpfish is based on a similar strategy. The elucidation of lumpfish immune responses and characterization of relevant bacterial pathogens are crucial for development of efficient vaccines. We have performed RNA sequencing to obtain a global transcriptional profile of the immunological responses of lumpfish upon bacterial challenge. This is the first presentation of transcriptomic data from lumpfish. We will present an updated overview of lumpfish pathogens, diseases and adaptive immune responses upon bacterial challenge and vaccination.

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Salmonella Typhimurium *rfaG* mutant shows reduced transcription of Toll-like receptor 4 in intestine of gnotobiotic piglets

Splichal, I., Splichalova, A., Slavikova, V.

Institute of Microbiology of the Czech Academy of Sciences, v.v.i., Novy Hradek, Czech Republic

Objectives: A lipopolysaccharide (LPS) is a virulence factor of Gram-negative bacteria. Shortening of its chain modifies type of LPS and decreases bacterial virulence. *Salmonella* Typhimurium (ST) is enteric pathogen that causes self-limiting gastroenteritis in the human and the pig.

Methods: One-week-old hysterectomy-derived germ-free piglets (GF) were infected with *S. Typhimurium* (ST) or its isogenic *rfaG* mutant for 24 hours. *Salmonella* CFU counting, TLR4, MyD88 and TRIF transcriptions in the ileum were evaluated by RT-qPCR, IL-8, TNF-alpha and HMGB1 proteins by ELISA.

Results: ST and its *rfaG* mutant comparably colonized the ileum of the gnotobiotic piglets. While TLR4 transcription was increased in ST group, the transcriptions in *rfaG* group were similar with GF piglets. MyD88 was decreased but TRIF increased in ST group while *rfaG* group was comparable with GF piglets. IL-8, TNF-alpha and HMGB1 were induced in ST piglets only.

Conclusions: TLR4 is the receptor for LPS. While ST induced high levels of IL-8, TNF-alpha and HMGB1 in ileum that may be harmful, the rough LPS mutant did not induce them at all. Future incorporation of other molecules related to LPS and TLR4 as MD-2, CD-14 and LBP would contribute to clarify relations of LPS type and TLR4-signaling.

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Synthesis of the *Mycobacterium tuberculosis* lipopeptide didehydroxymycobactin (DDM-838) for the study of CD1a-restricted T cells

Cheng, J.M.H.^{1,2,3}, Pellicci, D.G.^{2,3}, Reddiex, S.J.J.², van Rhijn, I.^{4,5}, Moody, D.B.⁴, Rossjohn, J.^{6,7,8}, Godfrey, D.I.^{2,3}, Williams, S.J.¹

¹Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, School of Chemistry, Melbourne, Australia,

²Peter Doherty Institute for Infection and Immunity, University of Melbourne, Department of Microbiology and Immunology,

Melbourne, Australia, ³University of Melbourne, Australian Research Council Centre of Excellence for Advanced Molecular Imaging, Melbourne, Australia,

⁴Brigham and Women's Hospital, Harvard Medical School, Division of Rheumatology, Immunology and Allergy, Department of Medicine, Boston, United States,

⁵Utrecht University, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht, Netherlands,

⁶Monash University, Department of Biochemistry and Molecular Biology, School of Biomedical Sciences, Clayton, Australia, ⁷Cardiff University School of Medicine, Institute of Infection and Immunity,

Cardiff, United Kingdom, ⁸Monash University, Australian Research Council Centre of Excellence for Advanced Molecular Imaging,

Clayton, Australia

A role for CD1a in mycobacterial immunity was discovered through its ability to present didehydroxymycobactins, a family of peptidolipids that were isolated from *Mycobacterium tuberculosis*, to CD1a-restricted T cells. The most potent didehydroxymycobactin for T cell stimulation is DDM-838, which is characterized by the presence of a (Z)-C20:1 fatty acyl chain. Using DDM-838 bound to CD1a tetramers, polyclonal T cell responses were detected *ex vivo* in tuberculosis patients, but the natural functions of these newly discovered, lipid-specific T cells remain unknown. Because DDM-838 cannot be acquired in sufficient quantities from natural sources, an efficient route for the synthesis of large quantities of DDM-838 is needed. We report a solution-phase synthesis of DDM-838, along with the synthesis of a series of analogues with unnatural stereochemistry of the lipopeptide backbone and the lipid portion. DDM-838 structure-activity relationships were studied by loading CD1a tetramers with the synthetic lipopeptides and measuring binding of CD1a-restricted T cell lines, as well as the ability of analogues to activate T cells. The natural form of DDM-838 was the most active species but various analogues also exhibited activity with certain T cell lines, indicating a spectrum of TCR recognition of these CD1a-presented lipid antigens. Our synthetic approach to DDM-838 will support structural and functional studies to understand how these antigens are recognized by the human immune system and permit large scale human clinical studies.

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Neutrophils differentially attenuate immune response to *Aspergillus* infection through complement receptor 3 and induction of myeloperoxidase

Goh, J.¹, Ravikumar, S.¹, Win, M.S.^{1,2}, Cao, Q.^{3,4}, Lim, J.¹, Leong, W.¹, Chai, L.^{1,4}

¹National University Health System, Singapore, Department of Medicine, Singapore, Singapore, ²National University Cancer Institute of Singapore, National University Health System, Department of Haematology-Oncology, Singapore, Singapore,

³Yong Loo Lin School of Medicine, National University of Singapore, Department of Anatomy, Singapore, Singapore, ⁴Yong Loo Lin School of Medicine, National University of Singapore, Department of Medicine, Singapore, Singapore

Invasive aspergillosis (IA) is a severe invasive mold infection affecting immunocompromised patients. Despite the use of antimicrobial drugs, IA remains a major cause of mortality and morbidity, partly due to the inability of the host immune system to respond appropriately to *Aspergillus*. One of the established risk factors for IA is neutropenia which is encountered by patients undergoing chemotherapy and stem cell transplantation. Here we investigate the role of neutrophils in modulating the host immunity to *Aspergillus*.

The co-incubation of PMN and PBMC with heat-inactivated (HI) *Aspergillus* markedly diminished IL1 β , IL6 and TNF α production. Using a transwell system to effect of separation between HI *Aspergillus*-stimulated PBMC and PMN, we observed the suppression of IL1 β production. Blocking CR3 with anti-CD11b, reversed the attenuation effect of IL1 β . On the other hand, TNF α production was not contact-dependent and might be influenced by neutrophil-secreted product instead. The addition

of myeloperoxidase (MPO) did suppress TNF α production, suggesting that the attenuative effect of TNF α is dependent on MPO, a neutrophil degranulatory product.

Our study suggests that neutrophils have an extended modulatory role besides its primary phagocytic function by dampening proinflammatory cytokine production by PBMC in response to *Aspergillus*. The absence of neutrophils and its inhibitory effect, may explain the elevated proinflammatory production seen in neutropenia. Our investigation has added a novel dimension to our current understanding on the pathogenesis of IA.

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Memory CD4 and CD8 T cells are dispensable for BCG-vaccine mediated protection

Steigler, P., Kirman, J.

University of Otago, Microbiology and Immunology, Dunedin, New Zealand

Despite nearly a century of widespread use, the only available tuberculosis (TB) vaccine, bacille Calmette-Guerin (BCG), has had little success lowering the global TB burden. Due to the urgent need for a more effective TB vaccine, newly developed TB vaccines are entering clinical trials without a solid understanding of the mechanism of protective memory immunity against TB. The current paradigm, that memory CD4 T cells provide resistance against TB, is based on adoptive transfer studies performed in immunodeficient mice that may be flawed due to T cell activation following adoptive transfer. Therefore, the protective ability of naturally arising memory CD4 T cells to protect against secondary TB infection remains to be shown. To determine the protective role of memory CD4 and CD8 T cells against mycobacterial infection, we developed a novel murine model of systemic memory T cell depletion. To measure the efficacy of BCG in the absence of T cells, BCG vaccinated mice were depleted of memory CD4 or CD8 T cells or both subsets, then intranasally challenged with mycobacteria and lung bacterial burden measured. Irrespective of whether memory T cells were present or not, all groups of BCG vaccinated mice were significantly protected against mycobacterial challenge compared to unvaccinated mice. Importantly, mice depleted of memory T cells of mice had similar lung bacterial burdens to non-depleted BCG vaccinated mice. These findings suggest that BCG mediated immunity can be mediated by cells other than memory CD4 or CD8 T cells and have major implications for TB vaccine design.

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Periodontopathic bacteria increases the risk of rheumatoid arthritis by affecting gut immune system

Sato, K.¹, Takahashi, N.², Nakajima, M.¹, Matsuda, Y.¹, Yamada, M.¹, Yokoji, M.¹, Kato, T.³, Ohno, H.³, Yamazaki, K.¹

¹Niigata University Graduate School of Medical and Dental Sciences, Division of Oral Science for Health Promotion, Niigata, Japan, ²Niigata University Graduate School of Medical and Dental Sciences, Division of Periodontology, Niigata, Japan, ³RCAI RIKEN Center for Integrative Medical Science, Laboratory for Intestinal Ecosystem, Yokohama, Japan

Periodontitis has been implicated as a risk factor for various diseases including rheumatoid arthritis (RA). *Porphyromonas gingivalis*, a representative periodontopathic bacterium, has drawn much attention because of expressing a bacterial peptidyl arginine deiminase which generates citrullinated proteins, major antigens in RA. However, clinical studies demonstrated inconsistent results and therefore, underlying mechanisms linking periodontal disease and RA remain elusive. We have demonstrated that *P. gingivalis* oral administration induced change of gut microbiota, reduced gene expression of tight junction protein in the ileum, and endotoxemia. Given that the change of gut microflora is associated with RA, we hypothesized that the association between periodontal disease and RA could be mediated by the modulation of gut microflora. DBA1/J mice were orally infected with *P. gingivalis* W83 were administered with collagen type II to induce arthritis. Arthritis development was evaluated by visual scoring of paw swelling and micro-CT analysis. IL-17 production of lymphocytes separated from spleen, mesenchymal lymph nodes, and inguinal lymph nodes were analyzed by flow cytometry and ELISA. Serum levels of IL-17 were measured by ELISA. *P. gingivalis* infection increased the incidence and severity of collagen-induced arthritis. IL-17 levels of serum and culture supernatants of lymphocytes from secondary lymphoid tissues were significantly higher in *P. gingivalis*-administered mice. *P. gingivalis* infection induced elevated IL-17 gene expression and decreased tjp-1 expression in the intestine. These results may provide novel mechanisms for the link between periodontitis and RA where periodontopathic bacteria affect gut immune system by modulating gut microbiota composition.

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Poly-functional CD4⁺ T cells specific to PE/PPE antigens are protective against pulmonary *Mycobacterium tuberculosis* infection

Sayes, F.¹, Bottai, D.², Brosch, R.¹, Majlessi, L.¹

¹Institut Pasteur, Genome and Genetics, Paris, France, ²University of Pisa, Ricerca Traslationale e delle Nuove Tecnologie in Medicina e Chirurgia, Pisa, Italy

Mycobacterium tuberculosis (*Mtb*), possesses at least three type VII secretion systems, ESX-1, -3 and -5 that are actively involved in pathogenesis and host-pathogen interaction. We recently showed that an attenuated *Mtb* vaccine candidate (*Mtb* Δ*ppe25-pe19*), which lacks the characteristic ESX-5-associated *pe/ppe* genes, but harbors all other components of the ESX-5 system, induces strong epitope-mediated cross-reactive CD4⁺ T-cell immunogenicity against non-ESX-5-associated PE/PPE protein homologs. These proteins are named after their characteristic N-terminal Pro-Glu (PE) or Pro-Pro-Glu (PPE) motifs and are unique to mycobacterial species.

Here, we have dissected the different components of this specific immune response and demonstrate the marked poly-functionality of the cross-reactive Th1 effector subsets specific to these shared PE/PPE epitopes in mice immunized with the *Mtb* Δ*ppe25-pe19* vaccine candidate. We provide evidence that the *Mtb* Δ*ppe25-pe19* strain, despite its significant attenuation, is comparable to WT *Mtb* with regard to: (i) its antigenic repertoire

related to the different ESX systems, (ii) the induced Th1 effector subset composition, and (iii) its particular features at stimulating the innate immune response. Indeed, significant contribution of PE/PPE-specific bi- and poly-functional Th1 effector cells in the protective immunity against pulmonary *Mtb* infection was found.

These results offer detailed insights into the immune mechanisms underlying the remarkable protective efficacy of the live attenuated *Mtb* Δ*ppe25-pe19* vaccine candidate, as well as the specific potential of PE/PPE proteins as protective immunogens.

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Otitis-prone children produce functional antibodies to pneumolysin and pneumococcal polysaccharides

Kirkham, L.-A.^{1,2}, Wiertsema, S.^{1,3}, Corscadden, K.², Mateus, T.¹, Mullaney, G.¹, Zhang, G.⁴, Richmond, P.^{1,2,5}, Thornton, R.^{1,2}

¹University of Western Australia, School of Paediatrics and Child Health, Perth, Australia, ²Telethon Kids Institute, Perth, Australia, ³Currently at Nutricia Research, Utrecht, Netherlands, ⁴Curtin University, School of Public Health, Perth, Australia, ⁵Princess Margaret Hospital for Children, Perth, Australia

Background: The pneumococcus is an important cause of otitis media (OM) but data are conflicting on whether otitis-prone children have impaired humoral immunity to pneumococcal antigens. We found similar anti-capsular and anti-protein antibody titres in healthy and otitis-prone children, however functional antibody levels are considered to better correlate with protection. We aimed to determine whether the anti-pneumococcal antibodies produced by otitis-prone children are functional by measuring anti-pneumolysin antibody neutralising titres and polysaccharide-specific opsonising titres. Antibody potency was compared between otitis-prone and healthy children.

Methods: The pneumolysin neutralising assay was conducted on cholesterol-depleted complement-inactivated sera from 165 otitis-prone children (cases) and 61 healthy age-matched controls. The multiplexed opsonophagocytosis assay was conducted on sera from 20 cases and 20 controls. Neutralising and opsonising titres were calculated with antigen-specific IgG titres to determine antibody potency for pneumolysin, vaccine polysaccharides (4, 6B, 14, and 23F) and non-vaccine polysaccharides (1, 5, 7F and 19A).

Results: There were no significant differences in functional antibody titres nor potency between cases and controls for the antigens tested. Anti-pneumolysin neutralising titres increased with number of episodes of OM but antibody potency did not. Pneumolysin antibody potency was lower in children colonised with pneumococci compared with non-carriers, and this was significant in the otitis-prone group ($p < 0.05$).

Conclusions: Production of functional anti-pneumococcal antibodies in otitis-prone children demonstrates that they respond to current conjugate vaccines and are likely to respond to pneumolysin-based vaccines as effectively as healthy children. Whether these functional circulating antibodies confer protection against pneumococcal OM requires further investigation.

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The transcription factor Ets1 cooperates with IL17 signaling to regulate antibacterial skin immune responses

Sunshine, A., Luo, W., Sinha, S., Garrett-Sinha, L.A.
State University of New York at Buffalo, Biochemistry, Buffalo,
United States

The transcription factor Ets1 regulates differentiation of lymphocytes and its loss leads to autoimmune disease. To identify roles for the cytokine IL17 in the autoimmune phenotype of mice lacking Ets1, we crossed Ets1^{-/-} mice to mice deficient in IL17RA that lack IL17 signaling. We unexpectedly found that Ets1/ IL17RA double knockout (DKO) mice do not show amelioration of autoimmune symptoms, but rather exhibit enhanced autoimmunity as evidenced by increased plasma cells and autoantibodies. In addition, DKO mice have strikingly elevated levels of serum IgG1 and IgE, suggesting a Th2 bias. By 4-6 months of age, DKO mice spontaneously develop severe inflammatory skin lesions colonized by *S. aureus* - a phenotype absent from single knockout mice. To determine if DKO mice are more susceptible to bacterial infection, we introduced a virulent, bioluminescent strain of *S. aureus* into surgical cuts on the skin of DKO mice before overt skin lesions develop. We monitored bacterial burden over time showing that DKO mice fail to clear the infections, which instead spread to distal areas. To address the mechanistic basis for this concerted role of IL-17RA and Ets1, we are currently examining several aspects of skin immunity including the innate effector functions of keratinocytes as well as the differentiation program of both conventional and gd T cells. We are also testing the potential role of autoantibodies in damaging the skin barrier function. The outcome of our studies will likely have important implications of understanding human skin inflammatory and infectious diseases.

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Differential response of dendritic cells upon co-infection with different *Aggregatibacter actinomycetemcomitans* serotypes

Rojas, L., Alvarez, C., Melgar-Rodriguez, S., Pujol, M., Monasterio, G., Garcia, K., Diaz-Zuñiga, J., Fariña, V., Hernandez, M., Vernal, R.
Universidad de Chile, Periodontal Biology Laboratory, Santiago,
Chile

Aim: *Aggregatibacter actinomycetemcomitans* is a periodontopathogenic bacteria involved in periodontitis. Based on the variable immunogenicity of the LPS-O-polysaccharide of *A. actinomycetemcomitans*, six different serotypes have been described. The serotype b induces higher levels of pro-inflammatory cytokines in stimulated human dendritic cells (DCs) compared with the other serotypes. Recently, it was reported that some periodontitis patients can harbour two or three different *A. actinomycetemcomitans* serotypes in their periodontal lesions; thus, it has been proposed variations of the immune response during these co-infections. This study aimed to analyze the response of DCs when co-infected with different combinations of the most prevalent *A. actinomycetemcomitans* serotypes (a-c).

Methods: PBMCs were differentiated into DCs and then stimulated with the *A. actinomycetemcomitans* serotypes a, b, or c and the combinations a+b, a+c, b+c, or a+b+c. The cytokines IL-1 β , IL-5, IL-6, IL-10, IL-12, IL-23, IFN- γ , and TNF- α and the chemokines and chemokine receptors CCL2, CCL5, CCL11, CCL17, CCR2, CCR3, CCR4, CCR5 were quantified by qRT-PCR. In addition, total MMP-2 was analyzed by ELISA, active MMP-9 was analyzed by fluorescence assay, and gelatinolytic activity of these MMPs was analyzed by gelatin-zymography. Finally, bone resorption activity was quantified by in-vitro bone resorption pits-assay.

Results: When DCs were stimulated with the different combinations of *A. actinomycetemcomitans* serotypes, significantly lower levels of cytokines, chemokines, chemokine receptors, MMPs, and bone resorption activity were detected compared with the same cells stimulated with serotype b.

Conclusions: On DCs, distinct *A. actinomycetemcomitans* serotype co-infections can modulate the immuno-stimulatory potential of serotype b.

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Immune response to *Mycobacterium leprae*: potential application for leprosy diagnosis

Fraga, L.¹, Marçal, P.^{2,3}, Zandim, P.³, Oliveira, L.⁴, Ottenhoff, T.⁵, Geluk, A.⁵, Teixeira, H.²

¹Universidade Federal de Juiz de Fora - Campus Governador Valadares, Basic Department of Health, Governador Valadares, Brazil, ²Universidade Federal de Juiz de Fora, Biological Sciences, Juiz de Fora, Brazil, ³Universidade Vale do Rio Doce, Ciências da Saúde, Governador Valadares, Brazil, ⁴Universidade Federal de Juiz de Fora - Campus, Basic Department of Health - Programa Multicêntrico de Bq e BM, Governador Valadares, Brazil, ⁵Leiden University Medical Center, Leiden, Netherlands

The clinical form of leprosy is characterized by dermatoneurological spectrum changes with different immune response patterns. The diagnosis of leprosy has been based on dermatoneurological examination, smear microscopy and histopathology. The development of specific and sensitive serologic test is very important for early diagnosis, preventing the disease transmission and the sequelae onset. The purpose of this study was to evaluate the serum samples from leprosy patients, subjects in contact with clinical leprosy and healthy subjects for reactivity against four specific recombinant proteins of *M. leprae* (ML0405, ML2055, ML2331 and Ag85B) using ELISA. The results showed that the group of multibacillary patients had higher IgG production compared to the healthy control and paucibacillary groups for all tested antigens. The group of subjects in contact with multibacillary leprosy showed higher production of specific IgG to the ML2055 and ML0405 antigens compared to the healthy control group (CS). IgM levels to both ML2055 and Ag85B antigens were higher in the MB contact group compared to the CS group. The IgM levels of the multibacillary group were higher than the levels of the CS group only for the ML2331 antigen. Levels of specific antibodies against the ML0405 antigen decreased after the implementation of multidrug therapy. Our results suggest that

a serologic test using the ML0405, ML2331, ML2055 and Ag85B antigens could be developed as an additional test for the early diagnosis of leprosy. The control of specific antibody levels for ML0405 could be used as an effective marker for monitoring the treatment progress. FAPEMIG, CNPq, FNS/MS.

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Characterization of secreted molecules from lactobacilli with systemic immune-modulatory activity

Mata Forsberg, M., Björkander, S., Johansson, M.A., Sverremark-Ekström, E.

Stockholm University, Department of Molecular Biosciences, Wenner-Gren Institute, Stockholm, Sweden

Lactobacilli are commensal lactic acid-producing bacteria with the capacity to influence and modulate mucosal and systemic immunity *in vitro* and *in vivo*. Early-life colonization with lactobacilli is associated with decreased risk of allergy development and supplementation of certain lactobacilli spp are known to ameliorate gut inflammatory diseases and improve epithelial barrier function. However, the direct mechanism of these bacteria on systemic immune responses remains elusive. Human PBMC were stimulated with *S. aureus* cell free supernatant (CFS) in the presence or absence of lactobacilli-CFS. Pro- and anti-inflammatory cytokine responses were evaluated using ELISA and flow cytometry. Classical bio-molecular/chemical techniques were employed to characterize and identify the bioactive molecules present in the lactobacilli-CFS.

We show that CFS of multiple lactobacilli spp dampen *S. aureus*-induced IFN- γ and IL-17A secretion. Size fractionation suggests that molecules of different size differentially dampen these cytokines. Heat-inactivation of the CFS prevented dampening of IL-17A but not of IFN- γ . Even though lactobacilli-CFS potently induced IL-10-production in PBMC, blocking of IL-10 did not prevent lactobacilli-mediated dampening. Addition of lactic acid to *S. aureus*-stimulated PBMC cultures selectively reduced the frequency of IFN- γ + unconventional T-cells and NK-cells in a dose dependent manner.

We conclude that lactobacilli-CFS induce and modulate both pro- and anti-inflammatory cytokine-responses in PBMC. Lactobacilli mediate dampening of IFN- γ by an IL-10-independent mechanism through the release of multiple molecules. Finally, lactic acid production may have a contributing role in the dampening of pro-inflammatory cytokine responses.

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Complement-opsonization of *A. fumigatus* modifies dendritic cell function

Steger, M.¹, Posch, W.¹, Lass-Flörl, C.¹, Haas, H.², Wilflingseder, D.¹

¹Medical University of Innsbruck, Division of Hygiene and Medical Microbiology, Innsbruck, Austria, ²Medical University of Innsbruck, Division of Molecular Biology, Innsbruck, Austria

Background: In this study, interactions of dendritic cells (DCs) with complement-opsonized and non-opsonized *Aspergillus fumigatus* strains and various mutants thereof were investigated. The opsonization pattern of the different strains

and mutants, the binding and internalization by dendritic cells as well as the cytokine secretion and initial signaling pathways were investigated.

Methods: Fungi were opsonized using normal human serum as complement source. The opsonization pattern, binding of conidia to DCs and internalization were characterized by FACS analyses. Inhibition of fungal growth in presence of DCs and interactions with complement receptors were detected using confocal microscopy. Furthermore, phosphorylation of ERK1/2 and p38 were detected by immunoblot analysis.

Results: We could demonstrate in this study that melanin and β -1,3-glucan have high impact on the fungal virulence compared to the wildtype *Aspergillus* strains. With respect to dendritic cell binding and internalization complement-opsonization of conidia enhanced these processes compared to their non-opsonized counterparts independent on the fungal strain used.

Conclusion: These data revealed, that melanin and β -1,3-glucan are key effectors of masking complement deposition and binding of conidia by DCs. However opsonization of swollen conidia enhanced internalization in DCs as well as production of pro-inflammatory cytokines, thereby resulting in a favorable TH1 immune response. These *in vitro* studies propose that the use of immune cells, like DCs or neutrophils, in combination with complement opsonins might act as potent vaccines against invasive aspergillosis.

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Elevated neutrophil TLR activation during acute severe leptospirosis

Lindow, J.^{1,2}, Montgomery, R.¹, Reis, E.², Tsay, A.¹, Wunder, E.^{1,2}, Araújo, G.², Nery, N.², Mohanty, S.¹, Shaw, A.¹, Reis, M.², Ko, A.^{1,2}

¹Yale University School of Medicine, New Haven, United States, ²Fiocruz, Salvador, Brazil

The role of the innate immune response in the pathogenesis of leptospirosis and progression to organ failure is poorly defined. To determine whether increased neutrophil function associated with renal and/or lung dysfunction, we used flow cytometry to quantitate the expression levels of neutrophil pathogen recognition receptors and activation markers in a cohort of leptospirosis patients with different disease outcomes. We found elevated levels of TLR2 on neutrophils from acute leptospirosis patients, with significantly higher levels in patients with organ dysfunction ($9.1 \pm 2.8\%$ v. $3.0 \pm 0.8\%$, $p=0.04$). However, we observed no significant differences in baseline activation of neutrophils in any patient group, though neutrophils from patients with organ dysfunction had higher activation responses to TLR4 stimulation. These results suggest excess inflammation from neutrophils may contribute to the organ pathogenesis observed in leptospirosis, and may represent a novel avenue for therapeutics in human leptospirosis.

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HIF-1 α stabilization through metabolic hypoxia via glutaminolysis in a murine model of pulmonary tuberculosis

Torres Rojas, M.¹, Abarca Rojano, E.², Huerta Yopez, S.¹, Hernández Pando, R.³, Rangel Santiago, J.¹, Mayoral Márquez, H.⁴, Domínguez López, M.L.⁵

¹Hospital Infantil de México Federico Gómez, Unidad de Investigación en Enfermedades Oncológicas, México, Mexico,

²Instituto Politécnico Nacional, Laboratorio de Respiración Celular, Escuela Superior de Medicina, México, Mexico, ³Instituto Nacional de Ciencias Médicas y Nutrición "Salvador Zubirán", Departamento de Patología Experimental, México, Mexico, ⁴Instituto Politécnico Nacional, de Respiración Celular, Escuela Superior de Medicina, México, Mexico, ⁵Instituto Politécnico Nacional, Laboratorio de Inmunoquímica I, Escuela Nacional de Ciencias Biológicas, México, Mexico

Tuberculosis is a chronic infection in which Mycobacterium tuberculosis triggers not only a complex immune response but also others modifications including metabolism of the host and the guest. For the study of these events many animal models had been developed. The murine model of pulmonary tuberculosis of Hernandez-Pando et al., which comprises 120 days after infection, is characterized by two immunopathogenic phases: the TH1 immune response which is protective or antibacterial with a climax on 21th day of infection and the TH2 immune response initiating the progressive phase of the disease on the 28th day of infection presenting pneumonia, large bacillary load and high mortality rates. Using immunohistochemistry essays we found out that HIF-1 α , an inflammatory transcription factor, is progressively higher through the advance of the infection days in the murine model, pattern that is also observed in the expression of glutaminolysis enzymes (glutaminase 1 and glutamate dehydrogenase) and in the expression of GPR91, a succinate receptor. According to these results we conclude that glutaminolysis might be functioning as an anaplerotic way of producing succinate, which in turn blocks the activity of the prolyl hydroxylase enzymes promoting the stabilization of HIF-1 α , finally leading to inflammation and a chronic or even a fatal progression of the infection.

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Group A streptococcus-induced macrophage death is through glycogen synthase kinase-3 beta activation and mitochondrial damage

Tsao, N.¹, Lin, W.-C.¹, Shen, Y.-C.¹, Kuo, C.-F.²

¹I-SHOU University, Department of Biological Science and Technology, Kaohsiung, Taiwan, Republic of China, ²I-SHOU University, Department of Nursing, Kaohsiung, Taiwan, Republic of China

Group A streptococcus (GAS) causes pharyngitis, scarlet fever, impetigo, necrotizing fasciitis, and streptococcal toxic shock syndrome in humans. In our previous study, we find that streptococcal pyrogenic exotoxin B (SPE B) and streptolysin S (SLS) both contributed to GAS evasion from the immune cell killing and mediated the GAS-infected macrophage

death. In this study, we explored the mechanisms involved in macrophage defense and GAS-induced cell death. The wild-type GAS-infected Raw 264.7 cells, but not in SLS or SPE B mutant-infected cells, manifested the increased levels of intracellular reactive oxygen species (ROS) and the decrease of mitochondrial membrane potential ($\Delta\psi_m$). Surveys of mitochondrial ROS production using MitoSOX indicated that the mitochondrial ROS was the primary source of cellular ROS after GAS infection, which regulated the decrease of $\Delta\psi_m$ and cell death in GAS-infected cells. Glycogen synthase kinase-3 beta (GSK-3 β) plays an important role in regulation of ROS-mediated cell death. The wild-type GAS infection induced GSK-3 β activation in Raw 264.7 cells. Inactivation of GSK-3 β not only decreased mitochondrial ROS production but also inhibited the cell death of infected macrophages. Furthermore, inactivation of GSK-3 β also enhanced the bactericidal activity of GAS-infected Raw264.7 cells. Taken together, we suggest that GAS infection-mediated GSK-3 β activation regulated mitochondrial ROS production and $\Delta\psi_m$ reduction, and further contributed to the bactericidal inhibition in GAS-infected Raw 264.7 cells.

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Vitamin D suppresses pro-inflammatory responses associated with *Streptococcus pneumoniae*

Hoe, E.¹, Nathanielsz, J.¹, Toh, Z.Q.¹, Spry, L.¹, Marimla, R.¹, Balloch, A.¹, Mulholland, K.^{1,2}, Licciardi, P.¹

¹Murdoch Childrens Research Institute, Melbourne, Australia,

²London School of Hygiene and Tropical Medicine, London, United Kingdom

S. pneumoniae is the most common bacterial respiratory pathogen and a leading cause of death in children under 5 years of age, mostly in developing countries. Risk factors for pneumococcal infections include nutrition, indoor air pollution and reduced exposure to sunlight. Recently, epidemiological evidence suggests that Vitamin D₃ (VitD₃) deficiency may also contribute to pneumococcal disease pathogenesis. VitD₃ has diverse immunomodulatory functions although its role during bacterial infections remains unclear. In this study, we examined the effects of VitD₃ pre-treatment of peripheral blood mononuclear cells (PBMCs) and purified immune cell subsets isolated from healthy adult volunteers on the response to specific innate [heat-killed pneumococcal serotype 19F (HK19F; TLR2) and LPS; TLR4] and adaptive (CD3/CD28) stimuli. We found that VitD₃ significantly reduced important pro-inflammatory cytokines TNF- α , IFN- γ , IL-8 and IL-1 β for all PBMC-stimulated ligands (3 to 53-fold), while anti-inflammatory IL-10 was increased (2-fold, p=0.016) in HK19F-stimulated monocytes. We also found that IFN- γ levels were higher (11.7-fold) in VitD₃-deficient adults stimulated with HK19F compared to VitD₃-sufficient adults. VitD₃ also shifted the T_H1/T_H2 balance towards an anti-inflammatory phenotype, increased the frequency of monocytes (3-fold, p=0.046) and CD4 expression on T cells (1.2-fold, p=0.008) which are important in protection against *S. pneumoniae*. These results demonstrate an important role for VitD₃ in the control of pneumococcal inflammation and supports further *in vivo* and clinical studies to confirm the potential benefits of VitD₃ in infants at high-risk of pneumococcal disease.

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Emerging roles of an innate immune regulator TAPE in Toll-like receptors, RIG-I-like receptors, and beyond*Ling, P.^{1,2}, Chen, K.-R.², Kao, C.-C.¹, Chuang, H.-C.³, Tan, T.-H.³*¹National Cheng Kung University, Microbiology and Immunology, Tainan, Taiwan, Republic of China, ²National Cheng Kung University, Institute of Basic Medical Sciences, Tainan, Taiwan, Republic of China, ³National Health Research Institutes, Immunology Research Center, Zhunan, Taiwan, Republic of China

Pattern-recognition receptors (PRRs) trigger innate immune defenses against pathogen infection via signaling pathways linking to inflammation and cell-autonomous immunity like phagocytosis and autophagy. IKK family kinases, IKK α and IKK β , function to relay PRR signals to proinflammatory cytokine production to amplify immune responses. TBK1, a non-canonical IKK kinase, links several nucleic acid sensors to type I interferon induction against viral infection and also regulates the autophagic clearance of intracellular bacteria. TBK1-Associated Protein in Endolysosomes designated TAPE, also known as CC2D1A, is an innate immune regulator acting upstream of Trif to regulate the TLR3 and TLR4 pathways, or upstream of MAVS to regulate the cytosolic RIG-I-like receptor (RLR) pathways. Notably, TAPE is a regulator implicated in both the endosomal TLR and cytosolic RLR pathways at such an early step. We are thus interested in investigating in vivo role of TAPE in innate immunity and molecular mechanisms by which TAPE regulates TLRs, RLRs, and possibly other PRRs. TAPE conditional knockout (cKO) mice, in which TAPE is selectively disrupted in immune cells, were generated for our study. Our results showed that upon influenza A virus infection, TAPE cKO mice exhibited a more severe mortality than wild type mice. Further, TAPE cKO mice were shown to be more susceptible to *Salmonella* infection but more resistant to LPS-induced septic shock. Ex vivo studies showed that TAPE was implicated in the autophagic clearance of *Salmonella*. Together, our data support a critical role for TAPE in regulating innate immune defenses through TLRs, RLRs, and autophagy.

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In vivo analysis of the interaction of antigen presenting cells (APC) in intestine of BALB/c mice with *Brucella abortus* administered intragastrically*López-Santiago, R.¹, Venegas-Brett, K.¹, Benito-Pineda, K.E.¹, Perea-Flores, M.J.², Sánchez-Argaes, A.B.¹, Gutiérrez-Hoya, A.^{1,3}*¹Instituto Politécnico Nacional, ENCB, Inmunología, México DF, Mexico, ²Instituto Politécnico Nacional, Centro de Nanociencias y Micro y Nanotecnologías, México DF, Mexico, ³FES-Zaragoza, UNAM, México DF, Mexico

Brucellosis is an infectious disease caused by *Brucella* spp. The infection causes abortions in the livestock and sterility in males. Humans acquire the infection through the ingestion of contaminated daily products.

In the 1970's it was observed in *in vitro* studies that *Brucella abortus* selectively attaches to B lymphocytes, which was used to identify lymphocytic leukaemia. The interaction was mediated by a lectin-like receptor and not by the BCR, but it was

not further studied the effect of the interaction on the B cell. So far, it is unknown whether *Brucella* binds to the B cell in an *in vivo* infection, and the role of the B cell in the pathogenesis or in the immune response to *Brucella*. The aim of the present work was to show the interaction of *B. abortus* with the antigen presenting cells (APC) in the Peyer's patches and the mesenteric lymph node of BALB/c mice infected intragastrically. *B. abortus* was captured mainly by B cells of both lymphoid organs, and by a small fraction of dendritic cells. The binding was already observed at 30 minutes after the infection. Images by laser scanning microscopy strongly suggested that the bacterium is internalized into the B cells by macropinocytosis. B cells which uptake the bacteria underwent activation as was shown by the cell surface markers CD40, CD80 and CD86. The phenotype and function of the APC infected with *B. abortus* must be defined, so it can be elucidated their role in the protection or the pathogenesis of brucellosis.

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Adults with bronchiectasis do not have reduced antibody titres to vaccine candidate antigens of nontypeable *Haemophilus influenzae* or *Streptococcus pneumoniae**Thornton, R.^{1,2}, Marsh, R.³, Wiertsema, S.¹, Mateus, T.^{1,2}, Granland, C.¹, Shorten, M.⁴, Thompson, P.⁴, Richmond, P.^{1,2,5}, Kirkham, L.-A.^{1,2}*¹University of Western Australia, School of Paediatrics and Child Health, Perth, Australia, ²Telethon Kids Institute, Perth, Australia, ³Menzies School of Health Research, Darwin, Australia, ⁴Institute for Respiratory Health, Perth, Australia, ⁵Princess Margaret Hospital for Children, Perth, Australia

Background: Nontypeable *Haemophilus influenzae* (NTHi) and *Streptococcus pneumoniae* are important in infective exacerbations in bronchiectasis patients and often persist in the lungs despite antimicrobial therapy. Impaired immune responses to these bacteria may contribute to persistence but little data on pathogen-specific immune responses exists. The aim of this pilot study was to measure antibody responses to vaccine candidate antigens for NTHi and *S. pneumoniae* in adults with bronchiectasis and compare to healthy controls.

Methods: Multiplex fluorescent bead-based assays were conducted on sera and saliva samples from 19 adults with bronchiectasis and 17 age and gender-matched healthy controls. IgA and IgG titres were measured against the pneumococcal protein vaccine candidates pneumolysin (Ply), pneumococcal surface protein A 1 and 2 (PspA1, PspA2) and choline binding protein A (CbpA); and NTHi vaccine candidate proteins P4, P6, P26 and protein D (PD).

Results: Significantly higher antibody titres were observed in adults with bronchiectasis compared to healthy controls for serum and salivary anti-P4 IgA (1066 vs 559 AU; $p=0.037$ and 29 vs 12 AU; $p=0.003$ respectively) and anti-P26 IgG (5864 vs 2030; AU $p=0.027$ and 1.2 vs 0.2 AU; $p=0.003$ respectively). No significant differences were observed between cases and controls for antibody to the other proteins.

Conclusions: In this small study, adults with bronchiectasis had similar anti-bacterial antibody titres to healthy controls. Where differences existed, adults with bronchiectasis had more antibody than their healthy counterparts. Whether antibodies

present are functional, or can be boosted to confer protection from acute exacerbations requires further investigation.

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B-cell epitope mapping of the *Helicobacter pylori* proteome

Adamsson, J.¹, Perkins, T.², Marshall, B.J.², Lundin, S.B.^{1,2}

¹Gothenburg University, Dept. of Microbiology and Immunology, Göteborg, Sweden, ²University of Western Australia, Marshall Centre for Infectious Diseases Research, Nedlands, Australia

The bacterium *Helicobacter pylori* is a major cause of diseases, including duodenal ulcer and gastric cancer. Its genome is highly variable, and strains harboring certain variants of virulence factors are conferring increased risk of gastric cancer development.

To increase understanding of the process of carcinogenesis, we aimed to study the B-cell response to *H. pylori* in extraordinary detail.

We mapped all linear B-cell epitopes of the *H. pylori* proteome using high-density peptide arrays. Overlapping 15-mer peptides were printed onto the chip surface, using an overlap of 10 aa, thereby covering the entire *H. pylori* proteome on each chip. Binding of IgG- or IgA-antibodies to each peptide on the chip was assessed by the use of serum pools (n=10 sera per pool) from *H. pylori*-infected or *H. pylori* uninfected individuals, followed by incubation with fluorescence-conjugated anti-IgG or anti-IgA antibodies.

The protein with the highest response, both regarding epitope numbers and intensity of response, was *cagA*. This agrees with the knowledge that *cagA* is an immunodominant antigen of *H. pylori*.

Around 20% of proteins tested had at least one epitope with a high antibody response in *H. pylori*-infected individuals. Consistent with the mucosal infection site, IgA-antibodies reacted to more than 80% of these epitopes, whereas IgG-antibodies bound to only around 30% of the epitopes.

Detailed mapping of the B-cell responses to *H. pylori* may provide an understanding of how the adaptive immune response to *H. pylori* influences gastric cancer development.

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Influence of the moricin-like antimicrobial peptide on perturbation of fungal cell membranes

Kim, S.-R., Choi, K.-H., Kim, S.-W.

National Academy of Agricultural Science, Department of Agricultural Biology, Wanju-gun, Korea, Republic of

Moricin like peptide (MLP) is a 33-residue antimicrobial peptide which was isolated from the Japanese oak silkworm, *Antheraea yamamai*. This peptide was shown to contain potent antimicrobial activities against several human pathogenic bacterial and fungal strains. In this study we investigated the mechanism of action towards human pathogenic fungi. To understand the antifungal mechanisms of MLP, flow cytometric analysis using PI staining and 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence analysis were conducted against *Candida albicans* cells. The results demonstrated that MLP perturbed

and disrupted the fungal plasma membrane. Furthermore, we confirmed that *C. albicans* cells treated with MLP showed several diagnostic markers of yeast apoptosis: accumulation of intracellular ROS, phosphatidylserine exposure, active metacaspase, dissipation of the mitochondrial membrane potential. In addition, nuclear fragmentation was seen by DAPI and TUNEL assay. These evidence strongly support antifungal property of MLP by promoting apoptosis in *C. albicans*.

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Influence of platelet-activating factor receptor (PAFR) on *Brucella abortus* infection: Implications for manipulating the phagocytic strategy of *B. abortus*

Kim, S.

Institute of Agriculture and Life Science, Gyeongsang National University, Jinju, Korea, Republic of

Brucella abortus is an intracellular pathogen which can infect and persist in host cells through multiple interactions. Above all, its interaction to host cell receptor is important to understand the pathogenic mechanisms of *B. abortus*. Accordingly, we demonstrated that platelet-activating factor receptor (PAFR) affects host cell response against *B. abortus* infection. First of all, *B. abortus* infection to macrophage induces secretion of platelet-activating factor (PAF), which is a PAFR agonist. The stimulation of PAFR by PAF remarkably increases *B. abortus* uptake into macrophages. It induces Janus kinase 2 (JAK2) and p38 α phosphorylation, indicating that PAFR-mediated activation of JAK2 signaling leads to enhanced uptake of *B. abortus*. Moreover, the dynamics of F-actin polymerization revealed that PAFR-mediated *B. abortus* uptake is related with the reorganization of F-actin and JAK2. Upon *B. abortus* phagocytosis, reduced PAFR in the membrane and subsequently increased levels of PAFR colocalization with endosomes were observed which indicate that *B. abortus* uptake into macrophages allowed PAFR trafficking to endosomes. This study demonstrated that PAFR has a compelling involvement in *B. abortus* uptake as a promoter of phagocytosis, which is associated with JAK2 activation. Thus, our findings establish a novel insight into a receptor-related phagocytic mechanism of *B. abortus*.

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Analysis of a molecular mechanism underlying the susceptibility to *Staphylococcus aureus* infection in Hyper-IgE syndrome

Wada, T., Nishikawa, Y., Minegishi, Y.

Tokushima University, Institute for Genome Research, Division of Molecular Medicine, Kuramoto, Tokushima, Japan

Introduction: Hyper-IgE syndrome (HIES) is a primary immunodeficiency, characterized by recurrent skin abscesses and pneumonia due to infection with *Staphylococcus aureus*. We identified that most cases of HIES are caused by dominant negative (DN) mutations in a single allele of STAT3 gene. However, the molecular mechanisms underlying the susceptibility to *S. aureus* infection remain unclear. To investigate the molecular mechanism of HIES, we established STAT3-DN knock-in mice as

a mouse model of HIES.

Methods and results: STAT3-DN mice and wild-type (WT) mice were epicutaneously infected with *S. aureus*, *S. epidermidis* or *Streptococcus pyogenes*, and the wound size and bacterial burden of the skin lesions at the site of infection were measured. STAT3-DN mice infected with *S. aureus* but not *S. epidermidis* or *S. pyogenes* developed larger skin lesions with higher bacterial burden than WT mice. In contrast to WT mice, histological examination revealed that accumulation of inflammatory cells were impaired in the skin lesions of STAT3-DN mice. Next, neutrophil influx and IL-17A production, which are essential for elimination of *S. aureus*, were evaluated in these lesions. Flow cytometric and real-time PCR analysis revealed that recruitment of neutrophils and production of chemoattractants were impaired. Furthermore, production of IL-17A by CD3+TCR $\gamma\delta$ + cells and CD3+CD4+ cells were also decreased compared to WT mice.

Conclusion: Impaired inflammatory cell response and IL-17A production might lead to the susceptibility to staphylococcal infection in HIES.

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The role of Interleukin-33 and its signaling in the development of infectious colitis

Mchedlidze, T., Kindermann, M., Mahapatro, M., Neurath, M., Wirtz, S.

University Erlangen, Medical Department 1, Erlangen, Germany

Infections with proteobacteria of the genus *Salmonella* are a significant health problem. Alarmin-like cytokine IL-33 is crucial in sensing damage during inflammatory conditions and therefore potentially plays an important role in immunity against infections. Previous studies implicated this cytokine in bacterial sepsis, but still the precise role of IL-33 in intestinal diseases remains poorly understood.

To identify IL-33 expression in the context of *S. typhimurium* infection, we employed IL-33-LacZ reporter mice and observed strong reporter gene activity after infection compared to steady-state. Upregulated intestinal IL-33 expression was also confirmed by qRT-PCR analysis. In order to assess the role of IL-33 pathway during *S. typhimurium* infection IL-33^{-/-} mice were compared to littermate controls. In fact, in IL33-deficient animals showed increased bacterial burden compared with controls. Besides, lack of IL-33 was associated with increased immune cell infiltration and tissue damage in cecum and ileum. Moreover, extensive epithelial cell loss was detected by immunofluorescent staining of β -catenin. The strong differences between the two groups was associated with increased mRNA levels of antimicrobial peptides such as Angiogenin 4, Defensin α 5, Cryptidin and Lysozyme demonstrated a substantial decrease in IL-33^{-/-} mice compared to controls. In line with this, the more severe pathology of knock-out animals was associated with decreased presence of polymorphonuclear granulocytes/neutrophils and macrophages. Similar results were observed in mice lacking the IL-33 receptor chain ST2 suggesting that IL-33 signal transduction protects from *S. typhimurium* infection-dependent pathology.

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Enhancing effect of Peptide-25 on the induction of functional activation of CD8 cytotoxic T lymphocytes

Tamura, T.¹, Shimohakamada, Y.^{2,3}, Umemura, M.⁴

¹National Institute of Infectious Diseases, Department of Mycobacteriology, Leprosy Research Center, Higashimurayama, Japan, ²National Sanatorium Tama-Zenshoen, Higashimurayama, Japan, ³National Institute of Infectious Diseases, Department of Mycobacteriology, Leprosy Research Center, Higashimurayama, Japan, ⁴Tropical Biosphere Research Center, University of the Ryukyus, Division of Molecular Microbiology, Department of Tropical Infectious Diseases, Nakagami, Japan

The effector cells involved in protective immune response against *Mycobacterium tuberculosis* are macrophages and CD8 cytotoxic T cells (CTL). Therefore, effectiveness of a vaccine against tuberculosis is mainly judged by its capability to induce memory CTL.

It has been reported that CD4 T cells are indispensable to induce functional activation of CTL. However, how CD4 T cells instruct or support functional activation of CTL during priming phase has not been resolved in detail. Therefore, we examined helper function of CD4 T cells in the CTL development.

Peptide-25 is the major T cell epitope of Ag85B of *Mycobacterium tuberculosis*. We found that this peptide induced Th1 differentiation even in the absence of IFN- γ and IL-12. Therefore, we examined whether Peptide-25 can enhance CTL activation. To examine the role of Peptide-25 in CTL development, we established *in vitro* analysis system by using Peptide-25, Peptide-25 specific CD4 T cells, OVA specific CD8 T cells and DC. By using this system, we found that CD4 T cells activated by Peptide-25 induced DC maturation even in the absence of IFN- γ and CD40 ligand association, and the mature DC induced the functional activation of CTL. To identify regulatory factors responsible for DC maturation, we analyzed gene expression profile of CD4 T cells and identified 27 genes. One of the genes was IL-17F. By using IL-17F^{-/-} mice, we found that Peptide-25 induced CTL activation was diminished in the absence of IL-17F. These results suggest that Th1-inducing peptide regulate the functional activation of CTL by inducing IL-17F production.

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Evaluating the host immune response to bacterial pathogens in children with obstructive sleep apnoea and recurrent tonsillitis

Mateus, T.^{1,2}, Kirkham, L.-A.^{1,2}, Coates, H.^{1,2}, Vijayasekaran, S.³, Richmond, P.^{2,3}, Thornton, R.^{2,3}

¹UWA, School of Paediatrics and Child Health, Perth, Australia,

²Telethon Kids Institute, Perth, Australia, ³UWA, Perth, Australia

Obstructive sleep apnoea (OSA) affects 2-3% of children under 10 years of age. The pathogenesis of OSA is not well understood but correlates with an increase in allergic response. We evaluated the antibody response to the following respiratory pathogens: nontypeable *Haemophilus influenzae* (NTHi), *Streptococcus pneumoniae* and Group A streptococcus (GAS). Serum IgG and IgA to vaccine candidate proteins from NTHi (P4, P6 and PD), *S. pneumoniae* (Ply, PspA1, PspA2 and CbpA) and GAS (ScpA) were

assessed in children with OSA (n=40), recurrent tonsillitis (RT, n=21), OSA+recurrent tonsillitis (OSA+RT, n=16) and healthy controls (n=55) using a multiplex bead based assay. Atopic status was assessed using total IgE levels and parental questionnaire. Our results showed that IgG and IgA titers against all bacterial proteins from NTHi, *S. pneumoniae* and GAS were significantly higher ($p \leq 0.003$) in children with OSA and OSA+RT compared to children with RT and healthy controls. Total IgE titers were not significantly different between the 4 groups. Whilst overall atopy appears similar across the cohorts, differences in pathogen specific IgE responses may still exist. The IgE pathogen specific responses and the relative abundance of the bacterial species in the adenoids, tonsils, nasopharyngeal and throat swabs of the same cohorts are currently under investigation.

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Immunopathogenesis of non-tuberculous mycobacterial infection in cystic fibrosis patients

Lutzky, V.¹, Ratnatunga, C.¹, Thomson, R.², Smith, D.^{2,3}, Reid, D.^{2,3}, Bell, S.^{2,4}, Miles, J.¹

¹QIMR Berghofer Medical Research Institute, Human Immunity Laboratory, Brisbane, Australia, ²The Prince Charles Hospital, Department of Thoracic Medicine, Brisbane, Australia, ³QIMR Berghofer Medical Research Institute, Lung Inflammation and Infection Laboratory, Brisbane, Australia, ⁴QIMR Berghofer Medical Research Institute, Lung Bacteria Laboratory, Brisbane, Australia

Non-tuberculous mycobacteria (NTM) are a family of bacteria found in food, soil and water distribution supplies. A number of NTM species are pathogenic in humans causing localized cutaneous lesions and pulmonary disease. NTM is a significant problem in cystic fibrosis (CF) patients affecting 20% of individuals. *Pseudomonas aeruginosa* is the major pathogen in the CF lung. Prevalence is high in CF and, once acquired, chronic infection will almost always ensue. Clinical outcomes from these lung pathogens are often poor, with increased risk of treatment toxicity and contra-indication for lung transplantation. Thus, there is an urgent need to understand the pathomechanisms behind NTM-driven pulmonary disease and develop effective therapies for treatment.

In this study, we have performed a comprehensive dissection of immune compartment composition in CF patients who have active NTM lung infection (CFA), previous NTM lung infection (CFN) or chronic *Pseudomonas* infection (CFC). Our results show that both CFA and CFN patients have higher number of circulating regulatory T cells, compared to chronic infected patients. In the CFA cohort there was also an increase in CD4+ displaying an exhausted phenotype. Additionally, CD8+ cells from patients exhibited aberrant function both at rest and post mitogen stimulation. In summary, we show that NTM infection in CF patients exhibit a distinct immune signature that can be used to identify future biomarkers and novel immune targets for use in immunotherapeutic modulation.

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Immunomodulatory muropeptide against *Candida albicans*

Win, M.S.^{1,2}, Lim, J.¹, Ravikumar, S.¹, Goh, J.¹, Sam, Q.¹, Tan, Z.¹, Huang, Z.³, Yue, W.³, Chai, L.^{1,4}

¹National University Hospital, Department of Medicine, Singapore, Singapore, ²National University Cancer Institute of Singapore, Department of Haematology-Oncology, Singapore, Singapore, ³Institute of Molecular and Cell Biology (IMCB), A*STAR, Singapore, Singapore, ⁴Yong Loo Lin School of Medicine, National University of Singapore, Department of Medicine, Singapore, Singapore

Invasive candidemia causes major morbidity in patients with weakened immune system. The interplay and balance of Th1 and Th2 response dictates the efficiency of adaptive immune response against *Candida*. We studied the modulatory effect of the muropeptide, muramyl dipeptide (MDP), on immune response to *Candida* in a murine candidiasis model. Balb/c mice were intravenously inoculated with *Candida albicans* (1×10^6 CFU/ml) after being primed with intravenous injection of MDP (100 µg) for 2 days. By 3 days, there was significant decrease in the kidney fungal load when compared to non-MDP-primed mice. Histopathological examination of the kidneys showed higher incidence of interstitial inflammation in MDP primed mice. MDP had induced higher IFN γ and TNF α production. Splenocytes of the MDP-primed mice stimulated with heat-killed *Candida* revealed augmented IFN γ , TNF α and IL6 production. mRNA expression of STAT1 & T-bet also showed up-regulation in MDP-primed mice while the expression of GATA-3 was down-regulated, indicating the augmentation of Th1 adaptive immunity in the MDP primed mice. NOD2 knockout mice shows similar reduction in the fungal load in the MDP primed mice and non-primed mice. This illustrates that priming effect of MDP may direct Th1 activation by NOD2 independent pathways warranting further study of the alternative pathways involved in the process.

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The expression of microRNA-146a is related with antibody titers and clinical symptoms in brucellosis patients

Shi, Y.¹, Yu, J.¹, Zheng, Y.¹, Han, X.²

¹Inner Mongolia Medical University, Inner Mongolia Key Laboratory of Molecular Biology, Hohhot, China, ²Inner Mongolia Medical University, School of Nursing, Hohhot, China

Brucellosis is one of the most common zoonosis worldwide. Human brucellosis is characterized by atypical symptoms, including fever, sweating, arthralgia/arthritis, constitutional symptoms, hepatomegaly, splenomegaly and host immune inhibition. MicroRNAs (miRNA) are endogenous 22-nucleotide RNAs that play important regulatory roles in gene regulation. miRNAs also modulate the innate and adaptive immune responses to pathogens. The potential applications of miRNAs as diagnostic or prognostic biomarkers have been demonstrated in various types of diseases. MicroRNA-146a (miR-146a) is involved in immune response and related with many infectious diseases. In this study, the expression of microRNA-146a in sera samples from 20 brucellosis patients and 20 healthy volunteers were analyzed by real time PCR assay. The mean expression

level of miR-146a in brucellosis patients was 0.71 μ M and ranged from 0.04 to 1.58 μ M. While that was 6.75 μ M and ranged from 0.18 to 22.53 μ M in healthy volunteers. The relation of expression level of miRNA-146a and antibody titer was analyzed by standard agglutination test (SAT), a confirmative serological assay for brucellosis. The 1:50 group showed an average of 4.6 ng/ul, and 1:100 showed 3.31, while 1:200 showed 1.54 ng/ul. The expression levels of the three groups differed significantly ($p < 0.01$). We also analyzed the possible correlation between miR-146a level and common clinical symptoms. Patients with symptom of fever and/or sweating showed lower level of miR-146a. The results indicate that the expression levels of miR-146a are significantly inhibited in brucellosis patients and negatively relate with serum antibody titers and clinical symptoms of fever and sweating.

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Colonization of resident pathobionts in the I κ B ζ signal-deficient skin accelerates atopic dermatitis-like syndrome

Kim, Y., Lee, Y.-S., Lee, S.-H., Yang, J.-Y., Kweon, M.-N.

University of Ulsan College of Medicine/Asan Medical Center, Seoul, Korea, Republic of

I κ B ζ , encoded by the *Nfkbiz* gene, is a member of the nuclear I κ B family of proteins that act as a transcriptional regulator via association with NF- κ B. Previous studies showed spontaneous development of atopic-like dermatitis in the I κ B $\zeta^{-/-}$ mice; however, underlying mechanism is still unveiled. In this study, we revealed that high levels of skin pathologic score, serum IgE Ab, and trans-epidermal water loss (TEWL) in the I κ B $\zeta^{-/-}$ mice than I κ B $\zeta^{+/+}$ mice. Predominant levels of pro-inflammatory cytokines (i.e., IL-1b, IL-6, and TNF- α) and chemokines (i.e., MCP-1, MIP-1 α , RANTES, and KC) were detected in skin homogenates of I κ B $\zeta^{-/-}$ mice. Importantly, numerous numbers of langerin⁺CD11b⁺CD103⁺ and langerin⁺CD11b⁻CD103⁺ cells were recruited into the inflamed skin of I κ B $\zeta^{-/-}$ mice. We also found that expansion of IFN- γ -, IL-17-, IL-22-secreting CD4⁺ T cells and IL-17-secreting TCR $\gamma\delta$ ⁺ T cells in the skin of I κ B $\zeta^{-/-}$ mice when compared to those of I κ B $\zeta^{+/+}$ mice. Of note, universal bacteria 16s rRNA levels were markedly increased and some *staphylococci* species were specifically expanded in the skin of I κ B $\zeta^{-/-}$ mice. Oral treatment of antibiotics containing cephalixin and enrofloxacin ameliorated skin inflammation. These results demonstrate that colonization of commensal *staphylococci* species might be one of causes of skin dysbiosis which provoked in I κ B $\zeta^{-/-}$ mice, and highlight that I κ B ζ gene has a potential regulatory role in microbiota-skin immunity axis.

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Role of renal CD169⁺ F480⁺ resident macrophages in acute systemic candidiasis

Teo, Y.J., Ruedl, C.

Nanyang Technological University, Singapore, Singapore

Systemic Candidiasis is one of the most frequent nosocomial infections to date, with mortality rate as high as 40%. Although the importance of neutrophils in this infection is well-established,

current understanding of the role of mononuclear phagocytic (MNPs) subsets, which includes DCs and macrophages, against invasive *Candida* infection is limited due to the lack of appropriate tools to specifically ablate mononuclear phagocytic cell subsets. Here, we reiterate the essential roles of neutrophils in host defense against invasive *Candida* infection, where the ablation of neutrophils *in vivo* causes rapid death of the mice as soon as 2 days post infection. Correspondingly, these mice display significantly higher fungal burden in kidneys and liver, with kidneys having the highest fungal load. To elucidate the role of tissue resident macrophages in the immunity against systemic candidiasis, we utilized CD169-DTR transgenic mouse in which CD169⁺F480⁺ cells can be specifically ablated in all organs upon DT injection. We observed that DT treated CD169-DTR mice consistently exhibit increased susceptibility to systemic *Candida* infection compared to the wild type mice. Importantly, these transgenic mice exhibited exceedingly high fungal burden only in the kidney while *Candida* presence was undetectable in all other organs. These observations suggest that renal CD169⁺F480⁺ resident macrophages are irreplaceable in the host defense against systemic candidiasis. Whether these renal CD169⁺F480⁺ resident macrophages are involved in clearing the fungal burden or regulating the host immune system against invasive *Candida* infection remains a question and is currently our main focus of study.

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Phospholipase C mediates the therapeutic effects of Trp-Lys-Tyr-Met-Val-D-Met against sepsis

Kim, H.S.¹, Lee, S.K.¹, Park, M.Y.¹, Kim, J.C.¹, Bae, Y.-S.^{1,2}

¹Sungkyunkwan University, Biological Sciences, Suwon, Korea, Republic of, ²Sungkyunkwan University, Health Sciences and Technology, Seoul, Korea, Republic of

Severe sepsis, a principal cause of death in intensive care units, results from a detrimental or damaging host reaction to infection. Previously we demonstrated that an immune stimulating peptide, Trp-Lys-Tyr-Met-Val-D-Met (WKYMVm), strongly induced therapeutic effects against polymicrobial sepsis by stimulating bacterial clearance and suppressing inflammatory cytokine production. We also reported that the peptide-induced anti-septic activity was mediated by an important leukocyte chemoattractant receptor, formyl peptide receptor 2 (FPR2). Here, we further investigated the mechanism involved in WKYMVm-induced therapeutic effects against sepsis focusing on putative role of phospholipase C (PLC). WKYMVm strongly stimulated mouse neutrophils, resulting in calcium increase which was completely blocked by a PLC-selective inhibitor (U-73122). PLC-specific inhibitor U-73122 markedly blocked the therapeutic effects of WKYMVm against sepsis. WKYMVm-induced inhibition of lung inflammation and lymphocyte apoptosis in cecal ligation and puncture were markedly blocked by the PLC-selective inhibitor, U-73122. We also found that WKYMVm-stimulated bactericidal activity and neutrophil migration to event area were also strongly inhibited by U-73122. Taken together our results, we suggest that PLC activity is required for the therapeutic activity of WKYMVm against sepsis.

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The role of IL-21 producing and nonproducing follicular helper T cells in mycobacterial infection*Shimohakamada, Y.^{1,2}, Tamura, T.², Umemura, M.³**¹National Sanatorium Tama-Zenshouen, Higashimurayama, Japan, ²Leprosy Research Center, National Institute of Infectious Disease, Department of Mycobacteriology, Higashimurayama, Japan, ³Tropical Biosphere Research Center, University of the Ryukyus, Molecular Microbiology Group, Development of Tropical Infectious Diseases, Nakagami, Japan*

T follicular helper (Tfh) cells are the specialized CD4 T cell subset that has evolved the appropriate mechanisms to induce the activation and differentiation of B cells into antibodies secreting cells. Therefore, Tfh cells are believed to be involved in induction of the humoral immunity. However, the involvement of Tfh cells in the protective immunity was not clearly demonstrated in mycobacterial infected mice. In this study, to verify the role of Tfh cells in the protective immunity in pulmonary *M. bovis* Bacille Calmette-Guerin (BCG) infection, we used IL21 GFP reporter mouse (IL21 GFP mouse) which was introduced sequence encoding GFP into the il21 locus, because Tfh cells are identified by elevated expression levels of IL-21 secretion. We have found that IL-21 producing (IL21 GFP+) and IL-21 non-producing (IL21 GFP-) cells were detected in a population of Tfh cells (CD4+ PD1+ CXCR5+ CD62L-) in the BCG infected mice. To evaluate of IL21 GFP- Tfh cells, we examined the transcription factors that could regulate the helper T cell function. We found that some of IL21 GFP- Tfh cells have foxp3 molecules.

These results suggest that mycobacterial infection may induce differentiation of inducible Treg cells from conventional naive CD4 T cells.

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Attenuation by platelets of macrophage inflammatory responses to bacterial endotoxin*Ando, Y., Oku, T., Tsuji, T.**Hoshi University, Microbiology, Tokyo, Japan*

Considerable evidence has been accumulated concerning the roles of platelets in immune responses. In this study, we focused on the cellular interaction between platelets and macrophages and examined the influences of platelets on the inflammatory responses of macrophages induced by bacterial components. When mouse bone marrow-derived macrophages (BMDMs) were co-cultured with platelets, BMDMs produced lower levels of nitric oxide (NO), TNF- α and IL-6 in response to a bacterial endotoxin (LPS) and zymosan. The attenuation in the macrophage susceptibility to LPS appeared to be mediated by soluble factors secreted from platelets. The mRNA levels of NOS2 (iNOS), TNF- α and IL-6 in LPS-stimulated BMDMs that had been cultured with a conditioned medium of unactivated or thrombin-activated platelets were also decreased as analyzed by RT-qPCR. The ability of the platelet-conditioned medium to suppress macrophage NO production was recovered in a

high-molecular-weight fraction (>670 kDa) after gel-filtration chromatography on a Superose 6 column. These results suggest that platelets control the susceptibility of macrophages to prevent excessive responses to LPS and provide mechanistic insight into the development of septicemia and new therapeutic strategies for septic shock.

Immunity to Parasites

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PD-1 dependent exhaustion of CD8⁺ T cells drives chronic malaria*Karunaratne, D.S.¹, Horne-Debets, J.M.¹, Faleiro, R.¹, Q. Liu, X.², E. Lineburg, K.¹, Meng Poh, C.³, Grotenbreg, G.M.³, Hill, G.R.¹, MacDonald, K.P.A.¹, Good, M.F.², Renia, L.⁴, Ahmed, R.⁵, Sharpe, A.H.⁶, Wykes, M.N.¹**¹QIMR Berghofer Medical Research Institute, Herston, Australia,**²Institute for Glycomics, Griffith University, Gold Coast Campus,**Gold Coast, Australia, ³Singapore Immunology Network, Agency for Science, Technology and Research (A STAR), Singapore,**Singapore, ⁴Singapore Immunology Network, Agency for Science, Technology and Research (A*STAR), Singapore, Singapore, ⁵Emory Vaccine Center, Atlanta, United States, ⁶Harvard Medical School, Department of Microbiology and Immunobiology, Boston, United States*

Malaria, caused by *Plasmodium* parasites, is a highly prevalent and devastating disease that can persist for years. There has been considerable difficulty in developing a malaria vaccine, highlighting our incomplete understanding of immunity against this disease. Antibodies and CD4⁺ T cells are thought to protect against blood-stage infections. We used an experimental rodent malaria model, to show that programmed death-1 (PD-1) mediates a 95% reduction in numbers and functional capacity of parasite-specific CD8⁺ T cells during acute malaria, driving chronic disease. Furthermore, we demonstrated PD-1 also affect CD4⁺ T cell function that, improved effector CD4⁺ and CD8⁺ T cell function during the chronic phase of infection, compared to wild-type mice. Importantly, in contrast to widely held views, parasite-specific CD8⁺ T cells are required to control both acute and chronic blood-stage disease even when parasite-specific antibodies and CD4⁺ T cells are present. Our findings provide a molecular explanation for chronic malaria which will be relevant to future malaria-vaccine design and may need consideration when vaccine development for other infections is problematic.

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Exogenous calreticulin, incorporated onto non-infective *Trypanosoma cruzi* epimastigotes, promotes their internalization into mammal hosts cells*Sosoniuk Roche, E.¹, Vallejos, G.¹, Aguilar Guzmán, L.¹, Pizarro Bäuerle, J.¹, Weinberger, K.¹, Rosas, C.¹, Valck, C.¹, Michalak, M.², Ferreira, A.¹**¹University of Chile, Faculty of Medicine, Immunology, Santiago,**Chile, ²University of Alberta, Department of Biochemistry, Alberta, Canada*

Chagas disease is an endemic pathology in Latin America, now emerging in developed countries, caused by the intracellular protozoan *Trypanosoma cruzi*. *T. cruzi* life cycle involves three stages: amastigotes, epimastigotes, and trypomastigotes. We have shown that, in infective trypomastigotes, *T. cruzi* Calreticulin (TcCRT), an endoplasmic reticulum (ER) resident chaperone, translocates to the cellular membrane, where it captures complement component C1q thus interfering with C4 activation, with consequent blocking of the classical pathway. Trypomastigote-bound C1q is detected as an "eat me" signal by macrophages and promotes the infective process. Thus, TcCRT is a new virulence factor. Different from infective trypomastigotes, non-infective epimastigotes express only marginal levels of TcCRT on their external membranes. We now show that epimastigotes bind exogenous rTcCRT to still unidentified moieties on their cellular membrane and, in the presence of C1q, this parasite form is internalized into normal fibroblasts. On the other hand, CRT- deficient fibroblasts show impaired parasite penetration. Consequently, CRTs, from both parasite and host cell origins, are important in the establishment of a C1q-dependent synapsis that allows the first contact between these two cell types. Perhaps, the epimastigote inability to infect mammal cells is due, at least partly, to an impaired capacity to translocate their CRT from the ER to the parasite surface. Financed by Regular FONDECYT project 1130099, from CONICYT-Chile.

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Characterisation of CD4⁺ T cell responses during malaria

Edwards, C.^{1,2}, *de Labastida Rivera, F.*¹, *Montes de Oca, M.*¹, *Ng, S.*^{1,3}, *Engwerda, C.*¹

¹QIMR Berghofer Medical Research Institute, Brisbane, Australia,

²University of Queensland, School of Medicine, Brisbane, Australia,

³Griffith University, School of Natural Sciences, Brisbane, Australia

CD4⁺ T cells are important in determining disease outcome during *Plasmodium* infection. Recently, a subset of CD4⁺ T cells which co-produce IFN γ and IL-10 (Tr1 cells) has been identified in mouse models of malaria. Importantly, Tr1 cell frequency has also been reported to significantly increase in response to *P. falciparum* infection in children living in malaria endemic regions of Africa. However, whether Tr1 cells are beneficial by prevention of inflammation and associated tissue damage or detrimental by suppression of anti-parasitic immune responses remains unclear. To better understand the role of Tr1 cells in malaria we isolated these cells from healthy and *P. falciparum* infected volunteers, as well as naïve CD4⁺ T cells and Th1 cells from the same individuals, using cytokine capture and cell sorting. Isolated cell populations were subjected to RNA-seq analysis to identify distinctive markers of Tr1 cells during malaria. Results from these studies will be presented and discussed.

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Reactive oxygen and nitrogen species levels produced by *Leishmania major*-infected phagocytes as host surrogate parasite virulence markers

Ben-Cheikh, A., Sghaier, M.R., Bali, A., Mkannez, G., Atri, C., Chourabi, K., Attia, H., Guerfali, F.Z., Laouini, D.
Institut Pasteur de Tunis, Transmission, Control and Immunobiology of Infections, Tunis-Belvedere, Tunisia

Zoonotic cutaneous leishmaniasis (ZCL) caused by *Leishmania (L.) major* shows various clinical manifestations, ranging from asymptomatic infection without apparent lesion, to extensive lesions that might cause severe disfiguring scars. This clinical expression is due to the host immune status, to the parasite intrinsic virulence or to both. Ability to predict clinical outcome is crucial when prescribing treatment or determining possible control measures in epidemiological studies.

During its life cycle, *Leishmania* encounters various hostile conditions such the two highly toxic radicals for *Leishmania*, reactive oxygen and nitrogen species (ROS and RNS) generated during phagocytosis by host cells

In this work, we sought to identify host cellular surrogate markers for *Leishmania* virulence that can discern between virulent and low virulent isolates i.e., the cellular levels of nitric oxide (NO) and reactive oxygen species production.

Using a selected couple of *L. major* isolates according to their contrasting virulence and three cell types i.e., human activated THP1 cells, human polymorphonuclear neutrophils (PMN) and murine macrophagic Raw 264.7 cells, we showed that virulent parasites are inducing higher NO and ROS amounts than non-virulent ones, contrasting with their corresponding parasite load. A collection of well-characterized isolates will be used to infect cells, to monitor NO and ROS produced amounts upon infection and to correlate them with lesion severity and pathogenicity in experimental animal model.

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TLR4 and RANTES triggered immunomodulation is critical for malaria disease outcome: a northeast India tribal population based study

*Bujarbaruah, D.*¹, *Kalita, M.P.*², *Hazarika, S.*³, *Baruah, V.*², *Basumatary, T.K.*², *Pathak, P.K.*⁴, *Mahanta, B.*⁴, *Bose, S.*²

¹Dimoria College, Zoology, Guwahati, India, ²Gauhati University, Biotechnology, Guwahati, India, ³Dimoria College, Botany, Guwahati, India, ⁴Community Health Centre, Rani, Guwahati, India

The study aimed to evaluate the critical role of TLR4 in association with serum-CD14 levels, its downstream immunomodulatory targets in the pathogenesis of malarial infection, and deciding the outcome of the disease.

Methods: Clinically proved malaria cases of tribal ethnicity (n=60) were enrolled on follow-up basis with all clinical details, pathogen load and informed consent; from northeast India. Blood collection was done on follow-up basis (baseline and third day). Differential TLR4 expression was evaluated by MUSE platform. TLR4 stimulators co-receptor sCD14 and chemokine RANTES levels were studied by ELISA. The differential expression of both TLR4 and RANTES at both protein and mRNA level was

correlated with downstream expression of pro-inflammatory and inflammatory markers NFkBp65, TNF α and IFN γ ; chemokine receptor CCR5; pathogen load, and association with disease susceptibility, severity and recovery. Statistical analysis was performed by SPSSV13.0 software.

Results: TLR4 expression increased in mild malaria cases(3.12 \pm 1.42%) compared to controls(1.02 \pm 0.36%) and severe malaria cases(1.22 \pm 0.98%). TLR4 expression increased significantly on recovery in paired cases(p=0.037). Expression of RANTES was significantly lower in severe malaria cases(15708.92ng/L) compared to mild(16147.74ng/ml)(p=0.046) and control cases (18587.2ng/L)(p< 0.001); and increased after recovery in individual paired mild malaria cases (p=0.106). TLR4 and RANTES expression inversely correlated with parasite load. NFkBp65, TNF α and IFN γ levels were significantly elevated on mild malaria cases compared to controls and severe malaria cases; TLR4, RANTES levels; and correlated with disease recovery at both protein and mRNA level.

Conclusions: TLR4 and associated immunomodulators plays a critical role in malaria susceptibility, severity and recovery, and has immense therapeutic implications.

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Severe acquired toxoplasmosis in children

Fitrani, D.A.

Harapan Kita Women and Children Hospital, Pediatrics, Jakarta-Barat, Indonesia

Introduction: Toxoplasmosis is a disease caused by the parasite *Toxoplasma gondii*. Toxoplasmosis can be postnatally acquired in children and the symptoms may vary.

Method: Case Presentation.

Results: We present a 14 months old boy with recurrent fever since he was 5 months old. His parents complained that his stomach got bigger and his face paler since he was 9 months old. He had history of severe anemia without bleeding. He was born full term without perinatal problems. The physical examination showed malnourished, gross motor delay, multiple lymphadenopathy and severe hepatosplenomegaly. Laboratory findings were anemia (5,9 g/dL), thrombocytopenia (64.000/ μ L), elevated ESR (102 mm/hour) and AST level (600 U/L). His procalcitonin level rised from 2 to 10 ng/mL. He received transfusions periodically but there were no significant changes and the platelets level was decreased more. He was first suspected had malignancy with secondary infection, but infection source was unconfirmed. Bone marrow puncture showed normoblastic marrow. The *Toxoplasma* IgM level was normal with elevated IgG level (39.00 IU/mL) and total IgE level (471.19 IU/mL). Biopsy of lymph gland showed reactive follicular hyperplasia suggesting infection confirmed by PCR *Toxoplasma* test which showed active toxoplasmosis. The patient received pyrimetamine and clindamycin for a few days but eventually he died due to severe blood loss caused by thrombocytopenia.

Conclusion: Severe multiple organ involvement is occasionally found in toxoplasmosis and can be misdiagnosed. The serodiagnostic of toxoplasmosis can be subsided and must be confirmed with physical examination and other diagnostic tests. Keywords: Toxoplasmosis, acquired, severe, children

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Human antibodies to *Plasmodium falciparum* merozoite surface antigens interact with Fc γ receptors to mediate immunity to malaria

Feng, G.¹, Wines, B.¹, Stubbs, J.¹, Richards, J.¹, Hogarth, M.¹, Osier, F.², Beeson, J.¹

¹The Burnet Institute of Medical Research and Public Health, Centre for Biomedical Research, Melbourne, Australia, ²KEMRI, Centre for Geographic Medicine Research, Kilifi, Kenya

Antibodies are known to protect against *P. falciparum* malaria by inhibiting parasite replication. The merozoite form of malaria parasites invades human red blood cells and replicates inside them, and is a major target of acquired antibodies. Recent studies have shown that opsonic phagocytosis of *P. falciparum* merozoites by monocytes, mediated by acquired human antibodies, is strongly associated with protective immunity to malaria. Fc γ -receptors have important roles in antibody-mediated opsonic phagocytosis and subsequent immune activation and regulation. Understanding how antibodies interact with Fc γ -receptors may be crucial in developing highly effective vaccines. We evaluated the role of different Fc γ -receptors in promoting opsonic phagocytosis using Fc γ -receptor blockers, and confirmed that mutation of the FcR-binding site of a recombinant human monoclonal antibody to a major merozoite surface protein ablated its ability to promote opsonic phagocytosis. Human immunization with a candidate vaccine induced antibodies that promote opsonic phagocytosis. To further investigate the roles of specific Fc γ receptors in immunity, we used recently developed Fc γ -receptor binding assays to define the ability of acquired human antibodies in engaging Fc γ RIIIa and Fc γ RIIIa in a longitudinal cohort of African children exposed to malaria. Together, our data suggest that activating Fc γ -receptors are key mediators in opsonic phagocytosis of merozoites and children with higher Fc γ -receptor binding activity are protected from malaria. Our findings support the potential of vaccines optimized to induce antibodies to merozoite surface antigens that interact with Fc γ -receptors, advance our understanding of the mechanisms and targets of protective immunity, and establish new assays for vaccine evaluation.

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Effects of helminth eradication on the immune system

Weisman, Z.¹, Mahdi, J.A.^{2,3}, Kalinkovich, A.^{1,4}, Stein, M.⁴, Greenberg, Z.^{4,5}, Borkow, G.¹, Adlerstein, D.^{1,2}, Bentwich, Z.^{1,2}

¹R. Ben-Ari Institute of Clinical Immunology, AIDS Center, Kaplan Medical Center, Rehovot, Israel, ²Ben Gurion University of the Negev, Microbiology Immunology and Genetics, BeerSheva, Israel, ³University of Gondar, Microbiology Immunology and Parasitology, Gondar, Ethiopia, ⁴Hebrew University, Hadassah Medical School, Rehovot, Israel, ⁵Public Health Laboratory, The Ministry of Health, Jerusalem, Israel

Background: Helminth infection has a profound effect on the immune system. However, the precise nature of the immune changes that are elicited by human helminth infection have not been sufficiently characterized. Furthermore, the reversibility of these changes after treatment has not been documented

sufficiently. We observed immune profiles of Ethiopian immigrants to Israel cohort.

Methods: A longitudinal follow up study involving different group of subjects were conducted. Each group had a baseline data for series of peripheral blood tests, including: IgE and Eosinophil levels, T-cell populations, T-cell receptors phenotypes, and cytokines measurement. These tests were all repeated at one year interval. Results were compared between newly-arrived-Ethiopian-Israelis (NEW-Eth-II), Long-stayed-Ethiopian-Israelis (OLD-Eth-II), and Non-immigrant-Israeli controls (NON-Imm-II).

Results: Out of the 184 individuals 111 were NEW-Eth-II, who had a high prevalence of helminth infection, the immunological changes were elevated IgE levels and eosinophil counts, decreased CD4/CD8 ratio, increased proportion of HLA-DR+CD3+, HLA-DR+CD4+ and HLA-DR+CD8+ cells, decreased proportion of CD45RA+CD4+ (naive) and CD28+CD8+ cells, increased proportion CD45RO+CD4+ (memory) cells, and increased secretion of IL-4 and IL-5 (Th2 type cytokines). In the OLD-Eth-II, who were all negative for helminth infection, we did not observe these immune changes and did not differ markedly from that of the NON-Imm-II controls. Significant normalizations in the above-mentioned variables were observed in 33 NEW-Eth-II who had received successful treatment but not in those who missed the treatment.

Conclusions: These findings demonstrate that helminth infection is associated with profound immune changes, and can be normalized within a short time after helminth eradication.

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Peroxybioflavonoids (MALSUP): a possible cure for severe cases of plasmodium falciparum malaria infection in Nigeria

Thornthwaite, J.T.¹, Akanni, E.O.², Ayankuule, A.A.³, Alli, O.A.T.²

¹Cancer Research Institute of West Tennessee, Henderson, United States, ²Ladoke Akintola University of Technology, Medical Laboratory Science Department College of Health Sciences, Osogbo, Nigeria, ³Ladoke Akintola University of Technology, Pharmacology & Therapeutics Department, Osogbo, Nigeria

Development of an antimalarial treatment capable of providing a permanent cure for malaria is presented. A trial of a novel formulation of Citrus Bioflavonoids and Artemisinin (PeroxyBioflavonoid™ or MALSUP™) was conducted in Osogbo, Nigeria following exciting reports from our previous study on malaria infected children for over 10 years in Haiti who are yet to record another episode of the infection. Children, ages 2-15, positive for P falciparum parasites, and whose parents gave consent, were among the 127 enrolled subjects treated with the MALSUP™ for 16 consecutive days. Serums were collected on days 0, 5, 10, 16, 30, and 60. The study shows that 90.2% of the patients showed no recurrence (termed "cured" in compliance with the literature definition where patients go at least 28 days without recurrence) by being parasite free by the standard blood tests at 30-60 days. Patient with serums (n=112) and buffy Coats (n=25) were obtained with 15 patients being lost to follow-up. Eleven so-called failures were reported, and three were from one family suggesting non-compliance. The cured patients

are currently being followed to confirm no recurrence of the infection. These data show for the first time a real possibility for a cure of Malaria in Nigeria. The one time, low dose, long term treatment minimizes the ability of the parasites to develop resistance. Having the serums and buffy coats obtained during the course of treatment will enable us to find the underlying antibody and cell-mediated immunological mechanism(s) of permanent immunity.

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Nod2/Rip2-mediated signaling contributes to shape adaptive immunity in visceral leishmaniasis

Nascimento, M.¹, Ferreira, M.², Quirino, G.³, Krishnaswamy, J.⁴, Liu, D.⁵, Berlink, J.⁵, Fonseca, D.³, Zamboni, D.³, Almeida, R.⁶, Carregaro, V.³, Cunha, T.³, Eisenbarth, S.⁵, Silva, J.S.³

¹Ribeirão Preto Medical School, Immunology, Ribeirão Preto, Brazil, ²Ribeirão Preto School of Medicine, University of São Paulo, Ribeirão Preto, Brazil, ³Ribeirão Preto Medical School, Ribeirão Preto, Brazil, ⁴Yale University School of Medicine, Immunology, New Haven, United States, ⁵Yale University School of Medicine, New Haven, United States, ⁶Federal University of Sergipe, Aracaju, Brazil

IFN-γ and IL-17A-producing cells are described to be related to protection against *Leishmania infantum* (*L. infantum*). How the immune system coordinates, or the parasite manipulates, the balance between Th1 and Th17 during visceral leishmaniasis (VL) is still unknown. We showed that Th17 is suppressed during *L. infantum* infection, and B cells are the major source of IL-17A. By using *Nod2*^{-/-} and *Rip2*^{-/-} mice we characterized this pathway as a negative regulator for Th17 cells in VL. On the other hand, the high level of Th1 induction was dependent on the NOD2-RIP2 signaling in CD8α⁺XCR1⁺ dendritic cells (DCs), which was crucial for IL-12 production through the phosphorylation of p38 and JNK. As a consequence, *Nod2*^{-/-} and *Rip2*^{-/-} mice showed a Th1 defective response, more Th17, and higher parasite loads compared to WT mice. Together, the data demonstrate that NOD2-RIP2 pathway plays a role in shaping adaptive immunity and promotes protection against VL caused by *L. infantum*.

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The programmed death-1-PD-L1 axis mediates lethality of malaria

Horne-Debets, J.^{1,2}, Karunarathne, D.¹, Faleiro, R.¹, Renia, L.³, Ahmed, R.⁴, Sharpe, A.⁵, Wykes, M.¹

¹QIMR Berghofer, Brisbane, Australia, ²The University of Queensland, The School of Medicine, Brisbane, Australia, ³A*STAR, Singapore, Singapore, ⁴Emory Vaccine Center, Atlanta, United States, ⁵Harvard Medical School, Boston, United States

Programmed death-1 (PD-1)-mediated dysfunction, or 'exhaustion' of T cells is a phenomenon that promotes cancer and chronic viral infections. Although the effects of PD-1 on chronic diseases have been well characterised, the role of PD-1 in severe infections has been less thoroughly examined. We previously showed that PD-1-dependent exhaustion of T cells resulted in incomplete protection against acute malaria, leading to chronic disease. In our current study, we utilised an acute,

lethal murine model of malaria to examine the effects of PD-1 on early T cell function in severe infections. We found from days 5-7 post-infection, PD-1 was expressed by a large proportion of T cells. Mechanistic studies showed mice deficient in PD-1, or administered with PD-1-blocking antibodies, survived lethal disease. Using PD-1-deficient mice that were also unable to express interferon-gamma or perforin, we showed a role for these effector molecules in protection. Adoptive transfer studies showed that dendritic cell-expressed PD-L1 was responsible for lethality. Overall, we demonstrated the capacity of the PD-1/PD-L1 axis to negate T cell immunity to this acute, lethal, systemic infection.

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MIF promotes classical activation and conversion of inflammatory Ly6C^{high} monocytes into Tip-DCs during murine toxoplasmosis

Ruiz-Rosado, J.D.D.¹, Olguín, J.E.¹, Saavedra, R.¹, Juárez-Avelar, I.¹, Robledo-Avila, F.H.², Fernández, J.¹, Vazquez-Mendoza, A.¹, Terrazas, L.I.¹, Satoskar, A.R.³, Partida-Sánchez, S.², Rodríguez-Sosa, M.¹

¹Universidad Nacional Autónoma de México, Biomedicine, Mexico City, Mexico, ²Nationwide Childrens Hospital, Research Institute, Columbus, United States, ³Ohio State University, Pathology, Columbus, United States

Toxoplasma gondii (*T. gondii*) is an obligate intracellular parasite that causes toxoplasmosis, a usually asymptomatic disease in healthy individuals but a potentially fatal illness in immunocompromised hosts. The cytokine Macrophage Migration Inhibitory Factor (MIF) is known to mediate immunity against *T. gondii* infection. However, the mechanism of MIF in the resistance to acute toxoplasmosis remains poorly understood. Here, we used *T. gondii*-infected WT and *Mif*^{-/-} mice to determine the role of MIF in the immune response against *T. gondii*. We found that the absence of MIF resulted in reduced numbers of IFN- γ producing Natural Killer (NK) cells in the site of infection and consequently impaired maturation of CD11b⁺ Dendritic Cells (DCs). Additionally, infected *Mif*^{-/-} mice showed increased parasitic burden in brain and significantly less numbers of both, TNF- α /iNOS producing CD11b⁺ DCs (TipDCs) and classically activated Ly6C^{high} monocytes, compared to acutely infected WT mice. The adoptive transfer of bone marrow WT or *Mif*^{-/-} Ly6C^{high} monocytes into *T. gondii*-infected mice, demonstrated that MIF is required for Ly6C^{high} monocytes differentiation to TipDCs, which increased their anti-microbial activity to *T. gondii*. Finally, administration of rMIF into *T. gondii*-infected *Mif*^{-/-} mice restored the numbers of TipDCs and reversed the susceptible phenotype of *Mif*^{-/-} mice. Collectively, these results demonstrate a novel role for MIF in promoting activation and functional differentiation of inflammatory monocytes into TipDCs and highlight the potential of rMIF as a therapeutic agent in toxoplasmosis or other protozoan infectious diseases.

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Differential regulation of TLR2 and associated immunomodulation is pivotal for deciding the fate of malaria infection

Kalita, M.P.¹, Bujarbaruah, D.², Begum, R.H.³, Pathak, P.K.⁴, Basumatary, T.K.¹, Bose, M.¹, Shyam, N.⁵, Bose, S.¹

¹Gauhati University, Biotechnology, Guwahati, India, ²Dimoria College, Zoology, Guwahati, India, ³Assam University, Diphu Campus, Lifescience and Bioinformatics, Diphu, India, ⁴Community Health Centre, Rani, Guwahati, India, ⁵Diphu Civil Hospital, Diphu, India

The present prospective study aimed to evaluate the role of differential TLR2 and co-receptor CD14 expression and resulting downstream cytokine modulation in susceptibility to malaria disease, and outcome of the disease.

Methods: Blood samples of clinically proven cases of malarial disease (N=55) were collected on follow-up basis (baseline, and after 3 days) along with healthy controls (N=60), from Assam. Differential TLR2 and mCD14 expression was studied by ELISA and MUSE analysis respectively. Serum level expression of cytokines NF κ B, TNF α , IL12, IL10, myd88 was evaluated by ELISA and RT-PCR. Statistical analysis was performed by SPSS software.

Results: Expression of TLR2 was found to be significantly lower in severe malaria cases (6676.66 \pm 1396.13pg/ml) compared to malaria (7399.125 \pm 2612.334pg/ml). The paired follow-up mild malaria cases after recovery showed an increased level of TLR2 (9358.12 \pm 2168pg/m). Expression of CD14 was in concurrence with TLR2 data as shown: Severe (3.1 \pm 0.846%) < Controls (3.32 \pm 1.03%) < mild (4.25 \pm 1.72%); and increased on recovery in mild malaria paired samples of individuals (5.69 \pm 1.84%). Serum level expression of TNF- α was found to be down regulated in severe malaria (3.022 \pm 0.456pg/ml) compared to both mild malaria (3.5816 \pm 0.1877)(p=0.212) and healthy controls (3.015 \pm 0.876)(p=0.382). NF κ B protein expression levels were found to be down-regulated in the severe malaria cases (0.145 \pm 0.062) compared to mild (0.523 \pm 0.4932 μ g/ml)(p=0.114) and controls (0.378 \pm 0.1213)(p=0.687). Both increase in IL12 and IL10 expression was noted in mild and severe malaria cases compared to controls. The protein expression data was concurrent with mRNA data. TLR2 expression correlated positively with mCD14, TNF α , NF κ B levels and recovery.

Conclusions: TLR2 mediates recovery from malaria through modulation of key immunomodulators in association with mCD14, and has prognostic and therapeutic significance.

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Effects of the host immune response against precedent infection on the establishment of successively infected gastrointestinal nematode

Ishiwata, K.

Jikei University School of Medicine, Department of Tropical Medicine, Tokyo, Japan

The fact that eggs of several species of soil-transmitted helminth are often detected in a sample of clinical stool examinations suggests that patients are usually infected with more than one species of gastrointestinal parasites. Host immune responses

against one species of infected parasite may affect other species of concurrently resident parasites. In this study, I examined effects of precedent infection of *Nippostrongylus brasiliensis* (Nb) on consecutively infected *Heligmosomoides polygyrus* (Hp). These two gastrointestinal nematodes dwell in the small intestine of mice, although the former is expelled by day 10 post-infection (pi) and the latter persists to infect over day 40 pi. Precedent Nb infection rendered both shortened infection period and impaired egg production performance (fecundity) of consecutively infected Hp. The impairment of fecundity was restored when the worms were transferred surgically into the small intestine of nude mice. This indicated that the impairment is reversible host immune-mediated effect. The impairment neither depends on antibodies nor mast cells, but depends on IL-4R signaling. When Nb was treated with pyrantel pamoate just before establishment of Hp in the intestinal lumen, the impairment was reduced. This suggested the importance of coexistence of Nb. The impairment is unlikely due to the nutritious competition between two species, because in IL-4Ra KO mice, that fail to expel Nb, the fecundity level of Hp of mixed infection was not significantly different from that of singularly infected Hp. These results suggest that precedent parasite infection disturbs prosperity of the consecutively infected parasite through the host immune response.

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Antibody and complement interactions in human immunity to malaria

Kurtovic, L.^{1,2}, Feng, G.¹, Reiling, L.¹, Fowkes, F.¹, Kazura, J.³, Dent, A.³, Beeson, J.^{1,2}

¹Burnet Institute, Centre for Biomedical Research, Melbourne, Australia, ²Monash University, Department of Immunology, Melbourne, Australia, ³Case Western Reserve University, Cleveland, United States

Malaria is a major cause of global morbidity and mortality, particularly in young children. An efficacious vaccine is urgently needed; however, this has proven to be challenging as the targets and mechanisms of protective immunity are undefined. The sporozoite developmental stage of the malaria parasite is an attractive target that prevents the onset of blood-stage parasitemia and may induce sterile immunity. Leading vaccine candidate, RTS,S, is based on the sporozoite antigen, CSP. RTS,S-induced anti-CSP antibodies are associated with protection, however, it is unknown how these antibodies function. Antibody functions may include invasion-inhibition/neutralisation, opsonisation, lysis, and complement activation via C1q-fixation. Here we investigated if anti-CSP antibodies could fix complement, and whether this is an important immune mechanism against sporozoites.

Using cohorts of malaria-exposed individuals we characterised the prevalence and acquisition of anti-CSP antibodies, and measured their ability to fix complement proteins. Anti-CSP antibodies were predominately IgG1, IgG3 and IgM, and could effectively fix C1q and subsequent complement proteins. Little C1q-fixation was observed for samples that lacked acquired anti-CSP antibodies, indicating that complement activation was antibody-dependent. Additionally, we identified a specific CSP

epitope that is targeted by C1q-fixing antibodies. We also show evidence that sporozoites are susceptible to C1q-fixation and complement activation. This utilised human antibodies, and vaccine-induced antibodies generated in rabbits. Antibody-complement interactions may be important in immunity to malaria, and could lead to sporozoite neutralisation, invasion-inhibition, or direct lysis.

Defining the key functions of antibody-complement interactions will be valuable for understanding immunity to malaria and developing highly effective malaria vaccines.

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Fighting at the frontier: chondroitin sulfate proteoglycans and the immune response to pathogens in the placenta

Randall, L.¹, Gunatillake, T.², Hasang, W.¹, Kirby, J.¹, Said, J.², Stephen, R.¹

¹The Peter Doherty Institute for Infection and Immunity and The University of Melbourne, Department of Medicine, The University of Melbourne, Australia, ²The University of Melbourne, Centre for Health, Research & Education, North-West Academic Centre, St Albans, Australia

During pregnancy, the maternal immune system is in an altered state. This allows a careful balance between the arms of the immune system so that the fetus is not rejected. Products produced by the placenta have a role in modulating the immune response. However, the placenta is also a site of choice for key pathogens that greatly impact the health of the mother and the infant. The malaria-causing parasite, *Plasmodium falciparum*, sequesters in the placenta by attaching to the glycosaminoglycan chondroitin sulfate A, which may lead to severe anaemia in the mother and poor outcomes in the baby. Proteins with glycosaminoglycan chains, known as proteoglycans, are often components of the extracellular matrix but they may have important immunomodulatory functions. Here, we characterise the chondroitin sulfate proteoglycans within the placenta. Chondroitin sulfate A and isolated placental chondroitin sulfate proteoglycans are tested for their ability to modulate the cellular immune response to *P. falciparum*-infected red blood cells, as well as to other parasite and bacteria products. The impact of infection on the expression of chondroitin sulfate proteoglycans in the placenta is also examined.

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IgG antibody and cytokines in *Plasmodium vivax* infected individuals living in a low malaria transmission area in Amazon region, Pará state, Brazil

Santos, M.C.^{1,2}, Silva, A.N.¹, Oliveira, C.F.¹, Póvoa, M.M.³, Ventura, A.M.^{2,3}, Ohnishi, M.D.², Cunha, M.G.¹

¹Universidade Federal do Pará, Belém, Brazil, ²Universidade do Estado do Pará, Belém, Brazil, ³Instituto Evandro Chagas, Ananindeua, Brazil

Introduction: The acquired antibody immune response in malaria has been reported to antigens from asexual stage of the Plasmodium. Among the best characterized antigens of this stage is the Merozoite Surface Protein 1 (MSP1). However,

few studies have evaluated the acquisition of IgG antibody and cytokines in *Plasmodium vivax* malaria.

Objective: This study was conducted to analyze the IgG antibody response and TNF- α , INF- γ and IL-10 cytokines levels among infected individuals living in low malaria transmission area, where *P. vivax* is the most prevalent species.

Methods: The IgG antibodies against MSP119 and cytokines were evaluated by ELISA in sera from 99 malaria patients living in Pará state, Brazil. The median age was 35 years, 70% male and the median parasitaemia was 6,140 parasites/ μ l. The samples sera were collected on the day of parasite detection by thick blood smear (day0, n=99), and day14 (n=63). At the moment of diagnosis all patients received treatment.

Results: The percentage of sera that recognized the recombinant protein (PvHis6MSP119) was 96% (day0) and 98% (day14). At day0 we detected IL-10 and INF- γ , but not TNF- α . After treatment (day14), only INF- γ was detected, but the levels decreased in comparison to patent infection. The levels of this cytokine (pg/ml) were 31,64 \pm 74,94(day0) and 17,80 \pm 57,19 (day14). Both IgG and INF- γ response were detected in the sera before and after treatment.

Conclusion: The MSP1 was immunogenic and antibody response occurred together with INF- γ production. It suggests that INF- γ can participate in effector mechanisms mediated by IgG antibodies in *P. vivax* malaria.

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The role of IL-17 in *Trichinella spiralis* infection

*Takamoto, M.*¹, *Yamanoi, M.*², *Nakayama, J.*²

¹Shinshu University School of Medicine, Department of Infection and Host Defense, Matsumoto, Japan, ²Shinshu University School of Medicine, Department of Molecular Pathology, Matsumoto, Japan

Differentiation of Th cell subsets influences one another. During nematode infection, Th2 immune response becomes dominant, therefore Th1 and Th17 cells are considered to play only limited role against nematode infection. In order to investigate the role of IL-17 in the protection against nematode infection, we infected mice lacking Th2 immune response (STAT6 KO) with an intestinal nematode, *Trichinella spiralis*. IFN- γ deficiency resulted in Th17 cell differentiation (Immunol Lett 127, 55-9, 2009), therefore STAT6 and IFN- γ receptor (GR) double KO (DKO) mice were compared to STAT6 KO littermates. Recovery of muscle larvae in DKO mice infected with muscle larvae were decreased compared to STAT6 KO mice. IL-17 production from the mesenteric lymph nodes was higher in DKO than in STAT6 KO mice. Mucus production and pathological change in the small intestine were now being investigated. Further experiments should be necessary also using GR KO and IL-17 KO mice.

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Malaria-induced bone disorder triggered by chronic inflammation in the bone

*Lee, M.S.J.*¹, *Maruyama, K.*², *Ishii, K.J.*^{3,4}, *Akira, S.*², *Coban, C.*¹

¹Immunology Frontier Research Center (IFReC), Osaka University, Laboratory of Malaria Immunology, Osaka, Japan, ²Immunology Frontier Research Center (IFReC), Osaka University, Laboratory of Host Defense, Osaka, Japan, ³Immunology Frontier Research Center, Osaka University, Laboratory of Vaccine Science, Osaka, Japan, ⁴National Institute of Biomedical Innovation, Laboratory of Adjuvant Innovation, Osaka, Japan

Malaria is a life-threatening infectious disease that puts almost half of the world's population at risk. The disease can quickly develop into complication and cause death, or in some cases may leave the survivors with life-long sufferings due to chronic infection. Physical growth retardation in Africa is found to be associated with the burden of infectious diseases, with high prevalence among malaria-infected children regardless of nutritional status. This observation raises the concern that malaria infection could impose a detrimental effect on growth development. However, the pathology of malaria in bone has been an overlooked area that currently there are no studies directly addressing the effect of malaria on bone. In order to comprehensively understand the effect of malaria infection on bone, mice were infected with *Plasmodium* parasite to study the bone development during the acute phase of infection and the recovery phase after parasite clearance. The suppression of bone cells differentiation during acute infection brings bone development to a halt, rendering impaired bone growth. Nevertheless, once the infection is resolved, the exacerbate bone resorption leads to further bone loss. The parasite metabolite was found to accumulate in the bone during the clearance of parasites and remain there permanently, thereby eliciting chronic inflammation in a MyD88-dependent manner. These studies suggest the necessity of bone therapy and supplementation alongside antimalarial treatment to mitigate the risk of malaria-induced bone loss.

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Plasmodium falciparum protein PfEMP1 modulates activation of transcription factors and dampens the cytokine and chemokine response from monocytes/macrophages

Sampaio, N.^{1,2}, *Eriksson, E.*^{1,2}, *Schofield, L.*^{1,3}

¹Walter and Eliza Hall Institute of Medical Research, Population Health and Immunity, Parkville, Australia, ²University of Melbourne, Department of Medical Biology, Parkville, Australia, ³James Cook University, Australian Institute of Tropical Health and Medicine, Townsville, Australia

Plasmodium falciparum-induced malaria is a serious global disease burden, with significant mortality rates amongst young children. Cells of the innate immune system are essential for anti-malaria immunity, where monocytes/macrophages play a central role in parasite killing and cytokine production. However, the cytokine response from these cells can also contribute to pathogenesis due to excessive inflammation.

Thus, understanding how protective and pathogenic cytokine responses are regulated is a vital step towards modulating this response in order to lower disease burden. *P. falciparum* Erythrocyte Membrane Protein 1 (PfEMP1) is an important virulence factor found on the infected red blood cell surface. It mediates parasite binding to host cells, and has high antigenic variation in order to evade detection by antibodies. Using transgenic PfEMP1-null parasite strains, we showed PfEMP1 significantly inhibits NF- κ B activation in RAW macrophages upon stimulus with trophozoite-stage infected red blood cells. PfEMP1 also modulated activation of CREB, IRFs and C/EBP- α , resulting in decreased expression of immune genes, and reduced TNF and IL-10 release from macrophages. Similarly, human primary monocytes released lower levels of IL-1 β , IL-6, IL-10, MCP-1, MIP-1 α , MIP-1 β and TNF in response to wild type PfEMP1-containing parasites, compared to PfEMP1-null parasites, suggesting this immune regulation by PfEMP1 is important in naturally occurring *P. falciparum* infections. Collectively this data indicates that PfEMP1 is an immune-inhibitory molecule that affects the activation of a range of transcription factors, dampening the cytokine and chemokine immune response. These findings could potentially have implications for management of cytokine-induced severe disease caused by *P. falciparum* infection.

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Investigating the role of malaria-specific T cells in malaria pathogenesis

Ghazanfari, N., Gregory, J., Fernandez-Ruiz, D., Mueller, S.N., Heath, W.

The University of Melbourne, at the Peter Doherty Institute for Infection and Immunity, Microbiology and Immunology, Melbourne, Australia

Human cerebral malaria causes a diffuse encephalopathy associated with coma and is responsible for most malaria-related deaths globally. The presence of CD8⁺ T cells is required for the development of cerebral malaria in mice. The mechanism underlying cerebral malaria pathogenesis is not well understood. To address this, we used intravital microscopy to image the brains in live animals undergoing malarial infection. We injected mice with antigen-specific PbT-I CD8⁺ T cells or antigen-specific PbT-II CD4⁺ T cells a day before infection with *Plasmodium berghei* ANKA (PbA) blood-stage parasites. Two-photon laser scanning microscopy was performed through a cover-slipped cranial window, which allows observation of the immune responses occurring in the brain in real time. CB57Bl/6 mice infected with PbA showed symptoms of cerebral malaria 5-7 days after infection. PbT-I and PbT-II T cells were both present in the brain blood vessels from day 4 post-infection and entered the brain parenchyma by day 5. Flow cytometric analysis after intravascular Ab labelling confirmed the accumulation of transgenic T cells as well as endogenous CD4⁺ and CD8⁺ T cells within the brain parenchyma of both acutely infected and long-term cured mice. The presence of PbT-I T cells in the brain of cured mice was also confirmed by intravital microscopy. These results suggest that both CD4⁺ and CD8⁺ T cells infiltrate the brain during experimental cerebral malaria, with some cells forming

long-term memory in this site. They also raise the possibility that infiltration of CD4⁺ T cells into the brain parenchyma may contribute to cerebral immunopathology.

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RIPK1 and PGAM5 control Leishmania replication through distinct mechanisms

Farias Luz, N.¹, Balaji, S.², Okuda, K.³, Bertin, J.⁴, Gough, P.⁴, Gazzinelli, R.³, Bozza, M.⁵, Borges, V.¹, Ka-Ming Chan, F.²

¹Centro de Pesquisas Gonçalo Moniz- Fundação Oswaldo Cruz, Imuno Regulation, Salvador, Brazil, ²University of Massachusetts Medical School, Department of Pathology, Worcester, United States, ³University of Massachusetts Medical School, Division of Infectious Diseases and Immunology, Worcester, United States, ⁴Pattern Recognition Receptor Discovery Performance Unit, Immuno-Inflammation Therapeutic Area, GlaxoSmithKline, Collegeville, United States, ⁵Universidade Federal do Rio de Janeiro, Departamento de Imunologia, Instituto de Microbiologia, Rio de Janeiro, Brazil

Leishmaniasis is an important parasitic disease found in the tropics and sub-tropics. Human infection is mediated by phlebotomic bites from sand flies. The two major forms of the disease affect the skin and visceral organs and are referred to as cutaneous and visceral leishmaniasis respectively. Cutaneous and visceral leishmaniasis affect an estimated 1.5 million people worldwide. Despite its human health relevance, relatively little is known about the host immune mechanisms that control Leishmania replication. Necroptosis is a recently identified form of cell death with potent anti-viral effects. RIPK1 is a critical kinase that mediates necroptosis downstream of death receptors and toll-like receptors. Heme, a product of hemoglobin catabolism during certain intracellular pathogen infections, is also a potent inducer of macrophage necroptosis. In this study, we examined the impact of heme and necroptosis on Leishmania replication. We found that although heme potently inhibited Leishmania replication in bone marrow derived macrophages (BMDMs), Leishmania did not induce extensive necroptosis. Surprisingly, inhibition of RIPK1 kinase activity dramatically enhanced parasite replication in BMDMs and in mice. We further found that phosphoglycerate mutase family member 5 (PGAM5), a downstream effector of RIPK1, was also required for inhibition of Leishmania replication. In mouse infection, both PGAM5 and RIPK1 kinase activity are required for IL-1 β expression in response to Leishmania. However, PGAM5, but not RIPK1 kinase activity, was directly responsible for Leishmania-induced IL-1 β secretion in BMDMs. Collectively, these results revealed that RIPK1 and PGAM5 function independently to exert optimal control of Leishmania replication

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IL-17 is associated with protection in human visceral leishmaniasis*Singh, O.P.¹, Ansari, N.², Kumar, R.¹, Nylen, S.³, Rai, M.¹, Sacks, D.², Sundar, S.¹*¹Banaras Hindu University, Medicine, Varanasi, India, ²National Institute of Allergy and Infectious Diseases, National Institute of Health, Bethesda, United States, ³Karolinska Institutet, Department of Microbiology Tumor and Cell Biology, Stockholm, Sweden

Visceral leishmaniasis (VL) is a potentially fatal parasitic disease that impose a huge health problem on the vulnerable population claiming the lives of 0.2- 0.4 million worldwide. Better understandings of disease pathogenesis that dampen the immune response are critical for controlling the disease. We had previously shown the role of IL-27 and IL-21 in differentiation and expansion of antigen-specific IL-10 producing T cells and inhibition of Th17 differentiation in VL patients. Here, we investigated the IL-17 association with protection in human VL. We found elevated mRNA expression of indolamine 3-3-dioxygenase in VL patients, the enzyme that negatively regulates T-cell effector function during infection. No up-regulation of IL-17 or ROR γ T mRNA expression in spleens cells and plasma IL-17 levels were detected in active VL, however significantly elevated mRNA levels of both subunits of IL-23 were detected in post treated VL splenic biopsies compared to active patients. IFN- γ or/and IL-10 neutralization does not affect IL-17 levels in whole blood cells of active VL and 6 months cured VL. However, plasma IL-17 and IFN- γ levels were elevated in 6 month cured VL which were further enhanced with SLA stimulation in whole blood. Analysis of Th17/Th1 cytokines response in subjects from a cohort of endemic healthy individuals who were protected against VL showed that IL-17 is strongly associated with protection against VL. These findings were further supported by enhance parasite clearance with recombinant IL-17 (rhIL-17) and rhIFN- γ by inducing TNF- α mRNA and down regulating IL-10 in monocyte derived macrophages infected with *L. donovani* amastigote.

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Infectious status of sporozoites evokes innate response, leading to cell-mediated protective CD8+T cell response against Plasmodia infection*Parmar, R., Patel, H., Yadav, N., Tyagi, R., Dalai, S. Institute of Science, Nirma University, Biotechnology, Ahemdabad, India*

Multiple immunizations with radiation-attenuated sporozoites has reportedly conferred sterile-protection against liver stage infection of plasmodia. It is reflective of immunity in endemic areas where people are repeatedly exposed to parasite and maintain their anti-malarial immunity. CD8⁺T cells are the major effector cell populations which extends protective immunity against liver-stage infection. The infectious status of sporozoite has played an instrumental role in liver stage immunity, and impact the host response, which leads to CD8⁺T cell response in rendering sterile-protection. Present study shows that innate immunity induced by infectious spz challenge is qualitatively

different, and helps rescue the loss of CD8⁺T cell response as has been observed in γ -spz immunization without intermittent challenge. The danger signals perceived from the pathogen attack are decisive in establishing the nature of innate immune response, leading to long-term protection. The initial host response against the pathogen might dictates the protective memory response, and encompasses an array of innate immune factors. The DCs are an indispensable component of priming and generation of CD8⁺T cell responses. However, their role in evoking the long-term protective immunity to *Plasmodia* liver stage infection is still not clear. Present study assesses *plasmodium* infection to measure host innate response by the generation of long-lived protective CD8⁺T cells in the liver. In conclusion, our results suggest that infectious status of sporozoites may influence the maintenance of protective CD8⁺T cell response by modulating the activation status of DCs.

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Acquisition and longevity of antibodies to Plasmodium vivax pre-erythrocytic antigens in western Thailand*Longley, R.^{1,2,3}, Reyes-Sandoval, A.⁴, Montoya-Diaz, E.⁴, Dunachie, S.^{5,6}, Kumpitak, C.², Nguitragoon, W.⁷, Mueller, I.^{1,3,8}, Sattabongkot, J.²*¹Walter and Eliza Hall Institute of Medical Research, Population Health and Immunity, Melbourne, Australia, ²Mahidol University, Mahidol Vivax Research Unit, Bangkok, Thailand, ³University of Melbourne, Department of Medical Biology, Melbourne, Australia, ⁴Jenner Institute, Oxford, United Kingdom, ⁵Mahidol University, Mahidol-Oxford Tropical Medicine Research Unit, Bangkok, Thailand, ⁶University of Oxford, Centre for Tropical Medicine, Oxford, United Kingdom, ⁷Mahidol University, Department of Molecular Tropical Medicine, Bangkok, Thailand, ⁸Barcelona Institute for Global Health (ISGlobal), Barcelona, Spain

Plasmodium vivax (Pv) is the dominant species causing malaria in Thailand, yet little is known about naturally acquired immune responses to Pv in this low-transmission region. The pre-erythrocytic antigens Pv CSP, TRAP and CelTOS are currently being assessed pre-clinically as potential vaccine candidates. We therefore aimed to assess naturally acquired antibody responses to these proteins in an endemic region of western Thailand. We utilized a cross-sectional survey of 546 volunteers to demonstrate a strong association of increasing IgG positivity and magnitude with age, and in individuals currently infected with *Plasmodium*. Of interest, for CSP only, we found a significantly higher IgG magnitude in asymptomatic Pv infected volunteers compared to those uninfected, but observed no increase in magnitude in symptomatic Pv cases. We also utilized samples from a longitudinal cohort study to investigate the stability of these IgG responses. Two groups of individuals were assessed: one group whom remained Pv-free (by qPCR detection, n=98) and another whom experienced two or more blood-stage Pv infections during the year of follow up (n=50). IgG positivity was maintained over the one-year period in the absence of Pv infections. In contrast, for most proteins we saw a slight but significant decline in IgG magnitude over time. The exception was for CSP, where IgG magnitude was long-lived in the absence of on-going exposure, and only decreased

significantly over time in exposed volunteers. These findings demonstrate the presence of antibody responses to pre-erythrocytic antigens in Thailand, and show that long-lasting humoral immunity can develop in low-transmission regions.

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TNF deficiency leads to splenomegaly following cutaneous infection with *Leishmania major*

Hu, S.^{1,2}, Marshall, C.¹, Darby, J.¹, Lyons, A.B.³, Körner, H.¹

¹Menzies Institute for Medical Research, University of Tasmania, Hobart, Australia, ²Institute of Clinical Pharmacology, Anhui Medical University, Hefei, China, ³School of Medicine, University of Tasmania, Hobart, Australia

Cutaneous leishmaniasis in resistant C57BL/6 (B6.WT) mice is resolved spontaneously by Th-1 mediated production of TNF- α and IFN- γ , with consequent induction of inducible nitric oxide synthase. Intriguingly, TNF gene knockout C57BL/6 mice (B6.TNF^{-/-}) show an uncontrolled fatal infection involving the liver and spleen, which are the two main targets in visceral leishmaniasis. The exact role of TNF or the specific mechanism, which is responsible for the fatal outcome of *Leishmania* infection in this gene-deficient mouse strain, is not yet understood.

B6.TNF^{-/-} mice spleens significant enlarged from Day 28 post-infection, with increasing more than 2-fold larger than B6.WT mice on Day 35 and Day 42. A limiting dilution assay showed *Leishmania major* (*L. major*) was present from Day 21 in B6.TNF^{-/-} mice, but none were evident in B6.WT mice. The splenic architecture of infected B6.TNF^{-/-} mice was disorganized, *L. major* detectable throughout the spleen and a significant lymphoid cell infiltration.

A growing population of inflammatory bone marrow-derived cells emerged in the spleens of *L. major*-infected B6.TNF^{-/-} mice, exhibiting a CD45⁺CD11b⁺CD11c^{high}CCR2⁺Ly6C⁺ phenotype and, surprisingly, a strong expression of F4/80. *In vitro*, at steady state, macrophages from B6.TNF^{-/-} mice showed no difference compared to B6.WT. After exposure with *L. major* promastigotes, they showed potential M2-like phenotype succumbing more easily to infection.

We are currently sorting this population from B6.TNF^{-/-} mice and examining the characteristics and potential downstream mechanisms that may affect the immunity against *L. major* infection in the absence of TNF.

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Clec9a⁺CD8⁺ DCs are key players in controlling *Plasmodium chabaudi chabaudi* AS infection

Lai, S.M.^{1,2}, Gupta, P.¹, Renia, L.², Ruedl, C.¹

¹Nanyang Technological University, SBS, Singapore, Singapore,

²Singapore Immunology Network (SigN), A*STAR, Singapore, Singapore

Plasmodium chabaudi chabaudi AS (*PccAS*) causes both acute and chronic malaria in mice. Dendritic cells (DCs), so-called immune "sentinels", are key players involved in establishing both acute-phase and parasite-specific adaptive immune responses. Functionally distinct and phenotypically heterogeneous DC

subsets have been identified, but their exact contribution in chronic malaria has yet to be elucidated. Using a Clec9A-diphtheria toxin receptor (DTR) transgenic mouse, wherein CD8⁺ and CD103⁺ DCs are specifically ablated following DT administration, has enabled us to study *in vivo* the role of this particular DC subset in controlling blood-stage *PccAS* infection. C57BL/6J-Clec9A-DTR mice exhibit high acute-phase parasitemia with significant recrudescence episodes, in stark contrast with wild-type mice which present sub-patent recrudescence. These mice also display significantly compromised IFN γ production, effector T-cell poly-functionality and an overall systemic skewing towards anti-inflammatory IL-10 response. Additionally, antibody isotype imbalance was clearly observed in Clec9A-DTR mice; with high levels of T_{h2}-skewed *Plasmodium*-specific IgG1 as opposed to strongly T_{h1}-inclined IgG2c responses in wild-type mice. In fact, infected Clec9A-DTR serum, when passively transferred into C57BL/6J wildtype recipients, was not as protective against *PccAS* infection as infected wildtype serum. Our findings suggest that Clec9a⁺CD8⁺ DCs are key players in mediating anti-*PccAS* immunity, culminating in robust effector T-cell functions and T_{h1}-inclined polarization of *Plasmodium*-specific antibody responses, both of which are required for protective immunity against *PccAS* infection.

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Immune-modulation by a Novel *Leishmania* antigen facilitates faster clearance of intracellular parasites from infected macrophages and synergizes with anti-leishmanial drugs

Arora, S., Tiwari, A.

PGIMER, Immunopathology, Chandigarh, India

Disturbed balance of host-parasite interaction during active disease and limited success of anti-leishmanial drugs rationale the use of immunomodulators as a feasible alternative. The high toxicity and developing resistance of anti-leishmanial drugs endorse the need for development of novel alternative approaches. We evaluated the immunotherapeutic properties of novel *Leishmania* recombinant antigens (recAg) in terms of macrophage activation and intracellular parasite clearance alone as well as in combination with standard anti-leishmanial drugs, in order to assess the potential use of immunomodulators for early clearance of intracellular parasites along with sub-optimal doses of anti-leishmanial drugs, that way reducing the toxicity also. Percentage of cells producing intracellular NO (14.25 \pm 2.76%) and ROS (30.6 \pm 3.51%) as assessed by flow cytometry using DAF-2DA and DCF-2DA dyes, were significantly increased among peritoneal macrophages when stimulated with recAg. Treatment of infected macrophages with recAg enhanced their phagocytic (47.00 \pm 3.46) as well as killing index (33.97 \pm 2.48) that coupled with increased intracellular parasite clearance. The recAg showed synergistic effect with miltefosine causing almost complete clearance of amastigotes at sub-optimal drug dose (10 μ M) at 48 h and significantly reducing

the number of infected cells. A concomitant increase in the production of inflammatory cytokines, IL-6 (3026.38±324.53), TNF- α (357.03±38.34) and MCP-1 (3574.74±784.62) was also observed when infected cells were stimulated with recAg. The leishmania recAg potentiates effector functions of parasitized macrophages and synergizes with antileishmanial drug miltefosine in early clearance of parasites at suboptimal drug dose, thus placing it as a promising candidate for adjunct therapy.

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IL-17 regulates permissiveness to infection with *Leishmania donovani*

Frame, T., Rivera, F., Engwerda, C., Beattie, L.

QIMR Berghofer Medical Research Institute, Herston, Australia

Visceral leishmaniasis occurs when *Leishmania* parasites infect tissue resident macrophages in the visceral organs, primarily in the liver, spleen and bone marrow. IL-17 is a pro-inflammatory cytokine that plays a crucial role in host defence against microbial infection. Studies in various pathogen infection models have shown control of pathogen growth to be dependent on IL-17 production. We have shown an increase in IL-17-producing cells in the liver early after *Leishmania donovani* infection, predominantly from CD4, CD8 and $\gamma\delta$ T cells. Unexpectedly, IL-17-deficient mice had lower liver parasite burdens, suggesting that IL-17 is playing a deleterious role in the control of *L. donovani*. The lower parasite burdens were independent of nitric oxide or super oxide induction in IL-17-deficient mice, indicating that a novel pathway for parasite control is at play. Using intravital 2-photon microscopy, we saw no difference in behaviour of IL-17-producing cells between naïve and treated mice. Flow cytometry allowed us to observe an enhanced immune cell response in the presence of IL-17, suggesting that IL-17 is not immune-suppressive. Instead, we hypothesise that IL-17 impairs parasite growth by regulating the availability of nutrients within macrophages, possibly via regulation of essential metals including iron, zinc and copper. These results will be discussed.

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Role of heme oxygenase-1 in the pathogenesis and immune regulation during *Fasciola hepatica* infection

Carasi, P.^{1,2}, Rodriguez, E.¹, Anegón, I.³, Freire, T.¹

¹Facultad de Medicina, Universidad de la Republica, Inmunobiología, Montevideo, Uruguay, ²Facultad de Ciencias Exactas, UNLP, Microbiología General, La Plata, Argentina, ³Centre Hospitalier Universitaire de Nantes, Faculté de Médecine, INSERM UMR 1064-ITUN, Nantes, France

Fasciola hepatica is a prevalent helminth parasite that affects mainly livestock and humans. Infection results in polarization of the host's immune response and generation of a modified type-2-helper (Th2) immune response characterized by the secretion of classical Th2 molecules and high quantities of potent regulatory cytokines (IL-10, TGF β). Heme oxygenase-1 (HO-1), an enzyme with anti-inflammatory and antioxidant

properties, has become an interesting target in transplantation, oncology and infectious diseases due to its ability to modulate immune responses. The aim of this work was to evaluate the involvement HO-1 in *F. hepatica* pathogenesis and in the host immune regulation induced by this parasite. Infected animals with *F. hepatica* expressed high levels of HO-1 in the liver and a recruitment of F4/80⁺ HO-1⁺ cells in the peritoneal cavity. To evaluate the immune-regulatory role of HO-1, the enzyme was chemically induced or inhibited, using CoPP and SnPP respectively, in *F. hepatica*-infected mice. SnPP-treated animals presented lower levels of liver damage and production of IL-4, IL-5 and IL-10. In addition, there was a significant decrease in the expression of the regulatory molecules TGF β and FIZZ by the livers from SnPP-treated animals. Finally, HO-1 induction by CoPP-treatment in infected animals was associated with higher levels of liver damage and TGF β -production by the liver as compared to infected-animals. Taken together these results suggest that HO-1 is involved in the immunoregulatory mechanisms elicited by *F. hepatica* in the mammalian host and in its pathogenesis.

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Dissecting the functional effector responses mediated by human IgG subclasses against *Plasmodium falciparum* merozoites

Irani, V.^{1,2}, Tan, P.S.³, Guy, A.^{1,4}, Andrews, D.¹, Sanders, P.¹, Feng, G.¹, Reiling, L.¹, Alagesan, K.^{5,6}, Kolarich, D.⁵, Lahoud, M.³, Beeson, J.^{1,2}, Ramsland, P.^{1,7}, Richards, J.^{1,2}

¹Centre for Biomedical Research, Burnet Institute, Melbourne, Australia, ²University of Melbourne, Department of Medicine, Parkville, Australia, ³Monash University, Department of Biochemistry and Molecular Biology, Melbourne, Australia, ⁴Monash University, Department of Immunology, Melbourne, Australia, ⁵Max Planck Institute of Colloids and Interfaces, Department of Biomolecular Systems, Potsdam, Germany, ⁶Institute of Chemistry and Biochemistry, Freie Universität Berlin, Berlin, Germany, ⁷School of Science, RMIT University, Melbourne, Australia

Naturally-acquired antibody responses against malaria antigens play an important role in protecting individuals from malaria endemic areas from symptomatic malaria. Some antibodies target merozoites, the extracellular form of blood-stage parasites, and are able to inhibit erythrocyte invasion. Naturally-acquired antibodies to different merozoite antigens are typically skewed towards IgG1 or IgG3 responses. Even though both subclasses are considered to be cytophilic antibodies, the specific differences in their effector responses are unclear and the observations that IgG3 responses are generally more strongly associated with clinical protection remain unexplained. It is therefore unclear whether optimal vaccine induced responses need to induce IgG1 or IgG3 responses, or if there are no significant functional differences.

To assess this, we chose to make IgG subclass switch variants in which chimeric recombinant antibodies that contained the same Fv region were combined with different human IgG subclass backbones. We chose well-characterised invasion inhibitory monoclonal antibodies to merozoite antigens that are

leading vaccine candidates (mAb R217 against EBA-175; mAb 1F9 against AMA-1; and mAb QA1 against Rh5). We expressed these subclass switch antibodies recombinantly in HEK293F cells and examined differences in effector responses in various *in vitro* functional assays. As expected, these subclass switch mAbs demonstrated the same epitope-specificity, affinity, and glycosylation patterns. Significant differences were observed for complement fixation. These findings suggest that human IgG subclasses do mediate differences in functional immunity against *P. falciparum*. This work increases our understanding of the IgG subclass contributions to natural immunity and helps design an effective vaccine against *P. falciparum*.

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Polymer nanoparticles in malaria: a dual role in targeted drug delivery and in vaccination approaches

Fernández-Busquets, X.^{1,2}, Marques, J.¹, Ranucci, E.³, Ferruti, P.³, Manfredi, A.³, Vilanova, E.⁴, Mourão, P.⁴

¹Barcelona Institute for Global Health (ISGlobal), Barcelona, Spain,

²Institute for Bioengineering of Catalonia (IBEC), Barcelona, Spain,

³Università degli Studi di Milano, Milano, Italy, ⁴Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

Most malaria vaccination strategies rely on antigens present in laboratory-maintained parasite strains. Because of the high clonal variability that *Plasmodium* exhibits in the wild, when these vaccines reach their clinical application after over a decade of product development and clinical trials, the original antigens might have disappeared from the parasites circulating in the human population. In addition, in each person are found several pathogen strains in a typical malaria infection in high-transmission endemic areas, which will dramatically reduce the efficacy of prophylaxis based on only a predetermined set of antigens. These could be some of the reasons why current malaria vaccines in clinical assays do not offer prospects of complete protection. Experimental evidence presented here and elsewhere has shown that malaria parasites are targeted by different sulfated polysaccharides and polyamidoamines, which in turn bind plasmodia from widely diverging malarias. The antiparasitic mechanism of these natural and synthetic polymers operates through inhibition of *Plasmodium* invasion of red blood cells. The failure of egressed parasites to quickly invade a new host cell will expose the pathogen to the immune system for a longer time, representing a new vaccination concept that requires the existence of an active malaria infection at the time of administration. This strategy is not a classical vaccine because it is not administered before contact with the pathogen, but the resulting prophylactic effect would provide protection against all the *Plasmodium* strains infecting the patient at the moment of treatment. Grants: 2013-0584 (Fondazione Cariplo, Italy), BIO2014-52872-R (MINECO, Spain, including FEDER funds).

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Human dendritic cell interactions with *P. falciparum* provide insight into the development of host immunity to malaria

Yap, X.Z.^{1,2}, Lundie, R.J.^{1,3}, Macri, C.F.³, Leal Rojas, I.⁴, Feng, G.¹, Radford, K.J.⁴, Beeson, J.G.^{1,2,3}, O'Keefe, M.³

¹Burnet Institute, Melbourne, Australia, ²University of Melbourne, Parkville, Australia, ³Monash University, Clayton, Australia, ⁴University of Queensland, Mater Research Institute, Woolloongabba, Australia

Malaria is an urgent global health problem and there is a need for effective malaria vaccines. However, interactions between the malaria parasite *P. falciparum* and host immune cells are poorly understood. Little is known about *P. falciparum* interactions with human dendritic cells (DCs). Due to the integral role of DCs in immune regulation, understanding how they respond to *P. falciparum* will be vital for development of malaria vaccines. Furthermore, due to the heterogeneity of the DC population it is important to understand how different DC subsets and tissue microenvironments affect antimalarial responses.

We investigated DC responses to *P. falciparum* using human peripheral blood DCs and spleen and bone marrow (BM) DCs from a humanised mouse model. DC responses were measured to two parasite stages: an extracellular stage (merozoite) and an intracellular stage (parasitized red blood cell; pRBC). We also investigated the effect of the parasite-to-DC ratio on DC activation.

Human blood DCs did not upregulate activation markers upon exposure to *P. falciparum* merozoites or pRBCs. Furthermore, pRBCs suppressed DC activation by TLR ligands in a dose-dependent fashion. For splenic and BM DCs, merozoites induced low levels of DC activation while pRBCs induced similar suppressive patterns to those seen in human blood DC.

Our findings reveal that DC activation in response to *P. falciparum* is limited and that pRBCs can suppress DC activation, even in the presence of other positive stimuli. This has key implications in understanding vaccine development and immunity to malaria.

Immunity to Viruses

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Highly pathogenic avian influenza virus causes severe symptoms due to insufficient contact between DCs and T cells

Ikejiri, A.¹, Yasui, F.¹, Kitabatake, M.², Sakaguchi, N.^{1,3}, Kohara, M.¹

¹Tokyo Metropolitan Institute of Medical Science, Department of Microbiology and Cell Biology, Tokyo, Japan, ²Nara Medical University, Department of Immunology, Nara, Japan, ³Osaka University, WPI Immunology Frontier Research Center, Osaka, Japan

Background: Highly pathogenic avian influenza virus (H5N1) infection causes severe symptoms resulting in an elevated mortality rate. However, the reason why patients cannot eliminate the viruses and succumb to them is not well known. We investigated temporal changes of the humoral immune response in mouse infected with low pathogenic pandemic

H1N1 or highly pathogenic H5N1 viruses. Mice infected with H5N1 could not induce sufficient neutralizing antibodies compared to H1N1. To clarify the insufficient induction of antibodies against H5N1, we focused on the antigen-presenting cells including dendritic cells (DCs) and macrophages.

Methods: BALB/c mice were infected with A/Tokyo/2619/2009 (H1N1) or A/Whooper swan/Hokkaido/1/08 (H5N1). To reinforce antibody responses after H5N1 infection, mice were inoculated intradermally with H5 HA protein-expressing recombinant vaccinia virus (rVV-H5 HA) 5 weeks before H5N1 challenge. After infection, spleens were collected to verify the localization of antigen-presenting cells by immunofluorescent staining.

Results and conclusion: The localization of macrophages in marginal zone and follicular DCs in germinal center were similar in the spleens of mice infected with H1N1 and H5N1. While the interaction of CD11c⁺ DCs with T-cells of mice infected with H5N1 decreased compare to H1N1. And the cell-cell contact between DCs and T-cells was loose and thin in H5N1 group. Furthermore, mice sensitized with rVV-H5 HA produce sufficient antibody and recovered the tight interaction of DCs with T-cells. These results imply that the severe symptom in H5N1 virus infection was associated with interaction of DCs with T-cells to induce efficient neutralizing antibodies.

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S1, a Cytomegalovirus encoded SLAMF6 homolog captured from the host by retrotranscription

Angulo, A.¹, Martinez-Vicente, P.², Farré, D.¹, Pérez-Carmona, N.¹, Engel, P.¹

¹University of Barcelona, Cell Biology, Immunology and Neurosciences, Barcelona, Spain, ²Institut d'Investigacions Biomèdiques August Pi i Sunyer, Barcelona, Spain

Cytomegaloviruses (CMVs) are ubiquitous, species-specific members of the herpesvirus family that have evolved multiple mechanisms to dampen host immune defenses and establish lifelong infections. Thus, CMVs contain a copious arsenal of gene products with immunomodulatory roles, including homologs of cellular immune proteins captured from their hosts during co-evolution. We have previously reported the presence of a number of homologs of signalling lymphocytic activation molecule (SLAM) family receptors in CMVs that infect human and other primates. Among them, squirrel monkey CMV (SMCMV) encodes a homolog of SLAMF6, a molecule expressed on the surface of NK, T and B cells and known to modulate immune functions through homotypic interactions. This viral homolog, S1, is a glycoprotein that exhibits an overall identity of 77% with the host SLAMF6 (*Saimiri boliviensis* SLAMF6, sbSLAMF6), including a nearly complete identity (97%) on its ligand-binding N-terminal immunoglobulin domain. Accordingly, we demonstrate that S1 specifically interacts with sbSLAMF6 and it is a self-binding protein. Furthermore, an S1-Fc fusion protein blocks sbSLAMF6 homotypic interactions. Interestingly, the interactions between the viral protein and sbSLAMF6 or between sbSLAMF6-sbSLAMF6 are stronger than human SLAMF6 homophilic interactions. We have generated a number of monoclonal antibodies (mAbs) that selectively recognize S1, sbSLAMF6 or both molecules, being some of them able

to target S1-sbSLAMF6 or sbSLAMF6-sbSLAMF6 interactions. Using anti-S1 mAbs we find S1 abundantly expressed at the cell surface during SMCMV infection. A detailed characterization of S1 should provide important information on how pathogens exploit in a novel manner SLAMF6-mediated pathways to suppress host surveillance.

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The role of intrinsic and aeroallergen-specific immune responses in the heightened susceptibility of atopics to respiratory viral infections: rat model studies

Jones, A.¹, Lauzon-Joset, J.-F.¹, Mincham, K.¹, Thomas, J.¹, Szakaly, R.², Rosenthal, L.², Bosco, A.¹, Holt, P.¹, Strickland, D.¹

¹Telethon Kids Institute, The University of Western Australia, Subiaco, Australia, ²University of Wisconsin School of Medicine and Public Health, Department of Medicine, Madison, United States

Background: Common (usually benign) respiratory infections exemplified by rhinovirus (HRV) can trigger asthma exacerbations requiring hospitalization in atopics. It is unclear whether sensitization plays a direct role in this context, or whether generic features of the underlying Th2high atopic immunophenotype are responsible for this effect.

Objective: To elucidate the cellular and molecular mechanisms underlying susceptibility to respiratory infection-induced asthma exacerbations in atopics, employing an animal model.

Methods: Our model comprised Th2high (atopic equivalent) BN rats and immunologically normal (non-atopic) PVG rats, unsensitized and OVA-sensitized. The animals were inoculated intranasally with attenuated HRV-like mengovirus, with or without aerosol exposure to OVA, and cellular and transcriptomic profiling was performed on bronchoalveolar lavage (BAL).

Results: Cellular profiling identified a strong neutrophilic response in PVG BAL at 24 hours post-infection, whilst the BN response was bimodal, characterised by peaks in macrophages (with minimal neutrophils) at 24 hours and 9 days post-infection. The acute BAL-cell transcriptome in infected PVGs displayed strong upregulation of genes associated with Th1/type1 interferon pathways, in contrast to a Th2-associated response in BNs including IL-33. OVA challenge of sensitized/infected BNs (not PVGs) triggered strong airways eosinophilia and exaggerated type1 interferon gene signatures, comparable to virus-associated exacerbations in human atopic asthmatics.

Conclusion: Intrinsic, and particularly acquired defects in anti-viral defence mechanisms triggered by co-exposure to aeroallergen, contribute to hypersusceptibility to infection-associated airways inflammation characteristic of the Th2high immunophenotype. These findings provide a plausible rationale for therapeutic targeting of aeroallergen-specific immunity in human atopics for prevention of virus-associated severe asthma exacerbations.

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Viral activation of RIG-I and STING pathways can induce necroptotic cell death

Schock, S., Chandra, N., Coscoy, L., Winoto, A.
UC Berkeley, Molecular and Cell Biology, Berkeley, United States

Necroptosis is a form of necrotic cell death that requires the activity of the death domain-containing kinase RIP1 and its family member RIP3. Necroptosis only occurs in apoptotic resistant cells and when RIP1 is de-ubiquitinated to form a complex with RIP3. Necroptosis may play a role in host defense through the release of inflammatory danger signals. We thus hypothesized that some viruses can induce necroptosis. We report here that among six different viruses surveyed, two of them, are capable of inducing necroptosis in the murine fibrosarcoma L929 cell line, especially when caspases are also inhibited. We found that one of the viruses induces cell death through the STING DNA sensor pathway in a TNF-dependent manner. In contrast, the other virus induces death through a TNF-independent manner. Knockdown of the RNA sensing molecule RIG-I or the RIP1 de-ubiquitin protein, CYLD but not STING, rescued cells from necroptosis induced by the latter virus. Mutant virus lacking some of the non-essential viral proteins is partially defective in its necroptotic activities. Together, these data identify viruses capable of programmed necrosis with divergent molecular pathways leading to death.

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Characterising nAb responses and B cell phenotypes in early primary HCV infection

Wu, B.-R., Walker, M.R., Eltahla, A., Lloyd, A.R., Bull, R.A.
University of New South Wales, Inflammation and Infection Research Centre, School of Medical Sciences, Faculty of Medicine, Sydney, Australia

Development of early and broad neutralising antibodies (nAbs) has recently been associated with clearance of hepatitis C virus (HCV) infections. However, it is unclear what factors are associated with the differential development of nAb responses in individual subjects. Successful nAb responses are derived from a robust B cell maturation and differentiation pathway. There is existing evidence to indicate that B cell development is skewed in late chronic HCV infection, with increasing immature and tissue-like B cells. In the present study, we aim to understand the correlation between HCV-specific B cell phenotypes, and the timing and breadth of nAbs in primary infection. Early incident subjects with peripheral blood mononuclear cells (PBMCs) and plasma were collected fortnightly over 24 weeks before clearance (n=7) or chronicity (n=7) is resolved. Neutralisation activity was determined against autologous HCV pseudoparticles (HCVpp) and a library of heterologous HCVpp representing all genotypes. A multichannel flow cytometric analysis is being performed to identify B lymphocyte subpopulations in the longitudinally-collected samples, using add some key markers. To-date, nAb responses targeting autologous virus have been measured in one subject that spontaneously cleared HCV. nAbs were detectable from 5 days post-infection (DPI) and gradually increased before

peaking at ~58 DPI and then persisted after clearance. The peak in nAb correlated with a decrease in viral load and subsequent clearance, suggesting nAbs contributed to clearance. We hypothesise that HCV-induced B cell skewing will be observed in those who become chronically infected and cause the loss of nAb responses thereby contributing to viral persistence.

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Abrogation of the interferon response promotes more efficient human cytomegalovirus replication

McSharry, B.P.¹, Forbes, S.K.^{1,2}, Ashley, C.¹, Avdic, S.¹, Randall, R.E.³, Wilkinson, G.W.G.⁴, Abendroth, A.¹, Slobedman, B.¹

¹University of Sydney, Infectious Diseases and Immunology, Sydney Medical School, Sydney, Australia, ²Westmead Institute for Medical Research, Centre for Virus Research, Sydney, Australia, ³University of Saint Andrews, School of Biology, Saint Andrews, United Kingdom, ⁴Cardiff University School of Medicine, Department of Medical Microbiology, Cardiff, United Kingdom

Human cytomegalovirus (HCMV) is a ubiquitous virus that is associated with severe morbidity and mortality in immunocompromised individuals and is the major infectious cause of birth defects in the developed world. Interferons (IFNs) are innate immune cytokines known to play a crucial role in controlling cytomegalovirus replication exemplified by the number of virally encoded gene products that limit the production of and/or the action of IFNs. However to our knowledge the effect of disabling the IFN response on HCMV replication has not been previously investigated. The effect of such an abrogation of the interferon response on HCMV infection and replication was examined using primary human fibroblast cell lines expressing either the V protein of Parainfluenza type V or the nPro protein of Bovine Viral Diarrhoea Virus that block the response to or the production of type I IFNs, respectively. In these IFN-defective cell lines, although there was no enhancement of the efficiency of initial infection, a number of different HCMV strains (both UL128L⁺ and UL128L⁻) produced significantly larger plaques compared to control cells. The virus also spread and replicated more rapidly than in parental fibroblasts demonstrating the key role of IFN- β in controlling viral replication. In addition, these cell lines facilitated the study of IFN stimulated gene (ISG) upregulation/induction post HCMV infection identifying ISGs directly regulated by HCMV independently of interferon e.g. ISG15. These cell lines demonstrate the vital role of IFNs in controlling HCMV replication and provide useful tools to further investigate the IFN response to HCMV.

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Varicella zoster virus ORF63 protects cells from staurosporine-induced apoptosis

Gerada, C., Steain, M., McSharry, B., Slobedman, B., Abendroth, A.
The University of Sydney, Infectious Diseases and Immunology, Camperdown, Australia

Varicella zoster virus (VZV) is a human alphaherpesvirus that causes varicella (chickenpox) during primary infection.

During varicella, VZV establishes life long latency in the dorsal root ganglia and can later reactivate to cause herpes zoster (shingles). The mechanisms by which VZV establishes latency and can later reactivate to cause herpes zoster are not fully understood; however it has been suggested that viral inhibition of neuronal apoptosis plays an important role. Apoptosis is a critical component of the intrinsic, innate and adaptive immune response to viral infection, as it allows for rapid elimination of infected cells without causing excessive inflammation. We have previously shown that VZV ORF63 can inhibit neuronal apoptosis, however the mechanism of action is unknown. To study the effects of VZV ORF63 on the apoptosis pathway, human foreskin fibroblasts (HFFs) were transduced with a novel lentivirus encoding VZV ORF63, with expression and functionality of ORF63 confirmed via western blotting and immunofluorescence assay (IFA) analysis. Apoptosis was induced through treatment with a protein kinase inhibitor, staurosporine, and the percentage of apoptotic cells was determined via the detection of cleaved caspase 3 (CC3) and DNA fragmentation (TUNEL) by IFA. Comparison of ORF63 expressing HFFs to parental transduced HFFs revealed a statistically significant reduction in CC3 and TUNEL positive cells with ORF63 expression. Together these data indicate that VZV ORF63 protects HFFs from staurosporine-induced apoptosis. Furthermore, this work validates a model that could be applied to determine the mode of action of VZV ORF63 in the protection of neurons from apoptosis.

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p62/sequestosome-1 supports dengue virus replication by inactivating NF- κ B-mediated IL-6 production

Chang, C.-P.^{1,2}, Li, C.-H.¹, Lin, Y.-S.^{1,2}

¹National Cheng Kung University, Department of Microbiology & Immunology, Tainan, Taiwan, Republic of China, ²National Cheng Kung University, Center of Infectious Disease and Signaling Research, Tainan, Taiwan, Republic of China

Dengue is a re-emerging disease which is endemic in more than 100 countries. Due to global warming effects, the dengue virus (DV) threat is broadening in the world. The symptoms are febrile illness called dengue fever to dengue haemorrhagic fever and dengue shock syndrome. A recent study showed that DV infection is able to reduce an autophagy adaptor protein p62, which may facilitates DV replication. However, our data here indicate that p62 seems to be required for DV replication. Compared to WT MEF cells, less virus proteins, released DV particles and DV RNA were detected in DV-infected p62-deficient MEF cells. Significant decreased DV protein translation was also observed in p62 knockdown DV-replicon stable expressing cells. This facilitating role of p62 to DV replication is not limited in MEF cells but also in hepatocytes and monocytes. There were no differences in virus cell binding, endocytosis, interferon activation and autophagy induction between DV-infected WT and p62-deficient cells. However, DV triggered high NF- κ B phosphorylation and nuclear translocation in p62-deficient cells. The reduced DV replication can be rescued by inactivation of NF- κ B with inhibitor BAY 11-7082 in p62-deficient cells. Moreover, we found that NF- κ B responsible cytokine

IL-6 was highly produced in DV-infected p62-deficient cells. Blocking IL-6 by neutralizing antibody can rescue DV replication in p62-deficient cells. In addition, recombinant IL-6 inhibits DV replication in vitro. Our findings uncover a new role of p62 in facilitating DV replication via inactivating NF- κ B-mediated IL-6 and also provide a potential therapeutic target for dengue infection.

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Antigen-presenting cell-like differentiation of microglia retard dengue virus-induced acute viral encephalitis

Lin, C.-F.^{1,2}, Tsai, T.-T.¹, Chen, C.-L.³

¹Taipei Medical University, Department of Microbiology and Immunology, Taipei, Taiwan, Republic of China, ²Taipei Medical University, Graduate Institute of Medical Sciences, Taipei, Taiwan, Republic of China, ³Taipei Medical University, Translational Research Center, Taipei, Taiwan, Republic of China

Mosquito-borne four serotypes of dengue virus (DENV) cause 390 million infections annually. Dengue patients may progress to severe dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), which can be fatal. Patients who succumb to DHF/DSS frequently present early altered neurological consciousness as similar with acute viral encephalitis acquired through an unknown mechanism. Here, we report that encephalitic DENV-infected mice exhibited progressive hunchback posture, limbic seizures, limbic weakness, paralysis, and lethality 7 days post-infection. These symptoms were accompanied by CNS inflammation, neurotoxicity, and blood-brain barrier destruction. Microglial cells surrounding blood vessels and injured hippocampus regions were activated by DENV infection. Pharmacologically depleting microglia unexpectedly increased viral replication, neuropathy, and mortality in DENV-infected mice. DENV infection increased expression of antiviral cytokines and infiltration of CD8 positive cytotoxic T lymphocytes (CTLs) in a microglia-dependent manner. DENV infection prompted antigen-presenting cell-like differentiation of microglia, which in turn stimulated CTL proliferation and activation. These results suggest that microglial cells are key factors in facilitating antiviral immune responses against DENV-induced acute viral encephalitis.

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Bromodomain-containing protein 3 (BRD3) selectively promotes IFN- β production via recruiting IRF3/p300 complex to the IFN- β promoter in macrophages

Wang, C., Ren, W., Sun, D., Cao, X.

Chinese Academy of Medical Sciences, Institute of Basic Medical Sciences, Beijing, China

Bromodomain containing protein 3 (BRD3) is a family member of bromodomain and extraterminal motif proteins (BETs) which function as protein scaffolds, mitotic bookmarks, cell cycle regulators and transcription regulators. However, the function of BRD3 in innate immune response remains unclear. Here we find that BRD3 is ubiquitously expressed in immune organs and immune cells, indicating that BRD3 might participate in

the regulation of innate immune response. The expression level of BRD3 is significantly down-regulated in macrophages upon stimulation with LPS or viruses. BRD3 knockout cells show significantly decreased production of IFN- β after LPS challenge and virus infection. However, the production of TNF- α and IL-6 is not affected. Importantly, we find BRD3 can bind to IRF3 and p300, and maintains the stability of IRF3/p300 complex upon virus infection. In addition, BRD3 recruits IRF3/p300 complex to the promoter of IFN- β , and enhances the acetylated histone3/histone4 promoter of IFN- β , leading to the promotion of type I interferon production. Therefore, our work reveals BRD3 as a selective positive regulator of innate IFN- β production, providing new mechanistic insight into the molecular mechanism for efficient activation of the innate immune response.

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Plasma expression profile and potential function of HCMV-encoded microRNAs in oral lichen planus patients

Zhang, C.¹, Ding, M.¹, Wang, X.², Wang, C.¹, Zen, K.³, Zhang, C.³

¹Jinling Hospital, Nanjing University School of Medicine, Clinical Laboratory, Nanjing, China, ²Nanjing Stomatological Hospital, Medical School of Nanjing University, Oral Medicine, Nanjing, China, ³Nanjing University, Life Sciences, Nanjing, China

Background: Oral lichen planus (OLP) is a T-cell-mediated autoimmune disease. The aetiology and molecular mechanisms of OLP remain unclear. Human cytomegalovirus (HCMV) infection is a causal factor in the development of various diseases, but the clinical relevance of HCMV in OLP has not been investigated. We examined HCMV-encoded miRNA expression profiles in plasma from OLP patients to reveal a potentially novel aetiology of OLP.

Methods and results: Twenty-three HCMV-encoded miRNAs were initially measured using RT-qPCR in a cohort of 21 OLP patients and 18 healthy controls. Five upregulated miRNAs in OLP patients, hcmv-miR-UL112, hcmv-miR-UL22a-5p, hcmv-miR-UL148D, hcmv-miR-UL36-5p and hcmv-miR-UL59, were confirmed in an additional cohort of 41 OLP patients and 33 controls. The levels of most of these miRNAs correlated significantly with anti-HCMV IgG concentrations in OLP patients. Additionally, by using a combination of luciferase reporter assays and western blotting, we demonstrated that cytomegalovirus UL16-binding protein 1, a molecule that mediates the killing of virus-infected cells by natural killer cells, is a direct target of hcmv-miR-UL59.

Conclusion: We report for the first time a distinct circulating HCMV-encoded miRNA profile and identify a novel HCMV miRNA-based immunoevasion strategy in OLP. Our results suggest a possible involvement of HCMV in the aetiology of OLP.

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The primary immune response to vaccinia virus vaccination includes cells with a distinct cytotoxic effector CD4 T cell phenotype

Munier, C.M.L.¹, Bailey, M.¹, Ip, S.¹, Xu, Y.¹, Alcantara, S.², Liu, S.M.³, van Bockel, D.¹, Suzuki, K.^{1,4}, Cooper, D.A.^{4,5}, Kent, S.J.^{2,6,7}, Zaunders, J.J.^{1,4}, Kelleher, A.D.^{1,4}, PHIIDO Study Group

¹The Kirby Institute, UNSW Australia, Immunovirology and Pathogenesis Program, Sydney, Australia, ²Peter Doherty Institute, University of Melbourne, Department of Microbiology and Immunology, Melbourne, Australia, ³The Garvan Institute, Sydney, Australia, ⁴St Vincent's Hospital, Sydney, Australia, ⁵The Kirby Institute, UNSW Australia, Sydney, Australia, ⁶Alfred Health, Central Clinical School, Monash University Melbourne, Melbourne Sexual Health Centre and Department of Infectious Diseases, Melbourne, Australia, ⁷ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, University of Melbourne, Melbourne, Australia

Smallpox was eradicated by global inoculation with vaccinia virus (VV). Robust VV-specific CD4 T cell responses during primary infection are likely to be essential to controlling VV replication. Although there is increasing interest in cytolytic CD4 T cells across a range of viral infections, the importance of these cells during acute VV infection is not understood. We undertook a detailed functional and genetic characterization of human VV-induced CD4 T cells during acute infection. VV-specific T cells were identified by up-regulation of activation markers *ex vivo* and through cytokine and co-stimulatory molecule expression during short *ex vivo* cultures. Thirteen days following VV inoculation, CD38^{high}CD45RO⁺ CD4 T cells were purified and analysed by microarray, quantitative RT-PCR and flow cytometry. We compared analyses of VV-specific CD4 T cells to studies on 12 subjects with primary HIV infection (PHI). A median 11.9% of CD4 T cells were CD38^{high}CD45RO⁺ at day 13 post-VV inoculation, compared to 3.0% prior to inoculation and 10.4% during PHI. Activated CD4 T cells had an up-regulation of genes related to cytolytic function, including Granzymes K and A, Perforin, Granulysin, granule marker TIA-1, and Rab27a. No difference was seen between CD4 T cell expression of perforin or TIA-1 in response to VV and PHI, however Granzyme K was more dominant in the VV response. We conclude that CD4 CTL are prominent in the early response to VV. Understanding the role of CD4 CTL in the generation of effective anti-viral memory may help develop more effective vaccines for diseases such as HIV.

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Altered neutrophil trafficking and viral pathogenicity resulting from genetic reassortment of influenza A virus PA and NA genes

Dlugolenski, D.¹, Tompkins, M.², Tripp, R.²

¹Deakin University, School of Medicine, Waurn Ponds, Australia, ²University of Georgia, Infectious Diseases, Athens, United States

Reverse zoonosis of the 2009 pandemic H1N1 virus into swine was identified soon after introduction into humans, and continued reassortment of the 2009 pandemic H1N1 influenza A virus (IAV) with current circulating strains of swine IAV has been observed. H3N2 variant (H3N2v) viruses resulted from introduction of the pandemic H1N1 M gene into triple reassortment internal gene (TRIG) swine IAV resulting in zoonotic transmission with enhanced morbidity. However, these viruses failed to transmit from human-to-human. Therefore, we sought to establish the pathogenic potential

of reassortment between current circulating strains of swine IAV and the pandemic H1N1 virus. Reassortants had altered pathogenic phenotypes linked to introduction of the swine PA and NA into pandemic H1N1. In mice, the swine H1N2 PA and NA mediated increased MIP-2 expression early post-infection resulting in substantial pulmonary neutrophilia with enhanced lung pathology and disease. These results show the potential for continued reassortment of the 2009 pandemic H1N1 virus with endemic swine IAV, and the potential for reassortants to have increased pathogenicity linked to the swine NA and PA genes which are associated with increased MIP-2 expression and pulmonary neutrophil trafficking.

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Biodegradable therapeutic nanoagents for influenza A virus by blocking viral entry via sialic receptor-targeting 3-aminophenylboronic acid

Yun, D.¹, Kim, H.-O.¹, Na, W.², Yeom, M.², Lim, J.-W.¹, Kim, J.¹, Chun, H.¹, Park, G.¹, Song, D.², Haam, S.¹

¹Yonsei University, Chemical and Biomolecular Engineering, Seoul, Korea, Republic of, ²Korea University College of Pharmacy, Sejong, Korea, Republic of

Influenza A virus (IAV) causes recurrent respiratory epidemics not only in humans but also birds and other mammals, threatening millions of lives every season. Due to the frequent gene reorganization or mutation of IAV, conventional therapeutic agents which are mostly neuraminidase (NA) inhibitors cause tolerance problems and side effects. Thus, development of biocompatible therapeutics free from resistance problems, types of virus as well as unwanted toxicity should be essential. In this study, we developed mucus-penetrating biodegradable nanoparticles (MPBNs) consisting of chitosan coated sialic receptor-targeting 3-aminophenylboronic acid (APBA) conjugated with Hyaluronic acid (HA) and poly (lactic-co-glycolic acid) (PLGA). MPBNs showed effective virus therapy by competitively blocking cellular infiltration of IAV as targeted cellular binding available nanoplatform *in vitro*. These results indicate that MPBNs with an easy administration method through mucous membrane could be an efficient treatment for various influenza viruses.

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Frequency and phenotype alterations in monocyte subsets from dengue patients: a main contribution of the non-classical subset

Naranjo-Gomez, J.-S.¹, Castillo, J.², Restrepo, B.³, Rojas, M.^{1,4}, Velilla, P.², Castaño, D.¹

¹Universidad de Antioquia, Facultad de Medicina, Instituto de Investigaciones Médicas, Grupo de Inmunología Celular e Inmunogenética, Medellín, Colombia, ²Universidad de Antioquia, Facultad de Medicina, Departamento de Microbiología y Parasitología, Grupo Inmunovirología., Medellín, Colombia,

³Instituto Colombiano de Medicina Tropical, Sabaneta, Colombia,

⁴Universidad de Antioquia, Unidad de Citometría de Flujo, Sede de Investigación Universitaria, Medellín, Colombia

About ten percent of patients with dengue virus develop severe manifestations, which can lead to death. It has been proposed that monocytes and their products must participate in severe dengue development. Considering that exist three different functional monocyte subsets (classical, intermediate and non-classical), they could be differentially involved in the immunopathogenesis. A flow cytometric approach was performed to evaluate in monocytes activation markers (CD86/CD68/HLA-DR/CD64), and molecules allowing interaction with endothelium (CD11a/CD11b/CD18/CD54), in 57 dengue patients.

An increased expression per cell of CD64, CD86, and CD54 was found on classical subset and of CD11b, CD18, CD54, CD64, and CD86 on intermediate subset, only in 4-7 days of illness without changes in the number of monocytes, compared to healthy controls. At day 8, the expression of these molecules decrease, except for CD64, indicating that these subsets may contribute with the immune responses to dengue mainly during the first days of illness.

It was observed a lower number of no-classical monocytes, with high expression of CD11b, CD54, CD68, CD64, CD86 and HLA-DR during illness evolution in particular, in dengue patients with alarm signs. Only this monocyte subset had augmented the percentage of TNF- α cells *ex vivo*. These results suggest that no-classical monocytes in dengue patients remain active and capable to interact with the endothelium during all days of illness; considering, these results were associated with severe manifestations, those changes could be evaluated in the future as prognostic tools.

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Dietary fatty acids alter T cell affinity for antigen and decrease anti-viral immunity

Kolawole, E.M., Evavold, B.D.

Emory University, Microbiology and Immunology, Atlanta, United States

There has been much investigation into the influence of dietary fatty acids with regard to modulation of immune function. Omega-3 polyunsaturated fatty acids have been shown to exert beneficial anti-inflammatory effects for both chronic and acute inflammatory disease. Based on studies suggesting incorporation of omega-3 fatty acids into lipid membranes, we assessed whether omega-3 fatty acids modulates T cell responses by altering the two dimensional membrane microenvironment of the TCR. Using the novel two dimensional (2D) micropipette adhesion frequency assay we determine T cell affinity for antigen on a single cell basis. Our data indicates that dietary fish oil reduces antigen affinity in T cell receptor transgenic cells. Moreover, we observed a decrease in the frequency of antigen specific (gp33) T cells by both tetramer and micropipette at peak anti viral immunity to lymphocytic choriomeningitis (LCMV) infection. These data indicate that a diet rich in omega-3 fatty acids decrease T cell affinity and expansion to viral antigen.

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Novel role for galectin-9 as a potent inhibitor of human cytomegalovirus infection

Machala, E.A., McSharry, B.P., Abendroth, A., Slobedman, B. University of Sydney, Infectious Diseases and Immunology, Sydney, Australia

Human cytomegalovirus (HCMV) is a large double stranded DNA virus of the herpesvirus family. While HCMV infection is generally asymptomatic in the immunocompetent, HCMV can have devastating consequences in immunocompromised individuals. Galectins are a family of cellular proteins characterised by the presence of a carbohydrate recognition domain. Members of this widely expressed protein family have been demonstrated to exert profound effects on host-pathogen interactions. Two of the best studied galectins, galectin-1 (Gal-1) and galectin-9 (Gal-9), have been implicated in a number of immune regulatory processes, and specific roles these for galectins in modulating both anti-viral immunity and regulating direct host-virus interactions have also recently emerged. Previous HCMV infection studies from the Slobedman laboratory have established potent upregulation of Gal-9 at the total protein level, dependent on the anti-viral cytokine IFN- β . The potential for galectins to directly modulate infection has not previously been studied in the context of HCMV. Infection studies utilising recombinant protein treatment, revealed Gal-9, but not Gal-1, as an anti-viral lectin that potently inhibited HCMV infection. Furthermore Gal-9 mediated inhibition of infection was carbohydrate recognition domain-dependent and blocked by anti-Gal-9 specific antibodies. Temperature-shift studies, separating the binding and entry stages of infection, identified Gal-9 specific inhibition as mediated primarily at the level of viral entry, and appeared to be dependent on binding to the virion rather than interacting with cellular ligands. This study provides the first evidence of a novel role for Gal-9 as an anti-viral, IFN- β induced protein, that exerts a profound inhibitory effect on HCMV infection.

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Influenza-specific antibody-dependent phagocytosis

Ana-Sosa-Batiz, F.¹, Vandervan, H.¹, Jegaskanda, S.¹, Johnston, A.², Rockman, S.^{1,3}, Laurie, K.⁴, Barr, I.⁴, Reading, P.^{1,4}, Lichtfuss, M.¹, Kent, S.J.¹

¹Peter Doherty Institute for Infection and Immunity at The University of Melbourne, Microbiology and Immunology, Melbourne, Australia, ²Monash Institute of Pharmaceutical Sciences, Monash University, Drug Delivery, Disposition and Dynamics Laboratory, Parkville, Australia, ³bioCSL Ltd, Parkville, Australia, ⁴WHO Collaborating Centre for Reference and Research on Influenza (VIDRL), Melbourne, Australia

Immunity to human influenza virus infection is only partially understood. Broadly reactive non-neutralizing antibodies may assist in reducing disease but have not been well characterized. Non-neutralizing antibodies have the potential to bind conserved regions of hemagglutinin (HA). Some of these antibodies can engage Fc receptor CD32 of effector cells and mediate antibody-dependent phagocytosis (ADP).

We measured internalization of opsonized, influenza protein-coated fluorescent beads and live influenza virus into a monocytic cell line to study antibody-dependent phagocytosis against multiple influenza HA subtypes. We analyzed influenza HA-specific ADP in healthy human donors, in preparations of intravenous immunoglobulin (IVIG), and following influenza infection of humans and macaques.

We found that both sera from healthy adults and IVIG preparations had broad ADP to multiple seasonal HA proteins. Many sera and all IVIGs also had weak cross-reactive ADP to non circulating HA proteins. Macaques experimentally infected with H1N1 and H3N2 viruses developed robust ADP. Further, the influenza virus phagocytosed in an antibody-mediated manner had reduced infectivity *in vitro*. Humans recently infected with H1N1 had a rise in ADP functional activity in sera to both homologous and heterologous HA proteins and influenza viruses.

Influenza infection in humans and macaques leads to the development of influenza-specific ADP that can clear influenza viruses. Repeated exposure of humans to multiple influenza infections likely leads to the development of ADP that is cross-reactive to strains not previously encountered. Further analyses of the protective capacity of broadly reactive influenza-specific ADP is warranted.

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The salmon pathogenic viruses IPNV, ISAV and SAV are differentially recognized by RIG-I-like receptors and induce different immune responses in a salmonid cell-line

Nerbøvik, I.-K.G., Solheim, M.A., Eggsetøl, H.Ø., Rønneseth, A., Jakobsen, R.A., Wergeland, H.I., Haugland, G.T. University of Bergen, Bergen, Norway

In fish, the type I interferons (IFNs) and their antiviral effect are well described, but less is known about the involvement of the RIG-I-like family receptors (RLRs) upon infection with whole viruses and the pro-inflammatory response during virus infection. Therefore, we infected a salmonid cell line, TO-cells, with infectious pancreatic necrosis virus (IPNV), infectious salmon anaemia virus (ISAV) and salmonid alphavirus (SAV) and monitored the responses of the RLRs RIG-I, MDA5 and LGP2 during infection. RIG-I, followed by LGP2, was highest upregulated by IPNV and ISAV at 1 and 4 days post infection, respectively, which correlates with the replication pattern of the viruses. During SAV infection, the opposite was observed, LGP2 was expressed significantly higher than RIG-I and MDA5. Furthermore, we investigated the innate immune responses by measuring the production of IFNs and pro-inflammatory cytokines at the transcriptional level. Among the interferons, IFN α was highest upregulated during infections with all viruses. Unexpectedly, the responses of pro-inflammatory cytokines upon infection with IPNV and ISAV were profoundly different from the SAV infection. During infection with the two aforementioned, TNF α 1 and α 2 was highly upregulated, while during SAV infection these cytokines were down-regulated. Similarly, one of the three IL12p40 homologues, IL12p40c, was down-regulated during SAV infection but not during infection with IPNV and ISAV.

Knowledge of virus recognition and the innate and adaptive immune responses during infection make the basis for development of prophylactic and preventive control measures and may help elucidate why and how some viruses can escape the immune system.

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Establishment of West Nile virus -neutralizing human monoclonal antibodies derived from the individuals vaccinated with inactivated Japanese encephalitis virus by ISAAC technology

Masaki, H.¹, Ozawa, T.², Takasaki, T.³, Aoyama, I.⁴, Yumisashi, T.⁴, Konishi, E.⁵, Kishi, H.², Muraguchi, A.²

¹Kinki University, Department of Biomedical Engineering, Faculty of Biology-Oriented Science and Technology, Kinokawa, Japan,

²University of Toyama, Department of Immunology, Graduate School of Medicine and Pharmaceutical Sciences, Toyama, Japan,

³National Institute of Infectious Diseases, Virology 1, Tokyo, Japan,

⁴Osaka Prefectural Institute for Public Health, Department of Infectious Diseases, Osaka, Japan, ⁵Osaka University, Department of Dengue Vaccine Research, Research Institute for Microbial Diseases, Suita, Japan

West Nile virus (WNV) belongs to the Japanese encephalitis virus (JEV) serocomplex of *Flaviviridae* family, which causes fatal encephalitis / meningitis mostly in the elderly. Currently, there is no specific therapeutics or vaccine for human use, thus, its development is urgent. Previously, we have reported that WNV-neutralizing antibodies are induced in some individuals immunized with inactivated JEV vaccine. And we have developed immunospot array assay on a chip (ISAAC) technology using microwell-array chip, which enables direct identification of antigen specific antibody-secreting cells from human peripheral blood lymphocytes (PBLs) and rapid cloning of antibody cDNA, leading to efficient and rapid production of human monoclonal antibodies (HuMoAbs). In the present study, we applied the ISAAC technology to establish WNV-neutralizing HuMoAbs from the PBLs of individuals vaccinated with inactivated JEV. As a result, we established three WNV E protein - specific HuMoAbs with high binding avidity as well as WNV-neutralizing activity. Competitive binding assay indicated no epitope sharing. Next, we examined protective effect of these HuMoAbs against WNV infection in vivo. WNV were inoculated to the C57BL/6 mice, to which HuMoAb was administrated subcutaneously on the day of WNV inoculation and 24 hours later, followed by monitor of their mortality and morbidity. Administration of the WNV-neutralizing HuMoAbs clearly postponed manifestation of the symptoms, indicating that these WNV-neutralizing HuMoAbs are protective against WNV infection. This is the first report that WNV-neutralizing HuMoAbs derived from JEV- vaccinated human can exert protection against WNV infection in vivo. Analysis of their epitopes is now under way.

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PTD fused Mx1 enhances protection against influenza virus infection

Jung, H.E., Lee, H.K.

Korea Advanced Institute of Science and Technology (KAIST), GSMSE, Daejeon, Korea, Republic of

Dynamine-like GTPase Mx1 is an intracellular antiviral protein that is induced by type I and type III interferons. A deficiency in the antiviral Mx1 protein increases susceptibility to influenza infection because the protein serves as a restriction factor and inhibits viral replication by blocking the transcription of viral RNA. Hence, although commercially manufactured anti-influenza drugs were primarily developed for inhibition of neuraminidase or the M2 protein, Mx1 could be another efficient target of antiviral therapy. To improve the delivery of Mx1 into cells, a polyarginine protein transduction domain (PTD) was fused to the C-terminus of Mx1. The engineered Mx1 was efficiently internalized in MDCK cells. Following influenza infection, viral replication and viral RNA expression levels in the infected cells were inhibited by treatment with the engineered Mx1. Further, intranasal administration of the engineered Mx1 improved the survival of mice infected with the PR8 influenza virus strain. These data reveal that engineered cell permeable Mx1 could be a novel therapeutic agent against mucosal influenza virus infection.

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HCMV glycoprotein gp39 downregulates host antiviral response

Choi, H.J., Park, A., Lee, S., Park, B.

Yonsei University, Seoul, Korea, Republic of

Human cytomegalovirus (HCMV) infects a large of percentage of the population worldwide and causes chronic infection. Type I interferon (IFN) response establishes an antiviral state in infected cells. However, it remains unclear how HCMV has evolved to evade the innate immune system for persistence. Here, we show that HCMV infection inhibits IFN- β induction during early and late times post-infection, when diverse viral genes are expressed. In particular, we identify HCMV glycoprotein gp39 that suppresses HCMV-induced IFN- β response. Localization of gp39 in the endoplasmic reticulum (ER) and mitochondria, important platforms for innate immune response, may be associated with its function in subverting host defense. Our finding provides a novel mechanism to understand HCMV pathogenesis and immune surveillance.

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Amphiphilic antiviral delivery nanocarrier possessing cell-targeting phenylboronic acid functional groups for virus therapy

Kim, H.-O.¹, Chun, H.¹, Na, W.², Yeom, M.², Lim, J.-W.¹, Yun, D.¹, Kim, J.¹, Park, G.¹, Song, D.², Haam, S.¹

¹Yonsei University, Chemical and Biomolecular Engineering, Seoul, Korea, Republic of, ²Korea University College of Pharmacy, Sejong, Korea, Republic of

Influenza, one of the most contagious diseases, has caused seasonal epidemics and pandemics being a consistent concern for global health. Despite laudable advances in antiviral agents and drugs, the vast majority of them have shown limited efficacy due to non-specificity and low viability in physiological or endosomal environment, especially in the case of intracellular drug. A nano platform, consisting of phenylboronic acid (PBA) pendant group polymer which has sialic acid-targeting property, gained greater access to the intracellular space transporting antivirals within the host cell. Amphiphilic copolymers made of pPhe-b-mPEG-PBA formed polymersomes which encapsulated hydrophilic antiviral agents in the core and hydrophobic drugs in the exterior layer. Combination of antiviral drug delivery using amphiphilic nanocarrier and cell-targeting functional group gives a better chance to improve transfection and intracellular distribution efficiency of therapeutic substances.

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Characterisation of the antibody response to hepatitis C virus by infected individuals

Allan, J.¹, Felsberger, G.¹, Silvia, L.^{2,3}, Hayley, C.⁴, Flexman, J.³, Watson, M.⁴

¹University of Western Australia, School of Medicine and Pharmacology, Perth, Australia, ²University of Western Australia, Pathology and Laboratory Medicine, Perth, Australia, ³Royal Perth Hospital and PathWest Laboratory Medicine, Perth, Australia, ⁴Murdoch University, Institute for Immunology and Infectious Diseases, Centre for Clinical Immunology & Biomedical Statistics, Perth, Australia

Hepatitis C virus (HCV) is a blood borne pathogen which is responsible for life long, chronic infection of the liver. It is expected that the optimal HCV vaccine will contain both cell mediated immunity and antibody stimulating epitopes. Our aim is to characterise antibodies against HCV elicited by infection that are genotype cross-reactive and infection neutralising *in vitro*.

Previously we have reported that 98% of a cohort of 100 chronic hepatitis C patients responded by ELISA to g2 (JFH-1) HCV prior to treatment. Analysis by denaturing western blot in a subset of this cohort revealed that each person recognised HCV core antigens but this was not found for the viral envelope glycoprotein, E2, which is the target of many infection neutralising antibody responses. Neutralisation of JFH-1 HCV infection of Huh7 cells *in vitro* was also common amongst the entire cohort irrespective of whether patients were infected with genotype 1, 2, 3 or 4 HCV. The presence of antibody to E2 has been tested by ELISA in a subset of patients using recombinant E2 for genotypes 1, 2, 3 and 4 produced in mammalian cells. Cross-reactivity of the antibody response between genotypes was common while the strength of the response to E2 was not directly related to the infecting HCV genotype. This information has been used to select donors whose HCV reactive B cells are captured in a modified ELISPOT for isolation of immunoglobulin sequences for diagnostic and vaccine development purposes.

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Characterisation of human CD14⁺ monocytes following Varicella-Zoster virus infection

Kennedy, J., Steain, M., Slobedman, B., Abendroth, A. University of Sydney, Infectious Diseases and Immunology, Sydney, Australia

Primary infection by Varicella-Zoster Virus (VZV) is presumed to involve transfer of virus from regional lymph nodes to peripheral blood mononuclear cells (PBMC) in support of dissemination throughout the host. The role CD14⁺ monocytes provide in this interplay is not fully elucidated and is a subject of conflicting reports. Here we demonstrate VZV infects CD14⁺ monocytes extracted from human blood, with 30% of cells expressing viral antigen within 24 hours of infection. CD14⁺ monocytes from at least 6 independent donors were co-cultured with VZV infected human fibroblasts and assessed by flow cytometry for viral antigen expression. By 24 hpi 20-60% of CD14⁺ monocytes were viral antigen positive. By immunofluorescence assays (IFA) CD14⁺ monocytes exhibited immediate-early, early and late VZV antigens; a full cascade of viral gene expression and hallmark of Herpesvirus infection. Concomitant with viral antigen expression, cell-surface analysis of monocytes revealed a trend of decreased cell-surface HLA-ABC at 72 hpi and statistically significant decreases in HLA-DR at 48 and 72 hpi. Interestingly the decrease in these cell markers coincided with a significant decrease in cell-surface CD14 at 72 hpi. These cellular changes may indicate infected monocytes are undertaking a polarising or differentiation event. Of publications that have previously investigated PBMC tropism, many have focused heavily on non-monocyte populations such as B and T lymphocytes or used less stringent indicators of infection. Thus this is the first demonstration of the full cascade of VZV gene expression during infection of human peripheral blood CD14⁺ monocytes.

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Immunostimulation reduces the severity of Influenza infection during pregnancy

Lauzon-Joset, J.-F., Scott, N.M., Mincham, K.T., Study, M., Holt, P.G., Strickland, D.H.

Telethon Kids Institute, University of Western Australia, Subiaco, Australia

Introduction: Pregnancy is associated with a heightened susceptibility to many pathogens and more severe clinical symptoms. The only preventive treatment currently available is vaccine, but the breadth of potential coverage is limited by vaccine availability. To circumvent this limitation, we were interested by a new class of treatment: the immunostimulation. Immunostimulation has been previously shown to limit infection severity, it had never been tested during pregnancy. As a proof-of-principle, we investigated the efficacy of an immunostimulant (OM-85) to protect from Influenza infection in a mouse model.

Methods: BALB/c mice were time-mated and fed OM-85 once a day from gestational day (GD) 0.5 until GD8.5. Mice were infected with a low dose of H1N1 influenza A virus (mouse adapted PR8 strain) at GD9.5 (corresponding to mid-late gestation). Disease

severity was assessed daily and the immune response was assessed at GD17.5 by flow cytometry in the airway tissues.

Results: The severity of Influenza infection was increased in pregnant mice compared to non-pregnant controls. In both pregnant and non-pregnant mice, Influenza infection was associated with an increased accumulation in the airway tissues of plasmacytoid dendritic cells and activated T cells, whereas the recruitment of inflammatory dendritic cells and granulocytes was only observed in pregnant mice. Interestingly, OM-85 preventive treatment did not have any adverse effect and it was able to normalize the inflammatory response to Influenza back to the level of non-pregnant.

Conclusions: Overall, we showed that OM-85 treatment prevent influenza-induced pregnancy complications by reducing inflammatory cell recruitment in the airways.

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Tregs influence kinetics of immunodominant antiviral CD8+ T cell responses

Chadderton, J.¹, L. La Gruta, N.^{1,2}

¹Peter Doherty Institute for Infection and Immunity at The University of Melbourne, Melbourne, Australia, ²Monash University, Department of Biochemistry and Molecular Biology, Melbourne, Australia

CD4+ regulatory T cells play a well characterised role in preventing autoimmune diseases where inflammatory immune responses have the potential to cause significant damage to the host, however their role in the regulation of T cell responses during viral infection is much less clear.

Previous studies have shown that removing or depleting regulatory T cells can modulate the antiviral CD8+ T cell immunodominance hierarchy by permitting the activation of low avidity CTLs that would normally be suppressed. The consequences of removing regulatory T cells may alternatively be beneficial in allowing increased numbers of CD8 T cells to participate in the immune response following vaccination. Thus we are studying the role of Treg cells in regulating both immunodominant and subdominant responses to influenza in C57Bl6 mice and asking whether removing Tregs during the acute antiviral response significantly alters CD8+ T cell responses and consequently influenza pathogenesis during infection. Experiments indicate that Tregs can respond during viral infection both in the lungs and periphery. Studies initially using PC61 antibody depletion suggest the size of NP₃₆₆ and PA₂₂₄ populations as well as the kinetics of the CD8 response are influenced by the presence of Tregs and this has been further confirmed using an influenza infection model in Treg depleted B6.Foxp3.GFP.DTR mice. This study is relevant to immunosuppressed individuals or those on immune therapy whose CD8+ T cells responses to acute viral infection may be significantly altered.

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CD160 plays a protective role during chronic HIV-1 infection and is critical to CD8+ T cell differentiation through T-bet pathway

Zhang, A.¹, Xu, J.¹, Zhang, L.¹, Qiu, C.¹, Qiu, C.¹, Wang, Y.², Ye, L.³, Li, T.⁴, Zhang, X.¹, Fu, Y.-X.⁵, Xu, J.¹

¹Fudan University, Shanghai Public Health Clinical Center, Shanghai, China, ²Shanghai CDC, AIDS Department, Shanghai, China, ³Third Military Medical University, Chongqing, China, ⁴Peking Union Medical College Hospital, Beijing, China, ⁵University of Texas Southwestern Medical Center, Dallas, United States

The role of CD160 during chronic HIV-1 infection remains controversial. Here we show that CD160 plays a protective role during chronic HIV-1 infection by enhancing CD8+ T cell functions through T-bet pathway. CD160+CD8+ T cells were enriched primarily in HIV-1+ slow progressors and their frequencies correlated with CD4 counts and inversely with plasma virus loads. CD160+CD8+ T cells were highly poly-functional and proliferative, which was further corroborated in a chronic LCMV infection in mouse model; triggering CD160 enhanced CD8+ T cell cytotoxicity and IFN- γ production in the context of HIV-1 antigen stimulation. Genetic ablation of CD160 resulted in the loss of T-bet expression in CD8+ T cells during chronic LCMV infection and thereby impaired CD8+ T cell functions and proliferation, and attenuated the containment of viral replication. Overall, we identified a novel CD160/T-bet pathway which plays a protective role during chronic infection through regulating CD8+ T cell function and proliferative capacity. These data suggest that CD160 represents a potential target for immune intervention in chronic infection.

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Retinoic acid regulates immune responses by promoting IL-22 production and modulating S100 protein in viral hepatitis

Sun, J.¹, Liang, Y.¹, Jie, Z.¹, Yi, P.¹, Tang, H.², Soong, L.¹, Cong, Y.¹, Zhang, K.²

¹University of Texas Medical Branch, Microbiology and Immunology, Galveston, United States, ²University of Texas Medical Branch, Pharmacology and Toxicology, Galveston, United States

The effector T cell responses promote viral clearance and disease resolution in viral hepatitis, but can also mediate tissue damage; however, much less is known as to how the liver protects itself against the injury. In this study, we have revealed a retinoic acid (RA)-mediated, hepatoprotective mechanism in adenovirus-induced hepatitis in mice. We found that RA treatment promoted hepatoprotective cytokine IL-22, but inhibited the pro-inflammatory cytokine IL-17, from gd T and double-negative T cells, and that the hepatic IL-17 and IL-22 production were regulated via the mTOR/PI3K signaling pathway. Moreover, we found that RA modulated the magnitude of antigen-specific T cell responses via down-regulating dendritic cell (DC) co-stimulatory molecule expression and its migratory capacity. Mechanistically, RA treatment inhibited calcium-binding S100 family proteins (S100A4/6/10) and the NF κ B/ERK signaling pathways on DCs. The inhibition of novel S100A4 resulted

in impaired DC migration from inflamed tissues to lymph nodes for T cell priming. Collectively, our study has revealed a previously unappreciated role and molecular mechanism of RA in modulating immune responses and protecting the liver from viral hepatitis.

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Highly cytotoxic effector CD8⁺ T cells in influenza A virus infection

*Kim, J., Lee, J., Lee, H., Kim, A.-R., Shin, E.-C.
KAIST, Daejeon, Korea, Republic of*

Influenza A virus is controlled by adaptive immune system in which cytotoxic CD8⁺ T cells target the cells with infection. However, it has been also reported that excessive activation of effector CD8⁺ T cells is harmful by destroying infected alveolar epithelial cells. With this study, we investigated CD8⁺ T cell responses in a mouse influenza model, using highly replicating and slowly replicating influenza A virus. Although influenza-specific CD8⁺ T cells were similar in frequency, effector T cells with same antigen specificity display significantly different function and phenotype during highly and slowly replicating virus infection. We found that effector CD8⁺ T cells from the highly replicating influenza-infected lungs exerted strong cytotoxicity and distinct phenotype. In contrast, effector CD8⁺ T cells with same antigen specificity presented intermediate cytotoxicity with IFN- γ secretion in the slow-replicating influenza-infected lungs. Interestingly, depletion of CD8⁺ T cells rescued survival during infection with highly replicating virus. These results indicate that cytotoxic effector CD8⁺ T cells are dominated in lungs during highly replicating influenza virus infection and they may contribute to lung injury.

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Lowering of influenza viral infectivity by using polypyrrole/polyaniline co-polymerized nanocomplex through control of cellular reactive oxygen species (ROS)

Kim, J.¹, Kim, H.-O.¹, Lim, J.-W.¹, Yun, D.¹, Chun, H.¹, Park, G.¹, Na, W.², Yeom, M.², Song, D.², Haam, S.¹

¹Yonsei University, Chemical and Biomolecular Engineering, Seoul, Korea, Republic of, ²Korea University, Pharmacy, Sejong, Korea, Republic of

Influenza A viral infection induces intracellular ROS generation to contribute to the virus propagation. In cytoplasm, ROS is generated by NADPH oxidase (Nox) whose expression is induced RNA and proteins in endocytosed virus through NADPH+O₂ (NADP⁺)+(H⁺)+2(O₂⁻). Plenty of ROS play a part in cell signal transduction to induce viral replication. The results of this study suggest that polypyrrole/polyaniline co-polymerized nanocomplex (PASome) has ROS controllability to block the viral replication. The ROS controllability comes from a character of Polypyrrole and polyaniline to be absorbing-emitting the hydrogen ion or electron by oxidizing-reducing themselves. As an antiviral agent, PASome has some strengths : it could be applied to all types of influenza A virus without the need to be modified or newly developed, and viruses don't acquire a

drug-resistance. We used a biocompatible amphiphilic polymer as template to form nanocomplex and to have the surface modified nanocomplex is bio-affinitive and stable in water. Thus we made effective ROS controllable nanocomplex to be suited for a medical application on a body. Furthermore, PASome could be applied to prevent or cure ROS-induced diseases like inflammation, arteriosclerosis, and cancer.

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The time-interval between infections and viral hierarchies are determinants of viral interference following influenza virus infection in a ferret model

Laurie, K.¹, Guarnaccia, T.^{1,2}, Carolan, L.¹, Horman, W.^{1,3}, Yan, A.³, Aban, M.¹, Petrie, S.³, Cao, P.³, Heffernan, J.^{3,4}, McVernon, J.^{3,5}, Mosse, J.², Kelso, A.⁶, McCaw, J.^{3,5}, Barr, I.¹

¹WHO Collaborating Centre for Reference and Research on Influenza (VIDRL), Melbourne, Australia, ²Federation University Australia, School of Applied and Biomedical Sciences, Gippsland, Australia, ³University of Melbourne, Melbourne, Australia, ⁴York University, Mathematics and Statistics and York Institute for Health Research, Centre for Disease Modelling, Toronto, Canada, ⁵Royal Children's Hospital Murdoch Childrens Research Institute, Melbourne, Australia, ⁶Formerly of WHO Collaborating Centre for Reference and Research on Influenza (VIDRL), Melbourne, Australia

Epidemiological studies suggest that following infection with influenza virus, there is a short period of time during which the host experiences a lower susceptibility to infection with other influenza viruses. This viral interference appears to be independent of the antigenic similarities between the two viruses. In this study, we used the ferret model of human influenza to systematically investigate viral interference and the role it plays in determining the outcome of consecutive infections. Ferrets were first infected then challenged 1 to 14 days later with pairs of influenza A (H1N1) pdm09, A (H3N2) and B viruses that were circulating in humans in 2009 and 2010. Challenge outcomes varied depending on the virus combination and time-interval between primary infection and challenge. Infection with A (H1N1) pdm09 virus was observed to prevent or delay infection with an influenza B or A (H3N2) virus. Infection with an A (H3N2) virus sometimes prevented infection with A (H1N1) pdm09 virus. Infection with an influenza B virus delayed the subsequent infection with either A (H1N1) pdm09 or A (H3N2) viruses. Co-infections occurred when short periods (1 or 3 day intervals) separated infections. Overall, these data indicate that the time-interval between infections and the sequential combination of viruses presented are important determinants of the degree of viral interference. Influenza viruses appear to have an ordered hierarchy according to their ability to block/delay infection, which may contribute to the dominance of different viruses often seen in an influenza season. Exploiting mechanisms that induce this temporary immunity may provide novel vaccine or therapeutic strategies to overcome influenza and other respiratory diseases.

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Immune recognition of respiratory syncytial virus (RSV) antigens and epitopes*Borochova, K.¹, Niespodziana, K.², Focke-Tejkl, M.², Valenta, R.²**¹Medical University of Vienna, Vienna, Austria, ²Medical University of Vienna, Division of Immunopathology, Department of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, Vienna, Austria*

Background: HRSV is an important causal agent of severe respiratory tract infections in children, elderly and immune compromised persons. There is still no active vaccine available for HRSV and there is a lack of serological diagnostic tests based on defined viral antigens and epitopes.

Materials and methods: The HRSV envelope proteins F and G were expressed in insect cells as C-terminally hexahistidine-tagged recombinant proteins and purified by Nickel-affinity chromatography. The purity and identity of both proteins were analysed by SDS-PAGE followed by Western-blotting using a monoclonal anti-His-tag antibody and mass spectrometry, whereas the correct folding was confirmed by circular dichroism analysis. Furthermore, synthetic peptides spanning the surface proteins were produced by solid phase synthesis. Enzyme-linked immunosorbent assay (ELISA) was used to investigate the occurrence of F- and G-specific as well as epitope-specific antibodies in healthy adult individuals.

Results and conclusion: We expressed and purified recombinant F and G proteins in very good yields. When we performed preliminary immunoassays, we found an interesting fine specificity of isotype and subclass specific immune responses. F protein was predominantly recognized by IgG1 whereas G protein reacted additionally with IgG4 by the tested subjects. Furthermore, recombinant intact F protein was exclusively recognized by the commercially available Palivizumab, a monoclonal antibody currently used for the prevention of severe HRSV infections in high risk infants. Our results suggest that recombinant HRSV-derived envelope proteins and their epitopes may be useful for the diagnosis and monitoring of HRSV infections.

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Effect of HBV-induced IL-23 on the biological behavior of hepatocarcinoma cells*Jiang, Q.¹, Ma, S.¹, Guo, Z.¹, He, Q.¹, Sun, Y.¹, Zhu, H.¹, Chen, R.¹, Ning, Q.², Cai, X.³, Lei, P.¹, Shen, G.¹**¹School of Basic Medicine, Tongji Medical College, Huazhong University of Science and Technology, Immunology, Wuhan, China,**²Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Infectious Disease, Wuhan, China,**³Wuhan General Hospital of Guangzhou Military, Wuhan, China*

Chronic infection with HBV is one of the major risk factors for development of HCC.

Published reports and our previous studies have indicated that HBV infection may induce the expression of IL-23, which exerts two-way regulation in carcinogenesis.

However, the reports about direct effects of IL-23 on tumor cells are rare and contradictory.

In present study, It was confirmed that HBV drove infected hepatoma cells to produce more inflammatory cytokines, especially IL-23. Furthermore, IL-23 had effects on the malignant properties of hepatoma cells in a concentration-dependent manner. At low concentration (0-20ng/ml), hrIL-23 could increase the proportion of stem/progenitor cells, promote proliferation and colony formation, reduce apoptosis and induce motility and invasivity of them. During exploration for the underlying mechanisms, HNF4a, which plays an important role in liver development and hepatocyte function, was found to be downregulated in HBV-infected hepatoma cells. Its expression was also decreased in cells treated by HepG2.215 culture supernatant, which could be abolished by supplement of anti-IL-23p19 Ab. Meanwhile, HNF4a level gradually decreased as hrIL-23 concentration increased in a certain range. Hence, it is speculated that HBV related IL-23 might regulate malignant properties of hepatoma cells through attenuation of HNF4a. The findings suggested that IL-23 could promote the development of HCC at a certain concentration, which might be potential targets of interventional strategies for treating hepatitis B patients.

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Influence of ectromelia virus infection on mitochondrial morphology and function in murine L929 fibroblast cell line*Gregorczyk, K.P.¹, Wyzewski, Z.¹, Szczepanowska, J.², Bossowska, M.¹, Mielcarska, M.¹, Struzik, J.¹, Szulc-Dabrowska, L.¹**¹Warsaw University of Life Sciences-SGGW, Faculty of Veterinary Medicine, Department of Preclinical Sciences, Warsaw, Poland,**²Nencki Institute of Experimental Biology, Department of Biochemistry, Laboratory of Bioenergetics and Biomembranes, Warsaw, Poland*

Ectromelia virus (ECTV), like other orthopoxviruses, affects mitochondria-associated processes including apoptosis. However, little is known about the impact of ECTV infection on morphology and functional status of mitochondria. In the present study we show that in infected L929 cells mitochondria gather around the viral factories, indicating a role for mitochondria during ECTV replication and/or morphogenesis. Therefore, maintaining mitochondrial functionality may be an important feature of ECTV survival, presumably, preservation of mitochondrial ability to produce ATP required in the viral replication process. Analysis of selected electron chain transport proteins, representing OXPHOS complexes, revealed no significant changes in their levels between control and infected cells. Moreover, the amount of chaperones (Hsp60, Hsp10) engaged in the maintenance of proper mitochondrial function increased during ECTV infection. On the other hand, virus infected cells displayed degradation of the mitochondrial network and increased level of reactive oxygen species (ROS). Extensive fragmentation of mitochondria resulted in decrease of mitochondrial mass. Furthermore, we observed increase of Fis1 and decrease of Opa1 proteins, responsible for mitochondrial fission and fusion, respectively. Also we found that in ECTV-infected cells the mitochondrial membrane potential was reduced, suggesting the induction of apoptosis. Interestingly, ECTV does not trigger apoptosis in the first 24 hours of

replication in L929 fibroblasts. Taken together, our results allow us to hypothesize that during ECTV infection mitochondria may engage the autophagy (mitophagy) process in order to protect infected cells from apoptosis and promote viral replication. Work supported by Grant No.2011/03/B/NZ6/03856 from National Science Centre, Poland.

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Leucocyte subsets in the fibroblast activation protein deficient mouse with influenza infection

Tan, S.-Y.^{1,2}, Chowdhury, S.^{2,3}, Weninger, W.^{1,2,4}, Gorrell, M.^{2,3}

¹Centenary Institute, Immune Imaging Program, Newtown, Australia, ²University of Sydney, Medical School, Sydney, Australia, ³Centenary Institute, Molecular Hepatology Laboratory, Newtown, Australia, ⁴Royal Prince Alfred Hospital, Department of Dermatology, Camperdown, Australia

Fibroblast activation protein (FAP), a serine protease with both post-proline dipeptidyl peptidase and endopeptidase activities, is highly upregulated in reactive stromal cells in embryogenesis, wound healing, fibrosis, tissue remodelling, inflammation and cancer. FAP⁺ stromal cells are thought to have a non-redundant, suppressive role in adaptive immunity. FAP might be involved via cleavage of its physiological substrates α 2-antiplasmin and neuropeptide Y. However, whether FAP itself exerts immunosuppressive functions in an inflammatory milieu, such as infection, is not known. We found that the major populations of leukocytes were unaltered in FAP-deficient mice; including splenic CD11c⁺ dendritic cells, CD19⁺ B cells, CD4⁺ and CD8⁺ T cells, CD25⁺CD44^{int} regulatory, CD25⁻CD44⁻ naive and CD25⁻CD44^{hi} activated subsets of CD4⁺ T cells, CD44⁺ and CD62L⁺ subsets of CD8⁺ T cells, and CD11b⁺Ly6G⁺ neutrophils. Although FAP expression was upregulated in the lungs and draining lymph nodes of influenza-infected mice, genetic ablation of FAP did not aggravate morbidity or mortality. In infected mice, FAP deficiency did not alter antigen-specific T cell proliferation or cytokine (IL-2 and IFN- γ) production or differentiation into CD44⁺CD62L⁻ effector T cells by donor OT-I cells. Anti-influenza antibody levels were unaltered in the absence of FAP. We conclude that FAP is dispensable for anti-influenza immunity.

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ADAMTS7: an essential enzyme for adaptive immunity

McMahon, M.¹, Ye, S.¹, Dlugolenski, D.^{1,2}, Tripp, R.², McCulloch, D.¹, Stambas, J.¹

¹Deakin University, Geelong, Australia, ²University of Georgia, Athens, United States

Clearance of influenza virus infection requires an effective and targeted immune response. Extracellular matrix (ECM) enzymes, such as the ADAMTS (A disintegrin-like and metalloproteinase with thrombospondin-1 motifs) family play a significant role in ECM remodelling. ECM remodelling facilitates immune cell migration, cell adhesion and cleavage of ECM proteins. ADAMTS7, a relatively uncharacterised metalloproteinase, has recently been found to play a role in influenza virus replication *in vitro*. To further validate these results, we assessed the

immune response in influenza-infected *Adamts7*^{-/-} mice. Upon infection, *Adamts7*^{-/-} mice displayed increased weight loss and virus titres at various time points post infection. This correlated with lower influenza-specific CD8⁺ T cell numbers at day 7 and 10 post infection. Moreover, secondary challenge with influenza virus resulted in increased weight loss and a diminished recall of influenza-specific CD8⁺ T cell immunity in *Adamts7*^{-/-} mice. These results suggest that ADAMTS7 expression is essential for optimal clearance of virus infection.

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Intrahepatic CXCL-10 is strongly associated with markers of liver fibrosis in HIV-HBV co-infection

Crane, M.¹, Tennakoon, S.¹, Harman, A.², Torresi, J.³, Avihingsanon, A.⁴, Lewin, S.¹

¹Peter Doherty Institute for Infection and Immunity, Melbourne, Australia, ²Westmead Institute for Medical Research, Sydney, Australia, ³Austin Hospital, Melbourne, Australia, ⁴Chulalongkorn University, Bangkok, Thailand

Liver disease progression is accelerated in HIV-HBV co-infection. HIV infection is associated with increased microbial translocation and chronic immune activation. We hypothesised that in HIV-HBV co-infection enhanced hepatic production of the chemokine CXCL-10 accelerates progression of liver disease. Liver biopsies and plasma were collected from co-infected patients naive to anti-retroviral therapy (ART; n=37) and from uninfected controls (n=8). Liver biopsies were analysed for CXCL-10, CXCR3, IFN α and - γ mRNA (qPCR). Plasma CXCL-10, sCD14 and CCL-2 were quantified by ELISA. Liver fibrosis was assessed by Fibroscan. Human hepatoma cell lines; HepG2, AD43 (HBV-negative) and AD38 (HBV-infected) were treated with IFN γ or IFN α and lipopolysaccharide (LPS) or PamCys (Invivogen) alone or in combination with HBsAg, HBeAg or HIV gp120 or following infection with HIV NL4.3 or vsv- pseudotyped NL4.3. Levels of CXCL-10 in the supernatant were measured by ELISA. Liver stiffness (kPa) was significantly correlated with intrahepatic CXCL-10 mRNA (p=0.017, r²=0.404), CXCR3 (p=0.0185 and IFN γ 0.0349). Intrahepatic CXCL-10 significantly correlated with intrahepatic CXCR3 (p=0.0005, r²=0.231), ALT (p=0.0055, r²=0.267), AST (p=0.001, r²=0.379) and circulating CXCL-10 (p=0.0047, r²=0.0218). *In vitro*, CXCL-10 production was increased 3 fold in HBV infected cells compared to HBV negative cells and vsv-pseudotyped HIV infection ablated CXCL-10 production. No effect of HIV or HBV proteins was observed. Intrahepatic markers of immune activation (CXCL-10, CXCR3 and IFN γ) were significantly associated with liver stiffness in HIV-HBV co-infected patients prior to ART. CXCL-10 and IFN γ should be considered as potential future targets to reduce liver stiffness in HIV-HBV co-infection.

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Varicella zoster virus inhibits necroptosis in HT-29 adenocarcinoma cells

Steain, M., McSharry, B., Avdic, S., Slobedman, B., Abendroth, A. University of Sydney, Infectious Diseases and Immunology, Sydney Medical School, Sydney, Australia

Varicella zoster virus (VZV) is the herpesvirus responsible for varicella and herpes zoster. Herpesviruses encode inhibitors of cell death pathways, including necroptosis. In cells expressing receptor interacting protein (RIP) 3, necroptosis can occur following death receptor signaling if caspase 8 activity is compromised. The impact of VZV on necroptosis has yet to be investigated. To address this we infected the necroptosis susceptible HT-29 cell line with VZV. Mock and VZV-infected HT-29s were treated with TNF (T), the smac mimetic BV-6 (S) and the pan-caspase inhibitor z-vad-fmk (V) to induce necroptosis and cell death was assessed by measuring ATP levels. Following the induction of necroptosis a significantly higher proportion of VZV-infected cells survived versus mock-infected cells (69% vs. 39%). IFA staining for VZV and phosphorylated mixed lineage kinase like protein (p-MLKL), which is induced during necroptosis, was also performed following T+S+V treatment. Phosphorylated-MLKL was readily observed in mock cells, however within the VZV-infected culture, p-MLKL was predominantly seen in uninfected bystander cells, and rarely within VZV antigen positive cells. Together these data suggest that VZV inhibits necroptosis. The ribonucleotide reductase (R1) encoded by herpes simplex virus (HSV)-1 and 2, encodes a RIP homotypic interaction motif (RHIM), which is essential for blocking necroptosis. VZV R1 shares homology with the C-terminus of HSV R1, however lacks the RHIM domain. We have identified a RHIM-like sequence within a capsid gene of VZV and are undertaking studies to determine if this viral gene product is responsible for the observed block in necroptosis mediated by VZV.

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Characterisation of the phenotypic profile of antigen specific cytotoxic T cell responses during primary HCV infection

Rasoli Pirozyan, M.¹, Leung, P.¹, Eltahla, A.¹, Rizzetto, S.¹, Nguyen, O.², Kedzierska, K.², Lloyd, A.¹, Bull, R.¹, Luciani, F.¹

¹University of New South Wales, Faculty of Medicine, School of Medical Sciences, Sydney, Australia, ²The University of Melbourne, Peter Doherty Institute for Infection and Immunity, Parkville, Australia

CD8+ T cells (CTLs) play a pivotal role in protection from viral infections. Several viruses infecting humans, such as HIV and HCV develop chronic infection, which are characterised by both immune escape and CTL exhaustion. TCR diversity and impaired differentiation are known to be associated with immune escape and exhaustion of CTLs, but the timing and mechanisms of these impairments remains unclear.

Exhaustion and differentiation profiles as well as TCR diversity, were assessed in unique longitudinal blood samples from primary HCV infection. Multicolor flow cytometry was employed for phenotypic characterisation of Antigen (Ag)-CTLs via activation, differentiation and inhibitory molecules. Single cells were sorted and analysed for transcriptomic profile (SmartSeq2) and TCR diversity using a novel computational workflow.

The majority of Ag-CTLs responses identified via IFN- γ ELSPOT were also characterised by a dominant population

of terminally differentiated effector memory cells (CCR7^{low}CD45RO^{high}KLRG1^{high}CD127^{low}), and elevated expression of co-inhibitory markers (PD-1, 2B4 and CTLA4) targeting both conserved as well as escaped HCV variants at the peak of immune response (as early as 70-90 days post infection). However, evidence of long-term central memory subpopulations with moderate IFN- γ production was identified in a subset of responses. There was an association of viral escape with the magnitude (IFN- γ production) of the response and ongoing diversification of TCR clonotypes, suggesting ongoing evolution of CTLs in response to prolonged viral exposure. These findings support the model of early onset of exhaustion, which compromises the successful establishment of a long-term memory repertoire in chronic HCV infection.

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Induction of CD137 expression by Epstein-Barr Virus facilitates immune escape of infected cells

Wu, M.^{1,2}, Schwarz, H.^{1,2}

¹National University of Singapore, Immunology Programme, Singapore, Singapore, ²National University of Singapore, Department of Physiology, Singapore, Singapore

The tumour necrosis factor (TNF) receptor family member CD137 (4-1BB, TNFRSF9) is a co-stimulatory molecule expressed on activated T cells. Its ligand CD137L (4-1BBL, TNFSF9) is constitutively expressed on most antigen-presenting cells (APCs), and reverse signalling through CD137L augments the activity of APCs. Previous studies in our lab had shown that ectopic CD137 expression on Hodgkin and Reed Sternberg (HRS) cells in Hodgkin lymphoma leads to a downregulation of surface CD137L on surrounding APCs, thus reducing T cell co-stimulation by CD137 and IFN- γ secretion, and enabling an escape of HRS cells from a Th1 immune response. Several intracellular pathogens are also known to induce CD137 expression and it has been reported that CD137 expression is induced in Epstein-Barr Virus (EBV)-positive cells from patients with EBV-positive T or NK cell lymphoproliferative diseases. In this study, we show that CD137 expression is upregulated shortly after nucleofection of mRNA encoding the EBV latent membrane protein 1 (LMP1) in peripheral T cells isolated from healthy donors and persists for several hours even after LMP1 expression has diminished. Furthermore, co-culture of these CD137-induced T cells with various CD137L-expressing monocytic and B cell lines leads to a decrease in surface CD137L expression on the cell lines. Our data suggests that similar to HRS cells, intracellular pathogens, such as EBV, utilise ectopic CD137 expression as a means to downregulate CD137L on APCs in order to evade a Th1 immune response.

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RNF122 suppresses antiviral type I interferon production by targeting RIG-I CARD to mediate RIG-I degradation*Jiang, M., Wang, W., Liu, S., Zhang, S., Liu, W., Ma, Y., Zhang, L., Zhang, J., Cao, X.**Chinese Academy of Medical Sciences, Institute of Basic Medical Sciences, Beijing, China*

As a cytoplasmic innate sensor for viral RNA, retinoic acid-inducible gene-I (RIG-I) is tightly regulated to avoid over activation and prevent excessive inflammation reactions. Post-translational modifications, particularly ubiquitination, are crucial for regulation of RIG-I activity. Increasing evidence suggest that E3 ligases play important roles in the regulation of various cellular processes, including cell proliferation and antiviral innate immune signaling. Here we report that RING finger protein 122 (RNF122), an E3 ubiquitin ligase, interacts with mouse RIG-I as demonstrated through screening the RIG-I-interacting proteins in RNA virus-infected cells using mass spectrometry. The transmembrane (TM) domain of RNF122 associates with the caspase activation and recruitment domains (CARDs) of RIG-I; this interaction effectively triggers RING finger domain of RNF122 to deliver the Lys-48-linked ubiquitin to the Lys115 and Lys146 residue of RIG-I CARDs and promote RIG-I degradation, resulting in a marked inhibition of RIG-I downstream signaling. Deficiency of RNF122 selectively increases RIG-I-triggered production of type I IFNs and proinflammatory cytokines in response to RNA virus infection both in vitro and in vivo, and RNF122-deficient mice exhibits longer survival after lethal VSV infection than control groups. Thus, we demonstrate that RNF122 acts as a selective negative regulator of RIG-I mediated antiviral innate responses by targeting CARDs of RIG-I.

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Dengue virus (DENV) inhibition of NETs is independent of neutrophil IL-8 synthesis*Moreno-Altamirano, M.M.B., Nava-Pérez, T.M., Rodríguez-Espinosa, O., Santiago-Ojeda, I.P.**Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Immunology, Mexico City, Mexico*

Introduction: Dengue is a disease caused by any of four serotypes of dengue virus (DENV-1, -2, -3 or -4). There is a striking correlation between increased serum levels of IL-8 and severe DHF. IL-8 stimulates the formation of neutrophil extracellular traps (NETs). Although the role of NETs in dengue patients, if any, is currently unknown, Since activated neutrophils produce IL-8 and this has proved to be an inducer of NETs.

The aim of this work was to explore if DENV-2 down-modulates the PMA-induced production of IL-8 and formation of NETs. Material and methods: neutrophils (PMN) were obtained from peripheral blood of healthy donors using heparin as anticoagulant and gradient centrifugation on Polymorphprep. The Neutrophils were recovered and resuspended in DMEM medium. For the formation of NETs and IL-8 detection, neutrophils (5X10⁵) were seeded on 6 well culture plates. Neutrophil were 8 hours incubated with 100 ng/mL PMA, with DENV and with PMA

plus DENV. After incubation, 50ul of supernatant was collected and the cytokines analyzed by using the human CBA kit (BD) PMN were fixed with 4% paraformaldehyde for 20 min, washed and stained with DAPI, mounted on Vectashield and observed by the fluorescence microscopy.

Results: showed that in vitro DENV-2 triggers the neutrophil production of IL-8. Thus, we propose that neutrophils should also been considered as an important source of IL-8 and that the mechanism(s) by which DENV-2 inhibits NET formation is independent of the synthesis of IL-8 by neutrophils. MMBMA and TMNP are supported by SIP 20150448/20161048.

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Enhancement of interferon and antiviral activities by staphylococcal enterotoxin superantigens and their mimetic peptides in vitro and in vivo*Mujtaba, M.¹, Johnson, H.²**¹Florida Gulf Coast University, Biological Sciences Department, Fort Myers, United States, ²University of Florida, Microbiology and Cell Science, Gainesville, United States*

The staphylococcal enterotoxins are a causative agent of food poisoning and have been implicated in other pathologies. They are classified as superantigens due to their potent stimulation of the immune system resulting in T cell activation and prodigious cytokine production. If superantigen activation of T cells and resulting cytokine production can be controlled, then the superantigen's beneficial properties can be harnessed. This study examined the ability of superantigens and their mimetic peptides to induce antiviral activity in vitro and in vivo. The superantigens SEA and SEB induced an antiviral activity in cells and prevented encephalomyocarditis (EMC) virus infection of L929 mouse cells in tissue culture. Furthermore, prophylactic treatment of mice in vivo with intraperitoneal injections of superantigens SEA and SEB prevented EMC virus lethality in 80% of the mice as compared to the control saline treated groups in which EMC virus was lethal to all mice. It was found that superantigens do not directly prevent EMC viral infection in mice, but rather indirectly via inducing IFN-gamma production in cells. Furthermore, development of a library of superantigen mimetic peptides showed one peptide (SEA3) having superantigen-like activity without the associated toxicity in vitro. If the negative side effects of superantigens can be eliminated then the beneficial effects of superantigens can be harnessed for prophylactic and therapeutic treatment of viral infections.

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Regulatory role of neutrophils in the inflammatory responses to paramyxovirus infection in mice*Cheemarla, N., Banos-Lara, R., Guerrero-Plata, A.**Louisiana State University, Department of Pathobiological Sciences, Baton Rouge, United States*

Neutrophils are the most abundant leukocytes (50% to 70%) in humans and are the first immune cell population recruited to the sites of infection. They are known to act in the first line of innate immune defense against invading pathogens, and more recently, to playing a crucial role in orchestrating adaptive immune responses. However, the role of neutrophils in the control of adaptive immune response during respiratory viral infections is still largely unknown. In order to elucidate the role of neutrophils in respiratory antiviral defense, we employed an experimental mouse model of human metapneumovirus (hMPV) infection. HMPV is a member of the Paramyxoviridae family, and a leading respiratory pathogen causing severe symptoms such as bronchiolitis and pneumonia in the young, elderly and immunocompromised patients. Our findings demonstrate that neutrophils are rapidly recruited in high numbers to the lungs of infected mice (>80%) during the acute phase of infection. Specific depletion of neutrophil *in vivo* using a monoclonal antibody and simultaneous infection with hMPV exhibited significantly higher levels of inflammatory cytokines, pulmonary inflammation and severe clinical disease compared to hMPV-infected competent mice. Interestingly, the lack of neutrophils altered the T cell responses in the lung, particularly the $\gamma\delta$ -T-cell subset. Finally, we also observed that the recruitment of neutrophils to the lung was controlled by the expression of the hMPV attachment protein. These novel findings demonstrate that neutrophils regulate T cell responses and are critically important for the control of the hMPV-induced inflammatory responses in the respiratory tract.

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Reshaping of the host immunopeptidome during virus infection*Croft, N.¹, Smith, S.², Wu, T.¹, Guan, J.³, Flesch, I.², La Gruta, N.³, Tschärke, D.², Purcell, A.¹*

¹Infection and Immunity Program, Monash Biomedicine Discovery Institute and Department of Biochemistry and Molecular Biology, Monash University, Biochemistry and Molecular Biology, Melbourne, Australia, ²Australian National University, John Curtin School of Medical Research, Canberra, Australia, ³University of Melbourne, Peter Doherty Institute for Infection and Immunity, Microbiology and Immunology, Melbourne, Australia

With the advent of rapid high-resolution mass spectrometry, our knowledge of the diversity of MHC-peptides presented on the surface of cells - the immunopeptidome - is becoming ever more complete. Our previous analyses have focused on the identification and quantification of virus-derived MHC-bound peptides presented during infection. However, in addition to substantial changes in the immunopeptidome elicited by viral peptides we have also observed changes in the host immunopeptidome generated by altered self-peptide

presentation.

We present data on the infection of cells with either of two different viruses (vaccinia virus, a large double-stranded DNA virus; and influenza A virus, a small single-stranded RNA virus) and find that the host immunopeptidome is reshaped following infection, with both the loss of many constitutively presented MHC-bound peptides and the subsequent gain of expression of previously undetected peptides. We describe data on the origin of these virus-induced self-peptides and discuss the clear implications in their potential autoreactivity as T cell targets.

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Evaluation of antiviral activity of *Carica papaya* aqueous leaf extract and its role in platelet augmentation*Sharma, N., Mishra, K.P., Ganju, L., Singh, S.B., Immunomodulation DIPAS, DRDO, Immunomodulation, Delhi, India*

Aim: Dengue disease is characterized by marked decrease in platelet count which is life threatening. We investigated the anti-viral activity of aqueous extract of *Carica papaya* (CP) leaves against dengue virus (DENV) and its effect on platelet augmentation.

Methods: Antiviral activity of CP in DENV infected THP-1 cells was determined by immunoblotting and flow cytometry. Its effect on platelet augmentation in cyclophosphamide induced thrombocytopenic rats was also examined. The rats were divided into four groups, normal control, thrombocytopenic control, thrombocytopenia induced rats in which CP dose was given prophylactically and in fourth group CP dose was given therapeutically after establishment of thrombocytopenia.

Results: CP significantly decreases the expression of envelope and NS1 protein in DENV infected THP-1 cells. Significant decrease in intracellular viral load by CP confirmed its antiviral activity. Platelet count of blood from retro orbital plexus of rats was examined at different time interval of 1st, 4th, 7th, 11th and 14 th day of study. Average platelet count in normal rats was $6.25 \times 10^5/\mu\text{l}$ on day 1. Platelet count in second group started to fall after Day 3 and remarkable thrombocytopenia developed after day 7 ($2.51 \times 10^5/\mu\text{l}$). However in third group interestingly, there was only mild decrease ($4.21 \times 10^5/\mu\text{l}$) even after day 7 and on day 11 it reaches normal level ($7.13 \times 10^5/\mu\text{l}$). CP also increases platelet count in the fourth group ($6.98 \times 10^5/\mu\text{l}$) at day 14.

Conclusion: Our findings suggest that CP can be a potent antiviral agent as it helps in platelet augmentation and exhibits excellent antiviral activity against DENV.

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Conjugated bilirubin regulates CD4+T effector cell and T regulatory cell function through outside-in and inside-out mechanisms: intracellular signalling and HAV receptor on cell surface*Corral-Jara, K.F.¹, Trujillo-Ochoa, J.L.², Realpe, M.³, Panduro, A.⁴, Gomez-Leyva, J.F.⁵, Rosenstein, Y.⁶, Roman, S.¹, Fierro, N.A.²*

¹Universidad de Guadalajara, Biología Molecular, Guadalajara, Mexico, ²Universidad de Guadalajara, Fisiología, Guadalajara, Mexico, ³Universidad de Guadalajara, Medicina Veterinaria,

Guadalajara, Mexico, ⁴Universidad de Guadalajara & Hospital Civil de Guadalajara Fray Antonio Alcalde, Clinicas Medicas, Guadalajara, Mexico, ⁵Instituto Tecnológico de Tlajomulco, Tlajomulco, Mexico, ⁶Universidad Nacional Autónoma de México & Instituto de Biotecnología, Cuernavaca, Mexico

We recently reported an immune-modulatory role of conjugated BR (CB) in Hepatitis A virus (HAV) infection. During HAV infection, the immune response relies on CD4+ T cells and may also be affected by the interaction of HAV with its cellular receptor (HAVCR1/TIM-1) expressed on T cell surface. Moreover, a six-amino-acid insertion (157insMTTVP) in *HAVCR1/TIM-1*, has been associated with the development of severe HAV infection. However, how BR may affect T cell function to define the clinical course of the disease has not been determined.

Herein, when CB was used to stimulate peripheral T lymphocytes (TLs) from healthy donors *in vitro*, changes in the degree of intracellular tyrosine phosphorylation relative to the concentration of CB were found. When side-by-side comparison between TLs from healthy and HAV-seropositive patients was conducted, changes in the degree of CREB, CD3e and Syk phosphorylation relative to discrete changes in CB levels were found. An increased activity of Tregs along with a Th17 cytokine profile and elevated proportion of Tregs expressing HAVCR1/TIM-1 on the cell surface were found in those patients which exhibited higher levels of CB. Moreover, a low frequency of the 157insMTTVP insertion in *HAVCR1/TIM-1* gene was found in patients and healthy donors suggesting that mild HAV-related disease is not associated with this mutation. Our data revealed that during HAV infection, CB plays a role in defining T cell function by modulating intracellular pathways and by inducing changes in the function of Tregs in a mechanisms related to expression of HAVCR1/TIM-1 on the cell surface.

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Effect of oxidative stress and rhinovirus infection on mitochondrial/endoplasmic reticular function in human primary bronchial epithelial cells

Pathinayake, P.S.¹, Hsu, A.C.-Y.¹, Parsons, K.¹, Loo, S.-L.¹, Fricker, M.¹, Wood, L.G.¹, Hansbro, P.¹, Wark, P.A.B.^{1,2}

¹Priority Research Center for Asthma and Respiratory Disease and Hunter Medical Research Institute, University of Newcastle, New Lambton Heights, Australia, ²John Hunter Hospital, Department of Respiratory Medicine, New Lambton Heights, Australia

Asthma is characterized by chronic airway inflammation and increased susceptibility to virus infections, such as Rhinovirus (RV), consequently the airway epithelium is exposed to high levels of oxidative stress. The Mitochondria play a central role in cellular metabolism and the immune response to virus infections. While mitochondrial dysfunction has been described in many chronic inflammatory diseases, little is known about its role in airway diseases.

Here we assessed the impact of the exposure of cigarette smoke, hydrogen peroxide (H₂O₂), tunicamycin (ER stress) and human rhinovirus infection (RV-1B) on mitochondrial function of human primary bronchial epithelial cells (pBECs) grown at the air liquid interface or submerged culture system. Mitochondrial functions were assessed by Seahorse XF⁹⁶ analyzer and

measuring cytochrome-C release. Pro-inflammatory cytokines were measured to evaluate the level of inflammation.

Infection with RV-1B demonstrated a reduced mitochondrial respiration and increased proton leak. This is accompanied with increased pro-inflammatory cytokines. Exposure of 1% CS increased the mitochondrial oxygen consumption rate, increased cytochrome-C release and increased virus replication compared with untreated cells. Induction of ER stress also increased the mitochondrial respiration. Exposure of 0.2mM H₂O₂ completely arrested the mitochondrial respiration with or without infection.

Taken together these results demonstrate that exposure of cigarette smoke or other forms of oxidative and ER stress change the mitochondrial function leading to impaired immune responses.

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The E3 ligase FBXW7 stabilizes RIG-I to mediate antiviral immunity by targeting SHP2 for ubiquitination and degradation

Song, Y.¹, Liu, Y.¹, Lai, L.¹, Zhang, Y.², Chen, Z.², Cao, X.³, Wang, Q.¹
¹Zhejiang University School of Medicine, Institute of Immunology, Hangzhou, China, ²Zhejiang University School of Medicine, Hangzhou, China, ³Chinese Academy of Medical Sciences, Beijing, China

RIG-I serves to detect viral RNA in cytosol and trigger a type I interferon-mediated response that protects the host against viral infection. Viruses can evade the host recognition by degradation of RIG-I or interference of the RIG-I signaling to establish persistent infections. However, the mechanisms how host cells stabilize RIG-I protein for avoiding its degradation is largely unknown. We report here that the E3 ubiquitin ligase FBXW7 stabilizes RIG-I to mediate antiviral immunity by targeting SHP2 for ubiquitination and degradation. The Lysm⁺FBXW7^{fl/fl} mice showed impaired antiviral ability when challenged with vesicular stomatitis virus (VSV) but not DNA virus. FBXW7 deficient macrophages showed a decrease in RIG-I protein level, produced less IFN- β and IFN- α 4 and downregulated the activation of NF- κ B, IRF3, AP-1 upon VSV and H1N1 infection. FBXW7 translocates from the nucleus into the cytoplasm upon VSV stimulation to interact with SHP2 and mediated the degradation and ubiquitination of SHP2, thus protecting RIG-I from degradation. The stabilization of RIG-I was enhanced in SHP2 deficient macrophages. Furthermore, we detected a significantly lower level of FBXW7 mRNA in peripheral blood mononuclear cells (PBMCs) from 70 cases of children with RSV infection when compared to that in PBMCs from control healthy children. Taken together, our results have identified the E3 ligase FBXW7 as an important interacting partner for RIG-I, which is crucial for the stability of RIG-I and RIG-I-triggered type I IFN production. These findings provide insights into the novel function of FBXW7 in antiviral immunity and its related clinical significance.

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Differentiation by cytokine signatures of acute hepatitis in Mexican population. The diagnostic value for independent, and HAV/HEV co-infection

Realpe, M.¹, Copado-Villagrana, E.D.², Meraz-Medina, T.³, Panduro, A.^{4,5}, Fierro, N.A.^{6,7}

¹Universidad de Guadalajara, Medicina Veterinaria, Guadalajara, Mexico, ²Universidad de Guadajara, Guadalajara, Mexico, ³Universidad Politecnica de la Zona Metropolitana de Guadalajara, Tlajomulco de Zuñiga, Mexico, ⁴Universidad de Guadalajara, Guadalajara, Mexico, ⁵Hospital Civil de Guadalajara Fray Antonio Alcalde, Servicio de Biología Molecular, Guadalajara, Mexico, ⁶Universidad de Guadalajara, Fisiología, Guadalajara, Mexico, ⁷Hospital Civil de Guadalajara Fray Antonio Alcalde, Unidad de Inmunovirología, Servicio de Biología Molecular, Guadalajara, Mexico

Hepatitis A virus (HAV) and hepatitis E virus (HEV) are the major causes of acute viral hepatitis worldwide. HAV infection use to resolve spontaneously whereas HEV infection initially defined as self-limiting, can take chronic courses under certain circumstances. Despite a major outbreak of HEV in Latin America occurred in Mexico, no information about incidence of single or co-infection has been available periodically for these two hepatotropic viruses. The detection of soluble immune mediators involved in specific immune responses against each of these viruses could provide valuable diagnostic tools.

The frequency of single and double infection was retrospectively analyzed in serum samples from Mexican patients clinically diagnosed with acute hepatitis. A total of 131 samples (seronegative for HBV and HCV), 8 without a known aetiological agent, and 30 samples from healthy individuals were screened for anti-HEV IgM and anti-HAV IgM. In addition, common and unique cytokines were identified by multiplexed immune assays. HAV-HEV co-infection, HEV- and HAV-single infections, and infections with no aetiological agent were found in 54%, 3%, 37% and 6% of the samples, respectively. IL-10 and IL-17 were commonly modulated in patients. A reduced secretion of cytokines was found in HEV-infected and HAV-HEV co-infected patients as compared to HAV single infection. Levels of IL-12, IL-13, IFN-gamma, and IL-4 were higher ($p < 0.05$) in HAV-positive samples as compared with other groups. Altogether, data highlighted the presence of HEV single infection and HAV-HEV co-infection in Mexico and revealed a virus-specific induction for specific host cytokines during acute viral hepatitis.

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Regulation of innate immune cell activation and the type I IFN response by interleukin enhancer binding factor 3 (ILF3) during HIV-1 infection

Nazitto, R.^{1,2}, Johnson, J.S.², Amon, L.², Aderem, A.^{1,2}

¹University of Washington School of Medicine, Immunology, Seattle, United States, ²Center for Infectious Disease Research, Immunology, Seattle, United States

Type I interferon (IFN) acts as a potent antiviral weapon. Almost all cells can produce type I IFN, but innate immune cells, such as myeloid dendritic cells (DCs), play key roles in IFN signaling

as they are positioned to “sense” pathogens and program adaptive immune responses. It is critical that we understand IFN regulation, since dysregulation occurs in disorders such as autoimmune disease, cancer, and chronic infections. In particular, HIV-1 is known to evade early detection in myeloid DCs, but as the disease progresses, IFN responses become exacerbated and correlate with the degree of pathogenesis. To uncover new regulatory nodes of IFN signaling, we have infected human monocyte-derived DCs (MDDCs) with HIV-1, and computationally analyzed the transcriptome. Here we describe that Interleukin Enhancer Binding Factor 3 (ILF3) acts as a negative regulator of HIV-driven innate immune responses. ILF3 is known to play roles in stress responses and regulation of host mRNA and miRNA processing in ribonucleoprotein (RNP) complexes. Knockdown of ILF3 in MDDCs led to increased basal activation and significantly potentiated maturation and IFN production during HIV-1 infection. By blocking reverse transcription during ILF3 knockdown, we inhibited maturation in response to HIV-1, supporting the hypothesis that viral DNA is sensed by MDDCs. Through combinatorial knockdown of ILF3 and cGAS, an innate intracellular DNA sensor, we abolished the activation phenotype and HIV-mediated induction of IFN. This work uniquely links host RNA processing complexes to modulation of innate antiviral responses, signaled through nucleic acid sensors of the myeloid immune compartment.

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Modulation of MHC class I expression by human cytomegalovirus encoded IL-10

Avdic, S., Ariyakumar, G., McSharry, B.P., Steain, M., Abendroth, A., Slobedman, B.

The University of Sydney, Discipline of Infectious Diseases and Immunology, Camperdown, Australia

Human cytomegalovirus (HCMV) infection induces both innate and adaptive immune responses, and whilst these responses ultimately lead to the resolution of productive infection, the clearance of replicating virus can take weeks or months. The capacity of HCMV to interfere with immune-mediated clearance is likely due to viral genes that target host immune responses, including a viral homolog of human interleukin-10 (hIL-10), termed cmvIL-10, which exerts a number of immunomodulatory functions.

We explored the impact of cmvIL-10 on MHC Class I (MHC I) expression by CD14⁺ monocytes. We included examination of NLRC5, a cellular protein which has recently been reported to play an important role in transactivation of MHC I genes. Whilst cmvIL-10 did not significantly alter surface expression of MHC I by freshly isolated CD14⁺ monocytes, transcription of NLRC5 and a range of genes associated with the MHC I antigen presentation pathway (HLA-A, HLA-B, B2M and LMP2) were significantly downregulated. These data indicate that cmvIL-10 may not rapidly alter constitutively expressed cell-surface MHC I, but may rather impair newly synthesized MHC I. To address this, we used LPS to stimulate surface MHC I by CD14⁺ monocytes in the presence of cmvIL-10. This analysis revealed inhibition of upregulation of surface MHC I protein as well as inhibited transcription of NLRC5 and MHC I associated genes.

Thus, cmvIL-10 can impair the MHC I biosynthesis pathway, including a key regulator of this pathway, NLRC5. These findings provide evidence of an additional mechanism by which HCMV may restrict immune recognition and virus clearance.

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MiR-22 promotes porcine reproductive and respiratory syndrome virus replication by targeting the host factor heme oxygenase-1

Xiao, S., Wang, X., Ni, H., Li, N., Yan, Y., Zhou, E.-M.

Northwest A&F University, College of Veterinary Medicine, Yangling, China

Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the most economically important viruses affecting the swine industry worldwide. Our previous research showed that PRRSV down-regulates the expression of heme oxygenase-1 (HO-1), a pivotal cytoprotective enzyme, post-infection and overexpression of HO-1 inhibits PRRSV replication. MicroRNAs (miRNAs) regulate gene expression at the posttranscriptional level and have recently been demonstrated to play vital roles in pathogen-host interactions. We have previously shown that miRNA miR-24-3p promotes PRRSV replication through the suppression of HO-1 expression. Given that multiple miRNAs can regulate expression of the same gene, we postulated that there may be other miRNAs modulating HO-1, and by doing so, regulate PRRSV replication. Using bioinformatic prediction and experimental verification, we demonstrate that HO-1 expression is regulated by miR-22. A direct interaction between miR-22 and HO-1 mRNA was confirmed using a number of approaches. miR-22 can downregulate HO-1 expression by directly targeting its 3' untranslated region. Suppression of HO-1 expression by miR-22 facilitates PRRSV replication. Collectively, these results suggested that miR-22 promotes PRRSV replication through suppression of HO-1 expression, which not only provides new insights into virus-host interactions during PRRSV infection, but also suggests potential new antiviral strategies against PRRSV infection.

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Bim deficiency prevents PD-1 mediated T cell attrition and rescues CD8 T cell exhaustion

Preston, S., Toe, J., Cooney, J., Pellegrini, M.

Walter and Eliza Hall Institute of Medical Research, Infection and Immunity, Parkville, Australia

Several host factors impede immunity to chronic infections including PD-1, CTLA-4 and type I interferons. These factors contribute to the exhaustion and attrition of virus specific T cells. During infection of mice with chronic LCMV docile the relative amounts of pro- and anti- apoptotic proteins are altered to favour the death of virus specific T cells. We show that loss of the pro-apoptotic protein Bim is sufficient to prevent the death of PD-1^{hi} virus specific T cells during chronic LCMV

infection. We found that conditional knock out mice deficient for Bim in their virus specific CD8 T cells had 10-40 fold more virus specific T cells compared to controls. These Bim deficient T cells were able to kill target cells as well as maintain their capacity to produce TNF α and IFN γ despite high expression of PD1. These findings are the first to demonstrate that keeping T cells alive, via inhibition of intrinsic apoptosis, rescues not only the quantity but also the quality of the T cell response. However, despite the ability of Bim deficient T cells to persist and retain immunological function their proliferative potential is impaired and this is not due to PD-1 signalling. Our data suggest that a PD-1-independent checkpoint blocks the proliferative capacity of pathogen specific T cells if they have escaped exhaustion/deletion. This work provides novel insights into the mechanisms of T cell death during chronic infections and could contribute significantly to development of immune modulating therapies that are desperately needed in many chronic overwhelming infections.

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Universal and heterologous CD8⁺ T cell immunity to influenza viruses

Koutsakos, M.¹, Gras, S.², Rossjohn, J.², Nguyen, O.¹, Kedzierska, K.¹

¹Peter Doherty Institute, University of Melbourne, Department of Microbiology and Immunology, Melbourne, Australia,

²Biomedicine Discovery Institute, Monash University, Infection and Immunity Program & Department of Biochemistry and Molecular Biology, Clayton, Australia

Influenza A and B viruses (IAV and IBV, respectively) remain a global health threat due to the occurrence of annual epidemics and the imminent emergence of novel pandemics. Thus, a CD8⁺T cell-inducing vaccine that can provide universal immunity across all influenza viruses would be of great health benefit. However, despite the breadth of work being done on immunity to IAVs, clinically relevant IBVs are greatly understudied. Here, to determine any cross-reactivity between IAVs and IBVs, we examined the conservation of known IAV CD8⁺T cell epitopes in IBV strains and identified a set of highly conserved epitopes. We found that a HLA-A02:01-restricted peptide from the PB1 protein (PB1₄₁₃₋₄₂₁ NMLSTVLGV) was 100% conserved in >99% of all human and avian influenza viruses. This A2/PB1₄₁₃₋₄₂₁ induces polyfunctional (IFN- γ , TNF and CD107a) responses in CD8⁺T cell lines from HLA-A2⁺ healthy donors, although appears to be subjected to a competition from the IAV A2/M1₅₈₋₆₆ epitope, at least in some donors. This first potential evidence of immunodominance in humans has implications for human CTL vaccines. We are further investigating the quality of the A2/PB1₄₁₃₋₄₂₁ response by determining the functional avidity of A2/PB1₄₁₃₋₄₂₁-specific CD8⁺T cells. Using a multiplex, paired single-cell PCR for TCR α repertoire, we have also determined the clonotype composition of A2/PB1₄₁₃₋₄₂₁ CD8⁺T cells. We are now evaluating the magnitude, quality and protective capacity of the A2/PB1₄₁₃₋₄₂₁ response in humanized HHD-A2 mice infected with IAV and IBV. Our studies demonstrate the potential for universal and protective CD8⁺ T cell-mediated immunity across IAV and IBV viral strains.

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TIPE2 negatively regulates hepatitis by inducing HBV-infected hepatocytes apoptosisCui, J.¹, Hu, Y.², Lian, K.³, Chen, Y.⁴, Liu, S.⁵¹Shandong University Medical of Sciences, Ji'nan, China, ²Binzhou Medical University, Department of Pathophysiology, Yantai, China, ³Shandong University School of Medicine, Ji'nan, China, ⁴University of Pennsylvania, Philadelphia, United States, ⁵Shandong University, Immunology, Ji'nan, China

Apoptosis, or type = 1 * ROMAN I programmed cell death (PCD), is considered as a controlled process of cell death involved in homeostasis. During HBV infection the apoptosis of infected-hepatocytes is very important for eliminating viruses. But the mechanism is not clear. Tumor necrosis factor- α -induced protein-8 like-2 (TNFAIP8L2, TIPE2) is a newly identified protein of TNFAIP8 family which can promote Fas/Fas Ligand (FasL)-induced apoptosis. Using gene expression microarray analysis, we found that TIPE2 deficiency could regulate the expression of apoptosis associated genes in liver tissues from hepatitis B mice models. TIPE2 protein was detected in TUNEL staining positive hepatocytes in HBV-infected C57 mice. Interestingly, the TIPE2 expressed hepatocytes were just the HBV infected cells. Furthermore, TIPE2 could upregulate the mRNA levels of FasL, Bim and TNFRsF1b which could promote cells death, when TIPE2 was transfected into HepG2 cells *in vitro*. As a result, TIPE2 overexpression cells showed a higher number of apoptotic cells and increased level of cleavage caspase3 compared to controls. Those results indicate that TIPE2 may participate in HBV infection by regulating apoptosis of virus-infected hepatocytes.

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MNV manipulation of the host immune response

Fritzlar, S., Mackenzie, J., Mackenzie Lab

Peter Doherty Institute, University of Melbourne, Microbiology and Immunology, Melbourne, Australia

Human Noroviruses are highly infective, single stranded RNA (ssRNA) viruses and the major cause of non-bacterial gastroenteritis worldwide. Due to the lack of a small animal or tissue culture model for Human Noroviruses, mechanisms of norovirus infections are so far poorly understood and drug treatments as well as efficient vaccines remain to be developed. With the discovery of the Murine Norovirus (MNV) and the introduction of an effective model for norovirus infection and replication, knowledge about infection mechanisms and its impact on the host immune response has progressed. We have recently discovered that MNV inhibits important effectors of the innate immune response, critical in controlling viral replication and systemic spread. We have observed that infection with MNV induces increased transcription of cytokines such as TNF α , IFN β and IL-6, however only a minimal amount of these cytokines is secreted into the culture medium. This observed manipulation of cytokine production obviously affects the autocrine and paracrine activity of these important immune mediators. We have observed that the virus-induced intervention is at the translation level as constitutive secretion is not impeded in MNV-infected cells. In addition, we have observed that MNV

interferes with the presentation of antigens on the surface of infected cells. We have shown that MNV-infected DCs and macrophages have a reduced surface expression of MHC class I proteins. Overall our observations indicate that MNV has a large array of strategies to avoid immune-mediated detection and clearance.

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Quantitative shifts in the influenza immunopeptidome reveal the relative contributions of direct and cross-presentation to the induction of T cell mediated antiviral immunityWu, T.¹, Guan, J.², Croft, N.P.¹, Tschärke, D.C.³, La Gruta, N.L.², Purcell, A.W.¹¹Monash University, Infection and Immunity Program & Department of Biochemistry and Molecular Biology, Melbourne, Australia, ²University of Melbourne, Department of Microbiology and Immunology, Melbourne, Australia, ³Australian National University, Research School of Biology, Canberra, Australia

T cell immunity directed against conserved epitopes from internal influenza proteins provides an alternative vaccination strategy to current antibody-based vaccines. However, the development of suitable peptide-based vaccines is currently limited by our incomplete understanding of the factors that control the relative immunogenicity of T cell epitopes. CD8+ T cells eradicate influenza virus through recognition of viral peptides in complex with major histocompatibility complex class I molecules that are presented on the surface of infected cells and professional antigen presenting cells.

We used a comprehensive mass spectrometry approach to identify and quantify naturally presented influenza epitopes following direct infection and cross-presentation of viral antigen *in vitro* and correlate this to the CD8+ response *in vivo* in C57/BL6 mice. In total, 22 viral peptides were identified including the two known immunodominant epitopes (NP₃₆₆₋₃₇₄ and PA₂₂₄₋₂₃₃), 11 known subdominant epitopes and 8 novel peptides. After direct infection of two murine cell lines, NP₃₆₆₋₃₇₄ abundance was the highest, whereas PA₂₂₄₋₂₃₃ abundance was the lowest, despite the co-dominant response to these epitopes during primary infection in mice. However, cross presentation data showed a 5-fold increase in PA₂₂₄₋₂₃₃ abundance, whilst the majority of other epitopes presented in lower abundance compared to direct presentation. This study provides novel insights into the relationship between peptide abundance and virus-specific CD8+ immunodominance hierarchies, with data suggesting that cross presentation may differentially affect the immunogenicity of influenza epitopes. This insight into the quantitative profiling of direct and cross-presented epitope abundance holds promise to optimise future antiviral vaccination strategies.

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Methyltransferase Dnmt3a upregulates HDAC9 to deacetylate TBK1 for activation of antiviral innate immunity*Li, X.^{1,2}, Zhang, Q.^{2,3}, Wang, Q.¹, Cao, X.^{1,2,3}*¹Zhejiang University School of Medicine, Hangzhou, China,²Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, China, ³Second Military Medical University, Shanghai, China

Epigenetic regulation of innate immunity needs further identification. Dnmt3a, a *de novo* DNA methyltransferase, is highly expressed in terminally differentiated macrophages, however, its role in innate immunity remains unknown. Here we report that deficiency of Dnmt3a selectively impairs PRR (Pattern Recognition Receptor)-triggered TBK1-dependent production of IFN α / β but not proinflammatory cytokine TNF α and IL-6. Dnmt3a-deficient mice exhibit more susceptibility to virus challenge than littermate controls. Although Dnmt3a does not directly regulate IFN α / β transcription, Dnmt3a strengthens TBK1/IRF3-initiated IFN α / β production by maintaining high expression of HDAC9 through antagonizing H3K27me3 at distal promoter of HDAC9. HDAC9 directly maintains deacetylation status of TBK1 at Lys241, enhancing TBK1 kinase activity. Our data add mechanistic insights into crosstalk of epigenetic regulation and posttranslational modification in regulation of innate signaling and activation of antiviral innate immune response.

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Spatiotemporal dynamics of innate immunity and lung pathogenesis during influenza virus infection*Jiang, X., Duan, E., Lu, C., Orian, J., Chen, W.**La Trobe Institute for Molecular Science, Melbourne, Australia*

Excessive pro-inflammatory immune responses are believed to contribute to lung pathogenesis during highly pathogenic influenza A virus (IAV) infections. Innate immune cells such as neutrophils and monocytes have been attributed both protective as well as pathological roles in the development of influenza-induced lung disease. However, current study methodologies such as flow cytometry and gene expression arrays require tissue digestion, which removes critical spatial information about influenza virus infection and clearance dynamics.

We utilize confocal imaging to study the spatial and temporal kinetics of influenza infection in mice following sublethal compared to lethal influenza virus infections. 2D and 3D imaging of influenza virus propagation and inflammatory immune cell recruitment revealed that the PR8 influenza virus infection produced a more diffuse and deeper spread in the lung parenchyma, even with lower initial viral doses compared to X-31. Critically, this pattern of virus infection was spatially associated with the formation of multiple foci of inflammatory innate immune cells. Investigation of influenza virus infection kinetics using the MyD88^{-/-} immunocompromised mouse model further showed that a lack of early inflammatory immune cell foci correlated with increased virus spread, triggering a paradoxical increase in inflammatory lung tissue responses

at day 6 and onwards post-infection. Altogether, our results demonstrate the importance of a spatiotemporal investigation of inflammatory immune cell responses during pathogen infection.

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Critical role of pDC in regulating gene expression and innate immune responses to human rhinovirus*Xi, Y.¹, Bosco, A.², Upham, J.W.^{1,3}*¹University of Queensland, Translational Research Institute, Lungand Allergy Research Centre, Woolloongabba, Australia, ²The

University of Western Australia, Telethon Kid's Institute, Perth,

Australia, ³Princess Alexandra Hospital, Department of Respiratory Medicine, Woolloongabba, Australia

Human rhinoviruses (HRV) are innocuous viruses in healthy people, but often trigger asthma exacerbations and this has been attributed to a defective type I interferon (IFN-I) response in asthma. We have previously shown that in HRV-stimulated peripheral blood mononuclear cell (PBMC), plasmacytoid dendritic cells (pDC) are responsible for more than 90% of IFN- α production, but the role of pDC in regulating gene expression patterns is unknown. In this project, we have further investigated the role of pDC using gene expression microarray. PBMC and pDC depleted PBMC were isolated from healthy volunteers (n=15) and cultured \pm HRV16 for 24h. HRV stimulated gene expression was determined using Illumina Human HT-12 microarrays: 3856 differentially expressed genes were identified when comparing pDC depleted PBMC versus intact PBMC. pDC depletion led to major changes in upstream regulators, many of which play an important role in IFN-I synthesis. RV16 stimulation triggered upregulation of 597 genes and downregulation of 236 genes; 70-80% of these appeared to be pDC dependant. Using RT-PCR and/or ELISA, we confirmed changes in the expression of selected key genes: pDC depleted PBMC expressed significantly lower *CD303*, *IL-27*, *IL-15RA*, *IRF7*, *EBI3*, *IFI27*, *IL-6*, and *IFN- γ* than PBMC. Adding recombinant *IL-27*, *IL-6*, *IL-15* and *IFN- β* to pDC-depleted cultures was able to successfully 'rescue' the IFN- α response.

In conclusion, we have demonstrated that HRV16 triggered immune response is highly regulated by pDC. The key changes in gene expression provide an important basis for developing novel therapies targeting anti-viral immunity in asthma.

Immunity to Viruses

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Immune responses to oral polio vaccine (OPV) in patients with X-linked agammaglobulinemia (XLA)

Wu, Y.¹, Mao, H.W.², Chan, S.M.¹, Lam, K.T.¹, Tu, W.¹, Lau, Y.L.¹

¹University of Hong Kong, Paediatrics and Adolescent Medicine, Hong Kong, Hong Kong, ²HKU-ShenZhen Hospital, ShenZhen, China

Background: XLA is characterized by impaired antibody response resulting from Bruton's tyrosine kinase (BTK) mutation. Unlike other primary immunodeficiency with no predisposition to extraordinary risk of viral infection, XLA renders a unique susceptibility to enteroviruses infection including poliovirus. OPV can induce paralytic poliomyelitis in XLA patients. Of great public concern is that the attenuated OPV can persist and revert to a virulent form in these patients, which renders a high risk of reintroducing virulent poliovirus into general population. It is known that BTK is involved in toll-like receptor 3 (TLR3) signaling, and TLR3-mediated type I IFN responses play critical roles in host protection against poliovirus. However, whether XLA patients have impaired type I IFN responses to OPV are not clear.

Hypothesis: BTK mutations in XLA impair TLR3-mediated type I IFN response, leading to the unique susceptibility to poliovirus in XLA patients.

Method: To investigate the response of XLA patients to OPV, monocyte derived macrophages (MDM) from XLA patients and healthy controls were infected with OPV Sabin strain 1 (OPV1). Further to examine the role of BTK in OPV infection, MDM from healthy controls were infected with OPV1 in the presence or absence BTK inhibitor.

Result: IFN-alpha production was measured by ELISA. OPV virus replication and IFN-inducible gene expressions including Mx1, Mx2, OAS1 were detected by qPCR. Results were compared between XLA patients and healthy controls

Conclusion: This study investigated the immune response of XLA patients to OPV and helped to reveal the role of BTK to control OPV infection.

Immunosuppression

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Ultraviolet-B irradiation of the skin for alteration of immunophenotype and functional responses in clinically isolated syndrome

Jones, A.P.¹, Kermod, A.G.^{2,3,4}, Nolan, D.^{3,5}, Lucas, R.M.^{1,6}, Hart, P.H.¹

¹Telethon Kids Institute, The University of Western Australia, Perth, Australia, ²Centre for Neuromuscular and Neurological Disorders, Western Australian Neuroscience Research Institute, The University of Western Australia, Sir Charles Gairdner Hospital, Perth, Australia, ³Institute for Immunology & Infectious Disease, Murdoch University, Perth, Australia, ⁴Sir Charles Gairdner Hospital, Department of Neurology and Clinical Neurophysiology, Perth, Australia, ⁵Royal Perth Hospital, Immunology Department, Perth, Australia, ⁶Australian National University, Canberra, Australia

Multiple sclerosis (MS) is an autoimmune disease characterised by demyelination in the CNS. T cells play a crucial role in the development of MS, with evidence of a T helper (Th)1/Th17 skewed phenotype, impaired regulatory T cell (Treg) function, and increased circulating follicular helper T cells (Tfh). Low exposure to ultraviolet-B radiation (UVB) is a strong MS environmental risk factor. UVB is immunosuppressive; inducing Treg and downregulating Th1/Th17 pathways. Due to the therapeutic use of UVB in psoriasis we are investigating its efficacy as a therapy in a neurological autoimmune condition.

We have a world first, NHMRC-funded, randomised controlled trial of narrowband UVB phototherapy for Clinically Isolated Syndrome - a first and single demyelinating event suggestive of MS. The hypothesis is that UVB phototherapy will slow or prevent the progression to MS via immunosuppression. Participants are randomised to receive, or not, 24 sessions of UVB phototherapy over 8 weeks. In addition, all participants receive vitamin D supplementation to control for the independent effects of vitamin D. Blood is collected at seven time points over the 12 month follow-up for measurement of serum 25(OH)D3 and bio-banking of peripheral blood mononuclear cells. Neurological examination and MRI scans are conducted to monitor clinical progression. Here we present longitudinal analysis of Th, Treg and Tfh phenotypes in n=12 participants and an equivalent number of age- and sex-matched controls. This study will inform on the biomarkers of progression of disease from a pre-MS condition, as well as the mechanisms by which UVB may be immunoregulatory.

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Solar-simulated ultraviolet radiation alters the formation of cell membrane-derived microvesicles in mouse plasma and skin draining lymph nodes

Ferguson, A.L.¹, Halliday, G.M.², Grau, G.E.R.³, Byrne, S.N.^{1,2}

¹University of Sydney, Cellular Photoimmunology Group, Infectious Diseases and Immunology, Sydney Medical School, Camperdown, Australia, ²University of Sydney, Dermatology Research Laboratories, Sydney Medical School, Camperdown, Australia, ³University of Sydney, Vascular Immunology Unit, Discipline of Pathology, Sydney Medical School, Camperdown, Australia

The ultraviolet (UV) radiation contained in sunlight is a powerful modulator of adaptive immune responses. UV can suppress immune responses both locally at the cutaneous site of exposure as well as systemically to affect distant organs and tissues. Modulation of immunity in this way partly explains how UV contributes to the development of skin cancer as well as protects from some autoimmune diseases like multiple sclerosis. We hypothesised that one way UV may be affecting immunity in distant tissues is by altering the number and type of microvesicles (MVs). MVs are cell membrane-derived vesicles that are involved in intercellular communication and are associated with diseases such as MS, cancer, diabetes and infections. MVs were isolated from peripheral blood as well as the lymph nodes that drain UV-exposed back skin (inguinal). Using a combination of fluorochrome conjugated antibodies together with flow cytometry we were able to identify the cellular origin of the MVs. We have discovered that exposure of mice to an immune suppressive dose of UV significantly alters the production and systemic circulating levels of microvesicles (MVs). Understanding the mechanisms by which UV affects immune responses in distant tissues will enable us to develop therapies that harness the power of UV to halt disease progression while eliminating any harmful side-effects.

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4-Methoxy TEMPO attenuates murine experimental colitis

Chami, B., Vallejo, A., Jeong, G., Varda, A., Witting, P
The University of Sydney, Sydney, Australia

Inflammatory bowel disease (IBD) is a chronic inflammatory condition, where MPO-mediated oxidative damage plays an important role in exacerbating tissue injury.

Aim: To test whether the MPO-inhibitor, 4-Methoxy TEMPO (MetT), can ameliorate the course of colitis.

Methods: Experimental colitis was induced in C57BL/6 mice by administering 3% v/v dextran-sodium-sulphate (DSS) in drinking water ad libitum over 9 days with mice also administered (i.p. injection) MetT (15mg/Kg) or vehicle control (10% DMSO+90% PBS) twice daily during DSS challenge.

Results: MetT attenuated bodyweight loss (50%, $p < 0.05$, $n=6$) and improved clinical score

(53%, $p < 0.05$, $n=6$) at day 9 of DSS challenge and inhibited the DSS-mediated increase in serum lipid hydroperoxide. Analysis of the inflamed colon revealed that damage to gut tissues and several inflammatory, including Ly6C⁺ monocytes and Ly6G⁺ neutrophils, were markedly decreased in MetT-treated mice. Constitutively expressed IL-10 (an anti-inflammatory cytokine) was significantly reduced in the colon of DSS-challenged mice; MetT-treated mice showed a trend to increased IL-10 expression. Levels of MPO were decreased in MetT-treated mice with DSS-induced colitis; likely a consequence of reduced neutrophil motility. Notably, crypt integrity was negatively correlated to MPO⁺ labelling ($p=0.011$, $n=24$), suggesting that increased MPO levels were associated with increased severity of colitis. MetT protected against MPO-producing neutrophil oxidative damage in a myocytes in an in vitro co-culture system.

Conclusion: MetT significantly reduced the severity of

experimental colitis, though it remains unclear whether MetT actions were mediated via inhibition of MPO or anti-inflammatory pathways, or both.

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Utilizing quantitative methods to study the interaction of immunosuppressive drugs in combination

Lye, B.K.^{1,2}, Heinzl, S.^{1,2}, Kan, A.^{1,2}, Hodgkin, P.D.^{1,2}

¹The Walter and Eliza Hall Institute of Medical Research, Parkville, Australia, ²The University of Melbourne, Medical Biology, Parkville, Australia

When naive T cells become activated, they undergo antigen-specific clonal expansion where their survival, proliferation and differentiation are governed by the antigenic signal, cytokines and co-stimulatory molecules received. This response is manipulated clinically by immunosuppressive drugs (ISDs). While the molecular targets of most ISDs are known, a comprehensive understanding of the kinetic changes on proliferation and survival is lacking and hence, predicting synergistic effects is difficult. Here, we utilize quantitative methods to investigate the effects of ISDs on CD8⁺ T cell activation, survival and proliferation in vitro. We report discrete effects that enable unique quantitative descriptions of each ISD that can be utilised for predicting combination therapies. Rapamycin and Mycophenolic acid lengthen division times and consequently reduce the number of division rounds cells undergo before they stop dividing. In contrast, Dexamethasone selectively affected survival, causing increased cell loss over time with unaffected division parameters. When combined, each drug acted independently, allowing net effects to be accurately predicted and their synergies evaluated. Examples include combining drugs affecting division times (Rapamycin and Mycophenolic acid) and survival (Dexamethasone), with progressively less cells undergoing fewer divisions. Interestingly, quantitative analysis of drugs affecting the same parameter such as Rapamycin and Mycophenolic acid showed that the effects of these drugs followed a simple rule of addition. Thus, we demonstrate that quantitative analysis of the effects of ISDs on T cell proliferation kinetic parameters to each fate provides a powerful platform to understand and potentially predict drug synergy.

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Residential memory regulatory T cells function on immune responses to influenza A virus infection

Lu, C.

La Trobe University, Biochemistry and Genetics, Melbourne, Australia

Recent studies on Tregs responses to influenza A virus (IAV) infections demonstrated that Tregs can be activated during IAV infection, thereby modulating antiviral immune response. Whereas the suppressive function of Tregs that have experienced primary IAV infection remain largely unexplored. To our observation in A/PR8/H1N1 infected C57BL/6 mouse model, there are a certain number of IAV infection experienced Tregs maintaining their residence in mediastinal lymph node (MedLN)

and lung. Moreover, Tregs that have experienced primary IAV infection show more rapid accumulation in bronchotracheo alveolar lavage (BAL), MedLN and lung during secondary infection when compared to naïve Tregs. Subsequently, MedLN and lung residential memory Tregs (RM Tregs) are expanded following by examining their function on immune responses to IAV infection. The expanded RM Tregs interact with bone marrow (BM) DCs resulting in downregulation of B7 molecules on DCs. RM Tregs interfere with antigen-presentation through downregulation of B7 molecules, therefore limiting memory CD4⁺ and CD8⁺ T cell responses. In contrast, blocking CTLA-4 on RM Tregs results in robust memory responses stimulated by DCs presenting IAV-antigens. Our results thus reveal that RM Tregs are more IAV-reactive and suppressive than naïve Tregs and their immunosuppression on IAV-induced immune responses may partially exert via CTLA4-B7 interaction.

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Translocation and dissemination of commensal bacteria as a source of infection after stroke

Wong, C.¹, Stanley, D.², Mackin, K.³, Lyras, D.³, Prakash, M.⁴, Nurgali, K.⁴, Hill, M.⁵, Moore, R.⁶

¹Monash University, Medicine, Melbourne, Australia, ²Central Queensland University, Rockhampton, Australia, ³Monash University, Melbourne, Australia, ⁴Victoria University, Melbourne, Australia, ⁵University of Calgary, Calgary, Canada, ⁶RMIT, Melbourne, Australia

Stroke is highly prevalent and is one of the leading contributors to morbidity and mortality worldwide. Despite the debilitating neurological deficits, bacterial pneumonia occur frequently and contributes significantly to disease mortality. Prophylactic administration of broad non-specific antibiotics may appear to be a viable option for treatment, but a recent large clinical trial did not support this. Most importantly, the source of infection post-stroke is unclear to date. In this study, we found the majority of the microorganisms detected in the blood of infected stroke patients were commensal bacteria which normally reside in the intestinal tracts. Using a mouse model of stroke, we found that post-stroke infection was only observed in mice born and raised in specific pathogen free facilities, and not germ free. Using high throughput 16S rRNA gene amplicon sequencing and relevant bioinformatics tools, the predicted source of bacteria forming the microbial community in the lungs (most clinically relevant site) of post-stroke mice was indeed the host small intestine. Additionally, stroke-induced gut barrier permeability preceded the dissemination of orally-inoculated bacteria to peripheral tissues. Taken together, we have identified an important source of infection after stroke that have been overlooked. Importantly, the findings of this study clearly indicate that stroke induces a rapid and aberrant intestinal response, permitting selective commensals to breach the gut epithelial barriers and contribute to the development of infectious complications following stroke.

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A two factor-cocktail: potential substitute for mesenchymal stromal cells in suppressing graft versus host disease

Fan, X.B.¹, Guo, D.Y.¹, Yap, C.S.¹, Bari, S.², Cheung, A.³, Li, S.³, Hwang, W.^{3,4,5}

¹Singapore General Hospital, Department of Clinical Research, Singapore, Singapore, ²National University of Singapore, Department of Pharmacy, Singapore, Singapore, ³Duke-NUS Graduate Medical School, Cancer & Stem Cell Biology, Singapore, Singapore, ⁴Singapore General Hospital, Department of Hematology, Singapore, Singapore, ⁵Singapore Cord Blood Bank, Singapore, Singapore

We identified two mesenchymal stromal cells (MSCs)-derived factors exhibited synergistic immunomodulation effect in mixed lymphocyte reaction. This two factor-cocktail (2F, patent pending) also showed excellent *in vivo* immunosuppressive effect in ameliorating graft versus host disease (GVHD) symptoms and improving survival. A moderate and severe GVHD animal model was created by injecting 200×10⁶cells/kg and 400×10⁶cells/kg of cryopreserved human PBMCs into NSG mice respectively. Four consecutive treatments were given on day-10, day-14, day-17 and day-21 post-transplantation. In moderate GVHD model, the immunosuppressive effect was comparable to BM-MSCs and single factors. However, in severe GVHD model, it exhibited excellent immunosuppressive effect as it could improve mice 36-day survival from 19.0% with severe symptoms to 61.9% with mild symptoms ($p < 0.01$). It was significantly better than BM-MSCs (8.3%, $p < 0.001$) and Cyclosporine A (26.1%, $p < 0.05$). Synergistic effect was again observed between those two factors (Factor-A, 18.2%; Factor-B, 9.1%; $p < 0.05$). The 2F cocktail treatment could elevate human regulatory T cells but also cytotoxic T lymphocytes (CTLs) and T helper 1 (Th1) cells. Meanwhile, it could increase anti-inflammatory cytokine IL-10 and pro-inflammatory cytokines IFN- γ , IL-8, MIP-1 β and MCP-1 in the circulation. These results indicated the 2F cocktail suppressed immune reaction probably through M2 macrophages and regulatory T cells. In conclusion, the efficacy of a novel identified 2F cocktail was validated in an *in vivo* GVHD model. It demonstrated a robust immunosuppressive effect and kept the development of GVHD under control. The 2F cocktail is a potential chemically defined substitute for MSC in GVHD therapy.

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Metabolic alarmins train monocyte tolerance

Uhle, E., Lichtenstern, C., Weigand, M.A.

Heidelberg University Hospital, Department of Anesthesiology, Heidelberg, Germany

The induction of tolerance is a crucial negative feedback mechanism of activated innate immune cells to counteract activation. The phenomenon of tolerance has been described for a variety of ligands (PAMPS, DAMPS and cytokines) and their corresponding pattern recognition receptors, with endotoxin tolerance being the most prominent one. During inflammation, metabolism of myeloid cells is shifted to aerobic glycolysis, resulting in a rapid energy provisioning necessary to fuel the

immune response. As a by-product, the reactive dicarbonyl compound methylglyoxal is formed, resulting subsequently in the generation of Advanced Glycation Endproducts (AGEs) by reacting with lipids and proteins. In recent studies, we were able to prove elevated levels MG as well as AGEs in both sterile (trauma) and infectious (sepsis) systemic inflammation.

To shed light on the consequence of its presence during disease, we performed in vitro experiments using the monocytic cell line MonoMac 6 and primary human monocytes. After priming of the cells with AGEs, we found a diminished cytokine response upon restimulation with both AGEs and LPS. The observed anergy was neither associated with a changed expression of the involved pattern recognition receptors TLR4 and RAGE, nor with increased cell death or cell cycle variations. It actually was associated with altered levels of intracellular TNF-alpha, despite no changes in the expression of the corresponding gene were measurable. In conclusion, we are able to prove a dual effect of AGEs: initially they act as pro-inflammatory mediators, secondarily training monocytes to a state of cross- and self-tolerance.

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Phenotypic characterization of LAG-3 expressing cells

Okazaki, I.-M., Okazaki, T.

Tokushima University, Institute for Genome Research, Tokushima, Japan

The activation of lymphocytes is tightly controlled by stimulatory and inhibitory co-receptors in addition to antigen receptors. These co-receptors provide not only a second signal for activation but also a balanced network of positive and negative signals to optimize immune responses against pathogens while limiting immune responses to self. We have been focusing on inhibitory co-receptors, PD-1 and LAG-3, which synergistically regulate T cell activation and autoimmunity. Although accumulating evidence suggests that LAG-3, a type I transmembrane protein with structural similarity with CD4 inhibits activation of T cells, the actual role of LAG-3 is still enigmatic. In this study, we tried to identify cell populations on which LAG-3 functions by single-cell gene expression analysis.

We selected 192 genes based on microarray analyses of bulk LAG-3 expressing cells, analyzed the expression levels of these 192 genes in each LAG-3 expressing cell using BioMark HD system (Fluidigm), and performed the hierarchical clustering analysis. We analyzed LAG-3 expressing cells induced by antigen immunization and those emerged upon autoimmunity. We found that LAG-3 expressing cells were heterogeneous in both conditions but apparently distinct cell populations were increased between two conditions. These results suggest that LAG-3 can regulate the function of variable cell types and that it may regulate the function of specific cell populations in the suppression of autoimmunity.

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Globo-H ceramide induced T cell immunosuppression via A2A receptor (A2A-R) and cAMP/PKA pathway

Cheng, J.-Y., Wang, S.-H., Hung, J.-T., Yu, A.L., Yu, J.

Chang Gung Memorial Hospital, Taoyuan, Taiwan, Republic of China

Globo-H ceramide (GHCer), the most prevalent antigen in epithelial cancers, is a hotly pursued target for cancer immunotherapy. Previously, we demonstrated that uptake of GHCer shed from cancer cells by endothelial cells promoted angiogenesis via binding of GHCer to TRAX, with consequent release of PLCb1 to trigger Ca²⁺ mobilization. In contrast, uptake of GHCer by lymphocytes significantly suppressed their activation via down-regulation of Notch1 signaling mediated by increased expression of *ID3* and *egr2/3* controlled *itch*. However, the link between GHCer and transcriptional regulation of *ID3/Itch* remains unclear. Here we provided the first evidence for GHCer induced immunosuppression through recruiting TRAX-KIF2A complex to A2A-R, thereby activating A2A-R signaling, which inhibits immune responses. We found that treatment of Jurkat cells, a human T-ALL cell line, with GHCer increased cAMP and CREB activity as determined by cAMP ELISA and CREB reporter assay, respectively, both of which were significantly blocked by pretreating cells with protein kinase A (PKA) inhibitor, H89, or A2A-R antagonist. Since A2A-R is known to be activated by binding to TRAX-KIF2A complex, we used Biacore system with immobilized TRAX on sensor chip CM5 to determine the binding of GHCer to TRAX. At concentrations of 9.77 nM to 5 mM, GHCer bound to TRAX with fast association rate, followed by slow dissociation ($K_D \sim 4.09 \times 10^{-8}$ M). Using immunoprecipitation and Mass Spectrometry, we showed that GHCer formed a complex with TRAX through its lipid-tail, which facilitated the recruitment of A2A-R to TRAX-KIF2A complex through its carbohydrate-head, with ensuing A2A-R signaling and immunosuppression.

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Pathogen non-specific bystander T cells were attenuated upon infection with malaria parasites

Suzue, K.¹, Amano, H.², Taniguchi, T.¹, Shimokawa, C.¹, Olia, A.¹, Onishi, R.¹, Hisaeda, H.¹

¹Gunma University Graduate School of Medicine, Department of Parasitology, Maebashi, Japan, ²Gunma University Graduate School of Medicine, Department of Dermatology, Maebashi, Japan

When infected with malaria parasites, infected host falls into immunosuppression state. As a result, allergic symptoms such as atopic dermatitis and contact hypersensitivity (CHS), autoimmune symptoms such as experimental autoimmune encephalomyelitis and collagen-induced arthritis, as well as the course of *Listeria* and *Leishmania* infection, were dramatically changed upon infection with malaria parasites. Our group has been reported that blood ATP concentration is markedly elevated concomitant with malaria parasite infection. Extracellular ATP and its metabolites are known to modulate immune response. Here we show that the extracellular ATP induced by the infection with malaria parasite modulates bystander immune

responses. To examine the role of extracellular ATP on bystander immune suppression, we monitored serum ATP concentration of *Plasmodium yoelii*-infected mice and parasite-non-specific T cell response. The serum ATP concentration was correlated with parasite rate, on the other hand, it was inversely correlated with T cell response and the outcome of CHS. Next, to examine the relationship between serum ATP and the outcome of DNFB-induced CHS, malaria parasite-infected mice were injected with carbenoxolone, an inhibitor for putative ATP secretion channel pannexin-1. Mice infected with malaria parasites showed milder outcome of CHS and relatively lower DNBS-specific T cell response than non-infected control mice. However, when injected with carbenoxolone, the suppressive effect of malaria infection on CHS outcome was diminished. These results suggested that the ATP, induced by malaria parasite infection, elicited bystander immune suppression.

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Human fibroblastic reticular cells of secondary lymphoid organs regulate T cell activation and proliferation through four overlapping mechanisms

Knoblich, K.¹, Siew, S.M.-L.¹, Parekkadan, B.², Turley, S.³, Fletcher, A.¹
¹University of Birmingham, Institute of Immunology and Immunotherapy, Birmingham, United Kingdom, ²Massachusetts General Hospital and Harvard Medical School, Center for Engineering in Medicine and Surgical Services, Boston, United States, ³Genentech, Department of Cancer Immunology, South San Francisco, United States

Fibroblastic reticular cells (FRCs) residing in secondary lymphoid organs are phenotypically and functionally distinct myofibroblasts of mesenchymal origin. Investigations into their role and function have yielded high impact findings regarding their manifold roles as immune regulators. FRCs provide pivotal regulation of lymphoid cells, particularly effector T cells. In mice, FRCs enable T cell activation but suppress effector T cell proliferation and acquisition of effector function, primarily through nitric oxide release, presumably to avoid damage to lymphoid organ infrastructure during an immune response. Accordingly, transfer of ex-vivo expanded mouse FRCs to models of severe inflammation prevents pathology and mortality, suggesting therapeutic potential. However, human FRCs are almost entirely unstudied. Here we show that primary human FRCs from lymph nodes and tonsils shut down human T cell proliferation through multifaceted mechanisms that are nitric oxide independent. Suppression was robust, dose-dependent and MHC independent. Specifically, human FRCs concomitantly utilised TGF β R, A2AR, PGE2 and IDO pathways to suppress both the proliferation and activation of human T cells and to alter their cell cycle and apoptotic properties. Signalling through these pathways increased LAG-3 expression by T cells in the presence of FRCs, while inhibition reversed FRC-mediated immunosuppression of T cell proliferation. FRCs were distinct from mesenchymal stromal cells and did not show trilineage potential in in vitro assays. These results show, for the first time, that human FRCs actively suppress the proliferation of human T cells and define the mechanisms at play, with implications both biological and therapeutic.

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Soluble CD52 is a negative regulator of innate immune cell-driven inflammation

Rashidi, M., Bandala-Sanchez, E., Zhang, Y., Ngui, K., Neale, A., Lawlor, K., Wentworth, J.M., Vince, J., Harrison, L.C.
 Walter & Eliza Hall Institute of Medical Research, Melbourne, Australia

Background: We showed that CD52, a glycosylphosphatidylinositol (GPI)-anchored cell surface glycopeptide, is released upon activation of T cells and suppresses bystander T cells via interaction with the sialic acid-binding immunoglobulin-type lectin 10. Whether soluble CD52 also influences innate immune function remains unexplored and is the focus of this study.

Methods: Human and mouse monocytes, dendritic cells and macrophages were cultured with different concentrations of CD52-Fc or control Fc in the presence of Toll-like receptor (TLR) and TNF receptor (TNFR) ligands for 24h at 37° C and the inflammatory response assessed using NF- κ B activation assays and cytokine ELISAs. Gene targeting of CD52 in the mouse was performed on a C57BL/6 background by homologous recombination of lox-p sites flanking exon 2. Mice were administered CD52-Fc or control Fc (i.v.), then LPS (i.p.), and assessed after 5 h.

Results: CD52-Fc potently inhibited cytokine secretion by different innate immune cells induced by a range of inflammatory stimuli, including both TLR and TNFR ligands, by inhibiting NF- κ B and ERK transcription factor activation. When injected into mice, CD52-Fc blunted the hypothermic and cytokine response to LPS. Moreover, the decrease in body core temperature and the increase in cytokine production upon LPS injection were more pronounced in CD52 knockout mice compared to wild-type littermates.

Conclusion: Our findings demonstrate the suppressive effect of CD52 on innate immune cell-driven inflammation and its potential as a therapeutic candidate for hyper inflammatory conditions such as septic shock.

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Involvement of caveolin-1 in quercetin-induced Nrf2 activation

Matsushima, M., Kusatsugu, Y., Oyabu, S., Ochi, H., Atsumi, K., Ogasawara, N., Takemura, K., Kawabe, T.
 Nagoya University Graduate School of Medicine, Department of Pathophysiological Laboratory Sciences, Nagoya, Japan

Quercetin is one of the flavonoids and has a wide variety of cytoprotective effects. We previously reported that quercetin exerted anti-allergic, anti-oxidant, and anti-fibrotic activities via heme oxygenase (HO)-1 activity. However, the mechanisms how quercetin can induce HO-1 to exhibit cytoprotective effects are poorly understood. We focused on cell membrane, the first line to interact with extracellular environment. Cell membrane has lipid rafts and caveolae, a subset of rafts which participate in the regulation of many cellular functions. Recent studies demonstrated that flavonoids including quercetin have a suppressive effect on the accumulation of lipid rafts. Moreover,

Caveolin-1 (Cav-1), a structural protein component of caveolae, has been reported to interact with Nrf2 and inhibited the expression of antioxidant enzymes, such as HO-1. In this study, we investigated the mechanisms underlying the induction of HO-1 by quercetin, focusing on lipid rafts and Cav-1. We found that quercetin changed lipid raft formation in NIH3T3 cells. Cav-1 was located in cell membrane with Nrf2 under resting condition, and Cav-1-Nrf2 complex was translocated to cytosol and nucleus after quercetin treatment. Treatment with M β CD induced HO-1 expression as much as quercetin. Quercetin dose-dependently increased the phosphorylation of Cav-1, ERK and JNK. These results suggested that the disruption of lipid rafts by quercetin would translocate Cav-1 and Nrf2 from cell membrane and to nucleus, leading to HO-1 induction.

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Soluble-CD40L, and/or CD40L-Ig could beneficially modulate disturbed costimulatory signaling between APCs and CD4+T cells

Royan, A.¹, Fazal, N.²

¹Chicago State University, College of Pharmacy, Chicago, United States, ²Chicago State University, Chicago, United States

Immunosuppression is a dreadful phenomenon in burn-septic patients. Although loss of T cell function that contributes to immunosuppression has been known in both burn-septic animal models and in patients, little is known about therapeutic approaches that could protect injured hosts from these conditions with high risks of morbidity and mortality. In a well-published rat model of burn and (Cecal-Ligation and Puncture)-CLP injury (Fazal, et al, 2006), we documented CD4+ T cell dysfunction and intestinal pathology (damaged microvascular/endothelial and mucosal/epithelial barrier). However, such concurrent T cell dysfunction and intestinal morbidity is not accompanied by any substantial mortality

(< ~20 %) in animals with burn or CLP alone. We designed studies to evaluate the tenet that T cell dysfunction and APC dysfunction are intimately linked in the pathogenesis of burn and/or sepsis injuries, such that injury-caused defective APCs can injure healthy T cells and defective T cells so generated can injure healthy APCs through disturbed signaling between the two immune cell systems. Such derangements could well be related to altered interactions between costimulatory ligands-receptors of APCs and T cells. In this series of experiments, we investigated whether costimulatory agonists, e.g., soluble-CD40L, and/or CD40L-Ig could beneficially modulate disturbed costimulatory signaling between APCs and CD4+T cells, or protect against APC's intracellular pathogenic mechanism developing after burn and/or CLP, could alleviate risks for morbidity and mortality in the burn and/or CLP injuries. Our results showed a partial reversal of immune suppression in these burn-injured animals leading to elimination of morbidity and mortality.

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Plant-derived heme-oxygenase 1 (HO-1) inducers modulate maturation and pro-inflammatory functions in human dendritic cells and macrophages

Campbell, N., Basdeo, S., Mahon, O., Fletcher, J., Dunne, A.

Trinity College Dublin, Biochemistry & Immunology, Dublin, Ireland

The stress response protein Heme-Oxygenase 1 (HO-1) has been identified as an important immunomodulator which is highly upregulated during inflammation. HO-1 catalyses the conversion of free heme to biliverdin, liberating carbon monoxide. Biliverdin is subsequently converted to bilirubin and all three products exhibit potent anti-inflammatory activity in various animal models of disease. The plant-derived polyphenolic compounds carnosol and curcumin induce the expression of HO-1 in many different cell types, and are considered potential anti-inflammatory compounds.

We have examined the effects of carnosol and curcumin in human monocyte-derived dendritic cells (DCs) and macrophages. Treatment of DCs with either compound inhibited LPS-induced expression of the maturation markers CD40, CD80, CD83 and CD86 and reduced the production of the pro-inflammatory cytokines IL-12 and IL-23, resulting in an impaired ability of treated DCs to stimulate allogeneic CD4+

T cells. Carnosol and curcumin treatment restored the phagocytic capacity of LPS-stimulated DCs, and this effect was blocked by addition of the HO-1 inhibitor, SnPP. Reduced activation of NF κ B in response to LPS was also observed in carnosol and curcumin treated DCs. Thus, carnosol and curcumin appear to maintain DCs in an immature, tolerogenic state and this effect may be mediated by HO-1. Macrophages treated with carnosol or curcumin demonstrated reduced expression of M1-associated genes, indicating that carnosol and curcumin may act to polarise macrophages towards an anti-inflammatory or tolerogenic phenotype. In summary, this study confirms that carnosol and curcumin have significant immunomodulatory effects in human innate cells and may have potential as treatments for inflammatory disease.

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Pre-malignant immune suppressive environment is dependent on HPV16E7-Rb interaction induced epithelium hyperplasia

Kuo, P., Tuong, K., Mattarollo, S., Frazer, I.

University of Queensland Diamantina Institute, Brisbane, Australia

Vaccines are available to prevent infection with cervical cancer-causing Human Papilloma Virus (HPV) 16 and 18. However, treatment for persisting HPV infection, the precursor to cancer, is still lacking. Using a transgenic mouse, which expresses the HPV16 viral oncoprotein E7 under the control of keratinocyte 14 promoter (K14E7), a chronic infection model that resembles cervical intraepithelial neoplasia grade 3 (CIN3) has been established. K14E7 skin is hyperproliferative, induced by the interaction between E7 and retinoblastoma protein (Rb), and is associated with an increased lymphocytic infiltration. Previous studies found an immune suppressive environment in HPV16E7-expressing skin, and this local immune suppression

limits transgenic skin graft rejection. To establish whether the expression of viral oncoprotein, or E7-Rb interaction induced hyperplasia, is responsible for the suppressive environment, we used an Rb mutation transgenic mouse model (K14E7Rb9), where the interaction of E7 and Rb is disrupted but normal Rb functions are preserved. K14E7Rb9 mice had, normal skin thickness, normal lymphocyte subpopulations and normal cytokine secretions, as found in wild type C57 and in contrast to K14E7 mice. Expression of E7 limited the effective immune response after OVA immunisation and induced multiple co-inhibitory markers, CTLA4 and PD-1, suggesting possible T cell exhaustion. RNAseq data revealed that chemokine and receptor pathways were highly correlated with E7-induced hyperplasia, which might account for lymphocyte recruitment to the skin. The K14E7Rb9 mouse enables differentiation of the non-Rb associated effects of E7 expression from the E7-induced hyperplasia, which induces locally immunosuppressive inflammation.

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The role of IL-4 in the development and function of regulatory T cells induced by B cells (Treg-of-B cells)

Lin, S.-Y., Chiang, B.-L.

National Taiwan University, College of Medicine, Graduate Institute of Immunology, Taipei, Taiwan, Republic of China

Naïve B cells could act as antigen-presenting cells to induce a subpopulation of Foxp3⁺ regulatory T cells, called Treg-of-B cells. Naïve B-cell-primed T cells is through cell-cell contact and independent of IL-10. The suppressive function of Treg-of-B cells partly requires close cell-cell proximity. Therefore, Treg-of-B cells is a population different from natural Treg cells (nTreg) and Type 1 regulatory T cells (Tr1). Recent studies have demonstrated that Treg-of-B cells could be developed as a therapeutic approach against transplant rejection, allergy and rheumatological diseases. However, the previous studies showed that the differentiation of Treg-of-B cells involved STAT6 phosphorylation and IL-4 secretion, the transcription factor and cytokine of Th2 cells. Hence, we hypothesized that naïve T-B cells co-culture might lead to two different populations. One differentiates to Th2 cells and the other to T cells with regulatory activities. This study was performed to examine the role of IL-4 signaling pathway in the development and function of Treg-of-B cells. We supposed that neutralizing IL-4 would produce higher purity of Treg-of-B cells and enhance regulatory function, in order to improve current therapeutic approaches.

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Inhibition of T cell activation by human LAG-3

Kajihara, T., Sugiura, D., Maruhashi, T., Okazaki, I.-M., Okazaki, T.

Institute for Genome Research Tokushima University, Tokushima, Japan

Inhibitory co-receptors regulate the activation of lymphocytes by dampening antigen receptor signaling. Because of the recent success of tumor immunotherapy targeting CTLA-4 and PD-1, inhibitory co-receptors are attracting high attention of

researchers in various fields. However, the precise mechanisms of inhibition and the interplay among multiple co-receptors are not fully understood.

We previously reported that the synergistic regulation of T cells by PD-1 and LAG-3 is critical for the regulation of autoimmunity. In this study, we analyzed the inhibitory capacity of human LAG-3 using mouse T cell hybridoma cells. Although the inhibitory function of mouse LAG-3 was readily observable, we could not observe the inhibitory effect of hLAG-3. We found that hLAG-3 soluble protein could bind to mouse antigen presenting cells and that the cytoplasmic region of hLAG-3 could suppress TCR signaling when fused with the extracellular region of mLAG-3. Unexpectedly, the expression level of hLAG-3 was much lower than that of mLAG-3 when their transcriptions were driven by retroviral LTR. When we increased the expression level of hLAG-3 by using a stronger promoter, substantial inhibitory effect was observed. We also found that two independent intracellular regions were required for the inhibitory activity of LAG-3 and that the relative contribution of these regions was different between human and mouse LAG-3. These results indicate that mouse ligand and signaling intermediates are cross-reactive to hLAG-3 and that the amount is critical for the inhibitory effect of LAG-3.

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Context-dependent inhibition of antigen-specific T cell activation by LAG-3

Maruhashi, T., Okazaki, I.-M., Sugiura, D., Okazaki, T.

Institute for Genome Research, Tokushima University, Tokushima, Japan

Stimulatory and inhibitory co-receptors play fundamental roles in the regulation of the immune system. We have been analyzing the inhibitory co-receptor, PD-1 which is especially important for the regulation of autoimmunity and tumor immunity as evidenced by the spontaneous development of autoimmune diseases in PD-1 deficient mice and the recent success of PD-1 blocking antibody in the treatment of various cancer patients. Recently, we found that PD-1 collaborates with another inhibitory co-receptor LAG-3 in the regulation of autoimmunity. Mice deficient for LAG-3 and PD-1 died of autoimmune myocarditis by 5 weeks of age on BALB/c background, and LAG-3 deficient NOD mice developed accelerated type 1 diabetes, indicating the critical regulatory role of LAG-3 in preventing autoimmunity.

Although accumulating evidence suggests that LAG-3, a type I transmembrane protein with structural similarity with CD4 is a critical regulator of T cells, its precise mechanism remains elusive. In this study, we tried to unravel the inhibitory mechanisms of LAG-3 against the antigen-dependent activation of T cells. By testing various cell lines overexpressing MHC class II and CD80 as antigen presenting cells, we found that some cell lines could not support inhibitory function of LAG-3 although they can activate TCR. By retroviral expression cloning, we identified a gene that endowed antigen presenting cells the capacity to support the inhibitory function of LAG-3. These results suggest that LAG-3 inhibits antigen-specific T cell activation in a context-dependent manner.

733**Status of immune system of patients with breast cancer in different stages***Bagina, U.S., Kovchur, P.I., Volkova, T.O.**Petrozavodsk State University, Petrozavodsk, Russian Federation*

Recent reports provide evidence that malignant tumors growth correlates with significant deviations in various components of the immune system. It is also well recognized that two possible effects of the immune system components on the tumor are either promoting or down-regulating tumor growth. To investigate connection between T cells and different stages of oncogenesis, specifically breast cancer, we assessed ratio of T cells. The goal of these studies was to investigate a possibility of using T-cell markers as prognostic parameters. In these studies, we used samples from patients with different stages of advanced breast cancer supported by histology analysis. The subsets of mononuclear cells in the peripheral blood were determined using antibodies to CD3, CD4, CD8, CD16, CD20, CD25, and CD95 antigens. We observed non-significant increase of CD16+ cells along with decrease of CD3+ and CD4+ T cells in patients with stage I breast cancer. At the same time, the percentage of CD8+ cells is increased thus suggesting a helper's effect. The percentage of CD3 CD4+ cells further decreases with progression of the disease in patients with stage II tumor. In contrast, the percentage of CD4+CD25+ (Treg) cells increases from 4% in normal controls to ~8% in stage III patients. These data allow for preliminary conclusions that using of T-cell markers for diagnosis of breast cancer might be promising and is worth continuation. Our long-standing goal is to improve the accuracy of diagnosis based on individual immunological pattern (or "portrait") of the patient. Project was supported by the grant №1713.

734**Erythroblasts (Er-suppressor cells) - a new type of suppressor cells that inhibit immune response***Kozlov, V.**Research Institute of Fundamental and Clinical Immunology, Laboratory of Clinical Immunopathology, Novosibirsk, Russian Federation*

In the literature, there is evidence of a pronounced immunosuppressive effect of stress, which stimulate erythropoiesis (erythropoietin, induction of phenylhydrazine anemia, high altitude hypoxia, hemorrhage). It was shown that the transfer of spleen cells from these experimental animals inhibits the production of antibodies in recipients. It turned out discovered that erythroblast cells have the immunosuppressive effect, and they were designated as Er-suppressor cells. It was found that erythroblasts express several cytokines, which are able to be responsible for immunosuppressive effect, including TGF- β . In addition, the erythroblasts have a cytotoxic effect in vitro on some tumor cells. It is assumed that reduced suppressor activity of erythroblasts is one of the mechanisms of autoimmune diseases development, for example. It was also shown that cells of embryonic liver transferring to the recipient mice also suppress the production of antibodies. It is likely that

the suppressor cells of erythroid origin are a major cause of the impossibility of immunogenesis in embryonic liver. Where, according to some authors, B cells are presented, but antibody forming cells completely absent. Perhaps Er-suppressor cells with cytotoxic effects are responsible for the lack of tumor growth during embryonic development.

735**Monocytic myeloid-derived suppressor cells from females, but not males, alleviate CVB3-induced myocarditis by increasing regulatory and CD4⁺IL-10⁺ T cells***Yue, Y., Xiong, S.**Soochow University, Suzhou, China*

Coxsackievirus group B type 3 (CVB3) is a common etiologic agent of viral myocarditis and often causes sexually dimorphic myocarditis with increased incidence and mortality in male. So far, the underlying mechanism for the high male prevalence is not well elucidated. In this study, we deciphered the role of myeloid-derived suppressor cells (MDSCs) in the gender bias in murine CVB3-induced myocarditis by comparing their frequencies, subsets as well as immune suppressive functions. We found that much more myocardial MDSCs were enriched in infected females than males, with dramatically higher percentage ratio of CD11b⁺Ly6G⁻Ly6C^{high} monocytic subset (M-MDSCs) to CD11b⁺Ly6G⁺Ly6C^{low} granulocytic subset (G-MDSCs). Interestingly, more potent suppression on T cell proliferation was also evidenced in female-derived M-MDSCs. Consistently, adoptive transfer of female- but not male-derived M-MDSCs efficiently alleviated CVB3-induced myocarditis in male recipient mice, and this protection could be ascribed to the increased induction of regulatory and CD4⁺IL-10⁺ T cells. Our study suggested that myocardial MDSCs were distinctively induced not only in quantities but also in phenotypes and immune suppressive functions in CVB3-infected males and females; and female-derived more suppressive M-MDSCs contributed to their insensitivity to CVB3-induced myocarditis.

736**Redox regulation of the critical immunoregulatory enzyme indoleamine 2,3-dioxygenase 1***Yeung, A.W.S.¹, Lim, Y.J.¹, Glaros, E.¹, Hastad, M.¹, Dang, L.¹, Freewan, M.¹, Orfi, L.^{2,3}, Keri, G.^{2,4}, Szabadkai, I.², Terentis, A.C.⁵, King, N.J.C.⁶, Witting, P.K.⁶, Thomas, S.R.¹**¹University of New South Wales, School of Medical Sciences, Sydney, Australia, ²Vichem Chemie Research Ltd., Budapest, Hungary, ³Semmelweis University, Department of Pharmaceutical Chemistry, Budapest, Hungary, ⁴Semmelweis University, Department of Medical Chemistry, Budapest, Hungary, ⁵Florida Atlantic University, Department of Chemistry and Biochemistry, Boca Raton, United States, ⁶University of Sydney, Charles Perkins Centre, Discipline of Pathology, Sydney, Australia*

The heme enzyme indoleamine 2,3-dioxygenase 1 (IDO1) is a critical immune regulatory enzyme capable of signalling for immune suppression in diverse pathological settings including chronic inflammation, infection and cancer. During inflammation, IDO1 is expressed within activated innate immune cells that can

produce the reactive oxygen species (ROS) hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) by the oxidative enzymes NADPH oxidase (Nox) and myeloperoxidase (MPO), respectively. We recently reported that H_2O_2 inhibits recombinant IDO1 dioxygenase activity by engaging the enzyme's heme peroxidase activity resulting in oxidative self-inactivation (*JBC* 2013;288:1548). To investigate if Nox-derived H_2O_2 or MPO-derived HOCl also control cellular IDO1 activity, we studied IFN- γ -stimulated human monocytes that co-express IDO1, Nox and MPO. Stimulation of monocytes with PMA increased cellular production of both H_2O_2 and HOCl that coincided with the post-translational inhibition of IDO1 activity. The ability of PMA to inhibit IDO1 was abrogated by inhibition of Nox-derived H_2O_2 production with the PKC inhibitor Bisindolylmaleimide or the flavoprotein oxidase inhibitor diphenyliodonium, but not by inhibition of MPO-derived HOCl with 4-aminobenzoic acid hydrazide. Interestingly, IFN- γ -stimulated human monocytes not only expressed the traditional phagocytic Nox isoform Nox2 but also the more recently discovered Nox4 isoform. While selective Nox4 inhibition reduced cellular H_2O_2 production by ~50%, it did not reverse the inhibitory activity of PMA on IDO1, suggesting that Nox2 but not Nox4-derived ROS control IDO1 activity. These studies establish that Nox-derived ROS controls IDO1 activity in innate immune cells and provide important new insights into the redox control of IDO1's immune regulatory actions.

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The effect of Wuzhi capsules on tacrolimus pharmacokinetics in renal transplant recipients

Li, Y., Chen, J., Yan, L., Ren, J., Shi, Y., Wang, L.

West China Hospital of Sichuan University, Chengdu, China

Objective: Wuzhi capsules (WZCs), a traditional Chinese medicine, which was used in renal transplant recipients to increase the concentration of tacrolimus, while the role of this drug on tacrolimus pharmacokinetics is still unknown.

Patients and methods: A total of 175 kidney transplantation recipients were included in this study, including three groups: Wuzhi group (used immediately after surgery, n=86), control group (always without use, n=40) and self-control group (used from two weeks after surgery, n=32). We measured the renal function and tacrolimus concentrations (7 days, 14 days, 21 days and 28 days after surgery). Additionally CYP3A5 and ABCB1 genotypes of each patient were analyzed.

Results: The mean dose and weight adjusted C₀ in the group with Wuzhi capsules was higher than the controls in the first month after transplant (P < 0.05). For the self-control group, we found their Tac dose and weight adjusted C₀ increased significantly after using Wuzhi capsules (P < 0.05). Multiple binary logistic regression analysis was carried out to assess the baseline clinical covariates (CYP3A5 and ABCB1 genotypes, age, gender, BMI of donors and recipients, HLA, PRA, dialysis time, coinfection with HBV). No modification appeared on the association between Wuzhi capsules and increased C₀. On the other side, there were no differences in the renal functions during those groups.

Conclusions: Wuzhi capsules have strong influence on

tacrolimus pharmacokinetics in the early period after transplantation and the same clinical outcome. This approach can reduce the economic burden of the recipients and maybe reduce tacrolimus-related toxicity.

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Anti-inflammatory activities of dangyuja (*Citrus grandis* Osbeck) on 12-O-tetradecanoylphorbol-13-acetate (TPA) induced skin inflammation

Herath, K.H.I.N.M.¹, Bing, S.J.¹, Cho, J.¹, Kim, A.², Kim, G.-O.³, Lee, J.-C.³, Lee, Y.¹

¹Jeju National University, Department of Veterinary Medicine, Jeju-si, Korea, Republic of, ²Jeju National University, Advanced Convergence Technology & Science, Jeju-si, Korea, Republic of, ³Jeju Diversity Research Institute, Seoqwoo-si, Korea, Republic of

Dangyuja (*Citrus grandis* Osbeck), which is widely used in traditional medicine for its potent anti-inflammatory effect is a citrus cultivated in South Korea. In this study, we examined the anti-inflammatory effect of 80% ethanol extract of *Citrus grandis* Osbeck (ECGO) on mouse edema model induced by 12-O-tetradecanoylphorbol acetate (TPA). For *in vitro* assays, murine splenocytes stimulated with concanavalin A (Con A) were treated with ECGO and evaluated for T cell population and production of inflammatory cytokines IL-2, IL-4 and IFN- γ . For *in vivo* assays, TPA-challenged mice ear skin were treated with ECGO topically and potent anti-inflammation of ECGO was assessed by edema induction, myeloperoxidase (MPO) activity, immunohistochemical localization of inducible nitric oxide (iNOS), cyclooxygenase (COX)-2, CD3⁺ T cells, F4/80⁺ macrophage and infiltration of inflammatory cytokines IL-1 β and TNF- α to the site of inflammation. ECGO-treated Con A-stimulated splenocytes showed a significant reduction in CD44/CD62L⁺ T cell population and a marked decrease in the production of inflammatory cytokines IL-2 and IFN- γ . Interestingly, topical treatment of ECGO significantly decreased the edema induction and MPO activity. Decreased expression of iNOS, COX-2, IL-1 β and TNF- α and suppressed homing of CD3⁺ T cells and F4/80⁺ macrophages to the site of inflammation further evinced the potent anti-inflammatory effect of ECGO. ECGO's anti-inflammatory activity disclosed in this study emphasizes the possibility of developing *Citrus grandis* Osbeck fruit extract as an alternative therapeutic agent for inflammatory edema. This work was supported by High Value-added Food Technology Development Program, Ministry of Agriculture, Food and Rural Affairs.

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Exhausted T cell signature enriched in HPV16 E7 mouse model and cervical intraepithelial neoplasia grade III

Tuong, Z.K., Kuo, P., Sinha, R., Tolley, L., Frazer, I.

UQDI, TRI, Brisbane, Australia

Background: High-risk human papillomavirus (HPV) infection is involved in the development of precancerous cervical intraepithelial neoplasia (CIN) and persistent lesions can eventually lead to cervical cancer. Overcoming immune cell

exhaustion is a highly topical field of research in immunology and the ability to target and reinvigorate exhausted T cells is a promising immunotherapy for a wide range of cancers. We have an established transgenic (K14 promoter) mouse model (C57.K14E7) of epithelial premalignancy in skin expressing HPV E7 oncoprotein (in keratinocytes) whereby the C57.K14E7 skin is hyperplastic and has increased immune cell infiltrate associated with a strong immunosuppressive microenvironment.

Aim: To define the transcriptome of the C57.K14E7 skin.

Method: Here we performed state-of-the-art RNA-seq deep-sequencing analysis coupled with Gene Set Enrichment Analysis and curated a set of ~300 upregulated genes as a 'K14E7' transcriptional signature.

Results: We show that this signature is significantly enriched in human CIN3 cohorts specifically. Furthermore, we show that C57.K14E7 skin express high levels of multiple co-inhibitory molecules by qPCR and FACS analysis and both C57.K14E7 skin and human CIN3 cohorts are significantly enriched for 'exhausted' CD8+ and/or CD4+ T cell gene signatures.

Conclusions: These results suggest that high-risk HPV exploit immune cell exhaustion during infection and future work should be directed towards the development of immune checkpoint blockade-based strategies for immunotherapy in HPV-related CIN.

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Identification of early myeloid progenitors as immunosuppressive cells

Pu, S.¹, Qin, B.¹, He, H.², Zhan, J.¹, Wu, Q.^{1,3,4}, Zhang, X.¹, Yang, L.^{1,3}, Lin, W.^{1,3}, Qu, C.², Zhou, Z.^{1,3,4}

¹Guangxi Normal University, School of Life Sciences, Guilin, China, ²Chinese Academy of Medical Sciences & Peking Union Medical College, State Key Laboratory of Molecular Oncology, Cancer Hospital/Institute, Beijing, China, ³Guangxi Normal University, Guangxi Universities Key Laboratory of Stem Cell and Biopharmaceutical Technology, Guilin, China, ⁴Guangxi Normal University, Research Center for Biomedical Sciences, Guilin, China

Background: Hematopoiesis in stress often exerts myeloid-biased alternations, resulting in accumulation of distinct myeloid lineages including early myeloid progenitors within the compartment of hematopoietic stem/progenitor cells (HSPCs). Despite growing evidence suggesting a crucial role for HSPCs in immunosurveillance, precise function of the specific myeloid progenitor subpopulations remains unclear.

Methods: Tumor model was established via injecting s.c. C57BL/6 mice with Lewis lung carcinoma (LLC) cells. We examined HSPC populations for their suppressive capabilities using T-cell proliferation assays. Gene expression was assessed by RT-PCR, and the levels of NO were determined with Greiss reagents.

Results: We show here that murine granulocyte/macrophage progenitors (GMPs) and common myeloid progenitors (CMPs) freshly isolated from tumor-bearing or naïve animals are capable of inhibiting polyclonal stimuli- and alloantigen-induced T cell proliferation, with tumor host-derived cells having elevated activities. Strikingly, both GMPs and CMPs even display much stronger suppressive capacity than the classical

myeloid-derived suppressive cells. Analysis of GMPs indicates that these cells express iNOS and can secrete high levels of NO. Further studies using iNOS specific inhibitors reveal that the immunosuppression of GMPs is, to a large extent, NO-dependent. Early myeloid progenitors can also efficiently induce regulatory T cell development. During tumor progression, GMPs but not CMPs are markedly expanded within both the bone marrow and blood of mice.

Conclusions: Together, these studies demonstrate that early myeloid progenitors can act as immunosuppressive cells in addition to being hematopoietic precursors. Our finding provides a unique insight into the functional diversity and plasticity of early myeloid progenitor cells.

Tolerance

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Understanding how infection of the thymus renders newly differentiated T cells tolerant to mycobacteria-specific antigens

Cerqueira-Rodrigues, B.^{1,2}, Nobrega, C.^{1,2}, Barreira-Silva, P.^{1,2}, Serre-Miranda, C.^{1,2}, Correia-Neves, M.^{1,2}

¹Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal, ²ICVS/3B's-PT Government Associate Laboratory, Braga/Guimaraes, Portugal

In mouse models of *Mycobacterium avium* systemic infection, bacteria colonize the thymus, the central organ for T cell differentiation, rendering newly generated T cells tolerant to mycobacterial peptides, but not unrelated proteins, indicating a pathogen-specific T cell tolerance.

Several mechanisms can be envisaged to explain this infection-induced T cell tolerance such as antigen-specific T cell deletion, increased frequency/activity of antigen-specific regulatory T cells (Tregs) and/or generation of anergic T cells. Our goal is to explore which of these mechanisms is/are responsible for such tolerance.

Infection of P25 T cell receptor (TCR) transgenic mice, a model where the vast majority of T cells recognize the Ag85 mycobacterial peptide, revealed no major alterations either on the total number of thymocytes, or on the proportion of the four main thymic cell populations, upon systemic infection. Making use of the P25-RAG-GFP mouse model, in which the recent thymic emigrants express GFP, we observed no difference in the number of exported newly generated mycobacteria-specific T cells (GFP⁺CD4⁺). Chimeric mice, transferred with a 10/90 mixture of P25/WT bone-marrow, revealed no differences between mycobacteria-specific and WT T cell differentiation. In addition, infection did not promote an obvious increase of Tregs, anergic T cells or T cells expressing a non Ag85-specific TCR.

Presently, we are testing the ability of these mycobacteria-specific T cells to confer protection upon *de novo* infection, analyzing molecular changes acquired on differentiating T cells and Treg suppressor ability acquired after infection, in order to understand the mechanism(s) responsible for infection-induced tolerance.

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Understanding the impact of patient-derived mutations on CTLA-4 expression and functionKennedy, A.¹, Hou, T.¹, Grimbacher, B.^{1,2}, Ikemizu, S.³, Walker, L.¹, Sansom, D.¹¹University College London, Institute of Immunity and Transplantation, London, United Kingdom, ²Universitätsklinikum Freiburg, CCI-Center for Chronic Immunodeficiency, Freiburg, Germany, ³Kumamoto University, Division of Structural Biology, Kumamoto, Japan

CTLA-4 maintains a critical immune checkpoint and is involved in the function of regulatory T cells. Mice lacking CTLA-4 die soon after birth of profound lymphoproliferation and fatal autoimmunity. Recently we and others have reported individuals with heterozygous (autosomal dominant) mutations in CTLA-4 who suffer from an immune dysregulation syndrome with wide ranging autoimmune features. In many cases these patients are identified as having immunodeficiency due to problems with class switched antibody production. Despite evidence for impaired CTLA-4 function, the impact of the various mutations identified are mostly not understood and may provide new information on the requirements for CTLA-4 function. To address this, we cloned and expressed CTLA-4 proteins based on a spectrum of mutations identified in patients. All missense mutations studied so far were capable of generating an expressed full length protein. We therefore performed a series of flow cytometric and microscopic analyses comparing the expression location, protein trafficking and ligand binding properties of each mutant. This reveals a number of discrete phenotypes of differing severity ranging from partial loss of ligand binding and failure to traffic correctly through to complete failure of the protein folding. This analysis may be used to predict the severity of different mutations, help immunological detection and possibly inform treatment strategies for these individuals.

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Effect of probiotic (Lactobacillus Reuterii) administration on regulatory T cell in SLE with mild manifestation

Mulya, D.

Gadjah Mada University, Internal Medicine, Yogyakarta, Indonesia

Background: In patient with Systemic Lupus Erythematosus (SLE) there are abnormality on T cell lymphocyte, including defect in regulatory T cells both in number and function. Giving probiotic (Lactobacillus reuterii) is expected to stimulate immune response by increasing the number of regulatory T cells and decreasing IL 6 production.

Method: Thirty SLE patients with mild manifestation, were given Lactobacillus reuterii (15 people) and placebo (15 people) for 8 weeks. We then analyzed the level of CD4+CD25+FoxP3+ and IL 6 before and after exposure.

Result: Administration of Lactobacillus reuterii for 8 weeks brought statistically significant improvement on CD4+CD25+Foxp3+ level (1,38%±8,36% VS 3,17%±3,71%; P=0,007; CI -3,91 - -0,74). There were decreased level of IL 6 in Lactobacillus reuterii group (4,76±5,75 pg/ml VS 3,71±3,36 pg/

ml; P=0,25; CI -0,83 - 2,9) and the placebo group (2,6±2,02 pg/ml VS 2,07±2,39 pg/ml; P=0,35; CI -0,57 - 1,5). However at the end of study those changes didn't make statistically significant difference on CD4+CD25+Foxp3+ and IL 6 level between two group.

Conclusion: A significant increase of CD4+CD25+Foxp3+ were found after 8 weeks Lactobacillus reuterii administration.

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Loss of TACI expression allows self-reactive MZ B cells to escape deletion but not energyLim, E.X.¹, Figgett, W.A.¹, Brink, R.², Mackay, F.¹¹Peter Doherty Institute for Infection and Immunity at The University of Melbourne, Department of Microbiology and Immunology, Carlton, Australia, ²The Garvan Institute of Medical Research, Darlinghurst, Australia

B cell-activation factor of the TNF family (BAFF) overexpression is known to subvert B cell tolerance, where self-reactive B cells are allowed to mature rather than being deleted. Transmembrane activator and cyclophilin ligand interactor (TACI) is a receptor for BAFF and loss of TACI protects against autoimmune manifestations mediated by excess BAFF. To further investigate the role of TACI in B cell self-tolerance, we crossed TACI^{-/-} mice onto switched-HEL (Sw_{HEL}) mice, whereby the BCR of B cells are modified to recognize hen egg lysozyme (HEL). We generated bone marrow chimeras using HEL-transgenic hosts (sHEL-Tg) reconstituted with Sw_{HEL} or TACI^{-/-}Sw_{HEL} BM to track the fate of anti-HEL self-reactive B cells in the presence or absence of TACI expression. Flow cytometry gating on HEL-binding B cell populations revealed skewing to a high proportion of TACI^{-/-} HEL-specific marginal zone (MZ) B cells remaining, compared to the TACI-sufficient Sw_{HEL}^osHEL-Tg control group in which mature HEL-specific B cells are efficiently deleted. In vitro BCR stimulation of splenic B cells with soluble HEL suggested that although the absence of TACI allowed HEL-binding B cells to escape deletion, the consequence of this breach of tolerance may be mitigated by the B cells being rendered anergic, as indicated by the failure to up-regulate CD86. TACI is expressed highly on MZ B cells and is where self-reactive B cells normally accumulate. These results suggest that loss of TACI is required for tolerance via self-reactive MZ B cell deletion, but is not required for tolerance via self-reactive B cell anergy.

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Neonatal tolerance to proinsulin is sufficient to prevent autoimmune diabetes*Jhala, G.^{1,2}, Chee, J.¹, Trivedi, P.¹, Selck, C.¹, Gurzov, E.¹, Graham, K.^{1,2}, Thomas, H.^{1,2}, Kay, T.^{1,2}, Krishnamurthy, B.^{1,2}*¹St. Vincent's Institute of Medical Research, Immunology and Diabetes Unit, Fitzroy, Australia, ²The University of Melbourne, Department of Medicine, Fitzroy, Australia

All individuals are born with a certain level of self-tolerance to tissue specific antigens partly due to presentation of self-antigens in the thymus. Those with genetic predisposition, who may have less robust self-tolerance, develop autoimmune disease. Augmenting the level of antigen-specific tolerance may prevent autoimmunity. We aimed to define whether augmentation of pre-existing tolerance for a limited period could be effective. For this, we expressed two different autoantigens, proinsulin and islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) in the antigen presenting cells (APCs) of autoimmune diabetes-prone non-obese diabetic (NOD) mice during defined periods and tracked self-antigen specific T cells. Proinsulin expression from birth until weaning was sufficient to completely protect NOD mice from diabetes, insulinitis and development of insulin autoantibodies. Insulin-specific T cells were significantly diminished (424 ± 50 vs. 181 ± 24), were naïve and did not express IFN γ ($14\% \pm 1.7$ vs. $4\% \pm 0.9$) when challenged. We tracked IGRP specific CD8⁺ T cells in NOD mice expressing IGRP in APCs. When IGRP was expressed until weaning, IGRP specific CD8⁺ T cells were not detected later in life (1797 ± 786 vs. 50 ± 4). Thus, islet specific auto-reactive T cells are uniquely produced in early life. Our finding that a brief exposure to proinsulin confined to neonatal life in NOD mice imparts long lasting protection from diabetes leads us to suggest that neonatal life is a vulnerable period for the escape of insulin-specific T cells. Therapies bolstering proinsulin antigen presentation during early life in high-risk human subjects may provide the best chance of prevention of diabetes development.

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Does dendritic-cell targeted antigen expression induce both T and B cell tolerance?*Brooks, J.¹, Al-Kouba, J.¹, Davies, J.², Wells, J.¹, Steptoe, R.¹*¹University of Queensland Diamantina Institute, University of Queensland, Woolloongabba, Australia, ²Institute of Health and Biomedical Innovation, Queensland University of Technology, Brisbane, Australia

Autoimmune and allergic diseases often involve an interplay of T and B cell responses and develop, potentially, through failure of tolerance in or between these compartments. Transgenic expression of antigen is robustly tolerogenic. Restricting antigen expression to dendritic cells (DC) or other antigen-presenting cells (APC) is equally tolerogenic, induces central and peripheral T-cell tolerance and prevents the development of autoimmune and allergic disease. The mechanisms through which DC- or APC-targeted antigen expression prevents disease development remain unclear. We asked whether DC, as specialized APC for T cells, might prevent disease purely through

induction of T-cell tolerance. To define the effects of targeted antigen expression, we aimed to compare models where OVA expression is restricted to DC (11c.OVA), MHC class II+ APC (MII.OVA) or expressed ubiquitously (actin.OVA). 11c.OVA and MII.OVA mice express a truncated form of OVA (OVA₁₃₉₋₃₈₆) and when immunized with full-length OVA mount strong T-cell and IgG1, 2a & 2b responses that appear to be directed against the unexpressed (aa1-138) portion of OVA. In contrast, actin.OVA mice that express full-length OVA have significantly dampened IgG and T cell responses. To define whether this reflects the effects of antigen targeting, expression level or the fragment of OVA expressed, we produced recombinant OVA₁₃₉₋₃₈₆ using *Escherichia coli* expression and his-tag purification. Studies underway will reveal the effects of targeted antigen expression using this recombinant protein. We conclude these transgenic models will be unique and valuable tools for defining T and B cell tolerance.

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Semi-mature dendritic cell generation by targeting acetyl-CoA carboxylase in the lipid metabolism process*Kim, J.-S., Thuy, N.P., Park, C.G.**Seoul National University College of Medicine, Seoul, Korea, Republic of*

The adoptive transfer of tolerogenic dendritic cells has great potential for treating autoimmune diseases and for preventing transplant rejection. In this study, we developed a novel method for generating tolerogenic semi-mature dendritic cells by targeting lipid metabolism pathway. Immature dendritic cells (imDCs) from C57BL/6 mice were generated by 8-day culture of the bone marrow-derived dendritic cell (BMDC) precursors in the presence of GM-CSF and IL-4. imDCs were matured with LPS in the presence or absence of CP-640186 (CP), an acetyl-CoA carboxylase (ACC) inhibitor for 24hr. Phenotypic and functional characteristics of BMDCs were examined for identifying the role of lipid metabolism pathway during DC maturation. ACC inhibition by CP-640186 resulted in decreased accumulation of neutral lipid in LPS-treated DCs. Expression level of costimulatory molecules including CD80, CD86 and CD40 and MHC II was greatly down-regulated by dose-dependant manners. Coincidentally, the secretion of pro-inflammatory cytokines (IFN- γ , IL-6, IL-12p70) was suppressed. Expression of CCR7, a mature DC homing receptor, was decreased. On the contrary, expression of CCR5, a receptor largely expressed in immature DCs, was reversely increased. Antigen uptake capacity was greatly increased in the presence of CP-640186 by a dose dependant manner. DCs generated in the presence of CP-640186 greatly down-regulated the CD4⁺T cell proliferation by an antigen-specific manner. In conclusion, these data demonstrated that semi-mature DCs can be generated by targeting acetyl-CoA carboxylase in the lipid metabolism pathway. We suggested here novel target for the induction of metabolism based-dendritic cell tolerance for preventing transplant rejection and treating autoimmune diseases.

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In vitro induction of serial transfer of allospecific suppression potential in human peripheral T cells*Uchida, K., Negishi, N., Bashuda, H., Kawai, F., Okumura, K., Habu, S. Juntendo University, Atopy Research Center, Tokyo, Japan*

Background: We have achieved clinical operational tolerance in transplantation via adoptive transfer of the ex vivo induced donor specific anergic cells. This accomplishment have proposed the life period of the anergic cells and maintenance mechanism or immunological tolerance. Based on the infectious tolerance concept, we have hypothesized naïve T cell inherit allospecific suppressive function when coexisted with the transferred anergic cells

Material and methods: Donor specific anergy in recipient PBMC was induced in mixed lymphocyte culture with irradiated donor PBMC in the presence of anti-CD80/86 antibodies. These generated anergy recipient cell were transferred into the next MLC to determine whether they have potential to sequentially transfer their suppressive function into new alloreactive PBMC. To distinguish the anergy and new naïve recipient cell after co-culture, new PBMC were labeled with CFSE.

Result: The proliferation and IL-2 expression of the recipient T cells were significantly suppressed under anti-CD80/86 blockade in the first MLR with significant increase of CD4⁺CD25⁺Lag3⁺FOXP3⁺ cells. These induced 1st anergic PBMC provided the donor-specific suppression activity to new naïve activated recipient cells with significant expansion of CD4⁺CD25⁺Lag3⁺ cells and low IL-2 expression. Moreover, such anergy status transferred into the third MLR recipient cells.

Conclusion: These results indicate the in vitro induced allospecific anergic T cells by blocking the 2nd TCR signal generate T cells with potential to suppress another activated T cells, suggesting the serial induction of allo-specific tolerance that may be promise. This observation established inheritance of allospecific suppressive function in human supporting immune tolerance.

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Ndfip1 limits autoreactive CD8⁺ T cell responses to high dose antigen*Wagle, M.V.¹, Goodnow, C.C.^{1,2,3}, Parish, I.A.¹**¹Australian National University, John Curtin School of Medical Research, Canberra, Australia, ²Garvan Institute of Medical Research, Immunology, Darlinghurst, Australia, ³University of New South Wales, St. Vincents Clinical School, Sydney, Australia*

As thymic selection is not a perfect process, peripheral tolerance mechanisms have evolved to inactivate rogue self-reactive T cells that escape into the periphery. While the molecular pathways that enforce CD4⁺ T cell peripheral tolerance are well studied, relatively little is known about the molecular basis of CD8⁺ T cell tolerance. Ndfip1 is a key immunoregulatory adaptor molecule required for the function of inhibitory ubiquitin ligases such as Itch, and Ndfip1 loss severely disrupts CD4⁺ T cell peripheral tolerance. In this study, we examined the role of Ndfip1 in CD8⁺ T cell peripheral tolerance using the transgenic RIP-OVAhi model of CD8⁺ T cell tolerance. In this model, ovalbumin (OVA)

is expressed as a neo-self-antigen within the insulin-producing pancreatic islet β -cells, and transfer of ovalbumin (OVA)-specific OT-I CD8⁺ T cells into RIP-OVAhi mice leads to OT-I tolerance. Surprisingly, Ndfip1 deficient OT-I cells were tolerised normally in the RIP-OVAhi model, with the transferred cells failing to cause diabetes. However, in the context of higher antigen doses within a peptide anergy model, Ndfip1 deficient OT-I cells displayed elevated expansion relative to their wild-type counterparts, and acquired effector function through enhanced Granzyme B expression and downregulated CD62L. Moreover, boosting antigen doses in the RIP-OVAhi model by peptide injection allowed mutant but not wild-type OT-I cells to breach tolerance and cause diabetes. Collectively, these data suggest that Ndfip1 regulates peripheral CD8⁺ T cell tolerance, but only in the context of high antigen doses.

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Involvement of TIGIT in serial transfer of anergy status in T cells*Negishi, N.¹, Uchida, K.¹, Sato, T.², Kametani, Y.³, Okumura, K.¹, Habu, S.¹**¹Juntendo University Graduate School of Medicine, Atopy (Allergy) Reserch Center, Tokyo, Japan, ²Tokai University School of Medicine, Department of Immunology, Isehara, Japan, ³Tokai University School of Medicine, Department of Molecular Life Science, Isehara, Japan*

The term “infectious tolerance” was referred to the serial suppression process in which anergy status is transferred from the anergic T cells to newly stimulated naïve cells. To confirm this phenomenon precisely, we established the in vitro experimental system, in which OVA specific TCR transgenic (TCR-Tg) mice that react super-antigen, TSST-1, through the TCR- β chain. The spleen cells from TCR-Tg mice receiving TSST-1 administration became anergic status restimulation of OVA/TSST-1. The induced 1st anergy T cells showed to induce newly stimulated naïve T cells to be the 2nd anergy T cells when both were co-cultured with OVA. And the 2nd anergy T cells also had a similar character. To elicit molecular mechanism of the anergic status transfer, we compared molecules between the anergy and activated T cells by flow-cytometric and microarray comparison of gene expression. Among the different expression molecules, we have focused TIGIT as a responsible molecule for serial transfer of the anergic status. TIGIT has been reported to function as the co-inhibitory molecules. TIGIT is negative in naïve status but expressed by stimulation. Then, we examined the time course of TIGIT-expression and -intensity in stimulated naïve T cells with or without anergy T cells. When naïve T cells were co-cultured with anergy T cells, stimulated naïve T cells showed higher and earlier TIGIT expression in comparison to that of their single culture without the anergy cells. Based on these results, we will discuss how TIGIT is linked to suppressing activation of stimulated naïve T cells.

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Modified signaling cascades in DAMP-tolerized human phagocytes

Freise, N., Ortkras, T., Vogl, T., Roth, J., Austermann, J.
University Hospital Muenster, Institute of Immunology, Münster, Germany

Background: Sepsis is still associated with high mortality and develops in two phases, an early strong inflammatory phase and a secondary hypo-reactive state which is called endotoxin-tolerance. We recently demonstrated that the DAMP (Damage associated molecular pattern) proteins S100A8/S100A9 are able to induce a Toll-like receptor 4 (TLR4) dependent hypo-responsiveness in phagocytes under sterile conditions, a mechanism called stress-tolerance (Austermann et al. Cell Rep. 2014). The goal of the present study was to analyse molecular mechanisms underlying stress-induced tolerance in phagocytes.

Methods: We investigated the impact of different kinase signaling pathways in the S100A8/S100A9 induced stress-tolerance of human monocytes. Impaired activity of kinases and the influence of kinase inhibitors on target proteins were analysed by western blot and ELISA. Protective effects of inhibitors *in vivo* were tested using the D-Gal model of septic shock.

Results: Activity of GSK-3 is down-regulated during S100-induced tolerance of human monocytes. Accordingly, endogenous NF- κ B antagonists I κ B α and BCL-3, two known targets of GSK-3, are up-regulated in tolerant monocytes. *In vivo* data show a protective effect of pharmacological tolerance induction by the use of the GSK-3 inhibitor CHIR99021 on the survival rate of mice during septic shock.

Conclusion: The inactivation of the GSK-3 and the resulting accumulation of I κ B α in tolerant human monocytes interfere with the translocation of the active transcription factor NF- κ B to the nucleus. *In vivo* data indicate that inhibiting GSK-3 and thus imitating a natural protective mechanism of the immune system could be used as a treatment in sepsis patients.

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Antigen-decorated engineered red blood cells as a novel immune tolerance-inducing agent

Pishesha, N., Bilate, A., Wibowo, M., Lodish, H., Ploegh, H.
Massachusetts Institute of Technology/Whitehead Institute for Biomedical Research, Cambridge, United States

Currently available therapies for autoimmune diseases are traditional immunosuppressive medications, exposing patients to higher risks of opportunistic infection and other discomforts. Immunoregulatory modalities, which are able to prophylactically educate or therapeutically re-educate the immune system to induce antigen-specific tolerance are desirable. Here we employed engineered red blood cells carrying desired synthetic or natural antigens to induce long term, drug-free, specific immunotolerance while maintaining immune competence. Utilizing genetic engineering and Sortase A-mediated protein modification strategies, we generated both mouse and human red blood cells covalently carrying the desired antigens. Covalent conjugation allows precise quantification of antigens

attached and sortagging method robustly attaches consistent amount of various antigens. These modification strategies do not inflict any damage to the red blood cells as the engineered red blood cells can circulate *in vivo* with normal half-life. As a proof of concept using the ovalbumin-specific T cell receptor transgenic mouse models, we found that administration of red blood cells that carry the relevant peptide epitopes leads to induction of tolerance, as inferred from the drastic reduction in number of adoptively transferred CD4⁺ and CD8⁺ T cells. We further illustrate the tolerance-induction capacity of these engineered red blood cells to a model of autoimmune diseases, experimental autoimmune encephalitis (EAE), a mouse model for multiple sclerosis, we showed that myelin oligodendrocyte glycoprotein (MOG)-decorated red blood cells could suppress, or at least delay the onset of, the disease. We have therefore highlighted the potential of hijacking the natural properties of red blood cells to curb autoimmune diseases.

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Dexamethasone and MPLA treatment of monocyte-derived dendritic cells from rheumatoid arthritis patients induce transcriptional modulation of genes related to cell recruitment, signaling and metabolism

García-González, P.A.^{1,2}, Schinnerling, K.^{1,2}, Sepúlveda, A.³, Ubilla-Olguín, G.^{1,2}, Soto, L.⁴, Neira, O.⁵, Medhi, A.M.⁶, Nel, H.J.⁶, Catalán, D.^{1,2}, Thomas, R.⁶, Verdugo, R.A.³, Aguilón, J.C.^{1,2}

¹Universidad de Chile, Immune Regulation and Tolerance Research Group, Programa Disciplinario de Inmunología, ICBM, Santiago, Chile, ²Millennium Institute on Immunology and Immunotherapy, Santiago, Chile, ³Universidad de Chile, Chilegenómico, Programa de Genética Humana, ICBM, Santiago, Chile, ⁴Universidad de Chile, Unidad del Dolor, Hospital Clínico de la Universidad de Chile, Santiago, Chile, ⁵Hospital del Salvador, Sección de Reumatología, Santiago, Chile, ⁶University of Queensland Diamantina Institute, Translational Research Institute, Queensland, Australia

Background: Despite growing interest in tolerogenic dendritic cells (tolDCs) as target for immunotherapy in autoimmunity, the molecular mechanisms driving differentiation of tolDCs towards a regulatory phenotype are poorly understood. We studied the transcriptional pattern of tolDCs from healthy subjects (HC) and rheumatoid arthritis patients (RA), modulated with dexamethasone (Dex) and activated with monophosphoryl lipid A (MPLA) (DM-DCs), to identify molecular regulators and pathways related with tolerogenicity.

Methods: Genome-wide transcriptional profiling of DM-DCs, Dex-modulated DCs (D-DCs), MPLA-matured DCs (M-DCs) and untreated DCs from HC and RA, was performed with Illumina HumanHT-12 v4 BeadChip Array. Differentially expressed (DE) genes were determined using a false discovery rate of 0.5 and a 1.5 fold change, compared to untreated DCs. Functional enrichment and pathway overrepresentation was performed using Ingenuity Pathway Analysis. Expression of genes of interest was confirmed through qPCR.

Results: Dex and MPLA exert a distinctive effect on DCs transcriptional program, which is similar on RA and HC. 814 DE transcripts were identified in DM-DCs, further grouped according to expression patterns, exhibiting downregulation of

maturation/inflammation-associated genes and upregulation of tolerance-related genes. Functional and pathway analysis revealed enrichment of genes involved in cellular movement, cell-to-cell signaling and interaction, and metabolism, particularly ROS production and zinc homeostasis; predicting the activation of chemotactic responses and proliferation of regulatory cells.

A network of gene-gene interactions, including potential transcriptional factors involved, was constructed.

Conclusion: DM-DCs exhibit a particular transcriptional programming in response to Dex and MPLA, supporting a regulatory profile on these cells.

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Liver sinusoidal endothelial cells induce the tolerogenic and regulatory feature of autoreactive CD4⁺ recent thymic emigrants

Ge, Q., Xu, X., Wang, K., Zhang, S.

Peking University Health Science Center, Beijing, China

Mechanisms of peripheral tolerance play a critical role in preventing T cells that escape from negative selection in the thymus from initiating autoimmune reactions. The purpose of this study was to investigate the tolerance of recent thymic emigrants (RTEs) in the liver. We found that a fraction of CD4⁺ RTEs were retained in the liver independent of the secondary lymphoid organs. Following transfer into RAG^{-/-} recipients, CD4⁺ liver RTEs induced more severe organ-specific inflammation and cell infiltration than lymph node RTEs, indicating that more autoreactive RTEs were retained in the liver. Compared to RTEs from the lymph nodes, more CD4⁺ RTEs from the liver acquired activated phenotype (CD44^{hi}CD62L^{lo}CXCR3^{hi}PD-1^{lo}Foxp3⁻) with high expression of LAG3, FasL, IL-10, and IFN- γ . These liver RTEs also acquired regulatory functions as they could suppress the proliferation of naïve T cells *in vitro*. We further found that liver sinusoidal endothelial cells could induce the tolerogenic and regulatory phenotype of liver RTEs via cellular interaction. The expression of MHC, Aire, and Notch all contributed to RTEs' acquisition of liver tolerance. These results suggest that the liver is the first checkpoint in the periphery to filter, retain, and enforce tolerance to autoreactive CD4⁺ thymic emigrants that escape from negative selection.

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Ablation of pathogenic memory T-cell responses by bone marrow-mediated gene therapy under immune-preserving conditions

Stephane, R.¹, Al-Kouba, J.¹, Coleman, M.¹, Jessup, C.², Starkey, M.³, Overgaard, N.¹, Bridge, J.¹, Horvat, J.³, Werder, R.⁴, Walters, S.⁵, Coates, P.T.⁶, Grey, S.⁵, Thomas, R.¹, Wells, J.¹, Hansbro, P.³, Phipps, S.⁴, Davies, J.⁷

¹The University of Queensland Diamantina Institute, Brisbane, Australia, ²Flinders University, Adelaide, Australia, ³The University of Newcastle and Hunter Medical Research Institute, Newcastle, Australia, ⁴The University of Queensland, Brisbane, Australia, ⁵Garvan Institute of Medical Research, Sydney, Australia,

⁶University of Adelaide, Adelaide, Australia, ⁷Queensland University of Technology, Brisbane, Australia

Established memory T cell responses represent a key hurdle to both protein and tissue replacement therapies as well as the application of tolerogenic immunotherapies in general. Targeting expression of antigen to antigen-presenting cells is a powerful means to induce T-cell tolerance. We have shown previously that genetic targeting of antigens to DC and other APC inactivates memory CD4⁺ and CD8⁺ T cells, but we now show this can be exploited for induction of therapeutic tolerance to 'turn-off' established but unwanted T-cell responses. We have combine the approaches of targeted antigen expression and bone marrow (BM) / hematopoietic stem cell transplantation under immune-preserving conditions to show antigen-specific, therapeutic termination of memory CD4⁺ and CD8⁺ T-cell responses. This approach has the capacity to turn off the pathogenic T-cell responses that underlie autoimmune rejection of antigen-expressing pancreatic islet transplants and Th2-mediated airways inflammation. Associated with this immune-related pathology related to these responses, such as airways inflammation and hyper-responsiveness is prevented or ameliorated. Immunological mechanisms associated with reversal of established memory T-cell responses by BM transfer under immune preserving conditions appear to be deletion and induction of unresponsiveness through T-cell 'adaption'. Refinement of BM-mediated gene therapy for tolerance such as this could lead to development of highly-effective therapies for T-cell mediated pathologies.

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Determining the molecular factors that control apoptotic cell disassembly

Tixeira, R.¹, Nedeva, C.¹, Atkin-Smith, G.¹, Phan, K.¹, Puthalakath, H.¹, Herold, M.², Hulett, M.¹, Poon, I.¹

¹La Trobe Institute for Molecular Science, Biochemistry, Melbourne, Australia, ²Walter & Eliza Hall Institute, Melbourne, Australia

Apoptosis is a key process in maintaining homeostasis in the body. Clearance of apoptotic cells is important, as inefficiency is linked to various diseases including autoimmune and inflammation. Numerous cells types are involved in the clearance of apoptotic cells including professional phagocytes namely, macrophages and dendritic cells, and non-professional phagocytes such as fibroblasts and epithelial cells. To achieve timely cell clearance, apoptotic cells undergo regulated disassembly. This apoptotic cell disassembly (ACD) process involves a series of coordinated morphological changes such as membrane blebbing (circular bulges of the plasma membrane), apoptopodia formation (string-like membrane extensions), and fragmentation to generate apoptotic bodies. Inhibitor based assays have shown Rho Kinase 1 (ROCK1) and P21 activated kinase 2 (PAK2) are involved in membrane blebbing, while Pannexin 1 (PANX1) is a negative regulator of apoptotic body formation. However, specific roles of these proteins in ACD are not well defined as most studies are based on pharmacological approaches. Moreover, how ACD could aid efficient cell clearance is also not well understood. Here, using CRISPR technology- based gene

disruption in Jurkat T cells as a model system; we are able to confirm that ROCK1 is important for membrane blebbing, while loss of PAK2 shows no phenotypic changes to ACD. Disruption of PANX1 leads to enhanced ACD. Furthermore, engulfment assays using dendritic cells and macrophages derived from peripheral blood monocytes show a preference for apoptotic bodies over apoptotic whole cells. These data suggests that apoptotic cell disassembly is a regulated process, which may have implication on efficient cell clearance.

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Tyrosine phosphatase SHP-1 as a critical switch in altering the sensitivity of Tcon cells to Treg mediated suppression

Mercadante, E., Lorenz, U.

University of Virginia, Carter Immunology Center and Department of Microbiology, Immunology and Cancer Biology, Charlottesville, United States

Immune tolerance requires a balance between T cell activation and regulatory T cell (Treg)-mediated suppression. While the number and functionality of Treg cells are known to influence immune responses, resistance of conventional T (Tcon) cells to Treg-mediated suppression can also contribute to autoimmunity. However, how Tcon resistance is modulated is poorly understood and here through several observations, we demonstrate a novel role for SHP-1 in regulating Tcon susceptibility to suppression. First, genetic ablation of SHP-1 specifically in T cells leads to resistance of Tcon cells to Treg-mediated suppression. Second, in a complementary approach, inhibiting the enzymatic activity of SHP-1 through sodium stibogluconate (SSG or Pentostam) also confers resistance to wild type Tcon cells indicating a

T cell intrinsic mechanism. Third, T cells deficient in SHP-1 protein or activity are hyper-responsive to TCR-mediated stimulation, as evidenced by hyper-proliferation. Fourth, in the absence of SHP-1 activity, a higher percentage of the T cell population respond to stimulation suggesting that SHP-1 regulates the threshold that separates response versus no response. Finally, analysis of downstream signaling events indicates altered activation of AKT under conditions of SHP-1 deficiency, consistent with resistance to Treg-mediated suppression. Collectively, these genetic and pharmacological approaches reveal a novel role for the signaling protein SHP-1 in regulating suppression of Tcon cells by Treg cells. Since resistance to Treg-mediated suppression is increasingly linked to the pathogenesis of autoimmune diseases, our data are relevant for autoimmunity, for potential new therapeutic options, and for tumor immunity where Treg cells hinder a productive immune response.

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Relevance of immunomodulatory factors in HSCT: HLA-G as a biomarker for graft versus host disease

Kanga, U.¹, Mourya, M.¹, Seth, T.², Chowdhury, K.¹, Mitra, D.K.¹, Coshic, P.³, Mahapatra, M.²

¹All India Institute of Medical Sciences, Transplant Immunology and Immunogenetics, New Delhi, India, ²All India Institute of Medical Sciences, Hematology, New Delhi, India, ³All India Institute of Medical Sciences, Transfusion Medicine, New Delhi, India

Introduction: HLA-G is a non-classical HLA-class I molecule with immunosuppressive property. Through interaction with receptors ILT2, ILT4 and KIR2DL4 on several immune cells, HLA-G mediates a micro-environment that induces tolerance. miRNAs interact with polymorphic sites in the HLA-G 3'UTR region and control post transcriptional regulation.

Material and methods: The present study was conducted on patients with leukaemia and aplastic anaemia who underwent Hematopoietic Stem Cell Transplantation (HSCT). We investigated HLA-G 3'UTR region- exon 8-14pb INDEL polymorphism and SNPs (miRNA interaction sites). Additionally, the levels of soluble HLA-G, Treg cell frequency and levels of Th1 (IL-2, IL-17, TNF- α , and IFN- λ) and Th2 cytokines (IL-4 and IL-10) were evaluated at pre and post transplant time points (days 15, 30, 90 and at GvHD).

Results: The Kaplan Meier analysis revealed that recipients with 14bp ins/ins genotype had lower incidence of GvHD in comparison of those with 14bp del/del genotype. The incidence of GvHD was higher in recipients with HLA-G 3'UTR +3027 CC, +3035CC other SNP genotypes. Of the 7 UTR haplotypes in North Indians, UTR1 and UTR 3 were associated with GvHD. The soluble HLA-G1/G5 levels, Treg cell frequency and Th2 cytokines levels were significantly lower at the time of GvHD, whereas levels of Th1 cytokines were higher.

Conclusion: HLA-G 3'UTR polymorphism impacts the soluble HLA-G levels that create an immunosuppressive micro-environment, modulates Treg cell frequency and Th1/Th2 cytokine balance and all this correlates with manifestation of GvHD and impacts transplant outcome. We propose HLA-G molecule as possible biomarker for predication of GvHD.

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Checkpoints for autoreactive B cells in peripheral blood of lupus patients assessed by flow cytometry

Jeganathan, V.¹, Malkiel, S.², Wolfson, S.², Manjarrez Orduño, N.², Marasco, E.², Aranow, C.², Mackay, M.², Gregersen, P.², Diamond, B.²
¹The Feinstein Institute for Medical Research, Bronx, United States,
²The Feinstein Institute for Medical Research, Manhasset, United States

Antibodies to nuclear antigens (ANA) are diagnostic in several autoimmune disorders, yet failure to achieve B cell tolerance in these diseases is still poorly understood. Although secreted ANA detected by an indirect immunofluorescent assay (IFA) are the gold standard for autoreactivity, there has been no convenient assay to measure the frequency of circulating B cells that recognize nuclear antigens (ANA + B cells) in patients. Our aim was to generate an assay to easily identify these B cells and to examine its utility in a study of autoreactive B cells in Systemic Lupus Erythematosus (SLE). We developed and validated a novel flow cytometry-based assay that identifies ANA + B cells using biotinylated nuclear extracts, and used it to examine B cell tolerance checkpoints in peripheral blood mononuclear cells (PBMCs) obtained from SLE patients and healthy controls (HCs). We show progressive selection against ANA + B cells as they mature from transitional to naïve to CD27 + IgD - memory cells in both healthy subjects and SLE patients; however SLE patients failed to anergize ANA + naïve B cells to

the same extent as healthy individuals. We also show anergy induction is restored in SLE patients treated with belimumab, an inhibitor of B cell-activating factor (BAFF). This assay will enable studies of large populations to identify potential genetic or environmental factors affecting B cell tolerance checkpoints in health and disease and permit monitoring of the B cell response to therapeutic interventions.

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Antigen-specificity of superior dominant encephalitogenic peptide confers inductivity, stability and hybrid signatures to CD69⁺CD103⁺ subset of Treg responsible for sustainable inhibition of CNS autoimmune diseases

Lin, Y.^{1,2}, Miyake, S.³, Yamamura, T.¹

¹National Institute of Neuroscience, National Center of Neurology and Psychiatry, Immunology, Tokyo, Japan, ²National Center Hospital, National Center of Neurology and Psychiatry, Neurology, Tokyo, Japan, ³Juntendo University Graduate School of Medicine, Immunology, Tokyo, Japan

Targeted monoclonal antibodies have dramatically improved the therapeutic efficacy for some autoimmune diseases, however, it is insufficient to completely inhibit disease activity and differs depending on the disease. Regulatory cells therapy is promising, but the heterogeneity influencing the stability and complications due to non-specific suppression are problematic. By focusing on experimental autoimmune encephalomyelitis (EAE), previously, we demonstrated that sensitization of superior dominant peptide sustained remission due to induction of CD69⁺CD103⁺ (DP)-subset of regulatory T cells (Treg) after EAE peak.

DP-subset of Treg was most potent, expressing maximum Treg-compatible markers, and harbored multiple hybrid signatures corresponding to each phase of EAE. DP-subset preferentially infiltrated into the central nervous system without loss of Foxp3 expression by retaining low IL-6R expression.

Furthermore, the DP-subset of Treg acquired high antigen-specificity that in turn maintained the hybrid signatures and IL-6R^{low} fraction, accounting for the stability. Antigen-specificity and hybrid signatures of Treg were also detected in CD69⁺CD103⁺ (103SP)-subset. Unlike the DP-subset, 103SP-subset expressed high IL-6R and also harbored the non-Treg fraction, exhibiting plasticity for the down-regulation of Foxp3 under inflammatory condition. TCR stimulation without proper antigen was unable to sustain hybrid signatures and increased the non-Treg fraction involved in EAE relapse.

Only for the superior dominant peptide, sensitization led to the suppression of the reactivity to other encephalitogenic peptides in a tissue-specific manner, unlike tolerance induction inhibiting in a peptide-specific manner. Such suppression was detected under non-EAE condition and in EAE-affected mice.

This mechanism can be used for alternative therapy to resolve the problems described here.

Transplantation

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P2X7 polymorphisms and the role of P2X7 in graft-versus-host disease

Adhikary, S.^{1,2}, Geraghty, N.^{1,2}, Belfiore, L.^{1,2}, Alexander, S.³, Sluyter, R.^{1,2}, Watson, D.^{1,2}

¹University of Wollongong, Wollongong, Australia, ²Illawarra Health and Medical Research Institute, Wollongong, Australia, ³The Children's Hospital at Westmead, Westmead, Australia

The P2X7 receptor has been implicated in the development of graft-versus-host disease (GVHD). The human *P2RX7* gene is highly polymorphic and P2X7 activity can be altered by single nucleotide polymorphisms (SNPs). We investigated donor human peripheral blood mononuclear cells (hPBMCs) for their *P2RX7* genotype and activity. The *P2RX7* gene was sequenced from genomic DNA from thirteen human donors. Seven known *P2RX7* gene SNPs including three gain-of-function and four loss-of-function P2X7 SNPs were found. The SNPs identified accounted for the five known haplotypes of the *P2RX7* gene (*P2X7-1* (wildtype), *P2X7-2*, *P2X7-3*, *P2X7-4*, and *P2X7-5*). Similar P2X7 activity was observed between CD3⁺, CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells within each donor. However, high P2X7 activity was observed in T cells from donors with gain-of-function *P2RX7* SNPs and haplotypes *P2X7-2*, *P2X7-4* and *P2X7-5*. Low P2X7 activity was observed in T cells from donors with loss-of-function SNPs and haplotype *P2X7-3*. hPBMCs from a *P2X7-2* haplotype donor were injected into NODSCIDIL-2Rg^{null} (NSG) mice and the effect of P2X7 blockade (with Brilliant Blue G, BBG) on the development of GVHD was examined. NSG mice injected with hPBMCs and BBG or saline (control) showed similar levels of hPBMC engraftment (predominantly T cells). Similar CD4⁺ and CD8⁺ T cell engraftment and intracellular IFN-gamma was observed in spleens. Both BBG and control mice developed GVHD. However BBG-injected mice demonstrated reduced serum IFN-gamma compared to control mice. This warrants further investigation into the role of P2X7 donor genotypes and P2X7 blockade on the development of GVHD in this humanised mouse model.

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The ligands of translocator protein (TSPO) inhibit human Th1 responses and prevent the rejection of murine skin allografts

Wu, C.¹, Zhang, Y.², Yu, S.², Yang, B.²

¹Institute of Immunology, Department of Immunology, Guangzhou, China, ²Institute of Immunology, Zhongshan School of Medicine, Guangzhou, China

The translocator protein (TSPO) may impact inflammatory and immune responses. However, the exact actions of TSPO on Th1 cell responses *in vitro* and *in vivo* are still unclear. In the current study, we found that TSPO ligands, FGIN1-27 and Ro5-4864, both suppressed the production of IFN- γ in a dose- and time-dependent manner, but only Ro5-4864 suppressed TNF- α production by human PBMCs after stimulation. FGIN1-27 inhibited the production of IFN- γ by memory CD4⁺ T cells and

the differentiation of naïve CD4⁺T cells into Th1 cells. In addition, TSPO ligands inhibited mixed lymphocyte reactions by human or murine cells. After the transplantation of allogeneic murine skin, injection of TSPO ligands prevented graft rejection and resulted in reduced levels of IFN- γ as measured *ex vivo* compared to the control. FGIN1-27 suppressed cell cycle, cell proliferation and activation of CD4⁺T cells. TSPO ligands also inhibited expression of the corresponding transcriptional factors including T-bet, pSTAT1, pSTAT4 and pSTAT5. Taken together, our data suggest that TSPO ligands inhibit Th1 cell responses and may be novel therapeutic drugs for the treatment of autoimmune diseases and prevention of transplant rejection.

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Chitosan encapsulated superparamagnetic iron oxide (SPIO) nanoparticles facilitating the graft tracking and immunosuppression in allo-transplantation

Shen, C.-R.^{1,2}, Wu, S.-T.¹, Liu, H.-Y.¹, Wang, J.-J.¹, Tsai, Z.-T.², Juang, J.-H.^{1,2}, Liu, C.-L.³

¹Chang Gung University, Taoyuan, Taiwan, Republic of China,

²Chang Gung Memorial Hospital, Taoyuan, Taiwan, Republic of China, ³Ming Chi University of Technology, New Tpei, Taiwan, Republic of China

In vivo imaging, being non-invasive, quantitative, and repetitive, of targeted macromolecules have recently attracted widespread interests. In particular, the use of superparamagnetic iron oxide (SPIO) leads to the combination of high spatial resolution of magnetic resonance (MR) imaging and the high specificity of magnetic markers. We had developed and demonstrated SPIO coated with γ -ray irradiated chitosan (CSPIO) as a great MR T2 contrast agent for grafted islet tracking. In this study, we intend to equip the CSPIO with immunosuppressive characteristics, and apply in allo-transplantation. After evaluating the transfecting efficiency of different CSPIO preparations, the CSPIO (HSPIO) encapsulated with a quaternized chitosan, N-[(2-hydroxy-3-trimethylammonium) propyl] chitosan chloride (HTCC), appeared to demonstrate a better potential to deliver gene expression. The transfecting efficiency of HSPIO could be even elevated by supplying poly-L-lysine (PLL) or lipofectamine, and such had no influence on cell growth and viability. In fact, the relaxivity of the HSPIO equipped with the reporter genes was also assessed, and no change was obtained on the T1 and T2 signals in MR imaging. It indicated that the interaction or intracellular uptake of such HSPIO with the cells resulted in the target gene expression. Finally, the utilization of IL-10 gene resulted in the satisfied amount of IL-10. Now the application of the HSPIO equipped with IL-10 is being tested for allo-transplantation. We anticipate that the newly developed contrast agents would not only benefit for graft tracking but also reducing immune rejection in cell or tissue transplantation.

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Fetal in-utero hepatocyte transplantation for immunodeficiency disease: a systematic review of case reports

Chibueze, C.E.¹, da Silva Lopes, K.¹, Ota, E.¹, Enosawa, S.², Umezawa, A.²

¹National Research Institute for Child Health and Development, Health Policy, Setagaya-ku, Japan, ²National Research Institute for Child Health and Development, Department of Reproductive Biology, Center for Regenerative Medicine, Setagaya-ku, Japan

Background: Fetal in-utero hepatocyte transplants for the treatment of prenatally diagnosed immunodeficiencies affords immune manipulation parallel to ongoing maturity without a need for immunosuppression and its attendant side effects. To evaluate available evidence on in-utero hepatocyte transplantation, we conducted a systematic review.

Methods: We searched multiple database for ongoing trials, case reports, published and unpublished studies on in-utero hepatocyte transplantation. Titles and abstracts were screened and ineligible studies excluded. Full texts were then evaluated for inclusion independently by two authors, eligible articles were then retrieved and data extracted and summarised.

Results: 2 studies conducted in 2 countries, France (1993) and Sweden (2002) were eligible for inclusion, this included 5 fetuses (2 SCID (severe combined immunodeficiency syndrome), 1 BLS (bare lymphocyte syndrome), 2 Thalassemia major). Hepatocytes were gotten from either dead or viable foetuses with ages ranging from 7 - 11.5 weeks. Fresh and cryopreserved hepatocytes at 0.02×10^9 at >78% viability were transplanted at 14 - 28 gestational weeks through the umbilical vein (3) and intraperitoneum (2). Reported outcomes included engraftment (5), immune reconstitution (4), live births (4), partial improvement in symptoms (3) or complete recovery (1), death (1). Due to evident heterogeneity between studies and a lack of detailed information, a meta-analysis was not possible.

Conclusions: Fetal in utero hepatocyte transplants provide a promising alternative to fetal abortions, as they result in partial or incomplete reconstitution of T cells usually prior to birth and exclude possible transplant rejection.

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Allele mismatch at the HLA-A locus is associated with high risk of relapse in unrelated umbilical cord blood transplantation

Chen, D.-P.^{1,2}, Tseng, C.-P.^{1,2}, Chang, S.-W.³, Wang, W.-T.¹, Jaing, T.-H.⁴

¹Chang-Gung Memorial Hospital, Laboratory Medicine, Taoyuan, Taiwan, Republic of China, ²Chang-Gung University, Taoyuan, Taiwan, Republic of China, ³Chang-Gung University, Clinical Informatics and Medical Statistics Research Center, Taoyuan, Taiwan, Republic of China, ⁴Chang Gung Children's Hospital, Department of Pediatrics, Taoyuan, Taiwan, Republic of China

Allogeneic hematopoietic stem cell transplantation is effective in the treatment of hematologic malignancy. Owing to the tolerance of immune reaction caused by one or two mismatched alleles in the HLA gene and the difficulty in finding matched HLA between donor and patient, unrelated umbilical cord blood transplantation (UUCBT) offers an attractive venue for

treatment of patients with rare HLA types. Nevertheless, which allele mismatch is relevant to the occurrence of graft-versus-host disease and relapse in UUCBT has not yet been unveiled. In this study, the HLA alleles in the HLA-A, -B, -C and -DR loci for 116 pairs of donor/patient undergone UUCBT with a minimum of one year follow-up were analyzed using the sequence-specific oligonucleotide probe method. Chi-square test was then used to compare the number of mismatched and non-mismatched donor-recipient pairs, the number of mismatched alleles, and the number of mismatched antigens at the HLA-A, -B, -C, and -DR loci. Our data revealed that both the number of mismatched donor-recipient pairs (P -value = 0.0182) and the number of mismatched alleles (P -value = 0.0253) at the HLA-A locus were significantly associated with the occurrence of relapse. The recipients with mismatched alleles were 2.2 times more likely to relapse when compared to those without mismatched alleles. Hence, matching of HLA-A alleles between recipient and donor is crucial to reduce the risk of relapse in UUCBT.

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Swine SIRP α (CD172) protein has specific IgV residues associated with recognition of human CD47 and can bind human CD47 *in vitro*

Powell, E.J.¹, Boettcher, A.¹, Cunnick, J.¹, Loving, C.L.², Tuggle, C.K.¹

¹Iowa State University, Ames, United States, ²USDA-ARS-NADC, Food Safety and Enteric Pathogens, Ames, United States

Superior success in human hematopoietic cell (hHSC) engraftment in the non-obese diabetic (NOD) mouse is due, in part, to the ability of the signal-regulatory protein α (SIRP α , CD172) receptor to recognize human CD47. Such binding causes an inactivation signal to be transduced into host SIRP α + cells, preventing phagocytosis and killing of graft cells. Pigs are more similar in size and physiology to humans than mice; therefore, a severe combined immunodeficient pig model for hHSC transplant research is under development. This includes evaluating the human CD47 interaction with porcine SIRP α to assess the potential for human-pig xenograft tolerance. Porcine SIRP α shares specific residues necessary for binding with the NOD mouse SIRP α and human SIRP α that are not found in human SIRP β or normal mouse SIRP α (not able to bind). Specifically, porcine SIRP α IgV, the domain critical for recognition of human CD47, contains twelve of nineteen residues that have been identified as significant for binding. To test the hypothesis that similarities between swine and NOD mice SIRP α are sufficient for binding to human CD47, we used flow cytometry to determine if recombinant human CD47 chimeric protein (hCD47Fc) could bind to swine peripheral blood mononuclear cells (PBMCs) *in vitro*. We also examined if cells bound by hCD47 were SIRP α +, which would be indicative of hCD47 binding to porcine SIRP α . Preliminary results indicate hCD47Fc does bind to SIRP α + porcine monocytes. This interaction suggests that SIRP α + porcine macrophages may tolerate human CD47+ cells, and further work underway to evaluate this interaction.

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Cytokine release syndrome in haploidentical stem cell transplantation in children with non-malignant disease: higher incidences of infusion-related febrile reaction but no acute graft-versus-host disease

Yeap, F.^{1,2}, Francisco, K.^{1,2}, Villegas, M.^{1,2}, Tan, P.-L.^{1,2}

¹Khoo Teck Puat-National University Children's Medical Institute, National University Health System, Singapore, Singapore,

²Department of Paediatrics, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore

Haploidentical SCT provides an important alternative for treating patients requiring an urgent SCT but with no HLA-matched donor available. However CRS is a feared complication in haploidentical SCT. We attempted to determine the incidence of CRS in pediatric patients with non-malignant conditions undergoing haploidentical SCT and its prognostic impact.

Our review consisted of 5 patients with non-malignant conditions (1 SCID, 1 ALD, 3 HLH), with 3 patients requiring a 2nd transplant. Patients were aged between 6 months to 15 years. All received myeloablative conditioning for their first transplant except 1 who became too septic. The mean CD34 cell dose was $26.9 \times 10^6/\text{kg}$ ($5.07\text{-}39.44 \times 10^6/\text{kg}$). These were all T cell depleted (CD3/CD45 RA depleted or TCR $\alpha\beta$ depleted). CRS was observed in 7 out of 8 transplants and 5 were grade 2 and above. 3 patients had grade 4 CRS; 2 required dual inotropic support and 1 had raised intracranial pressures and was given tocilizumab.

Six out of 8 cases met the criteria for IRFR and all except 1 developed engraftment syndrome. The median time for developing engraftment syndrome was +11 (9-13) days. Stem cell dose did not seem to influence the risk of CRS and we did not observe a correlation in the height of C-reactive protein or procalcitonin with the severity of CRS. With increased IRFR and ES seen in our small review of children compared to that previously reported,^{1,2} it was interesting that only 1 developed chronic graft-versus host-disease suggesting that GVHD may be dependent on the underlying disease.

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Impact of CD99 on T cell immune response following an allogeneic skin transplantation

Nam, G., Choi, E.Y.

Seoul National University College of Medicine, Biomedical Science, Seoul, Korea, Republic of

CD99 is expressed on most hematopoietic cells and has been intensively studied in leukocyte migration. However, its role has rarely been investigated in T cell-mediated immune response. Since allogeneic skin transplantation is a useful tool for studying the outcome of T cell immune response, we transplanted allogeneic BALB.B donor skin to wild type or CD99-deficient B6 recipient mice to examine the effect of the absence of CD99 on T cell reactivity during allogeneic graft rejection. We found that the progression of graft rejection based on the assessment of graft scores was delayed and median graft survival time was increased in CD99-deficient recipient mice, indicating the involvement of CD99 in allogeneic graft rejection. Contrary

to previous reports that blockade of CD99 stopped leukocyte transmigration, CD99-deficient CD8 T cells were able to migrate to the allograft antigen sites. However, the absence of CD99 in allo-antigen-specific CD8 T cells resulted in slower activation and weaker immune response than in CD99-sufficient CD8 T cells, suggesting that delayed graft rejection in CD99-deficient host was due to the insufficient activation of allo-reactive CD8 T cells, rather than inhibited migration. Results presented here provide new insight into CD99 as a regulator of T cell activation and its effector function following allogeneic tissue transplantation.

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Analysis of immune recovery following allogeneic stem cell transplantation

de Silva, H.¹, French, R.^{1,2}, Korem, M.^{3,4}, Paukovics, G.¹, Orłowski, E.¹, Curtis, D.^{5,6}, Spencer, A.^{5,6}, Avery, S.⁶, Patil, S.⁶, Morrissey, O.^{3,4}

¹Burnet Institute, Centre for Biomedical Research, Melbourne, Australia, ²Monash University, Department of Immunology and Pathology, Melbourne, Australia, ³Monash University Central Clinical School, Department of Infectious Disease, Melbourne, Australia, ⁴Alfred Health, Central Clinical School, Monash University Melbourne, Melbourne, Australia, ⁵Australian Centre for Blood Diseases, Monash University, Melbourne, Australia, ⁶Malignant Haematology and Stem Cell Transplantation Service, Alfred Health, Melbourne, Australia

Introduction: Allogeneic stem cell transplantation (SCT) is used to cure haematological malignancies but immune recovery is necessary to prevent infection. Data on immune responses in the current era of allogeneic SCT are limited.

Methods: Since Feb 2015, 18 allogeneic SCT recipients had blood collected at baseline, 3, 6, 9 months post-SCT for analysis by lymphocyte proliferation assays, Th1, Th2, Th17 cytokines in serum and culture supernatant, and immunophenotyping of T, B, NK, monocyte and neutrophil cell subsets.

Results: Median age of patients was 46 years, 65% were male and 50% had acute leukaemia. Baseline total white cell count was below normal in 37%, and 33%, 28% and 61% respectively had decreased baseline absolute neutrophil, monocyte and lymphocyte counts.

Median percent values for neutrophils (54%), monocytes (5.9%), T (14%) and NK cells (4.1%) were within normal range at baseline but B cells (0.7%) were decreased. At 3-months (n=6), neutrophils (68%), monocytes (8.3%) and NK cells (13%) were above baseline whereas B and T cell recovery was slower with only smaller increases by 6-months in median percent B (1.2%) and T cells (16%) respectively, (n=5).

Proliferative responses to mitogen decreased from baseline to 6-months (median stimulation-index 55 vs. 20) and antigen-specific responses were not seen until 9-months post-SCT. Th1, Th2, Th17 cytokines were detected in cell cultures following stimulation. Apart from CD40L (median=311pg/ml) serum had very low cytokine levels.

Conclusions: This pilot demonstrates the kinetics of immune recovery in the 9-months post allogeneic SCT. Further analysis in a larger cohort is required.

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Differential modulation of IL-12 family cytokines in islet graft rejection

Chen, H.-Y.¹, Chou, F.-C.², Sytwu, H.-K.^{1,2}

¹National Defense Medical Center, Graduate Institutes of Life Sciences, Taipei, Taiwan, Republic of China, ²National Defense Medical Center, Department and Graduate Institute of Microbiology and Immunology, Taipei, Taiwan, Republic of China

The relative contribution of T helper 1 (Th1) and T helper 17 (Th17) cells in graft rejection is inconclusive, based on evidence provided by different T cell-related cytokine-deficient animal models and graft types. We used novel antigen-presenting cell-specific IL-12p35 knockout (KO), IL-23p19 knockdown (KD) and IL-27p28 KD strategies to investigate T cell differentiation in islet graft rejection. In vitro dendritic cell-T cell coculture experiments revealed that dendritic cells (DCs) from IL-12p35 KO and IL-23p19 KD mice showed reduced ability to stimulate interferon- γ and IL-17 production by T cells, respectively. To further explore the T cell responses in islet graft rejection, we transplanted islets into streptozotocin-induced diabetic NOD/SCID recipient mice with IL-12-, IL-23-, or IL-27-deficient backgrounds and then challenged them with BDC2.5TCRtg T cells. The survivals of islet grafts were significantly prolonged in IL-12p35 KO and IL-23p19 KD recipients, compared with the control recipients. T cell infiltrations and Th1 cell populations were also decreased in the grafts, correlating with prolonged graft survival. Our results suggest that IL-12 and IL-23 promote and/or maintain Th1 cell-mediated islet graft rejection. Thus, blockade of IL-12 and IL-23 might act as therapeutic strategies for reducing rejection responses.

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Investigating the role of NK cells in regulating donor cell engraftment, rejection and the graft-versus tumour effect

Davis, J.^{1,2,3,4}, Jiao, Y.^{2,5,6}, Belz, G.^{5,7}, Koldej, R.^{1,2,3,4}, Huntington, N.^{5,7}, Ritchie, D.^{1,2,3,4,8}, Transplantation, NK cells

¹The Royal Melbourne Hospital, The ACRF Translational Research Laboratory, Melbourne, Australia, ²University of Melbourne, Department of Medicine, Melbourne, Australia, ³The Peter MacCallum Cancer Centre, Cancer Immunology Research, Melbourne, Australia, ⁴University of Melbourne, Sir Peter MacCallum Department of Oncology, Melbourne, Australia, ⁵Walter and Eliza Hall Institute of Medical Research, Molecular Immunology Division, Melbourne, Australia, ⁶Tsinghua University, School of Medicine, Beijing, China, ⁷University of Melbourne, Department of Medical Biology, Melbourne, Australia, ⁸The Royal Melbourne Hospital, Department of Clinical Oncology and Haematology, Melbourne, Australia

Allogeneic haematopoietic stem cell transplantation (allo-HSCT) is used to treat a range of haematological malignancies through an immunological anti-tumor effect. Allo-HSCT is limited by significant toxicity related to conditioning intensity, opportunistic infection, graft failure, and graft-versus host disease. Allo-HSCT outcome is dependent on immunological recovery, reducing toxicity and reliable and rapid marrow recovery. Natural killer (NK) cells contained within the donor

graft have been implicated in both the benefit and toxicity of allo-HSCT. The role of recipient NK cells in allo-HSCT outcome has not been explored. Using mouse models, we explored the manipulation of recipient NK cells on donor engraftment and the ability to exploit NK cell deficiencies to alter conditioning and donor T cell doses.

We used MHC-mismatched mouse models (BALB/c (H2K^d) into C57BL/6 (H2K^b)). Wild type (WT), perforin-deficient (PD) or NK-deficient (Mcl1^{fl/fl} Ncr1-Cre) (MCL) recipient mice received 2 x 600 rad before iv injection of 7.5^{×10⁶} donor BM + 1^{×10⁶} T cells. Compared to WT recipients, both PD and MCL mice had accelerated expansion of donor-derived T cells, rapid differentiation to effector memory T cell subtypes, and elevated pro-inflammatory serum cytokine levels (MCP-1, IFN γ and IL-6) resulting in a higher rate of fatal GVHD. In PD and MCL-1 recipients, reduction of irradiation doses to 800 rad was sufficient to achieve long-term engraftment, whereas WT mice rejected the graft.

These findings indicate that manipulation of recipient NK cell function can be utilized to lower conditioning intensity and promote engraftment. Exploration on how these findings impact on GVT is ongoing.

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Migration of allogenic T cells in the small intestine during graft-versus-host disease

Ottmueller, K.^{1,2}, Brede, C.¹, Friedrich, M.³, Mokhtari, Z.¹, Thusek, S.¹, Amich, J.¹, Brandl, A.¹, Jordán-Garrote, A.-L.¹, Kalleda, N.¹, Miller, M.⁴, Figge, M.T.⁵, Heinze, K.³, Beilhack, A.^{1,2}

¹University Clinic Wuerzburg, Wuerzburg, Germany, ²Graduate School of Life Sciences, University of Wuerzburg, Wuerzburg, Germany, ³Rudolf Virchow Center, University of Wuerzburg, Wuerzburg, Germany, ⁴Washington University School of Medicine, St. Louis (MO), United States, ⁵Hans Knoell Institute (HKI) Jena and Friedrich Schiller University Jena, Jena, Germany

Hematopoietic cell transplantation is a powerful treatment against hematologic malignancies. However, it is often hampered by the complication of graft-versus-host disease (GvHD), where alloreactive donor T cells attack healthy host tissues. Strikingly, only three specific target organs are infiltrated during acute GvHD: Beside liver and skin, the gut is a primary target organ of GvHD.

During priming of naive donor T cells in secondary lymphoid organs, upregulation of CCR9 on alloreactive donor T cells plays a pivotal role in their subsequent extravasation into the lamina propria of the small intestine where their vigorous inflammatory response destroys the host tissue. Infiltration occurs by both CD4 and CD8 conventional T cells, but also by populations of regulatory T cells and Th17 cells. However, the timing and proportion of these populations, as well as the basis for selective organ infiltration and guidance within the tissue are not well understood.

In the course of GvHD, we find increased intestinal CCL25 expression, which is an attractant for CCR9+ donor T cells. *In vivo* two-photon microscopy revealed that infiltration of CD4 T cells into the small intestine precedes CD8 T cell infiltration. These cells are influenced by the collagen network and migrate in a directed manner, confirming that after extravasation, T cells

are guided within the tissue towards a source of gradient such as chemokines. Manipulating this driving force is a promising strategy to avert GvHD without affecting beneficial anti-leukemogenic T cell toxicity.

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Transplantation equals organ and resident immune cell transfer

Prosser, A.¹, Larma, I.², Huang, W.H.³, Liu, L.¹, Nanayakkara, C.¹, Lu, B.⁴, Kallies, A.⁵, Lucas, M.⁶

¹University of Western Australia, Medicine and Pharmacology, Nedlands, Australia, ²University of Western Australia, Centre for Microscopy, Characterisation and Analysis, Nedlands, Australia, ³University of Western Australia, School of Surgery, Nedlands, Australia, ⁴St Vincent's Hospital, Immunology Research Centre, Melbourne, Australia, ⁵Walter & Eliza Hall Institute of Medical Research, Melbourne, Australia, ⁶University of Western Australia, Pathology and Laboratory Medicine, Perth, Australia

Transplantation of solid organs entails transplantation of immune cells residing within the organ, such as regulatory T cells, conventional effector T cells as well as circulating and tissue-resident memory cells. The fate and function of these cells after transplantation, however, is unknown.

Congenetic mice receive an orthotopic syngeneic liver transplant and organs (graft liver, spleen, LN and bone marrow; BM) are harvested at Day 1 and Day 14 post transplantation. The tissue homogenates are analysed for the presence of tissue resident T cells (Trm), innate lymphoid cells (NK and NKT), naive, central and effector memory CD8 and CD4 T cells by multi-parameter flowcytometry.

Shortly after transplantation, grafted livers are rapidly infiltrated by recipient lymphoid cells which consist predominantly of CD8⁺ T cells, some of which gain phenotypic characteristics of Trm cells by Day 14. In addition, there is an apparent expansion of donor cells with a Trm phenotype post transplantation. The numbers of donor and recipient conventional liver NK cells increase over time whereas donor NKT cell numbers remain stable and recipient NKT cells infiltrate the liver. Donor tissue resident immune cells that can be found at small numbers in LN, spleen and BM post transplantation surgery are maintained at low frequency over the study period.

Immune resident cells within organs are readily transferrable and are maintained within the new host. Recipient cells also contribute to the replenishment of the resident immune cell pools. The extent and quality of the cellular components transferred via the graft may influence transplantation outcome.

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Predictive immunological biomarkers of graft versus host disease

Moreno-Lafont, M.¹, Gutiérrez-Hoya, A.^{1,2}, Navarro-Hernández, I.C.¹, Vela-Ojeda, J.³, Montiel-Cervantes, L.³, Rodríguez-Cortés, O.⁴, Rosales-García, V.⁵

¹Instituto Politécnico Nacional, ENCB, Inmunología, México D.F., Mexico, ²FES-Zaragoza. UNAM, México DF, Mexico, ³Centro Médico Nacional La Raza, IMSS, México DF, Mexico, ⁴Instituto Politécnico Nacional, ESM, México DF, Mexico, ⁵Centro de Investigación y de

Estudios Avanzados, CINVESTAV, México DF, Mexico

Allogeneic hematopoietic stem cell transplantation (AHSCT) is used in patients with hematologic diseases; however, there are difficulties for the transplant to be successful: having a good mobilization of HSC and other cells, having a success graft, and no graft versus host disease (GVHD) develops. This work focused on the response to the HSC mobilization on donors, monitoring post-transplant patients, and the correlation of the data with the success or failure of the transplant by searching biomarkers to avoid GVHD. A panel of inflammatory biomarkers in healthy individuals, donors mobilized with Filgrastim® (G-CSF) and patients undergoing AHSCT were assessed by flow cytometry. At first, it was studied the donors after mobilize HSC, to know if this process induces a specific profile. An increase of cells with inflammatory profile CD8+IFN γ + ($p < 0.01$), CD4+IFN γ + ($p < 0.001$) and CD8+IL17+ ($p < 0.05$) as well as regulatory profile CD8+IL10+ ($p < 0.01$) were observed. So it was decided to make a correlation between patients who developed acute GVHD (aGVHD) and the percentages of IFN γ + T-cells that were infused. Patients which developed an aGVHD had received higher percentages of CD8+IFN γ + (Tc1) T-cells; however, this observation was not statistically significant. Patients were divided into 3 groups: with aGVHD, with controlled aGVHD, and without GVHD. In these cases the Tc1 ($p < 0.01$) and CD8+IL17+ T-cells (Tc17) ($p < 0.05$) are important in the development of GVHD. Moreover CD8+FoxP3+ Treg cells ($p < 0.01$) contribute in the process of regulating inflammation; so, these 3 subpopulations can be used as predictive biomarkers of GVHD.

775**Non-invasive prediction of renal transplant rejection using FOXP3 mRNA expression analysis in peripheral blood and urine: a prospective study**

Barabadi, M.¹, Keshavarz Shahbaz, S.¹, Hosseinzadeh, M.¹, Foroughi, F.², Nafar, M.³, Amirzargar, A.A.^{1,4}

¹Tehran University of Medical Sciences, Department of Immunology, Tehran, Iran, Islamic Republic of, ²Tehran University of Medical Sciences, Department of Immunology, School of Public Health, Tehran, Iran, Islamic Republic of, ³Shahid Labbafinejad Medical Center and Shahid Beheshti University of Medical Sciences, Chronic Kidney Disease Research Center and Department of Nephrology, Tehran, Iran, Islamic Republic of, ⁴Tehran University of Medical Sciences, Molecular Immunology Research Center, Tehran, Iran, Islamic Republic of

Background: FOXP3+ Regulatory T cells play a crucial role in immune response to rejection and the induction of allograft tolerance. This study focuses on the blood and urinary FOXP3 mRNA expression in renal transplant recipients, as an important factor in the development and function of regulatory T cells. We hypothesized that due to its regulatory function, FOXP3 gene expression alterations in patients undergoing renal transplantation can be used as an immunodiagnostic tool in predicting graft outcome.

Methods: we analyzed peripheral blood mononuclear cell and urinary FOXP3 mRNA expression in 212 blood and 159 urine samples taken from 53 kidney transplant recipients

in a prospective cohort study within first 6 months post transplantation. Measurements were performed using Taqman mRNA assay. Moreover, Receiver operating characteristic (ROC) analysis was performed to distinguish allograft dysfunction from well-functioning grafts.

Results: The expression level of transcription factor FOXP3 in patients developing allograft dysfunction ($n=25$) 6 months post-KTx was higher ($p < 0.001$) compared with well-functioning group ($n=27$). Analysis of receiver operating characteristic curves could discriminate graft dysfunction from normal functioning group by calculating the optimal FOXP3 mRNA cutoff value.

Conclusion: Results presented here provide the evidence that high expression of FOXP3 in blood and urine from kidney transplant recipients appears to be a useful non-invasive diagnosis of allograft dysfunction and graft outcome assessment.

Keywords: Kidney Transplantation, FOXP3, acute rejection.

776**Allogeneic transplantation associated 'Cytokine Storm' leads to early death in chemo-conditioning based murine model of graft versus host disease (GVHD)**

Mehta, M.^{1,2}, Majumdar, A.¹, Kumar, R.³, Gota, V.²

¹Bombay College of Pharmacy, Department of Pharmacology and Toxicology, Mumbai, India, ²Advanced Center for Treatment, Research and Education in Cancer, Department of Clinical Pharmacology, Navi-Mumbai, India, ³Tata Memorial Hospital, Department of Pathology, Mumbai, India

Background: The extent of release of pro-inflammatory cytokines and subsequent damage to target organs of GVHD following allogeneic transplantation in Busulfan - Cyclophosphamide conditioning based murine model was profiled.

Methods: Recipient Balb/c mice were treated with Busulfan (80mg/kg/4days) followed by Cyclophosphamide (100mg/kg/2days). On "Day 0" recipient mice were transplanted with 20*10⁶ bone marrow and 30*10⁶ spleen cells from MHC mismatched donor C57BL/6 mice. Non transplanted mice served as chemo-controls. At pre-defined time interval, mice were sacrificed and major internal organs were subjected for pathological evaluation. Serum cytokines were analyzed using Luminex xMAP platform.

Result: Levels of IFN-Gamma, TNF-Alpha, IL-4, IL-5, IL-1 Beta, IL-2, IL-10 and IL-17 were increased by 292, 2.89, 2.40, 2.84, 2.21, 2.96, 6.47 and 2.83 folds respectively compared to chemo-control group after transplantation. IL-6, IP-10 and IL-12 p70 remained unaffected. Aberrant rise of pro-inflammatory cytokines were accompanied by inflammatory changes in recipient's Liver, Skin and Gut. Liver was characterized by lobular inflammation and peri-portal and central vein chronic inflammation. Chronic active colitis with surface ulceration, crypt abscess, apoptosis and sloughing of epithelial lining revealed the damage in Gut. Inflammation along with apoptotic keratinocytes in epidermis and basal cell vacuolization were observed in Skin. Median survival of transplanted mice was 9 days compared to 15 days of chemo-control group [Hazard Ratio = 4.06 (3.39 - 39.75)].

Conclusion: Aberrant rise in levels of pro-inflammatory

cytokines leads to severe inflammatory changes and subsequent damage in target organs of GVHD and thus associated with early mortality in mice following allogeneic transplantation.

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Sterile inflammation in grafted organs and its systemic side effects

Larma, I.¹, Nanayakkara, C.², Prosser, A.², Huang, W.³, Liu, L.², Lucas, M.⁴

¹University of Western Australia, Centre for Microscopy, Characterization and Analysis, Nedlands, Australia, ²University of Western Australia, Medicine and Pharmacology, Nedlands, Australia, ³University of Western Australia, School of Surgery, Nedlands, Australia, ⁴University of Western Australia, Pathology and Laboratory Medicine, Nedlands, Australia

Sterile inflammation within grafted solid organs is an unavoidable technical consequence of transplantation. The corollaries of this early inflammation affect graft survival and the recipient's health overall.

We use a murine cardiac syngeneic transplant model in order to understand local and systemic cellular dynamics without adaptive responses to foreign tissue. Mice receive a graft heart besides their native heart. Hearts, draining lymph nodes (LN), spleen and bone marrow (BM) are analysed by multi-parameter flow-cytometry at day 1 (D1; n=6) or day 7 (D7; n=7) post-transplant.

The grafts increase in size at D1 due to an influx of monocytes (CD11b⁺CD11cLy6C⁺MHCII⁻), granulocytes (SSC^{high}CD11b^{high}Ly6G^{high}) and T cells accompanied by a proportional decrease of NK and B cells. These changes persist at D7 to a lesser extent. Native hearts show a lesser influx of neutrophils (but not monocytes) at D1 with an increase in ST2⁺CD3⁻NK⁻B220⁻CD11b⁻ cells on D1 and D7.

Draining LN and spleen show an immediate decrease in T, NK cells and pDCs while B cells and neutrophils (LN only) increase. In the BM T and NK cells increase overtime, while the proportion of B cells reduces by D7. There is an apparent early reduction in granulocytes and monocytes that is followed by an increase of these cells by D7.

Key changes occur predominantly within 24 hours post transplantation and are dominated by neutrophils and monocytes/macrophages. Understanding the nature and triggering of these early infiltrates after organ transplantation will contribute to new therapeutic developments extending graft longevity and reducing systemic side effects.

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New method of allogeneic hematopoietic stem cell transplantation: hematopoietic stem cell transplantation plus thymus transplantation for intractable diseases

Hosaka, N.^{1,2}

¹Kansai Medical University Kori Hospital, Neyagawa, Japan,

²Kansai Medical University, Hirakata, Japan

Although allogeneic hematopoietic stem cell transplantation (allo-HSCT) has become a valuable strategy for intractable diseases, a number of problems remain to be resolved. We have

developed a new HSCT method, HSCT + thymus transplantation (TT), which induces elevated T cell function with mild graft-versus-host disease (GVHD) in comparison to conventional HSCT alone and HSCT + donor lymphocyte infusion (HSCT + DLI) in mice. This method leads to improvement of immune function and the ability of engraftment. The results indicated that this method is effective for treatment of several intractable diseases and conditions, such as autoimmune diseases in aging, advanced malignant tumors, exposure to supralethal irradiation, multiple organ transplantation from different donors, and type 2 diabetes mellitus, for which conventional methods are ineffective. Our findings suggest that allo-HSCT + TT is preferable to conventional allo-HSCT alone or allo-HSCT + DLI. This method may become a valuable next-generation HSCT technique for clinical use.

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Identification of naturally presented allopeptides using immunoproteomics to determine their role in T cell cross-reactivity

Mifsud, N.^{1,2}, Rowntree, L.², Nguyen, O.³, Schittenhelm, R.¹, Illing, P.¹, Kotsimbos, T.^{2,4}, Purcell, A.¹

¹Monash University, Infection and Immunity Program, Monash Biomedicine Discovery Institute, Clayton, Australia, ²Monash University Central Clinical School, Melbourne, Australia, ³Peter Doherty Institute for Infection and Immunity at The University of Melbourne, Parkville, Australia, ⁴Alfred Hospital, Melbourne, Australia

Cross-reactive anti-viral memory T cells constitute a significant proportion of the alloresponse and potentially play a role in mediating adverse clinical outcomes in HLA mismatched allografts. Additionally, chronic DNA viruses including cytomegalovirus (CMV) contribute to significant post-transplant morbidity and mortality. We have identified CMV-reactive (HLA-A*02:01-NLVPMVATV) CD8⁺ T cells with a unique TCR signature (TRAV3TRAJ31_TRBV12-4TRBJ1-1; OTN5) that cross-react against specific HLA-B27 allomorphs with a defined hierarchy (B*27:09>B*27:07>B*27:05). This observation combined, with the failure of OTN5 to recognise TAP-deficient B27⁺ APC, highlights the peptide dependence of the allorecognition. However, the identity of the HLA-B27 restricted peptide(s) is unknown. In this study, we undertook an immunoproteomics approach for allopeptide identification. HLA B27-peptide complexes were captured by immunoaffinity chromatography from individual C1R-B27 transfectants (C1R-B*27:01, -B*27:02, -B*27:07 or -B*27:09), the bound peptide ligands were isolated and fractionated by RP-HPLC. An immunoreactive peptide fraction, common to all C1R-B27 transfectants, was identified by screening with SKW3 cells expressing the cross-reactive OTN5 TCR (SKW3.OTN5). Sequencing of the peptides within this fraction by mass spectrometry (LC-MS/MS) yielded 2646 unique B27-restricted peptides. Of these, a subset of 136 peptides were synthesised and either pooled (groups of 8-11 peptides) or individually tested to measure functional reactivity by SKW3.OTN5 cells. Whilst preliminary investigations have not as yet confirmed the presence of the allopeptide(s) within the first wave of selected peptides, this study demonstrates the

potential of immunoproteomics to identify naturally presented peptides in the context of specific HLA allomorphs in order to determine their role in mediating T cell cross-reactivity.

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Induction of transgene-specific immunological tolerance by hematopoietic microchimerism using gene-modified bone marrow transplantation in a gene therapy setting

Boyer, O.^{1,2}, Bourdenet, G.¹, Cohen, J.L.³, Mingozi, F.^{4,5}, Martinet, J.^{1,2}

¹Normandy University, Inserm U 905, Rouen, France, ²Rouen University Hospital, Department of Immunology, Rouen, France, ³UPEC, Inserm U 955, Creteil, France, ⁴Genethon, Evry, France, ⁵UPMC, Inserm U 974, Paris, France

Introduction: Gene therapy is a promising treatment option for hemophilia. Replacement therapy is hampered by a risk of immunization against recombinant coagulation factors and immune response against the transgene product is an obstacle to gene therapy. Induction of hematopoietic chimerism is a potent way for inducing immunological tolerance in experimental solid organ transplantation, including grafting of alloantigen expressing gene-modified syngeneic bone marrow. **Objective:** To elicit transgene-specific immunological tolerance by inducing a hematopoietic microchimerism in a gene therapy setting.

Material and methods: We developed lentiviral vectors encoding a strong immunogene composed of an immunodominant peptide of ovalbumin (OVA) linked to a MHC class I - beta-2-microglobulin fusion protein (LV-OVA), or factor IX cDNA under a pgk promoter (LV-FIX). C57BL/6 mice (Ly5.2) were lethally (10 Gy) or sub-lethally (5 Gy) irradiated and reconstituted with LV-transduced congenic Ly5.1 bone marrow cells.

Results: Less than 1% bone marrow microchimerism was sufficient to tolerize the CD8+ T cell compartment towards OVA and to allow persistent transgene expression after in vivo gene transfer. We could prevent the anti-FIX humoral response and achieve persistent production of circulating FIX after LV-mediated gene transfer in vivo.

Conclusion: This work provides proof of concept that inducing hematopoietic microchimerism by bone marrow transplantation under sub-lethal conditioning using gene-modified cells confers tolerance to a gene therapy product. This opens perspectives for further preclinical evaluation of a dual gene therapy approach using the same vector.

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Extracellular ATP as an early danger signal initiating allograft rejection

Amores-Iniesta, J., Martínez, C.M., Barberà-Cremades, M., Baroja-Mazo, A., Pelegrin, P.

Murcia's Biomedical Research Institute (IMIB-Arrixaca), Murcia, Spain

The immune system is activated in response to foreign non-self components that represent a danger to the host, such as pathogenic microorganisms. However, harmful agents for the host could also be of endogenous origin and activate immunity,

leading to 'sterile' inflammation. A challenging unexplored scenario for this model is the activation of the immune system in allogeneic transplantation, where a strong Th1 destructive immune response is developed against 'sterile' foreign non-self but 'not-dangerous' tissue grafts. Here we found that in allotransplants extracellular ATP concentration increase, suggesting that this nucleotide acts as an early endogenous danger signal released after innate sensing of allogeneic tissues. Antigen-presenting cells recognizing non-self antigens are responsible for the early elevation of extracellular ATP. In this process, P2X7 receptor plays a key role and activates the Nucleotide oligomerization domain-, Leucine-rich repeat-, and Pyrin domain-containing 3 (NLRP3) inflammasome. Furthermore, NLRP3 inflammasome induces the release of interleukin (IL)-18 that establish a Th1 response against the allotransplant via interferon- γ (IFN- γ) production. P2X7 receptor pharmacological inhibition, or genetic deficiency of P2X7 receptor or NLRP3 inflammasome resulted in a delayed graft rejection without complete immune paralysis. These data clarify the function of danger signals during non-self and 'non-dangerous' immune activation and have important implications for transplantation medicine, as pharmacological targeting of P2X7 receptor could improve the development of graft tolerance.

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Stem cell and novel biologic therapies to improve functional outcomes in limb transplantation

Salgar, S.¹, Kniery, K.²

¹Madigan Army Medical Center, Clinical Investigation, Tacoma, United States, ²Madigan Army Medical Center, Surgery, Tacoma, United States

Introduction: Vascular composite allotransplantation (VCA) such as limb and face require successful nerve regeneration and reinnervation of graft for optimal functional outcome.

Materials and methods: We investigated whether mesenchymal stem cells (MSCs), Granulocyte-Colony Stimulating Factor (G-CSF), and Dihexa can improve sensory and motor functional recovery in rat sciatic nerve transection repair and limb transplant models. Surgical procedures were performed under general anesthesia. Bone marrow derived syngeneic MSCs (5x10⁶), G-CSF (50 μ g/kg), (Dihexa 2 mg/kg) or Vehicle were administered topically and i.v./i.p. The motor function was assessed by walking track analysis and sensory function by cutaneous reaction test.

Results: Ex vivo expanded MSCs were CD29+, CD90+, MHC Class I+, CD34-, CD31-, Class II-, and pluripotent. At two weeks post-nerve repair, total sensory nerve function in all groups was ~1.5 on a scale of Grade 0-3 (0=No function; 3=Normal); however, peroneal nerve function ranged 2.6-3.0. By 4 weeks total sensory function was 2.2 \pm 1.0, 2.0 \pm 1.2, 2.2 \pm 1.1 and 1.8 \pm 1.3 in MSC, G-CSF, Dihexa and Vehicle groups, respectively. At 10 weeks, sensory function (~3) was restored to normal in all groups (n=8/group). Sciatic function index (SFI) a measure of motor function (0=normal function; -100=nonfunctional) during 5-16 weeks was markedly improved in G-CSF (-40 to -26) compared to MSC (-93 to -66), Dihexa (-85 to -57) or Vehicle (-110 to -45) group. In limb transplants, sensory function ranged 0.8-1.3 by

8 weeks, and 1.5-2.0 by 16 weeks post-surgery. Majority of the transplants (~60%) developed flexion-contractions.

Conclusions: MSC, G-CSF and Dihexa appear to promote functional recovery in VCA.

Treg

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ST2-expressing regulatory T cells in the colon express the repair mediator, Amphiregulin

Chomka, A.¹, Krausgruber, T.², Powrie, F.¹

¹University of Oxford, The Kennedy Institute of Rheumatology, Oxford, United Kingdom, ²CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria

Tissue-specific regulatory T cells (Tregs), in contrast to their lymphoid counterparts, develop specific adaptations enhancing their survival and function. Tregs are enriched in the intestinal lamina propria and represent a key immune cell type that controls the intestinal homeostasis. Recently we have identified a sizeable population of Tregs in the colon expressing the interleukin 33 (IL-33) receptor, ST2. We showed that these cells respond to the alarmin, IL-33, which is released by cells upon tissue damage.

To further characterize this population, we compared the mRNA expression profile of murine colonic ST2-expressing Tregs (ST2+) to colonic Tregs that do not express ST2 (ST2-). Amphiregulin, an EGF-like growth factor previously shown to be important for repair mechanisms, was significantly enriched among the ST2+ colonic Tregs. Amphiregulin was also significantly more abundant in the whole colonic tissue compared to lymphoid tissues, i.e. the spleen and the mesenteric lymph nodes. Finally, to investigate a direct link between IL-33 and the expression of Amphiregulin by Tregs, we performed both *in vitro* and *in vivo* experiments, which confirmed that upon IL-33 signalling in Tregs Amphiregulin production was substantially increased.

Together, these results highlight that intestinal Tregs, similarly to other tissue-specific Tregs, have a distinct phenotype and function. Through the enhanced production of Amphiregulin, ST2+ Tregs may represent a specialised population capable of mediating repair functions.

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Maternal dietary fiber intake during pregnancy affects on Foxp3⁺Treg differentiation in the thymus of offspring

Nakajima, A., Kaga, N., Miyake, S., Habu, S.

Juntendo University, Tokyo, Japan

Maternal diet intake during pregnancy is thought to be crucial for developing immune system of offspring. Recent studies have also shown that short-chain fatty acids (SCFAs), metabolites of dietary fiber have a potential role in inducing regulatory T cells (Tregs) in the gut. Moreover, accumulating evidence suggests that thymus-derived Tregs (tTregs) are important in suppressing immune response and homeostasis. Based on these evidences, we examined whether SCFAs metabolized in mother's gut influence on the development of tTregs in

offspring. For this purpose, purified high soluble fiber diet (HFD) or no fiber diet (NFD) was fed to mothers during pregnancy and then tTregs of offspring were analyzed. We then observed increased frequency of tTregs in the offspring born from HFD-fed mice when compared with those born from NFD-fed mice. In addition, SCFAs could also increase the number of tTregs in fetal thymus organ culture (FTOC) system. To address the mechanism by which SCFAs increase tTregs in offspring, we focused on the involvement of thymic microenvironment such as thymic epithelial cells (TECs). Given that the transcription factors Aire and Fezf2 especially expressed in TECs are related not only to negative selection of T cells but also to induction of tTregs, we analyzed the expression of Aire and Fezf2 in TECs. As expected, the expression of these genes was increased by SCFAs treatment in FTOC.

These results suggest that SCFAs, metabolites of commensal bacteria from mother's gut could affect Tregs development in the thymus of offspring.

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In follicular regulatory T cells NFAT2 is essential for homing to germinal centers

König, A.¹, Vaeth, M.¹, Müller, G.², Stauss, D.², Dietz, L.¹, Klein-Hessling, S.¹, Serfling, E.¹, Lipp, M.², Berberich, I.³, Berberich-Siebelt, F.¹

¹Institute of Pathology, Julius-Maximilians-University, Wuerzburg, Germany, ²Max-Delbrück-Center for Molecular Medicine (MDC), Department of Tumor Genetics and Immunogenetics, Berlin, Germany, ³Institute for Virology and Immunobiology, Julius-Maximilians-University, Wuerzburg, Germany

The humoral immune response represents one arm of adaptive immunity and is based on antibody-secreting plasma cells. Plasma cells, which secrete class-switched antibodies with high affinity, are the product of the germinal center reaction (GCR). The GCR depends on CD4⁺CXCR5⁺ follicular T helper cells (T_{FH}), while it is controlled by CD4⁺CXCR5⁺Foxp3⁺ follicular regulatory T cells (T_{FR}). Expression profiling revealed high expression of the 'Nuclear Factor of Activated T cells 2' (NFAT2) in follicular T cells, covering T_{FH} as well as T_{FR} cells. Therefore, we chose to investigate the role of NFAT2 in those two follicular T-cell subsets. The ablation of *Nfat2* in all T cells, but also in Foxp3-expressing cells only, led to an increase in the GCR. This effect was due to an impaired homing of the T_{FR} population to the B cell follicle, because T_{FR} cells specifically failed to upregulate the homing receptor CXCR5. In T_{FR} cells - in contrast to T_{FH} cells - 'B lymphocyte-induced maturation protein' (Blimp-1) is highly expressed in line with being a hallmark of effector Tregs. Our data indicated that, similar as in plasma cells, Blimp-1 represses the expression of *Cxcr5* in T_{FR} cells and that this is a direct effect. However, it supports the recruitment of NFAT2 to *Cxcr5* by protein-protein interaction, by those means cooperating with NFAT2 for transactivation of *Cxcr5*. In sum, NFAT2 is essential for overcoming Blimp-1-mediated repression of *Cxcr5* and therefore for homing of T_{FR} cells, which control the GCR, an essential part of the humoral immune response.

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IL-10 produced by B cell-induced regulatory CD4⁺Foxp3⁺ T cells via the activation of c-Maf*Chien, C.-H., Chiang, B.-L.**College of Medicine, National Taiwan University, Taipei City, Taiwan, Republic of China*

The role of B cells in the development of interleukin (IL)-10-producing regulatory T cells and Foxp3⁺ regulatory T cells have been emphasized recently. Previously, we demonstrated that the antigen-presenting splenic B cells converted naïve CD4⁺CD25⁻ T cells into CD4⁺CD25⁺ T cells with regulatory function without the expression of Foxp3. Here, we investigated the effect of OVA₃₂₃₋₃₃₉ peptide-presenting splenic B cells on the naïve DO11.10 splenic CD4⁺CD25⁻ T cells. We found that the levels of IL-10 and IL-4 increased gradually during the 3 rounds of B-T coculture. After 3 rounds of B-T coculture, B cell-induced CD4⁺CD25⁺ T cells increased the IL-10-producing population, the production of IL-10, the expression of c-Maf, and the expression of inducible T-cell co-stimulator (ICOS) as well as cytotoxic T-lymphocyte-associated protein 4 (CTLA4). We found that the major IL-10-producing population was confined to the c-Maf-expressing population in the B cell-induced regulatory T cells. By the blockade assay, not only IL-10 also CTLA4 played the roles in the suppressive mechanism of the B cell-induced regulatory T cells. We also found that the induction mechanism largely depended on cell-cell contact mechanism. These results suggested that the antigen-presenting B cells promote the development of IL-10-producing regulatory T cells via the activation of c-Maf.

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The phenotype, functions and cytokine properties of 5-Azacytidine (5AzaC) induced Treg-like FOXP3⁺ CD4⁺ T cells*Benfatto, C.¹, Seidl, T.¹, Perez Abellan, P.¹, Chung, S.S.¹, Ellis, R.², Mufti, G.¹, Thomas, N.S.B.¹, Kordasti, S.¹**¹King's College London, Haematological Medicine, London, United Kingdom, ²NIHR GSTFT/ KCL, Biomedical Research Centre, London, United Kingdom*

Myelodysplastic Syndrome (MDS) is a group of premalignant diseases characterised by ineffective erythropoiesis. Expansion of CD4⁺CD25^{hi}CD127^{low}FOXP3⁺ (Tregs) is one of the characteristic features of high risk MDS. Although the only curative treatment is bone marrow transplantation, treatment with the hypomethylating agent 5AzaC can enhance life expectancy. It has been shown by our group that following treatment with 5AzaC, the absolute number of Tregs is reduced.

This study focuses on the FOXP3⁺ T cells ("Treg-like" cells) derived from 5AzaC treated Tconv (CD4⁺CD25⁻). We wanted to investigate whether the features of this subpopulation were still Tconv or whether they polarised to another population. We have analysed the cell surface protein phenotype, functional analysis and cytokine secretion of "Treg-like" cells compared with Tregs, Tconv and Th-17 derived from a Th-17 polarising environment (*in-vitro* culture with IL-1b, IL-6 and IL-2).

Our data demonstrate: 1) "Treg-like" cells show an increase in secretion of IL-17 and expression of FOXP3 and have no significant immunosuppressive properties; 2) the ability of Tregs

to polarise towards Th-17; 3) the Treg-specific demethylated region (TSDR) within the FOXP3 gene in "Treg-like" cells is fully methylated compared with Tregs. Changes in the methylation status of the FOXP3 downstream genes of Tconv (+/- 5AzaC) versus Tregs from healthy donors are now being analysed. We used a mass-cytometry technology (CyTOF) to characterise the cell surface protein phenotype of "Treg-like" cells.

The phenotype and the methylome analysis will give an improvement in understanding how these "Treg-like" cells develop, behave and their potential use in therapy.

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The complementary role of FOXP3⁺ regulatory T cells and Tr1 cells in the maintenance of tolerogenic immune modifying nanoparticle (TIMP) peripheral tolerance*Podojil, J.R.¹, Getts, D.R.¹, Miller, S.D.²**¹Cour Pharmaceuticals Development Co, Chicago, United States,**²Northwestern University Feinberg School of Medicine, Chicago, United States*

Tolerogenic Immune Modifying Nanoparticles (TIMP) encapsulating self-antigens (TIMP) are highly effective for the prevention and treatment of autoimmunity. TIMP(PLP₁₃₉₋₁₅₁) treatment of naïve SJL/J mice totally abrogates the induction of PLP₁₃₉₋₁₅₁/CFA-induced R-EAE in an antigen-specific manner via synergistic mechanisms. First, deletion/inactivation of Tregs by treatment of with anti-CD25 at the time of tolerance partially reversed the protective effect of TIMP(PLP₁₃₉₋₁₅₁), and transfer of purified splenic

T cells from TIMP(PLP₁₃₉₋₁₅₁)-treated mice protects recipient SJL/J mice from PLP₁₃₉₋₁₅₁/CFA-induced EAE. Second, administration of TIMP(PLP₁₃₉₋₁₅₁) to 5B6 (PLP₁₃₉₋₁₅₁-specific) TCR transgenic mice was found to lead to the significant expansion of both antigen-specific Foxp3⁺ iTregs and Lag3⁺, IL-10⁺ Tr1 cells. Third, the supplementation of rIL-2 to *ex vivo* recall cultures from TIMP(PLP₁₃₉₋₁₅₁)-treated mice partially restored the proliferative response indicating a role for anergy. To test the effects of TIMP on regulation of previously activated T cells, we showed that administration of TIMP(p31) to NOD.SCID recipients of activated BDC2.5 TCR transgenic T cells (specific for the chromogranin A p31 mimotope peptide) led to long-term prevention from induction of T1D. TIMP(p31) infusion was dependent on the activation of Foxp3⁺ Tregs which acted to regulate the trafficking of the effector BDC2.5 T cells to the pancreas and causing their retention in the spleen of the recipient mouse. Tolerance was reversed by treatment with either anti-PD-1 or anti-CTLA-4. These findings indicate a role for Tregs, Tr1 cells, and T cell anergy in TIMP-induced tolerance.

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The role of regulatory T cells in T cell recovery under severe lymphopenic conditions in hemoblastosis patients*Batorov, E., Tikhonova, M., Kryuchkova, I., Batorova, D.,**Sergeevicheva, V., Sizikova, S., Ushakova, G., Gilevich, A., Ostanin, A., Chernykh, E.**Research Institute of Fundamental and Clinical Immunology, Novosibirsk, Russian Federation*

Introduction: Well-timed T-cell reconstitution is crucial for early restoration of anti-infectious and anti-tumor immune response. Homeostatic proliferation is important for the restoration of T cell count in immune competent host during the 1st year following autologous hematopoietic stem cell transplantation (AHSCT). Mature T cells involved in expansion may be sensitive for the balance between signals that mediate proliferation or suppression, e.g. from regulatory T cells (Tregs). We have investigated dynamics of CD4+FOXP3+ Tregs recovery following AHSCT and possible relationship between Tregs and clinical outcomes.

Patients and methods: 109 hemoblastosis patients underwent AHSCT in 2009-2014. The content of circulating CD4+FOXP3+ Tregs has been evaluated using flow cytometry before AHSCT, at the day of engraftment, and following 6 and 12 months.

Results: relative count of Tregs restored rapidly, reached initially high level at the time of engraftment, and then subsequently decreased for a year until it lowered to healthy donors' values. CD4+FOXP3+ Tregs at the time of engraftment were increased in patients with relapse or progression of disease during 6 and 12 months following AHSCT compared to non-relapsed patients. (12.1±5.6% vs. 7.3±3.9%; pU=0.016, and 10.7±5.0% vs. 6.9±3.7%; pU=0.024; data as Mean ± SD).

Conclusions: Our data of Tregs reconstitution may confirm the earlier assumption that the presence of Tregs during the period of immune recovery preserves optimal T cell receptors diversity. But excess of these cells leads to the inhibition of proliferative activity and immune response and is associated with early relapse.

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Inhibins regulate peripheral Treg induction through modulation of dendritic cell function

de la Fuente Granada, M.¹, Olguin Alor, R.¹, Bonifaz Alfonso, L.², García-Zepeda, E.A.¹, Soldevila, G.¹

¹Instituto de Investigaciones Biomedicas, Universidad Nacional Autónoma de México, Immunology, Mexico, Mexico, ²Hospital de Especialidades, Centro Médico Nacional 'Siglo XXI', Unidad de Investigación Médica en Inmunoquímica, Mexico, Mexico

Inhibins and Activins, members of the Transforming Growth Factor b superfamily, participate in the immune system as key regulators of several cellular functions. Despite some reports indicating that Activins may regulate DC maturation and function, the role of Inhibins in these processes remains elusive. Here, we investigated the role of Inhibins in DC maturation and function and its impact in Treg induction. Analysis of mature Inha-/- BMDCs obtained after lipopolysaccharide stimulation (mBMDC) showed a significant reduction in the surface expression of MHC II and CD86 (a semi-mature phenotype), and a concomitant increase in PD-L1, compared to WT mBMDC, suggesting a tolerogenic phenotype. In addition, the Inha-/- mBMDC showed reduced migration towards CCL19 and CCL21 by *in vitro* chemotaxis and *in vivo* adoptive competitive transfer assays. In addition, mBMDC from Inha-/- displayed reduced capacity to induce proliferation of allogeneic CD4+ naïve T cells. This correlated with an increased induction of iTregs (CD25+Foxp3+) from CD4+ naïve T cells in the presence of

TGFb, as well as with increased absolute numbers of iTregs and nTreg (CD4+CD25+Foxp3+Helios- and Helios+, respectively) in peripheral lymph nodes of 4 week-old Inha-/- mice compared to WT. Interestingly, Inha-/- T cells showed a reduced induction of Tregs in the absence of APCs, suggesting an intrinsic role of Inhibins in the differentiation of T cells. Our data demonstrate for the first time a direct role of Inhibins in the regulation of DC mediated functions and point out the importance of Inhibin signaling to maintain the balance between inflammation and tolerance.

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TET2 and TET3 play essential roles in Treg-specific DNA demethylation and Treg stability

Nakatsukasa, H., Yoshimura, A.

Keio University School of Medicine, Department of Microbiology and Immunology, Tokyo, Japan

It has been shown that DNA demethylation of Treg-specific demethylation regions (TSDRs) during Treg development plays essential roles in stable Foxp3 expression. Although DNA demethylating enzymes, ten-eleven translocation (TET) family proteins have been proposed to be involved in the DNA demethylation of TSDRs, actual contribution of TET family proteins in Treg stability and function remain to be elucidated. To investigate the role of TET proteins in Tregs, we generated Treg-specific Tet-deficient mice by crossing *Tet2^{flf}* and/or *Tet3^{flf}* mice with *Foxp3^{YFP-Cre}* mice. In Treg-specific *Tet2*- and *Tet3*-single KO mice, Treg development was normal and these mice did not show any spontaneous immunological diseases. While *Foxp3^{YFP-Cre}+Tet2^{flf}Tet3^{flf}* (FDKO) mice died at an average of 20 weeks of age with abnormal expansion of T cells. TSDRs of FDKO Tregs were hyper-, but not completely, methylated compared with WT Tregs, indicating that there are Tet2/3 dependent and independent mechanisms for TSDR demethylation. Treg transfer experiments into *Rag*-deficient mice revealed that Foxp3 expression in FDKO Tregs were more unstable than that in WT Tregs. In aged FDKO mice, both Foxp3⁺ and Foxp3⁻ T cells were expanded and produced high levels of IL-17, suggesting that the accumulation of exFoxp3 cells in FDKO mice. These data indicate that Tet2/3 are redundantly involved in TSDR demethylation and are important for the stability and homeostasis of Tregs.

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Regulatory T cell-dendritic cell interactions in the prevention of graft-versus-host disease

Bolton, H.^{1,2}, Pijning, A.¹, Terry, A.^{1,2}, Shklovskaya, E.^{1,2}, Zhu, E.^{1,3}, Fazekas de St Groth, B.^{1,2}

¹Centenary Institute, T Cell Biology, Camperdown, Australia, ²University of Sydney, Department of Dermatology, Camperdown, Australia, ³Childrens Medical Research Institute, Westmead, Australia

Regulatory T cells (Tregs) improve immune reconstitution after bone marrow transplant (BMT) by promoting a more diverse TCR repertoire and reducing the incidence of graft-versus-host disease (GVHD). How Tregs mediate these effects is unknown.

Using a mouse model of lymphopaenia-induced proliferation (LIP), we have shown that selective reconstitution of the Treg compartment inhibits spontaneous T cell proliferation in response to endogenous antigens, with T cells instead undergoing slow homeostatic division to repopulate the host with a diverse repertoire of naïve T cells. Inhibition of LIP in this model was primarily mediated via CTLA-4-dependent downregulation of CD80/CD86 on DCs. Importantly, the level of CD80/CD86 expressed by DCs was determined by the ratio of Tregs:DCs, suggesting that normalising the Treg:DC ratio rather than the Treg:Tcell ratio may be more important in promoting optimal immune reconstitution during lymphopaenia.

We tested the clinical relevance of these findings in situations in which treatment renders patients transiently lymphopaenic. Using an allogeneic BMT model, we have shown that the elevated DC costimulation in irradiated mice can be reduced by selective Treg-reconstitution of either donor-type Tregs or host-type Tregs. Critically, Treg-reconstitution of BMT recipients prior to transfer of conventional T cells was associated with complete protection against T cell-mediated GVHD, whereas Tregs co-transferred with conventional T cells provided minimal protection. These results indicate that optimal immune recovery from lymphopaenia should aim to achieve early Treg-reconstitution prior to transfer of conventional T cells.

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High regulatory T-cell predicts low CD4 recovery, and is associated with exacerbated CD4 turnover and inflammation in HIV-patients at risk of low CD4 recovery

Rosado-Sánchez, I.¹, Álvarez-Ríos, A.I.², Herrero, I.¹, Beltrán-Debón, R.³, Rafii-El-Idrissi, M.¹, Ruiz-Mateos, E.¹, Blanco, J.⁴, Vidal, F.³, Leal, M.¹, Pacheco, Y.M.¹

¹IBiS-HUVR, Seville, Spain, ²HUVR, Seville, Spain, ³Joan XXIII Hospital, Tarragona, Spain, ⁴IrsiCaixa, Barcelona, Spain

Peripheral Treg expansion has been associated with low CD4 T-cell counts, particularly in HIV-infected subjects. Our group and others have associated higher Treg frequency to HIV-infected patients with low CD4 recovery after antiviral-treatment (cART). However, this association has been found from post-treatment analysis, when their CD4 counts are significantly lower than any control group, thus it cannot be discarded that this feature could be simply a consequence of such low CD4 recovery.

To overcome this limitation, we analyzed before-to-cART matched samples (also by CD4 counts). In the Spanish CoRIS cohort, we selected samples of HIV-subjects starting cART with < 200 CD4/μL and achieving < 250 CD4/μL on cART (LR patients). Additionally, as control, we selected samples of HIV-subjects starting cART with < 200 CD4/μL but achieving ≥250 CD4/μL. We studied levels of pro-inflammatory biomarkers, cellular turnover and Treg frequencies.

We found that despite similar CD4 counts, LR patients showed an increased Treg frequency before-to-cART. Our results also showed elevated levels of pro-inflammatory biomarkers and exacerbated CD4 turnover. Moreover, these parameters were associated with Treg. Eventually, we performed a multivariate analysis to predict patients at risk

of low CD4 recovery. After adjusting, only Treg frequency was independently associated.

Conclusions: Before-to-cART, LR patients showed specific alterations despite similar CD4 counts. Multivariate analysis indicates that Treg could be used to early detect HIV-patients at risk of low CD4 recovery. Treg accumulation in this scenario could be due to exacerbated CD4 turnover and inflammation, and could play a pivotal role in low CD4 recovery.

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The lymphoproliferative disorder from depletion of regulatory T cells (Tregs) occurs independently of commensal microbial and dietary antigens and accompanies robust peripheral Treg generation

Yi, J.^{1,2}, Lee, J.^{1,2}, Han, D.¹, Surh, C.^{1,2,3}

¹Academy of Immunology and Microbiology, Institute for Basic Science, Pohang, Korea, Republic of, ²POSTECH, Department of Integrative Biosciences and Biotechnology, Pohang, Korea, Republic of, ³Division of Developmental Immunology, LIAI, San Diego, United States

Thymic regulatory T cells (tTregs) have been regarded as an important regulator of self-tolerance. However, the mechanism how tTregs prevent autoimmunity remains elusive. Since negative selection is believed to eliminate most of auto-reactive thymocyte clones in the thymus, molecular mimicry between foreign-antigens (Ags) and self-Ags has been proposed as possible explanation for most of autoimmune diseases. By utilizing Foxp3-diphtheria toxin receptor (Foxp3-DTR) mice, one group previously tested the role of Ags from intestinal microbiota for induction of lymphoproliferative disorder by depleting Tregs in germ-free (GF) Foxp3-DTR mice and found that lymphoproliferative disorder occurred unabated. In order to test the role of food Ags in inducing lymphoproliferative disorder upon Treg depletion, we generated Ag-depleted Foxp3-DTR bone-marrow chimeras in GF RAG-1 KO host mice that were raised with an ultra-filtered, elemental antigen-free (AF) diet. Strikingly, the AF Foxp3-DTR chimeras displayed equally strong lymphoproliferative disorder upon Treg depletion indicating that foreign Ags are not required for induction of this disease. In addition, we have discovered that the majority of effector CD4 T cells that emerge after Treg depletion originates from a small fraction of naïve CD4 T cells and display Th1 characteristics. More interestingly, the generation of effector CD4 T cells was accompanied with robust conversion of effector CD4 T cells into peripheral Tregs (pTregs). Such a transition applied to a significant fraction of effector CD4 T cells that display a high TCR affinity to self and also required the presence of IL-2. Our findings suggest existence of a strong homeostatic mechanism.

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Immunomodulatory functions of *Lactobacillus casei* surface proteins induced in interaction with intestinal epithelial cells

Keto, J., Ritamo, I., Haiko, J., Impola, U., Wacklin, P., Partanen, J., Valmu, L., Mättö, J., Lähteenmäki, K.

Finnish Red Cross Blood Service, Medical Services, Helsinki, Finland

T cell subtypes are critical in determination of the immunological balance in the intestine. Intestinal microbiota affects development and maturation of immunological cells. Many *Lactobacillus* species are considered beneficial for intestinal health, and knowledge of their potential effects on immune cell populations could lead to development of therapeutic applications for intestinal disorders. We have previously shown that *Lactobacillus casei* promotes maturation of regulatory T cells (CD4+CD25+FoxP3+) and induction of IL-10 in peripheral blood mononuclear cells (PBMC) more efficiently after cultivation with intestinal epithelial cells than after standard laboratory culture conditions (MRS broth)(Tiittanen et al.,2013,PlosOne,8(11),e78420).

In the current work, we completed a mass spectrometric characterization of the surface proteome of *Lactobacillus casei* ATCC334 with bacteria derived either from HT-29 intestinal epithelial cells or from MRS broth. The results indicated significant culture condition-dependent alterations in the bacterial surface proteome. We are currently analysing potential immunomodulatory effects of selected *Lactobacillus* proteins affected by epithelial cell cultivation. T cell population and cytokine profiling is performed after co-culture of PBMC with recombinant *L. casei* proteins and, for comparison, *Escherichia coli* lipopolysaccharide (LPS). Our initial results suggest similarities in PBMC responsiveness induced by LPS and *L. casei* glyceraldehyde-3-phosphate dehydrogenase (GAPDH), whereas two *L. casei* proteins with yet unknown function promote more immunosuppressive responses. Expression of these proteins was highly induced on the surface of bacteria cultivated with epithelial cells. The results suggest that simulation of bacterial contact with intestinal epithelium can be a valuable tool for identification and production of *Lactobacillus* proteins for therapeutic use.

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T cell tuning to self-reactivity generates a population of resting naïve Tregs that sensing recent activation of T conventional cells

Lee, J.Y.^{1,2,3}, Kim, J.H.^{1,2}, Yi, J.^{1,2}, Surh, C.^{1,2,4}

¹Institute for Basic Science, Pohang, Korea, Republic of, ²POSTECH, Pohang, Korea, Republic of, ³Division of Developmental Immunology, San Diego, United States, ⁴La Jolla Institute for Allergy and Immunology, Division of Developmental Immunology, San Diego, United States

Thymic selection of regulatory T cells (Tregs) is known to require a relatively strong TCR affinity for self-antigens (Ags). High affinity to self-Ags, however, does not induce all mature Tregs to eventually acquire effector/memory phenotype in the periphery and the mechanism regulating this peripheral differentiation remains elusive. Recently, it has been reported that Ly6c is expressed on naïve-phenotype Tregs and Ly6c⁺ Tregs exhibit impaired suppressive activity. However, the physiological significance of Ly6c⁺ Tregs and the factors regulating Ly6c⁺ Treg homeostasis are poorly understood. Based on the aforementioned findings, it has been deduced that Ly6c expression is associated with peripheral TCR tuning. Indeed, the Ly6c expression was found to inversely correlate

with the TCR signaling strength of Tregs, and Ly6c⁺ Tregs did not respond to self-Ags. We found that the proportion of naïve and effector Treg subsets changed dynamically depending on the age of mice. The proportion of Ly6c⁺ Tregs gradually increased from the perinatal periods to young adult age, but declined slowly with aging. The reduction of Ly6c⁺ Tregs in aged mice appears to be due to activation-induced down-regulation of Ly6c. Because effector T conventional (Tconv) cells are known to accumulate with aging, we speculated that the cytokines generated from effector Tconv cells are essential for awakening of resting Ly6c⁺ Tregs to transition into effector Tregs. Indeed, Ly6c⁺ Tregs underwent strong proliferation under conditions with elevated effector T cell. Therefore, our findings reveal novel characteristics of resting Tregs and suggest a new model of Tconv cell-Treg homeostasis.

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Requirement of full TCR repertoire for regulatory T cells to maintain intestinal homeostasis

Nishio, J.¹, Baba, M.¹, Atarashi, K.², Tanoue, T.², Negishi, H.¹, Yanai, H.^{1,3}, Habu, S.⁴, Hori, S.⁵, Honda, K.², Taniguchi, T.^{1,3}

¹Institute of Industrial Science, The University of Tokyo, Molecular Immunology, Meguro, Japan, ²RIKEN Center for Integrative Medical Sciences (IMS-RCAI), Laboratory for Gut Homeostasis, Yokohama, Japan, ³Max Planck-The University of Tokyo Center for Integrative Inflammation, Meguro, Japan, ⁴Juntendo University, Department of Immunology, Bunkyo, Japan, ⁵RIKEN Center for Integrative Medical Sciences (IMS-RCAI), Laboratory for Immune Homeostasis, Yokohama, Japan

The regulation of intestinal homeostasis by the immune system involves the dynamic interplay between gut commensal microbiota and resident immune cells. It is well known that a large and diverse lymphocyte antigen receptor repertoire enables the immune system to recognize and respond to a wide range of invading pathogens. There is also an emerging appreciation for a critical role the T cell receptor (TCR) repertoire serves in the maintenance of peripheral tolerance by regulatory T cells (Tregs). Nevertheless, how the diversity of the TCR repertoire in Tregs affects intestinal homeostasis remains unknown. To address this question, we studied mice whose T cells express a restricted TCR repertoire. We observed the development of spontaneous colitis, accompanied by the induction of T helper type 17 (Th17) cells in the colon that is driven by gut commensal microbiota. We provide further evidence that a restricted TCR repertoire causes a loss of tolerogenicity to microbiota, accompanied by a paucity of peripherally derived, Helios-negative Tregs and hyperactivation of migratory dendritic cells. These results thus reveal a new facet of the TCR repertoire in which Tregs require a diverse TCR repertoire for intestinal homeostasis, suggesting an additional driving force in the evolutionary significance of the TCR repertoire.

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Enhanced stability of the Foxp3 expression by forced expression of TET DNA demethylase catalytic domain in iTregs

Someya, K., Yoshimura, A.

Keio University School of Medicine, Microbiology and Immunology, Tokyo, Japan

Induced regulatory T cells (iTregs) are generated from naive T cells in the presence of transforming growth factor- β (TGF- β). Since iTregs can be produced in a large quantity *in vitro*, these cells are expected to be clinically used as an inducer of tolerance in various immunological diseases. However, unlike Tregs developed in the thymus, Foxp3 expression in iTregs is unstable and iTregs tend to become effector

T cells due to the lack of epigenetic modifications of Treg-specific demethylation regions (TSDRs). To facilitate demethylation in TSDRs, we have tried to overexpress the catalytic domain (CD) of the ten-eleven translocation (TET) protein, which catalyzes the steps of the iterative demethylation of 5-methylcytosine (5mC). By using retroviral gene transfer, we introduced the catalytic domain of the TET1 protein (TET1CD) or constitutively active form of STAT5a-TET1 CD fusion protein (STAT5aCA-TET1CD). We expected a specific recruitment of TET1CD to intronic element, CNS2 region, of the Foxp3 gene, since STAT5 has been shown to bind to CNS2. Unexpectedly, not only STAT5a-TET1CD expression but also TET1CD alone were sufficient for the introduction of demethylation of CNS2 in iTregs, and expression of Foxp3 was more stable in iTregs expressing TET1CD or STAT5aTET1CD than in control iTregs. TET1CD-iTregs exhibited stronger suppression activity in inflammatory bowel disease (IBD) and graft-versus-host disease (GVHD) models in mice. Our data suggest that induction of TET enzymes in iTregs would be an effective method for immunotherapy.

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T_{FR} cells regulate the germinal center to prevent the emergence of self-reactive and IgE-producing plasma cells

Gonzalez-Figueroa, P.¹, Papa, I.¹, Fernandez de Canete Nieto, P.¹, Roco, J.¹, Watchirs Connolly, L.¹, Dent, A.², G Vinuesa, C.¹

¹John Curtin School of Medical Research, Australian National University, Dept of Immunology and Infectious Disease, Canberra, Australia, ²Indiana University School of Medicine, Dept of Microbiology and Immunology, Indianapolis, United States

Stringent selection is required in the germinal center (GC) to ensure self-reactive B cells and IgE-expressing B cells are not selected as long-lived, high affinity plasma cells that may otherwise trigger autoimmune diseases or allergic reactions respectively. While B follicular helper T (T_{FH}) cells have been shown to be required for GC B cell selection, T follicular regulatory (T_{FR}) cells have been suggested to curtail GC reactions in response to both foreign and self-antigens. Whether T_{FR} cells play an important or selective role in preventing pathogenic antibody responses, as opposed to simply accelerating the demise of GC reactions, remains unclear. Here, we have taken advantage of mice that selectively lack T_{FR} cells and show that, in the absence of immunization, T_{FR}-deficient mice consistently

and spontaneously develop anti-nuclear autoantibodies characteristic of lupus, by 8 weeks of age. Furthermore, T_{FR} cell deficiency leads to spontaneous formation of GCs, hypergammaglobulinemia and high titres of total IgE. When immunized with protein antigens or sensitised to food allergens such as peanut extracts, T_{FR}-deficient mice develop exaggerated IgE responses, without marked differences in IgG production. Together, our results demonstrate that T_{FR} cells are actively involved in the suppression of self-reactive B cell clones and are critical to prevent the emergence of IgE-producing plasma cells. These findings suggest that manipulation of T_{FR} cells or their products may constitute the basis of powerful therapies against the growing numbers of autoantibody-mediated diseases, allergies and food intolerances.

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Optimized whole blood flow cytometry assay that improves separation of Foxp3+ and Foxp3- cells using 3G3 monoclonal antibody

Jinoch, P., Prouza, M., Tvarůžková, J., Benko, M., Suchánek, M.

EXBIO, Vestec u Prahy, Czech Republic

The central feature of the regulatory T cells (Treg) characterization is to detect the transcription factor Foxp3. The recommended flow cytometry staining protocols are usually optimized for a specific Foxp3 antibody clone and may differ depending on the character of the sample (whole blood vs. Ficoll-Hypaq purified PBMCs). Previously published data showed that Foxp3 staining with antibody clone 259D provides better separation of Foxp3+ and Foxp3- cells than clone 3G3. The results were the same for all tested commercial permeabilization buffer sets. In our study we demonstrate that the 3G3 staining combined with sample permeabilization with anionic detergents resulted in comparable signal to noise ratio and yield of Foxp3+ cells as obtained with the 259D and commercially available reagents. Based on this observation we developed a new Treg staining assay that defines Tregs as the CD4+CD25+Foxp3+ cells. The procedure uses the 3G3 clone and allows detection of other Treg markers such as Helios. We evaluated the protocol by measuring the proportion of Helios+ and Helios- Treg cells in umbilical cord blood and human adult blood. As expected the Helios-Foxp3+ events represented approximately one third of adult blood Tregs and were absent from the cord blood Tregs. The developed assay introduces a new tool for examining the quality and quantity of Tregs in the fields of rheumatology, organ transplantation and reproductive medicine.

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The transcriptional regulator, IRF4 is essential for the development of follicular T regulatory cells

Teh, P.P.^{1,2}, Vasanthakumar, A.^{1,2}, Tellier, J.^{1,2}, Sidwell, T.^{1,2}, Thelemann, C.¹, Nutt, S.^{1,2}, Kallies, A.^{1,2}

¹The Walter and Eliza Hall Institute of Medical Research, Parkville, Australia, ²University of Melbourne, Department of Medical Biology, Parkville, Australia

Follicular T regulatory (Tfr) cells are a specialised subset of

regulatory T (Treg) cells that display an effector phenotype and are found within the B-cell follicles of germinal centres (GC). Tfr cells play an important role in regulating humoral immunity by limiting GC formation and shaping affinity maturation of B cells during antigenic stimulation. Like their conventional counterparts, follicular T helper (Tfh) cells, Tfr cells express high levels of CXCR5 and PD-1, and rely on the expression of Bcl6 for their development. However, it remains unclear what signals or transcription factors other than Bcl6 drive Tfr cell development. Mice with a Treg-specific deletion of the transcription factor interferon regulatory factor (IRF) 4 develop severe immune pathology with large GC and increased antibody serum titres. Therefore, we hypothesised that IRF4 is involved in the development of Tfr cells. Indeed, using an IRF4-reporter mouse, we found that Tfr cells express an increased amount of IRF4. Furthermore, conditional deletion of IRF4 showed that Tfr cells strictly depend on IRF4. After viral infections, mice with IRF4-deficient Treg cells show a significant increase in Tfh cell numbers and form larger GC compared to their wild-type littermates. Importantly, we show that IRF4-deficient Treg cells were unable to induce the Tfr-specific transcriptional program, and demonstrate that multiple molecules required for Tfr development and function are regulated by IRF4. Our work provides fundamental insights into the essential role of IRF4 for the development of Tfr cells. This knowledge may help to develop protocols to further advance vaccination strategies.

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Characterisation and selection of highly suppressive alloantigen specific regulatory T cells from umbilical cord blood

Srivastava, S.^{1,2}, Navarrete, C.^{1,2}, Girdlestone, J.^{2,3}

¹NHS Blood and Transplant, Histocompatibility and Immunogenetics Research, London, United Kingdom, ²University College London, London, United Kingdom, ³NHS Blood and Transplant, Stem Cells and Immunotherapies, Oxford, United Kingdom

Ex-vivo expanded polyclonal CD4⁺CD25⁺FoxP3⁺ Regulatory T cells (Treg) have been used successfully in clinical trials to manage acute graft versus host disease (aGvHD). However pre-clinical data indicate that alloantigen specific Tregs (Ag-Tregs) are more efficient compared to polyclonal Tregs in suppressing allospecific responses. As limited cell numbers and phenotypic stability prevent the full clinical integration of Tregs, alternative and more directed strategies are required. In this study we have generated, expanded and characterised Ag-Tregs from umbilical cord blood (UCB).

CD4⁺CD25⁺ cells were directly isolated from UCB mononuclear cells using anti-CD25 magnetic beads. Ag-Tregs were generated by stimulating Tregs for 2 rounds with allogeneic monocyte derived dendritic cells (MDDCs) + IL-2. Phenotypic characterisation, including expression of FoxP3, was conducted by flow cytometry. Tregs were isolated with magnetic beads and tested for allospecificity using autologous UCB CD4⁺CD25⁻ cells co-cultured with the same allogeneic MDDC or a 3rd party stimulator.

Freshly isolated UCB Tregs show limited functional capacity

compared to adult Tregs, but improved following activation. Allostimulation of UCB Tregs resulted in a 62±34 fold expansion with Tregs providing effective suppression towards the alloantigen compared to a 3rd party stimulator. Characterisation of Ag-Tregs revealed the presence of a CD25^{high}CD45RO⁺CD39⁺ sub-population that expressed increased FoxP3, CTLA-4 and HELIOS. CD39⁺ Ag-Tregs showed improved suppression compared to total Ag-Tregs and CD39⁻ Ag-Tregs, with >50% suppression maintained at a 1:256 ratio.

The naivety of UCB Tregs can be advantageous for the development, expansion and selection of highly suppressive allospecific Tregs that could potentially be used in immunotherapy.

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IgG4 induced by regulatory T cells inhibits *in vitro* haemolysis and inversely correlates with clinical anaemia in infants

Gbenoudon Satoguina, S.J.¹, Tamadaho, R.¹, Adanho, C.¹, Vikou, R.¹, Biaoou, O.¹, Layland, L.², Hoerauf, A.²

¹University of Abomey Calavi, Biochemistry, Cotonou, Benin,

²Institute of Med. Microbiology, Immunology and Parasitology, IMMIP. Medical Faculty, University of Bonn, Bonn, Germany

Background: We previously showed regulatory T cells induced during chronic diseases and responsible for regulatory isotype IgG4 switch. IgG4 was induced by Tr cells from naïve and from memory B cells by mechanisms including IL-10, TGF-beta and receptor/ligand GITR and GITRL. IgG4 has neutralizing role and may capture antigen to prevent binding immune-active antibodies like IgG1 and IgG3. However, unlike other isotypes, IgG4 lacks physiological role. Here we demonstrate that IgG4 inhibits *in vitro* haemolysis induced artificially by homologous IgG1/IgG3 enriched human sera. Concordantly, we demonstrate that increasing IgG4 levels regulate actively inflammation and prevent anaemia in children with chronic malaria.

Hypothesis: We hypothesized that tolerance to parasite antigen, e.g. malaria parasite antigens, will involve active regulatory molecules to prevent severe disease, e.g. severe malaria. Therefore, if coinfecting with Tr and IgG4 inducing nematodes like hookworm, malaria induced clinical anaemia will be alleviated.

Objectives: We aim to show that IgG4 modulates clinical inflammatory events like anaemia.

Methods: Ex vivo and *in vitro* analysis, flow cytometry and ELISA on samples from children, 0-7 years with severe or asymptomatic malaria, were used and we measure specific IgG, isotypes IgG1-4, total protein, cytokines and Hb levels.

Results: We found severe anaemia prevalence in infants with single inflammatory infection compared to those with mixed infections, ($p < 0.0001$). The isotype IgG4 levels were highest with the increasing Hb levels. Using *in vitro* experience, IgG4 prevents haemolysis ($p=0.002$), while IgG1-IgG3 accelerate cell-lysis ($p=0.014$).

In conclusion, IgG4 modulates immune response and prevents inflammation induced burst of red-blood-cells.

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Pten-Foxo1 signaling prevents autoimmunity by reinforcing maintenance of T_H1 and T_{FH} responses*Shrestha, S.^{1,2}, Chi, H.^{1,2}*¹St. Jude Children's Research Hospital, Immunology, Memphis, United States, ²University of Tennessee Health Science Center, Memphis, United States

Regulatory T cells (Tregs) modulate the immune response to self-antigens, and dysfunctional Treg function can trigger autoimmune diseases. Although the transcriptional and epigenetic programs regulating Treg function have been extensively studied, the signaling and metabolic pathways underlying Treg stability and function are not fully understood. We have recently found that the phosphatase PTEN controls mTORC2 activation and metabolism within Tregs. In the absence of PTEN, mTORC2 function and glycolytic metabolism are elevated, and Treg stability and functional diversity are impaired. These alterations result in the development of a lymphoproliferative disease, spontaneous follicular T helper cell and germinal center B cell responses, and systemic autoimmunity characterized by elevated levels of autoantibodies. Mechanistically, hyper-activation of mTORC2 signaling in Pten-deficient Tregs suppresses the function of the transcription factor, Foxo1. Indeed, restoration of Foxo1 function in Pten-deficient Tregs ameliorates the autoimmune disease features described above. Our results establish PTEN-mTORC2-Foxo1 signaling axis is critical for controlling Treg stability and function necessary to prevent autoimmunity.

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Phosphorylation of Akt and Foxos induced by TGF- β negatively regulates the differentiation of induced regulatory T cells*Nagai, S.¹, Kurebayashi, Y.², Baba, Y.², Minowa, A.², Azuma, M.¹, Yoshimura, A.^{2,3}, Koyasu, S.^{2,4}*¹Tokyo Medical and Dental University (TMDU), Tokyo, Japan, ²Keio University School of Medicine, Shinanomachi Shinjuku-ku, Japan, ³CREST JST, Tokyo, Japan, ⁴Laboratory for Immune Cell System, RIKEN Center for IMS, Yokohama, Japan

TGF- β plays the pivotal role of preventing uncontrolled immune responses that result in lethal autoimmunity through inducing thymic development of naturally occurring regulatory T cells (nTregs) and peripheral differentiation of induced regulatory T cells (iTregs). Foxo transcription factors (Foxos) positively regulate this process to induce the expression of *Foxp3* gene by direct interaction with its promoter region. In the meanwhile, the function of Foxos are negatively regulated by PI3K/Akt signaling, which is activated by TGF- β in many kinds of cells. However, the role of TGF- β on Akt activity and its downstream substrates, including Foxos in CD4⁺ T cells, is still not clear. In this study, we demonstrate that TGF- β selectively induces Akt phosphorylation in a time-dependent manner in CD4⁺ T cells, resulting in the phosphorylation and inhibition of Foxos, which negatively regulates the differentiation of iTregs. The phosphorylation of Foxos induced by TGF- β required class I_A PI3K activity, and the deletion of p85 α , a regulatory subunit of class

I_A PI3K, increased the differentiation of iTreg but not of nTreg. These results indicate a novel negative regulatory mechanism involving Akt and Foxos in the iTreg differentiation process.

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A novel human T cell expansion technology for affordable cell therapy*Harding, F.¹, Delalat, B.¹, Guundsambuu, B.², Pardo, E.³, Huttmacher, D.³, Voelcker, N.¹, Barry, S.C.^{2,4}*¹CRC for Cell Therapy Manufacturing, Future Industries Institute, Adelaide, Australia, ²Molecular Immunology, Robinson Research Institute, Adelaide, Australia, ³CRC for Cell Therapy Manufacturing, Institute of Health and Biomedical Innovation, Brisbane, Australia, ⁴Women's and Children's Health Network, Gastroenterology, Adelaide, Australia

The demand for affordable cell therapy is rapidly rising, and the clinical imperative for tailored therapies with few side effects has placed cell therapy at the vanguard of modern medicine. While there is early promise for the use of T cells in cell therapy, the cost of manufacturing remains a hurdle to the wide scale use of cell therapy. The unmet need includes tolerisation in solid organ transplantation, prevention of graft vs. host disease in bone marrow transplantation autoimmune disease and cancer immunotherapy, which together account for thousands of patients a year who could benefit from cell therapy. In order to address this we are developing functionalised smart surface technologies that can be use in large scale expansion of T cells for clinical use. We have combined melt electrospin 3D scaffold production, epoxy plasma polymerisation and biological stimulation of T cells using surface immobilised antibodies to develop a T cell expansion platform that is scalable and affordable. Pilot data on this smart surface technology will be presented showing its ability to expand CD4⁺ T cells, regulatory T cells and CD8 T cells, giving utility for several immunotherapy approaches. We will present data on robust expansion of relevant human T cell subsets including CD8 T cells/CAR-T cells, CD4 T Cells and regulatory T cells.

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A new biomarker of stable regulatory T cell function which has diagnostic utility in type 1 diabetes*Mohandas, A.¹, Hill, D.¹, Hope, C.¹, Pederson, S.¹, Walsh, J.¹, Grose, R.², Zola, H.², Couper, J.³, Krumbiegel, D.², Sadlon, T.⁴, Barry, S.C.^{1,4}*¹Molecular Immunology, Robinson Research Institute, Adelaide, Australia, ²Leukocyte Biology, WCHRI, Adelaide, Australia, ³Diabetes and Endocrinology, WCHN, Adelaide, Australia, ⁴Women's and Children's Health Network, Gastroenterology, Adelaide, Australia

Natural Treg express the transcription factor FOXP3, but isolation using FOXP3 is not tractable for functional assays or for cell enrichment. A cell surface surrogate for FOXP3 is hence required. As part of a program to develop better biomarkers of immune homeostasis and its breakdown in disease, and also for immunotherapy, In search of a biomarker surrogate for FOXP3, we mined for up-regulated novel surface proteins(1),

and Peptidase Inhibitor 16 (PI16) was identified. Analysis of resting and stimulated Treg and Thelper cells demonstrated that PI16 was readily detectable on the surface of resting nTreg. In conjunction with CD25+, PI16 is expressed on both resting and stimulated FOXP3+ cells. Detailed characterisation of PI16+ve Treg cells reveals an antigen experienced memory phenotype. PI16+ CD25+ cells are highly suppressive *in vitro* and preliminary data suggest that they may be more potent than the CD25+ Treg pool. Importantly, stimulation of CD25-Thelper cells, which substantially up regulates CD25 expression and transiently induces FOXP3, does not induce detectable expression of PI16, suggesting that PI16 is able to segregate Treg subsets, as it is not expressed on CD25- derived iTreg. When a cohort of 30 type 1 diabetes samples were analysed for PI16+ Treg FOXP3 expression, we observed a significant decrease in FOXP3 expression levels in t1d, suggesting a loss of functional fitness in these Treg. We propose that PI16 identifies functional FOXP3+ve Treg and FOXP3-ve Thelper subsets with clinically relevant functional capacity in humans.

1) Sadlon et al JI, 185(2):1071-81.

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Regulatory T cells induced by B cells as a potent therapeutic approach for osteoporosis

Chen, S.-Y., Chiang, B.-L.

National Taiwan University College of Medicine, Graduate Institute of Clinical Medicine, Taipei City, Taiwan, Republic of China

Bone destruction in the metabolic bone diseases, including rheumatoid arthritis and osteoporosis, are triggered by activated immune responses. Regulatory T cells (Tregs) have been found to maintain immune homeostasis and self-tolerance. In addition to the regulatory activity, recent data demonstrated that Tregs suppressed the osteoclastogenesis both *in vitro* and *in vivo*, suggesting the different function of Tregs in protecting bone loss. Treg-of-B cell is a type of induced Tregs that could be generated by naïve B cells and possesses the immunosuppressive effect. In our previous study, mice of collagen induced arthritis receiving LAG3+ Treg-of-B cell treatment showed reduced osteolysis and tartrate-resistant acid phosphatase (TRAP) expression in the hind footpads. Hence, in this study, we aim to investigate the protective role of Treg-of-B cells in the animal model of osteoporosis. First, we perform the *in vitro* culture system to clarify the suppressive effect of Treg-of-B cells in osteoclastogenesis. Further, we develop the optimal potential iTreg-based therapy for ovariectomized mice as an osteoporosis model by adoptively transferring Treg-of-B cells. The osteoclastogenic factors and immunological parameters will be evaluated after mice are sacrificed. We hope this work not only enlighten the role and mechanisms of Treg-of-B cells in osteoclastogenesis, but also facilitate exploring novel regulatory T-cell-based therapies for controlling the bone loss in the metabolic bone diseases.

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Study on the role of galectins in the regulatory function of T-reg-of-B cells

Tsai, Y.C., Yang, C.C., Chiang, B.L.

Graduate Institute of Immunology, College of Medicine, National Taiwan University, Taipei, Taiwan, Republic of China

Regulatory T cells induced by B cells (Treg-of-B) are a subpopulation of regulatory T cells. They are different from other regulatory T cells on the expression of the major genes and cytokine functions. Different from natural regulatory T cells, T-reg-of-B cells are Foxp3 negative, and they are CD25 positive while type 1 regulatory T cells (Tr1 cells) are CD25 negative. However, T-reg-of-B cells suppressive function is not clearly understood, so we aim to further investigate the mechanisms how T-reg-of-B cells modulate immune responses. Galectins are b-galactoside proteins, and there are many researches showing that galectins play the roles in both innate and adaptive immunity. In this study, we assayed the suppressive function of Treg-of-B by T cell suppressive assay and checked the galectins gene expression of T-reg-of-B cells by real-time PCR and showed that galectin-2 is highly expressed in T-reg-of-B cells. We also checked galectin-2 expression at protein level by Western blot. We further tested the suppressive function with the addition of recombinant galectin-2. The results showed that the proliferation of either CD4+CD25- T cells was decreased in the presence of galectin-2. It suggested that galectin-2 might play a role in the regulatory function of Treg-of-B cells.

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Characterization of a novel mouse IL-33R (ST2)-specific monoclonal antibody

Wu, X., Ernst, D., Ejrnaes, M., Zhou, J.C., Kempton, L., Waterman, P.
BD Biosciences, R&D, San Diego, United States

Interleukin-33 (IL-33) is a novel and an unconventional member of IL-1 family. It is a ligand for IL-33R (ST2). The IL-33R exists in either a type I transmembrane or soluble glycoprotein form. These IL-33R forms are encoded by the *Il1rl1* (Interleukin-1 receptor-like 1) gene which belongs to the IL-1 Receptor family within the Ig superfamily. The IL-33R is expressed by subsets of T cells, including Th2-like cells and subset of regulatory T cells, and some innate lymphocytes, eosinophils, basophils, and mast cells. The IL-33/IL-33R (ST2) signaling has been studied in a wide range of inflammation, immunity and allergy for its crucial role in immune responses and tissue homeostasis. In order to further characterize the nature of cells that express IL-33R, we have developed a monoclonal antibody, U29-93, that specifically recognizes the mouse IL-33R. This antibody was used in multicolor flow cytometric analyses of mouse T cell subsets. U29-93 was found to stain either unfixed, or fixed and permeabilized mouse splenic leucocytes. The latter cells could be co-stained with a Foxp3-specific antibody to identify mouse T regulatory cells. The results showed that a small subset of T regulatory cells obtained from either C57BL/6 or BALB/c mice expressed IL-33R. The U29-93 monoclonal antibody should be useful in studying the functions of IL-33R+ T regulatory cells and other IL-33R+ cells.

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Foxp3⁺ regulatory T cells are maintained and induced by CD2-stimulation *ex vivo*

Kashiwakura, Y., Kanno, Y., Hashiguchi, M., Kobata, T., Kojima, H.
 Dokkyo Medical University School of Medicine, Department of Immunology, Shimotsuga, Japan

Regulatory T (Treg) cells are essential in the regulation of immune responses and the maintenance of immune homeostasis. We previously reported that CD2 was involved in the survival of peripheral Treg cells in mice. In this study, we examined whether the CD2 signaling solely affects the induction and function of Treg cells. CD48-Fc, which is composed of extra-cellular portion of murine CD48 and murine Fc γ , was used for CD2-stimulation. We found that CD2-stimuli upregulated Foxp3 expression and maintained the regulatory activity (functions) in Treg cells. Foxp3 upregulation in Treg cells is reported to be dependent on IL-2- and/or TCR-stimulation. We found that CD2-induced Foxp3 upregulation was partially dependent on IL-2. Furthermore, it was found that CD2- and TCR-stimulation synergistically increased the Foxp3 expression in Treg cells. These findings led us to examine whether the CD2-signaling is involved in generating the induced Treg (iTreg) cells. We found that CD2-stimuli induced Foxp3 expression in CD4⁺CD62L⁺CD25⁻ naïve T cells in the presence of TGF- β . It is well known that iTreg cells are induced by TCR- and/or IL-2-signaling in cooperation with TGF- β . It was confirmed that PI3K and JAK/STAT signals were involved in CD2-induced Foxp3 expression in naïve T cells by using inhibitors for these pathways. Importantly, the CD2-induced iTreg cells exerted regulatory effects on CD8⁺ T cell activation. Taken together, these results clearly suggest that CD2-stimuli complement the TCR/CD3-stimuli in Foxp3 expression and/or introduce a TCR/CD3-independent signal, which plays a key role in homeostasis and induction of Treg cells in the periphery.

45 Minute Oral**16:45:00 - 17:30:00****Computational Immunology****Every immune cell counts: The calculus behind lymphocyte decision-making**

Hodgkin, P.D.1,2, Marchingo, J.M.1,2, Kan, A.1,2, Zhou, J.H.1,2, Hawkins, E.D.1,2, Giang, A.1,2, Lye, B.K.1,2, Duffy, K.R.3, Heinzl, S.1,2

1The Walter and Eliza Hall Institute of Medical Research, VIC, Australia, 2The University of Melbourne, VIC, Australia, 3Hamilton Institute, National University of Ireland, Maynooth, Ireland

During the adaptive immune response T and B-lymphocytes receive signals from different sources that determine the strength and type of response they follow. We are investigating how multiple signals are integrated to direct cellular decisions. Initially, following stimulation, B and T cells respond by undergoing a series of divisions before returning to a small, quiescent state. The number of divisions the clonal families undergo is directly regulated by the strength and sum of the activating stimuli. For T cells costimulation by CD28, CD27 and cytokine signals (IL-2 and IL-12) extend the number of divisions undergone. These signals combine following a rule of simple arithmetic addition. Thus, integration of multiple inputs leads to a geometric increase in the size of immune responses due to the two-fold increase with each additional division round. This mode of regulation ensures the size of the response is highly sensitive to small changes in stimulatory inputs and that many combinations of signals can be 'added' to generate a strong immune response.

T Stress**Stressed Out: A Novel Approach to Cancer Immunotherapy**

Glimcher, L.H., Weiss Dean, S.
 Weill Cornell Medical College

Cancer cells induce a set of adaptive response pathways to survive in the face of stressors due to inadequate vascularization¹. One such adaptive pathway is the unfolded protein (UPR) or endoplasmic reticulum (ER) stress response mediated in part by the ER-localized transmembrane sensor IRE1 and its substrate XBP1. We have shown that the transcription factor XBP1 promotes intrinsic tumor growth directly in the setting of triple negative breast cancer, and now have established that this signaling pathway also regulates the host anti-tumor immune response. Dendritic cells (DCs) are required to initiate and sustain T cell-dependent anti-cancer immunity. However, tumors often evade immune control by crippling normal DC function. Constitutive activation of XBP1 in tumor-associated DCs (tDCs) drives ovarian cancer (OvCa) progression by blunting anti-tumor immunity. XBP1 activation, fueled by lipid peroxidation byproducts, induced a triglyceride biosynthetic program in tDCs leading to abnormal lipid accumulation and

subsequent inhibition of tDC capacity to support anti-tumor T cells. Accordingly, DC-specific XBP1 deletion or selective nanoparticle-mediated XBP1 silencing in tDCs restored their immunostimulatory activity *in situ* and extended survival by evoking protective Type 1 anti-tumor responses. Targeting the ER stress response should concomitantly inhibit tumor growth and enhance anti-cancer immunity, thus offering a unique approach to cancer immunotherapy.

Cell Trafficking

Imaging Immunity: Developing a Spatiotemporal Understanding of Host Defense and Disease

Germain, R.N.1, Laemmermann, T.1,2, Uderhardt, S.1, Liu, Z.1, Rudensky, A. Y.3,4, Levine, A. G.3,4, Li, W.1, Radtke, A.1, Yu, W.1, Pires da Silva Baptista, A.1, Gerner, M. Y.1,5

1Laboratory of Systems Biology, NIAID, NIH, Bethesda, MD USA 20892, 2Present address: Max-Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany, 3Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, New York, New York, USA, 4Immunology Program, Memorial Sloan-Kettering Cancer Center, New York, New York, USA, 5Present address: Dept. of Immunology, Univ. of Washington, Seattle, WA

Background: Immune responses involve cell-cell interactions within lymphoid tissues, trafficking of activated cells to sites of effector function, and the migration of effector cells within peripheral tissues. To gain insight into the relationships among cell movement, tissue architecture, and immune function, we have used intravital multiphoton microscopy and a novel multiplex immunohistochemical method we have developed called Histo-cytometry. Observations: Migrating T cells follow stromal pathways in lymph nodes, with chemokine cues facilitating interactions among rare antigen-presenting and antigen-recognizing cells. In tissue sites, effector cells stop when they perceive antigen and undergo transient activation and cytokine release, followed by tuning of their response to existing antigen levels. Innate immune (neutrophil) responses have been dissected at the molecular level. The role of cell localization in both innate and adaptive immunity has also been addressed using Histo-cytometry. With an ability to use as many as 14 different colors and antibodies not only to surface markers but to phospho-proteins and cytokines, and also to conduct imaging in large 3D volumes in a quantitative manner, our multiplex imaging technology facilitates analysis of the phenotype, number, location, signaling state, and function of immune cells and stromal elements in infected, inflamed, or tumor sites. **Conclusion:** This talk will illustrate the power of *in situ* imaging for the acquisition of a more accurate picture of the molecular, cellular, spatial, and temporal aspects of cell function and signaling events in host immune responses. This work was supported in part by the Intramural Research Program of the NIH, NIAID.

Thursday, 25 August 2016

30 Minute Oral

08:30:00 - 10:15:00

Late Breaker Program Lectures 2

3349

Gut microbiota-induced Immunoglobulin G controls systemic infection by symbiotic bacteria and pathogens

Zeng, M., Cisalpino, D., Nunez, G.

University of Michigan Medical School, Pathology, Ann Arbor, United States

The gut microbiota is compartmentalized in the intestinal lumen and induces local immune responses, but it remains unknown whether the gut microbiota can induce systemic response and contribute to systemic immunity. We report that selective gut symbiotic Gram-negative bacteria were able to disseminate systemically to induce Immunoglobulin G (IgG) response, which primarily targeted Gram-negative bacterial antigens and conferred protection against systemic infections by *E. coli* and *Salmonella*, by directly coating bacteria to promote killing by phagocytes. T cells and Toll-like receptor 4 on B cells were important in the generation of microbiota-specific IgG. We identified murein lipoprotein (MLP), a highly conserved Gram-negative outer membrane protein, as a major antigen that induced systemic IgG homeostatically in both mice and humans. Administration of anti-MLP IgG conferred crucial protection against systemic *Salmonella* infection. Thus, our findings reveal an important function for the gut microbiota in combating systemic infection through the induction of protective IgG.

1998

Beneficial effect of enhancing apoptotic cell clearance *in vivo*

Ravichandran, K., Lee, C.S.

University of Virginia, Department of Microbiology, Immunology and Cancer Biology, Charlottesville, United States

Proper apoptotic cell clearance is critical for regulating the inflammatory status in tissues. Very few apoptotic corpses are seen *in vivo*, even in tissues with high cellular turnover; it is often assumed that the capacity for engulfment *in vivo* is vast. Whether apoptotic cell clearance can be enhanced *in vivo* in mammals, and whether this can provide a functional benefit is not known. Here, using complementary genetic approaches, we have revealed that enhancing apoptotic cell clearance is possible *in vivo*, and that increasing the levels of even a single receptor can be beneficial. In an acute colonic inflammation model, endogenous level of the phagocytic receptor BAI1 was progressively downmodulated. Further, BAI1-deficient mice had more pronounced colitis and lower survival with many

uncleared apoptotic corpses and inflammatory cytokines within the colonic epithelium. Mice engineered to overexpress BAI1 had fewer apoptotic cells reduced inflammation, and attenuated disease. Remarkably, transgenic BAI1 expression specifically in the intestinal epithelial cells and enhancing their phagocytic potential was sufficient to lower the colonic inflammation. In contrast, transgenic mice overexpressing a signaling-deficient BAI1 mutant did not show this benefit. Collectively, these data provide genetic evidence that boosting apoptotic cell clearance is achievable *in vivo*, with potential to regulate tissue inflammation in specific contexts.

3762

Hobit and Blimp1 instruct a universal transcriptional program of tissue-residency in lymphocytes

Mackay, L.¹, Minnich, M.², Kragten, N.³, Seillet, C.⁴, Freestone, D.¹, Belz, G.⁴, Busslinger, M.², Shi, W.⁴, Carbone, F.¹, van Lier, R.³, Kallies, A.⁴, van Gisbergen, K.³

¹University of Melbourne, Peter Doherty Institute for Infection and Immunity, Melbourne, Australia, ²Research Institute of Molecular Pathology, Vienna Biocenter, Vienna, Austria, ³Sanquin Research and Landsteiner Laboratory AMC/UvA, Amsterdam, Netherlands, ⁴The Walter and Eliza Hall Institute of Medical Research (WEHI), Melbourne, Australia

Tissue-resident memory T cells (Trm) permanently localize to portals of pathogen entry, where they provide immediate protection against re-infection. To enforce tissue retention, Trm upregulate CD69 and downregulate molecules associated with tissue egress including CCR7 and S1PR1. Although suppression of the transcription factor KLF2 in Trm prevents S1PR1-driven migration, a Trm-specific transcriptional regulator has not been identified. Here, we show that the transcription factor Hobit is specifically upregulated in Trm and together with related Blimp1, mediates the development of Trm in skin, gut, liver and kidney. Importantly, the Hobit/Blimp1 transcriptional module is also required for other populations of tissue-resident lymphocytes including NKT cells and liver-resident NK cells, all of which share a common transcriptional program that includes repression of *Ccr7*, *S1pr1*, and *Klf2*. Our results identify Hobit and Blimp1 as central regulators of this universal program that instructs tissue retention in diverse tissue-resident lymphocyte populations.

Allergy

Why do we have mast cells and IgE? Roles in enhancing host defenses against venoms

Galli, S.
Stanford University

Mast cells (MCs) and IgE are thought to promote to host resistance to certain parasites, but other beneficial functions remained obscure. Margie Profet (1991) noted that many allergens are from sources which either might (e.g., nuts, seafood) or always (e.g., venoms) contain toxins, and hypothesized that allergic reactions evolved to allow sensitized hosts to respond

quickly to, and to neutralize and/or avoid, noxious substances indicative of potentially life-threatening situations. In mammals, venoms provoke innate inflammatory responses and pathology reflecting the activities of the contained toxins. Venoms also can induce allergic sensitization and development of venom-specific IgE antibodies. MCs can be activated directly by certain venoms, and, in mice, innate functions of MCs, including degradation of venom toxins by MC-derived proteases, can enhance host resistance to the venoms of certain arthropods and reptiles (Metz et al., 2006; Schneider et al., 2007; Akahoshi, Song, et al., 2011). Mice injected with sub-lethal amounts of honeybee venom or Russell's viper venom developed specific Th2 responses which increased their survival after subsequent challenge with potentially lethal amounts of that venom (Marichal, Starkl et al., 2013; Starkl, Marichal et al., 2016). Our data indicate that IgE antibodies, FcεRI, and probably MCs contribute to such acquired resistance to these venoms. The finding that IgE-dependent immune responses against venoms can enhance survival in mice supports the hypothesis that one important function of IgE is to help to protect the host against toxic substances.

Peptide immunotherapy in allergic disease: targeting T cells to induce tolerance

Larche, M.
McMaster University

Allergen-specific T cells play a key role in the pathogenesis of allergic diseases through provision of help for allergen-specific B cells and control of inflammatory responses. Allergen immunotherapy using intact allergen proteins (given either subcutaneously or sublingually) is clinically effective and demonstrates enduring efficacy (i.e. disease modifying). However, the requirement for monthly injections or daily sublingual administration (both for three years), combined with a high frequency of local and systemic adverse events, results in poor compliance. Targeting allergen-specific T cells with synthetic peptides representing dominant T cell epitopes markedly decreases treatment times (4-8 intradermal injections), reduces adverse events and provides efficacy for at least 2 years. We have developed peptide immunotherapies for allergies triggered by cats, house dust mites and grass pollen. Each of these consists of a mixture of seven peptides containing multiple dominant T cell epitopes and each have demonstrated statistically significant improvements in rhinoconjunctivitis symptom scores in controlled allergen challenge facilities. The mechanisms of action appear to involve increased IL-10 production, intra- and inter-molecular suppression, modulation of T cell receptor signaling pathways and down-regulation of chemokine pathways. In contrast, treatment does not appear to be associated with deletion of allergen-specific T cells, nor with the induction of allergen-specific IgG (as is seen with conventional whole allergen immunotherapy). Thus, peptide immunotherapy may provide a safe, effective and disease-modifying treatment for allergic disease.

Dendritic Cells

Epigenetic regulation of dendritic cell differentiation and function

Cao, X.

Department of Immunology, Chinese Academy of Medical Sciences Beijing, China; National Key Laboratory of Medical Immunology, Institute of Immunology, Second Military Medical University, Shanghai, China.

Epigenetic modifiers play fundamental roles in defining unique cellular identity through the establishment and maintenance of lineage-specific chromatin and methylation status. Now, increasing evidence demonstrate a critical role of chromatin modifications in controlling innate immunity and inflammation via controlling chromatin status and gene expression. Dendritic cells (DCs)-initiated innate inflammatory responses and adaptive immune response are essential for host defense against invading pathogens, but can also cause harmful immunopathology once uncontrolled. So we are interested in the epigenetic regulation of DC differentiation and function, trying to understand how the epigenetic modifiers regulate the developmental programs and control the transcriptional expression of inflammatory mediators in innate immune cells including DCs. The findings about several epigenetic modifiers in the regulation of the DC-initiated innate response and inflammation will be reported.

3886

Signaling from lysosomes through the TFEB-TRPML1 axis controls the migration of dendritic cells

Lennon-Dumenil, A.-M.

Institut Curie, Paris, France

Immature dendritic cells (DCs) patrol their environment by integrating antigen uptake to cell locomotion. Microbial sensing inhibits antigen internalization and allows mature DCs adopting a fast migration mode needed for them to reach lymph nodes (LNs) where they present microbial antigens to T cells. The signal(s) that triggers such migration changes upon innate sensing remain(s) unknown. We here identify a positive signaling feedback loop from lysosomes as responsible for this switch in DC migration. It involves the release of lysosomal calcium by the channel TRPML1 that

(1) promotes the retrograde flow of Myosin II to stabilize the predominant F-actin structure required for fast DC migration and

(2) triggers the translocation to the nucleus of the lysosomal transcription factor TFEB, which maintains TRPML1 expression. Activation of the TRPML1-TFEB axis is required for DC chemotaxis and arrival to LNs in vivo. We propose that control of DC migration by signaling from lysosomes, where processing of exogenous antigens takes place, establishes a checkpoint for selective arrival to LNs of DCs capable of activating T cells.

Mapping the human dendritic cell lineage

Ginhoux, F.

Singapore Immunology Network (SigN)

Conventional dendritic cells (cDC) are professional antigen-presenting cells that orchestrate immune responses and can be classified into two functionally distinct lineages named cDC1 and cDC2. Important questions on the origins and differentiation paths of human DC populations remain elusive. Here we combine two high-dimensional technologies, single-cell mRNA sequencing and Cytometry by Time-of-Flight (CyTOF) to define and characterize DC precursors (pre-DC) present in human blood. We show that a previously underestimated pre-DC population shares surface markers with plasmacytoid DC (pDC) but has distinct functional properties that were previously attributed to pDC. Finally, we trace the origin of DCs from BM to peripheral blood and reveal that pre-DC comprise three distinct sub-populations consisting of uncommitted preDC and pre-DC subpopulations committed to the cDC1 lineage or cDC2 lineage. The discovery of multiple committed pre-DC populations present in human peripheral blood confirms the existence of DCs as a distinct hematopoietic lineage and opens promising new avenues for the therapeutic exploitation of DC subset-specific targeting.

Innate Lymphoid Cells

Complementarity and redundancy of innate lymphoid cells

Vivier, E.

Centre d'Immunologie de Marseille-Luminy (Aix-Marseille University, INSERM, CNRS)

Innate Lymphoid Cells represent an emerging population of lymphocytes. ILCs include Natural Killer (NK) cells and three main subsets, ILC1, ILC2 and ILC3. In contrast to T and B cells, ILCs do not express antigen-specific receptors derived from gene rearrangements. Besides this major difference in their recognition repertoire, ILC and T cell subsets share striking similarities as ILC1, ILC2 and ILC3 are driven by T-bet, GATA-3 and ROR γ t transcription factors, and produce IFN- γ , IL-5/IL-13 and IL-17/IL-22 respectively. In addition, NK cells are driven by Eomes and T-bet, can be cytolytic and produce IFN- γ , inasmuch as CD8 $^{+}$ T cells. These common features led to suggest that ILCs might correspond to innate counterparts of T cells. Over the course of evolution, two highly parallel systems have thus emerged in which ILCs mimic the effector profile of T cell subsets. However, it is still unclear how the innate and adaptive immune systems integrate these two arms. We will review the emerging set of data showing that ILCs and T cells can exert redundant functions in natura in humans and in models of experimental disease in mice, and discuss how the overlapping functions of ILCs and T cells contribute to the robustness of immunity and hence to the fitness of the hosts.

Oral Abstract Sessions

10:30:00 - 12:10:00

NKT Cells

3427

Direct analysis of natural killer T (NKT) cell responses and their interactions with APCs *in situ*

Johnson, D.N.^{1,2}, Hor, J.L.^{1,2}, Zaid, A.^{1,2}, Pellicci, D.G.^{1,2}, Uldrich, A.P.^{1,2}, Mueller, S.N.^{1,2}, Heath, W.R.^{1,2}, Godfrey, D.I.^{1,2}

¹University of Melbourne, Microbiology & Immunology, Melbourne, Australia, ²ARC Centre of Excellence in Advanced Molecular Imaging, University of Melbourne, Melbourne, Australia

NKT cells recognise glycolipids presented in complex with CD1d molecules through semi-invariant TCRs and play important roles in health and disease. While many studies have investigated NKT cells, very few have determined their location and behavior in relation to other cell types *in situ*. Developments in immunohistological techniques have allowed for the detection of up to 10 different fluorochromes. In conjunction with histocytometry, a flow-cytometry-based analysis technique, this allows detection of many different cell populations *in situ* based on coexpression of multiple markers. With the use of 8 colour histo-cytometry, including CD1d tetramers loaded with a-GalCer, we have succeeded in detecting endogenous NKT cells, and subsets defined by CD4 and CD43 expression, in various tissues including spleen, thymus, LNs, and lungs. Furthermore, we have determined their location in relation to other populations of CD4 and CD8 T cells, B cells, DCs and macrophages. NKT cells were primarily located within the T cell zone of the spleen and LN, where CD4⁺ NKT cells were more common than

CD4⁺ NKT cells. Within the thymus most cells were located within the medulla with equal proportions of both subsets. Following a-GalCer injection, a rapid expansion in the number of either subset of NKT cells was observed throughout the spleen particularly within the T cell zone.

These techniques resulted in novel insights into the location of NKT cell subsets and their interactions with other immune cells in resting and activated states, and provide a means for future investigations into the behavior of innate-like T cells.

502

Neutrophils license NKT cells to regulate self-reactive B cell responses

Hägglöf, T.¹, Sedimbi, S.K.¹, Yates, J.L.², Lanthier, P.A.², Leadbetter, E.A.³, Karlsson, M.¹

¹Karolinska Institutet, Microbiology, Tumor and Cell Biology, Stockholm, Sweden, ²Trudeau Institute, Saranac Lake, United States, ³University of Texas Health Science Center at San Antonio, San Antonio, United States

The early innate responsiveness of the immune system is not only important for quick responses against pathogens but also

to initiate and shape the subsequent adaptive response. In mice, repeated injections of IL-18, a product of inflammasome activation, give rise to a rapid inflammatory response that includes autoreactive antibody production. As this cytokine is elevated in both allergic and autoimmune inflammatory disease we investigated the origin of the B cell response and how it is regulated. We identified an influx of neutrophils to the spleen following IL-18 injections that promote the B cell response. It is known that NKT cells block this B cell response and we found that neutrophils activated NKT cells to upregulate the transcription factor GATA3 and FAS ligand. Increased expression of these molecules was abrogated in neutrophil-depleted mice. We found that FAS ligand expressed specifically by NKT cells was required to restrict B cell activation and autoantibody production. IL-18-injected mice which were depleted of neutrophils also had a dramatic increase in B cell activation, along with autoantibody production, indicating that neutrophils license NKT cells to regulate potentially harmful autoreactive B cell responses in inflammasome-driven inflammation. These findings are relevant for designing treatments of inflammatory diseases that often display lower NKT cells numbers in combination with elevated IL-18 levels.

933

Dominant iNKT cells subsets differentially control immunity to allografts according to the site of T cell priming

Jones, N.D.¹, Chadha, R.², Dempsey, C.M.¹, Besra, G.³, Wood, K.J.², Nakamura, K.¹

¹University of Birmingham, MRC Centre for Immune Regulation, Birmingham, United Kingdom, ²University of Oxford, Nuffield Department of Surgical Sciences, Oxford, United Kingdom, ³University of Birmingham, School of Biosciences, Birmingham, United Kingdom

Invariant natural killer T (iNKT) cells are a rare but evolutionary conserved population of cells that display a marked immunomodulatory capacity. Although iNKT cells have documented roles in a number of diseases their responses frequently lead to different functional consequences in distinct settings.

In this study the effect of activating iNKT cells by injection of glycolipid agonists (e.g. α GalCer) during T cell responses to allografts was determined. We found that glycolipid administration led to prolonged cardiac allograft survival in mice in the absence of other immunosuppressive agents. In clear contrast, activated iNKT cells were found to facilitate rapid skin allograft rejection. The impact of iNKT cells on rejection of these different allografts (i.e. whether they were immunosuppressive or immunostimulatory) correlated with the composition of iNKT cell subsets at the site of T cell priming. IL-17-producing

iNKT cells were dominant in lymph nodes draining skin allografts resulting in the infiltration of neutrophils into grafts and rapid rejection. In contrast, IFN γ -producing iNKT cells were more prevalent in the spleen resulting in the deletion of alloreactive effector T cells primed by intravenous administration of alloantigen.

In conclusion, we clearly demonstrate that the make-up of iNKT cell subsets in the lymphoid tissue hosting T cell priming

dictates whether iNKT cells act in an immunosuppressive or immunostimulatory manner promoting graft survival or rejection, respectively. We also provide evidence that this differential impact of iNKT cells on T cell alloresponses is mediated through effects on myeloid cells rather than on the alloreactive T cells directly.

3412

Characterisation of human CD1 restricted T cells

Pellicci, D.G.¹, Nguyen-Robertson, C.V.¹, Souter, M.N.T.¹, Reddiex, S.J.J.¹, Cheng, J.M.H.², Rossjohn, J.³, Cheng, T.-Y.⁴, van Rhijn, I.⁴, Williams, S.J.², Uldrich, A.P.¹, Moody, D.B.⁴, Godfrey, D.I.¹

¹University of Melbourne, Microbiology and Immunology, Melbourne, Australia, ²University of Melbourne, School of Chemistry and Bio21 Molecular Science and Biotechnology Institute, Melbourne, Australia, ³Monash University, Dept. of Biochemistry and Molecular Biology, School of Biomedical Sciences, Clayton, Australia, ⁴Brigham and Women's Hospital Division of Rheumatology, Immunology and Allergy and Harvard Medical School, Boston, United States

Most studies of T cells have focused on those that respond to foreign peptides. However other T cells exist that recognise lipid antigens presented by the CD1 family. Whereas most published work focuses on CD1d and Natural Killer T cells, abundant populations of human T cells, which comprise up to 10 percent of T cells in the blood, recognize CD1a, CD1b or CD1c. Yet, little is known about their role in immunity. Notably, each CD1 molecule differs in their anatomical location, how they traffic within the cell and their expression levels on the cell surface. Moreover, each CD1 molecule possesses unique hydrophobic pockets that ultimately govern the types of lipid antigens that each CD1 molecule presents. We have produced CD1 tetramers and developed an innovative antigen discovery technique. By trapping lipid antigens in CD1-lipid-TCR complexes, we can specifically identify small molecules that ligate CD1-TCR binding. Using these unique tools, we have identified CD1 restricted T cells in healthy human blood. Moreover, we can detect CD1 restricted T cells that are specific towards Ags derived from *Mycobacterium tuberculosis*. The TCR repertoire of CD1a, CD1b and CD1c-restricted T cells is broad, thus allowing for the recognition of a broad array of antigens. We reveal the phenotypic characteristics of CD1-restricted T cells and also use CD1 mutagenesis to provide new insight into how different TCRs engages CD1-antigen complexes. Collectively, these studies will serve as a basis for future studies of lipid reactive T cells in health and disease.

4052

iNKT mediated anti-tumoral response activated by a Th1 biased agonist

Esteban, I.¹, Alari-Pahissa, E.¹, Lauzurica, P.², Castaño, A.R.¹

¹Institute of Biotechnology and Biomedicine, Universidad Autónoma de Barcelona, Barcelona, Spain, ²Instituto de Salud Carlos III, Madrid, Spain

Activation of iNKT cells by CD1d-presented agonists is a potentially potent immunotherapeutic tool. α -galactosylceramide

(α -GalCer) is the prototypic agonist but its excessive potency with simultaneous production of pro- and anti-inflammatory cytokines hampers its use. Synthetic agonists with a modified polar head activate iNKT cells, *in vitro* and *in vivo*. Significantly, direct recognition *in vitro* does not anticipate *in vivo* functionality. We published how a weak *in vitro* agonist, HS44, induced a strong Th1 response *in vivo*, inducing an efficient iNKT cell dependent antitumor response in the B16 mouse model. A new analog induced a stronger and absolutely specific Th1 response, with no traces of Th2 cytokines or capacity to induce a Th2 response. The characteristic cytokine storm produced by α -GalCer was not induced, which translates in increased antitumor response upon, both as a preventive and therapeutic treatment, more efficiently controlling the establishment of lung metastases. Secondary transactivation of immune cells is potently induced, with strong mobilization of adaptive and innate cells. Differential efficiency to α -GalCer depending on the susceptibility of target cell to NK lysis is shown. *In vivo* depletion experiments demonstrate the involvement of NK cells and M ϕ in tumor killing, depending on the target tumor and the agonist. A massive Th1 associated chemoquine production is induced, which may underline mechanistic differences in the antitumor response between both agonists. Finally, these analogs are also recognized by human iNKT cells, also showing differences in fine specificities with differential recognition of some analogs compared to murine iNKT cells.

713

The activation of iNKT cells induces alloreactive T-cell deletion through the generation of myeloid-derived suppressor cells

Nakamura, K.¹, Dempsey, C.M.¹, Chadha, R.², Besra, G.S.³, Cunningham, A.F.¹, Jones, N.D.¹

¹University of Birmingham, Immunology and Immunotherapy, Birmingham, United Kingdom, ²University of Oxford, Nuffield Department of Surgery, Oxford, United Kingdom, ³University of Birmingham, School of Biosciences, Birmingham, United Kingdom

Invariant natural killer T (iNKT) cells are rare cells that exclusively recognise certain glycolipids (the prototypical agonist being α -galactosylceramide (α GalCer)) presented by CD1d. We and others have shown that iNKT cells are indispensable for the induction of tolerance to allografts in mouse models suggesting that iNKT cell activation may be a worthwhile adjunct strategy to promote tolerance to allografts.

In this study the effect of specifically activating iNKT cells by α GalCer injection during a T cell response to alloantigen was investigated. We found that iNKT cell activation led to the prolonged survival of cardiac allografts in mice in the absence of other immunosuppressive agents. In order to dissect how iNKT cells mediated this suppressive effect, alloreactive, TCR-transgenic T cells (TEa) were transferred to syngeneic recipients and activated by injection of alloantigen with and without concomitant injection of α GalCer. TEa T cells were initially activated and proliferated following stimulation but were subsequently deleted in the presence but not absence of activated iNKT cells. iNKT cell activation resulted in increased numbers of myeloid-derived suppressor cells (MDSC) that were also suppressive *in vitro* and *in vivo* following transfer to

secondary hosts and the depletion of these cells resulted in TEa T cell survival similar to controls. Interestingly, iNKT cell-derived IFN- γ was found to play a key role in enabling the differentiation of such MDSC.

In conclusion, we demonstrate for the first time that glycolipid-activated iNKT cells delete alloreactive effector T cells via an MDSC intermediary in a process requiring the production of IFN- γ .

939

Ly9 (CD229) cell surface receptor controls NKT2 cell development

Cuenca, M., Balada, M., Engel, P.

University of Barcelona, Cell Biology, Immunology and Neurosciences, Barcelona, Spain

Invariant natural killer cells (iNKT cells) are prototypical innate-like T lymphocytes implicated in several pathologies, including microbial infections, allergic diseases, autoimmune diseases, and cancer. These cells produce high amounts of cytokines and have been further classified into three categories (NKT1, NKT2 and NKT17) based on the expression of distinct transcription factors. Signaling lymphocytic activation molecule family (SLAMF) receptors and the specific adapter SLAM-associated protein (SAP) modulate the development of innate-like lymphocytes. Ly9, a SLAM family member, has been shown to act as a negative regulator of iNKT cell development in the thymus. In this study, we show that the increase in iNKT cell numbers found in the thymus of *Ly9*^{-/-} mice was due to an expansion of NKT2 cells (CD1d-tetramer⁺ PLZF^{hi} T-bet^{neg}), while thymic NKT1 (CD1d-tetramer⁺ PLZF^{neg} T-bet^{hi}) were not detected. *Ly9* absence also lead to an enlargement of the iNKT cell pool in the spleen, and in contrast to what it was observed in the thymus, NKT1 cells were present in *Ly9*^{-/-} spleen, indicating that the homeostasis of the different iNKT cell subsets may have distinct requirements depending on their tissue localization. Furthermore, *in vivo* treatment with an agonistic monoclonal antibody directed against Ly9 significantly decreased iNKT cell numbers in the spleen, and this reduction was preferentially due to a depletion of NKT2 cells. Thus, anti-Ly9 targeting could represent a novel therapeutic approach to modulate NKT cell expansion.

3518

Wnt proteins drive the IL-12/IFN- γ -axis in response to the Natural Killer T cell antigen, α -galactosylceramide

Kling, J., Meiners, J., Blumenthal, A.

University of Queensland Diamantina Institute, Woolloongabba, Australia

Natural Killer T (NKT) cells are key regulators of immune responses in autoimmunity, infection and cancer. Upon recognition of glycolipid antigens, NKT cells rapidly produce cytokines, such as IFN- γ , thereby providing a defining cytokine milieu early during antigen challenge. NKT cell functions are critically influenced by cytokines released by antigen presenting cells (APCs), including IL-12, an established key driver of IFN- γ production. The nineteen mammalian Wnt proteins are known primarily for orchestrating cell differentiation and proliferation

during embryogenesis and tissue homeostasis. More recently, we and others have demonstrated that Wnt proteins are expressed by APCs and define inflammatory cytokine responses during infection and chronic inflammation. Our observation that NKT cells express Wnt signalling receptors, and our previous report that Wnt signalling perpetuated the IL-12/IFN- γ axis driven by antigen-recall of human T cells, led us to hypothesise that Wnt proteins contribute to IFN- γ production by NKT cells. *In vivo* challenge of mice with the glycolipid antigen, α -galactosylceramide (α -GalCer) led to differential expression of multiple Wnt ligands in liver tissue, an organ rich in NKT cells. Small molecule inhibitors that target functional bottle-necks of Wnt secretion and signalling decreased α -GalCer-induced expression of IFN- γ and IL-12p40. Moreover, reduced α -GalCer-induced IFN- γ and IL-12p40 responses were observed in mice with a myeloid-specific deletion of *Wntless*, a key chaperone in Wnt protein release. Taken together, our observations suggest that APC-derived Wnt proteins define early host responses to the NKT cell antigen, potentially by shaping the cytokine output from both APCs and NKT cells.

2377

Activity of sulfatide-reactive type II NKT cells from mouse lung

Pasquet, L., Kato, S., Xia, M., Berzofsky, J.A., Terabe, M.

NIH-NCI, Vaccine Branch, Bethesda, United States

Natural killer T cells are lymphocytes that recognize lipid antigens presented by the non-classical MHC-I molecule, CD1d. Type I NKT cells with semi-invariant TCR recognize α -galactosylceramide (α GalCer) while type II NKT cells with more diverse TCR repertoire recognize sulfatide. NKT cells are known to be frequent in the liver. By using sulfatide-loaded CD1d-tetramers, we observed that sulfatide-reactive type II NKT cells were enriched not only in the liver but also in the lung, a major site of metastasis. Type II NKT cells inhibit anti-tumor immunity, so understanding their function in lungs is critical. Here, we analyzed their activation either *in vitro* or *in vivo*. In the steady state, lung type II NKT cells expressed Granzyme A but not Granzyme B or perforin. By injecting sulfatide *in vivo*, we observed that lung type II NKT cells upregulated Granzyme A and Ki67 expression. However, it was difficult to detect responses *in vitro*, so we hypothesized that this might be due to inadequate antigen-presenting cells (APCs). Using syngeneic bone marrow-derived dendritic cells (BMDC) as APCs, *ex-vivo* stimulation of lung type II NKT cells with sulfatide induced the secretion of IFN- γ , IL-13 and IL-17. This was consistent with the expression of specific transcription factors, GATA-3 and ROR γ t by lung type II NKT cells. The ability to study responses of type II NKT cells *in vitro* can now help elucidate the mechanism by which these regulatory cells interact with other cells and modulate tumor immunity in the lung.

Transplantation 2

3262

Hematopoietic stem cells and their progenitors critically require autophagy to promote early engraftment following allogeneic stem cell transplantation

Lineburg, K.E.¹, Leveque-El Mouttie, L.¹, Le Texier, L.¹, Bianca, T.¹, Kuns, R.¹, Lane, S.^{1,2}, Hill, G.R.^{1,2}, MacDonald, K.P.A.¹

¹QIMR Berghofer Medical Research Institute, Brisbane, Australia,

²Royal Brisbane and Women's Hospital, Brisbane, Australia

Hematopoietic Stem Cells (HSC) are critical for the success of stem cell transplantation. Autophagy is an intracellular process that has an established role in the long-term survival and function of HSCs. We investigated the contribution of autophagy to HSC in the setting of allogeneic transplantation, in which GVHD results in a T cell derived cytokine storm early post-transplant. We demonstrate increased autophagy in donor HSC and progenitor cells in the setting of GVHD compared to non-GVHD. Competitive transplant experiments of 1:1 Atg5^{-/-} fetal liver (FL) with WT FL demonstrated that autophagy deficient cells display reduced capacity to reconstitute. In an MHC mismatch model of GVHD we demonstrated that while Atg5^{-/-} cells are capable of engraftment they are overcome in the presence of alloreactive T cells and undergo primary graft failure by day 10 post-transplant while WT cells survive and engraft. We confirmed this early graft failure in a second model, using donor VAV^{cre} x Atg7^{fl/fl} mice. The essential requirement for autophagy, specifically in early progenitors and HSC, was confirmed using LysM^{cre} Atg7^{fl/fl} mice. We demonstrate that autophagy is increased in the GVHD setting and that without autophagy early myeloid precursors fail to provide short term reconstitution leading to primary graft failure and mortality. This primary graft failure can be rescued by the administration of cyclosporine, which works to dampen the T cell induced cytokine storm post-transplant. Thus intervention to increase autophagy in these cells post-transplant may improve engraftment in the clinic.

2587

The IL-6/IL-17 pathway impairs the re-establishment of immune-equilibrium following autologous stem cell transplantation for multiple myeloma

Minnie, S.A.¹, Guillerey, C.², Chesi, M.³, Markey, K.A.¹, Gartlan, K.H.¹, Koyama, M.¹, Kuns, R.D.¹, Zhang, P.¹, Tey, S.-K.¹, Lane, S.W.⁴, Smyth, M.J.², Vuckovic, S.¹, Hill, G.R.¹

¹QIMR Berghofer Medical Research Institute, Bone Marrow Transplantation Laboratory, Brisbane, Australia, ²QIMR Berghofer Medical Research Institute, Immunology in Cancer and Infection Laboratory, Brisbane, Australia, ³Mayo Clinic, Comprehensive Cancer Center, Scottsdale, United States, ⁴QIMR Berghofer Medical Research Institute, Gordon and Jessie Gilmour Laboratory, Brisbane, Australia

Multiple Myeloma (MM) often escapes out of a long-standing monoclonal gammopathy, suggesting progression from a state of immune-equilibrium. Autologous stem cell transplant (auto-SCT) remains a standard of care for patients with MM, inducing a state of minimal residual disease and a prolonged

plateau phase of disease. We thus hypothesized that auto-SCT may overcome the acquired immune defects induced by myeloma and restore immune-equilibrium. Using a system in which B6.WT mice bearing clinically relevant Vk*MYC myeloma (Vk12653) were transplanted with syngeneic grafts, we demonstrated prolonged survival with a plateau phase when T cells were added to bone marrow (BM) grafts (BM+T vs. BM, gamma/albumin [M-band], 6 weeks post-transplantation 0.267 vs. 1.996, P=0.0008; median survival 106 vs. 58 days, P=0.0001). Myeloma 'experienced' T cells from mice in plateau phase (harvested >100 days post-transplantation) were able to transfer anti-myeloma immunity to secondary Vk*MYC-bearing recipients. This anti-myeloma immunity required both CD4⁺T and CD8⁺T cells but was independent of NKT or gd cells. T cell-mediated anti-myeloma immunity was mediated by IFN γ signalling within donor cells but was independent of perforin. Deletion of IL-6 and IL-17 within donor cells further enhanced anti-myeloma immunity (M-band, 8 weeks post-transplantation: WT vs IL-6^{-/-} donor 0.562 vs. 0.101; WT vs. IL-17^{-/-} donor 0.746 vs. 0.153; median survival was unreached at 120 days for IL-6^{-/-} and IL-17^{-/-} donor). These findings demonstrate the re-establishment of Th1-dependent immune-equilibrium after auto-SCT and highlight the opposing role of IL-6 and IL17 pathway in this process, providing a potential therapeutic strategy to improve outcomes.

Vuckovic, S. and Hill, G.R. contributed equally to this work

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Interleukin-35 mitigates development of rejection after murine pancreatic islet transplantation via regulation of Treg/Th17 ratio

Yin, Z.¹, Li, B.¹, Dongying, C.²

¹The First Hospital of China Medical University, Department of Hepatobiliary Surgery and Organ Transplantation, Shenyang, China, ²The First Hospital of China Medical University, Shenyang, China

IL-35 is a newly discovered inhibitory cytokine predominantly secreted by regulatory B (i35-Bregs) and T (iT35) cells and may have therapeutic potential in several inflammatory and autoimmune responses. The role of IL-35 in pancreatic islet transplantation (PIT) remains to be answered. To elucidate this, we investigated the kinetics of Treg/Th17 cell response in murine PIT model and measured the levels of IL-35 in human PIT patients. We found that Treg cells and pro-inflammatory (IFN- γ , IL-2, IL-17) cytokines were increased, however, anti-inflammatory (IL-10, IL-35, TGF- β) cytokines were decreased in PIT model. IL-35 administration effectively mitigated development of rejection after murine PIT, seemingly by repressing proliferation of Th17 and converting Th17 and conventional Treg cells into IL-35-yielded Treg cells, called iT35, indicating a phenotypic shift of Treg cells. Furthermore, serum IL-35 levels were decreased in human PIT patients compared to healthy controls, and in aGVHD grade 2-4 patients compared to time-matched patients with aGVHD grade 0-1. Our finding suggest that lack of IL-35 levels play a pivotal role in the development of rejection after PIT, and therapy with IL-35 should be investigated as the therapeutic strategy for PIT and other autoimmune diseases.

1469

Calcineurin inhibitor Tacrolimus impairs host immune response against urinary tract infection by affecting TLR4 negative regulators

Emal, D.¹, Tammaro, A.¹, Jansen, M.P.¹, Teske, G.J.¹, Claessen, N.¹, Florquin, S.^{1,2}, Leemans, J.C.¹, Dessing, M.C.¹

¹Academic Medical Center, University of Amsterdam, Department of Pathology, Amsterdam, Netherlands, ²Radboud University Nijmegen Medical Center, Department of Pathology, Nijmegen, Netherlands

Background: Calcineurin inhibitor Tacrolimus (TAC), is a potent immunosuppressive drug widely used in order to prevent acute graft rejection. Long term use of TAC increases susceptibility to bacterial infections. Urinary tract infection (UTI) is the most frequent infectious complication in renal transplant patients. The mechanism by which TAC suppresses the adaptive immunity is well known. There are indication that TAC also affects innate immunity, however it remains largely unknown how TAC affects host innate immune response against UTI.

Methods: To study this, we performed experimental UTI model by intravesical inoculation of uropathogenic E.coli in female wild type mice pre-treated with TAC or vehicle (CTR).

Results: We found that TAC pre-treated mice display higher bacterial loads than CTR mice in kidney and bladder homogenates after 24 and 48 hours. However, inflammatory mediators in kidney and bladder were similar between TAC and CTR group, indicating that TAC pre-treated mice respond relatively less to E. coli than CTR mice. Indeed, granulocytes from TAC pre-treated mice phagocytize less E. coli ex-vivo compared to CTR. Furthermore, whole blood of TAC pre-treated mice release less cytokines after LPS or heat-killed E.Coli stimulus. This tolerant state can be explained by upregulation of TLR4 negative regulators (IRAKM, SOCS1, A20, ATF3, BCL3 and TRIM30A) as observed in TAC pre-treated granulocytes.

Conclusion: We show that TAC affects TLR4-mediated responses in granulocytes by enhancing expression of negative regulators. This so called TAC-induced tolerant state can explain the impaired antibacterial defense mechanism against UTI in TAC pre-treated mice.

3223

Recipient bone marrow (BM) macrophages (Macs) are vital for haematopoietic stem cell (HSC) engraftment post autologous transplantation

Kaur, S.¹, Raggatt, L.J.¹, Jacobsen, R.N.¹, Millard, S.¹, Batoon, L.¹, Winkler, I.G.¹, Macdonald, K.P.A.², Perkins, A.C.¹, Hume, D.A.³, Levesque, J.P.¹, Pettit, A.R.¹

¹The University of Queensland, Mater Research Institute, Woolloongabba, Australia, ²Queensland Institute of Medical Research, Herston, Australia, ³University of Edinburgh, Roslin Institute, Scotland, United Kingdom

Use of BM-transplantation (Tx) in cancer treatment is constrained by substantial risks and limitations. Improvement in recovery and quality of HSC niches within BM will likely improve the BM-Tx risk-benefit ratio. BM-Macs support HSC niche homeostasis, consequently we examined whether BM-Macs play a role in Tx

success using an autologous Tx model. Recipient MacGreen mice (express GFP in myeloid cells) were lethally irradiated and transplanted with syngeneic HSC. Flow cytometry analyses of BM 2-16 weeks (wk) post-Tx confirmed more than 99% donor chimerism of monocytes and granulocytes validating ablation of recipient HSC. In contrast, GFP⁺F4/80⁺CD11b⁺Ly6G^{neg}CD169⁺VCAM-1⁺ER-HR3⁺ recipient BM-Macs were detected throughout the time-course. A significant 5.9 fold expansion of recipient BM-Macs occurred between wk 2 (4.5 x 10⁴ cells/femur) and 5 (27 x 10⁴ cells/femur) post-Tx which coincided with increased BM residence of long-term (LT) donor HSC. Recipient Macs in spleen displayed different frequency and longevity kinetics that correlated with transient post-Tx splenic extramedullary haematopoiesis. In situ, GFP⁺F480⁺ recipient BM-Macs localized to HSC niche-enriched perivascular microenvironments within both central BM and endosteal regions. We selectively depleted recipient BM-Macs using CD169-DTR mice transplanted with syngeneic ubiquitous GFP⁺ HSC. Depletion of recipient CD169⁺ Macs abated donor LT-HSC BM engraftment by 70% at 5 wk post-Tx and reduced BM reconstitution potential in competitive secondary transplants. Overall BM contains a myeloablation-resistant self-repopulating Mac subset that is necessary for efficient and/or sustained HSC BM engraftment in an autologous Tx setting. Therapeutically targeting resilient recipient BM-Macs may enhance stem cell engraftment and accelerate haematopoietic recovery post BM-Tx.

3975

Clinical grade multipotent adult progenitor cells suppress IL-7 driven stimulation of T cells *in vivo*

Carty, F.¹, Reading, J.², Tree, T.², Ting, A.³, Stubblefield, S.³, English, K.¹
¹National University of Ireland Maynooth, Maynooth, Co. Kildare, Ireland, ²Kings College London, London, United Kingdom, ³Athersys Inc., Cleveland, United States

In the setting of transplantation, T cells are deliberately depleted to prevent allograft rejection. In the lymphopenic environment which ensues, interleukin-7 (IL-7) is abundant, and competition is low. This IL-7 activates the proliferation of effector memory T cells, which are the predominant mediators of graft rejection. Multipotent adult progenitor cells (MAPC) have been previously shown to modulate the response of T cells to IL-7, *in vitro* but not *in vivo*. Here, for the first time, we demonstrate that MAPC suppress IL-7 driven proliferation and pro-inflammatory cytokine production by both CD4⁺ and CD8⁺ T cells in the spleen. In order to further investigate the effects of MAPC in this model, 140 cell populations in the spleen were examined following IL-7 and MAPC therapy, using a 16 colour flow cytometry immunophenotyping study. MAPC enhanced the frequency of regulatory T cells and transitional 1 B cells in the spleen, however this effect was only observed when MAPC were administered intraperitoneally (i.p.), rather than intravenously (i.v.). The effects of routes of administration on the bio-distribution of MAPC in our model of homeostatic proliferation was further clarified using novel 3D whole animal Cryo-imaging technology. Following i.p. administration, MAPC reside in the omentum tissue suggesting that MAPC mediate their effects in the spleen through trophic signalling.

Furthermore MAPC delivered i.p. may have contact-driven effects on cells in the omentum. These findings support ongoing studies showing the therapeutic efficacy of MAPC in both solid organ and hematopoietic cell transplantation, elucidating novel mechanisms for their effects *in vivo*.

2709

Monocyte derived dendritic cells promote Th polarization, while conventional dendritic cells promote Th proliferation

Chow, K.V.^{1,2,3}, Lew, A.M.^{1,2}, Sutherland, R.M.^{1,2}, Zhan, Y.^{1,2}

¹Walter & Eliza Hall Institute of Medical Research, Immunology, Parkville, Australia, ²University of Melbourne, Medical Biology, Parkville, Australia, ³Royal Melbourne Hospital, Nephrology, Parkville, Australia

Monocyte derived dendritic cells (moDCs) dramatically increase in numbers upon infection and inflammation. We found that this also occurs in response to allogeneic encounters, such as those that occur in the setting of organ transplantation. Despite their prominence in these settings, how emergent moDCs and resident conventional DCs (cDCs) divide their labor as APCs remain undefined. Since, unlike nominal antigens, transplant antigens can be recognized by direct and indirect presentation, we compared both direct and indirect antigen presentation by murine moDCs versus cDCs. We found that, despite having equivalent MHC-II expression and *in vitro* survival, moDCs were 20-fold less efficient than cDCs at inducing CD4⁺ T cell proliferation through both direct and indirect Ag presentation. Despite this, moDCs were more potent at inducing Th1 and Th17 differentiation (e.g. 8-fold higher IFN- γ , 2-fold higher IL-17A in T cell co-cultures) whereas cDCs induced 10-fold higher IL-2 production. Intriguingly, moDCs potently reduced the ability of cDCs to stimulate T cell proliferation *in vitro* and *in vivo*, partially through nitric oxide production. We surmise that such division of labor between moDCs and cDCs has implications for their respective roles in the immune response.

4353

A novel, blocking anti-CD40 monoclonal antibody prolongs non-human primate renal allograft survival in the absence of B-cell depletion or thromboembolic events

Cordoba, F.¹, Wiczorek, G.¹, Audet, M.², Schneider, M.¹, Espie, P.¹, Roth, L.¹, Heusser, C.¹, Bruns, C.¹, Patel, D.¹, Rush, J.¹

¹Novartis Institutes of Biomedical Research, Basel, Switzerland, ²Hôpital de Hautepierre, Strasbourg, France

CD40-CD154 pathway blockade using anti-CD40 antibodies has been shown to effectively prolong kidney allograft survival. However, these antibodies differed in their functional properties related to CD40 pathway activation, blockade as well as the ability to deplete CD40 expressing B cells. To address the question of the relative contribution of B-cell depletion to the efficacy of anti-CD40 blockade, we developed a novel, human Fc-silent anti-CD40 monoclonal antibody (CFZ533) that was incapable of antibody-dependent cellular cytotoxicity or complement-dependent cytotoxicity in MHC-mismatched cynomolgus monkey renal allograft transplantation. Well-

functioning allografts survived up to a pre-defined endpoint of 100 days in CFZ533-treated animals (100, 100, 100, 98, 78 days) in comparison to untreated monkeys (survival ~ 7 days; n = 9). Excellent graft morphology was observed in the CFZ533-dosed group, and the antibody suppressed a gene signature associated with acute rejection in a majority of animals. Further, CFZ533 completely disrupted splenic germinal centers in transplanted animals (indicating a full tissue pharmacodynamic effect), but failed to deplete peripheral blood B-cells depletion was only observed with the Fc-competent antibody. Importantly, CFZ533 was well-tolerated and there was no evidence of thromboembolic events. The data indicate that CD40 pathway blockade in the absence of B-cell depletion maintained very high allograft quality and function up to 100 days post-transplantation. Thus, use of the Fc-silent anti-CD40 antibody CFZ533 appears to be an attractive approach for preventing solid organ transplant rejection and treating autoimmune diseases involving T-cell-dependent humoral immune mechanisms.

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Keap1 regulates Mycobacterium avium - induced inflammatory signaling in human macrophages

Awuh, J.A.^{1,2}, Haug, M.^{1,2}, Mildenerger, J.^{1,2}, Marstad, A.^{1,2}, Ngoc Do, C.P.^{1,2}, Louet, C.^{1,2}, Stenvik, J.^{1,2}, Steigedal, M.^{1,2}, Damås, J.K.^{1,2,3}, Halaas, Ø.¹, Flo, T.H.^{1,2}

¹Norwegian University of Science and Technology, Cancer Research and Molecular Medicine, Trondheim, Norway, ²Norwegian University of Science and Technology, Center of Molecular Inflammation Research, Trondheim, Norway, ³St Olavs Hospital, Infectious Diseases, Trondheim, Norway

Inflammatory signaling is a central mechanism controlling host defenses to pathogens. However, how these mechanisms are in turn controlled remains poorly understood. Invading pathogens activate cytokine production and might induce the production of reactive oxygen species (ROS) that needs to be tightly controlled in cells. Kelch-like ECH-associated protein 1 (Keap1) plays a well-established role as a sensor for ROS for the protection of cells against oxidative damage. We recently demonstrated a role of Keap1 in regulating intracellular survival of *M. avium* in primary human macrophages. Keap1 associated with mycobacteria in a time-dependent manner while siRNA-mediated knockdown of Keap1, Cul3, and Rbx1 increased *M. avium*-induced expression of inflammatory cytokines and type I interferons. Keap1-Cul3-Rbx1 facilitates ubiquitination and degradation of IKK β and thus terminating IKK activity. Keap1 knockdown led to increased nuclear translocation of transcription factors NF- κ B, IRF1 and IRF5 driving the expression of inflammatory cytokines and IFN β . Finally, increased inflammatory responses in Keap1-silenced cells contributed to decreased intracellular growth of *M. avium* in primary human macrophages. Taken together we propose that Keap1 acts as a negative regulator for controlling inflammatory signaling in *M. avium*-infected macrophages. Although this might be important to avoid sustained or overwhelming inflammation, a negative consequence could be facilitated growth of pathogens within macrophages. Using

different ligands and other heat-killed bacteria we have new evidence that this mechanism is not specific for mycobacterial infections but rather an NF- κ B driven mechanism. Our results indicate that altered Keap1 gene expression may also have vital clinical implications for other inflammatory conditions.

2588

A dominant-negative mutation in TNFR1 results in increased susceptibility to pulmonary tuberculosis infection

Saunders, B.M.¹, Huch, J.², Whittle, B.³, Chan, B.², Cornall, R.⁴, Goodnow, C.^{5,6}, Britton, W.J.^{2,7}

¹University of Technology Sydney, School of Life Sciences, Broadway, Australia, ²Centenary Institute, Tuberculosis Research Program, Newtown, Australia, ³Australian National University, John Curtin School of Medical Research, Australian Phenomics Facility, Canberra, Australia, ⁴Oxford University, Nuffield Department of Medicine and Wellcome Trust Centre for Human Genetics, Oxford, United Kingdom, ⁵John Curtin School of Medical Research, Australian National University, Immunogenomics Laboratory, Canberra, Australia, ⁶Garvan Institute of Medical Research, Immunogenomics Laboratory, Darlinghurst, Australia, ⁷University of Sydney, Department of Immunology and Infectious Diseases, Sydney Medical School, Sydney, Australia

Tuberculosis remains a major global health challenge, interestingly only 10% of infected individuals progress to active disease and genetic variation contributes to this increased susceptibility. To examine this further, we undertook an unbiased genetic screen using pulmonary *M. tuberculosis* infection of mice following ENU mutagenesis. We identified a pedigree of mice (WT-007) with markedly increased susceptibility to *M. tuberculosis* infection. Deep sequencing identified a genetic variant in the extracellular domain of TNF receptor 1 (TNFR1) as the causative mutation. In humans this identical mutation results in the auto-inflammatory condition TNF receptor-associated periodic syndrome (TRAPS). In contrast to TNFR1 deficient mice, this mutation has a dominant-negative effect, as both homozygous and heterozygous mice displayed a gene dose-dependent increase in susceptibility to *M. tuberculosis* infection characterised by hyper-inflammation, failure to control bacterial growth and markedly reduced survival. This was associated with reduced circulating sTNFR1 levels, increased pro-inflammatory cytokine expression and dysregulated cellular recruitment. *M. tuberculosis*-infected mutant mice developed a robust antigen-specific T cell response, however this was insufficient to control the infection. Infection of 007-RAG^{-/-} chimeric mice demonstrated that expression of the TNFR1 mutation by both haemopoietic and stromal cells contributed to the phenotype of the 007 mice. Furthermore, while plasma sTNFR2 levels were elevated in the WT-007 mice, the hyper-inflammation and susceptibility were independent of TNFR2 expression. These data indicate that functional TNFR1 is critical to regulate TNF-induced inflammation and control of intracellular pathogens. Further, individuals with the TRAPS signalling defect may be at increased risk from intracellular pathogens.

819

Cathelicidin insufficiency in patients with fatal leptospirosis

Lindow, J.^{1,2}, Wunder, E.^{1,2}, Popper, S.³, Min, J.-N.², Mannam, P.², Srivastava, A.², Yao, Y.², Hacker, K.^{1,2}, Raddassi, K.², Lee, P.², Montgomery, R.², Shaw, A.², Hagan, J.⁴, Araújo, G.¹, Nery, N.¹, Relman, D.³, Kim, C.⁵, Reis, M.¹, Ko, A.^{1,2}

¹Fiocruz, Salvador, Brazil, ²Yale University School of Medicine, New Haven, United States, ³Stanford University, Stanford, United States, ⁴Centers for Disease Control and Prevention, Atlanta, United States, ⁵University of California, San Francisco, United States

Leptospirosis is an important zoonotic cause of morbidity and mortality worldwide due to life-threatening manifestations of Weil's disease and pulmonary hemorrhage syndrome. The role that the host response plays in disease progression and high case fatality (>10-50%) observed for these severe disease forms is poorly understood. We conducted a multiparameter investigation of patients with acute leptospirosis to identify mechanisms associated with case fatality. Whole blood transcriptional profiling revealed fatal cases had lower expression of the antimicrobial peptide, cathelicidin, and chemokines, but more abundant pro-inflammatory cytokine receptors (IL-18R and IL-1R2). In contrast, survivors generated strong adaptive immune signatures, including transcripts relevant to antigen presentation and immunoglobulin production. In an independent cohort (23 survivors, 22 deceased), low serum cathelicidin and RANTES levels during acute illness were independent risk factors for higher bacterial loads ($P=0.005$) and death ($P=0.04$), respectively. Additionally, administration of cathelicidin prior to lethal *Leptospira* infection in an experimental animal model of leptospirosis significantly increased survival and decreased bacterial loads. Together, these findings indicate that the host response plays a central role in disease progression during severe leptospirosis. Poor outcomes are associated with the inability to mount an immune response that controls systemic bacterial loads and the development of aberrant pro-inflammatory responses. Furthermore, the specific antimicrobial peptide, cathelicidin, is a key factor in mounting an effective bactericidal response against the pathogen and may represent a new therapeutic approach for leptospirosis.

2476

Structural basis of mycobacterium tuberculosis lipid antigen-CD1b complex recognition by T cell receptor

Gras, S.^{1,2}, Shahine, A.¹, Tan, L.¹, Van Rhijn, I.^{3,4}, Moody, D.B.³, Rossjohn, J.^{1,2,5}

¹Monash University, Infection and Immunity Program, Clayton, Australia, ²Monash University, Australian Research Council Centre of Excellence for Advanced Molecular Imaging, Clayton, Australia, ³Harvard Medical School, Division of Rheumatology, Immunology and Allergy, Boston, United States, ⁴Utrecht University, Department of Infectious Diseases and Immunology, Utrecht, Netherlands, ⁵Cardiff University School of Medicine, Institute of Infection and Immunity, Cardiff, United Kingdom

Recently, we have identified CD1b-reactive T cells recognising mycobacterium tuberculosis lipid antigen, namely glucose monomycolate (GMM) using tetramer staining (Van Rhijn et al.,

2013, *Nature Immunology*). We have isolated two sub-families of CD1b-GMM reactive T cells, exhibiting either high or low affinity. Sequence analysis of those high and low affinity TCRs, revealed a skewed TCR repertoire with a TRAV1-2 bias usage characteristic of the high affinity TCRs while a TRAV17 bias usage characterised the low affinity TCRs (Van Rhijn et al., 2014, *Journal of Immunology*).

Surprisingly the TRAV1-2 high affinity TCRs have a nearly invariant CDR3a sequences composed mainly of germline residues, therefore we named those receptors germline-encoded mycolyl-reactive or GEM TCRs. We have shown that the GEM TCRs exhibit an unusually high affinity of $\sim 1 \mu\text{M}$ for CD1b-GMM.

To further understand the molecular basis of CD1b-GMM recognition by TCRs, as well as to determine the role played by the different Va chains (TRAV1-2 and TRAV17) in TCR affinity, we performed a structural investigation of the CD1b-GMM in complex with a GEM TCR. The GEM TCRs will provide insight into a simplified CD1b-specific T cell repertoire and a specific understanding of T cell immunity from tuberculosis patients.

1360

Autoantibody production by murine B-1a cells stimulated with *Helicobacter pylori* urease through Toll-like receptor 2 signaling

Takeshita, H.¹, Watanabe, E.², Kobayashi, F.¹, Nakagawa, Y.², Yamanishi, S.¹, Norose, Y.², Shinya, E.², Kumagai, Y.², Ito, Y.³, Takahashi, H.²

¹Nippon Medical School, Departments of Microbiology/Immunology, and Pediatrics, Tokyo, Japan, ²Nippon Medical School, Departments of Microbiology and Immunology, Tokyo, Japan, ³Nippon Medical School, Departments of Pediatrics, Tokyo, Japan

Helicobacter pylori infection is associated with several autoimmune diseases, in which autoantibody-producing B cells must be activated. Among these B cells, CD5-positive B-1a cells from BALB/c mice were confirmed to secrete autoantibodies when cocultured with purified *H. pylori* urease in the absence of T cells. To determine the mechanisms for autoantibody production, CD5-positive B-1a cells were sorted from murine spleen cells and stimulated with either purified *H. pylori* urease or *H. pylori* coated onto plates (referred to hereafter as plate-coated *H. pylori*), and autoantibody production was measured by enzyme-linked immunosorbent assay (ELISA). Complete urease was not secreted from *H. pylori* but was visually expressed over the bacterium-like endotoxin. Urease-positive plated-coated *H. pylori* stimulated B-1a cells to produce autoantibodies, although urease-deficient isotype-matched *H. pylori* did not. Autoantibody secretion by B-1a cells was inhibited when bacteria were pretreated with anti-*H. pylori* urease-specific antibody having neutralizing ability against urease enzymatic activity but not with anti-*H. pylori* urease-specific antibody without neutralizing capacity. The B-1a cells externally express various Toll-like receptors (TLRs): TLR1, TLR2, TLR4, and TLR6. Among the TLRs, blocking of TLR2 on B-1a cells with a specific monoclonal antibody (MAb), T2.5, inhibited autoantibody secretion when B-1a cells were stimulated with plate-coated *H.*

pylori urease. Moreover, B-1a cells from TLR2-knockout mice did not produce those autoantibodies. The present study provides evidence that functional urease expressed on the surface of *H. pylori* will directly stimulate B-1a cells via innate TLR2 to produce various autoantibodies and may induce autoimmune disorders.

2607

Inhibition of TLR signaling by a bacterial protein containing immunoreceptor tyrosine-based inhibitory motifs

Yan, D.¹, Wang, X.², Luo, L.³, Cao, X.⁴, Ge, B.²

¹Fudan University, Department of Immunology, Shanghai, China, ²Clinical and Translational Research Center, Shanghai Pulmonary Hospital, Tongji University School of Medicine, Shanghai, China, ³Institute of Health Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences & Shanghai Jiaotong University School of Medicine, Shanghai, China, ⁴National Key Laboratory of Medical Immunology and Institute of Immunology, Second Military Medical University, Shanghai, China

The protein Tir (translocated intimin receptor) in enteric bacteria shares sequence similarity with the host cellular immunoreceptor tyrosine-based inhibition motifs (ITIMs). Despite the importance of Tir in pedestal formation, relatively little is known about the role of Tir and its ITIMs in the regulation of the host immune response. Here we demonstrate that Tir from enteropathogenic *Escherichia coli* (EPEC) interacted with the host cellular tyrosine phosphatase SHP-1 in an ITIM phosphorylation-dependent manner. The association of Tir with SHP-1 facilitated the recruitment of SHP-1 to the adaptor TRAF6 and inhibited the ubiquitination of TRAF6. Moreover, the ITIMs of Tir suppressed EPEC-stimulated expression of proinflammatory cytokines and inhibited intestinal immunity to infection with *Citrobacter rodentium*. Our findings identify a previously unknown mechanism by which bacterial ITIM-containing proteins can inhibit innate immune responses.

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Long noncoding RNA MEG3-4 tissue specifically regulates IL-1b in pulmonary infection by repressing microRNA-138

Li, R.¹, Wu, M.²

¹University of North Dakota, Grand Forks, United States, ²University of North Dakota, Biomedical Science Department, Medical School, Grand Forks, United States

Long noncoding RNAs (lncRNAs) modulate various biological processes; however, their function in host immunity response against infection has remained elusive. Here, we identify an intergenic lncRNA MEG3 (linc-MEG3) as a tissue specific regulator for pulmonary immunity during bacterial infection. Among the 10 transcripts of linc-MEG3, transcripts 1 and 4 are main regulators due to being the most downregulated in mouse lungs after bacterial infection. Overexpression of linc-MEG3-4 in mice led to intensified inflammatory response, severe lung injury, systemic infection dissemination, and ultimately, increased mortality. Alveolar epithelial cells and alveolar macrophages (AM) were major cell populations that are targeted by linc-MEG3-4. As its 3' sequences are complementary

to microRNA-138 (miR-138), linc-MEG3-4 competitively binds miR-138 and thus releases its target IL-1b mRNA, resulting in intensified inflammatory responses. Our findings characterize linc-MEG3 as a novel pulmonary inflammatory regulator of bacterial infection through a miR-138/IL-1b/NF- κ B circuit.

4029

FIBCD1 binds to *Aspergillus fumigatus* chitin and regulate epithelial inflammation in response to *Aspergillus fumigatus* cell wall components

Jepsen, C., Dubey, L.K., Møller, J.B., Christensen, K.B., Schlosser, A., Sørensen, G.L., Holmskov, U.

University of Southern Denmark, Odense, Denmark

Chitin is an essential cell-wall polysaccharide of fungal pathogens, that elicit pro- and anti-inflammatory responses *in vivo* and *in vitro*, but the receptors that directly interact with chitin to promote these responses are poorly understood. We have previously shown, that the membrane protein FIBCD1 expressed apically on gut epithelial cells binds crab chitin. Here we show that FIBCD1 is expressed in human airway epithelial cell and is upregulated during pulmonic *A. fumigatus* infection. We demonstrates that FIBCD1 binds to the composite cell wall extract alkaline insoluble fragment (AIF) from *Aspergillus fumigatus* (*Afu*) composed of chitin, beta-1-3-glucan and galactomannan. We show that FIBCD1 bind to *Afu* chitin but not to beta-1-3-glucan or galactomannan and that FIBCD1 recognizes chitin the rich zones like the septum, budding region, and bud scar in fungal cell wall. FIBCD1 expression inhibits AIF induces IL-8 secretion from in lung epithelial cells but it also dampens IL-8 production induced by non-FIBCD1 ligand including galactomannan, beta 1,3-glucan and TLR ligands. At the mRNA level FIBCD1 expression inhibits CCL20, GM-CSF-R-alpha, TNF-alpha TSLP Il-1b Il-8, MUC1, MUC13 MUC5AC and ZO-1, while IL-13, occludin and ICAM1 was upregulated. The anti-inflammatory role of FIBCD1 may be of benefit to the immunocompetent host as it protects against unnecessary collateral inflammation damage when the alveolar macrophages clear the opportunistic fungal pathogens. On the other hand the immunosuppressive effect of FIBCD1 may also favor *A. fumigatus* infection in the immune-compromised host.

4001

Characterization of the Interaction between M-ficolin and the fungal polysaccharides chitin and β -1,3-glucan

Jensen, K.¹, Lund, K.P.¹, Holm, A.T.¹, Moeller, J.B.¹, Jepsen, C.S.¹, Dubey, L.K.¹, Schlosser, A.¹, Galgózy, L.^{2,3}, Thiel, S.⁴, Holmskov, U.¹, Sorensen, G.L.¹

¹University of Southern Denmark, Institute of Molecular Medicine, Odense, Denmark, ²University of Szeged, Department of Microbiology, Szeged, Hungary, ³Innsbruck Medical University, Division of Molecular Biology, Innsbruck, Austria, ⁴Aarhus University, Department of Biomedicine, Aarhus, Denmark

M-ficolin is a secreted pattern recognition molecule of the innate immune system. It binds acetylated compounds, including N-acetylglucosamine (GlcNAc), and mediates complement

activation through the lectin pathway. The polysaccharides chitin and β -glucan are key structural components of fungal cell wall and are exposed during fungal growth thereby serving as PAMPs. We investigated the possible role of M-ficolin in the recognition of, and response to, these polysaccharides. M-ficolin was immune-localized to infiltrating granulocytes and monocytes in the periphery of the aspergilloma in invasive aspergillosis. Human recombinant M-ficolin (rM-ficolin) bound to the cell wall, hyphal septae and the primary buds of the growing hyphae of *Aspergillus fumigatus* (*A. fumigatus*). rM-ficolin binding to fungal germlings, chitin, β -1,3-glucan and a standard preparation of cell wall polysaccharides of *A. fumigatus*, the alkali-insoluble fraction (AIF), showed binding in a partially inhibited by EDTA and GlcNAc when evaluated by pull-down assays. Complement activation assays showed that the interaction between M-ficolin and chitin, β -1,3-glucan and AIF mediates complement activation through the lectin pathway.

In conclusion, this study suggests a role of M-ficolin in the innate recognition of, and response to, fungal polysaccharides with novel polysaccharide PAMPs chitin and the non-acetylated β -1,3-glucan.

Late Breaker Symposium 3

4731

Src-Like adapter protein (SLAP) regulates the proliferation of mast cells *in vivo*

Sharma, N., Patel, R., McGlade, C.J.

The Hospital for Sick Children, Cell Biology, Toronto, Canada

Mast cells (MCs) are innate immune effector cells found in peripheral tissues including skin and intestine and perform the role of a sentinel for invading pathogens. Homeostatic regulation of MCs is dependent upon a receptor tyrosine kinase, c-Kit and downstream signaling axis regulating the proliferation and survival of MCs *in situ*. Src-like adapter protein (SLAP) bearing structural similarities with Src kinase except lacking the kinase domain has been previously shown to interact with c-Kit and suppress downstream signaling via regulating the receptor stabilization. Here, we report that SLAP knockout (SLAP KO) mice showed reduced mast cells in peritoneum and dermis. In addition, peritoneal MCs in SLAP KO showed reduced proliferation while similar apoptosis compared to wild-type (WT) control. In addition, mast cell progenitors in spleen and bone marrow showed equal numbers between WT and SLAP KO mice. Furthermore, bone marrow-derived mast cells (BMMCs) from SLAP KO mice showed reduced proliferation compared to WT control in response to Stem cell factor, a ligand for Kit receptor. Moreover, SLAP KO BMMCs shows reduced activation of Erk and Jnk pathways downstream of KIT receptor. Therefore, SLAP regulates the proliferation of mast cells via regulating the Kit-Erk-Jnk pathways.

4466

The RNA-binding protein Mex-3B is required for IL-33 induction in the development of allergic airway inflammation

Yamazumi, Y.¹, Sasaki, O.², Imamura, M.², Oda, T.¹, Ohno, Y.¹, Shiozaki-Sato, Y.¹, Nagai, S.³, Suyama, S.¹, Kamoshida, Y.¹, Funato, K.¹, Yasui, T.⁴, Kikutani, H.⁴, Yamamoto, K.², Koyasu, S.⁵, Akiyama, T.¹

¹Institute for Molecular and Cellular Biosciences, The University of Tokyo, Laboratory of Molecular and Genetic Information, Tokyo, Japan, ²University of Tokyo, Department of Allergy and Rheumatology, Graduate School of Medicine, Tokyo, Japan, ³Tokyo Medical and Dental University, Department of Molecular Immunology, Graduate School of Medical and Dental Sciences, Tokyo, Japan, ⁴Osaka University, Research Institute for Microbial Diseases, Department of Molecular Immunology, Osaka, Japan, ⁵RIKEN Center for Integrative Medical Sciences, Laboratory for Immune Cell Systems, Yokohama, Japan

Allergic airway inflammation is one of the primary features of allergic asthma. Interleukin 33 (IL-33) is recognized as a key pro-inflammatory cytokine that mediates allergic airway inflammation and its expression is elevated in this condition, but little is known about the regulatory mechanisms underlying IL-33 induction. Here, we show that the RNA-binding protein Mex-3B plays a critical role in the induction of IL-33 in the development of allergic airway inflammation. We generated *Mex3b*^{-/-} mice and found that they develop significantly less airway inflammation than wild-type mice, due to reduced induction of IL-33. We further show that Mex-3B directly upregulates IL-33 expression by inhibiting miR-487b-3p-mediated repression of IL-33. Moreover, we show that inhalation of an antisense oligonucleotide targeting Mex-3B suppresses allergic airway inflammation. Our data identify a novel signaling pathway that post-transcriptionally regulates IL-33 expression and suggest that Mex-3B could be a promising molecular target for the treatment of allergic asthma.

4692

Induction of autoimmune disease by deletion of CTLA-4 in mice in adulthood

Klocke, K.¹, Sakaguchi, S.², Holmdahl, R.¹, Wing, K.¹

¹Karolinska Institutet, MBB, Stockholm, Sweden, ²Immunology Frontier Research Center, Osaka University, Osaka, Japan

Cytotoxic T lymphocyte antigen-4 (CTLA-4) is essential for immunological (self-) tolerance but due to the early fatality of CTLA-4 knock out mice its specific function in central and peripheral tolerance and in different systemic diseases remains to be determined. Here, we further examined the role of CTLA-4 by abrogating CTLA-4 expression in adult mice and compared the resulting autoimmunity that follows with that produced by congenital CTLA-4 deficiency. We found that conditional deletion of CTLA-4 in adult mice resulted in spontaneous lymphoproliferation, hypergammaglobulinemia and histologically evident pneumonitis, gastritis, insulinitis and sialadenitis, accompanied by organ specific autoantibodies. However in contrast to congenital deficiency this was not fatal. CTLA-4 deletion induced preferential expansion of

CD4⁺Foxp3⁺ Treg cells. Yet T cells from CTLA-4 deficient iKO mice were able to adoptively transfer the diseases into T cell-deficient mice. Notably, cell transfer of thymocytes *de novo* produced myocarditis, otherwise not observed in donor mice depleted in adulthood. Moreover, CTLA-4 deletion in adult mice had opposing impacts on induced autoimmune models. Thus while CTLA-4 deficient mice had more severe collagen induced arthritis (CIA) they were protected against peptide-induced experimental autoimmune encephalomyelitis (EAE) yet onset of protein-induced EAE was only delayed. Collectively this indicates that CTLA-4 deficiency affects both central and peripheral tolerance and Treg cell-mediated suppression.

4457

Targeting citrullination with a peptidyl arginine deiminase inhibitor is associated with a shift from Th17-mediated immune responses and abrogates collagen-induced arthritis

Kawalkowska, J.¹, Quirke, A.-M.¹, Ghari, F.², Subramanian, V.³, Thompson, P.³, Williams, R.¹, Fischer, R.⁴, La Thangue, N.², Venables, P.¹

¹University of Oxford, Kennedy Institute, NDORMS, Oxford, United Kingdom, ²University of Oxford, Laboratory of Cancer Biology, Department of Oncology, Oxford, United Kingdom, ³University of Massachusetts Medical School, Biochemistry and Molecular Pharmacology, Worcester, United States, ⁴University of Oxford, Target Discovery Institute, Nuffield Department of Medicine, Oxford, United Kingdom

Citrullination is catalysed by peptidylarginine deiminases (PADs) and hypercitrullination has been reported in several inflammatory diseases including multiple sclerosis, colitis, lupus, cancer and rheumatoid arthritis. In this study we examine the therapeutic potential of a PAD inhibitor (BB-CI-amidine) in immune-mediated arthritis and its effects on T helper differentiation.

Collagen-induced arthritis was induced in DBA/1 mice by immunization with bovine type II collagen. After disease onset, mice were treated daily with vehicle or BB-CI-amidine (10 mg/kg). On day 10, mice were culled, and paws, blood and lymph nodes collected for further analysis.

Treatment of arthritic mice with BB-CI-amidine resulted in a significant reduction in clinical scores. Histological scores in joints were almost completely normalised. Unexpectedly, while pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6) in serum remained unaffected by BB-CI-amidine, IL-4, IL-5 and IL-10 were significantly elevated. In line with this, IL-4⁺ Th2 cells were increased in the lymph nodes of BB-CI-amidine treated mice. Further analysis revealed a decrease in Th17 numbers in paws and lymph nodes with BB-CI-amidine. The addition of BB-CI-amidine to Th17 differentiation cultures (without APCs) lead to the inhibition of IL-17A. In addition, BB-CI-amidine reduced histone H3.2 citrullination which suggests that BB-CI-amidine may inhibit gene expression via an epigenetic mechanism.

In conclusion, BB-CI-amidine is therapeutic in CIA by inhibiting pro-inflammatory Th17-type responses. We propose that these effects are mediated by transcriptional regulation and that targeting PADs is a realistic strategy for the treatment of chronic inflammatory diseases in which PAD activity is dysregulated.

4370

MLKL mediates programmed hepatocellular necrosis independent of RIPK3 during inflammation-dependent hepatitis

Günther, C.¹, He, G.-W.¹, Kremer, A.¹, Murphy, J.², Amann, K.¹, Vandenabeele, P.³, Linkermann, A.⁴, Krautwald, S.⁴, Neurath, M.¹, Becker, C.¹, Wirtz, S.¹

¹University Hospital Erlangen, Erlangen, Germany, ²Walter and Eliza Hall Institute of Medical Research, Parkville, Australia, ³Ghent University, Ghent, Belgium, ⁴University Hospital Schleswig-Holstein, Kiel, Germany

Although the liver is an organ of remarkable regenerative capacity, cell death related compensatory tissue injury responses commonly culminate in liver fibrosis and eventually cirrhosis, a major cause of morbidity worldwide. With regard of this vital contribution of hepatocellular death to virtually all hepatic diseases, precise mechanistic knowledge of cell death regulation is essential for the understanding of the pathophysiology of liver disease and for the development of novel therapeutic approaches.

Given that the pseudokinase mixed lineage kinase domain-like protein (MLKL) has been identified as a key mediator and potential biomarker of receptor-interacting protein kinases (RIPK)-mediated regulated necrosis (necroptosis), we investigated the role of MLKL during inflammation-dependent hepatitis. Our data demonstrate that MLKL is strongly upregulated and activated in human autoimmune hepatitis and in a murine model of inflammation-dependent hepatitis (ConA-induced hepatitis). Using genetic (*Ripk3*^{-/-}, *Mkl1*^{-/-} mice) and pharmacological approaches (necrostatin-1s) we describe that hepatocellular necrosis in experimental hepatitis is driven by a previously unrecognized pathway of RIPK1-dependent MLKL activation that occurs independent of RIPK3. Thus, depending on the physiological context, MLKL has the capacity to promote RIPK3-mediated necroptosis or alternatively triggers IFN-mediated RIPK3-independent programmed necrosis. Moreover, we provide compelling evidence that the well-known cytotoxic-activity of the proinflammatory cytokine IFN- γ in hepatic inflammation is strongly connected to induction of MLKL expression via activation of the transcription-factor STAT1.

In summary, we discovered a previously undescribed type of MLKL-dependent programmed necrosis which is executed in the absence of RIPK3 and potentially promotes the pathogenesis of severe liver diseases.

4306

TLR9 polymorphisms induce autoimmunity in a murine model of SLE

Ruck, M.¹, Woo, J.², Laszik, Z.³, Hermiston, M.¹

¹UCSF, Department of Pediatrics, San Francisco, United States, ²UCSF, Diabetes Center, Division of Infectious Diseases, Department of Medicine, San Francisco, United States, ³UCSF, Department of Pathology, San Francisco, United States

The stochastic and heterogeneous nature of Systemic Lupus Erythematosus (SLE) supports a model whereby multiple genetic and/or environmental hits culminate in loss of tolerance

and autoantibody production. Consistent with this model, the phenotype of CD45E613R mice containing a single point mutation in the juxtamembrane wedge of CD45 depends on genetic context. Despite similar dysregulated phosphatase activity in all immune cells, CD45E613R mice on a C57Bl/6 (B6) background have no overt phenotype while BALB/c mice develop anti-double stranded (ds) DNA antibodies. An unbiased genome-wide screen for modifiers of autoantibody production between CD45E613R B6 and BALB/c mice identified two candidate loci: Wedge Associated Modifier (Wam) 1 on Chromosome (Chr) 9 encompassing *tlr9* and Wam2 on Chr 17 encompassing MHC H2. Previous work in knockouts showed the hyporesponsive BALB/c TLR9 allele permits anti-ds DNA antibodies while the B6 TLR9 allele is resistant. Here, we generate CD45E613R mice congenic for the TLR9 alleles. We demonstrate that one copy of the B6 TLR9 allele is sufficient to induce tolerance and decrease autoantibodies on the susceptible BALB/c background, while the BALB/c TLR9 allele is sufficient to induce autoantibodies and glomerulonephritis in the resistant B6 background. The signaling phenotype of the BALB/c TLR9 allele is recapitulated in B6 CD45E613R congenics and correlates with a defect in B cell tolerance prior to disease onset. These data indicate that the net signal strength between the TLR9 allele and the hyperresponsive CD45E613R mutation determines whether autoreactive B cells are deleted, resulting in tolerance, or survive, causing autoimmunity.

4481

Neuroprotection affected by immunomodulatory treatment with glatiramer acetate

Aharoni, R., Sela, M., Arnon, R.

The Weizmann Institute of Science, Immunology, Rehovot, Israel

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS), involving inflammation and neurodegeneration. Treatment strategies aim to reduce the inflammation and induce neuroprotective repair processes. The therapeutic effect of Copaxone (glatiramer acetate, GA), an approved drug for MS treatment, has been attributed to a shift from inflammatory Th1 towards anti-inflammatory Th2 pathway. Using the animal model of MS, experimental autoimmune encephalomyelitis (EAE), we studied the effect of GA on cell populations relevant to pathogenesis and repair within the injured CNS, and explored whether this immunomodulatory treatment can lead to neuroprotection.

We found that the secretion of the key inflammatory factors GM-CSF and IL-17 by spleen cells from EAE-induced mice was drastically reduced following GA treatment. In the CNS of GA-treated mice, Th-17 positive cell occurrence was significantly reduced, with parallel elevation in T-regulatory cells (Tregs). Furthermore, in transgenic mice in which Tregs express diphtheria toxin receptor facilitating their selective depletion, GA was less effective in disease suppression than in wild type mice, thus supporting the role of Tregs in its therapeutic effect. The consequences of GA treatment on the CNS injury inflicted by the disease were studied *in situ* using immunohistochemistry, electron microscopy, and magnetic resonance imaging. These analyses revealed reduced demyelination and neuroaxonal damages, as well as elevation in neuroprotective

repair processes such as neurotrophic factors expression, remyelination and neurogenesis. These combined findings indicate that immunomodulatory treatment can counteract the neurodegenerative disease course, supporting a linkage between immunomodulation, neuroprotection and therapeutic activity in the CNS.

4525

Augmented activation and IL-17 production of mucosal associated invariant T cells in multiple sclerosis

Willing, A., Jäger, J., Reinhard, S., Kursawe, N., Friese, M.A.

Zentrum für Molekulare Neurobiologie, Institut für Neuroimmunologie und Multiple Sklerose, Hamburg, Germany

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system involving focal T cell infiltration. The T cell derived cytokine interleukin (IL)-17 has been linked to MS pathogenesis, while the contribution of the well-characterised IL-17-producing CD4⁺ T helper (TH17) cells is controversial. Besides TH17 cells, mucosal associated invariant T (MAIT) cells produce IL-17 and carry a semi-invariant T cell receptor (TCR) recognizing bacterial and yeast antigens presented by the non-polymorphic, highly conserved major histocompatibility complex (MHC)-related protein 1 (MR1). We previously reported infiltration of MAIT cells in brain lesions and their decrease in the peripheral blood in MS. Here, we first investigated the surface expression of T cell activation markers in MS patients and healthy individuals and found a significant increase of IL-7-receptor-alpha-chain (IL-7Ra) on MAIT cells in MS, which was not observed for other T cell subsets. IL-7 is known to induce IL-17 production by CD4⁺ T cells and MAIT cells, which prompted us to investigate IL-7Ra and IL-17 expression in parallel in a second cohort of MS patients and healthy individuals. Notably, we could detect an elevated IL-7Ra surface expression on MAIT cells in MS, which correlated with an increased frequency of IL-17⁺ MAIT cells. In the same cohort IL-17⁺CD4⁺ T-cell-frequencies were not significantly different in MS patients in comparison to healthy individuals. Our findings implicate a novel role of IL-17-producing MAIT cells in MS pathogenesis driven by increased IL-7Ra expression. Factors inducing augmented MAIT cell activation in MS need to be further investigated.

4335

Gut microbiota drives autoimmune arthritis by unleashing the Tfh response

Teng, F.¹, Klinger, C.¹, Felix, K.¹, Bradley, P.¹, Wu, E.¹, Tran, N.¹, Umesaki, Y.², Wu, H.-J.J.^{1,3}

¹University of Arizona, Immunobiology, Tucson, United States,

²Yakult Central Institute, Tokyo, Japan, ³University of Arizona, Arizona Arthritis Center, College of Medicine, Tucson, United States

Gut microbiota profoundly affects diseases, both inside and outside (systemic) the gut, but the mechanism whereby it affects systemic diseases is unclear. Little is known about whether specific microbiota influences T follicular helper (Tfh) cells, whose excessive responses inflict antibody-mediated autoimmunity. Tfh cells need to be precisely regulated to avoid

autoimmune conditions and accordingly, IL-2 has been shown to dampen Tfh response by inhibiting Tfh differentiation. Using the K/BxN autoimmune arthritis model, we demonstrated that Peyer's patch (PP) Tfh cells are required for a gut commensal, segmented filamentous bacteria (SFB)- induced systemic arthritis despite auto-antibodies being produced predominantly in systemic lymphoid tissues, not PPs. We elucidated that SFB, by driving differentiation and egression of PP Tfh cells into systemic sites, boost systemic Tfh and auto-antibody responses that exacerbate arthritis. Specifically, by using a photoconvertible transgenic mouse line, we demonstrate that SFB increase the egression of PP Tfh cells into the spleen. SFB induce PP Tfh differentiation by limiting the access of IL-2 to CD4⁺ T cells, thereby enhancing Bcl-6, a master regulator of Tfh differentiation, in a dendritic cell-dependent manner. Thus, gut microbiota remotely regulates a systemic disease by driving the induction and egression of gut Tfh cells into systemic sites. These data have strong implications for public health, as we elucidate how a generally benign gut commensal can trigger autoimmune diseases outside the gut by tipping the delicate balance of Tfh response.

Mucosal Immunology 3

1585

MAIT cells reside in the female genital mucosa and are biased towards IL-17 and IL-22 production in response to bacterial stimulation

Gibbs, A.¹, Leeansyah, E.^{2,3}, Introini, A.¹, Paquin Proulx, D.^{2,4}, Hasselrot, K.^{1,5}, Andersson, E.⁶, Broliden, K.¹, Sandberg, J.K.², Tjernlund, A.¹

¹Karolinska Institutet, Unit of Infectious Diseases, Department of Medicine Solna, Center for Molecular Medicine, Karolinska University Hospital Solna, Stockholm, Sweden, ²Karolinska Institutet, Center for Infectious Medicine, Department of Medicine Huddinge, Karolinska University Hospital Huddinge, Stockholm, Sweden, ³Duke-NUS Graduate Medical School, Program Emerging Infectious Diseases, Singapore, Singapore, ⁴George Washington University, Department of Microbiology, Immunology and Tropical Medicine, Washington DC, United States, ⁵Danderyd Hospital, Department of Obstetrics and Gynecology, Stockholm, Sweden, ⁶Capio St. Göran Hospital, Clinical Pathology/Cytology, Stockholm, Sweden

Mucosal-associated invariant T (MAIT) cells are a novel, large innate-like T cell population that recognises microbial riboflavin metabolite antigens presented by the evolutionarily conserved major histocompatibility complex, class I related (MR1) molecules. Following microbial encounters, MAIT cells respond rapidly through cell-mediated cellular cytotoxicity, suppression of intracellular microbial growth, and secretion of pro-inflammatory cytokines. The female genital tract (FGT) mucosa is a critically important site for immune defense against microbes. The presence and role of MAIT cells in the FGT mucosa is unknown.

Here, we found that MAIT cells and MR1⁺ antigen-presenting cells were present in the upper and lower FGT, with distinct tissue localisation of MAIT cells in endometrium versus

cervix. MAIT cells from the FGT and blood displayed a distinct phenotype with expression of IL-18R α , CD127, α 4 β 7, PD-1, as well as the transcription factors PLZF, ROR γ t, Helios, Eomes, and T-bet. Their expression levels of PLZF and Eomes were lower in the FGT compared to blood. When stimulated with *Escherichia coli*, MAIT cells from the FGT displayed a bias towards IL-17 and IL-22 expression, whereas blood MAIT cells produced primarily IFN- γ , TNF, and Granzyme B. Furthermore, although both FGT- and blood-derived MAIT cells were polyfunctional following *E. coli* stimulation, deeper analyses revealed that MAIT cells displayed distinct polyfunctional profiles depending on their anatomical origin. Our data thus reveal that FGT-derived MAIT cells respond to bacterial stimuli in a fashion that is unique to the FGT, supporting their potentially important role in immunological homeostasis and control of microbes at this site.

3514

Conditioning of lung dendritic cells by dietary vitamin A and the gastrointestinal microbiota

Ruane, D., Manzanillo, P., Zhang, H., Diehl, L., Zeng, J., Kljavin, N., Sandoval, W., Xu, M., Ghilardi, N.

Genentech, South San Francisco, United States

Vitamin A deficiency (VAD) is one of the most common micronutrient deficiencies and is associated with profound defects within the adaptive immune system. VAD individuals are especially vulnerable to morbidity and mortality caused by respiratory and gastrointestinal (GI) viral infections. However the exact mechanism by which Vitamin A regulates the antiviral response within the pulmonary compartment remains to be fully elucidated. Here we found that VAD mice have increased susceptibility to influenza infection due to defects within lung dendritic cells (LDC). LDC had decreased capacity to induce IgA isotype switching in B cells and developmental defects due to loss of signaling by all-trans-retinoic acid (RA), a Vitamin A metabolite. Furthermore, we demonstrate that conversion of Vitamin A into RA depends on the presence of intestinal microbiota, but not microbiota in general. MyD88 signaling, presumably as a result of TLR activation by microbial components, induces expression of ALDH enzymes in intestinal epithelial cells (IEC), resulting in the generation of RA. Accordingly, depletion of intestinal microbiota phenocopied Vitamin A deficiency, and restoration of RA signaling in the absence of the GI-microbiota restored host resistance to influenza. Our results identify a role for the GI-microbiota in regulating the phenotype of DCs residing at other mucosal compartments in a RA dependent manner, and thus provide a mechanistic explanation of how Vitamin A deficiency results in host susceptibility to viral pulmonary infections.

3873

Pathogenic NK T cells attenuate clearance and exacerbate immunopathology in chlamydial infections

Armitage, C.¹, Carey, A.¹, Godfrey, D.², Beagley, K.¹

¹*Institute of Health and Biomedical Innovation, Brisbane, Australia,*

²*Australian Research Council Centre of Excellence for Advanced Molecular Imaging at the University of Melbourne, Melbourne, Australia*

Urogenital chlamydial infections continue to increase with over 100 million people affected annually, causing significant economic and public health pressures. Whilst the role of traditional MHCI and II peptide presentation is well defined in chlamydial infections, the role of lipid antigens in immunity remains unclear. Natural killer (NK) T cells are important effector cells that recognise and respond to lipid antigen during infections. Chlamydial infection of antigen-presenting cells facilitates presentation of lipid on the MHCI-like protein, CD1d, which stimulates NK T cells to respond. During urogenital chlamydial infection, wild type (WT) female mice had significantly greater chlamydial burden than CD1d $^{-/-}$ (NKT-deficient) mice, and also had significantly greater incidence and severity of immunopathology in both primary and secondary infections. WT mice had significantly more lymphocytic infiltrate and 59% more fallopian tube occlusion ($P < 0.0016$), and hence infertility, compared to CD1d $^{-/-}$ mice. Transcriptional array analysis of fallopian tubes day 6 post-infection revealed WT mice had elevated levels of IFN γ (6-fold), TNF α (38-fold), IL6 (2.5-fold), IL1 β (3-fold), and IL17 α (6-fold) mRNA compared to CD1d $^{-/-}$ mice. In infected females, both uterine and fallopian tissues had an elevated infiltration of CD4 $^{+}$ (but not CD8 $^{+}$) invariant NK T cells. Lipid mass spectrometry of infected macrophages expressing a cleavable CD1d protein revealed the chlamydial lipid profile presented to NK T cells. Taken together, these data reveal an immunopathogenic role for inflammatory NK T cells in chlamydial infections, facilitated by CD1d-restricted chlamydial lipid presentation by infected antigen-presenting cells.

911

PPAR γ in dendritic cells and T cells controls induction of pulmonary allergic inflammation

Nobs, S.P.¹, Schneider, C.², Van Rooijen, N.³, Kopf, M.¹

¹*ETH Zürich, Zürich, Switzerland, ²University of California, Department of Medicine, San Francisco, United States, ³Free University Medical Center, Amsterdam, Department of Molecular Cell Biology, Amsterdam, Netherlands*

Peroxisome Proliferator-Activated Receptor gamma (PPAR γ) is a well-established transcription factor which is required for development and function of alveolar macrophages. It is generally recognised as an anti-inflammatory mediator such as controlling polarization of macrophages into an anti-inflammatory or M2 phenotype. In the context of asthma PPAR γ agonists are suggested to reduce type 2 allergic airway inflammation. However, its precise role in this context and the underlying mechanism of action remains largely unclear. Using cell-type specific PPAR γ knockout mice to address the function of PPAR γ in pulmonary allergic inflammation in a more stringent manner, we could show that surprisingly specific absence of PPAR γ in either T cells or dendritic cells protected mice from ovalbumin/alum and house dust mite-induced asthma. PPAR γ was specifically required in lung DCs to polarize naive PPAR γ -sufficient

T cells towards a Th2 phenotype. Similarly, T cells lacking PPAR γ showed a cell intrinsic defect in polarization towards Th2 cells *in vivo*, while Th1 and Th17 polarization was unaffected. Thus we uncover a surprising pro-inflammatory role of PPAR γ in

pulmonary allergy, adding a novel layer of complexity to the biology of this important mediator.

1068

Dendritic cell-intrinsic molecular chaperone grp94/gp96 is critical for maintaining gut tolerance

Liu, B.¹, Yang, Y.¹, Sun, S.², Reizis, B.³, Li, Z.¹

¹Medical University of South Carolina, Department of Microbiology & Immunology, Hollings Cancer Center, Charleston, United States,

²Medical University of South Carolina, Department of Pathology and Laboratory Medicine, Charleston, United States, ³New York University Langone Medical Center, Department of Pathology and Medicine, New York, United States

The intestinal immunity and tolerance are orchestrated by both the innate and the adaptive immune system. Dendritic cells (DCs) are professional antigen-presenting cells that play a critical role in both innate and adaptive immune response. Intestinal DCs recognize and respond to the gut microbiota through multiple pattern-recognition receptors, including TLRs and NLRs. However, how DCs maintain mucosal homeostasis remains incompletely understood. Heat shock protein grp94, also known as gp96, is an essential immune chaperone for TLRs, integrins, and other vital innate receptors. By a genetic strategy, we report here that selective deletion of grp94 from DCs in mice results in alteration of DCs and regulatory T cells in the colonic lamina propria, loss of oral tolerance, and high susceptibility to chemical-induced colitis. Strikingly, DC-specific grp94 deficient mice develop spontaneous colitis. Our findings for the first time demonstrate that DC-intrinsic grp94 plays essential roles in maintaining gut tolerance to prevent inflammatory bowel diseases, and illustrate the importance of protein homeostasis in safeguarding against immune pathology.

1458

CD4+ T cell deficiency results in blunted inflammatory response during dysbiosis-triggered colitis

Roy, U., Strowig, T.

Helmholtz Centre for Infection Research, Braunschweig, Germany

In healthy individuals the intestinal microbiota, a complex microbial ecosystem, and the host maintain a mutually beneficial homeostasis. Changes in the host's health state and environmental factors may disrupt this homeostasis, resulting in dysbiosis. In numerous animal models of human diseases dysbiotic communities can directly modulate disease severity. Our aim is to understand the role of specific components of immune system that interplay with the dysbiotic microbiota to trigger pathologic changes. To do so we study the impact of a dysbiotic community (DysM) resulting from a deficiency of the Nlrp6 inflammasome on the host resulting in increased colitis severity. Important tools are isobiotic mouse lines in which the microbiota can be manipulated at will giving us an opportunity to study the functionalities of immune pathways in homeostasis and dysbiosis.

In this model, DysM-exacerbated dextran sulfate sodium induced colitis severity was drastically decreased in T cell deficient

mice (Tcrbd^{-/-}) compared to control mice suggesting a strong involvement of T cells. Furthermore, MHC class II-restricted TCR-transgenic and CD4-deficient mice were protected supporting that antigen-specific, potentially microbiota specific, CD4+ T cells are required in this model. Along these lines we observed increases in intestinal CD45+CD4+ T cells in DysM mice including both inflammatory IL17+ and Foxp3+ T cells further supported by local increases in proinflammatory cytokines IL-6 and IL-17. Future investigations of the functions of intestinal CD4+ T cells in DysM mice will be required to advance the understanding how a dysbiotic intestinal community results in alteration of disease severity.

2292

Functional role of intestinal dendritic cell subsets in the mucosal IgA response to rotavirus

Hütter, J.¹, Nakawesi, J.², Feng, N.^{3,4}, Greenberg, H.^{3,4}, Butcher, E.^{3,4}, Lahl, K.^{1,2}

¹Technical University of Denmark, National Veterinary Institute, Frederiksberg C, Denmark, ²Lund University, Department of Experimental Medical Science, Lund, Sweden, ³Stanford University, Stanford, United States, ⁴VA Palo Alto Health Care System, Palo Alto, United States

Rotavirus (RV) is a double-stranded RNA (dsRNA) virus that specifically infects epithelial cells of the small intestine causing severe diarrhea especially in infants under the age of 5 years. Intestinal dendritic cells play a crucial role in the recognition of mucosal pathogens but are also involved in maintaining tolerance toward commensal microorganisms. Conventional dendritic cells (cDCs) in the gut are divided into two major subsets. CD103⁺CD11b⁻ (cDC1) cells are well equipped to drive immunity toward viruses through cross-presentation of antigen and IL-12 driven induction of T_H1 responses. They specifically express the pattern-recognition receptor TLR3, which recognizes dsRNA. In contrast, CD103⁺CD11b⁺ (cDC2) cells were shown to promote T_H2 and T_H17 responses and express TLR5, the receptor for bacterial flagellin.

We have previously shown that plasmacytoid dendritic cells (pDCs) play an important role in the mucosal RV-specific IgA response. Secretory IgA clears the virus and also mediates long-term protection in mice and in humans. We here analyzed the function of cDC1 cells in the IgA response as well as the potential interplay of cDC1s and pDCs during RV infection. We show that BATF3-deficient mice that lack cDC1 cells shed virus longer and exhibit markedly reduced RV-specific IgA levels. Optimal activation and migration of cDC1 cells depends on TLR3 expression and is blunted in the absence of pDCs. These data reveal a crucial role for the coordinated action of cDC1 cells and pDCs in the immune response to RV, and a previously unappreciated role for cDC1 cells in the induction of mucosal IgA.

2617

Apoptotic epithelial cells control the abundance of regulatory T cells at barrier surfaces

Nakahashi-Oda, C., Udayanga, K.G.S., Nakamura, Y., Nakazawa, Y., Totsuka, N., Miki, H., Iino, S., Tahara-Hanaoka, S., Honda, S.-I., Shibuya, K., Shibuya, A.
 University of Tsukuba, Tsukuba, Japan

Epithelial tissues continually undergo apoptosis. Commensal organisms inhabiting the epithelium influence tissue homeostasis, in which regulatory T (T_{reg}) cells play a central role. However, the physiological significance of epithelial cell apoptosis and how T_{reg} cell number is regulated are both incompletely understood. Here, we show that apoptotic epithelial cells negatively regulate commensal-stimulated T_{reg} cell proliferation. Gut commensals stimulated $CX_3CR_1^+CD103^+CD11b^+$ dendritic cells (DCs) to produce interferon- γ (IFN- γ), which augmented proliferation of T_{reg} cells in the intestine. Conversely, phosphatidylserine exposed on apoptotic epithelial cells suppressed IFN- γ production by the DCs via CD300a-mediated inhibitory signal, thus suppressing T_{reg} cell proliferation. Our findings reveal a regulatory role of apoptotic epithelial cells in maintaining T_{reg} cell number and tissue homeostasis.

3537

Inhibition of Dectin-1 signaling ameliorates colitis by inducing Lactobacillus-mediated regulatory T cell expansion in the intestine

Tang, C.¹, Kamiya, T.^{1,2}, Iwakura, Y.¹

¹Center for Animal Disease Models, Research Institute for Biomedical Sciences, Tokyo University of Science, Noda, Japan,
²(Present address) Biomedical Research Institute, National Institute for Advanced Industrial Science and Technology-AIST, Tsukuba, Japan

Dectin-1, the receptor for beta-glucans, protects host from fungal infection. However, the role of Dectin-1 in intestinal immunity has not been elucidated completely. We found that Dectin-1-deficient (*Clec7a^{-/-}*) mice were refractory to both dextran sodium sulfate (DSS)- and CD45RB^{high}CD4⁺ T cell-induced colitis associated with an increase of regulatory T (Treg) cells. The proportion of lactobacilli, especially *Lactobacillus murinus* (*L. murinus*), in commensal microflora was increased in *Clec7a^{-/-}* mouse colon accompanied by a decrease of a group of antimicrobial peptides induced by Dectin-1 signaling. *L. murinus* colonization increased Treg cells in the colon. Oral administration of laminarin, a Dectin-1 antagonist, suppressed the development of DSS-colitis, associated with the increase of *L. murinus* and Treg cells. The proportion of closely related lactobacillus species was decreased in inflammatory bowel disease patients. These observations suggest that Dectin-1 regulates the homeostasis of intestinal immunity by controlling Treg cell differentiation through modification of microbiota.

Immunodeficiency

2105

Mycobacterial disease and myeloid dendritic cells defects in patients with SPPL2a deficiency

Martinez-Barricarte, R.¹, Kong, X.-F.¹, Kennedy, J.², Lazarov, T.³, Deenick, E.⁴, Ma, C.⁴, Mele, F.⁵, Trouillet, C.³, Bousfiha, A.⁶, Aytekin, C.⁷, Picard, C.^{8,9}, Salem, S.², Langlais, D.², Lasseau, T.¹, Migaud, M.⁸, Abel, L.^{8,9}, Boisson-Dupuis, S.^{1,8,9}, Hambleton, S.², Schröder, B.¹⁰, Geissmann, F.³, Tangye, S.⁴, Sallusto, F.^{5,11}, Bustamante, J.^{8,9}, Gros, P.², Casanova, J.-L.^{1,8,9,12}

¹Rockefeller University, New York City, United States, ²McGill University, Department of Biochemistry, Montreal, Canada, ³Memorial Sloan Kettering Cancer Center, Immunology Program, New York City, United States, ⁴Garvan Institute of Medical Research, Immunology Division, Darlinghurst, Australia, ⁵University of Italian Switzerland, Institute for Research in Biomedicine, Bellinzona, Switzerland, ⁶King Hassan II University, Ibn-Rochd Hospital, Clinical Immunology Unit, Department of Pediatrics, Casablanca, Morocco, ⁷Dr. Sami Ulus Maternity and Children's Health and Diseases Training and Research Hospital, Department of Pediatric Immunology, Ankara, Turkey, ⁸INSERM U 1163, Necker Hospital for Sick Children, Laboratory of Human Genetics of Infectious Diseases, Necker Branch, Paris, France, ⁹Paris Descartes University, Imagine Institute, Paris, France, ¹⁰Christian Albrechts University of Kiel, Biochemical Institute, Kiel, Germany, ¹¹University of Italian Switzerland, Center of Medical Immunology, Institute for Research in Biomedicine, Bellinzona, Switzerland, ¹²Howard Hughes Medical Institute, New York City, United States

Mendelian susceptibility to mycobacterial disease (MSMD) is a rare genetic condition predisposing otherwise healthy individuals to severe infection by weakly virulent mycobacteria. To date, 11 mutated genes, underlying 21 different genetic etiologies of MSMD, have been described. All of these mutations impair the production of, or response to, IL-12 or IFN- γ . Here we report 3 MSMD patients from two unrelated consanguineous families. All these patients carry essential splicing mutations in *SPPL2A* leading to a complete loss of SPPL2a enzyme expression, and causing the accumulation of SPPL2a's substrate, the N-terminal fragment of CD74, in B-cells and monocytes. Contrary to what is observed in *Spp12a^{-/-}* mice, these patients have normal B-cell frequency and function. *Spp12a^{-/-}* mice also have reduced numbers of CD11b⁺ myeloid dendritic cells (mDC) caused by the CD74 N-terminal fragment accumulation since the double knock out *Spp12a^{-/-} CD74^{-/-}* does not show this reduction. Resembling the mouse model and similarly to MSMD patients with autosomal dominant mutations in *IRF8*, SPPL2a deficient patients also have reduced CD1c⁺ IL-12 producing mDC (mDC₁). Furthermore, we demonstrated that the SPPL2a deficient humans' CD4 T-cells fail to produce IFN- γ in response to stimulation by mycobacterial antigens *in vitro*. In conclusion, our findings suggest autosomal recessive *SPPL2A* deficiency as a new genetic etiology of MSMD, show that human SPPL2a is essential for the homeostasis of IL-12 producing CD1c⁺ DCs and proposes a link between this DC population and T-cell IFN- γ mediated immunity against mycobacteria.

1940**Heterozygous mutations in IKAROS in patients with progressive loss of B cells and hypogammaglobulinemia***Boisson, B.*^{1,2}¹Rockefeller University, New York, United States, ²Institut Imagine UMR 1163, Paris, France

Common variable immunodeficiency disorders (CVID) are characterized by delayed onset of hypogammaglobulinemia in the absence of predisposing factors. The genetic etiology is unknown in the majority of cases and < 10% of patients have a family history of disease. Most patients have normal numbers of B cells but lack plasma cells. Whole exome sequencing and array comparative genomic hybridization were used to analyze a subset of CVID patients with low B cell numbers. Mutant proteins were analyzed for DNA binding using electrophoretic mobility shift assays (EMSA) and confocal microscopy. Flow cytometry was used to analyze peripheral blood lymphocytes and bone marrow aspirates. Six different heterozygous mutations in *IKZF1*, the gene encoding the transcription factor IKAROS, were identified in 29 individuals from 6 families. All of the mutations, 4 amino acid substitutions, an intragenic deletion and a 4.7 Mb multi-gene deletion, involved the DNA binding domain of IKAROS. The proteins bearing missense mutations did not bind target DNA sequences in EMSAs or by confocal microscopy but they did not inhibit the binding of wild type IKAROS. Family studies demonstrated progressive loss of B cells and serum immunoglobulins. Bone marrow aspirates revealed a marked decrease of early B cell precursors but the presence of plasma cells. Heterozygous mutations in the transcription factor IKAROS cause an autosomal dominant form of CVID associated with a striking decrease in B cell numbers.

2037**Human TYK2 deficiency: mycobacterial and viral infections without hyper-IgE syndrome***Boisson-Dupuis, S.**Rockefeller University, New York, United States*

Autosomal recessive, complete TYK2 deficiency was previously described in a patient (P1) with intracellular bacterial and viral infections and features of hyper-IgE syndrome (HIES), including atopic dermatitis, high serum IgE levels, and staphylococcal abscesses. We identified seven other TYK2-deficient patients from five families and four different ethnic groups. These patients were homozygous for one of five null mutations, different from that seen in P1. They displayed mycobacterial and/or viral infections, but no HIES. All eight TYK2-deficient patients displayed impaired but not abolished cellular responses to (a) IL-12 and IFN- α accounting for mycobacterial and viral infections, respectively; (b) IL-23, with normal proportions of circulating IL-17+ T cells, accounting for their apparent lack of mucocutaneous candidiasis; and (c) IL-10, with no overt clinical consequences, including a lack of inflammatory bowel disease. Cellular responses to IL-21, IL-27, IFN- γ , IL-28/29, and leukemia inhibitory factor (LIF) were normal. The leukocytes and fibroblasts of all seven newly identified TYK2-deficient patients, unlike those of P1, responded normally to IL-6, possibly

accounting for the lack of HIES in these patients. The expression of exogenous wild-type TYK2 or the silencing of endogenous TYK2 did not rescue IL-6 hyporesponsiveness, suggesting that this phenotype was not a consequence of the TYK2 genotype. The core clinical phenotype of TYK2 deficiency is mycobacterial and/or viral infections, caused by impaired responses to IL-12 and IFN- α . Moreover, impaired IL-6 responses and HIES do not appear to be intrinsic features of TYK2 deficiency in humans.

3889**Next generation TREC and KREC: quantification of human T- and B-cell replication from purified subsets and dried blood spots***Brooks, G.D.*¹, *Verstegen, R.H.J.*^{2,3}, *Bartol, S.J.W.*², *de Vries, E.*^{4,5}, *van Zelm, M.C.*^{1,2}¹Monash University, Alfred Hospital, Department of Immunology and Pathology, Melbourne, Australia, ²Erasmus Medical Center, Department of Immunology, Rotterdam, Netherlands, ³The Hospital for Sick Children, Department of Pediatric Rheumatology, Toronto, Canada, ⁴Jeroen Bosch Hospital, 4. Department of Pediatrics, 's-Hertogenbosch, Netherlands, ⁵Tilburg University, Scientific Center for Care and Welfare, Tilburg, Netherlands

Screening of newborns for primary immunodeficiencies is being implemented world-wide using quantitative PCR detection of signal joints on excision circles formed by T-cell receptor delta-deletion (TRECs) and immunoglobulin kappa-deletion rearrangements (KRECs). However technical limitations exist that prevent reproducible quantification of cells, and calculation of T-cell replication due to the lack of specific genomic markers, which impact on the sensitivity of these assays. Here, we have developed a new cell line control with TREC construct and a multiplex PCR to quantify T-cell receptor gamma (TRG) rearrangements to overcome these limitations.

A single copy of the TREC construct was retrovirally introduced into the DB01 cell line that was previously established with a KREC construct. We used DNA from this DB01-TREC cell line to correct for efficiencies between the control gene, KREC and TREC PCRs, and showed, from dried blood spots, that neonates with Down syndrome carried lower numbers of T- and B-cells. The multiplex TRG assay detected 90% of alleles in purified $\alpha\beta$ T-cells. Quantifications of TRG rearrangements and intronRSS-Kde coding joints were used to calculate T- and B-cell replication histories in combination with TRECs and KRECs. We showed that neonates with and without Down syndrome carried similar levels of proliferation for T-cells (2.5 divisions) and B-cells (0.6 divisions). Studies into replication histories of naive and memory T cells are ongoing.

These studies provide new insights into T- and B-cell replication in neonates, and our newly developed cell line and TRG assay will help to improve neonatal screening of primary immunodeficiencies world-wide.

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Life-threatening enterovirus 71 encephalitis in unrelated children with autosomal dominant TLR3 deficiency*KU, C.-L.¹, Kuo, C.-Y.², Lo, C.-C.¹, Lim, H.K.³, Zhang, S.-Y.³*¹*Chang Gung University, Graduate Institute of Clinical Medical Science, Taoyuan, Taiwan, Republic of China,* ²*Chang Gung Children's Hospital, Taoyuan, Taiwan, Republic of China,*³*Rockefeller University, New York, United States*

The spectrum of diseases of childhood caused by enterovirus 71 (EV71) is broad, ranging from asymptomatic infection or self-limited hand-foot-and-mouth diseases (HFMD) to life-threatening encephalitis. The molecular mechanisms underlying these different clinical presentations remain unknown. We hypothesized that EV71 encephalitis in children might reflect an intrinsic host single-gene defect of anti-viral immunity. We searched for mutations in Toll-like receptor 3 (TLR3), which have been previously found in children with herpes simplex encephalitis (HSE). We sequenced TLR3 and tested the impact of the mutation found. We tested dermal fibroblasts from TLR3-mutated patients and other patients with known genetic defects in TLR3 or related genes, for their response to both poly(I:C) stimulation and EV71 infection. We found that three children with EV71 encephalitis were heterozygous for the TLR3 W769X, E211K and R867Q rare mutations, which were all shown to have severe impact on TLR3 function. Moreover, fibroblasts from the W769X heterozygous patient displayed impaired but not abolished response to TLR3 agonist poly(I:C). We further showed that TLR3-deficient and TLR3 heterozygous W769X fibroblasts were highly vulnerable to EV71 infection. Autosomal dominant TLR3 deficiency may underlie severe EV71 infection with encephalitis. Human TLR3 immunity is non redundant against not only HSV, but also EV71. Children with severe EV71 infection, especially encephalitis, should be tested for inborn errors of TLR3 immunity.

1010

NFκB deficiency: new genes and new insights into CVID pathogenesis*Slade, C.^{1,2,3}, Tempny, J.^{1,2}, Fliegauf, M.⁴, Woon, S.-T.⁵, Scerri, T.^{1,2}, Leung, E.⁶, Bahlo, M.^{1,2}, van der Meer, J.⁷, Ameratunga, R.⁵, Grimbacher, B.⁴, Douglass, J.^{3,8}, Hodgkin, P.^{1,2}, Bryant, V.^{1,2}*¹*Walter & Eliza Hall Institute, Melbourne, Australia,* ²*University of Melbourne, Dept of Medical Biology, Melbourne, Australia,* ³*Royal Melbourne Hospital, Dept. Clinical Immunology & Allergy, Melbourne, Australia,* ⁴*University of Freiburg Medical Center, Center for Chronic Immunodeficiency, Freiburg, Germany,* ⁵*Auckland City Hospital, Dept. Virology & Immunology, Auckland, New Zealand,* ⁶*University of Auckland, School of Medical Sciences, Auckland, New Zealand,* ⁷*Radboud University Nijmegen Medical Center, Dept. Internal Medicine, Nijmegen, Netherlands,* ⁸*University of Melbourne, Dept. Medicine, Melbourne, Australia*

Common Variable Immunodeficiency (CVID), the most common Primary Immunodeficiency, both in Australia and worldwide, is characterised by hypogammaglobulinaemia (reduced serum IgG, IgA and/or IgM) and poor responses to antigens, and is largely a diagnosis of exclusion. As a result, CVID is a clinically heterogeneous disorder; many patients are diagnosed as adults,

despite 1 in 5 being symptomatic since childhood. Until recently the known genetic causes of CVID were mostly recessive, and exceedingly rare; the genetic basis of only 5-10% of CVID patients is known. Here, we identified, by whole-exome sequencing 5 families with CVID, 5 novel mutations in 3 genes within the NFκB pathway. In 3 families with heterozygous mutations in *NFKB1*, and a fourth family with a novel heterozygous *NFKB2* mutation truncated mutant proteins were expressed at reduced levels and were not processed to active mutant forms, but rapidly degraded. In a fifth family a novel homozygous mutation of *NFKBID* was identified. Amongst these individuals with defective NFκB signaling there exist overlapping clinical features, such as alopecia areata, and nodular regenerative hyperplasia of the liver. However there is variability of the severity of the immune defects observed, including the B cell phenotype, autoimmunity and the susceptibility to viral infection. This works suggests that defects in NFκB signalling could account for a significant proportion of CVID with autoimmune complications. Understanding the mechanisms of CVID pathogenesis and the polarising conditions that drive a given immunological phenotype will lead to novel therapeutic strategies for patients with this complex disorder.

1434

Cxcr4 desensitization is required for efficient humoral immune response and plasma cell trafficking*Natt, J.¹, Biajoux, V.¹, Freitas, C.¹, Alouche, N.¹, Sacquin, A.^{2,3,4}, Hemon, P.⁵, Gaudin, F.¹, Fazilleau, N.^{2,3,4}, Espéli, M.¹, Balabanian, K.¹*¹*UMR 996 - Inflammation, Chemokines and Immunopathology -, Inserm, Univ Paris-Sud, Université Paris-Saclay, Clamart, France,* ²*INSERM, U 1043, Toulouse, France,* ³*CNRS, U 5282, Toulouse, France,* ⁴*Centre de Physiopathologie de Toulouse Purpan, Université Toulouse III Paul-Sabatier, Toulouse, France,* ⁵*Plateforme d'Histologie (PHIC), Institut Paris-Saclay d'Innovation Thérapeutique (IPSIT), Clamart, France*

The Warts, Hypogammaglobulinemia, Infections and Myelokathexis Syndrome (WS) is a rare immuno-hematological disorder characterized by chronic pan-lymphopenia caused by heterozygous gain-of-function mutations in *CXCR4*. Patients mount an adaptive humoral immune response following vaccination, but fail to maintain it. The underlying mechanisms are unknown and often associated to the peripheral lymphopenia. We used the *Cxcr4^{+/1013}* knock-in mice that phenocopy WS-related pan-lymphopenia to assess how a gain-of-*Cxcr4*-function impacts on germinal center (GC) formation and vaccine responses. We showed that despite spleen follicular hypoplasia and absence of primary follicles in the lymph nodes (LNs), *Cxcr4^{+/1013}* mice mount a potent immune response against T-dependent antigen (Ag) as shown by increased numbers of Ag-specific GC B cells and plasma cells (PCs) in the spleen and LNs. Mechanistically, we unraveled that the gain-of-*Cxcr4*-function leads to enhanced signaling through Akt and exacerbated PC differentiation upon Cxcl12 exposure. Despite this and similarly to WS patients, the Ag-specific antibody titers were not maintained over time in *Cxcr4^{+/1013}* mice and Ag-specific PCs were almost completely absent from the BM. Surprisingly, we highlighted an accumulation of immature PCs in the BM,

potentially occupying the niches of long-lived Ag-specific PCs. So, failure to maintain the immune response in WS patients could be explained by the CXCR4-mediated deregulation of the adaptive immune response in secondary lymphoid organs, rather than the observed circulating lymphopenia. Therefore, a gain-of-function *Cxcr4* mutation intrinsically alters PC differentiation and homing, potentially accounting for the defective humoral immunity observed in WS patients.

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DOCK8-deficient CD4⁺ T cells are biased to a Th2 effector fate at the expense of Th1 and Th17 cells

Pillay, B.^{1,2}, Gray, P.³, Ziegler, J.B.³, Smart, J.M.⁴, Choo, S.⁴, Peake, J.⁵, Arkwright, P.D.⁶, Hambleton, S.⁶, Oswaldo Lugo Reyes, S.⁷, Freeman, A.F.⁸, Uzel, G.⁸, Su, H.C.⁹, Casanova, J.-L.¹⁰, Tangye, S.G.^{1,2}, Ma, C.S.^{1,2}

¹Garvan Institute of Medical Research, Sydney, Australia,

²University of New South Wales, St Vincent's Clinical School, Sydney, Australia,

³University of New South Wales School of Women's and Children's Health, Sydney, Australia, ⁴Royal Children's Hospital Melbourne, Department of Allergy and Immunology, Melbourne, Australia,

⁵Royal Children's Hospital Brisbane, Department of Paediatrics and Child Health, Brisbane, Australia, ⁶University of Manchester, Royal Manchester Children's Hospital, Manchester, United Kingdom,

⁷Immunodeficiencies Research Unit, and National Institute of Pediatrics, Mexico City, Mexico, ⁸Laboratory of Clinical Infectious Diseases, NIAID, NIH, Bethesda, United States,

⁹Laboratory of Host Defenses, NIAID, NIH, Bethesda, United States,

¹⁰St Giles Laboratory of Human Genetics of Infectious Diseases, Rockefeller Branch, Rockefeller University, New York, United States

Dedicator of cytokinesis 8 (DOCK8) deficiency is a combined immunodeficiency caused by autosomal recessive loss-of-function mutations in *DOCK8*. This disorder, often referred to as autosomal recessive hyper IgE, is characterised by elevated levels of serum IgE, severe recurrent cutaneous infections and atopic disease including anaphylaxis to certain foods. Here we investigated the peripheral CD4⁺ T cell compartment in DOCK8 deficient patients to determine whether there are defects present that can account for the disease phenotype in DOCK8 deficiency. We performed in depth analysis of the CD4⁺ T cell compartment of DOCK8-deficient patients with respect to naïve, central memory and effector memory CD4⁺ T cells, and Treg, Tfh, Th1, Th2 and Th17 subsets. Furthermore, cytokine production was determined after a 5-day culture with non-polarising stimulus. We found that DOCK8-deficient memory CD4⁺ T cells were biased towards a Th2 effector fate, characterised by high expression/secretion of IL-4, IL-5 and IL-13. This was at the expense of other Th cell subsets as evidenced by defects in IFN γ -expressing Th1 and IL-17A- and IL-17F-expressing Th17 cells. Thus, investigations into the CD4⁺ T cell compartment in DOCK8 deficient patients provided an explanation for some of the clinical signs of this disorder. On one hand, the Th2 bias is likely to contribute to atopic disease, such as eczema and food allergies, characteristic to DOCK8 deficiency. On the other hand, defects in Th1 and Th17 cells may compromise anti-viral and anti-fungal immunity, respectively in DOCK8-deficient patients.

1142

An autosomal recessive *TCF3* mutation underlies association of agammaglobulinemia and B-cell acute lymphoblastic leukemia

Barbouche, M.R.^{1,2}, Ben-Ali, M.^{1,2}, Yang, J.³, Chan, K.W.³, Mekki, N.^{1,2}, Ben-Mustapha, I.^{1,2}, Mellouli, F.⁴, Bejaoui, M.⁴, Yang, W.-L.³, Aissaoui, L.⁵, Lau, Y.L.³

¹Pasteur Institute of Tunis, Immunology, Tunis, Tunisia, ²University Tunis El Manar, Tunis, Tunisia, ³University of Hong Kong, Hong Kong, China, ⁴Bone Marrow Transplantation Center, Tunis, Tunisia, ⁵Aziza Othmana Hospital, Tunis, Tunisia

TCF3 (*E2A*) gene encodes E12 and E47 transcription factors which are essential in differentiation process of common lymphoid progenitors into B-lineage cells and are key regulators of B-cell development. Herein, we report the first patient with a homozygous mutation in *TCF3* gene, who presented with agammaglobulinemia, absent peripheral blood B cells and developed B-cell acute lymphoblastic leukemia (B-ALL).

The patient was born to Tunisian first cousin parents. He had recurrent pneumonia and meningitis since early childhood and mild facial dysmorphism. At age 7 years he developed pancytopenia and splenomegaly, with a diagnosis of B-ALL made. Complete remission was obtained under chemotherapy but at age 10 years he relapsed and died despite treatment.

Whole exome sequencing revealed a novel homozygous mutation within exon 9 of *TCF3* (c.C807T) resulting in a premature stop codon (p.Q270X). The resulting proteins are predicted to be deleterious since they lack two functional domains, the activation domain 2 and the bHLH domain. The parents were heterozygous for the variant.

Considering the crucial role of *TCF3* in the regulation of normal B cell development, it is not surprising that disruption of this transcription factor causes a profound B cell defect. Since *TCF3* is also known to be affected (translocations and deletions) in B-ALL, this could explain the clinical phenotype herein observed.

Allergy 2

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Mechanisms of anaphylaxis in human low-affinity IgG receptor locus knock-in mice

Gillis, C.M.^{1,2,3}, Jönsson, F.^{1,2}, Mancardi, D.^{1,2}, Tu, N.⁴, Beutier, H.^{1,2,3}, Van Rooijen, N.⁵, Macdonald, L.E.⁴, Murphy, A.J.⁴, Bruhns, P.^{1,2}

¹Institut Pasteur, Department of Immunology, Unit of Antibodies in Therapy & Pathology, Paris, France, ²INSERM U 1222, Paris, France, ³Université Pierre et Marie Curie, Paris, France, ⁴Regeneron Pharmaceuticals, Inc., Tarrytown, United States, ⁵VU Medical Center, Department of Molecular Cell Biology, Amsterdam, Netherlands

Background: Anaphylaxis can proceed through distinct IgE- or IgG-dependant pathways, which have been investigated in various mouse models. We developed a novel mouse strain in which the human low-affinity IgG receptor locus, comprising both activating (hFc γ RIIA, hFc γ RIIIA, hFc γ RIIIB) and inhibitory (hFc γ RIIB) hFc γ R genes, has been inserted into the equivalent murine locus, corresponding to a locus 'swap'.

Objective: We sought to determine the capabilities of hFcγRs to induce systemic anaphylaxis, and identify the cell types and mediators involved.

Methods: hFcγR expression on mouse and human cells was compared to validate the model. Passive systemic anaphylaxis was induced by injection of heat-aggregated human IVIG, and active systemic anaphylaxis following immunisation and challenge. Anaphylaxis severity was evaluated by hypothermia and mortality. The contribution of receptors, mediators or cell types was assessed by receptor blockade or depletion.

Results: The human to mouse low-affinity FcγR locus swap engendered hFcγRIIA/IIIB/IIIA/IIIB expression in mice comparable to that in humans. Knock-in mice were susceptible to passive and active anaphylaxis that was accompanied by downregulation of both activating and inhibitory hFcγR expression on specific myeloid cells. The contribution of hFcγRIIA was predominant. Depletion of neutrophils, but not monocytes/macrophages or basophils, protected against hypothermia and mortality. Both passive and active anaphylaxis relied on PAF, rather than histamine.

Conclusion: Low-affinity FcγR locus-switched mice represent an unprecedented model of cognate hFcγR expression. Importantly, IgG-anaphylaxis proceeds within a native context of activating and inhibitory hFcγRs; indicating that, despite robust hFcγRIIB expression, activating signals can dominate to initiate a severe anaphylactic reaction.

2080

FGD2 is a novel negative regulator of IgE-dependent mast cell activation

Mackay, G.A.¹, Kuek, L.E.¹, Heng, P.¹, Gavin, A.²

¹University of Melbourne, Dept of Pharmacology & Therapeutics and Lung Health Research Centre, Melbourne, Australia, ²Scripps Research Institute, Dept of Immunology and Microbial Science, La Jolla, United States

Introduction: The faciogenital dysplasia (FGD) family of proteins are guanine nucleotide exchange factors (GEFs) that selectively activate the small G-protein Cdc42. Little is known about the regulatory roles of family members such as FGD2. Given the well-established role of Cdc42 in regulating vesicular trafficking and cell signalling, this study aimed to examine the expression and function of FGD2 in mast cells.

Methods: Bone marrow-derived mast cells (BMMCs) were generated from FGD2^{-/-} mice and IgE-dependent degranulation (β-hexosaminidase release) and cytokine (IL-6) production compared with wild type BMMCs. A murine model of passive systemic anaphylaxis was used to examine the in vivo effects of FGD2 deletion. The expression of FGD2 was measured in BMMCs and the mast cell line RBL-2H3 by quantitative PCR (qPCR) and Western blotting.

Results: FGD2^{-/-} BMMCs showed enhanced IgE-dependent degranulation and IL-6 release. In keeping with this, FGD2^{-/-} mice exhibited a more profound systemic anaphylaxis response (as measured by a decline in core body temperature). FGD2 levels were dramatically down-regulated following FcεRI activation in both WT BMMCs and RBL-2H3 cells.

Discussion and conclusions: Our work identifies FGD2 as a

novel negative regulator of FcεRI signalling in mast cells. The marked down-regulation of FGD2 gene expression upon FcεRI activation would be predicted to reduce the impact of this negative regulating pathway enhancing the severity of allergic reactions. Ongoing work aims to more fully establish the effects of FGD2 at the mechanistic level with a view to harnessing its inhibitory actions as a novel anti-allergic strategy.

3933

Production of a hypoallergenic variant of the major peanut allergen Ara h 2 in the baculovirus insect cell system

Tscheppe, A.¹, Palmberger, D.², Bublin, M.¹, Radauer, C.¹, Palladino, C.¹, Gepp, B.¹, Lengger, N.¹, Grabherr, R.², Breiteneder, H.¹

¹Medical University of Vienna, Center for Pathophysiology, Infectiology and Immunology, Vienna, Austria, ²University of Natural Resources and Life Sciences, Department of Biotechnology, Vienna, Austria

Introduction: Ara h 2 is the most important peanut allergen. Currently peanut allergen-specific immunotherapy is not available for clinical use. We produced a hypoallergenic mutant, mtAra h 2, lacking the IgE-binding loops.

Methods: Trichoplusia ni BTI-TN5B1-4 "HighFive" cells were used to produce mtAra h 2 and wtAra h 2. Proteins were purified from the supernatants and their physicochemical characteristics were determined. IgE-binding to purified natural nAra h 2, wtAra h 2 and mtAra h 2 was tested by direct ELISA and inhibition ELISA using sera of five peanut allergic patients.

Results: Mass spectrometry confirmed the absence of post-translational modifications for the main fraction of wtAra h 2. Folding and N-terminal sequence of the wild-type recombinant corresponded to the natural protein. For mtAra h 2, mass spectrometry and N-terminal sequencing yielded results corresponding to those predicted from the sequence. CD-spectrometry showed a high alpha-helix content. Immunoblots with Ara h 2-sensitized patients' sera indicated lower IgE-binding to the mutant than to the wild-type and the natural proteins. Direct ELISA revealed 20-50% reduction of IgE-binding to mtAra h 2 compared with the wild-type allergen. Preincubation of sera with nAra h 2 and wtAra h 2 resulted in 98% and 56% inhibition of IgE-binding to immobilized nAra h 2 while the mutant showed significantly reduced inhibition (34%).

Conclusions: Our mutant is a promising template for designing next-generation hypoallergens. Slightly reduced IgE-binding of wtAra h 2 might be caused by the presence of altered post-translational modifications.

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Intensity of local skin memory response varying with the type of locally persisting T cell subsets

Murata, A., Yoshino, M., Hikosaka, M., Hayashi, S.-I.

Division of Immunology, Department of Molecular and Cellular Biology, School of Life Science, Faculty of Medicine, Tottori University, Tottori, Japan

In allergic contact dermatitis (ACD), previously ACD-experienced skin regions show an accelerated swelling upon re-exposure to the same hapten. This 'local skin memory (LSM)' is thought to involve locally persisting immune cells; however, it is unclear which cells determine the intensity of LSM. We found that ear skin of BALB/c but not C57BL/6 (B6) mice showed strong and prolonged LSM responses upon re-exposure to a hapten (1% TNCB).

To explain the difference, we compared immune cell subsets in ACD-experienced ear skin with the opposite vehicle-treated control ears, prior to TNCB re-exposure. In BALB/c mice, CD8⁺ T cells became localized in the epidermis, CD4⁺ and CD8⁺ T cells, and mast cells, increased in the dermis by the experience of ACD. There were few CD3⁺CD4⁺CD8⁻ cells in the skin of BALB/c mice. In B6 mice, by contrast, both ACD-experienced and control ears contained a substantial number of $\gamma\delta$ T cells (CD3⁺CD4⁺CD8⁻) in epidermis, few CD4⁺ or CD8⁺ cells, and increased dermal mast cells. The weak LSM response was impaired in B6-*Rag1*-KO but not mast cell-deficient B6-*Kit^{W-sh/W-sh}* mice, suggesting that LSM involved locally persisting T lineage cells.

These results suggest that,

- 1) strong LSM responses may be formed by locally persisting CD4⁺ and CD8⁺ T cells,
 - 2) locally persisting $\gamma\delta$ T cells generate only a weak LSM responses.
- We will discuss possibilities that the presence of skin $\gamma\delta$ T cells leads to suppression of $\alpha\beta$ T cell-based LSM formation and the strong human LSM responses involve the loss of skin $\gamma\delta$ T cells.

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Regulatory role of an oligopeptide transporter SLC15A4 in the inflammatory responses in mast cells

Kobayashi, T., Ohshima, D., Toyama-Sorimachi, N.

National Center for Global Health and Medicine, Tokyo, Japan

SLC15A4 is a proton-coupled amino acid/oligopeptide transporter that resides in the late endosome/lysosome of immune cells. We have reported that SLC15A4 is necessary for endo/lysosome-dependent inflammatory responses of DCs and B cells including TLR7/9- or mTORC1-mediated signaling events by controlling amino acids/proton concentration in the vesicles. Mast cells have specialized lysosomes, in which histamine, proteases and other inflammatory mediators are included, and secretion of these granules is the pivotal in their functions. We here investigated the requirement of SLC15A4 in the lysosome-dependent mast cell functions using SLC15A4-deficient mice.

SLC15A4^{-/-} mast cells showed striking alteration of lysosomal morphology. This was caused by enhancement of lysosome biogenesis, since nuclear localization of TFEB, a master regulator of lysosome biogenesis, which is regulated by mTORC1, was augmented in SLC15A4-deficient mast cells. Upon Fc ϵ RI crosslinking, SLC15A4^{-/-} mast cells released higher amounts of histamine than wild type (WT). Consistent with this, serum histamine levels were increased in SLC15A4-deficient mice in IgE-mediated passive anaphylaxis. In contrast, Fc ϵ RI-mediated cytokine productions such as TNF α or IL-6 in SLC15A4^{-/-} mice were comparable with WT mice. When mast cells were stimulated with IL-33, SLC15A4^{-/-} mast cells produced higher amounts of inflammatory cytokines than WT. Furthermore,

SLC15A4-deficient mice showed enhanced response than WT in airway inflammation induced by IL-33. Our results indicated that SLC15A4-mediated regulation of endo/lysosomal condition have an enormous impact on mast cell's effector functions and subsequent allergic responses. In this presentation, we discuss the mechanism of how SLC15A4-mediated regulation of endo/lysosomal condition regulate mast cell's effector functions.

1594

Posttranscriptional control of antigen-specific T cell proliferation and allergen induced airway inflammation by RNA binding protein HuR

Ellis, J.S.^{1,2}, Techasintana, P.^{1,2}, Glascock, J.^{1,2}, Ridenhour, S.², Atasoy, U.^{1,2}

¹University of Missouri, Molecular Microbiology & Immunology, Columbia, United States, ²University of Missouri, Surgery, Columbia, United States

RNA binding protein HuR (ELAVL1) governs transcript stability of IL-4, IL-13 and GATA-3 mRNAs, thereby controlling Th2 differentiation. We hypothesized that HuR plays a role in antigen induced T cell activation and initiation of allergic airway inflammation. We conditionally ablated HuR in T cells prior to activation (distal Ick-Cre.ROSA HuR^{fl/fl} mouse). Half of mature CD4⁺ T cells were HuR deficient in these mice. To determine the effects of HuR KO in T cells, mice were immunized with OVA, boosted 7 days later, and antigen specific proliferative responses were measured *in vitro*. Despite being capable of proliferation upon anti-CD3/CD28 stimulation, YFP⁺ (HuR KO) T cells did not proliferate in response to antigen while YFP⁻ control cells had no proliferative defects. We then used the OVA challenge model of airway inflammation to ascertain effects of HuR KO *in vivo*. HuR KO mice had significantly less total cellular inflammation and significantly reduced BAL IL-13. To determine the mechanism of suppression, we conducted *in vitro* activation experiments. HuR KO CD4⁺ T cells were sorted into YFP⁺ and YFP⁻ pools and activated. YFP⁺ T cells had significant decreases in IL-4, IL-5, IL-13 but not IFN γ protein. Gata-3, IL-4 and IL-2Ra (CD25) transcription were significantly reduced. KO T cells have reductions in pSTAT5 signaling which is required for robust Th2 differentiation. While only 50% of CD4⁺ T cells lack HuR, there was total suppression of Th2 differentiation in airway inflammation. These data elucidate a critical role for HuR in regulating Th2 differentiation and allergic airway inflammation.

2138

Tolerogenic immune modifying nanoparticles are a highly efficacious antigen-specific therapy for the treatment of Th2-mediated allergic airway inflammation

Smarr, C.¹, Shea, L.², Podojil, J.R.³, Getts, D.^{1,4}, Miller, S.D.¹

¹Northwestern University Feinberg School of Medicine, Chicago, United States, ²University of Michigan Medical School, Ann Arbor, United States, ³Cour Pharmaceuticals Development Co, Northbrook, United States, ⁴Cour Pharmaceuticals Development Co, Chicago, United States

Specific immunotherapy (SIT) is the most widely used treatment for allergic diseases, however, clinical applications of SIT require

a long period of dose escalation with soluble antigen and carry a significant risk of adverse reactions. We hypothesized that Tolerogenic Immune Modifying Nanoparticles (TIMP), which are in clinical development for treating celiac disease, would also be effective for the induction of immune tolerance in murine models of Th2-mediated allergic airway inflammation. Initially, using the model antigen, Ovalbumin we demonstrate that antigen-conjugated to the surface of polystyrene nanoparticles, while effective for the prophylactic induction of tolerance, induce anaphylaxis in pre-sensitized mice. Similarly, antigen-conjugated Immune Modifying Nanoparticles, derived from PLGA, are effective prophylactically. In contrast to the polystyrene nanoparticles, the PLGA nanoparticles are well tolerated by sensitized animals, but were not as effective at inhibiting airway inflammation when administered therapeutically. This efficacy, however, was overcome using TIMP. TIMP encapsulating the allergen were well tolerated, and most importantly TIMP inhibited OVA/Alum Th2 responses and airway inflammation when given either prophylactically or therapeutically. We show that this tolerance strategy may also be applied to Japanese Cedar Pollen airway inflammation, with TIMP containing pollen antigens abrogating JCP airway inflammation. In both models, this abrogation was not the result of immune deviation, but was correlated with a specific inhibition of antigen specific T cell responses and expansion of regulatory T cells. Thus, we illustrate the TIMP platform to be a safe and effective strategy to treat allergic airway inflammation without the need for nonspecific immunosuppression.

3864

Gut microbiota, bacterial metabolites and metabolite-sensing GPCRs determine mucosal tolerance and protection against food allergy

Macia, L.¹, Tan, J.², Mackay, C.²

¹University of Sydney, Charles Perkins Centre, Physiology, The University of Sydney, Australia, ²Monash University, Department of Biochemistry and Molecular Biology, Clayton, Australia

Incidence of food allergy has increased dramatically in recent decades particularly in Western countries. The diet hypothesis states that western diet enriched in fat and sugars while deprived in fibre contributes to development of western diseases such as allergy. Dietary fibre is potent prebiotic, reshaping beneficially gut microbiota. It is also fermented in the colon by anaerobic bacteria into short chain fatty acids (SCFA) that bind specific G-protein coupled receptors widely expressed in the host.

The aim of this study was to determine the impact of diet enriched in dietary fibre and SCFA on the development of food allergy in mice.

Mice were fed on diets either enriched or deprived in fibre in models of oral tolerance to peanut and of peanut allergy. In both models, dietary fibre was beneficial as enhanced oral tolerance and protection from food allergy were observed under high fibre feeding conditions. SCFA were behind these benefits as both acetate and butyrate protected from development of peanut allergy while propionate had no effects. Accordingly, mice knockout for GPR43 or for GPR109A, respectively preferential receptor for acetate and butyrate, were not protected from

food allergy development under high fibre feeding conditions. To determine the role of gut microbiota, germ free mice were reconstituted with microbiota isolated from high fibre vs zero fibre fed mice and we found that the first was protective in food allergy.

In conclusion, high fibre feeding protects from food allergy development by reshaping of gut microbiota and through the SCFA acetate and butyrate.

860

Contact hypersensitivity is ameliorated by 17,18-epoxy-eicosatetraenoic acid, a metabolite from eicosapentaenoic acid, in the skin

Nagatake, T.¹, Shioyama, Y.², Honda, T.³, Isobe, Y.⁴, Arita, M.⁴, Matsumoto, N.¹, Shimojima, M.¹, Tiwari, P.¹, Suzuki, H.¹, Yasutomi, Y.², Kiyono, H.⁵, Kabashima, K.³, Kunisawa, J.^{1,5,6,7}

¹National Institutes of Biomedical Innovation, Health and Nutrition, Laboratory of Vaccine Materials, Ibaraki, Japan, ²Tsukuba Primate Research Center, National Institutes of Biomedical Innovation, Health and Nutrition, Laboratory of Immunoregulation and Vaccine Research, Tsukuba, Japan, ³Kyoto University Graduate School of Medicine, Department of Dermatology, Kyoto, Japan, ⁴RIKEN Center for Integrative Medical Sciences, Laboratory for Metabolomics, Yokohama, Japan, ⁵Institute of Medical Science, University of Tokyo, Division of Mucosal Immunology, Department of Microbiology and Immunology / International Research and Development Center for Mucosal Vaccines, Tokyo, Japan, ⁶Osaka University, Graduate School of Medicine / Pharmaceutical Sciences / Dentistry, Suita, Japan, ⁷Kobe University, Graduate School of Medicine, Kobe, Japan

Our group has previously reported that dietary intake of ω 3 polyunsaturated fatty acids-rich linseed oil led to the increase of 17,18-epoxy-eicosatetraenoic acid (17,18-EpETE), a metabolite of eicosapentaenoic acid, in the body, which could inhibit the development of allergic responses in the intestine. In this study, we extend our study to assess the effects of 17,18-EpETE on contact hypersensitivity in the skin. When mice were preventively or therapeutically treated with 17,18-EpETE, the development of dinitrofluorobenzene-induced contact hypersensitivity was inhibited, which was associated with the decrease of neutrophil accumulation in the skin without affecting the dendritic cell-cluster formation and cytokine productions (e.g., IL-17A and IFN γ) from T cells. 17,18-EpETE suppressed Rac activation in neutrophils and consequently inhibited their infiltration to the skin. Unlike 17,18-EpETE, its metabolite 17,18-diHETE did not show any effects on contact hypersensitivity. It was also shown that therapeutic treatment of 17,18-EpETE ameliorated contact hypersensitivity in cynomolgus monkey. These results indicate that 17,18-EpETE could be applied to be a therapeutic agent for the control of allergic inflammatory diseases.

Dendritic Cells 2

2018

IRF8-dependent DCs play a key role in the regulation of CD8 T cell responses to epithelial-derived antigen in the steady state but not in inflammation

Joeris, T.^{1,2}, Gomez Casado, C.², Holmkvist, P.², Luda, K.², Tavernier, S.³, Lambrecht, B.N.³, Agace, W.W.^{1,2}

¹Danish Technical University - DTU, National Veterinary Institute - Section of Immunology and Vaccinology, Fredrighsberg C, Denmark,

²Lund University, Experimental Medical Sciences- Section for Immunology, Lund, Sweden, ³Inflammation Research Center (IRC), VIB-UGent, Ghent, Belgium

Along the process of epithelial self-renewal, antigens derived from apoptotic intestinal epithelial cells (IECs) are taken up by antigen presenting cells (APCs), transported to the gut-draining lymph nodes and cross-presented to CD8 T cells. In steady state, rapid tolerization of CD8 T cells reactive towards epithelial-derived antigens is crucial to maintain tissue homeostasis. In contrast, infection of IECs by intracellular pathogens requires induction of cytotoxic CD8 T cells (CTLs) towards epithelial-associated, pathogen-derived antigens. Currently, little is known about the regulation of CD8 T cells by intestinal APCs in these two different contexts. Since IRF8-dependent dendritic cells (IRF8-DCs) have superior cross-presenting capabilities, we aimed to investigate their role in this process. IFABP-tOva mice, expressing the model-antigen Ovalbumin (Ova) in IECs, were used as recipients to set up chimeras using either CD11c-cre. Irf8^{fl/fl} bone marrow, which cannot generate IRF8-DCs, or cre-negative Irf8^{fl/fl} control bone marrow. Whereas transfer of Ova-specific CD8 T cells (OT-I cells) to steady state control chimeras resulted in their rapid tolerization, OT-I cells transferred to CD11c-cre.Irf8^{fl/fl} chimeras spontaneously developed into CTLs, causing epithelial destruction and intestinal inflammation. However, when the TLR7-ligand R848 was applied as an inflammatory trigger mimicking viral infection in addition to OT-I transfer, expansion of CTLs occurred at similar rates in both, CD11c-cre. Irf8^{fl/fl} and control chimeras. Taken together, this demonstrates that IRF8-DCs are crucial for the rapid tolerization of CD8 T cells reactive towards epithelial-derived antigen in steady state, but are not essential for the induction of CTLs in an inflammatory setting such as found in infection.

4051

tRNAs: new regularors of immunity?

Arguello, R.J.¹, Reverendo, M.¹, Terawaki, S.¹, Combes, A.¹, Vu-Manh, T.P.¹, Santos, M.², Gatti, E.¹, Pierre, P.^{1,2}

¹CIML, Marseille, France, ²University of Aveiro, Aveiro, Portugal

Dendritic cells (DCs) respond to microbial cues by activating several coordinated metabolic and gene expression programs that altogether maximize their capacity to orchestrate immune responses. Translation of costimulatory molecules, cytokines and other induced mRNAs is an essential part of the program and it is tightly regulated. Recently, a connection between ribosomal speed of elongation, mRNA stability and protein expression has been found tumors and model

organisms. Analysing microarray data, we found that genes involved in tRNAs metabolism were coordinately regulated during the first hours after DC activation, suggesting a role connecting transcriptional and translational programs during DCs maturation. To test this hypothesis, we performed tRNA microarrays, codon usage analysis in DC transcriptomic data, and studied the effect of modulating tRNA metabolic pathways in the capacity of DCs mature in response to different stimuli. We have found, an essential inducible regulatory program in DCs that involves qualitative and quantitative changes in the tRNA pool and the presence of an evolutionarily conserved bias of codon usage in genes annotated to immune function. As we could show by using reporter with different codon usage his program affects both translation mRNA stability and antigen presentation. A deeper understanding on how the translation machinery is modified upon immune activation and how it affects decoding of mRNAs is required in order to further characterize this previously unexplored feature of immune gene regulation, which is embedded in the genetic code.

3176

Characterization of Sirpa+ dendritic cell paralysis following systemic inflammatory response syndrome

Valikhani, S., Mackay, L., Mueller, S., Roquilly, A., Villadangos, J. Doherty Institute, Microbiology & Immunology, University of Melbourne, Melbourne, Australia

Systemic/severe infections cause Systemic Inflammatory Response Syndrome (SIRS). The period of immunosuppression following SIRS (lasting several weeks) is associated with an increased risk of secondary infections. Impairment of dendritic cells (DCs) (primary initiators of T-cell immunity) clearly plays a prominent role in this inhibition. Our current studies have shown that SIRS triggers the lasting production of altered signals leading to sustained production of paralysed CD8+ DC; these have poor antigen presenting and T cell stimulating activity until the effect of SIRS decreases leading to return of normal signals. In this project, we aim to further characterise DC paralysis and extend the study to Sirpa+ (CD8-) DCs that are involved in classical antigen presentation. We used mouse models that utilized different SIRS triggers such as CpG (bacterial mimic), *E. coli* and Influenza A to initiate DC paralysis. Our results thus far show that newly formed Sirpa+ DCs (previously not exposed to SIRS trigger) were paralysed for up to 21 days following CpG-induced SIRS but not *E. coli*-induced SIRS probably due to the pneumonia model causing only local DC paralysis rather than systemic. Other functional defects that were found to be present in both DC subsets using the CpG model included decreased cytokine production and phagocytosis. Furthermore, transcriptomic analyses of paralysed Sirpa+ DC has identified several potential paralysis markers that could be used in future investigations. Our ultimate goal is to develop strategies to manage patients with SIRS-induced immunosuppression and to develop therapeutic treatments to prevent, shorten or overcome DC paralysis.

2159

T cell help amplifies innate signals in CD8+ DC for optimal CD8+ T cell priming

Greger, M.¹, Whitney, P.G.¹, Stock, A.T.¹, Davey, G.M.¹, Tebartz, C.¹, Bachem, A.¹, Mintern, J.D.², Strugnelli, R.A.¹, Turner, S.J.¹, Gebhardt, T.¹, O'Keeffe, M.³, Heath, W.R.¹, Bedoui, S.¹

¹The University of Melbourne, at the Peter Doherty Institute for Infection and Immunity, Department of Microbiology and Immunology, Melbourne, Australia, ²The University of Melbourne, Department of Biochemistry and Molecular Biology, Melbourne, Australia, ³Monash University, Department of Biochemistry and Molecular Biology, Melbourne, Australia

DC often require stimulation from CD4+ T cells to propagate CD8+ T cell responses, but precisely how T cell help optimizes the priming capacity of DC and why this appears to differ between varying types of CD8+ T cell immunity remains unclear. We show that CD8+ T cell priming upon HSV-1 skin infection depended on DC receiving stimulation from both IFN- α/β and CD4+ T cells to provide IL-15. This was not an additive effect, but resulted from CD4+ T cells amplifying DC production of IL-15 in response to IFN- α/β . We also observed that increased innate stimulation reversed the helper-dependence of CD8+ T cell priming and that the innate stimulus, rather than the CD4+ T cells themselves, determined how 'help' was integrated into the priming response by DC. These findings identify T cell help as a flexible means to amplify varying suboptimal innate signals in DC.

3238

Involvement of XCR1-expressing dendritic cells through the XCR1-XCL1 chemokine axis in intestinal immune homeostasis

Ohta, T.^{1,2}, Hemmi, H.^{1,2}, Kaisho, T.^{1,2}

¹Wakayama Medical University, Institute of Advanced Medicine, Department of Immunology, Wakayama, Japan, ²Osaka University, WPI Immunology Frontier Research Center, Suita, Japan

Intestinal immune homeostasis depends on innate and adaptive immune cells. Dendritic cells (DCs), which are professional Ag presenting cells linking innate and adaptive immunity, consist of several functionally distinct subsets. A DC subset expressing XCR1, a chemokine receptor conserved across mammalian species, is involved in protective immunity against microbial infection or tumors. However, it remains unknown how XCR1-expressing DCs are involved in the intestinal immune homeostasis. Here, we have generated and analyzed the mutant mice, XCR1-DTA mice, in which XCR1-expressing DCs are specifically ablated due to the expression of diphtheria toxin A subunit (DTA). In XCR1-DTA mice, all lamina propria and intraepithelial T cell populations were decreased concomitantly with defective survival, although splenic and lymph node (LN) T cells were normal. The remaining intestinal T cells exhibited defective intestinal T cell phenotype, i.e. higher and lower expression of CD62L and CD103. XCR1-DTA mice showed exaggerated manifestations of chemically induced colitis, indicating regulatory roles of XCR1-expressing DCs or intestinal T cells in inflammation. Furthermore, XCR1- or its ligand XCL1-

deficient mice also showed intestinal T cell decrease, suggesting that XCR1 is not just a marker, but involved in the intestinal homeostasis. The XCR1-XCL1 axis is also involved in homeostatic migration of XCR1-expressing DCs, because lamina propria and mesenteric LN XCR1-expressing DCs are increased and decreased, respectively, in XCR1- or XCL1-deficient mice. This study thus clarified that XCR1-expressing DCs play critical roles in the intestinal immune homeostasis through the XCR1-XCL1 chemokine axis, which is a novel regulatory point for intestinal immune system.

2232

Molecular basis for the efficient processing of antigens taken up by Clec9A, a DAMP receptor on dendritic cells

Tan, P.S.¹, Tullett, K.¹, Park, H.-Y.¹, Gruber, E.¹, Radford, K.², Nicola, N.³, Zhang, J.-G.³, Shortman, K.³, Caminschi, I.¹, Lahoud, M.¹

¹Monash University, Clayton, Australia, ²Mater Research Institute-University of Queensland, Brisbane, Australia, ³Walter & Eliza Hall Institute of Medical Research, Parkville, Australia

Dendritic cells (DC) use a variety of cell surface receptors to monitor the environment for potential dangers, including cells that have died of non-homeostatic causes (eg. infected cells), to induce appropriate immune responses. Clec9A is a DC-specific Damage-Associated Molecular Pattern receptor, that is expressed by mouse and human cross-presenting DC subsets. Clec9A recognises dead cells and plays an important role in the recognition and processing of dead cell-derived Ag. Our research has focussed on identification of Clec9A ligands, and determining the molecular basis of Clec9A recognition of dead cells. We identified that Clec9A recognises filamentous actin revealed by dead cells, to facilitate trafficking of dead cell-derived Ag. We sought to determine the proteins within DC that regulate Clec9A function and intracellular trafficking. We identified Clec9A interacts with an E3 Ubiquitin ligase, which regulates ubiquitination of Clec9A complexes, thereby targeting Clec9A complexes for downstream processing and regulating Clec9A function. Our research is currently focussed on elucidating the control of Clec9A by ubiquitination, and on characterising the molecular interactions that underpin Clec9A function and the role of these interactions in mediating immune responses.

3987

Transcription factor Ikaros regulates lineage choice in dendritic cell-committed progenitors

Roediger, B.^{1,2}, Mitchell, A.^{2,3}, Bailey, C.^{2,3}, Ashurst, T.⁴, Jain, R.^{2,3}, Shklovskaya, E.^{3,5}, Saunders, B.^{2,3,6}, Rasko, J.E.^{2,3,7}, King, N.J.C.⁴, Fazekas de St. Groth, B.^{2,3}, Holst, J.^{2,3}, Weninger, W.^{2,3,7}

¹Centenary Institute, University of Sydney, Sydney, Australia, ²Sydney Medical School, Sydney, Australia, ³Centenary Institute, Sydney, Australia, ⁴University of Sydney, Sydney, Australia, ⁵Sydney Children's Hospital, Sydney, Australia, ⁶University of Technology Sydney, Sydney, Australia, ⁷Royal Prince Alfred Hospital, Sydney, Australia

Dendritic cells (DCs) comprise a heterogeneous population of antigen presenting cells of which different subsets perform

specialized functions. How DC subset determination is regulated remains incompletely understood. Previous studies have shown that CD11b⁺ conventional (lymphoid) DCs require high levels of the transcription factor Ikaros for their development, but how and where Ikaros exerts its action within the DC lineage remains unknown. It also remains unclear what roles, if any, Ikaros plays in the other DC subsets. We have identified a cell-intrinsic requirement for Ikaros by both lymphoid-resident and migratory CD11b⁺ DCs, part of which is attributable to an upstream requirement in pre-DCs. We further demonstrate that DC progenitors with low levels of Ikaros are predisposed to differentiate into the CD8⁺/CD103⁺ DC pathway at the expense of CD11b⁺ DC development. This developmental bias offsets the pre-DC defect in the CD8⁺/CD103⁺ DC lineage but further compromises CD11b⁺ DC development. Collectively, our data identify a critical role for Ikaros in fate determination within the DC lineage, and lend support for a shared developmental pathway by lymphoid and non-lymphoid CD11b⁺ DCs.

1924

BCG skin infection triggers IL-1R-MyD88-dependent migration of EpCAM^{low} CD11b^{high} skin dendritic cells to draining lymph node during CD4⁺ T-cell priming

Bollampalli, V.P.¹, Yamashiro, L.H.¹, Feng, X.¹, Bierschenk, D.¹, Gao, Y.¹, Blom, H.², Henriques-Normark, B.¹, Nylén, S.¹, Rothfuchs, A.G.¹
¹Karolinska Institutet, MTC, Stockholm, Sweden, ²Royal Institute of Technology, Science for Life Laboratory, Stockholm, Sweden

The transport of antigen from the periphery to the draining lymph node (DLN) is critical for T-cell priming but remains poorly studied during infection with *Mycobacterium bovis* Bacille Calmette-Guérin (BCG). To address this we employed a mouse model to track the traffic of Dendritic cells (DCs) and mycobacteria from the BCG inoculation site in the skin to the DLN. Detection of BCG in the DLN was concomitant with the priming of antigen-specific CD4⁺

T cells at that site. We found EpCAM^{low} CD11b^{high} migratory skin DCs to be mobilized during the transport of BCG to the DLN. Migratory skin DCs distributed to the T-cell area of the LN, co-localized with BCG and were found in close apposition to antigen-specific CD4⁺ T cells. Consequently, blockade of skin DC traffic into DLN dramatically reduced mycobacterial entry into DLN and muted T-cell priming. Interestingly, DC and mycobacterial entry into the DLN was dependent on IL-1R-I, MyD88, TNFR-I and IL-12p40. In addition, we found using DC adoptive transfers that the requirement for MyD88 in BCG-triggered migration was not restricted to the migrating DC itself and that hematopoietic expression of MyD88 was needed in part for full-fledged migration. Our observations thus identify a population of DCs that contribute towards the priming of CD4⁺ T cells to BCG infection by transporting bacilli into the DLN in an IL-1R-MyD88-dependent manner and reveal both DC-intrinsic and -extrinsic requirements for MyD88 in DC migration.

3777

Immunoregulation by O-GlcNAc glycosylation in dendritic cells

Gupta, N.¹, Meuter, S.², Infusini, G.³, Zhan, Y.³, Mintern, J.D.¹, Villadangos, J.A.^{1,2}

¹Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, Department of Biochemistry and Molecular Biology, Melbourne, Australia, ²The Doherty Institute for Infection and Immunity, University of Melbourne, Department of Microbiology and Immunology, Melbourne, Australia, ³The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia

O-GlcNAc glycosylation is a unique post-translational modification found on nucleo-cytoplasmic proteins. It involves addition of a monosaccharide (GlcNAc) on serine or threonine residues of proteins such as kinases, phosphatases, metabolic enzymes, cytoskeletal proteins and transcription factors, often inhibiting or promoting phosphorylation of the same proteins. The enzymes O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) catalyse the addition and removal of O-GlcNAc respectively. O-GlcNAc glycosylation acts as a nutrient and environmental sensor and by engaging in an extensive crosstalk with phosphorylation, regulates various cellular processes.

Considering the transcripts of O-GlcNAc cycling enzymes are highly expressed in immune cells, we have investigated the role of O-GlcNAc glycosylation in dendritic cells (DCs). Genetic alteration of OGT/OGA expression in DCs affected T cell priming and modulated pro-inflammatory cytokine and chemokine production. Using biochemical and proteomic/mass spectrometric methods, we identified differential O-GlcNAcylation of proteins in resting and activated DCs, of which several proteins played key roles in signalling and metabolic processes.

Overall, we demonstrate that O-GlcNAcylation is a critical protein modification that regulates DC physiology and function.

Tumour Immunology 4

1288

Haematopoietic cell kinase activity in myeloid cells promotes colorectal cancer progression

Poh, A.¹, Love, C.¹, Masson, F.², Preaudet, A.¹, Khakham, Y.¹, Lessene, G.¹, Sieber, O.¹, Putoczki, T.¹, O'Donoghue, R.², Ernst, M.²

¹The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia, ²Olivia Newton-John Cancer Research Institute, Melbourne, Australia

Activation of the myeloid-specific Src-family kinase Hematopoietic Cell Kinase (HCK) promotes proliferation and survival of immune cells, and triggers hematological malignancies as a tumor cell-intrinsic oncogene. However, the role of HCK in the tumor stroma of solid cancers remains unexplored.

We analyzed the expression level of HCK in matched biopsies from sporadic colorectal cancer patients and observed elevated HCK phosphorylation in more than half of tumors compared to unaffected colons. Analysis of corresponding RNAseq data revealed a striking correlation between tumors with high HCK

gene expression and a gene signature of tumor-promoting alternatively-activated macrophages (AAMs). To functionally assess this observation, we subjected Hck^{CA} mice that express a constitutively active form of the kinase (Hck^{CA}) to a chemically-induced model of sporadic colorectal cancer. Hck^{CA} mice developed more and larger tumors compared to wild-type (WT) animals, and this was associated with a significant increase in AAMs in tumors of Hck^{CA} mice. Likewise, adoptive bone-marrow transfer experiments enhanced tumor formation and AAM differentiation in WT mice reconstituted with Hck^{CA} bone-marrow, with a reciprocal decrease of these parameters in Hck^{CA} mice reconstituted with WT bone-marrow. Accordingly, pharmacological inhibition of Hck activity suppressed AAM polarization and the growth of grafted colorectal tumors.

Together, our findings suggest that increased Hck activity in the tumor stroma promotes the progression of solid cancers by modulating the phenotype of tumor-associated myeloid cells. Thus, Hck represents a rational therapeutic target for macrophage re-education in solid cancers by limiting polarization of tumor-promoting AAMs.

3170

Oncolytic virus-mediated immunotherapy

Bourgeois-Daigneault, M.-C., Roy, D., Falls, T., Bell, J.
Ottawa Hospital Research Institute, Ottawa, Canada

The lack of treatment options for patients with chemotherapy-resistant cancers is pushing forward the rapid development of alternative therapies. One such option, especially for disseminated or recurrent diseases, is the use of oncolytic viruses, with the first candidate now approved for the use in patients. Oncolytic viruses are known to specifically destroy tumors by mediating direct killing and tumor vascular shutdown, but the induction of an efficient and persistent anti-tumor immune response is a facet that is underappreciated. Using the clinical trial candidate rhabdovirus Maraba MG1 in orthotopic murine breast tumor models, we demonstrate here the importance of this immune response. Our results show that the administration of MG1 is sufficient to control the growth of subsequent tumors and even cure some animals without the need for further treatments. The virus induces an efficient tumor-specific immune response and recruits immune cells to the tumor. Also, tumor-infiltrating lymphocytes are able to penetrate the tumor more profoundly. Importantly, treatment with MG1 causes the upregulation of PDL1 by tumor cells and active regulatory T cells were found in greater amounts. Given the recent success of immune checkpoint inhibitors, we investigated if this second treatment could further improve oncolytic virotherapy. Indeed, our data demonstrate the successful combination of MG1 with immune checkpoint inhibitors in tumor models that are usually resistant to the latter. We believe that our study is the first to reveal the extent of the potential of oncolytic viruses as immunotherapeutic agents.

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CIS is a potent checkpoint in NK cell-mediated tumour immunity

Huntington, N.
WEHI, Parkville, Australia

Natural killer (NK) cells have evolved to detect and kill aberrant cells with this activity being governed by the cytokine interleukin (IL)-15 and foreign and self-ligands. We have identified CIS (Cytokine-inducible SH2-containing protein; *Cish* gene) as the critical negative regulator of IL-15 signalling in NK cells. *Cish* was rapidly induced in response to IL-15 and deletion of *Cish* rendered NK cells hypersensitive to IL-15, as evidenced by superior proliferation, survival, IFN- γ production and cytotoxicity towards tumours. This was associated with enhanced JAK/STAT signalling in *Cish*-deleted NK cells. Correspondingly, CIS interacted with the tyrosine kinase JAK1, inhibiting its enzymatic activity and targeting JAK for proteasomal degradation. *Cish*^{-/-} mice were resistant to melanoma, prostate and breast cancer metastasis *in vivo*, and this was intrinsic to NK cell activity. This study has uncovered a potent checkpoint in NK cell-mediated tumour immunity and holds promise for novel immunotherapies directed at blocking CIS function.

2145

Mechanisms driving co-inhibitory receptor expression on T cells

Chihara, N.¹, Madi, A.¹, Kondo, T.¹, Singer, M.², Zhang, H.¹, Wang, C.¹, Kurtulus, S.¹, Burkett, P.¹, Regev, A.², Anderson, A.¹, Kuchroo, V.¹
¹Evergrande Center for Immunologic Diseases, Brigham and Women's Hospital and Harvard Medical School, Boston, United States, ²Broad Institute of MIT and Harvard, Cambridge, United States

The dysfunctional or exhausted CD8⁺T cells that arise in chronic disease settings such as cancer exhibit deficits in effector functions and express high levels of co-inhibitory receptors, such as PD-1 and CTLA-4. The expression of these receptors contributes to the maintenance of dysfunctional phenotype as blockade of these receptors has been associated with the recovery of effector T cell responses in multiple experimental tumor models. Indeed, the blockade of both CTLA-4 and PD-1 has now been successfully translated to the clinic for the treatment of several different cancers. In addition to CTLA-4 and PD-1, tumor-infiltrating lymphocytes (TILs) have been noted to co-express other co-inhibitory receptors (Tim-3, Lag-3, TIGIT), indicating shared regulatory mechanisms driving their expression. We have used cytometry by time of flight (CyTOF) to examine the degree of co-variance of co-inhibitory and co-stimulatory receptors on T cells at the single cell level both *in vitro* and *in vivo*. We have further identified an immunoregulatory cytokine that drives the expression of multiple known co-inhibitory receptors including Tim-3, Lag-3 and TIGIT. Our findings not only provide insight into the extracellular signals that drive the expression of co-inhibitory receptors in T cells but also provide a platform for the identification of novel molecules and the molecular mechanism underlying the co-expression of co-inhibitory receptors on T cells during T cell dysfunction.

963

Rescue of exhausted CD8 T cells by PD-1 targeted therapies is CD28-dependent

Kamphorst, A.O.¹, Wieland, A.¹, Yang, S.^{1,2}, Nasti, T.¹, Zhang, R.³, Barber, D.L.⁴, Konieczny, B.T.¹, Koenig, L.⁵, Yu, K.⁵, Sica, G.⁶, Owonikoko, T.K.⁵, Sharpe, A.H.⁷, Freeman, G.J.⁸, Blazar, B.R.⁹, Turka, L.A.³, Pillai, R.⁵, Ramalingam, S.S.⁵, Araki, K.¹, Ahmed, R.¹

¹Emory University School of Medicine, Microbiology and Immunology/Emory Vaccine Center, Atlanta, United States,

²Xiangya School of Medicine, Central South University, Changsha, China,

³Massachusetts General Hospital and Harvard Medical School, Department of Surgery, Boston, United States,

⁴National Institute of Allergy and Infectious Diseases, Laboratory of Parasitic Diseases, Bethesda, United States,

⁵Winship Cancer Institute, Emory University School of Medicine, Department of Hematology and Medical Oncology, Atlanta, United States,

⁶Emory University School of Medicine, Department of Pathology, Atlanta, United States,

⁷Harvard Medical School, Boston, United States,

⁸Dana-Farber Cancer Institute, Boston, United States,

⁹University of Minnesota, Division of Blood and Marrow Transplantation, Department of Pediatrics, Minneapolis, United States

T cell exhaustion was first described in mice during chronic lymphocytic choriomeningitis virus (LCMV) infection and later shown to occur in humans during persistent infections and cancer. Sustained expression of the inhibitory receptor programmed cell death-1 (PD-1) is a hallmark of T cell exhaustion, and therapeutic blockade of PD-1 reinvigorates exhausted T cells. PD-1 targeted therapies have shown efficacy for multiple cancer types, but the requirements for T cell rescue by blockade of the PD-1 pathway remain elusive. We analyzed T cells infiltrating human lung tumors, and found that PD-1^{hi} CD8 T cells were predominantly CD28 negative. To address the role of the CD28/B7 pathway on CD8 T cell rescue by PD-1 targeted therapies, we used the LCMV chronic infection model. When B7 signals were impeded by CTLA-4-Ig or B7 blocking antibodies, PD-1 pathway blockade failed to rescue virus-specific CD8 T cells. To investigate a cell intrinsic requirement of CD28 on exhausted CD8 T cells, we analyzed cells genetically deficient in CD28. We found that CD28-deficient LCMV-specific CD8 T cells do not expand after blockade of the PD-1 pathway. Furthermore, CD28-negative CD8 T cells were refractory to PD-1 targeted therapies; even when CD28-signaling was intact during priming, and eliminated by CD28-conditional gene deletion in exhausted CD8 T cells. These data demonstrate that CD28 signaling is required for CD8 T cell rescue following PD-1 blockade, and CD28 expression on tumor-specific CD8 T cells may constitute a predictive biomarker for PD-1 targeted therapies.

2088

NFκB1 suppresses intestinal-type gastric cancer development by preventing chronic inflammation in the stomach

O'Reilly, L.A.^{1,2}, Putoczki, T.P.^{1,2}, Mielke, L.^{1,2}, Lin, A.¹, Preaudet, A.¹, Low, J.T.^{1,2}, Herold, M.J.^{1,2}, Tai, L.¹, Ferrero, R.³, Hu, Y.^{1,2}, Grumont, R.⁴, Smyth, G.^{1,2}, Ernst, M.⁵, Waring, P.⁶, Gerondakis, S.⁴, Strasser, A.^{1,2}

¹The Walter and Eliza Hall Institute of Medical Research, Parkville,

Australia, ²The University of Melbourne, Department of Medical Biology, Parkville, Australia, ³Monash Institute of Medical Research, Clayton, Australia, ⁴School of Biomedical Sciences, Monash University, Department of Biochemistry and Molecular Biology, Clayton, Australia, ⁵Olivia Newton-John Cancer Research Institute, Heidelberg, Australia, ⁶The University of Melbourne, Department of Pathology, Parkville, Australia

Gastric cancer (GC) is a leading cause of cancer-related deaths world-wide. The often late-stage diagnosis has hindered understanding of early events in GC pathophysiology, resulting in a paucity of early diagnostic markers but limited targeted therapy development. We describe mice lacking NFκB1, a member of the NFκB family of transcription factors, as the first animal model of human intestinal-type GC (IGC) that faithfully recapitulates all histopathological disease stages, from inflammation (gastritis) to invasive adenocarcinoma and uniquely secondary metastasis in a time frame akin to human GC. Pertinently, reduced *nfk1* promoter activity resulting from polymorphisms/mutations is associated with human IGC but how loss of NFκB1 causes gastric cancer development is unknown.

We show that IGC in the *nfk1*^{-/-} mouse develops both independently of *H.pylori* infection and even occurs in an abiotic environment. We have generated bone marrow chimaeric mice to demonstrate that loss of NFκB1 in both inflammatory and gastric epithelial cells is required for the development of IGC. Transcriptome, protein expression and gene network analysis in pre-disease *nfk1*^{-/-} mice reveals a deregulation of cytokine receptor signalling and aberrant activation of STAT transcription factors, implicating these pathways in IGC. Accordingly, a gain of function mutation in gp130, the common chain of the IL-6, LIF and OSM receptors accelerates IGC development. These compound mutant mice constitute an ideal model to test novel therapeutics.

We conclude that NFκB1 suppresses GC development by preventing leukocyte infiltration, their cytokine overproduction, driving chronic gastric inflammation and conspires with epithelial cell intrinsic defects in their neoplastic transformation.

3006

The role of ACKR4 in melanoma biology and anti-tumour immunity

Foeng, J.¹, Harata-Lee, Y.¹, Gregor, C.¹, Pederson, S.², Brown, M.³, Smyth, M.⁴, Comerford, I.¹, McColl, S.¹

¹University of Adelaide, Molecular and Cellular Biology, Adelaide,

Australia, ²University at Adelaide, Bioinformatics Hub, Adelaide,

Australia, ³Royal Adelaide Hospital, Cancer Clinical Trials Unit,

Adelaide, Australia, ⁴QIMR Berghofer Medical Research Institute,

Immunology in Cancer and Infection Laboratory, Adelaide,

Australia

Australia has the highest incidence of melanoma worldwide where it represents the most common cancer amongst young Australians. In particular, metastatic melanoma is associated with high rates of mortality, highlighting an urgent need for new therapies. Melanoma progression and metastasis is influenced

by the chemokine family which includes typical and atypical chemokine receptors and their cognate ligands. Atypical chemokine receptors are uncoupled from classical signal transduction cascades and do not mediate cell migration. One of these receptors, ACKR4, has been implicated in melanoma progression. We have knocked down expression of ACKR4 in B16 melanoma cells, which results in tumour regression and complete survival in haematogenous metastases experiments. This is accompanied by immune cell infiltration, ectopic lymphoid neogenesis, loss of pigmentation and resistance to anoikis. To explore the mechanistic basis for this phenotype we performed genome-wide transcriptional analysis of B16 melanoma cells expressing an off-target shRNA or shRNA specific for ACKR4. The gene arrays show dramatic transcriptional changes between these two cell lines with 1303 differentially expressed genes exhibiting at least a 2-fold change. Many of the genes identified are involved in pigmentation, melanocyte differentiation and solute transport. Gene ontology term enrichment and pathway analyses indicates cell adhesion molecule and lipid binding, regulatory region nucleic acid binding and the TGF β pathway as some of the pathways and processes significantly impacted by ACKR4 knockdown. Further investigation is currently focusing on the mechanisms by which the knockdown of ACKR4 results in these transcriptional and phenotypic changes observed in the B16 ACKR4 knockdown model.

3959

Intravital 2-photon imaging of the anti-leukemia immunity in living bone marrow

Mizuno, H., Yamashita, E., Ishii, M.

Osaka University, Immunology and Cell Biology, Suita, Japan

Within a living organism, the adaptive immune system, e.g. cytotoxic T lymphocytes (CTLs), induce apoptosis in tumor cells, and therefore limit tumor development. Bone marrow is a mysterious hidden place for different tumor cells and the anti-tumor immunity in the bone marrow is important because the specific microenvironment had been considered to contribute to escape of tumor cells from immune attack. However, the practical mechanism of anti-tumor immune elimination by CTLs in the bone marrow is still unclear. In this study, to elucidate how the CTLs response against tumor cells in bone marrow, we observed the interaction of leukemic cells and CTLs as well as CTL-induced apoptosis in the bone marrow using an intravital two-photon microscopy.

For visualizing the adaptive immune system, we used a fluorescent FRET-probe that allows us to monitor caspase-3 activity and also used the ovalbumin-OT-I CTL system for inducing antigen specific T cell immunity.

First we visualized anti-tumor immune responses facilitated by CTLs in vitro. In results, leukemic cell death was dependent on the total cell number of CTLs. The CTLs took 6 hours on average to induce apoptosis after capturing.

Secondly in mouse leukemia model, we observed that CTLs inducing apoptosis in leukemic cells upon directed cell-cell contact in the bone marrow by intravital 2-photon imaging.

Visualizing how tumor cells are killed by CTLs in vivo, especially in bone marrow, offers new perspectives for understanding

anti-tumor immune elimination and the mechanism of escape from immune attack.

4017

Engaging deactivated tumor associated macrophages against cancer cells through TLR-3 ligand stimulation via IFN- $\alpha\beta$ signaling

Vidyarthi, A.¹, Khan, N.², Agnihotri, T.¹, Agrewala, J.¹

¹Institute of Microbial Technology, Immunology, Chandigarh,

India, ²McGill University, Dept Micro & Immunol, Montreal, Canada

Macrophages are known for their indispensable role in eliciting inflammation, pathogen clearance and anti-tumor immunity. However during tumor progression, macrophages shift their functional phenotype and acquire the properties of M2 subtype, which exhibit suppressive properties. M2 macrophages exert anti-inflammatory and pro-tumorigenic activities and therefore are attractive targets for therapeutic intervention. The role of TLR-3 has been extensively studied as a vaccine adjuvants for cancer immunotherapy. The current study demonstrates the crucial role of TLR-3 ligand in reversion of M2 macrophages to a protective subtype M1. TLR-3 signaling downregulated M2 specific marker CD206 and simultaneously upregulated CD86, CD80, CD40 and MHCII, which are highly expressed on M1 phenotype. Further, TLR-3 stimulated M2 macrophages shift their cytokine profile from anti-inflammatory to pro-inflammatory, as evidenced by significant increase in IL-6, IL-12 and TNF- α yield. Noteworthy, TLR-3 signaling altered the M2 profiling via IFN- $\alpha\beta$ signaling. TLR-3 triggered M2 macrophages incubated with anti-IFN- $\alpha\beta$ R Abs failed to acquire M1 property. Interestingly, we observed that triggering through TLR-3 suppressed the expression of TIM-3 on M2c macrophages. Administration of TLR-3 agonist in murine tumor model, reverted the M2 to M1 phenotype and regressed the tumor growth. Conversely, mice pre-treated with anti-IFN- $\alpha\beta$ R failed to respond to TLR-3 administration and thus no regression in tumor growth was noticed. Overall, the results indicate that the signaling through TLR-3 may be quite crucial in imparting protection against tumors by skewing macrophages to M1 subtype. Consequently, this strategy has enough potential to be explored for treatment of cancer.

45 Minute Oral

12:30:00 - 13:15:00

Antigen Presentation

The role of Endoplasmic Reticulum Aminopeptidase 1 in NK cell function

Fruci, D.¹, D'Alicandro, V.¹, Romania, P.¹, Cifaldi, L.¹, Melaiu, O.¹, Falco, M.², Pende, D.², Locatelli F.¹

¹Paediatric Haematology/Oncology Department, IRCCS, Ospedale Pediatrico Bambino Gesù, Rome, Italy, ²Istituto Giannina Gaslini, IRCCS, Genoa, Italy, ³IRCCS AOU San Martino-IST, Genoa, Italy

Endoplasmic reticulum aminopeptidase 1 (ERAP1) is an ER-resident aminopeptidase that generates optimal peptides for presentation by MHC class I molecules. Several functional genetic variations of this peptidase are known to be associated with a large number of immune-mediated diseases and resistance to infectious disease. In vitro and in vivo studies have demonstrated that an altered ERAP1 activity results in substantial change of the peptide repertoire presented by MHC class I molecules. Consistently, we have shown that in mice ERAP1 abrogation results in a conformational change of MHC class I molecules that stimulates both innate and adaptive immune responses leading to the concerted rejection of a murine t T-cell lymphoma, which is otherwise refractory to immune elimination. In human tumor cell lines, genetic or pharmacological inhibition of ERAP1 perturbs the engagement of inhibitory NK cell receptors by their specific ligands, in each case leading to NK cell killing. The protective effect of peptide-MHC class I complexes was restored in ERAP1-deficient settings by replacement of endogenous aberrant peptides with correctly trimmed high-affinity peptides, suggesting that ERAP1 was needed to positively modify the affinity of natural ligands. Notably, ERAP1 inhibition enhanced the ability of NK cells to kill freshly established human lymphoblastoid cell lines from autologous or allogeneic sources, thereby promoting NK cytotoxic activity against target cells that would not be expected due to KIR-KIR ligand matching. Therefore, these results identify ERAP1 as a modifier to leverage immune functions to improve the efficacy of NK cell-based approaches for cancer immunotherapy.

MAIT Cells

MAIT cells: Friend or Foe in recognising microbial vitamin metabolites presented by the MHC-I-related molecule MR1

McCluskey, J.¹, Corbett, A.J.¹, Eckle, S.B.G.¹, Chen, Z.¹, Wang, H.¹, Sun, S.¹, D'Souza, C.¹, Kostenko, L.¹, Reantragoon, R.¹, Meehan, B.¹, Birkinshaw, R.W.², Liu, L.³, Patel, O.², Mahony, J.⁴, Cao, H.¹, Jackson, D.¹, Williamson, N.A.⁵, Strugnelli, R.A.¹, Mak, J.Y.W.³, Van Sinderen, D.^{4,6}, Fairlie, D.P.^{3,7}, Kjer-Nielsen, L.¹, Godfrey, D.I.^{1,7}, Rossjohn, J.^{2,7,8}

¹Department of Microbiology and Immunology, Peter Doherty Institute for Infection and Immunity, University of Melbourne,

²Department of Biochemistry and Molecular Biology, School

of Biomedical Sciences, ³Division of Chemistry and Structural Biology, Institute for Molecular Bioscience, The University of Queensland, ⁴School of Microbiology, University College Cork, ⁵The Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, ⁶Alimentary Pharmabiotic Centre, University College Cork, ⁷Australian Research Council Centre of Excellence in Advanced Molecular Imaging, ⁸Institute of Infection and Immunity, Cardiff University,

T cells discriminate between foreign and host molecules by recognizing distinct microbial molecules, predominantly peptides and lipids. Riboflavin precursors found in many bacteria and yeast also selectively activate mucosal-associated invariant T (MAIT) cells an abundant population of innate-like T cells in humans. We have shown that MAIT-cell activation requires key genes encoding enzymes that form 5-amino-6-D-ribitylaminouracil (5-A-RU), an early intermediate in bacterial riboflavin synthesis. MAIT antigens formed as simple adducts by the reactions between 5-A-RU and glyoxal/methylglyoxal, 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU) and 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU), bound to MR1 as shown by crystal structures of MAIT TCR ternary complexes. Although 5-OP-RU and 5-OE-RU are unstable intermediates, they became trapped by MR1 as reversible covalent Schiff base complexes. Thus, MR1 is able to capture, stabilize and present chemically unstable pyrimidine intermediates, which otherwise convert to lumazines, as potent antigens to MAIT cells. These pyrimidine adducts are created by microbes such as bacteria and yeast but not by humans or other mammals who obtain vitamin B from the diet rather than endogenous synthesis. MR1-5-OP-RU tetramers specifically identified MAIT cells demonstrating that they were rapidly enriched in the lungs of C57BL/6 mice infected with *Salmonella enterica* var Typhimurium, comprising up to half of all $\alpha\beta$ T-cells after one week. MAIT cell accumulation in the lungs was MR1-dependent a

Innate Regulation

Orphan nuclear receptors and regulation of innate immunity

Jo, E., Yuk, J. M., Kim, S. Y., Lee, H. M.

Chungnam National University School of Medicine

Nuclear receptors (NRs) are critically involved in various physiological responses through the regulation of numerous target genes. Orphan NRs are a subset of NR superfamily which ligands and functions have not been fully characterized. Emerging evidence has accumulated that several orphan NRs play critical roles in regulation of innate immunity to prevent harmful inflammatory responses in the host. The orphan NR small heterodimer partner (SHP, NR0B2) is a well-known corepressor of numerous other NRs, and functions in lipid/glucose metabolic homeostasis and regulation of endocrine function. Our recent studies showed that SHP was an important negative regulator of NLRP3 inflammasome activation through interaction with NLRP3 and translocation into mitochondria. Estrogen-related receptor α (ERR α ; NR3B1) is the first identified orphan NR that plays an important

role in regulation of energy metabolism and mitochondrial biogenesis. We found that ERR α was a novel regulator of the toll-like receptor-induced inflammatory response, with the unique capacity to modulate Tnfrsf3 transcriptional induction and p65 acetylation through metabolic reprogramming via enhancement of mitochondrial function. Unveiling the new and existing functions of orphan NRs could accelerate develop and improve novel strategies against human inflammatory diseases.

Lymphocyte Development

Dissecting the transcriptional regulatory network for early T-cell commitment

Rothenberg, E.¹, Kueh, H.Y.¹, Hosokawa, H.¹, Ng, K.K.H.¹, Mehta, A.^{1,2}, Pease, S.S.¹, Romero-Wolf, M.¹, Ungerback, J.¹, Wang, X.¹, Yui, M.A.¹, Zeng, W.B.³, Elowitz, M.B.¹, Mortazavi, A.³

¹California Institute of Technology Division of Biology & Biological Engineering, ²David Geffen School of Medicine at UCLA, ³University of California, Irvine, Department of Developmental and Cell Biology

Multipotent precursors proliferating in the thymus become committed to a T-cell fate when their access to alternative cell fates is intrinsically and unconditionally closed. This process involves very dynamic changes in transcription factor expression and activity: PU.1 and other factors expressed in multipotent progenitors are downregulated and are usually permanently silenced, while T-cell transcription factors, especially Bcl11b, are sharply activated. The abrupt activation of Bcl11b from a silent state is not only a landmark in T-cell differentiation, marking commitment, but also a functional contributor to commitment. Key target genes affected by Bcl11b function at this particular stage will be described: many of them appear to be under the control of Bcl11b as a repressor. Recent proteomic characterization of Bcl11b-containing protein complexes indicates the corepressor and coactivator partners that it uses to mediate different types of regulatory activity at different genomic sites. Since Bcl11b is such a powerful factor, a central question is how the onset of expression of Bcl11b itself is controlled. The talk will describe the combination of trans-acting factors that are needed to open the Bcl11b locus and activate it, and show that these factors each play slightly different temporal and functional roles in the activation process. Interestingly, evidence will also be shown for an epigenetic component of Bcl11b regulation that further slows its activation but helps to make it irreversible.

Mucosal Immunology

Homing of Immune cells via afferent lymphatics

Förster, R.

Hannover Medical School

Homing of immune cells to lymph nodes via specialized high endothelial venules (HEV) has been intensively studied during the past two decades. In contrast, little is known about molecules and pathways that allow immune cells to home via the second port of entry to lymph nodes i.e. via afferent lymphatics. We have recently developed intra lymphatic transfer of immune cells to identify the route and molecules involved that guide the various immune cell subsets that arrive via afferent lymphatics into the lymph node parenchyma. In this presentation I will give new insights into cues that target dendritic cells and T cells from the afferent lymph into lymph nodes.

13:30:00 - 15:10:00

Veterinary & Comparative Immunology

3516

Evolution of innate versus adaptive lymphoid cells in vertebrates

Hirano, M., Li, J., Rosenberg, C.S., Cooper, M.D.

Emory University School of Medicine, Pathology and Laboratory Medicine, Atlanta, United States

Mammalian innate lymphoid cells (ILCs) are early responders to pathogen entry at barrier surfaces, modulate development and function of antigen-specific T and B lymphocytes and may facilitate wound healing by reaction to tissue injury. In order to determine when the ILCs evolved, we purified antigen receptor-positive and -negative lymphocytes in the sea lamprey, a jawless vertebrate representative, and examined their expression of genes shown to be important for ILC development and function in mammals. We first compared the gene expression profiles for four sorted lymphocyte populations: variable lymphocyte receptor (VLR) A⁺, VLRB⁺, VLRC⁺ and triple VLR⁻ cells by quantitative real-time PCR analysis. We next divided the VLR⁻ lymphocyte population into two subpopulations by using LysoTracker[®] to identify NK-like cells rich in acidic intracellular vesicles (i.e., lysosomes). The lysosome-positive (Lyso⁺) VLR⁻ lymphocytes preferentially expressed *CD56*, *interleukin 23 receptor*, *Runx2* and *granzyme K/A* orthologues, whereas the Lyso⁻ VLR⁻ lymphocytes preferentially expressed *PLZF*, *Id2*, *c-Kit*, *GATA3* and *RORalpha*. Furthermore, by staining with a lamprey TLR5-specific monoclonal antibody, we found that Lyso⁺ VLR⁻ lymphocytes expressed high levels of TLR5 on their cell surface. Electron microscopic analysis of the TLR5^{high}/Lyso⁺/VLR⁻ cells indicated a remarkable morphological resemblance to mammalian NK cells. Our composite findings suggest that the basic genetic programs for T-like cells, B-like cells, NK-like cells and ILC2-like cells were already present in a common vertebrate ancestor ~500 million years ago.

1786

New insights from amphioxus: on the origins of the adaptive immune system of jawed vertebrates

Huang, S., Yuan, S., Xu, A.

Sun Yat-sen University, Guangzhou, China

Amphioxus (or lancelets) is an extant basal lineage of chordates, which is important for understanding the vertebrate evolution, including the origins of adaptive immunity. By comparing two amphioxus draft genomes, we reconstruct the protoMHC region in amphioxus, which shares high syntenic conservation with the human paraMHC regions, but shows a local rearrangement rate twice the average genome-wide rearrangement rate. This observation agrees with the MHC "big bang" hypothesis, which suggests that the MHC regions are the "arsenal" for creating many novel domains and domain combinations used by adaptive immunity. Furthermore, we discover a large number

of ancient transposon families in lancelets, many of which have been lost in vertebrates. We show that one of these ancient transposons is the long-sought RAG transposon, the co-option of which by jawed vertebrates was crucial to the origin of V(D)J rearrangement.

2799

Divergence of bacteria sensing (TLR 4, 5) and virus sensing (TLR 3, 7) within the reptilian lineage

Priyam, M.¹, Rai, U.¹, Ghorai, S.M.²

¹University of Delhi, Department of Zoology, Delhi, India, ²Hindu College, University of Delhi, Delhi, India

Toll-like receptors (TLRs) are the best characterized pattern recognition receptors in all vertebrate classes, with the exception of reptiles. It was only earlier this year that the first reptilian TLR - TLR5, was functionally characterized to demonstrate the divergent nature of TLR ligand recognition. In the current study, the evolution of the bacteria sensing and virus sensing TLRs in the reptilian lineage was assessed with respect to selection pressure acting on extracellular ligand recognition domain (ECD) and intracellular Toll/interleukin-1 receptor (TIR) domain. Six TLRs (TLR2, 3, 4, 5, 7 and 13) from each TLR family were identified in the wall lizard, *Hemidactylus flaviviridis*. The expression of TLR2, 7 and 13 was omnipresent across the five selected tissues (lungs, liver, spleen, kidney and ovary). Screening of splenic transcriptome data of *H.flaviviridis* gave the potential full length coding sequences of four TLRs- bacteria sensing TLR 4, 5 and virus sensing TLR3, 7. Alignment wide selection studies showed positive selection for ECD of TLR3, 4 and 5 while high degree of conservation was noted for TIR domain. Episodic selection was distinctly noted in both virus and bacteria sensing TLRs of reptilian lineage. Most of the positively selected sites on the ECD of TLRs were mapped to the LRR regions pointing towards specie-specific divergence of ligand recognition in TLRs. Overall, our findings showed a higher degree of episodic diversifying selection for TLR3 and TLR4 as compared to TLR7 and TLR5.

3521

Plasticity of MHC-driven responses in the California sea lion: an eco-immunological approach

Montano-Frias, J.E.¹, Alvarez-Martinez, R.¹, Soto-Garcia, L.A.¹, Flores-Moran, A.¹, Acevedo-Whitehouse, K.^{1,2}

¹Autonomous University of Queretaro, Basic and Applied Microbiology Unit, Queretaro, Mexico, ²The Marine Mammal Center, Sausalito CA, United States

Studies based on the Major Histocompatibility Complex (MHC) have reported associations with resistance and susceptibility to certain pathogens and diseases, but little is known about MHC transcription in an ecological context. The California sea lion (CSL) is a good model to understand the eco-evolutionary dynamics of a marine predator's immune responses. Taking into account that their 'pathogenic environment' and food availability may vary spatially and temporally, and that implementing immune responses is energetically costly, we hypothesize that transcription of MHC-II *ZacaDRB* genes in CSL pups

1) varies spatially due to ecological differences among rookeries, 2) is limited by MHC diversity, body condition and pathogen exposure, and 3) influences other immune responses, such as inflammation.

Constitutive and expressed diversities were calculated as the number of *ZacaDRB* genes present in the genomic DNA and buffy coat cDNA, respectively. Expressed diversity was partly explained by constitutive diversity (GLM, $F=18.96$, $df=154$, $p<0.001$, $adj-r^2=10.4\%$), regardless of body condition, age and sex. Expressed diversity was influenced by ecological variables among rookeries in the Gulf of California, such as presence of enteric viruses (GLM_{ADV} $F=5.27$, $df=43$, $p<0.05$; GLM_{RTV} $F=5.71$, $df=35$, $p<0.05$). Expression of particular loci was influenced by circulating eosinophils when enteric viruses were included in the models. Finally, transcription of certain loci was linked to PHA-induced inflammation, with patterns that varied markedly between ages. Life history constraints and physiological processes associated with development, in conjunction with a changing 'pathogenic environment' of the marine ecosystem appear to explain the observed plasticity of the MHC.

529

V_H and V_L from polyspecific IgM and monospecific IgG antibodies contribute differentially to antigen recognition and virus neutralization function

Pasman, Y., Kaushik, A.

University of Guelph, Molecular and Cellular Biology, Guelph, Canada

We analyzed role of individual variable heavy (FdVH) and variable light (FdVL) domains in comparison with VH+VL pair (scFv) originating from a polyspecific bovine IgM, with an exceptionally long CDR3H (61 amino acids), and a monospecific IgG1 antibody in antigen (Ag) recognition and virus neutralization functions. To this end, recombinant FdVH, FdVL and scFv were constructed and expressed in *Pichia pastoris* from bovine polyspecific IgM and IgG1 encoding cDNA. The scFv1H12 showed polyspecific antigen recognition similar to parent IgM antibody with minor differences. Unlike variable light domain FdVL1H12, variable heavy domain FdVH1H12 recognized multiple antigens that differed from scFv1H12 and the parent IgM antibody. Nevertheless, role of FdVL1H12 in providing structural support to FdVH in antigen recognition is noted. By contrast, the individual FdVH073 and FdVL074, originating from induced BoHV-1 neutralizing IgG1 antibody, recognized target epitope on BoHV-1 relatively weakly when compared to VH+VL pair as scFv3-18L. Both VH and VL domains of induced IgG antibody are required to achieve BoHV-1 neutralization function. To conclude, there exist subtle functional differences in relative contribution of VH and VL from polyspecific IgM and monospecific IgG antibodies in antigen recognition and virus neutralization functions.

[Supported by NSERC Canada]

3748

Th1 paradigm: redirect the cell-mediated immune response in a typical Th2 disease such as Leishmaniasis

Scarpona, S.¹, Berardi, S.¹, Bordicchia, M.², Rossi, G.¹

¹University of Camerino, School of Biosciences and Veterinary Medicine, Matelica (MC), Italy, ²University of Sydney, University Veterinary Teaching Hospital, Sidney, Australia

Many diseases are the result of the Th2-phenotype immune response directed against an antigen, by triggering an immune-complex disease. In this situation, the manifestation of disease is not closely related to the pathogen, but to an inappropriate host immune response. Leishmaniasis is one of the most classic examples. We investigated the possibility of clinical recovery in dogs with Leishmaniasis following stimulation of the Th1-type immune response.

The immunomodulation was based on the exaltation of cell-mediated immune response, by extract of Mycobacteria, and the restraint of phlogosis that follows, by antiCOX-2 molecules. This combination gives a notable expansion of cell-mediated cytotoxic effect.

Naturally-infected dogs were enrolled and treated with the immunomodulatory therapy (group A) and a previous reported conventional therapy (group B). All animals were evaluated by clinical, parasitological and immunological criteria to monitor treatment efficacy.

Clinical and laboratory results demonstrated differences in both humoral and cell-mediated immune responses between the two groups. They highlighted a Th2/Th1-shift in group A, together with a significant improvement ($p<0.05$) in all parameters, whereas group B showed variable clinical improvement in absence of any immune shift.

The approach to diseases characterized by Th2 phenotype should not be limited to fight the pathogen, but also aim to restore an appropriate host immune response. The stimulation with antigens totally divorced from the biomolecular type of *Leishmania* antigens, and the subsequent control of the disease, indicate that modulation of the immunophenotypical attitude of the host response can be a cutting-edge approach both in veterinary and human medicine.

3887

The role of T cells in highly pathogenic avian influenza in the chicken

Layton, D., Butler, J., Stewart, C., Bruce, M., Rootes, C., Gough, T., Rohringer, A., Wang, J., Wong, F., Williams, D., Bingham, J., Bean, A. CSIRO, Health and Biosecurity, Geelong, Australia

Highly pathogenic avian influenza (HPAI) viruses with a H5 subtype arise from poultry and can cause severe morbidity and mortality in humans. In May 2014, a new assortant of HPAI H5N6 (A/Duck/Laos/XBY004/2014 (H5N6)) emerged in Laos and in China, causing the death of several hundred poultry and one individual. We have assessed the infectivity, pathobiology and immunological response associated with HPAI H5N6 infection in chickens. Infection caused 100 % mortality in all birds within 30-48 hours of inoculation, with clinical signs including facial swelling, depression, hunching and fluffed feathers. High

virus titers were observed at necropsy in blood and all tissues, indicating systemic infection. Pro-inflammatory cytokines were greatly elevated in birds infected with H5N6 compared to non-infected control birds. Furthermore, we showed a significant decrease in proportion of CD8 T cell and an increase in IgM low and IgY positive B cells. Our study is the first to describe disease in chickens caused by HPAI H5N6 and demonstrates several pathological and immunological responses comparable to HPAI H5 subtypes.

4071

Th2/Th17 cytokines are involved in the early stage of immune responses during PEDV infection

Chen, J., Liu, G.

Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Animal Immunology Group, Lanzhou, China

Porcine epidemic diarrhea (PED) has resulted significant economic losses for pig industry in Asia, North America, and some European countries in recent years. Porcine epidemic diarrhea virus (PEDV), a member within the family *Coronaviridae* of the order *Nidovirales*, initiates the infection via small intestinal villous enterocytes and infected enterocytes rapidly undergo acute necrosis, leading to marked villous atrophy in the small but not in the large intestine. In order to elucidate the immune responses induced by PEDV infection within the small intestine, a PEDV LIX strain was employed to infect the IPEC-J2 cell lines, intestinal porcine enterocytes isolated from the jejunum of a neonatal unsuckled piglet. A whole set of cytokines were measured by Real-time RT-PCR with 12 hours interval between 12 hpi and 72 hpi. The results showed that ISGs and type I and type III IFNs were down-regulated upon PEDV infection. On the contrary, the expression of IL2, IL4, IL10, IL12, IL15, TGF β 1, IL17A, and GM-CSF were significantly elevated post PEDV infection with a time-dependent manner. These data indicated that PEDV infection activate Th2, Th17, and Treg, as well as inflammatory responses within the small intestines. In conclusion, Th2/Th17 cytokines are involved in the early stage of immune responses during PEDV infection.

3287

Regulation role of metalloprotease ADAM17 on porcine reproductive and respiratory syndrome virus infection

Wang, Y., Feng, L.

Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, China

Metalloprotease ADAM17 has been implicated in the control of cancer, inflammation, and other pathologies. In this study, we demonstrated for the first time that ADAM17 plays a critical role on controlling PRRSV infection. First, we found that porcine alveolar macrophages produced a great amount of soluble TNF α upon PRRSV infection, and this soluble TNF α in turn has great anti-PRRSV effect. By using inhibitor and genetic modification methods, we addressed that the production of porcine TNF α was mediated by ADAM17. Next, we found that inhibition of ADAM17 activity with specific inhibitor/siRNA facilitated PRRSV infection in different target cells. Furthermore, we revealed that

virus infection-activated ADAM17 can cleave the PRRSV receptor CD163, which thus hinders virus entry. Lastly, we discovered that PRRSV treatment also can stimulate porcine neutrophils to produce soluble form of CD16 (sCD16) *in vivo* and *in vitro*, which might serve as a biomarker for PRRSV infection. We then discovered that the production of sCD16 is also an ADAM17-dependent ectodomain shedding event. Taken together, PRRSV infection can stimulate ADAM17, which subsequently plays an anti-virus role through cleaving its substrates. Our findings suggest that activation of ADAM17 may be useful in enhancing the porcine anti-PRRSV response.

3578

Long lasting immunity against *Pasteurella multocida* in mice and cattle vaccinated with inactivated *Pasteurella multocida* and herbal adjuvant 'DIP-HIP'

Bhushan, B.¹, Shweta, S.², Tanwar, H.¹, Yadav, A.P.¹, Singh, S.B.¹, Ganju, L.¹

¹Defence Institute of Physiology and Allied Sciences, DRDO, Immunomodulation, Delhi, India, ²Delhi University, Nanotechnology Lab Department of Zoology, Delhi, India

Pasteurella multocida (PM) which causes Hemorrhagic septicaemia (HS) disease in cattles is a gram-negative coccobacillus belonging to Pasteurellaceae family. *P. multocida* and transmitted by ingestion or inhalation, either during direct contact or via fomites such as contaminated feed and water. Currently, there are no broadly protective vaccines with long-lasting immunity are available against HS. Aluminium-based minerals salts (alum) continue to be the only immunologic adjuvant approved by the US Food and Drug Administration (FDA) other than surfactant based oil adjuvant. Herbal adjuvants are being evaluated extensively to replace the classical adjuvants. Hence, herbal immunomodulators can be ideal candidates to elicit effective and long lasting immune response with minimum side effects. In the present study, herbal adjuvant DIP-HIP was evaluated with inactivated PM (iPM) antigen as vaccine in mice and compared with classical alum adjuvant. The results suggest that after single booster DIP-HIP + iPM emulsion gave maximum neutralizing antibody titre in comparison to HS alone or Alum + iPM. The LD₅₀ dose of PM was found to be 1 X 10⁷ CFU/ml and mice were challenged with this dose to assess efficiency of DIP-HIP adjuvant with iPM antigen in providing protection against HS. Taking cue from these findings, finally, seronegative calves were also vaccinated with DIP-HIP + HS and Alum + HS to evaluate vaccine efficacy. DIP-HIP containing formulation turned out to be broadly protective against HS with long-lasting immunity in cattle. Hence, DIP-HIP is a potential adjuvant which can be used in HS vaccines to provide long lasting immunity.

Tolerance

1049

Somatic hypermutation serves two independent functions: antibody maturation away from binding self and towards binding foreign

*Burnett, D., Schofield, P., Christ, D., Brink, R., Goodnow, C.
Garvan Institute of Medical Research, Darlinghurst, Australia*

Purging of all self-reactive B-lymphocytes from the naïve repertoire would create “holes” in the immune repertoire. A compromise may be to retain anergic self-reactive B-lymphocytes in the repertoire, able to be reawakened to undergo somatic hypermutation away from self-reactivity.

We generated transgenic mice that allowed *in vivo* tracking of B cells carrying a knock-in BCR with physiologically low starting affinity for a self-antigen referred to here as “S”. In transgenic mice with S on the surface of all haematopoietic cells, anti-S B cells emigrate to the spleen but show an anergy profile including surface IgM downregulation and reduced lifespan.

These mice were then challenged with an antigen of identical primary sequence but displayed on foreign sheep red blood cells (S*), where the xenogeneic cell carrier elicits strong T follicular help. Anergic anti-S B-cells were activated to form germinal centres, which accumulated recurring V-region mutations that decrease affinity to S and S*. Despite mutating away from self, autoantibodies that retained S-binding were secreted into serum. In contrast, when S* differed from S by six contact residue amino-acids, progeny of anti-S B cells acquired different mutations that decreased affinity for S but concurrently increased affinity for S*. The serum now contained IgG with high binding to foreign S* but low S-binding.

We have shown a novel mechanism for maintaining self-tolerance while maximising the repertoire of antibodies against foreign antigens. The results show that the germinal centre serves to mutate B-cells away from self-reactivity, independently to its role increasing foreign antigen affinity.

1188

Antigen-specific suppression by TCR and scFv CAR-engineered CD4 Tregs and cytotoxic CD8's

*Kim, Y.C., Zhang, A., Parvatheni, K., Scott, D.W.
Uniformed Services University of the Health Sciences, Medicine,
Bethesda, United States*

Clinical application of expanded T regulatory cells (Tregs) offers great promise for the treatment of undesirable immune responses. However, treatment with polyclonal Tregs, which are not specific for a single epitope, could potentially lead to global immunosuppression. We have engineered human T cells to express chimeric antigen receptors (CARs) using either T-cell receptors from MS or hemophilia patients or specific single chain Fv's. Thus, we adapted the CAR approach utilized successfully for leukemia therapy (e.g. CD19 CAR CD8's) to create specific T regulatory cells recognizing myelin basic protein (for MS) or FVIII, in the case of hemophilia. Such cells can actively suppress effector T cell proliferation and cytokine formation, as well as antibody formation *in vitro*. These cells can mediate

“bystander” suppression of multiple epitopes expressed locally and function even on the presence of strong inflammatory signals. Mechanistic studies suggest both contact dependent and contact-independent pathways. Further development with B-cell targeting strategies have also been successful by expressing antigen domains in CD8 T cells that kill B cells specific for FVIII, for example. Application of these engineered T cells to modulate adverse antibody responses in hemophilia and pathogenic responses to CNS proteins will be presented, and the mechanisms further discussed.

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3014

Consequences of CD79B and MYD88 mutations for B cell tolerance and lymphomagenesis

Wang, J.Q.^{1,2}, Batchelor, E.², Kaya, S.^{1,2}, Goodnow, C.C.^{2,3}, Horikawa, K.^{1,2}

¹John Curtin School of Medical Research, Australian National University, Department of Cancer Biology and Therapeutics, Canberra, Australia, ²John Curtin School of Medical Research, Australian National University, Department of Immunology and Infectious Disease, Canberra, Australia, ³Garvan Institute of Medical Research, Immunology Division, Darlinghurst, Australia

Mechanisms of B cell tolerance include the selective elimination of self-reactive B cells with B cell receptors (BCRs) that constitutively bind to self-antigen and the inactivation of Toll-like receptors (TLRs) that bind to self-ligands. BCR and TLR signalling involve the adaptor proteins CD79A/B and MYD88 respectively. *CD79B* and *MYD88* are frequently and concomitantly mutated in activated B cell type diffuse large B cell lymphoma (ABC-DLBCL). Since the survival of some human ABC-DLBCL cells requires BCR engagement by self-antigen, we investigated the consequences of *CD79B* and *MYD88* mutations for self-antigen-binding B cells. We transduced mature hen egg lysozyme (HEL)-specific B cells with retroviral constructs encoding *CD79B* and *MYD88* mutations and tracked the transduced B cells when adoptively transferred into HEL-expressing *Rag1*^{-/-} recipient mice, where the B cells were constitutively stimulated by self-antigen. While B cells that carried a single *CD79B* or *MYD88* mutation were eliminated from the recipient mice, B cells that over expressed either wild type or mutant *CD79B* together with *MYD88* mutation escaped deletion and differentiated into antibody producing cells. Using retroviral constructs that regulated *CD79B* expression, we demonstrated the number of accumulating B cells correlated with *CD79B* mutation status and increasing surface expression of *CD79B* and IgM, suggesting that protection of self-antigen-binding B cells was conferred by elevated BCR expression. Conversely, *MYD88* mutation alone caused decrease surface *CD79B* and IgM expression. Overall, our results demonstrated the cooperative effects of the frequently overlapping *CD79B* and *MYD88* mutations for B cell tolerance and how self-antigen stimulation may contribute to ABC-DLBCL.

3490

High-affinity disease-ameliorating autoantibodies in APECED patients reveal their origins

Kisand, K.¹, Meyer, S.², Hertel, C.², Vlaicu, P.², Macagno, A.², Kärner, J.¹, Trebusak Podkrajsek, K.³, Battelino, T.³, Krohn, K.⁴, Ranki, A.⁴, Wolff, A.S.B.⁵, Husebye, E.S.⁵, Peterson, P.¹, Hayday, A.C.⁶

¹University of Tartu, Tartu, Estonia, ²ImmunoQure AG, Düsseldorf, Germany, ³University of Ljubljana, Ljubljana, Slovenia, ⁴University of Helsinki, Helsinki, Finland, ⁵University of Bergen, Bergen, Norway, ⁶King's College London, London, United Kingdom

APECED (autoimmune polyendocrinopathy candidiasis ectodermal dystrophy) patients are characterized by multiorgan autoimmunity and chronic mucocutaneous candidiasis. Their autoimmunity is explained by impaired central T cell tolerance due to recessive *AIRE* gene mutations resulting in defective negative selection of tissue-specific thymocytes. However, one of the most striking features of APECED patients is the presence of multiple high-titer autoantibodies that target certain cytokines. So far studies describing the B cell compartment in relation to impaired central T cell tolerance are lacking. Flow cytometric study of circulating B cell subpopulations and T follicular helper cells revealed only slight alterations in adult but not pediatric patients compared to age-matched healthy individuals indicating to secondary changes caused by e.g. inflammatory process throughout the life. Monoclonal auto-antibodies isolated from patients' single B cells recognized native and conformational epitopes of studied cytokines, were mostly of extremely high (sub-picomolar) affinity, and broadly neutralizing. The sequences in the complementarity determining regions of both the heavy and light chain of all the isolated autoantibodies were heavily mutated and the reversion of these mutations completely abolished the binding of these „germline ancestor antibodies“ to their targets. This is consistent with normal central B cell tolerance in APECED patients, and with dysregulated germinal centre reaction that drives the affinity maturation of B cells initially primed to exogenous antigens. Moreover, we found that neutralizing capacity of serum autoantibodies reactive to type I interferons correlated inversely with the clinical presentation of type I diabetes in APECED patients indicating to disease-ameliorating properties of these autoantibodies.

1000

Actively acquired self-tolerance by clonal redemption of autoantibodies during human immunization

Reed, J.¹, Jackson, J.¹, Christ, D.^{1,2}, Goodnow, C.^{1,2}

¹Garvan Institute of Medical Research, Immunology, Sydney, Australia, ²St Vincent's Clinical School, UNSW Faculty of Medicine, Sydney, Australia

B cell clonal anergy is an enigmatic mechanism for actively acquiring self-tolerance because no apparent purpose is served by retaining functionally suppressed B cells bearing autoantibodies. We propose a novel tolerance mechanism, clonal redemption, allowing reactivation of anergic B cells and somatic hypermutation of autoantibodies away from binding self. To test this hypothesis, we traced the somatic evolution of three IGHV4-34*01 IgG antibodies isolated from healthy

donors immunized against foreign RhD alloantigen or vaccinia virus. Human antibodies with IGHV4-34*01 heavy chains are of interest as autoantibodies because they bind to a well-defined self-antigen, poly-N-acetyllactosamine carbohydrates (I/i antigen) on erythrocytes and B lymphocytes, cause cold agglutinin disease, and are carried by 5% of naive B cells that are anergic. Each IGHV4-34*01 IgG was expressed and analysed either in hypermutated immune state or after reverting to its unmutated pre-immune ancestor. In each case the pre-immune ancestor was self-reactive, binding intensely to normal human B cells expressing I/i antigen. Self-reactivity was removed by a single somatic mutation that paradoxically decreased binding to the foreign immunogen, while additional mutations conferred increased foreign reactivity. These data show that potentially pathogenic autoantibodies on human B cells can evolve into foreign-specific antibodies by somatic hypermutation during the course of normal immunisation. Since 2.5% of switched memory B cells use IGHV4-34*01 and >43% of these have mutations that remove I/i binding, clonal redemption of anergic B cells is an efficient process for specificity maturation and mutation away from self-reactivity during physiological human antibody responses.

1518

Myelin Oligodendrocyte Glycoprotein induces incomplete tolerance of CD4 T cells specific for both a myelin and a neuronal self antigen

Axisa, P.-P.^{1,2,3}, Lucca, L.E.^{1,2,3}, Aloulou, M.^{1,2,3}, Perals, C.^{1,2,3}, Ramadan, A.^{1,2,3}, Rufas, P.^{1,2,3}, Kyewski, B.⁴, Derbinski, J.⁴, Fazilleau, N.^{1,2,3}, Mars, L.T.^{1,2,3}, Liblau, R.S.^{1,2,3}

¹INSERM, U 1043, Toulouse, France, ²CNRS, U 5282, Toulouse, France, ³Université Toulouse 3, CPTP, Toulouse, France, ⁴Developmental Immunobiology, Tumor Immunology Program, German Cancer Research Center, Heidelberg, Germany

The paradigm that each T cell can recognize a continuum of related ligands, defined as T-cell polyspecificity, implies that multiple antigens can tolerize T cells specific for a given self-antigen. We previously showed in C57BL/6 mice that part of the CD4 T-cell repertoire specific for the encephalitogenic antigen myelin oligodendrocyte glycoprotein (MOG) 35-55 also recognizes the neuronal antigen neurofilament medium (NF-M) 15-35. Such bi-specific autoreactive CD4 T cells are frequent, produce inflammatory cytokines after stimulation and are essential to disease progression.

Since T cells recognizing two self-antigens would be expected to be more likely to be tolerized, we were prompted to study how tolerance to bi-specific T cells is imparted. We found that similar to MOG, NF-M is expressed in the thymus by medullary thymic epithelial cells, i.e. in a potentially tolerogenic setting. Nevertheless, we found that the frequency, pro-inflammatory phenotype, and capacity to transfer experimental encephalomyelitis (EAE) of MOG₃₅₋₅₅-reactive CD4 T cells were increased in MOG-deficient but not in NF-M-deficient mice. This observation led us to investigate the efficiency of NF-M₁₅₋₃₅ presentation by antigen presenting cells, which we found of short duration, suggesting unstable MHC class II binding. Consistent with this hypothesis, introducing an MHC-

anchoring residue into NF-M₁₅₋₃₅ (NF-M₁₅₋₃₅ T20Y) increased its immunogenicity, and activated a repertoire able to induce EAE. Our results show that in C57BL/6 mice the expression of MOG, but not of NF-M, restrains bi-specific autoreactive CD4 T cells, but not to a level sufficient as to prevent EAE induction.

2221

PD-1 upholds the unresponsive phenotype of self-reactive TCR- $\alpha\beta$ CD4⁺ CD8⁻ (double negative) T cells

Rodriguez-Rodriguez, N.^{1,2}, Apostolidis, S.³, Martin Villa, J.M.², Tsokos, G.³, Crispin, J.C.¹

¹Instituto Nacional de Ciencias Médicas y Nutrición 'Salvador Zubirán', Immunology and Rheumatology, Mexico City, Mexico,

²Universidad Complutense de Madrid, Madrid, Spain, ³Beth Israel Deaconess Medical Center, Boston, United States

Self-reactive CD8 T cells that encounter cognate antigen in peripheral tissues become inactivated, downregulate CD8, and express high levels of PD-1 becoming PD-1⁺ DN T cells. Moreover, a similar subpopulation of PD-1⁺ DN T cells induced by self-antigens is found in healthy normal mice. Because PD-1 maintains immune tolerance, we investigated whether PD-1 plays a role in the generation of DN T cells induced by self-antigen, or in the maintenance of their phenotype. PD-1 deficiency or blockade did not affect the conversion of self-reactive CD8 cells into DN cells, although it was associated to expansion of both autoreactive populations (CD8 and DN). The expression of master regulator transcription factors (i.e. T-bet, ROR- γ t, Eomes) was likewise not affected by the absence of PD-1. On the contrary, steady state proliferation and cytokine production in DN T cells was released by the deficiency of PD-1 or its blockade. Interestingly, blocking PD-1 engagement abrogated the upregulation of several transcription factors (i.e. EGR2 and Helios) implicated in T cell inactivation. In addition, blocking PD-1 induced a partial recovery of CD8 expression in DN T cells in the presence of antigen. Overall, these results highlight the importance of PD-1 in controlling the expansion and pro-inflammatory potential of self-reactive DN T cells and suggest that dysregulation of this important immune checkpoint may underlie the increased numbers of DN T cells in certain autoimmune diseases such as Sjogren's syndrome and systemic lupus erythematosus.

2524

T cells specific for posttranslational modifications escape negative selection in thymus

Raposo, B.¹, Merky, P.¹, Yamada, H.², Niaudet, C.³, Urbonaviciute, V.¹, Pinto, S.⁴, Kyewski, B.⁴, Holmdahl, R.¹, Backlund, J.¹

¹Karolinska Institute, Medical Biochemistry & Biophysics, Section for Medical Inflammation Research, Stockholm, Sweden, ²Kyushu University, Medical Institute of Bioregulation, Division of Host Defense, Fukuoka, Japan, ³Karolinska Institute, Department of Medical Biochemistry and Biophysics, Division of Vascular Biology, Stockholm, Sweden, ⁴German Cancer Research Center (DKFZ), Division of Developmental Immunology, Tumor Immunology Program, Heidelberg, Germany

In the thymus, tissue-restricted antigen (TRA) expression is to a large extent dependent on the expression of Aire in medullary thymic epithelial cells (mTECs). This promiscuous gene expression contributes to the deletion of auto-reactive T cells. In the current study, we addressed the possibility that this central tolerance mechanism may exclude tolerance to TRAs that are normally subjected to post-translational modifications (PTM). We report that Aire-dependent central tolerance to a model self-antigen indeed targets T cells specific to the native antigen in an efficient manner. However, T cells specific for the PTM epitope variant escape tolerance, despite the PTM variant of the auto-antigen being the physiological form in the periphery. As the majority of self-antigens is subjected to different forms of post-translational modifications, our findings highlight a potential general mechanism, whereby T cells can escape an otherwise efficient tolerance mechanism and contribute to the development of autoimmune diseases.

3010

A highly conserved NF- κ B-responsive enhancer is critical for thymic expression of Aire in mice

Peterson, P., Haljasorg, U., Bichele, R., Saare, M., Guha, M., Maslovskaja, J., Kond, K., Remm, A., Pihlap, M., Tomson, L., Kisand, K., Laan, M.

University of Tartu, Tartu, Estonia

Autoimmune regulator (Aire) has a unique expression pattern in thymic medullary epithelial cells (mTECs), in which it plays a critical role in the activation of tissue-specific antigens. The expression of Aire in mTECs is activated by receptor activator of nuclear factor κ B (RANK) signaling; however, the molecular mechanism behind this activation is unknown. Here, we characterize a conserved noncoding sequence 1 (CNS1) containing two NF- κ B binding sites upstream of the Aire coding region. We show that CNS1-deficient mice lack thymic expression of Aire and share several features of Aire-knockout mice, including downregulation of Aire-dependent genes, impaired terminal differentiation of the mTEC population, and reduced production of thymic Treg cells. In addition, we show that CNS1 is indispensable for RANK-induced Aire expression and that CNS1 is activated by NF- κ B pathway complexes containing RelA. Together, our results indicate that CNS1 is a critical link between RANK signaling, NF- κ B activation, and thymic expression of Aire.

3601

Immunological tolerance mechanisms controlling human autoreactive CD8⁺ T cells in healthy and autoimmune conditions

Barnaba, V.¹, Martire, C.¹, Cammarata, I.¹, Citro, A.¹, Peruzzi, G.², Guerrieri, F.², Scrivo, R.¹, Valesini, G.¹

¹Sapienza, Internal Medicine e Specialità Mediche, Rome, Italy,

²Istituto Italiano di Tecnologia, CLNS@Sapienza, Rome, Italy

We report that apoptotic self-epitope (AE)-specific CD8⁺ T cells, derived from activated T cells undergoing apoptosis, maintain a naïve (N) phenotype in both healthy individuals (HIs) and

patients with autoimmune diseases (ADs) (rheumatoid arthritis or multiple sclerosis) showing a low disease activity (ADs^{lo} pts) or efficiently responding to anti-TNF- α therapy; whereas they show an effector memory or terminally-differentiated (EM or EMRA) phenotype, as well as a higher TCR avidity, in ADs^{hi} pts non-responding to the therapy. The combination of *in vitro* and *in vivo* evidences indicates that high peripheral levels of CD40L⁺ apoptotic T cells contribute to the selection of high avidity AE-specific CD8⁺ TEM/EMRA cells. In addition, at the light of the observation of a direct correlation between the percentage of activated (act)Tregs and AE-specific CD8⁺ TN cell frequency *in vivo*, and of a series of experiments on isolated Treg and AE-specific CD8⁺ TN or TEM/EMRA cell populations *in vitro*, we provide evidence that actTregs condition a naïve phenotype in AE-specific CD8⁺ T cells (that efficiently proliferate when made free from the Treg suppression), but they are unable to suppress the expansion of the differentiated ones. These data suggest that a high repertoire of autoreactive T cells is not deleted and is governed by various overlapping mechanisms to avoid intolerance by autoreactive T cells in HIs or ADs^{lo} pts. Gene expression profile analyses are in progress to define more in depth differences in this repertoire, and to identify the molecular pathway whereby Tregs control autoreactive CD8⁺ T cells.

Vaccines 3

3682

Oral vaccines for transmissible prion diseases for multiple animal species at risk of infection

Goni, E.¹, Elisei, A.², Yim, L.³, Marta-Ariza, M.¹, Mathiason, C.⁴, Hoover, E.⁴, Chabalgoity, J.A.³, Wisniewski, T.⁵

¹New York University School of Medicine, Neurology, New York, United States, ²Instituto de Virologia INTA, Virologia, Hurlingham, Argentina, ³Facultad de Medicina Universidad de la Republica, Biotechnology-Laboratory for Vaccine Research, Montevideo, Uruguay, ⁴College of Veterinary Medicine and Biomedical Sciences, CSU, Microbiology, Immunology and Pathology, Fort Collins, United States, ⁵New York University Langone Medical Center, Neurology, Pathology, Psychiatry, New York, United States

We have developed an oral vaccination with PrP molecules delivered by an attenuated Salmonella carrier that was successful in preventing transmission and infection of prionoses in susceptible mice (Goni et al 2008, Neuroscience). The methodology was scaled up for testing in white tail deer, where we were able to elicit a distinct mucosal and systemic antibody response to prion proteins and achieve a partial protection to an oral challenge with a pathological CWD-prion (Goni et al 2015 Vaccine). We have now constructed six different PrP-like peptides that are able in the same Salmonella vaccine delivery system, or by controlled polymerization, to produce a sustained response against prion proteins from different species, using a reduced number of inoculations. The proof of concept study included a preparation of the Salmonella vaccines 10 days before inoculation, which we document to be viable (>90%) for at least a week at 4°C and subsequently for 36 hours at room temperature. Wild type CD-1 mice and transgenic mice

expressing sheep, elk or human PrP were inoculated weekly for three weeks with the oral preparations. A week after the last inoculation the animals were bled and fresh feces was collected. Each cohort of mice produced antibodies (both IgA and IgG) that cross-react with PrPs from different species. Production of these new vaccine formulations could be scaled up to be used in larger animals of any species and administered in enclosed facilities or potentially as bait in the wild.

2167

A dual Chikungunya and smallpox vaccine derived from a novel, replication-incompetent poxvirus vaccine system provides mice with complete protection from Chikungunya virus and mousepox infection

Hayball, J.¹, Cooper, T.¹, Liu, L.¹, Eldi, P.¹, Tan, M.¹, Prow, N.², Suhrbier, A.², Howley, P.³

¹University of South Australia, Pharmacy and Medical Sciences, Adelaide, Australia, ²Queensland Institute of Medical Research, Berghofer Medical Research Institute, Brisbane, Australia, ³Sementis Ltd, Melbourne, Australia

Chikungunya virus (CHIKV) is a re-emerging pathogen responsible for febrile illness often accompanied by a rash, and arthralgia that may persist for years. Recent outbreaks in Asia, Europe and the Americas have highlighted the urgent need for a vaccine. The proprietary SCV vaccine delivery system consists of a live replication-defective Vaccinia virus vector (SCV) and a co-developed, CHO-derived, rescue cell line for virus production. The CHIKV structural polyprotein (C-E3-E2-Gp6K-E1) was inserted into SCV to produce SCV-CHIKV. Viral attenuation and an improved safety profile was demonstrated compared to the replication-competent counterpart (VACV-CHIKV) whereby immune deficient SCID mice remained healthy following infection with SCV-CHIKV while mice infected with the replication-competent counterpart (VACV-CHIKV) accumulated an increasing clinical score from 6 days post-infection. Vaccination of BALB/c mice with SCV-CHIKV (10⁶ pfu, i.p) was able to confer complete immunity against mousepox infection (3x10⁵ pfu, s.c), although a 10-fold higher dose was required compared to Vaccinia virus. Anti-CHIKV IgG1 and IgG2c antibodies (endpoint titres >2x10³) were detected three weeks after a single vaccination with 10⁶ pfu in C57BL/6 mice. Mice were then challenged with CHIKV (2x10⁴ CCID₅₀; s.c in both hind feet) and SCV-CHIKV vaccination completely protected mice from the viraemia and foot swelling produced in naive or Vaccinia vaccinated mice. The protective immune responses against CHIKV were equivalent to those generated by VACV-CHIKV at the dose used. In summary, SCV-CHIKV is a promising smallpox/CHIKV vaccine candidate that demonstrates the potential of the SCV delivery system in vaccine development for infectious diseases.

1308**Developing a non-human primate model for skin vaccination**Sandgren, K.¹, Kim, M.¹, Baharlou, H.¹, Bertram, K.¹, Pavelsky, T.², Botting, R.¹, Harman, A.¹, Cunningham, A.¹¹Westmead Institute for Medical Research, Centre for Virus Research, Westmead, Australia, ²University of North Carolina, Geological Sciences, Chapel Hill, United States

Dermal vaccination appears increasingly promising as the dense network of immune cells in the skin, especially dendritic cells (DC), allows for enhanced immune responses and the opportunity to tailor vaccine responses by targeting specific subsets for activation. A macaque model for skin vaccination will be valuable for dissecting the immune response to novel vaccines, incorporating both resident and migratory immune cells and may be critical for HIV challenge studies, where no small animal model of infection exists.

To inform accurate design and interpretation of such trials, we are comparing the anatomy and immune cell composition of pigtail macaque skin to human skin.

Macaque epidermis was significantly thinner than human which means devices aiming to deliver vaccines to the dermo-epidermal junction may not be accurate in macaques, although there were equivalent numbers of Langerhans cells. By fluorescence microscopy, similar populations of DCs (CD1a⁺, CD14⁺, XCR1⁺) and T cells were present in macaque and human dermis although some frequencies differed. We identified potential macaque equivalents of human BDCA-3⁺ cross-presenting DCs as XCR1⁺CADM1⁺ and further transcriptomic analysis will confirm this. These cells were the dominant dermal DC population in macaques, unlike humans, which could mean cross-presenting effects of vaccines tested in macaques are enhanced compared to humans. Langerin⁺ dermal DCs were rare in humans but present in marked numbers in macaques although the function of this subset is yet unknown.

These results will help inform design of delivery devices and vaccine formulations targeting specific DC subsets in skin, for testing in macaque models.

1324**Therapeutic vaccination enhances 4-1BB-mediated anti-tumor CD8 T cell immunity against poorly immunogenic haematological cancers**Doff, B., Soon, M., Kobayashi, T., McKee, S., Mattarollo, S.
The University of Queensland Diamantina Institute, Brisbane, Australia

Immunomodulators are effective in controlling haematological malignancy by initiating host anti-tumor immunity to otherwise poorly immunogenic and immune suppressive cancers. We questioned whether immune checkpoint modulation was sufficient to control poorly immunogenic pre-clinical models of non-Hodgkin's B cell lymphoma (B-NHL) and acute myeloid leukaemia (AML). Enhancing T cell co-stimulation with agonistic anti-4-1BB monoclonal antibody (mAb) was effective at suppressing the development of spontaneous Eμ-myc B-NHL in transgenic mice, and the growth of transplanted Eμ-myc

tumor clones that expressed ovalbumin. However, anti-4-1BB mAb treatment had limited efficacy against transplanted Eμ-myc tumors that had been 'immuno-selected'. In this setting, combination immunotherapy incorporating an alpha-Galactosylceramide (α-GalCer) based therapeutic vaccine with anti-4-1BB mAb was required to control these poorly immunogenic tumors. We observed potent synergy with vaccine + immune checkpoint treatment combination resulting in significantly enhanced survival, including durable complete responses in over 50% of mice. Elimination of lymphoma burden was associated with increased expansion and persistence of KLRG1⁺ CD8 T cell effector subsets upon combination treatment. Similar therapeutic efficacy was observed against AML-ETO9a tumors. In addition, anti-4-1BB mAb treatment led to significant increase in surface PD-1 expression on activated CD8 T cells and PD-L1 expression on myeloid cells, suggesting that sequential administration of anti-4-1BB mAb co-stimulation and anti-PD1 mAb blockage may provide further enhanced T cell-directed control of blood malignancies. Overall, our results demonstrate a powerful immune adjuvant effect of α-GalCer that acts in synergy with agonistic anti-4-1BB mAb therapy for control of poorly immunogenic lymphomas and leukaemias.

2684**Design of intracellular cleavable immune-conjugates**Kramer, K.^{1,2}, Young, S.L.², Walker, G.F.¹¹University of Otago, School of Pharmacy, Dunedin, New Zealand,²University of Otago, Department of Pathology, Dunedin, New Zealand

Antigen-adjuvant conjugates have shown to elevate immune-responses over mixtures, we hypothesised that antigen-adjuvant conjugates designed to be cleaved inside the cell may further enhance immunogenicity. Cytosine-phosphate-guanosine oligodeoxynucleotide (CpG) was conjugated onto the model antigen ovalbumin (OVA) using either stable or reversible disulphide linking chemistry. Two reversible conjugates were generated with different degrees of steric hindrance around the disulphide bond in order to vary the sensitivity to cleavage via disulphide exchange reactions. Stability towards intracellular as well as extracellular reducing conditions using glutathione (GSH) was characterised by size-exclusion chromatography. Stability studies showed that one disulphide linker was cleaved under extracellular reducing (extracellular triggered) conditions (10μM GSH) while the second disulphide linker was stable to the extracellular reducing environment however was cleaved by intracellular reducing (intracellular triggered) conditions (5mM GSH). Murine bone marrow derived dendritic cells (BMDC) were stimulated with either PBS, CpG/OVA mixture or a CpG-OVA conjugate. OTI and OTII cells were co-cultured with the stimulated BMDCs. Immunogenicity of the conjugates was measured by T-cell proliferation and interferon gamma (IFN-γ) production in the co-culture. The CpG/OVA mixture and the extracellular triggered conjugate induced a low percentage of proliferated T-cells as well as low IFN-γ production while the stable and intracellular triggered conjugates resulted in high immunogenicity as confirmed by high percentage of T-cell proliferation and high IFN-γ production. This work demonstrates

that the steric hindrance around disulphide linkers influences whether they are cleaved by extracellular or intracellular GSH concentrations and this stability correlates with activation of T-cells in cell culture studies.

3997

Influenza vaccination with HA mini stem for broad antibody immunity

Valkenburg, S.¹, Mallajosyula, V.V.A.², Li, O.T.W.³, Chin, A.W.³, Varadarajan, R.², Poon, L.L.³

¹The University of Hong Kong, School of Public Health, HKU Pasteur Pole, Pokfulam, Hong Kong, ²Indian Institute of Science, Molecular Biophysics Unit, Bangalore, India, ³The University of Hong Kong, School of Public Health, Pokfulam, Hong Kong

A universal vaccine against influenza is needed to overcome antigenic drift and provide broad protection against outbreak and pandemic influenza viruses. We adopted the approach of protein minimization of the HA protein to focus the antibody response towards the functionally conserved epitopes of the HA stem. This HA-mini stem was then assessed in a mouse model to determine the protective efficacy of HA-stem specific antibodies. The H5 HA mini-stem protein refolds as a trimer, in a highly stable pre-fusion conformation representing the native conformation of the HA stem. Importantly, vaccination of mice with the H1 and H5 HA mini-stem resulted in significant protection from morbidity and mortality against challenge with homologous and heterologous influenza viruses, including group 2 influenza, H3N2 challenge. Our study is the first report of a Group 1 HA-stem vaccine showing Group 2 protection. Furthermore, passive immune sera transfer to naïve mice challenged with H5N1 demonstrated protection. However, virus micro-neutralization was observed by vaccine immune sera for H1N1, H7N7 and H3N2 viruses, but not H5N1 viruses, despite clear protection in vaccinated and passive transfer experiments for H5N1 viruses. This raises important issues for the role of stem-specific antibodies, and whether neutralization is a requisite for broad spectrum protection. We believe that this observation will provide critical research directions for developing novel universal influenza vaccines.

1947

Peptide-lipid nanovaccine for tumor imaging and immunotherapy

Zhang, Z.¹, Qian, Y.², Huang, C.², Shen, G.², Luo, Q.²

¹Huazhong University of Science and Technology, Wuhan National Laboratory for Optoelectronics, Wuhan, China, ²Huazhong University of Science and Technology, Department of Immunology, Tongji Medical College, Wuhan, China

By adopting a lipoprotein-mimicking peptide self-assembly principle, we created the high density lipoprotein (HDL)-like peptide-phospholipid scaffold (HPPS), a sub-30 nm core-shell lipid nanoparticle stabilized by apoA-1 mimetic peptides. The size of HPPS nanoparticle is tunable between 10-30nm depending on cargo payloads, which are shielded from leakage at physiological conditions until released intracellularly. The HPPS nanocarrier adds a new dimension to the core-shell

nanoparticle families particularly valuable for the intracellular delivery of imaging agents and therapeutic peptides to tumor. *In vivo* delivery of antigen peptide (Ap) to a specific type of immune cells especially dendritic cells (DCs), the most potent antigen-presenting cells, has gained increased interests in cancer immunotherapy field. Recently, we developed HPPS as a carrier to deliver antigen peptides to mature DCs in lymph node, which successfully elicited desiring anti-tumor immune response. The injection of Ap-HPPS allowed long-term tracking of its flow into lymph nodes using NIR imaging. More importantly, Ap-HPPS exhibited the ability to activate immature DCs and could be directly used as an effective targeting vaccine for *in vivo* cancer prevention without the need of immunologic adjuvants.

1197

Is the sustained release of vaccine antigens beneficial or detrimental to the development of immunity?

Bobbala, S., Gibson, B., Hook, S.

University of Otago, School of Pharmacy, Dunedin, New Zealand

Immunological dogma suggests that the sustained release of vaccine may be fraught with danger. If antigen is released at low levels in the absence of an adjuvant, tolerance may result. If antigen presenting cells (APCs) take up adjuvant in the absence of antigen, then autoimmunity may result. If antigen and adjuvant release is too high for too long then immune exhaustion may occur. However if a sustained release vaccine formulation could be developed which reduced the requirement for booster immunizations then the benefits would be enormous in terms of increased immunization completion and reduced costs.

We have been investigating if development of a sustained release vaccine is possible. In order to circumvent the issue of asynchronous release of antigen and adjuvant, vaccine is incorporated into a nanoparticle before being loaded into the sustained release formulation. The theory behind this is that intact nanoparticles will be released from the depot and taken up by APCs. The issue of the time required for optimal immune stimulation and the avoidance of immune exhaustion has been explored using systems with varied release kinetics. We have found that it is possible to develop systems that stimulate potent, long lasting, antigen specific CD4 and CD8 effector immune responses. With these systems the matching of the nanoparticle to the sustained release formulation is important as interactions between the systems will impact on release and the subsequent immune response. Finally, with the depot-forming systems we have investigated, vaccine incorporation in a nanoparticle is not always required.

1832

Mycobacterial lipoprotein Z exerts protection against *Mycobacterium tuberculosis* infection mediated by increased innate and adaptive immunity

Chen, Y.¹, Li, W.¹, Xiao, J.¹, Xiao, Y.¹, Xiong, Y.², Fan, X.², Wang, S.¹, Ji, P.¹, Shen, H.^{1,3}, Lu, S.², Wang, Y.^{1,4}

¹Shanghai Institute of Immunology, Shanghai Jiao Tong University School of Medicine, Department of Microbiology and Immunology, Shanghai, China, ²Shanghai Public Health Clinical Center Affiliated

to Fudan University, Key Laboratory of Medical Molecular Virology of MOE/MOH, Shanghai, China, ³Perelman School of Medicine, University of Pennsylvania, Department of Microbiology, Philadelphia, United States, ⁴Chinese National Human Genome Center at Shanghai, Shanghai-MOST Key Laboratory of Health and Disease Genomics, Shanghai, China

Due to less efficacy of Bacillus Calmette-Guérin (BCG), developing new vaccines against *M. tuberculosis* (*M.tb*) infection is still an urgent task. In the present study we have identified *M.tb* derived lipoprotein Z (LppZ) with high humoral and cellular responsiveness in pulmonary TB (PTB) patients when compared to healthy controls (HC), demonstrated by more LppZ-specific IFN- γ producing cells and higher titer of LppZ-specific antibody levels in PTB patients. The immunogenicity of LppZ in mice was determined furthermore. More LppZ-specific IFN- γ producing cells were generated in the mice immunized with LppZ emulsified in dimethyl dioctadecylammonium bromide (DDA) and monophosphoryl lipid A (MPL) mixture adjuvant when compared to adjuvant only group. Polyfunctional CD4⁺ T cells expressing two or triple cytokines, including IFN- γ , IL-2 and TNF- α , were apparently induced in the spleens of LppZ immunized mice. LppZ also exhibited strong capacity to recruit inflammatory cells and induce proinflammatory cytokine generation in a murine air pouch model. More importantly, LppZ provided more significant protection in mice challenged with the virulent H37Rv stain of *M.tb* after LppZ immunization than adjuvant alone or BCG vaccination. Apparent LppZ-specific Th1 immune responses were observed in the lungs of H37Rv challenged mice. Altogether, LppZ induces strong immune responsiveness upon *M.tb* infection both in human and mice. Its capacity to trigger inflammation reaction also implies its immune protection through innate immunity. LppZ is subjected to a novel candidate for subunit vaccine development against *M.tb* infection largely due to its capacity to induce strong innate and adaptive immunity.

3457

A two-component synthetic peptide vaccine can protect against deep tissue infection with CovR/S mutant and wild type group A streptococci

Good, M.F.¹, Pandey, M.¹, Mortensen, R.^{2,3}, Calcutt, A.¹, Powell, J.¹, Dietrich, J.², Batzloff, M.R.¹

¹Griffith University, Institute for Glycomics, Gold Coast, Australia,

²Statens Serum Institut, Department of Infectious Disease Immunology, Copenhagen, Denmark, ³University of Copenhagen, Department of Immunology and Microbiology, Copenhagen, Denmark

Group A streptococci [GAS] are major human pathogens responsible for over 500,000 deaths annually mostly due to rheumatic heart disease or invasive (iGAS) disease. Two major virulence factors are the M protein and the IL8 protease, SpyCEP. Organisms that have a mutation within their 2-component negative regulatory system, CovR/S, are responsible for up to one half of all iGAS disease. The virulence of mutants is due in part to their ability to block neutrophil chemotaxis by upregulating SpyCEP. Although mice can be protected against WT organisms

by vaccination with a conserved M protein peptide (J8, linked to diphtheria toxoid [DT]), mutant organisms are vaccine-resistant. We show that vaccination with a large recombinant fragment of SpyCEP induces antibodies that protect IL8; furthermore we identify a 20-amino acid epitope from SpyCEP ('S2') and show that S2 linked to DT induces antibodies of equal potency. We combined S2-DT with J8-DT to create a combination vaccine. Vaccine resistance was completely overcome. Within 3 days of skin challenge the bio-burden in skin, blood and spleen of vaccinated mice was reduced by up to 99% with all organisms being cleared within 6 days. Histology demonstrated a significant increase of neutrophils at the infection site. Thus, the vaccine enables neutrophils to enter the site to destroy organisms that were opsonized by antibodies targeting the M protein. The data suggest that this vaccine that targets independent virulence factors would rectify the lack of immunity to hypervirulent GAS in humans. The vaccine is in clinical trials.

Immunity to Viruses 4

1597

Deposition, protection and re-call of influenza virus specific memory CD8 T cells in the upper respiratory tract

Wakim, L.¹, Pizzolla, A.¹, Smith, J.¹, Brooks, A.¹, Reading, P.^{1,2}

¹Doherty Institute, The University of Melbourne, Microbiology and Immunology, Melbourne, Australia, ²World Health Organization Centre for Reference and Research on Influenza, Melbourne, Australia

Although the upper respiratory tract (URT) is the first site of contact for inhaled pathogens such as influenza virus, little is known regarding the site-specific immunity that can be evoked within this tissue following infection. Here we investigated the deposition, protection and re-call response of influenza virus-specific memory CD8 T cells in the upper respiratory tract. Our findings show that influenza virus-specific resident memory T cells (Trm) develop within the URT following intranasal influenza virus infection and persist long term with minimal decay. Using infection conditions that generate Trm only in the URT, we demonstrate that these cells are sufficient to prevent transmission of influenza virus from the URT to the lung. Given that effective generation of Trm in the URT is therefore likely to impact intranasal vaccine regimes, we investigated CD8 T cell priming and recall responses in the URT. Surprisingly, the mucosal associated lymphoid structures of the upper respiratory tract, termed nasal associated lymphoid tissue (NALTs), served as a site for the recall expansion of memory T cells following influenza virus infection, but failed to support activation of naive CD8 T cells. Together, these studies describe a unique Trm population in the URT which could be targeted for the development of novel strategies to limit the impact of influenza.

1590

Immune responses during acute allergic asthma promotes host protection against influenza a virus*Samarasinghe, A.^{1,2}, Melo, R.³, Duan, S.⁴, Lemessurier, K.¹, Liedmann, S.⁴, Lee, J.⁵, Thomas, P.⁴, Mccullers, J.^{1,6}**¹University of Tennessee Health Science Center, Pediatrics, Memphis, United States, ²St. Jude Children's Research Hospital, Infectious Diseases, Memphis, United States, ³Federal University of Juiz de Fora, Cellular Biology, Juiz de Fora, Brazil, ⁴St Jude Children's Research Hospital, Immunology, Memphis, United States, ⁵Mayo Clinic, Biochemistry, Scottsdale, United States, ⁶St Jude Children's Research Hospital, Infectious Diseases, Memphis, United States*

Allergic asthma caused by fungal sensitization is a prominent subtype affecting 70% of severe asthmatics. Asthmatics are generally considered to be more susceptible to respiratory viruses, and were identified as an 'at risk' group for hospitalization during the 2009 influenza pandemic. Retrospective studies showing that asthmatics were less likely to be admitted to the intensive care unit or die from influenza compared to non-asthmatics raised questions as to possible mechanisms for these seemingly counterintuitive findings. As a first step, we created and characterized a mouse model system that could be used to study influenza in severe fungal allergy. Herein we found that the timing of influenza virus infection determined the disease outcome. Mice infected during peak allergic inflammation were protected from influenza morbidity and had enhanced viral clearance compared to mice infected at the resolution of allergic inflammation. Since eosinophils were a prominent cell type in the airways of mice that were protected from influenza, we hypothesized that eosinophils promote host protection by enhancing cellular immunity. Eosinophils were susceptible to virus infection and underwent piecemeal degranulation in response, but were resistant to virus-induced cell death. Virus-exposed eosinophils upregulated antigen presenting machinery and were able to induce CD8⁺ T cell activation and proliferation *in vitro* and *ex vivo*. Adoptive transfer of eosinophils resulted in reduced lung viral burden, enhanced lung compliance, and higher virus-specific CD8⁺ T cells. Eosinophils were also able to prime naïve CD8⁺ T cells. These data suggest a possible mechanism for host protection against influenza virus in asthmatics.

3135

Initial Interactions of herpes simplex virus with human skin dendritic cells*Kim, M.¹, Truong, N.R.¹, James, V.², Sandgren, K.J.¹, Bosnjak, L.², Harman, A.N.¹, Nasr, N.¹, Bertram, K.M.¹, Olbourne, N.³, Sawleshwarkar, S.⁴, McKinnon, K.⁵, Cohen, R.C.⁶, Cunningham, A.L.¹**¹The Westmead Institute for Medical Research and The University of Sydney, Westmead, Australia, ²The Westmead Institute for Medical Research, Westmead, Australia, ³Sydney Institute of Plastic and Reconstructive Surgery, Chatswood, Australia, ⁴Western Sydney Sexual Health Centre Health Centre, Westmead, Australia, ⁵Western Sydney Sexual Health Centre, Westmead, Australia, ⁶The Children's Hospital, Westmead, Australia*

HSV initially infects the stratified squamous epithelium of the

anogenital mucosa prior to entering nerve endings. We have previously reported that topical application of HSV-1 to human foreskin explants shows epidermal Langerhans cells (LCs) are initially infected, then emigrate into the dermis where they formed large cell clusters with BDCA3⁺ dermal dendritic cells (DCs) and DC-SIGN⁺ DCs/macrophages. HSV-expressing LC fragments were observed inside the dermal DCs/macrophages. No other infected epidermal cells interacted with dermal DCs (Kim et al., Plos Pathogens, 2015). Therefore, we isolated LCs and dermal DCs from large abdominal skin specimens by flow sorting. LCs were pulsed with GFP tagged HSV and co-cultured with BDCA3⁺ dermal DCs. All infected LCs showed markers of apoptosis at 18 hr p.i. Approximately 50% of BDCA3⁺ DCs co-localized with infected LCs and in some cases fragments of infected LCs were observed within the dermal DC cytoplasm. HSV infected LCs secreted CCL3, 4, 5, IL8, TNF α and IP10 and BDCA3⁺ DCs CCL2 and 5 [by cytometric bead assay] as potential 'find me' signals. Further changes in gene expression in HSV-LC-BDCA3⁺DC cultures during these processes are being examined by RNAseq. In genital herpes lesions the selective contact of CD8 T cells with BDCA3⁺ DCs rather than other dermal DCs was observed. Thus, we conclude that a viral antigen relay takes place where HSV infected LCs undergo apoptosis and are taken up by dermal DCs by phagocytosis for subsequent antigen presentation probably via different pathways for CD4 and CD8 T cells.

3761

An epithelial integrin regulates the amplitude of protective lung interferon responses through a TGF β -dependent mechanism*Meliopoulos, V.¹, Van de Velde, L.-A.¹, Thomas, P.², Sheppard, D.³, Murray, P.⁴, Schultz-Cherry, S.⁵**¹St Jude Children's Research Hospital, Infectious Diseases, Memphis, United States, ²St Jude Children's Research Hospital, Immunology, Memphis, United States, ³UCSF Medical Center, Medicine, San Francisco, United States, ⁴St Jude Children's Research Hospital, Infectious Diseases and Immunology, Memphis, United States, ⁵St Jude Children's Research Hospital, Memphis, Infectious Diseases, Memphis, United States*

Integrins facilitate intercellular movement and communication. Unlike the promiscuous activities of many integrins, $\beta 6$ integrin is restricted to damaged and developing epithelia and partners exclusively with integrin αV to modulate acute lung injury (ALI). Given that ALI is a complication of respiratory infection, we used mice lacking $\beta 6$ integrin ($\beta 6$ KO) to probe the role of the epithelial layer in controlling the lung microenvironment during infection. We found $\beta 6$ KO mice were resistant to a range of influenza virus strains, Sendai virus and displayed a remarkable resistance to *Streptococcus pneumoniae* infection after prior H1N1 infection; the main cause of death from influenza. Although there was no difference in overall viral load, the lungs of influenza virus infected $\beta 6$ KO mice had decreased inflammation, lower cytokine/chemokine levels, improved lung function and phenotypically distinct macrophages as compared to wild-type (WT) mice. Mechanistically, resistance in the absence of epithelial $\beta 6$ integrin was caused by intrinsic priming

of the lung microenvironment by type I interferons due to the absence of $\beta 6$ -activated latent TGF- β . Addition of exogenous TGF- β one day pre-influenza virus infection in $\beta 6$ KO mice resulted in morbidity, mortality and a lung microenvironment similar to WT mice. Our studies suggest that expression of $\beta 6$ on epithelia leads to activation of lung TGF- β that suppresses the production of interferons primarily from macrophages, leading to a dysregulated lung environment and increased lung injury. Timely modulation of $\beta 6$ integrin activity is therefore a means to improve outcomes in lung viral infections.

849

Human mucosal-associated invariant T cells contribute to anti-viral influenza immunity via IL-18-dependent activation

Loh, L.¹, Wang, Z.¹, Sant, S.¹, Nguyen, O.¹, Jegaskanda, S.¹, Corbett, A.¹, Rossjohn, J.², Xu, J.³, McCluskey, J.¹, Kedzierska, K.¹

¹The University of Melbourne at the Peter Doherty Institute for Infection and Immunity, Melbourne, Australia, ²Monash University, Department of Biochemistry and Molecular Biology, Clayton, Australia, ³Fudan University Shanghai Medical College, Shanghai, China

Mucosal-associated invariant T cells (MAIT) are innate-like T cells, which rapidly produce inflammatory mediators during microbial stimulation, thereby can contribute to the cytokine milieu and modulation of other immune populations. However, their role during viral infections remains unclear. We speculated that MAIT cells (CD161⁺Va7.2⁺) were activated in patients hospitalised with the novel avian H7N9 subtype influenza virus (N=16), as evidenced by dynamic changes in their frequencies during the course of a disease. To assess whether MAIT cells were activated and the mechanism underlying MAIT cell activation during an influenza virus infection, we utilized an *in vitro* co-culture system of influenza A virus (IAV)-infected human lung epithelial cells (A549) and human peripheral blood mononuclear cells (PBMC) with intracellular cytokine staining. We compared influenza-induced MAIT cell activation to the activation induced in NK cells. We showed robust upregulation of IFN- γ and GzmB after IAV co-culture with MAIT cells and NK cells, although responses varied across subjects (1-48% IFN- γ ⁺MAIT, N=22; 0.7-30% IFN- γ ⁺NK, N=22). The activation of MAIT cells was dependent on inflammatory mediators upregulated during IAV co-culture, whereas NK cells required cell-associated contact in addition to inflammatory milieu for their activation. Depletion of CD14⁺ monocytes and blocking of IL-18, but not IL-12, abrogated optimal MAIT cell activation. Overall, our data demonstrates the capacity for MAIT cells to be indirectly activated by IAV via an IL-18 dependent mechanism and highlight their potential to contribute to universal immunity and/or immunopathology in influenza virus-infected individuals.

1881

Knockdown of p65 subunit of NF- κ B transcription factor downregulates the induction of pro-inflammatory cytokines in human lung epithelial cells infected with influenza H9N2 virus

Haghpour, A.¹, Farzin, H.², Toroghi, R.²

¹Ferdowsi University of Mashhad, Mashhad, Iran, Islamic Republic of, ²Razi Vaccine and Serum research Institute, Mashhad, Iran, Islamic Republic of

Inflammation is a hallmark feature of many influenza virus infections. To obtain insight into the inflammatory mechanisms involved in influenza H9N2 infection of human lung epithelial cells, A549 airway epithelial cells were infected with H9N2 virus and the expressions of pro-inflammatory cytokines (IL-1 β , IL-6) and chemokine (IL-8) in these cells were examined by qPCR and ELISA. Moreover, the effect of silencing of p65 component of NF- κ B in A549 cells infected with H9N2 virus on the expression and secretion of pro-inflammatory cytokines and chemokine and virus replication were evaluated by qPCR, ELISA, Immunocytochemistry and western blotting.

H9N2 virus was able to cultivate in the human lung epithelial cell line (A549) and stimulate production of IL-1 β , IL-6 and IL-8. Expressions of cytokine and chemokine genes were down-regulated to a significantly lower level (IL-1 β after 24hours (P < 0.1) and 48hours (P < 0.01), IL-6 after 24 hours (p < 0.01) and IL-8 after 24 hours (p < 0.05)) in p65 knocked down A549 cells as compared with scramble shRNA cultured cells infected with H9N2 influenza virus. The amount of IL-6 and IL-1 β proteins secreted into the culture medium was also decreased after silencing the p65 component of NF- κ B in A549 cells infected with H9N2 influenza virus in different multiplicity of infections (MOI).

The results presented in this study provide the mechanism by which H9N2 virus induce inflammation in human lung epithelial cells. These findings will broaden our understanding of host innate immune mechanisms and the pathogenesis of H9N2 influenza viruses in human respiratory epithelium.

2392

Human TRIM5 is a functional restriction factor for tick-borne flaviviruses

Chiramel, A.¹, Meyerson, N.², McNally, K.¹, Mendes-Solis, O.¹, Montoya, V.¹, Robertson, S.¹, Sturdevant, G.¹, Sawyer, S.², Best, S.¹

¹Rocky Mountain Laboratories, NIAID, NIH, Hamilton, United States, ²University of Colorado Boulder, Molecular, Cellular, and Developmental Biology, Boulder, United States

Tripartite motif (TRIM) proteins function as interferon-inducible virus- and host-specific restriction factors that can bind to viral proteins and interrupt virus replication. The flaviviruses include globally significant human pathogens such as tick-borne encephalitis virus (TBEV), West Nile virus (WNV) and dengue virus. Previous studies demonstrated that TBEV replication is inhibited by TRIM30D, a rodent-specific protein that functions by binding to the viral RNA polymerase (called NS5) and targeting it for degradation. The closest human paralog of TRIM30D is TRIM5, a renowned restriction factor thought to

be retrovirus-specific. Our study reveals that both human (h) and rhesus (rh) TRIM5 restrict replication of viruses belonging to the TBEV serogroup but not WNV. The impact of ectopically expressed rhTRIM5 on TBEV was profound, reducing production of infectious virus by more than 99%. hTRIM5 overexpression imposed a 90% reduction whereas knock-down of hTRIM5 partially rescued the antiviral effects of interferon treatment. TRIM5 reduced production of both viral RNA and protein, suggesting that TRIM5 targets an early step in virus replication. However, unlike TRIM30D, TRIM5 did not interact with NS5 and instead interacts with and degrades NS3, an RNA helicase critical to virus replication. Moreover, TRIM5 colocalized with NS3 and dsRNA suggesting that TRIM5 disrupts the viral replication complex. This work demonstrates that TRIM5 can target two very different viral proteins, the HIV capsid and the TBEV helicase. Understanding the genetic trade-offs in TRIM5 that enable restriction of one virus versus another will illuminate how the evolution of host resistance is shaped by multiple pathogens.

3432

Vaccine-elicited preferential induction of polyfunctional Th cells is associated with protection against acute retroviral infection

Motozono, C., Tsuji-Kawahara, S., Takamura, S., Miyazawa, M.
Kindai University Faculty of Medicine, Department of Immunology, Osaka, Japan

Understanding functional characteristics of antigen-specific CD4⁺ T-cell responses contributing rapid elimination of pathogenic retroviruses is crucial for the development of effective antiretroviral vaccines. We previously identified an MHC class II- restricted epitope derived from Friend mouse retrovirus envelope glycoprotein, peptide i_{18} , that completely protected a highly susceptible strain of mice against fatal Friend virus (FV) infection by a single immunization. Using this model, we investigated the characteristics of antigen-specific CD4⁺ T cell responses associated with immune protection against acute retroviral infection. We found that i_{18} -specific CD4⁺ T cells became detectable much earlier than those specific for peptide fn , another envelope epitope that elicited less effective protection. Associated with higher affinity of i_{18} -specific TCRs in comparison with fn -specific ones, clonotypically restricted i_{18} -specific CD4⁺ T cells with individually different variable regions were detected after FV challenge, and showed the ability to produce polyfunctional cytokines. Moreover, the priming with i_{18} resulted in more prominent induction of CXCR5⁺ PD-1⁺ Tfh cells upon FV challenge than fn -immunization. As a result, FV-neutralizing antibodies were produced earlier in mice immunized with i_{18} compared with those given fn . Taken together, these results indicate that the use of an appropriate epitope preferentially induces polyfunctional Th responses in the early phase of acute retroviral infection that contribute to the effective antiviral protection.

3897

Orchestration of nucleic acid sensing pathways in HSV-specific CD8⁺ T cell priming

Bachem, A., Whitney, P.G., Greyer, M., Tebartz, C., Gebhardt, T., Bedoui, S.

The University of Melbourne at the Peter Doherty Institute for Infection and Immunity, Department of Microbiology and Immunology, Melbourne, Australia

Dendritic cells (DC) not only present antigens to T cells but also communicate pivotal information about the environmental context in which antigen uptake has occurred. Precisely how these signals are integrated by DC and then decoded by T cells remains unclear. We have addressed this question using an *in vivo* model of cutaneous Herpes Simplex virus (HSV) infection. Efficient HSV-1-specific CD8⁺ T cell priming relied heavily on the provision of type I interferon (IFN). However, it has been shown that a plethora of sensing pathways can induce the secretion of type I IFN. Unexpectedly, we demonstrate that the most prominent nucleic acid sensing pathways, such as those depending on STING, MAVS, MyD88 and Caspase-1/11 played redundant roles in HSV-specific CD8⁺ T cell priming. The TLR3-TRIF pathway, however, was essential for this process. Here, we untangle the type I IFN subtypes that were induced, the transcription factors that were required and the effects these factors have on IFN-stimulated genes, such as IL-15. These findings demonstrate that our immune system has evolved several independent mechanisms to translate the complex sensing of pathogens into effective adaptive immune responses.

2719

Memory B cells recognising avian H5 and H7 influenza are widely prevalent - potential role as vaccine targets or as an immunotherapy

Wheatley, A.K., Kristensen, A.B., Ana-Sosa-Batiz, F.E., Vandervan, H.A., Kent, S.J.

University of Melbourne, Department of Microbiology and Immunology, Melbourne, Australia

Endemic H1N1 and H3N2 influenza viruses cause recurrent seasonal infections and significant global morbidity and mortality. Recently, zoonotic transmission has resulted in human infections with avian influenza strains such as H5N1, H10N8 and H7N9. Such infections are associated with high pathogenicity and lethality, and a lack of population level immunity raises significant concerns about pandemic potential. Low titres of serum antibodies binding H5 or H7 have been previously reported in unexposed individuals, however the protective potential of these responses remains unclear. Here, we used fluorescently-labelled, recombinant hemagglutinin (HA) proteins as flow cytometric probes to identify B cells specific for avian influenza in healthy unexposed subjects (N=18). Low frequencies of H5+ (0.02-0.12%) and H7+ (0.03-0.08%) B cells were found in all individuals studied to date, expressed surface IgA, IgM or IgG and displayed a classical resting memory phenotype (IgD- CD27+ CD21+). Rare B cells binding both H5 and H7 (0.002-0.046%) were also observed. Administration of the 2015 trivalent influenza vaccine drove a significant but

modest expansion of B cells recognising avian HAs. H5+, H7+ and H5+H7+ B cells were sorted and B-cell receptor transcripts sequenced. Representative antibodies were expressed and demonstrated in vitro neutralisation activity and/or non-neutralising effector functions such as antibody-dependent cellular cytotoxicity and antibody-dependent phagocytosis. Our results suggest seasonal influenza exposure primes, albeit inefficiently, pools of highly cross-reactive memory B cells that could

- (a) form a substrate targetable by improved vaccines and
- (b) provide a source of cross-reactive HA-specific antibodies with therapeutic potential against future pandemics.

Dendritic Cells 3

2052

XCR1+ dendritic cells promote memory CD8+ T cell recall upon secondary infections

Alexandre, Y.^{1,2,3}, Ghilas, S.^{1,2,3}, Sanchez, C.^{1,2,3}, Le Bon, A.^{4,5,6}, Crozat, K.^{1,2,3}, Dalod, M.^{1,2,3}

¹Aix Marseille Université UM 2, Centre d'Immunologie de Marseille-Luminy, Marseille, France, ²CNRS, UMR 7280, Marseille, France, ³INSERM, U 1104, Marseille, France, ⁴Université Paris Descartes, Institut Cochin, Paris, France, ⁵CNRS, UMR 8104, Paris, France, ⁶INSERM, U 1016, Paris, France

A subset of mouse dendritic cells (DCs) specifically express the chemokine receptor XCR1 and excels at priming CD8+ T cells including through cross-presentation. Naive CD8+ T cell priming during tumor development or first encounters with many intracellular pathogens requires cross-presentation by XCR1+ DCs. Memory CD8+ T lymphocytes (mCTLs) harbor a lower activation threshold as compared to naive cells. However, whether their recall responses depend on XCR1+ DCs is unknown. By using a new mouse model allowing fluorescent tracking and conditional depletion of XCR1+ DCs, we demonstrate a differential requirement of these cells for mCTL recall during secondary infections by different pathogens. XCR1+ DCs were instrumental to promote this function upon secondary challenges with *Listeria monocytogenes*, Vesicular Stomatitis Virus or Vaccinia virus, but dispensable in the case of mouse cytomegalovirus. We deciphered how XCR1+ DCs promote mCTL recall upon secondary infections with *Listeria*. By visualizing for the first time the in vivo choreography of XCR1+ DCs, NK cells and mCTLs during secondary immune responses, and by neutralizing in vivo candidate molecules, we demonstrate that, very early after infection, mCTLs are activated, and attracted in a CXCR3-dependent manner, by NK cell-boosted, IL-12- and CXCL9-producing, XCR1+ DCs. Hence, depending on the infectious agent, strong recall of mCTLs during secondary challenges can require cytokine- and chemokine-dependent cross-talk with XCR1+ DCs and NK cells. This work was funded by the European Research Council under the European Community's Seventh Framework Programme (FP7/2007-2013 Grant Agreement no. 281225 for the SystemsDendritic project).

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RUNX2 mediates plasmacytoid dendritic cell egress from the bone marrow and controls viral immunity

Chopin, M.¹, Preston, S.², Lun, A.³, Tellier, J.¹, Pelligrini, M.², Visvader, J.¹, Corcoran, L.¹, Wu, L.¹, Nutt, S.¹

¹Walter & Eliza Hall Institute, Molecular Immunology, Parkville, Australia, ²Walter & Eliza Hall Institute, Infection and Immunity, Parkville, Australia, ³Walter & Eliza Hall Institute, Bioinformatics, Parkville, Australia

Dendritic cells (DCs) have evolved into a diverse family of cellular subsets, each with their own distinctive features. Plasmacytoid DCs (pDCs) represent a unique immune cell type that responds to viral nucleic acids through the rapid production of type I interferons. Within the hematopoietic system, the transcription factor RUNX2 is exclusively expressed in pDCs and is required for their peripheral homeostasis. Here we show that RUNX2 deficiency plays an essential role in promoting pDC localization and function. RUNX2 is required for the appropriate expression of the integrin-mediated adhesion machinery, enabling adherence to the extracellular matrix. We also show that bone marrow pDCs are highly responsive to the chemokine CXCL12, with the down-modulation of its receptor CXCR4 by RUNX2 being required for pDC egress into the circulation. RUNX2 also facilitates the robust response to acute viral infection through the direct control of IRF7, the major regulator of the type I interferon production. Mice lacking one copy of *Runx2* have reduced numbers of peripheral pDCs and IFN α expression, which might contribute to the reported difficulties of individuals with Cleidocranial Dysplasia, who are haploinsufficient for RUNX2, to clear viral infections.

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Dendritic cell expressed ADAM10: a novel target for reducing Th2 immune responses

Damle, S.¹, Martin, R.¹, Lownik, J.^{1,2}, Conrad, D.¹

¹Virginia Commonwealth University, Department of Microbiology and Immunology, Richmond, United States, ²Virginia Commonwealth University, Center for Clinical and Translational Research, Richmond, United States

Allergy and allergic asthma are significant health burdens in developed countries and are increasing in prevalence. We previously demonstrated therapeutic potential of ADAM10 inhibitors in murine allergic airway inflammation. In this study, we examined the phenotype of a DC-specific ADAM10 knockout mouse model (A10DCKO) as DCs are critical in the development of Th2 responses. Using house dust mite (HDM) extract induced model of allergic airway disease, we found that the A10DCKOs had significantly less airway resistance and fewer eosinophils than wildtype (WT). Further, the A10DCKOs had less IL-4 and IL-13 cytokine expression in the lung, HDM-specific IgG1 in serum, and histological lung inflammation. Upon challenge with inhaled antigen, the A10DCKOs demonstrated less stimulation of antigen-specific T cell proliferation in the spleen, but not in the mediastinal lymph node. We recapitulated this *in vitro* with sorted splenic DCs and found less T cell proliferation with A10DCKO CD172⁺ DCs compared to WT. Whereas CD24⁺ DCs

from both mice stimulated T cells to similar extents. Intriguingly, CD172⁺ DCs are reduced in the A10DCKOs, similar to previous reports of the DC-specific Notch2KO. We demonstrated alterations in lung DCs subsets in the HDM model, which may also contribute to the effects on Th2 responses seen. In addition, splenic DCs from A10DCKO exhibited elevated levels of the co-stimulatory molecules, ICOSL and OX40L, but not CD86. Overall these results point to a mechanism involving both Notch and co-stimulatory molecules in the phenotype observed and point to a novel strategy for modulating Th2 immune responses.

2230

Targeting CLEC9A can deliver antigen to human CD141⁺ DC for recognition by both CD4⁺ and CD8⁺ T cells

Tullett, K.M.^{1,2,3}, Leal Rojas, I.M.², Minoda, Y.², Tan, P.S.^{1,3}, Zhang, J.-G.^{4,5}, Smith, C.⁶, Khanna, R.⁶, Shortman, K.^{4,5}, Caminschi, I.^{1,3,5}, Lahoud, M.H.^{1,3}, Radford, K.J.²

¹Monash University, Melbourne, Australia, ²Mater Research Institute - University of Queensland, Brisbane, Australia, ³Burnet Institute, Melbourne, Australia, ⁴Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia, ⁵University of Melbourne, Melbourne, Australia, ⁶QIMR Berghofer Medical Research Institute, Brisbane, Australia

Targeting vaccine antigens (Ag) to the dendritic cells (DC) that initiate cytotoxic T lymphocyte (CTL) responses is an attractive strategy for tumor immunotherapy or viral clearance. The mouse CD8⁺DC-lineage and their human CD141⁺ DC equivalents cross-present Ag and initiate CTL responses. The C-type lectin-like receptor CLEC9A, that senses dead cells and facilitates Ag cross-presentation, is specifically expressed on CD141⁺DC, making it an ideal target for such immunotherapy. Indeed, when injected into mice the anti-Clec9A antibody (Ab) can deliver Ag to CD8⁺DC resulting in potent humoral and T cell responses. We therefore developed anti-human CLEC9A chimeric human IgG4 Ab and assessed its ability to deliver conjugated CMVpp65 Ag to CD141⁺DC for recognition by CD4 and CD8

T cells. For comparison we generated chimeric IgG4 Ab specific for DEC-205, a molecule expressed on CD141⁺DC and many human leukocytes; anti-DEC-205 has already been used to target human and mouse DC. The chimeric anti-CLEC9A and anti-DEC-205 Ab retained specificity for their target receptors and were accumulated equivalently by CD141⁺DC. Both chimeric Ab delivered Ag to CD141⁺DC for presentation to CD4⁺ T cells *in vitro*. However, anti-CLEC9A Ab were superior at delivering Ag for cross-presentation to CD8⁺ T cells *in vitro*. Anti-CLEC9A and anti-DEC-205 Ab were comparable at delivering Ag to splenic CD141⁺DC for cross-presentation *in vivo* in humanized mice comprising functional human DC. Therefore, anti-CLEC9A Ab are at least as effective as anti-DEC-205 at Ag delivery to CD141⁺ DC. The superior specificity of anti-CLEC9A Ab for this DC subset warrants further development for vaccines.

1352

Enhanced activation of human CD141⁺ DC and CD1c⁺ DC in vivo with combined TLR3/TLR8 ligation

Chang, K., Minoda, Y., Leal Rojas, I., Tullett, K., Radford, K. Mater Research Institute-University of Queensland, Brisbane, Australia

Mice reconstituted with human hematopoietic stem cells (humanised mice) are a practical model to study aspects of the human immune system. We developed a humanised mouse model in which fully functional human DC subsets, including CD141⁺ and CD1c⁺ DC and plasmacytoid (p)DC, develop from human CD34⁺ cord blood progenitor cells in immunodeficient mice. CD141⁺ DC are the human equivalents of the mouse CD8⁺ DC that are essential for the induction of cytotoxic T lymphocyte responses against cancers and many pathogens, making them attractive targets to exploit for the development of new vaccines. We used humanised mice to investigate activation of CD141⁺ DC by poly I:C and R848, agonists for TLR3 and TLR8 respectively, which are both expressed by CD141⁺ DC.

The transcriptome of CD141⁺ DC from humanised mice aligned with that of CD141⁺ DC found in human tissues, confirming this as a robust and practical model. Activation with either poly I:C or R848 upregulated costimulatory molecules CD40, CD80, CD83 and CD86 by CD141⁺ DC and CD1c⁺ DC to a similar extent *in vivo* and the combination of poly I:C and R848 further enhanced costimulatory molecule expression by both subsets. Poly I:C and R848 combined resulted in higher levels of IFN- λ and IL-12 in the serum compared to either agonist alone. Moreover, CD141⁺ DC activated with Poly I:C and R848 were more effective at priming naive antigen-specific CD8⁺ T cells compared to CD1c⁺ DC. These data suggest that poly I:C and R848 combined is an effective adjuvant for CD141⁺ DC.

3362

RelB-deficient dendritic cells have increased retinoic acid dehydrogenase activity and promote regulatory T cell differentiation in mice and humans

Nel, H.J., Ruscher, R., Boks, M., Thomas, R., O'Sullivan, B. UQDI, TRI, Brisbane, Australia

Peripherally-derived Foxp3⁺ regulatory T (pTreg) cell generation from naive T cells in the TGF- β -enriched intestinal environment depends on specialized dendritic cells (DCs). RelB-deficient mice have an increased proportion of Treg cells in the spleen despite their thymic atrophy and lack of lymph nodes and Peyer's patches. The mechanism by which RelB influences pTreg is incompletely understood. We demonstrate in RelB^{-/-} mice, that antigen-exposed RelB^{-/-} DCs induce significantly more Tregs from adoptively-transferred CD4⁺CD25⁻ precursors than RelB^{+/-} mice. This was recapitulated *in vitro*. Treg induction was TGF- β - and retinoic acid (RA)-dependent. RA-metabolizing *Raldh2* enzyme expression and ALDH activity were increased in RelB^{-/-} relative to RelB^{+/-} splenic DCs. In naive mice, these ALDH⁺ DCs developing in the absence of RelB generated pTreg cells in the spleen in the context of high TGF- β production. To determine the relevance to immunotherapy, we compared human monocyte-derived DCs generated in the absence or presence of calcitriol, a

selective suppressor of RelB. Similar to RelB^{-/-} DCs, ALDH activity, *ALDH1A2* and RA-responsive transcription factors *RARA* and *RXRA* expression were increased and LPS-induced costimulatory molecule expression was decreased in calcitriol-modified human DCs. In allogeneic mixed lymphocyte cultures, T cell proliferation decreased and IL-10⁺ regulatory T cells increased in response to calcitriol-modified, relative to untreated DCs. These data indicate that RelB deficiency or pharmacological inhibition promotes RA metabolism in DCs and RA- and TGF- β -dependent induction of regulatory T cells and support clinical application of DC targeting with RelB inhibitor and antigen.

1428

Developmental heterogeneity of splenic CD11b⁺ dendritic cells

Schraml, B.¹, van Blijswijk, J.², Snelgrove, K.², Rogers, N.², Reis e Sousa, C.²

¹Klinikum der Universität München, Walter-Brendel-Centre for Experimental Medicine, Planegg-Martinsried, Germany, ²The Francis Crick Institute, Lincoln's Inn Fields Laboratory, London, United Kingdom

Early life immune balance is essential for survival and establishment of healthy immunity in later life. In neonates, dendritic cells (DCs), which are versatile controllers of immunity, are qualitatively distinct from adults. Newborns have an underdeveloped DC compartment when compared to adults, containing not only fewer DCs, but also an altered ratio of DC subsets. Why such age-dependent differences exist is unclear but newborn DCs are considered underdeveloped and functionally immature.

We have recently generated a model to fate map conventional DC precursors (CDP) with yellow fluorescent protein (YFP) and have revisited the development of DCs during mouse embryogenesis, as well as in perinatal and adult mice. We found that cells resembling adult CD11c⁺MHCII⁺ DCs were present in spleen as early as embryonic day 17. Surprisingly, CD11c⁺MHCII⁺CD11b⁺ splenocytes, which phenotypically resemble CDP-derived CD11b⁺ DCs that label strongly with YFP in adult mice, were poorly labeled in embryonic and neonatal mice. However, the labeling frequency increased gradually overtime, reaching adult levels by five weeks of age, when the splenic DC pool is fully established. Thus, splenic CD11c⁺MHCII⁺CD11b⁺ DCs are predominantly CDP-derived in adults, yet cells with similar phenotypic characteristics but distinct ontogeny populate the spleen in young mice. Preliminary data indicate that these ontogenetically distinct DCs exhibit functional differences. Thus, our studies reveal a previously unappreciated developmental heterogeneity of splenic CD11b⁺ DCs in young mice. Characterizing how developmentally regulated DC poiesis shapes the unique features of early life immunity will provide novel insights into immune development.

2078

Defining CD16⁺ dendritic cells as a unique myeloid antigen presenting cell

Fromm, P.¹, Papadimitriou, M.¹, Hsu, J.¹, Larson, S.², Gibson, J.², Bradstock, K.³, Kupresanin, F.¹, Clark, G.¹, Hart, D.¹

¹ANZAC Research Institute, Dendritic Cell Research, Sydney, Australia, ²Royal Prince Alfred Hospital, Department of Haematology, Sydney, Australia, ³Westmead Hospital, Blood and Bone Marrow Transplant Service, Sydney, Australia

Dendritic Cells (DC) are phenotypically identified in human blood as HLA-DR⁺ cells, which lack major cell surface lineage markers. As part of the Human Leucocyte Differentiation Antigen Workshop, we demonstrated that myeloid antigen presenting cells including monocytes and DC display a continuum of CD14 and CD16 expression. This limits the robustness of accurate DC and monocyte identification, particularly when identifying cell subsets with little or no surface CD14. Application of poisson counting statistics established that rare cell types such as CD16⁺ DC are often overlooked in analyses powered to detect much larger populations such as classical and non-classical monocytes.

We used fluorescent and mass cytometry approaches, in conjunction with high dimensional clustering, to demonstrate that the continuum of CD14 expression separates CD14^{lo} "non-classical monocytes" and CD14⁺ DC. We have defined the CD14⁺CD16⁺ DC as both phenotypically and transcriptionally different to other CD16⁺ monocyte populations. The CD16⁺ DC have different functional responses to antigen-uptake, processing and presentation of antigen by MHC compared to other primary blood monocytes and DC populations. They have a limited capacity for further differentiation. We monitored leucocyte recovery in hematopoietic cell transplant patients after myeloablative conditioning and showed that the recovery of CD16⁺ DC followed similar kinetics to other myeloid cell populations. Interestingly, our analysis of reconstituting myeloid populations in transplant patients identified the early differentiation and activation of circulating CD16⁺ DC as predicting for the onset of acute graft versus host disease. Thus, CD16⁺ DC represent an important clinically relevant human DC population.

932

Tolerogenic dendritic cells generated by activin-A-induced regulatory T cells protect against allergic asthma through the instruction of Foxp3⁺ regulatory T cells

Semitekolou, M.¹, Morianos, I.¹, Sparwasser, T.², Xanthou, G.¹

¹Biomedical Research Foundation of the Academy of Athens, Athens, Greece, ²Institute of Infection Immunology, TWINCORE, Centre for Experimental and Clinical Infection Research, Hannover, Germany

Our previous studies have uncovered the cytokine activin-A as a novel inducer of regulatory T cells (activin-A-iTregs) that protect against experimental asthma. Still, the cellular and molecular mechanisms underlying the suppressive functions of activin-A-iTregs remain elusive. In the present study, we hypothesized that activin-A-iTregs inhibit Th2 cell-driven responses through

induction of tolerogenic dendritic cells (DCs). For this, we adoptively transferred allergen-primed activin-A-iTregs into immunodeficient mice and examined their effects on CD11c⁺DC responses.

Our findings reveal that act-Treg-modified DCs exhibit an immature phenotype, reflected by low expression of MHC-II, CD80, CD86 and CCR7, impaired capacity to uptake antigen and traffic to the draining lymph nodes and attenuated IL-12, IL-6 and TNF- α release in response to LPS stimulation. Moreover, act-Treg-modified DCs display poor immunostimulatory potential, exemplified by decreased ability to prime the proliferation and cytokine release by allergen-specific Tg(DO11.10) T responders *in vitro* and upon adoptive co-transfer *in vivo*. Importantly, administration of act-Treg-modified DCs protects against experimental asthma both in preventive and therapeutic protocols mainly through the *de novo* generation and expansion of Foxp3⁺ Tregs. In fact, depletion of CD4⁺Foxp3⁺Tregs in DEREK mice before act-Treg-modified DC transfer hampers the suppressive capacities of act-Treg-modified DCs, demonstrated by exacerbated asthma manifestations and overactive Th2 cell responses. Disruption of PD-1 signaling, hinders the capacity of activin-A-iTreg cells to generate tolerogenic DCs, highlighting the involvement of the regulatory PD-1/PDL-1 pathway. Our findings unravel a novel immunoregulatory circuit encompassing activin-A-iTreg cells, tolerogenic DCs and Foxp3⁺ Tregs critical for the regulation of allergic asthma.

4646

A long noncoding RNA attenuates dendritic cell function and decreases autoimmune disease susceptibility

Brodnicki, T.¹, Ashton, M.², Elso, C.¹, Mackin, L.¹, Chu, E.¹, Ford, S.³, Payne, N.³, Alsayb, M.¹, Thomas, H.¹, Mannering, S.¹, Kay, T.¹, Papenfuss, A.⁴, Kitching, A.R.³, Bernard, C.³, Acha-Orbea, H.⁵, Morahan, G.⁶, Shortman, K.⁴, O'Keeffe, M.³

¹St Vincent's Institute, Fitzroy North, Australia, ²Technische Universität Dresden, Dresden, Germany, ³Monash University, Clayton, Australia, ⁴The Walter and Eliza Hall Institute, Parkville, Australia, ⁵University of Lausanne, Epalinges, Switzerland, ⁶Harry Perkins Institute of Medical Research, Nedlands, Australia

A complex network of mechanisms allows appropriate immune responses to pathogens while preventing destruction of one's own tissues. Cell-based studies have demonstrated the potential for long noncoding RNAs (lncRNAs) to regulate innate immune responses, but their effect upon immune tolerance *in vivo* is poorly understood. lncRNAs are broadly defined as RNAs that are >200 nucleotides in length and perform molecular functions without encoding proteins. We have identified more than fifty putative murine lncRNAs that are differentially expressed in dendritic cells upon toll-like receptor (TLR) activation. We found that genetic disruption of one of these lncRNAs increases autoimmune disease susceptibility. This lncRNA is detected in the nucleus and cytoplasm of dendritic cells after induction by TLR activation, and binds SHIP1, an inositol phosphatase that regulates dendritic cell function. Upon TLR activation, dendritic cells deficient for this lncRNA exhibit enhanced cytokine production associated with decreased SHIP1. Our findings highlight a role for lncRNAs in promoting immune tolerance by

stabilizing regulatory proteins and attenuating specific innate immune responses that may otherwise precipitate pathological tissue destruction.

B Cells 3

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Where and how memory cells are reactivated in the secondary antibody response in the lymph node

Moran, L., Nguyen, A., Brink, R., Phan, T.G.

Garvan Institute of Medical Research, Immunology, Sydney, Australia

Despite the importance of immunological memory, the precise mechanism of memory B cell activation is still largely unknown. To address this our lab has undertaken *in situ* studies of unmanipulated memory T and B cells in their native environment in immune animals. Utilising two photon microscopy, we identified a novel population of memory CD4⁺ T cells, T follicular memory (T_{FM}) cells, that occupy a unique niche in the subcapsular region of the B cell follicle of the draining lymph node (dLN). Within the dLN, T_{FM} cells scan CD169⁺ subcapsular sinus (SCS) macrophages and may be reactivated by them to initiate the memory antibody response. We now describe an equivalent population of lymph node resident B follicular memory (B_{FM}) cells which can be re-activated by antigen captured and presented on SCS macrophages, mirroring the role of SCS macrophages in activating naive B cells to initiate the primary antibody response. These reactivated memory B cells expand and differentiate into short-lived plasmablasts in subcapsular proliferative foci (SPF), a novel structure that is distinct from CD35⁺ secondary germinal centres. Thus, these SPF facilitate the initial antigen reactivation of LN resident T_{FM} and B_{FM} cells by SCS macrophages. These studies highlight the importance of microanatomical location in the orchestration of a rapid and efficient secondary antibody response.

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ICOSL-controlled regulation of germinal center formation and relative affinity-based plasma cell fate determination

Liu, D.¹, Xu, H.^{1,2}, Shih, C.¹, Wan, Z.¹, Ma, X.³, Ma, W.¹, Luo, D.^{1,4}, Qi, H.¹

¹Tsinghua University, School of Medicine, Beijing, China, ²Cincinnati Children's Hospital Medical Center, Cincinnati, United States,

³Tsinghua University, School of Life Sciences, Beijing, China,

⁴Jiaotong University, College of Life Sciences & Bioengineering, Beijing, China

The germinal center (GC) reaction supports affinity-based B cell competition and generates high-affinity bone-marrow plasma cells (BMPC). Follicular T-helper (TFH) cells are specialized in regulating the GC formation and the high quality of the GC output, but the mechanism is not clear. A genetic deficiency in the inducible co-stimulator ligand (ICOSL) causes profound germinal center defects, leading to the view that ICOS-ICOSL signal induces the T_{FH} differentiation program to affect GC formation. Unexpectedly, we find that only ICOSL on follicular bystander B cells is sufficient and necessary for the GC formation.

As for antigen-specific ICOSL signal, intravital imaging aided by a calcium reporter reveals that ICOSL promotes an 'entangled' mode of T_{FH} -B cell interactions, characterized by brief but extensive surface engagement, productive T-cell calcium spikes, and B-cell acquisition of CD40 signals. ICOSL on GC B cells is upregulated by CD40 signals. Such an intercellular positive feedback between contact-dependent help and ICOSL-controlled entanglement promotes positive selection and BMPC development, as evidenced by observations that higher-affinity B-cell receptor variants are enriched in the ICOSL^{high} fraction, and that when GC competition proceeds without ICOSL, selection of high-affinity variants in GC reactions is impaired. By identifying different role of ICOSL in GC responses, our study reveals a pathway by which TFH cells control the quality of long-lived humoral immunity.

2496

NFκB1 prevents the development of multi-organ autoimmunity by limiting IL-6 production in follicular B cells

de Valle, E.^{1,2}, Grigoriadis, G.³, O'Reilly, L.⁴, Willis, S.⁴, Maxwell, M.¹, Tsantakis, E.¹, Cornish, J.¹, Fairfax, K.⁴, Vasanthakumar, A.⁴, Gugasyan, R.²

¹Central Clinical School, Monash University, Immunology, Melbourne, Australia, ²Burnet Institute, Centre for Biomedical Research, Melbourne, Australia, ³Hudson Institute of Medical Research, Centre for Cancer Research, Melbourne, Australia, ⁴Walter & Eliza Hall Institute of Medical Research, Melbourne, Australia

NFκB1 (p50/p105) is a member of the NFκB family of transcription factors, and signaling through this pathway regulates numerous target genes that control cell survival, proliferation and differentiation. Genetic inheritance and single nucleotide polymorphisms in the promoter region of *NFκB1* have been linked with Crohn's disease, ulcerative colitis and more recently common variable immunodeficiency syndrome. In this study we have shown that the absence of *NFκB1* in mice (*Nfkb1*^{-/-}) is associated with premature ageing and the development of multi-organ autoimmune disease. The pathogenesis of disease was primarily associated with the altered homeostasis and function of peripheral Follicular (Fo) B cells and enhanced B cell differentiation that contributes to the elevated production of immunoglobulins and autoantibodies. The study of mixed BM chimeric mice revealed that the Fo B cell intrinsic loss of NFκB1 led to the spontaneous generation of GC B cells. This was strongly linked to the excessive production of IL-6 by *Nfkb1*^{-/-} Fo B cells, which promotes the enhanced differentiation of *Nfkb1*^{-/-} germinal centre B cells and CD4⁺ follicular helper T cells (T_{FH}). The p50 component of NFκB1 was found to repress *Il-6* transcription in wild-type Fo B cells, with the absence of p50-NFκB1 in Fo B cells resulting in the uncontrolled expression of *Il-6* through RelA, the major dimer partner of p50. Collectively, these findings highlight a previously unrecognized role of p50-NFκB1 that serves to prevent multi-organ autoimmune disease in part through the negative regulation of the *Il-6* gene.

3148

A glucocorticoid alternative that interferes with B cell activation

Jones, S.A.¹, Toh, A.E.J.¹, Odobasic, D.¹, Oudin, M.A.V.¹, Chen, Q.¹, Lee, J.P.W.¹, White, S.J.², Russ, B.E.³, Infantino, S.⁴, Light, A.⁴, Tarlinton, D.M.⁴, Harris, J.¹, Morand, E.F.¹

¹Monash University, Centre for Inflammatory Diseases, School of Clinical Sciences, Clayton, Australia, ²Leiden University Medical Center, Leiden Genome Technology Center, Department of Human Genetics, Leiden, Netherlands, ³The Doherty Institute at The University of Melbourne, Department of Microbiology and Immunology, Parkville, Australia, ⁴The Walter and Eliza Hall Institute of Medical Research, Parkville, Australia

Glucocorticoids are routinely used to achieve control over autoimmune diseases, but their side effects cause unacceptable health and financial costs. GILZ is a glucocorticoid-induced transcriptional regulator and signal transduction modulator that we have found to recapitulate anti-inflammatory effects of glucocorticoids without the associated adverse effects. GILZ acts in multiple inflammatory cell types of mice and humans to provide a natural barrier to the acquisition of an activated state. In B cells, GILZ mediated activation-induced cell death and limited the extent of proliferation, class switching and plasma cell differentiation in response to stimulation. Down-regulation of GILZ occurred in germinal centre B cells, and naïve B cells lacking GILZ resembled germinal centre B cells in their transcriptional profile. Absence of GILZ was associated with development of a phenotype resembling systemic lupus erythematosus (SLE) in mice and in SLE patients, low GILZ induction in response to glucocorticoid treatment correlated with highest disease severity. Treatment of human B cells with a cell-permeable GILZ fusion protein potently suppressed their responsiveness to T-dependent stimuli. Thus we have shown GILZ to be an essential regulator of B cells, adding to the growing evidence that targeting this pathway may be a plausible and valuable alternative to the use of glucocorticoids for treatment of autoimmunity.

4027

IkBNS is an essential mediator of Toll-like receptor-induced B cell responses

Touma, M., Ishizuki, M.

Niigata University, Biology, Niigata, Japan

The activation of NF-κB is regulated by several IκB proteins, a family comprising classical (cytoplasmic) and atypical (nuclear) IκBs. A nuclear IκB protein, IκBNS, was originally identified in thymocytes undergoing negative selection and is now recognized as positive and negative regulator of cytokines in T cells, macrophages, and dendritic cells. In addition, we have shown that IκBNS deficiency results in a defective TI-antigen response and a developmental defect in peritoneal B1 B cells. Thus it appears an additional role for IκBNS in the B cell lineage. Further analysis revealed that the IκBNS deficiency leads to a significant reduction in IL-10-competent B10 cells and that IκBNS-deficient B cells failed to secrete TLR-induced IL-10. The impairment of IL-10 production by a lack of IκBNS was not

observed in macrophages or T cells. The *in vitro* suppressive function of LPS-stimulated B cells was impaired in the absence of IκBNS. We also found that two IL-10-producing B cell populations increase upon TLR-triggering: CD138⁺ and CD138⁻, and that IκBNS-deficient B cells are unable to generate IL-10-producing CD138⁺CD44^{hi} plasmablasts.

These results suggest that IκBNS is selectively required for IL-10 production in B cells responding to TLR signals. Overall, our findings indicate a significant role for IκBNS in the control of the TLR-mediated B cell responses. To address the *in vivo* impact of IκBNS-deficiency in B cells, investigations using IκBNS-deficient autoimmune-prone mice are ongoing.

2247

The antibody gene repertoire of the mouse lacks diversity and is highly skewed towards core VDJ sequences

Collins, A.¹, Edwards, R.¹, Jackson, K.²

¹University of New South Wales, School of Biotechnology and Biomolecular Sciences, Sydney, Australia, ²Stanford University, Department of Pathology, Stanford, United States

The mammalian immune system can create literally hundreds of billions of different antibodies from a relative handful of rearranging gene segments, but the small B cell population of the mouse allows it to express just a tiny fraction of this theoretical potential. We have used high throughput sequencing of immunoglobulin VDJ genes, to better understand how the small arsenal of the mouse can meet its immunological needs. Against all expectations, analysis shows the heavy chain IGHV gene repertoire of the C57BL/6 mouse strain to be almost completely different to that of the BALB/c strain. On the other hand, it is remarkably common for rearranged VDJ genes from the two strains to share identical complementarity determining region 3 (CDR3) sequences. Data will be presented demonstrating that a core group of sequences with short CDR3 regions are present at high frequency in the repertoires of both strains, and are often found in multiple individuals of each strain. We hypothesise that the mouse has evolved to limit the diversity of its repertoire, and that its limited repertoire is directed against anticipated pathogens. This would explain reports that the mouse can mount rapid and effective primary antibody responses to some viral infections. We further hypothesise that the divergent sets of IGHV genes seen in BALB/c and C57BL/6 mice are derived from different subspecies of the house mouse, as a result of their early breeding histories, and that the divergent gene sets evolved in response to the different pathogens faced by geographically separated mouse subspecies.

3037

The role of antigen and persistent germinal centers in recall memory responses

Hao, Y., Espinasse, M.-A., Weill, J.-C., Reynaud, C.-A.

Université Paris Descartes Faculté de Médecine, Institut Necker-Enfants Malades - INSERM U 1151/CNRS UMR 8253, Paris Cedex, France

The mouse B cell response against external challenges generates IgG and IgM memory B cells. We have proposed,

based on the AID-Cre-EYFP mouse reporter line (Dogan et al., 2009), that the IgG memory compartment was mainly involved in rapid plasma cell formation during recall responses, while the IgM compartment was mainly driven into new germinal center reactions. However, the behavior of IgM memory B cells upon successive challenges is still a debated issue. Based on the same mouse reporter line, we have tested three main parameters that likely impact the response: the presence of persistent germinal centers (which depends upon the nature of the antigen, soluble protein like NP-CGG or complex, particulate antigen, like sheep red blood cells (SRBC)); the level of circulating antibodies, specific for the relevant antigen (which will vary according to the elapsed time and the number of challenges), and the nature of the adjuvant (MPL vs. alum, i.e. TH1 vs. TH2 response bias). Our preliminary data, based on disruption of germinal centers by inhibition of the lymphotoxin-beta survival function, as well as prime-boost immunizations with NP-CGG and NP-SRBC in various combinations, allow us to delineate the relative impact of these different parameters on the outcome of the recall response, and to reconcile some divergent reports on memory B cell function.

3402

IL-10 secreting innate-like B cells migrate between the peritoneum and the inflamed skin

Geherin, S.¹, Gomez, D.¹, Glabman, R.¹, Ruthel, G.¹, Hamann, A.², Debes, G.¹

¹University of Pennsylvania, Philadelphia, United States,

²Deutsches Rheuma-Forschungszentrum Berlin, Berlin, Germany

The skin is an important barrier organ and frequent target of autoimmunity and allergy. We found innate-like B cells that express the anti-inflammatory cytokine IL-10 in the skin of humans and mice. Unexpectedly, innate-like B-1 and conventional B-2 cells show differential homing capacities with peritoneal B-1 cells preferentially migrating into the inflamed skin of mice. Importantly, the skin-homing B-1 cells includes IL-10 secreting cells. B-1 cell homing into the skin is independent of typical skin-homing trafficking receptors and instead requires alpha4-beta1 integrin. Moreover, B-1 cells constitutively express activated beta1 integrin and relocate from the peritoneum to the inflamed skin and intestine upon innate stimulation, indicating an inherent propensity to extravasate into inflamed and barrier sites. We conclude that innate-like B cells migrate from central reservoirs into skin, adding an important cell type with regulatory and protective functions to the skin immune system.

3474

The 'Ins and Outs' of Myc - Molecular regulation of division progression in B and T cells

Heinzel, S.^{1,2}, Giang, T.B.^{1,2}, Kan, A.^{1,2}, Corcoran, L.^{1,2}, Hodgkin, P.^{1,2}

¹Walter and Eliza Hall Institute, Parkville, Australia, ²University of Melbourne, Department of Medical Biology, Parkville, Australia

T and B lymphocytes integrate activating signals to trigger a controlled proliferation burst. While much is known about the

initiation signals, little is known about how the maintenance of cell division progression or the sudden loss of cells is regulated. Recent studies (Turner M, *Jl* 2008, Marchingo J, *Science* 2014) have shown that the number of times B and T cells divide upon stimulation before returning to quiescence is imprinted into the first generation and is dependent on the type and the strength of the stimulus. These features led to the hypothesis of a 'mitosis promoting factor' induced through activation and consequently diluted progressively to drop below a threshold level, at which division stops.

Here we have identified the proto-oncogene Myc as the division driving factor. Myc accumulates after activation in T and B cells and is then gradually lost over time. Myc expression levels correlate with stimulation strength and are not affected by cell division, identifying the molecular machinery as a division-independent timer. Variable sensitivity to signals is achieved by manipulating the kinetics of Myc production, directly integrating stimulating signals to determine clonal burst size. Forced overexpression leads to increased division rounds before cells return to quiescence. In addition, another division-independent timer controlling cell survival is induced through activation. These two cell intrinsic timers operate independently and together shape the early T and B cell response. Modelling reveals that small changes in these timed processes, as might occur during oncogenic transformation, synergize to significantly enhance cell numbers over time.

2355

The ion channel TRPM7 regulates actin organization and antigen internalization in B cells

Krishnamoorthy, M.¹, Buhari, H.², Wasim, L.², Ho, J.², Perraud, A.-L.³, Schmitz, C.³, Treanor, B.^{1,2,4}

¹University of Toronto, Department of Cell and Systems Biology, Toronto, Canada, ²University of Toronto Scarborough, Department of Biological Sciences, Toronto, Canada, ³University of Colorado, Department of Immunology and Microbiology, Denver, United States, ⁴University of Toronto, Department of Immunology, Toronto, Canada

The process of B cell spreading and contracting in response to membrane-bound antigen is important for collection of BCR-antigen microclusters for internalization and subsequent presentation to T cells. The spreading and contraction response is dependent on reorganization of actin, yet the molecular mechanism that leads to this reorganization is not well understood. Several studies have implicated the ion channel transient receptor potential member 7 (TRPM7) in regulating the cellular actomyosin network. TRPM7 is one of only two known channel proteins with a kinase domain. Interestingly, TRPM7 was recently found to phosphorylate PLC γ 2, a key regulator of B cell spreading and formation of antigen microclusters, and thus implicates TRPM7 in B cell signaling. Our studies have shown that TRPM7 plays an important role in early B cell activation through both channel and kinase activities. B cells that lack TRPM7 or express a phosphotransferase-deficient point mutant show enhanced antigen accumulation and prolonged signaling in addition to altered microcluster movement and contraction. Our findings suggest that this is due to alterations in the actin cytoskeleton. Importantly, antigen internalization is

defective in TRPM7-KO cells, and primary B cells treated with a pharmacological inhibitor of TRPM7 exhibit impaired antigen presentation and consequently T cell activation is compromised. Taken together, these results highlight TRPM7 as an important novel regulator of B cell activation.

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Suppression of metastases using a new lymphocyte checkpoint target for cancer immunotherapy

Smyth, M., Blake, S., Teng, M., Miles, J.

QIMR Berghofer Medical Research Institute, Herston, Australia

CD96 has recently been shown as a negative regulator of mouse NK cell activity, with *Cd96*^{-/-} mice shown to have hyper-responsive NK cells upon immune challenge. In this study we have demonstrated that blocking CD96 with a monoclonal antibody inhibited experimental metastases in multiple tumor models. The anti-metastatic activity of anti-CD96 was dependent on NK cells, CD226 (DNAM-1) and IFN γ , but independent of activating Fc receptors. Anti-CD96 was more effective in combination with anti-CTLA4, anti-PD1 or doxorubicin chemotherapy. Blocking CD96 in *Tigit*^{-/-} mice significantly reduced experimental and spontaneous metastasis compared to its activity in WT mice. Co-blockade of CD96 and PD1 potently inhibited lung metastases, with the combination increasing local NK cell IFN γ production and infiltration. Overall these data demonstrate that blocking CD96 is a new and complementary immunotherapeutic strategy to reduce tumor metastases. Ongoing studies in primary mouse and human tumours will be discussed.

706

The emerging role of tissue-resident memory (T_{RM}) CD8 T cells in antitumor immunity and malignant disease

Mami-Chouaib, F., Cognac, S., Djenidi, F., Boutet, M.

INSERM, Gustave Roussy, Villejuif, France

Tissue-resident memory (T_{RM}) T cells play an essential role in protecting human epithelial tissues against infectious and inflammatory diseases. They are highly activated T lymphocytes that reside within a variety of peripheral tissues including intestine, brain, skin and lung, and provide rapid and effective responses to viral reinfections. However, the presence of T_{RM} in human epithelial tumors and their role in the antitumor immunity have thus far not been systematically addressed. Accumulating evidence indicates that TGF- β 1, abundant at the tumor site, plays an essential role in the formation and maintenance of T_{RM} at least in part via induction of CD103 integrin. In this context, our results indicated that human lung tumor-infiltrating lymphocytes (TIL) include a homogeneous CD8⁺ T-cell population that displays a unique phenotypic and molecular signature associated with T_{RM} cells, which results in optimal effector functions within the tumor microenvironment. We also demonstrated that an enhanced CD103⁺ TIL subset correlates with improved early-stage non-small cell lung

carcinoma (NSCLC) patient survival and increased intraepithelial lymphocyte infiltration. Indeed, we provide evidence that CD103 is directly involved in T-cell recruitment within epithelial tumor islets and in local early T-cell signaling. Moreover, TGF- β upregulates CD103-mediated T-cell activities by promoting phosphorylation of integrin-linked kinase (ILK) by TGFBR1 and its subsequent binding to CD103, thereby initiating integrin inside-out signaling. These data emphasize the role of CD8⁺/CD103⁺ T_{RM} in promoting intratumoral cytotoxic T lymphocyte (CTL) responses and suggest their use in adoptive T-cell therapy or as a biomarker to predict patient response to checkpoint blockade immunotherapy.

1607

Dual-specific T cells and oncolytic virus eradicate large solid tumors

Slaney, C.Y.^{1,2}, Von Scheidt, B.^{1,2}, Beavis, P.A.^{1,2}, Davenport, A.J.^{1,2}, Westwood, J.A.^{1,2}, Mardiana, S.^{1,2}, Tschärke, D.C.³, Restifo, N.P.⁴, Darcy, P.K.^{1,2,5}, Kershaw, M.H.^{1,2,5}

¹Peter MacCallum Cancer Centre, Cancer Immunology Program, East Melbourne, Australia, ²University of Melbourne, Sir Peter MacCallum Department of Oncology, Parkville, Australia, ³Australian National University, Research School of Biology, Canberra, Australia, ⁴National Cancer Institute, National Institute of Health, Center for Cancer Research, Bethesda, United States, ⁵Monash University, Department of Immunology, Clayton, Australia

While immunotherapy can eliminate substantial burdens of leukemia, the ultimate challenge remains the consistent eradication of large solid tumors. Here we generate dual-specific CARaMEL T cells expressing a chimeric antigen receptor (CAR) reactive with Her2⁺ tumor and a TCR specific for the premelanocyte antigen (pMEL, gp100). Adoptive transfer of these T cells, together with systemic delivery of recombinant vaccinia virus expressing gp100, induced durable complete remission of a variety of Her2⁺ tumors, some in excess of 150 mm², in immunocompetent mice expressing Her2 as a self-antigen. Vaccinia virus effected changes to the tumor microenvironment and induced extensive proliferation of CARaMEL T cells. Tumor destruction mediated by dual-specific T cells and vaccinia occurred rapidly over a period of seven days and was associated with an extensive infiltrate of T cells. Mice that had rejected tumors were resistant to rechallenge with the same Her2⁺ tumor cells and partially resistant to rechallenge with Her2⁻ cells, indicating the formation of immune memory and epitope spreading. Transient toxicity against Her⁺ brain tissue was observed, which was associated with temporary accumulation of T cells in the cerebellum. This study supports a view that it is possible to design a highly effective combination immunotherapy for solid cancers, with acceptable transient toxicity, even when the target antigen is also expressed in vital tissues.

3699

SLAMF7 is a novel target for immunotherapy of multiple myeloma

Jenkins, M.¹, Rogers, A.¹, Canfield, R.¹, Robbins, M.², Bezman, N.², Darcy, P.¹, Kershaw, M.¹, Johnstone, R.¹, Ritchie, D.³, Prince, H.M.¹, Trapani, J.¹, Neeson, P.¹

¹Peter MacCallum Cancer Centre, Cancer Immunology Research, Melbourne, Australia, ²Bristol-Myers Squibb, Princeton, United States, ³Melbourne Health, Haematology, Melbourne, Australia

Elotuzumab (Elo) targets SLAMF7, a protein highly expressed on myeloma plasma cells and broadly on immune cell subsets. This project investigated the mechanisms whereby Elo activates NK cells and myeloma cell killing.

Elo and SLAMF7⁺ OPM-2 myeloma cells induce normal donor NK cell degranulation, IFN- γ production and myeloma cell ADCC. In the same conditions, MM patient NK cells are poor responders. Elo induced loss of the CD56^{lo}CD16⁺ NK subset in normal donors, but not in myeloma patients. This loss of the CD56^{lo}CD16⁺ NK subset was due to a combination of NK fratricide and ADAM-17 mediated cleavage of CD16a from the NK cell surface. Elo mediated dual activation of NK cells by binding directly to SLAMF7 and also via binding to CD16a. The CD16a binding was required for NK cell fratricide.

We then examined whether Elo altered the NK cell killing kinetics of myeloma target cells using time lapse live video microscopy (TLLVM). Normal donor NK cells used ADCC to kill OPM-2 cells in the presence of Elo, the killing kinetics was equivalent to the human IgG isotype control, delivery of the lethal hit occurring in under three minutes. Elo also mediated serial killing of OPM-2 cells. Interestingly, Elo delayed the NK cell detachment from dying myeloma plasma cell targets, this correlated with higher levels of secreted cytokines and pro-inflammatory chemokines. Taken together, this data indicates Elo-induced a profound change in the NK-mediated killing kinetics of myeloma cells which likely recruits other immune effectors to the myeloma killing site.

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Jak-STAT3 pathway triggers DICER1 for proteasomal degradation by ubiquitin ligase complex of CUL4A^{DCAF1} to promote colorectal cancer cells growth

Ren, W.¹, Shen, S.¹, Li, G.², Ma, J.^{1,2}

¹Central South University, The Third Xiangya Hospital, Changsha, China, ²Central South University, Cancer Research Institute, Changsha, China

Chronic intestinal inflammation is closely associated with colorectal cancer (CRC) development and STAT3 seems to take center stage in bridging chronic inflammation to CRC progress. Here, we discovered that DICER1 was significantly downregulated in response to IL-6 or LPS stimulation and identified a novel mechanism for DICER1 downregulation via proteasomal degradation by ubiquitin ligase complex of CUL4A^{DCAF1} in CRC cells. Meanwhile, PI3K-AKT signaling pathway phosphorylated DICER1 and contributed to its proteasomal degradation. The regulation of DICER1 by CUL4A^{DCAF1} affected cell growth and apoptosis which controlled by IL-6 activated

Jak-STAT3 pathway. Intervention of CUL4A^{DCAF1} ubiquitin ligase complex led to fluctuation in expression levels of DICER1 and microRNAs, and thus affected tumor growth in a mouse xenograft model. Furthermore, clinical specimens' analysis revealed that decreased DICER1 expression was negatively correlated with STAT3 activation and cancer progression in human CRC. DICER1 and p-STAT3 expression levels correlated with 5-year overall survival of CRC patients. Consequently, this study proposes that inflammation-induced Jak-STAT3 signaling leads to CRC cells growth through proteasomal degradation of DICER1 by ubiquitin ligase complex of CUL4A^{DCAF1}, which suggests a novel therapeutic opportunity for CRC.

2215

Therapeutic DNA vaccination against colorectal cancer by targeting the MYB oncoprotein

Ramsay, R.

Peter MacCallum Cancer Centre, Research, Melbourne, Australia

Cancers are often addicted to continued and relatively high expression of nuclear oncoproteins. This is evident in colorectal cancer (CRC) where the oncoprotein and transcription factor MYB is over-expressed and essential to continued proliferation and tumour cell survival. In adenoid cystic carcinoma MYB-NFIB fusion proteins are considered the key driver of this cancer. Historically, targeting transcription factors in the context of cancer has been very challenging. Nevertheless, we formulated a DNA vaccine to generate a MYB-specific immune response in the belief MYB peptides might be aberrantly presented on the cell surface of CRC cells. MYB, like many tumour antigens, is weakly immunogenic as it is a "self" antigen and is subject to tolerance. To break self-tolerance, a fusion vaccine was generated comprising a full-length MYB cDNA flanked by two potent CD4-epitopes derived from tetanus toxoid. Vaccination was achieved against tumours initiated by two distinct highly aggressive, syngeneic CRC cell lines that express MYB. We introduced multiple inactivating mutations into the oncogene sequence for safety and sub-cloned the cDNA into a FDA-compliant vector. We used low-dose cyclophosphamide (CY) to overcome T-regulatory cell immune suppression, and anti-PD-1 antibodies to block T-cell exhaustion. Anti-PD-1 administered alone slightly delayed tumour growth, while CY did not. We found that therapeutic vaccination elicits protection when tumour burden is low, mounts tumour-specific cell killing and affords enhanced protection when tumour burden is higher but only in combination with anti-PD-1 antibody and/or low dose CY. These data have now set the stage for a funded first-in-man clinical trial.

1036

Mismatch in epitope specificities between IFN inflamed and non-inflamed conditions may lead to escape from T lymphocyte killing in melanoma

Woods, K.^{1,2}, Knights, A.³, Anaka, M.³, Schittenhelm, R.B.⁴, Purcell, A.W.⁴, Behren, A.^{1,2}, Cebon, J.^{1,2}

¹Olivia Newton-John Cancer Research Institute, Cancer Immunobiology, Melbourne, Australia, ²La Trobe University, School

of Cancer Medicine, Melbourne, Australia, ³Ludwig Institute for Cancer Research, Cancer Immunobiology Laboratory, Melbourne, Australia, ⁴Monash University, Biochemistry and Molecular Biology, Melbourne, Australia

Degradation of cellular proteins by the proteasome is critical for generation of MHC-associated peptides. The constitutive proteasome and the IFN γ -induced immunoproteasome (IP) differ in the use of three catalytic β subunits, which alters production of MHC class I epitopes. The potential for a disparate repertoire of epitopes produced between inflammatory/non-inflammatory tumours therefore arises.

We have extensively investigated this phenomenon both broadly, in terms of the cancer cell as a whole, and specifically, by evaluating presentation of three NY-ESO-1 HLA-Cw3 restricted epitopes.

We profiled the immunopeptidome of a melanoma cell line under steady state or IFN γ -treated conditions; by mass spectrometry analysis of HLA Class I bound peptides. Profound changes in the overall epitope profile presented on HLA Class I molecules were observed.

We studied processing and presentation of three NY-ESO-1 HLA-Cw3 restricted epitopes by melanoma cell lines. We found changes in surface presentation of each of the three epitopes following processing through different proteasome subtypes. We further investigated processing of these epitopes by selective inhibition of the IP catalytic subunit LMP7 by siRNA knockdown, or direct inhibition.

In vivo, we manipulated melanoma cells to switch proteasome subtype following treatments which induced IFN γ at the tumour site.

Our data demonstrate broad changes in epitopes presented by melanoma cells under inflammatory versus non-inflammatory conditions. These results illustrate a little-studied mechanism of immune escape by tumour cells. Awareness of how individual cancer epitopes are processed by melanoma cells may be critical to inform development of therapies involving cancer vaccination, adoptive T-lymphocyte transfer, or combination treatments including these.

2545

Radiation dose and fractionation can influence the immune modulatory effects of radiotherapy and anti-cancer activity of checkpoint blockade therapy

Haynes, N.M.^{1,2}, Hagekyriakou, J.³, Halkerwal, S.¹, Darcy, P.K.^{2,4}, Chua, B.⁵, Johnstone, R.W.^{1,2}

¹Peter MacCallum Cancer Centre, Cancer Therapeutics Program, East Melbourne, Australia, ²The University of Melbourne, Sir Peter MacCallum Department of Oncology, Melbourne, Australia, ³Peter MacCallum Cancer Centre, Department of Physical Sciences, East Melbourne, Australia, ⁴Peter MacCallum Cancer Centre, Cancer Immunology Research, East Melbourne, Australia, ⁵Peter MacCallum Cancer Centre, Division of Radiation Oncology and Cancer Imaging, East Melbourne, Australia

Activation of host anti-cancer immune defenses is one mechanism by which radiation therapy can eliminate cancer cells. To promote radiation-induced immune responses to

cancer, radiotherapy is now being trialed in combination with immunotherapy, with promising success. In an effort to ensure that the full therapeutic potential of radiotherapy can be harnessed with immunotherapy, we have examined the impact of radiation dose and fractionation regimen on the immunomodulatory activity of radiotherapy, and its ability to support the anti-cancer effects of antibody-based immunotherapy. Importantly, we demonstrate that different fractionated radiation regimens are not equivalent in their ability to support the therapeutic activity of antibody-based checkpoint blockade therapy. A 3x4Gy (consecutive day) or 2x10Gy (10Gy/week) treatment regimen was significantly more effective than a single 12Gy or 20Gy (or 2x10Gy given over consecutive days) dose, respectively, in promoting T-cell based immune responses and the anti-cancer activity of anti-PD-1 therapy in a C57BL/6 model of triple negative breast cancer. Conversely, a single 20Gy dose of radiotherapy was most effective in supporting the growth inhibitory effects of anti-CTLA-4 therapy. Given that low dose (2Gy) fractionated radiotherapy is routinely used for the treatment of breast cancer we have also demonstrated that such fractionation regimens can enhance the combined curative activity of anti-PD-1 and anti-CTLA-4 therapy. Collectively these findings will help optimize the selection and method of integration of immunotherapeutic agents with radiation therapy and ensure that the burgeoning paradigm of cancer immunotherapy can be best capitalized on for the treatment of cancer.

3196

T cell mediated immune surveillance is essential for the control of spontaneous B cell lymphomas

Zotos, D.^{1,2}, Sterle, S.^{1,2}, Bernard, N.^{1,2}, Scherger, A.^{1,2}, Rodling, L.^{1,2}, Alsop, A.^{1,2}, Walker, J.^{1,2}, Masson, F.^{1,2}, Belz, G.^{1,2}, Corcoran, L.^{1,2}, O'Reilly, L.^{1,2}, Strasser, A.^{1,2}, Smyth, M.^{3,4}, Johnstone, R.^{5,6}, Tarlinton, D.^{1,2}, Nutt, S.^{1,2}, Kallies, A.^{1,2}

¹Walter & Eliza Hall Institute of Medical Research, Parkville, Australia, ²University of Melbourne, Department of Medical Biology, Parkville, Australia, ³QIMR Berghofer Medical Research Institute, Herston, Australia, ⁴University of Queensland, School of Medicine, Herston, Australia, ⁵Peter MacCallum Cancer Centre, East Melbourne, Australia, ⁶University of Melbourne, Sir Peter MacCallum Department of Oncology, Parkville, Australia

Non-Hodgkin lymphoma is a heterogeneous group of malignancies of lymphoid origin, the most common type being diffuse large B cell lymphoma (DLBCL), which represents 30-40% of cases. Despite improved treatment, 40-50% of DLBCL patients still succumb to their disease. A better understanding of DLBCL pathogenesis is therefore needed to identify new therapeutic approaches. Loss of function of the tumour suppressor PRDM1/Blimp1 or deregulated expression of the oncogene BCL6 are thought to be causative for a large proportion of DLBCL cases; however, mutations in either of these genes leads to only slow and infrequent development of overt lymphoma, and despite frequent mutations of the Bcl6 gene in activated B cells of healthy individuals lymphoma development is rare. Because B lymphomas show a strong association with immune suppression and immune deficiencies, it has been postulated that mutations that result in a breakdown of immune surveillance are necessary

to allow progression of lymphomas or other tumours. In the present study we show that Blimp1 deficiency or overexpression of Bcl6 in the B cell lineage leads to a pronounced accumulation of pre-plasmablast B cells, but does not result in overt lymphoma in the presence of a functioning T cell compartment. Removal of T cell control results in the rapid development of DLBCL-like lymphoma, which can be eradicated by polyclonal CD8 T cells in a Fas and T cell receptor/CD28-dependent manner. Thus, malignant transformation of mature B cells requires mutations that impair intrinsic differentiation processes and the escape from constant T cell-mediated tumour surveillance.

4074

Invariant NKT lymphocytes increase the development of intestinal tumors by promoting Treg cells and suppressing TH1 immunity

Wang, Y.¹, Sedimbi, S.², Löfbom, L.¹, Cardell, S.¹

¹University of Gothenburg, Dept of Microbiology and Immunology, Gothenburg, Sweden, ²Karolinska Institute, Dept of Microbiology Tumor and Cell Biology, Stockholm, Sweden

CD1d-restricted natural killer T (NKT) cells are potent immunoregulatory cells. Activation of invariant NKT (iNKT) cells suppresses tumor formation in murine models, and iNKT cells can naturally protect against spontaneous tumors, and also enhance immune responses to infections. Intestinal tumors develop in an environment of constant microbial pressure and inflammatory signals that increase tumor formation, raising the question whether NKT cells would suppress or promote tumor formation in this tissue. Here we have investigated the role for iNKT cells in intestinal tumor immunity. *APC^{Min/+}* mice develop intestinal polyps due to a mutation in the adenomatous polyposis coli (APC) gene, recapitulating early events in human colorectal cancerogenesis. Absence of iNKT cells in *APC^{Min/+}* mice decreased the total number of intestinal polyps with 60%. iNKT cells were present in the polyps of *APC^{Min/+}* mice, and showed a distinct phenotype compared to other organs, being CD4⁺, NK1.1⁻ CD44⁺, CD69⁺ and PD-1^o. They had an intermediate expression level of T-bet but were negative for the NKT cell transcription factor PLZF. In *APC^{Min/+} Jα18^{-/-}* mice, absence of iNKT cells was associated with a reduced frequency of Treg cells and lower expression levels of FoxP3 protein and transcript uniquely in the polyps. Moreover, in iNKT cell deficient *APC^{Min/+}* mice, expression of TH1-associated genes, such as *IFN-γ* and *Nos2*, was increased in polyps, concomitant with elevated frequencies of conventional CD4⁺ and CD8⁺ T cells in this tissue. The results suggest that iNKT cells promote intestinal polyp formation by enhancing Treg cells and local immunosuppression of anti-tumor TH1-immunity.

Macrophages 1

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Capacity of yolk sac macrophages, fetal liver and adult monocytes to colonize an empty niche and develop into functional tissue resident macrophages

van de Laar, L., Saelens, W., Scott, C.L., Saeys, Y., Lambrecht, B., Guillems, M.

Ghent University - VIB, Ghent, Belgium

Tissue-resident macrophages can derive from yolk sac macrophages (YS-MFs), fetal liver monocytes (FL-MOs) or adult bone marrow monocytes (BM-MOs). The relative capacity of these precursors to colonize a niche, self-maintain and perform tissue-specific functions is currently unknown. We simultaneously transferred traceable YS-MFs, FL-MOs and BM-MOs into the empty alveolar macrophage (AM) niche of neonatal *Csf2rb*^{-/-} mice. All subsets produced mature AMs, but in competition a preferential outgrowth of FL-MOs was observed, correlating with their superior GM-CSF reactivity and proliferation capacity. When transferred separately however, all precursors efficiently colonized the alveolar niche and generated AMs that were transcriptionally almost identical, self-maintained and durably prevented alveolar proteinosis. Mature liver, peritoneal or colon macrophages could not efficiently colonize the empty AM niche, whereas mature AMs could. Thus, precursor origin does not affect the development of functional self-maintaining tissue-resident macrophages and the plasticity of the mononuclear phagocyte system is largest at the precursor stage.

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Histone deacetylase 7 (HDAC7) regulates a sub-set of TLR-mediated pro-inflammatory responses in macrophages by both deacetylase-dependent and -independent mechanisms

Das Gupta, K., Shakespear, M., Tunny, K., Hohenhaus, D., J. Bokil, N., Iyer, A., P. Fairlie, D., J. Sweet, M.

Institute for Molecular Bioscience, The University of Queensland, Cell Biology and Molecular Medicine, Brisbane, Australia

Histone Deacetylases (HDACs), which deacetylate lysine residues of both histone and non-histone proteins, regulate inflammation and many other cellular processes. Broad spectrum and class-selective HDAC inhibitors (HDACi) are efficacious in animal models of several inflammatory diseases, however undesirable effects have also been reported. A clear understanding of which HDACs drive inflammatory pathways is required to improve drug design. This study highlights the role of one particular isoform, HDAC7, in sustaining macrophage inflammatory responses. We previously showed that inflammatory macrophages express elevated levels of HDAC7, and thus transgenic mice that selectively over-express HDAC7 in the myeloid compartment (mac-HDAC7 mice) were generated. Serum from naive mac-HDAC7 mice showed elevated levels of specific inflammatory mediators (e.g. IL-6, Ccl2), confirming the pro-inflammatory nature of HDAC7. Macrophages from these mice also displayed enhanced LPS-induced expression

of a sub-set of inflammatory mediators including IL-12, IL-6, Ccl2, and Edn1. These effects were also apparent in wild type primary macrophages, in which HDAC7 was over-expressed by retroviral transduction. The hyper inflammatory phenotype of macrophages from mac-HDAC7 mice was restricted to TLR1/2/4 agonists, since TLR3/7 responses were unaffected. Mechanistically, HDAC7 over-expression did not affect proximal TLR4 signalling, but down-regulated basal phosphorylation of AKT and ERK. Retroviral transduction of primary macrophages with enzyme-dead HDAC7 resulted in heightened LPS-inducible responses for some inflammatory mediators (e.g. IL-12, TNF), whereas others (e.g. Ccl2) were dependent on deacetylase activity. Overall these studies reveal both enzyme-dependent and -independent pro-inflammatory functions of HDAC7, which has important implications for development of selective HDAC inhibitors as anti-inflammatory agents.

1562

Identification and characterization of CD300H, a new member of the Human CD300 immunoreceptor family

Niizuma, K.^{1,2}, Tahara-Hanaoka, S.^{1,3}, Noguchi, E.⁴, Shibuya, A.^{1,3}

¹University of Tsukuba, Faculty of Medicine, Department of Immunology, Tsukuba, Japan, ²University of Tsukuba, School of Integrative and Global Majors, Ph.D. Program in Human Biology, Tsukuba, Japan, ³University of Tsukuba, Center for Tsukuba Advanced Research Alliance (TARA), Tsukuba, Japan, ⁴University of Tsukuba, Faculty of Medicine, Department of Medical Genetics, Tsukuba, Japan

Recruitment of circulating monocytes and neutrophils to infection sites is essential for host defense against infections. Here, we identified a previously unannotated gene that encodes an immunoglobulin-like receptor, designated CD300H, which is located in the CD300 gene cluster. CD300H has a short cytoplasmic tail and associates with the signaling adaptor proteins, DAP12 and DAP10. CD300H is expressed on CD16⁺ monocytes and myeloid dendritic cells. Ligation of CD300H on CD16⁺ monocytes and myeloid dendritic cells with anti-CD300H monoclonal antibody induced the production of neutrophil chemoattractants.

Interestingly, CD300H expression varied among healthy subjects, who could be classified into two groups according to "positive" and "negative" expression. Genomic sequence analysis revealed a single-nucleotide substitution (rs905709 (G→A)) at a splice donor site on intron 1 on either one or both alleles. The International HapMap Project database has demonstrated that homozygosity for the A allele of single nucleotide polymorphism (SNP) rs905709 ("negative" expression) is highly frequent in Han Chinese in Beijing, Japanese in Tokyo, and Europeans (A/A genotype frequencies 0.349, 0.167, and 0.138, respectively) but extremely rare in Sub-Saharan African populations.

Together, these results suggest that CD300H may play an important role in innate immunity, at least in populations that carry the G/G or G/A genotype of *CD300H*.

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Epigenetic and transcriptional regulation of macrophage polarization and endotoxin tolerance by Akt kinases and insulin signalling

Ieronymaki, E., Lyroni, K., Theodorakis, E., Patsalos, A., Daskalaki, M., Eliopoulos, A., Kampranis, S., Vaporidi, A., Tsatsanis, C.
University of Crete, School of Medicine, Heraklion, Greece

Macrophages possess different phenotypes in response to inflammatory stimuli described as M1 and M2 types, each of which includes an array of individual subtypes. Endotoxin tolerant macrophages is an M2 subtype characterised by hypo responsiveness to pro-inflammatory stimuli. Using mice lacking Akt1 or Akt2 kinase we demonstrated epigenetic and transcriptional events that regulate LPS responses, M1/M2 polarisation and endotoxin tolerance and are under the control of Akt kinases. Using siRNA library screening we showed that the methyl-transferase EZH2 and the demethylase UTX control endotoxin tolerance via methylation of Histone 3 on genes regulating endotoxin tolerance and macrophage polarisation. MiRNAs miR-155 and miR-146a, both contributing to endotoxin tolerance and macrophage polarization, were epigenetically controlled by H3 methylation/demethylation and transcriptionally regulated by C/EBP β , under the control of Akt kinases. Since Akt kinases mediate insulin signalling, we compared the RNAome of macrophages derived from diet-induced insulin resistant mice with those from Akt-deficient or wild type mice. We identified transcriptome networks that characterise a distinct subset of M2-like macrophages. Central role in these networks play metabolic processes that are under the control of Akt2 kinase. Exposure of diet-induced insulin resistant mice and Akt2 deficient mice in polymicrobial sepsis confirmed that changes in macrophage metabolism that occur upon development of insulin resistance mediate obesity-driven changes in the response to sepsis. Overall our work supports a central role of Akt kinases and insulin signaling in macrophages shaping their response phenotype via epigenetic, transcriptional and metabolic changes.

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Notch signaling plays a role in regulating IL-10 production in immune complex-stimulated macrophages

Wongchana, W.^{1,2}, Palaga, T.^{2,3}

¹Chulalongkorn University Faculty of Science, Graduate Program in Biotechnology, Bangkok, Thailand, ²Chulalongkorn University, Center of Excellence in Immunology and Immune-Mediate Diseases, Bangkok, Thailand, ³Chulalongkorn University Faculty of Science, Microbiology, Bangkok, Thailand

Macrophages are highly plastic during the effector phase, depending mainly on the stimuli and microenvironment. Classically activated macrophages are stimulated with IFN γ with TLR ligands such as LPS and referred to as M(LPS+IFN γ). They produce inflammatory mediators together with inflammatory cytokines such as IL-12 and low level of anti-inflammatory cytokine, IL-10. In the presence of immune complex, classically activated macrophages reduce the amount of IL-12 while increase IL-10 production. This type of activated macrophages is

referred to as M(IC). Previously, a well conserved Notch signaling is shown to regulate effector functions, including cytokine production in M(LPS+IFN γ). In this study, we investigated the role of Notch signaling in regulating IL-10 production in M(IC). Upon stimulation, M(IC) readily increased the level of cleaved Notch1, indicating that Notch signaling is activated. Cleaved Notch1 was observed at a similar level in M(LPS+IFN γ) and M(IC), regardless of the presence of immune complex. M(IC) also increased the expression level of Notch ligands, Jagged1, Jagged2 and DLL1, similar to M(LPS+IFN γ). CSL/RBP-J κ is a DNA-binding protein central to transcriptional activity of Notch signaling pathway. Macrophages with CSL/RBP-J κ KO produced significantly less IL-10. Similar impact of inhibiting Notch signaling using gamma-secretase inhibitor (GSI) was observed. The effect of GSI treatment on early signaling pathways, including PI3K/AKT, p38, Erk and SAPK/JNK, was not observed in M(IC). In contrast, defects in NF- κ B p50 nuclear localization was observed. Taken together, these results suggest that the Notch signaling pathway plays a role in regulating IL-10 production in M(IC), possibly through NF- κ B pathway.

2383

miR-155 promotes inflammatory macrophage phenotype signature

Guerau-de-Arellano, M., Jablonski, K., Amici, S., Webb, L., Gaudet, A., Popovich, P.

Ohio State University, Columbus, United States

Classical M1 (LPS + IFN-g) macrophage responses drive inflammation and tissue damage. Therefore, harnessing the pathways that drive inflammatory macrophage phenotype may be beneficial in inflammatory diseases. miRNA are small RNAs that post-transcriptionally suppress gene expression. Among several miRNAs associated with inflammatory responses miR-155 was the most highly up-regulated, with a 100-fold increase in M1 macrophages. To determine to what extent miR-155 is required for full expression of the classical inflammatory macrophage phenotype, we performed microarray analyses of wild-type (WT) and miR-155 knockout (KO) macrophages. This analysis revealed that miR-155 controls over 600 genes, corresponding to half of the WT M1 signature. Real-Time PCR and flow cytometry analyses on independent datasets showed that classical inflammatory markers IL-6, iNOS, IL-1b, TNF-a and IL-12 as well as the novel M1 marker CD38 were suppressed in miR-155 KO macrophages. In contrast, miR-155 deficiency did not reduce expression of genes associated with beneficial M2 macrophages (e.g., Arginase-1). The pro-inflammatory effects of miR-155 may be mediated by reduction in miR-155 targets Inpp5d, Mafk, c-Maf, Tspan14 and Il6ra, which were recovered in miR-155 KO M1 macrophages. Treatment with a miR-155 oligonucleotide inhibitor suppressed iNOS and TNF-a gene expressions in wild-type M1 macrophages. Overall, these data indicate that miR-155 plays an essential role in M1 macrophages inflammatory phenotype that miR-155 targeting may be beneficial in inflammatory disease.

4376

Understanding the anti-tumour potential of bisphosphonate drugs: inhibitory effects on macrophage populations outside the skeleton

Munoz, M.A.¹, Junankar, S.², Jurczyk, J.¹, Preston, A.¹, Zlatev, H.³, Auriola, S.³, Rogers, M.¹

¹Garvan Institute of Medical Research, Bone Biology, Darlinghurst, Australia, ²Garvan Institute of Medical Research, Darlinghurst, Australia, ³University of Eastern Finland, Kuopio, Finland

Bisphosphonates (BPs) are a class of calcium-seeking drugs used to inhibit bone destruction in cancer patients. BPs such as zoledronic acid (ZOL) act by inhibiting FPP synthase in osteoclasts, causing accumulation of the upstream metabolite IPP and preventing prenylation of small GTPase proteins required for bone resorption.

Evidence suggests that BPs have pleiotropic effects outside the skeleton, including anti-tumour activity. Using 2-photon intravital imaging of a fluorescently-tagged BP, we demonstrated that BPs diffuse from leaky vasculature and accumulate in the tumour tissue by binding to microcalcifications. These drug-bound microcalcifications are then efficiently engulfed by tumour-associated macrophages (TAMs). However, despite rapid drug uptake, we could not detect any defects in protein prenylation in TAMs from ZOL-treated animals, while by contrast, ZOL administration inhibited the prenylation of Rab proteins in CD11b⁺ peritoneal macrophages. To determine whether the lack of effect of ZOL on prenylation in TAMs was due to reduced drug activity of microcalcification-bound ZOL, we treated mice weekly for 4 weeks with free ZOL or an equivalent dose of liposome-encapsulated ZOL that is efficiently internalised by macrophages. We found that while treatment with free ZOL resulted in IPP accumulation in TAMs, only liposomal ZOL had a clear inhibitory effect on Rab prenylation.

Together, our data suggest that, at least in 4T1 tumours, engulfment of ZOL-bound microcalcifications leads to cytosolic ZOL concentrations that are sufficient to inhibit FPP synthase and cause accumulation of IPP, but insufficient to inhibit protein prenylation. How this affects TAM function *in vivo* remains to be determined.

3020

Swiprosin-1 (EFhd2) regulates the TLR4 signals for TNF- α production in macrophage

Wakabayashi, N.¹, Ogawa, M.¹, Ishikawa, S.¹, Hachiya, M.¹, Mielenz, D.², Katagiri, T.¹

¹University of Toyama, Dept. Biology (Faculty of Pharmaceutical Sciences), Toyama-shi, Japan, ²University of Erlangen-Nuremberg, Division of Molecular Immunology, Nikolaus-Fiebiger-Centre, Nuremberg, Germany

Swiprosin-1, also called as EFhd2, has a unique structure; single CC domain and tandem EF hand domains, which is reminiscent of the important role as a signal protein. In fact, it has been reported that Swip-1 involves antigen receptor signals in T cells and B cells. Nevertheless, there is no report about Swip-1 function in TLRs signals. In this study, we focused on TLR4 signals in macrophages. Swip-1 has also been reported as

cytoskeletal binding protein. However, Swip-1 knocked-down (KD) RAW264.7 was almost similar to the control cells that of the morphology and proliferation pattern. On the other hand, Swip-1 KD RAW264.7 showed a very low production of TNF- α even by LPS stimulation at high concentrations level. Under the same condition, RAW264.7, which was treated with control RNA, produced TNF- α to the same extent as normal signaling of TLR4. Then we tried to identify the target point of Swip-1 action in the signal that leads to TNF- α production. As a result of this analysis, at least partially, we could get the proof of suggesting a possibility to affect the breakdown of I κ B. In addition, we also have accumulated data that suggest Swip-1 is involved in the MAPK cascade. Furthermore, we found the results that Swip-1 KD RAW264.7 affected some of the phosphorylation pattern of tyrosine phosphorylated proteins, which are characteristic in TLR4 signal. As a conclusion, Swip-1 is important to regulate TNF- α production in macrophages, so our study allow develop new inflammation control.

1101

Lysophosphatidylcholine acyltransferase (LPCAT) 2 regulates macrophage inflammatory responses through acylation of key metabolic proteins

Abate, W., Sharma, V., Jackson, S.

Plymouth University, Plymouth, United Kingdom

We have previously shown that acyl-CoA: lysophosphatidylcholine acyltransferases (LPCAT) 2, which is involved in phospholipid remodelling, plays a key regulatory role in the inflammatory response to bacterial ligands. The mechanism through which LPCAT2 exerts this regulatory function on the inflammatory response in macrophages is not well defined. It has been demonstrated that modification of signalling proteins by covalent attachment of fatty acids promotes specific targeting of these proteins to membrane domains to facilitate signalling. In this work, we investigated the LPCAT2-mediated acylation of proteins in LPS-stimulated macrophages. The expression of LPCAT2 in RAW264 cells was suppressed using RNAi technology and the effect of LPCAT2 silencing on protein acylation was investigated using LC-MS based proteomics after isolation of acylated proteins. This study shows, for the first time, that LPS induces modification of key proteins by acylation dependent on LPCAT2 and LPCAT2 silencing inhibits the acylation of 20 proteins involved in cell metabolism. Our data thus suggest a novel mechanism for the regulation of macrophage inflammatory responses by LPCAT2.

1996

Increased glycolytic capacity in CD16⁺ monocyte subset-derived M2 macrophages is linked to foam cell formation

Lee, M.¹, Woollard, K.², Henstridge, D.¹, Palmer, C.³, Hamilton, J.A.⁴, Sviridov, D.¹, Chin-Dusting, J.¹, Murphy, A.¹

¹Baker IDI Heart and Diabetes Institute, Melbourne, Australia, ²Imperial College London, London, United Kingdom, ³Burnet Institute, Melbourne, Australia, ⁴University of Melbourne, Melbourne, Australia

Introduction: Macrophages scavenge lipids during atherogenesis. It remains unclear whether M1-inflammatory or M2-resolving macrophages have differing lipid-handling capacities. Intermediate (CD14+/16+) and non-classical (CD16+), but not classical (CD14+) monocytes, are inflammatory and significantly elevated in patients with cardiovascular disease. It is unknown if and how these specific subsets contribute to foam cell formation. We therefore aimed to determine the lipid-handling and metabolic phenotype of monocyte subset derived macrophages and to examine this in a mouse model of atherosclerosis.

Methods: The three human monocyte subsets were isolated from healthy donors by FACS and differentiated into macrophages with M-CSF and stimulated with LPS+IFN γ (M1) or IL-4 (M2) followed by incubation with oxLDL. Cell populations were analysed using flow cytometry, RT-PCR and XFe-96 Seahorse bioanalyzer. Ex vivo plaque macrophages were isolated from the atherosclerotic lesions of western-type diet fed Apoe^{-/-} mice.

Results: M2-macrophages from all human monocyte subsets express CD36, SR-A and LOX-1 receptors that take up oxLDL, whereas M1 macrophages do not. Interestingly, oxLDL-treated M2 macrophages derived from CD14+ and CD14+/16+ monocytes show upregulation of Abca1 mRNA levels which was not observed in macrophages derived from CD16+ monocytes, suggesting potential defects in cholesterol efflux capacity. Glycolytic activity was increased in CD16+ subset-derived M2-macrophages when stimulated with oxLDL. This glycolytic phenotype was also confirmed ex vivo in CD206+ (M2) macrophages in atherosclerotic plaques of Apoe^{-/-} mice.

Conclusions: We suggest that CD16+ subset-derived M2-macrophages are involved in foam cell formation through increased glycolytic metabolism. Studies exploring glycolytic pathways in atherosclerotic macrophage foam cells are warranted.

Allergy 3

1461

A helminth-derived suppressor of IL-33 in allergic asthma

McSorley, H.¹, Osbourn, M.¹, Toivakka, M.¹, Maizels, R.²

¹University of Edinburgh, MRC Centre for Inflammation Research, Edinburgh, United Kingdom, ²University of Glasgow, Wellcome Trust Centre for Molecular Parasitology, Glasgow, United Kingdom

The IL-33 pathway is strongly implicated in allergic asthma: IL-33 levels are increased in the asthmatic lung; genome-wide association studies implicate both IL-33 and the IL-33 receptor in development of disease; and administration of rIL-33 to mice results in an asthma-like phenotype. We previously showed that the excretory/secretory products (HES) of the helminth parasite *Heligmosomoides polygyrus* suppress both the release of IL-33 and expression of the IL-33 receptor in the *Alternaria* model of allergic asthma. More than 350 proteins can be identified in HES by proteomic analysis: to identify specific molecule in HES responsible for IL-33 suppression, we carried out size and charge fractionation, and identified candidate proteins in suppressive fractions by mass spectrometry. Expression of candidate

proteins in mammalian HEK293T cells, and testing in vitro, led to the identification of HpARI (*H. polygyrus* Alarmin Release Inhibitor), a protein which in recombinant form can replicate the IL-33 suppression shown by HES. In vivo, coadministration of HpARI with either *Alternaria* allergen or respiratory syncytial virus (RSV) (both of which are associated with the development of allergic asthma) results in reduced IL-33 release in the lung, in turn leading to reduced type 2 innate lymphoid cell cytokine production and ablation of BAL eosinophilia. The mechanism of action of HpARI and efficacy on human cells are presently being investigated, with an ultimate aim of developing HpARI for use as a novel treatment for human allergic disease.

2647

Role of interleukin-4 receptor alpha⁺ CD4⁺ T cells in chronic airway hyperresponsiveness

Kirstein, F., Nieuwenhuizen, N., Jayakumar, J., Horsnell, W., Brombacher, F.
University of Cape Town, Division of Immunology, Cape Town, South Africa

T-helper 2 (Th2) cells and their cytokines are associated with allergic asthma in humans and with mouse models of allergic airway disease. IL-4 signalling via interleukin-4 receptor alpha (IL-4Ra) on CD4⁺ T cells leads to Th2 cell differentiation *in vitro*, implying that IL-4Ra responsive CD4⁺ T cells are critical for the induction of allergic asthma.

This study defines the requirements for IL-4Ra responsive CD4⁺ T cells and the IL-4Ra ligands IL-4 and IL-13 during the onset and chronic phase of ovalbumin (OVA) induced allergic asthma in CD4⁺ T cell-specific IL-4Ra deficient BALB/c mice (Lck^{cre}IL4Ra^{-/lox}) mice, in the presence or absence of IL-4 or IL-13.

During acute allergic airway disease, IL-4 deficiency or absence of IL-4Ra responsive CD4⁺ T cells did not prevent the onset of Th2 immune responses, airway hyperresponsiveness (AHR) or goblet cell hyperplasia. In contrast, deficiency of IL-13 did prevent allergic asthma, irrespective of the presence or absence of IL-4Ra responsive CD4⁺ T cells. Importantly, IL-4Ra responsive CD4⁺ T cells were required to sustain chronic airway hyperresponsiveness and eosinophilic lung inflammation. Furthermore, deficiency in IL-4Ra responsive CD4⁺ T cells resulted in increased numbers of IL-17 producing T cells in Lck^{cre}IL4Ra^{-/lox} mice and consequently increased airway neutrophilia. However, airway hyperresponsiveness was not significantly altered by anti-IL-17 treatment.

In summary, IL-4-responsive T helper cells are dispensable for acute ovalbumin-induced airway disease but required to maintain chronic asthmatic pathology.

1223

B1 cells produce large amounts of IgE post-helminth infection and this IgE is protective against allergic disease

Martin, R.K., Damle, S.R., Conrad, D.H.

Virginia Commonwealth University, Microbiology and Immunology, Richmond, United States

Helminth infection is known for generating large amounts of non-specific IgE. This hinders parasite-specific IgE, thereby reducing

mast cell (MC) degranulation and anti-helminth immunity. Here we report that innate-like B1 cells are responsible for the non-specific IgE production during the helminth infection, *Nippostrongylus brasiliensis* (*Nb*). We utilize a mouse model that lacks B2 cells, but has equivalent IgE production to WT during *Nb* infection. Using this model with the NP-KLH system, B1 cells made no antigen-specific IgG1 nor exhibited memory. Notably, the B1 produced IgE is unable to induce MC degranulation in an active cutaneous anaphylaxis model and can block MC degranulation in a model of passive cutaneous anaphylaxis. Sorted B1 cells from *Nb*-infected WT mice make increased amounts of IgE in post-sort culture with IL-4, anti-CD40, and IL-5 over naïve B1-culture by ELISA, but equivalent secreted IgE message by RT-PCR. This is due to increased proliferative capacity of B1 cells in *Nb*-infected animals. Finally, we used RAG^{-/-} reconstitution to isolate the direct contribution of B1 cells alone, B2 cells alone, or B1 and B2 cells together. B1-only reconstituted RAG^{-/-} make large amounts of IgE in *Nb* infection and have higher fecal egg burdens than B2-only reconstituted RAG^{-/-}. Overall, these data represent a protective response that has evolved for the helminth. We hypothesize that human B1-IgE production could be harnessed as potential protection against allergic disease and may help explain the reduced atopy seen in parasite endemic populations.

2720

Serial mast cell tryptase measurements: sensitivity and specificity for a diagnosis of anaphylaxis in patients with shock and/or hypoxemia

Creamer, A.^{1,2}, *Fatovich, D.M.*^{1,2,3}, *Stone, S.F.*^{1,2}, *Macdonald, S.P.J.*^{1,2,3,4}, *Arendts, G.*^{1,2,3,5}, *Nagree, Y.*^{1,5}, *Mitenko, H.M.A.*^{1,6}, *Rajee, M.*^{1,7}, *Brown, S.G.A.*^{1,2,3,8,9}

¹Centre for Clinical Research in Emergency Medicine, Harry Perkins Institute of Medical Research, Perth, Australia, ²University of Western Australia, Crawley, Australia, ³Royal Perth Hospital, Perth, Australia, ⁴Armadale Kelmscott Memorial Hospital, Armadale, Australia, ⁵Fiona Stanley Hospital, Murdoch, Australia, ⁶South West Health Campus (formerly Bunbury Regional Hospital), Bunbury, Australia, ⁷Austin Hospital, Heidelberg, Australia, ⁸University of Tasmania, Hobart, Australia, ⁹Royal Hobart Hospital, Hobart, Australia

Background: Anaphylaxis is difficult to identify in cases presenting with atypical symptoms. Serial measurements of mast cell tryptase (MCT), looking for changes in MCT levels (delta-MCT) may increase the sensitivity of current diagnostic methods. The usefulness of this approach depends on the ability of delta-MCT to distinguish anaphylaxis from other critical illnesses, which is investigated in this study.

Methods: MCT was measured (ImmunoCAP®) in serum samples from patients with anaphylaxis (n=85) and non-anaphylactic critical illness (n=120) on at least three occasions: ED arrival, 1-2 hours, 3-4 hours, and 12-24 hours post-arrival where possible. Delta-MCT was calculated as the difference between the highest and lowest values regardless of time point, and analyzed using Receiver Operating Characteristic Curves. A positive delta-MCT was defined as >2.0 ng/mL.

Results: 48 (56%) of 85 anaphylaxis cases were positive,

compared to 31 (25%) of 120 cases of critical illness (including sepsis, cardiac arrest, trauma and toxicology). Non-anaphylaxis cases had delta-MCT values ranging from 0-22 ng/mL (2 cases had delta-MCT >10), whereas anaphylaxis cases had delta MCT values ranging from 0-114 ng/mL (22 cases had delta-MCT >10). The diagnostic specificity was 0.74 with a sensitivity of 0.56. Higher cutoff values provided higher specificity but lower sensitivity.

Conclusion: The specificity and sensitivity observed in this study indicate that delta-MCT measurements do not perform well in cases where anaphylactic shock has to be differentiated from other critical illnesses, which may also involve some mast cell degranulation. In this situation higher cut-offs are required, however this results in poor sensitivity.

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Deficit of Treg cells and vitamin D are predisposing factors for the establishment of cow's milk protein allergy in infants

*López-Abente, J.*¹, *Perezabad, L.*², *Alonso-Lebrero, E.*³, *Seoane Reula, E.*⁴, *Pion, M.*¹, *Correa-Rocha, R.*¹

¹Gregorio Marañón Health Research Institute, Laboratory of Immune-Regulation, Madrid, Spain, ²CIAL-CSIC, Department of Bioactivity and Food Analysis, Madrid, Spain, ³Hospital General Universitario Gregorio Marañón, Immunopediatric Division, Madrid, Spain, ⁴Hospital General Universitario Gregorio Marañón, Pediatric-Allergy Division, Madrid, Spain

Background: Cow's milk protein allergy (CMPA) is the most common food allergy in infants. However, little is known about which specific immune mechanisms are responsible of the CMPA onset. The objective was to investigate which immune alterations constitute the differential factors between allergy and tolerance, and hence would be responsible for the CMPA onset in infants.

Methods: Blood samples from infants younger than 9 months were obtained 1-4 days after the first adverse reaction to milk. We performed an extensive analysis of immune subsets, including Treg and cytokine-secreting cells previous to the definitive diagnosis of CMPA.

Results: Less than 4 days after milk exposure, infants with a CMPA diagnosis had decreased counts of Treg cells and an increased frequency of IL4-secreting CD4 T cells compared to controls. The deficit of Treg cells was not due to their decreased production by the thymus, but it was significantly correlated with decreased values of vitamin D in plasma. Values of Treg, IL4-secreting cells and vitamin D were good predictors of CMPA diagnosis.

Conclusion: After a first reaction to milk proteins, Treg cells from CMPA infants would have an impaired survival related to a vitamin D deficit. Decreased Treg numbers might favor the expansion of IL4-secreting CD4 T cells, and consequently these infants acquire an allergic phenotype to milk proteins. These immune alterations would be the crucial factor behind the CMPA onset, they could constitute predictive markers and might be a target for the prevention and treatment of this allergy in infants.

1664

Airway allergy can be controlled through dendritic cell subset manipulation in a mouse model

Takahashi, H.¹, Murakami, R.^{1,2}, Nakagawa, Y.¹, Shimizu, M.¹, Wakabayashi, A.¹, Negishi, Y.¹, Takachika, H.³, Shinya, E.¹, Ohkubo, K.²
¹Nippon Medical School, Department of Microbiology and Immunology, Tokyo, Japan, ²Nippon Medical School, Department of Otorhinolaryngology, Tokyo, Japan, ³Tokyo Metropolitan Institute of Medical Science, Department of Allergy and Immunology, Tokyo, Japan

Two major distinct subsets of dendritic cells (DCs) are arranged to regulate immune responses; DEC 205⁺ DCs drive Th1 polarization and 33D1⁺ DCs establish Th2 dominance. Th1 polarization can be achieved either by depletion of 33D1⁺ DCs with a 33D1-specific monoclonal antibody (mAb) or by activation of DEC-205⁺ DCs via intraperitoneal (i.p.) injection of α -galactosylceramide (α -GalCer). We studied the effect of 33D1⁺ DC depletion or DEC-205⁺ DC activation in vivo using an established mouse model of allergic rhinitis (AR). Mice were injected i.p. with OVA plus alum and challenged four times with daily intranasal (i.n.) administration of OVA. Immediately after the last challenge, allergic symptoms such as sneezing and nasal rubbing as well as the number of cells in the bronchoalveolar fluid (BALF) and nasal lavage fluid (NALF) were counted. The levels of serum OVA-specific IgG1, IgG2a and IgE were also determined by ELISA. The allergic symptom scores were significantly decreased in 33D1⁺ DC-depleted or DEC-205⁺ DC-activated AR mice. The levels of OVA-specific IgG1, IgG2a and IgE, and the number of NALF but not BALF cells were reduced in 33D1⁺ DC-depleted but not in DEC-205⁺ DC-activated AR mice. Moreover, the activated DEC-205⁺ DCs suppressed histamine release from IgE-sensitized mast cells, probably through IL-12 secretion. The manipulation of innate DC subsets may provide a new therapeutic strategy for controlling various allergic diseases by reducing histamine release from IgE-sensitized mast cells by driving the immune response towards Th1 dominance via activation of DEC-205⁺ DCs in vivo.

2766

Characterizing immune responses in severe T-cell mediated adverse drug reactions

Redwood, A.¹, Pavlos, R.¹, Strautins, K.¹, James, I.¹, Konvinse, K.², White, K.², Ergen, E.², Trubiano, J.³, Leary, S.¹, Koelle, D.⁴, Mallal, S.^{1,2}, Phillips, E.^{1,2}

¹Murdoch University, Institute for Immunology and Infectious Diseases, Murdoch, Australia, ²Vanderbilt University, Nashville, United States, ³Alfred Health, Department of Infectious Diseases, Melbourne, Australia, ⁴University of Washington School of Medicine, Seattle, United States

The severest of immune mediated adverse drug reactions (IM-ADR) are primarily class I HLA restricted, although only a small proportion of patients carrying an HLA risk allele develop an IM-ADR. To define the specific immunopathogenesis of these T-cell mediated ADRs we have drawn from a resource of HLA ABC DR DQ DP genotyped and cryopreserved paired PBMCs and blister fluid/skin biopsies developed from subjects with

severe IM-ADRs (Stevens-Johnson Syndrome/Toxic epidermal necrolysis (SJS/TEN), abacavir hypersensitivity, and drug reaction with eosinophilia and systemic symptoms (DRESS)). ELISpot and ICS were used to define drug specificity and T-cell involvement. Putative pathogenic TCRs were identified through single cell index sorting of drug reactive T cells and single cell T-cell receptor (TCR) sequencing. SNP analysis of genes involved in the antigen processing and presentation pathway was employed to refine genetic associations. A Jurkat T cell-based luciferase reporter assay was developed to further characterize the specificities of TCRs of interest. Immune responses differed with the drug, clinical phenotype/tissue specificity, longevity of immune response, CD4⁺, CD8⁺ and NK cell dependency, and the oligoclonality of drug specific T-cell populations. Two IM-ADR susceptibility genes were identified within the antigen presentation pathway, endoplasmic aminopeptidase 1 (ERAP1) and ERAP2. We hypothesize that an early immunodominant response to a chronic prevalent pathogen such as the human herpes viruses can later generate cross-reactive responses in the presence of drug-endogenous peptide and HLA risk allele and that this helps explain the short latency, long-lasting immunity and tissue specificity of many severe T-cell mediated ADRs.

3869

Regional variation in allergic sensitivity to subtropical and temperate grass pollen allergens; outcomes of the multicentre cross-sectional Grass Pollen Allergy Survey (GPAS)

Davies, J.¹, Timbrell, V.¹, Reibelt, L.², Simmonds, C.², Solley, G.³, Smith, W.⁴, Mclean-Tooke, A.⁵, van Nunen, S.⁶, Smith, P.⁷, Upham, J.⁸, Langguth, D.²

¹Queensland University of Technology, Brisbane, Australia, ²Sullivan Nicolaides Pathology, Brisbane, Australia, ³Watkins Medical Centre, Brisbane, Australia, ⁴Royal Adelaide Hospital, Adelaide, Australia, ⁵Sir Charles Gairdner Hospital, Perth, Australia, ⁶Royal North Shore Hospital and University of Sydney, Sydney, Australia, ⁷Queensland Allergy Services, Gold Coast, Australia, ⁸The University of Queensland and The Princess Alexandra Hospital, Brisbane, Australia

Grass pollens (GP) are major outdoor aeroallergens globally. Whilst allergen immunotherapy focuses on temperate (Pooideae) GP, allergens of subtropical (Panicoidae, Chloridoideae) GP show species-specific immunity. We aimed to evaluate regional variations in allergic sensitivity to temperate and subtropical GP.

Subjects (patients; 321, non-atopic; n = 31, other allergies; n = 42) were recruited with informed consent. Clinical history of allergic rhinitis and asthma and skin prick test (SPT) to GP extracts were evaluated (prior GP immunotherapy excluded). Total and specific serum IgE to GP and seven purified allergen components were measured by ImmunoCAP.

GP-allergic patients from Queensland showed higher SPT and IgE to Bahia and Bermuda GP as well as Pas n 1 and Cyn d 1, than Ryegrass pollen and Lol p 1. Patients from Adelaide and Sydney however, showed higher SPT and IgE to Ryegrass than Bermuda and Johnson GP. In Perth, SPT to Ryegrass was higher than Johnson GP, and IgE with Ryegrass was higher than with

Bermuda GP. In Adelaide, Sydney or Perth sensitivity to Bahia GP was similar to Ryegrass, but IgE to Lol p 1 was higher than IgE to Cyn d 1 or Pas n 1. Patients from Adelaide showed higher IgE reactivity than patients from QLD with the allergen (Lol p 5) specific to temperate GP.

Patients show regional differences in levels and patterns of allergic sensitivity with subtropical or temperate GP consistent with grass distributions. The outcomes provide insights for design of more specific diagnosis and treatment of GP allergy.

1368

Mucosal route of immunotherapy with transgenic rice seeds expressing hypoallergenic whole T cell epitopes of Cryj1 and Cryj2 - Investigation in murine model of cedar pollinosis

Kawauchi, H.¹, Aoi, N.¹, Yamada, T.², Takagi, H.³, Takaiwa, F.³

¹Shimane University, Faculty of Medicine, Otorhinolaryngology, Izumo, Japan, ²Shimane University, Center for Integrated Research in Science, Department of Experimental Animals, Izumo, Japan,

³National Institute of Agrobiological Sciences, Ministry of Agriculture, Tsukuba, Japan

For the last decade, we have been investigating the therapeutic effect of mucosal route of administration of transgenic rice (Tg-rice) seeds, which contain T-cell epitopes of Cryj1 and Cryj2, on murine allergic rhinitis models and reported its clinical efficacy to actually attenuate nasal symptoms. However, its mechanism remains to be further investigated and adverse events of this therapeutic approach should be very least with more sophisticated manners.

Results: Therefore, we have examined the effect of natural feeding with protein bodies (PB) of transgenic rice seeds expressing hypoallergenic whole T cell epitopes of Cryj1 and Cryj2 (PB-Tg rice), in comparison with whole Tg-rice, in a murine model of cedar pollinosis. The numbers of sneezing after final intranasal challenge in mice naturally fed with PB-Tg-rice powder were significantly decreased in a dose dependent manner, with less doses, in comparison with those of whole Tg-rice powder. Histopathological findings correspondingly demonstrated that the number of eosinophils infiltrating into nasal mucosa decreased and the damage of epithelial cells was less found in each group of mice. Sublingual route of administration is also effective to attenuate nasal symptoms.

Conclusion: Protein body fraction of Tg-rice more efficiently downregulated nasal symptom in murine model of cedar pollinosis with natural feeding or sublingual administration. These result implicates that intake of protein body form of Tg-rice can be more promising strategy and material to be utilized for mucosal route of immunotherapy to attenuate nasal symptoms of patients with cedar pollinosis.

1279

Thermal processing enhances IgE but not T cell reactivity of shellfish allergens

Abramovitch, J.^{1,2}, Lopata, A.³, O'Hehir, R.^{1,2}, Rolland, J.^{1,2}

¹Monash University, Immunology and Pathology, Melbourne, Australia, ²Alfred Health, Central Clinical School, Monash University

Melbourne, Allergy, Immunology and Respiratory Medicine, Melbourne, Australia, ³James Cook University, School of Pharmacy and Molecular Science, Townsville, Australia

Background: Shellfish allergy is a major cause of food-induced anaphylaxis. We showed previously that heating increases IgE reactivity of crustacean allergens. Here we investigate the effects of heating of crustacean extracts on cellular immune reactivity.

Methods: IgE reactivity of raw and cooked black tiger prawn, banana prawn, mud crab and blue swimmer crab extracts was assessed by ELISA. PBMC from shellfish-allergic (n=8) and control, non-atopic (n=4) subjects were cultured with the extracts, proliferation assessed by CFSE labelling and effector responses by intracellular IL-4 and IFN- γ . Regulatory T (CD4⁺CD25⁺CD127^{lo}Foxp3⁺) cell proportions in cultures were also compared.

Results: For each crustacean species, the cooked extract had greater IgE reactivity than the raw (mud crab p< 0.05, others p< 0.01). In contrast, there was a trend for higher PBMC proliferation to raw extracts. In shellfish-stimulated PBMC cultures, dividing CD4⁺ and CD56⁺ lymphocytes showed higher IL-4/IFN- γ ratios for shellfish-allergic subjects than for non-atopics (p< 0.001), but there was no difference between raw and cooked extracts. The percentage IL-4⁺ of dividing CD4⁺ cells correlated with total and allergen-specific IgE levels. There was a trend for higher proportions of regulatory T cells in cultures stimulated with raw compared with cooked extracts (mud crab p< 0.001, banana prawn p< 0.05).

Conclusions: Our novel findings confirm that raw and cooked shellfish extracts should be included in reliable specific-IgE diagnostic assays, but suggest that raw extracts are suitable for analysis of cellular responses. This has important implications for development of a safe T cell-targeted allergen-specific treatment for shellfish allergy.

Mini Oral Sessions

15:30:00 - 16:30:00

Transplantation

3808

Treg reconstitution before allogeneic bone marrow transplantation reduces T cell proliferation, activation status, and inflammatory cytokine secretion

Pijning, A.E., Bolton, H.A., Terry, A.M., Shklovskaya, E.V., Fazekas de St Groth, B.

Centenary Institute, University of Sydney, NSW, Sydney, Australia

Transfer of regulatory T cells (Tregs) has been proposed as a therapy for graft-versus-host disease (GVHD) in bone marrow transplant (BMT) recipients. The exact mechanism by which Tregs control GVHD is not yet well established. Previously, we used a Treg reconstitution approach in Rag-KO mice to show that Tregs prevent rapid oligoclonal lymphopaenia-induced proliferation, while promoting slow homeostatic repopulation of the naïve T cell pool. This process was critically dependent upon Treg-mediated down regulation of the co-stimulatory molecules CD80 and CD86 on DCs. Using a mouse model in which the Treg compartment of allogeneic BMT recipients is selectively reconstituted prior to transfer of allogeneic donor T cells, we showed that early Treg reconstitution completely protected against GVHD up to 120 days post-transplant.

Here we characterized the effect of Tregs on the early GVH response. When adoptively transferred after Treg reconstitution, allogeneic donor T cells underwent slow homeostatic division and retained a naïve phenotype. In contrast, in BMT recipients that were not Treg reconstituted, donor T cells underwent rapid proliferation and acquired an effector memory phenotype. Rapid proliferation of donor T cells was associated with increased expression of inflammatory cytokines, which were not seen in Treg reconstituted BMT recipients. These results provide a mechanism for the clinically improved outcome of BMT after Treg transfer, and stress the importance of achieving full Treg reconstitution before transfer of allogeneic T cells. In ongoing research, the effects of Treg reconstitution on T cell homing, proliferation, and phenotype in GVHD target organs are being investigated.

1301

Graft-versus-host disease precipitates cytomegalovirus reactivation after allogeneic bone marrow transplantation: lessons from the first preclinical model

Martins, J.P.¹, Fleming, P.^{2,3}, Kuns, R.D.¹, Ullah, M.A.¹, Varelias, A.¹, Koyama, M.¹, Andoniou, C.E.^{2,3}, Tey, S.K.¹, Degli-Espositi, M.A.^{2,3}, Hill, G.R.^{1,3}

¹QIMR Berghofer Medical Research Institute, Brisbane, Australia,

²Centre for Experimental Immunology, Lions Eye Institute,

Nedlands, Australia, ³Immunology and Virology Program, Centre for Ophthalmology and Visual Science, The University of Western Australia, Crawley, Australia

Cytomegalovirus (CMV) infection remains a significant complication after allogeneic bone marrow transplantation (BMT) and for reasons that are poorly understood, CMV seropositivity remains a major determinant of clinical transplant outcome.

Aim: To elucidate the mechanisms and immunological consequences of CMV reactivation in a newly established model.

Methods: Mice were infected with murine CMV (MCMV) and functional latency defined as the resolution of viremia in target organs and plasma. Latently infected mice were transplanted with Bone Marrow (BM) and T cells or Tcell-depleted (TCD) BM alone from MHC-disparate or MHC-matched uninfected donors to generate GVHD and non-GVHD conditions respectively. Reactivation was determined by qPCR on plasma and plaque assays in target organs after BMT.

Results: MCMV enters latency two months after primary infection with the resolution of viremia correlating with absence of replicating virus in organs by plaque assays. The reactivation of MCMV after BMT is GVHD-dependent: BALB/c→B6: 63% vs 17% (p< 0.05) and B6→BALB/c: 100% vs 15% (p< 0.0001) in GVHD vs non-GVHD recipients. This is corroborated by plaque assays, with virus replication detected in 54% vs 0% (p< 0.01) of livers and 46% vs 8% of lungs in the former model. GVHD is characterized in mice by an interferon-based cytokine storm early after BMT and recipients of IFN γ -/- grafts are more susceptible to CMV reactivation: wild type vs. IFN γ -/-: 20% vs 100% (p< 0.01) and 18% vs 100% (p< 0.001) in MHC-mismatched and MHC-matched systems, respectively.

Conclusion: CMV reactivation after BMT is GVHD-dependent and is inhibited by IFN γ signaling.

4084

Identifying cellular subsets diagnostic for severity and organ specificity chronic GVHD

Stikvoort, A.¹, Chen, Y.², Rådestad, E.¹, Törlén, J.¹, Sundberg, B.¹, Sundin, M.³, Mattsson, J.⁴, Brodin, P.², Uhlin, M.¹

¹Karolinska Institutet, Department of Oncology-Pathology, Stockholm, Sweden, ²Karolinska Institutet, Department of Medicine, Stockholm, Sweden, ³Karolinska University Hospital, Division of Paediatrics, Huddinge, Sweden, ⁴Karolinska University Hospital, Centre for Allogeneic Stem Cell Transplantation, Huddinge, Sweden

Chronic graft-versus-host-disease (cGvHD) is a severe late complication after hematopoietic stem cell transplantation, with a pathophysiology similar to severe auto-immune disorders.

We performed an extensive immune-phenotypic analysis on peripheral blood from 42 patients diagnosed with varying grades of cGvHD by flow cytometry (FC) and mass cytometry (CyTOF). Patients were divided into 4 groups according to the NIH-criteria: no (n=11), mild (n=8), moderate (n=12) and severe cGvHD (n=11). Additionally, the moderate and severe cGvHD patients were sub-grouped dependent on organ involvement.

An increased expression of activation marker CD38 on CD8+ T cells was associated with an increased severity of cGvHD. This was also correlated to a decreased frequency of mucosal-associated-invariant T (MAIT) cells. Additionally, patients with GI-tract-associated cGvHD displayed reduced frequencies of

MAIT cells. Expression of KIR CD158b was reduced in several NK cell subsets in patients with skin-associated cGvHD. Interestingly, CXCL10 was found to be increased in patients with liver-associated cGvHD. CyTOF analysis demonstrated both the ability to identify similar cellular subsets as found by traditional FC, as well as the ability to identify novel subsets of interest. These novel subsets consist of mostly small populations that appear conserved among patients. The role of these subsets needs further investigation.

In conclusion, we identified several subsets that could be correlated to cGvHD severity and localization. CyTOF may aid in identifying new markers vital to increase our knowledge on cGvHD pathophysiology. Subsequently, these markers may then be used in smaller FC panels for diagnostic purposes.

4098

Ex vivo immunological analysis following decidual stromal cell therapy in patients with acute graft-versus-host disease

Erkers, T.¹, Solders, M.^{1,2}, Verlang, L.¹, Nava, S.¹, Mollén, P.¹, Mattsson, J.^{2,3}, Ringdén, O.¹, Lundell, A.-C.⁴, Kaipe, H.^{1,5}

¹Karolinska Institutet, Laboratory Medicine, Stockholm, Sweden,

²Karolinska University Hospital Huddinge, Center for Allogeneic Stem Cell Transplantation, Stockholm, Sweden, ³Karolinska Institutet, Oncology-Pathology, Stockholm, Sweden, ⁴University of Gothenburg, Sahlgrenska Institute, Gothenburg, Sweden,

⁵Karolinska University Hospital Huddinge, Clinical Immunology and Transfusion Medicine, Stockholm, Sweden

Severe acute graft-versus-host disease (GVHD) is a common complication after hematopoietic stem cell transplantation. In this study, we present immunological surveillance data from 25 interventions in patients who were treated with placenta-derived decidual stromal cells (DSCs) for severe acute GVHD. Depending on clinical response, the patients were retrospectively divided into responders (n=17) or non-responders (n=8). Blood samples were obtained before and 3h, 1 week, 2 weeks, and 4 weeks after the DSC infusion. OPLS-DA and subsequent univariate analyses were used to discriminate and detect potential differences between the two groups based on data from 27 cytokines and 56 flow cytometry parameters. Interestingly, non-responding patients had significantly higher levels of IL-6 ($P=0.035$), IL8 ($P=0.048$) and IP-10 ($P=0.001$) compared to responding patients before DSC treatment. The frequency of CD4⁺T-cells expressing HLA-DR was reduced over time in the responding ($P=0.037$), but not in the non-responding patients. The frequency of naive (CD45RA⁺CCR7⁺) CD4⁺T-cells was higher in the non-responders at 1 week ($P=0.033$). The expression of the gut homing marker CCR9 in CD4⁺T-cells was higher in the responders compared to the non-responders at 4 weeks ($P=0.022$), and the frequency of $\alpha 4\beta 7^+$ expression in CD4⁺CD25⁺T-cells was increased after DSC-therapy in the responders over the time of measurement ($P=0.005$). To conclude, DSCs may induce changes in parameters that are of interest in GVHD pathophysiology. The data also suggests that IL6, IL-8 and IP-10 can be used to predict response to DSC-therapy.

4173

Placenta-derived decidual stromal cells alter IL-2R expression and signaling in alloantigen-activated T cells

Erkers, T.¹, Solders, M.¹, Verlang, L.¹, Bergström, C.¹, Stikvoort, A.², Rane, L.¹, Nava, S.¹, Ringdén, O.¹, Kaipe, H.¹

¹Karolinska Institutet, Therapeutic Immunology, Stockholm, Sweden, ²Karolinska Institutet, Oncology and Pathology, Stockholm, Sweden

Stromal cells are important in immune homeostasis. Due to their immunoregulatory functions, stromal cells have received increased attention in translational medicine as a cellular therapy for inflammatory conditions. In the present study, we examined how decidual stromal cells (DSCs) from term placentas affect the IL-2 pathway in alloreactive T cells ($n \geq 11$). We found that DSCs promoted significantly higher production of IL-2 from alloantigen-activated T cells compared to control mixed lymphocyte reactions (median 1.9 ng/ml vs 0.02 ng/ml, $P < 0.001$). The intensity of expression of the IL2R α on T cells was increased by DSCs ($P < 0.001$), whereas the expression of the signaling subunits IL-2R β and IL-2R γ_c was reduced ($P < 0.001$). This was accompanied by a decreased uptake of exogenously added ¹²⁵I-labelled IL-2 ($P < 0.01$) and a reduced STAT5-phosphorylation ($P < 0.05$) in T cells co-cultured with DSCs. The anti-proliferative effect of sirolimus (SRL), which targets the IL2 signaling pathway via mTOR, was diminished by DSCs *in vitro* ($P < 0.05$). This was not observed when combining cyclosporine A (CsA) and DSCs. To investigate the clinical importance of our findings, IL-2 levels, proportion of regulatory T cells, and IL2R α expression on T-cells were determined in 21 patients treated with DSCs for acute graft-versus-host disease (GVHD). No differences were detected before or after treatment, or between patients who received SRL or CsA as GVHD prophylaxis. To conclude, DSCs affect IL-2 production and signaling *in vitro*, and may influence the efficacy of immunosuppressive drugs. However, there were no indications that DSCs have any systemic effect on the IL-2 pathway *in vivo*.

1925

Downregulating cyclin-dependent kinase 9 of alloreactive CD4⁺T cells prolonged allograft survival

Hou, G., Zhan, Y.

School of Medicine Shandong University, Institute of Experimental Nuclear Medicine, Jinan, China

Allograft acute rejection has been the main barrier in clinic and it is urgent to find new target molecules varied significantly during allojection for diagnosis and therapy. A variety of inflammatory cells and molecules contribute to the development of allograft rejection. CDK9 (Cyclin-Dependent Kinase 9), a Positive Transcription Elongation Factor b (P-TEFb) kinase component, was reported to regulate several inflammatory diseases, including arthritis and atherosclerosis. However, the roles played by of CDK9 during allojection have not been described. This study aim to investigate whether the CDK9/Cyclin T1/RNA polymerase II pathway impacts CD4⁺T cells mediated -allograft rejection in mice models. The results showed that CDK9/Cyclin T1 were apparently up-regulated in

the allogeneic group than in the syngeneic group at the all time point (days 4, 8, 12, and 16) and positively associated with the severity of graft damage. Allografted SCID mice with adoptive transfer of CD4+ T cells pretreated with PHA767491 (Inhibitors of CDK9) revealed CDK9 inhibition in CD4+ T cell-mediated allojection model, the PHA767491-pretreated group showed prolonged survival than the PBS-pretreated group. This effect was associated with increase of IL-10, IL-4 and decrease of IFN- γ . Thus, our results demonstrated that CDK9/Cyclin T1/Pol II signaling regulated allojection in a mouse skin allograft model, which provide a new approach for prolong allograft through targeting CDK9.

1981

Lymphocyte expression of CD31 and leukocyte-derived CD31, two new markers of antibody-mediated rejection in human heart transplantation

Sannier, A.^{1,2}, Dorent, R.^{1,3}, Le Borgne, M.¹, Stroumza, N.¹, Gaston, A.-T.¹, Khallou-Laschet, J.¹, Deschildre, C.¹, Nataf, P.^{1,4}, Couvelard, A.², Caligiuri, G.^{1,3}, Nicoletti, A.¹

¹INSERM U 1148, Paris, France, ²AP-HP, Department of Pathology, Bichat University Hospital, Paris, France, ³AP-HP, Department of Cardiology, Bichat University Hospital, Paris, France, ⁴AP-HP, Department of Cardiosurgery, Bichat University Hospital, Paris, France

Background: Antibody-mediated rejection (AMR) severely shortens survival of heart allografts for which accurate non-invasive diagnostic tools are lacking. CD31 is a protein exclusively expressed at the blood-vessel interface. Its extracellular portion is cleaved upon immune stimulation thereby constituting a promising biomarker in AMR diagnosis.

Materials: Heart-transplanted (HTx) patients received a 5-day induction therapy of rabbit anti-thymocyte globulin (rATG) followed by a classical maintenance immunosuppression. AMR diagnosis was based on detection of anti-donor antibodies associated with pathological criteria of AMR on endomyocardial biopsy (pAMR2 in ISHLT classification).

Results: HTx patients (n=25) had significantly increased plasma levels of leukocyte-derived CD31 but patients diagnosed with AMR (n=5) had lower CD31 plasma levels and a higher proportion of CD4 T cells with a CD31+ recent thymic emigrant (RTE) phenotype. The initial lymphodepletion under rATG treatment was less efficient in patients that later developed an AMR (5-day post-transplantation lymphocyte count: 0.26 versus 0.17 G/l, P < 0.05). However, rATG binding assays on T cells and complement-mediated cytotoxicity did not differ between AMR and other HTx patients.

Conclusion: Among HTx patients, those with AMR patients have a higher proportion of RTE T cells. These patients are less depleted during the induction therapy but this is not due to a lower sensitivity to the rATG treatment. A better lymphocyte replenishment may be an additional factor explaining their larger RTE compartment. Their thymopoiesis and/or antigen-independent cytokine-induced peripheral homeostatic proliferation must now be addressed since both processes can promote the expansion of T cells with a RTE naïve phenotype.

B Cells 1

3547

Tfh-derived dopamine: motivation, learning and reward in the germinal centre

Papa, I.¹, Ponzoni, M.², Bustamante, S.³, Sweet, R.A.¹, Vohra, H.⁴, Canete, P.F.¹, Gonzalez Figueroa, P.¹, Hawley, N.¹, Doglioni, C.², Vinuesa, C.G.¹

¹John Curtin School of Medical Research - the Australian National University, Immunology and Infectious Disease, Canberra, Australia, ²San Raffaele Scientific Institute, Pathology, Milan, Italy, ³The University of New South Wales, Bioanalytical Mass Spectrometry Facility Mark Wainwright Analytical Centre, Sydney, Australia, ⁴John Curtin School of Medical Research - the Australian National University, Imaging and Cytometry Facility, Canberra, Australia

The germinal centre (GC) is a unique structure that develops in secondary lymphoid organs during immune responses to protein antigens, where high affinity antibodies and memory B cells are generated. In order to ensure fast selection of rapidly evolving clones in GC, follicular dendritic cells (FDCs), GC B cells and T cells engage in multiple short-lived interactions. So far, only co-stimulatory or inhibitory membrane immune receptor/ligand pairs and cytokines have been investigated and are known to be involved in this process. The speed and complexity of cellular interactions in this microenvironment is reminiscent of cellular connections and synaptic communication within the nervous system. Furthermore, molecules involved in axon growth and guidance have been found to be differentially expressed within GC B cells, suggesting that a neural-like transmission pathways may also be involved in GC B cell selection. Here we show that a subset of germinal centre T cells contains chromogranin B⁺ electron-dense granules typically found in neurons, and catecholamines, such as dopamine and noradrenaline. Furthermore RNA analysis and *in vitro* stimulation with dopamine shows this neurotransmitter is involved in a cross-talk among Tfh, GC B cells and FDCs. Our findings suggest a new co-stimulatory role of Tfh cell-derived dopamine during B cell maturation in germinal centres.

736

Interferon-g receptor and STAT1 signaling in B cells are central to spontaneous germinal center formation and autoimmunity

Domeier, P.P.¹, Chodisetti, S.B.¹, Soni, C.¹, Schell, S.L.¹, Elias, M.J.¹, Kitamura, D.², Rahman, Z.S.M.¹

¹Penn State University College of Medicine, Microbiology and Immunology, Hershey, United States, ²Tokyo University of Science, Research Institute for Biomedical Sciences, Tokyo, Japan

In Systemic Lupus Erythematosus (SLE), spontaneously developed germinal centers (Spt-GCs) harbor autoAb-producing B cells that promote disease, but the mechanisms that control Spt-GC development are not clear. Here we report that B cell-intrinsic IFN γ R and STAT1 signaling are essential for Spt-GC formation. The IFN γ R deficiency, however, does not significantly affect foreign Ag-induced GC and Tfh responses,

suggesting that two distinct mechanisms regulate foreign-Ag vs Spt-GC responses. We also demonstrate that IFN γ R-mediated STAT1 signaling drives T-bet expression, pro-GC gene expression and IFN γ production by B cells, which are critical for Spt-GC and Tfh development. To understand how IFN γ signaling regulates the Spt-GC response and associated autoAb production in SLE, we utilized B6.*Sle1b* mice that harbor the lupus-prone NZM2410 strain-derived SLAM locus. B6.*Sle1b* mice exhibit elevated Spt-GC and follicular helper T cell (Tfh) responses that associate with impaired B cell selection in GCs. B6.*Sle1b* mice with B cell intrinsic IFN γ R deficiency also have significantly reduced Spt-GC and Tfh responses resulting in a markedly lower number of IgG-producing antibody forming cells and diminished IgG_{2c} and IgG_{2b} autoantibody titers than B6.*Sle1b* mice. Additionally, the proliferation and differentiation of DNA-reactive B cells into a GC B cell phenotype require B cell-intrinsic IFN γ R signaling. Together, our data define a novel B cell-intrinsic IFN γ R signaling pathway in Spt-GC development and autoimmunity.

1328

LRRK1 plays an essential role in B-cell responses by regulating BCR-dependent NF- κ B signaling

Morimoto, K.¹, Baba, Y.^{2,3}, Shinohara, H.⁴, Kurosaki, T.^{2,3}, Toyofuku, T.⁵, Kumanogoh, A.¹

¹Osaka University Graduate School of Medicine, Department of Respiratory Medicine, Allergy and Rheumatic Disease, Suita, Japan, ²Osaka University, WPI Immunology Frontier Research Center, Laboratory for Lymphocyte Differentiation, Suita, Japan, ³RIKEN Center for Integrative Medical Sciences (IMS), Laboratory for Lymphocyte Differentiation, Yokohama, Japan, ⁴RIKEN Center for Integrative Medical Sciences (IMS-RCMI), Laboratory for Integrated Cellular Systems, Yokohama, Japan, ⁵Osaka University Graduate School of Medicine, Department of Immunology and Regenerative Medicine, Suita, Japan

Leucine-rich repeat kinase 1 (LRRK1), the homolog of famous Parkinson's disease-related gene LRRK2, has distinct functions including the regulation of intracellular trafficking of epidermal growth factor receptor, microtubule nucleation, autophagy, and osteoclast differentiation. In addition, LRRK1 has been shown to be expressed predominantly in B cells and monocytes in human peripheral blood, its contribution to the immune system remains to be elucidated. Here, we found that LRRK1 was expressed over the course of B cell development and *Lrrk1*^{-/-} mice showed altered B1a-cell development and impaired basal immunoglobulin production. Moreover, production of IgG3 antibody in response to

T cell-independent type 2 antigen was affected in *Lrrk1*^{-/-} mice due to defects in IgG3 class-switch recombination. In vitro analysis revealed that ablation of LRRK1 in B cells resulted in defective proliferation and survival in response to BCR stimulation, but not to LPS or anti-CD40 antibody. Further analysis revealed impaired BCR-mediated NF- κ B activation and reduced expression of NF- κ B target genes. Our results provide clues about how LRRK1 regulates B-cell mediated humoral immune response.

1997

Characterising CARD11 mutations in B cell malignancies

Horikawa, K., Hanioka, A., Kaya, S., Wang, J.

The John Curtin School of Medical Research, The Australian National University, Cancer Biology and Therapeutics, Acton, Australia

CARD11 is a multidomain adaptor protein essential for transmitting signal from B cell antigen receptor (BCR) to NF- κ B pathway. Numerous somatic mutations in *CARD11* are found in various types of lymphomas, which are addicted to NF- κ B enhanced by oncogenic mutant CARD11 proteins. However, it is not known if these mutations are oncogenic driver mutations or passenger mutations without significant function. We generated a library of vectors expressing CARD11 mutations derived from human malignancies and investigated the consequences of each CARD11 mutation with NF- κ B activity by reporter assay and B cell proliferation by retrovirus gene delivery. The 95 CARD11 mutations were classified as 5 very strong (> 20 fold compared to wild type), 19 strong (5~20 fold), 30 moderate (2~5 fold), and 41 weak (< 2 fold) based on NF- κ B activity. All (7/7) of the insertion or deletion mutations induced high NF- κ B activity (\geq 2 fold) compared to missense mutations (48/88). Potent mutations were enriched in a region between CARD and coiled-coil domains and several positions within the coiled-coil domains. B cell growth induced CARD11 mutations was not directly correlated with NF- κ B activity measured by reporter assay. The antigen stimulation generally turned CARD11 mutations into more potent mutations. This cooperation was cancelled by a BTK inhibitor. By investigating the effects of CARD11 mutations, we have shown that heterogeneity in lymphomas could be rooted deeply into mutated genes, mutation types, positions, and substitution residues. Characterising gene variants is essential to understand molecular mechanisms underlying lymphoma development, more importantly to provide rational therapies.

2612

A role for CD40 signalling in the formation of unswitched CD73+ memory B cell subsets

D'Souza, L.L., Bal, V., Rath, S., George, A.

National Institute of Immunology, New Delhi, India

Memory B cells are formed by germinal centre dependent or independent mechanisms and can be classified into unswitched or isotype-switched cells. The former retain the IgM BCR while the latter have switched to an IgG, IgA or IgE BCR. Unswitched memory is characterised by reduced mutations in germline sequences and can form germinal centres in recall responses as well as undergo both isotype switching and somatic hypermutation therein. The markers CD73, CD80 and CD273 are used to characterise multiple phenotypically and functionally distinct populations within this compartment. The factors, however, involved in the formation of these subsets remain unclear. Our analysis of multiple gene deficient strains points to reductions in unswitched CD73⁺ subsets in mice deficient in TCR β and Ii but not in TCR δ , ICAM-1, TAP-1, IL-4, and IL-10. This suggests that this subset may be generated in a T cell dependent manner. This finding was consolidated in TCR δ ^{-/-} mice, which also exhibited reduced frequencies of unswitched

CD73+ subsets and provision of T cells by adoptive transfer or in mixed bone marrow chimeras rescued this deficiency. We also report a role for CD40-CD40L signalling in this phenomenon, as CD73+ subsets were also reduced in CD40^{-/-} mice and in the CD40^{-/-} compartment of mixed bone marrow chimeras. The expression of CD73 on other lymphocyte populations remains unaffected in CD40-CD40L deficient environments, suggesting a restriction to the memory compartment. Put together, our findings suggest a role for T cells and germinal centres in the generation of subsets of unswitched memory B cells.

3523

STAT3 is a critical B-cell intrinsic regulator of functional antibody responses and IgE production

Kane, A.^{1,2}, Lau, A.^{1,3}, Brink, R.^{1,2}, Tangye, S.^{1,2}, Deenick, E.^{1,2}

¹Garvan Institute, Immunology, Darlinghurst, Australia, ²University of New South Wales, St Vincent's Clinical School, NSW, Australia,

³Bath University, Bath, United Kingdom

Autosomal dominant hyper IgE syndrome (AD-HIES) is a rare human primary immunodeficiency caused by heterozygous mutations in the gene encoding the transcription factor STAT3. Patients with AD-HIES suffer recurrent bacterial pneumonia and have decreased memory B cells, impaired antigen-specific antibody responses and aberrantly elevated IgE levels, suggesting a critical role for STAT3 in regulating antibody-mediated immunity. The mechanism underlying these defects are poorly defined. In particular it is not clear whether these defects arise from a B cell intrinsic requirement for STAT3 or are secondary to defects in other cell types. To resolve this, antigens specific B cell responses were determined in mice with conditional deletion of *Stat3* in B cells. Strikingly, STAT3 deficiency in B cells alone replicated the key humoral defects seen in AD-HIES including excessive IgE production demonstrating that B cell intrinsic STAT3 signaling is a critical regulator of optimal B cell clonal expansion, differentiation, affinity maturation, class-switching. Together these results reveal the pathophysiology of the humoral defects observed in AD-HIES.

1653

Sin1 mediated mTOR signalling to Akt activation in B cells

Su, B.^{1,2,3}, Li, M.¹, Li, F.¹, Larzorchak, A.², Liu, D.², Wang, J.¹, Ouyang, X.¹

¹Shanghai Institute of Immunology, Shanghai Jiao Tong University School of Medicine, Shanghai, China, ²Department of Immunobiology and The Vascular Biology and Therapeutics Program, Yale School of Medicine, New Haven, United States,

³XiangYa Hospital, Central South University, Changsha, China

The mechanistic target of rapamycin (mTOR) is a conserved protein kinase with central roles in regulating cell growth, metabolic and stress responses. It functions via at least two distinct mTOR protein complexes called mTOR complex (mTORC)1 and mTORC2. The mTORCs control many members of the protein kinase (PK)A/PKG/PKC (AGC) family. The AGC kinases are key regulators of multiple cellular functions and deregulation of many members of this family underlies numerous pathological conditions. One unique feature in the regulation of this family kinases is the phosphorylation of the

conserved motifs by mTORCs to allosterically augment their kinase activity, influence their substrate specificity, and promote protein maturation and stability. Activated AGC kinases in turn trigger the phosphorylation of diverse targets that ultimately control cellular response to a wide spectrum of stimuli. We found that Sin1, an essential component of mTORC2, plays specific role in B cell development and differentiation. We show specifically how Sin1 regulates Akt, a best-known member of AGC kinase, thus mediating specific mTOR signals for B cell mediated immune responses.

Innate Receptors and Inflammasomes 1

3792

Regulation of inhibitor of apoptosis (IAP) proteins by Toll-like receptors and type I interferons

Lawlor, K.E.^{1,2}, Feltham, R.^{1,2}, Conos, S.^{2,3}, Vince, J.E.^{1,2}

¹Walter & Eliza Hall Institute, Inflammation, Parkville, Australia,

²University of Melbourne, Medical Biology, Parkville, Australia,

³Walter & Eliza Hall Institute, Cell Signalling and Cell Death, Parkville, Australia

Toll-like Receptors (TLRs) detect pathogen molecules to mount immune responses required for microbial resistance. However, inappropriate TLR activation by host-derived danger molecules may also contribute to inflammatory diseases, such as atherosclerosis. Here we document that TLRs utilise the adaptor protein Myd88 to induce the degradation of the IAP protein cIAP1, and its binding partner TRAF2. This can result in two distinct cellular outcomes. Firstly, TLR-induced loss of cIAP1/TRAF2 triggers non-canonical NF- κ B activity to induce a subset of pro-inflammatory cytokines and chemokines. Secondly, in the absence of the related IAP protein, XIAP, TLR-induced cIAP1 degradation triggers activation of the NLRP3-caspase-1 inflammasome, which cleaves and activates IL-1 β and IL-18. Despite the fact genetic deletion of XIAP and cIAP1 in myeloid cells causes no overt phenotype in mice, co-deletion sensitises macrophages *in vitro*, or mice *in vivo*, to endotoxin induced IL-1 β production, compared to *Xiap*-deficient mice. This may explain why in response to pathogen infection, many XIAP mutant patients develop severe autoinflammatory disease characterised by enhanced inflammasome activity. Finally, we describe a novel type I Interferon (IFN) signalling mechanism that is able to protect against TLR-induced cIAP1 degradation, raising the prospect of treating pathological TLR signalling with anti-IFN therapies.

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Sterile signals generate weaker and delayed macrophage NLRP3 inflammasome responses relative to microbial signals

Coll, R.C., Bezbradica, J.S., Schroder, K.

Institute for Molecular Bioscience, The University of Queensland, Brisbane, St Lucia, Australia

Inflammation is the host response to microbial infection or sterile injury that aims to eliminate the insult, repair the tissue and restore homeostasis. Macrophages and the NLRP3

inflammasome are key sentinels for both types of insult. Although it is well established that the NLRP3 inflammasome is activated by microbial products and molecules released during sterile injury, it is unclear whether the responses elicited by these different types of signals are distinct. In this study, we used LPS and TNF as prototypical microbial and sterile signal 1 stimuli, respectively, to prime the NLRP3 inflammasome. We then used the bacterial toxin nigericin and a common product released from necrotic cells, ATP, as prototypical microbial and sterile signal 2 stimuli, respectively, to trigger the assembly of the NLRP3 inflammasome complex in mouse and human macrophages. We found that NLRP3 inflammasome responses were weakest when both signal 1 and signal 2 were sterile, but responses were faster and stronger when at least one of the two signals was microbial. Ultimately, the most rapid and potent responses were elicited when both signals were microbial. Together, these data suggest that microbial versus sterile signals are distinct, both kinetically and in magnitude, in their ability to generate inflammasome-dependent responses. This hierarchy of NLRP3 responses to sterile versus microbial stimuli likely reflects the urgent need for the immune system to respond rapidly to the presence of infection to halt pathogen dissemination.

3771

Nucleic acid recognition via MAVS is critical for robust adaptive immunity

Loetsch, C.^{1,2}, Warren, J.¹, Jandl, C.^{1,2}, King, C.^{1,2}

¹Garvan Institute of Medical Research, Darlinghurst, Australia, ²St. Vincent's Clinical School, University of New South Wales, Sydney, Australia

Innate immunity to RNA depends on recognition by RIG-I-like receptors within the cytosol. The subsequent interaction of these receptors with the mitochondrial adaptor protein (MAVS) is essential for the induction of type I IFN and pro-inflammatory cytokines. However, the impact of MAVS-mediated signalling on adaptive immunity remains incompletely understood.

The role of cytosolic detection of RNA in the T-dependent humoral immune response was investigated by utilising a novel mutant mouse strain (MAVS^{SNP}), which carries a point mutation within the MAVS transmembrane domain. Sheep red blood cells (SRBC) are a model polyvalent antigen for the analyses of T-dependent immune responses. To our surprise, MAVS^{SNP} mice exhibited reduced GC formation and antibody forming GC B cells, as well as cell-intrinsic defects in Tfh differentiation following immunisation with SRBC. This was associated with diminished production of type I (T1) IFN, type III IFN and IL-6. SRBC were found to contain microRNAs and were detected inside both macrophages and dendritic cells 30 min after immunisation, suggesting that phagocytosed material can enter the cytosolic MAVS pathway to influence the immune response. T1 IFN was found to be critical for optimal Tfh cell expansion and for upregulation of TLRs. Notably, blockade of TLR signalling induced a similar phenotype in MAVS^{WT} mice, suggesting an important role for early induction of T1 IFN by MAVS signaling in amplifying TLR pathways. These findings demonstrate that recognition of foreign RNA is central to the immune response

to SRBC and that cell intrinsic sensing of nucleic acids promotes adaptive immune responses.

348

Caspase-11 modulates inflammation and attenuates *Toxoplasma gondii* pathogenesis

Allen, L., Coutermarsh-Ott, S., Doran, J., Campbell, C., Williams, T., Lindsay, D.

Virginia Tech, Department of Biomedical Sciences and Pathobiology, Blacksburg, United States

Toxoplasma gondii is an obligate intracellular parasite that is the etiologic agent responsible for toxoplasmosis. Recent studies have identified inflammasome forming members of the NLR family of cytosolic sensors that are essential for modulating host resistance to *T. gondii*. Parasite infection results in canonical NLR inflammasome activation of caspase-1 and the subsequent cleavage of IL-1 β and IL-18. Recently, a non-canonical inflammasome has been characterized that functions through caspase-11 and appears to augment many biological functions previously considered to be dependent on caspase-1 and the canonical inflammasome. To better elucidate the function of this non-canonical inflammasome in toxoplasmosis, we utilized *Asc*^{-/-} and *Casp11*^{-/-} mice and infected these animals with Me49 strain *T. gondii*. Our data indicates that caspase-11 plays a critical role in modulating the innate immune response following *T. gondii* infection through a mechanism that is distinct from that currently described for the canonical inflammasome. *Asc*^{-/-} mice demonstrated increased disease pathogenesis during the acute phase of *T. gondii* infection, whereas *Casp11*^{-/-} mice demonstrated significantly attenuated disease pathogenesis and reduced inflammation. The attenuated host response was associated with reduced local and systemic cytokine production, including diminished IL-1 β . During the chronic phase of infection, caspase-11 deficiency resulted in increased neuroinflammation and tissue cyst burden in the brain. Together, our data suggests that caspase-11 functions to protect the host by enhancing inflammation during the acute phase of infection in an effort to minimize disease pathogenesis during chronic toxoplasmosis.

1233

Potential role of Toll-like receptors in calcific aortic valve disease: elevation of osteogenic activity of valvular cells by DAMPs

Meng, X., Ao, L., Fullerton, D.

University of Colorado Denver, Surgery, Aurora, United States

Calcific aortic valve disease (CAVD) affects elderly people and is becoming an increasingly important healthcare issue with the emerging longevity. Progressive aortic valve calcification causes heart failure. Currently, pharmacological intervention of the progression of this disease is unavailable. While CAVD is recognized as a chronic inflammatory disease, the pro-inflammatory mechanism of aortic valve calcification is poorly understood. Aortic valve interstitial cells (AVICs) are actively involved in valvular calcification. Our studies found that human AVICs express osteogenic proteins (including BMP-2 and

TGF- β 1) in response to stimulation of Toll-like receptor (TLR) 2, 3 or 4. Further, the TLR-mediated osteogenic response in human AVICs leads to pro-osteogenic reprogramming characterized by the expression of Runx2 and alkaline phosphatase, and formation of calcium deposits. These studies uncovered novel mechanistic roles of the innate immune receptors in aortic valve calcification. Our recent work identified several endogenous factors that can elicit the osteogenic response in human AVICs through TLRs, including oxidized low-density lipoprotein, biglycan and matrilin 2. While these factors utilize distinct TLRs, they induce the osteogenic response through common signaling pathways, mainly the NF- κ B and ERK1/2 pathways. Our findings demonstrate that DAMPs are capable of inducing the osteogenic response in human AVICs and that the innate immune receptors have novel functions in modulating the osteogenic response in human aortic valve cells. These findings suggest that TLRs may play a role in the pathogenesis of CAVD and that modulation of the common signaling pathways utilized by TLRs may have therapeutic potential for suppression of CAVD progression.

2214

Functional analysis of a novel E3 ubiquitin ligase in TLR4-mediated innate immune responses

Hsu, L.-C., Lai, T.-Y., Lee, C.-Y., Chou, C.-C., Chang, Y.-C., Ho, Y.-H.
National Taiwan University, Institute of Molecular Medicine, Taipei, Taiwan, Republic of China

The mammalian immune system is comprised of two branches: innate and adaptive immunity. During microbial infections, innate immune cells use pattern-recognition receptors, including Toll-like receptors (TLRs), to detect conserved microbial molecules termed pathogen-associated molecular patterns, and then mount a defense response to clear bacteria and control the initiation and determination of the adaptive immune response. Excess activation of pro-inflammatory responses has been associated with many inflammatory diseases including sepsis and infectious diseases. However, the precise mechanisms underlying the regulation of mammalian immune system remain fragmentary. Here, we identified a RING finger E3 ubiquitin ligase, which regulated TLR4-activated immune responses in bone marrow macrophages by modulating caveolin-1 (CAV1) expression. We demonstrated that this E3 ubiquitin ligase physically interacts with CAV1 in response to lipopolysaccharide and mediates ubiquitination and degradation of CAV1. Depletion of this E3 ubiquitin ligase in macrophages caused an increase in the expression of CAV1, which led to decreased GSK3 β activation resulting in enhanced production of pro-inflammatory cytokines and inhibition of anti-inflammatory cytokine IL-10. Likewise, overexpressing CAV1 in macrophages significantly suppressed GSK3 β activity in response to LPS. Importantly, mice with myeloid-specific the novel E3 ubiquitin ligase deficiency were significantly resistant to both LPS- and cecal ligation and puncture-induced septic death. Our data has identified this E3 ubiquitin ligase as a new regulator of TLR4-induced inflammatory responses and revealed a novel mechanism for the regulation of TLR4 signaling through CAV1.

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1379

Roles of MD-1 in inflammatory disorders

Tanimura, N., Miyake, K.

Institute of Medical Science, University of Tokyo, Tokyo, Japan

MD-1 is identified as an accessory molecule, which binds to the ectodomain on Radioprotective 105 (RP105), also known as CD180. The molecular structure of RP105/MD-1 is similar to that of Toll-like receptor 4 (TLR4)/MD-2, the endotoxin receptor, but is lacking its cytosolic Toll-and-interleukin-1 responding (TIR) domain. As MD-2 has a hydrophobic pocket to recognize lipopolysaccharide (LPS), MD-1 also has the pocket, which is supposed to bind self-derived bioactive lipids as well as pathogen-derived lipid agonists. Because the previous study of MD-1's crystal structure analysis was revealed that self-derived phosphatidylcholine is able to be enclosed in the pocket. Many bioactive lipids are released in inflammatory disorders *in vivo*. We have focused on the physiological role of RP105/MD-1 related to sterile inflammations.

To address the role of RP105 and MD-1 in inflammatory responses *in vivo*, we performed the 2,6,10,14-tetramethylpentadecan (TMPD)-induced peritonitis model by using RP105 or MD-1 deficient mice and dissect the peritonitis-bearing mice. In the peritonitis-bearing wild-type mouse, they showed elevations of serum MD-1 concentration. And we found that MD-1 or RP105 deficient mouse showed severe phenotype with large volumes of ascites fluid. This ascites was considered by the consequence of the abnormal elevation of vascular permeability with increased VEGF. These indicated that RP105 and/or MD-1 enrolled to regulate the responsiveness after tissue damages. It is suggesting that MD-1 and RP105 contribute to suppress exacerbation of inflammatory disorders by the consequence of modifying the bioactive lipid activities.

Macrophages

2479

High salt modulates cellular metabolic processes essential for macrophage activation

Binger, K.¹, Gebhardt, M.², Heinig, M.³, Rintisch, C.³, Schroeder, A.⁴, Kleinewietfeld, M.⁵, Schatz, V.⁶, Hubner, N.³, Jantsch, J.⁶, Titze, J.⁷, Muller, D.²

¹Baker IDI Heart and Diabetes Institute, Melbourne, Australia, ²Max Delbrück Center, Experimental and Clinical Research Center, Berlin, Germany, ³Max Delbrück Center, Berlin, Germany, ⁴University of Erlangen, Erlangen, Germany, ⁵Technical University Dresden, Dresden, Germany, ⁶University of Regensburg, Regensburg, Germany, ⁷Vanderbilt University, Nashville, Germany

High intake of dietary salt (NaCl) is an established environmental risk factor for hypertension, and more recently has been linked to chronic inflammation and autoimmune diseases. We have characterised the effects of high salt on immune cells: our first study showed that high salt promotes hyperactive responses in pro-inflammatory T cells (Th17). More recently, we reported that pro-inflammatory ("M1") macrophages have an augmented activation with high salt, while anti-inflammatory ("M2") macrophages are inhibited. Here, we have investigated the

molecular mechanism for the differential effect of high salt on macrophage activation. Briefly, mouse bone-marrow derived macrophages were generated from male C57Bl/6 mice and activated to M1 by stimulation with LPS, or to M2 by stimulation with IL-4 and IL-13 in isotonic ([Na⁺]=137mM) or high salt ([Na⁺]=177mM) media. Genome-wide gene expression, with subsequent gene ontology analyses, revealed alterations in genes important for cellular metabolism in both M1 and M2 macrophages activated in high salt. Several of these genes were confirmed by real-time qPCR, such as glucose transporter 1 (*Slc2a1*), which, in high salt, had augmented expression in M1, and reduced expression in M2 macrophages. Extracellular flux analysis indicated that both oxidative phosphorylation and glycolysis was blunted in M2 macrophages activated in high salt. Conversely, lactate production was boosted in M1 macrophages activated in high salt, indicating an increased glycolytic rate. We thus propose that changes in sodium concentrations within tissue microenvironments *in vivo* may directly affect the cellular metabolism of residing and recruited macrophages, subsequently affecting their response upon immune challenge.

3956

CX3CR1 reduces choline-deficient, ethionine-supplemented diet-induced liver injury and liver progenitor cell proliferation

Elsegood, C.^{1,2}, Domenichini, A.¹, Jackaman, C.¹, Ganss, R.³, Plant, G.⁴, Yeoh, G.^{2,3}, Olynyk, J.^{1,5}

¹Curtin University, School of Biomedical Sciences, Curtin Health Innovation Research Institute, Bentley, Australia, ²University of Western Australia, School of Chemistry and Biochemistry, Crawley, Australia, ³Harry Perkins Institute of Medical Research, Nedlands, Australia, ⁴Stanford University School of Medicine, Department of Neurosurgery, Stanford Partnership for Spinal Cord Injury and Repair, Stanford, United States, ⁵Fiona Stanley and Fremantle Hospitals, Department of Gastroenterology, Bull Creek, Australia

CX3CR1 and its cognate ligand, CX3CL1, is elevated in choline-deficient, ethionine-supplemented (CDE) diet-induced liver injury. ¹We assessed the role of CX3CR1 in liver injury and liver progenitor cell (LPC) proliferation by comparing the response of heterozygous CX3CR1^{gfp/+} (control) and homozygous CX3CR1^{gfp/gfp} (CX3CR1 knockout) mice to the CDE diet after 3 days. Heterozygous CX3CR1^{gfp/+} mice exhibited markedly reduced CDE diet-induced LPC proliferation when compared to homozygous CX3CR1^{gfp/gfp} mice. Furthermore, liver mRNA expression of the LPC mitogens, TNF α and lymphotoxin beta, were also significantly reduced in the heterozygous mice. In contrast, levels of IL-6, interferon γ , HGF, and TWEAK were not affected. Heterozygous CX3CR1^{gfp/+} mice developed less liver injury, steatosis, and inflammation compared to the homozygous CX3CR1^{gfp/gfp}, as serum alanine transaminase levels, oil red o staining, and the number of inflammatory cells such as neutrophils and B cells, respectively were reduced. However, the numbers of Kupffer cells and monocyte-derived macrophages were similar in the heterozygous and homozygous mice. In conclusion, CX3CR1 attenuates CDE diet-induced LPC proliferation. This may be a consequence of the reduction in liver injury and accompanying reduced

production of the LPC mitogens, TNF and lymphotoxin beta. Reference: 1. Viebahn CS, Benseler V, Holz LE, Elsegood CL, Vo M, Bertolino P, Ganss R, Yeoh GCT: Invading macrophages play a major role in the liver progenitor cell response to chronic liver injury. *Journal of Hepatology* 2010; 53: 500-507.

2407

Pdcd4 deficiency confers resistance to the assembly of stress granules in macrophages during high fat-diet challenge

Bai, Y., Dong, Z., Guo, C., Zhang, L., Wang, Q.
Shandong University School of Medicine, Department of Immunology, Jinan, China

Pdcd4, a translation repressor, has been reported to be implicated in obesity and associated stress responses. In response to various stresses such as oxidative and/or endoplasmic reticulum stresses, the cells assemble stress granules (SGs) that are translationally silent cytoplasmic ribonucleoprotein complexes. However, the possible link of Pdcd4 with SGs remains lacking. In this study we showed the critical roles of Pdcd4 in regulating SG assembly during high-fat diet (HFD) challenge. In HFD-fed mouse model, obvious TIA-1-positive SGs were detected in macrophages from wild type (WT) obese mice, whereas little were seen in those from Pdcd4-deficient lean mice, indicating Pdcd4 deficiency causes the resistance to SG formation in response to HFD challenge. Using oxidized low-density lipoprotein (ox-LDL) as a stress stimulus, we found the translocation of Pdcd4 from nuclei to cytoplasm that displayed granule-like expression and colocalized with SG markers TIA-1, FXR1 and eIF4A in WT macrophages. Accordingly, Pdcd4 deficiency also led to reduced SG assembly in ox-LDL-treated macrophages. Mechanistically, a marked elevation in eIF2 α phosphorylation, which is required for cap-dependent translation repression and SG assembly, was observed in ox-LDL-stimulated WT macrophages compared with non-treated ones, but abolished in Pdcd4-deficient macrophages in response to ox-LDL or HFD, suggesting that Pdcd4 is a key regulator during HFD factors-induced SGs, which is dependent on eIF2 α phosphorylation. These data link Pdcd4 with SG-associated stress responses, thereby proposing Pdcd4 as a potential target for obesity and associated diseases.

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Macrophages affected with exosome-carried miRNA-150 release secondary vesicles to inhibit the proliferation of effector T lymphocytes of mouse contact and delayed-type hypersensitivity

Nazimek, K.¹, Ptak, M.¹, Ptak, W.¹, Askenase, P.W.², Bryniarski, K.¹
¹Jagiellonian University Medical College, Department of Immunology, Krakow, Poland, ²Yale University School of Medicine, Department of Internal Medicine, New Haven, United States

Introduction: Mouse contact and delayed-type hypersensitivity reactions could be suppressed by

T CD8⁺ cell-derived miRNA-150 carried by exosomes

[Bryniarski et al. J Allergy Clin Immunol 2013;132:170-181.e9] acting through the antigen-presenting macrophages (Mf) [Nazimek et al. Immunology 2015;146:23-32]. Our research aimed to investigate the mechanism of Mf-dependent suppression of effector T lymphocytes.

Methodology: Peritoneal Mf from CBA/J, C57BL/6 wild-type or miRNA-150KO mice pulsed with T cell exosomes carrying miRNA-150 were cultured with effector T lymphocytes in direct or transmembrane contact, and incorporation of tritiated thymidine by proliferating T cells was measured. Supernatant from 48-hour culture of miRNA-150-pulsed Mf was ultracentrifuged, and resulting fractions were either tested for suppressive activity, in some instances after pre-incubation with monoclonal antibodies against MHC class II and antigen, or analyzed cytometrically.

Results: Regardless of the culturing conditions, miRNA-150-pulsed Mf suppressed proliferation of effector T cells. Pellet of ultracentrifuged culture supernatant of miRNA-150-pulsed Mf contained the CD9, MHC positive vesicles, which suppressive activity was blocked by pre-incubation with anti-MHC and anti-antigen antibodies. miRNA-150KO mouse Mf failed to generate inhibitory vesicles, unless they were pre-treated with miRNA-150-containing T cell exosomes from wild-type mice.

Conclusion: T suppressor cell-derived, exosome-carried miRNA-150 stimulate Mf to release secondary vesicles acting in a MHC and antigen-dependent manner to suppress contact and delayed-type hypersensitivity response through the inhibition of effector T cell proliferation. According to our preliminary data, reduced proliferation of effector T lymphocytes results from induction of their apoptosis and from impaired secretion of IL-2.

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Activation of L-type voltage gated calcium channel in macrophages during *Mycobacterium tuberculosis* infection leads to suppression of protective responses by the host

Sharma, D., Mehto, S., Tiwari, B., Antony, C., Natarajan, K.
University of Delhi, Dr. B.R Ambedkar Center for Biomedical Research, Delhi, India

With the emergence of drug resistant strains of *Mycobacterium tuberculosis* (*M. tb*) there is a need for understanding the intricacies of host pathogen interactions. In the recent times, Voltage Gated Calcium Channels have gained importance in the regulation of calcium homeostasis during *M.tb* infection. Our group, working on these lines have shown a suppressor role for VGCC during *M. tb* infections and recently reported the mechanisms of its regulation by *M. tb*. In this report, we characterized the role of VGCC in mediating defense responses by macrophages during mycobacterial infection. We report that activation of VGCC synergistically down modulates the generation of oxidative burst by macrophages. The route of calcium influx, protein kinase C (PKC) and mitogen activated protein kinase (MAPK) pathways regulates this attenuation. Activation of VGCC increases cell survival and down modulates autophagy. Concomitantly, the secretion of pro-inflammatory

cytokines (IFN- γ and IL-12) and the levels of their receptors on cell surface are inhibited. Finally, the ability of phagosomes to fuse with lysosomes in *M. bovis* BCG and *M. tb* H37Rv infected macrophages is also compromised by VGCC. The results point towards a well-orchestrated strategy adopted by mycobacteria to suppress protective responses mounted by the host. This begins with an increase in the surface levels of VGCC by mycobacteria and their antigens in well-controlled and regulated mechanisms. Activation of the up-regulated VGCC following tweaking of calcium levels by molecular sensors in turn mediates suppressor responses and prepares the macrophage for long-term persistent infection.

1567

Epigenetic regulation of the overexpressed STAT-1/Myd88 in macrophages from type 1 diabetic mice

Ribeiro Filgueiras, L.¹, L Brandt, S.², Raquel Oliveira Ramalho, T.¹, Jancar, S.¹, Henrique Serezani, C.²

¹University of São Paulo, Department of Immunology, Institute of Biomedical Science, São Paulo, Brazil, ²Indiana University School of Medicine, Department of Microbiology and Immunology, Indianapolis, United States

There is evidence that the sterile inflammation present in diabetic patients is involved in the development of several diabetes comorbidities. We have previously shown that in type 1 diabetic (T1D) mice, enhanced expression of the STAT-1/MyD88 axis in macrophages promotes the sterile inflammation. Because some long-term diabetes complications still develop in insulin treated diabetic patients, we hypothesized that epigenetic mechanisms are triggered during diabetes that promote macrophages MyD88 and STAT-1 expression. We found that the increased Myd88 and Stat1 gene expression in resident peritoneal macrophages from T1D mice persisted up to 6 days after incubation in low glucose medium. When macrophages were differentiated from bone marrow of T1D mice in both high and low glucose, the expression of Myd88 and Stat1 was enhanced compared to cells differentiated from bone marrow of non-diabetic animals in low glucose medium. The total histone acetyltransferase activity was found increased in resident peritoneal macrophages from T1D mice while the histone deacetylases activity was decreased. Macrophages from non-diabetic mice treated *in vivo* with histone deacetylase inhibitor presented increased Myd88 and Stat1 expression. ChIP analysis demonstrated that macrophages from T1D mice presented increased acetylation of MyD88 and STAT1 promoter region in histone H3 lysine 9 residue. Moreover, treating macrophages from T1D mice with histone acetylase inhibitor restored Myd88 and Stat1 expression to the same levels found in macrophages from non-diabetic mice. Together these results indicate an essential role of histone acetylation in the macrophages increased Myd88 and Stat1 gene expression in T1D mice.

3185

The role of mTORC1 on regulating tissue resident macrophage homeostasisDeng, W.^{1,2}, Yang, J.², Lin, X.¹, Shin, J.², Gao, J.¹, Zhong, X.-P.²¹Wenzhou Medical University, School of Laboratory Medicine, Wenzhou, China, ²Duke University Medical Center, Pediatrics, Division of Allergy and Immunology, Durham, United States

Alveolar macrophages (AM Φ) have the capacity of local self-renewal through adult life; however, mechanisms that regulate AM Φ self-renewal remain poorly understood. We found that myeloid specific deletion of Raptor, an essential component of the mammalian/mechanistic target of rapamycin complex 1 (mTORC1), resulted in a marked decrease of this population of cells accompanying altered phenotypic features and impaired phagocytosis activity. We demonstrated further that Raptor/mTORC1 deficiency did not affect AM Φ development, but compromised its proliferative activity at cell cycle entry in the steady state as well as in the context of repopulation in irradiation chimeras. Mechanically, mTORC1 confers AM Φ optimal responsiveness to GM-CSF induced proliferation. Thus, our results demonstrate an essential role of mTORC1 for AM Φ homeostasis by regulating proliferative renewal.

Immunity to Parasites 1

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***Mycobacterium indicus pranii* (Mw) in combination with heat induced promastigotes persuade host protection against *Leishmania donovani* infection: activation of IL-6+CD11c+ cDC and induction of CXCL10**

Dey, S., Mukherjee, D., Sultana, S., Mallick, S., Mandal, S., Dutta, A., Ghosh, J., Hussain, A., Pal, C.

West Bengal State University, Zoology, Kolkata, India

The protozoan parasite *Leishmania* causes leishmaniasis which threatens 350 million people worldwide. Chemotherapy is the only treatment currently available, however, clinical efficacies are found not so precise by severe side effects and emergences of resistant parasites.

The aim of this work is to design a successful adjuvant in combination with *Mycobacterium indicus pranii* (Mw) and heat induced *L. donovani* promastigotes (HIP) against experimental visceral leishmaniasis. Subcutaneous administration of Mw+HIP effectively reduced the hepatic and splenic parasite burden of *L. donovani*-infected BALB/c mice. Mw+HIP induced CD4+IFN γ + splenic T cells correspondingly reducing the CD4+IL-10+ T cells along with the upregulation of the Th1 killing cytokines (IL-12, TNF- α), chemokines (CXCL10, CCL4) and TLR2. The production of CXCL10 and CCL4, *in vitro*, depends on the cooperation and involvement of TLR2 and we speculated that the Mw + HIP combination therapy requires the cooperation of an integrated TLR~Cytokine~Chemokine loop. Dendritic cells (DCs) play a crucial role in determining the balance of CMI and humoral immunity by steering the T cell population towards a Th1 or Th2 response. We used this approach to identify IL-6 and IL-12p40 as critical cytokines that mediate anti-*Ld* host protection. Mw+HIP induced the expansions of CD11c+CD11b+ and CD11c+CD8a+

conventional splenic DCs, *in vivo*, along with the upregulated expressions of IL-6 & IL-12p40 in treated animals. Interestingly, Mw+HIP were also found effective against Miltefosine resistant-*L. donovani* (HePC-R) *in vitro* and *in vivo* as evidenced by the concomitant surge in the level of iNOS and limited expression of amastigote specific Ld-kDNA in macrophages.

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Liver-resident memory T cells can be harnessed for sterile protection against malariaFernandez-Ruiz, D.¹, Ng, W.Y.¹, Holz, L.¹, Ma, J.¹, Zaid, A.², Chun Wong, Y.³, Lau, L.S.¹, Mollard, V.⁴, Cozijnsen, A.⁴, Collins, N.⁵, Li, J.⁶, Davey, G.¹, Tay, S.S.³, Tan, P.S.³, Bowen, D.³, Koch-Nolte, F.⁷, Rissiek, B.⁷, Carbone, F.¹, Crabb, B.⁸, Lahoud, M.⁶, Cockburn, I.⁹, Mueller, S.¹, Bertolino, P.³, McFadden, G.⁴, Caminschi, I.⁶, Heath, W.¹

¹University of Melbourne, Peter Doherty Institute for Infection and Immunity, Microbiology and Immunology, Melbourne, Australia, ²Griffith University, Institute for Glycomics, Melbourne, Australia, ³Centenary Institute, Sydney, Australia, ⁴University of Melbourne, The School of Biosciences, Melbourne, Australia, ⁵University of Melbourne, Peter Doherty Institute for Infection and Immunity, Melbourne, Australia, ⁶Monash University, Department of Biochemistry, Melbourne, Australia, ⁷University Medical Centre Hamburg-Eppendorf, Institute of Immunology, Hamburg, Germany, ⁸Macfarlane Burnet Institute for Medical Research & Public Health, Melbourne, Australia, ⁹Australian National University, John Curtin School of Medical Research, Canberra, Australia

T cell memory allows for the rapid generation of effective immune responses to previously encountered pathogens. Although most memory T cells recirculate through the body, a recently discovered subset, the tissue resident memory T cells (T_{RM}), remains in the affected tissue after infection is cleared. By staying in the area most likely targeted by the pathogen in subsequent reinfections, T_{RM} have the potential to elicit faster, more focused, responses than recirculating memory T cell subsets. We have found that following vaccination with irradiated *Plasmodium berghei* ANKA sporozoites, a population of tissue-resident memory T cells forms in the liver. These memory cells patrol liver sinusoids and could be found in significant numbers more than 100 days after infection. A vaccination strategy involving dendritic cell priming and antigen recognition on hepatocytes was found to boost numbers of T_{RM} in the liver, resulting in high levels of sterile protection against live sporozoite challenge. Protection in vaccinated mice was lost when T_{RM} cells were depleted. Vaccination aimed at the generation of T_{RM} in the liver may be a more effective way to control liver-stage malaria than traditional strategies focused on generating circulating memory T cells.

3146

Identification of malaria vaccine targets using the online tool PlasmoSIP (*Plasmodium* Structure, Immunology and Polymorphisms)

Richards, J.S.^{1,2,3}, *Guy, A.J.*^{1,4}, *Irani, V.*^{1,2}, *Ramsland, P.A.*^{1,4,5}

¹Burnet Institute, Centre for Biomedical Research, Melbourne, Australia, ²University of Melbourne, Department of Medicine, Parkville, Australia, ³Royal Melbourne Hospital, Victorian Infectious Diseases Service, Parkville, Australia, ⁴Monash University, Department of Immunology, Melbourne, Australia, ⁵RMIT University, School of Science, Melbourne, Australia

Identification of appropriate vaccine targets for *Plasmodium falciparum* malaria is a major priority in the development of an effective malaria vaccine. We have recently developed a novel online tool called PlasmoSIP (*Plasmodium* Structure, Immunology and Polymorphisms), which integrates a number of experimental data and computational predictions into an interactive online tool to assess the suitability of potential vaccine candidates from the malaria proteome. We present here an analysis of a number of existing and potential vaccine candidates using this tool, and illustrate the applicability to novel vaccine targets. We have previously examined a number of vaccine candidates in a prospective cohort study in Papua New Guinea, and identified a number of proteins with a strong association with protective immune responses. These were examined using PlasmoSIP, and key features such as protein disorder, MHC binding and location and clustering of polymorphisms were collated. Furthermore, we examined known and predicted structural models in conjunction with genomic sequence data to identify particular protein regions which appear to be under immune selection pressure. This case study illustrates the applicability of this new tool to the field of vaccine development and design.

1556

Surfactant protein A promotes lung repair by enhancement of IL-4-mediated proliferation and activation of alveolar macrophages

Minutti, C.^{1,2}, *Jackson-Jones, L.*³, *Garcia-Fojeda, B.*⁴, *Rinqvist, E.*³, *Gillamat-Prats, R.*⁵, *Artigas, A.*⁶, *Zaiss, D.*³, *Stamme, C.*⁷, *Chroneos, Z.*⁸, *Allen, J.*³, *Casals, C.*⁴

¹Universidad Complutense de Madrid, Bioquímica y Biología Molecular I, Madrid, Spain, ²University of Edinburgh, Institute of Immunology and Infection Research, Edinburgh, United Kingdom, ³University of Edinburgh, Edinburgh, United Kingdom, ⁴Universidad Complutense de Madrid, Madrid, Spain, ⁵Universitat Autònoma de Barcelona, Sabadell, Spain, ⁶Universitat Autònoma de Barcelona, Sabadell, Spain, ⁷Leibniz-Center for Medicine and Biosciences, Borstel, Germany, ⁸Penn State University College of Medicine, Hershey, United States

Surfactant protein A (SP-A) is a critical lung defence factor against microbial pathogens but its role during pulmonary helminth infection is unknown. We found that SP-A protein levels increased following migration of *Nippostrongylus brasiliensis* through the lung and SP-A-deficient mice exhibited increased parasite burden and decreased resolution of tissue damage. The protective effect of SP-A during helminth infection

was explained by the ability of SP-A to enhance the effects of IL-4 receptor signalling in the lung. This was evidenced by reduced proliferation and alternative activation of alveolar macrophages in SP-A deficient mice following *N. brasiliensis* infection or IL-4 treatment. SP-A's effects on IL-4 signalling were also observed in vitro in alveolar macrophages isolated from mice, rats and humans, and occurred via Myo18A receptor. Critically, the in vitro effect of SP-A was restricted to alveolar and not peritoneal macrophages. Our studies indicated that SP-A is a tissue-specific factor that controls functional T_H2-type responses in the lung and that Myo18A receptor signalling functions to enhance IL-4 mediated proliferation and alternative activation.

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The contribution of IL-17RB in protective immunity to *Trichinella spiralis* infection

*Angkasekwinai, P.*¹, *Sodthawon, W.*¹, *Pattanapanyasat, K.*²

¹Thammasat University, Department of Medical Technology, Faculty of Allied Health Sciences, Pathumthani, Thailand, ²Mahidol University, Center of Excellence for Flow Cytometry, Office for Research and Development, Faculty of Medicine, Siriraj Hospital, Bangkok, Thailand

IL-25, an IL-17 family cytokine, derived from epithelial cells was shown to regulate Th2-type immune responses. We previously reported that IL-25 was critical in promoting immunity to *T. spiralis* infection; however the cellular target and function of IL-25 receptor (IL-17RB) during infection have not yet been addressed. Here, we investigated the role of IL-17RB during *T. spiralis* infection. The expression of IL-17RB was shown to be highly up-regulated in type-2 innate lymphoid (ILC2) and Th2 cells after infection. Mice deficiency of IL-17RB displayed delayed *T. spiralis* worm expulsion with markedly reduced ILC2 cell numbers and Th2 cytokine production. Thus, our data indicate that IL-17RB is required for promoting *T. spiralis* worm expulsion through the expansion and function of ILC2 and Th2 cells. This study should help to extend our knowledge of cytokine-cytokine receptor interaction in regulating parasite infection and may provide information for the design of future therapies for intestinal parasitic infection.

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Route of infection affects pathogenicity and visceral growth of *Leishmania major* in BALB/c mice

*Niknam, H.M.*¹, *Sarreshteh, E.*¹, *Rostamian, M.*¹, *Abolghazi, M.*², *Tat Asadi, M.*¹, *Abrishami, F.*¹

¹Pasteur Institute of Iran, Immunology Department, Tehran, Iran, Islamic Republic of, ²Qom Branch of Islamic Azad University, Department of Microbiology, Qom, Iran, Islamic Republic of

Leishmaniasis is a group of diseases caused by *Leishmania* parasite. Experimental models can be used for development of new methods of prevention and treatment for these diseases. Route of infection is one of the variables that have been reported to influence the immune responses as well as the disease outcome in experimental models of leishmaniasis. Aim of this research is to study the effect of infection route on the

virulence of *Leishmania (L.)* parasite. Low (10^3 parasites / mouse) or high (10^6 parasites / mouse) doses of *L. major* was injected subcutaneously into foot pad or intradermally into ear dermis of BALB/c mice. Results showed that subcutaneous infection route has substantial differences with intradermal infection route which results in higher pathogenicity of *L. major* in BALB/c mice as assessed by lesion diameter, parasite load in the draining lymph node, and dissemination of the parasite to spleen. The different pathogenicity of *L. major* in subcutaneous in comparison to intradermal may be due to presence of different immunoregulatory mechanisms such as IL-10 and CD4+CD25+ T_{REG} cells in these two infection routes.

1696

Oral formulations of *Brugia malayi* recombinant proteins elicited profound immune responses in mice against experimental lymphatic filariasis

Gangwar, M.¹, Banala, V.T.², Mishra, P.R.², Bajpai, P.³, Misra-Bhattacharya, S.¹

¹CSIR-Central Drug Research Institute, Division of Parasitology, Lucknow, India, ²CSIR-Central Drug Research Institute, Division of Pharmaceutics, Lucknow, India, ³Integral University, Department of Biotechnology, Lucknow, India

Human lymphatic filariasis (LF), world's most disabling and disfiguring disease is caused by mosquito-borne nematodes, *Wuchereria bancrofti*, *Brugia malayi* or *Brugia timori*. LF threatens 1.2 billion people in 81 countries with an estimated 120 million cases. The efficacy of protein vaccines may vary depending on the route or type of adjuvant. The oral route has numerous advantages in terms of improved safety and user compliance. Stabilization of proteins in delivery devices and design of appropriate protein carriers are major concern. We attempted to resolve these issues through biodegradable nanoparticles and liposome encapsulation that can continuously release the protein to enhance the oral absorption. In this study, male BALB/c mice were immunized with nano- and liposomal-formulations of *B. malayi* immunogenic recombinant proteins, trehalose-6-phosphate phosphatase (Bm-TPP) and heavy chain myosin (BmAF-Myo). Bm-TPP & BmAF-Myo loaded nanoparticles (SNEDDS) and liposomes in two doses (25 µg protein /dose) generated mixed Th1/Th2 immune responses as observed by ELISA, RT-PCR and flow cytometry. Nano-encapsulated and liposomal Bm-TPP led to the significantly increased accumulation of CD4+, CD8+ T-cells and CD19 B-cells in the spleen of immunized mice and also enhanced the production of both Th1 (IFN-γ, IL-12 and TNF-α) and Th2 (IL-13, IL-10 and IL-4) cytokines and IgG isotypes. On contrary, encapsulated preparation of BmAF-Myo did not show significant increase. The efficacy of nano-encapsulated Bm-TPP (65.5% protection) was superior to liposomal Bm-TPP (55.17%) with respect to control group. In conclusion, nano-formulation of Bm-TPP (25 µg, oral dose) in two doses showed improved protection against lymphatic filariasis.

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CD8⁺T cells that co-express RORγt and T-bet are functionally impaired and expand in patients with distal bile duct cancer

Chelappa, S.^{1,2}, Hugenschmidt, H.³, Hagness, M.⁴, Line, P.D.⁴, Labori, K.-J.⁵, Wiedswang, G.⁶, Tasken, K.^{1,2}, Aandahl, E.M.^{1,2,4}

¹University of Oslo and Oslo University Hospital, Centre for Molecular Medicine Norway, Nordic EMBL Partnership, Oslo, Norway, ²University of Oslo, Biotechnology Centre, KG Jebsen Centre for Inflammation Research and KG Jebsen Centre for Cancer Immunotherapy, Oslo, Norway, ³Oslo University Hospital, Section for Transplantation Surgery and Department of Hepato-Pancreato-Biliary Surgery, Oslo, Norway, ⁴Oslo University Hospital-Rikshospitalet, Section for Transplantation Surgery, Oslo, Norway, ⁵Oslo University Hospital, Department of Hepato-Pancreato-Biliary Surgery, Oslo, Norway, ⁶Oslo University Hospital, Department of Gastrointestinal Surgery, Oslo, Norway

CD8⁺ T cells that express RORγt (Tc17 cells) have recently been shown to promote pro-carcinogenic inflammation and contribute to a tolerogenic microenvironment in tumors. We investigated their phenotype and functional properties in relation to the pathogenesis of human distal bile duct cancer (DBDC). DBDC patients have an elevated level of type 17 immune responses and the frequency of CD8⁺RORγt⁺ T cells (Tc17 cells) was increased in peripheral blood. The CD8⁺RORγt⁺ T cells represented a highly activated subset and produced IL-17A in equal amount compared to CD4⁺RORγt⁺ T cells (Th17 cells). The majority of CD8⁺RORγt⁺ T cells also expressed T-bet, a lineage transcription factor for Th1 and Tc1 development, which suggested that CD8⁺RORγt⁺ T cells undergo plasticity towards a Tc17/1-like phenotype that led to co-production of the pro-inflammatory cytokines IL-17A and INFγ. In comparison to CD8⁺RORγt⁺ T cells, the CD8⁺RORγt⁺ T cells had significantly higher level of TCR signaling and were both phenotypically and functionally terminally differentiated and exhausted. The CD8⁺RORγt⁺ T cells also had impaired ability to re-express perforin after initial degranulation, which may affect their ability to sustain a cytotoxic immune response. These data reveal that CD8⁺RORγt⁺ T cells are pro-inflammatory and functionally impaired and may contribute to the pathogenesis of DBDC.

982

IL-15 activated NK cells overcame DC maturation defects induced by head and neck cancer microenvironment

Upreti, D., Zhang, M., Kung, S.K.P.

University of Manitoba, Winnipeg, Canada

Head and neck squamous cell carcinoma (HNSCC) patients have the lowest 5-year disease-free survival rate. There is need to develop novel therapeutics of HNSCC. Natural Killer (NK) cells play key roles in innate immunity against infections and transformed cells. Through interactions with dendritic cell (DC), NK cells can shape also subsequently induced adaptive T-cell immunity.

Here we used an immunocompetent mouse model (AT-84) to evaluate anti-tumor potential of IL-15 activated NK cells. A

direct injection of NK cells at tumor site significantly suppressed AT-84 tumor growth in vivo. It induced also protective memory responses against a secondary AT-84 challenge. AT-84 tumor cell was relatively resistant to IL-15 activated NK cells killing in vitro, suggesting that direct killing of AT-84 is unlikely the major underlying mechanism. We therefore hypothesized that IL-15 activated NK cells promoted anti-tumor activities via NK-DC crosstalk. Using bone marrow derived DC cultures; we observed that conditioned medium of AT-84 impaired DC maturation induced by TLR ligands in vitro. These DC were impaired in inducing T cell activations in vitro. Addition of IL-15 NK cells to the AT-84-exposed DC overcame the DC maturation and functional defects in vitro. These in vitro data was further corroborated by in vivo data obtained from the tumor infiltrating dendritic cells. Collectively, our data demonstrated that IL-15 activated NK cells were able to reverse the immunosuppressed DCs to an immunostimulatory state that correlated well with tumor regressions. It supported future development of IL-15 NK-based immunotherapy of HNSCC.

1710

An immune monitoring model that allows association between anti tumor immune surveillance and clinical response elicited by neoadjuvant chemotherapy in patients with breast cancer

Bernal-Estevez, D.^{1,2}, García, O.³, Sánchez, R.^{4,5}, Parra-López, C.¹

¹Universidad Nacional de Colombia, Microbiology, Bogota, Colombia, ²Fundación Salud de los Andes, Grupo de Investigación en Inmunología y Oncología Clínica, Bogota, Colombia,

³Instituto Nacional de Cancerología, Unidad de Seno y Tejidos Blandos, Bogota, Colombia, ⁴Clínica del Seno, Bogota, Colombia,

⁵Universidad Nacional de Colombia, Internal Medicine, Bogota, Colombia

In cancer animal models chemotherapy with anthracyclines (A/C) induce immunogenic cell death in tumors eliciting T cell responses that limits tumor growth in treated animals. Although neoadjuvant A/C is widely used in breast cancer patients, the relevance of its immune stimulating effect on clinical response has been difficult to prove. To evaluate this, we compared in blood samples before and after chemotherapy the behavior of APC and T cell compartments responsible of tumor immune-surveillance. We monitored by flow cytometry both ex vivo and after in vitro stimulation of PBMCs multiple parameters related to

- (i) levels of Tregs; MDSCs; myeloid and plasmacytoid dendritic cells;
- (ii) the responsiveness of T cells to anti-CD3 and APCs to pro-inflammatory stimuli and
- (iii) the immune-phenotype of naïve and tumor specific memory T cells.

The results show in samples from patients after three cycles of A/C, a T cell responsiveness and maturation of DCs not observed in pre-chemotherapy samples. Interestingly, the T cell responsiveness highly correlate with IL-12 secretion by CD83+ DCs. Using principal component analysis (PCA), our immune-monitoring model shows a significant difference among pre- and post-chemotherapy patients compared with healthy

donors that correlates with tumor regression.

The PCA lead us to propose an immune stimulating role attributed to A/C characterized by immune responsiveness of T and APC compartments to different stimuli in post-chemotherapy samples with levels close to those exhibited by healthy donors not observed in the pre-chemotherapy samples.

2056

Evaluating prediction strategies for identification of T cell responsive mutation-derived neo-epitopes in cancer

Andersen, S.R.¹, Bjerregaard, A.-M.², Fugmann, T.³, Donia, M.⁴, Bentzen, A.K.¹, Andersen, R.⁴, Szallasi, Z.², Neri, D.³, Svane, I.M.⁴, Eklund, A.C.², Hadrup, S.R.¹

¹Technical University of Denmark, Section for Immunology and Vaccinology, Frederiksberg C, Denmark, ²Technical University of Denmark, Center for Biological Sequence Analyses, Lyngby, Denmark, ³Philochem AG, Zürich, Switzerland, ⁴University Hospital Herlev, Center for Cancer Immune Therapy, Herlev, Denmark

Increasing evidences point to an important role of mutation-derived antigens in immune recognition of cancer. Current strategies for prediction of immunogenic neoepitopes results in large personalized peptide libraries, but only a minority (< 1%) elicit T cell responses at detectable levels. Neoepitopes are of potential valuable as predictors of response to therapy and targets for personalized immunotherapeutic approached. Consequently, there is an unmet need to understand the rules identifying immunogenic neoepitopes.

Both tumor mutation mapping via exome sequencing and mass-spectrometry-based elution for MHC class I presented peptides has been applied in different studies, combined with RNA sequencing to determine the expression level of relevant transcripts. Additionally, neoepitopes may be defined based on either autologous tumor cell lines or snapfrozen tumor material. We present here a study in which all the above mentioned strategies are assessed in three melanoma patients. Predicted large peptide libraries matching the HLA expression of the patients was identified and selected based on any of the strategies given above. This resulted in a total of ~3000 peptides for the three patients. We investigated the T cell recognition of these personalized peptide libraries using a new technology based on DNA-barcode labeled MHC multimers to detect multiple, potentially > 1000, different neoepitope specific T cell populations in a single sample. Through this unbiased comparison, we evaluate selection strategies for prediction of immunogenic cancer-associated neoepitopes, and identify rules for precise prediction. Precise prediction is essential for future application of neoepitopes both as predictors of responses to therapy and immunotherapeutic targets.

2537**Features of cancer associated fibroblasts that resemble circulating fibrocytes which constitute a unique subset of MDSCs***Gunaydin, G., Guc, D.**Hacettepe University Cancer Institute, Department of Basic Oncology, Ankara, Turkey*

The role of tumor stroma in the functional insufficiency of tumor-infiltrating T-cells has not yet been well determined. Circulating-fibrocytes represent a novel MDSC subset and they take part in the tumor immune escape. Fibroblasts turn into cancer-associated-fibroblasts(CAFs) in the tumor microenvironment. Our aim is to evaluate if CAFs demonstrate similar molecular/gene expression patterns and functional characteristics to the circulating-fibrocytes. N-Nitroso-N-Methylurea (NMU) induced breast cancer model was utilized. DNA damages due to NMU injections were evaluated by Comet-Assays. Fibroblasts were isolated using collagenase/hyaluronidase. Isolated CAFs and NFs were immunostained to investigate differential expressions of surface markers such as α -Smooth-Muscle-Actin (α SMA) and vimentin, in order to distinguish CAFs from NFs. CAFs/NFs were evaluated for their surface marker expressions by flow cytometry and for gene expression profiles by gene-set-enrichment-analysis. Cocultures of CAFs/NFs with PBMCs were performed and CFSE proliferation assays were used for functional analyses. Levels of DNA damage of tumor-bearing animals were similar to control levels about 2 months after injections. CAFs were spindle shaped cells unlike their circulating counterparts and had significantly higher levels of α SMA than NFs. CAFs did not express CD80, granulocytic or neutrophilic markers. Their MHC-II expression was lower than NFs. CAFs expressed the myeloid marker CD11b/c; however, its expression was lower than that on their circulating counterparts. They appeared to have developed in a milieu containing T_{HELPER}²-like cytokines. CFSE proliferation assays showed the immunosuppressive effects of CAFs similar to their blood-borne counterparts. In summary, CAFs resemble the circulating-fibrocytes that were reported to represent a novel MDSC subset.

3329**Regulation of IL-15 in the tumor microenvironment by the STING pathway***Rivas, S.¹, Anthony, S.¹, Pham, G.², Schluns, K.S.¹**¹University of Texas MD Anderson Cancer Center, Immunology, Houston, United States, ²University of Cincinnati College of Medicine, Cincinnati, United States*

Previous studies have shown that IL-15 expression within tumors correlates with increased tumor infiltrating T cells, decreased metastasis, and increased patient survival. Unfortunately, the regulation of IL-15 within the tumor microenvironment is not clear. Since we have recently shown IFN- α induces sIL-15 complexes and activation of the Stimulator of Interferon Genes (STING) pathway is important for regulating type I Interferons (IFN) in tumors, we set out to determine if sIL-15 complexes are produced in tumor microenvironment and are regulated by STING signaling. To examine this, B16-F10 tumors of various sizes

were isolated from mice and sIL-15 complexes were measured in homogenates from tumors and spleen. Interestingly, levels of sIL-15 complexes were high in homogenates from small tumors and low in larger tumors but were absent in IL-15Ra^{-/-} mice indicating sIL-15 complexes were derived from the tumor stroma. During the steady state, STING agonists were potent inducers of sIL-15 complexes in vivo and could directly stimulate production by BM-derived DCs. In addition, optimal induction of sIL-15 complexes in response to Ab-mediated T cell depletion or total body irradiation was dependent on the STING pathway suggesting products of cell death are a mediator of sIL-15 complexes in response to lymphodepletion. Furthermore, intratumoral delivery of STING agonists enhanced production of sIL-15 complexes in B16 tumors, which correlated increased T cells in the draining LN and decreased tumor growth. Altogether, these findings suggest soluble IL-15 complexes are a component of the inflammatory milieu of the tumor microenvironment that are regulated by the STING pathway.

3800**Interactions between CD4 T cells, regulatory T cells and B cells shape the immune response to tumours***Shklovskaya, E.¹, Terry, A.¹, Guy, T.¹, Bolton, H.¹, Brink, R.², Fazekas de St Groth, B.¹**¹Centenary Institute, University of Sydney, Sydney, Australia,**²Garvan Institute of Medical Research, Sydney, Australia*

Recent clinical studies have demonstrated that *de novo* immune responses to tumour-specific neo-antigens occur in melanoma patients treated with immune checkpoint inhibitors. We have developed a novel preclinical model in which the effects of current and future inhibitors and their combinations on anti-tumour responses can be tested and their effects on individual tumour-specific lymphocytes defined.

Our animal model is designed to measure the independent and collective contributions of CD4 T cells, regulatory T cells (Tregs) and B cells to anti-tumour immunity. The model uses B16 melanoma cells expressing a model antigen Hen Egg Lysozyme-Moth Cytochrome C (HELMCC), in combination with 5C.C7 CD4 T cells and Tregs (MCC-specific TCR-transgenic) and/or HEL-specific B cells capable of isotype switching. Our use of I-E-deficient tumours and I-E-restricted T cells mimics a common situation in which tumour antigen-specific CD4 T cells are unable to directly recognise an MHC class II-negative tumour. Adoptively transferred naive 5C.C7 T cells efficiently controlled growth of subcutaneous tumours in I-E⁺ RAG^{-/-} mice, or wild-type mice acutely depleted of Tregs, in an IFN γ -dependent manner. Co-transfer of 5C.C7 Tregs with naive 5C.C7 T cells inhibited Th1 effector differentiation and prevented efficient tumour rejection in RAG^{-/-} mice. Co-transfer of HEL-specific B cells with naive 5C.C7 T cells had a negative effect on CD4 T cell numbers and long-term survival of RAG^{-/-} tumour-bearing mice, although tumour-specific isotype-switched antibodies were protective in a lung metastasis model.

We are currently investigating the cellular mechanisms responsible for activation of anti-tumour immunity after treatment with immune checkpoint inhibitors.

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2255

A therapeutic neutralizing anti-G-CSFR antibody blocks G-CSF-induced neutrophilia but does not induce neutropenia in non-human primates

Scalzo-Inguanti, K.¹, Monaghan, K.¹, Edwards, K.¹, Nash, A.¹, Busfeild, S.², Andrews, A.¹

¹CSL Ltd, Melbourne, Australia, ²Nexvet, Melbourne, Australia

Neutrophils are the most abundant white blood cells and play an essential role in the clearance of pathogens. Tight regulation of neutrophil number and their recruitment to sites of inflammation is critical in maintaining a balanced immune response. In various inflammatory conditions, such as rheumatoid arthritis, vasculitis, cystic fibrosis and inflammatory bowel disease, there is an increase in serum G-CSF that correlates with neutrophilia and an enhanced neutrophil infiltration into the inflamed tissues, which contributes to disease pathogenesis. In this study, we describe a fully human therapeutic anti-G-CSFR antibody (CSL324) that is safe and well tolerated when administered to cynomolgus macaques. This antibody is effective in controlling G-CSF-mediated neutrophilia in a therapeutic and prophylactic manner. Surprisingly, CSL324 does not alter steady-state neutrophil numbers when administered in single or multiple doses. In addition, CSL324 completely neutralizes G-CSF-mediated pSTAT3 phosphorylation but does not impact on basic neutrophil functions such as phagocytosis and oxidative burst. This work suggests that targeting G-CSFR may provide a safe and effective way to control aberrant neutrophil-mediated inflammation in various inflammatory conditions.

3153

Loss of autophagy enhances macrophage migration inhibitory factor (MIF) release by macrophages

Lee, J.P.W.¹, Foote, A.¹, Huapeng, F.¹, Peral de Castro, C.², Lang, T.¹, Jones, S.A.¹, Mills, K.H.G.², Leech, M.¹, Morand, E.F.¹, Harris, J.¹

¹Monash University, Centre for Inflammatory Diseases, Clayton, Australia, ²Trinity Biomedical Sciences Institute, Trinity College, Dublin, Ireland

Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine expressed in multiple cells types, including macrophages. MIF plays a pathogenic role in a number of inflammatory diseases, including rheumatoid arthritis and systemic lupus erythematosus. MIF has also been linked to tumour progression in some cancers. Previous work has demonstrated that loss of autophagy in macrophages enhances secretion of the IL1 family cytokines IL-1 α , IL-1 β and IL-18. Here, we demonstrate that disruption of autophagy, either by pharmacological inhibition or siRNA silencing of *Atg5*, enhances MIF secretion by monocytes and macrophages. We further demonstrate that this is dependent on reactive oxygen species (ROS) released by mitochondria. Induction of autophagy with MTOR inhibitors had no effect on MIF secretion, suggesting that, unlike IL-1, MIF secretion is not directly regulated by autophagy. However, amino acid starvation did increase secretion. This was unaffected by *Atg5* siRNA but was

again dependent on mitochondrial ROS. Our data demonstrate that autophagic regulation of mitochondrial ROS plays a pivotal role in the regulation of inflammatory cytokine secretion in macrophages, with potential implications for the pathogenesis of inflammatory diseases and cancers.

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Cellular and molecular mechanism of resveratrol-mediated amelioration of experimental colitis and associated colon cancer

Singh, U.P.¹, Marpe, B.¹, Singh, N.¹, Murphy, E.A.¹, Price, R.¹, Mishra, M.K.², Nagarkatti, M.¹, Nagarkatti, P.¹

¹University of South Carolina School of Medicine, Pathology, Microbiology and Immunology, Columbia, United States,

²Alabama State University, Biological Sciences, Montgomery, United States

Ulcerative colitis (UC) and Crohn's disease (CD) are two forms of inflammatory bowel disease (IBD) result from chronic intestinal inflammation caused by activated effector immune cells. Resveratrol (3,4,5-Trihydroxy-trans-stilbene) is a naturally occurring polyphenol that has beneficial anti-inflammatory properties. We have investigated resveratrol-mediated cellular and molecular mechanisms in both acute and chronic experimental colitis and associated colon cancer. Resveratrol improve inflammation scores and various pathological markers of experimental colitis. It also reversed colitis-associated decreases in body weight, increased serum amyloid A (SAA) levels, while attenuating systemic and mucosal pro-inflammatory cytokine levels, as well as markers of inflammation, including cyclooxygenase-2 (Cox-2) and inflammatory stress (p53 and p53-Phospho-Serine 15). Resveratrol decreased the number of activated T cells in the colon lamina propria (LPL), restoring them to normal levels in spleens and mesenteric lymph nodes (MLNs). Frequency of macrophages and CXCR3⁺ T cells were decreased in spleens, MLNs, and colon LPL. However, as compared with controls, the percentages of MDSCs in LP lymphocytes and spleens were increased after resveratrol treatment. Resveratrol treatment also induced the silent-mating type of information regulation-1 (SIRT1) gene expression and decreased p-IkBa expression and nuclear transcription factor kappaB (NF-kB) activation. Tumor incidence also reduced to 80% in mice treated with resveratrol. This study demonstrates the chemopreventive properties of resveratrol, including inverse regulation of NF-kB, upregulation of SIRT1 and decreased numbers of CXCR3⁺ effector T cells in the colon. Our results suggest that resveratrol is a nontoxic complementary and alternative strategy to abate colitis and, potentially, colon cancer associated with colitis.

918

Two hit induced acute lung injury impairs cognitive function in mice

Naura, A., Sahu, B., Sandhir, R.

Panjab University, Biochemistry, Chandigarh, India

Acute lung injury (ALI) is a life threatening disorder characterized by accumulation of large numbers of neutrophils in the lungs. ALI survivors frequently present some cognitive deterioration at

discharge; such as impaired memory, attention, concentration and/or mental processing speed. The aim of present work was to investigate potential impact of ALI on cognitive impairment in mice for its potential application as a model system to study cross talk between lung and brain at molecular level. ALI in male Balb/c mice was induced by intra-tracheal administration of either 0.1 N HCl or LPS as single hits or both agents were administered to mimic 'two hit' model of ALI. Administration of LPS or HCl alone increased the neutrophils in bronchoalveolar lavage fluid (BALF) significantly but did not cause any impairment in the memory of mice as assessed by Morris water maze test 1 week after induction of injury. Interestingly 'two hit' mediated injury resulted in more pronounced increase in neutrophils in BALF and such injury leads to persistent decline in memory. It appears that exaggerated lung injury through two hits disrupts the threshold barrier to cause cognitive dysfunction. Indeed further analysis revealed that two hit mediated injury leads to disruption of blood brain barrier. Overall, our preliminary findings show that two hit mediated ALI leads to cognitive impairment in mice and hence the model can be used to examine lung-brain cross talk at molecular level.

2342

Hairy and enhancer of split 1 attenuates inflammation via regulating transcriptional elongation

Shang, Y.¹, Coppo, M.², Ning, F.¹, Yu, L.¹, He, T.³, Rogatsky, I.^{2,4}, Hu, X.¹

¹Tsinghua University, Institute for Immunology, Beijing, China,

²Hospital for Special Surgery, New York, United States, ³Peking University, Academy for Advanced Interdisciplinary Studies and Center for Quantitative Biology, Beijing, China, ⁴Weill Cornell Medical College, New York, United States

Most of the known regulatory mechanisms that curb inflammatory gene expression target pre-transcription initiation steps and evidence for regulation of inflammatory gene expression post initiation remains scarce. Here we show that transcription repressor hairy and enhancer of split 1 (Hes1) suppresses production of Cxcl1, a chemokine crucial for recruiting neutrophils. Hes1 negatively regulates neutrophil recruitment *in vivo* and attenuates severity of inflammatory arthritis. Mechanistically, inhibition of Cxcl1 expression by Hes1 does not involve modification of transcription initiation. Instead, Hes1 inhibits signal-induced recruitment of positive-transcription elongation complex P-TEFb, thereby preventing phosphorylation of RNA polymerase II on serine-2 and productive elongation. Thus, our results identify Hes1 as a homeostatic suppressor of inflammatory responses which exerts its suppressive function via regulating transcriptional elongation.

4158

Inhalation of granulocyte/macrophage-colony stimulating factor (GM-CSF) and GM-CSF antibody

Tazawa, R., Nakagaki, K., Ito, Y., Hashimoto, A., Tanaka, T., Akasaka, K., Nakata, K.

Niigata University Medical and Dental Hospital, Bioscience Medical Research Center, Niigata, Japan

Backgrounds: Autoimmune pulmonary alveolar proteinosis (aPAP) has been thought to be caused by long-lasting autoantibodies against endogenous granulocyte/macrophage-colony stimulating factor (GM-CSF). In contrast, subcutaneous administration of recombinant human GM-CSF (rhGM-CSF) has been reported to cause transient induction of GM-CSF antibody (GM-Ab) in patients with metastatic colon cancer. We hypothesized that repeated inhalation of exogenous GM-CSF induces neutralizing antibodies against GM-CSF.

Methods: NHPs (*Macaca fascicularis*) were administered intra-tracheally with 0.015 mg/body of non-glycosylated rhGM-CSF produced in *E. coli* (molgramostim group, n=2), or glycosylated rhGM-CSF produced in Chinese hamster ovary cells (CHO-GMCSF group, n=2) using catheter sprayer twice a week for 12 weeks. After the first 12-week GM-CSF treatment followed by 12-week cessation, the animals underwent the second 12-week treatment. GM-Ab levels were measured in serum (ELISA), and the neutralizing capacity was evaluated using TF-1, a GM-CSF-dependent cell line. Bronchoalveolar lavage fluids (BALF) were obtained and evaluated microscopically.

Results: In week 4, GM-Ab was first observed in the animals. GM-Ab increased to a higher level (< 60mcg/mL) in molgramostim group, while GM-Ab remained at a moderate level (20-30 mcg/mL) in CHO-GMCSF group. GM-Ab was decreased to the undetectable level after the 12-week cessation, and observed again in week 2 of the second treatment in both groups. At the end of the second GM-CSF treatment, neutralizing capacity was observed in one animal of both groups, and foamy macrophages and GM-Ab were observed in BALF.

Conclusions: Repeated inhalation of rhGM-CSF in NHPs induced GM-Ab which could react to both rhGM-CSF and primate GM-CSF.

3178

IL-37 requires the receptors IL-18Ra and IL-1R8 (SIGIRR) to carry out its multi-faceted anti-inflammatory program on innate signal transduction

Nold, C.¹, Lo, C.², Rudloff, I.¹, Elgass, K.³, Li, S.⁴, Gantier, M.¹, Lotz-Hayla, A.⁵, Gersting, S.⁵, Cho, S.¹, Lao, J.¹, Ellisdon, A.⁶, Rotter, B.⁷, Azam, T.⁴, Mangan, N.¹, Rosello, F.⁸, Whisstock, J.⁸, Bufler, P.⁵, Garlanda, C.⁹, Mantovani, A.¹⁰, Dinarello, C.¹¹, Nold, M.¹

¹Hudson Institute of Medical Research and Monash University, Clayton, Australia, ²Monash Institute of Pharmaceutical Sciences, Monash University, Clayton, Australia, ³Monash Micro Imaging, Clayton, Australia, ⁴UC Denver, Aurora Colorado, United States, ⁵Ludwig Maximilians University, Munich, Germany, ⁶Monash University, Clayton, Australia, ⁷GenXPro, Frankfurt am Main, Germany, ⁸Monash University, Clayton, Australia, ⁹Humanitas Clinical and Research Centre, Rozzano, Italy, ¹⁰Università degli Studi di Milano, Milano, Italy, ¹¹Radboud University Nijmegen Medical Center, Nijmegen, Netherlands

IL37 and IL-1 receptor 8 (IL-1R8, also called SIGIRR or TIR8) are orphan anti-inflammatory IL1-family ligand and receptor family members. Here, we demonstrate formation and function of the endogenous ligand-receptor complex IL37:IL-1R8:IL-18 receptor a (IL18Ra). Cell-surface interaction between IL37, IL-1R8 and IL18Ra on untransfected human PBMC was maximal 30 min after stimulation with LPS. The relative affinity of IL-37:IL-

18Ra was higher than that of IL-37:IL-1R8. Silencing of IL-1R8 or IL18Ra impaired the anti-inflammatory activity of IL37 in PBMC, macrophages and epithelial cells. Whereas IL37-transgenic mice (IL37tg) with intact IL-1R8 are protected from endotoxemia, IL-1R8-deficient IL37tg are not. Proteomic and transcriptomic investigations revealed that IL37 requires IL-1R8 to harness the anti-inflammatory properties of the signaling molecules Mer, PTEN, STAT3 and DOK1, to inhibit Fyn, Tak1, NF- κ B and MAPKs, as well as to exert a pseudo-starvational effect on the mTOR pathway. IL37 thus binds to IL-18Ra and exploits IL-1R8 to activate a multi-faceted intracellular anti-inflammatory program.

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Autoimmunity 4

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Micro-fluidics is the powerful tool for revealing a mechanism of autoreactive B cell-inducing inflammation in vasculitis

Yoshizaki, A., Nakamura, K., Ebata, S., Fukasawa, T., Saigusa, R., Takahashi, T., Taniguchi, T., Asano, Y., Sato, S.
University of Tokyo Graduate School of Medicine, Dermatology, Tokyo, Japan

Vasculitis is an inflammatory disease, characterized by necrosis of blood vessels. Cutaneous leukocytoclastic vasculitis (CLV) is one of the vasculitides of unknown etiology. Although oral corticosteroid and anticoagulation agents were commonly used, some resistant and recurrent cases require strong immunosuppressive therapy, which we named severe inflammatory CLV (SICLV).

Previous studies have shown that B lymphocytes play important roles in the pathogenesis of anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitis. Especially, regulatory B cells are known to be diminished in these vasculitides, suggesting that it is involved in the B cell abnormalities in ANCA associated vasculitis. However, the population of pathogenic B cells is so small that its function is not fully elucidated.

In present study, we developed original assay system utilizing micro flow channels that simulates the environment of blood vessels, by which the interaction of B cells with endothelial cells could be analyzed. Additionally, we utilized micro-enzyme-linked immunosorbent assay system that integrated immunoassay into a microchip in order to detect extremely small amounts of analytes. We found that the endothelium-specific B lymphocyte binds to endothelium in SICLV. This resulted in the increased expression of intercellular adhesion molecule 1 and programmed death-ligand 1 on endothelium, which can induce T cell recruitment and activation.

These findings show that B lymphocyte is the key player in SICLV whose pathophysiology is not fully understood to date. In addition, our original micro-fluidics methods can reveal new etiology of the inflammatory disease, which may lead the novel therapeutic strategies.

2353

Identifying autoimmune beta-cell epitopes in type 1 diabetes by HLA-peptidomics

Gonzalez Duque, S.^{1,2}, Verdier, Y.², Vinh, J.², Mallone, R.¹

¹INSERM U 1016, Intitut Cochin, CNRS UMR 8104-Université Paris Descartes, Paris, France, ²ESPCI ParisTech - CNRS-USR 3149, Paris, France

Type 1 diabetes (T1D) is an autoimmune disease in which autoreactive CD8+ T-cells destroy pancreatic insulin-secreting beta-cells. This destruction is triggered by the recognition of peptide epitopes presented at the beta-cell surface in the pocket of HLA Class I molecules. We aimed at identifying these epitopes by HLA peptidomics. This strategy consists in purifying the peptide-HLA complexes from immortalized human beta-cell lines and to analyze the eluted peptides by mass spectrometry. This workflow was validated based on: 1) the 9-11-aminoacid length of most (93%) peptides identified, which fits the HLA pocket constraints; 2) the peptide signature, which reflects the HLA haplotype expressed by the beta-cells; and 3) the identification of several preproinsulin peptides already described as major CD8+ T-cell epitopes.

We thus obtained a catalogue of 65 unique peptides derived from 49 beta-cell proteins. Twenty-three peptides were derived from known beta-cell protein antigens (preproinsulin, GAD, IA-2, ZnT8, IAPP and chromogranin-A), and novel candidate epitopes were identified within their sequences. The remaining 43 proteins represent novel candidate antigens. One recurrent feature shared by several known and putative antigens is their expression in insulin granules and their processing by proconvertases, which cleave protein precursors to obtain bioactive products. Intriguingly, several of the peptides identified map next to these proconvertase cleavage sites. The processing of protein precursors within the β -cell may thus represent a major source of autoimmune epitopes. The candidate epitopes identified may lead to the development of novel T-cell biomarkers.

2698

Anti-inflammatory effects of vitamin D are reduced in T-cells from the inflamed joints of rheumatoid arthritis patients

Jeffery, L.¹, Henley, P.¹, Filer, A.¹, Hewison, M.¹, Sansom, D.², Raza, K.^{1,3}

¹University of Birmingham, College of Medical and Dental Sciences, Birmingham, United Kingdom, ²University College London, Institute of Immunity and Transplantation, London, United Kingdom, ³Sandwell and West Birmingham Hospitals NHS Trust, Department of Rheumatology, Birmingham, United Kingdom

Vitamin-D has potent anti-inflammatory effects and low serum vitamin-D associates with inflammatory diseases such as rheumatoid arthritis (RA), thus vitamin-D supplementation is suggested for their treatment. However, whether immune responses to vitamin-D are efficient in patients is unclear. This study aimed to characterize the effect of active vitamin-D (1,25(OH)₂D₃) upon CD4+T-cells from the blood and synovial fluid(SF) of RA patients.

1,25(OH)₂D₃ significantly reduced IL-17 and IFN γ in antiCD3-stimulated CD4+Tcells from patient blood at levels similar to

healthy controls but SF CD4+T-cells showed reduced suppression of IL-17 and no inhibition of IFN γ . Since 98(\pm 1.8)% of SF CD4+T-cells were memory versus 49(\pm 14)% in blood, 1,25(OH) $_2$ D $_3$ responses in naïve and memory CD4+T-cells from blood were compared. Unlike SF CD4+T-cells, 1,25(OH) $_2$ D $_3$ suppressed IFN γ production by memory and naïve blood CD4+T-cells. However across 1,25(OH) $_2$ D $_3$ targets (IL-17, IFN γ , IL-21, CTLA-4, FoxP3), 1,25(OH) $_2$ D $_3$ had greatest effect upon naïve CD4+T-cells, despite equivalent vitamin-D receptor (VDR) expression in both populations. qPCR for regulators of 1,25(OH) $_2$ D $_3$ signaling (VDR, RXR, co-enhancers(DRIP205, NcoA1), co-repressors(NcoR1, NcoR2) and 1,25(OH) $_2$ D $_3$ -inactivating CYP24A1) in memory CD4+T-cells from RA blood and SF revealed raised 1,25(OH) $_2$ D $_3$ responsiveness in SF CD4+T-cells ex-vivo but no difference between SF and blood after stimulation, which increased responsiveness in both populations.

Collectively these data confirm a 1,25(OH) $_2$ D $_3$ -response system in CD4+T-cells from blood and SF of RA patients. However, whilst blood CD4+T-cells responded normally to 1,25(OH) $_2$ D $_3$, SF CD4+T-cells were less sensitive, partly through greater memory bias. Such insensitivity of SF CD4+T-cells to 1,25(OH) $_2$ D $_3$ has important implications for use of vitamin-D to treat RA.

3100

Convergent antibody repertoire in acquired thrombotic thrombocytopenic purpura patients

Skowronska, M., Schaller, M., Kremer Hovinga Strelbel, J.A. University of Bern, Department of Clinical Research, University Clinic of Hematology and Central Hematology Laboratory, Bern, Switzerland

Introduction: Autoantibodies neutralizing and/or accelerating the clearance of ADAMTS13 are hallmark of acquired thrombotic thrombocytopenic purpura (aTTP) and associated with risk of relapse in survivors of a first acute disease episode. To elucidate the pathophysiology and evolution of the anti-ADAMTS13 antibodies in aTTP, their genetic diversity, clonality and mutation status were studied.

Methods: Splenic mononuclear cells from eight patients splenectomized for frequently relapsing aTTP were sorted into naïve, switched as well as unswitched memory B-cell and plasma cell populations. Antibody cDNA libraries were deep sequenced on MiSeq Illumina. V(D)J gene alignments and junction were identified using IMGT/high V-QUEST. V(D)J pairings were visualized using Circos. From four patients anti-ADAMTS13 specific B-cells were individually sorted and IgG heavy/light chain genes were amplified. Additionally repertoire overlap analysis and diversity estimation were performed using VDJ tools.

Results: Deep sequencing of the total splenic B-cell repertoire revealed ~4 million functional antibody sequences divided into ~230 thousand different clones. Among the eight patients, 179 convergent and expanded clones were observed. Antibodies were encoded by V-genes from IGHV3 and IGHV4 families, J-genes from IGHJ4 and D-genes from IGHD3. Anti-ADAMTS13 specific B-cells revealed 80 unique clones with 15% antibodies encoded by IGHV1-69, compared to only 1% IGHV1-69

antibodies in the total repertoire.

Conclusion: The observed expanded convergent antibody sequences among 8 unrelated aTTP patients, as well as the enrichment of IGHV1-69 encoded anti-ADAMTS13 antibodies are highly unlikely to be coincidental and point at antigen-driven selection of autoantibodies in aTTP. Pathological relevance of the selected antibodies is under investigation.

3945

Identification and quantitation of autoantigen peptides targeted by CD8+ T cells on human islet beta cells

*Giam, K.^{1,2}, Dudek, N.L.¹, Croft, N.P.¹, Ayala, R.¹, Peakman, M.³, Purcell, A.W.¹
¹Monash Biomedicine Discovery Institute, Department of Biochemistry and Molecular Biology, Clayton, Australia, ²Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, Parkville, Australia, ³King's College London, Peter Gorer Department of Immunology, London, United Kingdom*

Type 1 diabetes (T1D) results from a targeted destruction of the insulin-secreting beta cells due to an islet-specific autoimmune response. Insulinitis, characterised by the infiltration of T cells into the islets, is predominantly driven by CD8+ T cells. Human leukocyte antigen (HLA) genes, specifically the class II HLA and to a lesser extent class I HLA genes, are strongly implicated in T1D. Class I HLA molecules constitutively present endogenous antigenic peptide fragments to incoming CD8+ T cells. Indeed, studies have documented the presence of circulating autoreactive CD8+ T cells reactive towards key islet epitopes in T1D patients. However, the presence of these epitopes on human islet beta cells have not been definitively shown.

We have used a reverse immunology strategy in combination with highly sensitive mass spectrometry to confirm and quantify the presentation of key HLA-A2 restricted preproinsulin epitopes, alongside the discovery of novel and posttranslationally modified autoantigenic HLA-bound peptides on human islets derived from a number of other candidate autoantigens including chromogranin A. This study represents the first characterisation of the class I restricted peptide repertoire that is naturally processed and presented by human islet beta cells. More importantly, we have identified a significant number of non-canonical HLA-bound peptides, posttranslationally modified and hybrid peptide species. Our current analysis of this complex peptidome data set and correlation with the proteome of the islets will allow us to start to understand the nature of the autoreactive immune response towards beta cells and provide a better understanding of T1D pathogenesis.

1015

CD4+ T cell-intrinsic TRAF5 negatively regulates Th17 cell-dependent experimental autoimmune encephalomyelitis

Nagashima, H.¹, Okuyama, Y.¹, Hayashi, T.¹, Asao, A.¹, Kawabe, T.¹, Yamaki, S.¹, Nakano, H.², Croft, M.³, Ishii, N.¹, So, T.¹

¹Tohoku University Graduate School of Medicine, Department of Microbiology and Immunology, Sendai, Japan, ²Toho University School of Medicine, Department of Biochemistry, Tokyo, Japan, ³La Jolla Institute for Allergy and Immunology, Division of Immune Regulation, La Jolla, United States

TNF receptor-associated factor 5 (TRAF5) is an adaptor protein that is highly expressed in lymphocytes. We have found that TRAF5 antagonizes IL-6 receptor signaling that is required for the differentiation of IL-17-producing inflammatory helper T (Th17) cells. However, it is not clear how TRAF5 controls Th17-driven autoimmunity such as experimental autoimmune encephalomyelitis (EAE). To test this, *Traf5*^{-/-} mice were immunized subcutaneously with a peptide of myelin oligodendrocyte glycoprotein (MOG) emulsified in complete Freund's adjuvant on day 0, and injected intraperitoneally with pertussis toxin on day 0 and day 2. Clinical signs of diseases were evident on day 11 and peaked between days 14 and 18. Compared with wild-type mice, *Traf5*^{-/-} mice developed EAE with a strongly enhanced clinical score and marked body weight loss in line with the favored Th17 differentiation. Consistent with this, significantly higher numbers of Th17 cells were accumulated in the central nervous system of *Traf5*^{-/-} mice. To investigate if the enhanced EAE was caused by TRAF5-deficiency in CD4⁺ T cells, we adoptively transferred wild-type or *Traf5*^{-/-} CD45.2⁺ CD4⁺ T cells into congenic CD45.1⁺ B6 mice that had been sublethally irradiated, then immunized the recipient mice with MOG peptide as described above. Recipient mice developed EAE in a CD4⁺ T cell-intrinsic manner, and recipients with *Traf5*^{-/-} CD4⁺ T cells had significantly higher EAE scores than those with wild-type CD4⁺ T cells. Thus, TRAF5 limits EAE pathogenesis through negative regulation of the IL-6-dependent Th17 program in T cells.

2289

GM-CSF induces multi-organ autoimmunity upon loss of regulatory T cell mediated suppression

Lanzinger, M., Croxford, A.L., Greter, M., Becher, B.

University of Zurich, Institute of Experimental Immunology, Zurich, Switzerland

The prevention of autoimmunity is largely mediated by regulatory T (T_{reg}) cells. Deletion of Foxp3⁺ cells in adult mice leads to severe multiple organ autoimmunity, which is driven by activated auto-aggressive helper T (T_H) cells. The nature and polarization pattern of these deregulated T_H cells is a matter of debate. We found that *in vivo* depletion of T_{reg} cells in Foxp3^{DTR} mice caused excessive GM-CSF and IFN- γ release by T_H cells. In order to assess the impact of individual T cell-derived proinflammatory cytokines, we generated Foxp3^{DTR} mice lacking either GM-CSF, IL-17A or IFN- γ . After T_{reg} cell depletion, GM-CSF emerged as a potent pathogenic mediator causing tissue damage in the absence of T_{reg} cells. The systematic application of the Foxp3^{DTR} mouse model to mice lacking either GM-CSF responsive cell types or the receptor for GM-CSF in a cell specific manner revealed that GM-CSF producing autoreactive T_H cells drive tissue inflammation through the licensing of pathogenic neutrophils.

Poster Thursday

15:30 - 16:30

Aging & Perinatal Immunology

812

Robust innate immunity underlies protection from acute endotoxemia in a long-lived mouse model of successful aging

Griffin, P.¹, Conover, C.², Bale, L.², Snyder, A.¹, Michel, J.¹, Vallejo, A.^{1,3,4}

¹University of Pittsburgh School of Medicine, Department of Pediatrics, Pittsburgh, United States, ²Mayo Clinic, Department of Medicine, Rochester, United States, ³UPMC Children's Hospital of Pittsburgh, Division of Rheumatology, Pittsburgh, United States, ⁴University of Pittsburgh School of Medicine, Department of Immunology, Pittsburgh, United States

We have shown that unlike healthy young adults and elderly persons with measurable disabilities, community-dwelling elders with high physical/cognitive function have a distinctive repertoire of oligoclonal T cells expressing highly diverse innate receptors. We propose therefore that innate immunity is a determinant of successful aging and long-term survivorship. To model successful aging, we examined mice deficient in pregnancy-associated plasma protein A (PAPPA) that we reported to have 40% lifespan extension and overall significant reduction in mid-life/end-of-life pathology. PAPPA is the enzyme that degrades inhibitory proteins bound to insulin-like growth factor (IGF) and thereby control the availability of bioactive IGF. Adopting the classic experimental platform of acute endotoxemia, we found that intraperitoneal doses of LPS that were lethal to 18 mos old wildtype (WT) mice, were survived by old *PAPPA*^{-/-} mice. Whereas moribund old WT mice showed classic LPS-induced cytokine storm, humoral profiles of LPS-treated old *PAPPA*^{-/-} mice showed significant global attenuation of cytokine levels. LPS induced high levels of Bcl-xL in all innate cell types of old *PAPPA*^{-/-} mice. Such *PAPPA*^{-/-} innate cells had nil evidence of apoptosis, and had significantly lower levels of intracellular TNF α , IL-6, and phospho-NF κ B, molecules linked to destructive inflammation. Significantly, old *PAPPA*^{-/-} mice have a distinctive transcriptomic signature of highly upregulated innate genes including antimicrobial peptides and inhibitors of TLR4 signaling. Collectively, our data underscore the relevance of immunologic factors in refining clinical definition(s) of successful longevity. Upmodulation of innate function could be an avenue for alternative innovations on immune intervention specifically for the elderly.

813

Thymic epithelial progenitor cell activation following sex steroid blockade

Lepletier, A.¹, Hammett, M.², Wong, K.², Hedger, M.³, Boyd, R.², Loveland, K.², P Chidgey, A.²

¹Queensland Institute of Medical Research, Brisbane, Australia, ²Monash University, Melbourne, Australia, ³Hudson Institute of Medical Research and Monash University, Melbourne, Australia

The decrease of naive T cell export and intrinsic defects in T cell activation characterized during aging has been broadly correlated with thymus atrophy. We have previously demonstrated that sex steroid blockade reverts thymic epithelial cells (TEC) numerical and compositional defects that accompany thymic involution and re-establishes thymocyte numbers in aged mice. The deleterious effects of androgens on thymus have been proposed to be associated with direct action on TEC, however, the mechanisms that underlie this phenomena remains unclear. Here we demonstrate that Activin A signalling in TEC progenitor cells (TEPC) plays an important role in thymus recovery of 9 month aged mice following castration (Cx). During thymus aging, follistatin (FST), a highly potent antagonist of Activin A, is increased in the thymic microenvironment. Castration re-establishes Activin A:FST ratio toward young thymus levels and at the same time influences upregulation of Activin receptors *Acvr1b*, *Acvr2a* and *Acvr2b* in progenitor cells of aged mice. Consistent with this, the addition of Activin A to aged TEPCs in our 3D in vitro culture system, increased differentiation into mTEC, whilst Activin A signalling blockade in young TEPC cultures decreased mTEC conversion. Young adult Activin A HET young mice have compromised mTEC development. Furthermore, thymi from E14.5 Activin A HET or KO mice grafted into athymic nude mice, generated reduced numbers of mTECs, total thymocytes and CD4+ T cells. In conclusion, sex steroid blockade unleashes an age-related block in TEPC differentiation, promoting mTEC recovery through an Activin A dependent mechanism.

814

Dendritic cells from aged subjects display impaired capacity to induce mucosal tolerance

Agrawal, A., Agrawal, S., Ganguly, S., Tran, A., Sundaram, P.
University of California, Medicine/Immunology, Irvine, United States

Aged subjects display increased susceptibility to mucosal diseases. Infections and diseases of the respiratory, gastrointestinal and urinary tract are prevalent in this population. Sterile inflammation of the mucosa is considered a major culprit. Retinoic Acid (RA) secreted by mucosal epithelial cells plays a major role in inducing tolerance in the mucosa. RA acts on Dendritic cells (DCs) to induce mucosal tolerance. Here we compared the response of DCs from aged and young individuals to RA with a view to understand the mechanisms underlying chronic sterile inflammation in aged mucosa. Our investigations revealed that compared to young DCs, RA stimulated DCs from aged subjects are defective in inducing T regulatory cells. Examinations of the underlying mechanisms indicated that impaired upregulation of CD141 expression on aged DCs by RA was a major factor in reduced induction of T regulatory cells. In summary, reduced response of aged DCs to RA enhances mucosal inflammation in the elderly, increasing their susceptibility to mucosal diseases. In addition, our data also suggests a novel mechanism of immunosuppression by RA in tumor microenvironment.

815

IGHV and IGHD encoding antibodies with exceptionally long CDR3H are most expressed at birth in the bovine neonate

Pasman, Y.¹, Merico, D.M.², Kaushik, A.¹

¹University of Guelph, Molecular and Cellular Biology, Guelph, Canada, ²Hospital for Sick Children, Centre for Applied Genomics, Toronto, Canada

We sequentially analyzed 18 RNA-seq libraries from three bovine calves at day 0, 7, 14 and 28, together with their dams (day 0 and 7) to delineate the development of neonatal immunity. A significant shift in neonatal transcriptome occurs within first week post-birth where a set of 717 genes is up-regulated. Global pathway analysis of the transcriptome showed 110 differentially expressed (DE) immune-related genes at birth as compared to day 7. These include immune related genes, such as, complement, *MHCII*, chemokine receptors, defensins and cytokines. As compared to adults, *IGKC* is expressed at higher levels in the neonates, despite predominant *IGLC* expression at birth. Two members of *IGKV2* family, *IGKV10* and *IGKV12*, are preferentially expressed in neonatal B cells. As for *IGLV1* family, *IGLV1b.1* and *IGLV1x.1* are most expressed in neonatal B cells. Another member, *IGLV3.4*, of rarely expressed *IGLV3* family is preferentially used in neonatal B cells. The *IGHM* and *IGHD* transcripts are expressed at birth and *IGHM* achieves adult levels by day 7. This is followed by *IGHA* and *IGHG* transcripts 14-28 days post birth. Interestingly, most 3' *IGHV1S1(BF4E9)* gene, known to encode antibodies with exceptionally long CDR3H, is preferentially expressed in neonatal B cells. In parallel, single longest *IGHD2(D_H2)* gene that encodes such an atypical CDR3H is expressed at high levels at birth. It seems that *IGHV1S1(BF4E9)* gene has an important functional role in the development and expansion of bovine neonatal B-cell repertoire. To conclude, the bovine neonate acquires IgM levels similar to adult by day 7.

816

Immune response and early transcriptional changes after primary and booster vaccination against hepatitis B in young and old adults

Weinberger, B.¹, Haks, M.C.², Katzgraber, F.³, Ottenhoff, T.H.M.², Grubeck-Loebenstien, B.¹

¹University Innsbruck, Institute for Biomedical Aging Research, Innsbruck, Austria, ²Leiden University Medical Center, Leiden, Netherlands, ³Public Health Department, Federal State of Tyrol, Innsbruck, Austria

Many currently used vaccines are less immunogenic and effective in the elderly compared to younger adults due to age-related changes of the immune system. Most vaccines utilized in the elderly contain antigens, with which the target population had previous contact. Therefore, most studies investigating vaccine-induced immune responses in the elderly do not analyze responses to neo-antigen but rather booster responses. It can be hypothesized that age-related differences in the immune response are distinct for primary and recall responses. We therefore aimed to investigate primary and recall responses using the same antigen in young and older adults

and chose Hepatitis B vaccine as a model antigen. Young (20-40 years) and elderly (>60 years) healthy volunteers received either a primary series (no prior vaccination) or a single booster shot (documented primary vaccination more than 10 years ago) of the registered vaccine Twinrix. Expression of immunity-related genes was measured before and 1 day after vaccination and antibody titers were determined at several time points with a long-term follow-up 6 months after the last vaccination. Antibody responses were lower and seemed to be delayed in the elderly compared to young adults. Non-responders after the 3-dose primary series were more frequent in the elderly group. Antibody titers after booster vaccination increased in all participants. Associations between early expression-profiles on day 1 and antibody responses were found.

817

Effects of Interleukin-1 receptor antagonist on airway remodeling and hyperreactivity in murine bronchopulmonary dysplasia induced by perinatal inflammation and hyperoxia

Royce, S.¹, Nold, M.², Bui, C.², Donovan, C.¹, Lam, M.¹, Lamanna, E.¹, Rudloff, I.², Bourke, J.¹, Nold, C.²

¹Monash University, Pharmacology, Clayton, Australia, ²Hudson Institute of Medical Research, Clayton, Australia

Bronchopulmonary dysplasia (BPD) is a chronic disease of extreme prematurity that has serious long-term consequences including increased asthma risk. We earlier identified Interleukin-1 receptor antagonist (IL-1Ra) as a potent inhibitor of murine BPD induced by combining perinatal inflammation (intra-peritoneal injection of lipopolysaccharide to pregnant dams) and exposure of pups to hyperoxia (FiO₂ 0.65).

The aim of this study was to determine whether airway remodeling and hyperresponsiveness similar to asthma are evident in this model, and whether IL-1Ra is protective. Pups received daily subcutaneous injections of vehicle or 10mg/kg IL-1Ra. At 28d, lungs were prepared for histology and morphometry of alveoli and airways, or inflated with agarose to prepare lung slices to visualise *ex vivo* airway contraction and relaxation by phase-contrast microscopy.

Pups reared in hyperoxia developed a severe BPD-like lung disease, with fewer, larger alveoli, and increased subepithelial collagen and airway α -smooth muscle actin. Following hyperoxia, methacholine elicited contraction with similar potency but increased maximum reduction in lumen area (air 44%; hyperoxia 89%), and the dilator potency of salbutamol was reduced. IL-1Ra treatment prevented hyperoxia-induced alveolar disruption and airway fibrosis, but surprisingly not the increase in methacholine-induced airway contraction.

The current study is the first to demonstrate *ex vivo* airway hyperreactivity and impaired dilator responsiveness caused by perinatal inflammation and hyperoxia and reveals further preclinical mechanistic insights of IL-1Ra as a treatment for BPD.

818

Targeting macrophages rescues age and tumor-induced T cell dysfunction in elderly mice

Jackaman, C.^{1,2}, Dye, D.E.^{1,2}, Crabb, H.G.^{1,2}, Gardner, J.K.^{1,2}, Grounds, M.D.³, Nelson, D.J.^{1,2}

¹Curtin University, Biomedical Sciences, Perth, Australia, ²Curtin University, Curtin Health Innovation Research Institute, Perth, Australia, ³University of Western Australia, School of Anatomy and Human Biology, Perth, Australia

Most cancers emerge in aging populations when immune function is declining. Macrophages make up a large percentage of the tumor microenvironment, a key site for effector tumour-specific T cells. Macrophage function may change with aging in multiple tissues and impact on T cell function, yet the consequences of macrophage interactions with T cells during aging is not understood. Flow cytometry showed that lymphoid tissues of healthy elderly mice (24 months, cf 70 human years) contained more anti-inflammatory IL-10⁺ macrophages than their younger counterparts (2 months, cf 16-18 human years). Interestingly, tumor-exposed elderly macrophages produced more suppressive TGF β and IL-4 than young macrophages; prevented young T cells from producing IFN γ ; and induced IL-10⁺ CD4⁺ T cells. These data imply that elderly macrophages are hypersensitive to suppressive signals and are more likely support tumour growth than younger counterparts. This was supported by data showing that mesothelioma tumours grew faster in elderly mice and that macrophage depletion inhibited tumour growth. We attempted to rescue tumor-exposed macrophages with LPS/IFN γ or IL-2/agonist anti-CD40 antibody immunotherapy. LPS/IFN γ upregulated CD40, but did not restore the ability of young or elderly tumour-exposed macrophages to secrete pro-inflammatory cytokines to the same levels as non-tumor-exposed, LPS/IFN γ controls. These macrophages induced young and elderly T cells to proliferate, however T cells did not secrete IFN γ . In contrast, tumor-exposed, IL-2/CD40-stimulated macrophages not only induced young and elderly T cells to proliferate, they also induced elderly T cells to produce IFN γ . These results support further studies examining macrophages in anti-cancer treatment the elderly.

819

Despite generating a weak tumour-specific CTL response during tumour progression chemotherapy is only effective in elderly mice if T cells are present

Nelson, D.¹, Spowart, J.¹, Crabb, H.¹, Dye, D.¹, Fox, S.², Metharom, P.³, Proksch, S.¹, Jackaman, C.¹

¹Curtin University, Biomedical Sciences, Perth, Australia, ²Curtin University, Pharmacy, Perth, Australia, ³Curtin University, Health Sciences, Perth, Australia

There is convincing evidence that the immune system protects from cancer. However, immune function declines with age and most cancers, including mesothelioma, emerge 30-40 years after exposure to the relevant carcinogen with most patients being greater than 60 years old. Studies in young mice (6-8 week old, cf 14-16 human years) have shown that several chemotherapeutic agents require a functional T cell

response to elicit an effective anti-cancer outcome, but T cells become increasingly dysfunctional with aging. We asked if chemotherapy in the elderly also requires T cells. We found that tumours grew faster in elderly mice (24-27 month old, cf 70-80 human years). This was associated with decreased muscle weight in the gastrocnemius and extensor digitorum longus, body weight loss, lower percentages of blood lymphocytes, higher percentages of blood granulocytes and increased liver damage, implying that elderly mice are more susceptible to cancer cachexia. Chemotherapy induced even more weight loss and liver damage in elderly but not young mice, and was not as effective in elderly mice as it was in young mice. We also found, using an in-vivo CFSE-based CTL assay, that, unlike their younger counterparts which generated a significant endogenous tumour-specific CD8+ CTL response, elderly mice generated only a weak tumour-specific CTL response during tumour progression. Nonetheless, T cell depletion studies showed that chemotherapy in elderly mice also required T cells to induce tumour regression. Future studies will determine if rescuing T cell function in the elderly improves their anti-cancer response to chemotherapy.

820

Refining anti-inflammatory therapy strategies in a murine model of bronchopulmonary dysplasia induced by perinatal inflammation and hyperoxia

Ngo, D.^{1,2}, Rudloff, I.^{1,2}, Nold, M.^{1,2}, Cho, S.^{1,2}, Bui, C.^{1,2}, McLean, C.³, Berger, P.^{1,2}, Nold, C.^{1,2}

¹Ritchie Centre, Hudson Institute of Medical Research, Clayton, Australia, ²Monash University, Department of Paediatrics, Clayton, Australia, ³The Alfred Hospital, Department of Anatomical Pathology, Melbourne, Australia

Bronchopulmonary dysplasia (BPD) is a common lung disease of premature infants, with devastating consequences. As no therapy for BPD exists, we investigated whether the anti-inflammatory protein C (PC) and interleukin 1 receptor antagonist (IL-1Ra) prevent murine BPD. BPD was precipitated by perinatal inflammation (intraperitoneal injection of lipopolysaccharide to pregnant dams) followed by postnatal exposure of pups to hyperoxia (65% or 85% O₂). Pups were treated daily with PC, IL-1Ra (10mg/kg or 100mg/kg), or vehicle (up to 28d). Vehicle animals in hyperoxia developed severe BPD (alveolar number and gas exchange-area decreased \leq 60%, alveolar size increased 4-fold). PC and IL-1Ra prevented structural lung damage in 65%, but not 85% O₂. Significant lung improvement was observed in mice treated with 10mg/kg of IL-1Ra immediately after birth compared to vehicle-treated hyperoxia group ($p < 0.001$) and in comparison to IL-1Ra (100mg/kg) and the delayed treatment with IL-1Ra ($p < 0.05$).

Perinatal inflammation and hyperoxia each triggered distinct pulmonary immune responses (20-fold increase), some reduced (\leq 94%) by PC or IL-1Ra or both. Pulmonary inflammatory mediators IL-1 β , MIP-1 α , and TREM-1, was found to play a critical role in murine BPD. Although the effectiveness of administering IL-1Ra to prevent alveolar lung damage is dose and timing dependent, our findings show that IL-1Ra and PC protect the

lungs of newborn mice from detrimental effects of hyperoxia and perinatal inflammation by attenuating the pulmonary immune response. Therefore, these two anti-inflammatory drugs could fundamentally change the prognosis of premature infants by treating or preventing the currently irremediable BPD.

821

Plasma vitamin D levels and the inflammatory status of preterm infants

Sava, F.¹, Treszl, A.¹, Hajdú, J.¹, Toldi, G.¹, Rigó Jrs, J.¹, Tulassay, T.², Vászrhelyi, B.³

¹Semmelweis University, First Department of Obstetrics and Gynecology, Budapest, Hungary, ²Semmelweis University, First Department of Pediatrics, Budapest, Hungary, ³Semmelweis University, Department of Laboratory Medicine, Budapest, Hungary

Background: Vitamin D has an important immunomodulatory role. We investigated whether vitamin D levels at birth may associate with immune status in preterm infants.

Methods: Cord blood samples were collected from 28 preterm infants born \leq 30 weeks of gestation. Infants were divided into groups below and above median vitamin D level. We measured plasma cortisol and cytokine levels and also assessed the peripheral prevalence of distinct immune cell subsets using flow cytometry. The mixed effect model was used to analyse the effects of vitamin D, plasma cortisol levels and gestational age on cytokine levels and immune phenotype.

Results: Vitamin D level in our cohort was 23.3 [18.7-26.6] ng/ml (median [IQR]). In infants with vitamin D level below the median the prevalence of CD4+ CXCR3+ (Th1) and CD8+ CXCR3+ cell subsets was higher, while the prevalence of CD4+ CCR4+ (Th2), CD8+ CCR4+ and plasmacytoid dendritic cell (pDC) subsets was lower than in those with vitamin D level above median. pDCs and Th2 lymphocytes were the only cell subsets which were only influenced by vitamin D levels, but not by plasma cortisol and gestational age. No association between vitamin D level and any of the tested plasma cytokine levels was detected.

Conclusions: Vitamin D levels may together with cortisol levels and gestational age have an effect on Th1/Th2 balance and the prevalence of plasmacytoid dendritic cells in the preterm newborn.

822

Impact of aging on calcium influx and potassium channel characteristics of T lymphocytes

Toldi, G.¹, Kollár, S.², Berta, L.¹, Rigó Jr, J.¹

¹Semmelweis University, First Dept of Obstetrics, Budapest, Hungary, ²Semmelweis University, Second Dept of Obstetrics, Budapest, Hungary

Adaptive immunity and T cell function are affected by aging. Calcium influx patterns, regulated by Kv1.3 and IKCa1 potassium channels, influence T cell activation. We aimed to compare calcium influx kinetics in CD8, Th1 and Th2 cells in human peripheral blood samples obtained from five different age groups (cord blood, 10-15 ys, 25-40 ys, 45-55 ys, 60-75 ys).

We evaluated calcium influx kinetics in peripheral lymphocytes by flow cytometry following activation with phytohemagglutinin in the above T cell subsets. We also assessed their sensitivity to specific inhibition of the Kv1.3 and IKCa1 potassium channels by margatoxin (MGTX) and triarylmethane (TRAM), respectively. Data were evaluated using an algorithm based on the calculation of a double-logistic functions for each recording (the FacsKin method).

Calcium influx was greater in Th1 cells of adults, however, its extent decreased again with aging. Importantly, these changes were not detected in Th2 cells. MGTX had a more pronounced inhibitory effect on calcium influx in Th2 cells in adults, while in Th1 cells the same was true for TRAM. Calcium influx of CD8 cells were inhibited to a similar extent by both applied inhibitors, and had no effect in the elderly.

Altered lymphocyte potassium channel inhibitory patterns, regulators of calcium influx kinetics, might contribute to the development of age-related changes of T cell function. The lower level of activation of Th1 cells in the elderly upon the same stimuli may play an important role in the increased susceptibility to infections in this age group.

823

Shedding light on preterm immunity

Lao, J.C.^{1,2}, Beker, F.³, Konig, K.³, Noble, E.³, Walsh, G.³, Malhotra, A.^{2,4}, Tan, K.^{2,4}, Yeoman, E.⁴, Mockler, J.^{1,5}, Woodhead, G.³, Nold-Petry, C.A.^{1,2}, Nold, M.F.^{1,2}

¹Ritchie Centre, Hudson Institute of Medical Research, Clayton, Australia, ²Monash University, Department of Paediatrics, Clayton, Australia, ³Mercy Hospital for Women, Mercy Health, Department of Paediatrics, Melbourne, Australia, ⁴Monash Newborn, Monash Health, Melbourne, Australia, ⁵Monash University, Department of Obstetrics and Gynaecology, Clayton, Australia

There is little knowledge on the immune system of extremely premature infants (born 24-29 weeks of gestation). This paucity of evidence impedes advances in combating bronchopulmonary dysplasia (BPD), a common, severe chronic lung disease that entails significant morbidity and mortality. No safe and effective treatment exists.

In order to improve our understanding on preterm immunity and to characterize the immunological hallmarks of BPD to enable early intervention, an observational study was set up to recruit a cohort of 75 extremely premature infants. Blood was collected from 20 extremely preterm infants at 5 timepoints [birth, days 1, 7 and 14 and 36 weeks corrected gestational age (WCGA)] to date. Following overnight in vitro stimulation with PMA/ionomycin, LPS or vehicle, flow cytometry was used to explore various immune cell subsets.

From the analysis of seven preterm babies, the ability of neonatal CD4+ T cells to produce IFN-gamma in response to cell stimulation increases over time, leading to a clearly positive but still attenuated response at 36 WCGA. Comparing the three infants that developed BPD with the four that did not at 36 WCGA, we observed a marked increase in monocytes (6-fold), dendritic cells (2-fold) and neutrophil activation (9-fold), but fewer circulating endothelial progenitor cells (0.3% vs 1.4% of viable cells).

These early data from preterm infants reveal that this first-of-its-kind study will revolutionise the understanding of preterm immunity. BPD appears associated with markedly increased cellular activation - a promising finding that may provide a basis for therapeutic innovations.

824

Aging induces increased expression of checkpoint inhibitory molecules on dendritic cells

Gardner, J.^{1,2}, Jackaman, C.^{1,2}, Mamotte, C.^{1,2}, Nelson, D.^{1,2}

¹Immunology and Cancer Group, Curtin University, Perth, Australia, ²CHIRI Biosciences Research Precinct, Curtin University, Perth, Australia

The incidence of most cancers increases with age. Recent studies have shown that immune function declines with age and this may contribute to tumour development and progression in the elderly. However, the effects of aging on dendritic cells (DCs) in healthy and tumour-bearing hosts are not yet well-characterised. The aim of this study was to compare changes in DC phenotype and function in young (6-8 weeks old, equivalent to 14-16 human years) and elderly (24-27 months old, equivalent to 70-80 human years) healthy and tumour-bearing mice. The proportions and phenotype of DCs in lymph nodes (LN) and tumours of young and elderly mice were assessed using flow cytometry. DC proportions of total cells increased in LN of healthy and tumour-bearing elderly mice, but decreased in elderly tumours, relative to young mice. Expression of MHC-II was reduced on elderly tumour-associated DCs, suggesting that tumour antigen presentation to CD4+ T-cells may be impaired in the elderly. Healthy and tumour-bearing young and elderly LN DCs showed similar expression of co-stimulatory molecules (CD40, CD80 and CD86) and cytokines (interferon- γ) involved in T-cell activation. However, elderly LN DCs expressed increased levels of the checkpoint inhibitory molecules PD-L1 and CD73 (an ecto-enzyme involved in adenosine production) and the immunosuppressive cytokine TGF- β , relative to young counterparts. These data suggest that elderly LN DCs are more likely to inhibit T-cell function and impair generation of anti-tumour immunity in the elderly.

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Comparison of intestinal innate lymphoid cell in neonatal mice with necrotizing enterocolitis to healthy controls and young adult mice

Cho, S.¹, Lao, J.¹, Rudloff, I.¹, Cheng, W.², Nold, M.¹, Nold, C.¹

¹Monash University, Hudson Institute of Medical Research, Clayton, Australia, ²Beijing United Family Hospital, Beijing, China

Background: The cytokine profile of innate lymphoid cells (ILC) resembles that canonically associated with adaptive immune responses, but their activation is not antigen-specific. ILC play important roles in intestinal immune homeostasis. Inflammatory bowel disease is linked to changes in intestinal ILC composition; e.g. increased IL-17 producing ILC3 are associated with Crohn's disease. However, little is known about developmental changes in ILC in neonatal intestinal disease. We aimed to explore

ILC regulation and function in a murine model of necrotizing enterocolitis (NEC).

Methods: 72h NEC model: formula feeding of newborn C57BL/6J pups combined with hypoxia and cold stress. Controls: dam-fed littermates. Both groups were compared to 10 weeks old mice and intestinal ILCs, analysed by flow cytometry.

Results: The intestinal NKp46⁻ ILC3 were markedly more abundant in neonatal than adult mice (43% vs 24%). In contrast, the NKp46⁺ ILC3 increased with age from 2.4% on d3 of life to 12% in young adults. The overall percentage of ILC2 was similar in pups and adults, but the fraction of KLRG1⁺ ILC2 was smaller in neonatal mice (2% vs 15% in adults). There was little change in intestinal ILC1 with age.

We observed an increase in NKp46⁻ ILC3 to 56%, and IL-17 and IL-22 in NEC on d3 of life when compared to dam-fed pups. The GATA3⁺ ILC2 and IL-33, were also markedly increased in NEC.

Conclusions: Murine NEC is associated with an increase in intestinal ILC2 and ILC3. The ILC repertoire changes markedly as the immune system matures.

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The depletion of regulatory T cells in the third trimester increases the susceptibility for LPS-induced preterm birth and causes adverse neonatal outcomes

Arenas-Hernandez, M.^{1,2,3}, Romero, R.², Balancio, A.¹, Mial, T.¹, Hassan, S.¹, Sanchez-Torres, C.³, Gomez-Lopez, N.^{1,2}

¹Wayne State University, OB/GYN, Detroit, United States, ²National Institute of Health, Perinatology Research Branch, Detroit, United States, ³Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Departamento de Biomedicina Molecular, Mexico City, Mexico

Introduction: The role of regulatory T cells (Tregs) in the third trimester of pregnancy is poorly understood. The aims of this study were to investigate whether the depletion of Tregs:

- 1) induces preterm birth (PTB);
- 2) increases the susceptibility for LPS-induced PTB, and
- 3) has adverse effects on pup survival and weight during the first and second pregnancy.

Methods: B6-Foxp3DTR females were impregnated by B6 (DTRs=syngeneic) or BALB/c males (DTRa=allogeneic). Tregs were depleted by injecting Diphtheria toxin (DT; n=16-19 each) daily from 14.5 days postcoitum (dpc) until delivery. Control mice were injected with [Glu52]-Diphtheria toxin (GluDT; n=11-14 each). DTRa females were impregnated again (DTRall) and injected with DT (n=10). A second group of DTRa dams was injected with DT or GluDT (n=10 each), and LPS was injected at 16.5 dpc. Following injection(s), dams were video recorded to calculate the rate of PTB, gestational age, pup survival and weight at 1-3 weeks postpartum.

Results:

- 1) The depletion of Tregs in the third trimester did not induce PTB neither in DTRs nor in DTRa dams; yet, DTRa dams delivered earlier than DTRs dams;
- 2) The depletion of Tregs increased the susceptibility for LPS-induced PTB in DTRa dams;
- 3) DTRa dams delivered leaner pups than controls;

- 4) DTRall dams delivered leaner pups than DTRa dams; and
- 5) DTRall dams delivered leaner and less viable pups than controls.

Conclusion: The depletion of Tregs in the third trimester increases the susceptibility for LPS-induced PTB and causes adverse neonatal outcomes in the first and second pregnancy.

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Aging with MCMV maintains TCR repertoire diversity in late life

Smithey, M.^{1,2}, Venturi, V.³, Davenport, M.³, Nikolich-Zugich, J.^{1,2}

¹University of Arizona College of Medicine, Immunobiology, Tucson, United States, ²University of Arizona, Arizona Center on Aging, Tucson, United States, ³University of New South Wales, Centre for Vascular Research, Kensington, Australia

Cytomegalovirus (CMV) is a ubiquitous persistent herpesvirus (worldwide infection rate 45-100%). CMV has coevolved with mammalian hosts for millennia. It has been postulated that lifelong persistent immune interactions between host and CMV may contribute to age-related immune senescence. We previously reported that mice aged with persistent murine CMV (MCMV) show impaired CD8 T cell function upon challenge with several microorganisms, including *Listeria monocytogenes* expressing the model OVA antigen (Lm-OVA) in late life. To further explore how lifelong persistent MCMV infection may influence aged immune responses, we challenged control adult and old mice, as well as lifelong MCMV-infected old mice (hereafter referred to as MCMV+) with Lm-OVA, and assessed the OVA-specific TCRb repertoire by single-cell sorting and TCRb CDR3 sequence analysis. We found that compared to old MCMV-negative animals, OVA-specific CD8 effector T cells generated in old MCMV+ mice contained diverse clonotypes exhibiting

- (i) non-canonical TCR genes;
- (ii) a loss of a conserved amino acid motif;
- (iii) reduced avidity for cognate antigen; and
- (iv) broader recognition of altered peptide ligands.

Thus, lifelong MCMV infection appeared to maintain/broaden diversity within the CD8 T cell response to a new infection in late life, by recruitment of clonotypes with broad cross-reactive antigen recognition capacity. These results have profound implications for our understanding of naive T cell maintenance over a lifespan, and suggest that our coevolution with CMV may include surprising, potentially positive impacts on adaptive immunity in late life.

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The roles of Krüppel-like factors (KLF) 9 and 10 in the regulation of the fetal naive T cell epigenome

Ng, M.¹, Burt, T.^{2,3}

¹University of California, San Francisco, Biomedical Science Ph.D., San Francisco, United States, ²University of California, San Francisco (UCSF), Division of Neonatology, San Francisco, United States, ³University of California, San Francisco (UCSF), Eli and Edythe Blythe Broad Center of Regeneration Medicine and Stem Cell Research, San Francisco, United States

The developing human fetal immune system achieves active immune tolerance, in part, through a dominant population of CD25⁺FoxP3⁺ regulatory T (Treg) cells present in secondary lymphoid organs. Crucially, fetal naïve T cells preferentially differentiate into Treg cells upon T cell receptor (TCR) stimulation. Krüppel-like factors (KLF) 9 and 10 have been identified as genes differentially expressed between adult and fetal naïve T cells respectively, and are likely candidates that control the fetal naïve T cell phenotypes. Overexpression of KLF9 in fetal naïve T cells thus inhibits their innate ability to differentiate into Treg cells and enhances their ability to produce IFN γ under Th0 conditions. Members of the KLF family operate to influence cell differentiation by directing epigenetic modifications. In addition to epigenetic modifications at the FOXP3 gene, modifications at other genes are thought to be required for the full maintenance and stability of the Treg cell suppressive phenotype. Sequencing downstream of the Assay for Transposase-Accessible Chromatin (ATAC-seq) has revealed that fetal naïve T cells share areas of chromatin accessibility with adult Treg cells that are distinct from adult naïve T cells, especially in genes such as IKZF2 (Helios) that are a part of the proposed Treg cell epigenome. A pre-existing epigenomic landscape within fetal naïve T cells similar to Treg cells might thus underlie the predisposition for naïve fetal T cells to differentiate into Tregs. Further work in this project will then characterize the roles of KLF9 and KLF10 on the acquisition, maintenance, and acquiescence of the fetal naïve T cell epigenome.

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Characterization of human TCR repertoire of CD4⁺ and CD8⁺ T cells and its implications in evaluation of T cell immunity and aging

Weng, N.-P.

National Institute on Aging, NIH, Baltimore, United States

A diverse TCR repertoire is considered as an essential property of T cell immunity to efficiently contain and eliminate any potential viral invasion. CD4⁺ and CD8⁺ T cells use the same genetic elements and process to generate a functional TCR but differ in their recognition of peptide bound to MHC class II and I, respectively. However, it is currently unclear to what extent the TCR repertoire of CD4⁺ and CD8⁺ T cells is different. Here, we report a comparative analysis of the TCR β repertoires of CD4⁺ and CD8⁺ T cells using a 5'-RACE/PCR/sequencing method. We found that TCR β diversity of CD4⁺ T cells ranges from 1.2-9.8 x10⁵ and is greater than that of CD8⁺ T cells in each study subject. TCR β sequences appeared to be distinct as there was little overlap in TCR β sequences between CD4⁺ and CD8⁺ T cells. Further analysis of CDR3 showed that CD4⁺ and CD8⁺ T cells exhibited distinct preferences for certain amino acids, and this was further confirmed by a supervised machine learning analysis, suggesting there are distinct and discernible differences between TCR β CDR3 in CD4⁺ and CD8⁺ T cells. Comparative sequence analysis also revealed 5-12% of the unique TCR β s that share an identical CDR3 with different V genes with an average of 3 V genes. Together, our findings reveal the distinct features of the TCR β repertoire between CD4⁺ and CD8⁺ T cells. Currently,

we are analyzing when and how the TCR repertoire change with age and the results will be presented at the congress.

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Age-related decline in natural IgM function: diversification and selection of the B-1a cell pool with age

Holodick, N., Vizconde, T., Hopkins, T., Rothstein, T.

Feinstein Institute for Medical Research, Oncology and Cell Biology, Manhasset, United States

Streptococcus pneumoniae is the most common cause of pneumonia, which claims the lives of people over the age of 65 seven times more frequently than those aged 5-49. B-1a cells provide immediate and essential protection from *S. pneumoniae* through production of natural immunoglobulin, which is germline-like due to minimal insertion of N-region additions. The germline-like Ig B-1a cells produce has been attributed to their unique development occurring mainly during fetal life while their persistence throughout adult life is maintained through self-renewal. Yet, B-1a derived IgM moves away from the germline with age. We demonstrate the fetal B-1a cell repertoire is diversified by addition of bone marrow-derived B-1a cells and more importantly, altered by selection-induced skewing of both peritoneal and bone marrow-derived B-1a cells with increasing age. As a result of this selection, the protective capacity of natural serum IgM against *S. pneumoniae* infection is significantly decreased in aged mice.

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Age-related profiling of DNA methylation in CD8⁺ T cells reveals changes in immune response and transcriptional regulator genes

Peterson, P., Tserel, L., Kolde, R., Limbach, M., Tretyakov, K., Kasela, S., Kisand, K., Saare, M., Vilo, J., Metspalu, A., Milani, L.

University of Tartu, Tartu, Estonia

Human ageing affects the immune system resulting in an overall decline in immunocompetence. Although all immune cells are affected during aging, the functional capacity of T cells is most influenced and is linked to decreased responsiveness to infections and impaired differentiation. We studied age-related changes in DNA methylation and gene expression in CD4⁺ and CD8⁺ T cells from younger and older individuals. We observed marked difference between T cell subsets, with increased number of methylation changes and higher methylome variation in CD8⁺ T cells with age. The majority of age-related hypermethylated sites were located at CpG islands of silent genes and enriched for repressive histone marks. Specifically, in CD8⁺ T cell subset we identified strong inverse correlation between methylation and expression levels in genes associated with T cell mediated immune response (LGALS1, IFNG, CCL5, GZMH, CCR7, CD27 and CD248) and differentiation (SATB1, TCF7, BCL11B and RUNX3). Our results thus suggest the link between age-related epigenetic changes and impaired T cell function.

832**Recombinant probiotic bacteria expressing allergen-chimers for the neonatal prevention of poly-sensitization**

Sarate, P.¹, Heini, S.², Daniel, C.³, Kozákova, H.⁴, Grabherr, R.², Schabussova, I.¹, Wiedermann, U.¹

¹Medical University of Vienna, Institute of Specific Prophylaxis and Tropical Medicine, Vienna, Austria, ²(c/o) CD Laboratory for Genetically Engineered Lactic Acid Bacteria, Department of Biotechnology University of Natural Resources and Life Sciences VIBT, Vienna, Austria, ³Institut Pasteur de Lille, Center for Infection and Immunity of Lille, Université Lille Nord de France, CNRS, UMR 8204, Institut National de la Santé et de la Recherche Médicale, Lille, France, ⁴Institute of Microbiology of Academy of Sciences of Czech Republic, V.V.I., Laboratory of Gnotobiology, Nový Hradek, Czech Republic

Poly-sensitization is becoming an increasing health issue in western countries, since poly-sensitized individuals are difficult to treat by conventional therapeutic measures. We recently developed a novel multi-allergen chimer of birch and grass pollen (Phl p 5-bet v 1-Phl p 1) and showed that allergic poly-sensitization was prevented by mucosal treatment with this novel allergen chimer in adult mice. An increasing number of studies report that immune responses induced in early life to environmental and dietary antigens including probiotic bacteria are decisive for future immune response to these antigens. With respect to neonatal interventions, we previously showed that neonatal colonization with recombinant lactic acid bacteria expressing the allergen Bet v 1 successfully prevented allergic responses.

With this background now we want to investigate whether the concept of neonatal colonization with recombinant probiotic bacteria expressing novel allergen constructs can be also used for prevention of allergic multi-sensitivities. Our first aim was to construct recombinant bacteria, constitutively expressing a birch (Bet v 1) and grass pollen (Phl p 1 and Phl p 5) chimer in order to test if this recombinant probiotic strains can be used to prevent poly-sensitization. The strains will be used for the neonatal colonization in

(a) conventional mice and in

(b) germ-free mice prior to poly-sensitization with allergens and test their immunosuppressive properties. Along with this we plan to investigate the gut-lung axis of the microbiota by testing the migration, colonization and interaction of orally or nasally applied bacteria with mucosal surfaces using an in vivo imaging system.

833**Immune mediators in the development of osteoarthritis**

Apinun, J.¹, Sengprasert, P.², Tanavalee, A.¹, Yuktanandan, P.¹, Ngarmukos, S.¹, Reantragoon, R.^{2,3}

¹King Chulalongkorn Memorial Hospital, Orthopedics, Bangkok, Thailand, ²Faculty of Medicine, Chulalongkorn University, Microbiology, Bangkok, Thailand, ³Faculty of Medicine, Chulalongkorn University, Center of Excellence in Immunology and Immune-Mediated Diseases, Bangkok, Thailand

Osteoarthritis is an age-related multifactorial disease

characterized by "joint failure" as pathologic changes of the disease can be detected in all joint components. A multitude of factors contribute to the pathogenesis and disease progression of osteoarthritis including immune mediators such as T cells. T cells have been shown to effect the progression of osteoarthritis in animal studies. However, their role in the development of osteoarthritis is yet unclear. Tissues of the joint that may partake in the development of osteoarthritis include the synovial tissue lining and more recently shown, the infrapatellar pad (Hoffa's pad), of which immune cells reside. Our aim was to investigate the role of T cells present in these joint tissues in the development of osteoarthritis. We therefore, isolated T cells from synovial tissue linings and the infrapatellar fat pad of patients undergoing total knee arthroplasty. Different composition of T cell subsets were observed at the local site of joint inflammation when compared to T cell subsets in the peripheral blood (of patient-matched). We also investigated expression of T cell receptors and other immune mediators that were present in the synovial tissue linings and infrapatellar fat pad and compared to those present in the systemic peripheral blood.

834**Long-term dynamics of individual T-cell receptor beta repertoires**

Britanova, O.V.^{1,2,3}, Shugay, M.^{1,2,3}, Merzlyak, E.M.¹, Staroverov, D.B.¹, Putintseva, E.V.^{1,4}, Turchaninova, M.A.^{1,2,3}, Mamedov, I.Z.^{1,3}, Pogorelyy, M.V.¹, Bolotin, D.A.^{1,2,3}, Izraelson, M.^{1,3}, Davydov, A.N.³, Egorov, E.S.^{1,2,3}, Kasatskaya, S.A.¹, Rebrikov, D.V.^{2,5}, Lukyanov, S.^{1,2}, Chudakov, D.M.^{1,2,3}

¹Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences, Moscow, Russian Federation, ²Pirogov Russian National Research Medical University, Moscow, Russian Federation, ³Central European Institute of Technology, Masaryk University, Brno, Czech Republic, ⁴Centre for Genomic Regulation, Barcelona, Spain, ⁵Vavilov Institute of General Genetics of Russian Academy of Sciences, Moscow, Russian Federation

The T-cell receptor repertoire diversity largely determine our ability to effectively withstand infections. Both thymic involution and skewed peripheral clonal selection events during aging lead to decrease of TCR diversity and limited T-cell immunity performance in the elderly. Here we implemented deep TCR beta repertoire sequencing with molecular barcoding to explore the long-term dynamics of T-cell immunity in healthy volunteers of all age groups (0-103 years old). The TCR diversity and publicity decreased in age groups from umbilical cord blood to aged individuals, whether private clonotypes expanded. We demonstrated unexpected repertoire stability: more than half of clonotypes detected in first timepoint persist and preserve observed high frequencies at timepoint 3 years later. Specifically, 880-960 of top 1000 most expanded clonotypes remained and underwent 1.8-2-fold expansion. We tracked behaviour of CMV- and EBV-specific TCR clonotypes in various age groups. While diversity of viral-specific clonotypes decreased with aging, average size of clones increased. Interestingly, we observed gender difference in aging-related repertoire skewing. Aging in 25-75 years range was related to more rapid shrinkage in naïve T cell pool in men than in women although the gender difference vanished in the oldest group (76-103 y.o.).

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Innate and adaptive immune defence molecules and cells of human milk

Trend, S.^{1,2,3}, Strunk, T.^{1,2,4}, Lloyd, M.L.^{1,5}, de Jong, E.^{1,2,6}, Hibbert, J.^{1,2}, Kok, C.H.^{1,4}, Metcalfe, J.², Geddes, D.T.⁷, Lai, C.T.⁷, Zhang, G.⁸, Kakulas, F.⁷, Doherty, D.A.⁹, Richmond, P.², Burgner, D.^{10,11}, Simmer, K.^{1,2,4}, Davidson, D.J.^{1,2}, Currie, A.J.^{1,2,6}

¹University of Western Australia, Centre for Neonatal Research and Education, Perth, Australia, ²University of Western Australia, School of Paediatrics and Child Health, Perth, Australia, ³Telethon Kids Institute, Centre for Health Research, Perth, Australia, ⁴King Edward Memorial Hospital, Neonatal Clinical Care Unit, Perth, Australia, ⁵University of Western Australia, School of Pathology and Laboratory Medicine, Perth, Australia, ⁶Murdoch University, School of Veterinary and Life Sciences, Perth, Australia, ⁷University of Western Australia, School of Chemistry and Biochemistry, Perth, Australia, ⁸Curtin University, School of Public Health, Perth, Australia, ⁹University of Western Australia, School of Women's and Infants' Health, Perth, Australia, ¹⁰Murdoch Childrens Research Institute, Parkville, Australia, ¹¹University of Melbourne, Melbourne, Australia, ¹²University of Edinburgh, MRC Centre for Inflammation Research, Queen's Medical Research Institute, Edinburgh, United Kingdom

Neonatal sepsis affects one-third of infants born very prematurely, and kills 320,000 infants worldwide annually. Lower human breast milk (BM) consumption is associated with sepsis but it is unclear whether preterm BM contains the same protective levels of immune factors as term BM. Supplementation of preterm BM with specific deficient immune factor/s may be a useful preventative strategy. We developed assays to measure and compare the levels of antimicrobial proteins and peptides (AMPs), leukocyte subsets, cytokines and soluble bacterial receptors in preterm and term BM. In BM from mothers of gestational age-matched pairs of preterm infants with and without sepsis, we investigated

(i) composition of BM at days 7 and 21 postpartum,
(ii) inhibitory effects of physiological levels of AMPs on bacterial growth, and

(iii) BM consumption in the first 28 days postpartum.

Contrary to our hypothesis, preterm birth was associated with higher concentrations of beta-defensin 1, lysozyme and soluble CD14 in BM. Infants with sepsis consumed less BM than their healthy counterparts from day two of life onwards. Lactoferrin (but no other tested AMPs) inhibited bacterial growth (>50%) at levels found in preterm BM. There was no effect of preterm birth on BM leukocyte concentrations, and only minor effects on frequencies of leukocyte subsets. These findings suggest that the prevalence of sepsis in preterm infants is not due to low concentrations of immune factors in BM, but may be related to low total consumption of BM. Increasing BM consumption and supplemental lactoferrin are potential therapeutic targets for future research.

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Early signaling changes with aging in human T cell subpopulations

Fulop, T.¹, Le Page, A.¹, Dupuis, G.², Larbi, A.³

¹Université de Sherbrooke, Medicine, Sherbrooke, Canada,

²Université de Sherbrooke, Biochemistry, Sherbrooke, Canada,

³Singapore Immunology Network (SigN), A*STAR, Singapore, Singapore

Aging is accompanied by many physiological changes and the immune system is not escaping this. This effect of time is affecting most cells of the immune system. Numerous changes have been described but most of them are phenomenological, a typical example is the change of phenotypes in T cells. We are interested since years to uncover the intracellular signaling associated with phenotypic and functional changes of various immune cells. We identified several signaling alterations starting from the early to the most downstream events in several cells like neutrophils, monocytes and T lymphocytes. We not only were interested in the forward signaling driven by kinases but also by the feedback or regulatory signaling mediated by phosphatases. We found profound alterations of regulatory phosphatases activity and phosphorylation (eg. SHP-1). The question was always whether these changes are occurring also uniformly in T cell or there are differences between the various subpopulations (naive to the continuum of memory T cells). Our studies indicate that there are changes according to the subpopulations and that basal hyper-phosphorylation status we observe may be explained by signaling crosstalks. We also observed that there is a resemblance between naive and TEMRA T cells, which differ much from CM and RM cells. In conclusion the different T cell subpopulations may have very specific changes distinguishing them from each other. This needs further investigations in relation to their functions.

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Monocytes from older men form foam cells more readily than those from younger individuals

Angelovich, T.^{1,2}, Shi, M.¹, Maisa, A.¹, Zhou, J.¹, Hearps, A.^{1,3}, Jaworowski, A.^{1,3,4}

¹Burnet Institute, Centre for Biomedical Research, Melbourne,

Australia, ²RMIT University, School of Applied Sciences, Melbourne,

Australia, ³Monash University, Department of Infectious Diseases,

Melbourne, Australia, ⁴Monash University, Department of Immunology, Melbourne, Australia

Aging is the strongest predictor of cardiovascular diseases such as atherosclerosis, which are leading causes of morbidity and mortality in older men. Monocytes migrate into artery walls at sites of inflammation and play an important role in atherosclerosis by forming foam cells (lipid-laden macrophages) and producing pro-atherogenic inflammatory cytokines (IL-6, TNF), which promote formation of atherosclerotic plaques. We have shown that inflammatory CD14⁺⁺CD16⁺ monocytes are expanded in older men and produce elevated levels of pro-atherogenic IL-6 and TNF; however, whether monocytes from older men are more atherogenic than those from younger individuals is unknown.

Using an *in vitro* model of monocyte transendothelial migration and foam cell formation, isolated monocytes from older men (median age [range]: 75 [58-85] yrs., n=20) formed foam cells more readily than those of younger individuals (32 [23-46] yrs., n=20) ($P < 0.001$) following migration across activated endothelial cells into a collagen matrix. Of the three subsets, inflammatory CD14⁺⁺CD16⁺ monocytes most readily formed foam cells ($P < 0.05$). Cholesterol efflux was impaired in monocytes from the elderly, associated with lower expression of the cholesterol transporter ABCA1 ($P=0.01$). Serum factors enhanced foam cell formation in older men; however, foam cell formation was not associated with altered serum lipid levels. We show that monocytes from older men have a higher propensity to form foam cells *in vitro* and conclude that they display higher atherogenic potential than those from younger individuals, which may contribute to increased risk of atherosclerosis in this population.

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Characteristics of age-related alteration of T cell repertoire, thymic recent output function and miRNAs

Xu, Y.¹, Xu, L.¹, Zeng, C.^{1,2}, Jin, Z.¹, Lu, S.², Yang, L.¹, Chen, S.¹, Li, B.¹, Li, Y.^{1,2,3}

¹Jinan University, Institute of Hematology, Guangzhou, China,

²Jinan University, Key Laboratory for Regenerative Medicine of Ministry of Education, Guangzhou, China, ³Jinan University, Department of Hematology, First Affiliated Hospital, Guangzhou, China

The expression of miR-17, miR-92a and miR-181a, the distribution pattern of the TCR repertoires, and the level of thymic recent output function (sjTRECs) were detected in PBMCs from 160 healthy individuals (HI) with different age (0 to 70 years old) and 20 case of cord blood mononuclear cells (CBMCs). The results showed that the expression levels of miR-17, miR-92a and miR-181a in PBMCs were down-regulated with aging. Compared with 0 ~ 9 ys HI group, miR-92a and miR-181a upregulated in the CBMCs, while miR-17 downregulated. The sjTRECs level in the PBMCs was inversely correlated with age, there was a largest decline of sjTRECs between 0~9 and 10~19 ys group. TCR V β 1, V β 2, V β 4~V β 11, V β 14~V β 16, V β 18, V β 20, V β 21 and V β 23 were expressed in 100%, while the remained V β families were absent in different frequency, the lowest expression frequency detected in V β 13 and V β 24 subfamily (both were 91.25%); polyclonality of TCR was the most common pattern in all the V β subfamilies, while oligoclonality was relatively high expressed in V β 6 and V β 23 (23.75%); the expression frequency of V β subfamilies were descend but the oligoclonal tendency were increased with aging. Similarly, the frequencies of TCR V γ and V δ were decreased by the increase of age, while oligoclonal proliferation tendency had no significant correlations with the ages, the distribution pattern of V δ was different from V β subfamilies, lower frequencies were found in V δ 5 and V δ 7 (30% and 18.75%). In conclusions, the study gives a more comprehensive description of the characteristics of the T cell immunosenescence.

Antigen Processing & Presentation

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Homozygosity for the HLA-DRB1*15 allele is associated with a responder profile in RhD-immunised blood donors

Tan, J.C.G.¹, Yuan, F.F.², Daley, J.³, Marks, K.⁴, Flower, R.L.⁵, Dyer, W.B.¹

¹Australian Red Cross Blood Service, Research and Development, Alexandria, Australia, ²University of Sydney, Faculty of Medicine, Camperdown, Australia, ³Australian Red Cross Blood Service, Manufacturing, Alexandria, Australia, ⁴University of New South Wales / Kirby Institute, Kensington, Australia, ⁵Australian Red Cross Blood Service, Research and Development, Kelvin Grove, Australia

Cases of haemolytic disease of the newborn have dropped significantly since the successful administration of prophylactic anti-D immunoglobulin (Ig) to susceptible RhD-negative pregnant women. Our Blood Service conducts an Anti-D Program to actively immunise selected RhD-negative blood donors with small volumes of RhD-positive red blood cells to stimulate anti-D Ig production. Donors (n=431) were considered Responders if serum anti-D Ig concentrations >1 IU/mL were recorded. However, 39.2% were Non-Responders and did not produce any anti-D Ig despite multiple exposures. We sought to examine donor genetic factors associated with antigen presentation and to identify genetic characteristics that could be utilised to identify Responders prior to immunisation. We found that female donors were more likely to be Responders ($p < 0.0001$). DNA was extracted from a subset of donors (n=272) and genotyped by restriction fragment length polymorphism-polymerase chain reaction for polymorphisms in genes that play an important role in antigen presentation and pathogen recognition (TLR2, TLR4, CD14 and Fc γ RIIA), although no statistically significant association was identified. Further work examined the association between Responders and the HLA-DRB1 allele, and revealed that donors with DRB1*15 allele were significantly associated with a Responder profile ($p=0.049$). High-resolution typing of donors with DRB1*15 alleles (n=78) demonstrated that 90% possessing two DRB1*15 alleles and 73.5% possessing one DRB1*15 allele had a Responder profile. This study has identified a potentially useful genetic characteristic (HLA-DRB1*15 allele) that could be used to screen new donors prior to entry into the Blood Service Anti-D Program.

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Biased T cell receptor usage by HLA-A*02-restricted CD8 T-cells specific for the yellow fever viral peptide NS4b₂₁₂₋₂₂₂

Bovay, A.^{1,2}, Fuertes Marraco, S.A.¹, Bulek, A.M.², Dolton, G.², Sewell, A.K.², Speiser, D.E.¹

¹Ludwig Cancer Research Center, Department of Oncology, University of Lausanne, Epalinges, Switzerland, ²Cardiff University School of Medicine, Cardiff, United Kingdom

The Yellow Fever vaccine YF-17D induces a dominant memory CD8 T-cell response against the HLA-A*02-restricted NS4b212-222 peptide LLWNGPMAV (hereafter, A2/LLW) that persists for at least 25 years. Curiously, naïve A2/LLW-specific CD8 T-cells are also extraordinarily frequent in unvaccinated donors. The only other system that enables direct visualisation of sizeable

populations of antigen-specific human naïve CD8 T-cells is the HLA-A*02-restricted melanoma derived epitope Melan-A/MART1. The high frequency of naïve Melan-A-specific CD8 T-cells arises in part as recognition of the cognate peptide EAAGIGILTV (A2/EAA) that occurs via contacts with amino acids in the germline encoded CDR1 loop of the TRAV12-2 gene utilised by most A2/EAA-specific T-cells. Major V α biases have also been observed for recognition of metabolites in the context of MR1 and in CD1d- α -galactocerimide. We examined the TCR chain usage of A2/LLW-specific CD8 T-cells in YF-17D-vaccinated individuals and naïve cells from unvaccinated donors and found that a single V α gene dominates both of these populations. Our work extends observations on V α -biased recognition of unconventional T-cell epitopes and melanoma-related A2/EAA to the recognition of viral-derived peptide epitopes. Ongoing structural and biophysical analyses of TCR-A2/LLW interactions are expected to reveal the underlying molecular reasons for this α -chain bias in A2/LLW-specific T-cell populations and to provide a potential mechanistic basis for the extraordinary frequency and immunodominance of T-cells with this specificity.

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Study of viral antigen processing and presentation by MHC class II using recombinant antibodies that mimic T-cell receptor specificity

Fridman, A., Reiter, Y.

Technion, Biology, Haifa, Israel

Influenza hemagglutinin (HA) is an antigenic glycoprotein found on the surface of the Influenza virus. By binding to the sialic acid on the cell surface, HA protein allows the Influenza virus to attach host cells. After vaccination with inactivated Influenza A virus (A/Aichi/2/1968-H3N2 strain), one can find elevated frequency of T cells directed towards Influenza HA antigen, residues 306-318, in immunized HLA-DR4 positive subjects. Based on these observations, we hypothesize that T Cell Receptor-Like (TCRL) recombinant Abs (rAbs) against the HLA-DR4/HA-306-318 viral epitope can be of high significance for the study of antigen processing and presentation on APCs during Influenza infection.

TCRL rAb against recombinant HLA-DR4/HA-306-318 complexes was isolated using a phage display approach. This TCRL, termed H1, was characterized in detail to demonstrate its fine TCR-like specificity. H1 exhibited specific staining of DR4+ APCs loaded with HA-306-318 peptide, compared to cells loaded with controlled peptides, and successfully compete with the cognate interaction of HA-306-318 specific DR4 restricted T cell hybridoma with HLA-DR4/HA-306-318 epitopes presented by APCs. Moreover, H1 demonstrated a positive binding towards F4/80+ cells derived from splenocytes of HA-immunized DR4 transgenic mice.

These results are a major step in the study of HLA-DR4/HA-306-318 epitope presentation during Influenza infection using H1 TCRL. This study will enable us for the first time to use direct visualization and quantitation tools to study the process of antigen processing and presentation of a native T cell epitope during the natural course of viral infection in the context of an animal model.

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Functional analysis of Immunodominant CD8+ T cell responses against unpredicted epitopes from Influenza virus

Di Carluccio, A., Chen, W.

La Trobe Institute for Molecular Science, Melbourne, Australia

CD8+ T cells recognise antigenic peptides of 8-12 amino acids which are presented by MHC-I molecules on the surface of infected cells. We are investigating the antigen processing and presentation characteristics of recently discovered Immunodominant CD8+ T cell epitopes derived from Influenza virus Nucleoprotein (NP). Interestingly, the selected epitopes are of varying lengths; including an 8mer (NP 219-226), 10mer (NP 172-181) and 12mer (NP 145-156). None of these selected epitopes were predicted by the online prediction programs IEDB (www.iedb.org/), NetMHC 4.0 (www.cbs.dtu.dk/services/NetMHC/) and SYFPEITHI (www.syfpeithi.de/), but were in fact Immunodominant in their respective donors. Each of these sites generated a different ranking order or 'score' of NP peptides most likely to bind to the HLA-ABC molecules from each individual donor. The ranking is based on the amino acids within the HLA binding groove, and the positions of potential binding residues within the NP protein, they also use published epitope anchors. To understand why these epitopes are not predicted, we characterised their antigen processing and presentation characteristics by performing functional kinetics of the CD8+ T cell response to infection, and confirmed the minimum epitope length by HPLC co-elution. HPLC fractions of the naturally presented NP 219-226 have also uncovered potential post translational modification of the epitope, which requires further investigation. It is still unclear which amino acids contribute to MHC-I binding in the ER during infection. Peptide-HLA binding assays will allow us to characterise any common rules associated with these Immunodominant epitopes, which could lead to improvement of the prediction programs.

843

QS-21 enhances cross-presentation of NY-ESO-1 by monocyte-derived dendritic cells *in vitro*

Pasam, A.^{1,2,3}, Woods, K.^{1,2,3}, Knights, A.², Gerard, C.⁴, Puaux, A.-L.⁴, Morel, S.⁴, Louahed, J.⁴, Cebon, J.^{1,2,3}

¹Olivia Newton-John Cancer Research Institute, Heidelberg, Australia, ²Ludwig Institute for Cancer Research, Olivia Newton-John Cancer and Wellness Centre, Austin Hospital, Heidelberg, Australia, ³La Trobe University, School of Cancer Medicine, Bundoora, Australia, ⁴GlaxoSmithKline Biologicals S.A., Rixensart, Belgium

Cancer-testis antigens serve as an attractive target for therapeutic cancer vaccines; based on their highly-restricted expression profile and also their frequent immunogenicity in cancer patients. NY-ESO-1 is an immunogenic cancer-testis antigen that has been extensively studied as a target for immunotherapeutic cancer vaccination. Dendritic cells (DC) take up and cross-present antigen to T cells, leading to their activation and stimulation of an immune response *in vivo*. Cross-presentation by antigen presenting cells, such as DC,

is therefore an essential element of any immunotherapeutic treatment. Several studies have described strategies to enhance antigen cross-presentation by DC. QS-21* is a naturally occurring saponin molecule which enhances T cell responses and is in current use as an immunostimulant in several clinical trials. We assessed the impact of QS-21 on the cross-presentation of three NY-ESO-1 epitopes by monocyte derived DC *in vitro*. In all cases, presence of QS-21 at the time of antigen delivery resulted in significantly enhanced cross-presentation. Strikingly, NY-ESO-1 epitopes could be cross-presented when delivered as a soluble protein only in the presence of QS-21, which suggests a novel role for QS-21 as a component of an immunotherapeutic vaccine.

Understanding these effects will be critical for evaluating the role of QS-21 as an immunostimulant, in addition to optimizing future immunotherapeutic cancer vaccine formulations.

**Quillaja saponaria* Molina, fraction 21 (Antigenics Inc, a wholly owned subsidiary of Agenus Inc., Lexington, MA, USA)

Funding source: GlaxoSmithKline Biologicals SA.

845

Immunoproteasome subunit LMP2 is crucial for lymphocyte survival and is a pro-survival factor for human multiple myeloma

Zanker, D.¹, Pang, K.², Oveissi, S.¹, Lu, C.¹, Neeson, P.³, Puthalakath, H.¹, Chen, W.¹

¹La Trobe Institute for Molecular Science, Bundoora, Australia,

²Walter & Eliza Hall Institute, Parkville, Australia, ³Peter MacCallum Cancer Centre, East Melbourne, Australia

Immunoproteasomes are specialized protein degradation machinery that are proposed to enhance immune responses due to altered cleavage motifs and increased processing rates compared to housekeeping proteasomes. Immunoproteasomes are constitutively expressed by antigen presenting cells such as dendritic cells and cytokines including IFN-gamma induce expression of LMP2 ($\beta 1_i$), LMP7 ($\beta 5_i$) and LMP10 ($\beta 2_i$) catalytic β -subunits that differentiate these two proteasome forms. Interestingly, investigation into immunoproteasome single-subunit deficient strains found LMP2^{-/-} mice exhibit dramatic decreases in all lymphocyte subsets. Using bone marrow chimeric mice, we have shown this not from thymic selection, but an intrinsic defect. We identified that lymphocytes die at various stages of development and in the periphery associated with antigen receptor stimulation or stress. Surprisingly, we identified immunoproteasomes in lymphocytes and this expression is upregulated following antigen-receptor stimulus. We postulated that LMP2 has a housekeeping role and is crucial for cleavage of particular proteins for either degradation or activation of function. Using mass spectrometry and RNAseq approaches, we identified cell-wide protein dysregulation with ~15% of proteins significantly changed. Changes in metabolic pathways, the NF κ B pathway and apoptosis pathways lead to a pro-apoptotic phenotype in LMP2^{-/-} lymphocytes. In vitro analysis found decreased or no degradation of specific pro-apoptotic proteins in LMP2^{-/-} MEFs. It is reported that various cancers over-express LMP2. To investigate whether LMP2 enhances cancer survival, using shRNA knock-down of LMP2 in various human multiple myeloma cell lines we found increased

apoptosis, decreased proliferation and increased sensitivity to chemotherapeutic drugs. We believe LMP2 is a viable anti-cancer therapeutic target.

45 Minute Oral

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MHCII with full peptide occupancy is a substrate for HLA-DM catalyzed peptide exchange

Reyes-Vargas, E.¹, Barker, A.P.^{1,2}, Zhou, Z.¹, He, X.¹, Jensen, P.E.^{1,2}

¹University of Utah, Pathology, Salt Lake City, United States, ²ARUP Laboratories, Pathology, Salt Lake City, United States

The major histocompatibility complex class II (MHCII) antigen-processing pathway is critical for adaptive immunity as the mechanism through which ligands are generated for recognition by CD4 T-cells. HLA-DM edits the repertoire of peptides that are presented to T-cells by catalyzing multiple rounds of peptide exchange on MHCII molecules. The catalytic potency of DM varies such that peptide exchange is markedly enhanced on some peptide-MHCII complexes and not others. This capacity to differentially edit complexes influences specificity and immunodominance in T-cell responses. The catalytic mechanism and rules that govern DM susceptibility are not fully understood. Evidence suggests that partial occupancy at the N-terminus of bound peptides is critical for peptide-MHCII complex sensitivity to HLA-DM. We employed a fluorescence polarization assay to independently assess the effective affinity and catalytic turnover, components in the catalytic mechanism of DM for peptide-MHCII complexes, in real-time kinetic measurements of DM activity. We found that complexes with an N-terminal truncated form of the immunodominant HA peptide, resulting in the loss of three H-bonds, showed an expected decrease in complex stability and increase in affinity for DM binding, but an unexpected increase in catalytic turnover when compared to the unmodified HA-MHCII complex. This result was even more pronounced when a conserved bidentate H-bonding residue near the P1 anchor of the HA-MHCII complex was disrupted by site-directed mutagenesis. Together, this implies that peptide-MHCII interactions near the N-terminal segment of a peptide are intact during the functionally active DM-peptide-MHCII catalytic complex. These results provide insight into the DM catalytic mechanism.

Oral Sessions

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EBV infection affects the processing of myelin oligodendrocyte glycoprotein in B cells - Implications for multiple sclerosis

Morandi, E.¹, Jagessar, A.², Gijon, M.³, 't Hart, B.², Gran, B.¹

¹University of Nottingham, Clinical Neurology Research Group, Division of Clinical Neuroscience, School of Medicine, QMC,

Nottingham, United Kingdom, ²Biomedical Primate Research Centre (BPRC), Immunobiology Department, Rijswijk, Netherlands,

³University of Nottingham, Academic Division of Clinical Oncology, School of Medicine, Nottingham, United Kingdom

Multiple Sclerosis (MS) is a neurodegenerative disease with an immune-mediated pathogenesis in which Epstein Barr Virus (EBV) is a risk factor. Our hypothesis is that EBV infection renders B cells potent antigen presenting cells (APC) for autoreactive T cells, through influences on intracellular proteolysis and post-translational modifications of self antigens. The aim of this study is to assess the effect of EBV infection in B cells on processing of myelin oligodendrocyte glycoprotein (MOG). We investigated the processing of MOG through SDS PAGE gel analysis in the presence or absence of cathepsin inhibitors and we determined the proteolytic activity of Cathepsin G by activity assay. We also studied the effect of citrullination of the immunodominant MOG₃₅₋₅₅ peptide in position 41 and 46. In addition, we measured the expression of peptidylarginine deiminase 2 by RT-PCR and LC3ii, an autophagy marker, by Western Blotting, in EBV infected and uninfected cells.

We found that EBV infection increases the activity of Cathepsin G, leading to increased degradation of the peptide MOG₃₅₋₅₅ by B cells. However, infection also rescues the whole extracellular rhMOG from total degradation. By contrast, inhibition of Cathepsin G or citrullination of Arg 46 abrogated the degradation of MOG₃₅₋₅₅.

We propose a model in which EBV infection induces autophagocytosis in B cells and activation of PAD which mediates citrullination of Arg. Citrullinated MOG₃₅₋₅₅ is protected from degradation and can be presented to T cells. This mechanism could facilitate presentation of a disease-relevant myelin autoantigen that may be involved in MS induction and progression.

848

VCG modulate innate and adaptive immune responses to vaccine antigens

Eko, F.O.¹, Russell, R.¹, He, Q.¹, Black, C.M.², Igiertseme, J.U.^{1,2}

¹Morehouse School of Medicine, Atlanta, United States, ²Centers for Disease Control and Prevention, Atlanta, United States

Vaccination strategies utilizing subunit antigens often rely on the incorporation of effective adjuvants to modulate immune responses. *Vibrio cholerae* ghosts (VCGs; genetically derived empty *V. cholerae* cell envelopes) constitute an effective delivery system that promotes the induction of protective immunity in the absence of external adjuvants. However, the mechanism by which VCGs enhance immunity has not been elucidated. We hypothesized that the immunostimulatory ability of VCGs involves dendritic cell (DC) activation and function. Thus, we investigated the ability of DCs to internalize VCGs and the immunomodulatory effect of internalized VCGs on the induction of innate immune responses, T cell proliferation, antigen presentation and enhancement of protective immunity. The results showed that VCGs were efficiently internalized by DCs without affecting their viability and modulated DC-mediated immune responses. VCG-pulsed DCs showed increased secretion of proinflammatory cytokines and expression of co-stimulatory molecules associated with DC maturation in response to stimulation with UV-irradiated *Chlamydia muridarum* elementary bodies (UV-EBs). This interaction resulted in effective chlamydial antigen presentation and enhancement

of protective immunity. The study demonstrated that VCGs activate the maturation of DCs leading to enhancement of innate and adaptive immunity to a co-delivered antigen. Thus, VCGs could be utilized as vaccine adjuvants for enhancement of protective immunity against microbial infections.

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March8 regulates thymic epithelial cell MHC II trafficking and CD4+ T cell development

Liu, H.¹, Gray, D.², Johnston, A.³, Villadangos, J.^{1,4}, Mintern, J.¹

¹University of Melbourne, Biochemistry and Molecular Biology, Melbourne, Australia, ²The Walter and Eliza Hall Institute of Medical Research, Molecular Genetics of Cancer Division and Immunology Division, Melbourne, Australia, ³Monash University, Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Melbourne, Australia, ⁴University of Melbourne, Department of Microbiology and Immunology, Melbourne, Australia

The development and activation of T cells depends on recognition of peptide:MHC II complexes displayed on the surface of antigen presenting cells, whose expression levels are tightly controlled. In dendritic cells and B cells, the E3 ligase Membrane Associated RING-CH 1 (MARCH1) downregulates MHC II via ubiquitination. MHC II is stabilized, on the other hand, by the immunoglobulin CD83, which is proposed to sequester MARCH1. CD83-deficient mice exhibit lower surface MHC II on DCs, B cells and thymic epithelial cells (TECs), and strongly reduced CD4+ T cell numbers. Interestingly, TECs do not express MARCH1, and TECs from MARCH1^{-/-} mice have normal MHC II levels, suggesting MARCH1 is not involved in MHC II regulation in TECs.

We now have identified MARCH8 as the E3 ligase controlling MHC II surface expression in TECs, particular in cortical TEC and AIRE- medullary TEC. TECs from MARCH8^{-/-} mice exhibit ~4-fold increased MHC II surface levels compared to wild type, while MHC II levels on DCs and B cells remain unchanged. Importantly, by deleting MARCH8 in CD83-deficient mice we restored CD4+ T cell numbers to wild type levels, implying that MARCH8, and not MARCH1, ubiquitinates MHC II in TECs.

Our results reveal a novel role for MARCH8 controlling surface MHC II in TECs in a physiological setting, and suggest that limiting MARCH8 activity is critical for the development of CD4+ T cells. Furthermore, our findings suggest that hematopoietic and non-hematopoietic antigen presenting cells employ different MARCH family ligases to regulate MHC II trafficking.

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Immuno-proteomic interrogation of antigen presentation during dengue infection reveals novel and HLA haplotype-specific MHC-I antigens

Swaminathan, K.¹, Lund, P.², Olsson, N.¹, Marceau, C.², Carette, J.², Davis, M.², Elias, J.¹

¹Stanford University, Chemical & Systems Biology, Stanford, United States, ²Stanford University, Microbiology & Immunology, Stanford, United States

Dengue (DEN) virus infection is a leading cause of hospitalization and death in the developing world and broadly effective vaccines or therapies remain elusive. Identifying infection-specific peptide antigens would open new avenues for developing T cell based interventions. Past efforts towards mapping viral antigens used computational predictions that only partially reflect actual antigens presented by the major histocompatibility complex (MHC). To identify DEN-specific antigens without relying on error prone predictions, we developed an immuno-proteomics approach for interrogating antigen presentation in DEN infected B-lymphocytes. This approach enabled three fundamental findings; First, we identified 86 viral MHC-I antigens (including 59 novel ones), and mapped them to presentation hotspots in the DEN genome. Second, we discovered post-translationally modified viral and host antigens and antigens derived from alternate reading frames. Predicting these antigens using computational methods alone would be infeasible. Third, we found antigens responsible for HLA-haplotype dependent immune responses against DEN infection: we genetically engineered B-lymphocytes to express HLA alleles associated with either strong or weak DEN-immune responses and identified corresponding allele specific antigens. Together these discoveries let us assay the immunogenic potentials of an unprecedented range of DEN-specific antigens. *Ex vivo* assays including ELISPOT and HLA tetramer staining supported our identification of immunogenic antigens in DEN-specific CD8⁺ T cells. These antigens have potential as both diagnostic tools to characterize DEN-specific T cell immunity, and serve as candidates for designing effective DEN vaccines.

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Studies on the binding affinities of *Yersinia pseudotuberculosis*-derived mitogen to MHC class II and TCR V β molecules

Kato, H.¹, Nunomura, W.², Takakuwa, Y.³, Uchiyama, T.¹, Imanishi, K.⁴

¹Tokyo Women's Medical University School of Medicine,

Department of Microbiology and Immunology, Tokyo, Japan,

²Akita University, Graduate School of Engineering and Resource Science Research Center for Engineering Science, Akita, Japan,

³Tokyo Women's Medical University School of Medicine, Department of Biochemistry, Tokyo, Japan, ⁴Japan University of Health Sciences, School of Health Sciences, Saitama, Japan

Yersinia pseudotuberculosis-derived mitogen (YPM) is a virulence factor of *Y. pseudotuberculosis* and a unique superantigen (SAG), which is identified from Gram-negative bacteria and has a smaller MW than other SAGs such as staphylococcal enterotoxins (SEs). The molecular basis of action of SAGs involves the binding of SAG to MHC class II and TCR V β molecules. It is known that YPM activates murine V β 7⁺ and V β 8⁺ T cells and that the tertiary structure of YPM is available. But little is known about the binding affinities of YPM to MHC class II and TCR V β molecules. In this paper, we compared two SAGs - YPM and SEA - with respect to their superantigenic activities and their binding affinities to MHC class II and TCR V β molecules. Their superantigenic activities in mice revealed a remarkable difference between them. Superantigenic activity of YPM is significantly lower than that of SEA *in vitro* and *in vivo*.

The binding affinity between SEA and TCR V β 3 molecules is nearly equal to that between YPM and TCR V β 7, while the affinity between SEA and I-A^b is 15 times stronger than that between YPM and I-A^b. These data suggest that the superantigenic activity seems to be highly associated with the binding affinity between SAG and MHC class II molecules. The difference in superantigenic activities between SEA and YPM is from 100 to 1000 times, while the difference of affinity between SEA or YPM and I-A^b is only around ten times. We will discuss this difference.

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NLRC5/CITA is a target for immune evasion of cancer

Kobayashi, K.

Texas A&M Health Science Center, Department of Microbial Pathogenesis and Immunology, College Station, United States

Cancer develops under immune surveillance, thus necessitating immune escape for successful growth. Loss of MHC class I provides a key immune evasion strategy in many cancers, although the mechanisms remain elusive. MHC-I transactivator (CITA)/NLRC5 has recently been identified as a master transcriptional co-activator of MHC-I genes. Here we show that the MHC-I transactivation pathway mediated by CITA/NLRC5 constitutes a major target for cancer immune evasion. *NLRC5* expression was highly correlated with the expression of MHC-I, cytotoxic T cell markers, and genes in the MHC-I pathway including *LMP2/LMP7*, *TAP1* and β 2-microglobulin in all 21 tumor types we examined. Epigenetic/genetic changes including promoter methylation and copy number loss were highly prevalent in the *NLRC5* gene, resulting in the impaired MHC-I pathway and evasion from CD8⁺ cytotoxic T cells. Strikingly, *NLRC5* expression was significantly associated with cancer patient survival. Thus, *NLRC5* constitutes a novel prognostic biomarker and potential therapeutic target of cancers.

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Immune modulation of Langerhans cells by microparticles from human papillomavirus type 16 E7 expressing keratinocytes

Zhang, T.¹, Burn, C.², Ly, K.¹, Young, K.¹, Wilson, M.^{1,2}, Braithwaite, A.¹, Baird, M.¹, Hibma, M.¹

¹University of Otago, Pathology, Dunedin, New Zealand, ²University of Otago, Microbiology and Immunology, Dunedin, New Zealand

Persistent infection with human papillomavirus type 16 (HPV-16) is a major risk factor for cervical cancer in females. The HPV-16 E7 oncoprotein is expressed in infected host keratinocytes and may contribute to viral persistence by suppressing the host immune response. Microparticles (MP) are small membrane fragments released by cells, that have been implicated in modulation of local immunity. The aim of this study is to determine the immunomodulatory effects of MP from HPV16 E7 expressing cells on Langerhans cells (LC).

MP were purified from control and an HPV16 E7 oncoprotein-expressing mouse keratinocyte cell line. Langerhans cell-like cells (LCLC) were differentiated from murine bone marrow derived cells and co-cultured with HPV16 E7 or control MPs

for 48h. Co-stimulatory molecule expression and cytokine production of LCLC was evaluated by flow cytometry analysis, and LCLC priming of CD8 T cells for antigen-specific cytotoxicity was investigated using an in vitro cytotoxic T lymphocyte (CTL) killing assay. Compared to cells incubated with control MP, HPV16 E7 MP-treated LCLC expressed significantly less CD40 and IL-12 following LPS-stimulation. Moreover, there was significantly reduced CTL killing in HPV-16E7 MP treated group compared with control MP treated cells.

MP from HPV16 E7-expressing cells therefore impair activation, cytokine secretion and the ability of LCLCs to prime an effective CTL response. This supports viral regulation of the local immune environment by E7 and MP in favor of tolerance, potentially contributing to viral persistence.

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Role of melanoma apoptotic vesicles in tumour immunity and coagulation

Muhsin-Sharafaldine, M.-R., Saunderson, S.C., Dunn, A.C., McLellan, A.D.

University of Otago, Microbiology & Immunology, Dunedin, New Zealand

During chemotherapy, tumour cells release apoptotic vesicles (ApoV) as part of programmed cell death pathways. Subcutaneous immunisation with 25 µg of ApoV prior to challenge (at day seven) protected mice from B16 tumour for up to 60 days. Furthermore, ApoV protection was significantly higher than that induced by extracellular vesicles released from living tumour cells (exosomes and microvesicles). We detected only trace amounts of the OVA antigen (< 1 µg/ dose) in OVA-pulsed ApoV and the immunisation of molar equivalents amounts of soluble OVA or latex bead bound did not induce a detectable cytotoxic immune response, demonstrating a role for ApoV as natural adjuvants for tumour antigens. Finally, in addition to their intrinsic immunogenicity, ApoV were found to be significantly more procoagulant than parental tumour cells, or other extracellular vesicles (exosomes and microvesicles). The procoagulant activity was dependent on tissue factor expression and phosphatidylserine exposure at the vesicle surface. Given that thrombotic events are the second highest cause of death in cancer patients, these results also highlight a potential role for ApoV in coagulopathies. Together, these results emphasise the complexities of interaction between the host and tumour vesicles, and suggest an important role for ApoV released during chemotherapy in immunity, as well as in cancer / chemotherapy-related thrombosis.

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Non-canonical MHC-I peptide binding enables a novel mechanism of HIV-1 escape

Pymm, P.¹, Illing, P.¹, Ramarathinam, S.¹, Hughes, V.¹, McLaren, J.², Sullivan, L.³, Price, D.², Brooks, A.³, Purcell, A.¹, Rossjohn, J.^{1,2}, Vivian, J.¹

¹Monash University, Biochemistry, Melbourne, Australia,

²Cardiff University School of Medicine, Cardiff, United Kingdom,

³Melbourne University, Melbourne, Australia

MHC-I molecules play a crucial role in adaptive and innate immunity by binding endogenous peptides from the cell and presenting them to CD8+ T-cells and natural killer (NK) cells for immune surveillance. In MHC-I the peptide-binding groove is closed at both ends, with the presented peptides being tethered in the groove at their N and C- termini. MHC-1 presented peptides extending C-terminally beyond the binding groove have occasionally been reported, although the extent to which other modes of peptide presentation occur is unknown. Analysis of the peptides eluted from HLA-B*57:01 revealed that 20% of the peptide repertoire formed N-terminally extended sets, in which multiple peptides are eluted that contain an identical "minimal motif" of at least nine amino acid residues. These nested sets shared a common motif at P2-P1. Crystal structures of HLA-B*57:01 presenting N-terminally extended peptides, including the well-characterised immunodominant HIV-1 epitope TSTLQEQIGW (TW10), showed that the N-terminus protruded out from the peptide-binding groove in a conserved manner. Notably, the common escape mutant TSNLQEQIGW bound HLA-B*57:01 in the canonical fashion without extension from the binding groove, therefore giving dramatically different peptide presentation compared with the wild-type epitope. Accordingly, this HIV-1 escape mutant causes a register shift of the TW10 epitope to enable immune evasion from T-cells. However, this shifted register also impacted on recognition by the inhibitory KIR3DL1 receptors expressed on NK cells. Collectively we define a previously uncharacterised feature of the HLA-I immunopeptidome with important implications for viral escape.

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The flanking region of cedar pollen peptide in complex with HLA-DP5 facilitates T-cell activation

Kusano, S.¹, Ueda, S.², Hamana, H.³, Kishi, H.³, Ohsawa, N.⁴, Wakiyama, M.⁴, Muraguchi, A.³, Yoshikai, Y.², Yamada, H.², Yamamoto, K.², Kukimoto-Niino, M.⁴, Nishimura, Y.⁵, Shirouzu, M.⁴, Sasazuki, T.², Yokoyama, S.¹

¹RIKEN Structural Biology Laboratory, Yokohama, Japan, ²Kyushu University, Fukuoka, Japan, ³University of Toyama, Toyama, Japan,

⁴RIKEN Center for Life Science Technologies, Yokohama, Japan,

⁵Kumamoto University, Kumamoto, Japan

Cryptomeria japonica (Cry j, Japanese cedar) 1, one of the major cedar pollen allergens, causes cedar pollinosis as a type I allergy. Our previous analysis of the association between cedar pollinosis and HLA class II alleles revealed that the frequency of HLA-DP5 is significantly higher in the pollinosis patients, and we identified an immunodominant peptide derived from Cry j 1 that induces Th2 restricted by HLA-DP5. Subsequently, we reported the crystal structure of HLA-DP5 (DPA1*02:02 and DPB1*05:01) in complex with a nine-residue antigenic peptide from Cry j 1 [1]. The 9 amino acid residues were accommodated by the peptide-binding groove, and thus the mechanism of the specific receptor antigen binding was established.

In this study, we identified the unique sequence located on the flanking region (FR) of the antigenic peptide associated with HLA-DP5. Based on this information, mutational analyses (point mutants and deletion mutants) for the FR of the Cry j 1

peptide (pCry j 1) were performed, using B cell-lines derived from the peripheral blood of pollinosis patients and TG40 cells expressing T-cell receptor restricted by the HLA-DP5-pCry j 1(FR) complex. Intriguingly, strong T-cell activation involving IL-2 production was observed when the 'distal' FR was included in the peptide. Therefore, the distal FR is responsible for the immune response. To elucidate the molecular mechanisms underlying this unconventional mode of the immune response, we solved the crystal structure of HLA-DP5 in complex with a pCry j 1 including the 'distal' FR.

[1] Kusano, S. *et al. J. Mol. Biol.* (2014)

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The spatial arrangement and mobility of molecules in the immunological synapse participate in regulation of the early immune response

Lin, W.^{1,2}, Suo, Y.³, Deng, Y.², Fan, Z.³, Qin, L.¹, Jiang, G.¹, Wei, X.³, Chu, Y.²

¹Shandong Academy of Medical Sciences, Jinan, China, ²Fudan University, Shanghai Medical College, Biotherapy Research Center of Fudan University, Shanghai, China, ³Shanghai Jiao Tong University, Med-X Research Institute and School of Biomedical Engineering, Shanghai, China

Immune regulation is the ability of the immune system to control and regulate its own responses. It plays an important role in properly balanced immune responses and this role is thought to be performed mostly by regulatory cells, inhibitory cytokines or inhibitory molecules. Are there any other factors involved in the regulation of the immune response? By using live-cell imaging, we found the localization and mobility of molecules in the immunological synapse (IS) between CD4⁺ T cells and Dendritic cells (DCs) participated in the regulation of early immune response:

(1) Cytotoxic T-lymphocyte antigen-4 (CTLA-4) in the IS promoted a suppressive synapse formation, which correlated with the low level of calcium releasing in T cell and the loss of the localization of TCR signaling molecules in the IS.

(2) The accumulation of the filamentous actin (F-actin) cytoskeleton in the IS regulated the calcium releasing in T cells by controlling the localization of the TCR signaling molecules and the calcium microdomains, such as ORAI1, PMCA and mitochondrion. Lack of adequate F-actin in the IS resulted in ineffective T-cell activation.

(3) The mobility of ICAM-1 in the IS formation participated in regulation of the calcium releasing in T cell. Loss of exclusion of ICAM-1 from the central of IS, could not induce the calcium releasing in T cell. Overall, the mobility and the localization of the molecules in the synapse formation participate in the regulation of early immune response. IS may play a regulatory role in balance of the early T-cell activation.

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TCR-HLA interactions implicate vimentin as a candidate autoantigen in pulmonary sarcoidosis

Kaiser, Y.¹, Dubnovitsky, A.², Sandalova, T.², Eklund, A.¹, Achour, A.², Grunewald, J.¹

¹Karolinska Institutet, Department of Medicine, Solna, Stockholm, Sweden, ²Science for Life Laboratory, Karolinska Institutet, Department of Medicine, Solna, Stockholm, Sweden

Sarcoidosis is a granulomatous disorder of unknown aetiology, characterised by accumulations of activated CD4⁺ T cells in the lungs. HLA variant DRB1*03 is closely associated with acute onset, distinct clinical features, rapid disease resolution and a good prognosis. In bronchoalveolar lavage fluid (BALF), but not blood, of HLA-DRB1*03⁺ patients, we identified increased frequencies of differentiated CD4⁺ T cells expressing T cell receptor (TCR) V genes Va2.3 and Vβ22. Sequencing of the TCR chain CDR3 region revealed a high degree of clonality, indicating expansion following antigen recognition. Molecular modelling of this particular HLA-TCR complex showed an ideal fit of a peptide derived from cytoskeletal protein vimentin into the peptide-binding cleft, implicating vimentin as a candidate autoantigen. Consequently, the aim of this project was to functionally characterise the antigen specificity of Va2.3⁺Vβ22⁺ T cells and the immunogenic potential of vimentin. We here show that anti-vimentin IgG and IgA antibodies can be detected in serum and BALF of sarcoidosis patients. Competition assays showed capability of vimentin-derived peptides to replace high-affinity HLA-DRB1*03 binder myoglobin from the peptide-binding cleft. Moreover, peptide N- and C-terminal ends had significant influence on competition efficiency, providing a potential target amino acid sequence. BLAST analysis found no similarity between vimentin candidate peptides and known microbial proteins. Ongoing stimulation tests strive to characterise the Va2.3⁺Vβ22⁺ T cell response to candidate vimentin peptides in terms of proliferation and cytokine production, with the long-term objective of identifying the causative agent in sarcoidosis and improving diagnostic, prognostic and therapeutic strategies in a clinical setting.

Autoimmunity

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Pathogenic Role of Anti-Voltage Gate Calcium Channel Antibodies in Mediating Beta Cells Stress in Type 1 Diabetes

Alshehri, A., Jackson, M.

Flinders University, Autoimmunity Research Laboratory, Department of Immunology, Adelaide, Australia

Type 1 diabetes (T1D) is an autoimmune disease caused by the loss of pancreatic beta cells, resulting in poor blood glucose regulation. Autonomic neuropathy is a common but poorly understood complication of T1D resulting in significant morbidity. We have previously described a functional autoantibody (Abs) in T1D directed against L type Voltage gated calcium channels (VGCC), which contributes to autonomic dysfunction of the gastrointestinal tract and bladder by increasing channel activity during target cell activation. The

presence in patients of these anti-VGCC Abs has recently been linked to immune responses against the Coxsackievirus B4, and anti-VGCC Abs have been shown to disrupt adherence, and promote apoptosis in cultured beta cells. In the current study, we have used an assay of reactive oxygen species production (ROS), as measured by DCFDA labeled, to demonstrate that anti-VGCC antibodies induce oxidative stress in beta cells, providing a potential pathogenic mechanism for Ab-mediated beta cell loss in T1D. The current assay of ROS generation confirmed the presence of pathogenic antibodies in patient samples which had previously been demonstrated to contain anti-VGCC Abs, suggesting that this assay may be a useful diagnostic tool for determining the presence of functional anti-VGCC Abs. We propose that increased VGCC activity resulting from the presence of pathogenic anti-VGCC Abs is a contributing factor in the beta cell dysfunction and loss associated with progression to insulin dependence in type 1 diabetes.

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S1P₁ deletion differentially affects TH17 and regulatory T cells

Eken, A.¹, Duhon, R.², Singh, A.K.³, Fry, M.³, Buckner, J.H.², Kita, M.², Bettelli, E.², Oukka, M.³

¹Erciyes University, Medical Biology Department, Kayseri, Turkey,

²Benaroya Research Institute, Seattle, United States, ³Seattle Children's Research Institute, Seattle, United States

Sphingosine-1 phosphate receptor 1 (S1P₁) is a G-protein coupled receptor critical for the egress of T and B cells out of lymphoid organs. Although S1P₁ agonists (such as fingolimod) are currently used for the treatment of multiple sclerosis (MS) little is known how S1P₁ signaling regulates Th17 and T_{reg} cell homeostasis. To study the impact of S1P₁ signaling on Th17 and T_{reg} biology, we specifically deleted S1P₁ in Th17 and T_{reg} cells using *IL-17A^{Cre}* and *Foxp3^{Cre}* mice, respectively. Deletion of S1P₁ in Th17 cells conferred resistance to experimental autoimmune encephalomyelitis (EAE) characterized by reduced Th17 cell distribution across peripheral organs and diminished Th17 cell generation. On the other hand, permanent deletion of S1P₁ in T_{reg} cells resulted in autoimmunity and acute deletion rendered mice more susceptible to EAE. Importantly, our study revealed that S1P₁ not only regulated the egress of T_{reg} cells out of lymphoid organs and subsequent non-lymphoid tissue distribution but also their phenotypic diversity. Most of the T_{reg} cells found in S1P₁-deficient mice had an activated phenotype and were more prone to apoptosis, thus converted to effector T_{reg}. The comparison of T_{reg} cells obtained from MS patients treated with fingolimod to those treated with other oral drugs confirmed the switch of T_{reg} cells into effector memory phenotype. Our results provide novel insight into the functions of S1P₁ and potential impact of long term fingolimod use on Th17 and T_{reg} cell biology and general health in MS patients.

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Apoptotic antigen-coupled splenocytes suppress autoimmune anti-myeloperoxidase glomerulonephritis in a tolerogenic manner

Godfrey, A.S.¹, Gan, P.-Y.¹, Kitching, A.R.^{1,2}, Holdsworth, S.R.^{1,2}

¹Centre for Inflammatory Diseases, Monash University, Department of Medicine, Clayton, Australia, ²Monash Health, Department of Nephrology, Clayton, Australia

Background: Glomerulonephritis, a major cause of end-stage renal failure, results from autoreactivity to the neutrophil derived enzyme myeloperoxidase (MPO). In most autoimmune conditions, treatment with apoptotic antigen-coupled splenocytes can induce peripheral T cell tolerance by facilitating host APCs to reprocess and present the Ag in a non-immunogenic manner. Recent discovery of immunodominant MPO peptides makes this approach relevant to anti-MPO glomerulonephritis.

Aim: To assess whether antigen-coupled ethylenecarbodiimide (ECDI)-fixed splenocytes (Ag-Sp) can induce tolerance to MPO and suppress anti-MPO glomerulonephritis.

Method: We compared disease between C57BL/6 (WT) mice receiving either MPO409-428-Sp or OVA323-339-Sp. A standard model of anti-MPO glomerulonephritis was used. Anti-MPO autoimmunity induced by MPO409-428 immunisations and glomerulonephritis triggered using low dose anti-glomerular basement membrane globulin.

Results: Glomerulonephritis and autoimmunity was less severe in mice treated with MPO409-428-Sp, prior to the induction of autoimmunity than in control mice (segmental necrosis 43±5 vs 19±1%), glomerular leukocytes (macrophages; 0.53 ± 0.08 vs 0.25 ± 0.02 cells per glomerular cross section [c/gcs]; CD4+ cells 0.47 ± 0.05 vs 0.24 ± 0.02c/gcs) Autoimmunity, assessed by MPO induced DTH was reduced in MPO409-428-Sp treated mice versus control (0.11 ± 0.02 vs 0.02 ± 0.01Δmm). Furthermore transfer of CD4+ cells from mice treated with MPO409-428-Sp to mice with established anti MPO autoimmunity significantly decreased induced glomerulonephritis (segmental necrosis 45.25 ± 4% vs 23.13 ± 3%) compared to controls receiving CD4+ cells from OVA323-339-Sp treated mice.

Conclusion: MPO409-428-Sp suppress anti-MPO glomerulonephritis by the induction of antigen specific tolerance mediated by Tregs and is relevant to human disease.

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Isolating small extracellular vesicles from synovial fluid

Foers, A.^{1,2}, Cheng, L.³, Chatfield, S.^{1,2,4}, Scicluna, B.^{3,5}, Hill, A.³, Pang, K.^{1,6}, Wicks, J.^{1,2,4}

¹The Walter and Eliza Hall Institute of Medical Research, Inflammation Division, Parkville, Australia, ²Melbourne University, Department of Medical Biology, Parkville, Australia, ³La Trobe University, Department of Biochemistry and Genetics, Bundoora, Australia, ⁴Royal Melbourne Hospital, Department of Rheumatology, Melbourne, Australia, ⁵University of Melbourne, Department of Biochemistry & Molecular Biology, Parkville, Australia, ⁶Murdoch Childrens Research Institute, The Royal Children's Hospital, Parkville, Australia

Extracellular vesicles (EVs) are lipid bilayer enclosed vesicles that

are found in all body fluids. EVs contain a cargo consisting of proteins, RNAs and lipids; this cargo can have functional effects when EVs come into contact with recipient cells. In rheumatoid arthritis, the profile, number and functional effects of EVs in synovial fluid have been reported to change with disease. These changes are believed to not only contribute to disease, but may also serve as clinical biomarkers. However, the complexity of biological fluids presents challenges in the isolation of pure EV populations. This is especially true of synovial fluid, where standard ultracentrifugation-based methods co-isolate large amounts of particulate material including non-EV proteins and cause artifactual vesicle aggregation. These limitations are confounders in studying the content and function of EVs in synovial fluid, bringing into question the accuracy of published work where EVs have been purified using standard isolation procedures.

We have applied a size exclusion chromatography method to purify small EVs, including exosomes and small microvesicles from synovial fluid of arthritis patients. The EVs have been characterised by immunoblotting for standard exosomal markers (flotillin 1, transferrin receptor and heat shock protein 70) and dynamic light scattering. Our method separates small EVs from the bulk of non-EV proteins that are also pelleted by ultracentrifugation. The ability to isolate EVs from synovial fluid will be useful for future investigations to define the precise contents and role of synovial fluid EVs in rheumatoid arthritis.

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The immunological effects of sitagliptin treatment in patients with psoriasis

Malara, A.^{1,2}, Lynch, M.³, Sweeney, C.^{1,2}, Awdeh, F.³, Kirby, B.³

¹University College Dublin, School of Medicine and Medical Science, Dublin, Ireland, ²St. Vincent's Hospital, Education and Research Centre, Dublin, Ireland, ³St. Vincent's Hospital, Department of Dermatology, Dublin, Ireland

Psoriasis is a chronic, immune-mediated skin disorder affecting approximately 2% of the population. The pathogenesis of psoriasis includes hyperproliferation and abnormal differentiation of keratinocytes and cutaneous inflammation. Pro-inflammatory cytokines such as TNF- α and IL-17 are central in the pathogenesis of psoriasis. Dipeptidyl peptidase-4 (DPP-4) is expressed on many cell types including keratinocytes and its activity is upregulated in psoriasis. DPP-4 is also expressed on T cells as CD26 and has been demonstrated to be crucial in the pathogenesis of autoimmune diseases. In this clinical study, we aimed to determine whether DPP-4 inhibition improves severity of psoriasis after 24 and 36 weeks of treatment in patients undergoing a course of narrowband ultraviolet-B phototherapy (NB-UVB). Patients were randomly allocated to be treated with both DPP-4 inhibitor sitagliptin and NB-UVB light therapy or with NB-UVB light therapy alone. Blood samples were collected at the baseline visit and after 24 and 36 weeks of treatment for analysis of cytokines in the serum. In addition, skin biopsies were taken to analyse expression pro-inflammatory cytokines. The preliminary results have shown that the serum level of pro-inflammatory cytokine IL-17A measured by ELISA decreased in patients treated with sitagliptin after 36 weeks (n=39). The

analysis of skin biopsies by quantitative PCR have demonstrated that the expression of IL-17 and TNF- α decreased after 24 weeks in patients treated with sitagliptin compared to patients undergoing NB-UVB therapy alone (n=11). These data show that DPP-4 inhibition has the capacity to modulate the expression of IL-17 and TNF- α in psoriasis.

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Immunological studies on an Australian cohort of patients diagnosed with systemic lupus erythematosus

Margery-Muir, A.¹, Nelson, D.¹, Wetherall, J.¹, Bundell, C.², Groth, D.¹

¹Curtin University, Biomedical Sciences, Bentley, Australia, ²Sir Charles Gardner Hospital, QEII Medical Centre, Nedlands, Australia

Systemic lupus erythematosus (SLE) is an important autoimmune disease affecting mainly females and is associated with anti dsDNA autoantibodies, and is potentially associated with defective clearance of apoptotic cells. Blood samples were collected from 56 patients with confirmed diagnoses of SLE for whom cellular and serological parameters are being investigated and compared with age/sex matched controls.

We observed that the inflammatory cytokine IL17, which has been implicated in tissue damage associated with SLE, was synthesized by regulatory T cells (Tregs), Th17 cells and gamma delta ($\gamma\delta$) T cells. The synthesis of IL17 by $\gamma\delta$ T cells was significantly higher in patients than healthy controls (p< 0.005). However, the percentage of Th17 cells was higher in controls relative to patients (p< 0.05). Interestingly, in contrast to the controls, myeloid DC2 cells were more frequent than myeloid DC1 cells in SLE patients (p< 0.05). In this study the $\gamma\delta$ T cells produced more IL17, IL23 and TGF β than Tregs and Th17 cells from patients.

In summary, an expected increased frequency of Th17 cells was not observed in SLE patients relative to controls. Intracellular IL17 in patients was produced mostly by $\gamma\delta$ T cells. The clinical management and drug therapies of the SLE patients are currently under review and may explain the results obtained.

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Rotavirus acceleration of type 1 diabetes depends on type I interferon signalling

Pane, J.^{1,2}, Fleming, F.¹, Graham, K.^{3,4}, Thomas, H.^{3,4}, Kay, T.^{3,4}, Coulson, B.¹

¹The University of Melbourne, Department of Microbiology at Peter Doherty Institute for Infection and Immunity, Melbourne, Australia, ²Skirball Institute of Biomolecular Medicine, NYU School of Medicine, Kimmel Center for Biology and Medicine, New York, United States, ³St Vincent's Institute, Fitzroy, Australia, ⁴The University of Melbourne, Department of Medicine at St Vincent's Hospital, Fitzroy, Australia

Background and aim: Rotavirus infection is associated with childhood progression to type 1 diabetes. Monkey rotavirus RRV acceleration of diabetes in NOD mice with pre-existing autoimmunity relates to regional lymph node (not pancreatic) infection and a Th1-specific immune response. RRV stimulation of plasmacytoid dendritic cells (pDCs) from NOD mice ex

vivo induces type I interferon (IFN) secretion and bystander lymphocyte activation, including islet-autoreactive T cells. We aimed to directly establish the dependence of RRV-induced diabetes acceleration on type I IFN signalling.

Methods: The requirement for type I IFN signalling was analysed in NOD.IFNAR1^{-/-} mice, which lack a functional type I IFN receptor. Rotavirus-infected adult female NOD, NOD.IFNAR1^{-/-} and C57BL/6 mice were studied. Rotavirus in lymph nodes and development of a Th1-specific antibody response were measured by ELISA, activation of DC and lymphocytes by flow cytometry, type I IFN-induced gene expression by quantitative PCR, and diabetes development by urinary and blood glucose monitoring.

Results: RRV infection of NOD mice induced pDC activation and strongly upregulated IFN-dependent gene expression, particularly within lymph nodes. Such pDC activation was absent following NOD mouse infection with porcine rotavirus, which does not modulate diabetes onset. RRV lymph node titres were slightly increased in NOD.IFNAR1^{-/-} mice over NOD mice. However, RRV-infected NOD.IFNAR1^{-/-} mice exhibited delayed pDC and lymphocyte activation, an absence of Th1 bias in RRV-specific antibody responses and unaltered diabetes onset.

Conclusion: Type I IFN signalling is required for the acceleration of type 1 diabetes onset by rotavirus infection in genetically-susceptible mice.

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ΔdblGATA mice are resistant to experimental autoimmune encephalomyelitis

Ciric, B., Hwang, D.

Thomas Jefferson University, Neurology, Philadelphia, United States

GATA-binding factor 1 (GATA-1) is a transcription factor expressed in certain hematopoietic cells, governing their development and function. GATA-1 is expressed in myeloid cells but not in lymphoid cells, such as T cells. Since knockout of GATA-1 is embryonic lethal, mouse lines lacking individual enhancers to the GATA-1 gene have been generated. Lack of a particular enhancer selectively reduces, or precludes GATA-1 expression in some cell types, while in others its expression remains normal. We use mice lacking dblGATA enhancer (ΔdblGATA mice), which are devoid of eosinophils and have reduced numbers and function of basophils. No other defects in these mice have been described to date.

We found that ΔdblGATA mice are resistant to both direct and adoptive experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis. ΔdblGATA mice develop weaker myelin-specific Th responses upon immunization for EAE induction, indicating a defect in priming immune responses. The resistance of ΔdblGATA mice to adoptive EAE indicates a deficiency in effector mechanisms of CNS inflammation. We have ruled out lack of eosinophils as the reason for EAE resistance of ΔdblGATA mice by demonstrating that another mouse strain (PHIL), which also lacks eosinophils, develops normal EAE. These findings show that ΔdblGATA mice have defects in both the priming and effector phases of disease. Additionally, immunized ΔdblGATA mice had fewer inflammatory monocytes and myeloid dendritic cells in blood, draining lymph nodes

and the CNS. Our findings thus far suggest that an unknown defect in monocytes/dendritic cells causes resistance to EAE in ΔdblGATA mice.

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NF-M is not critical to the development of MOG induced EAE

Lawrence, L., Blanchfield, L., Evavold, B.

Emory University, Microbiology and Immunology, Atlanta, United States

The 2D2 T cell receptor was reported to be cross-reactive to two neuropeptides, myelin oligodendrocyte glycoprotein epitope 35-55 (MOG35-55) and neurofilament medium polypeptide epitope 15-35 (NFM15-35). This information led us to consider cross-reactivity as a cause of demyelinating autoimmune disease. To tease out the functional relevance of NF-M for demyelinating disease, we investigated experimental autoimmune encephalomyelitis (EAE) in C57BL/6 mice. EAE could not be induced by the conventional induction protocol of NF-M emulsified in CFA and pertussis toxin. In addition, we made use of NF-M knockout (NFM-KO) mice to explore the functional contribution of the protein to disease. Before we began our experiments, we backcrossed the NF-M knockout onto a C57BL/6 background using a speed congenic process to identify individual mice from each litter with the highest amount of C57BL/6 genetic information. NFM-KO mice developed a severe EAE that was comparable to wildtype C57BL/6 mice after being challenged with MOG35-55. In conclusion, the presence of NF-M has a minimal contribution to the severity of MOG induced EAE.

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Exhaled methylacetate levels differ according to dysimmune diseases and existence of severe liver steatosis

Donatini, B., Le Blaye, I.

Medicine Information Formation, Research, Cormontreuil, France

Methylacetate (MA) is a volatile organic compound synthesized by the gut microbiota. It can be measured in breath and is decreased in Crohn's disease (CD).¹ We measured MA levels in other dysimmune diseases.

Methods: All patients with CD, hemorrhagic recto-colitis (HRC), multiple sclerosis (MS), Hashimoto thyroiditis (HT), rheumatoid arthritis (RA) or ankylosing spondylitis or psoriatic polyarthritis (AS/PP) were prospectively enrolled into a 24-months cohort study. Non Alcoholic SteatoHepatitis (NASH) was identified to avoid a bias because of the liver metabolism of MA. Breath test was performed with MX6 (Gazdetect® France). Comparisons of percentages were performed with a Student's t-test.

Results: 247 patients with dysimmune diseases were enrolled. All patients with NASH (38 cases) exhaled low levels of MA (< 2.6 ppm).

Patients with CD or HRC never presented with NASH. Patients with MA < 2.6 ppm and MS, RA, AS/PP or HT have a high incidence of NASH: 70.6%, 37.5%, 44.4% and 42.3% respectively.

The percentage of patients with MA levels ≥ 2.6 ppm differs according to diseases: CD (29.1% out of 86 cases), HRC (93% out of 28), MS (50% out of 34), RA (55.6% out of 18), AS/PP (34.1% out of 41), HT (35% out of 40). The difference was significant between CD and MS or RA or HRC ($p < 0.01$).

Conclusions: MA measure is interesting in the diagnosis of CD or NASH. Differences of MA levels between CD and MS or RA or HRC suggest differences in gut microbiota.

1. Donatini B, Le Blaye I. *J Biosciences Med.* 2015

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In patients with a medical history of allergy, steatohepatitis is more frequent in Hashimoto thyroiditis (HT) than in Crohn's disease (CD) or multiple sclerosis (MS)

Donatini, B., Le Blaye, I.

Medicine Information Formation, Research, Cormontreuil, France

We compared the percentages of medical history of allergy in patients with dysimmune diseases. We investigated whether Nonalcoholic steatohepatitis (NASH) is more frequent in some subgroups which may suggest differences in liver metabolism and perhaps a role of gut microbiota.

Methods: All patients with CD, hemorrhagic recto-colitis (HRC), multiple sclerosis (MS), Hashimoto thyroiditis (HT), rheumatoid arthritis (RA) or ankylosing spondylitis or psoriatic polyarthritis (AS/PP) were prospectively enrolled into a 24-months cohort study before any treatment with immunosuppressive agents. NASH was identified with blood tests and liver elastography. Comparisons of percentages were performed with a Student's t-test.

Results: 247 patients with dysimmune diseases were enrolled. 38 NASH were detected. Anamnesis retraces allergic diagnoses in 74 patients.

The percentage of patients with a history of allergy differs according to diseases: CD (9.3% out of 86 cases), HRC (10.7% out of 28), MS (5.9% out of 34), RA (50.0% out of 18), AS/PP (65.9% out of 41), HT (62.5% out of 40). The difference was significant between CD or MS and RA, AS/PP or HT ($p < 0.01$). NASH was detected in 16 out of the 74 patients with a history of allergy (21.6%) versus only 22 out of 171 patients without allergy (15.8%): $p < 0.01$. NASH is never associated with CD. NASH exists in 26% of patients with HT plus a history of allergy.

Conclusions: Allergy rarely precedes CD or MS. It however frequently precedes RA, AS/PP or HT.

NASH is more frequent in patients with HT and a history of allergy.

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Mannose receptor (MR) have a protective role in mannan induced psoriasis (MIP)

Hagert, C.¹, Sareila, O.¹, Jalkanen, S.¹, Holmdahl, R.^{1,2}

¹University of Turku, Turku, Finland, ²Karolinska Institutet, Stockholm, Sweden

Psoriasis and psoriasis arthritis are both poorly understood, but common diseases in human. They are induced by unknown

environmental factors and affects both skin and joints. Mannan induced psoriasis (MIP) is a novel mice model for psoriasis arthritis (induced by a single injection of mannan into mice) causing arthritic symptoms such as swelling and redness of the paws and the characteristic lesions of psoriasis. The model is dependent upon macrophage produced reactive oxygen species (ROS) and $\gamma\delta T$ cells. However, the receptor, important for the recognition and initiation of the disease, is unknown. This study focused on investigating one of the receptors binding mannan; the mannose receptor (MR). MR is a C-type lectin present on myeloid cells and lymphatics. To investigate its importance for MIP we utilized MR knock-out mice, showing that the lack of MR leads to more severe arthritis compared to wild type. We further concluded that a mutation leading to ROS deficiency will, as previously shown, exacerbate the disease. The importance of MR as a protecting pathway is diminishing if the mouse lacks ROS; either the protective pathway activated by MR is dependent upon ROS or the severity of MIP in a ROS deficient organism is too severe and the protective effect is therefore lost. In conclusion, these results indicate that MR scavenges mannan and thereby preventing it from binding to other receptors in the mouse, for example mannan binding lectin, hence protecting. Further studies are needed to conclude which receptor is important for the initiation of MIP.

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Effect of gene delivery plasmid encoding Interleukin-19 on rat experimental autoimmune myocarditis

He, C.¹, Yan, W.¹, Jun, Z.²

¹Xiamen Cardiovascular Hospital, Medical College of Xiamen University, Xiamen, China, ²Medical College of Xiamen University, Xiamen, China

Objectives: To evaluate the effect of gene delivery plasmid encoding Interleukin-19 on rat Experimental Autoimmune Myocarditis (EAM) and possible mechanism.

Background: IL-19 is a novel, recently identified member of the IL-10 family, however, little is known about the exact biological role in immunological regulation.

Methods: The rat was immunized on day 0 and were injected with plasmid encoding IL-19 on day 6, all the rats were sacrificed on day 17. The effect of IL-19 gene delivery was evaluated by measuring of heart weight/body weight and myocarditis area, The relative gene expression levels of heart failure markers atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) were detected by real-time RT-PCR. Cardiac Function were performed by Echocardiography. Furthermore, we examined the effect of serum containing IL-19 on the expression of immune-relevant genes in IL-1-stimulated Spleen cells cultured from EAM rats.

Results: IL-19 gene therapy was effective in controlling EAM as monitored by decreased ratio of heart weight / body weight and the myocarditis area, The level of ANP and BNP were significantly lower and cardiac function parameters improved in IL-19 treatment group than those in control group. The serum containing IL-19 significantly decreased the expression of IL-18, IL-1 β , IL-12p35, IFN- γ and upregulated IL-4 and IL-10 expression in IL-1-stimulated spleen cells cultured from EAM rats.

Conclusion: IL-19 effectively prevented progression of EAM by blocking related inflammatory immune genes expression. This might be a possible mechanism of the amelioration of EAM by IL-19 gene therapy.

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Serum IL-18 as biomarker in predicting long-term renal outcome in pediatric-onset systemic lupus erythematosus

Wu, C.-Y.¹, Yang, H.-Y.², Yao, T.-C.¹, Liu, S.-H.³, Huang, J.-L.¹

¹Chang-Guang Memorial Hospital, Pediatrics, Taoyuan, Taiwan, Republic of China, ²Chang-Guang Memorial Hospital, Nephrology, Taoyuan, Taiwan, Republic of China, ³Chang-Guang Memorial Hospital, Family Medicine, Taoyuan, Taiwan, Republic of China

Introduction: An urge of biomarker identification is needed to better monitor lupus nephritis (LN) disease activity and predict patient's long-term outcome. With the pro-inflammatory effect and its association with inflammasomes, the significance of interleukin-18 (IL-18) among pediatric onset systemic lupus erythematosus (pSLE) patient, especially, its importance in predicting long-term renal outcome was investigated.

Methods: In a pSLE cohort of 96 patients with an average follow up period of 10.39 ± 3.31 years, data were collected at time of disease onset and 6 months after treatment. Through Cox regression analysis, the parameters at baseline and post treatment were carefully analyzed.

Results: Average age of all cases was 12.74 ± 3.01 years old and 65 of them suffered from LN. Nine subjects (9.38%) progressed to ESRD and 2 cases (2.08%) died during follow up. Serum IL-18 level 24 weeks post treatment was found to be the most hostile factor associating poor clinical outcome despite patient's initial renal status. In addition, the presentation of serum IL-18 in its correlation with SLE global disease activity as well as the presence and severity of LN were all significant ($p < 0.001$, $p = 0.03$ and $p = 0.02$). The histological classification of LN, however, was not associated with the level of IL-18 among the pSLE patients.

Conclusions: The role of serum IL-18 as biomarker in pSLE patient representing global disease activity and status of renal flares was assured. Additionally, we've identified IL-18 at 24 weeks post treatment a novel marker for long-term outcome prediction.

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Antinuclear antibodies: quantitative prediction of Lupus-related and other morbidity and mortality

Bundell, C.^{1,2}, Preen, D.³, Lucas, M.^{1,2}, Hollingsworth, P.^{1,2}

¹PathWest Laboratory Medicine, Western Australia, Clinical Immunology, Nedlands, Australia, ²The University of Western Australia, School of Pathology and Laboratory Medicine, Crawley, Australia, ³The University of Western Australia, School of Population Health, Crawley, Australia

Background: The anti-nuclear antibody (ANA) indirect immunofluorescence assay is a screening test useful in the diagnosis and classification of SLE and other connective tissue/

rheumatic diseases. ANA results can be expressed in IU/lm, but are most commonly reported as titres and these are not standardised.

Aim: To determine the ability of ANA, precisely quantified in IU/ml, to predict inpatient morbidity and mortality in a Western Australian (WA) clinical cohort.

Method: Hospital morbidity and mortality data collected through the WA Department of Health were linked to PathWest Laboratory ANA data for individuals resident in WA who had their first ANA reported in the period 2000 - 2010 ($n = 58,700$). Multivariate logistic regression analysis was used to examine the relationship between ANA and morbidity and mortality adjusting for gender, Indigenous background, age at time of first ANA test and country of birth.

Results and conclusion: Hospitalisation for a diagnosis of systemic connective tissue disease (SLE, scleroderma, dermatomyositis and Sjogren's syndrome) was associated with an ANA level of $7 < 10$ IU/ml (OR=1.64; 95%CI 1.38-1.94) increasing to OR=21.91 (95% CI 18.07-26.57) for an ANA level of >30 IU/ml. Other conditions significantly associated with ANA included pulmonary embolism, pulmonary heart disease, chronic hepatitis, myasthenia gravis, glomerular disease and cancer of the oesophagus and of the lymph nodes. An ANA level of >30 IU/ml also predicted death (OR=1.69; 95% CI 1.35-2.11). ANA is quantitatively predictive of lupus and related connective tissue diseases and other morbidity and mortality.

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Neonatal expression of IGRP is sufficient to induce lifelong protection from autoreactive IGRP-specific T cells in NOD mice

Selck, C., Jhala, G., Chee, J., Thomas, H.E., Kay, T.W.H., Krishnamurthy, B.

St Vincent's Institute, Immunology and Diabetes Unit, Melbourne, Australia

Autoimmune disorders result from the failure of immune tolerance mechanisms. Antigen-specific therapy constitutes an attractive approach to re-establish a tolerant state, but has so far not been successful in clinical settings. One of the major hurdles is the timing of intervention since, at the time of disease diagnosis, naive T cells have differentiated into antigen-experienced memory cells. Notably, we and others have shown that inducing tolerance to self-antigens during the neonatal period has long-lasting benefit suggesting that the initial wave of T cell differentiation may include self-reactive cells likely to cause future autoimmunity. To address the fate of a dominant CD8+ T cell population in this system, we generated non-obese diabetic (NOD) mice with tetracycline-controlled IGRP (islet-specific-glucose 6 phosphatase catalytic subunit related protein) expression in antigen presenting cells allowing induction of tolerance to IGRP in a temporally regulated manner. IGRP expression did not affect the immune infiltration of islets of Langerhans. However, using the tetramer enrichment assay, we detected a 100-fold reduction of IGRP₂₀₆₋₂₁₄-specific CD8+ T cells in mice where IGRP was expressed throughout the whole life compared to control NOD mice. Importantly, the same effect was observed in animals where tolerance to IGRP was induced only until weaning. Thus, our findings indicate that autoreactive T cells develop predominantly during the fetal and neonatal

period suggesting that antigen-based therapeutic approaches have lasting benefits when applied for a short period in early life.

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Microbial fermentation product butyrate ameliorates autoimmune arthritis

Takahashi, D.¹, Yoshida-Hoshina, N.¹, Takahashi, Y.¹, Nakamura, M.¹, Tanabe, H.¹, Lockett, T.², Clarke, J.M.², Topping, D.L.², Suzuki, A.³, Morita, T.⁴, Komatsu, N.⁵, Takayanagi, H.⁵, Hase, K.¹

¹Keio University Faculty of Pharmacy, Minato-ku, Japan,

²Commonwealth Scientific and Industrial Research Organisation (CSIRO) Food and Nutritional, Preventative Health National Research Flagship, Adelaide, Australia, ³RIKEN Center for Genomic Medicine, Yokohama, Japan, ⁴Shizuoka University, Faculty of Agriculture, Shizuoka, Japan, ⁵University of Tokyo, Graduate School of Medicine and Faculty of Medicine, Bunkyo-ku, Japan

Gut microbiota and its metabolites have been implicated in autoimmune diseases, including rheumatoid arthritis (RA) and inflammatory bowel disease. We have been reported that a large bowel microbial fermentation product, butyrate, induces the differentiation of Foxp3-expressing regulatory T (Treg) cells in the colon, and ameliorate the development of mouse models of autoimmune colitis. Both systemic and organ-specific autoimmune diseases often result from an imbalance between Treg cells and pro-inflammatory IL-17-producing CD4⁺ T (Th17) cells. Treg cell functions to restrain excessive T-cell responses, while Th17 cell is a key player in the pathogenesis of autoimmune diseases. The aim of this study was to investigate the impact of butyrate on the development of collagen-induced arthritis (CIA) in mice, a model that shares many hallmarks with human RA. Here we show that mice fed butyrylated high amylose maize starch (HAMSB), which delivers butyrate to the colon in an effective manner, markedly suppressed the clinical and histologic signs in CIA. Treg cells were increased and Th17 cells were decreased in the draining lymph nodes of CIA mice fed HAMSB compared with CIA mice fed control diet. HAMSB also suppressed the production of collagen II-specific IgG. Adoptive transfer of Treg cells isolated from CIA mice fed HAMSB into CIA mice fed control diet followed by a booster immunisation prevented progression of the diseases. These data suggest that microbial metabolite butyrate play a key role in RA prevention and support that a potential protective role of butyrate in systemic autoimmune diseases.

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Increased serum IL-21 in systemic lupus erythematosus patients with vitamin D deficiency

Hirigoyen, D.¹, Alvarez, A.¹, Montalva, R.¹, Naves, R.², Iruetagoien, M.¹, Burgos, P.¹

¹Pontificia Universidad Catolica de Chile, Departamento de Reumatología, Facultad de Medicina., Santiago, Chile,

²Universidad de Chile, Programa de Inmunología, Instituto de Ciencias Biomédicas, Facultad de Medicina, Santiago, Chile

Systemic Lupus Erythematosus (SLE) is an autoimmune disease characterized by aberrant B cell signaling. Vitamin D (VD)

deficiency is highly prevalent in SLE patients and has been associated with clinical activity, but the mechanisms implied are not fully understood. Interleukin 21(IL-21) signaling pathway is involved in B cell differentiation into plasma cells and antibody production and has been suggested to play a role in SLE. Consistently, deficiency of IL-21 receptor (IL-21R) improves clinical symptoms in a SLE mouse model. In this study, we assessed VD status, serum IL-21 levels and IL-21R expression in B cells obtained from Chilean SLE patients. Serum VD and IL-21 levels and IL-21R expression were determined in 19 SLE patients and 6 healthy controls by tandem mass spectrometry, ELISA and flow cytometry, respectively. Average vitamin D levels in SLE patients were 19.7±6.85 ng/ml and 13.58±4.74 ng/ml in controls. The prevalence of hypovitaminosis D (serum VD levels below 30 ng/ml) is 89.5% in SLE patients. We found significantly higher levels of IL-21 in serum of SLE patients (226.3±54.11 pg/ml) compared to controls with hypovitaminosis D (37.1±22.53 pg/ml)

(Mann Whitney, $p < 0.005$). We observed no significant differences in IL-21R expression in B cells from patients when compared to controls (MFI 25,65±3.812 vs. 29.52±5.653).

Chilean SLE patients analyzed present a high hypovitaminosis D prevalence and increased IL-21 levels compared to controls with hypovitaminosis D. Further studies are required to establish the role of vitamin D in IL-21 levels and SLE.

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Increased salt exposure affects both lymphoid and myeloid effector functions, influencing antibody-induced arthritis but not autoimmunity

Vaartjes, D.¹, Raposo, B.¹, Nandakumar, K.S.¹, Holmdahl, R.^{1,2}

¹Karolinska Institutet, Section for Medical Inflammation Research, Department of Medical Biochemistry and Biophysics, Stockholm, Sweden, ²Southern Medical University, Section for Medical Inflammation Research, Guangzhou, China

Introduction: Salt is required to maintain the cell's homeostasis, helping to establish a membrane potential crucial for numerous cellular processes. In humans, high-salt intake is associated with elevated blood pressure, cardiovascular disease and insulin resistance. Recently, salt has been linked to the development of autoimmunity in mice. In the present study, we assessed how salt affects the activation and effector functions of murine lymphoid and myeloid cells in vitro, and how these effects can be translated in vivo using different models of inflammation.

Results: We show that a moderate increase in NaCl exposure significantly increases T cell proliferation while limiting the secretion of IFN- γ . After LPS stimulation of peritoneal macrophages, salt exposure reduced the production of pro-inflammatory cytokines as well as nitric oxide. Moreover, increased salt intake reduced the susceptibility to collagen antibody-induced arthritis, an acute arthritis model highly dependent on macrophage activation and inflammatory cell proliferation. However, a moderate increase of salt intake did not affect the development of the complex T cell dependent models of arthritis and multiple sclerosis.

Conclusion: Exposure of immune cells to moderate salt concentrations *in vitro* affects their proliferation capacity as well as their cytokine secretion profile. Even though salt had a suppressive effect on CAIA, its effects could not directly be translated into the T cell dependent models of autoimmunity CIA and EAE.

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Collagen specific CD4+ T-cells restricted by HLA-DRB1*0101 are selected into the repertoire and expand in disease in mice and patients with inflammatory arthritis

Jansen, D.T.S.L.¹, Nel, H.J.¹, Ramnoruth, N.¹, Tesiram, J.¹, Nagl, L.¹, Benham, H.¹, Scally, S.², Reed, H.H.², Rossjohn, J.², Thomas, R.¹

¹University of Queensland Diamantina Institute, Woolloongabba, Australia, ²Monash University, Department of Biochemistry and Molecular Biology, Clayton, Australia

The strong association between rheumatoid arthritis (RA) and HLA-DRB1, including HLA-DRB1*0101 and HLA-DRB1*0401 implicates autoreactive CD4+ T-cells in RA pathogenesis. Collagen-II is an arthritogenic autoantigen. Anti-collagen autoantibodies are present in 50% of early RA patients and bovine or human collagen-II is immunogenic in HLA-DRB1*0101-transgenic mice, with collagen₂₅₉₋₂₇₃ the dominant epitope. We characterized collagen₂₅₉₋₂₇₃-specific CD4+ T-cells using HLA-DRB1*0401- and HLA-DRB1*0101-collagen₂₅₉₋₂₇₃ tetramers. Collagen-specific CD4+ T-cells, binding tetramer with low affinity, were present at a frequency of 0.06% in blood, 0.11% in popliteal lymph node and 0.27% in spleen of naïve HLA-DRB1*0101-transgenic IA/IE^{-/-}B6xB10.BR mice. After bovine collagen immunization and onset of collagen-induced arthritis, the frequency of collagen-specific CD4+ T-cells almost doubled at each site, with expansion particularly among cells of higher binding affinity. High-affinity tetramer+ T-cells expanded markedly after *in vitro* stimulation of splenocytes from naïve or arthritic mice for 7 days with collagen₂₅₉₋₂₇₃. Expanded antigen-specific cells were CD4^{hi}CD44^{hi}CD62L^{lo} effector-memory T-cells. In peripheral blood of HLA-DRB1*0101+ or *0401+ RA patients, the frequency of collagen₂₅₉₋₂₇₃-specific CD4+ T-cells was 4-89/million PBMC. Tetramer+ cells were mostly CD45RO⁺CD69⁺CCR7^{+/+} and their frequency correlated with disease activity. These data indicate that collagen-specific CD4+ T-cells are selected into the repertoire of HLA-DRB1*0101 mice and are poorly controlled by peripheral tolerance mechanisms. Antigen-specific effector memory CD4+ T-cells expand with the development of autoimmune arthritis in mice and RA patients, associated with clinical inflammation. The collagen-specific CD4+ T-cells detected in RA patient blood at low frequency resemble CD4+ tissue-resident memory cells, likely trafficking between inflamed tissues.

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IRF7-dependent IFN- β production in response to RANKL is crucial for bone metabolism in CAIA arthritis model

Zhang, D.¹, Yu, Q.-W.¹, Chen, X.-H.², Chen, N.-N.^{1,2}

¹Shanghai Institute of Immunology, Shanghai Jiaotong University School of Medicine, Shanghai, China, ²Ruijin Hospital, Shanghai, China

IFN- β has significant immunomodulatory properties in RA, and IFN gene transcription is activated by IFN-regulatory factors (IRF) in response to innate sensor recognition, it may be a reactive attempt to inhibit inflammation and plays a very significant role in bone balance via RANKL-RANK pathway, which is the essential signal for osteoclasts differentiation. We established CAIA model on IRF7^{-/-} mice which results in exacerbated arthritis and decreased IFN- β expression in the gene and protein level. IFN- β treatment of IRF7^{-/-} CAIA mice resulted in decreased arthritis to similar to WT CAIA mice, including attenuated bone damage and TNF- α protein and gene level. The IRF7^{-/-} mice showed no difference in T cell numbers compared with control mice, while IFN- β led to significant reduction in CD4⁺Foxp3⁺Treg cells infiltration in IRF7^{-/-} mice. There have a significant decrease in the number of osteoclasts in IRF7^{-/-} mice compared with WT *in vitro*. And IFN- β had the strongest inhibitory effect on day 2 in osteoclastogenesis. IFN- β treatment of FLS with leads to decreased MMP-1, IKK ϵ and c-FOS, while increased production of IRF-7, but has limited direct IRF3, RANKL, NF- κ B regulating function. The partly increased expression of IFN- β in RA, and low expression of IFN- β exacerbated arthritis while replacement treatment with IFN- β decreased arthritis severity in CAIA mice suggests IFN- β might be useful for some RA patients where endogenous IFN- β expression is low. IFN- β plays an immunomodulatory mechanism that could inhibit synovial inflammation and alleviate bone damage.

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Atherosclerotic lesion regression is impaired in the K/BxN model of rheumatoid arthritis, independent of circulating cholesterol levels

Dragoljevic, D.^{1,2}, Whillas, A.^{1,2}, Kraakman, M.^{1,3}, Ngo, D.², Shihata, W.^{1,2}, van Doornum, S.⁴, Wicks, I.^{4,5}, Febbraio, M.^{1,6}, Chin-Dusting, J.^{1,2}, Murphy, A.J.^{1,2}

¹Baker IDI Heart and Diabetes Institute, Melbourne, Australia, ²Monash University, Melbourne, Australia, ³Columbia University, New York, United States, ⁴Royal Melbourne Hospital, Melbourne, Australia, ⁵Walter & Eliza Hall Institute of Medical Research, Melbourne, Australia, ⁶Garvan Institute of Medical Research, Sydney, Australia

Objective: Rheumatoid arthritis (RA) is associated with a ~2-fold elevated risk of mortality from cardiovascular disease (CVD). Chronic systemic inflammation is thought to be important but the precise mechanisms are not well understood. Peripheral blood in RA is often characterised by monocytosis and neutrophilia, which are known to play causal roles in atherosclerosis. We examined the effect of arthritis on atherosclerotic lesion regression when plasma cholesterol levels were controlled.

Methods: Low-density lipoprotein receptor knock-out mice were fed a high fat high cholesterol diet for 14wks to promote atherosclerotic lesion development. At 14wks, a subgroup of mice were sacrificed for baseline lesion characteristics and the remaining mice were placed on chow diet for 3 weeks to normalize plasma cholesterol and induce atherosclerotic regression. Simultaneously, a subgroup was rendered arthritic using passive transfer of K/BxN serum. Atherosclerotic lesion

size (H&E), lipid (Oil red O) and macrophage (CD68+) content were analysed. Flow cytometry was used to quantify leukocytes populations.

Results: RA impaired atherosclerotic regression, as lesions from the arthritic mice were larger than non-arthritic mice and appeared more rupture-prone as they contained more lipid and macrophages. This was independent of total serum cholesterol levels. Circulating monocyte levels were also elevated, predominantly the Ly6-Chi inflammatory subset that is considered more pro-atherogenic.

Conclusion: Inflammatory murine arthritis impaired atherosclerotic lesion regression. Enhanced monocyte production and entry into the atherosclerotic lesion may play a role in defective atherosclerotic lesion regression. Our findings suggest that control of lipid levels in RA patients is not sufficient in preventing atherosclerosis.

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Type I interferon associated immune responses in STING associated vasculopathy with onset in infancy (SAVI) patients

Gul, E.¹, Kara Eroglu, F.², Surucu, N.¹, Yalciner, C.³, Gursel, I.², Ozen, S.⁴, Gursel, M.¹

¹Middle East Technical University, Ankara, Turkey, ²Bilkent University, Ankara, Turkey, ³Acibadem University, Istanbul, Turkey, ⁴Hacettepe University, Ankara, Turkey

Response to cytosolic DNA depends on the expression of the adaptor STING, culminating in type I IFN production. Gain-of-function mutations in STING has recently been shown to trigger an interferonopathy characterized by neonatal-onset systemic inflammation with severe cutaneous vasculopathy (STING-associated vasculopathy with onset in infancy, or SAVI) leading to extensive tissue loss and interstitial lung disease. Herein, we present data on immune activation status of 3 new cases of SAVI syndrome. Genetic analysis by direct sequencing revealed N154S mutation in one patient and a novel compound heterozygous mutation of V155E/L170Q in TMEM173 in 2 others. N154S mutation results in constitutive activation of the STING-IRF3. qRT-PCR results demonstrate that unstimulated PBMC of the patient with N154S mutation have significant upregulation of interferon stimulated genes (ISGs) compared to healthy controls, while patients with V155E/L170Q mutations showed moderate level of upregulation of ISGs, suggesting that differences in STING-TBK1-IRF3 axis may exist in various TMEM173 mutations that may affect clinical features of this disease. Similar results were found in plasma IP-10 levels where patients have significantly higher levels of IP-10 compared to healthy controls. To assess cGAS activity, monocytes from HSV DNA stimulated samples were co-cultured with a STING reporter cell-line and gap-junction-mediated intercellular cGAMP transfer was determined. Results showed that DNA transfection stimulated patient cells to synthesize and then transfer cGAMP to the reporter cells, suggesting that cGAS activity was not compromised in SAVI syndrome. Collectively, these results could contribute to our understanding of mechanisms that contribute to the pathogenesis of this rare disease.

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Association of IL-27 gene polymorphisms (rs153109 and rs17855750) and its sera levels with risk of systemic sclerosis

Kamali Sarvestani, E.^{1,2}, Khalaj, F.^{1,2}, Nazarinia, M.A.³, Shenavandeh, S.³

¹Shiraz University of Medical Sciences, Autoimmune Diseases Research Center, Shiraz, Iran, Islamic Republic of, ²Shiraz University of Medical Sciences, Immunology, Shiraz, Iran, Islamic Republic of, ³Shiraz University of Medical Sciences, Rheumatology, Shiraz, Iran, Islamic Republic of

Introduction: IL-27 may play an important role in the pathogenesis of systemic sclerosis (SSc). Therefore, genetic variants and sera levels of IL-27 were studied in SSc.

Materials and methods: Two polymorphisms of IL-27p28 gene (rs153109 and rs17855750) were investigated in a total of 232 SSc patients and 234 controls by PCR-RFLP method. The IL-27 serum levels were also checked by ELISA.

Results: The rs153109-AA genotype and rs153109-A allele frequencies were significantly higher in patients compared to controls ($p=0.007$ and $p=0.002$, respectively). However, no significant differences were observed in genotype and allele frequencies of rs17855750 between patients and controls. In addition, after categorization of patients into rs153109-G and rs153109-non G carriers, rs153109-AA genotype was more frequent in patients with kidney involvements compared to cases without involvement ($p=0.03$). Moreover, AA carriers frequency was higher in CRP+ male patients compared to CRP- male patients ($p=0.03$). Furthermore, haplotype analysis showed AG haplotype was associated with increased SSc susceptibility ($p=0.001$) while GT was a protective haplotype ($p=0.007$). IL-27 serum levels showed an insignificant higher levels in patients compared to controls ($p=0.08$). Furthermore, IL-27 serum levels was higher in patients with increased FTP (>1 cm) compared to those with normal FTP (<1 cm, $p=0.003$).

Conclusion: In conclusion, functional SNP in IL-27p28 promoter (rs153109) and IL-27 levels may be associated with susceptibility and clinical manifestations of SSc.

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Splenic long-lived plasma cells act as antigen-presenting cells preferentially driving the development of follicular helper T cells

Jang, E., Youn, J.

Hanyang University, Laboratory of Autoimmunology, Department of Anatomy and Cell Biology, College of Medicine, Seoul, Korea, Republic of

Long-lived plasma cells (LLPCs) develop under the help of follicular helper T (T_{fh}) cells and reside mainly in the bone marrow. However, these cells are unusually abundant in the spleen of several autoimmune models including K/BxNsf mice, yet their pathogenic impact remains unknown. To investigate a previously unappreciated role of splenic LLPCs, we sorted splenic plasma cells (PCs) from K/BxNsf and K/BxN mice, corresponding to LLPCs and conventional short-lived PCs, respectively, and compared their phenotypes and ability to

prime and induce the differentiation of naive CD4⁺ T cells into effector cells *in vitro* and *in vivo*. We found that K/BxNsf PCs had lower levels of the Ag presentation machinery and costimulators than K/BxN PCs, and also a lower CD4⁺ T cell priming capacity. Autoantigen-pulsed K/BxNsf PCs selectively polarized cognate CD4⁺ T cells toward the expression of molecules necessary for Tfh development and function. As a result, the K/BxNsf PC-primed CD4⁺ T cells were more effective in stimulating B cells to produce autoantigen-specific IgGs than K/BxN PCs or even dendritic cells. Adoptive transfer of K/BxNsf PCs, but not K/BxN PCs, to K/BxN mice increased numbers of Tfh cells in draining lymph nodes. These results propose that abnormal accumulation of LLPCs in the spleen of autoimmune models drives the differentiation of autoantigen-primed CD4⁺ T cells to Tfh cells. This positive feedback loop between splenic LLPCs and Tfh cells may contribute to the persistence of humoral autoimmunity.

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Autoimmune evaluation in patients with idiopathic portal hypertension (IPH). Potential pathophysiological role and biomarkers

González-Navarro, E.A.^{1,2}, *Cerda Reyes, E.*^{2,3,4}, *Silva-Junior, G.*^{2,3,4}, *Seijo, S.*³, *Turon, F.*^{2,3,4}, *Baiges, A.*³, *Hernandez-Gea, V.*³, *Bosch, J.*³, *Juan, M.*^{1,2,5}, *García-Pagán, J.C.*^{2,3,4,5}

¹Hospital Clinic, Immunology Service, Barcelona, Spain, ²IDIBAPS, Barcelona, Spain, ³Hepatic Hemodynamic Lab. Liver Unit. Hospital Clinic, Barcelona, Spain, ⁴CIBERehd, Barcelona, Spain, ⁵Universidad de Barcelona, Barcelona, Spain

Background and aims: IPH is a rare clinical disorder consisting of intrahepatic portal hypertension in the absence of cirrhosis and other causes of liver disease. The pathophysiological mechanisms causing IPH remain largely unknown, although IPH is occasionally associated with autoimmune diseases (AD). This study evaluates the potential involvement of autoimmunity in the pathophysiology of IPH.

Methods: In serum samples prospectively collected from 39 patients with IPH and of 39 patients with liver cirrhosis (LC) matched by gender, signs of portal hypertension and liver function included as controls the 25 autoantibodies were determined. Antiendothelial cell antibodies (AECA) by CytoELISA (cell based ELISA using EA.Hy926 cells), immunohistochemistry (IH) and Western-blot on normal liver.

Results: Anti-TPO (Anti-Tiroperoxidase) and AECA were significantly more frequent in IPH patients than in patients with LC (41%vs10.3%;p=0.002 and 25.6%vs2.6%;p=0.003 respectively). These autoantibodies were represented in all IPH subgroups. Selectivity of AECA positivity was confirmed by IH showing reactivity against endothelial cells of normal livers. EA.Hy926 lysates were tested by Western blotting with serum samples, identifying a protein with a molecular weight of 68-72 kDa. Presence of anti-TPO and/or AECA had no prognostic value; patients had a similar clinical outcome during follow-up. In the current cohort of patients with portal hypertension (IPH+LC), presence of anti-TPO and/

or AECA had a specificity/sensitivity of 87.2%/64.1%; and a positive/negative likelihood ratio of 5/0.41 respectively for the diagnosis of IPH.

Conclusions: Our findings suggest a potential contribution of AECA and anti-TPO to IPH pathogenesis. and may be helpful for the diagnosis of IPH.

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The role of CD8+ T regulatory cells in the development of autoimmune thyroid diseases (AITD) and thyroid eye disease (TED)

Al-Ansari, F., Lahooti, H., Edirimanne, S., Wall, J., Department of Medicine, Sydney Medical School, Nepean

Autoimmune thyroid diseases (AITD) are one of the most common autoimmune diseases. Approximately 40% of AITD patients develop a manifestation called thyroid eye disease (TED) alongside their thyroid condition that involves the inflammation of the extra-ocular muscles and the surrounding connective tissue. Despite the fact that AITD were along the earliest autoimmune diseases that were addressed, their pathogenesis remains unclear with poor scientific evidence for a link of these diseases to the orbital inflammation seen in TED patients. In this study, we aimed to see whether the number and/or function of CD8+ T regulatory cells (Tregs) within the thyroid and/or peripheral blood are affected in patients with AITD. We also aimed to assess whether the number and/or function of CD8+ Tregs is affected among AITD patients who have developed TED.

Thyroid mononuclear cells (mncs) were isolated with enzyme digestion and filtration of the thyroid tissue followed by Ficol-Hypaque density gradient. The same density gradient was also used to separate peripheral blood mncs. The isolated cells were analysed using flow cytometry to evaluate the number of CD8+ Tregs. To assess the function of CD8+ Tregs, after magnetic cell sorting, CD4+CD25- T-responder cells (Tresps) were cultured either alone or in combination with CD8+ Tregs. Flow cytometry was then used to detect the difference in the expression of activation markers by Tresps.

Our data suggest that the development of AITD may be related to a decreased number and function of CD8+ Tregs within the thyroid.

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Immune mechanisms in chronic or relapsing autoimmune diseases investigated in the experimental rat model of autoimmune uveitis (EAU)

*Wildner, G.*¹, *Diedrichs-Möhrling, M.*¹, *Kaufmann, U.*^{1,2}, *von Toerne, C.*³, *Kungl, A.*⁴, *Thurau, S.*¹

¹Clinic of the University of Munich, Dept. of Ophthalmology, Section of Immunobiology, München, Germany, ²NYU Langone Medical Center, New York, United States, ³Helmholtz-Zentrum München, Research Unit Protein Science, Neuherberg, Germany, ⁴University of Graz, Institute of Pharmaceutical Sciences, Dept. of Pharmaceutical Chemistry, Graz, Austria

Experimental autoimmune uveitis (EAU) in rats is a model for human intraocular inflammatory disease. We can induce

either spontaneously relapsing-remitting disease with the interphotoreceptor retinoid-binding protein peptide R14 or a monophasic/chronic disease with the retinal S-Antigen peptide PDSAg. T cell lines were analyzed for gene and protein expression. Rats with EAU induced by both antigens were either treated with chemokine mutants or analyzed for intraocular T cell populations. Gene expression analysis revealed 26 genes upregulated in R14-specific T cells, all upstream or downstream of IFN-g signaling and belonging to pathways of wnt, antigen-presentation, secretion and cytokine expression. The role of IFN-g for recurrent disease was confirmed by synchronized relapses after intraocular injection of IFN-g in R14-mediated uveitis. PDSAg-specific T cell lines secreted more IL-6, IL-10, VEGF and CCL2. VEGF-secretion of PDSAg-specific T cells induced chorioretinal neovascularization as late complication in the monophasic uveitis. After application of CCL2- and CCL5-variants suppressive effects were only seen in PDSAg-, but not R14-induced EAU. During the course of EAU intraocular T cell populations co-express IFN-g, IL-17 and/or IL-10. Cells co-expressing these cytokines increased during monophasic and decreased during relapsing disease, and Foxp3-expression increased late in monophasic EAU. When rats were co-immunized with both antigens the monophasic EAU dominated with respect of the disease course and intraocular T cell populations. Analysis of autoreactive T cells in vitro, intraocular T cell populations in vivo and therapeutic responses revealed differences between both uveitis types and demonstrated that relapsing disease is strongly dependent on IFN-g.

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Shared genetic and environmental risk factors for the autoimmune diseases type 1 diabetes mellitus and multiple sclerosis

Parnell, G.¹, Ong, L.¹, Swaminathan, S.², Vucic, S.², Barnett, M.³, Goodnow, C.⁴, Booth, D.¹, Stewart, G.¹

¹Westmead Institute for Medical Research, Centre for Immunology and Allergy Research, Sydney, Australia, ²University of Sydney, Westmead Clinical School, Westmead, Australia, ³University of Sydney, Brain and Mind Research Institute, Sydney, Australia, ⁴The Garvan Institute, Immunogenomics, Sydney, Australia

Multiple Sclerosis (MS) and type 1 diabetes (T1D) are both autoimmune diseases with inflammatory, auto-antigen-specific T-cell, and decreased T-cell suppressor components, but they affect different organs and have marked differences in pathogenesis and clinical manifestations. Despite this, they co-occur more often than expected by chance, pointing to shared susceptibility factors that override the known differences in HLA association including protection against T1D by the MS risk genotype, HLA-DRB1*1501. Low sun exposure and low vitamin D (vitD) levels are common risk factors. Genome-wide association studies and candidate gene studies have identified shared non-HLA genetic risk factors, including variants in the vitD pathway gene, CYP27B1. Also, both MS and T1D risk variants are over-represented in the vitD receptor (VDR) cistrome of myeloid cells ($p < 0.005$, FDR < 0.05).

We compared linkage disequilibrium (LD) regions around the MS and T1D associated SNPs to better define the shared risk

genes. Eight genetic loci were identified as shared, with 15 genes in the LD-blocks. These genes are expressed in multiple immune subsets including myeloid, B, NK, and T-cells. There is also evidence that 5 of these haplotypes affect gene expression in immune cells. Six shared genes have VDR binding sites within 20kb.

We sought rare variants that may be contributing to disease risk using whole genome sequencing of genomic DNA from individuals with both T1D and MS. Variants were filtered by multiple criteria, including likely involvement in the vitD pathway. These data implicate shared, specific immune dysregulation between T1D and MS, and common targets for therapeutic intervention.

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Immune response to gingipain of *Porphyromonas gingivalis* in periodontitis and rheumatoid arthritis

Siao, S.-H.¹, Yen, J.-H.², Lee, Y.-H.², Sun, J.², Chang, Y.-C.³, Chen, J.², Lan, J.-L.², Tsai, J.¹, Zouali, M.⁴, Tsay, G.-J.²

¹Institute of Microbiology & Immunology, Chung Shan Medical University, Taichung, Taiwan, Republic of China, ²Division of Immunology and Rheumatology, Department of Internal Medicine, China Medical University Hospital, Taichung, Taiwan, Republic of China, ³Chung Shan Medical University, Department of Oral Medicine, Taichung, Taiwan, Republic of China, ⁴Inserm & University Paris Diderot, Sorbone Paris Cité, Paris, France

Objective: Several studies indicate an epidemiologic association between rheumatoid arthritis (RA) and periodontitis (PD) even after adjusting for risk factor of smoking. The aim of the study was to investigate the humoral immune response to Arginine-gingipain (RgpA) of *Porphyromonas gingivalis* in PD and RA.

Methods: Hemagglutinin domain and catalytic domain of RgpA of *Porphyromonas gingivalis* were cloned respectively. Serum samples were tested for the IgG and the IgG subclass including IgG1, IgG2, IgG3 and IgG4 anti-RgpA antibodies in patients with PD, RA and normal control by ELISAs.

Results: RA patients had the antibodies to hemagglutinin domain of RgpA, but not to the catalytic domain of RgpA. Total IgG anti-RgpA were detected in 32/153 (20.9%) patients with RA, and 9/46 (19.1%) patients with PD. The titers of anti-RgpA hemagglutinin domain antibodies in both RA and PD patients were significantly higher than that of the healthy normal control group ($p < 0.05$). IgG1 of anti-RgpA hemagglutinin domain antibodies were increased in patients with PD only. IgG2 of anti-RgpA hemagglutinin domain antibodies were increased in patients with both PD and RA. Both IgG3 and IgG4 of anti-RgpA hemagglutinin domain antibodies were not increased in both diseases.

Conclusion: Our results showed that the antibody responses in PD and RA are predominantly directed to the RgpA hemagglutinin domain, not the catalytic domain. The IgG2 of anti-RgpA hemagglutinin domain antibodies play an important role in the pathogenesis of both PD and RA. The relationships between anti-RgpA hemagglutinin domain antibodies and rheumatoid arthritis need to be further elucidated.

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Vaproic acid treatment suppresses autoimmune recurrence and allograft rejection in islet transplantation for type 1 diabetes

Lin, G.-J.¹, Lin, J.-R.¹, Yu, C.-C.¹, Huang, S.-H.^{1,2}

¹National Defense Medical Center, Department of Biology and Anatomy, Taipei, Taiwan, Republic of China, ²Tri-Service General Hospital, Department of General Surgery, Taipei, Taiwan, Republic of China

Type 1 diabetes mellitus (T1D) results from the destruction of insulin-producing β cells in the islet of the pancreas by leukocytes. It has been considered that T1D is a T cell-mediated autoimmune disease. CD4⁺ and CD8⁺ T cells are highly responsible for the destruction of β cells within the pancreatic islets of Langerhans. Previous studies have revealed that regulatory T cells (Tregs) play a key role in immune system homeostasis and tolerance to antigens, thereby preventing autoimmunity. Recent studies have found that HDAC inhibitor (HDACi) treatment *in vivo* increases the expression of forkhead box P3 (FOXP3), a critical transcription factor for Tregs, as well as the production and suppressive function of Tregs. Valproic acid (VPA), a branched short-chain fatty acid, is widely used as an antiepileptic drug and a mood stabilizer. Recently, VPA has been demonstrated to act as a HDAC inhibitor and VPA treatment increases Tregs function and decreases the incidence and severity of collagen-induced arthritis and experimental autoimmune neuritis. We treated a mouse model of islet transplantation in T1D (non-obese diabetic mice) with VPA. The survivals of the syngeneic and allogeneic islet grafts were significantly prolonged. This treatment increased the production of TGF- β in the splenocytes. Our study demonstrated a therapeutic potential of VPA treatment in islet transplantation for T1D.

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Blockade of IL-7Ra alleviates collagen-induced arthritis via inhibiting Th1 cell differentiation and CD4⁺ T cell migration

Cai, L., Xu, H., Nie, H.

Shanghai Institute of Immunology, Shanghai JiaoTong University School of Medicine, Shanghai, China

IL-7/IL-7R axis shows the importance on CD4⁺ T cell response, including proliferation, differentiation, survival and migration. But whether blockade of IL-7/IL-7R axis can relieve rheumatoid arthritis and the treatment mechanisms have remained unclear. In this study, we established collagen-induced arthritis (CIA) model and observed the effect of IL-7Ra antibody in the treatment of CIA mice. The data showed IL-7Ra antibody treatment significantly alleviated clinical symptoms of CIA mice, accompanied by reduced number of CD4⁺ T cells in both spleen and joints. We found that CII-specific CD4⁺ T cell proliferation decreased and the mRNA expression of inflammatory cytokines reduced in the IL-7Ra antibody-treated mice. Subsequently, IL-7Ra antibody treatment *in vivo* reduced the percentage of Th1 and Th17 cells and the mRNA expression of T-bet and ROR γ t gene. Moreover, we found that IL-7 promoted Th1 differentiation *in vitro*, but no effect on Th17 differentiation. In addition, administration of IL-7Ra antibody reduced the

mRNA expression of chemokine receptors (CCR7, CXCR3, CXCR6 and XCR1) on CD4⁺ T cells and chemokine CXCL2 in joints. The results suggested that the treatment effect of IL-7Ra antibody on CIA was via the inhibition of CII-specific CD4⁺ T cell proliferation, reduction of Th1 differentiation and restraint of CD4⁺ T cell migration to joint lesion site. Our data indicated that IL-7Ra maybe a potential therapeutic target for rheumatoid arthritis. (This project is supported by grants from National Natural Science Foundation of China (81273307) and Shanghai Municipal Education Commission (14ZZ106).)

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CXCR3 chemokine axis regulates T cell function in autoimmune cholangitis

Ma, H.-D., Liu, Q.-Z., Lian, Z.-X.

University of Science and Technology of China, Hefei, China

CXCR3 is a chemokine receptor that is highly expressed on effector T cells and plays an important role in T cell trafficking and function. Autoimmune cholangitis, primary biliary cirrhosis (PBC) among which, is a progressive autoimmune liver disease. As a mouse model of PBC, dnTGF- β R II mice fully mimic the clinical character of PBC patients. Our study aims at investigating how CXCR3 regulate the effector cells-T lymphocyte function in autoimmune liver disease. First, we detected that there is a high level of CXCR3 ligands-CXCL9 and CXCL10 in the liver of PBC model-dnTGF- β R II mice (TG mice). By flow cytometry we found that the frequency and number of CXCR3 expressing T cells are higher in liver of TG mice compared with wild type mice. In order to determine the role of CXCR3 in the progress of PBC, we crossed CXCR3 knockout mice with TG mice. Histological analysis showed that TGC3 (CXCR3^{-/-} dnTGF- β R II) mice have more severe infiltrations in the moderate portal vein in liver. Serological analysis showed that the level of anti-mitochondria antibody, which is the serological marker of this disease, and inflammatory cytokines are higher in TGC3 mice. Moreover, T cells, especially IFN- γ producing T cells and terminated KLRG1⁺ T cells increased after CXCR3 knockout in TG mice. From the above results, we know that CXCR3 chemokine axis may balance the choice between driving inflammation and dampening over-exuberant responses in liver tolerance. Further study is needed to illustrate the detailed mechanism of this regulation.

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Unraveling autoimmune diabetes by using genetically modified mouse models: from mechanism dissection to clinical application

Sytwu, H.-K.

National Defense Medical Center, Microbiology and Immunology, Taipei, Taiwan, Republic of China

Autoimmune diabetes is a T cell-mediated autoimmune disease. To delineate the protective roles of some immune modulatory molecules, including soluble decoy receptor 3 (DcR3), cytotoxic T lymphocyte antigen 4 (CTLA4), program death ligand 1 and 2 (PD-L1 and 2), heme oxygenase 1 (HO-1), and chemokine receptor D6 in the autoimmune process and to search for potential preventive and/or therapeutic targets in this disease,

we have generated

(a) insulin promoter (plns)-sDcr3 transgenic non-obese diabetic (NOD) mice,

(b) plns-single chain anti-CTLA4 transgenic NOD mice,

(c) plns-single chain anti-4-1BB transgenic NOD mice,

(d) plns-PD-L1 transgenic NOD mice,

(e) plns-HO-1 transgenic NOD mice, and

(f) plns-D6 transgenic NOD mice and demonstrated their immunomodulatory potential and underlying mechanisms.

Meanwhile, to explore the modulatory potential of interleukin-12, 23 and 27 on autoimmune diabetes, we have generated following transgenic, knockout and knockdown NOD mice:

(1) Th1 and Th2 doubly transgenic

(2) IL-12 knockout

(3) IL-23 knockdown

(4) IL-27 knockdown NOD mice.

Our results revealed that 20% IL-12-deficient NOD mice still developed autoimmune diabetes, the diabetic incidence of IL-23 knockdown NOD mice is lower than that of control littermates, and the number and percentage of Th1 cells are dramatically decreased and Th17 cells are increased in IL-27 knockdown mice, indicating a differential role of IL-12 cytokine family in modulating Th1 and Th17 cell development during autoimmune diabetogenic process. Making full use of these unique mouse strains, we are quantitatively and qualitatively investigating the immunopathogenic mechanisms of autoimmune diabetes and providing valuable information for the development of novel immunotherapies.

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CD1d and lipid regulation of Vdelta1 and Vgamma9 T cells in systemic sclerosis

Bank, L., Migalovich Sheikhet, H.

Sheba Medical Center, Ramat Gan, Israel

Vδ1+ T cell (TC) receptors recognize CD1d- presented lipids, and are activated oligoclonally in peripheral blood (PB) and organs in systemic sclerosis (SSc). To determine effects of the antigenic lipid (cardiolipin)- in SSc, mononuclear cells (MC) were isolated from PB of 12 SSc patients and 8 healthy controls (HC) and cultured in IL2C (RPMI-1640 with 10% FBS + 100 IU of IL-2), CARDC or OCHC [IL2C+cardiolipin 2.5µg/ml or OCH 50ng/ml (analogue of α-galactosyl ceramide) respectively]. Zoledronate (zol, 2µM), a Vγ9+ γδTC stimulant that induces their CD1d expression, was added to parallel cultures (labeled ILCzol etc.). Flow-cytometry (FC) after 4-5 day showed that Vδ1+ and Vγ9+TC consisted 11.85±2.0% and 14.7±3.5% vs 10.42±2.4% and 7.08±1.2% of the CD3+ TC in SSc and HC IL-2C respectively (not significantly different). In HC, %CD25+TC was not significantly altered by lipids absent of zol. However, IL2Czol increased CD25 expression, which was significantly further amplified on Vδ1+ TC in CARDCzol. In SSc, OCHC and CARDC slightly enhanced %CD25+Vδ1+TC versus IL2C whereas CARDC significantly suppressed %CD25+Vγ9+TC. In IL2Czol and CARDCzol, %CD25+Vγ9+TC increased. By contrast %CD25+Vδ1+TC decreased in IL2Czol relative to IL2C (p< 0.028) but increased in CARDCzol. 50-70% of SSc and HC

Vδ1+TC in IL2C reacted with native and OCH-bound CD1d tetramers, which was reduced in IL2Czol in SSc samples. Furthermore, blocking mAb against CD1d abolished CARDCzol induced elevated expression of CD25 in SSc Vδ1+ TC (n=5) specifically. Thus, CD1d mediated interactions with Vδ1+TC and lipid antigens may play a role in immune-dysregulation in SSc.

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Development of a model for antigen-specific tolerising immunotherapy and response in NOD mice

Buckle, L., Steptoe, R.J., Thomas, R., Hamilton-Williams, E.E.

University of Queensland Diamantina Institute, Translational Research Institute, Brisbane, Australia

Type 1 diabetes (T1D) results from autoimmune destruction of insulin-producing pancreatic β cells, involving CD4+ and CD8+ T cells. Non-obese diabetic (NOD) mice are a model for human T1D. As islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) is a major diabetogenic antigen in NOD mice, we developed a model to test IGRP antigen-specific immunotherapy. We showed previously that liposomes co-encapsulating antigen and NF-κB inhibitor induced antigen-specific suppression of CD4+ T cells in mice. These liposomes passively target and deliver their payload to dendritic cells in lymphoid organs, suppressing NF-κB activation.

For the current model, we injected NOD mice with liposomes co-delivering IGRP₂₀₆₋₂₁₄ peptide and NF-κB inhibitor. After adoptive transfer of CTV-labelled IGRP-specific CD8+ T cells, we analysed proliferation in lymphoid organs, enumerated adoptively-transferred and endogenous antigen-specific T cells using K^d-IGRP tetramers, and measured IFN-γ effector function by intracellular staining ex vivo.

To induce antigen-specific T cell activation, mice were immunized with IGRP₂₀₆₋₂₁₄ peptide and adjuvant. 5 days after immunization, IGRP-specific CD8+ T cells dramatically expanded and production of IFN-γ increased. Twenty four hours after liposome injection to naïve mice, we observed uptake of liposomes specifically by antigen presenting cells (APCs). Delivered peptide was presented by these APCs as CTV-labelled IGRP-specific CD8+ T cells proliferated in draining lymph nodes after subcutaneous delivery of liposomes co-encapsulating IGRP₂₀₆₋₂₁₄ and NF-κB inhibitor.

These data establish a model in NOD mice for analysis of diabetogenic antigen presentation and response to antigen-specific liposome immunotherapy to complement studies of immunotherapy to prevent or treat diabetes.

896

CD40-pathway activation in ectopic lymphoid structure (ELS)-resident B cells contributes to disease pathology in primary Sjögren's syndrome

Wieczorek, G., Bigaud, M., Pfister, S., Hoersch, S., McMichael, K., Afatsawo, C., Hamburger, M., Texier, C., Cojean, C., Henry, M., Rush, J.

Novartis Institutes of Biomedical Research, Basel, Switzerland

T cell-dependent antibody responses are a key effector arm of the adaptive immune system. These responses often occur in germinal centers (GCs) within secondary lymphoid organs.

Similar GC-like structures can be found in affected tissue in various autoimmune diseases, including salivary glands of primary Sjögren's syndrome (pSS) patients. Previous work has implicated CD40 signalling in the regulation of GC function, and we wanted to investigate whether it also played a role in ELS maintenance and function in pSS. Histological analysis of salivary gland biopsies from pSS patients revealed evidence of CD40 and CD154 expression on ELS-resident B and T cells respectively. These results suggested that there might be ongoing T-B cell collaboration in these ELS and we subsequently examined whether there was evidence of CD40 pathway activation *in situ*. Using a published microarray dataset generated using pSS parotid gland biopsies we could demonstrate upregulation of a B cell-specific CD40 pathway gene signature, suggesting that the CD40 pathway was activated in ELS-resident B cells. To directly address the role of CD40-CD154 in pSS ELS, we used an anti-CD154 antibody in NOD mice, a preclinical model of pSS. Therapeutic treatment of NOD mice resulted in disaggregation of established ELS as well as a reduction in the frequencies of T and B lymphocytes and IgG secreting cells in salivary glands. Collectively our data indicate that CD40 pathway signalling is essential for the maintenance of salivary gland ELS, supporting the notion that blockade of CD40-CD154 interactions may provide therapeutic benefit in pSS patients.

897

Variants of the pro-inflammatory P2X7 receptor on platelets are associated with the acute attack of multiple sclerosis

Gu, B.¹, Krupa, M.², Ou, A.¹, Slee, M.², Wiley, J.¹

¹University of Melbourne, The Florey Institute of Neuroscience, Melbourne, Australia, ²Flinders Medical Centre/Flinders University, Department of Neurology, Adelaide, Australia

Multiple sclerosis (MS) is an autoimmune inflammatory disease of the central nervous system characterized by demyelination and neuronal death. In discovery cohorts of Australasian patients with multiple sclerosis (total 2941 patients, 3008 controls) we examined the associations of twelve functional polymorphisms of P2X7, a microglial/macrophage receptor with proinflammatory effects when activated by extracellular ATP. In discovery cohorts, rs28360457, coding for Arg307Gln was associated with MS and combined analysis showed a two-fold lower minor allele frequency compared with controls (1.11% for MS and 2.15% for controls, $p=0.0000071$). A meta-analysis of three Australasian and four European cohorts indicated that Arg307Gln confers a 1.8-fold protective effect on MS risk (OR 0.57, $p=0.0000024$) [1]. In contrast a gain of function variant of P2X7, Ala348Thr was associated with an increased risk of MS (OR 1.10, $p=0.0006$) [1]. Fresh human monocytes heterozygous for Arg307Gln have >85% loss of 'pore' function of the P2X7 receptor measured by ATP-induced ethidium uptake. Flow cytometric analysis of both resting and activated human platelets showed binding of the L4 mAb specific for the extracellular domain of the P2X7 receptor. Microvesicles derived from activated platelets have been shown in MS blood [2], and these may arise from the action of ATP on platelet P2X7 to induce cell blebbing. Our data show the protective effect against MS of a loss of function variant of P2RX7 and suggest a role for platelet P2X7-

function in the acute MS relapse.

[1] Gu, BJ et al., *HumMolGen*, 2015;24:5644-5654

[2] Marcos-Ramiro, B et al., *BMCNeurosci*, 2014;15:110-123

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High throughput screening co-culture assay to identify novel targets in IBD

Stallen, J.¹, Legent, A.¹, Schluep, D.¹, van Berge, L.¹, Kartono, A.¹, de Pril, R.¹, van Es, T.¹, Vandeghinste, N.², Brys, R.², Janssen, R.¹

¹Galapagos, Leiden, Netherlands, ²Galapagos, Mechelen, Belgium

Inflammatory bowel disease (IBD) is an autoimmune disease characterized by inflammation at the intestinal epithelium which acts as a barrier to antigens and bacteria in the gut lumen and is involved in immune cell regulation. Dendritic cells (DC) as antigen-presenting cells play an important role due to their function of antigen presentation to T cells and secretion of proinflammatory cytokines. As the interaction between intestinal epithelium, DC and the bacteria in the lumen is crucial, we developed a high throughput co-culture assay and screened this with our adenoviral shRNA library to identify novel targets inhibiting the proinflammatory response to DC cytokines in response to *E.coli*. Caco-2 cells, cultured in 96 well transwell plates until polarized, were either transduced with adenoviral shRNA constructs or not transduced for compound screening. Monocyte derived DC were added to the basolateral compartment and the system was triggered with UV irradiated *E.coli* in the culture medium at the basolateral compartment. Compound treatment was performed by apical and basolateral addition to the culture medium prior to trigger addition. Chemokines/cytokines were measured in both apical and basolateral supernatants on the Flexmap3D with CCL20 as primary readout. Barrier function was assessed using FITC labelled dextran leakage through the epithelial barrier. Several novel targets that inhibited CCL20 secretion and prevented barrier disruption were identified using adenoviral shRNA and/or compounds. A robust high throughput screening assay was developed to identify and validate targets in a disease relevant co-culture system for IBD. Known targets were confirmed and novel targets identified.

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Identification of new therapeutic targets for the control of autoreactive plasma cells in inflamed kidneys in systemic lupus erythematosus

Natt, J.¹, Mathian, A.², Rucker-Martin, C.³, Amoura, Z.², Espéli, M.¹

¹UMR 996 - Inflammation, Chemokines and Immunopathology, Inserm, Univ Paris-Sud, Université Paris-Saclay, Clamart, France, ²Service de Médecine Interne 2, Centre de Référence National pour le Lupus et le Syndrome des Antiphospholipides, Institut E3M, Groupement Hospitalier Pitié-Salpêtrière, Assistance Publique-Hôpitaux de Paris, Paris, France, ³Centre Chirurgical Marie Lannelongue, Inserm UMR_S 999, LabEx LERMIT, Medical Research Department, Le Plessis-Robinson, France

Increased incidence of autoimmune diseases is a public health concern. Despite medical advances, they remain incurable and

are associated with a poor quality of life and vital prognostic. This is particularly true for systemic lupus erythematosus (SLE) that is a systemic autoimmune inflammatory disease involving several organs. One of the worst clinical manifestations of SLE is lupus nephritis (LN), where kidney damages progressively lead to an irreversible renal failure. Autoantibodies produced by autoreactive plasma cells (PCs) play a central role in the development of kidney inflammation. These autoreactive PCs are resistant to most therapies commonly used to treat autoimmune diseases. Moreover, we previously showed that they persist into specific niches within the inflamed kidneys. Controlling or eliminating autoreactive PCs within the inflamed kidney are thus promising therapeutic approaches for SLE.

Our project aims to identify and validate new therapeutic targets for the specific depletion of inflamed kidney PCs by focusing on their migratory potential and their survival niches. Firstly, we characterized the migratory barcode of PCs from SLE patients and a murine model of LN, the NZB/W F1 mice. Our current results highlight the potential role of two chemokine receptors. Secondly, we investigated the PC survival niche within the inflamed kidneys, with a specific focus on myeloid cells. We are currently determining the type of support, in particular the secretion of survival factors.

Altogether our results may open new therapeutic paths for the treatment of SLE and probably for other autoimmune diseases.

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Regulation of murine lupus nephritis and nephritic T cell responses by Ox40/Ox40L

Sitrin, J., Suto, E., Chu, F., Austin, C.D., Lee, W.P., Behrens, T.W. Genentech, South San Francisco, United States

Ox40L locus polymorphisms are strongly linked with risk for systemic lupus erythematosus (SLE) in multiple ancestries. However, exactly where and how Ox40L contributes to SLE pathology remains unclear, limiting our understanding of the underlying biology and the potential clinical value in targeting this pathway as a therapeutic modality. In this study, we evaluated the contribution of the Ox40L and Ox40 receptor pathway using a combination of *in vivo* antagonist and agonist approaches in the classical spontaneous and interferon alpha-accelerated NZB/W murine model of SLE and lupus nephritis (LN). Ox40 was expressed by CD4⁺ T cells in the spleen and kidney on cells with an activated/memory phenotype, accumulating in a pattern correlating to disease severity. Administration of an antagonist Ox40:Fc reagent limited the pathogenicity of interferon alpha-accelerated disease, including a delayed onset of severe proteinuria and extended survival. Administration of an agonist anti-Ox40 monoclonal antibody provoked systemic and nephritic T cell responses, impacted the relative presence of glomerular and interstitial T cells, and exacerbated renal disease. Notably, the renal pathogenicity of anti-Ox40 treatment was limited to older mice with underlying mild renal disease. Both the antagonist and agonist approaches proceeded without altering serum dsDNA autoantibody titers. Collectively, these data support a scenario in which the Ox40/Ox40L pathway regulates LN and renal disease, likely by impacting nephritic T cell responses. Our findings support a pathogenic role for T

cells in SLE and LN, and highlight the potential clinical value in targeting the Ox40/Ox40L pathway as a therapy for SLE.

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CD8⁺ T cells effect glomerular injury in experimental anti-myeloperoxidase glomerulonephritis

Ooi, J.D.¹, Chang, J.¹, Eggenhuizen, P.J.¹, O'Sullivan, K.M.¹, Holdsworth, S.R.^{1,2}, Kitching, A.R.^{1,2,3}

¹Monash University, Centre for Inflammatory Diseases, Clayton, Australia, ²Monash Health, Dept. of Nephrology, Clayton, Australia, ³Monash Health, Dept. of Paediatric Nephrology, Clayton, Australia

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis is an autoimmune disease that manifests as focal and necrotizing glomerulonephritis and renal failure. Observations in patients suggest that CD8⁺ T cells participate in disease, but there is no experimental functional evidence of their pathological involvement. Myeloperoxidase (MPO) is a well defined autoantigen in ANCA-associated vasculitis and following ANCA-induced neutrophil localization, deposited MPO within glomeruli is recognized by autoreactive T-cells that contribute to injury.

The current studies test the hypothesis that CD8⁺ T-cells mediate disease in experimental ANCA-associated vasculitis. CD8⁺ T-cells were depleted in experimental murine anti-MPO glomerulonephritis using monoclonal antibodies. Candidate CD8⁺ T-cell epitopes were identified *in silico* using the IEDB and their immunogenic and cytotoxicity assessed using flow cytometric tetramer based and cytotoxicity assays. The pathogenicity of MPO-specific CD8⁺ T-cells was determined by transferring T-cell clones into *Rag1*^{-/-} mice.

CD8⁺ T-cell depletion in the effector phase of disease attenuated injury in murine anti-MPO glomerulonephritis. This was associated with decreased intrarenal IFN- γ and TNF- α , reduced inflammatory chemokines and fewer glomerular macrophages. A pathogenic CD8⁺ T-cell MPO epitope (MPO₄₃₁₋₄₃₉) was identified and transferred MPO₄₃₁₋₄₃₉-specific CD8⁺ T-cell clones exacerbated disease when co-transferred with MPO-specific CD4⁺ cells. Transfer of MPO₄₃₁₋₄₃₉-specific CD8⁺ T-cells, without CD4⁺ T-cells, could mediate glomerular injury when MPO was planted in glomeruli. These results demonstrate a pathogenic role for MPO-specific CD8⁺ T-cells, provide evidence that CD8⁺ T-cells are a therapeutic target in ANCA-associated vasculitis and suggest that a molecular hot spot within the MPO molecule contains important CD8⁺, CD4⁺ and B cell epitopes.

902

Elevation of serum Sema4A in neuromyelitis optica spectrum disorders (NMOSD)

Sakakibara, K.¹, Okuno, T.^{1,2}, Sakakibara, S.^{2,3}, Kikutani, H.^{2,3}, Mochizuki, H.¹, Kumanogoh, A.^{2,4}

¹Osaka University Graduate School of Medicine, Department of Neurology, Suita, Japan, ²World Premier International Immunology Frontier Research Center, Osaka University, Suita, Japan, ³Research Institute for Microbial Disease, Osaka university, Department of Molecular Immunology, Suita, Japan, ⁴Osaka University Graduate School of Medicine, Department of Respiratory Medicine, Allergy and Rheumatic Disease, Suita, Japan

Background: Sema4A is a class IV semaphorin, which plays an important role in the activation of Th cells. We previously established ELISA system to measure Sema4A and reported that serum Sema4A levels are significantly higher in patients with multiple sclerosis (MS) than those with other neurological diseases. Although Th cells as well as anti-aquaporin 4 antibodies (AQP4ab) play important roles in neuromyelitis optica spectrum disorders (NMOSD), the involvement of Sema4A in NMOSD remains to be elucidated.

Methods: Serum Sema4A levels were analyzed by ELISA in 38 patients with NMOSD and 129 patients with MS. All NMOSD patients were positive for serum AQP4ab. MS was diagnosed according to MacDonald criteria. We compared clinical characteristics of patients with high Sema4A levels to those with low Sema4A.

Results: The serum Sema4A levels were significantly higher in NMOSD patients than those with MS. The mean age of onset in patients with high Sema4A levels was significantly younger than those with low Sema4A levels. Patients with higher Sema4A levels had significantly less severe the Expanded Disability Status Scale (EDSS) score during exacerbations and remissions. Their relapse rate tended to decrease but this difference was not significant.

Conclusions: The levels of serum Sema4A in NMOSD is as high as those in MS, suggesting that Sema4A plays important roles in the pathogenesis of NMO.

903

Irradiation on NZBWF1 mice is an effective therapy for SLE-like morbidity

Fujita, K.^{1,2,3}, Kuwabara, T.⁴, Tome, Y.¹, Ito, K.^{1,2}, Tsukimoto, A.^{1,5}, Yoshida, Y.^{1,6}, Wang, B.³, Tanaka, K.³, Mikami, T.², Akasaka, Y.²

¹Tsukuba International University, Medical Technology, Tsuchiura, Ibaraki, Japan, ²School of Medicine, Toho University, Pathology, Tokyo, Japan, ³National Institute of Radiological Sciences, Research Center for Radiation Protection, Chiba, Japan, ⁴School of Medicine, Toho University, Immunology, Tokyo, Japan, ⁵Graduate School of Engineering, Chiba Institute of Technology, Narashino, Chiba, Japan, ⁶Graduate School of Comprehensive Human Sciences, University of Tsukuba, Doctoral Program in Sports Medicine, Tsukuba, Ibaraki, Japan

CD180 molecule expressed on mature B cells is a key regulator of cell proliferation and death, showing a protective effect against radiation-induced apoptosis *in vitro*. In SLE patients, the number of CD180-negative cells increased in peripheral blood, and appeared to be associated with the activity of SLE. Furthermore, production of autoantibodies was found by CD180-negative cells from SLE patients. We previously showed that CD180-negative cells were more radiosensitive than CD180-positive cells *in vivo*. In SLE-model NZBWF1 mouse, we also reported the increase in number of CD180-negative B cells in parallel with development of SLE morbidity, the production of autoantibodies by CD180-negative cells, and pathogenesis of lupus-like nephritis caused by CD180-negative cells. In the present study, we attempted to examine the effects of irradiation on CD180-negative cells in NZBWF1 mice. After the onset of symptoms, mice were paired according to their conditions. In each pair, one was irradiated

with 4 Gy, and the other was sham-irradiated. Compared to the sham-irradiated mice, the mean number of CD180-negative cells was significantly decreased, the mean life span was markedly extended, and the nephritis was suppressed in irradiated mice. The results suggest that radiation could effectively eliminate CD180-negative cells, leading to reduction of autoantibody production, and consequently relieve the SLE symptoms in the mouse. These findings suggest that the radiotherapy of autoimmune diseases associated with the expression of CD180-negative cells is a novel candidate for practical application.

904

Rethinking Rowell syndrome

Sacco, K.¹, Pongdee, T.²

¹Mayo Clinic, Internal Medicine, Jacksonville, United States, ²Mayo Clinic, Allergy and Immunology, Jacksonville, United States

Introduction: Patients meeting diagnostic criteria for systemic lupus erythematosus (SLE) but also having erythema multiforme-like lesions are diagnosed as suffering from Rowell Syndrome (RS). We present a case and review the literature discussing the relevance of this diagnosis to clinical immunology.

Methods: Case report (exempt from IRB approval) and literature review.

Case report: A 51 year old female presented with a 6 week history of a facial rash, oral ulcers, diffuse joint pain and malaise. She had no significant past medical history, no known allergies and family history was unremarkable. The rash was diffusely targetoid and superficially desquamative compatible with a diagnosis of erythema multiforme. Immunoserology was positive for ANA and anti-Sm antibodies. Thus the patient met American College of Rheumatology criteria for diagnosis of SLE. She was started on prednisone 40mg daily with a tapering regime, and hydroxychloroquine was added 1 week later with marked clinical improvement noted after 12 days.

Conclusion: RS typically affects middle-aged women similar to SLE. The majority of patients show a speckled anti-nuclear antibody pattern on autoantibody testing with rheumatoid factor and anti-Ro/anti-LA antibody positivity being reported to a lesser extent. Clinical remission is achieved with similar immunosuppressant medications used in SLE, namely prednisone, azathioprine, antimalarials and cyclosporine. No distinct immunopathogenesis or prognostic outcome between SLE and RS have been described to date. Thus, questions remain as to whether RS is an overlap syndrome, RS is a subset of SLE or a chance event, and if RS influences patient outcomes and disease morbidity.

905

Autoimmune associated missense mutation in the human gene *TNIP1* does not affect protein cellular localization

Simpfendorfer, K.R.¹, Armstead, B.E.¹, Diamond, B.², Lee, A.¹, Gregersen, P.K.¹

¹Feinstein Institute for Medical Research, Robert S. Boas Center for Genomics and Human Genetics, Manhasset, United States,

²Feinstein Institute for Medical Research, Center for Autoimmune and Musculoskeletal Diseases, Manhasset, United States

A risk haplotype (H1) spanning 30kb of the *TNIP1* gene is associated with risk for Myasthenia Gravis, Systemic Lupus Erythematosus and Systemic Sclerosis in genome-wide association studies. A missense mutation within the H1 risk haplotype is the lead candidate causal mutation and converts a proline to alanine at amino acid 151. Association of the *TNIP1* P151A mutation with multiple B-cell mediated autoimmune diseases across ancestral groups suggest that *TNIP1* P151A is involved in a common mechanism for B-cell mediated autoimmune disease. We confirmed that *TNIP1* is most highly expressed in B cells, compared to other leukocyte populations in human peripheral blood. The TNIP1 protein is reported to function as a co-regulator of NF κ B signaling, as well as a nuclear co-receptor with retinoic acid receptors RAR and PPAR. Amino acid 151 is located near a nuclear export sequence in the TNIP1 protein and substitution of amino acid 151 with an alanine is predicted to be deleterious to the stability of the TNIP1 protein. Given the differing functional roles of TNIP1 in the cytoplasm and nucleus we first investigated if the missense mutation 151A affects the localization of TNIP1 to the nucleus or cytoplasm, there was no difference. Our future studies will investigate the affect of the 151A mutation on NF κ B signaling in B cells from human peripheral blood.

906

TREM-1 knockout promotes lupus-like syndrome in mice

Sun, K.-H.¹, Liu, C.-J.²

¹National Yang-Ming University/Taipei City Hospital, Department of Biotechnology and Laboratory Science in Medicine/Department of Education and Research, Taipei, Taiwan, Republic of China,

²National Yang-Ming University, Department of Biotechnology and Laboratory Science in Medicine, Taipei, Taiwan, Republic of China

Systemic lupus erythematosus (SLE) is a multi-organ autoimmune disease. Inflammatory responses induced by innate immune system play important roles in disease pathogenesis. Triggering receptor expressed on myeloid cells-1 (TREM-1) is a novel innate immune receptor which amplifies TLR-induced inflammatory response. TREM-1 plays important role in many infectious diseases and aseptic inflammatory diseases. However the precise functions of TREM-1 in diseases remain controversial. Blockage of TREM-1 reduced the inflammation in mice with sepsis and collagen-induced arthritis (CIA) but promoted microbial-induced sepsis, pneumonia and liver abscess. Moreover, levels of soluble form of TREM-1 (sTREM-1) were found elevated in sera of SLE patients. These results suggest that TREM-1 may be involved in the progression of SLE. In this study, we established TREM-1^{-/-}.Lpr mice to investigate the role of TREM-1 in SLE. We found upregulated TREM-1 expression in spleen and lymph nodes of B6.Lpr mice. In addition, we found TREM-1^{-/-}.Lpr mice exhibited significantly lower survival rate and more severe syndromes, including higher spleen weight, lymph node weight, proteinuria, anti-dsDNA, anti-Sm/RNP antibodies, and renal immune complex deposition. Peripheral lymphocytes as well as myeloid dendritic cells, plasmacytoid dendritic cells, B cell and T cell subpopulations in both spleen and lymph nodes of TREM-1^{-/-}.Lpr mice were greatly increased. Importantly, the expansion of follicular B cells and plasma cells were consistent

with elevated serum B-cell activating factor (BAFF) levels in TREM-1^{-/-}.Lpr mice. The results suggest TREM-1 deficiency promotes lupus progression.

907

Fc receptor-like proteins (FCRL) 3 polymorphisms in Hashimoto's disease

Kalantar, K.¹, Ghandehari, F.², Dabbaghmanesh, M.H.², Malek Hosseini, S.³, Amirghofran, Z.¹

¹School of Medicine, Shiraz University of Medical Sciences, Immunology Department, Shiraz, Iran, Islamic Republic of,

²Endocrinology and Metabolism Research Center, Namazi

Hospital, Shiraz University of Medical Sciences, Shiraz, Iran, Islamic Republic of, ³Shiraz University of Medical Sciences, Immunology

Department, Shiraz, Iran, Islamic Republic of

Hashimoto's disease (HD) is a archetypal organ specific autoimmune diseases. The substantial contribution from genetic factors in susceptibility to HD has been well-defined. The Fc receptor-like3 (FCRL3) gene is one of the genes that have recently shown a significant association with HD. To determine the possible role of FCRL3-169 C/T and FCRL3-110 A/G gene polymorphisms in the development of HD in Iranian patients, 220 HD patients and 302 healthy subjects were genotyped by polymerase chain reaction-restriction fragment length polymorphism. No significant difference was found in genotype and allele frequencies of FCRL3-169 C/T between patients and controls (Pv=0.55). In regards to position -110 A/G also no significant difference was found for genotype and allele (Pv= 0.6) between two groups of study. In conclusion, the results of this study showed no significant association between FCRL3-169 C/T and FCRL3-110 A/G polymorphism and susceptibility to HD.

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Autophagy is suppressed by immune complex and TNF-alpha in glomerular endothelial cells

Wang, L., Law, H.K.W.

Hong Kong Polytechnic University, Department of Health

Technology and Informatics, Faculty of Health and Social Science, Hong Kong, China

Background: Lupus nephritis (LN) is a common complication of SLE. Glomerular endothelial cell (GEC) is one of the three components of the glomerular filtration barrier, which is the structural foundation of renal functions. However, GECs have been little studied and the precise mechanisms by which GECs are injured in LN remain unknown. Autophagy is a conserved metabolic process and shows protective roles in many cell types under stresses. Our previous results showed that heat-aggregated gamma globulin (HAGG), a substitute for immune complex, led to suppressed autophagy and injuries in GECs. TNF-alpha is elevated in serum and overexpressed in kidney in LN patients. Thus, in present study, we investigated the roles of TNF-alpha on GEC autophagy and dysfunctions.

Methods: GECs were incubated with TNF-alpha alone or in combination with HAGG. Proteins related to autophagy,

including LC3, p62, and mTOR, were determined by western blotting. Endothelial NOS (eNOS) expression was measured to evaluate endothelial function.

Results: TNF- α led to increased expressions of p62 and phosphorylated-mTOR (Ser2448) in GECs. Intriguingly, the ratio of LC3 II/I was slightly elevated. GECs were injured as phosphorylated-eNOS (Ser1177) expression was decreased. Co-stimulation of GECs with TNF- α and HAGG led to further decrease in phosphorylated-eNOS expression.

Conclusions: Our results showed that TNF- α suppressed autophagy in GECs through an mTOR-dependent pathway and led to cell injury. Co-stimulation of GECs with TNF- α and HAGG further damaged the cells. The suppressed autophagy in GECs may provide new insights for the mechanisms of renal impairments in LN patients.

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Characterization of the Systemic Lupus Erythematosus associated single nucleotide polymorphism RS1143678 in integrin α M(CD11b) subunit

Ong, L.T., Tan, S.M.

Nanyang Technological University, School of Biological Sciences, Singapore, Singapore

Systemic Lupus Erythematosus (SLE) is a chronic autoimmune disease that affects skin, joints, kidneys, brain and other organs. Genome wide analysis studies have identified single nucleotide polymorphisms in integrin α M β 2 (CD11bCD18) that are associated with SLE. Integrin α M β 2 is involved in various myeloid immune responses, including cell migration, phagocytosis, oxidative burst, and tolerance. Herein, we examined the single nucleotide polymorphism RS1143678 that substitutes Pro1146 to Ser in the cytoplasmic tail of integrin α M. Using reconstituted bone marrow-derived macrophages, we showed that integrin α MP1146S β 2 promoted cell adhesion and spreading, exhibited faster kinetics in terms of ERK1/2 phosphorylation and augmented IL-6 production in the presence of LPS compared with wild-type α M β 2 cells. Neutrophils expressing integrin α MP1146S β 2 also showed elevated adhesive capacity when stimulated with PDBu compared to cells expressing wild-type α M β 2. Inhibition of SFK and Syk pathways blunted integrin α MP1146S β 2 induced ERK1/2 activation. The different properties of integrin α MP1146S β 2 compared with wild-type α M β 2 may be attributed to a higher propensity of α MP1146S β 2 to cluster. Altogether, these data suggest that RS1143678 (integrin α MP1146S β 2) confers a pro-inflammatory property to myeloid cells.

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The avidity of T cell receptor for self peptide-MHC complex dictates the diabetogenicity of CD8⁺ T cells in autoimmune diabetes

Yeh, L.-T., Chen, Y.-P., Sytwu, H.-K.

National Defense Medical Center, Taipei, Taiwan, Republic of China

Ptpn22 encodes PEST domain-enriched tyrosine phosphatase (Pep), a negative regulator of TCR proximal signaling molecules. We previously generated a T cell-specific *Ptpn22* transgenic

mouse model and demonstrate different modulation of *Ptpn22* in effector and regulatory T cells leads to attenuation of autoimmune diabetes in transgenic nonobese diabetic (NOD) mice. Recently, we observe that transgenic Pep attenuates the proliferation and diabetogenicity of CD8⁺ T cells in NOD mice but not in NOD8.3 TCR transgenic mice. Moreover, the TCR-induced cell proliferation of NOD8.3 CD8⁺ T cells is higher than that of NOD T cells, suggesting the regulatory potential of transgenic Pep on CD8⁺ T cells may be affected and/or masked by a selection bias of 8.3 TCR transgenic T cells. Recent studies demonstrate that the avidity of TCR for self peptide-major histocompatibility complex (self-pMHC) can be reflected in CD5 expression, further dictates the responses of naive T cells to foreign antigens. We observe a higher expression of CD5 in NOD8.3 CD8⁺ T cells than in NOD CD8⁺ T cells. Moreover, the CD5 expression on NOD CD8⁺ T cells positively correlates with the expressing profile of effector molecules and the response of cells to TCR stimulation, suggesting that the high self-recognition cells dominate the immune response to autoantigens in autoimmune diabetes. Taken together, the avidity of TCR for self-pMHC dictates the diabetogenicity of CD8⁺ T cells and masks the regulatory potential of transgenic *Ptpn22* in autoimmune diabetes.

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Autoimmune manifestations of chronic Hepatitis C virus infection

Deshpande, P.¹, Bundell, C.^{2,3}, Mckinnon, E.⁴, Hellard, M.^{5,6,7,8}, Ffrench, R.^{9,10}, Wilkinson, A.⁵, Drummer, H.^{9,10,11,12}, Gaudieri, S.^{1,4,13}, Lucas, M.^{2,3,4,14}

¹University of Western Australia, School of Anatomy, Physiology and Human Biology, Perth, Australia, ²PathWest, Laboratory Medicine, Department of Clinical Immunology, Perth, Australia, ³University of Western Australia, School of Pathology and Laboratory Medicine, Perth, Australia, ⁴Institute for Immunology and Infectious Diseases, Perth, Australia, ⁵Burnet Institute, Center for Population Health, Melbourne, Australia, ⁶Infectious Diseases Unit, The Alfred Hospital, Melbourne, Australia, ⁷Monash University, Department of Epidemiology and Preventive Medicine, Melbourne, Australia, ⁸Burnet Institute, Centre for Research Excellence in Injecting Drug Use, Melbourne, Australia, ⁹Monash University, Department of Immunology, Melbourne, Australia, ¹⁰Centre for Biomedicine, Melbourne, Australia, ¹¹Monash University, Department of Microbiology, Melbourne, Australia, ¹²Peter Doherty Institute for Infection and Immunity at The University of Melbourne, Department of Microbiology and Immunology, Melbourne, Australia, ¹³Vanderbilt University, Division of Infectious Diseases, Department of Medicine, Nashville, United States, ¹⁴School of Medicine and Pharmacology, Harry Perkins Building, University of Western Australia, Perth, Australia

Background: Hepatitis C virus (HCV) infection has been linked to systemic auto-immunity. This may have prognostic implications in the clinical management of HCV infection. Despite multiple observational studies, there does not appear to be any study that has systematically evaluated the prevalence rate of serological markers relevant to autoimmune manifestations in HCV infection compared to their prevalence in a general population.

Method: This study examines a panel of diagnostic autoantibodies (AABs) in subjects with chronic HCV infection (n=142) and age and gender matched general Australian population (n=198). AABs tested were: anti-nuclear antibodies (ANA), extractable nuclear antigen, anti-neutrophil cytoplasmic antibodies (ANCA), myeloperoxidase, proteinase 3, glomerular basement membrane, smooth muscle antibody (SMA), mitochondrial, liver kidney microsomal, β 2 glycoprotein I IgG/M/A, cardiolipin, tissue transglutaminase, parietal cell, intrinsic factor, thyroid peroxidase and cyclic citrullinated peptide.

Results: HCV subjects showed increased positivity for at least one autoantibody as compared to controls ($p < 0.0001$), with no additional gender effect. ANA followed by SMA were the most frequently detected autoantibodies within the HCV cohort. The SMA was commonly classified as mainly non-specific vessel and glomerular staining and not directed against the F-actin, which is specific for autoimmune hepatitis. ANCA were detected more frequently in the HCV cohort compared to the general population. Variation at IL-28B was not associated with AAB positivity.

Conclusion: A systemic comparison between two cohorts shows significant differences in autoimmune manifestations. However, due to the low level and lack of high specificity of some of the AABs they are unlikely to impact clinical outcomes.

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Restraint of autoreactive T cell expansion is defective in female BALB/c ZAP70^{W163C} mutant (SKG) mice

Rahman, M.A., Montizaan, D., Bhuyan, Z.A., Rehaume, L.M., Thomas, R.
The University of Queensland Diamantina Institute, Brisbane, Australia

In the context of reduced TCR signaling in the BALB/c ZAP70^{W163C} mutant (SKG) mouse, auto-reactive CD4⁺ T cells are increased and female mice develop arthritis, psoriasis-like skin inflammation and ileitis within weeks after intra-peritoneal injection of microbial β -glucan (curdlan). Curdlan-triggered disease is mild in BALB/c mice and male SKG mice. It is not known how systemic adjuvant promotes T-cell-mediated tissue inflammation or the influence of gender. To elucidate where CD4⁺ T cells expand, syngeneic luciferase-expressing CD4⁺ T cells were adoptively transferred, and tracked *in vivo* post-curdlan in female SKG mice using *in vivo* bioluminescence imaging. Bioluminescence increased in spleen and inguinal lymph nodes 3 days post-curdlan, followed by focal signals from the ankles, tail, ears and abdomen at day 7, and wrists at day 10. Without curdlan, CD4⁺ T cells expanded to a lesser extent in lymph nodes and spleen by day 7, but did not traffic to joints. Signal from transferred SKG T cells increased at low level in spleen of curdlan-treated but not naive female BALB/c recipients from day 7-10 and subsequently disappeared. In male SKG recipients, signal from transferred SKG T cells increased at low level in spleen of curdlan-treated SKG but not BALB/c recipients. These data indicate that self-reactive SKG T cells spontaneously expand in lymphoid organs of female SKG mice. Systemic curdlan increases T cell activation and tissue infiltration in females, but autoreactive T cell activation is restrained in BALB/c hosts and male SKG host mice, implying deficient peripheral tolerance specifically in female SKG hosts.

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Targeted delivery of withaferin-A incorporated mannosylated liposomes to the synovial macrophages suppresses the inflammatory immune response in adjuvant-induced arthritic rats - a potential therapeutic approach

Sultana, F., Rasool, M.

VIT University, School of BioSciences and Technology, Vellore, India

Rheumatoid arthritis is characterized by a chronic inflammation of the joint synovium where macrophages are activated and proliferate in an abnormal way, leading to the production of pro-inflammatory cytokines and inflammatory enzymes. To diminish inflammation and to prevent irreversible joint damage, selective counteraction of macrophage activation seems to be an appropriate approach. Therefore, our study was designed to explore whether withaferin-A, a steroidal lactone incorporated mannosylated liposomes are effective in suppressing the inflammatory immune response in the synovial macrophages of adjuvant-induced arthritic rats. Arthritis was induced by intradermal injection of complete Freund's adjuvant (0.1ml) into the right hind paw of the Wistar albino rats. The rats were treated intravenously with either withaferin-A incorporated mannosylated liposomes (10mg/kg/b.wt) for 3 days (11th, 14th, 17th day) or free withaferin-A alone (30mg/kg/b.wt) or saline for 8 days (from 11th to 18th day) after adjuvant injection. Withaferin-A incorporated mannosylated liposomes suppressed the paw swelling and levels of oxidative stress markers (ROS and NO) and pro-inflammatory cytokines (TNF- α , IL-6, and IL-1 β) in synovial macrophages of arthritic rats. Consistently, the expression of pro-inflammatory cytokines (TNF- α , IL-6, and IL-1 β), inflammatory enzymes (iNOS and COX-2) and transcription factor NF-kBp65 was also found downregulated at both the gene and protein level. These results indicate that the efficacy of withaferin-A incorporated mannosylated liposomes suppressed inflammatory immune response in arthritic synovial macrophages more effectively than the free drug withaferin-A.

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The potential role of human endogenous retrovirus HERV-K10 in the pathogenesis of rheumatoid arthritis through molecular mimicry

Trela, M.¹, Nelson, P.¹, Ryland, P.²

¹University of Wolverhampton, Faculty of Science and Engineering, Wolverhampton, United Kingdom, ²Royal Wolverhampton New Cross Hospital NHS Trust, Wolverhampton, United Kingdom

Human endogenous retroviruses (HERVs) constitute 8% of the human genome and represent ancient retroviral infectious agents that have since been incorporated into our DNA. HERVs have then been inherited through successive generations in a Mendelian manner. Whilst many HERV families are defective, some can become activated and produce viral particles and products.

HERV-K10 is one such virus that has been linked with rheumatoid arthritis (RA). Preliminary analysis of HERV-K10 has identified a highly antigenic epitope within the Gag matrix region of this virus. Following the development of a representative synthetic

peptide (GKELK; defined as MAG1), significant IgG antibody reactivity to this epitope has been observed in patients with RA as compared to controls. A homologous region (GKEYK) within IgG1Fc, a key auto-antigen in RA, was revealed. On developing a polyclonal antibody (PAbMAG1) to the viral synthetic peptide, we have demonstrated immunological cross-reactivity in a range of immunoassays to IgG1Fc. We have also shown that the epitope on IgG1Fc, recognized by PAbMAG1, is targeted by Rheumatoid Factor (RF) auto-antibodies generated in RA.

These results confirmed immune reactivity to both virus and auto-antigen thus highlighting the potential for molecular mimicry between HERV-K10 Gag matrix and IgG1Fc. Consequently, HERV-K10 could trigger and/or augment reactivity to IgG1Fc in RA patients and provide a mechanism which contributes to RA pathogenesis.

Finally our results raise the possibility of HERV-K10 peptide or antibody as potential therapeutic blocking agents of rheumatoid factors which could transform patient therapy. A novel diagnostic/prognostic test for RA may also be provided.

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Unravelling the multiple sclerosis complex disease trait through an immune transcriptional regulatory network approach

Jordan, M.A.¹, Gresle, M.², Laverick, L.², Stanley, D.³, Smith, L.¹, Spelman, T.², Field, J.⁴, Johnson, L.⁴, Butzkueven, H.², Baxter, A.G.¹

¹Comparative Genomics Centre, James Cook University, Molecular and Cell Biology, Townsville, Australia, ²University of Melbourne, Department of Medicine, Melbourne, Australia, ³Central Queensland University, Rockhampton, Australia, ⁴The Florey Neuroscience Institute, Melbourne, Australia

Multiple Sclerosis (MS), the most common disabling neurological disease affecting young adults in developed countries, is a complex genetic disease associated with both environmental and genetic risk factors. In most cases, the risk factors' individual associations with MS are so weak that any meaningful understanding of the disease will require the identification of molecular pathways that contribute to MS liability. We therefore hypothesised that the complex genetic phenotype is driven by a co-ordinated expression of transcriptional regulatory networks. To test this, we generated a weighted gene co-expression network based on 712 pooled Affymetrix Human Gene 1.0 ST array analyses of magnetic bead sorted B cells, CD4 and CD8 T cells, NK cells and monocytes, from 67 untreated relapsing/remitting MS patients and 102 Healthy Controls (HC). Sixteen relatively independent gene modules were identified. For each leukocyte population, the strength of differential expression between patients and HC was assessed, by ranking genes by Mann Whitney U test and ANOVA, and each transcript was tested across the network to identify modules of interest. A group of transcripts we named the "Black" module was most significantly associated with MS in monocytes & was strongly down-regulated in patients. Twelve highly differentially expressed genes with high centrality were identified and the top annotation clusters comprised the immune processes: Natural killer cell mediated cytotoxicity & Antigen processing and presentation. We propose that manipulating the module

as a whole may provide a new perspective on the aetiology of complex genetic diseases and offer novel therapies for MS.

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Role of PD-L2 in B lymphocytes during the development of lupus

Hidalgo, Y.¹, Fuenzalida, M.J.¹, Roseblatt, M.², Sauma, D.¹, Bono, M.¹

¹Universidad de Chile, Biology, Santiago, Chile, ²Fundación Ciencia & Vida, Biology, Santiago, Chile

Introduction: Systemic lupus erythematosus is an autoimmune disease characterized by activation of autoreactive T and B lymphocytes, autoantibody production and immune complex deposition, which causes damage to various tissues and organs. Autoimmune diseases are caused by the loss of immunological tolerance. Tolerance is regulated by a balance between stimulatory and inhibitory signals between antigen presenting cells and T lymphocytes. PD-L2 is an inhibitory ligand expressed on antigen presenting cells that binds to PD-1 expressed in T lymphocytes.

Materials and methods: We evaluated the expression of PD-L2 in the B lymphocytes of mice susceptible to the development lupus (BWF1).

Results: Our results demonstrate that during the development of the disease there is an increase in the number of B cells in the spleen. Moreover, we observed a significant rise in the percentage of B cells in the thymus. In both, thymus and spleen, B cells show a reduced expression of PD-L2. To assess the role of PD-L2 on B cells in the development of lupus we compare the ability of B cells before and after the development of lupus to inhibit the proliferation of antigen-specific CD4+ T lymphocytes and their capacity to differentiate into cells secreting anti-DNA autoantibodies.

Conclusions: Together our results suggest that expression of PD-L2 in B lymphocytes exert a protective role in the development of lupus.

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A novel drug targeting dihydroorotate dehydrogenase for treating inflammation and chorioretinal neovascularization in relapsing-remitting and chronic experimental autoimmune uveitis

Diedrichs-Möhrling, M.¹, Niesik, S.¹, Lebn, J.², Strobl, S.², Obermayr, F.³, Thurau, S.¹, Wildner, G.¹

¹Clinic of the University of Munich, Dept. of Ophthalmology, Section of Immunobiology, Munich, Germany, ²4SC Discovery GmbH, Planegg-Martinsried, Germany, ³Panoptes Pharma GmbH, Vienna, Austria

Experimental autoimmune uveitis (EAU) in rats is a suitable model for the respective intraocular inflammatory disease in humans. We investigated the effect of the dihydroorotate dehydrogenase inhibitor PP-001 in two EAU models: a spontaneously relapsing-remitting model and a monophasic/chronic disease resulting in chorioretinal neovascularization.

Daily oral applications of PP-001 after immunization with retinal S-Antigen peptide PDSAg (induces monophasic uveitis and neovascularizations) or the interphotoreceptor retinoid-binding protein peptide R14 (induces spontaneously relapsing-remitting EAU) abrogated both types of EAU. Uveitis was graded clinically and histologically.

Oral treatment after onset or peak of PDSAg-induced EAU significantly reduced neovascularizations (as determined by histology), and a significant reduction of the number and intensity of relapses in R14-induced EAU was observed when PP-001 treatment was initiated after resolution of the first attack of uveitis. Intraocular injection of PP-001 after resolution of R14-induced EAU also suppressed the number and intensity of relapses. PP-001 showed no toxic effect on rat eye tissues after intraocular injection.

Proliferation of autoantigen-specific rat T-cell lines and secretion of IFN- γ , IL-17, IL-10, IP10 and VEGF were efficiently suppressed by PP-001. PP-001 also suppressed proliferation and cytokine secretion of PHA-stimulated human PBL without affecting the viability of the cells. Treating the human retinal pigment epithelial cell line ARPE-19 with PP-001 no suppressive effect on proliferation, viability and VEGF production was observed.

Here we present a novel drug for systemic and local treatment of autoimmune uveitis without adverse effects on resident ocular cells.

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The pemphigus-differentially expressed miR-223-3p induces p38 MAPK phosphorylation and IL-8 secretion possibly through direct inhibition of DUSP10

Cipolla, G.A.¹, Park, J.K.², Tadra-Sfeir, M.Z.³, Almeida, R.C.¹, Malheiros, D.¹, Souza, E.M.³, Roselino, A.M.⁴, Lavker, R.M.², Petzl-Erler, M.L.¹

¹Federal University of Parana, Department of Genetics, Curitiba, Brazil, ²Northwestern University Feinberg School of Medicine, Department of Dermatology, Chicago, United States, ³Federal University of Parana, Department of Biochemistry, Curitiba, Brazil, ⁴University of São Paulo, Division of Dermatology, School of Medicine of Ribeirão Preto, São Paulo, Brazil

MicroRNAs (miRNAs) are small non-coding RNAs that fine tune gene expression, being involved in virtually all signaling pathways. Pemphigus is an autoimmune skin disease characterized by IgG autoantibodies capable of activating different signaling pathways that ultimately lead to loss of keratinocyte intercellular adhesion, the disease hallmark. To understand the role of miRNAs in pemphigus, we first established the differentially expressed miRNAs between lesional and non-lesional skin biopsies of three pemphigus foliaceus (PF) patients through small RNA-sequencing. We identified miR-223-3p as the potentially most upregulated miRNA in PF lesions ($P=0.09$; fold change 7.58), and further confirmed this result in a larger sample ($n=7$) comprising also two pemphigus vulgaris biopsy pairs ($P=0.02$; fold change 6.77). Transfection of miR-223-3p mimic in HaCaT cells followed by western blot revealed that p38 phosphorylation was 5.5-fold increased. Downstream of p38 activation after

miR-223-3p transfection, we found increase of both HSP27 protein levels and IL-8 mRNA levels ($P<0.01$), the latter also being augmented in HaCaT culture media. IL-8 mRNA levels were rescued after miR-223-3p transfection in the presence of a well-known p38 inhibitor ($P=0.03$). We hypothesized that miR-223-3p downregulates a negative regulator of the p38 pathway and therefore tested protein levels of three dual-specificity phosphatases (DUSP) predicted to be direct targets of miR-223-3p. Preliminary results suggest DUSP10 as direct target of miR-223-3p. In conclusion, we show that miR-223-3p overexpression increases HSP27 and IL-8 production by inducing p38 MAPK phosphorylation, a key factor contributing to pemphigus onset/progression. Ultimately, this may allow development of miRNA-based pemphigus therapy.

Keywords: microRNA profiling; pemphigus; autoantibodies; miR-223-3p; p38 MAPK; HSP27; DUSP10.

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Anti-GSTT1 antibodies and graft rejection in liver and kidney transplantation

Español-Rego, M.¹, García-Ormaechea, M.^{1,2}, Martorell, J.¹, Palou, E.¹, Ercilla, G.¹, Viñas, O.¹

¹Hospital Clínic de Barcelona, Immunology, Barcelona, Spain,

²Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain

Introduction: Approximately 20% of Caucasian population lacks Glutathione S-transferase Theta 1 (GSTT1), an enzyme involved in cellular detoxification that can induce the production of anti-GSTT1 antibodies in transplanted patients with GSTT1 mismatch between a positive donor and a null receptor. These antibodies may influence graft dysfunction in liver and kidney transplantation.

Aim: The aim of our study was to identify, retrospectively, whether the presence of anti-GSTT1 antibodies was associated to a higher risk of developing greater graft dysfunction and or rejection, compared to controls; and validate the ELISA test with the human GSTT1-recombinant protein, using indirect immunofluorescence (IIF) as gold standard method.

Methods: We included 26 (10 kidney and 16 liver) transplanted patients from Hospital Clínic, Barcelona, from 2010 until 2015, with no donor anti-HLA specific antibodies. Ten patients had a positive IIF characteristic pattern of anti-GSTT1 antibodies.

Results: Preliminary results showed that 1/4(25%) of anti-GSTT1 positive kidney transplant and 4/6(66.7%) of GSTT1 positive liver transplanted patients experienced graft rejection vs 2/6(33.3%) of kidney and 1/10(10.0%) liver control patients ($p=0.5320$ and $p=0.0216$ respectively). After adjust the cutoff, IIF positive samples were also positive through the ELISA.

Conclusion: Although the study group is small, a 100% correlation was obtained between IFI and ELISA detection of anti-GSTT1 antibodies, and the results confirm previous studies showing a higher risk of graft rejection in liver transplant patients with anti-GSTT1 antibodies and suggest that

monitoring the presence of anti-GSTT1 antibodies in recipients of GSTT1 mismatch liver transplant could help to predict liver graft rejection.

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CD141⁺ CLEC9A⁺ dendritic cells are enriched in an active state in the inflamed synovium and contribute to synovial inflammation in rheumatoid arthritis

Canavan, M.¹, O'Rourke, M.², Orr, C.², Basdeo, S.³, Fletcher, J.³, Veale, D.², Fearon, U.¹

¹Trinity College Dublin, Trinity Biomedical Sciences Institute, Molecular Rheumatology, Dublin, Ireland, ²Centre for Arthritis and Rheumatic Diseases, St Vincent's University Hospital, Translational Rheumatology Research Group, Dublin, Ireland,

³Trinity College Dublin, Trinity Biomedical Sciences Institute, School of Immunology and Biochemistry, Dublin, Ireland

Studies identifying dendritic cell (DC) subsets in human disease are limited. Most focus on blood with few characterising DC from inflamed tissues. The aim of this work was to characterise DC from the synovium in Rheumatoid Arthritis. RA synovial fluid (SF), synovial tissue (ST) & whole blood (WB) were collected & used for DC characterisation. RA patients had significantly less mDC in WB compared to HC, suggesting these cells are recruited to the site of inflammation. Upon examination of DC in the synovium we identified an increase in CD11c⁺ HLADR⁺ myeloid cells in ST compared to WB. These cells had increased expression of CD80/CD40 & expressed monocytic markers CD64 & CD14. Additionally, upon examination of mDC in SF, they exhibited a more mature phenotype compared to WB (increased CD80/CD40). There was a significant increase in CD141⁺ DC within the SF compared to WB & these cells expressed the immune amplification receptor TREM1. SF CD141 DC expressed higher levels of CD80/CD86 & CD40 compared to WB & had a heightened response to TLR3 stimulation. To examine the effect of the synovial microenvironment on DC, MoDC were cultured in the presence of SF which induced a significant increase in CD80. Finally DC treated with SF & cocultured with CD4⁺ T cells had enhanced IFN γ production & proliferation. Our data suggests that unique DC subsets can be found in RA. These cells display a more activated phenotype than DC in blood & the inflammatory nature of the joint contributes to this activation.

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Increased circulating follicular T helper (Tfh) cells in children with type I diabetes

Izad, M.¹, Arab, M.¹, Razzaghi-azar, M.², Salehi, Z.¹, Mehdi shekarabi, Maryam Keshavarz, Ensieh Nasli

¹School of Medicine, Tehran University of Medical Sciences, Immunology, Tehran, Iran, Islamic Republic of, ²Shariati Hospital, Tehran University of Medical Sciences, Endocrinology and Metabolism Research Center, Tehran, Iran, Islamic Republic of

Background: Type 1 diabetes (T1D) is an autoimmune disease resulting from the damage of pancreatic β -cells because of autoreactive CD4⁺ and CD8⁺ T cells activation. In recent years, follicular T helper (Tfh) cells have been recognized as

a subpopulation of CD4⁺ T cells providing help for B cells differentiation and antibody production. So far, several studies have been investigated the role of circulating Tfh cells in autoimmune diseases. In this study, we examined the frequency of circulating Tfh cells and aglutamic acid decarboxylase autoantibodies (GAD65) and islet cell autoantibodies (ICA) serum level in children with type I diabetes.

Methods: We analyzed the percentage of Tfh cells within peripheral blood mononuclear cells in T1D patients (\leq 300 days from disease onset; n=20; Mean age 6.8 ± 4.6 years) and healthy individuals (n=18; Mean age 8.8 ± 2.2 years) by flowcytometry. Anti-GAD and islet cell autoantibodies (ICA) level were determined using enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence (IF) respectively.

Results: We found that the frequency of CD4⁺CXCR5⁺ and CD4⁺CXCR5⁺ICOS⁺ Tfh cells were increased significantly in the peripheral blood of patients, compared with that of healthy controls ($P < 0.0001$); Furthermore, an increased level of Anti-GAD and ICA was seen in Patients compared to controls ($P=0.001$ and $P=0.02$ respectively). There was no correlation between Tfh cells frequency and the autoantibody levels.

Conclusion: Our results demonstrated the possible involvement of Tfh cells in T1D and suggest that Tfh cells could be considered as a therapeutic target in type 1 diabetes.

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Correlation of miR-146a and miR-155 with regulatory T cell in systemic lupus erythematosus patients

Rastin, M.¹, Mahmoudi, M.², Hasannejad, A.¹, Sahebari, M.³, Rezaei Yazdi, Z.³, Tabasi, N.⁴, Ekrami, Z.⁴, Jaber, S.⁴, Yazdanpanah, E.⁴, Esmaili, A.⁴, Khazaei, M.⁴

¹Bu Ali Research Institute, Faculty of Medicine, Mashhad University of Medical Sciences, Immunology Research Center, Mashhad, Iran, Islamic Republic of, ²Mashhad University of Medical Sciences, Immunology Research Center, Mashhad, Iran, Islamic Republic of, ³Rheumatic Disease Research Center, Internal Medicine Department, Ghaem Hospital, Faculty of Medicine, Mashhad University of Medical Sciences, Internal Medicine Section, Mashhad, Iran, Islamic Republic of, ⁴BuAli Research Institute, Faculty of Medicine, Mashhad University of Medical Sciences, Immunology Research Center, Mashhad, Iran, Islamic Republic of

Introduction: Systemic Lupus Erythematosus (SLE) is an autoimmune disease characterized by the presence of pathogenic autoantibodies. Dysregulated number and function of regulatory T cells are implicated in the pathogenesis of SLE. MicroRNAs are small noncoding RNAs that regulate the expression of the genes involved in immune responses regulation. These molecules are concerned as anticipant biomarkers for diagnosis, prognosis and treatment of SLE. In this study we purposed to investigate the expression of microRNAs and its association with regulatory T cell in systemic lupus erythematosus patients.

Materials and methods: In the current study, 20 healthy controls and 20 patients with SLE were studied. Their PBMCs were isolated, and regulatory T cells were analyzed by flow cytometry. MiRNAs was extracted, cDNA synthesized and the gene expression of miR-146a, and miR-155 were assessed by

Real-Time PCR method.

Result: in SLE patients the expression level of miR-146a decreased compared to controls (1.83 ± 2.1 versus 4.01 ± 5.26), while the expression of miR-155 increased significantly compared to controls (11.07 ± 4.51 versus 7.77 ± 3.72) ($p < 0.001$). Decreased expression of miR-146a had positive correlation with regulatory T cells ($p = 0.029$).

Conclusion: MicroRNAs could control cellular events such as cell growth, and differentiation. They have influence on the regulatory T cell population. It seems that by influencing regulatory cells these molecules can control immune responses in SLE patients.

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Correlation of TLRs with autoantibodies profile in systemic lupus erythematosus patients

Rastin, M.¹, Mahmoudi, M.², Ekrami, Z.², Sahebari, M.³, Rezaei Yazdi, Z.³, Hasannejad, A.¹, Jaber, S.¹, Yazdanpanah, E.¹, Tabasi, N.¹, Esmaili, A.¹

¹BuAli Research Institute, Faculty of Medicine, Mashhad University of Medical Sciences, Immunology Research Center, Mashhad, Iran, Islamic Republic of, ²BuAli Sq, Ferdousi Sq, BuAli Research Center, Immunology Research Center, Mashhad, Iran, Islamic Republic of, ³Rheumatic Disease Research Center, Internal Medicine Department, Ghaem Hospital, Faculty of Medicine, Mashhad University of Medical Sciences, Internal Medicine Section, Mashhad, Iran, Islamic Republic of

Introduction: Systemic lupus erythematosus (SLE) is a severe systemic autoimmune disease with heterogeneous clinical manifestations. Recent experimental and clinical studies have placed new emphasis on the role of Toll-Like Receptors (TLRs) in the pathogenesis of SLE. TLRs are important innate immune receptors for the identification and clearance of pathogens. TLRs by deleting apoptotic cells play an important role in the control of the production of pathogenic autoantibodies and development of clinical features of SLE.

Materials and methods: In the current study, Peripheral Blood Mononuclear Cells (PBMCs) from 20 SLE patient and 20 healthy controls were separated. RNAs was extracted using Tripure, cDNA was synthesized, and the expression levels of TLR3, and TLR8 genes were assessed by Real-Time PCR method. Autoantibodies profile was screened on serum by using a commercial kit.

Results: The expression level of TLR3 was up-regulated in SLE patients compared to controls (5.96 ± 2.47 VS, 5.05 ± 3.44), while the expression of TLR8 was down-regulated (0.17 ± 2.89 VS, 3.18 ± 5.34) ($p = 0.03$). Ninety percent of SLE patients were ANA positive, and anti-SSA was the most prevalent autoantibody, followed by anti-dsDNA (59.1%) and anti-nucleosomes (57.9%). Our findings showed that the increased expression of TLR3 was significantly correlated with the presence of anti-nucleosomes autoantibodies in SLE patients ($p = 0.03$).

Conclusion: Our results showed that TLR3 and TLR8 has differential expressions in SLE patients compared to controls, we also showed that TLR3 may play an important role in the pathogenesis of SLE by power on the production of some autoantibodies.

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In experimental autoimmune anti-glomerular basement membrane glomerulonephritis, HLA-DR15 inhibition blocks autoreactivity to the immunodominant T cell epitope, $\alpha 3_{135-145}$, and prevents disease

Huynh, M.¹, Eggenhuizen, P.J.¹, Olson, G.L.², Bhaskara Rao, N.², Self, C.R.², Sun, Y.², Holdsworth, S.R.^{1,3}, Kitching, A.R.^{1,3,4}, Ooi, J.D.¹

¹Monash University, Centre for Inflammatory Diseases, Clayton, Australia, ²Provid Pharmaceuticals, Monmouth Junction, United States, ³Monash Health, Department of Nephrology, Clayton, Australia, ⁴Monash Health, Department of Paediatric Nephrology, Clayton, Australia

Anti-glomerular basement membrane (GBM) disease is an autoimmune disease that manifests as rapidly progressive glomerulonephritis. It has a strong HLA association with the DR15 allele (odds ratio: 8.5) and the immunodominant, DR15-restricted T-cell epitope has been defined. A DR15 specific inhibitor, PV-267, has potential as a targeted therapy, as current treatments are non-specific and have detrimental side effects. Using humanised HLA transgenic mice expressing DR15, this study tests the hypotheses that administration of PV-267 will inhibit autoreactivity to the immunodominant T cell epitope, $\alpha 3_{135-145}$ and prevent disease in experimental autoimmune anti-GBM disease. Autoreactivity to $\alpha 3_{135-145}$ was determined by immunizing mice with $\alpha 3_{135-145}$, then measuring recall responses *ex vivo* 10 days later by [³H]-T proliferation and IFN- γ and IL-17 ELISPOTs. Experimental anti-GBM disease was induced by immunizing DR15 transgenic mice (backcrossed onto an Fc γ R11b^{-/-} background) with three weekly injections of $\alpha 3_{135-145}$, then functional and histological endpoints measured at 6 weeks.

Compared with mice that received vehicle control, DR15 transgenic mice that received PV-267 daily (30mg/kg) from day -1 developed markedly reduced $\alpha 3_{135-145}$ -specific proliferation (SI: 9.5 ± 0.9 vs 2.2 ± 0.5 , $P < 0.001$) as well as reduced numbers of IFN- γ and IL-17 spots. In experimental anti-GBM disease, DR15 transgenic mice that received PV-267 (30mg/kg) on alternate days from day -1 had markedly reduced 24 hour albuminuria (mg: 1064 ± 313 vs 42 ± 29 , $P < 0.01$) and reduced glomerular segmental necrosis and glomerular crescent formation. In conclusion, PV-267 effectively prevents experimental autoimmune anti-GBM disease, and highlights the potential for specific MHCII inhibitors as a targeted therapy for autoimmune disease.

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Changes of MMP-2 and MMP-9 levels in arthritis rats after inhibiting of LOX

Liu, R.¹, Han, M.²

¹General Hospital of Ningxia Medical University, Department of Rheumatology and Immunology, Yinchuan, China, ²Ningxia Medical University, Department of Immunology, Yinchuan, China

Objective: Inhibiting lysyl oxidase (LOX) with β -aminopropionitrile (BAPN), detected the levels of the MMP-2 and 9 in spleen and thymus of arthritis arthritis rat.

Methods: The SD rats were divided into control, model

and intervention group, Rats in model group were injected intradermally bovine type II collagen with complete Freund's adjuvant into tail, back and paw to induce arthritis. Rats in intervention group were induced arthritis using the same way as the model group, injected BAPN 100mg/kg.day for 40 days intraperitoneally. Rats in the control group were injected with normal saline. The morphological changes of spleen and thymus were observed through HE staining. The levels of MMP-2 and MMP-9 in the Spleen and Thymus were determined by immunohistochemistry.

Results: Cells in the areas of cortex of thymus and white pulp of spleen become more in model group and intervention group. Levels of MMP-2 and MMP-9 in the spleen and thymus of model group and intervention group were significantly higher than the control group ($P < 0.05$). Expression of MMP-2 and MMP-9 in the spleen and thymus of the intervention group were significantly lower than the model group ($P < 0.05$).

Conclusion: High expression of MMP-2 and MMP-9 in the spleen and thymus of rats with arthritis, the levels of MMP-2 and MMP-9 were decreased after inhibiting LOX. Suggest that inhibition of LOX may reduce the pathological reaction that MMP-2 and MMP-9, and LOX may be as a new target for the treatment of arthritis.

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The gluten-specific T cell repertoire is stable in coeliac disease irrespective of age

Hardy, M.Y.^{1,2}, Girardin, A.¹, Pizzey, C.^{3,4}, Cameron, D.J.^{3,5}, Watson, K.A.⁶, La Gruta, N.L.^{6,7}, Anderson, R.P.⁸, Tye-Din, J.A.^{1,2,4}

¹Walter and Eliza Hall Institute of Medical Research, Immunology Division, Melbourne, Australia, ²The University of Melbourne, Department of Medical Biology, Melbourne, Australia, ³Murdoch Childrens Research Institute, The Royal Children's Hospital, Melbourne, Australia, ⁴The Royal Melbourne Hospital, Department of Gastroenterology, Melbourne, Australia, ⁵The Royal Children's Hospital, Department of Gastroenterology, Melbourne, Australia, ⁶The Peter Doherty Institute for Infection and Immunity, Department of Immunology, Melbourne, Australia, ⁷University of Melbourne, Department of Microbiology and Immunology, Melbourne, Australia, ⁸ImmusanT, Inc, Cambridge, MA, United States

Antigen-specific approaches for the diagnosis and treatment of coeliac disease (CD) require detailed understanding of pathogenic CD4⁺ T cells. The pathogenic gluten peptides are well established in adults with CD, but few studies from children suggest the T-cell response is more diverse than in adults. We aimed to characterize the *in vivo* recall T-cell response following short-term oral wheat and/or barley challenge in paediatric CD. Children with HLA-DQ2.5⁺ CD (aged 3-17 years) consumed wheat bread or barley biscuits for 3 days. Peripheral blood was collected on day 6 and T-cell responses were measured by IFN- γ ELISpot to a set of wheat, barley, and rye peptides previously shown to be immunogenic in CD adults.

Gluten-specific responses were detected in 33/45 (73%) children. Recognition of peptides relevant in adults with CD

was highly consistent in children, and like adults, deamidation was important for bioactivity. Homozygosity for HLA-DQ2.5 was associated with response magnitude, but age and time since diagnosis was not. Gluten-specific T cells from children demonstrated comparable levels of cross-reactivity to wheat, rye, and barley peptides as those from adults. α -gliadin-specific T cells showed similar T cell receptor usage with biased TRBV7-2 expression, public CDR3 β sequences, and conserved Arg residues. In adults, public α - and ω -gliadin-specific CDR3 β 's were detected following challenge with two different grains (wheat and barley).

In paediatric CD, the peptide hierarchy, deamidation dependence, cross-reactivity, and T-cell repertoire induced by *in vivo* grain challenge are similar to adults, suggesting additional utility of peptide-based applications designed in adults with CD.

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Methyltransferase Ash1l controls T cell autoimmunity by upregulating Smad3 to polarize iTreg cell generation

Xia, M.¹, Liu, J.², Liu, S.², Chen, K.³, Xue, Y.², Cao, X.¹

¹National Key Laboratory of Medical Molecular Biology & Department of Immunology, Institute of Basic Medical Sciences, Peking Union Medical College, Chinese Academy of Medical Sciences, Beijing, China, ²National Key Laboratory of Medical Immunology & Institute of Immunology, Second Military Medical University, Shanghai, China, ³Institute of Immunology, Zhejiang University School of Medicine, Hangzhou, China

Transforming growth factor- β (TGF- β)-dependent regulatory T (Treg) cells are important for maintenance of immune homeostasis and prevention of autoimmune diseases, but the mechanisms especially the epigenetic controls on their polarization still remain unclear. Here we report H3K4 methyltransferase Ash1l promotes TGF- β -mediated induced Treg (iTreg) cell generation. Less iTreg cells are generated from Ash1l-silenced CD4⁺ T cells, and Ash1l-silenced mice are more susceptible to T cell-mediated colitis with decreased iTreg cell generation. Mechanistically, Ash1l upregulates Smad3 expression by directly binding and modifying the Smad3 promoter. Furthermore, we identify a lncRNA, namely lnc-Smad3, which locates nearby the Smad3 promoter and silences its transcription at steady state. Under TGF- β stimulation, Smad3 suppresses lnc-Smad3 transcription, thereby recovering the Smad3 promoter accessibility to Ash1l. Our data add mechanistic insights into antagonistic crosstalk of histone modification and lncRNA in regulation of Treg cell fate by revealing the opposite role of Ash1l and lnc-Smad3 in epigenetic regulation of Smad3 expression.

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Augmented AIRE expression paradoxically induces polymyositis-like autoimmunity in mice

Nishijima, H., Mouri, Y., Morimoto, J., Matsumoto, M.

Institute for Enzyme Research, Tokushima University, Tokushima, Japan

Autoimmunity is prevented by the function of transcriptional regulator Aire, which promotes the expression of wide

varieties of tissue-restricted antigens from medullary thymic epithelial cells (mTECs) and from a subset of peripheral antigen-presenting cells (APCs). We hypothesized that augmented expression of Aire would ameliorate autoimmune diabetes in NOD mice. We observed, in contrast, that mice overexpressing Aire developed muscle-specific autoimmunity associated with incomplete maturation of mTECs. This led to failure of deletion of autoreactive T cells together with dramatically reduced production of Tregs in the thymus. In peripheral APCs, costimulatory molecule expression was augmented by the additive Aire expression. Our results highlight the importance of coordinated action between central tolerance and peripheral tolerance under the common control of Aire. We suggest that autoimmune diseases develop not only by the loss of function of Aire but also by its augmented expression, which could occur in some forms of human autoimmune disease.

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Impaired expansion of regulatory T cells in a neonatal thymectomy-induced autoimmune mouse model

Yamada, A., Ushio, A., Arakaki, R., Kudo, Y., *Ishimaru, N.*
Tokushima University Graduate School, Tokushima, Japan

Neonatal thymectomy (Tx) in certain mouse strains is known to induce organ-specific autoimmunity due to impaired functions of T cells, including Foxp3⁺ regulatory T (T_{reg}) cells in the thymus. The precise mechanism underlying the induction of autoimmunity by neonatal Tx remains unclear. One possibility is that depletion of T_{reg} cells breaks down peripheral tolerance. We examined the functions of T_{reg} cells by using a murine Sjögren's syndrome (SS) model, NFS/sld mice that underwent neonatal Tx. The ratio of T_{reg} cells to effector memory phenotype T cells in Tx mice was significantly lower than that of non-Tx mice. In addition, *in vitro* induction of peripherally induced T_{reg} cells by transforming growth factor- β (TGF- β) using naïve T cells from SS model mice was severely impaired. The mRNA expression of TGF- β receptor I, II, and Smad3 and -4 in the TGF- β -induced signal transduction pathway of T_{reg} cells in this SS model were lower than those of control mice. In addition, T_{reg} cells in this SS model exhibited an IFN- γ -producing Th1-like phenotype that resembled effector T cells. In conclusion, these results suggest that abnormal expansion and differentiation of T_{reg} cells and inflammatory cytokines produced by T_{reg} cells contribute to the development of autoimmunity.

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Single cell gene expression studies in lupus patient monocytes reveal novel patterns reflecting disease activity, interferon, and medical treatment

Jin, Z.¹, Fan, W.², Jensen, M.A.¹, Dorschner, J.M.¹, Vsetecka, D.M.¹, Amin, S.¹, Makol, A.¹, Ernste, F.¹, Osborn, T.¹, Moder, K.¹, Chowdhary, V.¹, *Niewold, T.B.*¹

¹Mayo Clinic, Rochester, United States, ²Ren Ji Hospital, Shanghai Jiao Tong University, Shanghai, China

Background: Our previous studies have shown that different cell types from the same sample demonstrate diverse gene

expression, and important findings can be masked in mixed cell populations. In this study, we examine single cell gene expression in SLE patient monocytes and determine correlations with clinical features.

Methods: CD14⁺⁺CD16⁻ classical monocytes (CLs) and CD14^{dim}CD16⁺ non-classical monocytes (NCLs) from SLE patients were purified by magnetic separation. The Fluidigm single cell capture and RT-PCR system was used to quantify expression of 87 monocyte-related genes.

Results: Both CLs and NCLs demonstrated a wide range of expression of IFN-induced genes, and NCL monocytes had higher IFN scores than CL monocytes. Unsupervised hierarchical clustering of the entire data set demonstrated two unique clusters found only in SLE patients, one related to high disease activity and one related to prednisone use. Independent clusters in the SLE patients were related to disease activity (SLEDAI 10 or greater), interferon signature, and medication use, indicating that each of these factors exerted a different impact on monocyte gene expression that could be separately identified. A subset of anti-inflammatory gene set expressing NCLs was inversely correlated with anti-dsDNA titers ($\rho = -0.77$, $p=0.0051$) and positively correlated with C3 complement ($\rho = 0.68$, $p=0.030$) in the SLE patient group.

Conclusion: Using single cell gene expression, we have identified a unique gene expression patterns that reflect the major clinical and immunologic characteristics of the SLE patients which are not evident in bulk cell data, supporting the critical importance of the single cell technique.

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Analysis of follicular helper T cells in a mouse model for Sjögren's syndrome

*Kunihiro, O.*¹, Masako, S.¹, Aya, U.¹, Takaaki, T.², Yasusei, K.¹, Rieko, A.¹, Naozumi, I.¹

¹Tokushima University Graduate School, Department of Oral Molecular Pathology, Tokushima, Japan, ²Tokushima University Graduate School, Department of Pathology and Laboratory Medicine, Tokushima, Japan

Objective: Follicular helper T cells (T_{fh}s) play a key role in the pathogenesis of various autoimmune diseases. T_{fh}s contribute to the formation and maintenance of germinal center (GC) in the lymphoid tissues or inflammatory lesions. Sjögren's syndrome (SS) is an autoimmune disorder that affects salivary and lacrimal glands. The precise mechanism of the onset of autoimmune lesions and autoantibody production through T_{fh}s in a SS model remains unclear. In this study, we evaluated how T_{fh}s contribute to the pathogenesis of SS to understand the differentiation of T_{fh}s, GC B cells, and autoantibody production in a SS model.

Methods: NFS/sld mutant mice thymectomized at 3 days after birth were used as SS model mice, and cervical lymph nodes (CLN), spleen (Sp) and sera were investigated by immunological and pathological analyzes such as flow cytometry, real-time RT-PCR, immunofluorescence staining, and ELISA.

Results: The proportion of CD4⁺CD62L⁻PD-1⁺CXCR5⁺ T_{fh}s and CD19⁺PNA⁺IgD⁻ GC-B cells in CLN and Sp of SS model was significantly increased compared with that of control mice in parallel with the upregulation of autoantibodies. In addition,

more enhanced GC formation in SS model was found by immunofluorescence analysis. Moreover, mRNA expression level of transcriptional factor Bcl-6, master regulator of GC reaction, in CLN and Sp of SS model was higher than that of control mice. **Conclusion:** These results suggest that Tfh₁ and GC-B cells might play an important role in the autoimmune response during the pathogenesis of SS.

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Clozapine ameliorates experimental autoimmune encephalomyelitis in a CD4 independent fashion

Zareie, P.¹, La Flamme, A.^{1,2}

¹Victoria University of Wellington, Wellington, New Zealand,

²Malaghan Institute of Medical Research, Wellington, New Zealand

Recent research has revealed a neuroinflammatory component to psychiatric disorders including schizophrenia and depression, and this neuroinflammation has been shown to be reduced by several atypical anti-psychotics such as clozapine, which is used to treat schizophrenia. We have shown previously in mice that treatment with clozapine reduces disease severity in experimental autoimmune encephalomyelitis, an animal model for multiple sclerosis. In this study, we assessed the effect of treatment on CD4 T cells during EAE as a potential mechanism by which clozapine is able to reduce disease. We found that while clozapine treatment effectively ameliorated EAE, this protection was not associated with defective proliferation, expansion and encephalitogenicity of antigen-specific CD4 T cells. Instead our results suggest that clozapine may target myeloid cells as its mechanism of action in suppressing EAE.

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Autoantigen microarray profiling identified IgA antibody clusters associated with autoimmune clinical manifestations in Systemic lupus erythematosus, systemic scleroderma and idiopathic inflammatory myositis

Li, Q.-Z.¹, Yan, M.¹, Zhao, H.², Zuo, X.²

¹University of Texas Southwestern Medical Center, Immunology,

Dallas, United States, ²Central-South University of China,

Rheumatology, Changsha, China

Systemic lupus erythematosus (SLE), systemic scleroderma (SSc) and idiopathic inflammatory myositis (IIM) are systemic autoimmune diseases characterized by autoantibodies (AutoAbs) against various self-antigens. In this study, we measured the serum IgA AutoAbs against 124 self-antigens in a cohort of 128 SLE, 73 SSc, 75 IIM and 140 healthy controls (HC) using autoantigen microarrays. Our result indicated that IgA AutoAbs were highly prevalent in SLE and SSc compared with IIM and NC. 58.4% SLE and 41.7% SSc have 5 or more IgA AutoAbs compared with 16% in IIM and 14.1% in NC. 9 IgA AutoAbs most significantly elevated in SLE were anti-DNA antigens including dsDNA (54.7%), ssDNA (51.1%), and chromatin (46%). 32 IgA AutoAbs were significantly increased in SSc with highest reactivity to Scl-70 (55.6%). IIM showed lower prevalence of IgA AutoAbs with highest in anti-Jo-1 (20%), anti-muscarinic receptor (8%) and anti-B2-microglobulin (6.7%). Significant correlation was observed between IgA and IgG isotypes on most

AutoAbs. 3 IgA autoAbs (anti-gDNA, anti-C1q and anti-dsDNA) positively correlated with SLEDAI Score, but only anti-C1q IgA was significantly elevated in lupus nephritis. 14 anti-nuclear IgA AutoAbs negatively correlated with serum complement C3 and/or C4 levels. Our study indicated that autoreactive IgA AutoAbs against nuclear components, in parallel with IgG AutoAbs, are highly prevalent in SLE and SSc patients. IgA AutoAbs could be used as biomarkers for diagnosis and prognosis of systemic autoimmune diseases, and the molecular mechanisms underlining IgA AutoAbs production in systemic autoimmune diseases warrant further study.

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High-throughput discovery of T cell epitopes in type 1 diabetes using DNA barcode labelled peptide-MHC multimers

Lyngea, R.¹, Kai Bentzen, A.¹, Julie Overgaard, A.², Piciot, F.², Størling, J.², Reker Hadrup, S.¹

¹Veterinary Institute, DTU, Section of Immunology and Vaccinology,

Fredriksberg C, Denmark, ²Copenhagen Diabetes Research Center,

University Hospital Herlev, Department of Pediatrics E, Herlev,

Denmark

Type 1 diabetes (T1D) is characterized by a CD8⁺ lymphocyte-mediated selective destruction of the insulin-producing β -cells causing clinical diabetes. Several autoantigens including glutamic acid decarboxylase 65kDa (GAD65), insulin, protein tyrosine phosphatase (IA-2) and zinc transporter 8 (ZnT8) have been identified based on reactivity in sera from T1D individuals. Here we investigate if post-translational deamination of arginine in the form of citrullination plays a role in T cell recognition of T1D autoantigens. Citrullination may lead to generation of neo-epitopes, which has been described as

T cell targets in other autoimmune diseases. We used netMHC prediction algorithm to identify 764 epitopes from Insulin, GAD65, IA-2 and ZnT8 restricted to HLA-A2, A24, B8 and B15. Among these 91 peptide sequences were susceptible for citrullination. We evaluate the MHC-affinity of both the citrullinated and non-citrullinated library, to identify potential neo-epitopes and to understand the impact of citrullination on MHC affinity. In parallel we will analyse peripheral blood lymphocytes from 50 T1D patients for immune reactivity against the full library. The large library screen will be conducted applying a novel technology where the selection of MHC-multimer binding T cells is followed by amplification and sequencing of MHC multimer-associated DNA barcodes revealing their recognition. This technique enables simultaneous detection of >1000 specificities. Identifying post translational modifications capable of eliciting autoreactive T cell responses in T1D patients is highly relevant for understanding the underlying mechanisms leading to T1D.

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Abnormal germinal center (GC) reaction in autoimmunity*Saito, M., Otsuka, K., Ushio, A., Kurosawa, M., Yamada, A., Kudo, Y., Arakaki, R., Ishimaru, N.**Tokushima University Graduate School, Institute of Biomedical Sciences, Department of Oral Molecular Pathology, Tokushima, Japan*

The hallmark of follicular helper T cells (T_{FH}) is the ability to help B cell generation and induction of germinal center (GC) reactions, including production of high-affinity antibodies. Recent studies suggest that abnormal function of T_{FH} plays a crucial role in the generation of autoantibodies which inflict autoimmune pathology. However, the molecular mechanisms how T_{FH} promote GC formation and antibody production in autoimmune response remains unclear. Here we investigated the distribution and function of T_{FH} and GC-B cell using MRL/lpr, a mouse model of autoimmunity such as Rheumatoid arthritis and Sjögren's syndrome. The number of $CD4^+CD62L^-CXCR5^+T_{FH}$ and $CD19^+PNA^+IgD^-$ GC-B cell in MRL/lpr mice was significantly increased compared with that in MRL/+ control mice. In addition, higher expression levels of Egr2 in MRL/lpr mice, which is required for Bcl6 expression during T_{FH} cell differentiation, was observed comparing with that in control mice. Moreover, the expression of CXCL13, the ligand for CXCR5, and GC formation in the cervical lymph nodes of MRL/lpr mice were significantly enhanced compared with those in control mice together with increasing autoantibodies. These findings indicate that Egr2 and CXCL13 might positively regulate GC formation and promote the production of autoantibody following the onset of autoimmune disease.

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Identification of self-reactive marginal-zone B cell like cells in lymph nodes of mice*Palm, A.-K.E., Friedrich, H.C., Kleinau, S.**Uppsala University, Cell and Molecular Biology, Uppsala, Sweden*

Marginal-zone (MZ) B cells represent a distinct subset of innate B cells that have been associated with autoimmunity in animal models. The MZ B cells express low-affinity polyreactive B cell receptors that recognize both foreign and self-structures. Murine MZ B cells have been confined to the spleen, but we demonstrate here that B cells with a MZ B-cell phenotype also exist in the subcapsular sinuses of lymph nodes of mice. The nodal MZ (nMZ) B cells display high levels of costimulators and toll-like receptors, and are represented by virgin and memory cells, with memory cells containing both IgM^+ and switched B cells. The frequency of nMZ B cells is about 1-6 % of nodal B cells depending on mouse strain, with higher numbers in older mice and a trend of increased numbers in females. Notably, there is a significant expansion of nMZ B cells following immunization with an autoantigen, but not after likewise immunization with a control protein or with the adjuvant alone. The nMZ B cells produce autoantibodies upon activation and can efficiently present autoantigen to cognate T cells in vitro, triggering T-cell proliferation, IFN- γ and IL-17 secretion. The existence of self-reactive MZ B cells in lymph nodes may be an additional

source of antigen-presenting cells that in an unfortunate environment may activate T cells leading to autoimmunity.

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HtrA2 suppresses autoimmune arthritis and regulates activation of STAT3*Lee, S.H.¹, Moon, Y.-M.¹, Seo, H.-B.¹, Kim, S.-Y.¹, Kim, E.-K.¹, Nam, M.-K.², Rhim, H.², Cho, M.-L.¹**¹Rheumatism Research Center, Seoul, Korea, Republic of, ²College of Medicine, Catholic University of Korea, Department of Biomedicine and Health Sciences, Seoul, Korea, Republic of*

Rheumatoid arthritis (RA) is an autoimmune disorder inducing dysregulated inflammation. RA can result in promotion of Th17 cells secreting IL-17 associated with the activation of signal transducer and activator of transcription (STAT) 3. High temperature requirement protein A (HtrA) 2 is a serine protease involved in apoptosis and neurodegeneration. This protein is related with non-response to RA treatment. The aim of this study was to determine that HtrA2 overexpression may reveal therapeutic effects on RA. We observed that T helper cell (Th) 17 differentiation, osteoclastogenesis and lymphocyte activation is promoted in mnd2 mutant mice compared to wild type. Moreover, inhibitor of HtrA2 increases in T helper cell (Th) 17 differentiation. On the other hand, the overexpression HtrA2 induces cleavage of STAT3 and HtrA2 overexpression attenuates collagen-induced arthritis (CIA) in mice model. Additionally, HtrA2 overexpression inhibited development of plaque formation and differentiation of Th17 in ApoE^{-/-} mice with proteoglycan immunization for animal model of hyperlipidemia based RA. The therapeutic function of HtrA2 in inflammatory diseases is related with the targeting of Th17 development and STAT3 pathway in splenocytes. These results suggest that HtrA2 takes part in immunomodulatory activity and upregulation of HtrA2 may shed light on therapeutic approaches to inflammatory disorder such as RA and hyperlipidemia.

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Natural killer cells are required for the development of imiquimod-induced psoriasis-like skin inflammation*Yang, S.-J.¹, Liu, M.-F.², Shieh, C.-C.³, Sytwu, H.-K.⁴, Wu, C.-L.⁵, Shiau, A.-L.⁶**¹National Cheng Kung University, Institute of Basic Medical Sciences, Tainan, Taiwan, Republic of China, ²National Cheng Kung University, Section of Allergy, Immunology, and Rheumatology, Department of Internal Medicine, Tainan, Taiwan, Republic of China, ³National Cheng Kung University, Institute of Clinical Medicine, Tainan, Taiwan, Republic of China, ⁴National Defense Medical Center, Department and Graduate Institute of Microbiology and Immunology, Taipei, Taiwan, Republic of China, ⁵National Cheng Kung University, Department of Biochemistry and Molecular Biology, Tainan, Taiwan, Republic of China, ⁶National Cheng Kung University, Department of Microbiology and Immunology, Tainan, Taiwan, Republic of China*

Psoriasis is a common chronic immune-mediated inflammatory disease that causes erythema, scaling and thickening of the skin.

Th1- and Th17-mediated mechanisms have been demonstrated to be involved in the pathogenesis of psoriasis. However, the exact pathogenesis of psoriasis, which likely combines genetic, immunological and environmental factors, remains to be fully elucidated. Natural killer (NK) cells and macrophages, which play important roles in innate immunity, have been suggested to contribute to the pathogenesis of psoriasis. Whether NK cells are pivotal for the initiation/development of psoriasis still remains to be elucidated. Here, we used nonobese diabetic (NOD) mice, which are defective in innate immunity, to investigate the role of NK cells in the development of psoriasis-like dermatitis induced by imiquimod (IMQ). Our results show that while BALB/c mice treated with IMQ exhibited skin lesions resembling human psoriasis, NOD mice were protected from IMQ-induced psoriasis-like dermatitis. NOD mice had defects in the cytotoxic activity of NK cells and phagocytic activity of macrophages. Depletion of NK cells ameliorated IMQ-induced skin lesions in BALB/c mice. Taken together, our findings underline the importance of NK cells in the development of psoriasis and have implications for new therapeutic strategies targeting NK cells for psoriasis.

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Toll-like receptors in primary Sjögren's syndrome

Karlsen, M.¹, Hansen, T.^{2,3}, Jakobsen, K.¹, Nordal, H.H.^{1,4}, Brun, J.G.^{2,4}, Jonsson, R.^{1,4}, Appel, S.¹

¹University of Bergen, Broegelmann Research Laboratory, Department of Clinical Science, Bergen, Norway, ²University of Bergen, Department of Clinical Science, Bergen, Norway, ³Haukeland University Hospital, Department of Immunology and Transfusion Medicine, Bergen, Norway, ⁴Haukeland University Hospital, Department of Rheumatology, Bergen, Norway

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease of unknown etiology. It is characterized by chronic inflammation in salivary and lacrimal glands resulting in dry mouth and eyes, and the presence of autoantibodies. Many patients suffer from various additional symptoms due to extraglandular manifestations. The female to male ratio is 9:1, and the age of onset is usually between 40 and 60 years of age. We analysed Toll-like receptor (TLR) expression and function in peripheral blood mononuclear cells (PBMC), B cells and B cell subsets. TLR7 and -9 were expressed at similar levels in B cells of pSS patients and healthy controls. Naïve B cells expressed less TLR7 and -9 compared to memory and pre-switched memory B cells. We next analyzed the effect of B cell stimulation via TLR7 and -9. In general we found upregulated levels of surface markers and cytokines after stimulation with both TLR7 and -9 ligands in pSS patients and controls. B cells from pSS patients secreted higher amounts of IL-8, IL-15, IFN- α , IL-1RA, MCP-1 and IL-2R in both unstimulated and stimulated cells. We also found less intracellular IL-10 in pre-switched memory B cells in pSS patients compared to healthy controls. Evaluating expression levels of all ten TLRs in PBMC, we found that pSS patients expressed less TLR9 and more TLR8 at mRNA level, and less TLR5 and more TLR7 at protein level compared to controls. These results indicate that TLRs might indeed be involved in the pathogenesis of pSS.

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Oligoclonally expanded CD4+ T cells recognising citrullinated vimentin in peripheral blood of rheumatoid arthritis patients

Law, S.C.¹, Nel, H.J.¹, Scally, S.W.², Mehdi, A.¹, Le Cao, K.-A.¹, Rossjohn, J.^{2,3}, Reid, H.H.², La Gruta, N.L.⁴, Thomas, R.¹

¹The University of Queensland Diamantina Institute, Translational Research Institute, Brisbane, Australia, ²Monash University, Department of Biochemistry and Molecular Biology, Melbourne, Australia, ³Institute of Infection and Immunity, Cardiff University School of Medicine, Cardiff, United Kingdom, ⁴Department of Microbiology and Immunology, The University of Melbourne, at the Peter Doherty Institute for Infection and Immunity, Melbourne, Australia

RA is strongly associated with HLA-DRB1, including the high-risk allele HLA-DRB1*0401, and with the development of autoantibodies specific for citrullinated-self-antigens. The preferential binding of citrullinated-vimentin to HLA-DRB1*0401, and a correlation between RA disease activity and the frequency of citrullinated-vimentin-specific T-cells suggests citrullinated-vimentin selects and expands autoreactive T-cells in HLA-DRB1*0401+ RA patients. Expanded CD4+ T cell clones have been identified in inflamed synovial compartment, however the antigen specificities of these clones are unknown. Furthermore, no studies have determined how presentation of citrullinated-vimentin shapes the T cell receptor (TCR) repertoire in HLA-DRB1*0401+ individuals, and the impact of RA on that process. We profiled TCR repertoire diversity among a total of 267 and 763 single peripheral blood (PB) vimentin₅₉₋₇₁-cit64-specific CD4+ T cells from 6 healthy controls (HC) and 7 recent-onset RA patients. We used pHLA tetramers and multiplex clonotypic analysis of single cells to simultaneously amplify expressed CDR3 α and CDR3 β segments from individual antigen-specific T cells. The repertoire encoding TCR α and TCR β among these was highly diverse and private in all individuals and included a large number of unique CDR3 sequences. Repeated sequences of individual clonotypes were observed in 2 patients, but no HC. This expansion was present pre- and post RA-treatment, and exclusively derived from CD25+CD127+ effector CD4+ T cells. These data demonstrate that a wide range of possible TCRs recognize the vimentin₅₉₋₇₁-cit64 epitope in PB CD4+ T cells of RA patients and HC. Repeated clonotypic sequences only among RA patients strongly suggest selective citrullinated self-antigen-driven effector T cell expansion in disease.

942

Elevated GITRL is associated with multi-organ involvement and increased disease activity of primary sjogren's syndrome (pSS) and promotes pathogenic Th17 differentiation

Gan, Y., Sun, X., Li, L., He, J., Li, Z.

Peking University People's Hospital, Department of Rheumatology and Immunology, Beijing, China

Methods: 78 pSS patients and 44 healthy controls were recruited, and the serum GITRL level was measured by ELISA, and serum levels of IL-17A, IL-17E, IL-17F, IL-6, IL-22 and IL-23 were

determined by multiplex cytokine assays. CD4+T lymphocytes were isolated from PBMCs from healthy donors and cultured in TexMACS™GMP medium with anti-CD3 mAb, anti-CD28 mAb and rhIL-2. For Th17 differentiation and pathway exploration, CD4+T cells were treated either with recombinant-human GITRL or with the medium control. Surface marker, intracellular cytokine and phosphorylated signal protein were evaluated by flow cytometry.

Results: Serum levels of GITRL were significantly higher in pSS patients. There is a negative correlation between elevated levels of GITRL and WBC, Neutrophils, PLT, C3 and C4, and a positive correlation with Lymphocytes, IgG and RF. pSS patients with overt hypothyroidism showed higher level of GITRL comparing to those with subclinical hypothyroidism and normal thyroid function. pSS patients of moderate to high disease activity (ESSDAI \geq 5) showed higher level of GITRL. Moreover, Serum GITRL level was positively correlated with Th17-related cytokines, some of which had been shown to be the causal agents increasing autoimmunity and organ involvement in pSS. After GITRL treatment, we found the expansion of Th17 cells in vitro, and increased activation of mTOR and STAT5 and STAT3 signaling in Th cells.

Conclusions: Our results identified the clinical significance of GITRL in exacerbating disease activity in pSS patients and its pathogenic roles in enhancing the differentiation of Th17 cells and the possible involved signaling pathways.

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Serum and intra-renal CD44 Levels correlate with disease activity in patients with lupus nephritis

Yung, S., Au, K.Y., Zhang, Q., Chau, M., Tse, W.W., Chan, T.M.

University of Hong Kong, Department of Medicine, Hong Kong, Hong Kong

Lupus nephritis is a severe manifestation of systemic lupus erythematosus and often portends poor prognosis. CD44 is a cell surface receptor for hyaluronan and is involved in lymphocyte activation and extravasation, and tissue inflammation. We investigated serum CD44 level and its renal expression during lupus nephritis.

Serum CD44 level was measured in paired sera obtained during active disease and remission respectively from patients with biopsy-proven severe proliferative lupus nephritis using ELISA. Controls included patients with non-lupus glomerular diseases and healthy subjects. CD44 expression in human lupus nephritis kidney biopsies and NZBWF1 mice was assessed by cytochemical staining. Mesangial cells were isolated from NZBWF1 mice to investigate the mechanism of CD44 synthesis. Serum CD44 levels were significantly higher during active lupus nephritis compared with remission samples, the non-lupus glomerular disease group and healthy subjects ($P < 0.001$ for all). In lupus nephritis patients, serum CD44 level correlated with that of anti-dsDNA antibody ($r=0.43$, $P < 0.001$) and creatinine ($r=0.46$, $P < 0.0001$), and inversely correlated with C3 ($r=-0.45$, $P < 0.001$). Strong CD44 staining was observed in infiltrating and resident renal cells in glomeruli and renal tubules in human and murine active lupus nephritis renal specimens. In contrast, minimal CD44 expression was observed in healthy kidney tissue.

CD44 was constitutively expressed in cultured NZBWF1 mice mesangial cells, and its expression was significantly induced after adding exogenous hyaluronan, IL-6, IL-1 β and TNF- α .

Our data suggest that CD44 contributes to the pathogenesis of lupus nephritis through its effect on both infiltrating inflammatory cells and resident kidney cells.

944

An autoimmune disease-associated gene, Lnk/Sh2b3 controls inflammation in adipose tissue and reduces the risk for onset of diabetes

Mori, T., Yamazaki, N., Takaki, S.

Research Institute, National Center for Global Health and Medicine, Department of Immune Regulation, Research Center for Hepatitis and Immunology, Chiba, Japan

Lnk/Sh2b3 is an adaptor protein that regulates cytokine signaling in lymphohematopoiesis. In human, a nonsynonymous SNP in *LNK/SH2B3* has been reported as a risk variant for several autoimmune diseases including diabetes. We have shown that Lnk/Sh2b3 plays a role in preventing pathogenic expansion and activation of CD8⁺ T cells leading to intestinal tissue damage in the physiological levels of IL-15 (Eur J Immunol 44:1622-32, 2014). We also revealed that Lnk/Sh2b3 regulates DC production, and that the ability of DCs to support Th1 or Treg cells was altered by Lnk-deficiency in response to GM-CSF and IL-15 (J Immunol 193:1728-36, 2014). In this study, we investigated Lnk/Sh2b3 functions in tissues related to pathogenesis of diabetes. We found that adult *Lnk*^{-/-} mice showed slightly but significantly elevated blood sugar levels in steady state compared to WT mice. While serum insulin levels were not reduced, glucose tolerance and insulin sensitivity were impaired, indicating that *Lnk*^{-/-} mice spontaneously showed some symptoms observed in type2 diabetes. In adipose tissues of *Lnk*^{-/-} mice, CD45⁺ immune cells such as NK cells, CD8⁺ T cells and M1 macrophages were accumulated and inflammatory cytokines were up-regulated. When *Lnk*^{-/-} mice were crossed with *Il15*^{-/-} mice, NK cells disappeared, CD8⁺ T cells in adipose tissues decreased, and glucose intolerance and adipose inflammation were ameliorated. Thus, Lnk/Sh2b3 plays a role in preventing adipose tissue inflammation by controlling the expansion and function of IL-15-dependent cells, and maintains the threshold for onset of diabetes.

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Serum acetate levels correlate with disease activity in patients with lupus nephritis

Chan, T.M., Au, K.Y., Zhang, Q., Chan, P.L., Chau, M., Yung, S.

University of Hong Kong, Department of Medicine, Hong Kong, Hong Kong

Introduction: Lupus nephritis patients have impaired immune tolerance to self-antigens resulting in immune-mediated kidney injury. It is speculated that gut microbiota or its products are involved in the aetiology or pathogenesis of autoimmune diseases. Bacterial metabolites from the gut can enter the circulation and modulate inflammatory responses. Acetate is

a short-chain fatty acid produced by the gut microbiota. We measured serum acetate level and renal expression of its cell surface receptors GPR-41 and GPR-43 in lupus nephritis patients to investigate the potential relationship between the gut microbiota and serological and clinical parameters of disease activity.

Methods: Serum acetate, IL-6, IL-8, MCP-1 and LPS levels were measured in patients with biopsy-proven severe proliferative lupus nephritis, patients with non-lupus glomerular diseases and healthy controls. Renal expression of GPR-41 and GPR-43 was determined by cytochemical staining.

Results: Serum acetate level was higher in lupus nephritis patients compared with non-lupus renal disease group ($P < 0.05$) and healthy subjects ($P < 0.01$); and in lupus nephritis serum acetate level was higher during disease flare ($P = 0.04$ compared with remission). Furthermore, acetate level in lupus nephritis patients correlated with serum LPS ($r = 0.34$, $P < 0.0001$), MCP-1 ($r = 0.21$, $P < 0.0001$), and IL-8 levels ($r = 0.22$, $P < 0.0001$), and also proteinuria ($r = 0.29$, $P < 0.01$), but not serum IL-6 level. Renal GPR-41 and GPR-43 expression was markedly increased in active lupus nephritis kidney biopsies, and was predominantly detected in the tubulo-interstitium.

Conclusions: Our data suggest that acetate may contribute to the pathogenesis of lupus nephritis, and may have a role in tubulo-interstitial disease.

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IFN- γ -receptor is required for intrafollicular localization of autoreactive marginal zone-B cells in the spleens of lupus-prone MRL+/+ mice

Machida, T.¹, Omori, T.¹, Sakamoto, N.¹, Zhang, X.², Gilkeson, G.², Sekine, H.¹

¹Fukushima Medical University, Department of Immunology, Fukushima, Japan, ²Medical University of South Carolina, Division of Rheumatology, Department of Medicine, Charleston, United States

It has been reported that IFN- γ and/or IFN- γ -receptor-1 (IFNGR1) is required for development of murine lupus-like disease, however, their role in auto-Ab production is not defined. We previously demonstrated that splenic marginal zone-B (MZ-B) cells of lupus-prone MRL+/+ mice express high levels of IFNGR1 compared to their follicular-B (FO-B) cells or MZ-B cells of non lupus-prone C57BL/6 mice. In this study, we further investigated the role of IFNGR1 in the development of autoreactive MZ-B cells and their localization in the spleens of MRL+/+ mice.

Flow cytometric analysis showed that MRL+/+ mice had significantly increased numbers of splenic MZ-B cells compared to C57BL/6 mice, while there was no difference in the numbers of FO-B cells between these strains. ELISPOT assay showed significantly increased frequency of anti-dsDNA IgM-producing cells in MZ-B cells of MRL+/+ mice compared to their FO-B cells. However, there was no difference in total numbers of MZ-B cells or frequency of anti-dsDNA IgM-producing MZ-B cells between WT and *Ifngr1*^{-/-} MRL+/+ mice. Strikingly, immunofluorescence analysis of spleen sections revealed significantly increased frequency of MZ-B cells inside the lymphoid follicles of WT MRL+/+ mice compared to *Ifngr1*^{-/-} MRL+/+ mice or C57BL/6

mice.

These results indicate that IFNGR1 contributes to intrafollicular localization of MZ-B cells in the spleens of MRL+/+ mice. From these results, we propose the hypothesis that the intrafollicular autoreactive MZ-B cells are involved in auto-Ab production by presenting auto-antigen to T_{FH} cells and/or differentiating into auto-Ab-producing cells in the spleens of MRL+/+ mice.

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The decreased expression of miR-10a in mesangial cells treated with anti-dsDNA antibodies and in lupus nephritis kidney tissues

Thammasate, B.¹, Tangtanatakul, P.², Leelahavanichkul, A.¹, Avihingsanon, Y.³, Hirankarn, N.¹

¹Faculty of Medicine, Chulalongkorn University, Center of Excellence in Immunology and Immune Mediated Diseases, Department of Microbiology, Bangkok, Thailand, ²Chulalongkorn University, Medical Microbiology Interdisciplinary Program, Graduated School, Bangkok, Thailand, ³Faculty of Medicine, Chulalongkorn University, Center of Excellence in Immunology and Immune Mediated Diseases, Department of Medicine, Bangkok, Thailand

Lupus nephritis (LN) is a serious complication in systemic lupus erythematosus (SLE). It is a major cause of morbidity and mortality in SLE patients. MicroRNAs (miRNAs) are small non-coding RNAs that act as epigenetics regulator. In order to screen for the potential miRNA involved, primary human mesangial cells were incubated with anti-dsDNA IgG antibodies purified from LN patients (N=20) compared to pool normal IgG control (N=20). The stem-loop RT PCR result showed that anti-dsDNA IgG antibodies down-regulated miR-10a expression in mesangial cell significantly ($p < 0.05$). MicroRNA-10a (miR-10a) plays roles in cell proliferation and inflammatory pathway and founded to be decrease in kidney from ischemic reperfusion mouse model and SLE peripheral blood mononuclear cells (PBMCs). Next, we investigated the level of miR-10a in kidney tissues from LN patients. In order to test the hypothesis, 26 kidney-tissue samples from biopsy-proven active LN and 6 kidney-tissue samples from cadaveric donors as control were used. The RNAs were extracted and miR-10a was specifically amplified using real-time PCR normalized with RNU44 expression. The results showed that miR-10a expression in LN kidney tissue was significantly decreased compared to control group (fold change = 0.19, SD = 0.56; p -value = 0.01). However, the fold change of miR-10a expression in kidney was not correlated with creatinine clearance (CCr) ($n = 20$, $R^2 = 0.0247$; p -value = 0.508). Since miR-10a is important in regulating cell proliferation and inflammation, which are the main pathology in LN kidney, the use of miR-10a mimic to regain control might serve as a novel therapeutic target for LN.

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Is generation of reactive oxygen species (ROS) in pristane induced lupus responsible for development of autoimmunity?

Minhas, U.^{1,2}, Bhatnagar, A.²

¹University of Allahabad, Biochemistry, Allahabad, India, ²Panjab University, Biochemistry, Chandigarh, India

Pristane induced murine model of lupus is a well established model of lupus like disease which is characterized by development of antibodies against nuclear antigens like dsDNA, ssDNA along with glomerulonephritis and arthritis. Reactive oxygen species are produced in huge amounts during inflammation and stress mediated through these reactive intermediates is being highlighted for their role in pathogenesis of autoimmune disorders. Oxidative Stress (OS) not only causes damage to cellular components but also induces cellular responses like gene activation which may contribute to pathogenesis of autoimmunity. The mechanism of disease development in pristane induced lupus is not completely understood and ROS mediated OS might play some role. We have recently reported generation of ROS in the peritoneal macrophages in response to pristane which remain high even six months after the pristane treatment. The persistent ROS generation during chronic inflammation might be the cause of weakening of antioxidant defense leading to oxidative stress. Oxidative modification of nuclear components may direct formation of neo-antigens which may further lead to production of autoantibodies against them. We propose that oxidative modification of DNA may lead to its mistaken identity as non self and thus the production of anti DNA antibodies in the model. The deposition of immune complexes is implicated in the pathogenesis of this systemic disorder.

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Alphaviruses mediated arthritis in humans: a possible role for autoimmunity through molecular mimicry

Janakiraman, V., Nandakumar, A., Kannan, V.

Indian Institute of Technology Madras, Department of Biotechnology, Chennai, India

The mechanisms responsible for initiation and pathogenesis of autoimmune diseases remain unclear. Several theories have been proposed including infections. Microbial determinants that mimic host antigens can trigger self-reactive T cell clones and antibodies to destroy host tissue. Alpha virus infections are foremost in causing pathogenic conditions resulting in rheumatic arthritis like symptoms. The chronic stage of these infections is characterized by joint inflammation with arthritic like symptoms and the basis for this rheumatic outcomes is not clear. We have investigated the possible involvement of auto immune process in alpha viral induced disease pathogenicity through an *in silico* analysis. We have analysed the proteins from 23 characterized strains of alphaviruses (all of which had documented arthritic symptoms). Our results show presence of consensus regions of significant length in their proteomes that are conserved across the strains of arthritogenic alpha viruses only. Comparative analysis of these regions of consensus

with the human proteome identified some human proteins that shared homology with the structural and non-structural proteins of alpha viruses. Interestingly these proteins have also been implicated in auto immune responses. We have further mapped the plausible immunogenic domains on these regions of homology and have found that they harbour both T and B cell epitopes and are capable of binding to multiple HLA alleles. We speculate that these cross reactive epitopes between alpha virus proteins and human proteins could be responsible for inducing auto immune responses that might play a role in rheumatic like manifestations of arthritogenic alphaviral infections.

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CD4+ T cells show a distinct epigenetic signature between disease states in Multiple sclerosis patients

Maltby, V.¹, Lea, R.², Miles, B.², Sanders, K.^{3,4}, Tajouri, L.⁴, Rodney, S.^{1,5}, Jeannette, L.-S.^{6,7}

¹University of Newcastle & Hunter Medical Research Institute, School of Biomedical Sciences and Pharmacy, Callaghan, Australia,

²Institute of Health and Biomedical Innovation, Brisbane, Australia,

³University of Newcastle & Hunter Medical Research Institute, Callaghan, Australia, ⁴Bond University, Faculty of Health Sciences

and Medicine, Gold Coast, Australia, ⁵Hunter Area Pathology Service, Molecular Genetics, Newcastle, Australia, ⁶University of Newcastle & Hunter Medical Research Institute, School of Medicine

and Public Health, Callaghan, Australia, ⁷John Hunter Hospital, Neurology, Newcastle, Australia

Background: Multiple Sclerosis (MS) is a T-cell mediated autoimmune disorder. Pathogenesis is due to a genetic predisposition triggered by environmental factors. Epigenetics, such as DNA methylation and non-coding RNA (ncRNA), provide a logical interface for environmental factors to influence the genome. Our previous work identified a differentially methylated region (DMR) at the HLA-DRB1 locus in CD4+ T cells of relapsing-remitting MS (RRMS) patients that was not present in CD8+ T cells or CD19+ B cells.

Aims:

1) Determine if these DMRs are also present in secondary progressive MS (SPMS).

2) Determine if there are changes in expression of ncRNAs between RRMS and SPMS patients.

3) Correlate these changes with clinical outcomes

Methods: DNA and RNA from CD4+ T cells was extracted from 28 RRMS, 21 SPMS patients and 40 healthy age and sex matched controls. DNA was bisulphite converted and hybridised to Illumina 450K arrays. RNA was hybridized to Illumina HumanHT12 expression arrays or used for microRNA Next Generation Sequencing (NGS) respectively.

Results and discussion: RRMS and SPMS patients have distinct and non-overlapping DNA methylation profiles, and the HLA-DRB1 DMR is unique to RRMS patients. Of the differentially methylated regions 63% are hypomethylated in RRMS patients, whereas 75% are hypermethylated in SPMS patients. This could be explained by a decrease of DNMT1 in RRMS patients and increase in DNMT3b in SPMS patients. Additionally, miR-29b, which targets DNMT3b, is downregulated in SPMS patients. In conclusion, we have demonstrated that differences in

epigenetic may be contributing to MS disease pathology and progression.

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Defective regulatory T cell function results in autoimmune progression in New Zealand Black mice

Yang, W.C.^{1,2}, Kuo, Y.Y.¹, Chen, Y.Y.¹, Tan, Y.Y.^{1,3}, Chang, C.C.¹, Cho, Y.C.¹, Chan, T.H.¹, Liu, C.L.⁴, Hwang, Y.S.⁵, Shen, C.R.^{1,2,5}

¹Chang Gung University, Graduate Institute and Department of Medical Biotechnology and Laboratory Science, Taoyuan, Taiwan, Republic of China, ²Chang Gung University, Graduate Institute of Biomedical Sciences, Taoyuan, Taiwan, Republic of China, ³Chang Gung University, Department of Biomedical Sciences, Taoyuan, Taiwan, Republic of China, ⁴Ming Chi University of Technology, Department of Chemical Engineering and Graduate School of Biochemical Engineering, Taoyuan, Taiwan, Republic of China, ⁵Linkou Chang Gung Memorial Hospital, Department of Ophthalmology, Taoyuan, Taiwan, Republic of China

Simultaneous increment of Tregs in both numbers and frequencies with AIHA progression have been observed in New Zealand Black (NZB) mice, which spontaneously develop autoimmune hemolytic anemia (AIHA) as they age. However, supplement of CD4⁺CD25⁺ T cells from congenic mice helped ameliorating AIHA progression, implicating a defective role of Tregs in NZB mice during autoimmune progression. We designed both *in vitro* and *in vivo* suppression assay to understand the integrity of Tregs in NZB mice. Judging by the fluctuating percentages of Foxp3 expressing cells among CD4 T cells after anti-CD3 plus anti-CD28 antibody mediated stimulation *in vitro*, the expanded Tregs pool in NZB mice may result from the instable expression of Foxp3 within CD4 T cells after TCR engagement. In addition, enriched Tregs demonstrated inferior suppressive capabilities when suppressing Tregs *in vitro*. The defective phenotype of Tregs from NZB mice *in vivo* is evident by the decreased hematocrit values and increased Coombs' titers in the T cell deficient host receiving co-transferring of both Tregs and Tregs from NZB mice. Instead of Tregs from NZB mice, co-transferring of Tregs from BALB/c mice provided the recipients with effective protection against AIHA progression. We found the limited retention of Tregs from BALB/c mice when they were transferred into immune competent NZB mice may be the reason that the single infusion of Tregs provided the recipient with effective but temporal protection. Our study clearly demonstrated that the capabilities of Tregs from NZB mice in maintaining immune homeostasis is disrupted, thereby leading to autoimmune progression.

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Opposing roles of tyrosine kinase receptors Mer and Axl determine clinical outcome in a mouse model of nephritis

Shao, W.

Temple University, School of Medicine, Medicine, Philadelphia, United States

Systemic lupus erythematosus (SLE) is an autoimmune disorder with unknown etiology. Lupus nephritis remains one

of the most severe manifestations of SLE, with considerable morbidity and mortality. There remains a major void in the successful management of lupus nephritis. TAM (Tyro-3, Axl and Mer) receptor tyrosine kinases play an important role in the maintenance of immune homeostasis. Mer is constitutively highly expressed on glomerular endothelial cells; Axl expression is inducible in kidney under inflammatory conditions. To investigate the functions of Axl and Mer in lupus nephritis, we induced glomerulonephritis in WT, Axl-KO, Mer-KO, and A/M-KO (Axl/Mer-double knockout) mice by injection of nephrotoxic serum (NTS). Mer-KO mice developed severe glomerulonephritis, with significantly decreased survival rate and increased blood urea nitrogen (BUN) levels as compared to WT mice given the same treatment. Two weeks after injection, necrotic cell death was evident in the glomeruli of Mer-KO mice. However, Axl-KO mice presented with significantly increased survival rate and reduced BUN as compared to the WT, Mer-KO and A/M-KO mice injected with the same serum. Interestingly, the A/M-KO mice developed kidney inflammation comparable to the WT mice. Western blot analysis revealed significantly increased Stat3 phosphorylation and caspase-1 activation in the kidney of Mer-KO mice after NTS injection. In contrast, Axl deficient NTS-injected mice developed decreased Akt phosphorylation and Bcl-xl upregulation. The reciprocal activation of Axl and Mer receptor tyrosine kinases may have a major impact on renal inflammation. Results from these studies may lead to novel therapeutic drug development targeted to lupus nephritis.

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Deleting the BAFF receptor TACI protects against systemic lupus erythematosus without extensive reduction of B cell numbers

Figgett, W.¹, Deliyanti, D.², Fairfax, K.^{1,3}, Quah, P.S.^{1,2}, Wilkinson-Berka, J.², Mackay, F.¹

¹The University of Melbourne, Melbourne, Australia, ²Monash University, Melbourne, Australia, ³The Walter and Eliza Hall Institute of Medical Research, Parkville, Australia

B cell-activating factor of the TNF family (BAFF) is an essential B cell survival factor, however, high levels of BAFF promote systemic lupus erythematosus (SLE) in mice and humans. Belimumab (anti-human BAFF) limits B cell survival and is approved for use in SLE patients. Surprisingly, the efficacy of rituximab (anti-human CD20) in SLE remains controversial, despite depleting B cells more potently than belimumab. This raises the question of whether B cell depletion is really the mechanism of action of belimumab. In BAFF transgenic mice, SLE development is T cell-independent but relies on innate activation of B cells via TLRs, and TLR expression is modulated by the BAFF receptor TACI. Here, we show that loss of TACI on B cells protected against BAFF-mediated autoimmune manifestations, and B cells were retained. Therefore, B cell sparing blockade of TACI may offer a more specific and safer alternative to B cell depletion in SLE.

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Next-generation sequencing demonstrates dynamic recirculation of B cell clones in ectopic lymphoid structures of Sjögren's syndrome

Murray-Brown, W.¹, Carlotti, E.¹, Floyd, J.¹, Sutcliffe, N.¹, Tappuni, A.², Vartoukian, S.², Fortune, F.², Mehr, R.³, Pitzalis, C.¹, Bomabardieri, M.¹

¹Queen Mary University of London, Experimental Medicine and Rheumatology, London, United Kingdom, ²Queen Mary University of London, Department of Oral Medicine, London, United Kingdom, ³Bar-Ilan University, The Mina and Everard Goodman Faculty of Life Sciences, Ramat-Gan, Israel

Sjögren's syndrome (SS) is characterised by immune cell infiltration in the salivary glands (SG) leading to xerostomia and exocrine dysfunction. B-cells play a central role in SS pathogenesis, whereby autoreactive B-cells populate ectopic germinal centres (EGC) in SS-SG and undergo somatic hypermutation (SHM) and class-switch recombination of the immunoglobulin (Ig) genes. However, the capacity of specific B-cell clones to seed EGC in different SG and undergo clonal diversification is unclear.

To unravel B-cell recirculation dynamics, we investigated immunoglobulin heavy-chain (IgH) gene rearrangements and patterns of SHM using a high-throughput next-generation sequencing approach for 4 pairs of mSG biopsies from 4 SS patients with high B-cell infiltration and EGC. Total RNA was used to generate Ig-specific cDNA for use in PCR library preparation and barcoded primers were used to allow multiplex sequencing and to assist in bioinformatic filtering of sequences.

In total, we generated ~166,000 reads >350bp, and we detected 1631 clonotypes (reads with same IgHV and IgHJ gene usage and equal CDR3 length). Between 5 and 9 shared clonotypes were observed among paired SG biopsies, demonstrating that B-cells can recirculate between different glands. Furthermore, lineage tree analysis revealed B-cells undergo further rounds of SHM in adjacent glands and highlight distinct patterns of B-cell circulation between biopsies. Finally, the level of B-cell recirculation appears to be related to the Ig-isotype, as IgG-rich samples appeared to recirculate less often. Although further study is needed to confirm these observations, these findings demonstrate the dynamic nature of B-cell affinity maturation in SS within EGCs.

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Dendritic cells engineered for *de novo* synthesis of calcitriol and retinoic acid prime gut-homing regulatory T cells and rapidly arrest progression of ongoing experimental colitis

Xu, Y.¹, Cheng, Y.¹, Chan, C.¹, Chelliah, H.¹, Li, C.-H.¹, Wang, X.¹, Qin, X.^{1,2}, Lau, W.^{1,2}, Baylink, D.¹, Tang, X.¹

¹Loma Linda University, Medicine, Loma Linda, United States, ²Jerry L. Pettis Memorial Veterans Affairs Medical Center, Loma Linda, United States

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder in gastrointestinal tract (GUT) and does not have a permanent cure. Recent immunological interventions, e.g. TNF- α and α 4 β 7 blockers, have improved specificity in the control of

gut inflammation and slowed disease progression. However, off target immune suppression is still causing unbearable adverse side effects, e.g. infections and cancers. Since deficiency of immune regulation in intestines is a major mechanism underlying IBD, this study aims to induce gut-homing regulatory T (Treg) cells in the peripheral lymphoid tissues such that Treg cells in the intestines of IBD patients are selectively augmented to further increase specificity of immunotherapy. Here we showed that enhanced generation of gut-homing Treg cells was achieved through immunization with dendritic cells engineered for *de novo* synthesis of both the active vitamin D metabolite and retinoic acid (RA). In addition, immunization with such engineered DCs rapidly arrested progression of experimental colitis induced by 2,4,6-trinitrobenzene sulfonic acid (TNBS).

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Mild electrical stimulation with heat shock suppresses skin hyperplasia in imiquimod-induced psoriasis model

Tsurekawa, Y., Kai, Y., Morita, M., Takada, M., Moriuchi, M., Okita, G., Suico, M.A., Shuto, T., Kai, H.

Kumamoto University, Molecular Medicine, Kumamoto, Japan

Biophysical stimulations, such as shear stress, mild electrical current and heat shock (HS), affect various biological functions. We have comprehensively studied the biological effects of optimized combination treatment of mild electrical stimulation (MES) (0.1-ms pulse width, 55-pulse per second; pps) and HS at 42°C. Specifically, we have shown that MES+HS suppressed the production of inflammatory cytokines in mouse models of diabetes and chronic kidney disease (Morino, et al., *PLoS One*, 2008; Koga, et al., *PLoS One*, 2012). These findings prompted us to investigate the effects of MES+HS on skin inflammatory diseases such as psoriasis. In addition, our data from microarray analysis of healthy mouse skin tissues suggest that MES+HS may positively affect wound healing, and regulate cell proliferation and differentiation. Here, we examined the effect of MES+HS on the imiquimod-induced psoriasis mouse model. Interestingly, MES+HS treatment (10 min, daily) significantly suppressed skin hyperplasia in the psoriasis mouse model compared with the untreated group despite a minimal impact on the size of spleen and the mRNA expression of inflammatory cytokines in the skin tissue. Although the mechanism of MES+HS in ameliorating the progression of psoriasis still needs to be elucidated, our study provides information on the possible therapeutic application of MES+HS on psoriasis skin condition.

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Blimp-1 deficiency exacerbates the pathogenic processes of autoimmune diabetes in NOD mice harboring islet antigen-specific TCR repertoire

Liu, Y.-W.¹, Lin, M.-H.², Fu, S.-H.³, Sytwu, H.-K.³

¹National Defense Medical Center, Taiwan International Graduate Program, Graduate Institute of Life Sciences, Taipei, Taiwan, Republic of China, ²Kaohsiung Medical University, College of Medicine, Institute of Medicine, Department of Microbiology and Immunology, Kaohsiung, Taiwan, Republic of China, ³National Defense Medical Center, Department and Graduate Institute of

Microbiology and Immunology, Taipei, Taiwan, Republic of China

Since Blimp-1 is a master suppressor in the terminal differentiation of T cell lineages, its deficiency in T cells tightly leads to the massive expansion of effector T cells (CD4^{hi}CD62L^{lo}) in peripheral lymphoid organs and further causes the development of severe inflammatory colitis. However, it is still elusive how Blimp-1 immuno-modulates the pathogenesis of autoimmune diabetes. In this study, to examine whether Blimp-1 ablation affects the pathogenesis of autoimmune diabetes, we currently established T cell-specific Blimp-1 conditional knockout (CKO) non-obese diabetic (NOD) mice to further investigate the underlying molecular mechanisms of disease. NOD mice spontaneously develop autoimmune diabetes, resulting from the autoreactive T cell-dependent destruction of insulin-producing islet cells of pancreas. Unexpectedly, Blimp-1 CKO NOD mice were completely resistant to the development of autoimmune diabetes. In contrast, these mice were highly susceptible to the pathogenesis of inflammatory colitis. Our accumulating results indicated that CKO NOD mice have increased amounts of Th1 (CD4⁺IFN- γ ⁺) and Th17 (CD4⁺IL-17⁺) cells in mesenteric lymph nodes (MLNs), pancreatic lymph nodes (PLNs) and spleen but not in pancreas-infiltrating lymphocytes (PILs). By the contrary, BDC2.5 Blimp-1 CKO NOD mice, bearing islets antigen-specific TCR repertoire, have massive amount of Th1 cells in PILs. Moreover, Treg cells (CD4⁺Foxp3⁺) in the PILs of BDC2.5 Blimp-1 CKO NOD mice are comparable to BDC2.5 control mice. In summary, islet antigen-specific TCR repertoire might drive the recruitment of Th1 cells in pancreas-infiltrating lymphocytes of Blimp-1 CKO mice and subsequently these inflammatory cells aggravated the pathogenesis of autoimmune diabetes of mice.

Dendritic Cells

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Effects of α 1-adrenergic receptor stimulation on pro- and anti-inflammatory cytokine production by dendritic cells

Back, C.¹, Stumbles, P.^{1,2,3}, Drummond, P.⁴

¹Murdoch University, Veterinary and Life Sciences, Perth, Australia,

²Telethon Kids Institute, Perth, Australia, ³University of Western

Australia, Perth, Australia, ⁴Murdoch University, Psychology and

Exercise Science, Perth, Australia

Adrenergic receptors (AR) include the α - and β - classes and their subtypes, and are targets for the catecholamines noradrenaline, adrenaline and dopamine. Adrenergic receptors are expressed on a range of peripheral tissues, and can influence immune cell function. To date, most studies have examined the role of β -AR on immune cell function, with less known regarding α -AR. However, animal models suggest a role for α 1-AR in inflammatory disease pathogenesis. Signalling through α 1-AR can modulate cytokine production by macrophages and monocytes, but their effect on dendritic cells (DC) remains unclear. As DC control immune responses in part by the cytokines they produce during T cell activation, we hypothesized that α 1-adrenoceptor signalling would alter pro- or anti-inflammatory cytokine production by DC dependent on the timing of signalling and

their maturation status. To examine this, DC were derived from the bone marrow (BMDC) of C57BL/6 mice by culture in GM-CSF, then pre-incubated with bacterial lipopolysaccharide (LPS) for 18h prior to the introduction of the α 1-adrenoceptor specific agonist phenylephrine (PHE) for 6h or 24h. Culture supernatants were then analysed by 27-plex immunomagnetic bead assay. Exposure to PHE inhibited the production of the pro-inflammatory cytokines IL-1 α , IL-12p70 and p40, KC, MIP-1 α and MIP-1 β by LPS matured BMDC after 24h (but not 6h), with the anti-inflammatory cytokines IL-10 and TGF β less affected. This is the first study to show inhibition of pro-inflammatory cytokine production by DC after α 1-AR signalling, and provides a basis for further investigations into the potential role of α 1-AR in treating inflammatory disease.

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GSK-J4 a potent and selective inhibitor of the H3K27 demethylase JMJD3/UTX limits inflammation by favoring a tolerogenic potential in dendritic cells

Doñas, C.^{1,2}, Carrasco, M.^{1,2}, Fritz, M.^{1,2}, Prado, C.¹, Tejón, G.³, Osorio-Barrios, F.¹, Manriquez, V.³, Bono, M.R.³, Pacheco, R.¹, Loyola, A.¹, Roseblatt, M.^{1,2,3}

¹Fundación Ciencia & Vida, Santiago, Chile, ²Universidad Andrés

Bello, Santiago, Chile, ³Universidad de Chile, Santiago, Chile

Introduction: Epigenetic modifications on immune cells have been implicated in the development of several inflammatory diseases. Histone H3 lysine 27 (H3K27) demethylase JMJD3 plays an important role in the inflammatory response and appears as an interesting target for the treatment of inflammation. Recently, GSK-J4, a selective and potent JMJD3 inhibitor was synthesized, however, its effects on T cells and dendritic cells in the context of inflammation have not been reported.

Materials and methods: DCs purified from C57BL/6 mice and activated with LPS in the presence or absence of the drug were co-cultured with purified naïve CD4⁺ T cells from Foxp3-GFP mice under different polarizing conditions. DCs maturation and tolerogenic markers, as well as cytokines secretion were evaluated. C57BL/6 WT mice were treated with pMOG to induce EAE and subsequently treated with systemic administration of GSK-J4, with GSK-J4-generated Treg or with dendritic cells pre-treated with GSK-J4 and then, disease onset and severity were determined.

Results: GSK-J4 induced a tolerogenic phenotype on dendritic cells in vitro, increasing Treg generation and improving their phenotypic stability and suppressive function without affecting Th1 and Th17 differentiation. Administration of the drug in vivo ameliorates the severity of EAE. Moreover, adoptive transfer of Treg generated in the presence of GSK-J4 or of GSK-J4-treated DCs into EAE mice reduced the clinical manifestation of the disease.

Conclusions: Our data indicate that GSK-J4 attenuates inflammation through an effect on DCs. This drug may be a promising approach for the treatment of inflammatory diseases.

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Individual and cocktails of TLR ligands influence APCs *in situ* in human skin explants*du Mez, E.¹, Feisst, V.^{1,2}, Locke, M.³, McIntosh, J.^{1,2}, Brooks, A.^{1,2}, Didsbury, A.^{1,2}, Graham, S.^{4,5}, Angel, C.^{1,2}**¹University of Auckland, School of Biological Sciences, Auckland, New Zealand, ²University of Auckland, Maurice Wilkins Centre, Auckland, New Zealand, ³University of Auckland, Department of Surgery, South Auckland Clinical Campus, Middlemore Hospital, Auckland, New Zealand, ⁴University of Auckland, Centre for Brain Research, School of Medical Sciences, Faculty of Medical and Health Sciences, Auckland, New Zealand, ⁵University of Auckland, Department of Pharmacology and Clinical Pharmacology, School of Medical Sciences, Faculty of Medical and Health Sciences, Auckland, New Zealand*

Antigen presenting cells (APCs) are the sentinel cells of the immune system and can initiate strong, directed immune responses against foreign pathogens. In humans, APCs are found at sites susceptible to pathogen infiltration, including the skin. Cutaneous APCs are therefore ideal vaccine targets as they are accessible by injection and can stimulate immune responses.

APCs can detect invading pathogens via Toll-like receptors (TLR), and recognition by these receptors enables APCs to stimulate an appropriate immune response to that pathogen. We can exploit this mechanism by including synthetic TLR ligands in vaccines to augment an immune response against vaccine antigens.

We have conducted immunohistochemical analyses of human skin, which demonstrates that CD1a⁺ and CD14⁺ dermal APCs express TLR3 and TLR7 proteins respectively, whilst both APC populations express TLR9 protein.

We next developed an intradermal injection protocol using human skin explants to test the response of APCs to TLR stimulation *in situ*. Initial experiments established which concentrations of individual or cocktails of TLR3 (Poly I:C), TLR 7/8 (R848) and TLR9 (ODN2006, ODN2216 and ODN2396) ligands induced minimal toxicity, and identified optimal timepoints. In subsequent experiments the influence of TLR ligand/s on migratory APCs was assessed using flow cytometry, and four-colour immunohistochemistry was used to study the APCs remaining in the skin. Our findings show that stimulation with TLR ligand/s affects the phenotype and distribution of APC subsets in human skin.

Collectively these findings identify TLR ligand/s that could be incorporated into vaccines targeting APCs in human skin.

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T cell priming ability by activated, *Nlrc5*-deficient DCs is unaffected despite partially reduced MHCI levels*Rota, G.¹, Ludigs, K.¹, Siegert, S.², Tardivel, A.¹, Morgado, L.¹, Reith, W.³, De Gassart, A.¹, Guarda, G.¹**¹University of Lausanne, Epalinges, Switzerland, ²Ludwig Center for Cancer Research of the University of Lausanne, Epalinges, Switzerland, ³University of Geneva, Geneva, Switzerland*

NLRC5, a member of the NOD-like receptor protein family, has recently been characterized as the master transcriptional

regulator of major histocompatibility class I (MHCI) molecules in lymphocytes, in which it is highly expressed. However, its role in activated dendritic cells (DCs), which are instrumental to initiate T cell responses, remained elusive.

Using *Nlrc5*^{-/-} BMDCs, we showed that the increase of MHCI mRNA upon exposure to inflammatory stimuli is largely dependent on NLRC5. Despite the strong defect in transcript and intracellular protein levels of MHCI, surface display was nearly normal in *Nlrc5*^{-/-} DCs. Importantly, this discrepancy between a strong intracellular and a mild surface defect in H2-K levels was observed also in DCs with H2-K transcription defects independent of *Nlrc5*.

Hence, alongside with demonstrating the importance of NLRC5 in MHCI transcription in activated DCs, these data highlight a general, NLRC5-independent compensatory mechanism maintaining surface MHCI levels in cells with reduced MHCI mRNA.

Accordingly, with the decreased amount of neosynthesized MHCI, *Nlrc5*^{-/-} DCs exhibited a defective capacity to display endogenous antigens. However neither T cell priming by endogenous antigens, nor crosspriming ability were substantially affected in activated *Nlrc5*^{-/-} DCs.

Altogether, these data show that *Nlrc5*-deficiency, despite significantly affecting MHCI transcription and antigen display, is not sufficient to hinder T cell activation, underlining the robustness of the T cell priming process by activated DCs.

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Distribution of the potential DC-markers DEC205, Langerin, DC-LAMP and DC-SIGN within tissues of Atlantic salmon (*Salmo salar* L.) and their response upon challenge with microbes*Eggestøl, H.Ø.¹, Wegeland, H.I.¹, Rønneseth, A.¹, Tollefsen, K.E.², Haugland, G.T.¹**¹University of Bergen, Department of Biology, Bergen, Norway, ²Norwegian Institute of Water Research (NIVA), Oslo, Norway*

In mammals, dendritic cells (DCs) are the sentinels of the body and the main organizers of the immune system essential for initiation and regulation of immune responses. Until recently, it was debated whether DCs are present in teleost fish, but functional and morphological studies from different species suggest that fish do indeed have DCs. However, little is yet known about the role of DCs in the fish immune system and in which organs and tissues they are present. We have, indirectly, by targeting the potential DC-markers DEC205 (CD205/Ly75), Langerin (CD207), DC-LAMP (CD208) and DC-SIGN (CD209A, D and E), studied the distribution of DCs within 17 tissues of Atlantic salmon (*Salmo salar* L.) using RT-qPCR. The highest level of DEC205 was detected in gut, but it was also abundant in head kidney, body kidney, thymus and gills. Surprisingly, high level of DEC205 was also measured in the brain. The highest level of Langerin and DC-LAMP was detected in spleen, but also in head kidney and epithelial surfaces like skin, gill and gut. Atlantic salmon has three DC-SIGN homologues. CD209A

was highest in PBL, spleen and the posterior part of the kidney, CD209D was highest in the kidney tissues while the expression of CD209E was evenly distributed in all organs. Further, we have investigated the response of these DC-markers, that all belong to the family of C-type lectin receptors (CLRs), upon challenge with virus, bacteria and fungus.

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The phenotypical and functional effects of dendritic cells by calyx extract of a new variety *Hibiscus sabdariffa* with superior anthocyanin

Chuang, J.-J.¹, Tseng, H.-Y.¹, Chou, W.-M.²

¹National Chiayi University, Department of Microbiology, Immunology, and Biopharmaceuticals, Chiayi, Taiwan, Republic of China, ²National Chiayi University, Department of Biochemical Science and Technology, Chiayi, Taiwan, Republic of China

Dendritic cells (DCs) are professional antigen-presenting cells with high immunostimulatory abilities, can joint primary and secondary immune responses. Roselle (*Hibiscus sabdariffa*) possess rich anthocyanin, has recently been reported to induce multiple pharmacological effects. One γ -ray induced new roselle mutant designated Chia-yi Univ. 1 with extra superior anthocyanin was found in calyx extracts. In the present study we evaluated the effect of Chia-yi Univ. 1 on phenotype and function of dendritic cells. The toxicity of the calyx extract was first assessed by cytotoxicity assay. The result showed that roselle Chia-yi Univ. 1 expressed low toxicity to DCs. Under 0.5 mg/ml calyx extract could not induce the cell death of DCs. After analyzing cytokine production of treated immature DCs, we observed that the expression of TNF- α was relatively induced. Besides, the calyx extract of Chia-yi Univ. 1 obviously suppressed the ability of mature DCs to produce IL-10 and TGF- β and could increase the IL-6 in mature DCs. Additionally, after FACS analysis, we demonstrated that the expression of CD40, CD86 and IA/IE (MHC II) on mature DCs were further increased by Chia-yi Univ. 1. Thus, the results suggested that the effects of DCs by calyx extract of Chia-yi Univ. 1. The treated DCs may prove to be a potential tool for inducing of immunity control.

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Investigating the role of dendritic cells in a mouse model of Myelodysplasia

Pooley, J.¹, Cafarella, M.¹, Guirguis, A.², Curtis, D.^{3,4}, O'Keeffe, M.¹

¹Monash University, Clayton, Australia, ²Brigham and Women's Hospital, Cambridge, United States, ³Australian Centre for Blood Diseases, Monash University, Melbourne, Australia, ⁴Alfred Health, Central Clinical School, Monash University Melbourne, Dept. of Clinical Haematology, Melbourne, Australia

NHD13 mice are a mouse model resembling human Myelodysplasia (MDS). NHD13 mice express the fusion protein NUP98-HOXD13 (NHD13) of the chromosomal translocation t(2;11)(q31;p15) which is expressed in hematopoietic cells under control of the *vav* promoter. Mice bearing this transgene develop leukemia resembling human Acute Myeloid Leukemia. The role of dendritic cells in MDS, inflammatory responses in MDS or in the progression of MDS to leukemia is unknown. However, DC are greatly reduced in the blood of patients with MDS and in the BM of patients with multiple different forms of MDS. We have determined that mice with Myelodysplasia have a reduced number of dendritic cells (DC) as early as 4 weeks of age. The DC present in young NHD13 mice are activated but in mice older than 6 months of age the DC are in an immature state relative to wildtype. We additionally show that the DC of NHD13 mice are functionally defective with respect to their production of cytokines including interferons.

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Effect of past radiation exposure on the circulating dendritic cell populations in A-bomb survivors

Kajimura, J.¹, Lynch, H.E.², Geyer, S.³, Yamaoka, M.⁴, Shterev, I.²,

Kyoizumi, S.⁴, Sempowski, G.D.², Nakachi, K.⁴, Kusunoki, Y.⁴
¹Radiation Effects Research Foundation, Department of Molecular Biosciences, Hiroshima, Japan, ²Duke University Medical Center, Durham, United States, ³University of South Florida, Tampa, United States, ⁴Radiation Effects Research Foundation, Hiroshima, Japan

Previous A-bomb survivor studies suggested that radiation exposure might induce long-lasting immunological changes, such as enhancement of aging-related T-cell phenotypes, in the survivors. However, radiation effects on dendritic cells (DC) that may be a key factor behind the immunological changes remain unknown. In this study, we investigated numerical and functional alterations associated with radiation and aging in the circulating DC populations among A-bomb survivors.

Method and materials: Peripheral blood samples were collected with informed consent from 229 participants in the Adult Health Study at Radiation Effects Research Foundation. The two major DC populations, conventional DC (cDC) and plasmacytoid DC (pDC), were numerically analyzed and sorted by flow cytometry. The sorted cDC and pDC were stimulated with TLR3 and TLR7 ligands, respectively; and then, alterations in cytokine production levels and gene expression profiles of the DC populations were determined, using a bead-based multiplex assay and a PCR array, respectively.

Results and discussion: In female survivors, both the number

and functions of cDC decreased with age but not radiation dose, whereas the number of pDC decreased but function of pDC increased with age and radiation exposure dose. However, no significant numerical and functional changes associated with age or radiation dose were observed in male survivors. Our results suggest that aging-associated alterations in the DC populations, especially those in pDC, might be accelerated by past radiation exposure in a gender-dependent manner.

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IRF8 dependent classical dendritic cells are essential for intestinal T cell homeostasis

Luda, K.M.¹, Joeris, T.^{1,2}, Persson, E.K.³, Rivollier, A.^{1,2}, Demiri, M.¹, Sitnik, K.M.², Pool, L.², Holm, J.B.⁴, Melo-Gonzalez, F.^{5,6,7}, Richter, L.⁸, Lambrecht, B.N.³, Kristiansen, K.⁴, Travis, M.A.^{5,6,7}, Svensson-Frej, M.¹, Kotarsky, K.¹, Agace, W.W.^{1,2}

¹Lund University, Lund, Sweden, ²Technical University of Denmark, Copenhagen, Denmark, ³Ghent University - VIB, Ghent, Belgium, ⁴University of Copenhagen, Copenhagen, Denmark, ⁵University of Manchester, Manchester, United Kingdom, ⁶Manchester Collaborative Centre of Inflammation Research (MCCIR), Manchester, United Kingdom, ⁷Wellcome Trust Centre for Cell-Matrix Research, Manchester, United Kingdom, ⁸Oslo University Hospital, Oslo, Norway

The role of dendritic cells (DCs) in intestinal immune homeostasis remains incompletely defined. Here we show that mice lacking IRF8 dependent DCs have reduced numbers of T cells in the small intestine (SI), but not large intestine (LI), including an almost complete absence of SI CD8ab⁺ and CD4⁺CD8aa⁺ T cells; the latter requiring b8 integrin expression by migratory IRF8 dependent CD103⁺CD11b⁻ DCs. SI homing receptor induction was impaired during T cell priming in mesenteric lymph nodes (MLN), which correlated with a reduction in aldehyde dehydrogenase activity by SI derived MLN DCs, and inefficient T cell localization to the SI. Finally, mice with a DC deletion in IRF8 lacked intestinal T helper 1 (Th1) cells, and failed to support Th1 cell differentiation in MLN and mount Th1 responses to *Trichuris muris* infection. Collectively these results highlight multiple non-redundant roles for IRF8 dependent DCs in the maintenance of intestinal T cell homeostasis.

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Coordinate regulation of dendritic cell migration by the DOCK family of Rac guanine exchange factors

Kunimura, K., Fukui, Y.
Kyushu University, Division of Immunogenetics, Medical Institute of Bioregulation, Fukuoka, Japan

Dendritic cells (DCs) are specialized antigen presenting cells that play a critical role in initiation of adaptive immune responses. Upon antigen exposure, DCs phagocytose antigens in peripheral tissues, and migrate via the afferent lymphatic vessels into the draining lymph nodes to stimulate T cells. During this process, DCs switch their sessile sampling behavior to a highly migratory one, which is characterized by the acquisition of a polarized morphology. This morphologic polarity is regulated by Rac,

a member of the small GTPases that cycle between inactive GDP-bound and active GTP-bound states. Although Rac is activated by means of guanine exchange factors (GEFs), the Rac GEFs critical for DC migration remains to be determined. We found that DCs express all DOCK-A subfamily members, DOCK1, DOCK2 and DOCK5 that are known to act as Rac GEFs. By developing single, double and triple KO mice, we show in this study that DC migration is coordinately regulated by these DOCK family GEFs.

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Identification of unique dendritic cell and macrophage subsets in human anogenital tissues

Bertram, K.M.¹, Botting, R.A.^{1,2}, Baharlou, H.^{1,2}, Sandgren, K.¹, Kim, M.¹, Cunningham, A.L.^{1,2}, Harman, A.N.¹

¹Westmead Institute for Medical Research, Centre for Virus Research, Westmead, Australia, ²University of Sydney, Medicine, Sydney, Australia

Dendritic cells (DCs) and macrophages present within anogenital tissues are two of the first cell types to encounter pathogens during sexual intercourse, which they detect via C-type lectin receptors (CLR) expressed on their surface. Furthermore, these cells play a direct role in transmission of some viruses such as HIV. Surprisingly however, very little human data is available regarding the specific DC and macrophage subsets that are present within different anogenital tissues.

We have established access to all the tissues that pathogens may encounter during sexual intercourse (labia, vagina, cervix, glans penis, foreskin, anus and rectum). Using complex multicolour flow cytometry we have determined the population frequency of all known DC and macrophage subsets within these tissues and also determined which CLR they express. We find that different anogenital tissues differ significantly in the subsets of these cells they contain and, importantly, we have identified several novel subsets. For example in foreskin and labia, in addition to Langerhans cells, we have identified an additional CD1a⁺ epidermal DC subset and two CD11c⁺ dermal myeloid subsets that can be discriminated by CD1c expression and are negative for all other DC and macrophage markers (CD1a, CD14, CD141 and CD123). In the anal epithelium we have identified a further epidermal DC subset that expresses langerin but not CD1a and these cells are also present in rectal but not anal lamina propria.

Identifying unique anogenital DC and macrophage populations and their CLR expression is important in understanding the transmission and immune control of sexually transmitted pathogens.

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GMP-production of an allogenic DC-based cancer vaccine (INTUVAX) for treatment of patients with metastatic kidney- or primary liver cancer. Comparison of two production platforms for DC-generation

Adamson, L.¹, Andersson, B.², Kiessling, R.¹, Nasman-Glaser, B.¹, Karlsson-Parra, A.³

¹Karolinska Institutet, Dept of Oncology & Pathology, Stockholm,

Sweden, ²Gothenburg University, Immunology, Gothenburg, Sweden, ³Uppsala University, Dept of Immunology, Uppsala, Sweden

The aim of INTUVAX[®] phase I/II studies in patients with metastatic kidney- or primary liver cancer is to evaluate adverse effects and anti-tumor effects of a therapeutic vaccine-concept that is based on intratumoral injections of activated allogenic dendritic cells (DCs) produced from healthy donors. The strong allogenicity of such cells and their sustained production of pro-inflammatory mediators is expected to create a local inflammatory environment that promotes recruitment, antigen-loading and activation of endogenous DCs.

Clinical studies require protocols where a sufficient number of well characterized DCs are produced according to Good Manufacturing Practice (GMP) guidelines. We validated two different platforms for production of activated allogeneic DCs. In the COMBIG ("COMBined toll-like receptor Interferon-Gamma") GMP platform, ELUTRA[®] -enriched monocytes from leukapheresis products from healthy donors were used as starting material. In the other, CliniMACS[®] purified monocytes from pooled Buffy Coats were used as starting material. Collected fractions were phenotypically analysed for cell content by flow cytometry. The enriched monocyte fractions were differentiated into immature DC in culture bags using GM-CSF and IL-4 and matured for 18 hours using a cocktail of Poly-IC, R 848 and IFN γ . DC-generation was determined using phenotypic markers and expression of cytokines.

Our results show that these two COMBIG vaccine production platforms are comparable, very robust and reproducible resulting in high initial monocyte purity, high recovery and efficient differentiation/maturation of phenotypically and functionally mature DCs. Delivered frozen vaccine cells show a high and reproducible quality after thawing.

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Ectromelia virus-induced down-regulation of genes associated with myeloid dendritic cell activation and maturation is mouse-strain independent

Szulc-Dabrowska, L.¹, Cymerys, J.¹, Toka, F.N.^{1,2}, Struzik, J.¹, Nowak, Z.³, Gierynska, M.¹

¹Warsaw University of Life Sciences-SGGW, Faculty of Veterinary Medicine, Department of Preclinical Sciences, Warsaw, Poland,

²Ross University School of Veterinary Medicine, Department of Biomedical Sciences, Basseterre, Saint Kitts and Nevis, ³Warsaw University of Life Sciences-SGGW, Faculty of Animal Sciences, Department of Genetics and Animal Breeding, Warsaw, Poland

Resistance and susceptibility to ectromelia virus (ECTV) infection in mice are controlled by many genetic factors and are associated with the preferential development of Th1 and Th2 cytokine immune responses, respectively. Myeloid dendritic cells (mDCs) isolated from various inbred mouse strains have a different ability to react to pathogen infection/exposure and, consequently, stimulate protective or non-protective Th immune responses. In the present study we evaluated the

effect of ECTV infection on transcriptional response of innate and adaptive immune genes in bone marrow-derived DCs (BMDCs) obtained from BALB/c (susceptible) and C57BL/6 (resistant) mice. Our results showed that ECTV similarly affects mRNA expression of chemokines and cytokines, and mRNA encoding proteins engaged in antigen uptake, processing and presentation in BMDCs generated from BALB/c and C57BL/6 mice. In both cases, ECTV does not stimulate genes, such as *cd80* and *cd86*, related to maturation of BMDCs, moreover it down-regulates several genes associated with their innate and adaptive immune functions, with the exception of *il10* that is significantly up-regulated. These data strongly suggest that *in vitro* modulation of BMDCs gene expression profile by a poxvirus occurs independently of the mouse strain susceptibility to infection. Inhibition of the activation and/or maturation of mDCs may be a strategy of immune evasion used by ECTV to reduce the capacity of mDCs to stimulate activation and proliferation of antigen-specific T lymphocytes. It is likely that this inhibitory effect is more pronounced in susceptible mouse strains under *in vivo* conditions.

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A endogenous retroviral noncoding RNA is highly enriched in dendritic cell exosomes and transferred to recipient cells *in vitro* and *in vivo*

Barrios, M.^{1,2}, Garnham, A.³, Wakefield, M.³, Scicluna, B.J.^{4,5}, Huntington, N.^{2,6}, Masters, S.^{1,2}, Pang, K.C.^{1,2,7}

¹Walter and Eliza Hall Institute of Medical Research (WEHI), Inflammation, Melbourne, Australia, ²University of Melbourne, Medical Biology, Melbourne, Australia, ³Walter and Eliza Hall Institute of Medical Research (WEHI), Bioinformatics, Melbourne, Australia, ⁴University of Melbourne, Department of Medicine, Department of Biochemistry and Molecular Biology, Parkville, Australia, ⁵La Trobe University, Department of Biochemistry and Genetics, La Trobe Institute for Molecular Sciences, Bundoora, Australia, ⁶Walter and Eliza Hall Institute of Medical Research (WEHI), Molecular Immunology, Melbourne, Australia, ⁷Royal Children's Hospital, Murdoch Childrens Research Institute, Parkville, Australia

Exosomes are nano-scale vesicles that are released from cells into the extracellular environment. There is increasing interest in the role of exosomal RNAs as mediators of intercellular communication, but there is minimal evidence that intercellular RNA transfer occurs *in vivo*. To explore this issue, we profiled exosomal RNAs from mouse dendritic cells (DCs) using RNAseq, and identified 230 exosome-enriched RNAs. Unexpectedly, the RNA that showed the greatest enrichment in exosomes (~200-fold) and was also the third most abundant exosomal RNA was the VL30 long noncoding RNA (lncRNA), which is derived from a mouse-specific endogenous retrovirus and has previously been shown to function as an important transcriptional regulator. Next, we tested whether VL30 lncRNA could be transferred to recipient cells *in vitro* by co-culturing DC-derived exosomes with various human cell lines. In each case, we found evidence of exosome-mediated VL30 lncRNA transfer. Finally, to determine

whether VL30 lncRNA can be transferred to recipient cells *in vivo*, we generated humanized mice whose T and B cells are of human origin and assessed whether VL30 lncRNA can be found in either the human T or B cells, whose genomes lack VL30 orthologs. Strikingly, VL30 was readily detected in human B cells from these mice, indicating that VL30 is also transferred *in vivo*. Taken together, this work suggests that VL30 lncRNA is being selectively loaded into exosomes and subsequently transferred to recipient cells. How this selective loading occurs and the functional consequences of VL30 lncRNA transfer are now being investigated.

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The influence of immunosuppression on intraepithelial airway mucosal dendritic cell

Shevchenko, M.¹, Troyanova, N.¹, Servuli, E.¹, Fedorina, A.², Mirzoev, R.¹, Bolkhovtina, E.¹, Sapozhnikov, A.¹

¹Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Immunology, Moscow, Russian Federation, ²Lomonosov Moscow State University, Biology, Moscow, Russian Federation

Intraepithelial airway mucosal dendritic cells due to their location can sense inhaled pathogens, including conidia of opportunistic fungi *Aspergillus fumigatus*. Susceptibility to *Aspergillus*-induced infection is usually a result of immunosuppression that mainly leads to neutropenia, but also affects some other leukocyte populations.

In the present study we investigate whether immunosuppression influences intraepithelial airway mucosal dendritic cells.

BALB/c mice were subjected to immunosuppression by two ways: two injections of cyclophosphamide and cortisone acetate or one injection of anti-Gr-1 depletion antibodies. 24 hours after mice were sacrificed; main bronchi were microdissected and subjected to immunostaining and confocal imaging as whole-mount airways. Airway mucosal dendritic cells were identified according to MHCII expression, morphology - special dendritiform shape and location in a close proximity to epithelial cells. Specimens were scanned in Z-stack mode and intraepithelial airway mucosal dendritic cells were quantified per square millimeter of epithelium.

MHCII positive cells with dendritiform shape were identified in the airways of both intact mice and mice with induced immunosuppression. Statistical analysis revealed no significant differences between the numbers of intraepithelial dendritic cells per square millimeter of epithelium in intact mice (340±125), mice that received cyclophosphamide and cortisone acetate in combination (239±98) and mice that were injected with depletion antibodies (255±91).

Thus, immunosuppression does not influence morphology and number of intraepithelial airway mucosal dendritic cells. Functional analysis of the population in condition of immunosuppression is to be investigated.

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Human and canine dendritic cell responses triggered by *Brucella canis* and *Escherichia coli* lipopolysaccharide

Pujol, M.¹, Arias, J.², Alvarez, C.², Rojas, L.², Ferreira, A.³, Vernal, R.²

¹Universidad de Chile, Facultad de Ciencias Veterinarias y Pecuarias, Santiago, Chile, ²Universidad de Chile, Facultad de Odontología, Santiago, Chile, ³Universidad de Chile, Facultad de Medicina, Santiago, Chile

The aim of this study was to evaluate the cytokine profile production by canine and human dendritic cells (DCs) when stimulated *in vitro* with *B. canis* Lipopolysaccharide (LPS) or *E. coli* LPS, as compared to the activating capacity of LPS purified from a naturally rough *Brucella*.

Brucella spp. can lead to chronic intracellular infections due to their low stimulatory activity on immune cells, in part mediated by their LPS. Naturally smooth *Brucella* LPS is several times less effective at inducing the innate immunity than enterobacterial LPS. However, there is much less knowledge about naturally rough *Brucella* LPS.

Monocytes were purified from canine and human blood samples, using magnetic cell sorting, and then differentiated to immature DCs, upon induction with GM-CSF and IL-4. DCs were stimulated with LPS purified from *B. canis* or *E. coli*. The mRNA expression levels for IL-1 β , IL-4, IL-5, IL-6, IL-10, IL-12p35, IL-17A, IFN- γ , TNF- α and TGF- β 1 were quantified by qRT-PCR.

Canine DCs stimulated with *B. canis* LPS increased expression of IL-6 and IL-12p35, while *E. coli* LPS increased IL-12p35 expression. *B. canis* LPS increased IL-12p35 expression in human DCs, while *E. coli* LPS increased IL-1 β and TNF- α expression, as compared with non-stimulated DCs.

No significant differences were detected in canine DCs stimulated with LPS from *B. canis* vs *E. coli*. However IL-1 β expression in human DCs with *E. coli* LPS was higher.

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Identification of human DC precursors through the integration of high dimensional strategies

See, P.¹, Dutertre, C.-A.^{1,2}, Chen, J.¹, Günther, P.³, McGovern, N.¹, Gunawan, M.⁴, Duan, K.¹, Händler, K.³, Sumatoh, H.R.B.¹, Malleret, B.¹, Beyer, M.³, Irac, S.E.², Low, I.¹, Shadan, N.B.¹, Lum, J.¹, Zolezzi, F.¹, Larbi, A.¹, Poidinger, M.¹, Renia, L.¹, Schlitzer, A.^{1,3}, Schultze, J.L.³, Newell, E.¹, Haniffa, M.⁴, Ginhoux, F.¹

¹Singapore Immunology Network (SiGN), Singapore, Singapore, ²Duke-NUS Graduate Medical School, Singapore, Singapore, ³Life and Medical Science (LIMES) Institute, University of Bonn, Bonn, Germany, ⁴Institute of Cellular Medicine, Medical School, Newcastle University, Newcastle upon Tyne, United Kingdom

Dendritic cells (DC) are professional antigen-presenting cells that mediate immune responses. Important questions on the origins and differentiation paths of human DC populations remain unanswered. Here we combined two high-dimension techniques, single-cell mRNA sequencing and Cytometry by Time of Flight mass spectrometry to define and characterize

human DC precursors (pre-DC) present in blood. We showed that the previously-underestimated pre-DC population share several surface markers with plasmacytoid DC (pDC), and we revealed how some properties previously attributed to pDC are in fact restricted to pre-DC present in the same cultures. We also identified the whole DC lineage from BM to peripheral blood and revealed that pre-DC comprised of three distinct lineage-committed sub-populations, and demonstrated their presence and functional distinctions in peripheral blood. Hence, this opens promising new avenues for investigation of therapeutic potential of DC subset-specific targeting.

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Modulation of dendritic cells by exosomes derived from human breast milk and plasma

Larssen, P., Eldh, M., Gabrielsson, S.

Karolinska Institutet, Department of Medicine, Translational Immunology Unit, Stockholm, Sweden

Exosomes are small extracellular vesicles, 30-100 nm in diameter, produced and secreted by most cells including immune cells. They are present in body fluids such as plasma and breast milk. Breast milk derived exosomes can induce foxp3 expression in PBMCs indicating that they may induce tolerance, thereby possibly influencing allergy development. It is not known how plasma- or milk exosomes affect dendritic cells. Therefore, we aimed to compare the immune modulatory properties of exosomes derived from human breast milk and plasma on monocyte derived dendritic cells (MDDC). We show that plasma-derived exosomes strongly increase the HLA-DR expression on MDDC, while breast milk-derived exosomes do not. Furthermore, plasma exosomes but not milk derived exosomes induced cytokine secretion such as; IL-10, IL-6 and TNF α , suggesting a more pro-inflammatory role of plasma exosomes. Instead breast milk exosomes may be important in regulation of immune responses towards induction of tolerance, which we will further investigate. We conclude that these exosomes have diverse immunological properties and this contributes to the understanding of the role of exosomes in immunological processes.

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Characterization of dendritic cells of gingival mucosa in subjects with periodontal disease

Lizárraga-Verdugo, E.R.¹, Jiménez-Gastélum, G.R.², Gastélum-Rosales, X.G.¹, Romero-Quintana, J.G.², Ayala-Ham, A.D.R.², Ramos-Payán, R.², Flores-Romo, J.L.³, Aguilera-Galaviz, L.A.⁴, Sánchez-Schmitz, G.⁵, Aguilar-Medina, E.M.²

¹University Autonomous of Sinaloa, Biotechnology Masters Regional Program. Chemistry and Biology Science Faculty, Culiacan, Mexico, ²University Autonomous of Sinaloa, Immunogenetics and Molecular Biology Academic Group. Chemistry and Biology Science Faculty, Culiacan, Mexico, ³CINVESTAV IPN, Department of Cellular Biology, Mexico City, Mexico, ⁴Autonomous University of Zacatecas, Odontology Faculty, Zacatecas, Mexico, ⁵Harvard University, Division of Infectious Diseases, Boston Children's Hospital and Harvard Medical School, Boston, United States

Introduction: Periodontal disease (PD) is one of the most common chronic inflammatory diseases in humans it involves many factors; mainly the composition of the microbes in the subgingival plaque, and the host immune response. A high rate of monocytes may be found in chronic lesions as T cells, B cells, macrophages and dendritic cells (DC). The role of DCs it's not well described in this pathological condition.

Methodology: Gingival biopsies samples were obtained from healthy individuals, patients with gingivitis and chronic periodontitis. HLA, CD11c, CD83 and CD80 were analyzed by flow cytometry. In other hand, one fraction of biopsies were oriented in Ployfreeze and frozen in liquid nitrogen and stored -80 °C to analyze CD1a, CD-80 and -83 by fluorescence IHC. Sub- and supra-gingival plaque were collected in order to characterize the microflora and make an association with the state of the cells.

Results: DCs CD11c+ were higher in healthy and gingivitis subjects (9.55 and 8.93%) whereas in periodontitis subjects are 6.17%. CD80+ were low expressed in periodontitis subjects (4.99%) and higher in gingivitis and healthy subjects (7.50 and 10.19%) by flow cytometry.

Conclusion: Due to the chronic inflammation DCs in a normal state remains low in periodontitis subjects. Sameway, activated DCs are low in periodontitis subjects which show that the labor of present antigen in DCs are not good under periodontitis.

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Column-based untouched magnetic separation of human pDCs yields superior performance and functionally unaffected cells

Morrissey-Wettey, F.R., Angerer, C., Kurig, S., Richter, A., Dzionek, A. Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

Plasmacytoid dendritic cells (pDC) play a crucial role in innate immune defense by recognition of conserved pathogen-associated molecular patterns (PAMPs). PAMPs activate pDCs via pattern recognition receptors such as Toll-like receptors (TLR). In particular, CpG motifs of bacterial DNA bind specifically either endosomal or lysosomal TLR9, thereby inducing secretion of IFN- α or proinflammatory cytokines and classical DC maturation, respectively. However, as observed in autoimmune settings, TLR9 also recognizes vertebrate CpG DNA-motifs as danger-associated molecular patterns (DAMPs) that activate pDCs. Routinely pDCs are isolated by indirect magnetic cell separation for *in-vitro* research. Here we describe the influence of the isolation method on the functionality of the purified pDCs. We compared Miltenyi Biotec's column-based with a column-free system from another supplier. Not only achieved the former a superior separation performance, it also enriched 10- and 30-fold less DNA-containing dead cells and debris, respectively. Both procedures yielded phenotypically comparable pDCs instantly after isolation. Whereas pDCs isolated using Miltenyi's procedure were functionally normal, cells isolated with the column-free system were pre-activated as evident from elevated expression of CD80, -83, -86 and HLA-DR and significantly increased IFN- α production after 24h of culture without stimulation. Moreover, upon stimulation with CpG-B pDCs isolated with the column-free method secreted extraordinary high amounts of IFN- α ,

whereas pDCs isolated with Miltenyi's system exhibited normal responses to CpG-A and CpG-B.

These results clearly demonstrate that unspecific enrichment of dead cells and debris may significantly influence the activation status of the isolated pDCs and therefore affect the results of downstream applications.

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Importance of lung migratory DC in Influenza immunity

Ng, S.L., Ruedl, C., Erik Karjalainen, K.

Nanyang Technological University (NTU), Singapore, Singapore

Migratory CD103⁺ DC, which is specialized in cross-presenting extracellular antigen to cytotoxic T lymphocytes (CTLs), is indispensable for the induction of protective CTL response against Influenza A virus (IAV) infection. Using Clec9a-DTR transgenic mouse which allows us to specifically ablate CD103⁺ DCs upon DT administration, we aim to investigate the detailed mechanisms on how CD103⁺ DC-deficiency contributes to reduced accumulation of IAV-specific CTLs in the lung. We demonstrated in our Clec9a-DTR mouse that ablation of CD103⁺ DCs led to increased susceptibility to IAV infection and IAV-infected Clec9a-DTR mice harbored drastically fewer number of IAV-specific CTLs. Contrary to what we expected, in the absence of CD103⁺ DCs, the number of IAV-specific CTLs cells in the mediastinal lymph node (mLN) increased despite attenuated cross-presentation *in situ*. This observation suggests that efficient migration of CTLs from the mLN requires CD103⁺ DCs. Additionally our data showed that in IAV-infected Clec9a-DTR mouse, IAV-specific CTLs in the lung were less viable and adoptive transfer of CD103⁺ DCs improved the survival of these effector T cells suggesting direct involvement of CD103⁺ DCs in supporting CTLs survival. Moreover, our investigations also revealed significant reduction of memory CTL (KLRG1- IL7R⁺) number in the absence of CD103⁺ DCs and IAV-primed Clec9a-DTR mice were more susceptible to re-infection by serotypically distinct IAV strain. We reason that this reduction can be partially explained by the reduction in the frequency of CXCR3 (neg) effector CTLs in which these cells have been associated with high memory potential.

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XCR1 expression by dendritic cells promotes NK cell activation during viral infection

Ghilas, S., Dalod, M., Crozat, K.

Centre d'Immunologie de Marseille-Luminy, Marseille, France

A subset of mouse dendritic cells (DCs) specifically express the chemokine receptor XCR1 and excels at priming CD8⁺ T cells including through cross-presentation. XCR1⁺ DCs are also highly efficient for the activation of natural killer (NK) cells and invariant NK T cells. XCL1, the ligand of XCR1, is selectively and strongly expressed by NK, NK T and activated or memory CD8⁺ T cells. Hence, we are investigating whether and how the XCL1/XCR1 axis regulates the cross-talk between cytotoxic lymphocyte subsets and XCR1⁺ DCs.

Here we examined whether XCR1 deficiency affected NK cell

responses to mouse cytomegalovirus (MCMV) infection. XCR1-deficient mice harbored high viral loads at day 5 after infection, at a time when viral replication had become undetectable in WT animals. XCR1-deficient mice also harbored diminished XCR1⁺ DC IL-12 production and NK cell responses. Finally, in infected XCR1-deficient mice, NK cells and XCR1⁺ DCs failed to cluster together in the marginal zone of the spleen. Hence, signaling through XCR1 may orchestrate in time and space the interactions between XCR1⁺ DCs and NK cells during MCMV infection, thereby promoting their mutual activation and the downstream control of the virus.

This work was funded by Aix Marseille Université (scholarship from the University President to S.G.), Association pour la Recherche sur le Cancer (ARC) and the European Research Council under the European Community's Seventh Framework Programme (FP7/2007-2013 Grant Agreement no. 281225 for the SystemsDendritic project).

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Interferon response factor-3 is required for the pro-Th2 activity of mouse myeloid CD11b⁺ dendritic cells

Janss, T., Mesnil, C., Pirottin, D., Lemaitre, P., Schyns, J., Marichal, T., Bureau, F., Desmet, C.

University of Liège, Liège, Belgium

The transcription factors required for antigen-presenting cells (APCs) to induce T helper type 2 (Th2) responses are less well defined than for other types of T helper responses. Using mouse lung resident conventional CD11b⁺ dendritic cells (CD11b⁺ cDCs) in the context of house-dust mite (HDM)-driven allergic airway sensitization as a model, we aimed here to identify transcriptional events regulating the pro-Th2 activity of dendritic cells. Transcriptomic profiling of CD11b⁺ cDCs exposed to HDM *in vivo* revealed first that HDM triggered a prominent antiviral defense-like response and second that most of the HDM-induced transcriptional changes in CD11b⁺ cDCs depended on the transcription factor Interferon Response Factor-3 (Irf3). Validating the functional relevance of these observations, genetic deficiency in Irf3 diminished the pro-allergic activity of CD11b⁺ cDCs. The reduced pro-allergic activity of Irf3-deficient CD11b⁺ cDCs was not related to impaired antigen uptake or migratory activity. Instead, following direct contact with naive T cells, Irf3-deficient CD11b⁺ cDC induced less Th2, more regulatory T cell, and similar Th1 differentiation compared to their wild-type counterparts. The altered APC activity of Irf3 CD11b⁺ cDCs was associated with reduced expression of specific costimulatory molecules and was phenocopied by blocking the activity of the same molecules in wild-type CD11b⁺ cDCs. Altogether, these results establish Irf3, mostly known for its antiviral activities, as a transcription factor required for the induction of Th2 responses through appropriate costimulation in lung CD11b⁺ cDCs.

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Gene profiling of murine splenic dendritic and myeloid subsets*Hey, Y.Y., O'Neill, H.C., O'Neill, T.J.**Bond University, Gold Coast, Australia*

Phenotype studies have been widely used to identify and characterise splenic myeloid and dendritic cell (DC) subsets. However, these studies are limited by the availability of antibodies, and the expression level, and specificity of markers. Multiple subsets can express the same marker. For example, F4/80, a red pulp macrophage marker, is also expressed on monocytes and DC. In order to delineate the lineages of DC and tissue macrophages more completely, recent studies have employed transcriptome analysis to generate gene profiles unique to macrophages and DC, respectively. A novel dendritic-like cell (L-DC) described recently resembles a myeloid cell on the basis of a CD11b^{hi}CD11c^{lo}CD43⁺MHCII⁻Ly6C⁻Ly6G⁻Siglec-F⁻CX3CR1^{lo} phenotype, but a DC on the basis of antigen presenting function. To fully elucidate the relationship between L-DC, conventional DC (cDC) and myeloid subsets, transcriptome analysis was undertaken. Principal components analysis showed close grouping of monocytes, L-DC and cDC in the first principal component, but separation of L-DC and monocytes from cDC subsets in the second principal component. In addition, hierarchical clustering indicates a close relationship between L-DC and resident monocytes, and then with inflammatory monocytes. Lists of common splenic cDC and monocytes genes were generated, respectively. L-DC expressed most of the common cDC and monocytes genes, suggesting that L-DC mirror both the cDC and monocyte lineages. Further analysis of L-DC gene profile also showed upregulation of genes reflecting both DC and myeloid lineages. Lastly, a comparison of gene profiles between L-DC and monocyte subsets, revealed CD300e to be a distinct marker for L-DC.

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The role of inflammatory stimuli in dendritic cell biology*Tian, Z.¹, Villadangos, J.^{2,3}, Roquilly, A.⁴, Mintern, J.³, Jose Villadangos*

¹University of Melbourne, Peter Doherty Institute for Infection and Immunity, Melbourne, Australia, ²University of Melbourne, Peter Doherty Institute for Infection and Immunity, Department of Microbiology and Immunology, Melbourne, Australia, ³University of Melbourne, Department of Biochemistry and Molecular Biology, Melbourne, Australia, ⁴University Hospital of Nantes, Nantes, France

Infection-induced inflammation drives direct DC activation by encountering pathogen associated/ danger associated molecular patterns (PAMPs/ DAMPs) directly, and indirect DC activation by encountering secondary inflammatory signals released by PAMPs/ DAMPs- exposed cells. Directly activated DC and indirectly activated DC are two distinct DC populations with differential characteristics and immunological roles. However, due to the lack of effective markers to discriminate these two DC populations, the study on the characteristics and immunological roles of the two DC populations is limited. This project aims to identify the membrane markers that discriminate directly vs.

indirectly activated DC, and to characterize the T cell response to indirectly activated DC, as well as to investigate the interest of antigen targeting to indirectly activated DCs to boost immune response during inflammation. Based on this study, we will be able to describe the specific phenotypes of directly vs indirectly activated DCs, to assess the immune functional properties of indirectly activated DC, as well as to assess the formation of directly and indirectly activated DCs *in vivo*, under both steady-state and inflammatory conditions. The results will also assist rational designing of vaccines to maximize the immune response, as well as strategies to minimize autoimmunity.

Stem Cells & Immunity

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Increasing stem cell dose promotes immune reconstitution following stem cell transplant*Xu, N., Shen, S., O'Brien, T., Dolnikov, A.**Sydney Children's Hospital, Cord and Marrow Transplant Laboratory, Randwick, Australia*

Immunologic reconstitution following allogeneic stem cell transplantation is a critical component of successful outcome. The effect of stem cell dose on immune reconstitution was examined using immunocompromised NSG mice transplanted with different numbers of T-cell depleted cord blood derived CD34⁺ stem cells. Infusion of large numbers of stem cells promoted early B-, T- and myeloid cell engraftment and increased the magnitude of immune reconstitution at late stages post-transplant. Infusion of larger numbers of stem cells promoted CD8⁺ T cell differentiation and delayed late memory T cell skewing in expanse of naive T cells resulting in increased diversity in the T cell repertoire and stronger immune responses. We have also shown that the immune responses against xenografted leukaemia cells correlate with the magnitude of T-cell engraftment in stem cell reconstituted mice highlighting the importance of stem cell dose in the transplant to reduce the risk of leukaemia relapse particularly in T cell depleted haploidentical stem cell transplants. Additionally, increased expansion of adoptively transferred T cells generating potent anti-tumour effects against mismatched leukaemia cells was associated with higher stem cell engraftment. Therefore, increasing stem cell dose in the transplant prior to adoptive T cell transfer may promote graft-versus-leukaemia effect.

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Investigating myeloid development in the context of a spleen microenvironment*Short, C., O'Neill, H., Tan, J.**Bond University, Robina, Australia*

The spleen contains multiple myeloid cell populations with varying functions in immunity and wound healing. The differentiation of hematopoietic stem cells (HSC) and progenitors into myeloid CD11b⁺CD11c^{lo} antigen presenting cells (APC) has been previously demonstrated *in vitro* in the context of a spleen stromal microenvironment. This same

subset is also produced indefinitely in spleen long-term cultures (LTC), suggesting they represent a spleen endogenous immune cell population derived from tissue-resident HSC. However, the precise correlation between culture-derived myeloid APC and naturally-occurring APC in spleen has been difficult to establish, although they are clearly distinct from conventional dendritic cells. This study now addresses whether common monocyte progenitors (CMoP) differentiate to give this distinct subset of myeloid CD11b⁺CD11c^{lo} APC in the context of a spleen stromal microenvironment, and whether these represent monocytes or a unique cell lineage. Findings will be presented demonstrating *in vitro* spleen stromal co-cultures of CMoP from sources including adult bone marrow, and adult and neonatal spleen. Co-cultures of HSC over spleen stroma, and cytokine-induced (SCF, LIF, IL-3 and IL-6) cultures of CMoP, will serve as controls which produce myeloid CD11b⁺CD11c^{lo} APC and CD11b⁺Ly6C^{hi} monocytes, respectively. Results from this study specifically allow the establishment of a relationship between co-culture and LTC-derived myeloid-APC, and cells arising from the monocyte lineage defined by the CMoP precursor.

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Single cell sequencing reveals heterogeneity within murine bone marrow derived stem and progenitor population

Zalcenstein, D., Tian, L., Schreuder, J., Naik, S.

Walter & Eliza Hall Institute of Medical Research, Molecular Medicine, Parkville, Australia

In blood cell development (haematopoiesis) the prevailing paradigm is that haematopoietic stem cells (HSCs) generate multi-potent progenitors (MPPs) that later asymmetrically split to initiate the generation of cell type diversity. However, recent evidence suggests the classic 'tree' diagram of haematopoiesis is overly simplistic and, instead, HSC/MPP fate is highly heterogeneous and dictated by gene expression heterogeneity. Here, we isolated HSC/MPPs from mouse bone marrow, stained them with a panel of surface markers, and used single cell index sorting by FACS, which records each cell's phenotype, and then performed single cell RNA sequencing of the same cell. In this way, we have linked phenotypic and fate information per cell for hundreds of cells. Using novel computational methods, we could correlate gene expression heterogeneity to phenotypic heterogeneity. Differentially expressed genes between HSC/MPP subpopulations included both novel and known genes involved in lineage specification. This work is consistent with emerging evidence that lineage specification occurs much earlier in haematopoiesis than previously appreciated.

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LL-37 upregulates genes related to stemness in breast cancer cells

Pinheiro da Silva, E., Martins de Lima, T., Tude Coelho Neto, G., Cesar Machado, M.

University of Sao Paulo, Emergency Medicine, Sao Paulo, Brazil

Introduction: Antimicrobial peptides (AMPs) are among the rare examples of very ancient molecules that emerged millions

of years ago and have been preserved during the evolutionary process of living organisms. Components of this large family of over 2,300 peptides can be found wherever there is life. Probably due to their prolonged existence in our planet, AMPs evolved a multitude of exquisite functions that go beyond our understanding. Indeed, despite their bactericidal capacity, AMPs have been intriguing the scientific community, since they are able to bind lipopolysaccharide (LPS) and other pathogen-associated patterns (PAMPs), bind membrane receptors, alter a variety of signaling pathways in different cell types, induce chemotactic responses, stimulate angiogenesis, promote wound healing, activate the inflammasome, trigger apoptosis and other mechanisms of cell death, such as NETosis, among other of their immunoregulatory and non-immune functions.

Objective: Investigate the role of LL-37 in cancer biology.

Results and discussion: Here, we show that LL-37 knockout in breast cancer cells (SKBr3) leads to down regulation of several genes related to stemness, such as Telomerase reverse transcriptase, Forkhead box D3 and Nestin and decreased production of mammospheres.

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Delineation of cellular niches which support hematopoiesis in spleen

O'Neill, H.¹, Lim, H.K.², Tran, V.², Petvises, S.², Periasamy, P.², O'Neill, T.³

¹Bond University, Faculty of Health Sciences and Medicine, Robina, Australia, ²Australian National University, Research School of Biology, Canberra, Australia, ³Bond University, Business School, Gold Coast, Australia

Hematopoietic stem cell (HSC) niches in bone marrow have been described in terms of distinct cell types including osteoblastic, endothelial and perivascular reticular cells. However, niches which support extramedullary hematopoiesis in other sites like spleen remain to be elucidated. Previous studies have described splenic stromal cells which support hematopoiesis *in vitro* from purified HSC and multipotential progenitors (MPP) leading to the production of dendritic-like cells. Stromal cell lines were originally derived from splenocyte cultures, and later as freshly isolated stromal subsets prepared by enzymatic digestion and sorting. Transcriptome analysis has revealed that stroma which support hematopoiesis express many genes in parallel with perivascular cells described in bone marrow. They share a common mesenchymal lineage with perivascular cells in bone marrow, as well as mesenchymal progenitor cells and CXCL12-abundant reticular cells. They reflect osteoprogenitors in that cells can be induced to osteogenesis, although not adipogenesis or chondrogenesis, when cultured in defined media. Cell surface markers common to these cells include CD105, CD29, VCAM1, CD51, CD140a/b and CD90. A number of new markers and genes have been identified which delineate this cell type and determine their ability to support hematopoiesis. These studies have employed inhibitors and gene knockdown to block stromal cell signaling and interaction with HSC in order to identify novel regulators of *in vitro* hematopoiesis.

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Combination of T-cell secreted cytokines promotes long-term muscle stem cell expansion

Fu, X., Xiao, J., Wei, Y., Gu, H., Wang, H., Hu, P.

Shanghai Institute of Biochemistry and Cell Biology, Shanghai, China

Muscle stem cells (MuSCs, satellite cells) are the major contributor to muscle regeneration. Like most adult stem cells, long-term expansion of MuSCs *in vitro* is difficult. The *in vivo* muscle regeneration abilities of MuSCs are quickly lost after culturing *in vitro*, which prevents the potential applications of MuSCs in cell-based therapies. Here, we found that muscle regeneration was delayed in Rag1 KO mice, which could be rescued by adoptive transfer of activated T cells. We further identified that the combination of four pro-inflammatory cytokines, IL-1 α , IL-13, TNF- α , and IFN- γ , secreted by T cells was able to stimulate MuSC proliferation *in vivo* upon injury and promote serial expansion of MuSCs *in vitro*. The expanded MuSCs can replenish the endogenous stem cell pool and are capable of repairing multiple rounds of muscle injuries *in vivo* after a single transplantation. The establishment of the *in vitro* system provides us a powerful method to expand functional MuSCs to repair muscle injuries (Cell Research, 2015, 25:655-73. Cover Story).

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Study on the role of PPAR- γ in the immune regulatory activities during mesenchymal stem cell differentiation

Kuo, H.-L., Chiang, B.-L.

National Taiwan University, Department of Immunology, Taipei, Taiwan, Republic of China

Mesenchymal stem cells (MSCs) are self-renewable multipotent progenitor cells that can differentiate into a variety of cell types including adipocytes, osteocytes and chondrocytes. Recent studies have demonstrated that MSCs could exert an immunosuppressive activity. However, many obesity-related metabolic diseases such as type II diabetes are attributed to adipocyte-induced inflammation. Macrophages recruited by adipose tissue-derived hormones or chemokines, play the key roles on the chronic inflammation. We hypothesized that some mediators might be changed during adipogenesis of MSCs. Especially, the role of PPAR- γ in inflammation and anti-inflammation remain still unclear. Hence, this study was performed to examine the gene and cytokine profiles of MSCs in different differentiation process. Along with MSCs adipogenesis, more inflammatory cytokines, including IL-6, TNF- α , and *mcp1* were secreted and expressed. But the expression of IL-1Ra was negatively correlated with adipogenic process of MSCs. Furthermore, peroxisome proliferator-activated receptors (PPARs) can promote adipogenesis by inhibiting IL-1Ra expression. The results here provided rational mechanisms clarifying the role of adipogenesis-related genes and molecules in the differentiation and regulatory activities of MSCs.

Keywords: Mesenchymal stem cells; adipogenesis; inflammation; PPAR- γ ; IL-1Ra

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Linking the gene expression profile of a cell with its fate

Creus, L., Naik, S., Schreuder, J., Zalcenstein, D.

Walter & Eliza Hall Institute of Medical Research, Melbourne, Australia

In blood cell development the prevailing paradigm is that haematopoietic stem cells (HSCs) generate multi-potent progenitors (MPPs) that later asymmetrically split to initiate the generation of cell type diversity. However, recent evidence suggests the classic 'tree' diagram of haematopoiesis is overly simplistic and, instead, HSC/MPP fate is highly heterogeneous and dictated by gene expression heterogeneity. Linking the gene expression profile of a cell with its fate is challenging, because measurement of the first (e.g. through single cell RNA-seq) necessarily destroys the chance of testing the second (e.g. in clonal fate assays). Here we demonstrate that daughters of a single cell (a clone) when separated into separate mice, or wells in a tissue culture plate, largely inherit the same fate. This indicates that daughters can be used as surrogates of the mother. Therefore, we first sorted single HSC/MPPs, allowed a brief pre-expansion then tested daughter cells for B-, T-, DC- and myeloid potential separately, and some daughters for RNA-sequencing. In this way, we could link gene expression heterogeneity with fate heterogeneity. These preliminary results will be presented.

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Lineage priming in early hematopoietic progenitors as revealed by *in vitro* clone splitting experimentsTran, J.¹, Schreuder, J.¹, Zalcenstein, D.¹, Kocovski, N.¹, Tian, L.²,Sargeant, T.², Alexander, W.², Metcalf, D.², Naik, S.¹¹WEHI, Naik Lab/Molecular Medicine, Parkville, Australia, ²WEHI, Parkville, Australia

Recent data has tabulated the nature of HSC and progenitor heterogeneity utilizing single cell transfers *in-vivo* or cellular barcoding technologies. While these studies valiantly examined cell fate *in-vivo* rather than *in-vitro*, these assays measure 'outcome' rather than 'potential', as it can be difficult to exclude the effects of niche where the cells engraft. Instead, we have performed large-scale clonal assays on total Sca1+CD117+ progenitors *in-vitro* across the B cell, T cell, dendritic cell and myeloid lineages. However, unlike examination of potential of single cells directly in a single condition, we first pre-expanded the progenitors for 3 days, then split the daughter cells into wells testing the potential of each lineage in replicate wells for each to exclude any stochastic effects. In this way we observed a large diversity in fate per clone. Also, paired wells were largely conserved in their fate, which indicates that the progenitor heterogeneity reflects an intrinsic and heritable program rather than a stochastic acquisition. In addition to splitting the daughter cells into the different lineage conditions, we also assessed some of these cells by RNA-seq. This made it possible to correlate the transcriptome of these progenitors to their fate; subsequently we were able to identify novel genes which might be involved in the earliest priming events.

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Directing therapeutic stem cells to the inflamed CNS to augment repair and limit neuroinflammation in experimental MS

*Wilson, J., Foyle, K., Foeng, J., McColl, S., Comerford, I.
The University of Adelaide, Adelaide, Australia*

The potential for cell-based therapies to promote tissue repair and locally control immune responses in autoimmune and inflammatory neurodegenerative diseases such as multiple sclerosis (MS) is limited by restricted entry of transplanted cells to the injured central nervous system (CNS). Adult stem cell (SC) therapies have demonstrated anti-inflammatory, protective and reparative effects in a variety of experimental settings. However, SC-based therapies for MS are limited by entry of SCs into the inflamed CNS following systemic transplantation; a challenge for successful neural repair and local immunomodulation. To address this, we explored the utility of forced expression of chemokine receptors [CCR2 and CCR6] known to drive pathogenic leukocytes into the inflamed CNS, to redirect therapeutic SCs to this site in an experimental model of MS, experimental autoimmune encephalomyelitis (EAE). Expression of CCR6 by SCs significantly enhanced migration towards CCL20, a cognate chemokine ligand expressed at homeostasis and highly upregulated during inflammation within the CNS. An apparent redistribution of CCR6 expressing SCs into the inflamed CNS following systemic transplantation was accompanied by a significant inhibition in clinical EAE and pathogenic CD4⁺ T cells infiltrating the CNS. These findings suggest that conferring CNS tropism on adult stem cells enhances their anti-inflammatory and potentially neuroprotective effects in order to inhibit an important step in progression of EAE pathogenesis. This strategy advances the potential for cell-based immunotherapies tailored to redirect transplanted cells to target tissues defined by their inflammatory signatures; offering an additional level of control to halt the progression of inflammatory and autoimmune diseases.

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Mesenchymal stem cell infusion modulates systemic inflammation in patients with chronic obstructive pulmonary disorder (COPD)

Armitage, J.^{1,2}, Tan, D.^{1,2}, Moodley, Y.^{1,2,3}

¹University of Western Australia, Medicine and Pharmacology, Perth, Australia, ²Sir Charles Gardiner Hospital, Institute of Respiratory Health, Perth, Australia, ³Fiona Stanley Hospital, Department of Respiratory Medicine, Perth, Australia

Background: COPD is characterised by chronic pulmonary and systemic inflammation. Recently, MSC infusions have shown success in clinical trials for improved several aspects of inflammatory-driven diseases including improving repair. Although promising, the reparative mechanisms by MSC and their acute effects post-infusion are poorly characterised, particularly in COPD. MSC possess anti-inflammatory properties, and have been shown to enhance the function of immunosuppressive T regulatory (Tregs) cells. Therefore, we hypothesise that

MSC infusion will alleviate chronic inflammation in patients with COPD.

Aims:

- 1) To assess trafficking of infused MSC by radiology
- 2) To evaluate inflammatory biomarkers following MSC infusion.
- 3) To assess phenotypic changes of Tregs post-infusion

Methods: Radiolabelled MSC were infused intravenously into 9 patients with stable COPD and tracked by CT scan across the first week post-infusion. Systemic inflammatory (sTNFR1, CRP, IL-6, IP-10) and oxidative stress (F2-Isoprostane) markers were measured in plasma across 7 days post-infusion by ELISA and GC-MS respectively. HLA-DR-expressing FoxP3+CD25⁺ Tregs were quantified by flow cytometry.

Results: MSC first localised in the lungs from 0-24 hours post-infusion and then trafficked to the liver and spleen at day 1-7. Levels of F2-isoprostanes, IL-6 and IP-10 were significantly lower, while CRP and sTNFR1 significantly increased after MSC infusion. HLA-DR⁺ Tregs were significantly higher after MSC infusion.

Summary and conclusions: We provide novel data showing MSC therapy in COPD patients modulating systemic inflammation within a week of infusion. Further characterisation of the systemic immunological changes will provide a deeper understanding of immune regulation during MSC therapy.

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Enhanced immunoregulation of mesenchymal stem cells by IL-10-producing type 1 regulatory T cells in collagen-induced arthritis

Lim, J.-Y.¹, Im, K.-I.¹, Kim, N.¹, Nam, Y.-S.¹, Song, Y.J.¹, Jeon, Y.-W.^{1,2}, Cho, S.-G.^{1,2}

¹Institute for Translational Research and Molecular Imaging, The Catholic University of Korea College of Medicine, Seoul, Korea, Republic of, ²Department of Hematology, Catholic Blood and Marrow Transplantation Center, Seoul St. Mary's Hospital, Seoul, Korea, Republic of

Mesenchymal stem cells (MSCs) possess immunomodulatory properties and have potential, however, there have been conflicting reports regarding their effects in rheumatoid arthritis (RA), which causes inflammation and destruction of the joints. Through a comparative analysis of regulatory T (Treg) and IL-10-producing type 1 regulatory T (Tr1) cells, we hypothesized that Tr1 cells enhance the immunoregulatory functions of MSCs, and that a combinatorial approach to cell therapy may exert synergistic immunomodulatory effects in an experimental animal model of rheumatoid arthritis (RA). A combination of MSCs and Tr1 cells prevented the development of destructive arthritis compared to single cell therapy. These therapeutic effects were associated with an increase in type II collagen (CII)-specific CD4⁺CD25⁺Foxp3⁺ Treg cells and inhibition of CII-specific CD4⁺IL-17⁺ T cells. We observed that Tr1 cells produce high levels of IL-10-dependent interferon (IFN)- β , which induces toll-like receptor (TLR) 3 expression in MSCs. Moreover, induction of indoleamine 2,3-dioxygenase (IDO) by TLR3 involved an autocrine IFN- β that was dependent on STAT1 signaling. Furthermore, we observed that production

of IFN- β and IL-10 in Tr1 cells synergistically induces IDO in MSCs through the STAT1 pathway. These findings suggest co-administration of MSCs and Tr1 cells to be a novel therapeutic modality for clinical autoimmune diseases.

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Dental pulp stem cells inhibit encephalitogenic T cell responses and suppress disease in an animal model of multiple sclerosis

Foyle, K.^{1,2}, Wilson, J.¹, Koblar, S.², McColl, S.¹, Comerford, I.¹

¹University of Adelaide, School of Biological Sciences, Adelaide, Australia, ²South Australian Health and Medical Research Institute, Adelaide, Australia

Mesenchymal stem cells (MSCs) have been proposed as a potential therapy for autoimmune diseases due to their capacity to both participate in the tissue repair process and modulate immune responses. In the autoimmune disease multiple sclerosis (MS), encephalitogenic T cells enter the central nervous system (CNS) and initiate an inflammatory cascade that destroys neuron-insulating myelin and current therapies are limited. The therapeutic capacity of dental pulp-derived MSCs (DPSCs) is unexplored in the context of MS or its animal model, experimental autoimmune encephalomyelitis (EAE), and little is known about the immunomodulatory properties of DPSCs in autoimmune settings. DPSCs represent an abundantly available source of human MSCs for cell based therapy and may have particular utility in degenerative disorders of the nervous system. In this study we show that human DPSCs suppress MOG-induced chronic EAE and inhibit disease relapse in PLP-induced relapsing EAE. In chronic EAE, DPSC treatment led to a reduction in the emergence of CCR2⁺CCR6⁺ Th17 cells expressing GM-CSF in the spleen, a cell subset previously identified to be a key pathogenic T cell subset in EAE. Additionally, the CNS was infiltrated with fewer Th17 cells following DPSC-treatment and there was a corresponding reduction in frequency of activated microglia, indicative of less inflammation in the CNS. Therefore DPSCs are able to inhibit the differentiation of pathogenic T cell populations in EAE, which significantly suppresses development of disease. The molecular mechanisms enabling DPSCs to inhibit the pathogenic immune response in EAE are under investigation.

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Autotaxin a cytokine modulator, is a critical regulator of satellite cell functions and skeletal muscle repair

Ray, R.^{1,2}, Raj, V.¹

¹Institute of Life Science, Vascular Immunology Group, Infectious Disease Biology Department, Bhubaneswar, India, ²Manipal University, Manipal, India

Satellite cells are a heterogeneous population of stem and progenitor cells that are required for the growth, maintenance and regeneration of skeletal muscle and possess noteworthy regenerative capacity for the repair of muscle damage in injuries or muscular disorders. Injury response in skeletal muscles is commenced by various cytokines secreted by

infiltrating immune cells, specifically IL-6 and are mediated by a network of myogenic transcription factors, including Pax3/7, Myf5, MyoD, and Myogenin. The endogenous phospholipid lysophosphatidic acid (LPA), a cytokine modulator generated by Autotaxin, regulates fundamental cellular processes and implicated in homeostatic and pathological conditions. Here we uncover, that Autotaxin dramatic expression and activity is essential for satellite cell differentiation and ATX-LPA regulated IL-6 release controls satellite cell compartment, myogenic differentiation. Ablation of ATX in satellite cells resulted in inhibition of differentiation process *ex vivo* affecting IL-6 release. The effects of ATX inhibition on cell fate and differentiation were conserved in human. Next, we demonstrate, Atx genetic deletion or intramuscular injection of ATX inhibitor resulted in a marked inhibition of muscle repair after cardiotoxin injury *in vivo* modulating IL-6. This study identifies ATX, as an essential master regulator in satellite cells, indispensable marker for muscle differentiation and its role in of ATX-LPA dependent IL-6 signalling may allow the development of novel strategies for the control of muscular dysfunctions.

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Interplay between macrophages and human endometrial stem/stromal cells on mesh implants in a mouse model

Darzi, S.¹, Deane, J.¹, Edwards, S.², Gough, D.³, Werkmeister, J.², Gargett, C.E.¹

¹Monash University/Hudson Institute of Medical Research, Obstetrics & Gynaecology, Melbourne, Australia, ²CSIRO Manufacturing Flagship, Melbourne, Australia, ³Hudson Institute of Medical Research, Centre for Cancer Research, Melbourne, Australia

Pelvic Organ Prolapse (POP) is the herniation of pelvic organs into the vagina. POP is treated by mesh augmented surgery but the complication rate is unacceptable. Human endometrial mesenchymal stem cells (eMSC) are a new MSC, which are purified using SUSD2 antibodies. The anti-inflammatory properties of eMSC are poorly understood. Our aim was track eMSC delivered on novel polyamide/gelatin (PA+G) mesh to examine their modulatory role on macrophage phenotype in immunocompromised mice.

SUSD2⁺ eMSCs were isolated from endometrial biopsies by magnetic beads sorting. A mCherry lentiviral plasmid was used to permanently label eMSC. mCherry⁺SUSD2⁺eMSC were seeded onto PA+G mesh and implanted in an abdominal fascial defect. Tissues were harvested at 7, 14, 30 and 90 days. Macrophage markers used for immunofluorescence were F4/80 (Pan), CCR7 (M1) and CD206 (M2). ELISA was used to detect IL-1 β , IL-6 and TNF- α .

Transduction efficiency of eMSC was >80%. Small numbers of mCherry⁺ eMSC were identified in mouse tissue after 7, but not 14 days. Many M1 macrophages was observed around mesh filaments at 7 and 14 days but the M1/M2 ratio reduced at later time-points. Less IL-1 β was found in eMSC/PA+G explants than PA+G after 7 days, but TNF- α and IL-6 were similar for all time-points.

This study shows that human mCherry-labelled eMSC were traceable in-vivo and that their immunomodulatory effects on M1-macrophage mediated inflammation was limited by their

short lifespan in immunocompromised mice. Ongoing studies will evaluate the interaction between eMSCs and macrophages associated with the foreign body response to mesh.

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Evaluation the capacity of immune tolerance induction on tissue-derived stromal cells in allogeneic setting

Chou, S.-H., Lee, W., Huang, B.-Y.

FU JEN Catholic University, Life Science, New Taipei, Taiwan, Republic of China

The induction of donor-specific tolerance in patients after allogeneic hematopoietic stem cell transplantation is major criteria for success of transplantation. One of the successes of allogeneic tolerance induction is determined on supported elements derived from the microenvironment during hematopoiesis. Health stroma elements have been reported that not only supporting donor cell hematopoiesis but also instruct tolerance to alloantigen. The purpose of this study is evaluating several tissue-derived stromal cells on allogeneic tolerance induction after allogeneic bone marrow transplantation. We first established stromal cell lines derived from spleen, thymus, bone marrow, two perinatal tissues (amniotic fluid and placenta) from C57BL/6 donor mice, respectively. The results of characterization on stromal cells indicated that all types of stromal cells positively expressed Sca-1, CD34, CD44, CD29, CD44 surface markers and exhibited different degrees of hematopoietic supportive activity. By used single injection of stroma cells into 13-14 day gestation BALB/c fetuses, bone marrow or perinatal-derived stromal cells are highly engrafted into thymus and bone marrow, the major tolerance education site. In addition, thymus, bone marrow or perinatal of stroma cells had shown the strong immunosuppressive activity in comparison to allogeneic positive control. In allogeneic HSCT murine model, co-injected bone marrow or perinatal-derived stromal cells enhance allogeneic donor cell engraftment. Importantly, results of donor skin graft, mixed leukocyte reaction, and adoptive cell transfer had showed that tissue-derived stromal cells may act as immune cell instructors during hematopoiesis. In conclusion, co-injection of bone marrow or perinatal-derived stromal cells with donor cells did induce the specific alloantigen tolerance.

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A dual role for alveolar macrophages in mesenchymal stem/stromal cell therapy

Mendonca, S.¹, D'Rozario, J.¹, Payne, N.², Hammett, M.¹, Khong, S.¹, Mathias, L.¹, Siatskas, C.¹, Boyd, R.¹, Heng, T.^{1,3}

¹Monash University, Anatomy and Developmental Biology, Melbourne, Australia, ²Monash University, Australian Regenerative Medicine Institute, Melbourne, Australia, ³Monash Biomedicine Discovery Institute, Infection and Immunity Program, Melbourne, Australia

The reparative and immunomodulatory properties of multipotent mesenchymal stromal cell (MSCs) are currently being investigated in >350 clinical trials on various inflammatory conditions. The majority of trials utilize systemically infused

MSCs, which localize to the lungs and do not survive for long, raising the question of these cells mediate their anti-inflammatory effects elsewhere in the body. The broad use of MSCs without clear understanding of their immunogenicity and mechanisms of action has led to many controversies in the field. We therefore investigated the *in vivo* fate of infused MSCs to better understand how their immunogenicity and survival impact on their therapeutic effects. We found that MSCs disappeared shortly (~3 days) after intravenous administration in either immunocompetent or immunodeficient mice, discounting a role for the adaptive immune system in cell clearance. MSC survival is further influenced by the route of administration, as MSCs injected into the peritoneum showed increased survival. Despite the limited survival of MSCs, MSC infusion in a mouse model of allergic asthma led to a reduction in disease, even upon re-exposure to allergen several weeks later. Intranasal depletion of alveolar macrophages prolonged MSC survival in the lungs, but paradoxically abrogated the beneficial effects of MSCs on asthma. Our data suggest that alveolar macrophages act as a double-edge sword in limiting the survival of MSCs and activating downstream immune cells to inhibit asthma. Our studies provide insight into how MSCs modify the host response to therapeutic effect, which is crucial for the design of safer and smarter MSC-based therapies.

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Probiotic-derived lactate accelerates intestinal stem cell-mediated epithelial regeneration and ameliorates injury

Lee, Y.-S., Kim, Y., Lee, S.-H., Yang, J.-Y., Kang, M.-H., Park, Y.-Y., Kweon, M.-N.

University of Ulsan College of Medicine/Asan Medical Center, Seoul, Korea, Republic of

The probiotics play an indispensable role in gut homeostasis but underlying mechanisms are still unveiled. In order to clarify an exact role of probiotic for intestinal stem cells (ISCs)-mediated epithelial regeneration, we fed mice for five days with human-use probiotics (VSL#3) including *Bifidobacterium*, *Lactobacillus*, and *Streptococcus spp.* Interestingly, short-term administration of probiotics significantly increases the growth of epithelium including expansion of Lgr5⁺ ISCs, paneth cells, and goblet cells. Similar results were found in *ex vivo* gut organoid culture in the presence of culture supernatant from probiotic. Among several candidates, we identified that lactate directly stimulated ISCs through Wnt/-catenin signals. In this regards, lactate receptor Gpr81 were highly expressed on the Lgr5⁺ ISCs in the steady-state condition and blockade of Gpr81 during gut organoid culture in the presence of lactate significantly reduced epithelial regeneration. Most importantly, pre-treatment with probiotic or lactate *in vivo* protected mice against intestinal injury provoked by treatment with irradiation and methotrexate. Our findings demonstrate that lactate produced by probiotics play a pivotal role to promote gut stem cell-mediated epithelial regeneration by direct stimulating Lgr5⁺ ISCs in Gpr81-dependent manner.

1001**Protective effect of phloroglucinol against gamma radiation-induced oxidative stress in hair follicle***Kim, A.¹, Bing, S.J.², Cho, J.², Herath, K.H.I.N.M.², Jee, Y.²**¹Jeju National University, Advanced Convergence Technology & Science, Jeju-si, Korea, Republic of, ²Jeju National University, Department of Veterinary Medicine, Jeju-si, Korea, Republic of*

When exposed to gamma-rays, hair follicle stem cells, immediately go through apoptosis, which injures their rapid differentiation essential for the regeneration of hair. Phloroglucinol (PG) is a phenolic compound of *Ecklonia cava*, brown algae abundant in Jeju island, Korea. Containing plentiful polyphenol, PG is known for its instructive effects by inhibiting apoptosis, scavenging oxygen radicals, and protecting cells against oxidative stress. In this study, we demonstrate that PG rescues radiosensitive hair follicle stem cells from gamma radiation-induced apoptosis and DNA damage. To identify the hair follicle protection capability of PG, we irradiated gamma-rays to the whole body of C57BL/6 mice at day 6 after depilation with or without PG. PG or EpiCeram® (EC) was applied once at 17.8 hours before irradiation. At 8 hours after gamma-rays irradiation, PG not only decreased apoptosis which was suppressed by radiation as shown in control but also decreased morphological changes of hair follicles. Our results suggest that PG presents radioprotective effects by inhibiting apoptosis of radiosensitive hair follicle stem cells and can PG protects hair follicle stem cells from gamma-ray induced damage. This research was a part of the project titled "Development of functional materials derived from marine living resources for regulation of immune diseases", funded by the Ministry of Oceans and Fisheries, Korea.

1002**Study on the nutrients on the adipogenesis and immune regulatory activities of mesenchymal stem cells***Wang, Z.T., Lin, B.F.**National Taiwan University, Taipei, Taiwan, Republic of China*

Introduction: Mesenchymal stem cells (MSCs) are multipotent stem cells, which can differentiate into adipocytes and osteocytes. Recently, MSCs are reported to have capacity to modulate the immune responses. During the adipogenic differentiation of MSCs, they might lose the ability of immunomodulatory function. Therefore, we investigated the effect of nutrients such as all trans-retinoic acid, 1 α , 25-(OH)₂D₃ and folate on the adipogenic differentiation and also immune modulatory ability of MSCs.

Materials and methods: MSCs were isolated from 4-week-old BALB/c bone marrow and characterized by surface marker. They were treated by different concentrations of all trans-retinoic acid (10, 100, 1000 nM), 1 α , 25-(OH)₂D₃ (0.1, 1, 10 nM) and folate (20, 200 μ M). Adipogenic differentiation of MSCs were determined on days 2, 7, 14, 21. Lipid accumulation and adipogenic genes expression of C/EBP α and PPAR- γ 2 were measured by Oil Red O staining and real-time PCR. The cytokines levels of IL-1 β , TNF- α and IL-6 were also detected.

Results: All trans-retinoic acid and folate inhibited adipogenic differentiation of MSCs

after 14 days induction. In addition, 1 α , 25-(OH)₂D₃ inhibited that after 7 days' culture. The results of C/EBP α and PPAR- γ 2 gene expression were downregulated by folate, all trans-retinoic acid and 1 α , 25-(OH)₂D₃. We will further clarify that IL-1 β , TNF- α and IL-6 of MSCs would also be decreased by all three nutrients.

Conclusion: These data showed that trans-retinoic acid, 1 α , 25-(OH)₂D₃ and folate affected the adipogenic differentiation of MSCs. We will further assay all these nutrients on the immune regulatory activities of MSCs.

T Cell Development

1003**TCR induced transcription factors RelA and IRF4 co-operatively regulate the differentiation of effector T_{reg} cells***Vasanthakumar, A.¹, Yang Laio, Y.¹, Gloury, R.¹, Sidwell, T.¹, Teh, P.¹, Mielke, L.¹, Belz, G.¹, Shi, W.¹, Grigoriadis, G.², Banerjee, A.², Kallies, A.¹**¹The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia, ²Hudson Institute of Medical Research and Monash University, Melbourne, Australia*

TCR signaling is pivotal for the development of Foxp3⁺ regulatory (T_{reg}) T cells in the thymus. These cells continue to require TCR signals in the periphery for their homeostasis and function. Since TCR signaling and NF- κ B transcription factors are linked, we interrogated the role of NF- κ B family members in peripheral Treg cells. Surprisingly, deletion of c-Rel or NF κ B1/p50 did not have a major impact on Treg homeostasis and function. In contrast, RelA deficiency in T_{reg} cells led to fatal multi-organ autoimmune pathology, accompanied by the expansion of inflammatory adaptive and innate immune cells. Transcriptional profiling of RelA deficient T_{reg} cells revealed the loss of a gene signature associated with the development of activated or effector Treg (eT_{reg}) cells. In agreement with this notion, RelA-deficient T_{reg} cells failed to differentiate into eT_{reg} cells. We have previously shown that the TCR-induced transcription factor IRF4 is required for eT_{reg} cell differentiation in a non-redundant manner. Although neither RelA nor IRF4 were transcriptional targets of each other, the set of genes regulated by either of these transcription factors in T_{reg} cells largely overlapped. Together our observations suggest that the transcription factors RelA and IRF4 co-operate to establish the eT_{reg} cell differentiation program in a TCR dependent manner.

1004**Apoptosis regulates thymic progenitor fitness and progression to T cell acute lymphoblastic leukemia***Policheni, A.¹, Grabow, S.², Boulliet, P.³, Strasser, A.³, Gray, D.⁴**¹Melbourne University, Medical Biology, Melbourne, Australia, ²The Walter and Eliza Hall Institute of Medical Research, Molecular Genetics of Cancer, Melbourne, Australia, ³The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia, ⁴The Walter and Eliza Hall Institute, Melbourne, Australia*

Cell competition is a process whereby "new" or "fit" cells replace "old" or "unfit" cells to maintain tissue integrity. Cell competition

among thymic T cell precursors was recently found to be essential for suppression of malignancy. Disruption of thymic cell competition can be achieved by grafting thymi from wildtype mice into immunodeficient mice, creating a situation where host progenitors are incapable of displacing donor. The ensuing reliance upon self-renewal of 'old' thymic progenitors for T cell differentiation leads to cancer with the cellular and molecular hallmarks of T cell acute lymphoblastic leukemia (T-ALL). The processes that govern competitive fitness or displacement in the thymus are unknown. We explored whether the intrinsic pathway of apoptosis in thymic progenitors plays a role in cell competition and leukaemogenesis.

Thymic lobes from mice deficient in the pro-survival proteins BCL-2 (*Bcl2*^{-/-}) or MCL-1 (*Mcl1*^{+/-}) were grafted into immunodeficient *Rag2*^{-/-}/*gC*^{-/-} mice to measure whether reducing donor cell survival would diminish progenitor fitness and restore competition to prevent T-ALL. Surprisingly, we found that these mice succumbed to T-ALL at an accelerated rate (average latency of 100 days and 100% penetrance) as compared to *Rag2*^{-/-}/*gC*^{-/-} mice grafted with wildtype thymi (average latency of 250 days). Furthermore, we have identified a cellular phenotype associated with "fit" and "unfit" thymic progenitors that will aid dissection of the cellular processes leading to T-ALL in situations of disrupted cell competition. This study implicates the intrinsic pathway of apoptosis as a major regulator of thymic progenitor cell competition and subsequent leukaemia development.

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Apoptosis regulates thymic progenitor fitness and progression to T cell acute lymphoblastic leukemia

Policheni, A.^{1,2}, Grabow, S.^{1,2}, Bouillet, P.^{1,2}, Strasser, A.^{1,2}, Gray, D.^{1,2}

¹The Walter and Eliza Hall Institute of Medical Research, Molecular Genetics of Cancer, Melbourne, Australia, ²The University of Melbourne, Department of Medical Biology, Melbourne, Australia

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days). Furthermore, we have identified a cellular phenotype associated with "fit" and "unfit" thymic progenitors that will aid dissection of the cellular processes leading to T-ALL in situations of disrupted cell competition. This study implicates the intrinsic pathway of apoptosis as a major regulator of thymic progenitor cell competition and subsequent leukaemia development.

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The histone acetylase HBO1 directs expression of the autoimmune regulator AIRE in thymic epithelial cells

Heinlein, M.^{1,2}, Jain, R.^{1,2}, Voss, A.^{2,3}, Thomas, T.^{2,3}, Gray, D.^{1,2}

¹The Walter and Eliza Hall Institute of Medical Research, Molecular Genetics of Cancer, Melbourne, Australia, ²The University of Melbourne, Department of Medical Biology, Melbourne, Australia, ³The Walter and Eliza Hall Institute of Medical Research, Development and Cancer Division, Melbourne, Australia

The autoimmune regulator, AIRE, induces the transcription of a panoply of peripheral tissue self-antigens in thymic epithelial cells (TECs). This activity mediates self-tolerance via the clonal deletion of auto-reactive thymocytes and/or the selection of Foxp3⁺ regulatory T cells. The molecular mechanisms by which AIRE orchestrates transcriptional regulation in TECs remain poorly defined. Post-translational modifications of histone proteins, such as acetylation, play an important role in the transcriptional regulation of genes. HBO-1 is a histone acetylase that can promote or repress transcription at a range of loci, prompting us to examine whether HBO-1 plays a role in the epigenetic regulation of transcription in TECs. We took a conditional genetic deletion approach, ablating a floxed allele of *Hbo1* specifically in TECs using *Foxn1*^{Cre}. Functional deletion of HBO-1 was confirmed by the complete loss of histone 3 lysine 14 acetylation only in TECs from these mice. Young *Foxn1*^{Cre} *Hbo1*^{fl/fl} mice exhibited thymic hypo-cellularity but without a major reduction in overall TEC numbers. Yet, there was a substantial reduction in medullary TEC subsets, coincident with an increase in the cortical TEC subset. AIRE-positive medullary TECs were greatly diminished in *Foxn1*^{Cre} *Hbo1*^{fl/fl} mice and there was a reduced level of AIRE expression in this population. Increased T cell activation in young *Foxn1*^{Cre} *Hbo1*^{fl/fl} mice and mild lymphocytic infiltration of the lung in older mice suggested tolerance was perturbed. Together, these data reveal a role for histone 3 lysine 14 acetylation mediated by HBO-1 for establishing a normal thymic microenvironment, AIRE expression and the establishment of self-tolerance.

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EGF regulation of MCL-1 is indispensable for thymic epithelial cell survival and thymic function

Jain, R.^{1,2}, Sheridan, J.^{1,2}, Policheni, A.^{1,2}, Kupresanin, F.¹, Fu, N.Y.^{2,3}, Hollander, G.⁴, Strasser, A.^{1,2}, Gray, D.^{1,2}

¹The Walter and Eliza Hall Institute of Medical Research, Molecular Genetics of Cancer, Melbourne, Australia, ²The University of Melbourne, Department of Medical Biology, Melbourne, Australia, ³The Walter and Eliza Hall Institute of Medical Research, Stem Cells and Cancer Division, Melbourne, Australia, ⁴University of Oxford, Weatherall Institute for Molecular Medicine, Oxford, United

Kingdom

The differentiation of haematopoietic precursors into functional T cells that are tolerant of self-tissues is driven by interactions with thymic epithelial cells (TEC). Despite the importance of TEC for adaptive immunity and tolerance, very little is known about the molecular control of TEC function and dysfunction. We aim to understand the molecular mechanisms that govern life and death decisions in TEC, with a view to developing new strategies to restore thymic function in immunodeficiency. Guided by expression profiling, we created conditional genetic models deleting various pro-survival genes only in TEC using *Foxn1^{Cre}*. We found that, although the pro-survival proteins BCL-2 and BCL_x_l were dispensable for TEC homeostasis and thymic function, TEC-specific loss of MCL-1 (i.e. *Mcl-1^{ΔFoxn1}* mice) caused early thymic involution, with near complete loss of the thymus in 2 month-old mice. Loss of thymic function in *Mcl-1^{ΔFoxn1}* mice reduced their peripheral T cell compartment by two thirds. Defects in TEC composition of *Mcl-1^{ΔFoxn1}* mice were evident early in life, with the progressive loss of Aire⁺ medullary TEC, followed by disruption of other TEC subsets. In a screen of TEC trophic factors in thymic organ cultures, we found that only EGF upregulated MCL-1, providing a molecular mechanism for mesenchymal support of TEC. We conclude that MCL-1 is essential for TEC survival and function, highlighting an important mesenchymal/epithelial signalling axis with bearing on thymic involution and injury.

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Homeostatic proliferation maintains recent thymic emigrants' phenotype of naïve T cells and TCR signaling affects «thymic» phenotype of memory T cells in hemoblastosis patients

Batorov, E., Tikhonova, M., Kryuchkova, I., Sergeevicheva, V., Batorova, D., Sizikova, S., Ushakova, G., Gilevich, A., Ostanin, A., Chernykh, E.

Research Institute of Fundamental and Clinical Immunology, Novosibirsk, Russian Federation

Numerous studies have shown that chemotherapy and autologous hematopoietic stem cell transplantation (AHSCT) led to a profound immunodeficiency in hemoblastosis patients. Thymus begins to fill up T cell repertoire approximately from the 6th post-transplant month. We evaluated thymic activity by measuring amounts of CD31⁺ naïve T cells, i.e. "recent thymic emigrants" (RTEs).

87 patients underwent AHSCT. Circulating CD4⁺CD45RA⁺CD31⁺ and CD4⁺CD45RO⁺CD31⁺ cells were evaluated by flow cytometry before AHSCT and during 1st post-transplant year. Mononuclear cells (MNCs) were cultured separately with IL-2, IL-7, αCD3.

Pre-transplant count of CD4⁺CD45RA⁺CD31⁺ T cells was lower comparing to controls and did not reach donors' values during 12-month period. We found high levels of CD4⁺CD45RA⁻ T cells expressing CD31 in patients, since this molecule was infrequent on memory subsets in healthy controls. Amount of CD4⁺CD45RA⁻CD31⁺ T cells highly correlated with CD4⁺CD45RO⁺CD31⁺ population. CD4⁺CD45RO⁺CD31⁺ T cells recovered intensively, reached pre-transplant level during the 1st month and remained

it for the observation period. *In vitro* studies showed the growth of CD4⁺CD45RA⁺CD31⁺ T cells counts upon stimulation of donors and patients MNCs with IL-7 (homeostatic proliferation trigger) but not IL-2 (peripheral expansion trigger) or anti-CD3 (TCR trigger). CD4⁺CD45RO⁺CD31⁺ T cells elevated upon stimulation with anti-CD3 but not IL-2 or IL-7.

Homeostatic proliferation might decrease the significance of CD4⁺CD45RA⁺CD31⁺ T-cells as a marker of RTEs. The biological role of CD31 on memory T cells remained unclear. Presumably, lymphocyte stimulation through CD31 might prevent their hyper-reactivity by increasing the activation threshold of TCR signaling and thus provide their survival.

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Assessing the role of CXCR4 in the CD8+ T cell response to vaccinia virus with mathematical modeling

Körber, V.¹, Wencker, M.², Djebali, S.², Arpin, C.², Marvel, J.², Graw, F.¹

¹Heidelberg University, Center for Modeling and Simulation in the Biosciences (BIOMS), BioQuant-Center, Heidelberg, Germany,

²International Center for Infectiology Research (CIRI), INSERM U 1111, Lyon, France

Migration and motility are essential properties of the cellular immune response, playing an important role for development and effective function. Genetic defects affecting chemokine receptor (CR) expression can lead to inappropriate migratory patterns of developing and mature immune cells resulting in ineffective immune responses, increased sensitivity to infections and autoimmune diseases. However, the exact interplay between cell differentiation and CR expression has not been determined so far.

Using a mouse model with a gain-of-function mutation in CXCR4, we studied the role of this CR on the dynamics of CD8+ T cell responses following infection with Vaccinia Virus. We found that overexpression of CXCR4 leads to a shift in the generation of memory CD8+ T cell subsets, as well as a delayed appearance of CD8+ T cells in the blood during acute infection. Using a mathematical model, we estimated a delayed dynamics of ~1 day.

In order to assess the role of CXCR4 in more detail, we developed an agent-based model following individual cell proliferation, differentiation and migration in the lymph node (LN) and the blood, explicitly accounting for LN structure. Testing different hypotheses our model suggests that the observed altered CD8+ T cell phenotype dynamics in the blood can be explained by a changed dwell time of cells within the LN medulla, or by a smaller pool of naïve T cells in the LN at the start of infection.

Our model provides a systematic framework for an integrative analysis of cell differentiation and migration in the context of the CXCR4-receptor.

1011**Superior activation of human naïve CD27^{hi}CD45RA⁺CD8⁺ T cells in the presence of CD27 co-stimulation**

Grant, E.¹, Nussing, S.¹, Bird, N.¹, Jaworowski, A.², Schuessler, A.³, Smith, C.³, Khanna, R.³, Al-Shamkhani, A.⁴, Bharadwaj, M.¹, Kedzierska, K.¹

¹The University of Melbourne at the Peter Doherty Institute for Infection and Immunity, Melbourne, Australia, ²Burnet Institute, Centre for Biomedical Research, Melbourne, Australia, ³QIMR Berghofer Medical Research Institute, Brisbane, Australia, ⁴University of Southampton, Faculty of Medicine, Southampton, United Kingdom

CD27 is constitutively expressed on naïve and majority of circulating memory-T cells in humans at any stage of life. While it is well-known that CD27 co-stimulation enhances primary, secondary and recall CD8⁺ T-cell responses in murine models, little is known about the effect of CD27 co-stimulation on human T-cells. Using a combination of *ex vivo* phenotypic staining and stimulation, we firstly assessed the functional capacity of different human T-cell subsets, as defined by CD27 and CD45RA. We found that CD27^{lo}CD45RA⁻ and CD27^{lo}CD45RA⁺ T-cells have superior polyfunctional and cytotoxic potential, respectively. Interestingly, quantitation of gene expression in these CD8⁺ T-cells using qPCR revealed novel gene expression profiles in CD27^{hi} and CD27^{lo} CD8⁺ T-cells. To understand the activation of CD27^{hi} CD8⁺ T-cells subsets, we performed a comprehensive kinetic analysis to directly assess the effect of CD27 co-stimulation on human CD8⁺ T-cells over time. CD8⁺ T-cell populations were stimulated by anti-CD3 cross-linking alone, or with additional co-stimulation using plate bound CD70 (CD27 ligand) or anti-CD28 (pCD70/pCD28, respectively) for up to 7 days. Co-stimulation with pCD70 was required for naïve CD27^{hi}CD45RA⁺CD8⁺ T-cell proliferation and accelerated Granzyme B acquisition. Furthermore, pCD70 co-stimulation enhanced phenotypic plasticity of naïve and central-like memory CD27^{hi}CD45RA⁺CD8⁺ T-cells and their cytokine secretion potential. The effect of anti-CD27 stimulation was superior in comparison to anti-CD28 stimulation in human T-cells. As expected, CD27^{lo}CD8⁺ T-cells were unaffected by pCD70 or pCD28 co-stimulation. Overall, these data provide new evidence that CD27 co-stimulation mediates superior activation of human CD27-expressing naïve CD8⁺ T-cells.

1012**Although Gtf2h4 is highly expressed in double positive thymocytes there is no fundamental role regarding negative selection and T cell development**

Beil-Wagner, J.¹, Prazeres da Costa, O.², vom Berg, J.¹, Specht, S.¹, Heikenwälder, M.³, Buch, T.¹

¹University of Zurich, Institute for Laboratory Animal Sciences, Schlieren, Switzerland, ²Technische Universität München, ²Institute for Medical Microbiology, Immunology and Hygiene, Munich, Germany, ³Deutsches Krebsforschungszentrum, Heidelberg, Germany

During T cell development negative selection facilitates elimination of self-reactive thymocytes. It is controlled by the

avidity of the interaction between the TCR and the self antigen which means that cells which interact with a low avidity to self antigen can survive and those with a high avidity die by apoptosis. However, the exact molecular pathway enabling negative selection is not yet known. Therefore we were looking for candidate transcription factors that are highly expressed at the double positive stage of T cell development. Microarray analyses and RT-PCR identified, besides others, Gtf2h4 as candidate gene. To analyse its role in T cell development we followed two approaches: First, we cloned it under the control of the CD4 promoter to generate a mouse line overexpressing Gtf2h4 in T cells specifically. Second, by using the CRISPR/Cas9 we generated a knockout strain. Both mouse strains were deeply analysed with regard to T cell development by flow cytometry and histology. Unexpectedly, homozygous knockout mice were found to be embryonic lethal. Heterozygous mice did not show any alterations. Also the overexpression strains did not present with changes in T cell development. In summary, although Gtf2h4 is expressed at remarkable height at the double positive stage compared to other thymic development stages it does not seem to play a key role in T cell development and specifically negative selection.

1013**CD4⁺CD8⁺ double-positive T-cells regulate CD8⁺ single-positive T cell function in the skin**

Gonzalez Cruz, J.L.¹, Overgaard, N.H.^{1,2}, Bridge, J.A.¹, Jung, J.-W.¹, He, J.¹, Nel, H.J.¹, Watson, K.³, Frazer, I.H.¹, La Gruta, N.L.³, Steptoe, R.J.¹, Wells, J.W.¹

¹The University of Queensland Diamantina Institute, Translational Research Institute, Brisbane, Australia, ²National Veterinary Institute, Technical University of Denmark, Copenhagen, Denmark, ³The University of Melbourne and The Peter Doherty Institute for Infection and Immunity, Department of Microbiology and Immunology, Melbourne, Australia

CD4⁺CD8⁺ double-positive mature peripheral T-cells (DPs) are detectable in a variety of tissues. However, despite their relevant implications in autoimmune and malignant skin disorders, such as atopic dermatitis and cutaneous T-cell lymphoma, very little is known about their role or function; likely due to their very low abundance. Additionally, technical difficulties that confound distinguishing DP single-cells from CD4⁺CD8⁺ T-cell aggregates makes them difficult to study. We have overcome these issues using a novel isolation strategy which allows us to obtain >95% pure DPs. To determine whether peripheral DPs arise from CD4⁺ T-cells, we adoptively transferred highly purified single-positive T-cells into Rag1^{-/-} mice. Under these conditions we observed that subsets of CD4⁺ T-cells become DP T-cells with the peculiarity that these cells express CD8 α but not CD8 β , which is in contrast to DPs isolated from wild-type mice. To study the effect of DPs on the regulation of skin CD8⁺ T-cells, we used a skin graft rejection model in which skin engineered to express Ovalbumin (OVA) under the K5 promoter was grafted onto wild-type recipient mice. As expected, grafts were rejected from untreated recipients around 20-30 days post-grafting. However, transfer of a low number of DPs, but not CD4⁺ or CD8⁺ single-positive T-cells, suppressed CD8⁺ T-cell

dependent rejection of OVA-expressing grafts. Interestingly, the DP inhibitory phenotype depended on their activation status since only activated and not naïve DPs prevented skin graft rejection. Our data suggests a powerful immunoregulatory role for DPs in controlling CD8⁺ T-cell function in the skin.

1014

CBAP promotes thymocyte negative selection by facilitating T cell receptor proximal signaling

Ho, K.-C.¹, Chiang, Y.-J.¹, Lai, A.¹, Liao, N.S.², Chang, Y.J.¹, Yang-Yen, H.-F.², Yen, J.¹

¹Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan, Republic of China, ²Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan, Republic of China

T cell receptor (TCR)-transduced signaling is critical to thymocyte development at the CD4/CD8 double-positive stage, but the molecules involved in this process are not yet fully characterized. We previously demonstrated that GM-CSF/IL-3/IL-5 receptor common β -chain associated protein (CBAP) modulates ZAP70-mediated T cell migration and adhesion. Based on the high expression of CBAP during thymocyte development, we utilized a CBAP knockout mouse to investigate the function of CBAP in thymocyte development. CBAP-deficient mice showed normal early thymocyte development and positive selection. In contrast, several negative selection models (including TCR transgene, superantigen staphylococcal enterotoxin B, and anti-CD3 antibody treatment) revealed an attenuation of TCR-induced thymocyte deletion in CBAP knockout mice. This phenotype correlated with a reduced accumulation of BIM upon TCR crosslinking in CBAP-deficient thymocytes. Loss of CBAP led to reduced TCR-induced phosphorylation of proteins involved in both proximal and distal signaling events, including ZAP70, LAT, PLC γ 1, and JNK1/2. Moreover, TCR-induced association of LAT signalosome components was reduced in CBAP-deficient thymocytes. Our data demonstrate that CBAP is a novel component in the TCR signaling pathway and modulates thymocyte apoptosis during negative selection.

1015

C-Myc regulation by Notch signaling modulates T cell differentiation

Song, J., Haque, R., Song, X., Lei, F.

Penn State University College of Medicine, Hershey, United States

Notch signaling is essential for T lineage commitment and further required during early phases of thymocyte differentiation. Active Notch signaling during early stages of T cell development inhibits other lineage potentials, such as B cell and myeloid cell (e.g., dendritic cell) potentials. However, the intracellular signaling pathways by which Notch signaling regulates T cell differentiation remain unknown. Here we show that the transcriptional factor c-Myc is controlled by Notch signaling which regulates T cell differentiation. In a well-established *in vitro* differentiation of T lymphocytes from stem cells, Notch signaling directly controls c-Myc expression. Overexpression of active c-Myc promotes while dominant-negative (dn) c-Myc

inhibits early cell differentiation. Moreover, c-Myc expression mediated by Notch signaling modulates survivin, an inhibitor of apoptosis (IAP) protein, which is crucial for T cell development. Overexpression of active c-Myc increases while dn c-Myc reduces survivin expression, which corresponds to T cell differentiation within the *in vitro* differentiation system. These results identify c-Myc, together with survivin, as regulators of the differentiation of T cells from Notch signaling.

1016

Two waves of thymic deletion distinguished by differential dependence on thymic antigen-presenting cell subsets

Yap, J.Y.¹, Howard, D.R.¹, Wirasinha, R.C.^{1,2}, Goodnow, C.C.^{1,3}, Daley, S.R.²

¹The Australian National University, The John Curtin School of Medical Research, Department of Immunology, Canberra, Australia, ²Monash University, Clayton Campus, Department of Biochemistry and Molecular Biology, Melbourne, Australia, ³Garvan Institute of Medical Research, Immunology Division, New South Wales, Australia

Distinct antigen-presenting cell (APC) types cooperate to delete strongly self-reactive thymocytes to establish central tolerance. Here, we ascertained deletion defects caused by perturbing bone marrow (BM)-derived APCs (BM-APCs), and/or Aire, which drives expression of certain self-antigens in non-BM-derived APCs. Deletable thymocytes, identified by Helios expression, were resolved into two subsets: immature Helios⁺ CCR7⁻ cells characterised as wave 1 of thymic deletion, and mature Helios⁺ CCR7⁺ cells as wave 2. Wave 1 deletion was reduced 40% by MHCII-deficiency within BM-APCs versus 18% by Aire-deficiency within non-BM-derived APCs. Aire contributes to wave 1 indirectly in a mechanism requiring BM-APCs. However, Aire-dependent direct presentation deletes thymocytes at wave 2, including thymocytes that escape wave 1, and ~35% of wave 2 requires Aire. The different stage and extent of impairments caused by perturbing BM-APCs versus Aire shows that thymic deletion is not confined to a single stage of thymocyte development in the natural TCR repertoire.

1017

Tespa1 regulates late thymocyte development through interacting with inositol 1,4,5-triphosphate (IP3) receptors

Liang, J., Lv, J., Zheng, M., Li, D., Lu, L., Jun Lv, Mingzhu Zheng, Dan Li and Linrong Lu

Zhejiang University, School of Medicine, Hangzhou, China

T cell antigen receptor (TCR) ligation on the surface of DP thymocyte induces the formation of TCR proximal LAT signalosome complexes in the cell, which is critical for mediating subsequent signal transduction pathways that direct its late stage development into Single positive cells. We've previously shown that, Tespa1, a newly- identified adaptor protein, is also recruited to the LAT signalosome and participate to the activation of ERK kinases and calcium flux. However, the precise function of Tespa1 remains to be elucidated. Here we found that Tespa1 interacted with the N-terminal of IP3R1 through its PFF domain, which enable it to recruit IP3R1 to the TCR complexes after TCR stimulation. We also found that

the recruitment of IP3R1 by Tespa1 enabled the membrane-proximal phosphorylation of IP3R1 on tyrosine 353 by Fyn and was critical for optimal TCR-proximal calcium flux signaling. Disruption of Tespa1-IP3R1 interaction by mutagenesis led to substantial decrease of calcium flux signals. Finally, we proved that the interaction between Tespa1 and IP3R1 was essential for the positive selection of thymocytes in Teaps1 PFF mutant transgenic mice. In sum, our work uncovered a new molecular mechanism that regulates the calcium signaling in T cells.

1018

Different extracellular regions of Notch ligands, DII1 and DII4, are necessary for supporting T cell development

Hozumi, K., Hirano, K.-I.

Tokai University School of Medicine, Immunology, Isehara, Japan

We have shown that DII4 is absolutely necessary and efficiently induces T cell development. However, it has not been clarified what is unique characteristics in DII4 among NotchLs. It was known that the DOS motif, observed into the 1st and 2nd EGF repeats of the extracellular region, is widely conserved in NotchLs from invertebrates to mammals, but mammalian DII4 does not possess this motif. To investigate and compare the significance of DOS motif with another functional region, DSL, we make six chimeric molecules between DII1 and DII4 and check their abilities. The improvement of their function is only found in DII4-derived molecules with DOS region of DII1, which effectively induces the signaling and supports T cell development in vitro. These results indicate the significance of DOS motif for the functional modification of DII family members, but suggest that the superiority of DII4 cannot be explained by the difference in DSL/DOS regions. On the other hand, we reveal that the N-terminus (MNNL) of DII4 is critical for DII4, but not for DII1, to trigger the signaling and support T cell development. Taken together, it is demonstrated that different components of the extracellular region of DII family members are required to function, and that MNNL region of DII4 can be linked with the superiority of DII4 over DII1.

1019

CXXC finger protein 1 is critical for T cell intrathymic development through regulating H3K4 trimethylation

Wang, L., Cao, W., Guo, J., Wen, X.

Zhejiang University School of Medicine, Institute of Immunology, Hangzhou, China

T cell development in the thymus is largely controlled by an epigenetic program involving in both DNA methylation and histone modifications. Previous studies have identified Cxxc1 as a regulator of both cytosine methylation and histone 3 lysine 4 trimethylation (H3K4me3). However, it is unknown whether Cxxc1 plays a role in thymocyte development. Here we show that T cell development in the thymus is severely impaired in Cxxc1-deficient mice. Furthermore, we identify genome-wide Cxxc1 binding sites and H3K4me3 modification sites in wild-type and Cxxc1-deficient thymocytes. Our results demonstrate that Cxxc1 directly controls the expression of key genes important

for thymocyte survival such as ROR γ t and for TCR signaling including Zap70 and CD8, through maintaining the appropriate H3K4me3 on their promoters. Importantly, we show that ROR γ t, a direct target of Cxxc1, can rescue the survival defects in Cxxc1-deficient thymocytes. Our data strongly support a critical role of Cxxc1 in thymocyte development.

1020

T cell receptor recognition of the non-classical MHC molecule HLA-E

Sullivan, L.¹, Nguyen, O.¹, Kedzierska, K.¹, Cai, Z.¹, Gray, J.¹, Widjaja, J.¹, Gras, S.², Farenc, C.², Malmberg, K.³, Rossjohn, J.², Brooks, A.¹

¹Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Department of Microbiology and Immunology, Melbourne, Australia, ²Biomedicine Discovery Institute, Monash University, Department of Biochemistry and Molecular Biology, Clayton, Australia, ³Karolinska University Hospital, Department of Hematology, Stockholm, Sweden

The non-classical MHC class I molecule (MHC-I) HLA-E has a restrictive peptide-binding groove; largely binding peptides derived from the leader sequences of other MHC-I. The human cytomegalovirus glycoprotein UL40 contains a sequence that is identical to the HLA-E-binding peptide found in many HLA-C alleles and the HLA-E/UL40 peptide complex is recognised by the T cell receptor (TCR) of a subset of CD8⁺ T cells (UL40-specific T cells). The ability of UL40-specific TCR to recognise the UL40 peptide represents a potential specificity challenge for the immune system. In order to avoid self-reactivity, UL40-specific TCR must distinguish between the UL40 peptide (VMAPRTLIL) and near-identical self-peptides (e.g. VMAPRTLVL encoded by HLA-A2), which may differ by only a single methyl group. Limited knowledge exists on how TCRs are capable of such discrimination or how TCRs recognise HLA-E in general. We used single-cell multiplex-nested RT-PCR to assess the TCR $\alpha\beta$ repertoire diversity of UL40-specific TCR in healthy donors. We showed that the TCR repertoire capable of recognising HLA-E/UL40 was highly restricted, with repeated selection of TRBV14 in several unrelated donors. Furthermore, we generated soluble recombinant forms of these TCRs and assessed their ability to bind to HLA-E in complex with the UL40 peptide and self-HLA derived peptides by surface plasmon resonance (SPR). SPR analysis indicated that the affinity of the TCRs for HLA-E/UL40 was consistently higher than the affinity of TCRs for HLA-E-self peptide complexes. These findings provide fundamental insights into the generation of the T cell repertoire, self/non-self discrimination and the requirements for activation.

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A critical role for proper fucosylation in T cell development

Yabas, M.^{1,2}, Roots, C.M.², Goodnow, C.C.^{2,3}, Enders, A.²

¹Faculty of Engineering, Trakya University, Department of Genetics and Bioengineering, Edirne, Turkey, ²The John Curtin School of Medical Research, The Australian National University, Department of Immunology and Infectious Disease, Canberra, Australia, ³Immunology Division, Garvan Institute of Medical Research, Sydney, Australia

Fucosylation, a type of glycosylation, is the attachment of a fucose residue to *N*-glycans, *O*-glycans and glycolipids, and is critical for posttranscriptional regulation of many essential pathways. The defects in the genes encoding enzymes involved in the GDP-fucose synthesis have been implicated in Notch-dependent lymphoid cell development in the immune system. Here we describe a mouse strain with an *N*-ethyl-*N*-nitrosourea (ENU)-induced point mutation in the gene encoding an enzyme involved in fucosylation. The mutant mice displayed growth retardation and increased postnatal mortality with a median survival of 5 weeks. Despite the presence of normal numbers of double negative (DN) thymocytes mutant mice at 3 weeks of age had reduced numbers of double positive (DP), CD4 single positive (SP) and CD8SP cells. Interestingly, the presence of B cells, T cells and myeloid cells in the spleen of mutant mice at 3 weeks of age seemed to be normal. Analysis of thymi from *Rag1*^{-/-} chimeras reconstituted with mutant bone marrow cells revealed a partial arrest at the DN stage of T cell development with an approximately two-fold reduction in thymocyte numbers compared to WT *Rag1*^{-/-} control chimeras. Furthermore, mixed bone marrow chimera experiment demonstrated that mutant cells were unable to compete with wild-type cells from the DP stage of T cell development in the thymus onwards. This inability to compete results in a complete absence of mutant derived peripheral T cells in the recipient mice. These findings highlight an important role for proper fucosylation in T cell development.

1022

PP2A C subunit isoform α (PP2Ac) is essential for early T cell development

Zheng, M., Li, D., Lu, L.

Institute of Immunology/Zhejiang University, Hangzhou, China

The development of T cells is critically dependent on signaling through the TCR and can be disrupted by deficiencies in the receptor itself and in many signaling molecules and mediators. Protein phosphatase 2A is a highly regulated serine/threonine phosphatases family implicated in cell growth and signaling, which contains three subunits: A(scaffold subunit), B(regulatory subunit) and C(catalytic subunit). T cells from SLE patients express high levels of PP2Ac. However, the function of PP2Ac that mediates the maturation, differentiation and activation in T cells remained undefined. Here we generated T-cell-specific PP2Ac conditional knockout mice. We found that *Ppp2ca* gene deletion in the T-cell-lineage resulted in aberrant thymocytes development including reduced number of total thymocytes, T-cell arrest from DN4 to DP. PP2Ac deficient thymocytes showed decreased proliferation and enhanced apoptosis *in vivo*. Thus our results indicate that PP2Ac is a critical component involved in the T cell developmental program and maturation.

1023

Elucidating T cell fate using microfabricated cell culture platforms

Charnley, M.^{1,2}, Ludford-Menting, M.², Pham, K.², McArthur, S.¹, Russell, S.^{1,2}

¹Swinburne University of Technology, Melbourne, Australia, ²Peter

MacCallum Cancer Centre, Melbourne, Australia

An effective immune response depends upon the coordinated proliferation and differentiation of T cells, with each cell performing its own designated role. Current research indicates that this diversification is controlled by asymmetric cell division (ACD); a process that orchestrates fundamental aspects of stem and immune cell biology and is disrupted in cancer. ACD occurs when there is intracellular polarity in a dividing cell resulting in two daughter cells with different molecular compositions. My group has definitively revealed for the first time that ACD also controls the differential inheritance of cell fate determinants in T cell precursors (thymocytes).

This project aims to tease out the external cues responsible for the initiation of ACD. To achieve this I have developed a simple and robust model using functionalised surfaces to individually present proteins to the thymocytes. With this model I can study the effect of different cues on the development of individual immune cells in real time. Excitingly, the Notch-1 ligand, Delta-like-1 (DL-1), induced the polarisation of cell fate proteins during interphase and mitosis. On-going work is focused on exploring the synergistic interactions between the molecular players that control ACD and its impact on cell development. Thus, this approach provides unique opportunities to hone in on the mechanisms by which the microenvironment can influence cell fate. This is expected to impact on our understanding of the immune response and will have profound implications for the development of therapeutic and diagnostic solutions for immune disorders, improved vaccination, and T cell leukaemias.

1024

The Redox function of apurinic/apyrimidinic endonuclease1/redox factor-1 (Ape1/Ref-1) modulates helper T cell response through antigen presenting cells

Akhter, N.¹, Takeda, Y.¹, Nara, H.¹, Araki, A.¹, Asao, N.², Ishii, N.³, Asao, H.¹

¹Yamagata University, Faculty of Medicine, Immunology, Yamagata, Japan, ²Tohoku University, Advance Institute for Material Research, Sendai, Japan, ³Tohoku University Graduate School of Medicine, Immunology, Sendai, Japan

Ape1/Ref-1 is a multifunctional protein possessing DNA repair, redox control and transcriptional regulatory activities. In immune system Ape1/Ref-1 is essential for immunoglobulin class-switch recombination and for CD40-mediated B cell activation. However, specific roles of Ape1/Ref-1 in helper T cell response are largely unknown. In this study, the function of Ape1/Ref-1 redox control activity in helper T cell immune response was analyzed using an Ape1/Ref-1 redox specific inhibitor, E3330. When OT-II cells, which are CD4⁺ T cells with ovalbumin (OVA) specific TCR, were activated with OVA-pulsed antigen presenting cells (APC) in the presence of E3330, IFN- γ -producing OT-II T cells were significantly increased. E3330 doesn't enhance IFN- γ producing CD4⁺ T cells, which were activated with anti-CD3 and anti-CD28 antibodies without APC. Moreover, E3330-pre-treated and OVA-pulsed APC could enhance IFN- γ production from OT-II T cells. These results suggest that E3330 enhances Th1 response through the modification of

APC function. E3330 has no effect on CD80/CD86 and MHC-II expression in APC. Therefore, we focused on the IL-12. E3330 up-regulated the *Il12a* and *Il12b* gene activation in TLR ligand-stimulated APC and increased IL-12 surface expression on APC, but decreased the secretion. These data were confirmed with Ape1/Ref-1 knockdown APC. Next, to elucidate the mechanisms how Ape1/Ref-1 modulates APC functions, we examined the signal transduction. Amusingly E3330 augmented TLR ligand-induced p38 MAPK activation suggesting that the activated p38 MAPK may enhance the IL-12 gene activation. This is the first study to demonstrate that the redox function of Ape1/Ref-1 modulates APC function and inhibits Th1 cell differentiation.

1025

MiR-205 maintains T cell output from the thymus by positively regulating Foxn1 expression

Hoover, A.¹, MacLeod, J.¹, Dozmorov, I.¹, de la Morena, M.T.^{2,3}, van Oers, N.^{1,4}

¹UT Southwestern Medical Center, Immunology, Dallas, United States, ²UT Southwestern Medical Center, Pediatrics, Dallas, United States, ³Childrens Health, Allergy and Immunology, Dallas, United States, ⁴UT Southwestern Medical Center, Microbiology, Dallas, United States

T cells are a critical component of the adaptive immune system, developing within the thymus. Immature thymocytes interact with an interconnected meshwork of thymic epithelial cells (TECs) to establish the T cell repertoire. This developmental process is extremely stress sensitive, with the thymus undergoing a rapid involution in response to inflammatory and endocrine mediators. The type of stress predicates whether TECs and/or thymocytes mediate this process. Several microRNAs (miRs) have been identified in the thymus based on their ability to mitigate stress damage. We identified miR-205 as a stress responsive miR specifically expressed in TECs. The conditional ablation of miR-205 in TECs results in an age and sex-dependent thymic hypoplasia in mice beginning at 8 weeks. During type I interferon responses (dsRNA mimic; polyI:C), the TEC-miR-205 deficient mice displayed a severe thymic atrophy compared to littermate controls, including a delayed recovery of single positive CD4 and CD8 thymocytes. qPCR and gene expression comparisons revealed a number of putative miR-205 targets with significant changes in chemokine/chemokine receptors and antigen processing pathways. The addition of miR-205 mimics in fetal thymic organ cultures lacking miR-205 restored normal levels of Foxn1, the master regulator of TEC development and function. Interestingly, miR-205 is encoded within a long non-coding RNA (lncRNA), 4631405K08Rik. Current experiments will reveal the mechanisms by which the miR and lncRNA are transcriptionally regulated, and how these affect Foxn1 expression to support thymopoiesis.

1026

Scribble is an important regulator of T cell development

Novita, N.¹, Dew, A.¹, Durrant, M.¹, Johnson, C.¹, Walkley, C.R.^{2,3}, Purton, L.E.^{2,3}, Darcy, P.K.¹, Ellis, S.L.¹

¹Peter MacCallum Cancer Centre, East Melbourne, Australia, ²St Vincent's Institute, Fitzroy, Australia, ³University of Melbourne, Department of Medicine at St Vincent's Hospital, Fitzroy, Australia

As a scaffolding protein, Scribble regulates multiple intracellular signalling pathways that control cell proliferation, migration, and apoptosis. Scribble is pivotal to the development of epithelial tissues and its deregulation has been linked to multiple epithelial cancers. Scribble's role in haematopoiesis and haematopoietic malignancies is an exciting area of burgeoning interest. Using multiple approaches, we show that Scribble is expressed in haematopoietic stem and progenitor cells (HSPC) and their differentiated progeny, implying a putative role for Scribble in blood production. We investigated the effect of Scribble loss in haematopoiesis through two inducible knockout mouse models and an extensive phenotyping labelling regime in conjunction with multi-color flow cytometry. Surprisingly, we observed no effect of loss of Scribble on either the proportion or numbers of HSPC, or cells from the B, myeloid, or erythroid lineages. Mature differentiated cells in the peripheral blood were also unaffected by loss of Scribble. However, mice lacking Scribble had a significantly smaller thymus compared to controls, which was caused by a significant decrease in the number of DN1, DN2, DN3, single positive and double positive thymocytes. Our data suggests Scribble may regulate thymocyte development and/or the migration of the early progenitors from the bone marrow to the thymus. We are currently investigating these hypotheses and the underlying mechanism/s involved. Our research will improve the understanding of T cell development and will form the basis of future studies aimed at investigating how Scribble impacts T cell leukaemia.

1027

Screening of mouse mutagenesis pipeline for cytotoxic T lymphocyte function

Barton, P., Smart, E., Griffiths, G., Wellcome Trust - 3i Consortium CIMR, Cambridge, United Kingdom

As part of the 3i consortium - an expansion of the immunological phenotyping carried out at the Mouse Genetics Project at the Wellcome Trust Sanger Institute (WTSI) - we have developed a screen to assess cytotoxic function of CD8+ T-cells in mutant mouse lines. CD8+ T cells kill target cells through the polarised release of cytotoxic granules¹, the contents of which initiate apoptosis and the subsequent release of lactate dehydrogenase (LDH) from the target cell. Release of LDH from target cells can be quantified to ascertain the cytotoxicity of T cells². Furthermore, the presence of the lysosomal associated proteins LAMP1 and 2 (CD107a and CD107b), proteins found within the cytolytic granule membrane, can be detected at the cell surface using flow cytometric quantification to indicate the level of cytolytic degranulation³. Combining cytotoxicity and degranulation assays, alongside a flow cytometric analysis of

the ratio of CD4⁺:CD8⁺ cells within the cultured population allows for a comprehensive screen of T cell function from these mutant mouse lines, in order to identify novel T cell phenotypes.

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1028

Quantification of the peripheral T cell repertoire that escapes negative selection due to attenuated TCR signalling

Wirasinha, R.C.^{1,2}, Singh, M.³, Archer, S.⁴, Daley, S.R.^{1,2}

¹Monash University, Infection and Immunity Program, Monash Biomedicine Discovery Institute, Clayton, Victoria, Australia,

²Monash University, Department of Biochemistry and Molecular Biology, Clayton, Victoria, Australia, ³Garvan Institute of Medical Research, Immunology Division, Darlinghurst, Sydney, Australia,

⁴Monash University, Monash Bioinformatics Platform, Clayton, Victoria, Australia

Genetic mutations that attenuate intracellular TCR-signalling are associated with T-cell lymphopaenia, immunodeficiency and autoimmune disease. During T cell development in the thymus, TCR-signalling thresholds play a critical role in central tolerance. T cells that receive no TCR signal undergo death by neglect, weak TCR signals drive positive selection and strong TCR signals trigger negative selection. One suspected cause of autoimmune disease invokes shifting of these T-cell signalling thresholds, whereby strong TCR binding is incorrectly interpreted and transmitted as a weak intracellular TCR signal. This "selection shift" hypothesis proposes that TCR binding events that would normally trigger negative selection instead induce positive selection. The fraction of the peripheral T cell repertoire composed of negatively-selectable TCRs due to the selection shift has not been examined. To address this, we cell sorted thymic and peripheral T cell subsets from fixed TCR-beta chain, wild-type or TCR-attenuated mouse strains bearing hypomorphic alleles of genes encoding Zap70 or SIp-76, and performed deep-sequencing of the TCR-alpha chain repertoire. Negatively-selectable thymocytes were identified as CCR7⁺ CD4^{lo}CD8^{lo}PD-1^{hi}. We found that < 5% of wildtype peripheral naive CD4⁺ T cells were negatively-selectable, versus up to 70% in TCR-attenuated mutants. Further analysis of TCR-attenuated strain peripheral effector/memory CD4⁺, regulatory T cells and naive CD8⁺ splenocytes, were also enriched in negatively-selectable TCRs, albeit to a lesser extent than naive CD4⁺ splenocytes. These findings are the first quantitative estimate of the selection shift, and strongly support that attenuated TCR signals can lead to negatively-selectable TCRs dominating the repertoire exported from the thymus.

1029

Bone morphogenetic protein type-II receptor mutation affects circulatory T cell subsets but is dispensable for B cell development in mouse models of pulmonary arterial hypertension

Jafri, S.¹, Moore, S.D.¹, Morrell, N.W.¹, Ormiston, M.L.²

¹University of Cambridge, Department of Medicine, Cambridge, United Kingdom, ²Queens University, Departments of Biomedical and Molecular Sciences, Kingston, Canada

Bone morphogenetic protein (BMP) signalling is involved in lymphocyte development. Mutations in BMP type-II receptor (BMPRII) predispose to pulmonary arterial hypertension (PAH), which is also associated with immune dysfunction. We aimed to investigate the effect of a disease-relevant BMPRII mutation on mouse T and B cell development.

Wild type (WT) and *Bmpr2*^{+/R899X} mice, bearing a PAH-associated premature stop mutation, were exposed to chronic hypoxia (21 days, 10% O₂) as a model of pulmonary hypertension. T and B cell development was characterised in bone marrow, spleen, thymus and blood by flow cytometric analysis. *Bmpr2*^{+/R899X} mice spontaneously develop pulmonary arterial hypertension with age, and these were also used to characterise T and B cell development.

Naïve *Bmpr2*^{+/R899X} mice do not have altered T or B cell development compared to WT. Hypoxic *Bmpr2*^{+/R899X} mice exhibit an expansion of absolute circulating CD8⁺ memory T cells. This is accompanied with increased follicular splenic B cells. However, no early stage T or B cell differences are observed between mice genotypes. Aged *Bmpr2*^{+/R899X} mice also trend towards increased circulating CD8⁺ memory T cells, manifest unaltered B cell subsets, and an increase in the common lymphoid progenitor population in bone marrow.

Our data show a PAH-relevant BMPRII mutation does not considerably affect mouse B cell development, even with hypoxia as a trigger. However, an expansion of circulating CD8⁺ memory T cells, particularly in *Bmpr2*^{+/R899X} mice following hypoxia, suggests that elevated T cell activation secondary to BMPRII dysfunction may contribute to disease development.

1030

Functional analysis of a developmental stage specific enhancer in the CD4 locus

Fujii, C.¹, Littleton, S.¹, Kasapi, M.¹, Zapatero, Z.¹, Sarafova, S.^{1,2}

¹Davidson College, Biology, Davidson, United States, ²Duke University, Immunology, Durham, United States

CD4 helper T cells coordinate the immune response and are highly dependent on the expression of the *Cd4* gene for proper development and function. The function of a promoter, an enhancer and a silencer have been well documented and together explain how the *Cd4* gene gets turned on in CD4 T cells and off in CD8 T cells. However once turned on, the amount and timing of *Cd4* expression varies during T cell development and activation. This modulation of CD4 surface levels is essential for proper lineage specification and T cell function. Yet, how subtle changes of CD4 expression are regulated remains unclear. We have recently identified a novel positive

cis-acting transcriptional regulatory element (NCE) in the *Cd4* locus that is inactive at the double-positive stage and becomes activated during positive selection. Here we investigate the *in vivo* function of NCE by introducing modified versions of the CD4 locus as a BAC transgene into mice deficient for CD4 and beta-2 microglobulin and look for deviations from the normal T cell development program. In addition we have generated a version of the BACs that contain a destabilized version of EGFP as a reporter, so we can observe the changes in transcription level directly. This set of mice will allow us to interrogate the transcriptional regulation of CD4 in other cells that express CD4, such as NKT and CD4+ DCs.

1031

Poly(A) shortening of ASK1 mRNA contributes to positive selection of thymocytes through impairment of TCR-induced stress response

Kureha, T.¹, Akiyama, T.², Morita, M.³, Ichijo, H.⁴, Yamamoto, T.⁵

¹Okinawa Institute of Science and Technology Graduate University, Cell Signal Unit, Okinawa, Japan, ²Institute of Medical Science, University of Tokyo, Department of Cancer Biology, Tokyo, Japan, ³McGill University, Department of Biochemistry, Montreal, Canada, ⁴The University of Tokyo, Laboratory of Cell Signaling, Tokyo, Japan, ⁵Okinawa Institute of Science and Technology Graduate University, Cell Signal Unit, Onna-son, Japan

The CCR4-NOT complex is the major deadenylase that removes poly(A) tails from mRNAs. Recently, we found that the number of mature T cells was reduced in mice hetero-deficient for CNOT3, a subunit of the CCR4-NOT complex. The data suggest an involvement of CNOT3 in T cell development and/or maintenance. To clarify the role of CNOT3 in T cell, we generated mice devoid of CNOT3 in T cell specific manner (T-CNOT3^{-/-} mice). We found fewer mature thymic CD4⁺ and CD8⁺ T cells in T-CNOT3^{-/-} mice than in wild type mice. To identify the stage at which thymocyte development is first affected in T-CNOT3^{-/-} mice, we quantified cells at five distinct developmental stages defined by the expression of CD3 and the activation marker CD69. We found that numbers of immature CD3^{lo}CD69^{lo} and CD3^{int}CD69^{lo} cells did not differ between T-CNOT3^{-/-} mice and control mice. However, we observed fewer CD3^{int}CD69^{high}, CD3^{hi}CD69^{hi} and CD3^{hi}CD69^{lo} cells in T-CNOT3^{-/-} mice. Furthermore, we found the survival defect occurred in the presence of TCR stimulation. These results indicated that the defect of DP differentiation in T-CNOT3^{-/-} mice was caused by the cell death after TCR stimulation. We found that the CCR4-NOT complex post-transcriptionally attenuates expression of ASK1, thereby preventing prolonged activation of Jnk and p38 after TCR stimulation, which leads TCR-induced cell death of DP thymocytes. Our results identified the CCR4-NOT complex-mediated deadenylation of mRNAs is a critical factor during the positive selection of DP thymocytes.

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The impact of the selecting MHCI molecule on epitope-specific CD8 T cell repertoire and function

Sng, X.Y.X.^{1,2}, Quinn, K.M.^{1,2}, Guan, J.^{1,2}, La Gruta, N.L.^{1,2}

¹Monash University, Biochemistry, Melbourne, Australia, ²University of Melbourne, Microbiology and Immunology, Melbourne, Australia

Up to 10% of naïve CD8 T cell precursors (CTLps) are known as alloreactive cells that recognise major histocompatibility complex class I (MHCI) variants that are not expressed during thymic selection. This indicates that MHCI restriction is not absolute, however the degree to which non-restricting MHCI affect epitope-specific T cell repertoire and function is not well delineated. We used naïve tetramer enrichment to characterise H-2D^b restricted epitope (influenza A virus derived PA₂₂₄)-specific CTLps in mice lacking H-2D^b (the restricting MHCI) or H-2K^b, the non-restricting MHCI. CTLps were enumerated, their clonal composition determined and their function assessed on a single-cell level. Intriguingly, although D^bPA₂₂₄-specific CTLp numbers were substantially lower in H-2D^b KO compared to wildtype and H-2K^b KO, they were reproducibly present, indicating the ability of H-2D^b reactive cells to be selected on H-2K^b.

The median fluorescence intensity of tetramer straining on CTLps from H-2D^b KO mice was lower, suggesting poorer avidity of TCR-MHCI interactions. Assays for intrinsic and TCR dependent functionality will be performed to further dissect TCR-MHCI avidity. TCR repertoire analysis revealed similar TRAV usage across the different mouse strains but markedly diminished TRBV29 usage in H-2D^b KO. Altogether, these data demonstrate that epitope-specific CTLps

- (i) can be selected on distinct MHCI alleles,
- (ii) are largely defined by their selecting MHCI ligand, and
- (iii) are intrinsically poorer at epitope recognition when selected on mismatched MHCI molecule, thus likely affecting their functionality.

These data have implications for clinically significant diseases (e.g. GvHD) and treatments mediated by alloreactive CD8 T cells.

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CRTAM is differentially regulated in naïve CD4 T-cells by IL-6 and IL-27

Liu, X., Twohig, J.P., Cardus, A., Andrews, R., Jones, G.W., Jones, S.A.
Cardiff University, Infection and Immunity, Cardiff, United Kingdom

Aim: Class-I MHC-restricted T-cell associated molecule (CRTAM) is an activation-induced surface receptor that regulates T-cell development and proliferation. CRTAM+CD4+ T-cells secrete IFN- γ and express cytotoxic T-cell-related genes. Currently, the mechanism of CRTAM regulation remains unknown. Here, using an unbiased transcriptomic approach, we investigated the role of IL-6 and IL-27 on CRTAM expression in CD4+ T-cells and examined the impact of CRTAM+CD4+ T-cells in inflammatory arthritis.

Methods: Naïve CD4 T-cells were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies in the presence or absence of IL-6 or IL-27. Changes in CRTAM together with markers of

T-cell activation, cytokine release and proliferation/survival were analysed by flow cytometry, qPCR and ELISA. The in vivo regulation of CRTAM was monitored by immunofluorescence of joint sections from antigen-induced-arthritis treated mice.

Results: Activation of naïve CD4 T-cells caused a temporal increase in CRTAM surface expression. Maximal expression was observed 18hrs post-stimulation. Treatment with IL-6 significantly impaired CRTAM expression (50-60%). A similar early inhibition of CRTAM expression was also observed using IL-27, however in contrast to IL-6, IL-27 promoted a later induction of CRTAM (48-96hrs post-stimulation). Here, regulation of CRTAM by IL-6 and IL-27 was associated with a differential control of Zeb1, a negative regulator of CRTAM. Data will be presented showing the in vivo regulation of CRTAM in synovitis from IL6r^{-/-} and IL27r^{-/-} mice.

Conclusions: Our findings highlight the differential regulatory effects of IL-6 and IL-27 on CRTAM expression in naïve CD4 T-cells and provide the first evidence of CRTAM+CD4⁺ T-cells within the inflamed synovium.

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CD4 T cell help for a more potent CD8 T cell response against hepatocyte-expressed antigens

English, K.^{1,2,3}, Sierro, F.^{1,2,3}, Tay, S.S.^{1,2,3}, Wood, N.A.W.^{1,2,3}, McGuffog, C.^{1,2,3}, McCaughan, G.W.^{1,2,3}, Bowen, D.G.^{1,2,3}, Wong, Y.C.^{1,2,3}, Bertolino, P.^{1,2,3}

¹Centenary Institute, Liver Immunology Program and AW Morrow Gastroenterology and Liver Centre, Camperdown, Australia, ²Royal Prince Alfred Hospital, Camperdown, Australia, ³University of Sydney, Camperdown, Australia

CD4 T cell help is required to elicit potent CD8 T cell responses to clear liver-tropic pathogens. The kinetics of the CD4 T cell responses and mechanism by which CD4 T cell help enhances liver-specific CD8 T cell responses are, however, unknown. We developed a recombinant adeno-associated viral vector system to express a fusion protein containing both CD4 and CD8 T cell epitopes in mouse hepatocytes. Using this approach, we investigated the CD4 T cell response against the hepatocyte-expressed antigen and how it affects the antigen-specific CD8 T cell response at different time points in liver, lymph nodes (LNs), spleen and blood.

While activation of antigen-specific CD8 T cells was initiated in both liver and LNs, naïve CD4 T cells were preferentially activated and proliferated in the liver-draining LNs. These activated CD4 T cells re-circulated to the liver and all secondary lymphoid organs (SLOs) and their numbers peaked at 7 days post antigen exposure. Although activated CD4 T cells did not influence the early proliferation of CD8 T cells in the liver and SLOs, they promoted a 5-15 fold increase in the number of antigen-specific CD8 T cells at day 7. These 'helped' CD8 T cells also displayed an enhanced functionality.

Our results suggest that CD4 T cells increase both the number and function of antigen-specific CD8 T cells after recirculation to the liver, an effect that will likely promote faster clearance of antigen-expressing hepatocytes. This model will help to induce a better CD8 T cell immunity against hepatotropic infections.

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Drinking Jeju ground water containing vanadium leads to immune activation in chronically stressed mice

Cho, J.¹, Bing, S.J.¹, Ha, D.¹, Joo, H.¹, Kim, M.J.¹, Kim, A.², Herath, K.H.I.N.M.¹, Jee, Y.¹

¹Jeju National University, Department of Veterinary Medicine, Jeju-si, Korea, Republic of, ²Jeju National University, Advanced Convergence Technology & Science, Jeju-si, Korea, Republic of

Vanadium, an essential trace element for mammals, has been studied frequently in recent time cause its biological and pharmacological properties. We previously demonstrated that low doses of ammonium metavanadate (NaVO₃) activate peripheral immune system. It is well known that immune system, which protects host bodies from foreign invaders, is influenced by stress. Exposure to stress, through the action of stress hormones, has detrimental effects on immune function by reducing NK cell activity, lymphocyte populations, lymphocyte proliferation and antibody production. In this study, we aimed to investigate the immunomodulatory effects of Jeju water containing vanadium (Jeju water) on immunosuppression in stressed mice. We used various stressors such as physical exercise (swim), environmental factors (heat, sound), deprivation of freedom (restraint) and supplied mice with tap water (control) or Jeju water of varying vanadium concentrations for 28 days; S₁(vanadium 24.0 ± 2.0 µg/L), and S₂(vanadium 26.0 ± 2.0 µg/L). All Jeju water drinking groups showed increased single-positive (CD4⁺CD8⁻ or CD4⁺CD8⁺) thymocytes compare to control. Cell proliferation of splenocytes showed significant increase in Jeju water drinking groups, and the numbers of CD4 positive T cells, B cells, macrophages and granulocytes in spleen were also increased. Additionally, Jeju waters promoted IgG production in splenocytes of stressed mice. Taken together, Jeju waters promote T cell development in thymus and immune activation against immunosuppression by stress through the enhancement the splenocyte proliferation and its function. This research was supported by Jeju Special Self-Governing Province Development Corporation (JPDC) in 2016.

T Cell Memory

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Acute influenza A virus (IAV) infection in humans leads to expansion of highly diverse CD8 T cell repertoires crossreactive with persistent Epstein Barr virus (EBV)

Gil, A., Mishra, R., Aslan, N., Selin, L.

University of Massachusetts Medical School, Pathology, Worcester, United States

The competence of T cell responses predominantly depends on how efficient T cell receptors (TCRs) are at recognizing antigenic epitopes. We show here that during acute severely symptomatic IAV infection there was an expansion of IAV-M1/EBV-BRLF1 and IAV-M1/EBV-BMLF1 double-tetramer+ cells directly ex-vivo in 5 HLA-A2+ patients. We questioned whether this expansion specific to these two different crossreactive responses would lead to alterations in the TCR repertoire of the IAV-M158, EBV-BRLF1109 and -BMLF1280 from before, during and following

acute IAV infection. Using staining with VB mAb we found that T cell responses generated to these epitopes became surprisingly more polyclonal, with the sharing of Vb between M1, BMLF1 and BRLF1 populations which is not seen in healthy donors and which decreased 2 months later consistent with crossreactive expansion. Furthermore, by using single-cell analysis of TCR α and TCR β repertoire of tetramer sorted IAV-M1 cells we showed dramatic changes in specific clonotype usage and in JA and JB family usage during acute IAV infection compared to before infection. In summary, these changes in TCR repertoire during acute symptomatic IAV infection suggest that during severe infection there is a preferential expansion of highly diverse crossreactive responses between IAV and the persistent virus, EBV, which leads to permanent changes in TCR repertoires to both of these two viruses (NIH AI049320 and NIH AI109858).

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The molecular basis of CD4 help during CD8⁺ T cell memory formation

Cullen, J., Olshansky, M., Turner, S.

Peter Doherty Institute for Infection and Immunity, Department of Microbiology and Immunology, Melbourne, Australia

A long-lived pool of memory cells is the defining feature of adaptive immunity. Memory CD8 T cells offer protection for the life of the host due to their unique capabilities to survive in the absence of antigen and respond rapidly to secondary challenge. Therefore, effective CD8 T cell memory is the goal of cell-mediated vaccination strategies. While it is well established that CD4 help is required for CD8 T cell memory formation, it is unclear when during CD8 differentiation this help is required. Further, the affect that CD4 help has on the transcriptional profiles of CD8 T cells and the molecular pathways they use during the generation of memory CD8 T cells remains elusive. Using a mouse model of Influenza infection, where priming occurs in the presence or absence of CD4 T cell help, we have pinpointed that help is required for priming of CD8 T cells, and not during memory maintenance or recall. Genome wide RNA-sequencing analysis of the transcriptional signatures between resting "helped" and "unhelped" memory CD8 T cells reveals few differentially expressed genes. However, upon reactivation, "helped" memory CD8 T cells exhibited greater transcriptional up regulation than "unhelped" counterparts, and utilization of alternate molecular pathways. Our analysis revealed that CD4 help during initial priming is essential for establishing a memory cell pool with enhanced transcriptional potential. Thus, CD4 T cell dependent programming likely underpins rapid responsiveness, a key characteristic of memory CD8 T cells. Analysis into intriguing metabolic differences based on this RNA-sequencing data are underway.

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Antigen-specific CD8⁺ human memory stem T-cell clones: decoupling T-cell differentiation and division

Verdon, D.J.^{1,2}, Brooks, A.E.S.^{1,2}, Sheppard, H.M.¹, Ho, Y.J.¹, Dunbar, P.R.^{1,2}
¹University of Auckland, School of Biological Sciences, Auckland, New Zealand, ²Maurice Wilkins Centre for Molecular Biodiscovery, Auckland, New Zealand

Memory stem T-cells (T_{SCM}) have recently been characterised in mice, non-human-primates and humans as the least-differentiated subset of antigen-experienced T-cells, retaining a naive-like phenotype and proliferative potential. *In vivo* T_{SCM} can both self-renew, to retain a T_{SCM} pool and give rise to diverse memory and effector progeny. These characteristics make T_{SCM} the most potent and efficacious cellular resource for adoptive cell therapy in the treatment of cancer and protection from latent viruses.

In this study we describe the development of a cytokine regimen for reliably generating CD8⁺ T_{SCM} clones specific for epitopes derived from tumour-associated- and cancer-testis-antigen-derived epitopes. T-cell clones generated retained T_{SCM} phenotype, polyfunctional cytokine production and homeostatic cytokine responsiveness through serial expansions and ≤ 40 population doublings. Interestingly, these characteristics were lost during matched expansion in IL-2 alone. Dynamic tracking of memory-phenotype-associated cell surface markers revealed that these T_{SCM} clones do enter an 'effector phase' post-stimulation, but regain T_{SCM} phenotype following cessation of expansion, without evidence of population contraction. T_{SCM} clones do not constitutively express all cytotoxic granule proteins, but are able to rapidly upregulate these proteins and lyse epitope-matched melanoma cells on co-culture, without the need for prior cell division.

This study represents the first description of *in vitro*-generated epitope-specific CD8⁺ T_{SCM} clones and provides important insights into the regulation of T-cell memory phenotype-associated protein expression. Analysis of the surface phenotype and effector function of T_{SCM} populations throughout expansion and maintenance demonstrates that T-cell differentiation status is not determined by proliferative history, but is cytokine-responsive.

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A novel IL-17-dependent positive control loop influencing human Th17 physiology and cutaneous inflammation

Pascoal Ramos, M.I.¹, Kakeda, M.², Paul-Gilloteaux, P.³, Hivroz, C.³, Puel, A.^{4,5,6}, Casanova, J.L.^{4,5,6,7,8}, Iezzi, G.⁹, Yawalkar, N.², Padovan, E.¹⁰

¹Instituto Gulbenkian de Ciencia, Oeiras, Portugal, ²Inselspital - Dermatology, Bern, Switzerland, ³Institut Curie, Paris, France, ⁴France Imagine Institute, Paris, France, ⁵Descartes University, Paris, France, ⁶Necker Hospital, Paris, France, ⁷St. Giles Laboratory, New York, United States, ⁸The Rockefeller University and HHM Institute, New York, United States, ⁹Basel University Hospital and University of Basel, Basel, Switzerland, ¹⁰University of Basel, Basel, Switzerland

IL-17 immunity is required for host immune protection, but can also be pathogenic. In our studies, we observed a decreased

frequency of blood-circulating Th17 cells in a patient lacking functional IL-17RA. Furthermore, by studying plaque psoriasis as skin disease model, we observed that psoriatic lesional skin, contained IL-17+ and CD3+ cells conjugated with CD68+/IL-6+ Macrophages (Mf). Based on these observations, we hypothesized that IL-17 cytokine could contribute to Th17 memory responses through a bidirectional interaction between M ϕ and Th17 cells. We demonstrated that pro-inflammatory M1 Mf were powerful inducers of Th17 responses, as compared to anti-inflammatory M2 Mf. We further showed that M1 Mf responded to activated Th17 by releasing IL-6. CD40 crosslinking on M1 Mf with sCD40L was necessary for IL-6 induction, but concomitant stimulation with IL-17A enhanced Mf cytokine release. The presence of Mf lacking functional IL-17RA and antibody blocking IL-17 hampered IL-6 release and Th17 expansion from memory T cell precursors. Moreover, conditioned media from M1 Mf activated by sCD40L and IL-17 consistently expanded Th17 cells from CD4+ T lymphocytes activated through CD3. Contrary to M1 cells, M2 Mf responded to activated Th17 cells by producing IL-10 and had no effect on Th17 expansion. Collectively, we demonstrated that IL-17, in synergy with CD40L, feeds back M1 Mf establishing a positive control loop on Th17 responses, thus identifying new pathways for the fine-tuning of IL-17 immunity and the maintenance skin homeostasis *in vivo*.

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Effect of gain-of-function mutations in PI3 kinase p110d on the phenotype and function of EBV-specific CD8⁺ T-cells

Edwards, E.S.J.^{1,2}, Cole, T.³, Wong, M.⁴, Uzel, G.⁵, Tangye, S.G.^{1,2}

¹Garvan Institute of Medical Research, Immunology Division, Darlinghurst, Australia, ²St Vincent's Clinical School, University of New South Wales, Sydney, Australia, ³Royal Childrens Hospital Melbourne, Department of Allergy and Immunology, Melbourne, Australia, ⁴Childrens Hospital at Westmead, Department of Allergy and Immunology, Sydney, Australia, ⁵NIAID, NIH, Immunopathogenesis Section, Laboratory of Clinical Infectious Diseases, Bethesda, United States

CD8⁺ T-cells are largely responsible for controlling infection caused by the common herpes virus EBV. Due to the efficacy of CD8⁺ T-cells, primary EBV infection in healthy individuals is often asymptomatic. However, individuals with primary immunodeficiencies characterised by uncontrolled EBV viremia have been identified. These include patients with heterozygous, germline gain-of-function mutations in *PIK3CD* (which encodes the p110d catalytic subunit of PI3-kinase). *PIK3CD* is predominantly expressed in leukocytes and is critical for lymphocyte function. Previous studies reported an altered CD8⁺ T-cell phenotype in patients, with a skewing towards a T_{EM}/T_{EMRA} phenotype, at the expense of naïve CD8⁺ T-cells, consequent with hyperactivation of the PI3K-Akt-mTOR pathway. In order to understand how *PIK3CD* GOF mutations contribute to EBV susceptibility, we have utilized polychromatic FACS to enumerate the functional and phenotypic attributes of total and EBV-specific CD8⁺ T-cells in patients and healthy controls. Results show that EBV-specific CD8⁺ T-cells are present at similar frequencies in patients and controls. Total and EBV-specific CD8⁺

T-cells are hyperactivated in patients, as indicated by significant upregulation of regulatory markers CD95, CD160 and 2B4, as well as cytotoxic marker Granzyme B. In addition, CD57 and PD-1 are overexpressed indicating immunosensence of CD8⁺ T-cells. Preliminary experiments suggest dysregulated cytokine and cytolytic marker expression in affected individuals, and by extension suspected dysfunction of cytotoxicity towards EBV-infected targets. Further investigation will provide new insights into the generation and function of CD8⁺ T-cells in the control of EBV, which will be important in the treatment of EBV-associated diseases, and EBV vaccine development.

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Tissue resident memory T cells closely interact with a 3D macrophage network for immune surveillance of salivary gland

Thelen, F.¹, Ficht, X.¹, Stolp, B.², Page, N.³, Merkler, D.³, Stein, J.V.¹

¹University Bern, Theodor Kocher Institut, Bern, Switzerland, ²Universitätsklinikum Heidelberg, Ruprecht-Karls-Universität Heidelberg, Infektiologie, Heidelberg, Germany, ³University of Geneva, Pathology and Immunology, Geneva, Switzerland

After clearing of infections, distinct memory T cell populations persist in the host for efficient protection upon pathogen re-encounter. Re-activation of resident CD8⁺ memory T cells (T_{RM}) is particularly relevant for a fast response to viral and microbial infections on a tissue level. However, the requirements for efficient tissue protection by T_{RM} are not fully understood. Here, we used multiphoton intravital imaging, confocal imaging and light sheet microscopy of submandibular salivary glands (SMG) to dissect memory-mediated tissue surveillance during acute and memory phase of lymphocytic choriomeningitis virus (LCMV) infections in mice. SMG is an exocrine gland with ductal and acinar structures, which are anchored via extracellular matrix (ECM) sheets. We discovered in SMG and lacrimal glands an extended tissue macrophage network, which facilitates migration of T_{RM} between different epithelial and stromal compartments by extending protrusions through ECM and densely packed epithelial cells. These protrusions create gaps in the ECM layer that are used by T_{RM} as guidance cues for efficient scanning of ductal and acinar structures. Depletion of the macrophage network resulted in significantly reduced T_{RM} motility and patrolling ability. After viral re-challenge of the SMG, we found that the macrophage network was essential for fast clearance of infected cells by T_{RM}. In sum, we describe a close cooperation between the resident macrophage network and T_{RM} in exocrine glands that ensures effective tissue surveillance by granting T cell access to epithelial structures sheathed by ECM.

1042**CD8⁺ T cells require LFA-1 to patrol the liver and protect against hepatic pathogens***McNamara, H.A., Cai, Y., Enders, A., Cockburn, I.A.**Australian National University, John Curtin School of Medical Research, Canberra, Australia*

CD8⁺ T cells in the liver play a critical role in protection against pathogens such as Hepatitis C and malaria. However, the behaviour of CD8⁺ T cells within the liver has yet to be clearly defined. Using multiphoton microscopy we imaged the behaviour of effector and memory CD8⁺ T cells following immunization with the malaria parasite *Plasmodium*. At early time points most CD8⁺ T cells in the liver were found to be temporarily trapped in the liver or flowing in the blood stream. However by 28 days post-immunization the majority of CD8⁺ T cells had adopted a characteristic patrolling behaviour within the hepatic sinusoids. Flow cytometry analysis of CD8⁺ T cells at memory time points revealed a population of tissue resident memory-like cells which were CD69^{hi} CXCR3^{hi}, KLRG1^{lo}. Intriguingly these cells also expressed exceptionally high levels of LFA-1 (CD11a/CD18). We therefore investigated whether LFA-1 was critical for the migration and homing of T cells in the liver. We found that antibody blockade of ICAM-1 - a major LFA-1 ligand - reduced the migratory speed of CD8⁺ T cells in the liver. Furthermore, CD8⁺ T cells deficient in LFA-1 did not exhibit patrolling behaviour and failed to home efficiently to the liver. Finally, we found that cells deficient in LFA-1 were unable to efficiently protect against malaria parasite challenge despite retaining cytotoxic activity. Collectively these observations demonstrate a clear role for LFA-1 in CD8⁺ T cell patrolling of the hepatic sinusoids, and their ability to protect against liver pathogens.

1043**Reduction in pathogen-specific memory responses after antibiotic treatment increases susceptibility to secondary infection***Benoun, J.¹, Fogassy, Z.¹, McSorley, S.^{1,2}*¹*University California, Immunology, Davis, United States,*²*University California, Department of Anatomy, Physiology and Cell Biology, School of Veterinary Medicine, Davis, United States*

Salmonella enterica serovar Typhi causes recurrent and relapsing infection in antibiotic-treated individuals, suggesting that rapid bacterial clearance hinders the development of adaptive immunity. We have developed an antibiotic-treatment model in mice to examine this issue. This model demonstrates poor secondary protection after antibiotic treatment and allows the use of antigen-specific reagents to examine this issue in detail. Infection with *Salmonella* Typhimurium (BRD509-2W1S) caused expansion of *Salmonella*-specific CD4 T cells that were detected in peripheral blood, lymphoid tissues, and various non-lymphoid tissues. In contrast, antibiotic-treated mice had lower CD4 clonal expansion that persisted for months after infection. This reduced clonal frequency of *Salmonella*-specific CD4 T cells correlated with diminished protective immunity to secondary infection. In order to examine the protective contribution

of circulating and non-circulating CD4 T cells, parabiosis experiments were performed whereby mice previously infected with BRD509-2W1S were paired with naïve mice for 30 days. Previously infected mice were more capable of controlling secondary infection compared to naïve parabionts or naïve control mice, indicating that protection is dependent upon the tissue resident memory cells. Current experiments are focused on examining tissue resident memory cells in antibiotic-treated and untreated mice. Greater understanding of how antibiotics hinder CD4 memory development may allow for therapeutics to boost protective immunity to secondary or relapsing *Salmonella* infections. (Funding: NIH 5P01AI056172-09).

1044**Protein tyrosine phosphatase non-receptor type 2 regulates development and function of tissue resident memory T cells***Hochheiser, K.¹, Wiede, F.², Tigani, T.², Gebhardt, T.¹*¹*University of Melbourne, Microbiology and Immunology, Melbourne, Australia,* ²*Monash University, Department of Biochemistry and Molecular Biology, Clayton, Australia*

T cells play a central role in immune protection against pathogens. Upon resolution of an infection, memory T cells provide enhanced immune surveillance and recall responses towards re-infection. Central memory T cells (T_{CM}) and effector memory T cells (T_{EM}) circulate through secondary lymphoid organs or non-lymphoid tissues, respectively. Recently, a distinct subset of memory T cells was discovered, which permanently remains in tissues after infection without recirculation through the blood. These tissue resident memory T cells (T_{RM}) provide a high degree of protection against localized infection. However, they may also drive aberrant immune responses associated with autoimmunity. Here, we investigated the role of the Protein tyrosine phosphatase non-receptor type 2 (ptpn2) in T_{RM} formation and function in a model of Herpes simplex virus skin infection. We observed a profound defect in ptpn2-deficient T cells to form a stable T_{RM} population in vivo. Related to this, we found a relative reduction of KLRG1⁺ T_{RM} precursors after priming. This defect was reversed by in vitro instead of in vivo activation, a situation that exclusively induces KLRG1⁺ cells. In vitro activated ptpn2-deficient T cells were able to form T_{RM} when lodged into the skin and reacted more strongly towards viral challenge in comparison to ptpn2-competent T cells. Our study reveals a dual role for ptpn2 in skin T_{RM} promoting T_{RM} development by enhancing precursor frequencies and regulating potentially detrimental effector functions.

1045**CCR2 Is critical for memory CD8 T cell generation following influenza A virus infection***Fenix, K.¹, Kara, E.¹, Bastow, C.¹, Gregor, C.¹, McKenzie, D.¹, Norton, T.¹, Seilet, C.², Alsharifi, M.¹, Belz, G.², Comerford, I.¹, McColl, S.^{1,3}*¹*The University of Adelaide, Adelaide, Australia,* ²*Walter and Eliza Hall Institute of Medical Research (WEHI), Parkville, Australia,*³*Centre for Molecular Pathology, Adelaide, Australia*

Memory CD8⁺ T cells are important for controlling reinfection

against intracellular pathogens, yet the migratory signals required for CD8⁺ T cell memory formation are poorly understood. In this study, we show that the chemokine receptor CCR2 is expressed on CD8⁺ T cells following influenza A infection. CCR2-deficient antigen-specific CD8⁺ T cells displayed normal effector cell differentiation and cytokine production but impaired development of memory precursor effector cells (MPECs). Furthermore, CCR2^{-/-} CD8⁺ T cells had reduced proliferation during the late stages of infection. This led to enhanced CD8⁺

T cell contraction alongside a cell intrinsic defect in the formation of central and tissue-resident memory CD8⁺ T cells. Consistent with these observations, CCR2^{-/-} memory CD8⁺ T cells failed to efficiently control secondary infection against intracellular pathogens. These data uncover a novel CCR2-dependent pathway critical for generating memory CD8⁺ T cells.

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Molecular control of tissue resident memory T cell generation in the liver

Yang, K.^{1,2}, Mackay, L.K.², Prosser, A.³, Liao, Y.¹, Man, K.^{1,2}, Lucas, M.³, Shi, W.¹, Gisbergen, K.V.⁴, Kallies, A.^{1,2}

¹Walter and Eliza Hall Institute of Medical Research, Molecular Immunology, Melbourne, Australia, ²University of Melbourne, Department of Medical Biology, Melbourne, Australia, ³The University of Western Australia, School of Pathology and Laboratory Medicine, Crawley, Australia, ⁴AMC, Department of Experimental Immunology, Amsterdam, Netherlands

CD8 memory T cells can be classified into three types, central memory T cells (T_{cm}), effector memory T cells (T_{em}) and resident memory T cells (T_{rm}). While T_{cm} and T_{em} recirculate through secondary lymphoid organs, T_{rm} reside predominantly in non-lymphoid tissues, and do not enter circulation or get replenished from blood or lymphoid tissues. T_{rm} in most tissues, in particular T_{rm} associated with epithelia, express a set of surface molecules including CD69 and CD103 that prevent egress and mediate retention in the tissue. We have characterized the phenotype and transcriptional profiles of multiple circulating and resident memory T cell populations, isolated from various lymphoid and non-lymphoid tissues, including the skin, gut and liver.

In the course of this work we identified a population of memory CD8 T cells in the liver with T_{rm}-like properties. Similar to other T_{rm}, this memory T cell population expressed CD69 and CD49a and was dependent on the transcription factors Hobit and Blimp1. Indeed, transplantation experiments revealed that these cells resided long-term in the liver and were in disequilibrium with circulating memory T cells. However, in contrast to T_{rm} in the skin or intestine, liver T_{rm} did not express CD103, and had access to the blood. Importantly, liver T_{rm} can protect locally against viral infection. We are now testing the molecular requirements and functional properties of liver T_{rm} generation.

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Infection of mice with influenza A virus generates a dichotomy of long-term, local immunity and local immune tolerance of antigen-specific T cells

Eriksson, M., Grönvik, K.-O.

National Veterinary Institute, Department of Microbiology, Uppsala, Sweden

Aim: To study localization and function of influenza virus-specific memory T cells and of APCs.

Methods: Mice were infected intranasally with a sublethal dose of H1N1 PR8 influenza A virus. Between 3 - 17 months post challenge with a lethal dose of homologous virus T cells and APCs from mediastinal lymph nodes (MLNs), lungs and spleens were obtained from sacrificed mice. Viral RNA in tissues was detected by RT-PCR. Purified T cells and infected APCs were co-cultured in vitro. Cytokines in cell-free culture supernatants were determined with Gyrolab Bioaffy, and cell proliferation by uptake of 3H-thymidine.

Results: Replicating influenza virus was restricted to sensitive tissues of the respiratory tract such as MLNs and lungs whereas no viral RNA was detected in spleen. At 12 - 17 months post infection localization of infected, autologous APCs determined immunity versus immune tolerance of influenza-virus specific memory T cells. Immune T cells of MLNs produced significantly less TH1 cytokines when co-cultured with infected, autologous APCs of MLNs than co-culture with infected, autologous APCs of spleen. Conversely immune spleen T cells produced significantly less TH1 cytokines upon stimulation with infected APCs of spleen than when co-cultured with infected APCs of MLNs.

Conclusions: Long-term memory T cells display a localization into different lymphoid organs in a similar way to the localization of differentiated $\gamma\delta$ -T cells into different tissues. This may be an important mechanism for maintenance of peripheral immunological tolerance and for establishing a more energy-efficient state of tissue-resident, memory T cells.

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Pulmonary immunization with recombinant influenza A virus stimulates *M. tuberculosis*-specific CD4⁺ tissue resident memory T cells in the lung

Flórido, M.¹, Muflihah, H.¹, Lin, L.¹, Stambas, J.², Palendira, M.¹, Britton, W.J.^{1,3}

¹Centenary Institute, Tuberculosis Research Program, Newtown, Australia, ²Deakin University, School of Medicine, Geelong, Australia, ³University of Sydney, Sydney Medical School, Camperdown, Australia

The recently identified tissue resident memory T cells (TRMs) are retained within tissues and are phenotypically and functionally distinct from the circulating effector and memory T cells. They have been associated with protection against several pathogens. We have determined that pulmonary immunization of mice with recombinant influenza A virus (rIAV) expressing the p25 CD4⁺ T cell epitope of the *Mycobacterium tuberculosis* (*Mtb*) Ag85B protein (PR8.p25) induced strong p25-specific CD4⁺ T cell responses and protected mice against *Mtb* challenge. Using adoptive transfer of P25 TCR transgenic CD4⁺ T cells

and intravascular staining, we found that p25-specific T cells induced by PR8.p25 vaccine persist in the lung parenchyma long after the virus is cleared. After 6 wk, more than 90% of the P25 T cells were present in the lung parenchyma and expressed higher levels of CD69, a marker of TRMs, compared to P25 cells in the vasculature. The presence and location of these cells in the lungs following PR8.p25 immunization were confirmed by 2-photon imaging. Moreover, transcriptional analysis of the CD69⁺ P25 T cells isolated from the lungs 6 weeks after PR8.p25 immunization revealed down-regulation of the TRM-associated transcription factors, Klf2 and S1pr1, as compared to splenic effector or naïve P25 CD4 T cells. Thus, immunization with PR8.p25 stimulates retention in the lungs of *Mtb*-specific CD4⁺ T cells with the characteristics of TRMs. One potential advantage of the pulmonary delivery of TB vaccines is the induction of protective CD4 TRMs at the site of *Mtb* infection in the lung. Support: NHMRC.

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Phenotypic and functional alterations in memory CD8 T cells with time after primary infection

*Martin, M.*¹, *Kim, M.*², *Shan, Q.*³, *Xue, H.-H.*^{2,3}, *Harty, J.*^{2,3}, *Badovinac, V.*^{1,2}
¹University of Iowa, Pathology, Iowa City, United States, ²University of Iowa, Interdisciplinary Graduate Program in Immunology, Iowa City, United States, ³University of Iowa, Microbiology, Iowa City, United States

Memory CD8 T cells confer increased protection upon pathogen re-encounter. The level of protection provided depends on the numbers, functional abilities, and location of memory cells present at the time of re-infection. While primary memory CD8 T cells can be maintained for life, the extent of phenotypic and functional changes that occur over time after initial antigen (Ag) encounter remains poorly characterized. We show that critical properties of circulating primary memory CD8 T cells, including location, phenotype, maintenance, Ag-driven secondary proliferation and generation of secondary memory, mitochondrial function, and inflammation-induced bystander activation change with time after initial infection. Interestingly, phenotypic and functional alterations in the memory population are not due solely to shifts in the ratio of effector (CD62L^{lo}) and central memory (CD62L^{hi}) cells, but also occur within defined CD62L^{hi} memory subsets. CD62L^{hi} memory cells retain the ability to efficiently produce cytokines with time after infection. However, the gene expression profiles of CD62L^{hi} memory cells change, phenotypic heterogeneity decreases, and mitochondrial function and proliferative capacity in either a lymphopenic environment or in response to cognate Ag increase with time. Importantly, protection provided against chronic LCMV clone-13 infection increases over time for both circulating memory CD8 T cell populations and for CD62L^{hi} memory cells. Taken together, the data reveal that memory CD8 T cells continue to change with time after primary infection and suggest that the outcome of vaccination strategies designed to elicit protective memory CD8 T cell responses using single or prime-boost immunizations depends upon the timing between antigen encounters.

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Distinct recirculation potential of phenotypically disparate CD69⁺CD103⁻ and CD103⁺ thymic memory T cells

Park, S., Mackay, L., Gebhardt, T.

Peter Doherty Institute for Infection and Immunity at The University of Melbourne, Department of Microbiology and Immunology, Melbourne, Australia

Unlike central and effector memory T cells that transit through blood, non-recirculating tissue-resident memory T (T_{RM}) cells permanently occupy peripheral and lymphoid tissues where they confer superior protection against infection. Whilst epithelial CD8⁺ T_{RM} cells co-express CD69 and CD103, CD103⁻ memory cells have been described in diverse organs and are often presumed non-recirculating based on their expression of CD69 alone. We found that both CD69⁺CD103⁻ and CD69⁺CD103⁺ memory cells populated the thymus upon transfer of CD8⁺ effector T cells into uninfected recipients, demonstrating that these subsets can form in the absence of both local antigen and inflammation. Transcriptionally and phenotypically, CD103⁺ thymic cells resembled non-lymphoid T_{RM} cells whereas CD69⁺CD103⁻ cells displayed an intermediate profile more closely related to recirculating cells, indicating that these memory subpopulations are fundamentally distinct. Although CD69 was required for optimal CD103⁺ T_{RM} cell formation, its expression alone did not identify permanently resident cells, as the CD69⁺CD103⁻ subset was largely eradicated from the thymus following antibody-mediated depletion of recirculating cells. In contrast, CD69⁺CD103⁺ thymic cells were selectively retained despite antibody treatment, confirming that they are *bona fide* T_{RM} cells. Our findings highlight a distinct migration potential of phenotypically divergent thymic CD8⁺ memory T cells and emphasise the inadequacy of CD69 as an absolute marker of tissue residency.

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Non-classical CD8⁺ T cells restricted by HLA class II DRB1 emerge in HIV infection and show antiviral efficacy and atypical TCR usage

*Ranasinghe, S.*¹, *Lamothe-Molina, P.*¹, *Soghoian, D.*¹, *Kaizer, S.*¹, *Shalek, A.*¹, *Cole, M.*², *Yosef, N.*², *Jones, B.*³, *White, J.*⁴, *Crawford, F.*⁴, *Sidney, J.*⁵, *Sette, A.*⁵, *Carrington, M.*⁶, *Streeck, H.*⁷, *Kaufmann, D.*⁸, *Picker, L.*⁹, *Kappler, J.*^{4,10}, *Walker, B.D.*^{1,10}

¹Ragon Institute of MGH, MIT and Harvard, Cambridge, United States, ²University of California, Berkeley, United States, ³George Washington University, Washington DC, United States, ⁴National Jewish Health, Denver, United States, ⁵La Jolla Institute for Allergy & Immunology, La Jolla, United States, ⁶National Cancer Institute, National Institutes of Health, Frederick, Maryland, United States, ⁷University of Duisburg-Essen, Essen, Germany, ⁸Centre de Recherche du Centre Hospitalier de l'Université de Montréal, Montreal, Canada, ⁹Oregon Health Sciences University, Oregon, United States, ¹⁰Howard Hughes Medical Institute, Chevy Chase, United States

Background: CD8⁺ T cells typically recognize infected cells through viral peptides presented on HLA class I. However, class II-restricted CD8⁺ T cell responses have been reported

in CD4 knockout mice and in a macaque AIDS vaccine model. This raises a critical question: do class II-restricted CD8+ T cell responses exist in natural HIV-infection?

Methods: We detected class II-restricted CD8+ T cells in 3% of treatment-naive HIV-infected individuals (3/101 screened) using a novel 'CD8 HLA-DR ELISpot'. CD8+ T cells targeted HIV Gag37 or Gag41 peptides presented by LCL stably expressing recombinant human DR01 and DR11, respectively. Antibody blocking of class I and II, and flow cytometric staining with class II tetramers confirmed the restriction. TCR sequencing was conducted from tetramer-sorted cells.

Results: Class II-restricted CD8+ T memory cells exist in HIV-infection. Although rare, in one individual it was the most immunodominant CD8+ response encompassing 12% of total CD8+ T cells directly ex vivo. These CD8+ TEMRA cells were Perforin+GranzymeB+ and efficiently lysed autologous HIV-infected CD4+ T cells and macrophage targets ($p < 0.01$). Furthermore, HLA-DR-restricted CD8+ T cells showed atypical patterns of TCR usage, characterized by two distinct co-expressed TCR alpha chains (TRAV26 and TRAV6), and a single TCR beta clonotype shared with CD4+ T cells targeting the same HLA-DR-epitope. Indeed, TCR beta clonotype TRBV2 was shared between 100% of CD8+ and 73.9% of CD4+ T cells targeting DR11-Gag41.

Conclusions: HLA-DR-restricted antigen-specific CD8+ T cells exist in natural HIV infection, demonstrating that the established T cell recognition paradigms are not absolute.

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Reprogramming of T cells from effector to stem cell memory by Notch signaling

Kondo, T.¹, Morita, R.¹, Kassai, Y.², Sekiya, T.¹, Chikuma, S.¹, Gotou, Y.², Kanamori, M.¹, Miyazaki, T.², Kubo, M.³, Yoshimura, A.¹

¹Keio University, Tokyo, Japan, ²Takeda Pharmaceutical Company Limited, Kanagawa, Japan, ³Tokyo University of Science, Chiba, Japan

Immunological memory is important for vaccinations and the suppression of neoplasia, yet it remains unclear how this memory function is maintained in the long term. Stem cell memory T (T_{SCM}) cells have recently been proposed as a novel memory T cell population which possesses the potential of long lifespan, self-renewal and the ability to produce large number of effector T cells. However, methods of T_{SCM} cells generation from effector T cells for clinical use have not been established. In this study, we have developed a method for the *in vitro* generation of induced T_{SCM} -like (iT_{SCM}) cells and characterized their function in antitumor immunity. We found that Notch signaling can generate iT_{SCM} cells from CD4+ or CD8+ activated effector T cells. iT_{SCM} cells not only supplied effector, classical central and effector memory T cell populations (T_{CM} and T_{EM}) by antigen re-stimulation, but also possessed long-lived and self-renewing potentials. Both CD4+ and CD8+ iT_{SCM} cells suppressed tumor growth much more efficiently than did T_{CM} or effector T cells in mouse models. Notch signaling also converted peripheral memory T cells to more potent antitumor iT_{SCM} cells. Our data suggest that Notch signaling confers "stemness" to effector T cells, and converts them into potent anti-tumorigenic T cells.

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Dissecting T cell repertoires at the human placenta by fusing high definition gene profiling with TCR massive parallel sequencing

Watkins, T.S.¹, McGuire, H.², Darko, S.³, Haigh, O.¹, Ransier, A.³, Santner-Nanan, B.⁴, Nanan, R.⁴, Douek, D.C.³, Miles, J.J.¹

¹QIMR Berghofer Medical Research Institute, Human Immunity, Brisbane, Australia, ²Centenary Institute, Sydney, Australia, ³Vaccine Research Center, National Institute of Allergy and Infectious Disease, NIH, Bethesda, United States, ⁴Nepean Centre for Perinatal Care, Sydney Medical School, Sydney, Australia

During pregnancy a semi-allogenic foetus develops within the uterus of its HLA mismatched mother. The decidua is able to secrete a milieu of cytokines and growth factors that ultimately permits development of the highly immunogenic foetus through potent immune suppression, whilst also preparing the foetus' immune system for subsequent challenge. Previous studies have highlighted the role of a number of important cytokines in establishing and maintaining pregnancy, as well as in foetal immune priming. The majority of this work however has focused on the functions of these molecules acting individually or in simple combinations. To date, detailed immune phenotyping at the decidua and in newborns has not been undertaken. Here, we performed high definition profiling using T cell pathway-focused gene profiling that quantified markers of survival, apoptosis, transcription, migration, adhesion, differentiation and effector function. We combined this phenotypic analysis with massive parallel sequencing of the human TCR repertoire. We identified atomically specific signatures in both gene profiling and TCR repertoire composition at different anatomical sites. Identifying immune "baselines" in different human tissues could be expanded in future to define immune correlates of disease in pregnancy-related complications where altered T cell cytokine expression has already been implicated.

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Signaling through PD-1 on CD8 T cells is critical for antigen-independent maintenance of immune memory

Yuzefpolskiy, Y.¹, Baumann, F.M.², Penny, L.A.², Kalia, V.^{3,4}, Sarkar, S.^{3,4}

¹Penn State University, Seattle, United States, ²Penn State University, University Park, United States, ³University of Washington School of Medicine, Seattle, United States, ⁴Seattle Children's Research Institute, Seattle, United States

PD-1 is highly expressed on CD8 T cells during activation, both in acute and chronic viral infections. Inhibitory signaling in cytotoxic T cells through the PD-1 axis is well characterized during chronic infections and has led to the development of potent blockade therapies that restore function to otherwise exhausted CD8 T cells. However, the functional significance of transiently increased PD-1 expression on CD8 T cells early after activation and its rapid down-regulation following clearance of antigen during acute infections, remains to be characterized. The enigma is - expression of PD-1 (an immunological brake) during activation temporally coincides with rapid proliferation and production of copious amounts of signature effector

cytokines (IFN- γ and TNF- α). We generated PD-1^{-/-} antigen-specific CD8 T cells to study its cell-intrinsic role during acute infection with LCMV. Unexpectedly, PD-1^{-/-} cells did not exhibit increased proliferation early during infection, or enhanced accumulation at the peak of expansion. PD-1^{-/-} CD8 T cells were also unaltered in their polyfunctionality and granzyme B expression. Despite robust effector properties, PD-1^{-/-} CD8 T cells underwent precipitous contraction, leading to near ablation of the memory pool. Mechanistically, *in vivo* analysis of PD-1^{-/-} memory cells showed a severe defect in antigen-independent homeostatic proliferation due to decreased sensitivity to IL-2, -7, and -15 signals, without evident change in the expression of common gamma-chain cytokine receptors. These studies demonstrate a previously unrecognized role of PD-1 signaling in programming of memory CD8 T cell maintenance and could make it a novel target for manipulating vaccine-induced memory T cell longevity.

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Differentiation of resident memory CD8⁺ T cells in the liver

Wong, Y.C.^{1,2,3}, Sierro, F.^{1,2,3}, Lu, B.⁴, Tay, S.S.^{1,2,3}, Wood, N.A.W.^{1,2,3}, McGuffog, C.^{1,2,3}, McCaughan, G.W.^{1,2,3}, Bowen, D.G.^{1,2,3}, Bertolino, P.^{1,2,3}

¹Centenary Institute, Liver Immunology Program and AW Morrow Gastroenterology and Liver Centre, Camperdown, Australia, ²Royal Prince Alfred Hospital, Camperdown, Australia, ³University of Sydney, Camperdown, Australia, ⁴St. Vincent's Hospital, Melbourne, Australia

Tissue-resident memory T cells (T_{RM}) have been identified in different organs including skin and lung. Whether T_{RM} can differentiate in the liver is unknown. To induce intrahepatic CD8⁺ T cell responses, recipient mice were transferred with OVA-specific transgenic T cells and treated with recombinant adeno-associated viral vectors (rAAV) that target mouse hepatocytes to induce de novo OVA expression in the liver. We have recently shown that a low rAAV dose induced a low antigen load and allowed the development of intrahepatic effector T cells. We investigated here whether these cells differentiated into T_{RM}. When 1-5% of hepatocytes expressed OVA, functional memory OVA-specific CD8⁺ T cells expressing CD69, a common T_{RM} marker, were established in the liver. Intra-vital multi-photon microscopy experiments revealed that these memory cells moved slowly and against the blood flow, suggesting that they were patrolling the hepatic sinusoids. To test the ability of these cells to recirculate, livers containing memory T cells were transplanted into naive recipients. Most donor memory CD8⁺ T cells were detected within the transplanted livers and very few recirculated into lymphoid tissues, suggesting that they were liver resident. Importantly, when OVA presentation was restricted to hepatocytes, naive CD8⁺ T cells became effectors but failed to develop into T_{RM}.

In summary, low numbers of antigen-expressing hepatocytes promote the differentiation of liver CD8⁺T_{RM}. Although hepatocytes are critical in providing antigen, the differentiation of intrahepatic CD8⁺ T_{RM} requires non-hepatocyte antigen-presenting cells. Our results have important implications for vaccine development and for the treatment of hepatotropic infections.

Th Subsets

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CRTAM instructs the CD4⁺ cytotoxic T lymphocyte lineage

Takeuchi, A.¹, Gadelhaq Badr, M.E.S.¹, Miyauchi, K.², Kubo, M.^{2,3}, Saito, T.^{1,4}

¹RIKEN Center for Integrative Medical Sciences, Laboratory for Cell Signaling, Yokohama, Japan, ²RIKEN Center for Integrative Medical Sciences, Laboratory for Cytokine Regulation, Yokohama, Japan, ³Tokyo University of Science, Research Institute for Biomedical Science, Division of Molecular Pathology, Chiba, Japan, ⁴Osaka University, WPI Immunology Frontier Research Center, Osaka, Japan

Naïve T cells differentiate into various effector T cells including CD4⁺ helper T cell subsets and CD8⁺ cytotoxic T cells (CTL). Cytotoxic CD4⁺T cells (CD4⁺CTL) also develop from naïve T cells, but the mechanism of development is elusive. Class-I restricted T cell associated molecule (CRTAM) is an activation-induced surface receptor molecules predominantly expressed on activated CD8⁺T cells. However, tiny fraction of activated CD4⁺T cells also express CRTAM and has characterized these unique CD4⁺T cells. Micro array data revealed that the CRTAM⁺ CD4⁺T cells have the characteristics of both CD4⁺ and CD8⁺T cells. These cells particularly secrete IFN γ , express CTL-related genes such as eomesodermin (Eomes), granzyme B and perforins, and exhibit cytotoxicity after cultivation. These results suggest that CRTAM⁺ T cells are the precursor of CD4⁺CTL. Furthermore, ectopic expression of CRTAM in T cells also induced the production of IFN γ , expression of CTL-related genes, and cytotoxic activity. This result indicates that the induction of CD4⁺CTL and IFN γ production requires CRTAM-mediated intracellular signaling. CRTAM⁺ T cells traffic to mucosal tissues and inflammatory sites, and developed into CD4⁺CTL. These cells are involved in mediating protection against infection as well as inducing inflammatory response, depending on the circumstances, through IFN γ secretion and cytotoxic activity. These results reveal that CRTAM is critical to instruct the differentiation of CD4⁺CTL through the induction of Eomes and CTL-related gene.

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Using immunodeficiency patient cells in *in vitro* cultures to provide insight into T-helper cell differentiation

Pillay, B.A.^{1,2}, Ma, C.S.^{1,2}, Casanova, J.L.³, Uzel, G.⁴, Tangye, S.G.^{1,2}

¹The Garvan Institute of Medical Research, Immunology Division, Darlinghurst, Australia, ²The University of New South Wales, St Vincent's Clinical School, Sydney, Australia, ³The Rockefeller University, Laboratory of Human Genetics of Infectious Diseases, Rockefeller Branch, New York, United States, ⁴NIAID, NIH, Laboratory of Clinical Infectious Diseases, Bethesda, United States

CD4⁺ T-cells play a broad role in host protection against disease. To achieve this, specialised subsets of effector CD4⁺ T-cells have evolved to orchestrate the diverse jobs required. These include defence against viruses, bacteria and fungi, as well as being involved in immune regulation and providing "help" to B-cells for their differentiation into memory and antibody-secreting

plasma cells. It is believed that all CD4⁺ T-cell subsets originate from a common precursor: the naïve CD4⁺ T-cell. Upon receipt of signals provided by specific antigen, antigen-presenting cells and the priming microenvironment, naïve T-cells differentiate into effector subsets via a process that is regulated by specific transcription factors induced by these stimulatory signals at the time of T-cell activation.

Our study of monogenic primary immunodeficiencies caused by mutations in cytokine receptors (*IL21R*, *IL12RB1*, *IL12RB2*, *IFNGR1*, *IFNGR2*) and proteins involved in downstream signalling (*STAT1*, *STAT3*, *DOCK8*) showed a CD4⁺ memory compartment with increased production of cytokines characteristically produced by Th2 cells (IL4, IL5, IL13). The intrinsic nature of this apparent Th2 skewing has been explored using *in vitro* cultures to polarise naïve CD4⁺ T-cells towards Th1, Th2 and Th17 differentiation pathways and assessing transcription factor and cytokine expression.

We found that the ability of some of these patient cells to differentiate into Th1 and Th17 cells in an *in vitro* system was unaffected and this suggests that *in vivo* an element of the environment during differentiation is responsible for Th2 skewing. Further investigation is being undertaken to elucidate the nature of this environmental element.

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Immunological signature predictive of rituximab response in a subgroup of focal segmental glomerulosclerosis patients

Chan, C.Y.^{1,2}, Liu, I.D.^{1,2}, Resontoc, L.P.^{1,2}, Ng, K.H.^{1,2}, Chan, Y.H.³, Lau, Y.W.^{1,2}, Than, M.^{1,2}, Jordan, S.C.⁴, Lam, K.P.^{1,5}, Yeo, W.S.^{1,2}, Yap, H.K.^{1,2}

¹National University of Singapore, Paediatrics, Singapore, Singapore, ²Khoo Teck Puat-National University Children's Medical Institute, National University Health System, Singapore, Singapore, ³National University of Singapore, Biostatistics Unit, Singapore, Singapore, ⁴Cedars Sinai Medical Center, CA, United States, ⁵Bioprocessing Technology Institute, Agency for Science, Technology and Research (A*STAR), Singapore, Singapore

Rituximab has been used with variable success in focal segmental glomerulosclerosis (FSGS) patients, but the immunological basis of its efficacy is poorly characterized. This study aimed to characterize T-cell subsets in FSGS patients in order to identify an immunological signature predictive to favourable rituximab treatment.

Twenty-two FSGS patients (median age 14.4 years, range 6.2-25.0 years) were recruited prospectively to receive rituximab and were followed up longitudinally. Clinical parameters and immunological subsets were monitored at baseline and followed up until relapse. Baseline immunological subsets were compared to 22 patients with minimal change nephrotic syndrome (MCNS) in relapse and 30 healthy controls, and subsequently examined for association with response to rituximab.

Of the 22 patients, 12 (54.5%) responded to rituximab therapy. *In-vivo* expression of baseline B-cells, T-cells, natural killer cells, helper T-cell, cytotoxic T-cell and regulatory T-cell in FSGS rituximab responders were comparable to non-responders. However, mitogen-stimulated CD154⁺CD4⁺CD3⁺, IFN- γ ⁺CD3⁺

and IL-2⁺CD3⁺ expressions before rituximab were significantly lower in responders compared to non-responders and controls. The IFN- γ ⁺CD3⁺ and IL-2⁺CD3⁺ expressions in FSGS rituximab responders were also significantly lower compared to MCNS in relapse. At six months post-rituximab treatment, T-cells activation level in the responders were restored. Using ROC curve analysis, activated CD154⁺CD4⁺CD3⁺ (AUC 0.81, 95% CI 0.61-1.01), IFN- γ ⁺CD3⁺ (AUC 0.90, 95% CI 0.75-1.05) and IL-2⁺CD3⁺ (AUC 0.78, 95% CI 0.57-0.98) were good predictors of response to rituximab.

We have identified prognostic markers which define a subgroup of FSGS patients bearing an immunological signature of hyporesponsiveness to T-cell stimulation, with good response to rituximab therapy.

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Innate effector function of Th2 cells is regulated by EGF-R expression

Zaiss, D., Minuti, C., Blair, N., Maizels, R.

University of Edinburgh, Institute of Immunology and Infection Research, Edinburgh, United Kingdom

Host resistance to helminth infection is critically dependent on cytokine production at the site of infection, mediated by both innate immune cells and cells of the adaptive immune system. Recently, it was revealed that Th2 cells contribute to helminth resistance, both in an antigen-dependent and - independent manner; however, to which either mechanism contributes to helminth resistance remains unknown.

Here we show that EGF-R expression on activated Th2 cells critically contributes to host resistance to helminths, and directly correlates with IL13 production at the site of infection in mice. IL33 induces "innate" IL13 production in Th2 cells, dependent on the formation of a signaling complex between T1/ST2, the IL33-R, and EGF-R. T cell transfer into MHC-II deficient mice confirmed that the innate capacity of Th2 cells to expel helminth infections was dependent on EGF-R expression. In addition, while EGF-R expression in activated Th2 cells was i) transient, ii) induced by either antigen or STAT5-signalling inducing cytokines, and iii) rapidly lost upon drug-induced pathogen clearance or transfer of Th2 cells into naïve mice, T1/ST2 expression was stable over time of infection.

Taken together, these data suggest a mechanism by which pathogen-specific Th2 cells are activated in an antigen-specific manner within draining lymph nodes, which results in an up-regulation of the EGF-R. This EGF-R expression then opens a window of opportunity within which activated Th2 cells can secrete IL13 at the site of infection in an antigen-independent way upon exposure to IL-33, and so contribute to helminth expulsion.

1060**MINK1 kinase suppresses Th17 cell differentiation and autoimmune inflammation via inhibition of the TGF- β pathway***Fu, G., Lu, L.**Zhejiang University, Hangzhou, China*

T-helper type 17 (Th17) cells that produce the cytokines interleukin-17A (IL-17A) and IL-17F are implicated in the pathogenesis of several autoimmune diseases. The differentiation of Th17 cells is instructed and critically regulated by different cytokines, which drives intracellular signaling cascades and transcriptional signatures that determines the cell fate. We report that MINK1, a germinal center kinase (GCK) family kinase, is a negative regulator of IL-17A production in the periphery. Mice deficient in MINK1 developed exacerbated inflammation during EAE. MINK1 directly phosphorylated SAMD2 at T324 residue, which inhibited the activation of SMAD2 and suppressed the induction of Th17 cells. Antioxidants have been shown to compromise the activation of MINK1 in other cells. Here we show that antioxidant N-acetyl cysteine (NAC) boosts Th17 cells generation *in vitro* in a MINK1-dependent manner. Moreover, mice with NAC treatment develop a more severe form of EAE through a cell-intrinsic mechanism. These data identify MINK1 as a suppressor of Th17 cells and provide a molecular insight into a mechanism by which an antioxidant (health product) such as continued NAC supplementation triggers Th17 development and promotes autoimmunity.

1061**Promotion of mouse autoimmune arthritis via TLR4/ICOS/IL-17 axis by mycoplasma superantigen***Mu, H.-H., Nourian, M., Jiang, H.-H., Wang, J., Cole, B.**University of Utah School of Medicine, Salt Lake City, United States*

Mycoplasma arthritidis mitogen (MAM), a potent superantigen (SAg) secreted by *M. arthritidis*, is known to contribute to mouse arthritis models that are related to the pathogenic Th17 cells. We previously demonstrated that IL-17 mobilizes, recruits and activates inflammatory cells to increase local inflammation in arthritic mice infected with *M. arthritidis*, and an early engagement of TLR4 by MAM is necessary for the enhanced disease severity that is associated to the increased IL-17. ICOS, with its counterpart ICOS ligand (ICOSL) that is expressed on activated T cells. In the present study, we found injections of MAM to a collagen-immunized mice aggravated collagen-induced arthritis (CIA), initiating an early onset of disease, enhancing incidence and severity of arthritis. The increased arthritis was accompanied by an increase in anti-CII Ig levels. Enhanced level of ICOS expression was observed in the inflamed synovial tissue as well as in the draining lymph nodes (LNs) that are associated with increased IL-17 and Th17-associated cytokines and chemokines. Furthermore, blocking antibody against ICOSL significantly ameliorated arthritis in the joints. The number of T cells expressing ICOS in lymphoid tissues were remarkably reduced by the anti-ICOSL treatment. Serum anti-CII IgG1, IgG2a, and IgG2b levels were also reduced. Our results demonstrates a beneficial effect of the ICOSL blockade on mouse

arthritis through suppression of Th17-mediated pathogenic effect, suggesting that the ICOS/ICOSL co-stimulatory pathway plays an important role in the pathogenesis of inflammatory/autoimmune arthritis and thus the targeting of this pathway can be a useful therapeutic strategy for treating human rheumatoid arthritis.

1062**Itk is required for Th9 differentiation via TCR-mediated induction of IL-2 and IRF4***Gomez Rodriguez, J.¹, Meylan, F.², Handon, R.¹, Hayes, E.², Anderson, S.¹, Kirby, M.¹, Siegel, R.², Schwartzberg, P.¹**¹NIH/NHGRI, Bethesda, United States, ²NIH/NIAMS, Bethesda, United States*

T helper 9 (Th9) cells produce IL-9, a cytokine implicated in allergic asthma and autoimmunity. We show that Itk, a mediator of T-cell receptor signalling required for Th2 immune responses and the development of asthma, is a positive regulator of Th9 differentiation *in vivo* and *in vitro*. In a papain-induced model of allergic lung disease, Itk-deficient mice have reduced pulmonary inflammation and decreased IL-9 production by T cells and innate lymphoid type 2 cells (ILC2), despite normal early induction of ILC2s. *In vitro*, naive *Itk*^{-/-} CD4⁺ T cells essentially fail to produce IL-9 and have reduced levels of IRF4, a critical transcription factor for effector T-cell function. Both IL-9 and IRF4 expression are rescued by either IL-2 or expression of constitutively-active STAT5, but not NFATc1. We further find that STAT5 binds the *Irf4* promoter, indicating one mechanism by which IL-2 can rescue weakly-activated T cells. Interestingly, naive WT CD4 T cells cultured in presence of the Itk inhibitor show very high levels of IL-9 production when IL-2 is added to the cultures, suggesting complex effects of Itk inhibitors. Thus, inhibiting Itk as a therapeutic strategy for treating or preventing symptoms of asthma and other diseases in which IL-9 participates still remains an important question.

1063**Th17 plasticity and transition towards a pathogenic cytokine signature is regulated by Cyclosporin after allogeneic-SCT***Gartlan, K.H.^{1,2}, Varelias, A.^{1,2}, Koyama, M.¹, Markey, K.¹, Kuns, R.¹, Raffelt, N.¹, Olver, S.¹, Lineburg, K.¹, Teal, B.¹, Cheong, M.¹, Smyth, M.¹, Tey, S.-K.¹, Macdonald, K.¹, Hill, G.¹**¹QIMR Berghofer, Immunology Department, Brisbane, Australia,**²University of Queensland, School of Medicine, Brisbane, Australia*

Th17 cells have been widely implicated as drivers of autoimmune disease. In particular, Th17 cytokine plasticity and acquisition of an IL-17A⁺IFN γ ⁺ cytokine profile is associated with increased pathogenic capacity. Donor Th17 polarization is known to exacerbate GVHD after allogeneic stem cell transplant (allo-SCT), however donor Th17 cytokine co-expression and plasticity have not been fully characterized. Using IL-17 'fate-mapping' reporter mice, we identified IL-6-dependent Th17 cells early after allo-SCT, characterized by significantly elevated expression of pro-inflammatory cytokines, IL-17A, IL-22, GM-CSF and TNF. This

population did not maintain lineage fidelity, with a marked loss of IL-17A and IL-22 expression late post-transplant. Th17 cells could be further segregated based on IFN γ co-expression and IL-17A⁺IFN γ ⁺ Th17 displayed an enhanced pro-inflammatory phenotype. This cytokine plasticity and IFN γ production was critically dependent upon donor derived IL-12/IL-23 and calcineurin inhibition via Cyclosporin treatment regulated this differentiation pathway. This observation was highly concordant with clinical samples from recipients receiving Cyclosporin-based immune suppression where although the IFN γ negative-Th17 subset predominated, IFN γ ⁺-Th17 cells remained present. In sum, Th17 polarization and ensuing differentiation are mediated by sequential inflammatory signals which are modulated by immunosuppressive therapy, leading to distinct phenotypes within this lineage.

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IFN-g and IL-21 producing Th1 cells dependent IgG2 responses gave a protection to H5N1 pandemic influenza virus

Miyauchi, K.¹, Ishige, A.², Takahashi, Y.³, Takemori, T.², Kubo, M.^{1,4}

¹RIKEN Center for Integrative Medical Sciences, Laboratory for Cytokine Regulation, Yokohama, Japan, ²RIKEN Center for Integrative Medical Sciences (IMS), Drug Discovery Antibody Platform Unit, Yokohama, Japan, ³National Institute of Infectious Diseases, Department of Immunology, Tokyo, Japan, ⁴Research Institute for Biomedical Science, Tokyo University of Science, Division of Molecular Pathology, Chiba, Japan

Two major distinct helper T cell subsets, Tfh and Th1 cell are developed with influenza A virus infection. The contribution of Tfh on producing neutralizing antibody, which takes a pivotal role in virus protection, has become a consensus. However, Th1 cell's contribution in protective humoral response is controversial. The mice carrying loxP-flanked Bcl6 exon allele and crossing with cd4-cre (Bcl6f/fCd4cre), Tfh deficient mice, give us an opportunity to ask about Th1 function in anti-viral IgG induction. Vaccinated Bcl6f/fCd4cre mice showed normal protection against H1N1 seasonal and H5N1 pandemic virus that relied on virus specific IgG2 antibodies. Thus, Tfh cells are dispensable in virus specific IgG2 response. Further experiments revealed that IFN-g and IL-21 from Th1 cells were required for anti-virus IgG2. Moreover, the transfer of IL-21 producing Th1 cells augmented anti-virus IgG2 titer. Taken together, the distinct Th1 subset producing IFN-g and IL-21 takes significant roles in influenza virus specific humoral responses. Plasticity and lineage decision of Tfh and Th1 cells are also debatable. In our experimental setting, transferred Th1 cells retained Th1 signature for two weeks, which suggests that Th1 and Tfh lineage are rather independent. Th1 induction could be a promising strategy in novel vaccine design to tackle H1N1 and H5N1 influenza virus pandemic.

1065

Vaccination induced ICOS⁺ circulating memory T_{FH} cells share similar characteristics to T_{FH} cells but are dysfunctional and Th2 skewed in chronic HIV-1 infection

Abudulai, L.¹, Hunter, M.^{2,3}, Post, J.^{2,3}, French, M.^{1,4}, Fernandez, S.¹

¹The University of Western Australia, School of Pathology and Laboratory Medicine, Perth, Australia, ²University of New South Wales, Prince of Wales Clinical School, Sydney, Australia, ³Prince of Wales Hospital, Department of Infectious Diseases, Sydney, Australia, ⁴Royal Perth Hospital and PathWest Laboratory Medicine, Department of Clinical Immunology, Perth, Australia

HIV infection increases the risk of pneumococcal disease, probably related to B-cell and/or T follicular-helper (T_{FH}) cell dysfunction. We have shown that decreased production of pneumococcal polysaccharide (PcP)-specific IgG2⁺ antibody secreting cells (ASC) in HIV patients 7 days after vaccination with unconjugated PcPs is associated with impaired expansion of ICOS⁺ circulating memory (cm)T_{FH} (CD4⁺CD45RA⁻CD27⁺CXCR5⁺PD-1⁺) cells. Here, we sought to further characterise these cells in antiretroviral therapy (ART)-treated (n=27) and ART-naive (n=11) HIV patients and non-HIV subjects (n=20). Intracellular expression of Bcl-6, Blimp-1 and BATF, and surface expression of CCR6, CCR7, CXCR3, BTLA and IL-6Ra were examined in unstimulated cells while production of IL-21 and IFN- γ were assessed following *in vitro* stimulation. After vaccination, ICOS⁺ cmT_{FH} cells in HIV patients and non-HIV subjects displayed characteristics of canonical T_{FH} cells, with BTLA, IL-6Ra and Bcl-6 expression and IL-21 production all observed. However, IL-21 production by ICOS⁺ cmT_{FH} cells was decreased in ART-naive (p=0.02) and ART-treated (p=0.03) patients compared to non-HIV subjects. Furthermore, production of IgG2 ASCs specific for PcP serotypes 4, 6B, 9V and 14 correlated with ICOS⁺ cmT_{FH} cells that displayed a Th1 phenotype (CXCR3⁺CCR6⁻) in non-HIV subjects (R \geq 0.42, p \leq 0.06) but a Th2 phenotype (CXCR3⁻CCR6⁻) in ART-treated HIV patients (R \geq 0.44, p \leq 0.02). Thus, after vaccination with PcPs, ICOS⁺ cmT_{FH} cells were skewed from Th1 to Th2 and displayed impaired production of IL-21 in ART-treated HIV patients. These findings provide new evidence that impaired antibody responses to PcPs, and possibly other antigens, in HIV patients receiving ART may result from residual T_{FH} cell dysfunction.

1066

Bach2-Batf interactions control Th2-type immune response by regulating the IL-4 amplification loop

Yamashita, M.¹, Ochi, M.¹, Sawasaki, T.², Kuwahara, M.¹

¹Ehime University, Department of Immunology, Graduate School of Medicine, Toon, Japan, ²Ehime University, Proteo-Science Center, Matsuyama, Japan

Although Bach2 plays an important role in regulating the Th2-type immune response, the underlying molecular mechanisms remain unclear. We herein demonstrate that Bach2 associates with Batf family transcription factors and binds to the regulatory regions of the Th2 cytokine gene loci, which contain AP-1 motifs. The Bach2-Batf complex antagonizes the recruitment of the Batf-Irf4 complex to AP-1 motifs and suppresses Th2 cytokine production. Furthermore, we found that Bach2 also regulates

the *Batf* and *Batf3* expressions via two distinct pathways. First, the Bach2-Batf complex directly binds to the *Batf* and *Batf3* gene loci and reduces transcription by interfering with the Batf-Irf4 complex. Second, Bach2 suppresses IL-4-induced augmentation of the *Batf* and *Batf3* expressions through the inhibition of IL-4 production. These findings suggest that IL-4 and Batf family transcription factors form a positive feedback amplification loop to induce Th2-type immune response, and Bach2-Batf interactions block formation of this amplification loop.

1067

Multivariate modeling of human T helper differentiation

Grandclaudeon, M., Perrot, M., Trichot, C., Raieli, S., Soumelis, V.
Institut Curie, Paris, France

In the past decade, 2 main levels of complexity have emerged in the field of T helper (h) differentiation: the multiplicity of "input" signals interacting together to control Th differentiation, and the multiplicity of secreted "output" Th cytokines. In this work, we studied the mechanisms that control T helper cell differentiation taking into account these 2 levels of complexity. We built a statistical model on 600 single observation points of 42 T cell "inputs" measured on dendritic cells (DC) and 19 "outputs" measured on Th cells, in human heterologous DC - naïve CD4 T cells co-cultures. From this dataset we derived a sparse partial least square 2 model able to predict the outputs based on the input dataset with a low error score assessed computationally by 10 fold cross-validation. Such an unbiased modeling strategy was able to identify known regulators of T helper cell differentiation, for instance, IL-12 inducing Th1 or IL-23 inducing Th17. This strategy also led to the identification of several new input-output relationships that are currently investigated for functional validation. Multivariate modeling already enabled us to test novel hypotheses in silico and will bring new insights into the mechanistic understanding of Th cell differentiation. Ultimately, our strategy will help to identify optimal vaccine and immunotherapy strategies in order to induce specific Th cytokine profiles.

1068

Peripheral memory OX40⁺CXCR5⁺PD-1⁺ Bcl6⁺ Flu-specific T follicular helper cells elicited by vaccination correlated with anti hemagglutinin antibody responses in healthy individuals

Brezar, V.^{1,2,3}, Dahlke, C.⁴, Wiedemann, A.^{1,2,3}, Hani, L.^{1,2,3}, Godot, V.^{1,2,3}, Tcherakian, C.^{5,6}, Lelièvre, J.D.^{1,2,3,7}, Addo, M.⁴, Lévy, Y.^{1,2,3,7}, Seddiki, N.^{1,2,3}

¹INSERM U 955 Eq 16, Créteil, France, ²Université Paris-Est Créteil, Faculté de Médecine, Créteil, France, ³Vaccine Research Institute (VRI), Créteil, France, ⁴University Medical Center Hamburg - Eppendorf, Hamburg, Germany, ⁵Service de Pneumologie, Hôpital Foch, Suresnes, France, ⁶UFR des Sciences de la Santé Simone Veil, Université de Versailles Saint-Quentin-en-Yvelines, Montigny-Le-Bretonneux, France, ⁷AP-HP, Hôpital H. Mondor - A. Chenevier, Service d'Immunologie Clinique et Maladies Infectieuses, Créteil, France

OX40 signals promote memory CD4⁺ to express Tfh-cell molecules and become functional B-cell helpers.

We have previously shown that peripheral memory Ag-specific CD4⁺ can be detected by their dual expression of OX40 and CD25 after stimulation with cognate Ag. These cells include effector cells and CD39⁺Foxp3⁺ Ag-specific regulatory T cells (Tregs). Here we report that CD4⁺CD25⁺OX40⁺ also include Tfh cells.

By using blood samples from 15 healthy individuals prior and after (3, 7, 14 and 50 days) post-seasonal influenza (Flu) vaccination, our data reveal the presence of Flu-specific OX40⁺CD25⁺CXCR5⁺PD-1⁺ Tfh expressing the transcription factor Bcl6; these cells peaked at day 7 post-vaccination. CD4⁺OX40⁺ CD25⁺CXCR5⁺PD-1⁺Bcl6⁺ Flu-specific Tfh but not *ex vivo* bulk CD4⁺CXCR5⁺PD-1⁺CCR7^{low}Tfh, correlated significantly with Hemagglutinin inhibition (HI) titers ($r=0.4$, $p=0.04$ and $r=0.04$, $p=0.8$, respectively), suggesting that the former subset is associated with vaccine responses. Moreover, we show the presence of OX40⁺CD25⁺CXCR5⁺PD-1⁺-cells expressing both Bcl6 and Foxp3 transcription factors, which indicate their T-follicular regulatory (Tfr) phenotype. The proportion of Tfr varies between days 7, 14 and 50 post-vaccination with a decrease at day-7. More characterization of these cells is under way. Moreover, current co-culture experiments will demonstrate whether Flu-specific OX40⁺CD25⁺CXCR5⁺PD-1⁺Bcl6⁺ Tfh are able to help memory B-cells to produce immunoglobulins. Taken together our data demonstrate for the first time the presence of Flu-specific peripheral memory OX40⁺CD25⁺CXCR5⁺PD-1⁺ Tfh that express Bcl6. These cells peak at day-7 post-vaccination and contain low proportions of dual Bcl6⁺Foxp3⁺ Tfr as compared to day-0 or day-50 post-vaccination. Other vaccine models are currently tested in this context.

1069

Non-cognate stimulation of CD4 T cells contributes to the resolution of intracellular bacterial infections

Pham, O.H.¹, O'Donnell, H.², Li, L.¹, Mooney, J.³, Kerrinnes, T.³, Tsois, R.³, Davies, H.⁴, McSorley, S.J.¹

¹University of California, Center for Comparative Medicine, Davis, United States, ²Pastuer Institute, Paris, France, ³University of California, Department of Medical Microbiology and Immunology, Davis, United States, ⁴University of California, Division of Infectious Diseases, Irvine, United States

Salmonella-specific CD4 T cells acquire the ability to rapidly produce IFN- γ in response to non-cognate stimuli during active infection. Treatment of *Salmonella*-infected mice with PAMPs caused increased production of multiple cytokines, as detected by ELISA, Luminex array, or DNA microarray. Mixed bone marrow chimera experiments indicate that IL-18 and IL-33 are essential for optimal IFN- γ production while several other cytokines are dispensable. T cell-intrinsic MyD88 signaling was required for maximal IFN- γ production and optimal clearance of *Salmonella*, *Chlamydia*, and *Brucella* infection, demonstrating the importance of non-cognate stimulation in vivo. Interestingly, Th1 cells generated by immunization did not respond to non-cognate stimuli in vivo, despite surface expression of IL-18R.

Together, these data suggest that non-cognate activation of CD4 T cells occurs in a variety of infectious disease models and contributes to the resolution of infection. Current studies are exploring how non-cognate stimulation contributes to in vitro background responses and whether this information can be utilized to uncover target antigens of *Salmonella*-specific T cells in vitro.

1070

Long noncoding RNAs: new players in plasticity and new therapeutic opportunities in human immune system

Panzeri, I.¹, Lorenzo, M.¹, Gruarin, P.¹, Dardanelli, A.¹, Marchesi, S.¹, Ranzani, V.¹, Arrigoni, A.¹, Bonnal, R.J.¹, Rossetti, G.¹, Abrignani, S.², Pagani, M.²

¹Fondazione INGM, Milan, Italy, ²Fondazione INGM - University of Milan, Milan, Italy

The human genome encodes thousands of long non-coding-RNAs that are emerging as key molecules in modulating differentiation events and maintenance of cell identity in different cellular contexts. As little is known on long intergenic non-coding-RNAs (lincRNAs) in the human immune system, we investigated lincRNAs in thirteen T and B lymphocyte subsets by RNA-seq analysis and de-novo transcriptome reconstruction. Hundreds of new lincRNAs were identified and lincRNAs signatures were described. Expression of linc-MAF-4, a chromatin associated TH1 specific lincRNA, was found to anti-correlate with MAF, a TH2 specific transcription factor, and down-regulation of linc-MAF-4 associated to skewing of T cell differentiation toward TH2 phenotype. We demonstrated a long distance interaction between linc-MAF-4 and MAF regions, and found specific association of linc-MAF-4 with LSD1 and EZH2, suggesting linc-MAF-4 regulated MAF transcription by recruiting chromatin modifiers on MAF promoter. Our findings further support the key role of lincRNAs in regulating lymphocyte differentiation. As immune mediated diseases often arise from an imbalance among the different subsets in the immune system, we are now investigating the possibility to exploit lincRNAs as new therapeutic targets to modulate T cell plasticity and to restore immune system function in pathological settings. In this perspective, we are mainly focusing on lincRNAs specifically expressed in human CD4+ regulatory T cells, which have a prominent role in the etiology of such diseases. We are therefore applying several parallel approaches to investigate the functional role of signature Treg cell lincRNAs in both auto-immune and tumor contexts.

1071

GITRL enhances p38 MAPK/STAT3 signaling to promote Th17 cells differentiation in autoimmune arthritis

Tang, X.¹, Xu, H.², Wang, S.³

¹Affiliated People's Hospital of Jiangsu University, Zhenjiang, China,

²Jiangsu University, Zhenjiang, China, ³Affiliated People's Hospital of Jiangsu University, Clinical Laboratory, Zhenjiang, China

The glucocorticoid-induced TNFR family-related protein (GITR) and its ligand play a critical role in the pathogenesis of

autoimmune arthritis by enhancing the Th17 cell response, but their molecular mechanisms remain largely unclear. This study aims to define the role of p38 mitogen-activated protein kinases (MAPK) and signal transducer and activator of transcription 3 (STAT3) signaling in GITRL-induced Th17 cells in autoimmune arthritis.

We found that the p38 phosphorylation was enhanced by GITRL in activated CD4⁺T cells, and the p38 inhibitor restrained the GITRL-induced Th17 cell expansion in a dose-dependent manner. Moreover, there was decreased STAT3 activity on Tyr705 and Ser727 with the p38 inhibitor in vitro. Notably, the p38 inhibitor could prevent GITRL-treated arthritis progression and markedly decrease the Th17 cell percentages. The phosphorylation of the Tyr705 site was significantly lower in the GITRL-treated CIA mice treated with the p38 inhibitor. A significantly higher phosphorylation of p38 was detected in RA patients and had a positive relationship with the serum level of anti-cyclic citrullinated peptide (anti-CCP) antibody.

Taken together, these findings indicate that GITRL could promote Th17 cell differentiation by p38 MAPK and STAT3 signaling in autoimmune arthritis, which is considered a central pathogenesis of autoimmune disorders.

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TGFb induces fractalkine receptor CX3CR1 expression during T cell polarization

Dong, L., Hertel, B., Haller, H., von Vietinghoff, S.

Hannover Medical School, Nephrology and Hypertension, Hannover, Germany

Background: Fractalkine receptor CX3CR1 is the only known CX3 cytokine receptor with a single known ligand, fractalkine. Monocytes have the highest absolute expression levels and their response to fractalkine has received most interest over the last years. However, its expression on other leukocytes including T cells is well documented. We here investigated T cell CX3CR1 expression during T cell polarization.

Methods: Murine splenocytes were polarized towards TH1, TH17 and TREG phenotypes on plate-bound purified anti-CD28 and anti-CD3 with IL-12 and anti-IL-4 or IL-6, TGFb and IL-23 or TGFb and IL-2. Polarization was assessed by flow cytometry after intracellular staining for IFN γ , IL-17A and FoxP3, respectively. Cell proliferation was assessed by CFSE dilution. CX3CR1 expression was investigated by reporter gene expression in CX3CR1gfp/gfp and mRNA expression in wildtype cells. TGFb signaling was inhibited by SMAD3 inhibitor SB431542.

Results: TH17 polarization or stimulation with TGFb alone increased T cell CX3CR1 reporter gene expression. TGFb induced CX3CR1 mRNA expression in wildtype cells in a dose and time dependent manner. This was reverted by SMAD3 blockade. Less TH17 cells were obtained from CX3CR1^{-/-} than wildtype splenocytes by in vitro polarization.

Conclusion: T cell CX3CR1 can be induced by TGFb during both TH17 and TREG polarization.

1073**The RNA-binding protein HuR is necessary for IL-2 homeostasis and CD4⁺ T cell differentiation**

Atasoy, U., Glascock, J., Techasinta, P., Ellis, J., Ridenhour, S. University of Missouri, Surgery, Molecular Microbiology and Immunology, Columbia, United States

The RNA binding protein, HuR, (ELAVL1), controls gene expression in activated T cells by interacting with 3' UTR AU-rich elements (AREs) and altering transcript stability or translation. IL-2 and many Th2 cytokine mRNAs contain AREs. We hypothesized that HuR may play a critical role in early CD4⁺ T cell differentiation by regulating IL-2 signaling pathways. We conditionally ablated HuR in CD4⁺ T cells (distal Ick-Cre ROSA HuR^{fl/fl}) to test this hypothesis. HuR KO T cells developed and normally egressed from the thymus, but upon activation were unable to shut off IL-2 production and had defects in both Th2 differentiation and cytokine expression. HuR null T cells displayed proliferation defects, decreased p-stat5, and reductions in CD25 and blimp-1, which is involved in IL-2 transcriptional repression. In the absence of HuR, *Il2ra* (CD25) mRNA recruitment to heavy polysomes is reduced, resulting in decreased protein translation. This suggests that HuR regulates T cell activation and IL-2 homeostasis by controlling CD25 expression. Activated HuR KO cells still expressed IL-2 (97%) on day 5 with large increases in both *IL-2* mRNA (30 fold) and protein (7 fold) but scant amounts of Th2 cytokines. We investigated other T cell lineages in HuR null T cells. Under Treg, Th17, and Tfh differentiation conditions, HuR KO T cells had reduced levels of transcription factors Foxp3, RoRyt, and Bcl6, as compared to controls. Thus, we conclude that HuR plays an indispensable role in normal IL-2 homeostasis and CD4⁺ T cell differentiation via influencing CD25 mRNA translation.

1074**Vitamin D modulates the expression of HLA-DR and CD38 after in vitro activation of T lymphocytes**

Aguilar-Jimenez, W., Villegas-Ospina, S., Gonzalez, S.M., Rugeles, M.T.

Grupo Inmunovirologia, Facultad de Medicina, Universidad de Antioquia UdeA, Medellin, Colombia

The steroid hormone Vitamin D (VitD) acts as an immunomodulatory molecule through specific responsive sequences on genes of the immune response. VitD modulates several cell populations towards an anti-inflammatory state while promoting pathogens clearance. Contrary to that anti-inflammatory effects, previous evidence show that VitD induces the activation marker CD38 under certain stimuli; in addition, the HLA-DR gene carries VitD responsive sequences in its promoter. However, the direct effect of VitD on the expression of these activation markers remains unclear. We studied the effects of its active form, calcitriol, at three concentrations (10⁻¹¹M, 10⁻⁹M and 10⁻⁷M), on the expression of these activation markers in PHA/IL-2-activated CD4⁺ and CD8⁺ T lymphocytes. When analyzed individually, a significant dose-dependent increase of CD38⁺ cells was seen in both CD4⁺ and CD8⁺ T cells and a decrease of HLA-DR⁺ cells within CD8⁺ T cells.

The coexpression of these markers showed a dose-dependent increase of CD38⁺HLA-DR⁻ and a decrease of CD38⁺HLA-DR⁺ subpopulations in both CD4⁺ and CD8⁺ cells, as well as decreases in the CD4⁺CD38⁺HLA-DR⁻ and CD8⁺CD38⁺HLA-DR⁺ subpopulations. Furthermore, calcitriol treatment significantly increased in a dose-dependent manner the number of CD38 molecules per cell within the CD38⁺HLA-DR⁺ and CD38⁺HLA-DR⁻ subpopulations of CD4⁺ and CD8⁺ T cells, as measured by their mean fluorescence intensity. Our results support the role of calcitriol as a modulator of the immune activation, by inducing CD38 while reducing HLA-DR on T lymphocytes. These results highlight the potential of the VitD as an alternative therapeutic strategy in pathogenic immune disturbances.

1075**RORgt and RORa signature genes in human Th17 cells**

Castro, G.¹, Liu, X.², Ngo, K.², De Leon, A.², Xue, X.², Zhao, S.², Fourie, A.², Fung-Leung, W.-P.³

¹Janssen Research and Development, LCC, San Diego, United States, ²Janssen R&D, Immunology, San Diego, United States,

³Janssen Research and Development LCC, San Diego, United States

Retinoid-related orphan nuclear receptor *gt* (RORgt) is a transcription factor involved in the differentiation of IL-17 producing, pro-inflammatory T helper 17 (Th17) cells. These cells have been implicated in the pathology of autoimmune diseases such as psoriasis, and novel therapeutics targeting IL-23 or IL-17A have shown benefit in reducing disease in psoriasis patients. RORgt is therefore a novel oral therapeutic target of interest for psoriasis treatment. The goal of our study was to identify a RORgt specific gene signature that could be used to develop biomarkers for studying RORgt modulators in clinical studies as well as to explore additional disease indications where RORgt modulation could be beneficial. We studied the global gene expression profile of human CD4⁺ T cells activated under Th17-skewing conditions and examined the effects of treatment with either specific siRNAs to knock down RORgt expression or antagonistic RORgt compounds. In addition to RORgt, RORa has been suggested to participate in Th17 cell differentiation and we therefore also included RORa specific siRNAs in our studies. Gene expression of human T cell samples at the mRNA level was profiled using Affymetrix DNA microarrays, RNA-sequencing, and quantitative RT-PCR. Cytokine production from Th17 cells was measured with Luminex multiplex assays. Our studies identified RORgt and RORa specific signature genes. These results support an important role for RORgt in Th17 cell differentiation and cytokine production and provide critical information to inform biomarker strategies for studying RORgt modulators in patients.

1076**CD155 (PVR/Necl5) mediates a costimulatory signal in CD4⁺ T cells and regulates allergic inflammation**

Yamashita-Kanemaru, Y.¹, Bernhardt, G.², Shibuya, A.¹, Shibuya, K.¹

¹University of Tsukuba, Tsukuba, Japan, ²Hannover Medical School, Hannover, Germany

Although Th1 and Th2 cells are known to be involved in allergic inflammatory diseases, the molecular mechanisms underlying their differentiation are incompletely understood. Here, we identified CD155 as a costimulatory molecule on CD4⁺ T cells. Importantly, CD155-mediated signaling induced Th1 development in both humans and mice, as evidenced by production of interferon- γ and upregulation of *Tbx21* transcription; these effects were independent of interleukin-12 but dependent on NF κ B-induced autocrine interferon- γ that triggered positive-feedback via STAT1 activation. Mice genetically deficient in CD155 or treated with anti-CD155 antibody exhibited attenuated Th1-type contact hypersensitivity. Thus, CD155 plays an important regulatory role in helper T-cell differentiation and allergic diseases.

Friday, 26 August 2016

30 Minute Oral

08:30:00 - 10:15:00

Zika

Zika Virus Program at Butantan Institute

Kalil, J., Ho, P., Precioso, A. R.

Instituto Butantan

Zika vírus (ZIKV) has recently aroused global attention due to its rapid spread since its first detection in May 2015 in Brazil to 22 other countries and other territories in the Americas. ZIKV outbreak in Brazil, has also been associated with a significant increase in the number of notifications of newborn with microcephaly and neurological disorders, and has been declared a "Global Emergency" by the World Health Organization. Instituto Butantan in other to contribute to overcome the challenges posed of the explosive spread of ZIKV in Brazil has developed the following strategic programs: 1) Development of ZIKV vaccines and vaccination strategies: a) Pentavalent live attenuated vaccine (4 dengue serotypes+zika virus): Partnership between NIH and Butantan; b) Inactivated vaccine: Butantan R&D program; 2) Development of serum against ZIKV (horses immunization); 3) Development of ZIKV live attenuated vaccine: Butantan R&D program; 4) Diagnostic assays: Development of sensitive, specific, and rapid clinical diagnostic tests for ZIKV: Butantan R&D program;) Development of animal model to study ZIKV pathogenesis and evaluate candidate therapeutics and vaccines; 6) Development and manufacturing of neutralizing human anti-zika monoclonal antibodies. To aid Brazil in developing a vaccine to protect people from Zika virus infection, U.S. Department of Health and Human Services (HHS) is joining the World Health Organization (WHO) and international public health groups in providing funding and technical assistance to Butantan Institute to produce ZIKV inactivated vaccine. Biomedical Advanced Research and Development Authority (BARDA), part of the HHS Office of the Assistant Secretary for Preparedness and Response (ASPR), will work along with Butantan Institute providing not only financial support, through its existing cooperative agreement with the World Health Organization, but also technical assistance whenever it is required.

B Cells

Control of B-cell immunity and leukemia by the transcription factor Pax5

Busslinger, M., Smeenk, L., Malin S., Schindler, K.

Research Institute of Molecular Pathology (IMP), Vienna Biocenter, Vienna, Austria

B-cell commitment and development depend on the transcriptional factor Pax5. Conditional inactivation identified a critical role for Pax5 in maintaining B-cell identity and function in late B-lymphopoiesis. At the molecular level, Pax5 represses B-lineage-inappropriate genes and activates B-cell-specific genes. PAX5 is also a prominent tumor suppressor gene in B-cell acute lymphoblastic (B-ALL) leukemia, while chromosome translocations in a subset of leukemia patients generate novel PAX5 fusion proteins. By analyzing a PAX5-ETV6 mouse model, we could demonstrate that this fusion protein arrests early B-cell development, regulates genes implicated in pre-B cell receptor signaling and cooperates with loss of the Cdkn2a/b tumor suppressor in promoting B-ALL development. Hence, PAX5-ETV6 is a potent oncoprotein. By investigating the role of Pax5 in late B-lymphopoiesis, we could show that B-1, marginal zone, follicular (FO) and germinal center B-cells are rapidly lost upon Pax5 inactivation, whereas follicular (FO) B-cells tolerate the loss of Pax5 for some time. Pax5-deficient B-cells furthermore fail to generate plasma cells, indicating that all B-cell immune responses are lost in the absence of Pax5. Upon stimulation with anti-CD40 and IL-4, Pax5-deficient FO B-cells undergo normal proliferation, whereas they fail to proliferate in response to anti-IgM plus IL-4 stimulation. Unexpectedly, B-cell receptor signaling pathways from the cell surface to the nucleus are largely normal in the absence of Pax5. However, many immediate early genes are only minimally induced in Pax5-deficient

Instructive selection of germinal center B cells into the memory compartment

Kurosaki, T.

WPI Immunology Frontier Research Center, Osaka University

The germinal center (GC) B cells undergo one of two terminal fates; differentiation into memory B cells and plasma cells that provide protection against recurrent infection. The mechanism of how these memory B cells are selected and generated remains unclear. Here, we show that light zone GC B cells with lower affinity BCRs express relatively high Bach2 expression, being prone to enter memory B cell pool. Conversely, inhibition of Bach2 expression led to suppression of memory B cell generation. The levels of Bach2 in GC cells are inversely correlated with the strength of T cell help. Thus, we propose an instructive model in which weak T cell help maintains relatively high expression of Bach2, thereby predisposing GC cells to enter the memory pool.

Inflammation

Innate and adaptive IL-17-secreting T cells in autoimmunity

Mills, K.H.G., Edwards, S.C., McGinley, A.M., McGuinness, N.C., Raverdeau, M., Sutton, C.E.

School of Biochemistry and Immunology, Trinity Biomedical Sciences Institute Trinity College

Cells of the adaptive immune system, especially IL-17-secreting CD4+ T cells (Th17 cells), are considered to be the key pathogenic lymphocytes in mediating inflammatory pathology in autoimmune diseases, such as multiple sclerosis, psoriasis and rheumatoid arthritis. However, there is increasing evidence that innate-like lymphocytes, including $\gamma\delta$ T cells, NK cells, NKT cells and innate lymphoid cells (ILC) play a key role in the pathogenesis of many autoimmune diseases. We have shown that IL-1 β and IL-18 produced by TLR- and NLR-activated dendritic cells and macrophages synergize with IL-23 to activate IL-17A, IL-17F, IL-21 and GM-CSF production by $\gamma\delta$ T cells without TCR engagement. We found that $\gamma\delta$ T cells are key IL-17-producing T cells in the CNS of mice with experimental autoimmune encephalomyelitis (EAE). We have also discovered a novel subset of V γ 4+ T cells that play a protective role in infection and are pathogenic in EAE by providing an early source of IL-17A and related cytokines. IL-17A is pathogenic in EAE, not the effector stage, but in promoting the induction of Th17 cells. Furthermore, early IFN- γ from NK cells plays a role in the induction phase of EAE by promoting M1 macrophage activation and VLA-4 expression on CD4+ T cells, thus conferring encephalitogenic activity on T cells. Rather than acting as effector cytokines, it appears that the primary function of IL-17A and IFN- γ produced by innate cells early in inflammatory responses is to promote the induction of pathogenic Th17 and Th1 cells.

Immunodeficiency

Dysregulated PI3K signaling causes primary immunodeficiencies

Durandy, A., Deau, M.C., Heurtier, L., Kracker, S.

INSERM U 1163, Institut Imagine, Université Sorbonne, Paris Cité, Paris, France

We herein show that hyperactivation of the PI3K signaling in lymphocytes causes an autosomal dominant primary immunodeficiency, affecting mostly, but not only, antibody production. Gain-of-function mutations in PIK3CD, the gene encoding the catalytic subunit of PI3K δ , p110 δ , lead to activated PI3 kinase delta syndrome (APDS1) whereas heterozygous mutations in PIK3R1, the gene encoding the regulatory subunit p85a, although they do not impair either p110 δ -p85a interaction or p110 δ stability, are responsible for a similar phenotype (APDS2). Both syndromes have a very variable clinical and immunological expression, from subtle defect in immunoglobulin subclass through common variable immunodeficiency- or hyper-IgM-like syndromes to combined immunodeficiency. Respiratory infections leading in some cases to bronchiectasis are the main feature of the disease.

Massive lymphoproliferation in spleen or lymph nodes, autoimmunity and development of B cell lymphomas are frequent complications. Extra-immunological features (short stature, neurodevelopmental delay) can also be observed. The immune deficiency is mainly characterized by decreased levels of IgG and IgA, B lymphopenia (with decreased numbers of switched B cells and expansion of transitional B cells), defective naïve T CD4 and CD8 T cells' counts with expansion of effector memory CD8 T and senescent CD57 T cells. Hematopoietic stem cell transplantation, shown to be successful in a few cases, is a therapeutical option in severe forms. Immunosuppressive treatment using rapamycin, an inhibitor of the mTOR pathway, is largely used with efficacy in patients presenting with lymphoproliferation. Administration of specific p110 δ inhibitors could be a possible first choice treatment in a near future.

Intrinsic functional defects in human lymphocytes: insights into disease pathogenesis of primary immunodeficiencies

Tangye, S.

Garvan Institute of Medical Research

Intracellular signaling pathways induced by interactions between surface receptors and their cognate ligands facilitate the generation of successful immune responses. This underlies the efficient neutralization and clearance of pathogens, and the establishment of long-lived immunological memory. The corollary of this is that perturbations to signaling pathways compromise the differentiation and effector function of immune cells, underlying the clinical features of, and disease pathogenesis in, primary immunodeficiencies.

We have been studying lymphocyte development, differentiation and effector function in individuals with monogenic mutations resulting in the loss- or gain-of of function of the encoded protein. These genetic lesions render affected individuals susceptible to infection with specific pathogens, as well as impaired long-term humoral immunity. This has provided an opportunity to ascribe non-redundant and lineage-specific functions of defined genes, and to identify defects in lymphocyte biology caused by specific disease-causing mutations. Our studies reveal the value of examining "Experiments of Nature" to not only delineate signaling pathways required for human lymphocyte differentiation and effector function and to understand disease etiology in primary immunodeficiencies, but more importantly provide a framework to identify molecules and signaling pathways that could be targeted for intervention to improve immune function in affected individuals.

Oral Abstract Sessions

10:30:00 - 12:10:00

Innate Receptors & Inflammasomes 3

1699

HBV inhibits LPS-induced NLRP3 inflammasome activation and IL-1 β production

Yu, X.¹, Han, Q.¹, Zhang, J.¹, Tian, Z.², Zhang, C.¹

¹*Institute of Immunopharmacology & Immunotherapy, School of Pharmaceutical Sciences, Shandong University, Jinan, China,*

²*Institute of Immunology, School of Life Sciences, University of Science and Technology of China, Hefei, China*

Hepatitis B virus (HBV) constitutes a global public health problem involving chronic infection of the liver, which can cause liver disease and is linked with liver cancer. Although HBV has developed multifaceted strategies to evade the surveillance of immune responses, the precise mechanisms involved remain unclear. In the current study, by utilizing an HBV-persistent mouse model, it was demonstrated that Kupffer cells expressed a considerable amount of NLRP3 and IL-1 β after LPS stimulation; whereas, chronic HBV infection not only suppressed LPS-induced NLRP3 and pro-IL-1 β expression, but also repressed caspase-1 activation and IL-1 β maturation. Furthermore, by utilizing differentiated THP-1 cells, it was found that inhibitory activity is mediated by HBeAg, but not by HBsAg, and is involved in NF- κ B signal pathway inhibition. Additionally, the inhibitory effect of HBeAg was confirmed in patients with chronic hepatitis B and hepatocellular carcinoma by comparing the levels of IL-1 β and NLRP3-related proteins in para-carcinoma tissues from HBeAg-positive or negative patients. Moreover, chronic HBV infection increases the susceptibility of mice to *S. typhimurium* infection, possibly via inhibiting the activation of NLRP3 inflammasome and the production of IL-1 β . The present study provides a novel mechanism of HBV-mediated suppression of innate immune responses and identifies new therapeutic targets for chronic HBV infection and related diseases.

There is no conflict interest.

Macrophages 2

2787

Macrophage death following influenza vaccination promotes IL-1a-dependent dendritic cell activation and antigen presentation in draining lymph nodes

Chatziandreou, N.^{1,2}, Farsakoglu, Y.^{1,2}, D'Antuono, R.^{1,2}, Palomino-Segura, M.^{1,2}, Pizzagalli, D.U.^{1,3}, Sallusto, F.^{1,2}, Lukacs-Kornek, V.⁴, Turley, S.J.⁵, Lanzavecchia, A.^{1,2,6}, Carroll, M.C.⁷, Gonzalez, S.F.^{1,2}

¹*Institute for Research in Biomedicine, Bellinzona, Switzerland,*

²*Università della Svizzera Italiana, Lugano, Switzerland,* ³*Università della Svizzera Italiana (USI), Institute of Computational Science, Lugano, Switzerland,*

⁴*Saarland University, Department of Internal Medicine II, Homburg, Germany,* ⁵*Genentech, Cancer Immunology and Stromal Biology, San Francisco, United States,* ⁶*ETH Zürich,*

Institute for Microbiology, Zurich, Switzerland, ⁷*Harvard Medical School, Department of Microbiology and Immunology, Boston, United States*

The inflammatory response that follows vaccination has an important role in immunity. However, the mechanism by which it influences the adaptive response is not fully understood. In this work we characterised the role of lymph node (LN) subcapsular sinus macrophages (SSM) as inducers of the inflammatory mechanism that is triggered following vaccination with inactivated influenza virus. We found that, in response to vaccination, SSM undergo inflammasome-independent necrosis-like death that is reliant on MyD88 expression. Activated SSM produced IFN- β that induced the secretion of IL-1a by LN-resident dendritic cells (LNDC). We found that IL-1a activates DC and induces the expression of IL-6, which in turn promotes the expression of the secreted form of IL-1R antagonist that limits the duration and intensity of the effect of IL-1a. Importantly, macrophage depletion affected the capacity of LNDC to capture virus and limited their ability to present antigen to CD4+ T cells. We also observed that upon SSM death, germinal centre DC migrated to the subcapsular sinus, in a mechanism that involved the upregulation of the CCR2 receptor in this cells and the secretion of MCP-1.

1500

Bone marrow derived monocytes give rise to self-renewing and fully differentiated Kupffer cells

Scott, C.^{1,2}, Zheng, F.^{3,4}, De Baetselier, P.^{3,4}, Martens, L.^{1,5}, Saeys, Y.^{1,5}, De Prijck, S.^{1,2}, Lambrecht, B.^{1,5}, Beschin, A.^{3,4}, Guillems, M.^{1,2}

¹*VIB-UGent, Inflammation Research Center Zwijnaarde, Ghent, Belgium,* ²*Ghent University, Department of Biomedical Molecular Biology, Ghent, Belgium,* ³*VIB-VUB, Myeloid Cell Immunology, Brussels, Belgium,* ⁴*Vrije Universiteit Brussel, Cellular and Molecular Immunology Research Group, Brussels, Belgium,* ⁵*Ghent University, Department of Internal Medicine, Ghent, Belgium*

Tissue-resident macrophages are found in all organs of the body where they are adapted to perform numerous functions required for tissue homeostasis. Contrary to most immune cells, they develop prenatally from embryonic progenitors, have self-renewal mechanisms and exist, at least in most tissues, independently of adult hematopoietic stem cells (HSCs). These insights have undermined the concept of the mononuclear phagocyte system, where the circulating monocyte was seen as the central progenitor of all tissue macrophages. Importantly, despite their common origin, tissue-resident macrophages are highly specialised to the tissue of residence. However, macrophages that develop from adult HSCs after irradiation neither display the full gene signature of their embryonic counterparts. Here, using a new model of selective diphtheria toxin-mediated depletion of Clec4F-positive liver-resident Kupffer cells, we found that short-lived circulating adult monocytes engrafted in the adult liver following both full and partial depletion of embryonically-derived Kupffer cells. These monocytes differentiated into bona fide self-renewing Kupffer cells, which adopted the full transcriptional profile of the depleted tissue-resident Kupffer cells, as well as

their morphological and functional features. Highlighting the physiological relevance of our findings we found that, contrary to recent studies, bone marrow-derived monocytes engrafted in the liver and spleen during the period of normal organ growth in the first weeks of life. Thus in conclusion, we find that nurture by the tissue, rather than nature of the progenitor, is the principal factor controlling macrophage life-span and organ-specific functions. This concept paves the way for monocyte-based cellular therapy.

1604

Monocyte derived alternatively activated macrophages prevent experimental autoimmune encephalomyelitis

Ruiz-Rosado, J.D.D.^{1,2}, Terrazas, C.³, Robledo-Avila, F.¹, Rodriguez-Sosa, M.², Terrazas, L.I.², Guerau de Arellano, M.⁴, Partida-Sanchez, S.^{1,5}

¹Research Institute at Nationwide Children's Hospital, Center for Microbial Pathogenesis, Columbus, United States, ²Universidad Nacional Autonoma de Mexico, FES-Iztacala, Tlalnepantla, Mexico, ³Ohio State University, Department of Pathology, Columbus, United States, ⁴Ohio State University, School of Health and Rehabilitation Sciences, Columbus, United States, ⁵Ohio State University, Department of Pediatrics, Columbus, United States

Helminth parasites trigger polarized Th2-type immune responses in their hosts. These worms have been recently used to ameliorate ongoing inflammatory conditions. Although the anti-inflammatory mechanism is poorly understood, a role for alternatively activated macrophages (AAMφs) has been suggested. We previously demonstrated that the helminth *Taenia crassiceps* induces a population of AAMφs in mice. Here, we aimed to define the origin of these AAMφs and their potential role in modulating experimental autoimmune encephalomyelitis (EAE). We first established the phenotypic profile of AAMφs from C57BL/6 and CX3CR1^{EGFP/+} mice infected with *T. crassiceps*. To determine the origin of these AAMφs, we transferred Ly6C⁺ monocytes from a CX3CR1^{EGFP+/-}/CD45.1 mice into a C57BL/6/CD45.2 mice infected with *T. crassiceps*, and determined their differentiation into AAMφs. To assess the role AAMφs in modulating autoimmune diseases, we transferred AAMφs into EAE induced C57BL/6 mice and monitored clinical scores. Our results showed two populations of AAMφs after *T. crassiceps* infection, PDL1⁺ PDL2⁻ cells that did not express chemokine receptors CX3CR1 or CCR2 and PDL1⁺ PDL2⁺ cells that expressed both CCR2 and CX3CR1 receptors. These data suggested that whereas AAMφs PDL1⁺ PDL2⁻ may originate from resident macrophages, AAMφs PDL1⁺ PDL2⁺ subpopulation originated from blood-derived monocytes. This later hypothesis was confirmed by adoptively transferring of Ly6C⁺ monocytes, which gave rise only to AAMφs PDL1⁺ PDL2⁺ in *T. crassiceps* infected mice. Strikingly, adoptive transfer of AAMφs PDL1⁺ PDL2⁺ into EAE induced mice, completely prevented the clinical signs of disease, indicating these cells play a critical role in the regulation of autoimmune disorders.

1281

Tissue-resident macrophages as a front line of defense in *Plasmodium* infection

Gupta, P., Lai, S.M., Ruedl, C.

Nanyang Technological University (NTU), School of Biological Sciences, Singapore, Singapore

Tissue-resident macrophages exhibit diverse functions, ranging from the maintenance of tissue homeostasis, including clearance of senescent erythrocytes and cell debris, to modulation of inflammation and immunity. However, their contribution to the control of blood-stage malaria infection is unclear. Here, we show that tissue-resident macrophages regulate the outcome of *Plasmodium berghei* ANKA (PbA) infection by restraining the parasite burden and dramatically decreasing malarial-related pathology and morbidity. In the absence of tissue-resident macrophages, PbA infection results in significantly increased parasite sequestration leading to vascular occlusion, leakage and augmented tissue deposition of the malarial pigment hemozoin. This leads to widespread tissue damage, including in the lung, liver, heart, kidney and brain, culminating in multiple organ inflammation and failure. Thus, the capacity of tissue-resident macrophages to contain the parasite burden and its sequestration into different tissues is crucial to their role in mitigating malaria infection and pathogenesis.

This work was supported by National Medical Research Council grant NMRC.1307.2011 to C.R.

Disclosure of Interest: None Declared

981

A tissue-resident macrophage specific coinhibitory molecule promotes regulatory T cell differentiation and stability

Fu, W., Yuan, X., Dong, Y., Yang, B.-H., Sanogo, F.

University of California San Diego, La Jolla, United States

CD4⁺ Foxp3⁺ regulatory T (Treg) cells are key players in immune tolerance and tissue homeostasis. Their impact on tissue homeostasis has been further reinforced by recent studies showing that Tregs in tissue control non-immunological processes. However, how Treg cell differentiation and function are influenced by tissue-derived signals remain poorly understood. We here show that a specific subset of tissue-resident macrophages can promote the differentiation and stability of Tregs. This subset of tissue-resident macrophages distinctively express complement receptor of the immunoglobulin superfamily (CRIg). The phenotype of these macrophages are F4/80^{hi} CRIg⁺. We find that in addition to dampening effector T cell proliferation, CRIg promotes the conversion of induced Treg cells, by synergizing with TGFβ signaling. CRIg also stabilizes Foxp3 expression in induced Tregs. In vivo CRIg-Ig fusion protein treatment increases Treg proportion, particularly in tissue (islets) of NOD mice, a commonly used animal model for autoimmune diabetes. Together, these data reveal a novel mechanism of shaping Treg pool and function by tissue-resident macrophages. CRIg may represent a novel means to generate 'stable' iTregs, which remains unmet need in Treg-based therapy.

4095

Hypoxia reprograms human macrophages towards a proinflammatory direction*Raggi, F.¹, Pelassa, S.¹, Pierobon, D.², Giovarelli, M.², Cangelosi, D.¹, Varesio, L.¹, Bosco, M.C.¹**¹G.Gaslini Institute, Laboratory of Molecular Biology, Genova, Italy, ²Cerms, AUO Città della Scienza, Department of Molecular Biotechnology and Health Sciences, Torino, Italy*

Mononuclear phagocytes are recruited from the circulation as primary monocytes to sites of infection, inflammation, and tumor growth, where they undergo terminal differentiation into macrophages. Macrophages can be polarized into classically activated macrophages (M1) or alternatively activated macrophages (M2) which are characterized by a proinflammatory or an anti-inflammatory phenotype, respectively. M1 and M2 polarization is regulated by microenvironment factors. A common feature of pathologic situations is represented by hypoxia. Little is known about the impact of hypoxia on M1/M2 polarization. To address this issue, M1 (CD80⁺) and M2 (CD206⁺) macrophages were generated by culturing human monocytes with LPS or IL4 for 24h under normoxia (20%O₂) or hypoxia (1%O₂). We present data showing that hypoxia amplifies the proinflammatory state of M1 macrophages and reprograms M2 macrophages towards a proinflammatory direction by increasing the production of inflammatory and proangiogenic M1 type cytokines/chemokines. The hypoxic pathologic microenvironment can finely tune the expression of immunoregulatory signaling (IRS) receptors, whose deregulated expression may result in amplification of inflammation or establishment of immune escape situations. We demonstrate that hypoxia strongly upregulates the expression of one of such receptors, TREM-1, in both M1 and M2 macrophages. Engagement of TREM-1 by agonist Ab triggers further production of M1-type cytokines/chemokines in both macrophage populations. These results suggest the role of the hypoxic environment present at pathologic sites in skewing macrophages towards a M1-like proinflammatory phenotype by inducing TREM-1, highlighting the potential of targeting TREM-1 as a strategy to counteract inflammation in inflammatory disorders and in tumors.

3524

Elucidating the molecular mechanism of apoptotic cell disassembly for drug targeting*Atkin-Smith, G., Tixeira, R., Jiang, L., Paone, S., Caruso, S., Poon, I. La Trobe University, Biochemistry and Genetics, Melbourne, Australia*

Apoptosis (programmed cell death) occurs in essentially all tissues as part of development, homeostasis, and pathogenic processes including infection and cardiovascular disorders. Apoptotic cells often disassemble into smaller membrane-bound particles called apoptotic bodies (a process known as apoptotic cell disassembly). Under normal physiological conditions, the generation of apoptotic bodies during apoptosis can facilitate efficient removal of apoptotic cells by phagocytes, to prevent intracellular factors leaking from dying cells and

promoting unwanted inflammation. However, under certain pathological conditions, cellular materials such as cytokines, cell surface molecules and microRNA can be packaged into apoptotic bodies as a mechanism to regulate immunity and tissue repair. Since billions of cells undergo apoptosis daily, the importance of apoptotic cell disassembly and clearance for health and disease is fundamental, yet the mechanisms involved in the formation of apoptotic bodies are poorly understood. These mechanisms also represent attractive targets for the development of novel therapeutics. Here, we describe two new mechanisms of cell disassembly by apoptotic T lymphocytes and monocytes via the formation of a novel membrane protrusion called apoptopodia. Mechanistically, we have identified ROCK1 kinase and pannexin 1 membrane channels as key regulators of apoptotic cell disassembly. Additionally, we have identified a novel selection of drugs that can modulate apoptotic body formation. Understanding the mechanistic basis of this process will generate fundamental knowledge of the downstream consequence of cell death and has significant implication in health and diseases.

712

M3 switch macrophage phenotype: implication in the cancer immunotherapy*Malyshev, I., Lyamina, S., Kalish, S.**Moscow State University of Medicine and Dentistry, Pathophysiology, Moscow, Russian Federation*

The presentation will describe key phenomena of macrophage reprogramming, analyze signalling mechanisms of reprogramming and give an idea of the specific features of these mechanisms, which provides a fundamental characteristic of macrophages and immune response in general - plasticity. Macrophages play the key role in carcinogenesis. Depending on the microenvironment macrophages acquire or, in other words, reprogram themselves into either a pro-inflammatory, antitumor M1 phenotype or an anti-inflammatory, protumor M2 phenotype. Many tumors produce anti-inflammatory cytokines, which reprogram the antitumor M1 phenotype into the protumor M2 phenotype. We have hypothesized that the problem of protumor macrophage reprogramming could be solved using a special M3 switch phenotype. The M3 switch phenotype, in contrast to the M1 phenotype, should respond to anti-inflammatory cytokines by increasing production of proinflammatory cytokines to retain thereby its antitumor properties. The aim of the study was to verify this hypothesis.

Results:

1. Activation of M1 reprogramming pathways and inhibition of M2 reprogramming pathways programs the M3 phenotype of macrophages,
2. M3 macrophages exerted an effective anti-tumor effect *in vitro* and *in vivo* and
3. The antitumor effect of M3 macrophages was due to an anti-proliferative rather than a cytotoxic effect, and accompanied by pro-inflammatory reprogramming of tumor microenvironment.

Conclusion: Development of new biotechnologies for restriction of tumor growth using in vitro reprogrammed M3 switch macrophages is very promising.

737

Unprimed, M1 and M2 macrophages differentially interact with *Porphyromonas gingivalis*

Lam, R., O'Brien-Simpson, N., Holden, J., Lenzo, J., Fong, S.B., Reynolds, E.

The University of Melbourne, Oral Health CRC, Carlton, Australia

Tissue macrophages are likely to be one of the first immune cells to encounter to *Porphyromonas gingivalis*, a major pathogen associated with chronic periodontitis in humans. Naïve macrophages (M0) differentiate into a classical inflammatory (antibacterial) M1 macrophage upon IFN- γ priming and TLR ligation or anti-inflammatory (tissue-repair) M2 macrophage upon IL-4 priming. Previous work has shown that *P. gingivalis* infection induced M1 macrophages phenotype. The aims of this study were to determine the ability of M0, M1 and M2 macrophages (different subsets) to phagocytose *P. gingivalis* and investigate how this interaction affects both the bacterial cell and the macrophage. M1 and M2 macrophages were found to have enhanced phagocytic capacity compared to unprimed (M0) macrophages, however only the M0 and M1 macrophages were able to produce a respiratory burst in phagosome to clear the bacteria. *P. gingivalis* was found to persist in M2 macrophages for 24 hours, however not at the same levels of persistence as *S. Typhimurium*, a bacterium well known for survival inside macrophages. Phagocytosis of *P. gingivalis* also induced high levels of TNF- α and IL-27 in M1 macrophages, but not in M0 or M2 macrophages. Furthermore, infection of macrophages with *P. gingivalis* at high bacteria to cell ratios, while inducing an inflammatory response, was also found to be deleterious to macrophage longevity, with high levels of apoptotic death found in macrophages after infection. The activation of M1 macrophages observed in this study would contribute to the initiation and maintenance of a pro-inflammatory state during chronic periodontitis.

Tumour Immunology 6

764

Therapeutic potential anti-CD137 mAbs in multiple myeloma

Guillerey, C.^{1,2}, de Andrade, L.F.^{1,3}, Miles, K.¹, Vuckovic, S.⁴, Chesi, M.⁵, Bergsagel, L.P.⁵, Geoffrey R., H.⁴, Martinet, L.^{1,6}, Smyth, M.J.^{1,2}

¹QIMR Berghofer, Immunology in Cancer and Infection Lab, Herston, Australia, ²The University of Queensland, School of Medicine, Herston, Australia, ³Dana-Farber Cancer Institute, Department of Cancer Immunology and AIDS, Boston, United States, ⁴QIMR Berghofer, Bone Marrow Transplantation Lab, Herston, Australia, ⁵Mayo Clinic, Comprehensive Cancer Center, Scottsdale, United States, ⁶Cancer Research Center of Toulouse, INSERM UMR 1037, Toulouse, France

Multiple myeloma (MM) is a blood cancer that arises in the bone

marrow from malignant plasma cells. MM remains an incurable disease responsible for approximately 80 000 deaths per year worldwide. CD137 is a costimulatory molecule expressed on activated T cells and NK cells. Here, we report that early treatment with agonist monoclonal antibodies (mAbs) against CD137 (Rat IgG2a, clone 3H3) significantly reduces tumour burden and improves survival in the V κ^* myc mouse model of MM. We demonstrated that anti-CD137 mAb therapeutic activity requires the presence of both CD8 T cells and NK cells but is independent of Fc receptors. We found that anti-CD137 mAb treatment loses efficacy when given more than 3 weeks after V κ^* MYC cell injection. The depletion of CD4 cells or Foxp3⁺ Tregs restored the efficacy of late anti-CD137 mAb treatment, indicating that Treg-mediated suppression is a major hindrance. In line with these data, a single injection of anti-CD137 mAbs stimulated Treg proliferation and led to a rapid increase in spleen Treg numbers. Interestingly, we observed higher levels of TNF and IFN- γ and lower levels of IL-10 in the serum of responders compared with non-responders to late anti-CD137 mAb treatment. Finally, we established that, when combined with the chemotherapeutic bortezomib, anti-CD137 mAbs achieve high efficacy against late MM without adverse event. Collectively, our data emphasize the therapeutic benefit of anti-CD137 mAbs and suggest that low doses of anti-CD137 mAbs combined with chemotherapy could allow efficient anti-tumour responses at late MM stages with limited toxicity.

2365

Growth regulation of chronic lymphocytic leukemia by IL-10 produced constitutively by leukemic cells

Bondada, S.¹, McKenna, M.K.¹, Rangnekar, V.R.^{1,2}, Byrd, J.C.³, Muthusamy, N.⁴, Alhakeem, S.S.¹

¹University of Kentucky, Microbiology, Immunology, and Molecular Genetics and Markey Cancer Center, Lexington, United States, ²University of Kentucky, Radiation Medicine, Lexington, United States, ³Ohio State University, Internal Medicine and Comprehensive Cancer Center, Columbus, United States, ⁴Ohio State University, Internal Medicine and Comprehensive Cancer Center, Lexington, United States

B-cell chronic lymphocytic leukemia (B-CLL) is characterized by a slow and progressive accumulation of abnormal lymphocytes in blood, bone marrow and secondary lymphoid organs. There is evidence for immunosuppression in CLL. We studied the basis of this immunoregulation using the transgenic E μ -Tcl1 mouse, which spontaneously develop B-CLL due to a B-cell specific expression of the oncogene, Tcl1. We showed that E μ -Tcl1 CLL cells constitutively produce IL-10, an anti-inflammatory cytokine. Here we studied the role of IL-10 in CLL cell survival *in vitro* and the development of CLL *in vivo*. Neutralization of IL-10 using anti-IL-10 antibodies or blocking the IL-10 receptor (IL-10R) using anti-IL-10R antibodies did not affect the survival of CLL cells *in vitro*. Adoptively transferred E μ -Tcl1 cells grew at a slower rate in IL-10R KO mice vs. wild type (WT) mice. There was a significant reduction in CLL cell engraftment in the spleen, bone marrow, peritoneal cavity and liver of the IL-10R KO compared to WT mice. IL-10 appeared to be playing a role in the tumor microenvironment possibly by affecting anti-tumor immunity.

There was a reduction in the activation of CD8⁺ T cells as well as a significantly lower production of IFN- γ by CD4⁺ T cells purified from WT mice compared to the IL-10R KO mice injected with CLL. Currently, we are testing if neutralization of IL-10 with monoclonal anti-IL-10 antibody in WT mice will have similar enhancement of anti-leukemic effects. These studies support the concept that CLL cells suppress host anti-tumour immunity via IL-10 production.

3785

Tumour-infiltrating T cells leave tumours to provide effector functions in draining lymph nodes

Torcellan, T.^{1,2}, Hampton, H.R.^{1,2}, Bailey, J.¹, Tomura, M.³, Brink, R.^{1,2}, Chtanova, T.^{1,2}

¹Garvan Institute of Medical Research, Department of Immunology, Sydney, Australia, ²St. Vincent's Clinical School, UNSW Australia, Sydney, Australia, ³Osaka Ohtani University, Laboratory of Immunology, Faculty of Pharmacy, Osaka, Japan

Tumour-infiltrating T cells are a key component of the tumour microenvironment and their presence within the tumour can dramatically alter anti-tumour responses. While CD8⁺ T cells can have an anti-tumour role and destroy tumour cells, regulatory T cells (Treg cells) play a pro-tumour role and promote tumour growth by impairing anti-tumour immunity. Despite their impact on tumour growth, the fate of tumour-infiltrating cells is largely unknown. We hypothesise that tumour-infiltrating cells leave primary tumour deposits and play a key role in tumour immunity in draining lymph nodes.

Using Kaede photoconvertible mice we developed a method to label and track tumour-infiltrating cells and showed that these cells leave primary tumours and accumulate in draining lymph nodes. Flow cytometric analysis revealed that over 60% of tumour-egressing cells in draining lymph nodes are T cells, of which 80% are antigen experienced. While Treg cells represented a minor fraction of the migratory population, $\gamma\delta$ T cell subsets were enriched substantially. Compared to total lymph node T cells, tumour-egressing T cells showed enhanced effector functions: $\gamma\delta$ T cells showed increased IL17A⁺ and decreased IFN- γ ⁺ proportions, whereas CD8⁺ T cells had enriched IFN- γ ⁺ proportions and expressed increased cytotoxicity marker CD107a. Finally, tumour-conditioned T cell migration to draining lymph nodes involved G protein-coupled receptors but was not dependent on CCR7 or CD62L alone.

In summary, we demonstrate for the first time that tumour-infiltrating T cells leave tumour sites and migrate to the draining lymph node to perform effector functions that may modulate anti-tumour immune responses.

1202

D133p53 isoform - a novel adjuvant for tumour vaccination

Braithwaite, A., Slatter, T., Wilson, M., Baird, M.
University of Otago, Dunedin, New Zealand

p53 is widely known as a tumour suppressor that responds to DNA damage by causing cell cycle arrest and repair, senescence or apoptosis. However, recent data from our lab has shown

that p53 is required to generate a protective response against B16 melanoma using a prophylactic vaccine model¹. Bone marrow derived APCs from p53 null mice pulsed with tumour antigen (BMAPC/tumour Ag) fail to generate in vivo cytotoxicity and prevent tumour growth, due to a lack of IL-12 expression. These data suggest that p53 plays an important role in tumour suppression by regulating the production of key cytokines. The p53 gene also encodes a number of transcriptional variants (isoforms) due to alternate promoter usage and splicing. We created a transgenic mouse model of human D133p53 isoform, designated D122p53². These mice express constitutively high levels of pro-inflammatory cytokines and upregulate the NF κ B pathway in response to pathogens³. BMAPC from these mice have a pro-inflammatory phenotype so we investigated the protective effect of a D122p53 BMAPC/tumour Ag prophylactic vaccine against melanoma. Consistent with the constitutive elevation of cytokines, we found that this vaccine not only provoked an antigen-specific anti-tumour immune response, but also induced a marked degree of non-specific protection. These results suggest that augmentation of antigen presentation using pro-inflammatory D133p53 BMAPC might be a useful adjuvant in anti-cancer vaccines.

1. Slatter *et al* (2015) *Oncol Immunology* DOI: 10.1080/2162402X.2015.1112941.
2. Slatter *et al* (2011) *Blood* 117, 5166-5177.
3. Wei *et al* (2012) *Proc Natl Acad Sci USA* doi/10.1073/pnas.1205664109

3111

In vitro neutralization of IL-17A sensitizes dendritic cells to vinblastine in Langerhans cell histiocytosis

Olsson Åkefeldt, S.^{1,2}, Ismail, M.B.^{2,3}, Belot, A.^{2,4,5}, Lourda, M.¹, Valentin, H.⁶, Maise, C.^{2,7}, Salvatore, G.^{2,8}, Bissay, N.², Svensson, M.¹, Arico, M.⁹, Henter, J.-I.¹, Delprat, C.^{2,6,10,11}

¹Childhood Cancer Research Unit, Karolinska Institutet, Center for Infectious Medicine, Karolinska University Hospital Solna, Department of Women's and Children's Health, Stockholm, Sweden, ²CNRS, UMR 5239, Laboratoire de Biologie Moléculaire de la Cellule, Ecole Normale Supérieure de Lyon, Lyon, France, ³Laboratoire de Microbiologie Santé et Environnement, Université Libanaise, Tripoli, Lebanon, ⁴Hôpital Femme Mère Enfant, Bron, France, ⁵CIRI, Centre International de Recherche en Infectiologie - International Center for Infectiology Research, Lyon, France, ⁶Centre de Recherche en Cancérologie de Lyon, Inserm 1052 - CNRS 5286, Lyon, France, ⁷INRA-UCBL-ENVL, UMR 754, Rétrovirus et Pathologie Comparée, Lyon, France, ⁸Università degli Studi di Firenze, Firenze, Italy, ⁹Azienda Sanitaria Provinciale 7, Ragusa, Italy, ¹⁰Université de Lyon 1, Lyon, France, ¹¹Institut Universitaire de France, Paris, France

Langerhans cell histiocytosis (LCH) is an inflammatory neoplasm that damages tissues by accumulating death-resistant dendritic cells (DCs) and giant cells. Death resistance of lesional DCs is both controlled by BRAFV600E mutation and the microenvironment. Monocyte-derived DCs (Mo-DCs) are one possible source for pathological DCs. Because we have previously demonstrated that IL-17A induces survival and fusion of Mo-DCs, we investigated whether inhibiting IL-17A may represent a new

strategy to optimize chemotherapy in LCH.

Using ELISA, significant higher levels of plasma IL-17A were detected in young patients with LCH compared to healthy donors. Plasma from LCH patients with sequelae contained higher IL-17A levels than plasma from LCH patients without sequelae, suggesting that IL-17A may be pathogenic. In Mo-DCs from LCH patients, we observed abnormal and correlated intracellular expressions of IL-17A and BCL2A1, a pro-survival BCL2 family member. Both proteins correlated to DC survival, while MCL1, the myeloid pro-survival BCL2 member, did not. *In vivo*, BCL2A1 expression was highly detected by immunohistofluorescence in CD1a-positive pathogenic DCs and giant cells of LCH granulomas. Neutralizing IL-17A impaired both BCL2A1 expression and survival in Mo-DCs from LCH patients. Vinblastine is the main cytotoxic compound of the current front-line standard of care of LCH. Vinblastine had no effect on IL-17A and BCL2A1 expression, while it can decrease MCL1 and depolymerize microtubules in Mo-DCs, depending on doses. Vinblastine synergized with IL-17A neutralization to kill Mo-DCs from LCH patients. These results strongly suggest that targeting IL-17A may represent an interesting strategy to improve efficiency of chemotherapy in LCH.

1152

Cancer immunotherapy: translation from mice to human clinical trials

Berzofsky, J.A., Terabe, M., Wood, L.V.

National Cancer Institute, National Institutes of Health, Vaccine Branch, Bethesda, United States

We have developed cancer immunotherapeutic vaccines dependent on either CD8⁺ T cells or antibodies, and translated these from mice to human clinical trials with promising early results. We modified epitopes of the TARP prostate cancer antigen to increase binding to HLA-A2, and elicited human T cells that killed human tumor cells. These peptides were tested in stage D0 prostate cancer patients, whose tumors have been removed but have rising PSA indicative of recurrence. Minimal disease is an ideal setting for cancer vaccine efficacy, but usually not amenable to measuring tumor regression. In D0 prostate cancer, the rate of change of PSA is a validated predictor of clinical outcome. Of 29 patients immunized with TARP peptides assessable at 48 weeks, 74% had a reduced PSA slope compared with their pretreatment slope ($p = 0.0004$). Based on this unexpectedly strong effect, we have opened a randomized placebo-controlled phase II trial that will be discussed. We also developed an adenovirus vaccine expressing HER2 extracellular/ transmembrane domains, which cured large 2-cm established mammary tumors and lung metastases in mice, dependent on antibodies that inhibited HER2 phosphorylation, not FcR binding like trastuzumab. This vaccine is being tested in a clinical trial in patients with various advanced metastatic cancers expressing HER2. A high frequency of objective responses have been seen in the second and third dose levels. Thus, mouse immunotherapeutic cancer vaccines dependent on either T cells or antibodies can be successfully translated to human cancer patients with promising clinical results.

2656

Melanoma cell lysosome secretory burst neutralises the CTL-mediated cytotoxicity at the lytic synapse

Khazen, R.¹, Müller, S.¹, Gaudenzio, N.^{1,2}, Espinosa, E.¹, Puissegur, M.-P.¹, Valitutti, S.^{1,3}

¹Inserm, U 1043, Toulouse, France, ²Stanford University School of Medicine, Department of Pathology, Stanford, United States, ³Institut Universitaire du Cancer-Oncopole de Toulouse, Department of Pathology, Toulouse, France

Human melanoma cells express various tumor antigens that are recognized by CD8⁺ cytotoxic

T lymphocytes (CTL) and elicit tumor-specific responses *in vivo*. However, natural and therapeutically enhanced CTL responses in melanoma patients are of limited efficacy. The mechanisms underlying CTL effector phase failure when facing melanomas are still largely elusive.

Here we show that, upon conjugation with CTL, human melanoma cells undergo an active late endosome/lysosome trafficking, which is intensified at the lytic synapse and is paralleled by cathepsin-mediated perforin degradation and deficient granzyme B penetration. Abortion of SNAP-23-dependent lysosomal trafficking, pH perturbation or impairment of lysosomal proteolytic activity restores susceptibility to CTL attack.

Inside the arsenal of melanoma cell strategies to escape immune surveillance, we disclose a self-defense mechanism based on exacerbated lysosome secretion and perforin degradation at the lytic synapse. Interfering with this synaptic self-defense mechanism might be instrumental to potentiate CTL-mediated therapies in melanoma patients.

2965

Predicted neoantigens serve as attractive targets for tumor infiltrating lymphocytes in pediatric leukemias

Zamora, A.¹, Dash, P.¹, Abdelsamed, H.¹, Dallas, M.², Geiger, T.³, Carter, R.⁴, Youngblood, B.¹, Thomas, P.¹

¹St. Jude Children's Research Hospital, Immunology, Memphis, United States, ²St. Jude Children's Research Hospital, Bone Marrow Transplantation and Cellular Therapy, Memphis, United States, ³St. Jude Children's Research Hospital, Pathology, Memphis, United States, ⁴St. Jude Children's Research Hospital, Computational Biology and Bioinformatics, Memphis, United States

The use of tumor-reactive T lymphocytes as a means of adoptive immunotherapy has emerged as a promising therapeutic against various cancers. Currently, several approaches towards achieving tumor specificity are beginning to bear fruit, including the use of: tumor infiltrating lymphocytes, chimeric antigen receptor and T cell receptor engineered T cells. In this study, we aimed to determine the clonality, differentiation status, and phenotype of tumor infiltrating lymphocytes from pediatric patients with acute lymphoid leukemia (ALL) and acute myeloid leukemia (AML). Additionally, we generated a list of predicted neoantigens and determined the HLA-binding potential of each of these neoantigens using computational algorithms. We next determined the tumor-associated T cell receptor repertoire with single cell resolution, allowing us to determine the structure of

tumor-associated TCR repertoires and their propensity to target neoantigen. Finally, we tested which predicted neoantigens elicited the best T cell responses measured by TCR activation. Our results expand on studies aiming to better characterize the phenotypic and developmental changes that enhance the efficacy of adoptive T cell therapies and provide a platform for designing therapeutic interventions, including engineered T cells, against identified neoepitopes, which serve as attractive anti-tumor targets.

4050

PD-1 blockade induces quantitative and qualitative changes within a vast and common antigen-specific T cell repertoire in melanoma treated patients

Simon, S.^{1,2,3,4}, Vignard, V.^{1,2,3,5}, Florenceau, L.^{1,2,3,4,5}, Khammari, A.^{1,2,3,5}, Lang, F.^{1,2,3,4}, Labarrière, N.^{1,2,3,4}

¹University of Nantes, Nantes, France, ²INSERM - U892, Nantes, France, ³CNRS 6299, Nantes, France, ⁴LabEx IGO, Nantes, France,

⁵Nantes Hospital, Nantes, France

Therapeutic strategies using anti-PD-1 antibody reported unparalleled effectiveness for cancer immunotherapy. Understanding mechanisms involved in clinical benefit remains crucial to improve patients' management.

Despite its negative role in anti-tumor immunity, PD-1 first identifies reactive tumor-specific T-cells. We previously demonstrated that PD-1^{pos} melanoma specific T-cell clones exhibited a better functional avidity than their PD-1^{neg} counterpart. We further documented *in vitro* that PD-1 blockade during the selection and amplification process of melanoma specific T-cells from patients' PBMC, resulted in the proliferation of specific T-cells with a biased TCRVbeta repertoire exhibiting a better functional avidity (*Simon et al., Oncoimmunol, 2016*).

We assumed that this bias in antigen specific T-cell repertoire also occurs *in vivo* for patients treated with anti-PD-1 antibody. We compared Melan-A specific T-cell repertoire diversity from melanoma patients before and after anti-PD-1 therapy. We documented, for all patients tested, a bias in Melan-A-specific T-cell repertoire after treatment with the preferential amplification of clonotypes highly expressing PD-1. We characterized the most represented Vb subtypes (reactivity against melanoma cell lines and functional avidity) and analyzed results according to the expression of additional inhibitory receptors to allow discriminating between highly reactive and exhausted T-cells.

We are currently correlating experimental results with clinical outcomes of treated-patients to identify new biomarkers associated with anti-PD-1 therapy efficiency.

In addition to the emergence of neo-antigen specific T-cells previously documented upon anti-PD-1 therapy, our work describes qualitative and quantitative changes within an antigen-specific T-cell repertoire and offers new prospects for the monitoring of patients upon anti-PD-1 therapy.

Aging & Perinatal Immunology

2410

A thymus-sparing conditioning regime for tolerance induction in aged recipients

Anticevic, C.¹, Morison, J.¹, Hammett, M.¹, Lister, N.¹, Homann, J.¹, Barbuto, J.¹, Boyd, R.¹, Heng, T.^{1,2}

¹Monash University, Department of Anatomy and Developmental Biology, Melbourne, Australia, ²Monash Biomedicine Discovery Institute, Infection and Immunity Program, Melbourne, Australia

Allograft tolerance induction avoids the need for long-term dependence on immunosuppressive drugs. A promising approach being explored clinically is mixed chimerism via graft-matched haematopoietic stem cell (HSC) transplantation. Aged recipients, however, exhibit reduced engraftment of transplanted HSCs, which is further compounded by poor T cell recovery due to age-related thymic atrophy. We therefore investigated minimal conditioning regimes that would mitigate the damage to the thymus and allow for long-term allogeneic tolerance in aged mice. In contrast to low-dose irradiation or cyclophosphamide, busulfan caused only minimal damage to the thymus and facilitated highly efficient engraftment of donor HSCs for durable mixed chimerism. Chimeric mice were tolerant to full MHC-mismatched donor skin grafts and rejected third-party grafts, indicating immune tolerance and competence. Surprisingly, thymectomy did not prevent the establishment of mixed chimerism and tolerance in this model. Instead, tolerance induction was associated with an expansion in Gr-1⁺CD11b⁺ myeloid-derived suppressor cells and Foxp3⁺ regulatory T cells; depletion of the latter affected donor graft integrity. Thus, our conditioning protocol operates via peripheral regulation and donor-specific tolerance could be induced despite thymic atrophy and immunosenescence. Whilst busulfan-based conditioning was less effective in aged mice compared to young, we established a transplantation threshold for donor HSC dose and further determined that host HSC mobilisation could increase the sensitivity of aged HSCs to busulfan treatment. Our findings are especially relevant for the clinical application of chimerism-based transplantation tolerance in older patients, which the majority of transplant recipients.

2599

Metabolic and epigenetic mechanisms of age-related functional defects in naïve CD8 T cells

Quinn, K.M.^{1,2}, Russ, B.E.^{1,2}, Dagley, M.^{3,4}, Olshansky, M.^{1,2}, Saunders, E.^{3,4}, Sng, X.Y.X.^{1,2}, Harland, K.², Fox, A.², McConville, M.J.^{3,4}, Turner, S.J.^{1,2}, La Gruta, N.L.^{1,2}

¹Monash University, Biomedical Sciences, Clayton, Australia, ²University of Melbourne, Department of Microbiology and Immunology, Melbourne, Australia, ³University of Melbourne, Department of Biochemistry, Melbourne, Australia, ⁴Bio21, Melbourne, Australia

Immune dysfunction occurs with ageing, and particularly impacts upon primary CD8 T cell responses that are needed to control novel intracellular infections and cancers. Ageing causes a decrease in the number of naïve CD8 T cells, an increase in

the proportion of these cells exhibiting a semi-differentiated “virtual memory” phenotype and a decrease in their intrinsic functionality. We have recently defined the dynamics of epitope-specific CD8 T cell loss (Quinn et al., 2016, PNAS), and we now examine molecular mechanisms that underpin the phenotypic shift and loss of function. To this end, we assessed metabolic, epigenetic and transcriptional differences across true naïve (T_N) and virtual memory (T_{VM}) CD8 T cells from young and aged mice. We found that aged CD8 T_{VM} cells were the least functional, with aberrant cytokine production and minimal proliferative capacity. These cells had metabolic indicators of diminished functionality, including lower glucose uptake, high rates of reactive oxygen species production and minimal induction of glycolytic pathways upon activation. Upon adoptive transfer of cells, the phenotypic and functional profiles of young and aged T_N and T_{VM} were stable regardless of the age of their environment, suggestive of epigenetic programming. We performed ChIP-Seq of T_N and T_{VM} cells from young and aged mice, targeting H3K9 acetylation, to define this program and will correlate this with the transcriptional profile of the cells. These data highlight the molecular constraints, both metabolic and epigenetic, on aged CD8 T_{VM} cells and suggest cellular targets to improve function.

3217

Rules governing homeostasis, repertoire mobilization and protective immunity change with old age

Nikolich-Zugich, J.¹, Uhrlaub, J.¹, Pulko, V.¹, Rudd, B.², Davies, J.¹, Renkema, K.³, Li, G.¹, Padilla, J.¹, Nikolich-Zugich, D.¹, Contreras, N.¹, Smithey, M.¹

¹University of Arizona, Immunobiology, Tucson, United States,

²Cornell University College of Veterinary Medicine, Immunology, Ithaca, United States, ³University of Minnesota Medical School, Laboratory Science, Minneapolis, United States

Age-related defects in production, maintenance and function of lymphocytes are commonly described in quantitative terms, as a reduction in molecules, cells and/or processes that govern various facets of the immune function. Qualitative changes often also ensue from these quantitative changes, most frequently in the form of insufficient or dysregulated immune responses that lead to sickness and/or death. The opposite is also possible, so that qualitative alterations of cells and molecules and of their spatial arrangement can lead to quantitatively diminished responses.

Results presented here will provide evidence for the third possibility - that the very rules that govern maintenance and function of the adult immune system change with age, and therefore orchestrate different types of responses. Specifically, such results will illustrate age-related changes in

- (i) homeostatic rules that enforce selection of different types and classes of naïve T cells with aging;
- (ii) epigenetic changes that drive the process of selection and survival of some, but not other T cell clones during the primary immune response; and
- (iii) changes in polarization of the immune response against acute viral infection. Broader implications of such “rule breakers” associated with immune aging will be discussed.

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A tumor suppressor Menin controls CD8 T cell senescence by regulating energy metabolism

Suzuki, J.^{1,2}, Kuwahara, M.², Yasukawa, M.¹, Yamashita, M.²

¹Ehime University, Department of Hematology, Clinical Immunology and Infectious Diseases, Graduate School of Medicine, Toon, Japan, ²Ehime University, Department of Immunology, Graduate School of Medicine, Toon, Japan

Age-induced alterations of immune system are designated as immunosenescence, which is involved in increasing the susceptibility of elderly individuals to infectious diseases and certain cancers. We previously reported that deficiency in Menin, a tumor suppressor protein, induces premature senescence of CD4 T cells. In this study, we investigated the role of Menin in the regulation of CD8 T-cell senescence. The characteristic features of cellular senescence including the early cycle arrest, increased expression of the inhibitory receptors, augmented expressions of pro-inflammatory factors and increase in SA- β -Gal activity were rapidly induced in the *Menin*-deficient activated CD8 T cells. It has been recently reported that the change of the metabolic pathways and the induction of cellular senescence are intimately connected. We performed a metabolic profiling of 116 metabolites and found that *Menin*-deficient activated CD8 T cells had higher rate of anaerobic glycolysis and glutaminolysis than that in the WT activated CD8 T cells. Furthermore, premature CD8 T cell senescence in *Menin*-deficient CD8 T cells was partially inhibited by the treatment with rapamycin or inhibitors for glutamine metabolism. These results suggest that Menin controls CD8 T-cell senescence in part by regulating the energy metabolism.

1870

Significance of age-associated changes in miRNA expression regarding human CD4+ T cells

Frackowiak, J.E.¹, Wicik, Z.², Lisowska, K.A.¹, Jasiulewicz, A.¹, Mikosik, A.¹, Ruckemann-Dziurdzinska, K.³, Bartoszewski, R.⁴, Witkowski, J.M.¹

¹Medical University of Gdansk, Department of Pathophysiology, Gdansk, Poland, ²Mossakowski Medical Research Centre, Polish Academy of Sciences, Department of Human Epigenetics, Warsaw, Poland, ³Medical University of Gdansk, Department of Pathology and Experimental Rheumatology, Gdansk, Poland, ⁴Medical University of Gdansk, Department of Biology and Pharmaceutical Botany, Gdansk, Poland

Successful aging depends on a number of factors. A healthy immune system is of vital importance for this process. The phenotype of immune cells in the context of aging undergoes multiple changes which reflects in their function. CD4+ T helper cells modulate the function of other types of immune cells and are therefore especially important as they shape immune response. Activation and subsequent proliferation of CD4+ T cells accompanied by cytokine production is pivotal to their function. The impact of miRNAs on differentiation and function of human immune cells has recently been described in several publications. Furthermore, these small RNA species have been associated with human aging. Our research is among the few

which study miRNAs in an extreme age range.

Using Next Generation Sequencing (Illumina) we established the expression profile of mature miRNAs in FACS-sorted peripheral resting CD4+ cells obtained from Polish centenarians and three healthy control age groups: below 35 years of age, in their 60s and individuals aged 70-89.

Data analysis revealed significant age-associated changes in the expression profile of miRNAs. miRNA target prediction using *in silico* algorithms and ontological analysis revealed pathways significantly affected by age, including GNRHR, Wnt, CCKR and EGF receptor signaling. We also singled out several miRNAs, including miR-432-3p, for further study in qRT-PCR experiments. The studied miRNAs may influence homeostasis in resting cells and their sensitivity to T cell receptor activation, resulting in age-related changes in CD4+ T cell function.

1745

The visceral adipose tissue contributes to the generation of pro-inflammatory B cell subsets in aging mice and humans

Frasca, D., Diaz, A., Romero, M., Vazquez, T., Blomberg, B.

University of Miami Miller School of Medicine, Microbiology and Immunology, Miami, United States

Age-related decrease in B cell function is associated with chronic low-grade systemic and metabolic inflammation and with an increase in visceral adipose tissue (VAT). Adipocyte-derived chemokines are involved in the chemotaxis of immune cells which infiltrate the VAT and contribute to the inflammatory process. To identify contributors to the phenotypic and functional changes observed in aged B cells, we studied B cells in the VAT of obese mice and humans (epididymal and abdominal VAT, respectively). We found macrophages, B cells and T cells in VAT in a ratio of 1:3:5. As to B cells, we measured the major B cell subsets and found higher percentages of pro-inflammatory and less percentages of anti-inflammatory B cell subsets in VAT as compared to peripheral B cells. B cells isolated from VAT express higher levels of inflammatory immune activation markers (TNF- α /IL-6/IL-8), significantly higher NF- κ B activation and phospho-STAT3, and secrete higher amounts of Ig antibodies specific for fat antigens as compared to peripheral B cells. To evaluate the ability of adipocytes from the VAT to promote inflammation and induce pro-inflammatory B cell subsets, we co-cultured adipocytes from the VAT with peripheral B cells. Results show that co-culture for 72 hrs significantly changed the relative percentages of the B cell subsets, leading to a higher percentage of pro-inflammatory B cell subsets, similar to what we have observed in the fat. Moreover, we found that the adipocytes produce several pro-inflammatory chemokines. These results are the first to show a direct effect of adipocytes on pro-inflammatory B cells.

3499

Respiratory syncytial virus targets a fetally developed B cell population with regulatory functions which predicts disease severity

Zhivaki, D.¹, Lemoine, S.¹, Rameix-Welti, M.-A.², Lim, A.¹, Descamps, D.³, Eleouet, J.-F.³, Riffault, S.³, Zhang, X.⁴, Tissières, P.⁵, Lo-Man, R.¹

¹Institut Pasteur, Immunology, Paris, France, ²Hôpital Ambroise Paré, Boulogne-Billancourt, France, ³INRA, Jouy-en-Josas, France, ⁴Institut Pasteur of Shanghai, Chinese Academy of Sciences, Molecular Virology and Immunology, Shanghai, China, ⁵Hopital Bicetre, Kremlin Bicetre, France

Human Respiratory Syncytial Virus (RSV) is the commonest viral cause of severe lower respiratory tract infection in children under 5 years of age, leading to over 3 million hospitalizations related to severe bronchiolitis. Newborns and very young infants are highly susceptible to infections and poorly responsive to vaccines with a biased T cell response towards Th2 polarization in response to RSV infection. We analyzed neonatal blood for B cell population, including regulatory B cells (nBregs) and analyzed their susceptibility to infection and their impact on T cell immune responses *in vitro* and *in vivo* in infected human newborns.

We identified a fetally developed B cell population with regulatory properties and with a biased Ig repertoire. Using a recombinant RSV expressing the red fluorescent Cherry protein, we showed that RSV infects the neonatal B cells, with Breg cells subset in particular being the most permissive to RSV infection. Gene expression microarray analysis revealed an activation signature of Breg population upon RSV stimulation involving the BCR pathway. We also described an immunoregulatory mechanism by which upon RSV exposure, these nBregs can modulate neonatal CD4 Th1 development through the production of a high level of IL-10. These *in vitro* data were confirmed in patients suffering of acute bronchiolitis in which high nBregs frequency correlated with disease severity. Thus, we uncovered neonatal regulatory B cells as new target for RSV and how hijacking the newborn immune system contributes to disease severity and reduced viral clearance.

2011

What role do immune cells play in preterm newborn brain injury?

Zhang, X., Jabin, D., Zhou, K., Nazmi, A., Zhu, C., Mallard, C., Wang, X.

University of Gothenburg/Department of Neuroscience and Physiology, Gothenburg, Sweden

Infants with sepsis have increased incidence of brain abnormalities, and these are especially common in low birthweight newborn infants. Immune cells derived from the systemic circulation are a key feature of many diseases of the central nervous system. The aim of this study was to investigate whether or not immune cells contribute to the development of preterm brain injury using a mouse model of sepsis.

C57BL/6J wild-type (WT), T-cell receptor delta knockout (deltaTCRKO), and TCR beta knockout (betaTCRKO) mice were subcutaneously administered sterile saline or

lipopolysaccharide (LPS) (5 mg/kg) at postnatal day (PND) 2. Mouse brains were examined at PND12. Motor function and anxiety were evaluated at PND 26-30, using DigiGait analysis and an elevated plus maze.

LPS causes the loss of brain white-matter volume in WT and betaTCRKO mice, compared to that of the saline-treated group. However, deltaTCRKO mice did not have any white-matter tissue loss after LPS compared to the WT controls. The different treatments and genotypes had no effect on anxiety behavior in mice as determined with the elevated plus maze. DigiGait analysis showed that there were increases in stance, stride, and stride length and a decrease in stride frequency after LPS treatment in the WT and betaTCRKO mice, but no such effect was observed in the deltaTCRKO mice.

LPS causes sepsis-induced damage to the white matter in the mouse brain. Gamma/delta T cells, but not alpha/beta T cells, contribute to sepsis-induced brain injury and mild motor abnormalities in early life.

3753

Interleukin-22Ra1 signaling attenuates the severity of necrotizing enterocolitis and enhances expression of antimicrobial peptides

Good, M.¹, Parks, O.², Hodzic, Z.², Ma, C.¹, Kumar, P.², Horne, W.², Kolls, J.²

¹University of Pittsburgh School of Medicine/Children's Hospital of Pittsburgh, Pediatrics/Newborn Medicine, Pittsburgh, United States, ²University of Pittsburgh School of Medicine/Children's Hospital of Pittsburgh, Pediatrics, Pittsburgh, United States

Necrotizing enterocolitis (NEC) is the leading cause of death from gastrointestinal disease in premature infants and is characterized by an exaggerated inflammatory response, intestinal epithelial barrier disruption and impaired mucosal healing. Recently, interleukin (IL)-22 signaling has proven to play a critical role in attenuating intestinal inflammation, maintaining the gut barrier and promoting intestinal wound healing. Thus, we hypothesized that IL-22Ra1 signaling plays an important role in NEC pathogenesis. To test this hypothesis, we subjected wild type (WT) and IL-22Ra1 intestinal specific knock out mice (*villin-cre x IL22Ra1^{fl/fl}*, termed IL-22Ra1^{ΔIEC}) to an experimental model of NEC using gavage formula feeds and intermittent hypoxia. Mice were administered rIL-22 (4ug IP daily) and NEC severity was assessed by intestinal mucosal expression of pro-inflammatory markers, IL-6 and iNOS and histology. NEC resulted in disruption in the intestinal mucosal architecture and upregulation of pro-inflammatory cytokines and IL-22Ra1^{ΔIEC} mice demonstrated earlier NEC-related mortality than WT mice. Strikingly, administration of rIL-22 attenuated NEC severity and decreased pro-inflammatory markers. To determine the mechanisms involved, mouse primary intestinal stem cell enteroids were isolated and cultured with or without rIL-22. rIL-22 enhanced the growth of enteroids *ex vivo* and RNA sequencing on these enteroids demonstrated upregulated gene expression of antimicrobial peptides and defensins. Taken together, these data suggest IL-22 signaling may play an important role in attenuating the immune response in NEC pathogenesis and raise the possibility of novel therapeutic

approaches to this devastating disease by exploring the efficacy of IL-22 treatment for NEC and further defining the mechanisms involved.

Treg 2

2957

Chemokine production by regulatory T cells is required for therapeutic attenuation of autoimmunity and allograft rejection

Pesenacker, A.M.^{1,2}, Patterson, S.J.^{1,2}, Wang, A.Y.^{1,2}, Mojibian, M.³, Morishita, K.^{2,4}, Tan, R.^{2,5,6}, Kieffer, T.J.³, Verchere, C.B.^{1,2}, Panagiotopoulos, C.^{2,4}, Levings, M.K.^{1,2}

¹University of British Columbia, Surgery, Vancouver, Canada, ²Child & Family Research Institute, Vancouver, Canada, ³University of British Columbia, Cellular & Physiological Sciences, Vancouver, Canada, ⁴University of British Columbia, Pediatrics, Vancouver, Canada, ⁵University of British Columbia, Pathology & Laboratory Medicine, Vancouver, Canada, ⁶Sidra Medical and Research Center, Pathology, Doha, Qatar

Regulatory T cells (Tregs) control immune homeostasis by preventing inappropriate responses to self and non-harmful foreign antigens. Tregs use multiple mechanisms to control immune responses, all of which require Tregs to be near their targets of suppression, but how Treg-to-target proximity is controlled is unknown. We found that Tregs produce chemokines to attract CD4⁺ and CD8⁺ T cells close to their proximity *in vitro* and *in vivo*. Mouse and human lineage committed Tregs, as well as murine *in vitro* induced Tregs, produced CCL3 and CCL4 at message and protein level. Chemokine producing Tregs had an activated Treg phenotype with high expression of multiple proteins associated with suppressive function. Furthermore the human CCL3 and CCL4 promoter could be transactivated by FOXP3. *In vitro* and *in vivo* migration experiments indicated that CCR5 was the major receptor required by target cells. CCL3 and CCL4 deficient Tregs were impaired in their ability to prevent experimental autoimmune encephalomyelitis or islet allograft rejection, but suppressive in a standard *in vitro* assay. Moreover, Tregs from subjects with established type 1 diabetes were impaired in their ability to produce CCL3 and CCL4. These results demonstrate a previously unknown facet of Treg function and suggest that chemokine secretion by Tregs is a fundamental aspect of their *in vivo* function and therapeutic effect in autoimmunity and transplantation.

3582

Transcriptional landscape of human tissue lymphocytes unveils uniqueness of tumor-infiltrating T regulatory cells

De Simone, M.¹, Arrigoni, A.¹, Rossetti, G.¹, Gruarin, P.¹, Ranzani, V.¹, Politano, C.¹, Bonnal, R.J.¹, Panzeri, I.¹, Vaira, V.¹, Bosari, S.², Palleschi, A.³, Santambrogio, L.³, Bovo, G.⁴, Zucchini, N.⁴, Totis, M.⁴, Gianotti, L.⁵, Geginat, J.¹, Abrignani, S.⁶, Pagani, M.⁶

¹Fondazione INGM, Milan, Italy, ²Fondazione IRCCS Policlinico di Milano - University of Milan, Milan, Italy, ³Fondazione IRCCS Policlinico di Milano, Milan, Italy, ⁴San Gerardo Hospital, Monza, Italy, ⁵San Gerardo Hospital - Milano Bicocca University, Monza,

Italy, ⁶Fondazione INGM - University of Milan, Milan, Italy

T cell immunity is key to protective immune responses against tumors; however, antitumor immunity is often compromised by the recruitment in the tumor microenvironment of immunosuppressive regulatory cells. Discoveries regarding regulation of T cell responses have provided key principles for the development of promising immunomodulatory strategies in antitumor therapies, however characterization of the immune landscapes in different tumor contexts is still largely incomplete. Here we provide the first comprehensive RNA-sequencing analysis of CD4⁺ effector cells (Th1 and Th17) and regulatory T cells (Treg) infiltrating colorectal cancer (CRC) and non-small cell lung cancer (NSCLC). These data were integrated by transcriptomic data obtained from the same subsets isolated from adjacent healthy tissues to obtain the expression landscape of transcription factors, membrane receptors, cytokines and immune checkpoints of tumor infiltrating lymphocytes in NSCLC and CRC samples. We show that tumor infiltrating Treg cells display the more pronounced difference between normal and tumor tissues and are described by the expression of a subset of specific signature genes. Single-cell gene expression analysis show distinct expression frequencies between Treg cells infiltrating CRC and NSCLC suggesting an underlying heterogeneity in tumor infiltrating Treg cells that cannot be appreciated at the population level. These findings illustrate the importance of studying Treg cells contextually at tumor sites to better elucidate the underlying mechanisms of localized immune responses and to optimize the efficacy of current immune-based cancer therapy.

1629

Molecular control of Foxp3⁺ regulatory T cell survival and death during chronic viral infection

Gray, D.^{1,2}, Teh, C.^{1,2}, Preston, S.^{2,3}, Ebert, G.^{2,3}, Strasser, A.^{1,2}, Pellegrini, M.^{2,3}

¹The Walter and Eliza Hall Institute of Medical Research, Molecular Genetics of Cancer, Melbourne, Australia, ²The University of Melbourne, Department of Medical Biology, Melbourne, Australia, ³The Walter and Eliza Hall Institute, Infection and Immunity Division, Melbourne, Australia

The immunosuppressive function of Foxp3⁺ regulatory T (Treg) cells prevents immune-mediated damage from autoimmunity and chronic viral infections; however, the death and survival programs that control the Treg cell pool during these states are unknown. We examined how two major pathways of apoptosis (the intrinsic and extrinsic pathways) impinge upon Treg cell homeostasis and function under steady-state conditions and during chronic inflammatory conditions.

We generated mice with Treg cell-specific deletion of key mediators of the intrinsic (*Bax* Δ ^{FoxP3}*Bak*^{-/-}) or extrinsic (*Casp8* Δ ^{FoxP3}) apoptotic pathways. Unexpectedly, Treg cell-specific ablation of either resulted in a marked expansion of Treg cell number, mainly among FoxP3⁺ Treg cells with an effector phenotype (CD62L^{low}PD1^{hi}ICOS^{hi}), suggesting that dual control mechanisms maintain Treg cell homeostasis. Infection with the fast-replicating chronic lymphocytic choriomeningitis virus

(LCMV) Docile strain triggered massive expansion in the Treg cell population in wild-type and *Bax* Δ ^{FoxP3}*Bak*^{-/-} mice eight days post-infection. By contrast, infection of *Casp8* Δ ^{FoxP3} mice resulted in a striking loss of Treg cells and increased CD8⁺ and CD4⁺ cell activation, indicating a pro-survival role for this caspase in Treg cells only during chronic inflammation. We explore the role of necroptosis in this cell death switch in Treg cells, to better understand the divergent roles of the apoptotic pathways in the control of Treg cell function during chronic inflammation. Understanding how these pathways alter Treg cell homeostasis and the immune response is pivotal because drugs that target these pathways are currently in clinical trials for treatment of cancers and autoimmune disease.

3725

Low-dose interleukin-2 selectively modulates CD4⁺ T cell subsets in SLE

He, J.¹, Zhang, X.¹, Wei, Y.², Yu, D.³, Li, Z.¹

¹Peking Univ. People's Hospital, Beijing, China, ²Shandong Analysis and Test Center, Shandong Academy of Sciences, Shandong, China, ³Monash University, Melbourne, Australia

Background: Systemic lupus erythematosus (SLE) is a chronic autoimmune disease with multi-system involvement. We hypothesized that low-dose IL-2 treatment of SLE would result in selective expansion of regulatory CD4⁺ T (Treg) cells, while suppressing follicular helper T (Tfh) cells and IL-17-producing helper T (Th17) cells.

Methods: Forty patients with active SLE were recruited to a prospective open label study. The primary end points were the SLE Responder Index (SRI) and safety at week 12. Secondary end points were the effects of the therapy on Treg, Tfh and Th17 cells.

Results: An SRI response was seen in 34/38 patients (89.5%) at week 12. At week 12, resolution of clinical activity present at baseline was observed in multiple domains, including rash (20/24 patients), alopecia (13/14), arthritis (10/11), fever (3/3), leukopenia (18/19) and thrombocytopenia (4/4). No severe adverse events were observed. Significant reductions of anti-dsDNA titres ($p < 0.001$) and proteinuria ($p = 0.005$), and increased levels of C3 ($p < 0.001$) and C4 ($p < 0.001$), were observed at week 12. Immunological analysis revealed that low-dose rhIL-2 administration was associated with selective expansion of Treg cells and conversely with reductions of Tfh and Th17 cells ($p < 0.001$).

Conclusion: Low-dose IL-2 was well tolerated in active SLE, and was associated with improvements in disease activity accompanied by selective modulation of CD4⁺ T cell subsets.

1495

Regulatory T cells modulate dendritic cell maturation and function at a transcriptional level*Mavin, E., Nicholson, L., Dickinson, A., Wang, X.-N.**Institute of Cellular Medicine, Haematological Sciences, Newcastle, United Kingdom*

Regulatory T cells (Treg) are able to prevent effective T cell priming through modulation of dendritic cell function, arresting them in a semi-mature status. However, little is known about the molecular mechanisms underlying this effect. Using microarrays, we examined the gene expression profile of LPS-stimulated monocyte-derived dendritic cells either with Treg treatment (Treg-moDC), or without (mat-moDC) and compared them to unstimulated immature moDC (imm-moDC) controls. Hierarchical clustering showed a significant difference between all three DC populations at a global gene expression level. Treg-moDC had reduced levels of NFκB signalling genes and decreased phosphorylation of the Ser536 residue of the RelA subunit, suggesting attenuation of the NFκB signalling pathway which is essential for DC maturation. We also observed Treg-DC had intermediate levels of the NFκB-regulated genes CD80, CD86, CD83 and CD38, which was then confirmed by qPCR and flow cytometry. Treg-moDC had a distinct morphology, resembling that of imm-moDC with an impaired ability to uptake antigen. Functional analysis demonstrated that Treg-moDC skewed naïve T cell polarisation from a Th1 to a Treg-type response, possibly due to their increased surface expression of TGFβ and decreased secretion of IL-12p70 and IL-6. Furthermore Treg-moDC had an impaired ability to induce allo-reactive CD8+ T cell activation and proliferation. Using a unique human skin explant model of graft-versus-host reactions we demonstrated the clinical relevance of Treg modulation of DC function. This study provides the first evidence that Treg modulation of DC function occurs, at least in part, through attenuation of NFκB signalling.

1540

Suppression by human FOXP3⁺ Treg cells requires FOXP3/TIP60 interaction*Bin Dhuban, K.¹, d'Hennezel, E.¹, Xiao, Y.², Nagai, Y.², Ben-Shoshan, M.³, Mazer, B.³, Ochs, H.⁴, Nicholas, B.⁵, Park, M.⁵, Torgerson, T.⁴, Greene, M.², Piccirillo, C.^{1,6}*

¹McGill University, Microbiology and Immunology, Montreal, Canada, ²University of Pennsylvania, Pathology and Laboratory Medicine, Philadelphia, United States, ³McGill University, Pediatrics, Montreal, Canada, ⁴University of Washington, Pediatrics, Seattle, United States, ⁵McGill University, Medicine, Montreal, Canada, ⁶McGill University, Program in Infectious Diseases and Immunology in Global Health, Research Institute of the McGill University Health Centre, Montreal, Canada

CD4⁺FOXP3⁺ regulatory T cells are critical mediators of immune tolerance, and their deficiency due to mutations in the transcription factor *FOXP3* results in severe autoimmunity in IPEX patients. However, different *FOXP3* mutations result in a wide range of disease severity, highlighting the complexity of *FOXP3* interactions. Here, we used IPEX-derived *FOXP3*

mutations to identify specific molecular interactions through which *FOXP3* orchestrates the transcriptional and cellular profile of human Treg cells. Using a sensitive single-cell cloning strategy of primary cells derived from IPEX patients, as well as lentiviral overexpression of WT and mutated *FOXP3* in healthy naïve CD4⁺CD25⁻ cells, we analyzed the molecular and cellular defects caused by the most common IPEX mutation, pAla384Thr. We show that this mutation selectively impairs the suppressive capacity of Treg cells while sparing *FOXP3*'s ability to mediate repression of inflammatory cytokines such as IFN-γ, IL-2 and IL-17, and to induce Treg-associated surface markers such as CD25 and CTLA-4. Microarray and biochemical analyses revealed that *FOXP3*^{A384T} has a diminished ability to interact with TIP60, a histone acetyltransferase previously shown to bind to, and acetylate, *FOXP3* leading to an increased stability of the *FOXP3* protein. We were able to correct this specific defect using allosteric modifiers that enhance *FOXP3*/TIP60 interaction leading to a significant rescue in the suppressive capacity of *FOXP3*^{A384T}-transduced cells. These findings highlight the critical role of TIP60 in Treg biology and provide potential targets for therapeutic manipulation of Treg activity.

485

USP21 prevents the generation of T-helper-1-like exTreg cells*Li, B., Li, Y.**Instut Pasteur of Shanghai, Unit of Molecular Immunology, Shanghai, China*

FOXP3⁺ Regulatory T (Treg) cells harbor immune suppressive capacity and are crucial for the maintenance of immune homeostasis and control of dominant immune tolerance¹⁻³. Treg cells are considered to be heterogenic, where compromised *FOXP3* expression results in the generation of exTreg cells and acquisition of alternative effector or regulatory/effector hybrid fates⁴⁻¹⁰. However, direct tracing of *FOXP3* protein and its stability in exTreg cells in vivo remains unclear. Here we report that the E3 deubiquitinase USP21 prevents the depletion of *FOXP3* protein and restricts T-helper-1-like exTreg cell generation. Mice lacking USP21 in Treg cells display immune disorders characterized by spontaneous T cell activation and excessive T helper type 1 (Th1) skewing. USP21 stabilizes *FOXP3* protein by mediating its deubiquitination and therefore helps to maintain the expression of Treg signature genes. Moreover, at inflamed tissue loci, Treg-specific deletion of USP21 leads to potent induction of Th1-like exTreg cells. Our results demonstrate how USP21 prevents *FOXP3* protein depletion and controls Treg lineage stability under inflammation. These findings support the notion that instability or loss of *FOXP3* in Treg cells potentially induces exTreg cell generation in vivo.

1776

Divergent target specificity of human Foxp3+ regulatory T cells and Th2 effector cells poses a risk for allergy development

Bacher, P.¹, Heinrich, F.¹, Stervbo, U.², Nienen, M.², Vahldieck, M.³, Iwert, C.⁴, Kollet, J.³, Babel, N.², Sawitzki, B.⁴, Schwarz, C.⁴, Kniemeyer, O.⁵, Brakhage, A.⁵, Scheffold, A.¹

¹Charite Universitätsmedizin Berlin and DRFZ, Berlin, Germany,

²Ruhr University Bochum, Bochum, Germany, ³Miltenyi Biotec GmbH, Bergisch Gladbach, Germany, ⁴Charite Universitätsmedizin Berlin, Berlin, Germany, ⁵Hans Knoell Institute (HKI) Jena and Friedrich Schiller University Jena, Jena, Germany

Foxp3+ regulatory T cells (Treg) are thought to play a central role in maintaining tolerance against harmless antigens at mucosal sites. However, which antigens are actually recognized by Treg in particular in humans is so far unknown. We established a highly sensitive enrichment system based on antigen-induced CD154 (CD40L) versus CD137 (4-1BB) expression, to detect human antigen-specific conventional T cells (Tcon) and Treg directly *ex vivo* in peripheral blood. We show that the ubiquitous airborne fungal antigen *A. fumigatus* activates a dominant population of CD4+CD25+CD127-Foxp3+Helios+ Treg in peripheral blood of healthy donors, with demethylated TSDR and potent *in vitro* suppressive activity. In cystic fibrosis patients allergic to *A. fumigatus*, this Treg dominance is abrogated, due to a massive expansion of conventional Th2-type memory cells, despite the presence of functional Treg. Using a panel of 15 different *A. fumigatus* proteins we show, that in allergic donors, antigen-specific Treg surprisingly have non-overlapping target-specificities with Th2 cells, suggesting divergent target specificity of human Treg and Th2 effector cells as a potential risk for allergy development.

Our data identify innocuous airborne antigens as a target of human Treg and provide direct evidence that antigen-specific Treg are potent suppressors of allergy development. Furthermore we provide an explanation how allergen-specific Th2 responses can escape Treg control due to selective targeting of *A. fumigatus* proteins not protected by a specific Treg response.

4204

Human regulatory T cells control TCR signaling and susceptibility to suppression in CD4+ T cells

Chelappa, S.^{1,2}, Lieske, N.V.^{1,2}, Hagness, M.^{1,2,3}, Line, P.D.³, Tasken, K.^{1,2}, Aandahl, E.M.^{1,2,3}

¹University of Oslo and Oslo University Hospital-Rikshospitalet, Centre for Molecular Medicine Norway, Nordic EMBL Partnership, Oslo, Norway, ²University of Oslo, Biotechnology Centre, KG Jebsen Centre for Inflammation Research and KG Jebsen Centre for Cancer Immunotherapy, Oslo, Norway, ³Oslo University Hospital-Rikshospitalet, Section for Transplantation Surgery, Oslo, Norway

Human CD4+CD25^{hi}FOXP3+ regulatory T cells maintain immunologic tolerance and prevent autoimmune and inflammatory immune responses. Regulatory T cells undergo a similar activation cycle as conventional CD4+ T cells upon antigen stimulation. Here, we demonstrate that T cell receptors

and costimulation are required to activate the regulatory T cell suppressive function. Regulatory T cells suppressed the T cell receptor signaling in effector T cells in a time-dependent manner that corresponded with inhibition of cytokine production and proliferation. Modulation of the activation level and thereby the suppressive capacity of regulatory T cells imposed distinct T cell receptor signaling signatures and hyporesponsiveness in suppressed and proliferating effector T cells and established a threshold for effector T cell proliferation. The immune suppression of effector T cells was completely reversible upon removal of regulatory T cells. However, the strength of prior immune suppression by regulatory T cells and corresponding T cell receptor signaling in effector T cells determined the susceptibility to suppression upon later reexposure to regulatory T cells. These findings demonstrate how the strength of the regulatory T cell suppressive function determines intracellular signaling, immune responsiveness, and the later susceptibility of effector T cells to immune suppression and contribute to unveiling the complex interactions between regulatory T cells and effector T cells.

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1474

Inflammatory monocytes contributes to the restriction of Legionella in a mouse model of Legionnaires' disease

Massis, L.M.^{1,2}, Lima-Junior, D.S.², Pereira, M.F.², Manin, G.¹, Zamboni, D.S.²

¹University of Sao Paulo, Biochemistry and Immunology, Ribeirao Preto, Brazil, ²University of Sao Paulo, Cell Biology, Ribeirao Preto, Brazil

Legionella species, in particular *Legionella pneumophila* and *Legionella longbeachae*, are mainly found in environmental sources like water reservoir and soil. *Legionella* can be an opportunistic pathogen of humans causing a severe pneumonia. The response to *Legionella* infection leads to a recruitment of immune cells to the lung that contributes to control of infection. CCR2 receptor and monocyte chemoattractant protein-1 (MCP-1) are critical for monocyte recruitment to the lungs in response to bacterial infection. In this context the aim of this study was to evaluate the importance of inflammatory monocytes in Legionnaires' disease. To address this question we infected *ccr2*^{-/-} mice and C57BL/6 mice with *L. pneumophila* flagellin deficient strain or with *L. longbeachae* strain. Our results demonstrated that MCP-1, IFN- γ and TNF are increased in the infection with both *Legionella* strains. Furthermore, *ccr2*^{-/-} mice are more susceptible to *L. pneumophila* and *L. longbeachae* infection compared to C57BL/6 mice. The infection also triggers a CCR2-dependent inflammatory monocyte migration to the lungs. To confirm the importance of inflammatory monocytes we adoptively transferred inflammatory monocytes to *ccr2*^{-/-} mice that rescue the restriction of the infection found in C57BL/6 mice. These results indicated that inflammatory monocytes participate in the control of *Legionella* infection. Therefore, CCR2-dependent inflammatory monocytes are important cells involved in inflammation and in the control

of the two most prevalent *Legionella* strains that cause pneumonia in humans.

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3357

***Mycobacterium tuberculosis* expresses noncoding RNAs to regulate mycobacterial and eukaryotic gene expression during infections**

Srivastava, S.¹, Raj, P.², Dozmorov, I.², Wakeland, E.², Gumbo, T.¹, van Oers, N.²

¹Baylor Scott & White Health, Baylor Institute for Immunology Research, Dallas, United States, ²UT Southwestern Medical Center, Immunology, Dallas, United States

Mycobacterium tuberculosis (*Mtb*) presently infects 1/3 of the World's population, causing active and latent pulmonary tuberculosis (TB). The pathogenic mechanisms used by *Mtb* remain poorly defined. We used a small RNA sequencing strategy on RNA extracted from *Mtb*-infected THP-1 macrophages to characterize the changes in eukaryotic microRNAs (miRs) and identify any mycobacterially-encoded small RNAs. A large number of mammalian microRNAs were differentially regulated during the infection course. Target prediction programs indicated that the mRNAs regulated by these miRs were involved in immune processes, metabolic pathways, cell communication, and developmental events. In addition, 35 *Mtb*-encoded small RNAs (18-30 nucleotides, *Mtb*-mRs) were uncovered. Several of these were also detected in lung tissue biopsies from TB infected monkeys. The 100-150 nucleotides surrounding the 35 *Mtb*-encoded small RNAs had extensive secondary RNA folding capabilities, including hairpins and antisense stretches more characteristic of eukaryotic pre-microRNAs. Two such small RNAs, *Mtb*-mR-1 and *Mtb*-R-6 were transcribed in *Mycobacterium avium* complex and *Mtb* strains in a hairpin loop and antisense sequence-dependent manner. These mRs were not expressed in *Mycobacterium smegmatis*, revealing a restricted expression to pathogenic strains. *Mtb*-encoded mR-1 and mR-6 modulated both prokaryotic and eukaryotic gene expression. Taken together, our findings reveal novel pathogenic processes deployed by *Mtb* to modulate infectivity.

703

Inflammatory properties and adjuvant potential of synthetic glycolipids homologous to mycolate esters of the cell wall of *Mycobacterium tuberculosis*

Tima, G.¹, Lehebel, P.¹, Denis, O.¹, Baird, M.², Huygen, K.¹, Romano, M.¹

¹Scientific Institute of Public Health, Immunology, Brussels, Belgium, ²Bangor University, School of Chemistry, Bangor, United Kingdom

The cell wall of mycobacteria is characterized by glycolipids composed of different classes of mycolic acids (MAs) (alpha-, keto- and methoxy-) and sugars (trehalose, glucose, and arabinose). Several studies using mutant strains have shown that the structure of MAs bound to trehalose influence the inflammatory potential of this glycolipid. Nevertheless, these studies were performed with mixtures of isolated glycolipids.

Here we were interested in dissecting the contribution of single class of mycolate esters in the inflammatory potential of *Mtb* cell wall using synthetic analogues. The synthetic compounds tested here vary in terms of their carbohydrates moiety (trehalose, glucose or arabinose) and in terms of the number, nature and class of mycolic acids bound to it. Our results showed that synthetic trehalose dimycolate (TDM), trehalose-, glucose- and arabinose-monomycolates (TMMs, GMMs and AraMMs) activate BMDCs in term of proinflammatory cytokines (IL-6 and TNF- α), ROS productions, upregulation of costimulatory molecules and activation of NLRP3 inflammasome by mechanism dependent on Mincle pathway. These results indicate that Mincle receptor can recognize pentose esters and contradict the hypothesis that the production of GMM is an escape mechanism used by pathogenic mycobacteria to avoid the recognition by the innate immune system. Finally, our data showed that TMM and GMM as well as TDM can induce Th1 and Th17 responses in mice in an OVA immunization model. Taken together, these data provide an interesting lead in the development of new adjuvant for subunit vaccine.

4002

Leptin and IL-17 mediated responses in vaccination against *Helicobacter pylori*

Becher, D.^{1,2}, Ortega, J.³, Raghavan, S.⁴, Sjökvist Ottsjö, L.⁴, Wijburg, O.¹, Strugnell, R.A.¹, Walduck, A.⁵

¹University of Melbourne, Microbiology and Immunology, Melbourne, Australia, ²Glaxo Smith Kline, Melbourne, Australia, ³MIT University, Melbourne, Australia, ⁴University of Gothenburg, Sahlgrenska Institute, Microbiology and Immunology, Gothenburg, Sweden, ⁵MIT University, School of Sciences, Health Innovations Research Institute, Melbourne, Australia

Infection with *Helicobacter pylori* is the greatest risk factor for gastric cancer. Candidate *H. pylori* vaccines tested in volunteers to date have generally been poorly effective and adverse effects are reported. Indeed there is evidence that "post immunisation gastritis" occurs because the tightly controlled inflammatory response in the gastric mucosa is unbalanced.

Vaccination is effective in animal models, and CD4⁺ T cells, and the adipokine leptin (Ob) and interleukin 17 (IL-17) have been show to be important for vaccine-induced protection against *H. pylori*. Leptin is pro-inflammatory, and impacts CD4⁺ T cell, regulatory CD4⁺ T cell (T_{reg}), and neutrophil function. IL-17 promotes neutrophil recruitment into tissues, and inflammatory cytokine production by macrophages.

Aims: To investigate the roles of leptin receptor (ObR) signalling and IL-17 in the vaccine- induced immune responses against *H. pylori*.

Methods: We studied responses in vaccinated wild-type C57BL/6 and ObR-signalling deficient C57BL/6 CgObR db/db mice (db/db).

Results: Vaccinated db/db mice are not protected from *H. pylori* challenge, gastric IL-17 production was also reduced in these mice. In the stomachs of wild-type mice, both leptin and ObR are expressed on epithelial, CD4⁺ T cells, CD4⁺ Treg, macrophages and neutrophils. Further, bone marrow chimaera studies revealed that functional ObR on BM-derived cells was

essential for vaccine-induced protection.

Conclusions: Our data support a link between leptin and Th-17 responses in gastric inflammation. We hypothesize that T-cell derived leptin impacts both Treg function and IL-17 production, regulating protective responses in the vaccinated stomach.

1935

NRAMP (Natural Resistance Associated Macrophage Protein, Slc11a1) plays a critical role in the resistance of mice to *Francisella tularensis*

Powell, D., Frelinger, J.

University of Arizona, Immunobiology, Tucson, United States

NRAMP (Natural Resistance Associated Macrophage Protein, Slc11a1) is a major mediator of resistance to *Salmonella*, *Listeria*, and *Mycobacterium*. Murine NRAMP has two known alleles, a functional NRAMP^{Gly169}, in DBA2/J, NOD, and 129p3/J related strains, and a non-functional NRAMP^{Asp169}, in C57BL/6 (B6) and BALB/c mice. B6 mice congenic for NRAMP^{Gly169} (B6-NRAMP^{G169}) show a marked resistance to intracellular pathogens. Here we examined the pathogenesis of *Francisella tularensis* Strain LVS in B6-NRAMP^{G169} mice. When intranasally (i.n.) challenged B6-NRAMP^{G169} mice showed no weight loss or signs of disease. In contrast B6 mice lost significant amounts (~15%) of weight upon challenge, as previously described. Organ burdens differed between B6-NRAMP^{G169} and B6 mice. Three days post infection all B6-NRAMP^{G169} mice had no detectable *Francisella* in the lung, liver or spleen. B6 mice had burdens approaching 1x10⁶ Colony Forming Units (CFU) in all three organs. To further examine the degree of resistance imparted by NRAMP^{Gly169} expression we further challenged mice deficient in TLR2, TLR4, and TLR9, expressing NRAMP^{Gly169} (B6-NRAMP^{G169} TLR2/4/9^{-/-}). While TLR4 has no effect on *Francisella* pathogenesis, TLR2 has been shown to be crucial in B6 mice surviving *Francisella* infection. Surprisingly, B6-NRAMP^{G169} TLR2/4/9^{-/-} mice showed no notable weight loss upon i.n. challenge. 80% of B6-NRAMP^{G169} TLR2/4/9^{-/-} mice showed no detectable *Francisella*. The mice that did show bacteria were still almost 100 fold less than B6 wild type mice. These data taken together serve to highlight that functional NRAMP^{Gly169} is a critical player in murine resistance to *Francisella* infection and interaction with the host immune response.

2845

Commensal microbial modulation of immunity in the male urogenital tract

Murphy, S., Done, J., Schaeffer, A., Thumbikat, P.

Northwestern University, Urology, Chicago, United States

Staphylococcus epidermidis, a skin commensal, modulates local immune responses homeostatically and during inflammation. The human prostate harbors small numbers of bacteria that are considered to be clinically insignificant. These include gram-positive bacterial species such as *S. epidermidis* that are isolated from both patients and healthy men. Chronic pelvic pain syndrome (CPPS) in men has unknown etiology but is primarily associated with pain and alterations in prostate immunity. CPPS can be modeled using murine experimental prostatitis (EAP)

where we have shown that CD4+ve IL17A+ve T-cells play a critical role. Here we report that a specific *S. epidermidis* strain (designated NPI (non pain-inducing)) from a healthy human prostate ameliorates local immune activation and pain in the EAP model. The strain was isolated from the expressed prostatic secretion of a healthy human male and intra-urethally instilled into mice with EAP. Instillation reversed the influx of CD4+ve IL17A+ve T-cells associated with EAP by locally increasing the level of suppressive FoxP3+ve IL10 producing T-regs. In another murine model of CPPS, a human uropathogenic *E. coli* (UPEC) strain (designated CP1) induces chronic pain in mice that remains after bacterial clearance. Prophylactic instillation with NPI prevented CP1 colonization of the prostate and induction of pain, and therapeutic intervention with the commensal strain post-infection with CP1, ameliorated pain responses and reversed bacterial colonization. These results identify a new immunomodulatory niche for *S. epidermidis* and provide evidence for existence of a commensal flora of the prostate that interacts directly with the host immune response in health and disease.

3664

Guanabenz protects against tuberculosis infection by targeting ESAT-6 of *Mycobacterium tuberculosis*

Samten, B., Jung, B.-G., Yi, N., Wang, X.

University of Texas Health Science Center at Tyler, Pulmonary Immunology, Tyler, United States

Tuberculosis remains as a major public health crisis due to lack of an effective vaccine and development of drug resistance by *Mycobacterium tuberculosis* (*Mtb*). Therefore, development of better vaccine and novel therapeutics are urgently needed for tuberculosis control. Early secreted antigenic target 6 kDa (ESAT-6) of *Mtb* is an essential virulence factor while it has been the focus of tuberculosis vaccine research. We have shown previously that ESAT-6 inhibits T-cell IFN- γ production via p38 mitogen activated protein kinase. Since ESAT-6 was shown to induce untranslated protein response (UPR) of lung epithelial cells and guanabenz, a centrally acting α 2-adrenergic antihypertensive drug, protects cells by suppressing UPR, we tested whether guanabenz protects from ESAT-6 inhibition of T cells and suppresses *Mtb* growth in human macrophages *in vitro* and in mouse infected through aerosol route. Our results show that guanabenz reversed ESAT-6 inhibition of T-cell IFN- γ production and proliferation dose dependently without the involvement of either α 2-adrenergic receptor or UPR-induced endoplasmic stress responses as determined by pathway specific inhibitors. Guanabenz also reduced *Mtb* growth in human monocyte-derived macrophages and inhibited *Mtb* growth and pathology of the lungs of *Mtb*-infected mice with no direct effect on *Mtb* growth in broth. Compared with controls, guanabenz also reduced inflammatory cytokines in the lungs of *Mtb*-infected mice and enhanced immune responses of T cells isolated from the spleens and the mediastinal lymph nodes of *Mtb*-infected mice. In conclusion, we demonstrated that guanabenz protects from tuberculosis infection probably by targeting ESAT-6, a major virulence factor of *Mtb*.

4205

Phosphoinositide-mediated oligomerization by defensins induces fungal and tumour cell lysis*Hulett, M., Poon, I., Lay, F., Baxter, A., Phan, T.K., Mills, G., Bleakley, M., Anderson, M., Kvangsakul, M.**La Trobe Institute for Molecular Science, La Trobe University, Department of Biochemistry and Genetics, Melbourne, Australia*

Cationic antimicrobial peptides (CAPs) such as defensins are ubiquitously found innate immune molecules that often exhibit broad activity against microbial pathogens and mammalian tumor cells. Many CAPs act at the plasma membrane of cells leading to membrane destabilization and permeabilization. Here we describe a novel cell lysis mechanism for fungal and tumor cells by plant and human defensins that act via direct binding to the plasma membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2). We determined the crystal structure of the plant defensin NaD1 in complex with PIP2, revealing a striking oligomeric arrangement comprising seven dimers of NaD1 that cooperatively bind the anionic head-groups of 14 PIP2 molecules through a unique "cationic grip" configuration. Site-directed mutagenesis of NaD1 confirms that PIP2 binding and oligomerization are important for fungal and tumor cell permeabilization. We have shown that human beta-defensin 3 also uses a similar mechanism for cell lysis. These observations identify a conserved innate recognition system by defensins for direct binding of PIP2 that permeabilizes cells via a novel membrane disrupting mechanism.

2284

Neutrophil-producing IFN- γ eases tissue injury by driving macrophages to phagocytose apoptotic neutrophils during *Listeria monocytogenes* infection*Wang, G.¹, Lin, A.¹, Zhao, H.¹, Han, Q.¹, Zhang, C.¹, Tian, Z.^{1,2}, Zhang, J.¹*¹*Institute of Immunopharmaceutical Sciences, School of Pharmaceutical Sciences, Shandong University, Jinan, China,*²*University of Science and Technology of China, School of Life Sciences, Hefei, China*

IFN- γ plays a critical protective function against acute *Listeria monocytogenes* (Lm) infection, which was thought to be predominantly produced by NK cells in innate response. However, we found IFN- γ production was decreased mildly in Lm-infected Rag1^{-/-} and NK cell-depleted mice, while neutrophils were dominant IFN- γ -producing cells especially in blood and peritoneal cavity. IFN- γ was important in anti-*Listeria* immune response, which was indicated by poor survival, high bacteria loads in liver and spleen, severe tissue injury and the reduced number of splenic immune cells in GKO mice. IFN- γ was autocrinely released and effected on neutrophils during Lm infection, promoting bacteria clearance and inhibiting listeria toxin-induced neutrophil apoptosis. In addition, IFN- γ promoted the activation of macrophages and impaired *Listeria* replication. Importantly, IFN- γ could effectively drive macrophages to phagocytose apoptotic neutrophils, which would release tissue damage associated factors such as ROS and myeloperoxidase (MPO), and accompanied by TGF- β secretion. The uptake of

MPO by macrophages further promoted pathogen elimination. To sum up, we showed that neutrophil was an important source of early IFN- γ , which could promote bacteria clearance and ease tissue injury by driving macrophages to phagocytose apoptotic neutrophils.

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Cellular sources of tumor necrosis factor in disease and cell type-restricted cytokine targeting*Nedospasov, S.^{1,2,3}*¹*Engelhardt Institute of Molecular Biology, Molecular Mechanisms of Immunity, Moscow, Russian Federation,* ²*Lobachevsky University, Experimental Immunology, Nizhni Novgorod, Russian Federation,*³*German Rheumatism Research Center (DRFZ), Leibniz Institute, Inflammation Biology, Berlin, Germany*

Proinflammatory cytokines are involved in pathogenesis of autoimmune diseases and cancer. As a result systemic inhibition of these cytokines may have a therapeutic effect. Our study in experimental arthritis suggests that TNF from one particular cellular source may play a protective, anti-inflammatory role. Another study suggests the importance of TNF from restricted cellular sources for resistance to infections that may be compromised during systemic TNF ablation. Finally, we and others have provided evidence that in several diseases pathogenic TNF is produced by myeloid cells. Thus, in the same disease the contribution of TNF may be opposite, depending on the source. Based on these findings we are developing bi-specific antibodies that would attach to the cell surface of a particular type of immune cells, and capture TNF released by these cells, preventing its dissemination and bioavailability. TNF from other sources should not be affected. Our constructs are based on single domain antibodies (V_HH) specific for human or mouse TNF and for cell type-specific markers, in particular, expressed by macrophages. We find that such antibodies, called MYSTI (myeloid-specific TNF inhibitors) can effectively attach to the macrophage cell surface, capture and retain the released TNF. Using mice humanized for the TNF system and macrophages isolated from such mice we assessed activity of these constructs *in vitro* and *in vivo*. Our findings may serve as a basis for bioengineering of a new type of cytokine inhibitors. Supported by Russian Ministry of Science and Education (14. Z50.31.0008), Russian Science Foundation (#14-50-00060) and Deutsche Forschungsgemeinschaft (NE 1466/2).

1884

Interleukin-27 inhibits synovial lymphoid neogenesis in inflammatory arthritis*Jones, G.W.¹, Bombardieri, M.², Greenhill, C.J.¹, McLeod, L.³, Nerviani, A.², Rocher-Ros, V.², Cardus, A.¹, Williams, A.S.¹, Pitzalis, C.², Jenkins, B.J.³, Jones, S.A.¹*¹*Cardiff University, Institute of Infection & Immunity, School of Medicine, Cardiff, United Kingdom,* ²*Queen Mary University of London, William Harvey Research Institute, London, United Kingdom,* ³*Hudson Institute of Medical Research, Clayton, Australia*

from early inflammatory arthritis patients has enabled disease classification based on local pathology. For example, synovitis can be classed as predominantly fibroblast- (pauci-immune), myeloid- or lymphoid-rich. Lymphoid histopathology can either comprise diffuse lymphocytic infiltrates or ectopic lymphoid-like structures (ELS) reminiscent of the follicular compartments of secondary lymphoid organs. ELS often develop at sites of chronic inflammation and autoimmunity where they contribute to immune-mediated pathology. However, mechanisms governing ectopic lymphoneogenesis are poorly defined. Evaluation of synovial tissue from rheumatoid arthritis (RA) patients revealed that reduced synovial interleukin-27 (IL-27) expression corresponded with the clinical presence of ELS and gene signatures associated with their development and activity. Induction of inflammatory arthritis in mice deficient in the IL-27 receptor (IL-27R) promoted synovial ELS formation, which was associated with increased synovial expression of pro-inflammatory cytokines (*Il17a*, *Il21*) homeostatic chemokines (*Cxcl13*, *Ccl21*) and transcriptional regulators (*Bcl6*, *Prdm1*) linked with lymphoneogenesis. In both clinical and experimental RA, synovial ELS coincided with the heightened local expression of signature cytokines and transcription factors of the Th17 and T follicular helper (Tfh) cell lineages, and the presence of podoplanin-expressing T cells within lymphoid aggregates. IL-27 inhibited the differentiation of T helper cells into podoplanin-expressing Th17 cells, and an increased number of these cells were observed in the draining lymph nodes of IL-27R-deficient mice with inflammatory arthritis. Thus, IL-27 appears to negatively regulate ELS development in RA through control of effector T cell responses, which opens new opportunities for improved patient stratification and treatment.

4150

Ongoing classical IL-6 signaling is required for Th17 maintenance and the pathogenesis of colitis

Harbour, S.¹, Maynard, C.¹, Zindl, C.¹, Jones, S.², Jones, G.², Weaver, C.¹

¹University of Alabama at Birmingham, Birmingham, United States, ²Cardiff University, Institute of Infection and Immunity, Cardiff, United Kingdom

Th17 cells reactive to the enteric microbiota are central to the pathogenesis of certain types of inflammatory bowel disease. Factors involved in early Th17 differentiation include IL-6, TGF- β and IL-1, however it is unknown to what extent factors other than IL-23 are important in the late development and maintenance of mature Th17 cells.

While early Th17 development is mediated by binding of IL-6 to membrane bound IL-6Ra (classical signaling), activated Th17 cells cleave surface IL-6Ra and are only responsive to soluble IL-6Ra complexed to IL-6 (trans signaling). Using two T-cell dependent models of colitis, we found that both naïve T cells and Th17 cells deficient in IL-6Ra, which cannot signal via classical IL-6, were unable to induce colitis in recipients. Lack of disease was associated with failure to induce and maintain a Th17 response respectively, with reduced numbers of recovered cells and inability to maintain an IL-17A+ phenotype. Further, co-transfer of IL-6Ra deficient Th17 cells into an inflammatory

environment driven by WT Th17 cells was unable to rescue the phenotype of these cells, despite the absence of defects in apoptosis, proliferation or trafficking, implying that the inability of IL-6Ra Th17 cells to drive disease is due to a Th17-intrinsic mechanism which cannot be compensated for by other Th17 factors including IL-23 and IL-21.

These results indicate that ongoing IL-6 signaling is required for the maintenance of Th17 cells, and this process is contingent on IL-6 classical signaling, as IL-6 trans signaling is not sufficient for development or induction of inflammation.

1293

Understanding the role of pro-inflammatory cytokines in the development of gastric cancer

Low, J.T., Putoczki, T., Strasser, A., O'Reilly, L.A.

Walter & Eliza Hall Institute, Melbourne, Australia

NF κ B is an important transcriptional regulator of many genes involved in tumour-promoting inflammation, cell proliferation and survival. This pathway is frequently found to be over-activated in infiltrating immune cells in gastritis lesions and in gastric cancer (GC). Our laboratory has discovered that aging NF κ B1-deficient (*nfkb1^{-/-}*) mice develop GC mimicking the progression of human invasive intestinal-type GC (IGC). These mice develop chronic gastritis, gastric mucosal atrophy or dysplasia, with a high incidence of invasive gastric adenocarcinomas. We observed abnormally increased infiltration of F4/80-positive macrophages and other CD45-positive leukocytes in the gastric mucosa of young (pre-disease) *nfkb1^{-/-}* mice. Elevated levels of pro-inflammatory cytokines and chemokines were found in the serum of young *nfkb1^{-/-}* mice, and also in established tumours of these mice. This suggests that deregulated expression of pro-inflammatory cytokines or chemokines may contribute to IGC development in these animals.

Preliminary studies in *nfkb1^{-/-}* mice indicate that NF κ B1 loss results in abnormal STAT1/STAT3 stimulation, which contributes to deregulated cytokine production. To characterise the role of deregulated cytokines in IGC development in *nfkb1^{-/-}* mice, we crossed mice lacking functional alleles for IL-11Ra, IL-22 or IL-6 to *nfkb1^{-/-}* mice. IL-6, IL-22 and IL-11 are STAT3-activating pro-inflammatory cytokines implicated in GC development. Their levels are abnormally high in the serum and gastric tumour tissue of *nfkb1^{-/-}* mice. Early survival analysis indicates that loss of IL-6 may not improve survival of *nfkb1^{-/-}* mice. In contrast, loss of IL-11Ra or IL-22 prolongs survival of *nfkb1^{-/-}* mice, suggesting that these cytokines play an important role in IGC development.

1796

Target organ-directed nanoparticles effectively deliver IL-27 for arthritis therapy

Meka, R.¹, Venkatesha, S.¹, Ruoslahti, E.², Moudgil, K.¹

¹University of Maryland School of Medicine, Microbiology and Immunology, Baltimore, United States, ²Sanford Burnham Prebys Medical Discovery Institute, La Jolla, United States

Rheumatoid arthritis (RA) is a debilitating autoimmune disease characterized by synovial inflammation, pain, and joint damage.

Many potent anti-arthritis drugs are available for arthritis therapy, but their prolonged use leads to severe adverse effects. These drugs are given orally or by injection, and thereby are distributed to multiple organs in the body besides the intended target, the inflamed joints, resulting in unwanted toxicity. Therefore, search continues not only for better therapeutic agents, but also for novel drug delivery approaches that preferentially target the inflamed joints. In this context, our previous studies in the rat adjuvant-induced arthritis (AA) model of RA revealed that IL-27, a new member of the IL-12 family, had immunomodulatory attributes (JBC, 2011), and that a phage encoding a 9-amino acid peptide denoted as "ADK" preferentially homed to inflamed joints following intravenous injection into arthritic rats (PNAS 2011). Peptide ADK was identified using a combination of *ex vivo* and *in vivo* screening of a phage peptide-display library in rats having AA. This peptide was distinct from the well-known RGD motif-bearing peptide in regard to its receptor-binding characteristics. Therefore, we employed peptide ADK to direct IL-27-entrapping liposomes to inflamed arthritic joints of rats. This nanoparticle-based therapy was effective in significantly reducing the severity of arthritis at a much lower dose than that of IL-27 in solution. Furthermore, the nanoparticles were tolerated well without any adverse effects. We believe that with appropriate modifications, a similar targeted therapeutic approach can be adapted for human RA therapy in the near future.

2655

Regulators of inflammation and wound formation induced by trehalose 6,6'-dimycolate (TDM) from *Mycobacterium tuberculosis* *in vivo*

da Silva Domingues, J.F., Ducasse, L., Peixoto, A.

Institut de Pharmacologie et de Biologie Structurale - CNRS, Toulouse, France

Trehalose 6,6'-dimycolate (TDM) is a glycolipid found in the cell wall of *Mycobacterium tuberculosis* that alone can induce an inflammatory and granulomatous response *in vivo*, similar to a natural infection. These properties of TDM make it a potent adjuvant for subunit vaccine, yet excessive inflammation and wound formation are adverse side effects often observed upon immunization. So far the cellular and molecular mediators of the pathology associated with the response to TDM challenge remain poorly defined. Herein, we dissected the cellular dynamics of the inflammatory response to TDM challenge in the skin of mice and the cell-cell interactions occurring at the site of challenge by intravital multiphoton microscopy. In parallel, with the use of histology we established the cellular organization of the evolving wound upon challenge. In combination with the depletion of specific immune cells we provide evidence that TDM is not toxic by itself but induces significant immunopathology mediated by myeloid cells that are attracted to the site of challenge and promote wound formation. Altogether our results provide novel insights into the mechanisms behind the pathology induced by a potent adjuvant such as TDM that are important to guide the development of better adjuvants for vaccine research.

3274

Haematopoietic cell kinase promotes myeloid endotypes underpinning the development of COPD and tumour progression in mice

O'Donoghue, R.^{1,2}, Jarnicki, A.³, Poh, A.^{1,4}, Masson, F.^{1,2}, Faux, M.⁵, Nowell, C.⁶, Bozinovski, S.⁷, Anderson, G.⁸, Ernst, M.^{1,2}

¹Olivia Newton-John Cancer Research Institute, Cancer and Inflammation Laboratory, Heidelberg, Australia, ²La Trobe University, Cancer Medicine, Bundoora, Australia, ³University of Melbourne, Pharmacology and Medicine, Melbourne, Australia, ⁴Walter & Eliza Hall Institute of Medical Research, Inflammation, Melbourne, Australia, ⁵Walter and Eliza Hall Institute of Medical Research, Structural Biology, Melbourne, Australia, ⁶Monash University, Monash Centre of Pharmaceutical Sciences, Melbourne, Australia, ⁷RMIT University, Health Innovations Research Institute, Bundoora, Australia, ⁸University of Melbourne, Pharmacology and Medicine, Melbourne, Australia

Chronic obstructive pulmonary disease (COPD) is the third leading cause of death in the world and is an independent risk factor for the development of lung cancer. Polymorphisms and mutations in haematopoietic cell kinase (Hck) have been observed in patients with COPD and lung cancer while aberrant activation of Hck ($Hck^{Up/Up}$) promotes the development of COPD hallmarks in the lungs of mice. Adult $Hck^{Up/Up}$ mice develop pulmonary inflammation compared to Wt mice, which is linked to emphysema from 12 weeks of age. This phenotype was independent of the adaptive immune system since lymphocyte deficient ($Hck^{Up/Up}; Rag1^{-/-}$) mice were similar to $Hck^{Up/Up}$ mice. Phenotypic analysis of inflammatory cells in the lungs of mutant mice showed enhanced numbers and enrichment of Cd11b+:Ly6g+:Csfr1+ myeloid-derived suppressor cells (MDSCs) and Cd11b+:F4/80+:Arg1+ alternatively activated macrophages (AAMs). These cells were implicated in promoting disease after adoptive bone marrow transfers of Wt bone marrow alleviated disease and reduced MDSCs and AAMs in $Hck^{Up/Up}$ host mice. Pulmonary inflammation was reduced in $Hck^{Up/Up}; Gcsf^{-/-}$ and $Hck^{Up/Up}; Il6^{-/-}$ mice consistent with the ability of these cytokines to promote MDSC and AAM differentiation respectively. The tumour enhancing properties of Hck was confirmed after lung tumour burden was significantly larger in $Hck^{Up/Up}; Kras^{LSL-G12D/+}$ mice than in $Kras^{LSL-G12D/+}$ and adenoma formation was promoted in $Hck^{Up/Up}; Scgb1a1-Cre; Kras^{LSL-G12D/+}$ mice compared to the bronchiolar hyperplasia observed in control mice. In summary, Hck activity within pulmonary macrophages promotes MDSC accumulation and AAM differentiation to drive a COPD-like inflammatory pulmonary disease and lung tumour progression in mice.

535

GM-CSF primes cardiac inflammation during Kawasaki disease

Stock, A., Wicks, I.

The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia

Kawasaki Disease (KD) is the leading cause of pediatric heart disease in developed countries. KD patients develop cardiac

inflammation, characterized by an early infiltrate of neutrophils and monocytes that precipitates coronary arteritis. While the early inflammatory processes are linked to cardiac pathology, the factors that regulate cardiac inflammation and immune cell recruitment to the heart remain obscure. Using a murine model of KD (induced by a water-soluble cell-wall fraction of *Candida albicans*, CAWS), we identified an essential role for GM-CSF in orchestrating these events. GM-CSF was rapidly produced by cardiac fibroblasts following CAWS challenge, precipitating cardiac inflammation. Mechanistically, GM-CSF acted upon the local myeloid compartment, driving the expression of inflammatory cytokines and chemokines that facilitated neutrophil and monocyte recruitment into the heart. Our findings describe a novel role for GM-CSF as an essential initiating cytokine in cardiac inflammation, and implicate GM-CSF as a potential target for therapeutic intervention in KD

1312

Regulation of CCL5 by Runx/CBF β is essential to maintain lung homeostasis

Seo, W., Taniuchi, I.

RIKEN IMS Center for Integrative Medical Sciences, Laboratory for Transcriptional Regulation, Yokohama, Japan

Recent findings suggest that Runx/CBF β transcription factors might directly control inflammation in addition to their roles during hematopoiesis. Indeed, we previously reported that mice with impaired Runx/CBF β function showed IL-4 dysregulation and airway inflammation with allergic characteristics. However, the spontaneous lung infiltration of Runx/CBF β -deficient mice was much more severe than that of the previously reported IL-4 transgenic mice, suggesting that there might exist other inflammatory molecules contributing to the observed lung pathology. The search for other dysregulated cytokines and chemokines from Runx/CBF β mutant mice revealed that CCL5, a potent inflammatory chemokine normally secreted from activated CD8⁺T cells, is over-expressed by activated CD4⁺T cells, indicating that Runx/CBF β not only represses CCL5 during the steady state but also control cell-type specific CCL5 expression. Elimination of CCR5, the major receptor for CCL5, rescued the lung pathology, confirming that CCL5-CCR5 axis is one of the main causes of the lung pathology. Our ChIP assays identified a conserved Runx/CBF β binding region located upstream of CCL5 promoter. Deletion of this Runx/CBF β binding region abrogated CCL5-GFP BAC reporter expression as well as endogenous CCL5 expression, indicating that this region contains a critical enhancer against which Runx/CBF β may counteract to regulate CCL5 expression in CD4⁺T cells. We propose that Runx/CBF β is involved in the intricate regulation of CCL5 by direct binding to an essential cis-regulatory region.

Innate Receptors & Inflammasomes 3

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Protein kinase D1 links IRAK1 and TRAF6, but not TRAF3, in the MyD88-dependent Toll-like receptor signaling pathway

Yi, A.-K.¹, Park, J.-E.²

¹*University of Tennessee Health Science Center, Microbiology, Immunology and Biochemistry, Memphis, United States,*

²*University of Tennessee Health Science Center, Pediatrics, Memphis, United States*

The initial proinflammatory responses to *Saccharopolyspora rectivirgula* (SR), which causes farmers' lung disease, is dependent on Toll-like receptor 2 (TLR2) and MyD88, and MyD88 is absolutely required for SR-induced protein kinase D1 (PKD1) activation. We found that SR-mediated proinflammatory responses and neutrophil infiltration in the lungs are ablated in mice treated with PKD inhibitor or lacking the PKD1 gene. However, the mechanisms by which PKD1 is activated and functions to regulate TLR signaling are incompletely understood. In the present study, we found that upon TLR ligand stimulation, the receptor signaling complex composed of MyD88, IRAK4, IRAK1, TRAF6, and PKD1 is formed in membrane lipid rafts, and that IRAK4 and IRAK1, but not TRAF6, are required for recruitment of PKD1 to the receptor complex and for activation by TLR ligands. In addition, MyD88-dependent translocation of TRAF6 into lipid rafts, ubiquitination of TRAF6, degradation of IRAK1, activation of TAK1, NF- κ B and MAPKs, and subsequent expression of proinflammatory cytokines/chemokines are dependent on PKD1. However, PKD1 does not interact with TRAF3 and is dispensable for TLR-mediated type I interferon expression. Our findings indicate that PKD1 is a downstream effector of IRAK1, but an upstream modulator of TRAF6. Together with the known ability of PKD1 to shuttle between membrane lipid rafts and the cytosol, our results further support the hypothesis that PKD1 functions as a molecular shuttle that brings TRAF6 to IRAK1 in membrane lipid rafts and then aids in translocation of the IRAK1/TRAF6 complex to the cytosol, where TRAF6 interacts with its downstream effectors.

1183

TLRs and FcRs acting together to eradicate infections

Bakema, J.¹, Tuk, K.², van Vliet, S.J.², Bruijns, S.C.², Vos, J.B.², Letsiou, S.³, Brenkman, A.B.³, van Egmond, M.²

¹*VUMC, Department of Otolaryngology/Head-Neck Surgery, Amsterdam, Netherlands,*

²*VUMC, Molecular Cell Biology and Immunology, Amsterdam, Netherlands,*

³*UMCU, Department of Metabolic Diseases and Netherlands Metabolomics Centre, Utrecht, Netherlands*

Cell-mediated mechanisms of the host defense are crucial for the control of infectious pathogens. Whereas pattern recognizing receptors, like Toll-like receptors (TLRs), play an essential role during primary responses, pathogen-specific antibodies play a key role during re-encounter with the same pathogen by inducing rapid Fc-receptor (FcR)- mediated uptake and clearance by immune effector cells. The individual importance

of these different classes of receptors is well studied. However, during binding and uptake of antibody-opsonized bacteria, ligands for both receptor systems are involved, and potential cross-talk between these two receptor classes received relatively little attention.

We therefore investigated whether antibody-opsonized bacteria could evoke simultaneous activation of FcRs and TLRs and demonstrated cross-talk between MyD88-signalling dependent TLRs and ITAM-bearing FcRs on various immune cells. Cross-talk between TLRs and FcRs on DCs, for instance, induced a profound altered secretory profile of inflammatory cytokines and eicosanoids. Moreover, this resulted in skewing of IFN γ producing TH-1-subset towards IL-17 and GM-CSF producing TH-cells. The observed altered cytokine profile, induced by cross-talk between the two receptors types, was regulated on both transcription and post-transcriptional levels.

In conclusion, our findings demonstrate functional cross-talk between pathogen recognizing TLRs and antibody binding FcRs. These observations generate novel insights in pathogen elimination during secondary infections which induce fundamentally different immune responses compared to non-antibody mediated primary immune responses. This knowledge could be helpful to develop and refine new therapies for inflammatory diseases as well as for induction of anti-tumor immune responses during antibody mediated treatment of cancer.

2223

Cre-dependent DNA recombination activates cGAS-STING innate immune response

Pépin, G.¹, Ferrand, J.¹, Höning, K.², Gough, D.J.³, Williams, B.R.G.³, Hornung, V.⁴, Gantier, M.¹

¹Hudson Institute of Medical Research, Centre for Innate Immunity and Infectious Diseases, Clayton, Australia, ²University of Bonn, Institute of Molecular Medicine, Bonn, Germany, ³Hudson Institute of Medical Research, Centre for Cancer Research, Clayton, Australia, ⁴Ludwig-Maximilians University, Gene Center and Department of Biochemistry, Munich, Germany

Gene-recombinase technologies, such as Cre/*loxP*-mediated DNA recombination, are important tools in the study of gene function, but have potential side effects due to damaging activity on DNA. Here we show that DNA recombination by Cre instigates a robust antiviral response in mammalian cells, independent of legitimate *loxP* recombination. This is due to Cre-dependent DNA damage and the accumulation of cytoplasmic DNA, which recruits the cytosolic cyclic GMP-AMP (cGAMP) synthase (cGAS)-STING innate immune pathway. Importantly, we establish a direct interplay between this antiviral response and cell-cell interactions, indicating that low cell densities could be useful to help mitigate these effects of Cre *in vitro*. Taking into account the wide range of interferon stimulated genes that may be induced by the cGAS-STING pathway together with widespread use of the Cre/*loxP* based system, these results call for caution in the interpretation of data from Cre/*loxP*-based studies that do not include appropriate Cre-only controls.

2329

A mitochondrial target of imiquimod is involved in Nlrp3 inflammasome activation and cancer cell growth arrest

Gross, C.J.¹, Mishra, R.¹, Dittlein, D.C.², Wettmarshausen, J.³, Ruland, J.¹, Traidl-Hoffmann, C.², Perocchi, F.³, Gross, O.¹

¹Technische Universität München, Institut für Klinische Chemie und Pathobiochemie, Munich, Germany, ²Technische Universität München, Institut für Umweltmedizin, Munich, Germany, ³Ludwig-Maximilians-Universität, Genzentrum, Munich, Germany

Imiquimod is a topical immunomodulator that is used to treat genital warts and basal cell carcinoma. Its ability to activate Toll-like receptor 7 (TLR7) for the production of type I interferon and pro-inflammatory cytokines is presumed to underlie its efficacy. However, TLR7-independent effects of imiquimod, including growth arrest and apoptosis in cancer cells and NLRP3 inflammasome activation in myeloid cells, may contribute to its efficacy or adverse effects. We investigated the mechanism of NLRP3 inflammasome activation by imiquimod, including the involvement of endolysosomal leakage, ROS, and K⁺ efflux. Our results challenge the current model of Nlrp3 inflammasome activation. Further chemical proteomics and functional studies identified a direct mitochondrial target of imiquimod that is known to have an important metabolic role in immune cells and cancer cells. We present evidence that inhibition of this target by imiquimod is important for the ability of imiquimod to activate the Nlrp3 inflammasome in myeloid cells, and to trigger growth arrest in cancer cells.

1117

NOD/RIP2 signaling in response to non-peptidoglycan containing pathogen contribute to development of a TH1-biased response and resistance against *L. major* infection

Lima-Junior, D.S., Silva, J.S., Zamboni, D.S.

University of Sao Paulo, Ribeirão Preto, Brazil

The intracellular sensors Nod1 and Nod2 have key role in the host responses. During activation, these proteins signals via the adapter molecule Rip2, which is a protein kinase that leads to activation of NF- κ B and MAPK. Also, Nod1 and Nod2 participate in the detection/control of several pathogens as they sense PAMPs contained in the cell walls of Gram-negative and Gram-positive bacteria. However, the role of Nod1 and Nod2 during *Leishmania* infection is unknown. Here, we investigated the participation of Nod/Rip2 pathway in host response during *L. major* infection. Using *Nod1*^{-/-}, *Nod2*^{-/-} and *Rip2*^{-/-} BMDMs we observed an impaired induction of NF- κ B-dependent products in response to *L. major* infection. Moreover, IL-12p40 production and surface molecules expression were decreased in parasite infected-*Nod1*^{-/-}, *-Nod2*^{-/-}, *-Rip2*^{-/-} BMDCs. On the other hand, *Nod1*^{-/-}, *Nod2*^{-/-} and *Rip2*^{-/-} BMDCs presented an increased IL-4 production after infection with *L. major*. Nod1 and Nod2 activation was crucial for *in vivo* parasite replication control and resolved cutaneous lesions. Nod/Rip2-dependent response was required for dendritic cells activation and induction of effective Th1 response *in vivo*. Additionally, Rip2-dependent signaling in radio-sensitive compartments was required for the control of the infection and induction of Th1 response. These studies

indicate that Nod/Rip2-dependent responses account for host resistance against *L. major* infection by mechanisms dependent of Th1 cytokine. Importantly, this study shows that the Nod-Rip2 axis effectively participate of the induction of adaptive immune responses against a *Leishmania* parasite, thus providing a novel function for Nod-like receptors family or proteins in parasite-host interactions.

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3323

TLR13 signaling on cardiomyocytes and macrophages is critical to establish protective immunity to Coxsackievirus B3 infection

Qian, Q., Xu, W.

Soochow University, Institutes of Biology and Medical Sciences, Suzhou, China

Toll like receptor 13 (TLR13) is an unclarified TLR member responsible for the recognition of 23S bacteria rRNA. Little is known, however, of the impact of TLR3 on antiviral host defenses. Here we examined the role of TLR13 in the innate response to Coxsackievirus B3 (CVB3), a prevalent human pathogen associated with viral myocarditis and chronic dilated cardiomyopathy (DCM). We confirmed TLR13 as the most robustly up-regulated cardiac TLR in C57BL/6 mice following CVB3 infection. TLR13-deficient mice developed substantially higher viral load in the heart and spleen by day 3 following CVB3 infection thus had significantly increased mortality compared with their controls. However, survived mice had reduced heart inflammation and improved left ventricular dysfunction related with reduced cardiac T and CD11b+ leucocytes infiltration. TLR13 deficiency enhanced IFN γ +Th1 response but dramatically reduced T and neutrophil population in the spleen which may be related with the failure of viral elimination and increased mortality of mice. Cardiomyocyte deficient of TLR13 are highly susceptible to viral replication for reduced IFN- β production via IRF-7 activation; while CVB3 up-regulation of TLR13 in macrophages enhanced inflammatory cytokine production by promoting NF- κ B activation. Taken together, TLR13 innate response plays important role in host antiviral defense. TLR13 signaling in cardiomyocytes and splenocytes is critical for survival by promoting type I IFN production and neutrophil function; while macrophage TLR13 triggers local inflammatory mediators and promote immune infiltration into the heart. The protective versus pathogenic contribution of TLR13 in CVB3 infection may have profound impact on the development of viral myocarditis.

2393

BAD-LAMP a novel actor of TLR9 trafficking in human plasmacytoid dendritic cells

Combes, A.¹, N'guessan Sari, P.¹, Arguello, R.¹, Camosseto, V.¹, Bendriss-Vermare, N.², Pierre, P.¹, Gatti, E.¹

¹Centre d'Immunologie de Marseille-Luminy, Marseille, France,

²Centre de Recherche en Cancérologie de Lyon, Lyon, France

The brain and DC associated LAMP-like molecule (BAD-LAMP/Lamp5) is a newly identified member of the family of lysosome-

associated membrane proteins (LAMPs). BAD-LAMP expression in most animals is confined to neurons. However, we could demonstrate that in humans, BAD-LAMP can specifically be found in the type I interferon-producing plasmacytoid DCs (pDCs). Human BAD-LAMP is localized in the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) of freshly isolated CD123⁺ pDCs. Upon unmethylated cytosine-phosphate-guanine (CpG) oligonucleotides activation of pDCs, BAD-LAMP accumulates in endosomes with Toll Like Receptor 9 (TLR9) prior being strongly down-regulated. We further show that BAD-LAMP and the endosomal TLRs chaperone protein UNC93B1 co-localize and influence reciprocally their intracellular trafficking suggesting that BAD-LAMP could play a role in TLR trafficking in activated pDCs. The restricted pattern of BAD-LAMP expression allows for the rapid identification of normal and leukemic human pDCs in tissues and blood. We also could show that pDCs infiltrating human breast cancers have sustained BAD-LAMP expression, compared to their blood circulating equivalents. BAD-LAMP stands therefore as a potential accessory molecule contributing to the control of TLR signaling in pDCs both during exposure to nucleic acids and in pathological conditions like cancer.

3551

Molecular arrangement and activation of procaspases-1 and -8 at inflammasomes

Vajjhala, P.¹, Lu, A.^{2,3}, Brown, D.⁴, Sagulenko, V.¹, Schroder, K.⁴, Stow, J.⁴, Wu, H.^{2,3}, Stacey, K.^{1,4}

¹The University of Queensland, School of Chemistry and Molecular Bioscience, Brisbane, Australia, ²Harvard Medical School, Department of Biological Chemistry and Molecular Pharmacology, Boston, United States, ³Boston Children's Hospital, Program in Cellular and Molecular Medicine, Boston, United States, ⁴The University of Queensland, Institute for Molecular Bioscience, Brisbane, Australia

The inflammasome is a multi-protein complex that activates caspases-1 and -8 to mediate cell death and inflammatory responses to pathogens and cellular stress signals. Excessive activation of inflammasomes contributes to the pathology of many diseases thus a detailed understanding of inflammasome assembly and function may lead to the development of novel therapeutic agents. During inflammasome assembly, activated receptors of the NLR or PYHIN families, recruit the adaptor protein ASC and induce polymerization of its pyrin domain (PYD) into a filament. The caspase recruitment domains (CARD) of ASC are exposed on the ASC PYD filament and interact to condense the complex into a perinuclear speck. ASC CARD recruits procaspase-1 to the complex while ASC PYD recruits procaspase-8 via its death effector domains (DED). We show that ectopically expressed procaspase-8 DEDs form filaments that extend from an ASC cluster. However, full-length procaspase-8 localises within the centre of the ASC speck suggesting that catalytic domains from different filaments interact to condense procaspase-8. In contrast, procaspase-1 bundles ASC filaments into a tight ring, with which it co-localizes and also extends into the core of the ASC ring. Overexpression of ASC induces spontaneous specks that can recruit procaspases-1 and -8, but

caspase processing differs from that in ASC specks induced by the PYHIN protein AIM2. Our data suggest that filament formation is important for processing of procaspases into active forms at inflammasomes but additional interactions govern optimal processing and activation.

Therapeutic Antibodies

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Monoclonal antibody against dengue virus nonstructural protein 1 wing domain protect mice from dengue virus infection

Lai, Y.-C.¹, Chuang, Y.-C.², Yeh, T.M.³

¹National Cheng Kung University, Institute of Basic Medical Sciences, Tainan, Taiwan, Republic of China, ²National Cheng Kung University, Medical Laboratory Science and Biotechnology, Tainan, Taiwan, Republic of China, ³National Cheng Kung University, Medical Laboratory Science and Biotechnology, Tainan, Taiwan, Republic of China

Dengue virus (DENV) infection is the most common mosquito-transmitted disease which may cause life-threatening dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS). Two main characteristic features of DHF/DSS are hemorrhage and vascular leakage. Previously, it has been shown that DENV nonstructural protein 1 (NS1) may play important roles in the pathogenesis of DHF/DSS by itself or by its antibodies which can cross-react with platelets and endothelial cells. Sequence homology analysis shows that C-terminal region amino acid 271-352 of DENV contains most of the cross-reactive epitope and passive immunization with antibodies against C-terminal deleted NS1 can protect mice from DENV infection. In this study, we further studied the mechanism and tested the possibility that whether monoclonal antibodies (mAb) against NS1 can be a potential therapy to protect mice against DENV infection. We first showed that NS1 is indispensable in the disease development in an established DENV-hemorrhagic mice model. Then we generated several anti-NS1 mAbs from NS1-immunized mice and screened for the ones that did not cross react to host proteins. Among them, we found mAb 33D2, which recognized all four serotypes of DENV NS1, could reduce viral load in both complement dependent and independent manner *in vitro*. Passive immunization of mAb 33D2 reduced all four serotypes of DENV-induced local skin hemorrhage and prolonged bleeding time in mice. Epitope mapping by phage-displayed random peptide library showed that mAb 33D2 recognized wing domain of NS1. These results suggest mAb 33D2 may represent a therapeutic antibody to prevent DHF/DSS.

3619

Ultra-potent neutralization of GM-CSF by multispecific antibodies

Piccoli, L.¹, Corti, D.², Lanzavecchia, A.¹

¹Institute for Research in Biomedicine, Immune Regulation Laboratory, Bellinzona, Switzerland, ²Humabs Biomed SA, Bellinzona, Switzerland

Granulocyte-macrophage colony stimulating factor (GM-CSF) is a pro-inflammatory cytokine involved in the activation of monocytes, macrophages and granulocytes in several inflammatory and autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, Crohn's disease, psoriasis, asthma and atopic dermatitis. GM-CSF is therefore a potential target for anti-inflammatory and autoimmune therapies.

We recently showed that autoantibodies to GM-CSF, causing autoimmune pulmonary alveolar proteinosis, potently neutralize the cytokine by synergistic action. In particular, a combination of three non-cross-competing antibodies completely neutralizes GM-CSF activity *in vitro* by sequestering the cytokine in high-molecular-weight complexes, and *in vivo* promotes the rapid degradation of GM-CSF-containing immune complexes in an Fc-dependent manner.

These findings prompted us to design a panel of anti-GM-CSF multispecific antibodies originated by different combinations of four non-cross-competing GM-CSF autoantibodies. Surprisingly, such multispecific antibodies have significantly higher neutralizing activity not only compared to single GM-CSF autoantibodies and to therapeutic antibodies MOR103 and Namilumab, but also compared to combinations of GM-CSF antibodies. These results suggest that multispecific antibodies chelate and sequester irreversibly GM-CSF from the interaction with its cognate receptor. These multispecific antibodies can be used at extremely low dosage to treat several GM-CSF-dependent inflammatory and autoimmune diseases.

3901

Evaluation of a novel passive immunotherapy approach targeting HMGB1 in the treatment of sepsis

Stevens, N.¹, Fraser, C.², Chapman, M.³, Kuchel, T.², Diener, K.^{1,4}, Hayball, J.^{1,5}

¹University of South Australia, Experimental Therapeutics Laboratory, Adelaide, Australia, ²South Australian Health and Medical Research Institute, Preclinical, Imaging and Research Laboratories, Adelaide, Australia, ³Royal Adelaide Hospital, Department of Intensive Care Medicine, Adelaide, Australia, ⁴University of Adelaide, Robinson Research Institute, School of Medicine, Adelaide, Australia, ⁵University of Adelaide, School of Medicine, Adelaide, Australia

Sepsis is characterised by systemic inflammatory response syndrome (SIRS) triggered by infection from various pathogens which may benefit from passive immunotherapy during emergency care. Nuclear protein high-mobility group box 1 (HMGB1) has emerged as a key mediator in SIRS, where it acts as an alarmin signalling primarily through RAGE and TLR's. HMGB1 may also play a role in secondary immunosuppression and nosocomial infection which is a significant cause of mortality in septic patients. These contrary roles of HMGB1 have been linked to the redox status of two cysteine residues present on the A-box domain of the circulating protein.

From a clinical perspective, analysis of plasma samples taken from a cohort of adult septic patients revealed significantly higher levels of HMGB1 in non-survivors compared to survivors and healthy adults. To determine whether inhibition of HMGB1 could improve survival from sepsis, potent HMGB1-

neutralising ovine polyclonal antibodies were generated following similar methodology to currently available critical care therapies. Administration of anti-HMGB1 antibodies to mice in preclinical models of sepsis confers a survival advantage in a murine endotoxin model, reduces morbidity and circulating proinflammatory cytokines in a caecal ligation and puncture peritonitis model, and significantly increases survival following a secondary infection of survivors in a two-hit model. Further studies will assess HMGB1 redox status in sepsis patients, and determine the effect of anti-HMGB1 treatment in conjunction with clinically-relevant supportive interventions in an intensive care model of ovine sepsis. Together, these works will establish the validity of targeting HMGB1 by passive immunotherapy in sepsis.

548

Application of an ELISA based competitive binding assay to measure concentration of anti-TNF biologics and neutralising anti-drug antibodies in the clinical laboratory

Keating, P.¹, Hock, B.², Barclay, M.³, Stamp, L.⁴, Spellerberg, M.¹, O'Donnell, J.¹

¹Canterbury Health Laboratories, Immunology, Christchurch, New Zealand, ²Christchurch Hospital, Haematology Research Group, Christchurch, New Zealand, ³Christchurch Hospital, Department of Clinical Pharmacology, Christchurch, New Zealand, ⁴Christchurch Hospital, Department of Rheumatology, Immunology and Allergy, Christchurch, New Zealand

Introduction: There is increasing interest in measuring both drug and anti-drug antibody (ADA) levels in patients receiving anti-TNF treatment as part of algorithms for guiding therapeutic strategies.

Aim: Application of ELISA based methods to measure drug concentration and ADA in patients receiving either adalimumab or infliximab in the clinical laboratory to aid clinicians in their treatments.

Methods: An ELISA to specifically detect drug concentrations in patient sera with no evidence of interference by rheumatoid factor was developed. A subset of patients with low drug concentrations were tested for neutralising ADA by competitively inhibiting the in vitro binding of drug to solid phase TNF. The assays were made available to clinicians within New Zealand.

Results: In our experience, 62% of samples (n=182) had levels of drug less than 7mg/L, a suggested threshold for drug activity. Samples received were predominantly from those with Inflammatory Bowel Disease and disease activity was higher in those with drug concentrations < 7mg/L. Our method of neutralising ADA detection was more sensitive than commercially available bridging ELISA formats. Of the samples with drug concentration < 1mg/L, 40% of those taking adalimumab (n=15) and 30% of those taking infliximab (n=23) had developed ADA. Clinicians used the assay results to alter treatments.

Conclusion: Monitoring of anti-TNF biologic drug levels and ADA was well received by clinicians. The results confirm published data showing trough concentrations and presence of neutralising ADA are correlated with disease activity. The available assays have influenced the management of disease.

2089

Novel ways of targeting PKCθ reveals new molecular clues about T cell signaling

Ozay, E.I.^{1,2,3}, Trombley, G.^{2,4}, Torres, J.^{1,2}, Chikkegowda, P.^{2,5}, Tew, G.N.^{1,3}, Minter, L.M.^{1,2,5}

¹UMass Amherst, Graduate Program in Molecular and Cellular Biology, Amherst, United States, ²UMass Amherst, Veterinary and Animal Sciences, Amherst, United States, ³UMass Amherst, Polymer Science and Engineering, Amherst, United States, ⁴UMass Amherst, Biochemistry and Molecular Biology, Amherst, United States, ⁵UMass Amherst, Graduate Program in Animal Biotechnology and Biomedical Sciences, Amherst, United States

Protein kinase C theta (PKCθ) activates several transcriptional programs in T cells responsible for the induction of normal immune responses. PKCθ can be phosphorylated on several residues and essentially, Threonine 538 (Thr538) phosphorylation leads to full T cell activation. There are studies showing PKCθ hyperphosphorylation leads to abnormal immune responses. For instance, patients with aplastic anemia, an immune-mediated bone marrow failure, express higher levels of Thr538 phosphorylation compared to healthy controls. Furthermore, Threonine 219 (Thr219) phosphorylation is necessary for PKCθ to translocate to immunological synapse (IS) strengthening T cell activation. PKCθ functions in the cytosol and in the nucleus; thus, its localization in T cells may result in differential responses. Here, our objective is to elucidate PKCθ-associated molecular signals and function by modulating its phosphorylation status and cellular localization. To achieve this, we delivered an intracellular antibody against Thr538 phosphorylation which blocked nuclear import, we used a small molecule inhibitor to prevent Thr538 phosphorylation, and we used a PKCθ-specific inhibitor to impair IS recruitment. Our results show that different means of targeting PKCθ reveal its distinct molecular mechanisms with regard to T cell signaling. Elucidating these mechanisms in detail may provide a greater understanding of specific PKCθ functions in order to modulate particular immune responses.

3103

Preclinical development of a bispecific antibody that safely and effectively targets CD47 for the treatment of B cell cancers

Johnson, Z.¹, Papaioannou, A.¹, Cons, L.¹, Chatel, L.¹, Chauchet, X.¹, Hatterer, E.¹, Daubeuf, B.¹, Cosimo, E.², Shang, L.¹, Salgado-Pires, S.¹, Nelson, R.¹, Michie, A.², Fischer, N.¹, Kosco-Vilbois, M.¹, Masternak, K.¹, Ferlin, W.¹

¹Novimmune, Geneva, Switzerland, ²Glasgow University, Glasgow, United Kingdom

Immunotherapy represents the most promising new cancer treatment approach in decades and to date most novel immunotherapies rely on harnessing the killing power of T and NK cells to eliminate malignant cells. Exploiting patients' macrophages to kill tumor cells is gaining interest as an alternative, or complementary, approach that holds potential for the future. Many cancers up-regulate the expression of CD47 in order to evade immune surveillance and killing. This

'don't eat me' signal is a molecular means for healthy cells to limit their phagocytic elimination by macrophages and other innate immune cells and represents an interesting therapeutic target. However, the development of anti-CD47 monoclonal antibodies may be hindered due to the ubiquitous expression of CD47, leading to rapid drug elimination kinetics through target-mediated drug disposition, an unfavorable pharmacokinetic profile and target related toxicity including severe anemia. To address this, we have developed a bispecific antibody, NI-1701, that consists of a CD47 antibody arm with an affinity that affords rapid engagement/disengagement kinetics when binding CD47 in a monovalent setting. This arm is paired with a high affinity anti-CD19 arm in a fully human bispecific format resulting in an agent that selectively targets and kills CD19+ B cells. We demonstrate that this novel bispecific antibody is effective at inducing phagocytosis of a range of B cell cancer cell lines as well as patient derived cells, is effective at controlling human B cell tumour growth in mouse xenograft models, and circumvents the clinical pharmacology liabilities seen with a monoclonal antibody targeting CD47.

3680

Anti-conformation monoclonal antibody effective in pre-clinical treatment of full Alzheimer's disease animal models by targeting pathological oligomeric forms of A β and modified Tau

Goni, F.¹, Marta-Ariza, M.¹, Herline, K.¹, Boutajangout, A.¹, Mehta, P.², Drummond, E.¹, Prelli, F.¹, Wisniewski, T.³

¹New York University Langone Medical Center, Neurology, New York, United States, ²NY State Institute for Basic Research in Developmental Disabilities, Immunology, New York, United States, ³New York University Langone Medical Center, Neurology, Pathology, Psychiatry, New York, United States

We have previously demonstrated that a non-self β -sheet oligomerized peptide could elicit an anti-conformational therapeutic response in Alzheimer's Disease (AD) animal models (Goni et al 2013, J Neuroinflammation) and serve as the basis for producing monoclonal antibodies that recognize pathological forms of A β and Tau in human AD brains and brains of AD animal models (Goni et al 2015 Alzheimer & Dementia pp485-6). We have now selected one of these monoclonals, mAb23B, with reactivity to both oligomeric Tau and A β , to treat 16 month old 3xTg AD mice with extensive preexisting A β and Tau pathology. Two groups of 10 animals, of comparable age, were inoculated i.p. biweekly for three weeks and weekly thereafter for 5 weeks with either 100 μ g of mAb23B in 100 μ L of sterile saline or vehicle control. Both groups were then tested for locomotor performance and radial arm maze cognitive analysis. The animals were then sacrificed and the brains harvested for histochemistry and biochemical analysis. No adverse reactions were observed during the infusion period or locomotor differences between groups. The mAb23B infused animals showed significant cognitive rescue compared to controls. This was associated with a non-significant decrease in A β plaques and intracellular PHF, but a significant decrease of the soluble oligomeric forms of A β and Tau. These results indicate that a humanized version of mAb23B would have potential as a treatment for AD.

3726

Selective IL-2 immunotherapy

Boyman, O., Arenas-Ramirez, N., Woytschak, J.

University of Zurich, University Hospital Zurich, Department of Immunology, Zurich, Switzerland

At steady state, interleukin-2 (IL-2) is produced at low levels and serves to maintain CD4⁺ regulatory T (Treg) cell homeostasis. During immune responses, IL-2 levels acutely increase and also stimulate CD8⁺ T cells and natural killer cells. This notion has been translated to clinical use where low-dose recombinant IL-2 has been used for Treg cell-based immunosuppressive strategies in immune pathologies, while high-dose IL-2 has shown clinical efficacy in certain types of metastatic cancer. The detailed characterization, in the recent years, of functional, biophysical and structural properties of IL-2 has allowed the development of IL-2 formulations, including IL-2/anti-IL-2 monoclonal antibody complexes (briefly, IL-2 complexes) and IL-2 muteins. These IL-2 formulations selectively enhance IL-2's immune stimulatory versus inhibitory properties by acting preferentially on CD8⁺ T cells and natural killer cells versus Treg cells. I will discuss selective IL-2 immunotherapy by integrating these data into a framework and highlight recent developments in this field.

45 Minute Oral

12:30:00 - 13:15:00

NK

The secret life of NK cells – the regulatory functions of NK cells are essential to balance immune responses and avoid immune pathology

Schuster, I.S., Coudert, J.D., Fleming, P., Andoniou, C.E., Degli-Esposti, M.A.

University of Western Australia and Lions Eye Institute

Key to effective immune responses is the ability to balance protective immunity with the risk of inappropriate, misdirected or dysregulated immune responses that can cause pathology. Effective immunity requires the coordinated activities of innate and adaptive immune responses. Natural killer (NK) cells are central players in innate immunity. The most acknowledged functions of NK cells are their cytotoxic potential and their ability to release pro-inflammatory cytokines early in the response and without prior activation. Recently however, it has become clear that NK cells are more than assassins. Indeed, NK cells appear capable of mediating a sophisticated series of activities that impact on the functions of both innate and adaptive immunity. Our studies have focused on defining the impact of NK cells on cytokine responses, virus-specific T cell responses and immunopathology. These studies have revealed important interactions between NK cells and macrophages and shown that these are essential for protecting tissues from immune-mediated pathology. We have also defined the impact of NK cell responses on T cell mediated immunity and how this affects the control of chronic viral infection. More recently we have extended this work to show that chronic cytomegalovirus infection is critical in the development of autoimmunity and have highlighted an unexpected regulatory role of NK cells in this process.

Cytokines

New insights into interleukin-1beta release, inflammasome activation and cell death

Pelegri, P.¹, Hodgkin, P.D.^{1,2}, Marchingo, J.M.^{1,2}, Kan, A.^{1,2}, Zhou, J.H.^{1,2}, Hawkins, E.D.^{1,2}, Giang, A.^{1,2}, Lye, B.K.^{1,2}, Duffy, K.R.³, Heinzl, S.^{1,2}

¹Murcia Biomedical Research Institute - University Hospital Virgen Arrixaca,

²The Walter and Eliza Hall Institute of Medical Research, VIC, Australia., ³The University of Melbourne, VIC, Australia., ³Hamilton Institute, Nat

Inflammatory diseases affect over 80 million people worldwide and accompany many diseases of industrialized countries, being the majority of them infection-free conditions. We now know that innate immunity is the main coordinator and driver of inflammation through the secretion of cytokines and other signaling proteins upon innate immune cell activation by

pathogen associated molecular patterns. The activation of purinergic P2X7 receptors in immune cells by extracellular ATP is a novel and increasingly validated “sterile” pathway to initiate inflammation. P2X7 receptor induces the activation of the NLRP3 inflammasome and caspase-1, leading to the release of IL-1beta via unconventional protein release. In the last years, we have gain substantial insights into the release of IL-1beta and other proteins that constitute the secretome of P2X7 receptor activation, being some of them dependent on caspase-1 driving pyroptotic cell death. Extracellular ATP, the physiological P2X7 receptor agonist, is a crucial danger signal released by stressed or injured cells, and one of the most important mediators of infection-free inflammation. We have recently translated this knowledge to human clinical pathology, where the development of selective P2X7 receptors with a suitable clinical profile, increase the therapeutic window to treat inflammatory, metabolic and degenerative diseases.

Innate Receptors

Molecular Mechanism controlling TLR7 responses in plasmacytoid dendritic cells

Miyake, K.

University of Tokyo

The Toll family of receptors have critical roles in microbial recognition and activation of defense responses. Nucleic acid (NA) is a major Toll-like receptor (TLR) ligand. TLR7/8 and 9 are localized in intracellular organelle and sense single strand (ss) RNA and ssDNA, respectively. TLR9 directly binds to short ssDNA fragment, which is generated by DNase II. The structure of TLR8 with ssRNA shows that TLR8 binds to Uridine and di-ribonucleotide UG instead of a ssRNA fragment. TLR7 and 8 are synergistically activated by ssRNA with Guanosine or Uridine, respectively. RNA sensing by TLR7/8 is distinct from DNA sensing by TLR9. Plasmacytoid dendritic cells (pDCs) sense viral infection by TLR7/9, and rapidly produce a large amount of type I interferon (IFN) upon viral infection. TLR9 is shown to traffic upon ligand stimulation and TLR9 trafficking is required for type I IFN. Much less is known about the molecular mechanism underlying TLR7 trafficking and type I IFN induction in pDCs. We here show the molecular mechanism specifically controlling ligand dependent TLR7 trafficking and TLR7-dependent type I IFN induction in pDCs. TLR7 ligand induced rapid cytoskeletal changes and anterograde TLR7 trafficking from the perinuclear region to the cell periphery. The molecules controlling TLR7 trafficking were required for type I IFN induction by TLR7 but dispensable for TLR9 responses. These results demonstrate that innate immune responses to DNA and RNA are differentially regulated in pDCs.

13:30:00 - 15:10:00

Neuroimmunology 2

2863

Identification of eomes-positive T helper cells as a pathogenic factor in chronic neuroinflammation

Raveney, B.¹, Oki, S.¹, Nakamura, M.², Lin, Y.^{1,2,3}, Sato, W.^{1,2}, Yamamura, T.^{1,2,3}

¹National Institute of Neuroscience, National Center of Neurology and Psychiatry, Department of Immunology, Kodaira, Japan,

²National Center Hospital, National Center of Neurology and Psychiatry, Multiple Sclerosis Center, Kodaira, Japan, ³National Center Hospital, National Center of Neurology and Psychiatry, Department of Neurology, Kodaira, Japan

Multiple sclerosis (MS), the autoimmune disease of the central nervous system (CNS), frequently manifests a relapsing/remitting course (RRMS), later shifting to a secondary progressive form (SPMS). Pathogenesis of SPMS is poorly understood and lacks effective therapies. We have previously reported that NR4A2 is upregulated by circulating T cells in RRMS patients and is NR4A2 is expressed by CNS-infiltrating Th17 cells during experimental autoimmune encephalomyelitis (EAE).

Mice with T cell-specific NR4A2 deficiency (NR4A2 cKO) did not develop early/acute clinical EAE symptoms, but instead developed an unexpected late/chronic EAE. Disease reduction was associated with reduced NR4A2-dependent Th17 cells, whereas in late disease, non-Th17 CNS-infiltrating T cells from NR4A2 cKO were pathogenic on transfer.

The absence of NR4A2-dependent T cell responses revealed a novel population of CD4⁺ T cells accumulating in CNS during late/chronic EAE that expressed the transcription factor eomes. Eomes RNAi treatment *in vivo* and CD4-specific eomes knockout suppressed the late/chronic symptoms in NR4A2 cKO. Such eomes⁺Th cells were implicated in granzyme B-mediated cytotoxicity potentially acting directly on PAR-1 receptors expressed by neurons; treatment with PAR-1 activating peptides restored late/chronic EAE in the absence of eomes. Strikingly, eomes⁺ Th cells were also remarkably increased in peripheral blood and further enriched in cerebrospinal fluid of SPMS patients.

Our findings raised the possibility that these previously unappreciated eomes⁺CD4⁺ T cells may be a novel therapeutic target for SPMS. NR4A2 cKO may also provide a useful animal model for investigating pathogenesis of late/chronic CNS autoimmunity.

328

General control non-derepressible 2 (GCN2) controls T_{reg}-mediated remission of acute neuroinflammation via chemokine signaling

Sonner, J.¹, Keil, M.¹, Lanz, T.^{1,2}, Oezen, I.¹, Bunse, T.¹, Bittner, S.³, Meyer, H.⁴, Meuth, S.⁵, Wick, W.^{2,6}, Platten, M.^{1,2}

¹German Cancer Research Center, DKTK CCU Neuroimmunology and Brain Tumor Immunology, Heidelberg, Germany, ²University

Hospital Heidelberg, Department of Neurology and National Center of Tumor Diseases, Heidelberg, Germany, ³University Medical Center of the Johannes Gutenberg-University Mainz, Department of Neurology, Mainz, Germany, ⁴European Molecular Biology Laboratory, European Bioinformatics Institute, Hinxton, United Kingdom, ⁵University of Muenster, Department of Neurology, Muenster, Germany, ⁶German Cancer Research Center, DKTK CCU Neurooncology, Heidelberg, Germany

Multiple sclerosis (MS) is an inflammatory disorder of the central nervous system (CNS) that is caused by an imbalanced immune response to self-antigens. Spontaneous remission from acute neuroinflammation is characterized by infiltration of regulatory T cells (T_{regs}). The tryptophan-degrading enzyme indoleamine 2,3-dioxygenase (IDO) has been implicated in restriction of autoreactive T cell responses as seen in neuroinflammation. Local depletion of tryptophan upon upregulation of IDO expression in response to inflammatory processes is thought to restrict T cell responses partly via activation of the stress kinase general control non-derepressible 2 (GCN2).

Our data show that GCN2 expression in T cells facilitates T_{reg} accumulation in the CNS to alleviate acute neuroinflammation. T cell-specific GCN2 knockout mice presented with a specific failure of spontaneous remission and impaired remission was associated with reduced T_{reg} frequencies in the inflamed CNS, while infiltration by T helper (T_H) 1 and T_H17 cells was not altered. This T_{reg}-specific defect was due to impaired migration towards a chemokine gradient.

In conclusion, these results identify GCN2 as a key player for T_{reg} infiltration into the CNS during acute neuroinflammation and point to a novel molecular mechanism that regulates chemokine signaling via GCN2 activation.

1470

Assessment of vasoactive neuropeptide receptor expression in varying severities of chronic fatigue syndrome/myalgic encephalomyelitis (CFS/ME)

Johnston, S.^{1,2}, El-Zahar, M.², Nguyen, T.^{1,2}, Knauth, E.¹, Staines, D.¹, Marshall-Gradisnik, S.^{1,2}

¹Menzies Health Institute Queensland, National Centre for Neuroimmunology and Emerging Diseases, Parklands, Australia,

²Griffith University, School of Medical Science, Parklands, Australia

Vasoactive neuropeptides (VN) are implicated in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) symptomatology due to their involvement in neurological-immunological communication as they are able to modulate the immune system. Preliminary research findings have reported significant changes in VN receptor numbers in T cells, however, the T cell phenotype(s) are yet to be investigated. The aim of this study was to investigate the level of VN receptor expression in T cells phenotypes, such as CD4 and CD8 cells among healthy individuals compared to CFS/ME patients with varying severities of this illness. Participants were classified as control (n=15, age 43.47±3.46), moderate CFS (n=15, age 39.47±2.82) or severe CFS (n=7 age 41.71±3.71). All CFS/ME participants meet the International Consensus Criteria (ICC) and were further classified as moderate or severe based on the Dr

Bell's CFS Disability scale. VN receptors VPAC1, VPAC2 and PAC1 expression on CD4 and CD8 T-cell subsets was investigated in stimulated and unstimulated conditions using flow cytometry. Severe CFS/ME patients showed a significant increase in VPAC2 expression on CD4+ T-cells when stimulated compared to healthy controls with trends being observed in expression of other receptors between groups. An increase in VPAC2 expression indicates dysregulation of the Th1/Th2 pathway. The dysregulation of the Th2 pathway may result from changes in VPAC2 expression in severe CFS/ME participants and their possible clinical presentation.

1979

CD8 T cell-mediated killing of orexin-producing neurons induces a narcolepsy-like phenotype in mice

Bernard-Valnet, R.^{1,2}, Yshii, L.¹, Queriaux, C.¹, Matthys, A.¹, Bauer, J.³, Pignolet, B.¹, Dauvilliers, Y.⁴, Peyron, C.⁵, Liblau, R.^{1,2}

¹Center for Pathophysiology Toulouse Purpan, Toulouse, France,

²CHU de Toulouse, Toulouse, France, ³Medical University of

Vienna, Center for Brain Research, Vienna, Austria, ⁴Gui-de-Chauliac Hospital, CHU de Montpellier, Department of Neurology,

Montpellier, France, ⁵Center for Research in Neuroscience of Lyon, Lyon, France

Narcolepsy with cataplexy is a rare and severe sleep disorder caused by destruction of orexinergic neurons. The disease is associated with genetic, environmental and biological factors pointing to an autoimmune origin. However, current animal models of narcolepsy, based on genetic disruption of the orexinergic neurotransmission or genetic destruction of the orexin⁺ neurons, do not allow studying its aetiology. Our goal was to generate a mouse model in order to decipher the immune mechanisms leading to orexin⁺ neurons loss and narcolepsy development. To this end, mice expressing influenza virus hemagglutinin (HA) in hypothalamic orexin⁺ neurons (called Orex-HA) were developed. The consequences of an autoimmune attack on these neurons were evaluated by adoptive transfer of effectors T lymphocytes specific for HA. Considering, the tight association of narcolepsy with the HLA-DQB1*06:02 allele, we first tested the pathogenic contribution of CD4 Th1 cells. However, although Th1 cells penetrated the hypothalamic and triggered local inflammation, they did not promote loss of orexin⁺ neurons or clinical manifestations of narcolepsy. In stark contrast, the transfer of cytotoxic CD8 T cells (CTLs) led to both T cells infiltration and specific destruction of orexin⁺ neurons. In situ, CTLs interacted directly with MHC class I-expressing orexin⁺ neurons, resulting in cytolytic granule polarization toward neurons. This neuronal loss caused manifestations mimicking human narcolepsy in the Orex-HA mice. The phenotype was further aggravated upon repeated injections of CTLs. This work demonstrates the potential role of CTLs as final effectors of the immunopathological process in narcolepsy.

2663

EphA2 is necessary for the development of experimental cerebral malaria

King, T.¹, Mimche, P.¹, Bray, C.¹, Brady, L.¹, Meyer, E.², Galinski, M.², Lamb, T.¹

¹Emory University, Pediatrics Infectious Disease, Emory University School of Medicine, Atlanta, United States, ²Yerkes National Primate Research Center, Emory Vaccine Center, Atlanta, United States

Cerebral malaria (CM) is a leading cause of death from *Plasmodium falciparum* infection, yet the mechanisms of disease pathology are poorly understood. Eph receptors, the largest family of receptor tyrosine kinases in humans, have been linked to several neurological disorders although their roles in CM remain unknown. Experimental cerebral malaria (ECM) is a lethal condition resulting from *Plasmodium berghei* ANKA (PbANKA) infection of C57BL/6J mice that recapitulates key features of human CM including sequestration of leukocytes and parasitized red blood cells (pRBCs) in the brain along with blood-brain barrier (BBB) breakdown. Here we show that upregulation of EphA2 mRNA in brains of PbANKA-infected C57BL/6J mice is a feature of ECM absent in non-lethal *Plasmodium* infections including PbNK65. PbANKA-infected EphA2^{-/-} mice, unlike EphA2^{+/+} mice, have an intact BBB at ECM onset. This is likely because accumulation of CD8⁺ T cells, which mediate ECM development, is greatly reduced in brains of PbANKA-infected EphA2^{-/-} mice despite normal splenic expansion. Unlike EphA2^{+/+} mice, PbANKA-infected EphA2^{-/-} mice are protected from death even with peripheral and organ-sequestered parasite levels similar to EphA2^{+/+} mice suggesting BBB integrity, not parasite burden, is responsible for improved survival in EphA2^{-/-} mice. Therapeutically targeting EphA2 also leads to increased survival of PbANKA-infected C57BL/6J mice. Along with preliminary data showing *P. falciparum*-induced upregulation of ephrin-A mRNA in human PBMCs and EphA2 mRNA in human brain endothelial cells, our data suggests a novel, crucial role for EphA2 in ECM with potential translation to human CM.

3350

Regulation of CD4 T cell-mediated inflammation by cognate interactions with B cells in experimental autoimmune encephalomyelitis

Parker Harp, C.R.¹, Archambault, A.S.², Sim, J.³, Russell, J.H.³, Wu, G.F.^{1,2}

¹Washington University School of Medicine, Pathology & Immunology, St. Louis, United States, ²Washington University

School of Medicine, Neurology, St. Louis, United States,

³Washington University School of Medicine, Developmental Biology, St. Louis, United States

The mechanisms by which B cells direct CD4 T cells in an antigen-specific, major histocompatibility complex II (MHCII)-dependent manner during inflammatory demyelination of the central nervous system (CNS) are unclear. Previously, we demonstrated that antigen presentation exclusively by B cells is sufficient to propagate CNS inflammatory demyelination in the animal model of multiple sclerosis, experimental autoimmune

encephalomyelitis (EAE). However, a delay in onset of disease is observed when B cells are the exclusive antigen presenting cell during EAE. These results raise questions regarding the timing and location of cognate interactions between B cells and CD4 T cells during secondary phases of autoimmune neuro-inflammation.

Passive EAE using encephalitogenic CD4 T cells from wild-type (WT) mice was induced by transfer into WT mice and mice with MHCII expression restricted to B cells that express a higher frequency of myelin oligodendrocyte glycoprotein (MOG)-specific receptor (termed IgH^{MOG}xB^{MHCII}). EAE was also induced in mice with temporal regulation of MHCII expression by antigen-specific B cells, (termed IgH^{MOG}xB^{Tam-MHCII}).

Tamoxifen administration rapidly induced maximal MHCII expression within three days. Disease was dependent on VLA4 expression by B cells. IgH^{MOG}xB^{Tam-MHCII} mice were resistant to passive EAE until tamoxifen administration 30 days post cell transfer, resulting in EAE onset within three days. Furthermore, decreasing the duration between cell transfer and tamoxifen administration increased the time to disease onset. Our findings indicate that B cells are capable of accessing CNS myelin targets and eliciting antigen-specific CD4 T cell-dependent neuro-inflammation to dictate the tempo of neuro-inflammation during EAE.

3529

Inflammatory monocytes recruited from the bone marrow in West Nile virus or dengue encephalitis have distinctive differentiative fates in the CNS

Vu, L.D.¹, King, N.J.C.²

¹University of Sydney, Charles Perkins Centre, Pathology, Camperdown, Australia, ²University of Sydney, Pathology, Camperdown, Australia

West Nile (WNV) and Dengue viruses (DENV) are both flaviviruses associated with significant neurological disease. In WNV encephalitis we have shown that infiltrating Ly6C^{hi} monocytes directly correlate with disease development and mortality, but the differentiation and function of these cells remain contentious. Comparing lethal WNV and non-lethal DENV encephalitis in mice, we found two distinct differentiation patterns of bone marrow-derived monocytes (BMM) infiltrating the brain. During WNV encephalitis, BMM differentiated into Ly6C^{hi}CD11c^{low}MHCII^{int} inflammatory macrophages in an inoculation route-independent manner, while in DENV encephalitis, BMM gave rise to Ly6C^{hi}CD11c^{hi}MHCII^{hi} dendritic cells (DC). Intravenous adoptively transferred BMM from WNV-infected mice into DENV-infected mice and vice versa, differentiated in the brain into DC and inflammatory macrophages, respectively, indicating that the brain, not the bone-marrow milieu, drives the fate of infiltrating BMM. Intriguingly, however, in the DENV model, differentiation of BMM into DC was abrogated when WNV donor cells were injected intracranially, suggesting a requirement for 'priming' during leukocyte extravasation for subsequent downstream differentiation in the brain. Pathway analysis of gene transcription between DENV- and WNV-infected brains showed greater activation of the transforming growth factor-beta (TGF- β) -signalling pathway, including TGF- β , vasoactive

intestinal peptide and IL-16, in DENV- than WNV-infected brains. This pathway may modulate differentiation to favour generation of tolerogenic DC. We hypothesise that the local balance between pro- and anti-inflammatory responses, beginning at the endothelium, governs the subsequent differentiation of inflammatory monocytes infiltrating into the CNS. Understanding mechanisms underlying this differentiation may suggest potential therapeutic opportunities to control CNS inflammation.

1357

Neuroinflammation drives the neuronal degenerative phenotype in a rat model of Ataxia-telangiectasia

Quek, H.¹, Luff, J.¹, Cheung, K.^{1,2}, Kozlov, S.¹, Gatei, M.¹, Lee, C.S.³, Bellingham, M.⁴, Noakes, P.⁴, Lim, Y.C.², Barnett, N.¹, Dingwall, S.⁵, Wolvetang, E.⁵, Mashimo, T.⁶, Roberts, T.^{1,3}, Lavin, M.¹

¹The University of Queensland, Centre for Clinical Research, Brisbane, Australia, ²QIMR Berghofer Medical Research Institute, Brisbane, Australia, ³Western Sydney University, Ingham Institute for Applied Medical Research, Liverpool, Australia, ⁴The University of Queensland, School of Biomedical Sciences, Brisbane, Australia, ⁵The University of Queensland, AIBN, Brisbane, Australia, ⁶Osaka University Graduate School of Medicine, Osaka, Japan

Neuroinflammation contributes to disease progression in neurodegenerative disorders including Alzheimer's disease and Amyotrophic Lateral Sclerosis. Ataxia-telangiectasia (A-T), an autosomal recessive disease caused by mutations in the ATM gene is characterised by progressive neurodegeneration which has been poorly recapitulated in *Atm* mutant mice. Consequently, pathways leading to neurodegeneration in A-T are poorly understood. Here we show that *Atm* knockout rats display a neuroinflammatory phenotype. Loss of *Atm* in neurons leads to accumulation of cytosolic DNA, increased cytokine production and constitutive activation of microglia. Rats lacking ATM had significant loss of motor neurons and microgliosis in the spinal cord, consistent with onset of paralysis. Betamethasone treatment extended *Atm* knockout rats' lifespan, prevented microglial activation and significantly decreased neuroinflammation and motor neuron loss. These findings provide a basis for neurodegeneration in A-T patients and rationale for anti-inflammatory drugs as treatment options for A-T patients and more generally in neurodegenerative diseases.

3866

Protection against development of a mouse model of multiple sclerosis by a parasite-derived 68-mer peptide

Dixit, A.¹, Donnelly, S.², Lund, M.A.², Dalton, J.P.³, Greer, J.M.¹

¹The University of Queensland, UQ Centre for Clinical Research, Brisbane, Australia, ²University of Technology Sydney, The School of Life Sciences, Sydney, Australia, ³Queen's University Belfast, School of Biological Sciences, Belfast, United Kingdom

Helminths (parasitic worms) can exert protective effects on autoimmune diseases by modulating the type of immune response, and deliberate infection with helminths is being

explored as a potential therapeutic strategy for autoimmunity. However, the use of live helminths as therapeutic agents for autoimmune disease has a number of drawbacks, and it would be preferable to instead identify and use purified immunomodulatory components of the helminths. Previously it has been shown that the immunomodulatory activity of the liver fluke *Fasciola hepatica* resides in its excretory-secretory products (FhES), and further analysis of FhES has identified 3 major components: a 68 amino acid alpha helical cathelicidin-like peptide (FhHDM1), a cathepsin L-cysteine protease (FhCL1), and peroxiredoxin (FhPrx). In the current study, the ability of these three components to modify the course of a relapsing-remitting experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis (MS) was tested. FhHDM1 was the most effective, significantly ($p < 0.0001$) reducing the overall severity of the disease and the number of EAE relapses compared to mice treated with vehicle alone or mice treated with FhPrx. The effects were long-lasting, with mice continuing to show benefits for up to 70 days following a single course of FhHDM1 treatment. Preliminary investigation of the mechanism of action of FhHDM1 suggests that it does not affect the adaptive arm of the immune response, but instead exerts its effects by modulation of innate pro-inflammatory responses. The data suggest that this parasite-derived peptide has potential as a novel therapeutic agent for treatment of MS.

1675

Protective roles for microglia in neuroinflammation

Włodarczyk, A.¹, Holtman, I.R.², Bruttger, J.³, Yogev, N.³, de Boer-Bergsma, J.J.², Nolling Jensen, K.¹, Martin, N.A.⁴, Karram, K.³, Boddeke, E.W.G.M.², Waisman, A.³, Eggen, B.J.L.², Owens, T.¹

¹Institute of Molecular Medicine, University of Southern Denmark, Odense, Denmark, ²University Medical Center Groningen, University of Groningen, Groningen, Netherlands, ³Institute for Molecular Medicine, University Medical Center of the Johannes Gutenberg University, Mainz, Germany, ⁴Institute of Clinical Research, Odense University Hospital, Odense, Denmark

Microglia are central nervous system (CNS)-resident immune cells. They are often referred to as brain macrophages and indeed share many phenotypic features with them. However, it is now recognized that they are of a separate lineage and are not replaced from blood-derived precursors, at least under normal circumstances. Microglia are implicated in neuroinflammatory and neurodegenerative diseases including multiple sclerosis (MS). We have shown that in a mouse model for MS-experimental autoimmune encephalomyelitis (EAE) numbers of microglia expressing CD11c significantly increase. These CD11c⁺ microglia are effective antigen presenting cells, but poor inducers of Th1 or Th17 responses. Interestingly, CD11c⁺ microglia express neuroprotective insulin-like growth factor 1 which suggests a neuroprotective rather than proinflammatory role. Here we show that CD11c⁺ microglia predominated in the neonatal brain and expressed genes governing neuronal and glial survival, migration and differentiation. These cells were localized in sites of primary myelination such as cerebellum and corpus callosum. They expanded rapidly after birth and then contracted to become almost undetectable in the adult CNS.

Neonatal and microglia from EAE CNS differed in their gene expression profiles, showing neurogenic and immune response gene signatures, respectively. Moreover, neonatal microglia showed a transient stem-cell like phenotype that was partially recapitulated by cells repopulating the adult CNS after microglia ablation. Interestingly, transplantation of neonatal microglia suppressed EAE. CD11c⁺ microglia therefore can deliver signals necessary for neurogenesis and myelination.

Metabolism

3536

Impaired cellular metabolism and inhibitory receptor expression during chronic infection are linked by IRF4

Man, K.¹, Kallies, A.²

¹The Walter and Eliza Hall Institute of Medical Research, Parkville, Australia, ²The Walter and Eliza Hall Institute, Parkville, Australia

During chronic infection T cells acquire an exhausted phenotype that is characterized by high expression of inhibitory receptors and down-modulation of effector function. While this is required for the protection of the organism from excessive immunopathology, it also prevents successful immunity against persistent viruses or tumor cells. The molecular mechanisms that lead to T cell exhaustion are only incompletely understood. Here, we demonstrate that exhausted T cells exhibited impaired cellular metabolism, which was mediated by the TCR-dependent transcription factor IRF4. Blockade of inhibitory PDL1-PD1 interactions restored aspects of metabolic function, which was critical for improved population expansion and CD8 T cell function, thereby revealing a molecular circuit that links cellular metabolism and immune checkpoints. Thus, in addition to its critical role in promoting clonal population expansion and effector differentiation in the early phase of an immune response, IRF4 is required for the adoption of the exhausted T cell phenotype during chronic infection.

4177

CD28 induces metabolic fitness in long-lived plasma cells through Slp76 for reactive oxygen species-dependent survival

Utle, A.¹, Carlson, L.², Peng, P.², Mahpour, A.¹, Maharaj, S.¹, Ventro, D.¹, Murray-Dupuis, M.³, Lee, K.¹

¹Roswell Park Cancer Institute, Immunology, Buffalo, United States, ²Roswell Park Cancer Institute, Buffalo, United States, ³Duke University Medical Center, Raleigh, United States

Sustained humoral immunity is dependent upon the continual production of antigen specific antibodies by plasma cells. Upon activation, B cells develop into short-lived plasma cells (SLPCs) which home to secondary lymphoid organs and live up to several weeks before undergoing apoptosis. In a second non-mutually exclusive model, plasma cells can home to the bone marrow where they reside in competitive survival niches and can exist the lifetime of an organism as long-lived plasma cells (LLPCs). We have shown that CD28, the canonical T cell co-stimulatory molecule, is required for LLPC survival

and antibody production; however, the mechanistic basis for this is unclear. Upon T cell activation, CD28 is known to induce glycolysis at the expense of mitochondrial respiration. To our great surprise, CD28 increases mitochondrial respiration and the spare respiratory capacity in LLPCs through the adaptor molecule Slp76. Furthermore, although CD28 does not directly affect glycolysis, the glycolytic capacity and glycolytic reserve are increased in LLPCs. Paradoxically, mitochondrial respiration derived reactive oxygen species (ROS) are required for CD28-mediated LLPC survival. CD28 activates NF κ B through ROS for Irf4 mediated upregulation of cMyc, which subsequently drives mitochondrial biogenesis. This leads to a self-reinforcing model wherein CD28 induces metabolic fitness in LLPCs through ROS-dependent signaling to NF κ B and subsequent Irf4-cMyc mediated mitochondrial biogenesis. Due to the highly biosynthetic nature of plasma cells and the limited nutrient availability in the bone marrow microenvironment, CD28 may govern the ability of LLPCs to compete in the survival niche for survival and continual antibody production.

1390

Chronically stimulated CD4 T cells exhibit a defective glycolytic metabolism yet produce rapid effector function after PD-1 blockade

Braun, M.¹, Bettonville, M.¹, D'Aria, S.¹, Porporato, P.², Sonveaux, P.²
¹Universite Libre de Bruxelles, Institute for Medical Immunology, Gosselies, Belgium, ²Universite Catholique de Louvain, Pole of Pharmacology and Therapeutics (FATH), Brussels, Belgium

Continuous exposure to antigen profoundly modifies T cell function and chronically-stimulated T cells develop a state of unresponsiveness characterized by a weakened capacity to proliferate and mount effector function. Unresponsiveness is maintained by engagement of cell-surface inhibitory receptors, such as PD-1, and immunotherapies targeting these receptors have proven to be effective in restoring T cell function. How persistent stimulation influences metabolic requirements for T cell function is unknown. We have developed a mouse model of chronic antigen exposure in which antigen-specific CD4 T cells are adoptively maintained in recipients expressing a systemic antigen. Proteomic analysis indicated that many enzymes involved in cellular metabolism were down-regulated in these cells. Consequently, the cells displayed low basal metabolism compared to naive or effector T cells. Moreover, they also exhibited reduced GLUT1 expression and mitochondrial mass. Consequently, upon activation, the cells carried out limited glucose uptake and ATP synthesis. Though blockade of PD-1/PD-L1 inhibitory pathway fully restored rapid effector function, such as IFN γ secretion, it did not switch metabolism towards aerobic glycolysis. Interestingly, PD-1 blockade also stimulated mitochondrial superoxide production in chronically-stimulated T cells and specific scavenging of superoxide, though promoting cell survival, inhibited IFN γ production. Thus, 1) chronically-stimulated CD4 T cells have a glycolysis-independent capacity to produce rapid IFN γ responses; 2) PD-1 blockade promotes mitochondrial dysfunction in these cells which stimulates IFN γ production. These observations should be considered when blocking PD-1/

PD-L1 for the treatment of chronic diseases.

1999

SHIPi alters innate immunity in VAT to control obesity

Kerr, W.¹, Sudan, R.², Youngs, C.², Howard, K.³, Russo, C.³, Engelman, R.⁴, Chisholm, J.³, Srivastava, N.²

¹SUNY Upstate Medical University, Microbiology & Immunology and Pediatrics, Syracuse, United States, ²SUNY Upstate Medical University, Syracuse, United States, ³Syracuse University, Chemistry, Syracuse, United States, ⁴Moffitt Cancer Center, Comparative Medicine and Pathology, Tampa, United States

Low-grade chronic inflammation has been established as the key etiological phenomenon responsible for the initiation and perpetuation of obesity and its associated metabolic disorders. Novel therapeutic approaches that can specifically target inflammatory pathways are very much needed to avert this looming epidemic of obesity & diabetes. Genetic & chemical inhibition of SH2 domain containing inositol phosphatase (SHIP)1 has been associated with systemic expansion of immunoregulatory cells that promote a lean-body state however its function in immunometabolism has never been assessed. This led us to investigate the role of SHIP1 in metabolic disorders during excess caloric intake in mice. Using a small molecule inhibitor of SHIP (SHIPi), here we show that SHIPi treatment in mice significantly reduces body-weight and fat content and improves control of blood glucose and insulin sensitivity as well as increases energy expenditure, despite continued consumption of a HFD. Additionally, SHIPi reduces age-associated fat accumulation in mice. We find that SHIPi treatment reverses diet-associated obesity by attenuating inflammation in the visceral adipose tissue (VAT). SHIPi treatment increases IL4-producing eosinophils in VAT and consequently increases both AAM and MDSC. In addition, SHIPi decreases the number of γ -IFN producing T-cells and NK-cells in VAT. Thus, SHIPi represents a novel approach that permits control of obesity and diet-induced metabolic syndrome without apparent toxicity that could benefit the health of millions worldwide.

3444

Lymphocyte activation gene 3 controls metabolic profile of naïve and activated CD4⁺ T cells

Piganelli, J.D.¹, Previte, D.M.², Menk, A.V.³, Delgoffe, G.M.³

¹University of Pittsburgh School of Medicine/Children's Hospital of Pittsburgh, Surgery, Immunology and Pathology, Pittsburgh, United States, ²University of Pittsburgh/Children's Hospital of Pittsburgh, Surgery, Pittsburgh, United States, ³University of Pittsburgh School of Medicine, Immunology, Pittsburgh, United States

LAG-3 is an inhibitory receptor surfaced expressed on CD4⁺ T cells that is upregulated upon MHCII/TCR engagement and eventually cleaved, for optimal CD4⁺ T cell activation. LAG-3 negatively regulates T cell homeostatic expansion, and LAG-3^{-/-} naïve T cells exhibit increased expansion in lymphopenic hosts as well as accelerated diabetes in NOD mice. Current interest in cellular metabolism has revealed that CD4⁺ T cell function is tightly coupled to its metabolic profile. Therefore, given that

LAG-3 is a regulator of CD4⁺ T cell proliferation and effector function, we hypothesized LAG-3 expression controls the metabolic profile of CD4⁺ T cells. Naïve T cells primarily rely on oxidative phosphorylation for basal metabolic needs. Naïve CD4⁺ T cells were isolated from wildtype (WT) and LAG-3^{-/-} animals, and oxygen consumption was measured. LAG-3^{-/-} CD4⁺ T cells showed enhanced basal respiration (OCR) and mitochondrial biogenesis measured by the ratio of mitochondrial to nuclear DNA ($p < 0.05$) and transcription factor TFAM levels ($p < 0.05$). The AMPK/Sirt-1 signaling pathway was also enhanced. Upon activation, CD4⁺ T cells alter their metabolic profile, becoming highly glycolytic. Activated LAG-3^{-/-} CD4⁺ T cells compared to WT demonstrated increased glucose uptake, lactate production ($p < 0.01$), and increased expression of glycolysis-associated genes. This heightened activation suggests that the metabolic phenotype exhibited in naïve LAG-3^{-/-} CD4⁺ T cells may be advantageous during activation. These results indicate LAG-3 as a potent regulator of cellular metabolism in naïve and activated CD4⁺ T cells, thus providing new avenues to exploit for targeted therapeutics to inhibit aberrant immune responses.

3977

Cytomegalovirus infection enhances development of glucose intolerance and insulin resistance in obesity

Šestan, M.¹, Valentić, S.¹, Reichel, J.J.², Brizić, I.², Jonjić, S.^{1,2}, Wensveen, F.M.¹, Polić, B.¹

¹University of Rijeka, School of Medicine, Department of Histology and Embryology, Rijeka, Croatia, ²University of Rijeka, School of Medicine, Center for Proteomics, Rijeka, Croatia

Obesity and its complication Diabetes Mellitus type 2 (DM2) represent global health problems. It is well established that low-grade chronic inflammation, which originates in obese visceral adipose tissue (VAT), is an underlying cause of insulin resistance (IR) and DM2. It is characterized by accumulation of Th1-type immune cells in VAT and secretion of pro-inflammatory cytokines. Thus, obese VAT resembles immune response to a viral infection. Although obese people are very often exposed to viral infections, very little is known how a viral infection contributes to the progression of DM2. Cytomegaloviruses are species-specific beta-herpesviruses. Majority of humans are infected with HCMV and after acute infection they establish a life-long latency. Infection of mice with MCMV represents a well-accepted model for HCMV infection.

In this work we investigated how MCMV infection influences the development DM2 in well-established mouse model of diet induced obesity (DIO). The infection was associated with a rapid accumulation of activated NK cells, which was followed by a dramatic increase of M1 macrophages, CD4 and CD8 T cells. MCMV infection itself did not induce GI in mice fed with normal chawing diet. However, it induced fast progression of GI and IR after only 4 - 6 weeks of high fat diet treatment in comparison to the DIO mice. Depletion of NK cells reduced accumulation of M1 macrophages in VAT, suggesting that NK cells were mostly responsible for the induction. Altogether, our results show that MCMV infection can cause aggravation of VAT-inflammation and induce progression of DM2 in obesity.

2666

Saturated fatty acids-induced metabolic stress leads to biased effector memory CD4⁺ T cell differentiation via mTOR-independent, PI3K p110d/Akt-mediated signals

Smith, J.¹, Mauro, C.¹, Cucchi, D.^{1,2}, Coe, D.¹, Fu, H.¹, Bonacina, F.³, Baragetti, A.³, Catapano, A.^{3,4}, Ammirati, E.⁵, Longhi, M.P.¹, Okkenhaug, K.⁶, Norata, G.D.³, Marelli-Berg, F.¹

¹Queen Mary University of London, William Harvey Research Institute, London, United Kingdom, ²Istituto Pasteur, Fondazione Cenci Bolognetti, Rome, Italy, ³Università degli Studi di Milano, Department of Pharmacological and Biomolecular Sciences, Milan, Italy, ⁴IRCCS Multimedica, Milan, Italy, ⁵Niguarda Hospital, Milan, Italy, ⁶Babraham Institute, Laboratory of Lymphocyte Signalling and Development, Cambridge, United Kingdom

Obesity is associated with low-grade systemic inflammation, which is believed to contribute to the development of its cardiovascular complications. Smouldering inflammation in obesity involves activation of adaptive immunity and infiltration of adipose and vascular tissue by T cells with an effector phenotype. However, the signals leading to T cell activation and targeted tissue infiltration during obesity are only beginning to be understood.

We have tested the hypothesis that high-fat diet (HFD)-induced metabolic stress directly affects the differentiation and trafficking of CD4⁺ T cells to non-lymphoid tissues. We found that memory CD4⁺

T cells primed in HFD-fed donors preferentially migrate in response to the pro-inflammatory chemokine CXCL10 *in vitro* and to non-lymphoid inflammatory sites *in vivo*. The existence of a differentiation bias during priming in HFD-fed animals was confirmed by experiments showing that CD4⁺ T lymphocytes priming during HFD regimen preferentially develop into CD44^{hi}-CD62L^{lo}-CCR7^{lo}-CXCR3⁺-LFA1⁺ effector-like memory T cells in mice. A similar CD4⁺ T cell phenotype was observed when a cohort of n=1,172 subjects selected from a large survey of free-living people was systematically analyzed.

Mechanistically, we show that this developmental bias is independent of any crosstalk of CD4⁺ T cells with dendritic cells but is mediated by direct exposure of CD4⁺ T cells to the saturated fatty acids, i.e. palmitate - enriched in HFD -, leading to increased activation of a PI3K p110d/Akt-dependent and -mTOR-independent pathway downstream of TCR engagement. The observed developmental bias of CD4⁺ T cells can be corrected by selective inactivation of the PI3K p110d/Akt pathway.

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Precision immunometabolic checkpoint treatment of lethal murine sepsis: targeting the NAD⁺ and sirtuin 1-dependent homeostasis network

Liu, T.^{1,2}, Vachharajani, V.³, Zhang, X.¹, Xu, J.¹, McCall, C.²

¹Scientific Research Center, Shanghai Public Health Clinical Center, Fudan University, Shanghai, China, ²Wake Forest University School of Medicine, Molecular Medicine, Winston-Salem, United States, ³Wake Forest University School of Medicine, Anesthesiology, Winston-Salem, United States

Sepsis inflammation kills millions each year worldwide and all anti-inflammatory treatments have failed. Molecular-based precision medicine is urgently needed to reduce this global health care crisis. Toward meeting this goal, we developed a precise molecular-based treatment approach for improving sepsis outcome in mice by targeting an epigenetic immunometabolic checkpoint, which is controlled by NAD⁺ sensor nuclear sirtuin 1 (SIRT1). Mechanistically, nuclear SIRT1 couples with nuclear SIRT6 and mitochondrial SIRT3 to switch the glycolysis-dependent immune effector state (cytokine storm) to a fatty acid oxidation-dependent immune repressor state. In a successful sepsis response, the SIRT1 immunometabolic checkpoint and its network links resolve sepsis by restoring homeostasis. During lethal sepsis, the SIRT1 checkpoint becomes inflexible, resulting in sustained immune repression and failure of pyruvate to fuel the mitochondrial electron transport chain. This immunometabolic paralysis promotes infection and multiorgan failure. We previously reported that precisely inhibiting SIRT1 during immunometabolic failure retrieves checkpoint function, rebalances homeostasis and rescues septic mice from death. Here, we show that opening the pyruvate mitochondrial portal for glucose oxidation by inhibiting the repressor function of pyruvate dehydrogenase kinase—a downstream node in the nuclear SIRT1 and SIRT6 checkpoint network—also relieves immunometabolic paralysis, promotes bioenergy homeostasis and rescues septic mice from death. We conclude that precision medicine can improve sepsis outcomes by targeting immunometabolic checkpoints that impede homeostasis retrieval.

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Pdcd4 inhibits macrophage lipoautophagy and promotes foam cell formation and atherosclerosis in mice

Wang, L., Jiang, Y., Zhang, L.

Shandong University, Immunology, Jinan, China

Macrophage foam cells, a major component of the atherosclerotic lesion, play vital roles in the development of atherosclerosis. Lipoautophagy, a type of autophagy characterized by selective delivery of lipid droplet for lysosomal degradation, may impact atherosclerosis by regulating macrophage foam cell formation. Previously, we reported that programmed cell death 4 (PDCD4), a tumor suppressor, negatively regulated autophagy in tumor cells. However, its roles in macrophage lipoautophagy, foam cell formation, and atherosclerosis remain to be established. Here, we found that Pdcd4 deficiency clearly improved oxidized low-density lipoproteins (ox-LDL)-impaired autophagy efflux, promoted autophagy-mediated lipid breakdown in murine macrophages, and thus prevented macrophage conversion into foam cells. Importantly, Pdcd4 deficiency in mice significantly upregulated macrophage autophagy in local plaques along with attenuated lipid accumulation and atherosclerotic lesions in high fat-fed Apolipoprotein E (ApoE) knockout mice. Bone marrow transplantation experiment demonstrated that Pdcd4-mediated autophagy in hematopoietic cells contributed to the development of atherosclerosis. These results indicate that endogenous PDCD4 promotes for macrophage foam cell formation atherosclerosis development via inhibiting autophagy

and provides new insights into atherogenesis, suggesting that promoting macrophage autophagy through down-regulating Pdcd4 expression may be beneficial for treating atherosclerosis.

4038

Modulating lipopolysaccharide-induced cytokine secretion profiles of monocytes by manipulating metabolic pathways

Jones, N., Thornton, C.A.

Institute of Life Science, Swansea University Medical School,

Swansea, United Kingdom

Monocyte activation demands energy to fuel effector functions such as pro-inflammatory cytokine production with ATP. Glycolysis and oxidative phosphorylation are the two main energy-producing pathways within mammalian cells. Many types of human leukocytes undergo activation-induced metabolic rewiring preferentially toward aerobic glycolysis, a process initiated with a six-carbon monosaccharide precursor, typically glucose. This metabolic switch is known as the Warburg effect. However, little is known about whether monocytes possess this glycolytic switch.

Here we investigate the involvement of different respiratory pathways in IL-1 β , IL-6, IL-10 and TNF α production by lipopolysaccharide (LPS)-stimulated monocytes; respiratory pathways were inhibited with 2-deoxy-D-glucose (2-DG; glycolysis), oligomycin (oxidative phosphorylation), and etomoxir (fatty acid oxidation). The effect of different monosaccharide fuels (glucose, fructose, mannose and galactose) was also considered.

Inhibition of glycolysis and fatty acid oxidation reduced LPS-stimulated production of all cytokines but only IL-10 production was reduced with inhibition of oxidative phosphorylation. By measuring LPS-induced changes in extracellular acidification rates it was shown that monocytes do possess the glycolytic switch and utilise glucose as a primary energy source. Monocytes can also metabolise other monosaccharides especially mannose and to a much lesser extent, fructose and galactose. In contrast, LPS-stimulated cytokine outputs were increased with fructose and galactose compared to glucose and mannose.

Understanding the differential pathways and metabolic fuels contributing to monocyte function could have potential implications for understanding and treating inflammatory conditions.

Immunity to Parasites 2

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Liver resident memory T cells express CXCR6 and patrol in the sinusoids

Holz, L.^{1,2}, Fernandez-Ruiz, D.¹, Zaid, A.^{1,2}, Ng, W.Y.^{1,3}, Wong, Y.C.⁴, Mollard, V.⁵, Cozijnsen, A.⁵, Davey, G.¹, Bowen, D.⁴, Tay, S.S.⁴, Lahoud, M.^{3,6}, Bertolino, P.⁴, Mueller, S.^{1,2}, McFadden, G.⁵, Caminschi, I.^{3,6}, Heath, W.^{1,2}

¹Peter Doherty Institute, University of Melbourne, Parkville, Australia, ²ARC Centre of Excellence in Advanced Molecular Imaging, University of Melbourne, Parkville, Australia, ³Macfarlane Burnet Institute for Medical Research & Public Health, Melbourne,

Australia, ⁴Centenary Institute and AW Morrow Gastroenterology and Liver Centre, Newtown, Australia, ⁵The School of Biosciences, University of Melbourne, Parkville, Australia, ⁶Monash University, Clayton, Australia

Malaria is the most prevalent parasitic infection in the world and a major cause of mortality, with approximately 600,000 deaths in 2013. The liver stage of malaria represents a bottleneck for the parasite so we have focused on developing a vaccine targeting this phase in mice. Using a novel approach we have generated large numbers of tissue-resident memory cells (T_{RM}) in the liver that can protect against malaria. Briefly, PbT-I/uGFP cells (CD8⁺ transgenic T cells specific for malaria) are transferred into B6 mice and primed in lymphoid tissues by treating the recipient mice with an adjuvant and a malaria peptide conjugated to anti-Clec9A. A day later the mice are infected with adeno-associated virus expressing the same malaria epitope, allowing the PbT-I/uGFP cells to interact with antigen in the liver where they eliminate infected hepatocytes and form T_{RM} . These liver T_{RM} display a unique cell surface phenotype and express a gene signature associated with other resident memory populations. Intravital imaging of the liver one month post-vaccination revealed a mixed population of cells, one population patrolling the sinusoids and a second rapidly transiting the liver. Using PbT-I T cells expressing CX3CR1-GFP or CXCR6-GFP, we show that the rapidly transiting T cell population are those PbT-I cells that develop into circulating effector memory whereas the patrolling population are T_{RM} . Using CXCR3-specific antibodies, we specifically eliminate PbT-I T_{RM} which abrogates protection against sporozoite challenge. This suggests liver T_{RM} cells represent an effective weapon in the arsenal for malaria vaccine development.

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Interleukin-6 and type I Interferon-signalling control ICOS-mediated humoral immunity to malaria

Sebina, L.¹, James, K.¹, Soon, M.¹, Best, S.¹, Hertzog, P.², Engwerda, C.¹, Haque, A.¹

¹QIMR Berghofer Medical Research Institute, Brisbane, Australia,

²Hudson Institute of Medical Research, Melbourne, Australia

Parasite-specific antibodies are effective at controlling blood-stage *Plasmodium* infections. However, generating these in humans has proven difficult, via natural exposure or vaccination. Explanations for this have focused on antigenic variation by the parasite, but have not considered whether host generation of antibody responses is sub-optimal. As a result, host immune factors that serve to control antibody responses remain poorly characterized. Using mouse models of blood-stage malaria, we explored roles for *Plasmodium*-induced innate cytokines, interleukin-6 (IL-6) and Type I Interferon (IFN-I), in controlling CD4⁺ T cell-dependent humoral immunity. Firstly, we demonstrated that CD4⁺ T cells and ICOS-signalling were crucial for germinal centre (GC) B cell and plasmablast responses. Next, we found that IL-6 was essential for optimal parasite control, and for production of parasite-specific, complement-fixing antibodies, IgM and IgG2b. Moreover, GC B cells, plasmablast responses, and ICOS expression by emerging and established

Tfh cells all required IL-6-signalling. In contrast, IFN-I signalling, which proceeded via conventional dendritic cells, but not T or B cells, suppressed parasite control, production of parasite-specific IgM and IgG, as well as GC B cell, plasmablast and Tfh cell responses. IFN-I-signalling acted early, by limiting ICOS expression on activated CD4⁺ T cells, and restricting their movement towards B cells that were required to cement the emerging Tfh phenotype. Finally, IFN-I blockade boosted early B cell responses in a manner partially dependent on IL-6. Thus, we reveal innate cytokine responses, long-established to occur during blood-stage *Plasmodium* infection, as possible targets for improved antibody-mediated immunity to malaria.

1227

Immune-driven alterations in mucin glycosylation are essential for *Trichuris muris* worm expulsion

Hasnain, S.Z.¹, Lourie, R.¹, Hutson, P.G.², Dawson, P.³, Grencis, R.K.⁴, Thornton, D.J.⁵

¹Mater Research Institute-University of Queensland, Immunity, Infection and Inflammation Group, Brisbane, Australia, ²Mater Research Institute-University of Queensland, Mater Pathology, Brisbane, Australia, ³Mater Research Institute-University of Queensland, Developmental Disorders Group, Brisbane, Australia, ⁴University of Manchester, Manchester Immunology Group, Manchester, United Kingdom, ⁵University of Manchester, Wellcome Trust Centre for Cell-Matrix Research, Manchester, United Kingdom

Mucins are heavily glycosylated proteins that play an important role in rejection of the nematode, *Trichuris muris* (*T. muris*). The glycans decorating the mucin protein core can alter the protective properties of the mucus barrier. Therefore, we investigated changes in glycosylation during *T. muris* infection, using different infection doses and strains of mice that are resistant (high dose infection in BALBc/C57BL6 mice) or susceptible (high dose infection in AKR and low dose infection in BALBc mice) to *T. muris* infection. During chronicity, within the immediate vicinity of the *T. muris* nematode the goblet cell thecae contained mainly sialylated mucins. In contrast, maintained sulphation on the mucins was apparent in the goblet cells within the epithelial cell crypts in the resistant models. Examination of human appendices with chronic enterobiosis showed unglycosylated mucin accumulation within the endoplasmic reticulum compared to parasite free controls, consistent with a parasite-induced disruption of mucin processing. The maintained mucin sulphation (controlled by a T_H2 -immune response) contributed to the protective properties to the mucus layer, making it less vulnerable to degradation by *T. muris* exo-products. Interestingly, *T. muris* egg hatching was diminished in the sulphate anion transporter1^{-/-} mice, which were susceptible to a chronic infection despite strong T_H2 -immune response. This study highlights the complex process by which immune-driven alterations in glycosylation occur following *T. muris* infection, which contributes to resistance to chronic parasitic infection.

2454**HIF-1 α impairs monocyte's effector function and induces M2 macrophage polarization during chronic *Leishmania donovani* infection**

Hamami, A., Abidin, B.M., Charpentier, T., Fabié, A., Heinonen, K., Stäger, S. Institut Nationale de la Recherche Scientifique INRS, Institut Armand Frappier IAF, Laval, Canada

Leishmania donovani impairs the immune response by triggering a hypoxic micro-environment that facilitates the establishment of a chronic infection. Under this condition, HIF-1 α , the master regulator of the response to low oxygen tension, is stabilized in all cell populations. The effect of hypoxia on the immune response to *Leishmania* has not yet been investigated. Particularly, the role of HIF-1 α in macrophage and monocyte function during chronic visceral leishmaniasis remains unknown. Here, we investigate the role of HIF-1 α in inflammatory monocyte and dendritic cell functions, and in the modulation of macrophage polarization during chronic *L. donovani* infection. To this end, we generated CD11c-specific HIF-1 α knock-out mice that were then infected with *L. donovani*. Our data demonstrate that HIF-1 α limits inflammatory monocyte recruitment to the spleen and induces M2 macrophage polarization in response to extracellular lactate. The ablation of HIF-1 α in CD11c⁺ cells resulted in decreased intracellular lactate concentrations, lower expression of M2 macrophage markers, greater expansion and effector capacity of monocytes, and enhanced IFN γ production by CD4⁺ T cells. Moreover, mice with a targeted depletion of HIF-1 α in CD11c⁺ cells had a significantly lower splenic parasite burden, suggesting that induction of HIF-1 α in CD11c⁺ cells may represent a key factor adopted by *Leishmania* parasites to establish persistent infections.

3787**PD-L2 is crucial for establishing effective CD4⁺ Th1 immunity against malaria**

Wykes, M. QIMR Berghofer, Brisbane, Australia

Malaria is caused by *Plasmodium* parasites, which after introduction into the host by mosquitoes proceed to infect hepatocytes followed by red blood cells. It is the blood-stage infection that causes the symptoms and lethality associated with malaria. Many pathogens, including *Plasmodium* spp., exploit the programmed death-1 (PD-1)/PD-1 ligand-1 (PD-L1) pathway to 'deactivate' T cell functions but the role of PD-1/PD-L2 remains unclear. We studied malarial infections to understand the contribution of PD-L2 to immunity. Here we show that higher PD-L2 expression on blood dendritic cells (DC), from *Plasmodium falciparum*-infected individuals, correlated with lower parasitemia. Mechanistic studies in mice showed that PD-L2 was indispensable for establishing effective CD4⁺ Th1 immunity. Importantly, administration of soluble PD-L2 to mice with lethal malaria was sufficient to dramatically improve immunity and survival (92% vs 0%). These studies show a new function for PD-L2, which has potential to be translated into an effective treatment for malaria and other diseases where T cell immunity is ineffective or short-lived due to PD-1.

1742**Protein tyrosine phosphatase inhibition enhances the generation of protective Tr1 cells and attenuates the brain sequestration of pathogenic T cells during experimental cerebral malaria**

*Van Den Ham, K.^{1,2}, Richer, M.¹, Olivier, M.^{1,2}
¹Mc Gill University, Microbiology and Immunology, Montréal, Canada, ²Research Institute of the McGill University Health Centre, Montréal, Canada*

Neuropathology induced by *Plasmodium berghei* ANKA infection is dependent on the sequestration of cytotoxic CD8⁺ T cells within the brain microvasculature and augmentation of the inflammatory response. Inflammation is integral to controlling parasitemia, but can result in tissue damage if unregulated. IL-10 has the capacity to suppress inflammation and has also been implicated in limiting tissue parasite burden. Recently, IL-10-producing Foxp3⁺ CD4⁺ T cells were identified in *P. falciparum*-infected children and were determined to have a role in the regulation of pathogenic inflammation. Modulation of protein tyrosine phosphorylation has the capacity to alter the immune response and has previously been demonstrated to mitigate pathology in models of leishmaniasis, asthma and cancer. Here we determined that pharmacological inhibition of protein tyrosine phosphatase (PTP) activity markedly protected mice from developing experimental cerebral malaria (ECM). Protection was concomitant with an increase in IL-10⁺ Foxp3⁺ CD4⁺ T cells, which were shown to be largely comprised of LAG-3⁺CD49b⁺ type 1 regulatory cells. Moreover, the enhanced IL-10 production was established to be a major component of the protection afforded by PTP inhibition. Additionally, the decreased incidence of ECM was associated with significantly reduced sequestration of CD8⁺ T cells within the brain. And this attenuated accumulation correlated with decreased cell surface expression of CXCR3 on splenic CD8⁺ T cells. Overall, our study suggests that pharmacological modulation of host PTPs could provide a novel mechanism for the development of new immunotherapies to treat parasitic infections.

3324**The role of TREM-1 in the severity of human cutaneous leishmaniasis through a translational study**

*Ampuero, M.^{1,2}, Nunes, S.¹, Feijó, D.¹, Khouri, R.¹, Boaventura, V.^{1,2}, Barral, A.^{1,2}, Brodskyn, C.^{1,2}, Tavares, N.³
¹Fundação Oswaldo Cruz - FIOCRUZ, Centro de Pesquisas Gonçalo Moniz - CPqGM, Salvador, Brazil, ²Universidade Federal ba Bahia, Salvador, Brazil, ³Fundação Oswaldo Cruz - FIOCRUZ, Salvador, Brazil*

Leishmaniasis is a neglected disease caused by Leishmania. The immune response is critical for parasite killing, but it also accounts for inflammation and disease severity. The Triggering Receptors Expressed on Myeloid Cells (TREM) was recently identified as an amplifier of the innate immune response, which synergize with Toll-like receptors (TLRs) in the production of proinflammatory cytokines. TREM-1 is mainly expressed in neutrophils and its signaling depends on the adapter protein DAP12, resulting in cell degranulation, production of reactive oxygen species (ROS)

and activation of NFκB. We used bioinformatics tools to analyze public transcriptome data of lesions from patients infected with *Leishmania braziliensis*. The expression of all factors involved in TREM-1 pathway is increased. We validated these findings by Real-Time PCR with fresh samples of blood and biopsies. The expression of DAP12 and TLR4 messenger RNA (mRNA), but not of TREM-1 and TLR2, is significantly increased in biopsies. The expression of these mRNA is not altered in peripheral blood mononuclear cells (PBMC). However, there is a down regulation in TREM-1, DAP12, TLR2 and TLR4 expression in resting neutrophils. Moreover, TREM-2, that act as counterregulatory molecule, attenuating inflammation, was not detected in any of these samples. In conclusion, TREM-1 is differentially regulated between different compartments and its modulation depends on the presence of inflammatory stimulus. Preliminary data from flow cytometry suggests that TREM-1 expression is increased in infected macrophages. Further ongoing assays will be important to define the role of TREM-1 in the balance between inflammation and parasite killing.

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Omics profiling of *Leishmania major*-infected human macrophages: a systems-level approach

Atri, C., Mkannez, G., Bali, A., Sghaier, M.R., Attia, H., Laouini, D., Ghouila, A., Guerfali, F.Z.

Institut Pasteur de Tunis, Immunology/Laboratory of Transmission, Control and Immunobiology of Infections, Tunis-Belvedere, Tunisia

Leishmania (L.) are intracellular protozoan parasites able to survive and replicate in the hostile phagolysosomal environment of infected macrophages in human host. To establish a successful infection and ensure their own survival, *Leishmania* have developed sophisticated strategies to subvert the host macrophage responses. As a result, a complex interplay takes place between the parasite and the macrophage, that subtends for a large part the clinical manifestations of leishmaniasis diseases.

Despite huge community efforts, only a small number of molecules are known to play a role during the infectious process. In order to provide the most complete profiling of invaded cells during this host-parasite conflict at different levels, we used transcriptomics (microarrays), regulomics (miRNAs) and proteomics profilings through a kinetic of infection during the first 24 hours of contact between monocyte-derived human macrophages and *L. major* metacyclic promastigotes. Using different available analyzing tools we identified molecules, networks, top functions and canonical pathways to be deregulated during the infection processes, tightly related to chemokine signaling, Toll Like receptor signaling or apoptotic pathways. This comprehensive approach is likely to allow the identification of new molecules and functional pathways that could be targeted by new treatments to this parasitic infection.

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During infection, native *Trypanosoma cruzi* calreticulin mediates growth inhibition of a mammary tumor

Abello, P., Pizarro Bäuerle, J., Rosas, C., Maldonado, I., Sosoniuk Roche, E., Ferreira, A.

University of Chile, Faculty of Medicine, Immunology, Santiago, Chile

Decades ago, researchers from the former Soviet Union, detected that *Trypanosoma cruzi* (the agent of American Trypanosomiasis) infection inhibits tumor development. The molecular basis underlying this phenomenon has remained largely unknown. We propose that, in *T. cruzi* infected animals, native Calreticulin (nTcCRT), an endoplasmic reticulum (ER)-resident chaperone, translocated and externalized by the parasite, mediates, at least an important part of this anti tumor effect. Previously, we showed that, *in vitro*, TcCRT by virtue of its capacity to interact with Complement component C1 is a potent inhibitor of its classical pathway. Moreover, inactive C1q, still bound to TcCRT, acts as an 'eat me' signal, thus promoting infectivity (defined here as the capacity to contact and infect mammal host cells). This property is important for the parasite capacity to interact with both endothelial and a tumor cell line (the aggressive murine mammary adenocarcinoma TA3-MTXR). In agreement with this proposal, we have specifically neutralized the anti tumor properties of infection, with polyclonal anti-rTcCRT F(ab')₂ antibody fragments, devoid of their Fc-dependent capacity to recruit C1q. Moreover, these antibody fragments were used to specifically reverse the capacity of rTcCRT to inhibit EAhy926 endothelial cells proliferation. Thus, angiogenesis may be important in this tumor development. Moreover, anti-rTcCRT antibodies reversed the antitumor effect of epimastigotes (that express but do not translocate TcCRT) extracts. In synthesis, during *T. cruzi* infection, nTcCRT mediates important anti-tumor effects.

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Allergy 4

1586

Eosinophil lineage commitment and IL-5-dependent expansion is regulated by IL-33 in mice

Johnston, L.¹, Hsu, C.-L.¹, Krier-Burris, R.¹, Chhiba, K.¹, Chien, K.¹, McKenzie, A.², Berdnikovs, S.¹, Bryce, P.¹

¹Northwestern University Feinberg School of Medicine, Department of Medicine, Division of Allergy-Immunology, Chicago, United States, ²MRC Laboratory of Molecular Biology, Cambridge, United Kingdom

Eosinophils are important in the pathogenesis of many diseases, including asthma, eosinophilic esophagitis and eczema. While IL-5 is necessary for the maturation of eosinophil progenitors (EoP) into mature eosinophils (EoM), the signals that promote commitment to the eosinophil lineage are unknown. The IL-33 receptor, ST2, is expressed on several inflammatory cells, including eosinophils, and is best characterized for its role during the initiation of allergic responses in the peripheral tissues. Recently, ST2 expression was described on

hematopoietic stem cells, where its function remains unclear. Here, we sought to determine whether IL-33 and ST2 contribute to hematopoietic lineage decisions. We found that both IL-33- and ST2-deficient mice exhibited diminished peripheral blood eosinophils at baseline. Correspondingly, IL-33 administration increased EoM as well as IL-5 in the blood and bone marrow in WT and IL-33-deficient but not ST2-deficient mice. Blocking IL-5 with a neutralizing antibody prevented IL-33-expanded EoP from maturing into EoM, while transgenic overexpression of IL-5 in ST2-deficient mice resulted in significantly lower hypereosinophilia than transgenic IL-5 mice. Finally, we observed that IL-33, but not IL-5, specifically expanded EoP and upregulated IL-5Ra on EoP as well as increased IL-5 after bone marrow was cultured for three days. Our findings identify a basal defect in eosinophilopoiesis in IL-33- and ST2-deficient mice. Furthermore, we establish unappreciated roles for IL-33 and ST2 in eosinophil development via progenitor regulation and define a mechanism whereby IL-33 licenses commitment into the eosinophil lineage by driving both responsiveness to IL-5 and IL-5 production.

3690

Pulmonary NK cells prevent allergic airway sensitization: regulation by CB2-active eicosanoids

Roberts, K., Ferrini, M., Jaffar, Z.

University of Montana, Biomedical & Pharmaceutical Sciences, Missoula, United States

In allergic asthma, inhalation of airborne allergens such as house dust mite (HDM) effectively activates both innate and adaptive immunity in the lung mucosa. The contribution of pulmonary NK cells to the innate events preceding sensitization of the airways to the common house dust mite (HDM) allergen remains unclear. Cannabinoid CB2-active mediators are known to impact innate immunity including responses by monocytes and NK cells. To determine the role of the CB2-active eicosanoids during allergic sensitization, allergic airway inflammation was induced in mice by sensitization via the airways by intranasal HDM instillation, and responses in wild type (WT) and CB2-deficient (CB2^{-/-}) mice were compared. Mice lacking CB2 receptor exhibited elevated numbers of pulmonary CD3⁺CD19⁺NK1.1⁺ NK cells but were resistant to the induction of allergic airway inflammation and showed diminished airway hyperreactivity, pulmonary eosinophilia, T_H2 cytokine production and mucus secretion after HDM allergen inhalation. Cellular depletion and adoptive transfer studies were undertaken to dissect the mechanisms involved. Depletion of NK cells restored HDM responsiveness. Conversely, transfer of CB2^{-/-} NK cells into WT mice suppressed the allergic airway inflammation and was associated with a reduction in monocyte-derived dendritic cells but elevated CX3CL1 release and recruitment of Ly6C⁺CX3CR1⁺ monocytes. These studies demonstrate a hitherto unknown role for CB2 activation in allergic lung disease and reveal a role for NK cells in preventing allergic sensitization to HDM allergen.

1956

In vivo optical imaging of skin allergy inflammation

Zhang, Z., Liu, Z., Luo, M., Luo, Q.

Huazhong University of Science and Technology, Wuhan National Laboratory for Optoelectronics, Wuhan, China

Optical molecular imaging is the most promising tools for investigating the function and motility of immune cells *in vivo*. Immuno-Optimaging as a new interdisciplinary research area has been developed for a decade. Here, we developed a multi-scale optical imaging approach to evaluate the spatio-temporal dynamic behavior and properties of immune cells from the whole field of organs to the cellular level at the inflammatory site: the recruitment of leucocytes was directly visualized using whole-field fluorescent imaging at the organ and tissue levels; the dynamic distribution of the leukocytes was directly visualized using large-scale scanning microscopy at the single-cell level; the motility and migration dynamics of leukocytes were visualized in real time and analyzed using time-lapse confocal imaging or two-photon excitation microscopy at the single-cell level with high temporal resolution. Meanwhile, some multi-color labeling mouse models based on the exogenous labeling with fluorescent protein and the endogenous labeling with fluorescent dye were established. The dynamic properties and function of immunocytes

(e.g. monocyte-macrophages and neutrophils) in the skin allergy inflammation (e.g., the delayed type hypersensitivity reaction or contact hypersensitivity) were visually investigated using multi-scale optical imaging techniques combined with the multi-color labeling mice model.

3557

Intrinsic- and extrinsic- mediated CTLA-4-signals shape the T_H2 response: implications for allergic disorders

Pierau, M., Brunner-Weinzierl, M.C.

Otto-von-Guericke University, Children`s Hospital, Magdeburg, Germany

T helper (T_H) 2 lymphocytes play a crucial role in the initiation, progression and persistence of IgE-mediated allergic diseases. During the sensitization phase, the differentiation and clonal expansion of allergen-specific CD4⁺ T_H2-cells producing IL-4, IL-5, IL-10 and IL-13 is essential. During this step, a memory population of allergen-specific T-cells is also generated. Memory immune responses are long lived, antigen specific, and are characterized by a rapid and robust cytokine response to allergens. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is an inhibitory co-stimulatory molecule expressed by regulatory T-cells (Tregs) and activated T-cells. In this study, we will determine the role of intrinsic (CTLA-4 on CD4⁺ T_H2-cells) and extrinsic (CTLA-4 on Tregs) mediated CTLA-4-signals during the generation of effector and memory T_H2-subpopulations, with special emphasis on the role of Tregs.

For the characterisation of the T_H2-effector and -memory pool *in vitro* and *in vivo* we analysed multifunctional T-cells which synchronously produce several cytokines by boolean gating flow cytometry as well as expression of typically hallmark T_H2 molecules.

We found that intrinsic as well as extrinsic mediated CTLA-4-signals modulate the quality of T_H2-effector and -memory cells in vitro and in vivo.

Summarizing the different T_H2-cytokine-subpopulations in vitro revealed that CTLA-4^{-/-} T_H2-cells show an increased frequency of single-, double-, triple- and quadruple- producers. In vivo, quantity and quality of T_H2-effector cells in the memory pool was mainly dependent on extrinsic-mediated CTLA-4 signals triggered by Tregs during the priming phase.

Modulation of CTLA-4 signal transduction could be an option for therapeutic interventions of allergic diseases.

993

Unravelling modes of HLA-drug-TCR interaction in HLA-associated adverse drug reactions

Illing, P.¹, Mifsud, N.¹, Fettke, H.¹, Lai, J.¹, Ho, R.¹, Kwan, P.², Purcell, A.¹

¹Monash University, Infection and Immunity Program, Monash Biomedicine Discovery Institute, Biochemistry and Molecular Biology, Clayton, Australia, ²The Royal Melbourne Hospital, The University of Melbourne, Parkville, Australia

Adverse drug reactions (ADRs) impose a major economic burden on our health care system, and preclude certain individuals from utilising routine medications. A subset of ADRs are associated with specific alleles of the Human Leukocyte Antigen genes. The strongest associations reported are between HLA-B*57:01 and abacavir hypersensitivity syndrome, HLA-B*58:01 and allopurinol hypersensitivities, and HLA-B*15:02 and carbamazepine induced Stevens-Johnson Syndrome/Toxic Epidermal Necrolysis. A weaker association also exists between carbamazepine hypersensitivities and HLA-A*31:01. We have previously shown that abacavir causes a major alteration in the array of self-peptide ligands presented by HLA-B*57:01 via stable co-occupation of the antigen binding cleft, and stimulates robust, polyclonal CD8+ T cell responses. Here, we expanded this study to explore modes of interaction between allopurinol and carbamazepine (including structurally related drugs and metabolites) and ADR-associated HLA molecules. We found that carbamazepine and oxypurinol (the major metabolite of allopurinol) appear to interact in a more labile fashion with the ADR-associated HLA than abacavir and have a limited impact on peptide presentation. Paired analysis of T cell receptor (TCR) alpha and beta chains using a novel multiplex nested RT-PCR methodology at a single cell level, indicated that CD8+ T cell responses against carbamazepine were more restricted than those to abacavir. Responses to oxypurinol showed some diversity. Expression of identified TCRs in TCR-negative SKW3 T cell lines allowed confirmation of specificity and exploration of the mechanisms of drug presentation. These data suggest alternate modes of HLA-peptide-drug-TCR interaction that may cause a spectrum of diversity in the T cell response.

1722

FIBCD1: a novel regulator of Th2 immune responses

Pilecki, B.¹, Fuchtbauer, D.¹, Thomsen, L.K.¹, Gotfred-Rasmussen, H.¹, Schlosser, A.¹, Sorensen, G.L.¹, Moeller, J.B.^{1,2}, Holmskov, U.¹

¹University of Southern Denmark, Institute of Molecular Medicine,

Odense, Denmark, ²Cornell University, Weill Cornell Medical College, New York, United States

FIBCD1 is a transmembrane protein highly expressed by intestinal and pulmonary epithelial cells. We have previously identified and characterized FIBCD1 as an endocytic receptor that binds acetylated structures including fungal and helminth-derived chitin, suggesting its involvement in host defence. Moreover, we have recently generated FIBCD1-deficient mice and shown their susceptibility to Th1/Th17-mediated intestinal inflammation induced by dextran sodium sulfate.

In the current study we aim to investigate FIBCD1 role in Th2-mediated intestinal and airway inflammation. B6N wild-type mice and FIBCD1-deficient littermates were subjected to

1) oxazolone-induced inflammatory bowel disease or

2) ovalbumin (OVA)-induced allergic asthma models.

We found that FIBCD1-deficient mice were protected from Th2-dominated gut and lung inflammation. Oxazolone-induced weight loss and decrease in colon length as well as histopathological signs of intestinal inflammation were all strongly attenuated in FIBCD1-deficient mice compared to wild-type littermates. Furthermore, we observed that FIBCD1-deficient mice were partially protected from ovalbumin-induced airway hyperresponsiveness and inflammation, major hallmarks of allergic asthma.

Obtained data suggest FIBCD1 involvement in Th2-like immune response. While the detailed mechanisms underlying FIBCD1 action are currently being investigated, our results indicate that FIBCD1 is a novel immune regulator and may be required for the optimal mobilization of a normal Th2 response.

3827

Exploring the IL7-IL7R pathway: a comparative approach confirms reduced gene expression in atopic dogs and a function in prenatal immune system development of chicks

Mazrier, H.¹, Vogelnest, L.J.¹, Kohl, A.², Taylor, R.M.¹, Sela-Donenfeld, D.², Wei, J.¹, Williamson, P.¹

¹School of Life and Environmental Sciences, Faculty of Veterinary Science, University of Sydney, Australia, ²Koret School of Veterinary Medicine, Robert H. Smith Faculty of Agriculture, Food and Environment, Hebrew University of Jerusalem, Rehovot, Israel

The signalling pathway of interleukin-7 (IL7) is known to regulate T-lymphocyte development. Accumulating evidence supports its involvement in inherited human immune-dysfunction diseases. Variants of the IL7-receptor alpha subunit gene (*IL7R*, or *CD127*) are associated with risk for human multiple sclerosis, diabetes, atopic dermatitis (AD), and some T-cell lymphomas. Considering the homology between dog and human gene sequences, complex-inherited canine immune diseases may serve as a useful animal model to study the role of this gene-pathway in human. Canine AD is a genetically-linked hypersensitivity which has early onset and shares similar clinical signs to human AD. Recent human studies suggest that the pathogenesis of AD involves immune dysregulation, orchestrated by cytokines associated with T-lymphocytes and skin barrier impairment. Our previous studies of canine AD revealed that IL7 was the most altered plasma cytokine

(elevated concentrations; 19 cytokine/chemokine multiplex bio-assay; $p=0.003$) while the IL7R gene showed the greatest differential expression (mRNA expression array of leukocytes; $p=0.001$) between affected and controls. The current study used a comparative approach to further explore the role of the IL7-IL7R pathway in canine AD and in-ovo. Several genes belonging to the IL7-IL7R pathway had decreased leukocyte expression patterns in atopic dogs, using quantitative real-time PCR (qRT-PCR). Immunofluorescence and qRT-PCR studies of normal chick embryos (E2.5 to E9) established expression of *IL7R* in early thymus development, suggesting a developmental function. Understanding the involvement of the IL7-IL7R pathway in canine AD, and its role in perinatal immune development may provide novel insights to its function in human immune-related diseases.

4189

IL-33 enhances IL-9-producing mucosal mast cell function in IgE-mediated food allergy

Wang, Y.-H., Liu, J., Shik, D., Smith, A.

Cincinnati Children's Hospital Medical Center, Cincinnati, United States

Food allergy affects approximately 2-4% of older children and adults and the prevalence of this disease is increasing significantly in developed nations. Little is known about the mechanisms that underlie the propensity to develop food allergy-induced, life-threatening anaphylaxis. We have identified a novel population of intestinal IL-9-producing mucosal mast cells (MMC9s) in mice that functions in promoting an anaphylactic response to ingested antigens. MMC9s are scarce in immunologically naïve mice, but accumulate in the gut of mice with TSLP-induced atopic dermatitis following ingestion of food antigens. Repeated intragastric antigen challenge induces significant accumulations of both MMC9 and CD4⁺T_{H2} cells, which correlates positively with symptoms and susceptibility to experimental food allergy. Mechanistically, following ingestion of food antigens, IL-33 expression is induced in mouse intestinal epithelium. MMC9s can produce high levels of IL-9, IL-13 in response to IL-33 and mast cell proteases 1 (chymase) and 6 (tryptase) in response to antigen/IgE complex crosslinking. By producing significant amounts of IL-9, MMC9s cause pronounced intestinal mastocytosis and the production of mast cells. Indeed, mice overexpressing intestinal IL-33 are more prone to develop gastrointestinal manifestations after repetitive intragastric OVA challenge; on the contrary, mice deficient of IL-33 or IL-1R1 (ST2) exhibit attenuated pathology of IgE-mediated intestinal anaphylaxis. Finally, atopic patients that develop food allergy display increased intestinal expression of *Il9* and MC-specific transcripts. These findings suggest that epidermal TSLP-initiated CD4⁺T_{H2} immune response drives MMC9 development in the gastrointestinal (GI) tract and that intestinal epithelial-derived IL-33 then amplifies MMC9 function to promote IgE-mediated food allergy.

1091

The environment alters the immune response to ragweed pollen

Liu, S.-H.¹, Debiasi, M.¹, Anea, C.B.¹, Karrer, G.², Chaturvedi, P.³, Bellaire, A.⁴, Weckwerth, W.³, Epstein, M.M.¹

¹Medical University of Vienna, Department of Dermatology, DIAID, Vienna, Austria, ²University of Natural Resources and Applied Life Sciences, Vienna, Austria, ³University of Vienna, Department of Molecular Systems Biology, Vienna, Austria, ⁴University of Vienna, Department of Botany and Biodiversity Research, Vienna, Austria

Ambrosia artemisiifolia (ragweed) is a highly invasive plant with pollen that causes allergy. Using an experimental mouse model of ragweed pollen-induced allergic disease, we sought to determine whether environmental factors e.g., climate and pollution exacerbate pollen allergy. Ragweed pollen from several sources including some collected from urban and rural areas in Austria were repeatedly administered intranasally (i.n.) to female BALB/c mice. Pollen were either untreated or treated with low pH, high temperature and various pollutants including ozone to mimic acid rain, extreme heat and drought periods, and ground and air pollution. When treated or untreated ragweed pollen suspensions (10 µg) were administered i.n. 6 times over a 3-week period, mice developed lung and airway inflammation, mucus hypersecretion, and high serum ragweed-specific IgG1 titres in a dose-dependent fashion. Interestingly, disease was more severe with pollen from urban compared with rural areas. Similarly, *in vitro*-treated pollen generated differential *in vivo* responses. Taken together, these data demonstrate that the environment alters the allergenicity of ragweed pollen. These results have serious ramifications on environmental health and well-being and underscore the importance of addressing climate change and air quality issues by policy makers. Further studies are necessary to elucidate the mechanism underlying the environmental effect on pollen.

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Sex-related differences in the interaction of CD4⁺ T cells with CD8⁺ T cells lead to female-dominant allergic airway inflammation

Miyasaka, T.¹, Ito, C.¹, Dobashi-Okuyama, K.¹, Masuda, C.¹, Kawano, T.¹, Ohkawara, Y.¹, Kikuchi, T.², Takayanagi, M.¹, Ohno, I.¹

¹Tohoku Pharmaceutical University, Department of Pathophysiology, Sendai, Japan, ²Niigata University Graduate School of Medical and Dental Sciences, Department of Respiratory Medicine and Infectious Diseases, Niigata, Japan

The severity of asthma, which is correlated positively with the intensity of type 2 T-helper (Th2)-driven airway inflammation, in adults are higher in women than men. The number of interleukin (IL)-13-producing T cells in peripheral blood lymphocytes is increased in female asthmatics compared with male asthmatics. However, it remains to be clarified how sex differences in Th2-oriented immune responses are generated in asthma. In the present study, we addressed sex-related regulation of Th2 immune responses by CD8⁺ T cells using a murine model of allergic asthma. Airway inflammation characterized by the accumulation of eosinophils and lymphocytes in the airways

and IL-4 contents in the lungs were significantly higher in female than male wild type (WT) mice, whereas no such sex differences were observed in CD8 knock-out (KO) mice. In WT mice, IL-4 production by CD4⁺ T cells prepared from bronchial lymph nodes were almost equivalent between male and female mice. Interestingly, IL-4 production by male CD4⁺ T cells was significantly reduced in the presence of male CD8⁺ T cells, but not female CD8⁺ T cells, while the production by female CD4⁺ T cells was not affected by the presence of either CD8⁺ T cells. Furthermore, the transfer of male CD8⁺ T cells, but not female CD8⁺ T cells, into male CD8KO mice significantly reduced the accumulation of inflammatory cells compared with saline transfer. These results suggest that the higher production of IL-4 in female mice may be attributable to sex-related difference in the suppressive activity of CD8⁺ T cells.

Vaccines 4

1879

Out-manipulating the manipulator: development of a live attenuated human cytomegalovirus vaccine

Tomic, A.¹, Varanasi, P.R.², Borst, E.M.¹, Golemac, M.³, Malic, S.³, Mischak-Weisinger, E.M.², Riese, P.⁴, Guzman, C.⁴, Krmpotic, A.³, Jonjic, S.³, Messerle, M.¹

¹Hannover Medical School, Department of Virology, Hannover, Germany, ²Hannover Medical School, Department of Hematology, Hannover, Germany, ³Faculty of Medicine, University of Rijeka, Department of Histology and Embryology, Rijeka, Croatia, ⁴Helmholtz Centre for Infection Research, Department of Vaccinology, Braunschweig, Germany

Human cytomegalovirus (HCMV) is a major cause of morbidity in congenitally infected newborns and immunocompromised individuals. Thus, there is an utmost need to develop a HCMV vaccine to protect these vulnerable groups. CMVs have unique impact on the immune system and are therefore considered as promising vaccine candidates. In a mouse model expression of a host NKG2D ligand by CMV turned out to be a successful strategy to develop a live attenuated vaccine vector. Insertion of the NKG2D ligand not only contributed to the early control of the virus by NK cells, but also improved

T cell responses.

Results of this study show for the first time that a similar approach can be applied to HCMV. The expression of an NKG2D ligand in HCMV improved the safety profile and contributed to the preservation of immunogenicity of the virus as shown in *ex vivo* stimulation assays using primary human cells and *in vivo* in a humanized mouse model. Surprisingly, expression of the NKG2D ligand led to concomitant activation of innate and adaptive immunity, accomplished by distinct mechanisms. NK cell activation was only partially dependent on NKG2D, while activation of CD8⁺ T cells involved NKG2D. This mode of regulation was mediated by an unexpected influence of the NKG2D ligand on expression of MHC class I molecules.

These results indicate that a recombinant HCMV expressing NKG2D ligand can be used and further developed as a potent vaccine vector.

4223

Lipovaxin, a versatile chelating liposomal vaccine platform for surface-loading of recombinant antigens to generate self-adjuncting immune-stimulatory particles for improved immune responses

MacLennan, N., Price, J., Rusden, A., Gosling, K., Atmosukarto, I. Lipotek Pty Ltd, Canberra, Australia

Lipotek have developed a liposome platform that utilises chelating lipid-based immune-stimulatory nanoparticles for the co-delivery of recombinant antigens and immune potentiating agents. Antigens are attached to the surface of Lipovaxin liposomes by way of the commonly used poly-histidine tag engineered in the recombinant protein, which anchors the protein to a proprietary chelating lipid included in the liposome bilayer. As a result, antigens are all anchored in the same orientation and are displayed as highly ordered array on the surface of the liposomes, a conformation that mimics the display of surface antigens on viral particles. Chelating liposomes can be prepared with various immune-stimulatory properties, by including TLR ligands to achieve disease-relevant vaccine design.

This approach has been tested successfully with a number of antigens, including the model antigen OVA, a fusion of the tuberculosis antigen fusion ESAT6-Ag85C and the malaria antigen MSP2. In combination these studies have confirmed that particle delivered-surface anchored antigens are effectively drained to the lymph node resulting in greater antigen presentation. Furthermore we have demonstrated that the immune-stimulatory content of the particles increases internalisation by antigen presenting cells, the maturation of dendritic cells and the secretion of a range of cytokines. In mouse models humoral and CD4 and CD8 T cell responses have been induced successfully. The data presented will highlight the use of the Lipovaxin as a versatile vaccine platform.

2109

Targeting dendritic cells with heterologous prime-boosting to enhance immunity

Li, J.^{1,2}, Lahoud, M.¹, Shortman, K.^{3,4}, Heath, W.^{2,5}, Caminschi, I.¹

¹Monash University, Infection and Immunity Program, Monash Biomedicine Discovery Institute, Melbourne, Australia, ²University of Melbourne, Peter Doherty Institute for Infection and Immunity, Department of Microbiology and Immunology, Melbourne, Australia, ³Macfarlane Burnet Institute for Medical Research & Public Health, Centre for Biomedical Research, Melbourne, Australia, ⁴Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia, ⁵ARC Centre of Excellence in Advanced Molecular Imaging, University of Melbourne, Melbourne, Australia

Vaccine regimens typically involve multiple immunisations to boost the immune response for more potent, long-lived immunity. However, many pathogens, such as malaria and HIV, have so far proven difficult to generate protective immunity against with conventional vaccines. In an effort to improve vaccine efficacy, we have delivered antigen directly to dendritic cells (DCs), the cells that initiate T cell responses. CD8⁺ DCs specialise in the activation of cytotoxic T cell responses, and

targeting antigen to these DCs greatly enhances primary cytotoxic T cell responses. However, targeting DCs also induces a potent humoral immune response, which was found to prevent boosting by neutralising the targeting construct within 1 hour after secondary immunisation. Indeed, the greater the primary anti-construct antibody response generated, the more difficult it proved to boost. To reduce the reactivity of the primary response to the boosting construct, we attempted heterologous prime-boosting with different forms of the targeting constructs. We compared combinations of different constructs recognising the same molecule, Clec9A, as well as constructs that recognise another CD8+ DC-restricted molecule, XCR1, for their ability to induce T cell responses. Our findings indicate that the most effective prime-boosting strategy requires changing the form of not just the targeting component, but also the antigen itself in the boosting construct. As new advances create more potent vaccines, strategies to minimise the interference from the primary response must be considered if effective boosting, and long-lasting memory, is to be achieved.

2987

Dendritic cell responses to pneumococcal vaccines are impaired in HIV+ subjects

De La Rosa, I.¹, Rodriguez-Barradas, M.², Corry, D.¹, Mendoza, D.¹

¹Baylor College of Medicine, Medicine, Houston, United States,

²Center for Translational Research on Inflammatory Diseases, MEDVAMC, Houston, United States

Background: HIV+ subjects are more susceptible to invasive pneumococcal disease (IPD) than HIV- subjects in the antiretroviral (ART) era. Moreover, the pneumococcal polysaccharide (PPV) and conjugate (PCV) vaccines have reduced efficacy in HIV+ patients. Dendritic cells (DC) promote immune responses through cytokine production; however, their role in responses to PS is not very clear. We hypothesize that DC produce cytokines in response to pneumococcal vaccines and that HIV infection impairs this function.

Methods: We performed clustering analysis of 41 cytokines in supernatant of healthy PBMC exposed to PPV and PCV using Luminex technology. We exposed PBMC of HIV+ patients off ART, HIV+ patients on ART and age-matched HIV- controls to PCV and PPV ex vivo to measure specific cytokines production in DC by flow cytometry. We assessed if pre-vaccine DC production of cytokines ex vivo predicted post-vaccine serum opsonophagocytic killing activity (OPA) by linear regression. OPA is an in vitro correlate of protection against IPD.

Results: We found that DC produced IL-6 and IL-8 against both pneumococcal vaccines. IL-6 and IL-8 are known to induce chemotaxis, inflammation and B cell differentiation. Interestingly, the production of IL-6 and IL-8 after PCV and PPV was impaired in DC from HIV+ patients regardless of ART and predicted post-vaccine OPA. These results indicate a novel mechanism that could explain persistent poor vaccine responses and that could be exploited to improve vaccine efficacy in HIV+ subjects.

Keywords: Streptococcus pneumoniae, Dendritic cells, Vaccine

2804

Vectorization in an oncolytic vaccinia virus of an antibody, a Fab and a scFv against PD-1 allow their intratumoral delivery and an improved tumor-growth inhibition

Fend, L.¹, Kleinpeter, P.¹, Thioudellet, C.¹, Geist, M.¹, Sfronato, N.¹, Koerper, V.¹, Brandely, R.¹, Villeval, D.¹, Rittner, K.¹, Silvestre, N.¹, Erbs, P.¹, Zitvogel, L.², Quemeneur, E.¹, Prévaille, X.¹, Marchand, J.-B.¹

¹TRANSGENE S.A., Illkirch Graffenstaden, France, ²Institut Gustave Roussy, Villejuif, France

We report here the successful vectorization of a hamster monoclonal IgG (namely J43) recognizing the murine Programmed cell death-1 (mPD-1) in Western Reserve (WR) oncolytic vaccinia virus. Three forms of mPD-1 binders have been inserted in the virus: whole antibody (mAb), Fragment antigen-binding (Fab) or single-chain variable fragment (scFv). mAb, Fab and scFv were produced and assembled with the expected patterns in supernatants of cells infected by the recombinant viruses. The 3 purified mPD-1 binders were able to block the binding of mPD-1 ligand to mPD-1 *in vitro*. Moreover, mAb was detected in tumor and in serum of C57BL/6 mice when the recombinant WR-mAb was injected intratumorally (IT) in B16F10 and MCA 205 tumors. The concentration of circulating mAb detected after IT injection was up to 1900-fold higher than the level obtained after a subcutaneous (SC) injection (*i.e.* without tumor) confirming the virus tropism for tumoral cells and/or that tumoral microenvironment allows virus escape from immune surveillance. Moreover, the overall tumoral accumulation of the mAb was higher and lasted longer after IT injection of WR-mPD-1, than after IT administration of 10 µg of J43. Interestingly, in the MCA 205 tumor model, WR-mPD-1 (both mAb and scFv) induced a therapeutic control of tumor growth similar to unarmed WR combined to systemically administered J43 and superior to that provided by an unarmed WR. These results pave the way for next generation of oncolytic vaccinia armed with immunomodulatory therapeutic proteins such as mAbs.

4048

Vaccine encoding SIV accessory antigens protects against pathogenic challenge

Holst, P.J.

University of Copenhagen, Center for Medical Parasitology, Institute of Immunology and Microbiology, Copenhagen, Denmark

Conventional HIV vaccine strategies have not been successful. Here we immunized rhesus macaques intramuscularly and rectally with an SIV vaccine regimen based on a heterologous adenovirus prime-boost regimen and the use of the MHC class II associated invariant chain as a molecular adjuvant for the encoded antigens. The immunizations induced strong and broad T cell responses against the normally weakly immunogenic accessory antigens tat, vif, rev and vpr. Following repeated low dose intrarectal challenges, vaccinated animals demonstrated significantly reduced risk of acquisition ($P=0.04$, likelihood ratio test for leaky effect), and suppression of early viral replication in animals that became infected ($P=0.02$ combined effect on early replication). Infected vaccinees had better protection of the CD4+

T cell population in blood and mucosal tissues, maintenance of naïve CD4⁺ T cells, reduced T cell exhaustion and reduced T follicular helper cell expansion. Induction of potent T cell responses against weakly immunogenic accessory antigens is capable of preventing viral transmission, markedly diminishing early viral replication and reducing immune hyperactivation associated with disease progression. These results indicate that a vaccine that does not incorporate envelope proteins may be an effective strategy for protection against HIV infection and for reduction of infectiousness during early infection. Early results on the integration of accessory antigen vaccines into virus vectored gag and env based vaccines is expected.

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1247

Pulmonary immunisation with recombinant influenza A viruses against *Mycobacterium tuberculosis*

Muflihah, H.¹, Flórido, M.¹, Triccas, J.², Xia, Y.³, Stambas, J.³, Britton, W.^{1,2}

¹Centenary Institute, Tuberculosis Research Program, Newtown, Australia, ²University of Sydney, Sydney Medical School, Sydney, Australia, ³Deakin University, School of Medicine, Geelong, Australia

The current *Mycobacterium bovis* BCG vaccine provides inconsistent protection against pulmonary infection with *Mycobacterium tuberculosis* (*Mtb*). Immunity induced by subcutaneous BCG wanes and does not promote early recruitment of T cell to the lungs after *Mtb* infection. Pulmonary immunisation may increase and prolong immunity at the site of *Mtb* infection. We have shown that intranasal (i/n) immunisation with a single dose of recombinant IAV (rIAV) expressing an *Mtb* CD4⁺ T cell epitope was immunogenic and protective against *Mtb* challenge. To enhance this effect, we constructed different strains of rIAVs, PR8 H1N1 and X31 H3N2, expressing the IA^b-restricted p25 CD4⁺ T cell epitope of *Mtb* Ag85B₂₄₀₋₂₅₄ (PR8-p25, X31-p25). Sequential i/n immunisation with these rIAVs significantly increased the frequency of IFN- γ secreting T cells specific to Ag85B₂₄₀₋₂₅₄ in the lungs and spleen, and resulted in protection against aerosol *Mtb* infection. To investigate whether the rIAVs could boost prior BCG immunisation, mice were immunised with BCG 12 weeks prior to the sequential rIAVs. This resulted in increased frequency of IFN- γ -secreting specific T cells and polyfunctional CD4⁺ T cells compared to the BCG alone ($p < 0.001$, $p < 0.05$). Using this approach, there was a consistent trend towards increased protection at 4 weeks after *Mtb* challenge, compared to BCG alone. Therefore, pulmonary immunisation with sequential rIAVs after BCG increases *Mtb*-specific T cell responses and may enhance protection at the site of *Mtb* infection. These results suggest the potential use of rIAVs for a new tuberculosis vaccine candidate.

1901

A cytolytic DNA vaccine to target cross-presentation of dendritic cells

Wijesundara, D.¹, Grubor-Bauk, B.¹, Gargett, T.², Yu, W.², Gowans, E.¹
¹The Basil Hetzel Institute, Surgery, Woodville, Australia, ²The Centre for Cancer Biology, Adelaide, Australia

The use of cost-effective vaccines capable of inducing robust CD8⁺ T cell immunity will contribute significantly to the development of prophylactic and therapeutic strategies aimed at eliminating persistent viral infections and cancers worldwide. Hence, DNA vaccines are ideal candidates for this purpose because they are inexpensive and easy to manufacture on a large scale even in low-income countries where many people suffer from absolute poverty. We have now generated published data to suggest that harnessing the cytolytic capacity of perforin (PRF) in a unique bi-cistronic cytolytic DNA vaccine system can significantly augment anti-viral T cell (including CD8⁺ T cell) immunity in mice as well as pigs which share a closer immune system to humans than mice. This highlights promising translational prospects especially for the Hepatitis C virus (HCV) therapeutic clinical trial that we plan to conduct soon using this vaccine. To further exploit this vaccine and expand its clinical applications, we decided to understand how this vaccine operates mechanistically to result in an immune adjuvant effect. In ICI 2016, I will discuss our progress in deciphering this mechanism and show exciting *in vivo* data to suggest that expression of our novel cytolytic DNA vaccine increases the capacity of dendritic cells (DCs) to mature and cross-present antigen to naïve CD8⁺ T cells compared to canonical DNA vaccines. Since DCs are crucial for initiating CD8⁺ T cell immunity, we anticipate that these findings will have important implications to effectively harness the immune system to target pathogens and cancers.

Immunosuppression

3307

Understanding the molecular control of immune cell survival for the development of tailored immunotherapy

Carrington, E.^{1,2}, Zhan, Y.^{1,2}, Brady, J.^{1,2}, Sutherland, R.^{1,2}, Vikstrom, I.^{1,2}, Anstee, N.^{1,2}, Schenk, R.^{1,2}, Herold, M.^{1,2}, Lew, A.^{1,2}

¹The Walter and Eliza Hall Institute of Medical Research (WEHI), Parkville, Australia, ²University of Melbourne, Department of Medical Biology, Parkville, Australia

Apoptosis is critical for a normal functioning immune system, purging unwanted, harmful or superfluous cells from the repertoire. Too little apoptosis can lead to immunopathology and cancer, too much and immune system failure ensues. The Bcl-2 family of proteins are crucial regulators of immune cell apoptosis, with a balance between pro-apoptotic and anti-apoptotic members thought to determine survival outcome. Using mice over-expressing or genetically deficient in individual proteins, or treated in combination with mimetic inhibitors targeting selective Bcl-2 family members, we interrogated the survival requirements of various immune cell subsets. Interestingly, in contrast to the pro-apoptotic proteins,

manipulation of anti-apoptotic members (BCL-2, BCL-XL, BCL-W, A1, MCL-1) leads to differential survival of immune cell subsets. For example, plasmacytoid dendritic cells (pDC) were found to be dependent on BCL-2 for survival, whereas conventional dendritic cells (cDC) were not. We therefore advocate that these intrinsic survival requirements may be exploited in order to achieve cell-targeted immunosuppression for the tailored treatment of disease.

1877

Identification of a functional TGF-beta mimic secreted by the helminth *Heligmosomoides polygyrus*

Smyth, D.J.¹, Johnston, C.¹, McSorley, H.¹, Maizels, R.^{1,2}

¹University of Edinburgh, Edinburgh, United Kingdom, ²University of Glasgow, Glasgow, United Kingdom

The helminth parasite *Heligmosomoides polygyrus* can drive expansion of immunosuppressive regulatory T cells through its secretory/excretory products (HES), however the identity of the active molecule remained elusive. Data from an in vitro TGF-beta bioassay (cell line MFB-11) indicates that HES contains an orthologue or mimic which signals through the mammalian TGF-beta receptor. We have now identified a novel molecule, TGM, from the excretory secretory products of *H. polygyrus* that is a functional TGF-beta mimic. TGM shares no homology to mammalian TGF-beta (and is not a member of the TGF-beta superfamily), is acid stable (to pH 3) and remains fully active after 28 days at 37 degrees Celsius. TGM induces Foxp3 expression in murine T cells at similar concentrations (approx. 1 ng/ml) as mammalian TGF-beta, and induced Foxp3+ cells are functionally suppressive in vitro. An inhibitor of the TGF-beta receptor signalling kinase ALK5 (SB431542) completely ablates TGM activity, while neutralizing antibody against mammalian TGF-beta has no effect. Remarkably, TGM can induce FoxP3 expression of human peripheral blood CD4+ T cells at similar concentrations to mammalian TGF-beta, indicating that TGM could translate directly into human disease. We are now applying TGM to several mouse models of inflammation (asthma, colitis, autoimmunity and transplant tolerance) to assess its therapeutic potential and/or its involvement in immune modulation through the TGF-beta pathway.

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Ultraviolet irradiation establishes an immune suppressive microenvironment within the skin-draining lymph nodes

Tse, B.¹, Marsh-Wakefield, F.¹, Kok, L.F.¹, Halliday, G.², Byrne, S.^{1,2}

¹University of Sydney, The Cellular Photoimmunology Group, Infectious Diseases and Immunology, Sydney, Australia, ²University of Sydney, Dermatology Research Laboratories, Sydney, Australia

The ultraviolet (UV) component of sunlight is a major contributor to skin carcinogenesis. However, multiple epidemiological and empirical studies support a role for UV in protection from diseases including metabolic syndrome, cardiovascular disease and a number of autoimmune diseases. While evidence of this protective effect is strongest for multiple sclerosis, the mechanism responsible for this protection is not well understood.

As UV is a strong modulator of adaptive immune responses, we hypothesised that UV-induced immune suppression is one way in which UV protects from CNS-targeted autoimmunity. In our previous work, exposure of mice to a dose of UV, which protected mice from experimental autoimmune encephalomyelitis (EAE), resulted in the preferential accumulation of leukocytes within the lymph nodes draining the site of UV irradiation. Furthermore, there was a concomitant decrease in the number of leukocytes in the CNS-draining lymph nodes. To determine the effect of UV on the skin-draining lymph nodes, UV-induced molecular changes were investigated. Using a combination of OpenArray and quantitative PCR, it was discovered that UV enhanced the expression of genes involved in immune suppression including CCR8 and GITR. Flow cytometry analysis confirmed this upregulation at the protein level, revealing that UV significantly increased the expression of these molecules on a CD4^{hi}CD8^{int} double positive T cell population. These results indicate that UV establishes an immunoregulatory environment within the skin-draining lymph nodes, which is likely to play an important role in UV-protection from autoimmunity.

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Alarmin S100A8/A9 dependent accumulation of myeloid-derived suppressor cells (MDSC) and their relevance in rheumatoid arthritis

Kuhlencord, M., Zenker, S., Roth, J., Vogl, T.

University of Münster, Institute of Immunology, Münster, Germany

The differentiation and activation of MDSC in tumor conditions has been studied extensively over the last years. Various tumor-derived factors have been identified in promoting the accumulation of this suppressive cell population. Recent findings identified the two alarmins S100A8 and S100A9 as important factors in the differentiation of MDSC during tumor conditions. However, little is known about the expansion and activation, as well as the relevance of these cells in autoimmune diseases such as rheumatoid arthritis.

Our in vitro studies reveal that differentiation of myeloid progenitor cells in the presence of S100A8 promotes the accumulation of immature cells that phenotypically as well as functionally resemble monocytic MDSC (CD11b+ Ly6C+). These cells show strong suppressive effects on both, CD4 and CD8 positive T cells, mainly mediated via the production of nitric oxide (NO). The accumulation of MDSC by extracellular S100A8 was found to be mediated via the Toll-like receptor 4 (TLR4).

In addition, lack of intracellular S100A8/A9 results in a decreased number of MDSC indicating an intracellular role of this alarmin in MDSC development. In a mouse model of rheumatoid arthritis, first in vivo data indicate a correlation of high systemic S100A8/A9 level and the accumulation of MDSC, implying the importance of the two S100 proteins in facilitating MDSC expansion.

Our in vitro results clearly show a S100A8/A9 dependent accumulation of cells that phenotypically as well as functionally resemble monocytic MDSCs. Preliminary in vivo data strongly support the importance of these in vitro findings for the disease outcome in autoimmune diseases.

344

Resveratrol-induced myeloid-derived suppressor cells attenuate experimental autoimmune encephalomyelitis by regulating microRNA profile

Singh, N., Singh, U., Nagarkatti, P., Nagarkatti, M.
University of South Carolina School of Medicine, Pathology,
Microbiology and Immunology, Columbia, United States

Resveratrol (trans-3,5,4'-trihydroxystilbene; RES), is a plant-derived natural polyphenolic compound that possesses anti-inflammatory, antioxidant, and anticancer properties. Myeloid derived suppressor cells (MDSCs) constitute the recently discovered regulatory cells that possess potent immunosuppressive properties. In the current study, we investigated resveratrol-mediated epigenetic mechanisms leading to suppression of Experimental Autoimmune Encephalomyelitis (EAE) in mice. We observed that administration of RES into C57BL/6 mice immunized with MOG35-55 peptide to induce EAE, caused significant attenuation of the clinical symptoms and neuroinflammation, when used before or after the onset of EAE symptoms. Interestingly, we observed significant increases in MDSCs in the CNS of mice with EAE treated with RES, when compared to EAE mice treated with vehicle (VEH). Moreover, RES-induced MDSCs suppressed EAE, when adoptively transferred into mice. Also, RES-induced MDSCs showed suppressive effects on T cell proliferation *in vitro*. Upon analysis of microRNA (miR) profile in RES-induced MDSCs, we observed altered expression of a large number of miRs, when compared to MDSCs from VEH-treated mice with EAE. Upon analysis of these miRs, we observed their significant role in the regulation of MDSCs as well as various other molecular mechanisms and pathways including apoptosis, immunological pathways, cellular growth and proliferation. Data from the present study demonstrate for the first time the role of RES-induced MDSCs in the suppression of EAE through regulation of miRs. Thus, treatment with RES and/or RES-generated MDSCs may have potential in clinics to treat autoimmune diseases including, Multiple Sclerosis (NIH grants P01AT003961, R01AT006888, R01ES019313, R01MH094755, P20GM103641 and Veterans Affairs Merit Award BX001357).

3105

A Non-hematopoietic erythropoietin analogue, ARA 290, prolonged allogeneic islet graft survival in a mouse model

*Watanabe, M.¹, Yao, M.H.¹, Cerami, A.², Brines, M.², Ericzon, B.-G.¹,
 Lundgren, T.¹, Kumagai-Braesch, M.¹*

¹Karolinska Institutet, Transplantation Surgery, Stockholm, Sweden, ²Araim Pharmaceuticals, Tarrytown, United States

Erythropoietin exerts anti-inflammatory, anti-apoptotic, and cyto-protective effects. We have recently shown that ARA 290, a non-hematopoietic erythropoietin analogue, protected isolated pancreatic islets from cytokine-induced damages and ameliorated inflammatory responses, resulting in engraftment of the even fewer transplanted islets in a syngeneic mouse model. In this study we further investigated the effect of ARA 290 on mouse allogeneic islet transplantation (PITx).

Methods: Immunomodulatory effects of ARA 290 were assessed by allogeneic mouse mixed lymphocyte culture (MLC) and bone marrow derived dendritic cells (DCs). Balb/c mice (H-2d) islets (3-400 islets) were transplanted of streptozotocin-induced diabetic C57BL/6N mice (H-2b) via the portal vein. Recipients were given ARA 290 (120 µg/kg) intraperitoneally just before and at 0, 6, and once per day during 14 days after PITx. Blood glucose level was monitored and graft survival was assessed. Immunological status was assessed at 5 days after PITx.

Results: The treatment with ARA 290 significantly prolonged graft survival time compared to non-treated control group (MGT; 15.0 days vs. 5 days, p=0.02, n=10 in each group). The frequency of allo-reactive IFN-γ producing precursors in the spleen was significantly lower in the ARA 290 treatment group animals. *In vitro* study, ARA 290 did not inhibit T cell proliferation nor IFN-γ production in allogeneic MLC. While, ARA 290 treated DCs showed less LPS driven CD86 up-regulations, and IL-6, TNF-α, and IFN-γ production.

Conclusion: ARA 290 significantly prolonged islet grafts survival following allogeneic PITx. With having anti-inflammatory and anti-apoptotic properties, ARA 290 may become a promising modality in clinical PITx.

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Antigen-specific exosomes carrying miRNA-150 interact with ovalbumin-derived peptides to suppress the delayed-type hypersensitivity reaction in mice

*Bryniarski, K.¹, Nazimek, K.¹, Ptak, M.¹, Ptak, W.¹, Askenase, P.W.²,
¹Jagiellonian University Medical College, Department of Immunology, Krakow, Poland, ²Yale University School of Medicine, Department of Internal Medicine, New Haven, United States*

Introduction. T suppressor (Ts) cell-derived exosomes carrying miRNA-150 to suppress mouse contact and delayed-type hypersensitivity (DTH) responses are coated with specific antibody light chains (LC) from B1a cells [Bryniarski et al. J Allergy Clin Immunol 2013;132:170-181]. Current studies aim to investigate the exact role of LC ensuring antigen-specificity of exosome action in suppression of protein-induced DTH in mice. **Methodology.** CBA/J, Balb/c or pan-immunoglobulin deficient JH-KO mice were injected intravenously with ovalbumin-conjugated red blood cells (OVA-MRBC) to induce Ts cell production of exosomes and then were injected intradermally with OVA-saline solution to activate B1a cells to generate OVA-specific LC. Supernatant of lymph node and spleen cell culture was filtered and ultracentrifuged (100.000g) and pelleted exosomes were tested for their suppressive activity in active or adoptively transferred DTH induced by OVA or KLH antigens. In some instances, exosomes were pre-incubated with tryptic OVA-peptides, or JH-KO-derived exosomes were coated with LC separated on Sepharose column conjugated with tryptic OVA-peptides.

Results. OVA-specific exosomes administered intraperitoneally at 24-hour peak of DTH response alleviated subsequent ear swelling in CBA mice actively immunized with OVA but not with KLH. Pan-immunoglobulin-deficient JH-KO mice-derived exosomes were non-inhibitory unless they were pre-incubated with OVA-specific LC eluted from OVA-peptide column.

Incubation of OVA-specific exosomes with OVA-peptides prior to treatment of adoptively transferred DTH effector cells blocked their suppressive activity.

Conclusions. Above data proved that suppressive exosomes act antigen-specifically, likely due to the interaction of their surface LC with antigenic peptides complexed with MHC on antigen-presenting cells.

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Coexpression of BTLA and PD-1 identifies a novel CD4⁺T-cell exhaustion phenotype in human hepatocellular carcinoma

Zhao, Q., Su, S., Zhang, M., Su, D., Wang, M.-L., Gao, Z.

Sun Yat-sen University, Guangzhou, China

Although immunotherapy targeting programmed cell death 1/programmed cell death ligand 1 (PD-1/PD-L1) pathway is being applied in clinic, the response outcomes are heterogeneous, suggesting existences of distinctive subsets within PD-1-expressing T cells that react differently to PD-1/PD-L1 blockade. However, markers to demarcate these subsets in human cancers remain unclear. Here, we found that both PD-1 and B and T lymphocyte attenuator (BTLA) were significantly up-regulated on CD4⁺ T cells from HCC tumor compared with those from paired nontumor liver. Interestingly, over 85% BTLA⁺ CD4⁺ T cells were PD-1-expressing cells and represented about 50% PD-1⁺ CD4⁺ T cells in tumors, and that level of BTLA⁺PD-1⁺ tumor CD4⁺ T cells were selectively associated with disease progression of patients. BTLA⁺ identified highly dysfunctional PD-1-expressing CD4⁺ T cell subset, whereas BTLA⁻ defined PD-1⁺ CD4⁺ T cells undergoing activation in HCC. Importantly, blockade of PD-L1 could restore the IFN- γ /TNF- α production in BTLA⁺PD-1⁺ tumor CD4⁺ T cells but partially suppressed the activation of BTLA⁻PD-1⁺ CD4⁺ T cells. Moreover, we provided evidence that interaction of BTLA and its ligand herpesvirus entry mediator (HVEM) also participated in suppressing CD4⁺ T cell function in HCC tumor. BTLA could identify distinct function of PD-1 expressing CD4⁺ T cells in human cancer, which might not only advance our understanding of inhibitory receptor blockade, but also provide new targets for clinical predictors of response to these immunotherapies.

3963

Kinetics of the Foxp3⁺ cell response in kidney tissue of both autoimmune and non-autoimmune induced hyperglycemia in mice

Varli, S., Thorvaldson, L., Sandler, S., Singh, K., Stellan Sandler
Uppsala University, Uppsala, Sweden

Diabetes causes an elevation of the blood glucose level and a long-term hyperglycemia that contributes to kidney damage, i.e. diabetic nephropathy (DN). DN exhibits signs of inflammation and infiltration of mononuclear cells. Foxp3⁺ T cells maintain the homeostasis of immune system. Herein, we determined the numbers of Foxp3⁺ Treg cells in the kidneys of multiple low dose streptozotocin (MLDSTZ) treated male mice, NOD female mice

and single dose STZ (SDSTZ) treated mice. MLDSTZ and NOD mice were used as models for autoimmune T1D and the SDSTZ model was studied as a model for non-autoimmune induced hyperglycemia. Surprisingly, we found an increase in the numbers of Foxp3⁺ Treg cells and an infiltration of mononuclear cells in the kidneys of SDSTZ, MLDSTZ, and NOD mice. Thus, our data reveal that Treg cells are increased in kidneys of mouse models of both hyperglycemia and T1D. However, the upregulation of Treg cells did not protect against both hyperglycemia and infiltration of mononuclear cells in kidneys. In addition, administration of the novel anti-inflammatory cytokine IL-35 prevented the mononuclear cell infiltration in kidneys of MLDSTZ and NOD diabetic mice, suggesting that IL-35 could be investigated to prevent mononuclear cell infiltration in kidneys and perhaps DN.

1439

PM2.5 induces oxidative stress in splenic CD11b⁺ cells in mouse

He, C.¹, Ichinose, T.², Song, Y.¹, Wang, D.¹, Morita, K.¹, Kanazawa, T.¹, Yoshida, Y.¹

¹University of Occupational and Environmental Health, Department of Immunology and Parasitology, Kitakyushu, Japan,

²Oita University of Nursing and Health Sciences, Department of Health Sciences, Oita, Japan

Epidemiological studies demonstrated that ambient PM2.5 (particulate matter, PM with aerodynamic diameter less than and equal to 2.5 μ m) exposure was associated with mobility of respiratory diseases, cardiovascular disease, diabetes, and mortality. Evidences indicate PM2.5 contributes to systemic influence rather than respiratory restricted dysfunction. Immune system plays essential roles in PM exposure related adverse health outcomes; however effects of PM2.5 on immune system and established mechanisms remain to be elucidated. In the present study, we investigated the splenic response in PM2.5-exposed mice. PM2.5 used in the present study was collected from the atmosphere at Shenyang in China. BALB/c mice were intratracheally administered of PM2.5 or saline four times at 2-week intervals, and sacrificed at 24 hours after the last administration. Cell viability and mitogen-induced proliferation in splenocytes were decreased in PM2.5-exposed mice compared to control mice. Additionally, ConA-induced IL-2 and LPS-induced TNF- α production in splenocytes from PM2.5-exposed mice were lower than that of control mice. Immunoblotting demonstrated increased expression of Heme Oxygenase-1 (HO-1) in the splenocytes after PM2.5 exposure. Activation of ERK was also identified in splenocytes from PM2.5-exposed mice. Particularly, increased HO-1 expression and ERK activation were detected in CD11b⁺ cells from PM2.5-exposed mice, but not in CD4⁺ or CD8⁺ or B220⁺ cells. Furthermore, antioxidant N-acetylcystein partially rescued suppression effects on splenocytes from PM2.5-exposed mice. Taken together, these results suggest that administration of PM2.5 induces suppression of splenocytes with oxidative stress, and CD11b⁺ cells work as responder cells.

Innate Lymphoid Cells

1133

Down-regulating E protein function augments ILC2 production in the thymus

Sun, X.-H.¹, Wang, H.¹, Qian, M.L.¹, Zhao, Y.¹, Zhuang, Y.², Urban Jr, J.³, Fung, K.-M.⁴

¹Oklahoma Medical Research Foundation, Oklahoma City, United States, ²Duke University Medical Center, Durham, United States,

³U.S. Department of Agriculture, Agriculture Research Service, Beltsville Human Nutrition Center, Beltsville, United States, ⁴University of Oklahoma Health Sciences Center, Pathology, Oklahoma City, United States

Innate lymphoid cells (ILCs) have gained recognition as important regulators in various immune responses. How these cells are generated is just beginning to be understood. Current paradigm states that all ILCs originate from common lymphoid progenitors (CLP) in the bone marrow through an array of downstream ILC progenitors. *Id2*, a member of the *Id* family that inhibits E protein transcription factors, is expressed in these ILC progenitors and is indispensable for ILC differentiation. Unexpectedly, we found that down-regulating E protein function in the thymus by *Id1* expression or conditional deletion of two E protein genes resulted in massive expansion of ILC2s in the thymus as well as other organs where ILC2 normally reside. Consequently, the mutant mice exhibit augmented spontaneous infiltration of eosinophils and heightened responses to allergen, papain, in the lung. Furthermore, these mice also display increased ability in the expulsion of helminth parasites, *N. Brasiliensis*. These results raise an interesting question as to whether the thymus is a natural site for ILC2 production and the phenotypes of our mutant mice simply highlight such capacity. Indeed, lineage tracing experiments using *lck-Cre/ROSA26-stop-tdTomato* mice demonstrate that *tdTomato* specifically marks a fraction of ILC2s in the thymus as well as in the lung, suggesting that thymus-derived ILC2s preferentially home to the lung. We also found that thymic progenitors were able to differentiate into ILC2 cells *in vitro*. Together, our data reveal a new ILC2 differentiation program occurring in the thymus, which enriches our knowledge about ILC2 ontogeny and has potential biological significance.

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Differential roles of T helper 2 cells and group 2 innate lymphoid cells in allergic airway inflammation

Hendriks, R.W.¹, Li, B.¹, Stadhouders, R.², Rao, T.N.³, Beerens, D.¹, Brem, M.¹, De Bruijn, M.¹, Lukkes, M.¹, Bergen, I.¹, Hoogsteden, H.¹, Kool, M.¹, Fehling, H.J.³

¹Erasmus MC, Pulmonary Medicine, Rotterdam, Netherlands,

²Center for Genomic Regulation, Laboratory of Hematopoietic Stem Cells, Transdifferentiation and Reprogramming, Barcelona, Spain, ³University Clinics, Ulm, Germany

Both T helper-2 (Th2) cells and group 2 innate lymphoid cells (ILC2) are major producers of IL-5 and IL-13 in house dust mite (HDM)-driven allergic airway inflammation. We found that in HDM-challenged mice the induction of ILC2s required prior

sensitization with HDM allergen. ILC2 induction was dependent on T cells, whereby activation of ILC2s and Th2 cells was concomitant. Here, we present the characterization of a GATA3-YFP knock-in mouse model that allows direct *in situ* visualization of GATA3⁺ ILC2s and Th2 cells. In these animals, the expression level and function of the transcription factor GATA3 are unperturbed, which is critically important since we previously demonstrated an essential and dose-dependent role for GATA3 in ILC2s. Confocal microscopy revealed that ILC2s and T cells occupied distinct locations in lungs from mice with HDM-driven allergic airway inflammation: ILC2s were scattered underneath the mucosa and Th2 cells were present within clusters of lymphocytes. In addition, we generated epigenome maps of FACS-sorted GATA3⁺ ILC2s and Th2 cells isolated from mediastinal lymph nodes and broncho-alveolar lavage of GATA3-YFP mice with allergic airway inflammation. Chromatin immunoprecipitation and deep-sequencing (ChIP-Seq) experiments revealed remarkably similar genome-wide histone-3 lysine-4 dimethylation (H3K4Me2) active chromatin signatures among *in vivo* activated ILC2s and Th2 cells. However, specific loci showed striking differences between the two cell types, including loci implicated in allergic inflammation, such as *Alox5* (encoding 5-lipoxygenase), *Csf2* (GM-CSF), *Tnfrsf4* (Ox40), and several chemokine (*Cxcl*) genes. Taken together, these findings indicate novel biological roles for ILC2s in asthma - distinct from Th2 cells.

1051

Deciphering the innate lymphoid cell developmental program reveals transient requirement of the transcription factor Nfil3

Seillet, C.¹, Mielke, L.¹, Zalcenstein, D.¹, Su, S.¹, Gao, J.¹, Almeida, F.¹, Shi, W.¹, Ritchie, M.¹, Naik, S.¹, Huntington, N.¹, Carotta, S.², Belz, G.¹

¹Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia, ²Boehringer-Ingelheim, Vienna, Austria

Innate lymphoid cells (ILCs) are enriched at mucosal surfaces where they provide immune surveillance. Several groups have described ILC progenitor populations, however, their potential to generate all ILC lineages remains unclear. Here we describe the *c-kit*⁺ CILP that generated all ILC subsets, including NK cells, and the CD25⁻ ILC2-restricted *Sca-1*⁺ precursor. Using CEL-seq to determine the global transcriptional changes that occur during ILC development we identified several novel pathways in the differentiation process and established the hierarchical interactions amongst *Id2*, *Nfil3* and *Tcf7* utilized by progenitor cells for generation of ILC populations. Importantly, we showed that only transient expression of *Nfil3* was required for commitment to the ILC lineage prior to induction of *Id2* and *Tcf-1* expression that are key downstream regulators of the *c-kit*⁺ CILP. Collectively, our computational and functional mapping of transcription factor activity at the earliest stages of ILC differentiation revealed the molecular complexity underlying ILC commitment.

3162**Flt3L controls the number of innate lymphoid cells in fetal and adult mice**

Bärenwaldt, A.¹, von Burg, N.¹, Kreuzaler, M.², Sitte, S.³, Horvath, E.¹, Peter, A.¹, Vöhringer, D.³, Rolink, A.², Finke, D.¹

¹University Children's Hospital Basel, University of Basel, Department of Biomedicine, Basel, Switzerland, ²University of Basel, Department of Biomedicine, Basel, Switzerland, ³University Clinic of Erlangen, Friedrich Alexander University Erlangen-Nuremberg, Department of Infection Biology, Erlangen, Germany

Fms-like tyrosine kinase 3 ligand (Flt3L) is an important cytokine for hematopoietic progenitors in the bone marrow (BM) and differentiation of dendritic cells (DCs) in the periphery. While the effect of Flt3L on B cells, T cells and myeloid cells is well studied, the role of Flt3L for the different groups of innate lymphoid cells (ILCs) is not clear.

To investigate the effect of Flt3L on ILC development we analyzed ILC numbers in *flt3l*^{-/-}, *flt3l*-tg and Flt3L-treated mice. Deletion of Flt3L led to reduced numbers of fetal intestinal ILC3s, resulting in impaired Peyer's patch development. In the adult intestine, loss of Flt3L reduced the number of all groups of ILCs. For ILC3s, this reduction was even more pronounced as in *IL7*^{-/-} mice. Conversely, Flt3L transgenic mice showed increased numbers of ILCs. Furthermore, administration of recombinant Flt3L could increase all ILCs in WT mice and ILC1s and ILC3s in *IL7*^{-/-} mice, but was unable to rescue ILC numbers in *Flt3L*^{-/-} mice. To test whether ILC numbers were indirectly affected by Flt3L-dependent DCs, we analyzed DC-deficient mice. These animals had normal or even elevated ILC numbers under steady state conditions arguing against a role for DCs in regulating ILC numbers. Finally, we could show that Flt3L influenced ILC numbers by regulating the precursor pool of ILCs in the fetal liver and the bone marrow and, in contrast to IL-7, acts mainly on early stages of ILC differentiation.

224**Differential ID2 in the group 3 innate lymphoid cells control the development of different gut associated lymphoid tissues**

Wang, W.¹, Li, Y.¹, Fu, Y.-X.², Guo, X.¹

¹Tsinghua University, School of Medicine, Beijing, China, ²University of Texas Southwestern Medical Center, Department of Pathology, Dallas, United States

Gut associated lymphoid tissues (GALTs), including the mesentery lymph nodes (mLNs), Peyer's patches (PPs), and isolated lymphoid follicles (ILFs), are important locations for the initiation of adaptive immune responses in the intestine. Group 3 Innate lymphoid cells (ILC3s) are defined by ROR γ t expression and include lymphoid tissue inducer (LTi) cells, which play a critical role in the development of GALTs. However, the function of ID2 in these well-differentiated ILCs is still unclear.

In our study, *Rorc*^{cre}/*Id2*^{fl/fl} mice showed normal mLNs, but completely lacked PPs and ILFs compared with WT mice. Furthermore, ILC3s were significantly reduced in the gut of *Rorc*^{cre}/*Id2*^{fl/fl} mice compared with WT mice, but were actually increased in mLNs, indicating that the development or maintenance of ILC3s in the

gut, but not mLNs, requires ID2 regulation.

Moreover, ID2 deficient ILC3s exhibited significantly reduction of the lymphotoxin expression in the gut, which is important for the formation of GALTs. Furthermore, we found IL-7 stimulation could induce lymphotoxin expression in WT ILC3s, while the IL-7Ra expression was dramatically reduced in ID2 deficient ILC3s, suggesting that ID2 may regulate the lymphotoxin expression through IL-7 signaling. On the other hand, we found that the expression of Rank was upregulated in ID2 deficient ILC3s from the gut and mLNs, which indicates that the elevated Rank may rescue ILC3s' survival and lymphotoxin expression through NF- κ B pathway. Taken together, our results suggest that ID2 could regulate the function and maintenance of ILC3s through IL-7 and Rank signaling to further determine the differential development of GALTs.

954**Tissue-microenvironment dictates the fate and tumor-suppressive activity of type 3 ILCs**

Nussbaum, K.¹, Burkhard, S.H.¹, Klose, C.S.², Arnold, S.J.³, Diefenbach, A.^{2,4}, Becher, B.¹

¹University of Zurich, Institute of Experimental Immunology, Zurich, Switzerland, ²University of Freiburg Medical Center, Institute of Medical Microbiology and Hygiene, Freiburg, Germany, ³University of Freiburg Medical Center, Department of Internal Medicine IV, Freiburg, Germany, ⁴University of Mainz Medical Center, Institute of Medical Microbiology and Hygiene, Mainz, Germany

ROR γ t-dependent innate lymphoid cells (ILC3s) have been implicated in the control and suppression of solid tumors. While intestinal ILC3s promote tumor progression in the presence of IL-23, we showed that lymphoid IL-12-activated *RORc*^{fate map(fm)+} ILCs significantly inhibit tumor growth. The IL-12/IL-23 axis, hence, influences the tissue-specialization that ILCs acquire to gain tumor-suppressive or -promoting capacities. Thus far, the impact of the tissue-microenvironment on their phenotype and function remains largely elusive. We show clearly demarcated tissue specification across lymphoid and non-lymphoid organs. While the intestinal *RORc*^{fm+} ILCs retain their ILC3 phenotype, lymphoid-derived *RORc*^{fm+} ILCs acquire an NK cell/ILC1-like phenotype. Strikingly, adoptively transferred *RORc*^{fm+} ILCs distributed among various organs and phenotypically adapted to their new tissue-environment.

However, only the lymphoid *RORc*^{fm+} ILCs were tumor-protective while intestinal *RORc*^{fm+} ILCs failed to suppress tumor growth. Yet, despite their lymphoid origin and phenotypic similarities, *bona fide* type 1 ILCs (*RORc*^{fm} ILC1s/NK cells) also failed to suppress tumor growth, indicating that the ontogeny of the cells supersedes the phenotypic variations.

We thus propose that the tissue-microenvironment combined with ontogeny provides the specific function, whereas the phenotype provides merely a snapshot of some ILC properties with only a small predictive value to translate into functional properties.

1497**Redefining human ILC using high dimensional single cell analysis***Simoni, Y.¹, Fehlings, M.², Ginhoux, F.¹, Newell, E.²**¹A*STAR, SgN, Singapore, Singapore, ²A*STAR, Singapore, Singapore*

Animal models highlight the importance of innate lymphoid cells (ILC) in many immune responses. However, technical limitations have hampered adequate characterization of ILCs in humans. Here, we used mass-cytometry including a broad range of surface makers and transcription factors to accurately identify and profile these cells across twelve different healthy and inflamed tissue types. High dimensional analysis with t-Distributed Stochastic Neighbor Embedding (t-SNE) allowed for clear phenotypic delineation of ILC2 and ILC3 subsets. In the same analysis, ILC1 cells were not detected in any tissue, but we did identify iILC1-like cells that appear to be representative of a broader category of NK cells present in mucosal and non-mucosal inflamed tissues. In addition, our analysis shows that human ILC are highly heterogeneous between individuals and between tissues. This is the first study giving a global, comprehensive and detailed description of ILC heterogeneity in humans across patients, tissues at steady state and in inflammation.

4103**Cross-talk between pancreas resident innate lymphoid cells and T cells in autoimmune diabetes***Heuts, F.¹, Wardzinski, L.¹, Wang, C.J.¹, Ono, M.², Ovcinnikovs, V.¹, Kenefeck, R.¹, Kogimtzis, A.¹, Ryan, G.³, Withers, D.³, Walker, L.¹**¹University College London, Institute of Immunity & Transplantation, London, United Kingdom, ²Imperial College London, Department of Life Sciences, London, United Kingdom, ³University of Birmingham, College of Medical & Dental Sciences, Birmingham, United Kingdom*

During the initiation of autoimmune diabetes T cells enter the pancreas and orchestrate the destruction of insulin-producing beta cells. While staining for CD25+ regulatory T cells in the pancreas of diabetic mice we identified a lineage-negative CD25+ population. These cells express the phenotypic markers of ILC2 cells including CD127, ST2, ICOS, Thy1, klrp1, IL-13, IL-5, IL-6 and the transcription factor GATA3. The ILC population was present independent of disease as it could be detected in the pancreas of normal non-diabetic animals and rag gene deficient animals. Co-culture of T cells with pancreas-derived ILC altered the pattern of CD4 T cell cytokine production. Conversely, exposure of the ILC2 population to T cells during the development of diabetes modulated gene expression and phenotype of pancreas resident ILCs whilst lung ILC2s in the same animal did not show such changes. Collectively these data support a model of bidirectional communication between innate lymphoid cells and T cells in the pancreas which may have implications for the development of autoimmune diabetes.

1168**GATA3 regulates the development and functions of innate lymphoid cell subsets at multiple stages***Zhong, C.¹, Yagi, R.¹, Northrup, D.², Cui, K.², Wilhelm, C.³, Bouladoux, N.³, Hu, G.², Mao, K.³, Belkaid, Y.³, Zhao, K.², Zhu, J.¹**¹NIAID, NIH, Laboratory of Immunology, Molecular and Cellular Immunoregulation Unit, Bethesda, United States, ²NHLBI, NIH, Bethesda, United States, ³NIAID, NIH, Bethesda, United States*

Both IL-7R α -expressing innate lymphoid cells (ILCs) and CD4 T helper (Th) cells are critically involved in immune responses to pathogens by producing a similar set of cytokines. Similar to its essential function in CD4 T cell development in the thymus, GATA3 is indispensable for the development of IL-7R α -expressing ILC subsets. Residue ILCs found in the conditionally *Gata3*-deficient mice are exclusively lymphoid tissue inducers (LTis), consistent with a selective loss of PLZF-expressing ILC progenitors in the bone marrow of these mice. Nevertheless, *Gata3*-deficient mice do not develop lymph node structure suggesting that GATA3 also regulates the functions of LTis. After the ILCs had fully developed, GATA3 remains essential for the maintenance and functions of ILC2s. Genome-wide anti-GATA3 ChIP-Seq and gene expression (RNA-Seq) analyses indicate that GATA3 directly regulates a similar set of cytokines and receptors in Th2 cells and ILC2s. Furthermore, despite of its low expression, GATA3 plays a critical role in modulating the homeostasis, further maturation, and functions of the ILC3 subsets by directly regulating the expression of several important genes including *Il7r*, *Rorc* and *Il22*. Thus, GATA3 regulates the development and functions of innate lymphoid cell subsets at multiple stages. The work was supported by the Intramural Research Program of NIH, NIAID and NHLBI.

2270**Balance of environmental factors modulates cell fate decision of adaptive lymphocytes and innate lymphoid cells***Koga, S.^{1,2,3}, Hozumi, K.⁴, Hirano, K.-I.⁴, Yazawa, M.^{4,5}, Koyasu, S.^{2,6}, Moro, K.^{1,2,7,8}**¹RIKEN Center for Integrative Medical Sciences (IMS), Laboratory for Innate Immune Systems, Kanagawa, Japan, ²RIKEN Center for Integrative Medical Sciences (IMS), Laboratory for Immune Cell Systems, Kanagawa, Japan, ³Yokohama City University, Department of Supramolecular Biology, Kanagawa, Japan, ⁴Tokai University School of Medicine, Department of Immunology, Kanagawa, Japan, ⁵Tokai University, Department of Biochemistry, Kanagawa, Japan, ⁶Keio University School of Medicine, Department of Microbiology and Immunology, Tokyo, Japan, ⁷Yokohama City University Graduate School of Medicine, Department of Medical Life Science, Kanagawa, Japan, ⁸Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Agency, Tokyo, Japan*

Group 2 innate lymphoid cells (ILC2 cells) derive from CLP and the progenitors and transcription factors involved in their development are well reported. However, the microenvironment that regulates the expression of transcription factors and promotes specific differentiation of ILC2 cells remains unclear. To identify the external factor that induces specific differentiation

of lymphocytes, we isolated CLP and co-cultured with TSt4-DLL1 stromal cells under different concentrations of IL-7. We found that the concentration of IL-7 differentially regulates T cell and ILC2 differentiation. Next, we established TSt4 Tet-off DLL stromal cells in which DLL expression levels can be regulated by changing the concentration of Doxycycline (Dox). The strength and duration of Notch signaling divided commitment to T cell, B cell and ILC2 lineages in CLP co-cultured under different concentrations and exposure times of Dox. Furthermore, to clarify the specific environment that supports ILC2 development *in vivo*, we focused on the mesentery, the most abundant source of ILC2 cells. We identified ILC progenitor cells in the fetal mesentery that had potential to differentiate into all ILC subset but not T cells and B cells under the TSt4 co-culture system. We also observed in fetal mesentery KLRG1⁻ immature ILC2 cells, which have not acquired the ability to produce IL-5 and IL-13 under IL-33 stimulation in the absence of STAT5 activators. Our findings demonstrate that concentration of IL-7, and strength and duration of Notch signaling regulate lymphocyte lineage determination and the mesentery plays an important role in the differentiation and maturation of ILC2 cells.

B Cells 4

3091

PRMT5 regulates B cell development and germinal center homeostasis

Litzler, L.C.^{1,2}, Zahn, A.¹, Methot, S.P.^{1,3}, Bois, T.¹, Richard, S.⁴, Di Noia, J.M.^{1,2,3}

¹Institut de Recherche Clinique de Montréal, Montréal, Canada,

²Université de Montréal, Biochimie, Montréal, Canada, ³McGill University, Experimental Medicine, Montréal, Canada, ⁴Lady Davis Institute, Montréal, Canada

B cells develop in the bone marrow, where the rearrangement of immunoglobulin genes leads to the expression of a functional B cell receptor. Mature B cells then populate the periphery, where their activation by cognate antigen prompts the formation of germinal centers (GC). GC B cells undergo somatic hypermutation, underpinning the production of high affinity antibodies, and isotype switching. Arginine methylation is a post-translational modification, still understudied in B cells, which is involved in many cellular processes. Here, we investigate the role of protein arginine methyl transferase 5 (PRMT5) in B cell development and antibody diversification.

We found that, *in vivo*, PRMT5 expression is induced during early B cell development. Indeed, PRMT5 ablation in pro-B cells causes a total block in development at that stage. PRMT5 is induced *in vivo* in GC from immunized mice, as well as *ex vivo* in activated mouse primary B cells, compared to resting follicular B cells. In immunized mice, conditional ablation of PRMT5 in activated B cells results in a three-fold decrease in GC B cells and drastically impairs antigen-specific antibody production. *Ex vivo*, depletion of PRMT5 in activated mouse primary B cells reduces class switch recombination, cell proliferation and viability.

Overall, PRMT5 is necessary for B cell development and antibody diversification. We are investigating the mechanism

of action behind these phenotypes. Since PRMT5 is overexpressed in B cell lymphomas, our research will lead to a better understanding of PRMT5's role in both normal and malignant B cells.

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Single molecular force sensitivity and threshold for the activation of B cell receptors

Wan, Z., Liu, W.

Tsinghua University, Beijing, China

B lymphocytes show remarkable mechanosensing capability by utilizing B cell receptors (BCRs) to sense antigens. However, the sensitivity and threshold for the activation of BCRs resulting from the stimulation by mechanical forces are unknown. Here we systemically addressed this question using a ds-DNA based tension gauge tether (TGT) system serving as a predefined single molecular force gauge ranging from 12 to 56 pN to restrict BCR and antigen tension. Utilizing total internal reflection fluorescence microscopy, we observed that IgM-BCR activation is sensitive to single molecular forces. We observed an obvious multi-threshold effect with a low-end 12-16 pN force which triggered a weak activation, whereas a 23-43 pN force initiated a medium-level activation, and a high-end 50-56 pN force produced a strong activation. Such patterned dependence on mechanical forces with multi-threshold effects did not strictly rely on myosin IIA, dynein and integrin. However, the break-through of the high-end but not the medium- or low-end mechanical force threshold in IgM-BCR activation required myosin IIA and the inside-out activation of integrin. Surprisingly, we found that the activation of the isotype-switched IgG-BCR or IgE-BCR only requires an extremely low threshold of less than 12 pN, providing an explanation for their rapid activation in response to antigen stimulation. Mechanistically, we found that the cytoplasmic tail of the IgG-BCR heavy chain is both required and sufficient for the low mechanical force threshold to activate the IgG-BCR. These results defined the single molecular force sensitivity and threshold that are required to activate different isotypic BCRs.

3757

Transcriptional and epigenetic regulation of germinal centre and plasma cell differentiation

Good-Jacobson, K.^{1,2}, Di Pietro, A.¹, Pupovac, A.¹

¹Biomedicine Discovery Institute, Monash University, Infection and Immunity Program and Biochemistry and Molecular Biology, Clayton, Australia, ²Walter and Eliza Hall Institute, Parkville, Australia

The production of high-quality antibody is central to adaptive immunity. Affinity is increased through iterative rounds of mutation, selection and expansion of selected clones. These functions are physically segregated in the dark zone (mutation) or light zone (selection) of the germinal centre (GC). Crucially, differentiation into plasma cells is repressed until properly selected clones exit the GC. It is critical to understand activation and repression mechanisms that allow these germinal processes

to occur. Deletion of the transcription factor c-Myb in mature B cells led to altered affinity maturation and aberrant up-regulation of plasma cell transcription factors within the GC after immunisation with NP-KLH in alum. Indeed, a subset of cycling cells within the GC secreted antibody and were CD138⁺Blimp-1-GFP⁺. The transcription factor T-bet, normally suppressed in our Th2-biased model, was also upregulated. Deletion of T-bet in c-Myb-deficient mice suppressed the formation of aberrant plasma cells within the GC, thus identifying c-Myb and T-bet as crucial regulators of differentiation. Chromatin accessibility studies of the T-bet promoter revealed that there was a significant increase in chromatin accessibility in flu-infected c-Myb-deficient GC B cells compared to wild-type. Therefore, we hypothesise that histone modifications are critical in GC function and repression of differentiation. Indeed, deletion of the histone acetyltransferase MOZ results in a significantly impaired dark zone. We are currently investigating polycomb group proteins that are expressed in different zones of the GC, and in particular EED, the binding partner of EZH2 which is critical for GC formation.

2742

G1-phase nucleotide paucity causes AID-induced mismatch repair to be mutagenic in germinal centre B cells

Thientosapol, E.^{1,2}, Boznjak, D.^{1,2}, Durack, T.¹, Stevanovski, I.¹, Jolly, C.^{1,2}

¹Centenary Institute, Sydney, Australia, ²University of Sydney, Faculty of Medicine, Sydney, Australia

Somatic hypermutation of Ig genes in activated B cells requires the generation of "founder" U:G mismatches via deamination of genomic C by Activation-Induced cytidine Deaminase (AID). Founder mutations can be further processed by uracil base excision repair (BER), or by mismatch repair (MMR). Both processes result in a mix of faithful repair, and further mutagenesis - of the founder base pair, and/or of nearby base pairs. Processing by MMR results in downstream mutations introduced by DNA polymerase η , largely at A:T base pairs, but also at G:C base pairs. Since pol η is not involved in canonical S/G2-phase MMR, the basis of its recruitment during antibody hypermutation has been a mystery. We recently showed that BER excises AID-induced dU predominantly in G1-phase (Sharbeen et al. 2012 J. Exp. Med. 209:965-74). Here we present data suggesting that AID-induced MMR also occurs in G1-phase. Using genetic tools, we enhanced G1-phase synthesis of dNTPs, inhibited G1-phase dNTP degradation, or both, in activated B cells in mice, and substantially reduced the incidence of MMR-dependent Ig mutations, while having no obvious effect on the incidence of AID-induced founder mutations, or upon AID-induced class switching. Our data suggest that both BER- and MMR-induced mutagenesis downstream of AID is the result of DNA repair recruitment in "non-canonical" cell cycle phases. We propose that MMR-induced Ig mutagenesis results from attempting DNA repair under conditions of severe nucleotide paucity.

3172

The impact of Arginine methylation on BCR signaling

Infantino, S.^{1,2}, Light, A.¹, O'donnell, K.^{1,2}, Belz, G.³, Richard, S.⁴, Tarlinton, D.^{1,2}

¹WEHI, Immunology, Parkville, Australia, ²Monash University, Immunology, Melbourne, Australia, ³WEHI, Molecular Immunology, Parkville, Australia, ⁴Lady Davis Institute for Medical Research, McGill University, Montreal, Canada

Signals through the B cell receptor (BCR) are transmitted by a multitude of molecules, many of which undergo post-translational modification. In BCR signaling, arginine methylation, a characterized post-translational modification, has been suggested to control B cell development. Specifically, Protein Arginine Methyl-Transferase 1 (PRMT1) has been shown to methylate Ig- α and consequently to modulate BCR signal. We have examined the consequence of a conditional deletion of the *prmt1* gene in B cells. We find that PRMT1 regulates B cell maturation by shaping the pre-B cell compartment and modulating the subsequent transition to the immature B cell stage. By deleting PRMT1 at the CD23⁺ stage of B cell development, we find that both follicular and marginal zone B cells were not affected. When activated, PRMT1-deficient B cells displayed an altered proliferation and survival capacity. Arginine methylation mediated by PRMT1 is required in B cells for humoral immunity. Indeed, in response to protein antigen, B cells lacking PRMT1 failed to form germinal centre. Moreover, the T-independent immune response against a polysaccharide antigen was also severely impaired in mice lacking PRMT1 in B cells. Thus PRMT1 plays a critical, B-cell intrinsic role in specifying the outcome of humoral immune responses and in fine-tuning B cell development.

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What determines B cell fate during infection?

Sutton, H., Cai, Y., Singh, M., Enders, A., Cockburn, I.

John Curtin School of Medical Research, Canberra, Australia

When naïve B cells are activated by antigen, they undergo clonal expansion and can adopt 3 main cell "fates": plasmablasts, germinal center B cells and early memory B cells. However, how B cell fate is determined is poorly understood, especially during infection. To address this question we characterized the B cell response against the central repeat region ((NANP)_n) of the circumsporozoite protein (CSP), a major *Plasmodium* surface antigen. We used B cell tetramers to sort different (NANP)_n-specific B cell populations and then used next generation sequencing to gain a snapshot of the entire (NANP)_n-specific BCR repertoire after *Plasmodium* immunization. We determined that, the immune response was highly restricted, utilizing just 1-3 different Vh regions. Utilizing this restricted repertoire, it was possible to identify B cell clones based on their shared CDR3s. We were able to detect B cell clones that could differentiate into multiple cell fates, suggesting that differentiation is somewhat stochastic. To test the impact of BCR affinity on B cell fate, we used a novel knock-in mouse expressing the heavy chain of an anti-(NANP)_n antibody. This mouse had a highly expanded number of (NANP)_n specific cells, which, crucially had different

affinities for the antigen based on pairing with different light chains. We found that strong BCR-antigen interactions lead to differentiation into the plasmablast pathway. As a result a model is proposed which states that strong or weak BCR-antigen interactions trigger differentiation into particular cell fate while intermediate interactions lead to stochastic fate determination.

1835

Distinct germinal center selection at local sites shapes cross-protective memory B cell response to viral escape

Adachi, Y., Ato, M., Takahashi, Y.

National Institute of Infectious Diseases, Department of Immunology, Tokyo, Japan

Respiratory influenza virus infection induces cross-reactive memory B cells targeting invariant hemagglutinin (HA) regions of viral escape mutants. However, cellular events dictating the cross-reactive memory B cell responses remain to be fully defined. Here we dissected the development of cross-reactive germinal center (GC) / memory B cells following influenza virus infection using two types of HA probes. Somatic mutated GC B cells developed in several organs, but lung GCs were superior to those in secondary lymphoid organs for persistence, proliferation rate, and cross-reactivity. Moreover, the persistent lung GCs selected preferentially cross-reactive B cells into lung memory compartment.

To gain further insight into the lung GCs, we detected the persistent GCs by immunohistochemistry using GL7 as the marker of GCs. GL7⁺ B cell clusters were readily detectable in the MLN and spleen. In contrast, iBALT-like structures containing both less dense GL7⁺ B cell clusters and CD4⁺ T cells, but lacking defined T cell area, were observed in the lungs. Furthermore, viral RNA was detectable in the infected lung for up to 30 days after infection, implying viral persistence may lead to distinctive feature of GCs in the lungs.

Taken together, our data illustrate the unique features of the persistent local GCs to select cross-reactive B cells, which likely contribute to broad protection against antigenically evolving pathogens like influenza virus.

1522

TAPP adaptors control Akt-dependent metabolic activation of germinal center B cells and suppress autoimmunity via binding to the SHIP product PI(3,4)P2

Jayachandran, N.¹, Landego, I.¹, Hou, S.¹, Meija, E.², Sheikholeslami, K.¹, Hatch, G.², Alessi, D.³, Marshall, A.¹

¹*University of Manitoba, Immunology, Winnipeg, Canada,*

²*University of Manitoba, Pharmacology and Therapeutics, Winnipeg, Canada,* ³*University of Dundee, Dundee, United Kingdom*

The phosphoinositide phosphatase SHIP regulates B cell activation by converting the PI 3-kinase product PI(3,4,5)P3 to PI(3,4)P2. While this activity of SHIP appears critical for generation of effective adaptive immunity while avoiding autoimmunity, the function of PI(3,4)P2 in B cells remains relatively unexplored. Tandem PH domain containing proteins

(TAPPs) are adaptor proteins that specifically bind to PI(3,4)P2 via their C-terminal PH domains, targeting them to the plasma membrane. Mice bearing inactivating mutations in the PH domains of both TAPP1 and TAPP2, uncoupling them from PI(3,4)P2, exhibit hypergammaglobulinemia and develop lupus-like characteristics including anti-DNA antibodies and deposition of immune complexes in kidneys. Here we find that TAPP KI mice develop chronic germinal centres (GCs) and age-associated increases in B cell expression of activation, exhaustion and memory markers, all of which are B cell intrinsic and dependent on the co-stimulatory molecule ICOS. TAPP KI B cells exhibit elevated phosphorylation of Akt and its target GSK3, and increased survival. Strikingly, TAPP KI B cells exhibit increased metabolic activity associated with elevated oxygen consumption rate, increased extracellular acidification rate, increased expression of glucose transporter Glut1 and increased glucose transport rate. Together our findings suggest that TAPP-PI(3,4)P2 interaction is important for regulating signalling via Akt, B cell metabolism and development of autoimmunity.

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T-helper signals restore B-cell receptor signaling in autoreactive anergic B-cells by upregulating CD45 phosphatase activity

Szodoray, P.¹, Stanford, S.M.², Bottini, N.², Nakken, B.¹

¹*University of Oslo and Oslo University Hospital-Rikshospitalet, Department of Immunology, Oslo, Norway,* ²*La Jolla Institute for Allergy and Immunology (LIAI), Division of Cellular Biology, La Jolla, United States*

Background: We recently identified a human B-cell population that is naturally autoreactive and tolerized by functional anergy, termed BND-cells.

Objective: To identify the molecular mechanism how anergic, autoreactive BND-cells escape functional anergy and whether this process is altered in lupus patients.

Methods: Isolated peripheral blood naïve and BND-cells were cultured with various stimuli and their activation status was determined by intracellular Ca²⁺-mobilization assay. Lyn and Syk kinase activity were assessed by phospho-flow analysis. CD45 phosphatase activity was determined with a novel flow-based assay, which takes advantage of the fluorogenic properties of phosphorylated coumaryl amino propionic acid, an analog of phosphotyrosine, which can be incorporated into peptides. Real-time qPCR was utilized to quantitate LYN, SYK and CD45 mRNA.

Results: T-helper signals reversed the state of anergy allowing BND-cells to fully respond to antigenic stimulation by restoring signaling through the B-cell receptor. The mechanism was dependent on increased activity of the tyrosine phosphatase CD45, and CD45-dependent activation of Lyn and Syk kinases. CD45 phosphatase activity was increased by T-cell help both in BND and naïve B-cells. Furthermore, we found that BND-cells obtained from SLE patients exhibited increased CD45 activity and B-cell receptor signaling capacity, thus being less tolerized, than BND-cells from healthy controls.

Conclusion: Our findings suggest that CD45 is a key regulator of B-cell receptor signaling thresholds mediated

by T-cell help. This raises the possibility that BND-cells could represent precursors of autoantibody-secreting plasma cells and suggests a role for these autoreactive B-cells in contributing to autoimmunity if not properly controlled.

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Human B cell memory defined by tyrosine phosphatase CD45 activity

Nakken, B.¹, Stanford, S.M.², Bottini, N.², Szodoray, P.¹

¹University of Oslo and Oslo University Hospital-Rikshospitalet, Department of Immunology, Oslo, Norway, ²La Jolla Institute for Allergy and Immunology (LIAI), Division of Cellular Biology, La Jolla, United States

We previously discovered that CD45 phosphatase activity was upregulated upon T cell help in naïve human B cells, enhancing B cell receptor signaling in naïve B cells and restoring antigen-signaling in anergic B cells through activation of early B cell receptor signaling kinases. This novel connection between T cell help and upregulation of CD45 phosphatase activity suggested that CD45 phosphatase activity may be important for B cell memory, allowing heightened responses upon re-exposure to antigen.

We report herein that B cell subsets expressing the classical memory B cell marker CD27 exhibited a robust increase in CD45 phosphatase activity compared to their naïve counterparts. Moreover, this finding also included the CD27-IgG⁺ memory subset in addition to the CD27-IgM⁺ memory subset, showing that increased CD45 phosphatase activity encompass memory B cell subsets not included by conventional memory marker. The highest level of CD45 phosphatase activity was found within the IgG class-switched memory B cells, which have undergone most somatic hypermutation, express the highest-affinity B cell receptors and are postulated to have received the most efficient T cell help.

Functional analyses revealed that increased CD45 phosphatase activity in memory B cells translated into increased B cell receptor signaling capacity and ability to differentiate into plasma cells. These results explain in part the heightened recall responses of memory B cell subsets and emphasize the role of CD45 phosphatase activity in the B cell memory phenotype. Understanding the mechanisms resulting CD45 phosphatase activity may prove important in understanding immunity to infection and development of vaccines.

Mini Oral Sessions

15:30:00 - 16:30:00

Dendritic Cells 2

1050

The contribution of interferon lambda to systemic lupus erythematosus

Macri, C.¹, Pang, E.S.¹, Pooley, J.¹, Fancke, B.², O'Keefe, M.¹

¹Monash University, Biomedicine Discovery Unit, Clayton, Australia,

²Burnet Institute, Centre for Biomedical Research, Melbourne, Australia

Systemic lupus erythematosus (also called SLE, or lupus) is an autoimmune disease that is characterised the production of autoantibodies, resulting in a broad range of symptoms that can affect any organ in the body. Recent studies have identified an "interferon (IFN) signature" that is carried by many SLE patients. This was evidenced by the expression of IFN-stimulated genes (ISGs) that are typically induced by type I IFN (encompassing IFN- α and IFN- β). Interferon lambda (IFN- λ) is a recently identified cytokine that is secreted by dendritic cells (DCs). IFN- λ shares functional similarities with type I IFN by activating a STAT-dependent signaling pathway that leads to ISGs expression. Plasmacytoid DCs are a major source of IFN- λ in response to Toll-Like receptor (TLR)-7 and TLR-9 stimulation. Mouse CD8+ DCs and their human equivalents, CD141+ DCs, also produce large amounts of IFN- λ in response to TLR-3 ligand. We have observed that IFN- λ plays a crucial role in DC innate immune response. We have also investigated the role of IFN- λ in the development of autoimmunity. High levels of IFN- λ were detected in the serum from different lupus-prone mice and in the serum from some lupus patients. Furthermore, DCs from lupus-prone mice over-express IFN- λ in response to different stimuli. Altogether, these data suggest that IFN- λ is a potential harmful cytokine during lupus that may represent a target of interest for the design of new treatments.

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Targeting infectious disease related antigen to Clec9A enhances antibody responses

Park, H.Y.^{1,2}, Tan, P.S.^{1,2}, Caminschi, I.^{1,2}, Alonso, S.³, Shortman, K.^{2,4}, Lahoud, M.H.^{1,2}

¹Monash University, Biochemistry and Molecular Biology, Melbourne, Australia, ²Burnet Institute, Centre for Biomedical Research, Melbourne, Australia, ³National University of Singapore, Yong Loo Lin School of Medicine, Singapore, Singapore, ⁴Walter & Eliza Hall Institute of Medical Research, Immunology, Melbourne, Australia

Targeting antigen (Ag) to dendritic cells (DCs), by injecting Ag coupled to a monoclonal antibody (mAb) specific for a DC surface molecule, is an established experimental strategy for enhancing immune responses and has the potential for improving the efficiency of vaccines. We have focused on emerging infectious

diseases, Influenza and Hand foot and mouth disease (HFMD) where potent Ab responses are required for protection. We have generated targeting constructs consisting of anti-Clec9A Ab genetically fused to key pathogen derived Ags; (1) anti-Clec9A-Influenza M2e and (2) anti-Clec9A-HFMD Enterovirus 71 SP70. To determine whether the candidate Ag targeted to Clec9A could induce immune responses, mice were injected with the targeting Ab or a control Ab, then production of anti-specific Ag Ab response was measured. Our results indicate small amount of the anti-Clec9A-Influenza M2e mAb produces enhanced and prolonged Ab responses in the absence of adjuvants or additional signals for DC activations whereas the anti-Clec9A-HFMD Enterovirus 71 SP70 mAb which presents Ag to only B cell epitope produced moderate Ab responses. In addition, we have utilized SP70 to know whether the B cell epitopes, as well as the T cell epitopes, must be targeted to the Clec9A-bearing DC. For maximal Ab response, both B and T cell epitopes need to be targeted to Clec9A bearing DC, suggesting the DC presents Ag to B cells as well as T cells. Our current focus is to develop Clec9A-targeting strategy for the enhancement of vaccine effectiveness, because protection against many infectious diseases depends on Ab production.

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Role of dendritic cells in the induction of immune response by lentiviral vector

Cousin, C.¹, Felix, T.¹, Cara, A.², Negri, D.², Fabrega, S.³, Dadaglio, G.¹, Leclerc, C.¹

¹Institut Pasteur, Unité de Régulation Immunitaire et Vaccinologie, Inserm U 1041, Paris, France, ²Istituto Superiore di Sanita di Roma, Roma, Italy, ³Plateforme Vecteurs Viraux et Transfert de Gènes, SFR Necker, US 24, UMS 3633, Paris, France

Non-replicating integrase deficient lentiviral vector (IDLV) can transduce dendritic cells (DC) leading to their activation, cytokine production and strong cytotoxic T lymphocyte (CTL) activity. However, the role of the different DC subsets in the *in vivo* induction of immune responses by IDLV is not established. Thus, we aim to determine the mechanisms by which IDLV induce strong and persistent CTL responses.

First, we showed that IDLV induced a strong *in vivo* activation of both conventional and plasmacytoid DCs (cDCs and pDCs respectively) and the production of a large panel of seric inflammatory cytokines and chemokines. To assess the role of DC subsets in the induction of immune responses, we used mouse strains deficient in pDCs or cDCs. We first established that cDC activation is not affected in pDC-deficient mice. In contrast, in cDC-deficient mice, we showed that pDC activation induced by IDLV vaccination is fully abolished. In addition, we demonstrated that the induction of specific CTL responses is abrogated in the absence of cDCs, but is not affected by pDCs depletion.

IDLV has a ssRNA genome which could be detected by various intracellular receptors (TLR, RIG-I and DNA sensors). We have established that IDLV signaling is independent of all TLR potentially involved in the activation of innate responses, as well as of RIG-I and DNA sensors.

In conclusion, we showed that cDCs are mandatory for the

induction of both innate and adaptive responses by IDLV. The pathways involved in the induction of immune responses by IDLV are under analysis.

1353

High mobility group box 1 protein contributes to the enhancement of co-stimulatory molecule-expression and cross-presentation by mucosal DCs through oral administration of cholera toxin

Wakabayashi, A., Yonekawa, M., Ishii, K., Kuroki, K., Shinya, E., Takahashi, H.

Nippon Medical School, Department of Microbiology and Immunology, Tokyo, Japan

We have already reported that oral administration of an antigen plus cholera toxin (CT) induced antigen-specific CTLs in the mucosal tissues. In this study, we examined if activation and cross-presentation by DCs are achieved by high mobility group box 1 protein (HMGB1), a damage associated molecular pattern molecule (DAMP) released from the damaged mucosal tissues by CT. We found that expression of CD80 and CD86 was enhanced on CD11c⁺ DCs of the mesenteric lymph nodes (MLNs) when C57BL/6 (B6) mice are orally administered with CT. In these mice, HMGB1 was increased in both fecal extracts and plasma. MLN cells were cultured with HMGB1 or CT and the expression of CD80 and CD86 on DCs was analyzed by flow cytometry. Then their expression was enhanced by the addition of HMGB1 but not CT and such *in vitro* DC activation using HMGB1 was inhibited by the addition of glycyrrhizin (GL), an inhibitor of HMGB1. Thus, we orally administered OVA plus CT to B6 mice treated with either intravenous (i.v.) injection or oral administration of GL. Subsequently, both i.v. and oral treatment with GL effectively suppressed the mucosal DC activation and proliferation of OVA-specific OT-I T cells. These results suggest that both i.v. or oral treatment of GL can efficiently act as an inhibitor of HMGB1 released from the damaged mucosal sites through CT stimulation. In conclusion, we show here that HMGB1 released from the damaged mucosal tissues by CT seems to contribute to DC activation and cross-priming of CTLs in the mucosa.

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The role of dendritic cells in sexual transmission of HIV

Harman, A.N., Bertram, K.M., Botting, R.A., Baharlou, H., Sandgren, K.J., Cunningham, A.

The Westmead Institute, Centre for Virus Research, Westmead, Australia

Human anogenital mucosae contain various tissue types, which represent unique opportunities and barriers for transmission of viruses, and each of these tissues should be considered separately in terms of how transmission occurs. Dendritic cells (DCs) and macrophages present in these tissues are two of the first cell types to encounter viruses during sexual intercourse. Furthermore, these cells play a direct role in the transmission of some viruses such as HIV where they transfer the virus to T cells in which explosive replication occurs.

Different anogenital tissues are likely to contain unique

combinations of DC and macrophage subsets however very little human data is available concerning these differences. Therefore, we have gained access to all the anogenital tissues that HIV encounters during transmission (labia, vagina, cervix, glans penis, foreskin, anus and rectum). Using flow cytometry and fluorescence microscopy we have thoroughly characterised the immune populations found in these tissues. Critically, we have identified key differences in specific immune cell subsets present in different anogenital sites and in the viral binding receptors they express.

Understanding the differences in composition of immune cells that interact with viruses in anogenital tissues is critical in the development of strategies to block transmission and in mucosal vaccine development. This is particularly the case for anorectal tissue as there is little knowledge available on its immune cell composition. This represents a key gap in our understanding as transmission across this tissue is hundreds of times more efficient in the case of HIV.

2481

Dendritic cell activity is dependent on circadian rhythmicity and mitochondrial dynamics

Cervantes-Silva, M.P., Sánchez-García, F.J.

Escuela Nacional de Ciencias Biológicas-Instituto Politécnico Nacional, Immunology, Mexico City, Mexico

Dendritic cells (DCs) capture, process and present antigens, thus initiating primary immune responses, all of which take place in a timely-ordered fashion. It has been recognized that immune responses such as T cell activation and cytokine production are dependent on the time of the day (i.e., circadian rhythm-dependent). Strikingly, mice mortality rate in LPS-induced endotoxic shock depends on whether LPS is applied during daylight or at night.

There is increasing evidence of a close relationship between immune response and cell metabolism, including a role for mitochondria in processes such as immune synapse, and antigen processing and presentation.

Mitochondrial activity correlates with circadian rhythmicity, and crosstalk between circadian rhythmicity, mitochondrial dynamics and macrophage bactericidal activity has been observed.

Here, we analysed *in vitro* DCs function in the context of both circadian rhythmicity and mitochondrial dynamics. Murine (balb/c) bone marrow-derived dendritic cells (GM-CSF-differentiated) were synchronized with dexamethasone; antigen uptake, antigen processing, mitochondrial dynamics, expression of clock proteins, and mitochondrial fusion- and fission-related proteins, were determined at different time points spanning at least a whole circadian cycle (24 h). At critical time points of DCs activity (antigen processing), DCs were stimulated with LPS for 12 h, fixed, and used for allogeneic T cell (from C57 mice) proliferation (antigen presentation assays).

Results showed that DCs function is regulated by both circadian rhythmicity and mitochondrial dynamics, contributing to the understanding of the fine-tune balance between immunity, cell metabolism, and circadian rhythmicity.

1176

Induction of tolerogenic dendritic cells by nasopharyngeal carcinoma-derived exosomes

Renaud, S.¹, Aboussemdai, H.¹, Mustapha, R.¹, Mrizak, D.¹, Ingelaere, C.¹, Mason, J.², Fitzpatrick, Z.³, Moralès, O.¹, Delhem, N.¹
¹CNRS, Lille, France, ²University of Notre Dame, Elkhart, United States, ³Harvard Medical Sciences University, Boston, United States

Background: A characteristic of the nasopharyngeal carcinoma (NPC) micro-environment is the presence of immunosuppressive exosomes released by tumor cells. Our team has recently shown that NPC-derived exosomes, which carry Galectine-9, favor the recruitment and suppressive activity of human regulatory T cells (Treg), thus contributing to NPC immune escape (Mrizak et al, JNCI, 2015). In this study, our objective is now to evaluate whether these NPC-derived exosomes could promote the emergence of tolerogenic immature dendritic cells (tolDC) able to induce regulatory T cells from naïve CD4⁺ T cells ultimately contributing to the tolerance of tumor cells.

Methods and results: We performed a complete phenotypical and functional study comparing the effect of NPC and healthy donor-derived exosomes on DC maturation. This study includes (i) cell morphological analysis by photonic microscopy, (ii) transcriptomic study by RTqPCR, (iii) flow cytometric analysis of the expression of specific markers (phenotypic DC and co-stimulatory markers), (iv) a preliminary DC functional study by western blotting (IDO) and finally (v) a secretome analysis by ELISA (IL-10; TGF- β , TNF- α). Taken together our results strongly suggest that the presence of NPC-derived exosomes favors the emergence of semi-mature DCs seemingly tolerogenic.

Conclusion: Despite the importance of immature DCs as mediators of cancer immune escape, no other studies have shown the impact of tumor exosomes on the maturation of human DCs. Thus, these promising results should open new prospects for antitumor immunotherapies based on the inhibition of factors involved in the emergence and activation of Treg.

B Cells 2

732

TGF- β -induced regulatory T cells directly suppress B cell responses through a non-cytotoxic mechanism

Zheng, S.G., Wang, J., Olsen, N.
 Penn State University College of Medicine, Hershey, United States

Foxp3⁺ regulatory T cells (Treg) playing a crucial role in the maintenance of immune tolerance and prevention of autoimmune diseases consist of thymus-derived naturally-occurring CD4⁺Foxp3⁺ Treg cells (nTreg) and those that can be induced *ex vivo* with TGF- β (iTreg). Although both Treg subsets share similar phenotypes and functional characteristics, they also have potential biologic differences on their biology. The role of iTreg in regulating B cells remains unclear so far. The suppression assays of Treg subsets on activation, proliferation and antibodies production of B cells were measured using a Treg and B cell co-culture system *in vitro*. Transwell and antibody blockade experiments were performed to assess the

roles of cell contact and soluble cytokines. Treg were adoptively transferred to lupus mice to assess *in vivo* effects on B cells. Like nTreg, iTreg subset also directly suppressed activation and proliferation of B cells isolated from C57BL/6. nTreg subset suppressed B cell responses through cytotoxic manner related to expression of Granzyme A, Granzyme B and Perforin; while the role of iTreg subset on B cells did not involve in cytotoxic action, but depending on TGF- β signaling. Furthermore, iTreg subset can significantly suppress antibody produced by lupus B cells *in vitro*. Comparison experiments using microarray assay on autoantibodies demonstrated that adoptive transfer of iTreg had a superior effect than nTreg subset on suppressing lupus B cell responses *in vivo*. Our data implicate a role and advantage of iTreg subset in treating B cell-mediated autoimmune diseases, boosting the translational potential of these findings.

4634

Deregulation of PAX5 causes abnormal B cell lymphopoiesis and spontaneous precursor B cell lymphoma development in mice

Boast, B.¹, Reed, J.², Bergmann, H.¹, Yabas, M.^{1,3}, Roots, C.¹, Young, C.^{1,2}, Nutt, S.⁴, Goodnow, C.^{1,2}, Enders, A.¹

¹The Australian National University, The John Curtin School of Medical Research, Department of Immunology, Canberra, Australia, ²The Garvan Institute of Medical Research, Department of Immunology, Sydney, Australia, ³Trakya University, Department of Genetics and Bioengineering, Edirne, Turkey, ⁴Walter and Eliza Hall Institute of Medical Research, Molecular Immunology Division, Melbourne, Australia

Pax5 knock out mice have a complete block in early B cell development and die at approximately 3 weeks of age due to neurological complications. Through ENU mutagenesis, we have discovered a strain of mice with a mutation in *Pax5* that impairs protein function and B cell development but allows enough PAX5 expression for mice to survive into adulthood. Mutations in PAX5 are also implicated in approximately 30% of paediatric B cell acute lymphoblastic leukaemia (B-ALL). Our ENU-induced *Pax5* mutant mice spontaneously develop precursor B cell lymphomas. In agreement with published data about cooperation between activation of STAT5 and PAX5 in tumour development, we find that STAT5 is highly phosphorylated in tumour cells from the PAX5 mutant mice. The incorrect function of the mutant PAX5 protein results in deregulation of target proteins and abnormal B cell lymphopoiesis before tumour development. Mutant mice have an abnormal population of CD19⁺B220⁻ precursor B cells in the bone marrow that have not previously been described. These abnormal cells are highly proliferative and are partially able to rearrange the *Igh* locus. The phenotype of these cells appears to be very similar to that of tumour cells that develop later on. We therefore hypothesise a relationship between these abnormal cells, and subsequent tumour development in mutant mice. In summary, we present a mouse strain with abnormal PAX5 function, highlighting the crucial role of the affected protein domain. Mutant mice spontaneously develop precursor B cell lymphomas that might eventually be used as a model for human B-ALL.

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IL-4-treated mast cells activate regulatory B cells in a cross-talk involving microvesicles*Marsh-Wakefield, F.^{1,2}, O'Sullivan, C.², Leighton, S.², Gillis, C.^{1,2}, Halliday, G.², Byrne, S.^{1,2}*¹University of Sydney, Cellular Photoimmunology Group, Infectious Diseases and Immunology, Sydney, Australia, ²University of Sydney, Dermatology Research Laboratories, Sydney, Australia

The ultraviolet (UV) component of sunlight is most well known for its contribution to the development of skin cancers. This is in part due to its capability of suppressing the adaptive immune response, although the exact mechanism by which UV achieves this is not fully understood. Mast cells as well as a subset of regulatory B cells have been found to be essential for UV-induced immune suppression. To investigate the potential role of mast cells in regulatory B cell activation our laboratory developed a co-culture system whereby IL4-treated bone marrow-derived mast cells are mixed with B cells. The activated B cells were in turn found to be functionally suppressive against a murine model of allergic contact dermatitis. It was also discovered that mast cell-derived microvesicles could activate regulatory B cells. The molecular mechanism involves an upregulation of IL10, IL13 and subunits of IL35 (EBI3 and p35). However the mast cells but not the B cells were the primary cells responsible for producing these cytokines. As the microvesicle-activated B cells could in turn promote mast cell production of IL13, an important cross-talk is suggested to induce immune suppression. Revelation of the nature of mast cell-B cell interactions following UV exposure could lead to novel therapeutic targets aimed at either enhancing the therapeutic benefits of phototherapy for skin diseases, or preventing UV-induced immune suppression to mitigate the risks of photocarcinogenesis.

2020

A disintegrin and metalloprotease 10 (ADAM10) regulates inducible costimulatory ligand (ICOS-L) levels on B-cells*Lownik, J.^{1,2}, Cooley, L.^{1,2}, Damle, S.¹, Conrad, D.¹*¹Virginia Commonwealth University, Department of Microbiology and Immunology, Richmond, United States, ²Virginia Commonwealth University, Center for Clinical and Translational Research, Richmond, United States

Up to 40% of the population of developed countries suffer from atopic disease and that number is expected to rise over the coming years. Recently, we have shown the importance of ADAM10 in allergic disease, with conditional deletion of ADAM10 in B-cells (ADAM10^{B-/-}) in mice leading to a decreased asthmatic phenotype. In addition, B-cells from asthmatic patients had increased protein and mRNA levels of ADAM10. Here we report that ADAM10 regulates ICOS-L on B-cells. ADAM10^{B-/-} mice exhibit approximately 10-fold increase in ICOS-L surface levels on naïve B-cells compared to wildtype mice, which persists after stimulation with anti-CD40, whereas ICOS-L mRNA message levels are 10-fold reduced in ADAM10^{B-/-} B-cells. Similar increases in ICOS-L expression are seen when WT mouse or human B-cells are cultured in the presence of ADAM10 inhibitors. Flow analysis indicates that T-cell ICOS levels in ADAM10^{B-/-} mice is essentially

equivalent to the isotype control staining levels, indicating that the high ICOS-L expression causes strong downregulation of T-cell ICOS. ICOS-L-ICOS engagement has an important role in T-cell responses, particularly for T_{FH} cells. Consistent with the negligible ICOS expression on T-cells from ADAM10^{B-/-} mice, they also have reduced numbers of T_{FH} cells and impaired antibody production. Given the importance of ICOS-L/ICOS in the induction of asthmatic disease and our results demonstrating alterations in this pathway in ADAM10^{B-/-} mice, we hypothesize that abnormal regulation of ICOS-L by ADAM10 explains the decreased allergic phenotype seen in ADAM10^{B-/-} mice and that ADAM10 inhibitors may provide new way to modulate T_{FH} and Th2 responses.

2973

Myosin 1g participates in CD44-recycling in B-lymphocytes*López-Ortega, O.^{1,2}, Santos-Argumedo, L.¹*¹Centro de Investigación y de Estudios Avanzados del IPN, Biomedicina Molecular, Ciudad de Mexico, Mexico, ²Facultad de Medicina Universidad Nacional Autónoma de México, Ciudad de Mexico, Mexico

Recycling of adhesion molecules is an important process for cell adhesion and cell migration. This phenomenon is actin-cholesterol- and sphingolipid-enriched lipid rafts-dependent, involving several proteins such as actin, tubulin, GTPases, kinesins and dyneins; however, the motor proteins associated in these processes are unknown. Class I myosins are proteins with a conserved structure that consists of three regions: head, neck and tail. There are 8 class I myosins in mammals, ranging from myosin 1a to 1h, which are molecular motors that interact both with actin fibers and plasma membrane phosphoinositides. Myosin 1g (Myo1g) binds primarily to PIP2 and PIP3; these phosphoinositides are significantly present in several types of vesicles. In this work, we identified Myo1g as the important motor protein that drives the recycling of CD44 in B lymphocytes. We demonstrated that the lack of Myo1g decreases the levels of CD44 and GM1 at the surface of the cell. In cells lacking Myo1g, the recycling of adhesion molecules is delayed at the initial step of the recycling pathway. Defects in the lipid-raft-recycling in Myo1g-deficient cells have a significant impact in cell capping and cell migration. Both processes require the transport of lipid rafts to the surface of cell, to deliver the signaling components and the extra membrane essential for the expansion of the cell surface. Thus, Myo1g is important for the recycling of the lipid rafts in the membrane, affecting the associated proteins that regulate adhesion and migration of B lymphocytes.

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3983

IL-27 directly influences germinal center B cell development via STAT1*Vijayan, D.^{1,2,3}, Mohd. Redzwan, N.², Avery, D.², Wirasinha, R.², Brink, R.¹, Walters, G.^{4,5,6}, Adelstein, S.⁷, Kobayashi, M.⁸, Gray, P.⁹, Elliott, M.¹⁰, King, C.^{2,3}, Vinuesa, C.⁴, Ghilardi, N.¹¹, Ma, C.^{2,3}, Tangye, S.^{2,3}, Batten, M.^{2,3}*¹QIMR Berghofer Medical Research Institute, Immunology,

Brisbane, Australia, ²Garvan Institute of Medical Research, Immunology, Sydney, Australia, ³University of New South Wales, St Vincents Medical School, Sydney, Australia, ⁴John Curtin School of Medical Research, Australian National University, Department of Immunology and Infectious Disease, Canberra, Australia, ⁵Canberra Hospital, Department of Renal Medicine, Canberra, Australia, ⁶Australian National University, Australian National University Medical School, Canberra, Australia, ⁷Royal Prince Alfred Hospital, Clinical Immunology, Sydney, Australia, ⁸Hiroshima University, Department of Pediatrics, Hiroshima, Japan, ⁹University of New South Wales School of Women's and Children's Health, Sydney, Australia, ¹⁰University of Sydney, Sydney Medical School, Sydney, Australia, ¹¹Genentech, Department of Immunology, San Francisco, United States

Dysregulated germinal center (GC) activity can result in antibody driven autoimmune disease, such as lupus. IL-27 has a pro-inflammatory role in germinal center (GC) responses. We previously demonstrated that IL-27 effects on T follicular helper cells (T_{FH}) are essential for supporting GC. The present study provides the first demonstration that IL-27 signals also directly enhance the differentiation of human B cells towards a CD20⁺CD38⁺CD27^{low}CD95⁺CD10⁺ phenotype, consistent with that of GC B cells. Loss-of-function mutations in STAT1, but not STAT3, prevented IL-27 mediated differentiation of human GC-like B cells. The GC-driven *Roquin*^{san/san} lupus mouse model was used to determine the importance of IL-27 signals to B cells *in vivo*. *Il27ra*^{-/-}.*Roquin*^{san/san} mice exhibited amelioration of lupus pathology. Significantly reduced GC B cells and anti-DNA IgG2a(c)+ switched B cells were observed and mixed bone marrow chimeras confirmed that IL-27 signaling promotes GC activity through both B and CD4⁺ T cell-intrinsic mechanisms. Together, our data provides the first evidence of a direct role for IL-27 in GC B cell differentiation. The amelioration of lupus pathology in *Il27ra*^{-/-}.*Roquin*^{san/san} mice suggests that modulating IL-27 functions in GC responses could be an attractive therapeutic strategy in the treatment of some lupus patients.

2788

CD21^{low}- age-associated B cells are memory B cells in autoimmunity

Höök, N.¹, Aranburu, A.¹, Grimsholm, O.^{1,2}, Gerasimcik, N.¹, Mårtensson, I.-L.¹

¹University of Gothenburg, Gothenburg, Sweden, ²Bambino Gesù Children Hospital, Rome, Italy

A B cell subset (CD21^{-low}) expressing low levels of complement receptor 2 (CR2, CD21) has been described in conditions of chronic immune stimulation in humans, e.g. autoimmune diseases. A CD21^{-low} B-cell subset has also been described in mice where it accumulates with age, and a similar subset is enriched under autoimmune conditions, e.g. in lupus prone mice and in those that lack a pre-BCR (*SLC*^{-/-}). However, it is unclear what type of cells they are. Here we demonstrate that in autoimmunity, in *SLC*^{-/-} mice, a majority of the splenic CD21^{-low} subset are memory B cells that express IgM and have undergone somatic hypermutation. About half of these CD21^{-low} cells express CD11c. Hybridomas established from these

cells produce autoantibodies that recognize DNA and nuclear antigens. Thus, under autoimmune conditions, the CD21^{-low} B cells are memory B cells.

Innate Receptors & Inflammasomes 2

3833

Inflammasome function in autoimmunity

Thygesen, S.J., Sester, D.P., Sagulenko, V., Stacey, K.J.

The University of Queensland, School of Chemistry and Molecular Biosciences, St Lucia, Australia

Inflammasomes are molecular complexes activated by infection and cell stress, responsible for activating caspase-1 that subsequently facilitates IL-1 β processing and cell death. We have discovered inflammasome deficiencies in the autoimmune NZB mouse strain. NZB has a point mutation leading to loss of expression of inflammasome initiator NLRP3, and is also deficient in AIM2 inflammasome function. NZB mice develop anti-erythrocyte antibodies and haemolytic anaemia, and also anti-nuclear antibodies typical of lupus. We hypothesise that the inflammasome deficiencies in NZB alter the interaction of the host with both microflora and pathogens, promoting prolonged production of cytokines such as type I IFN, which contribute to loss of tolerance. The progeny of NZB and NZW mice (NZB/W F1) have a more severe lupus-like phenotype, developing anti-nuclear antibodies and immune complex-mediated glomerulonephritis at around 5-6 months of age, dying around 10-12 months from kidney failure. We have found that NZW has normal inflammasome function, and macrophages from NZB/W F1 mice have an intermediate inflammasome response compared to the parental strains, although there is no sign of deficiency when the stimulus strength is high. NLRP3 may have contrasting roles at different stages of disease. The partial NLRP3 deficiency in NZB/W F1 mice could contribute to the initiation of autoimmunity. However, with abundant self danger molecules as triggering stimuli later in disease, NLRP3 inflammasome may exacerbate kidney damage.

3080

The role of SSc5D in pathogen recognition and inflammation

Santos, R.F.^{1,2}, Mendes, S.¹, Bessa-Pereira, C.^{1,2}, Oliveira, L.¹, Carmo, A.M.¹

¹IBMC/i3S, Porto, Portugal, ²University of Porto/ICBAS, Porto, Portugal

Scavenger receptor cysteine-rich (SRCR) receptors are a group of membrane-attached or secreted proteins that contain one or more domains structurally similar to the membrane distal domain of the type I macrophage scavenger receptor. These receptors are thought to mediate protein-protein interactions within the immune system and/or to serve as pathogen pattern recognition receptors. However, the overall function of the SRCR family is still poorly understood. Soluble scavenger receptor with 5 domains (SSc5D) is a secreted SRCR glycoprotein that we found to be abundant in the human serum and whose expression was

detected in cells of myeloblastic, monocytic and lymphocytic origin. By immunohistochemical staining of sections of different organs, we additionally identified the presence of SSc5D in epithelial cells of the gastrointestinal and genitourinary tracts. Furthermore, SSc5D was strongly detected in human placenta, most noticeably in syncytiotrophoblast cells, but interestingly it was almost undetectable in tissue samples displaying signs of infection, thus suggesting a possible role in fetal immunity. While we describe that SSc5D is able to bind to pathogenic bacteria and that bacterial challenges induce SSc5D secretion from certain cell types, several inflammatory mediators also induce significant changes in SSc5D protein levels in human cell models, suggesting also a role for SSc5D on inflammation. Overall, our results indicate that SSc5D is involved in fighting infectious agents as well as in inflammatory responses.

3961

Fungal immunomodulatory proteins LZ-8 and GMI activate the NLRP3 inflammasome via stimulating ROS generation

Hsu, S.-Y., Lu, C.-Y., Chen, C.-J.

National Taiwan University, Department of Biochemical Science and Technology, Taipei, Taiwan, Republic of China

The inflammasome is a multiprotein complex which mediates the processing and production of proinflammatory cytokines IL-1 β and IL-18. The NLRP3 inflammasome complex is composed of NLRP3, ASC, and procaspase-1, in which caspase-1 is autoactivated and processes pro-IL-1 β and pro-IL-18 into mature cytokines. LZ-8 and GMI are fungal immunomodulatory proteins (FIPs) derived from *Ganoderma lucidum* and *Ganoderma microsporum*, respectively. FIPs are known to stimulate the activation of dendritic cells and T lymphocytes, and in animals, FIPs exhibit adjuvant and antitumor functions. In this study, we investigated the immunostimulatory mechanisms of FIPs and found that LZ-8 and GMI can stimulate inflammasome activation and IL-1 β production in murine macrophages. LZ-8 and GMI stimulated caspase-1 activation and IL-1 β production in peritoneal macrophages and BMDMs, which was strongly enhanced when cells were co-stimulated with LPS. FIP-stimulated IL-1 β production in LPS-primed BMDMs was markedly attenuated when cells lacked the expression of NLRP3, ASC, or caspase-1, indicating that FIPs activate the NLRP3 inflammasome. FIP-induced IL-1 β production was inhibited in the presence of high extracellular KCl or inhibitors targeting the membrane pores P2X7 and pannexin-1, indicating the requirement of potassium efflux through ion channels. FIPs stimulated ROS generation in BMDMs, and blocking ROS production by N-acetylcysteine significantly inhibited FIP-induced IL-1 β production. Overall our findings provide evidence that FIPs are new type of protein ligands that activate the NLRP3 inflammasome via stimulating ROS generation, and this activity may contribute to the immunomodulatory functions of FIPs.

1126

Inhibition of TLR3 signalling by single stranded oligonucleotides

Järver, P.¹, Dondalska, A.¹, Lennox, K.², Behlke, M.², EL-Andaloussi, S.³, Spetz, A.-L.¹

¹Stockholm University, Department of Molecular Biosciences, Wenner-Gren Institute, Stockholm, Sweden, ²Integrated DNA Technologies Inc, Coralville, United States, ³Karolinska Institutet, Department of Laboratory Medicine, Stockholm, Sweden

TLR3 is a pattern recognition receptor found in cellular endosomes and it recognizes dsRNA, which can be found in some viral genomes, produced during viral replication cycles, or released by dying cells. TLR3 activation is engaged in defences against viral infections. However, TLR3 hyperresponsiveness has been implicated in certain pathologic and inflammatory conditions. To address TLR3-induced inflammation, we have previously used single stranded oligonucleotides (ssONs) and showed that they can inhibit TLR3-mediated activation of human monocyte-derived DCs (MoDCs), as well as decrease cytokine production in the airways of cynomolgus macaques. We have now further investigated the mechanism(s) behind this inhibition in human MoDCs. We provide data showing that ssONs might be direct inhibitors of TLR3 signalling as they interact directly with TLR3, as well as compete for the same binding site as the dsRNA analogue, Poly I:C. Furthermore, the binding of ssONs is pH and length dependent and the observed inhibition appears to be sequence independent. Stability modifications are not essential for inhibition of TLR3 signalling, but seem necessary for retained activity. The biokinetics of the ssON-mediated inhibition appear to follow that of clathrin-mediated endocytosis and certain structural features are required for ssONs's inhibitory activity. Finally, we propose that the observed inhibitory effect could be a negative feedback-loop preventing over-activation of TLR3 from endogenous dsRNA. Our data provide the basis for elucidating the molecular mechanism(s) involved in the inhibition of TLR3 signalling which, could give further insight into therapeutic methods for TLR3-mediated immunopathogenesis.

3141

Bacterial DNA sensed by STING activates guanylate-binding proteins via type I IFN signaling pathway enhancing host cell defense

Costa, M.¹, Marim, F.¹, Marinho, F.¹, Harms, J.², Splitter, G.², Smith, J.³, Kanneganti, T.⁴, Barber, G.⁵, Oliveira, S.¹

¹Federal University of Minas Gerais / Institute of Biological Sciences, Biochemistry and Immunology, Belo Horizonte, Brazil, ²University of Wisconsin-Madison, Pathobiological Sciences, Madison, United States, ³University of Wisconsin-Madison, Pediatrics, Madison, United States, ⁴St Jude Children's Research Hospital, Immunology, Memphis, United States, ⁵University of Miami Miller School of Medicine, Cell Biology, Miami, United States

Immunity against invading microbes depends on the recognition of pathogen-associated molecular patterns by innate receptors. Signaling pathways triggered by *Brucella abortus* DNA involves TLR9, AIM2 and STING. Previously, our research group showed

that *Brucella* DNA-induced IFN- β is dependent on STING. In this study, we observed by microarray analysis that several type I IFN associated genes are down-regulated in STING KO macrophages infected with *Brucella* or transfected with bacterial DNA compared to C57BL/6 cells. Among these genes are guanylate-binding proteins (GBPs), CXCL10, CXCL11, Mx1. We confirmed that expression of GBP2, GBP3 and GBP5 is dependent on type I IFN signaling using IFNAR KO mice. Further, we determined whether GBPs affect intracellular survival of *Brucella* using BMDM from GbpChr3 and Gbp5 KO mice. Luminometry of lux expressing *Brucella* indicated that BMDM of GbpChr3 and Gbp5 KO mice did not control bacterial replication as well as the wild-type BMDM. Further, macrophages derived from STING KO mice showed reduction in IL-1 β secretion and caspase-1 activation when infected with *Brucella* or stimulated with bacterial DNA. These findings demonstrate that STING mediated type I IFN pathway is critical for engagement of GBP-mediated killing of *Brucella* which may result in the release of bacterial DNA for the activation of AIM2. Finally, STING and AIM2 KO mice are more susceptible to *Brucella* infection suggesting these DNA sensors are critical to induce an effective innate immune response. Funding: This study was supported by funding agencies CNPq, FAPEMIG and CAPES.

3036

Regulation of innate immune response to mycobacteria through DAP12 versus FcR γ -coupled myeloid receptors that recognize different mycolic acid-containing lipids

*Iizasa, E.*¹, *Uematsu, T.*², *Kubota, M.*³, *Kiyohara, H.*⁴, *Chuma, Y.*⁴, *Matsuzaki, G.*⁵, *Yamasaki, S.*⁶, *Yoshida, H.*³

¹Kagoshima University, Immunology, Kagoshima, Japan, ²Kitasato University Medical Center Hospital, Department of Biomedical Research, Saitama, Japan, ³Saga University, Saga, Japan, ⁴Japan BCG Laboratory, Tokyo, Japan, ⁵University of Ryukyus, Naha, Japan, ⁶Kyushu University, Fukuoka, Japan

Myeloid immunoreceptor tyrosine-based activation motif (ITAM)-coupled receptors (ITAMRs) activate innate immunity against fungi or bacteria through the recognition of pathogen-associated molecular patterns (PAMPs). Recent studies have reported that several FcR γ -associated receptors recognize PAMPs of mycobacteria. For instance, Mincle and MCL recognize trehalose-dimycolate (TDM).

Mycobacterial cell wall contains various type of MA-containing lipids, which include non-glycosylated MAs, such as free MA itself and glycerol monomycolate (GroMM), and glycosylated MAs, such as TDM and glucose monomycolate (GMM). These cell-wall lipid constituents dynamically change depending on mycobacterial conditions, such as proliferating or dormant status, and regulate host immune responses. However, how host recognize and respond to MA-containing lipids other than TDM, remains obscure.

Recently, we have identified a DAP12-associated ITAMR named IgSFR2 as a novel receptor recognizing MA-containing lipids. We found that macrophage stimulation with the non-glycosylated MAs produced the chemokine MCP-1 but only few or no inflammatory cytokines such as TNF and IL-6, while the glycosylated MAs induced both of them. The non-glycosylated

MA-induced chemokine production was abolished by IgSFR2 deficiency, while the glycosylated MA-induced cytokine and chemokine production was abolished by Mincle deficiency, indicating that IgSFR2 and Mincle are innate receptors for non-glycosylated and glycosylated MAs, respectively. Interestingly, IgSFR2 also could bind to glycosylated MAs but its binding inhibited cytokine response through Mincle. These results suggest that IgSFR2 and Mincle distinctively recognize MA-related lipids for the induction of different immune responses and hence their balance may regulate immune response to different conditions of mycobacteria.

3665

Cross-regulation between the inflammasome and linear ubiquitination machinery

*Douglas, T.*¹, *Champagne, C.*², *Lapointe, J.-M.*³, *Saleh, M.*⁴

¹McGill University, Microbiology and Immunology, Montreal, Canada, ²McGill University, Medicine, Montreal, Canada, ³McGill University, Comparative Medicine and Animal Resources Centre, Montreal, Canada, ⁴McGill University, Biochemistry, Montreal, Canada

The linear ubiquitin assembly complex (LUBAC) is a tripartite E3 ubiquitin ligase complex which plays key roles in mediating cellular life or death decisions downstream of various innate receptors. Mice that lack the adaptor of LUBAC, Sharpin, develop a severe multi-organ inflammatory disease termed chronic proliferative dermatitis in mice (*cpdm*) which shares pathological hallmarks with atopic dermatitis in humans. While skin inflammation in this context has been shown to be driven by TNF α , the relative contribution of additional inflammatory pathways remains poorly characterized. We have recently identified the Nlrp3 inflammasome to be critical for driving dermatitis in *cpdm* mice by mediating the production of inflammatory cytokines and through the induction of apoptosis and necroptosis. Given these findings we hypothesized that there may exist a cell-intrinsic interaction between LUBAC and the inflammasome. Here, we identify LUBAC to be a novel negative regulator of inflammasome activity in keratinocytes by mediating the linear ubiquitination of caspase-1. Furthermore, the central E3 ligase of LUBAC, HOIP, is cleaved by caspase-1 during apoptosis, impeding substrate linear ubiquitination. These findings provide further insight into the complex interplay between the inflammasome and cell death machinery and add to our understanding of inflammasome regulation.

Neuroimmunology

2718

Analysis of the binding specificity of antibody to dopamine-2 receptor in paediatric autoimmune movement and psychiatric disorders

Sinmaz, N., Pilli, D., Merheb, V., Amatoury, M., Pathmanandavel, K., Ramanathan, S., Dale, R., Brilot, F.

Brain Autoimmunity Group, Institute for Neuroscience and Muscle Research, The Kids Research Institute, Children's Hospital at Westmead, Discipline of Paediatrics and Child Health, University of Sydney, Sydney, Australia

Autoantibodies to neuronal surface antigens have been associated with brain disorders. The identification of these autoantibodies is important as they can be used as biomarkers for diagnostic purposes. We recently identified dopamine-2 receptor (D2R) autoantibodies in children with autoimmune movement and psychiatric disorders, however the target epitope(s) remain unknown. To assess anti-D2R antibody specificity, we subcloned D2R mutants modified in their extracellular domains and expressed them in human embryonic kidney cells. Confocal microscopy and flow cytometry were used to assess cell-surface expression levels of mutant D2R. Using a flow cytometry live cell-based assay, we found 0/35 (0%) D2R antibody-positive patient sera bound to N^{TermD1R}cD2R, suggesting the autoantibody epitope was within D2R N-terminus. Next, we examined autoantibody immunoreactivity to D2-22 D2R and found 25/35 (71%) patients recognised an epitope in this region. As this extracellular D2R domain also included an N-glycosylation site at position 23, we next assessed autoantibody to the D2R N-glycosylation. Out of the 25 patients positive for D2-22 D2R binding, 22/25 (88%) recognised N23Q with a 40% reduced immunoreactivity. Furthermore, D26E/A29P was subcloned to emulate mouse D2R, and 15/25 (60%) patients recognised this construct with a 45% decreased immunoreactivity. In order to abolish epitope binding, we subcloned R20K/P21G/F22W/N23Q/D26E/A29P mutant; 11/25 (44%) did not recognise this construct and the rest of the patients (14/25, 56%) bound the mutant, but with a substantial 79% decrease in immunoreactivity compared to D2-22 D2R. Defining the D2R antigenic region is important to elucidate functional effects of antibodies and provide precise testing for patients.

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Role of the cholinergic system in experimental pulmonary tuberculosis

Islas-Weinstein, L.^{1,2}, Barrios-Payán, J.¹, Mata-Espinosa, D.³, Marquina-Castillo, B.³, Hernández-Pando, R.¹

¹National Institute of Medical Sciences and Nutrition, Experimental Pathology, Mexico City, Mexico, ²National Autonomous University of Mexico, Mexico City, Mexico, ³National Institute of Medical Sciences and Nutrition, Mexico City, Mexico

Acetylcholine is an endogenous nicotinic and muscarinic receptor agonist that is secreted in the lungs of BALB/c mice under pathological conditions. Specific interactions of

acetylcholine with cholinergic receptors have been associated with macrophage deactivation, increased Th2 activity and decreased Th1 and Th17 responses in mouse models of pulmonary inflammation. Using high performance liquid chromatography (HPLC), lungs of BALB/c mice infected with *Mycobacterium tuberculosis* strain H37Rv revealed specific lung acetylcholine elevations particularly during late active disease that may contribute to protective immune decay and disease progression. Lung and brain tissue were also analyzed to determine the kinetics of specific acetylcholine receptors during the course of the disease. In order to study the contribution of the cholinergic system to the disease evolution, mice received either: saline solution (control group) or nicotinic selective antagonists coinciding with periods of acetylcholine exacerbation. Mice lungs were obtained after treatment and parameters of disease progression such as: granuloma formation, bacilli loads (colony-forming units) and pneumonia were measured. Our results showed that the administration of specific nicotinic receptor antagonists resulted in improved immune protection permitting better control of bacterial growth when compared to the control group. Thus, the cholinergic system contributes to decreased inflammation during late stages of disease through the partial suppression of cell-mediated immunity, permitting bacilli growth. Its pharmacological manipulation using anticholinergic agents may hence emerge as a novel therapeutic approach in the treatment of this significant infectious disease.

1625

Association of naive or differentiated human CD8+ T cells frequency with EBV and HHV-6 load in RRMS patients in relapse phase

Salehi, Z.¹, Beheshti, M.², Khosravani, P.³, Nomanpour, B.⁴, Naseri, M.⁵, Sahraian, M.-A.⁶, Izad, M.^{6,7}

¹Faculty of Medicine, Tehran University of Medical Sciences, Immunology Department, Tehran, Iran, Islamic Republic of, ²Pathophysiology Laboratory, Sina Hospital, Tehran University of Medical Sciences, Tehran, Iran, Islamic Republic of, ³Flow Cytometry Laboratory, Royan Institute, Department of Stem Cell and Developmental Biology, Tehran, Iran, Islamic Republic of, ⁴Kermanshah University of Medical Science, Microbiology Department, Tehran, Iran, Islamic Republic of, ⁵School of Public Health, Tehran University of Medical Sciences, Virology Department, Tehran, Iran, Islamic Republic of, ⁶MS Research Center, Neuroscience Institute, Tehran University of Medical Sciences, Tehran, Iran, Islamic Republic of, ⁷Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran, Islamic Republic of

Viruses such as Epstein-Barr virus (EBV) and Human herpesvirus 6 (HHV-6) have recently considered as potential causes of multiple sclerosis (MS). CD8+ T cells are the essential mechanism to handle with viral diseases. T CD8+ lymphocytes can be subdivided in to different subtypes; naïve, early-, intermediate- and late-differentiated, according to the expression of CD27, CD28, CD45 and CD57, the phenomena may induced by viruses. Our aim was to determine the EBV and HHV-6 load and their relation with CD8+ T cells composition change in RRMS patients. In this study we performed quantitative detection of

EBV and HHV-6 DNA in plasma in RRMS patients, in active phase (n=23) and healthy age- and gender-matched controls (n=19) using Real-time PCR. CD8+ subset frequency with Anti-CD27, CD28, CD45 and CD57 monoclonal antibodies were measured using flowcytometry. Although higher EBV viral load was found in MS patients compared to controls (250.95 copies/ml and 4.15 copies/ml respectively), it was not significant (P=0.1). There was no difference between patients and controls in HHV-6 copy numbers (P=0.9). Notably, we demonstrated higher frequency of early-differentiated CD8+ cells (CD8+ CD27+ CD28+ CD45RO+ CD57-) in RRMS patients (P=0.03). We also identified higher proportion of CD27+ CD28- CD8+ T cells in HHV-6 positive compared to HHV-6 negative patients (P=0.01). In addition, CD27+ CD28+ CD45RO- CD57- CD8+ frequency in RRMS patients was positively correlated with HHV-6 viral load (P=0.009). The present study indicates further evidence for changes in CD8+ T cells subsets composition that may be associated with persistent antigenic stimulation.

3079

Innate receptor signaling and Type I interferons in glial response in the central nervous system

Khoroshi, R.M.H.¹, Zaucha Sørensen, M.¹, Wais, V.¹, Dieu, R.¹, Thorsen Mørch, M.¹, Nistal-Villán, E.², González-Aseguinolaza, G.², Webster, G.³, Lienenklaus, S.⁴, Owens, T.¹

¹Institute of Molecular Medicine, University of Southern Denmark, Neurobiology Research, Odense, Denmark, ²Centro de Investigación Médica Aplicada (CIMA), Gene Therapy and Regulation of Gene Expression Program, University of Navarra, Pamplona, Spain, ³Innate Immunotherapeutics, Auckland, New Zealand, ⁴Institute for Laboratory Animal Science, Hannover Medical School, Hannover, Germany

Type I interferons (IFN), including IFN- β , play a central role in regulation of inflammation. IFN- β is used as a therapeutic for multiple sclerosis (MS), an inflammatory demyelinating disease in central nervous system (CNS). IFN- β is induced by ligation of innate receptors, including Toll-like receptors (TLRs). Microglia and astrocytes express innate receptors and are known to be involved in the regulation of inflammation. Previous studies identified microglia as a major source of endogenous Type I IFN in the CNS, and induction of microglial type I IFN by a TLR3 agonist administered directly into the cerebrospinal fluid protected against experimental autoimmune encephalomyelitis, a mouse model of MS. To further study glial responses and induction of type I IFN we have administered a range of innate receptor agonists directly to the CNS of mice. These include agonists to TLR2, 3, 4, 7, 9, a construct (MIS416) composed of ligands to NOD2-TLR9, as well as an AAV-vector encoding 2CARD-MAVS200. We present results from reporter mice showing in vivo expression of IFN- β , as well as cellular source and glial response. Administration of a TLR3 ligand transiently induced strong up-regulation of IFN- β , within hours, whereas a TLR2 ligand was ineffective. TLR3 ligation did not induce IFN- β in astrocytes. IFN response factors and IFN-inducible chemokines were upregulated in astrocytes and microglia. Our studies support a general hypothesis that microglia initiate a regulatory response via type I IFN that acts on astrocytes.

3485

Insight into regulatory mechanisms induced by a novel immunospecific therapy with synthetic multi-epitope targeting agent modulate MS-like disease

Kaushansky, N., Kaminitz, A., Allouche-Arnon, H., Ben-Nun, A. The Weizmann Institute of Science, Rehovot, Israel

Specific neutralization of the pathogenic autoimmune cells is the ultimate goal in therapy of autoimmune diseases. Among all approaches proposed for MS therapy, an approach that neutralizes only the pathogenic T cells, while leaving the innocent immune cells intact, is the ultimate goal in the immune-specific therapy for MS. We recently showed that a concomitant "multi-epitope-targeting" approach, using a specifically designed artificial multi-epitope protein (Y-MSPc) is required for effective antigen-based immune-specific therapy of EAE. Strikingly, Y-MSPc was consistently more effective than treatment with the single peptide or with the peptide cocktail, both in suppressing the development of "classical" or "complex" EAE and in ameliorating ongoing disease. Overall, the modulation of EAE by Y-MSPc was associated with anergizing the autoreactive T-cells, downregulation of Th1/Th17 cytokine secretion and upregulation of TGF- β secretion. Moreover, administration of Y-MSPc is associated also with a remarkable increase in a unique subset of myeloid-derived dendritic-cells (DCs), CD11c+CD11b+Gr1+ in both spleen and CNS of treated mice. These DCs, are functional in down-modulation of MS-like-disease displayed increased production of IL-4, IL-10 and TGF- β and low IL-12. Functionally, these DCs suppress the in-vitro proliferation of T-cells and were functional in-vivo, as their adoptive transfer into EAE induced mice resulted in suppression of the disease, associated with a remarkable induction of CD4+FoxP3+ regulatory cells.

These results, which highlight the efficacy of "multi-epitope-targeting" agent in induction of functional regulatory CD11c+CD11b+Gr1+myeloid DCs, further indicate the potential role of these DCs in maintaining peripheral tolerance and their involvement in downregulation of MS-like-disease.

3613

Cytokine production of CD4⁺ responder and regulatory T cells and the effect of IL-21 in co-culture in myasthenia gravis patients

Alahgholi-Hajibehzad, M.¹, Durmus, H.², Aysal, F.³, Gulsen-Parman, Y.², Oflazer, P.², Deymeer, F.², Saruhan Direskeneli, G.⁴

¹Istanbul University, Istanbul, Turkey, ²Istanbul University, Istanbul Medical Faculty Department of Neurology, Istanbul, Turkey, ³Bakirkoy Research and Training Hospital for Psychiatric and Neurological Diseases, Istanbul, Turkey, ⁴Istanbul University, Istanbul Medical Faculty Department of Physiology, Istanbul, Turkey

Impairment in the suppressive function of regulatory T cells (Treg) was reported in myasthenia gravis (MG). In this study, mechanisms that underlie the defect of Treg were investigated in patients with anti-acetylcholine receptor antibody positive MG (AChR-MG). The proliferation and cytokine production were measured in a suppression assay system performed with

polyclonal activation of responder T cells (Tresp). Restoring effect of IL-21 on suppression was evaluated in vitro in co-culture.

Tresp from MG patients secreted significantly lower levels of IL-2, whereas the proliferation did not differ from the controls. In Tresp/Treg co-cultures, IL-4, IL-6, IL-10 and IL-17 levels were increased in the presence of Treg, whereas IFN- γ was decreased in both patient and control groups. In MG patients, IL-2 levels did not change with the addition of Tregs to cultures, whereas it decreased significantly in controls. The addition of IL-21 to co-cultures increased the proliferation of Tresp and decreased the secretion of IL-4 and IL-17 in MG and controls. However IL-21 did restore the proliferation of Tresp from MG patients as in the controls and the production of cytokines was not effected by IL-21 in vitro differentially in the co-cultures.

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The transcription factor T-bet negatively regulates germinal centre responses and humoral immunity to malaria

Ly, A.^{1,2}, Ryg-Cornejo, V.^{1,2}, Chiu, C.Y.^{1,2}, Ioannidis, L.J.^{1,2}, Jacobson, K.³, Kallies, A.^{1,2}, Hansen, D.S.^{1,2}

¹The Walter and Eliza Hall Institute of Medical Research, Parkville, Australia, ²The University of Melbourne, Department of Medical Biology, Parkville, Australia, ³Monash University, Department of Biochemistry and Molecular Biology, Clayton, Australia

Malaria, the infectious disease caused by the *Plasmodium* parasites, claims the lives of almost half a million people annually. A large body of evidence suggests that naturally acquired immunity to malaria develops after many years of repeated infections. This form of protection is not sterilizing, and only prevents the development of symptomatic disease by substantially reducing blood-stage parasite densities. Immunological studies suggest that antibodies to *Plasmodium* antigens are inefficiently generated and responses wane rapidly in the absence of on-going parasite exposure. Moreover, individuals residing in areas of high malaria transmission have been found to exhibit a delayed development of parasite-specific memory B cells. These findings lend support to the notion that acute malaria may impede the generation and maintenance of malaria-specific B cell responses. However, this hypothesis has not been extensively investigated. Using the *P. berghei* ANKA rodent malaria model, we found that severe malaria infection impairs germinal centre responses and inhibits T follicular helper (Tfh) cell differentiation in the spleen. Our findings revealed that infection induces a high frequency of Tfh cell precursors that displayed low levels of PD-1 and CXCR5 and co-expressed T helper 1 associated molecules, including the transcription factor T-bet and chemokine receptor CXCR3. Moreover, genetic deletion of T-bet significantly restored Tfh cell differentiation and resulted in efficient germinal centre formation, as well as improved parasite-specific antibody responses to infection. Together, these results demonstrate that the same pro-inflammatory pathways that drive severe

symptomatic malaria have detrimental effects on the induction of protective humoral immunity.

3133

Inflammatory monocyte- and neutrophil-derived CXCL10 impairs efficient control of blood-stage malaria infection and promotes severe disease

Ioannidis, L.J.^{1,2}, Nie, C.Q.³, Ly, A.^{1,2}, Ryg-Cornejo, V.^{1,2}, Chiu, C.Y.^{1,2}, Hansen, D.S.^{1,2}

¹The Walter and Eliza Hall Institute of Medical Research, Infection and Immunity Division, Victoria, Australia, ²The University of Melbourne, The Department of Medical Biology, Victoria, Australia, ³The University of Melbourne, Office for Research Ethics and Integrity, Victoria, Australia

Cerebral malaria (CM) is a severe and often fatal complication of *Plasmodium falciparum* infection. This syndrome results from the sequestration of parasitized red blood cells in the brain microvasculature and strong inflammatory responses to infection. Among the inflammatory factors produced in response to infection, the chemokine CXCL10 has been identified as a biomarker of CM. Consistent with this, we have previously shown that CXCL10 blockade alleviates brain intravascular inflammation and protects malaria-infected mice from CM. In addition to organ-specific effects, here we found that a lack of CXCL10 during infection also reduces parasite biomass. To identify the cellular sources of CXCL10 involved in these processes, wild-type and CXCL10^{-/-} mice were irradiated and reconstituted with bone marrow from either wild-type or CXCL10^{-/-} mice. Chimeric mice were then infected and brain parasite and leukocyte sequestration, as well as parasite biomass was assessed. This revealed that hematopoietic cell-derived CXCL10 impairs efficient control of parasite biomass, regulates leukocyte recruitment to the brain and is responsible for the development of CM. Inflammatory monocytes and neutrophils were identified as the main cellular sources of CXCL10 responsible for the induction of these processes. Moreover, the improved control of infection observed in the absence of CXCL10-mediated trafficking was associated with a preferential accumulation of CXCR3⁺CD4⁺ T follicular helper cells in the spleen and enhanced antibody responses to infection. These findings indicate that some inflammatory responses elicited during malaria infection contribute to the development of high parasite densities involved in the induction of severe disease in target organs.

1579

Evaluation of immunomodulatory activities of lentinan in combination with miltefosine in Balb/c mice against visceral leishmaniasis

Shivhare, R.¹, Ali, W.¹, Singh, U.S.¹, Khattri, S.², Puri, S.K.³, Gupta, S.³

¹King George's Medical University, Department of Pathology, Lucknow, India, ²King George's Medical University, Department of Pharmacology and Therapeutics, Lucknow, India, ³CSIR-Central Drug Research Institute, Division of Parasitology, Lucknow, India

Visceral leishmaniasis (VL), a vector-borne infection, caused by protozoan parasite *Leishmania* is the second largest parasitic

killer after malaria. VL is potentially fatal disease in which parasite distorts the normal immune function of the host. Single-drug therapy against VL is far from satisfaction since existing antileishmanials have several limitations. Miltefosine, only oral drug, is the mainstay of the VL elimination program but its teratogenic effect, narrow therapeutic window and resistance issue raise the concern. Thus, combinations of existing drugs and/or immunomodulators are the options for the successful treatment of VL. In our study in *Leishmania donovani*/mouse model of VL, we have assessed the optimum dose (2.5 mg/kg×5 days, intraperitoneal route) of lentinan, a biological defense modifier and tested in combination with short dose (2.5 mg/kg×5 days, oral) of miltefosine and detailed immunological parameters (Th1/Th2 cytokines, IgG antibody, nitric oxide, splenocyte proliferation and phagocytic activity) were investigated at days 7 and 14 post treatment. Animals that received combination therapy showed sterile cure (~100% inhibition of parasite) of VL in both liver and splenic cells. Combination group displayed ($p < 0.001$) increased level of Th1 cytokines, IFN- γ , IL-12 and TNF- α along with nitric oxide and significantly suppressed level of Th2 cytokines, IL-10, IL-4 and TGF- β . Furthermore, maximum IgG2 antibody level, splenocyte proliferation and induced phagocytic ability of macrophages were recorded during combination therapy when compared with other treated groups. Collectively, lentinan has synergistic effect on miltefosine efficacy and its protective effect against VL is host-mediated and involves potentiation of Th1 mediated immune responses.

834

Combining immunoproteomics and *in silico* MHC-II epitopes prediction for the identification of potential protective antigens against *Echinococcus granulosus* infection in mice

Miles, S., Navatta, M., Dematteis, S., Mourglia-Ettlin, G.

Universidad de la República, Faculty of Chemistry - Department of Biosciences - Laboratory of Immunology, Montevideo, Uruguay

Echinococcus granulosus is the parasite responsible for cystic echinococcosis. In intermediate hosts, induction of specific antibodies play important roles in susceptibility/resistance phenomena. Identification of the targets of such antibodies is extremely important for the assignment of protective antigens. Therefore, we have analyzed the differential recognition of tegumental antigens from *E. granulosus* protoscoleces (termed PSEx) by antibodies from early-infected mice belonging to high (Balb/c) and low (C57Bl/6) susceptible strains. Through 2DE and immunoblots we described remarkable differences in the recognition patterns by IgM, IgA and IgG subclasses. Then, by means of mass spectrometry we identified 10 proteins from PSEx which were solely recognized by antibodies from the most resistant mouse strain. Gene ontology term enrichment classified most of them as cytoplasmatic proteins involved in metabolic processes with catalytic activity. On the other hand, since T-dependent antibody responses depends on the collaboration between B and T cells via antigens presented in MHC class II molecules, we further analyzed MHC class II epitopes in the set of proteins identified. Through the online prediction software NetMHCII, we discriminated such epitopes

between C57Bl/6 and Balb/c alleles (H2-IAb and H2-IAd, respectively), and our results predicted that 9 out of 10 proteins have a larger number of epitopes with higher binding affinity towards the allele in C57Bl/6. In summary, our results suggest that the genetic predisposition to generate better T-dependent antibody responses against particular (and thus potentially protective) antigens might be a key factor influencing the susceptibility of the host to the infection by *E. granulosus*.

2687

Perforin is required for edema and blood-brain barrier disruption in vital regions of the brain in experimental cerebral malaria

Johnson, A.¹, Huggins, M.², Jin, F.²

¹Mayo Clinic, Immunology and Neurology, Rochester, United States, ²Mayo Clinic, Immunology, Rochester, United States

Human cerebral malaria (HCM) is a serious complication of *Plasmodium falciparum* infection, resulting in coma, neurological deficit, and death. Recently, the first large-scale MRI study in humans identified brain swelling/edema as the most prominent biomarker of fatal HCM. We therefore investigated the mechanism by which edema and blood-brain barrier (BBB) disruption occurs in the *Plasmodium berghei* ANKA (PbA) infection model of HCM. We developed T2-weighted and gadolinium-enhanced T1-weighted small animal magnetic resonance imaging (MRI) platforms to study edema and CNS vascular permeability, respectively. T2-weighted MRI analysis of symptomatic PBA infected C57Bl/6 mice revealed significant edema. In addition, gadolinium enhanced T1-weighted MRI analysis of these animals revealed punctate CNS vascular permeability in vital regions of the brain and brain stem. Confocal microscopy confirmed CNS vascular permeability in these brain regions, with FITC albumin leakage from vasculature co-localizing with disrupted BBB tight junction proteins, occludin and claudin 5. Importantly, perforin deficient C57Bl/6 mice, despite having identical levels of PbA infected erythrocytes and brain infiltrating CD8 T cells, are completely resistant to edema and BBB disruption in these studies. We conclude the onset of brain edema in experimental cerebral malaria is mediated by perforin, providing a mechanism for how a significant biomarker of fatal HCM occurs in this deadly neurological disease.

3032

***Echinococcus granulosus* infection ameliorates airway inflammation in ovalbumin-induced mouse allergic asthma model**

Zhang, W., Wang, H., Li, J., Wen, H.

The First Affiliated Hospital of Xinjiang Medical University, Urumqi, China

Background: Cystic echinococcosis (CE) is a near cosmopolitan zoonosis caused by the larval stage of the dog tapeworm *Echinococcus granulosus*. The infection induces a polarized T-helper type 2 (Th2) systematic immune response in its intermediate hosts. However, it is not known whether the

infection modulates lung inflammation by regulating local immune response. In this study, we examined the effects of *E. granulosus* infection on mouse ovalbumin (OVA)-induced asthma model.

Methods: BALB/c mice were intraperitoneally transplanted with 50 small *E. granulosus* cysts cultured in vitro. At 3 months post-inoculation, the mice were sensitized and challenged with ovalbumin (OVA). For histopathological studies, hematoxylin eosin and periodic acid schiff staining was used to examine the inflammatory cells infiltration and goblet cells hyperplasia, respectively. Cytokine levels were measured by mouse cytometric bead array (CBA) Kit and quantitative qPCR and other molecular biological approaches.

Results: *E. granulosus* infection significantly increased Th2 and Treg cytokine levels in serum and lung tissues, but down-regulated the expression of IL-5 in the lungs and IL-17A in serum and lung tissues of asthmatic mice sensitized and challenged with OVA. Histological staining of lung tissues showed that *E. granulosus* infection significantly reduced the severity of OVA-induced airway inflammation including reduction of eosinophil cell infiltration and mucus production. The infection also reduced eosinophil accumulation induced by OVA in bronchoalveolar lavage fluid and also ameliorated airway hyperresponsiveness, a hallmark symptom of asthma.

Conclusions: *E. granulosus* infection remarkably reduces the severity of OVA-induced airway inflammation likely through enhancing IL-10 and down-regulation of IL-5 and IL-17A.

Tumour Immunology 5

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Costimulatory signals derived from myeloid leukemia cells contribute to functional exhaustion in CD4⁺ T cells

Ozkazanc, D.¹, Buyukasik, Y.², Esendagli, G.¹

¹Hacettepe University Cancer Institute, Ankara, Turkey, ²Hacettepe University Medical Faculty, Ankara, Turkey

Expression of costimulatory molecules on myeloid leukemia cells has been associated with disease severity and bad prognosis. Since the effector T cell responses under chronic stimuli are shadowed by exhaustion, a state of hypo-responsiveness, this study explores the contribution of myeloid leukemia-derived costimulatory signals to Th cell exhaustion as an indirect immune escape mechanism. Found on leukemia cells, CD86 and ICOS-LG costimulatory molecules supported Th activities however, under prolonged stimulation, T cells were directed into an exhaustion-related state. Alternatively, Th cells co-cultured with peripheral blood monocytes preserved their functional responsiveness. The in vitro-generated exhausted Th cells were characterized by the upregulation of PD-1, CTLA-4, LAG3 and TIM-3 surface molecules. These TIM-3⁺ Th cells were reluctant to proliferate upon restimulation and displayed attenuation in IL-2, TNF- α and IFN- γ production. IL-2 supplementation restored the proliferation capacity of exhausted Th cells. When the costimulatory signals were blocked with CTLA-4-Fc and ICOS-Fc recombinant proteins or CD86⁺ leukemia cells were depleted, the percentage of exhausted Th cells was significantly decreased. Moreover, in the bone marrow samples of acute

myeloid leukemia (AML) or myelodysplastic syndrome (MDS) patients, PD-1⁺/TIM-3⁺/LAG3⁺ Th cells were identified together with myeloid blasts expressing CD86 and/or ICOS-LG, in vivo. Collectively, the constitutive costimulation provided by myeloid leukemia cells contributes to T cell exhaustion as a novel immunogenic escape mechanism.

1044

Seromic profiling of melanoma patients using a custom protein microarray platform

Duarte, J.^{1,2,3}, Woods, K.^{1,2}, Tsao, S.C.-H.^{1,2}, Cebon, J.^{1,2}, Blackburn, J.³

¹Olivia Newton-John Cancer Research Institute, Cancer Immunobiology Laboratory, Heidelberg, Australia, ²La Trobe University, School of Cancer Medicine, Heidelberg, Australia, ³University of Cape Town/Institute of Infectious Disease and Molecular Medicine, Blackburn Laboratory, Cape Town, South Africa

There is increasing evidence that the aberrant expression of cancer-testis (CT) antigens - a family that are auto-immunogenic and mainly restricted to tumours in various types of human cancers - makes them attractive immunotherapy targets, as well as possible cancer diagnostic markers. Therefore, we aimed to measure differences in CT antigen-specific antibody repertoires between melanoma patient samples, and assess whether these could identify novel diagnostic or prognostic biomarkers, which could aid in the detection and management of cancer.

We carried out a retrospective serological study of antibody titres across a large cohort of eighty-eight malignant melanoma patients undergoing a variety of distinct cancer treatments (surgery, chemotherapy, radiotherapy, immunotherapy or none), using our recently developed and validated novel CT antigen microarray platform.

We successfully identified abundant antibody titres towards two antigens, NY-ESO-1 and CTAG2 - a highly homologous pair - expressed across 48% (n = 42/88) and 45% (n = 40/88) of all patients, respectively, with reported diagnostic and clinical efficacy predictive biomarker potential. Additionally, when considering treatment specific antibody patterns, the highest antibody levels were observed for treatment naïve patients. Furthermore, the overlap in antibody titres of this pair validated our array platform, showing consistency among our obtained results across patients.

In conclusion, we showed that our novel protein microarray platform represents a sensitive, high-throughput and customizable means to detect and quantify the presence of large panels of cancer-specific human antibodies in serum, obtaining consistently robust, high quality and reproducible data, and demonstrating its potential feasibility and inferred biological significance.

1734

Differential expression of the inhibitory receptors PD-1, CTLA-4 and BTLA on effector T cells is a requirement for the development of intestinal type gastric cancer

Morales-Alvarez, A.¹, Gómez, M.², Burgos, R.³, Parra-López, C.¹

¹Universidad Nacional de Colombia, Medical School, Bogota,

Colombia, ²Hospital el Tunal E.S.E Nivel III, Gastroenterology Service, Bogotá, Colombia, ³Hospital el Tunal E.S.E Nivel III, Pathology Service, Bogotá, Colombia

Gastric cancer is a multistep process influenced by chronic inflammation, where initial states such as severe atrophic gastritis or intestinal metaplasia are associated to high risk of adenocarcinoma development. It has also been shown that gastric cancer patients have an immunosuppressive status; however, the timeframe of how this status evolved remains unclear. Using multi-color flow cytometry here we developed a strategy to screen the antigen presenting-cells (APC) and T cell compartments in PBMC from individuals in different stages of the carcinogenesis process (healthy donors (n=12), gastritis (n=20), metaplasia (n=18), early cancer (n=15) and advanced cancer (n=22)). The levels of Tregs, MDSCs, myeloid and plasmacytoid dendritic cells (DCs) *ex vivo* and the immune-phenotype of DCs and T cells (including the induction of effector Th17 vs Tregs) after *in vitro* stimulation were measured. In some individuals the findings in PBMC were compared to the contexture of immune-cell infiltrate in biopsies by immunohistochemistry. Our results show that patients with cancer and metaplasia compared with gastritis patients and healthy donors, have a marked immune-suppression phenotype evidenced by an increase in Treg and MDSCs *ex vivo*, DCs refractoriness to pro-inflammatory stimuli and poor T cell response to TCR stimulation accompanied by a differential expression of PD1, BTLA and CTLA-4 inhibitory receptors. This study support that the transition from metaplasia to intestinal type of gastric cancer might be in part explained by differential expression of inhibitory receptors in effector T cells.

2090

The role of CD169 in tumour metastasis

Muhsin-Sharafaldine, M.-R., Saunderson, S.C., Dunn, A.C., D. McLellan, A.

University of Otago, Microbiology & Immunology, Dunedin, New Zealand

Tumour spread (metastasis) is the leading cause of death in cancer patients. Tumour soluble factors including extracellular vesicles may condition host organs for metastatic tumour spread. The CD169 (sialoadhesin) molecule expressed in lymph node is an important uptake and immune-modulator system for sialic acid-rich extracellular vesicles. In order to study the role of CD169 in melanoma metastasis, we first developed a model for metastatic melanoma spread from the skin to the lymph nodes. Subcutaneous implantation of 10⁶ B16 (F1 or F10) into the ventral carpal aspect of forelimb resulted in melanoma infiltration into the axillary and brachial lymph node within 12 days. Models using intradermal and subcutaneous implantation in the flank or ear pinnae either did not yield consistent metastasis or resulted in a higher welfare cost to the animals. Metastasis was quantified by Image-J analysis of frozen sections of lymph nodes and tumour infiltrated areas were confirmed by exclusion of the majority of CD45 labelled leukocytes. Although CD169^{-/-} mice displayed a lower levels of metastatic lymph node lesions over the course of the two studies, this failed to reach statistical significance in one experiment. CD169^{-/-}

displayed higher immune responses to tumour extracellular vesicles, and protection against subcutaneous melanoma was enhanced beyond that observed with control C57BL/6 mice. Together, these findings suggest that capture of tumour extracellular vesicles by CD169 may participate in tumour escape mechanisms.

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The cold shock protein YB-1 (Y-box binding protein 1) regulates resistance against apoptosis in T-lymphocytes

Meltendorf, S.¹, Gieseler-Halbach, S.¹, Handschuh, J.², Pierau, M.¹, Mertens, P.R.³, Thomas, U.², Brunner-Weinzierl, M.C.¹

¹Universitätskinderklinik Magdeburg, Experimentelle Paediatric, Magdeburg, Germany, ²Leibniz Institute for Neurobiology, Department of Neurochemistry and Molecular Biology, Magdeburg, Germany, ³Otto-von-Guericke University Magdeburg, Department of Nephrology and Hypertension, Diabetes and Endocrinology, Magdeburg, Germany

The cold shock protein YB-1 is highly expressed in tumours, such as breast cancer, and associated with hyperproliferation and resistance against apoptosis. Enhanced YB-1 expression at the transcript and nuclear protein levels have been shown to correlate with poor prognosis for tumour patients and resistance to chemotherapy. Its role in T-lymphocytes is not understood so far.

Human CD4⁺ T-lymphocytes isolated from PBMCs were stimulated with anti-CD3/anti-CD28 coupled beads. YB-1-signaling was manipulated by overexpression of YB-1^{wt} and various mutations using lentiviral transduction of FuGW-GFP-constructs or reduction of YB-1 using specific YB-1shRNA. Localisation within T-lymphocytes was determined by microscopy. Apoptosis was induced by anti-CD95 cross-linking and analyses carried out six to eight days after initial stimulation. Expression of pro- and anti-apoptotic molecules was monitored by FACS, Western blot, and real-time PCR.

Transduction of GFP-YB-1^{wt} constructs and mutant variants thereof into primary human CD4⁺ T-cells yielded around 40% GFP positive cells. Various YB-1 mutant proteins exhibited distinct subcellular localisations. We observed that GFP-YB-1 overexpression reduced apoptosis by 60% compared to the control. Additionally, the percentage of apoptotic cells was decreased with specific inhibitors (QVD) for apoptosis in empty vector-transduced cells but not in YB-1^{wt} overexpressing cells. Furthermore, we observed substantial differences in the expression of the pro- and anti-apoptotic molecules Bax and Bcl2. The reduction of YB-1 with YB-1shRNA leads to elevated apoptosis in T-Lymphocytes. Thus, YB-1 tightly controls and inhibits apoptosis in T-cells and could therefore serve as a major player to induce apoptosis resistance in various types of cancers, such as T-ALL.

3375

B7-H1 (PD-L1) is expressed in the Tasmanian devil facial tumor microenvironment and is strongly upregulated in response to IFN- γ *Flies, A.^{1,2}, Woods, G.¹, Lyons, A.B.³, Hayball, J.²**¹Menzies Institute for Medical Research, University of Tasmania, Hobart, Australia, ²University of South Australia, Adelaide, Australia, ³School of Medicine, University of Tasmania, Hobart, Australia*

In 1996 a clonal, transmissible tumour was discovered in wild Tasmanian devils and this devil facial tumour disease (DFTD) has been a primary cause of a greater than 85% decline in the wild devil population. In 2015 a second transmissible tumour was discovered in wild Tasmanian devils, further threatening the survival of the species. Although the second transmissible tumour (DFT2) has not been fully characterized, in the nearly 20 years of ongoing transmission the first transmissible tumour (DFT1) has accumulated greater than 17,000 somatic mutations. Evidence from human clinical trials and mouse models suggests that blocking PD-1:B7-H1 interactions is an effective treatment against a wide range of cancers and that blocking the PD-1:B7-H1 pathway is most effective in cancers with a high number of mutations. We hypothesized that the DFTD cells use the PD-1:B7-H1 pathway to evade the anti-DFT responses. We developed a panel of devil specific monoclonal antibodies against PD-1 and B7-H1, some capable of blocking PD-1 binding to B7-H1. Immunohistochemistry of devil tissues revealed that B7-H1 was not normally expressed on primary tumours, but was present in the microenvironment of metastatic tumours. *In vitro* stimulation of several DFTD cell lines with IFN- γ resulted in upregulation of B7-H1 on the cell surface. Altogether our results suggest that B7-H1 is transiently upregulated by IFN γ and that abrogating the effects of B7-H1 in the tumour microenvironment and lymphoid organs might be critical to ongoing development of the vaccine to treat DFTD cancers.

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Characterising T-cell responses against mutated tumour neo-antigens*Chee, J.¹, Ma, S.¹, Creaney, J.^{1,2}, Robinson, B.^{1,2,3}**¹University of Western Australia, National Centre of Asbestos Related Diseases, School of Medicine and Pharmacology, Nedlands, Australia, ²Sir Charles Gairdner Hospital, Australia Mesothelioma Tumour Bank, Nedlands, Australia, ³Sir Charles Gairdner Hospital, Department of Respiratory Medicine, Nedlands, Australia*

A feature of carcinogen-induced cancers is the accumulation of mutations in the cancer cell. Cytotoxic T lymphocytes (CTLs) recognise mutated proteins (neo-antigens), and in some cases, boosting CTL responses specifically against neo-antigens causes tumour regression. We have previously interrogated mutations in asbestos induced mesothelioma with RNA and exome sequencing. By applying MHC-binding algorithms on the sequencing data, we described mutated predicted CTL neo-antigens. We detected immune responses against one of the predicted neo-antigens: mutated *Uqrc2* (Uq2) in mesothelioma

bearing animals. It is unclear if Uq2-specific CTL responses are important for immunosurveillance, or if boosting them will be effective for tumour regression. To understand the role neo-antigen-specific T cells play in tumour immunity, we tracked the development of Uq2-specific CTLs during tumour growth, and how they changed after immunotherapy.

The frequencies of neo-antigen specific T cells are low and restricted to only one or a few mutated neo-antigens. Uq2-specific CTLs were < 0.1% of total CD8⁺. Conventional flow cytometry techniques were not sensitive enough to detect them. Using an ELISPOT assay, we demonstrated that the frequencies of Uq2-specific responses varied between tumour bearing mice, and generally increased after different therapies (e.g. checkpoint-blockade immunotherapy, chemotherapy or Treg depletion). For effective translation, e.g. using neo-antigen vaccines, assays with increased sensitivities, or an additional T cell expansion step must be used to detect and phenotype neo-antigen specific T cells. To that extent, we have optimised sensitive pMHC tetramer enrichment assays, and *in vitro* T cell expansion protocols to further analyse neo-antigen specific cells.

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1410

Oxidative stress and age related impairments in the maintenance of immunological memory*Pangrazzi, L.¹, Meryk, A.¹, Trieb, K.², Grubeck-Loebenstien, B.¹**¹University Innsbruck, Research Institute for Biomedical Aging Research, Innsbruck, Austria, ²Klinikum Wels-Grieskirchen, Wels, Austria*

Aging induces a basal level of inflammation throughout the body, a condition known as inflammaging, which contributes to immunosenescence. New strategies to counteract immunosenescence in the elderly are needed, in particular by improving the maintenance of immunological memory. It has been demonstrated that memory T cells and long-lived plasma cells home to bone marrow niches, well organized structures which promote the survival of these cells through homeostatic proliferation. CD4⁺ and CD8⁺ effector memory T cell survival is promoted by IL-7 and IL-15. IL-7 is believed to be important for long-lived memory T cells while IL-15 is mostly important for short-lived CD28⁻ senescent T cells, of which accumulation is associated with mortality in old age. The expression of effector memory T cells and proinflammatory factors were investigated in bone marrow mononuclear cells (BMMC) using qPCR and FACS, finding that, with age, IL-7 decreases while IL-15, IL-6, TNF α , IFN γ and IL1 β increase. Stimulation of peripheral blood mononuclear cells (PBMC) with IFN γ and TNF α lead to increased IL-15 expression in CD11c^{hi} cells and monocytes, well-known producers of this cytokine. A correlation was found between ROS levels and IL-15 expression in the same cell populations. Incubation of stimulated PBMCs with ROS scavengers N-acetylcysteine and vitamin C completely neutralized the effects of proinflammatory molecules in CD11c^{hi} cells and monocytes. This indicates that oxidative stress may contribute to the age-related impairments in the maintenance of immunological memory. Antioxidant treatment may be an

important strategy to counteract immunosenescence, reducing the level of proinflammatory cytokines in old age.

2874

The role of IL-1 and IL-23 signaling in tumor elicited inflammation in colon cancer

Grivennikov, S., Dmitrieva, O., Hou, V.

Fox Chase Cancer Center, Cancer Prevention and Control, Philadelphia, United States

Many solid tumors are characterized by the presence of immune infiltrates and are able to enhance the expression of inflammatory cytokines - a process referred to as tumor elicited inflammation (TEI). In colorectal cancer (CRC), early oncogenic events lead to deterioration of the integrity of epithelial barriers, allowing translocation of the microbial products and induction of pro-inflammatory cytokine expression, particularly of IL-23 and IL-17A. The intricate mechanistic details of TEI remain to be worked out, however we and others showed that microbial-driven expression of IL-23 stimulates the production of its downstream pro-inflammatory and pro-tumorigenic effector cytokines (e.g. IL-17A, IL-17F and IL-22). We hypothesized that members of IL-1 family (IL1a, b) cooperate with IL-23 in induction of TEI and colorectal cancer progression. Using conditional IL-1R, IL-23R and ligand knockout mice, we demonstrated that IL-1 and IL-23 responsive T cells and Innate lymphoid cells (ILC) type 3 are required to integrate upstream microbiota and stressed tissues released signals to enhance pro-tumorigenic IL-17 production. By multiphoton imaging we have demonstrated the dynamics of IL-17 producing cells in tumor and normal tissue. We also determined that strongly adhesive bacteria, capable only of colonizing tumor tissue are sensed by monocytes and macrophages to induce IL-1 and IL-23 production. Altogether, we established the role of microbiota and IL-1/IL-23 signaling in induction of Tumor elicited inflammation and immunity in CRC tumors.

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OPG may protect bleomycin-induced pulmonary fibrosis by inhibiting SMAD2/3 dependent TGF- β 1 activation

Weng, D.¹, Li, H.¹, Ge, B.²

¹Shanghai Pulmonary Hospital, School of Medicine, Tongji University, Respiratory Medicine, Shanghai, China, ²Shanghai Pulmonary Hospital, School of Medicine, Tongji University, Clinical and Translational Research Center, Shanghai, China

Rationale: Idiopathic pulmonary fibrosis (IPF) is a highly heterogeneous and lethal pathological process with limited therapeutic options. The pathogenesis of IPF is a complex process with continual injury, chronic inflammation and wound healing response. We found OPG (osteoprotegerin) were significantly increased in IPF patients than that in healthy controls.

Methods: To investigate the role of OPG in the pathogenesis of IPF, we used WT and OPG^{-/-} mouse to establish the model of pulmonary fibrosis by bleomycin. We analyzed the histopathology and evaluated expression of various key

cytokines. We also investigate the adaptive immune response by flow cytometry. Finally, important signaling pathway was determined by western blotting.

Results: We found that OPG-deficiency led to exacerbation of inflammation and fibrosis during fibrosis. OPG^{-/-} mice showed increased Th1, Th17, Th2 and decreased Tregs at early stage of BLM-induced fibrosis. OPG^{-/-} mice showed increased inflammatory cytokines at early stage of BLM-induced fibrosis. OPG^{-/-} mice have decreased Th1, Th17 and Bregs at late stage of BLM-induced fibrosis. OPG^{-/-} KO mice showed increased inflammatory cytokines, TGF- β and decreased IL-17A at late stage of BLM-induced fibrosis. OPG may inhibit TGF- β 1-dependent activation of SMAD2/3 signaling in pulmonary fibrosis.

Conclusions: Our data provided clear evidences that OPG might play a critical role in the pathogenesis of idiopathic pulmonary fibrosis. OPG may protect mice from bleomycin-induced pulmonary fibrosis by inhibiting SMAD2/3 dependent TGF- β 1 activation.

Keywords: OPG, pulmonary fibrosis, SMAD2/3, TGF- β 1

Conflicts of interest: The authors confirm that there are no conflicts of interest.

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Socs1 and Socs3 degrades Traf6 via polyubiquitination in LPS induced acute necrotizing pancreatitis

Zhou, X.¹, Liu, Z.², He, Y.³

¹Affiliated Hospital of Sichuan Medical University, Department of Vascular and Thyroid, Luzhou, China, ²University of Texas MD Anderson Cancer Center, Houston, United States, ³Affiliated Hospital of Sichuan Medical University, Luzhou, China

Mechanisms involved in inflammatory development during acute pancreatitis (AP) are largely vague, especially in the transformation of acute edematous pancreatitis (AEP) into acute necrotizing pancreatitis (ANP). This current study aims to investigate the functions of Traf6 in different acute pancreatitis models in vitro and in vivo, and to identify the possible regulatory mechanism in the progression of inflammation from mild to severe. Our data revealed that the level of Traf6 expression was significantly increased in the mild AP induced by caerulein, and the up-regulation of Traf6 played a protective role in acinar cells against caerulein-induced apoptosis. In contrast, only Traf6 protein but not mRNA was downregulated in the severe ANP induced by combination treatment of caerulein and LPS. Mechanistic studies showed that LPS upregulated the levels of Socs1 and Socs3 expressions in acinar cells, Socs1 and Socs3 interacted Traf6 directly and degraded Traf6 protein via polyubiquitination, thereby counteracted the protective function of Traf6. In vivo study further showed that combination treatment of caerulein and LPS failed to induce an ANP model in the TLR4 knockout mice, and the level of Traf6 expression in the pancreatic tissues remained the same as that from the AEP mouse. Taken together, our study reveals that Traf6 functioned as a protective factor in the progression of AP, and LPS induced Socs1 and Socs3 exacerbate mild AP to severe AP, which provides evidence for developing a new therapeutic target to combat AP.

2974**ATF3 is a key regulator of macrophage interferon-responses**

Labzin, L.I.¹, Schmidt, S.V.², Masters, S.L.^{3,4}, Beyer, M.², Krebs, W.², Klee, K.², Stahl, R.¹, Lütjohann, D.⁵, Schultze, J.L.², Latz, E.^{1,6,7}, De Nardo, D.^{3,4}

¹Institute of Innate Immunity, University of Bonn, Bonn, Germany,

²Life and Medical Sciences Institute (LIMES), University of Bonn, Bonn, Germany, ³The Walter and Eliza Hall Institute of Medical Research (WEHI), Inflammation Division, Parkville, Australia,

⁴The University of Melbourne, Department of Medical Biology, Parkville, Australia, ⁵Institute of Clinical Chemistry and Clinical Pharmacology, University of Bonn, Bonn, Germany, ⁶University of Massachusetts Medical School, Department of Infectious Diseases and Immunology, Worcester, United States, ⁷German Center for Neurodegenerative Diseases, Bonn, Germany

Cytokines and IFNs downstream of innate immune pathways are critical for mounting an appropriate immune response to microbial infection. However, the expression of these inflammatory mediators is tightly regulated, as uncontrolled production can result in tissue damage and lead to chronic inflammatory conditions and autoimmune diseases. Activating Transcription Factor 3 (ATF3) is an important transcriptional modulator that limits the inflammatory response by controlling the expression of a number of cytokines and chemokines. However, its role in modulating IFN responses remains poorly defined. Here, we demonstrate that ATF3 expression in macrophages is necessary for governing basal IFN β expression, as well as the magnitude of IFN β cytokine production following activation of innate immune receptors. We found that ATF3 acted as a transcriptional repressor, and regulated IFN β via direct binding to a previously unidentified specific regulatory site distal to the *Irfn1* promoter. Additionally, we observed that ATF3 itself is a type I IFN-inducible gene, and that ATF3 further modulates the expression of a subset of inflammatory genes downstream of IFN signaling, suggesting it constitutes a key component of an IFN negative feedback loop. Consistent with this, macrophages deficient in *Atf3* showed enhanced viral clearance in LCMV and VSV infection models. Our study therefore demonstrates an important role for ATF3 in modulating IFN responses in macrophages by controlling basal and inducible levels of IFN β , as well as the expression of genes downstream of IFN signaling.

3944**Constitutive AHR signaling-mediated modulation of antiviral IFN response**

Yamada, T.¹, Kameyama, T.^{1,2}, Watts, T.H.³, Matthews, J.^{4,5}, Takaoka, A.^{1,2}

¹Division of Signaling in Cancer and Immunology, Institute for Genetic Medicine, Hokkaido University, Sapporo, Japan, ²Molecular Medical Biochemistry Unit, Graduate School of Chemical Sciences and Engineering, Hokkaido University, Sapporo, Japan,

³Department of Immunology, University of Toronto, Toronto, Canada, ⁴Department of Pharmacology and Toxicology, University of Toronto, Toronto, Canada, ⁵Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that mediates the toxicity of many environmental xenobiotics including dioxins, and also has important biophysiological roles. However, its role in innate immune responses during viral infection is not fully understood. Type I interferons (IFNs) are produced in response to viral infection and are crucial cytokines for the activation of antiviral immune responses. The induction of type I IFNs is triggered by the detection of the viral nucleic acids through pattern recognition receptors. In this study, we show that constitutive AHR signaling in the steady state modulates type I IFN response during infection with various types of virus. The type I IFN response was markedly enhanced in AHR-deficient mouse embryonic fibroblasts (MEFs) during infection with various types of virus. Such an excessive response was also observed upon pharmacological inhibition with an AHR antagonist or a TDO inhibitor. Furthermore, we identified TIPARP as an AHR target gene that is essential for AHR-mediated regulation of type I IFN production. Mechanistic studies revealed that TIPARP interacts and post-translationally ADP-ribosylates TBK1, resulting in the inhibition of its kinase activity. Thus, our findings demonstrate a novel link between AHR signaling and innate signaling for the modulation of IFN-mediated antiviral response during viral infection, suggesting a physiological role of constitutive activation of the AHR-TIPARP axis mediated by endogenous ligands such as tryptophan metabolites, in restraining the IFN-dependent host antiviral defense system.

3389**Potent antitumor effect of two self-limiting adeno-associated viruses**

Vásquez, M., Aranda, F., Paredes, V., Fioravanti, J., Gomar, C., Ardaiz, N., Berraondo, P.

Center for Applied Medical Research (CIMA), University of Navarra, Division of Immunology and Immunotherapy, Pamplona, Spain

Interferon alpha (IFN α) is a cytokine approved for the treatment of several malignancies but the serious adverse effects induced by the high doses of the cytokine limit its clinical utility. We have developed a murine model of colon cancer liver metastases that reproduce the clinical outcome of the IFN α therapy: high doses of IFN α encoded by an adeno-associated virus (AAV-IFN) induce a potent antitumor effect associated with lethal toxicity. Low doses of AAV-IFN are well tolerated but do not induce an antitumor effect and mice die due to the colon cancer liver metastases. Co-administering an adeno-associated virus that encodes for an anaphylactic peptide that activates several danger signals, we were able to trigger a potent antiviral response that partially eliminated the AAV genomes once the tumor was eradicated. Thus, liver metastases of colon cancer could be eradicated without the toxicity associated to long-term interferon alpha expression.

Vaccines 2

3672

Salmonella porins are a multivalent vaccine candidate against typhoid, paratyphoid, and non-typhoidal salmonellosis

Pérez-Toledo, M.^{1,2}, Valero-Pacheco, N.^{1,2}, Aguilar-Salvador, D.I.¹, Leonardo-Reza, M.¹, Dionicio-Martínez, N.¹, Pastelin-Palacios, R.³, Cunningham, A.F.⁴, Isibasi, A.¹, López-Macías, C.^{1,5}

¹Mexican Social Security Institute, Medical Research Unit on Immunochemistry, Mexico City, Mexico, ²Instituto Politécnico Nacional, National School of Biological Sciences, Mexico City, Mexico, ³Universidad Nacional Autónoma de México, Faculty of Chemistry, Mexico City, Mexico, ⁴University of Birmingham, Institute of Immunology and Immunotherapy, Birmingham, United Kingdom, ⁵University of Oxford, Nuffield Department of Medicine, Oxford, United Kingdom

Salmonella-related diseases account for approximately 1 million deaths around the world. *S. Typhi* and *S. Paratyphi* cause typhoid and paratyphoid fever, respectively, while *S. Typhimurium* and *S. Enteritidis* are associated to non-typhoidal salmonellosis. Although different vaccines are employed to combat *S. enterica* infections, nowadays there is no vaccine that targets typhoidal and non-typhoidal serovars. Porins are outer membrane proteins that have shown to be safe and immunogenic. They have also shown to be a protective target for antibody responses during *Salmonella* infection. Since porins are well conserved among Gram-negative bacteria, we asked whether these proteins could be used as a potential vaccine candidate against typhoid, paratyphoid, and non-typhoidal salmonellosis. Through an *in silico* analysis, we confirmed that OmpC, OmpF, and OmpD porins share a high degree of amino acid identity amongst Typhi, Typhimurium, Enteritidis, and Paratyphi A serovars. The co-immunisation of *S. Typhi* and *S. Typhimurium* porins in mice elicited long-lasting IgG and IgM antibody responses that bound to whole inactivated bacteria. In primed and challenged mice, we found significantly lower bacterial numbers in spleen and liver for all serovars compared to the naïve controls. We also found that opsonisation of bacteria with anti-porins serum lowered bacterial burden in organs when compared to naïve mice. Overall, these results suggest that the mixture of porins is a potential vaccine candidate against typhoid, paratyphoid, and non-typhoidal salmonellosis.

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Exceptionally long CDR3H of bovine scFv antigenized with BoHV-1 B-epitope generates specific immune response against the targeted epitope

Pasman, Y., Kaushik, A.

University of Guelph, Molecular and Cellular Biology, Guelph, Canada

Earlier, our laboratory discovered that some bovine antibodies are among the largest known to exist in a species with an exceptionally long CDR3H (>48 amino acids) with multiple cysteine amino acids. Such CDR3H generates a unique antigen-binding site with a "stalk-knob" structure capable of providing configurational diversity via variable intra-CDR3H

disulfide bridging. Given the large CDR3H size, unlike mouse and human immunoglobulins where CDR3H is relatively shorter, these provide suitable platform for antigenization with large configurational B-epitopes. We first identified a B-epitope on gC envelope protein of bovine herpes virus type-1 (BoHV-1) recognized by a neutralizing bovine IgG1 antibody. A 156 amino acid long gC fragment (gC156) (UL44; NCBI Genome NC_001847 position 16,818-17,285) was expressed as a recombinant protein in *P. pastoris*. Subsequently, an scFv fragment with a 61 amino-acid long CDR3H (scFv1H12) was constructed where gC156 was grafted into the CDR3H with short flanking flexible linkers (gC156scFv1H12). The gC156scFv1H12 was successfully expressed in *P. pastoris*. The grafting of BoHV-1 B-epitope in bovine scFv with an exceptionally long CDR3H sustained configuration similar to native glycoprotein on BoHV-1 envelope as these could be recognized by BoHV-1 neutralizing scFv3-18L in solid phase. Free recombinant gC156 and its antigenized form, gC156scFv1H12, were characterized and used to immunize bovine calves. The antigenized scFv, gC156scFv1H12, induced higher antibody response as compared to free recombinant gC156 fragment in the calves.

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Epithelial ovarian cancer stem cells: a potential vaccine for ovarian cancer

Wu, D.¹, Wang, J.², Zhang, Y.², Cai, Y.², Ren, M.², Dou, J.¹

¹School of Medicine, Southeast University, Department of Pathogenic Biology and Immunology, Nanjing, China, ²Zhongda Hospital, School of Medicine, Southeast University, Department of Gynecology & Obstetrics, Nanjing, China

Epithelial ovarian cancer (EOC) is the most malignant type of gynecological tumor due to its high recurrence rate following initial treatment in the China. Though EOC is a highly chemosensitive disease, it is a difficultly cured owing to the presence of drug-resistant cancer stem cells (CSCs) that are responsible for EOC initiation, metastasis, chemo-resistance, and recurrence. The aim of this work was to investigate the effects on the inhibition of EOC growth by vaccination of CSC vaccine. The CD117+CD44+CSCs were isolated from human EOC SKOV3 and HO8910 cell lines by using a magnetic-activated cell sorting system, respectively. The nude mice were subcutaneously inoculated with the inactivated SKOV3 or HO8910 CD117+CD44+CSC vaccine three times before the mice were challenged subcutaneously with SKOV3 or HO8910 cells. The inhibition tumor efficacy of CSC vaccine was assessed by the tumorigenicity, immune efficient analysis by flow cytometer, and enzyme-linked immunosorbent assays, respectively. The results showed that, compared to the non-CSC vaccine, the inhibition tumor efficacy of SKOV3 or HO8910 CD117+CD44+CSC vaccine was significantly increased in the xenograft mice. Vaccination of SKOV3 CD117+ CD44+CSCs resulted in increasing cytotoxic activity of natural killer cells, enhancing serum IFN- γ , and decreasing TGF- β levels in the mice. The SKOV3 CD117+CD44+CSC vaccine significantly reduced the CSC and the aldehyde dehydrogenase 1 positive cell populations in the ovarian cancer tissues. The data provided the first evidence that the human SKOV3 or HO8910 CD117+CD44+CSC-based

vaccine may be an attractive therapeutic approach to excitation of anti-tumor immunity for treatment of ovarian cancer.

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Development of a BCG challenge model for the testing of vaccine candidates against bovine tuberculosis

Villarreal-Ramos, B.¹, Berg, S.¹, Blunt, L.¹, McShane, H.², Muller, J.², Ramasamy, A.², Spiropoulos, J.¹, Hicks, D.¹, Clifford, D.¹, Hewinson, R.G.¹, Vordermeier, H.M.¹

¹Animal and Plant Health Agency, Bacteriology/Bovine TB Research, Addlestone, United Kingdom, ²Jenner Institute, Oxford, United Kingdom

Despite the application of the test and slaughter policy, the incidence of bovine tuberculosis (BTB) in the UK has been increasing; this is thought to be due to the existence of a wildlife reservoir. Vaccination is being considered as part of a sustainable strategy for the control of BTB. The live attenuated *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) has been used experimentally to vaccinate cattle against BTB. However, BCG only confers partial protection against BTB and therefore, there is a need to develop improved vaccines. BTB vaccine efficacy experiments require BSL3 facilities which are expensive to maintain, generally oversubscribed and represent a bottle neck for the testing of vaccine candidates. It is reasonable to propose that if a vaccine is to be successful in conferring protection against *M. bovis* it should also confer protection against BCG. In this work we have evaluated intranodal BCG challenge as a model for the selection of vaccine candidates. Intranodally injected BCG was detected in vivo for at least 21 days after injection. As a group, cattle vaccinated with BCG presented lower BCG cfu counts at 21 days after intranodal BCG challenge than non-vaccinated cattle. Importantly, within the BCG vaccinated group, cattle could be divided into protected and non-protected according to their bacterial load. This model could be used to prioritize between competing vaccine candidates; furthermore this model could be used to explore the nature of the mycobacteriocidal immune responses elicited by BCG, which could help understand why BCG sometimes protects and sometimes does not.

4642

Differential expression of the IL17 cytokines and receptors in cells isolated from the female reproductive tract of mice during and after a genital *Chlamydia* infection

Carey, A., Trim, L., Armitage, C., Beagley, K.

Institute of Health & Biomedical Innovation, School of Biomedical Sciences, QUT, Kelvin Grove, Australia

Chlamydia trachomatis is often referred to as a silent infection, due to its asymptomatic nature and the devastating consequences of infection on reproductive health. *Chlamydia* infection can cause pelvic inflammatory disease, tubal obstruction and infertility in women. The mechanism of pathology development in the female reproductive tract is not fully understood. The production of IL17A in the oviducts of mice has proven to be a double edged sword, both enhancing

chlamydial vaccine induced immunity and being involved in oviduct pathology development. The cellular source of IL17 and associated receptor expression throughout chlamydial infection is unknown. We used our female mouse model of immunization (chlamydial major outer membrane protein/Iscomatrix) and genital infection to examine the cellular sources of the IL17 group of cytokines (IL17A-F) and corresponding receptors (IL17RA-E) at two, six and 35 days post infection, with or without immunization. Cells (CD4⁺, CD8⁺, NKT, $\gamma\delta$ TCR⁺, Ly6G⁺, F4/80⁺, NK and epithelial) were isolated from cervix/vaginal tissues and oviducts and collected via flow cytometry sorting at each time point. The mRNA expression of each IL17 cytokine and receptor was examined at each time point using digital droplet PCR. This highlighted that the cellular source and levels of the IL17 group of cytokines and receptors changed throughout infection and that immunization altered this expression. This greater understanding of the role of IL17 will underpin the development of vaccines that protect, without collateral tissue damage and provide a basis for trials of experimental IL17 inhibitors as a potential treatment for chlamydial disease.

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The pig as a large preclinical model for therapeutic human anti-cancer vaccine development

Overgaard, N.H.¹, Frøsig, T.M.¹, Welner, S.¹, Rasmussen, M.², Ilsøe, M.¹, Sørensen, M.R.¹, Andersen, M.H.³, Buus, S.², Jungersen, G.¹

¹Technical University of Denmark, National Veterinary Institute, Frederiksberg C, Denmark, ²University of Copenhagen, Department of International Health, Immunology and Microbiology, Copenhagen N, Denmark, ³Copenhagen University Hospital, Herlev, Center for Cancer Immune Therapy, Department of Hematology, Herlev, Denmark

Immunotherapy has increased overall survival of metastatic cancer patients, but appropriate tailoring of vaccine formulations to mount cytotoxic T cell (CTL) responses towards co-delivered antigens is crucial. Development of therapeutic cancer vaccines has largely been based on rodent models and the majority failed to establish therapeutic responses in clinical trials. Since the porcine immunome is closely related to the human counterpart, we introduce pigs as a large animal model for human cancer vaccine development via the use of our unique technology for swine leukocyte antigen (SLA) production. 20-mer overlapping peptides covering the porcine IDO protein sequence, a cancer antigen important in human disease, was administered to pigs formulated in CTL-inducing adjuvants. 136 candidate IDO-derived 9-11 mer peptides were identified in silico and peptide-SLA class I complex stability measurements revealed 89 stable ($t_{1/2} \geq 0.5$ hour) complexes. We showed that using our immunization strategy it was possible to break the peripheral tolerance and induce a cell mediated response to an endogenous antigen. The body size and metabolism of the pig allow dose translation to humans; therefore we designed a titration study with pigs receiving either 1 μ g, 10 μ g or 100 μ g of each peptide covering the majority of IDO-derived potential CTL epitopes formulated in CAF09 adjuvant and delivered by the intra-peritoneal route. Immune responses were tracked by IFN- γ ELISpot and peptide-MHC tetramers. Determining the

optimal dose is crucial to mount a proper Th₁ response, and we believe pigs will be a highly beneficial model for the subsequent design of clinical trials.

Poster Friday

15:30:00 - 16:30:00

Allergy

1077

The influence of vitamin D and cotinine on the T regulatory cells in children with asthma

Wawrzyniak, A.¹, Lipińska-Opałka, A.¹, Kalicki, B.¹, Zdanowski, R.², Lewicki, S.², Murawski, P.³

¹Military Institute of Medicine, Department of Pediatrics, Nephrology and Allergology, Warsaw, Poland, ²Military Institute of Hygiene and Epidemiology, Warsaw, Poland, ³Military Institute of Medicine, ICT, Warsaw, Poland

Background: Asthma is one of the most common chronic respiratory disease in children. It is claimed that vitamin D inhibits immunological reactions with Th1 and Th17 lymphocytes. The influence of vitamin D on T-regulatory cells (Treg) is not clear. Literature data evaluating the effect of cotinine on the immune system in children with asthma are poor.

Aim of the study: The aim of this study was to analyze the effect of vitamin D and cotinine on Treg Foxp3 and other immune parameters (phenotype CD3, CD4, CD8, CD19, CD16/56, anti-CD3 HLA -DR3) in patients with asthma. It was also investigated the correlation between the concentration of vitamin D and cotinine and severity of asthma.

Material and methods: The study involved 25 children diagnosed with asthma. The evaluation of severity was carried out using the Asthma Control Test and spirometry. The control group consisted of 15 healthy children. The study was performed using flow cytometry with monoclonal antibodies.

Results: It was found significantly lower levels of vitamin D in children with asthma compared to healthy children ($p < 0.005$). In children with asthma has been found significantly lower number of regulatory T cells in serum compared with the control group ($p < 0.002$). There were no significant differences in other immunological parameters. The study showed no significant correlation between vitamin D3, cotinine and the course of asthma and Treg.

Conclusions: These results do not confirm the influence of vitamin D and cotinine on Treg and clinical course of allergic diseases.

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The influence of vitamin D and cotinine on the selected immune parameters in children with atopic dermatitis.

Preliminary reports

Lipińska-Opałka, A.¹, Wawrzyniak, A.¹, Kalicki, B.¹, Zdanowski, R.², Lewicki, S.², Murawski, P.³

¹Military Institute of Medicine, Department of Pediatrics, Nephrology and Allergology, Warsaw, Poland, ²Military Institute of Hygiene and Epidemiology, Warsaw, Poland, ³Military Institute of Medicine, ICT Department, Warsaw, Poland

Background: Atopic dermatitis is a chronic inflammatory skin disease, with the key role of immune system disorders. Decreasing number of regulatory T cells (Foxp3) seems to be the major abnormality. Numerous reports pointed the role of vitamin D in modulation of the immune system function. However there are a small number of data regarding the effects of cotinine on the immune system.

Aim of the study: The aim of the study was to analyze the percentage of lymphocytes (CD3+, CD4+, CD8+, CD4/CD8 ratio, CD19+, CD16+/56+, CD3 +anti HLA -DR+) and natural regulatory T lymphocytes in patients with atopic dermatitis. Additionally the correlation between immune parameters and vitamin D levels and cotinine was performed.

Material and methods: The study involved 28 children with a diagnosis of atopic dermatitis. The control group consisted of 15 healthy children. The phenotype of lymphocytes was evaluated by flow cytometry.

Results: In children with atopic dermatitis significantly lower number of regulatory T cells in serum compared with the control group ($p < 0.002$) has been found. There were no differences in the other studied parameters between examined groups. The study revealed a negative correlation between serum vitamin D levels and the percentage of NKT cells ($p < 0.04$). Moreover, there was no significant correlation between vitamin D, cotinine levels and the severity of the disease.

Conclusions: The results confirmed the role of regulatory T cells in the pathogenesis of atopic dermatitis. Effects of vitamin D and cotinine on the severity of the disease has not been proven.

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IL-18 has a critical role in the maturation of pathogenic eosinophils in disease state

Mishra, A., Venkateshaiah, S.U., Manohar, M., Verma, A.K., Mishra, A., Allergy

Tulane University School of Medicine, Medicine/Pulmonary, New Orleans, United States

Eosinophils are multifunctional leukocytes that are critically involved in host defense, promoting allergic inflammation and allograft rejection. These diverse functions of eosinophils indicate that they may generate and mature differently in allergic condition. Herein, we show that both *in vivo* and *in vitro* eosinophil also be generated independent to endogenous IL-5 by IL-18. IL-18 is reported induced in all allergic diseases and a number of reports indicate that overexpression promotes tissue eosinophilia. We first time demonstrate that both rIL-5 and rIL-18 differentiate distinct subsets that have different structural and morphological characteristics, including size, shape and granular content levels. The microarray analysis detected highly induced CD274 transcripts in rIL-18 differentiated eosinophils compared to rIL-5 differentiated eosinophils. The CD274 expression was furthest validated and two distinct CD274+ and CD274- eosinophil subsets identified in the blood of healthy mice and humans. Notably, no CD274+ eosinophil develops *in vivo* or *in vitro* in the absence of endogenous IL-18. Most importantly, we observed that the CD274+ subset exclusively increases in the blood of various allergic patients including asthma. In addition, we report that all tissue eosinophils (CCR3+/Siglec-F+) in the

airway of asthmatic mice and most eosinophils (CCR3+/Siglec-8+) in the nasal lavages of asthma/rhinitis patients and esophageal biopsies of eosinophilic esophagitis patients are solely express CD274+ subset. Taken together, we report CD274+ and CD274- eosinophil in health and disease and provide evidence that IL-18 is critical the maturation of pathogenic CD274+ eosinophils. These investigations provide novel diagnostic and therapeutic target strategies for eosinophil-associated allergic disorders.

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Sensitization to inhaled allergens and risk factors for allergic diseases (including asthma) in Brunei Darussalam

Chieng, C.¹, Sharif, H.^{2,3}, Taib, R.⁴, Taib, S.⁵, Cunningham, A.C.²

¹RIPAS Hospital, Immunology Laboratory Services, Bandar Seri Begawan, Brunei Darussalam, ²Universiti Brunei Darussalam, PAPRSB Institute of Health Sciences, Gadong, Brunei Darussalam, ³Imperial College London, Allergy & Clinical Immunology, NHLI, London, United Kingdom, ⁴RIPAS Hospital, Dept of Paediatrics and Neonatology, Bandar Seri Begawan, Brunei Darussalam, ⁵RIPAS Hospital, Department of Laboratory Services, Bandar Seri Begawan, Brunei Darussalam

Allergic diseases to inhaled allergens are on the rise in the ASEAN region (Lai, Beasley *et al.* 2009). Studies in Western countries indicate that *Dermatophagoides pteronyssinus* (DerP) is the major allergen in 50% of patients, however this seems to be higher in urban tropical settings (ranging from 70-98%). A number of studies in SE Asia have demonstrated that children with respiratory and allergic symptoms (including allergic rhinitis, asthma, and atopic dermatitis) are predominantly sensitized to house dust mite (HDM) in Hong Kong (Leung *et al.* 1997), Indonesia (Baratawidjaja *et al.* 1999), Thailand (Daengsuwan *et al.* 2003), and Korea (Kim *et al.* 2015). Interestingly, in Singapore there appears to be a mono-specific IgE response to DerP which is associated with increased prevalence of allergic airway diseases (Andiappan *et al.* 2014). There are no published studies on IgE sensitization to inhaled allergens in Brunei.

Specific IgE levels to a panel of defined inhaled allergens were measured in patient serum by fluoroenzymeimmunoassay using the Phadia 100 platform. Laboratory data will be compared to electronic clinical records. 503 positive results were seen in 1740 samples in 1 calendar year (Jan - Dec 2015). The highest levels of sensitisation observed was to HDM (d1, d2, d3; 61%-81%), the storage mite *Blomia tropicalis* (75%) followed by cockroach, bermuda grass and acacia.

The significance of this study is the identification of risk factors for potential morbidities (eg asthma) with the aim of improving patient management (allergen identification and avoidance) plus raising awareness on allergy in Brunei Darussalam.

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Proteomic identification of moesin upon exposure to smoking irritant

JANG, A.-S.¹, Lee, P.-H.², Kim, B.-G.²

¹Soonchunhyang University Bucheon Hospital, Internal Medicine, Bucheon, Korea, Republic of, ²Soonchunhyang University Bucheon Hospital, Bucheon, Korea, Republic of

1097**cDNA cloning and in silico characterisation of ML domain protein-homolog allergen from the house dust mite *Suidasia pontifica****Bajao, J.E.¹, Ramos, J.D.²*¹University of Santo Tomas, Graduate School, Manila, Philippines,²University of Santo Tomas, College of Science, Manila, Philippines

Suidasia pontifica (Sp) is a house dust mite (HDM) that was proven to cause sensitization of up to 75% of tropical allergic patients. Characterization of specific allergens from Sp is a significant step in elucidating the clinical importance of this HDM. Here, we describe the cDNA cloning of a gene from Sp with significant homology to ML lipid binding domain protein, or the HDM group 2 allergen. The cDNA was synthesized from the isolated total RNA of pure Sp culture. The gene encoding for the ML domain protein from Sp (Sui p 2) was amplified and sequenced from the cDNA using gene-specific primers. Sequence and structural characterization of Sui p 2 gene was done in silico. Three clones were produced and all of them contains a 405 bp open reading frame. Among the other HDM group 2 allergen, sui p 2 gene exhibits highest percent identity to *Tyrophagus putrescentiae* (65.84%) while *Dermatophagoides farinae* (50.26%) exhibits the lowest. Deduced amino acid sequence showed that Sui p 2 exhibits 47.62% sequence homology to *Dermatophagoides farinae*. Predicted tertiary structure showed an immunoglobulin fold, consisting of two anti parallel β strands, three conserved disulfide bridges, and a lipid binding cavity, which is distinctive to the ML protein family. Ten possible epitope forming amino acid has also been seen. Thus, Sui p 2 might have a role in HDM induced allergy and innate immunity exacerbation. Characterization of Sui p 2 is important to properly evaluate its use for allergy component resolved diagnosis and allergen specific immunotherapy.

1098**Mast cell-eosinophil crosstalk reveals potential therapeutic strategy for atopic dermatitis***Naidoo, K.^{1,2}, Jones, A.¹, van den Elsen, L.¹, Lee, J.³, Le Gros, G.¹, Forbes-Blom, E.¹*¹Malaghan Institute of Medical Research, Wellington, New Zealand,²Victoria University of Wellington, Wellington, New Zealand,³Mayo Clinic, Scottsdale, United States

Atopic dermatitis (AD), an inflammatory skin disease, has become a significant health concern worldwide. It impacts their quality of life and imposes a life long burden, particularly amongst children. Treating this disease remains a challenge, as current management strategies for AD are nonspecific. Therefore there is a vital need for pharmacotherapy that calms the immunological storm associated with AD. Previous investigations demonstrate that mast cells and eosinophils are key effector cells driving allergic inflammation. We sought to elucidate mast cell - eosinophil interactions during AD to identify putative targets for treatment. Our aim was to suppress these key cell types or block the mediators released such as eosinophil peroxidase (EPO) and interleukin 9 (IL-9). To address this we established a model of chronic allergic skin

inflammation, with the use of MC903. This murine model of allergic skin inflammation was characterized by significant skin thickening, pronounced inflammatory cell infiltrate and elevated cytokine production that resembles AD in humans. The effects of blocking IL-9 and EPO were examined by the use of anti-IL-9 and resorcinol respectively. Our data revealed IL-9 blockade as well as the use of resorcinol significantly ameliorated disease. These results suggest MC903-induced AD is IL-9 dependent and the contribution of mast cells and eosinophils is strongly associated with disease severity. Thus, these effector cells are attractive targets for the treatment of AD.

1099**The effect of ORMDL3 overexpression in mast cells***Ogi, K., Takabayashi, T., Yamada, T., Sakashita, M., Narita, N., Fujieda, S.*

Fukui University, Division of Otorhinolaryngology Head and Neck Surgery, Faculty of Medical Science, Fukui, Japan

Purpose: In recent years, the patients with allergic rhinitis (AR) have been increasing on a worldwide level. Single-nucleotide polymorphism studies have linked the chromosome 17q12-q21 region, where human Orosomucoid like 3 (ORMDL3) gene is localized, to the risk of asthma. We have reported that genetic variants in the 17q21 are significantly associated with AR in the Japanese population. Although mast cells are involved in development of AR, the contribution of ORMDL3 to mast cell physiology is unknown. The purpose of this study is to reveal the function of ORMDL3 in mast cells.

Method: To examine the function of ORMDL3 in mast cells, we transfected the cDNA of ORMDL3 into RBL-2H3 cells. We generated stable clones overexpressing ORMDL3 from these transfected cells. Using these stable clones, we analyzed IgE-mediated degranulation by β -hexosaminidase assay and cytokine production by real-time quantitative PCR. We stimulated the cells by optimal or low concentrated antigen, IgE only without antigen (monomeric IgE). Moreover, we investigated intracellular signal transduction by immunoblotting.

Result: Our data showed that overexpression of ORMDL3 had no effect on degranulation induced by antigen in optimal concentration, but enhanced degranulation by low concentrated antigen. Moreover, we found that overexpression of ORMDL3 enhanced inflammatory cytokine production induced by not only optimal concentrated antigen stimulation but also low concentrated antigen stimulation and monomeric IgE stimulation. Immunoblotting analysis revealed that phosphorylation of extracellular signal regulated kinase is enhanced in stable cell lines overexpressing ORMDL3.

Conclusion: ORMDL3 acts as an enhancer of inflammatory cytokine production in mast cells.

1100**Identification of nasal resident bacteria as causative allergens in eosinophilic chronic rhinosinusitis**

Takeda, K.^{1,2}, *Sakakibara, S.*^{1,3}, *Motooka, D.*⁴, *Nakamura, S.*⁴, *Yamashita, K.*³, *Standley, D.M.*^{3,5}, *Hayama, M.*², *Shikina, T.*², *Inohara, H.*², *Kikutani, H.*^{1,3}

¹Research Institute for Microbial Diseases, Osaka University, Suita, Japan, ²Osaka University Graduate School of Medicine, Department of Otorhinolaryngology-Head and Neck Surgery, Suita, Japan, ³WPI-Immunology Frontier Research Center, Osaka University, Suita, Japan, ⁴Genome Information Research Center, Research Institute for Microbial Diseases, Osaka University, Suita, Japan, ⁵Institute for Virus Research, Kyoto University, Kyoto, Japan

Eosinophilic chronic rhinosinusitis (ECRS) is a refractory allergic disease and associated with nasal polyps (NPs) characterized by intensive eosinophilic infiltration. High levels of IgE are detected in NPs of ECRS patients, nonetheless, causative allergens have been unidentified. By using NP tissues and lymphocytes from patients, we have attempted to identify causative allergens. In the immunohistochemical analysis, clusters of IgE⁺ cells were observed in NPs. A fraction of IgE⁺ cells displayed the plasmablastic morphology and expressed activated-induced cytidine deaminase (AID), a key factor for somatic hypermutation (SHM) and class-switch recombination (CSR), indicating that these IgE plasmablasts were differentiated within NP tissues. Immunoglobulin cloning from sorted plasmablasts of NPs revealed that in most cases, the variable region of IgE contained a relatively large number of mutations, implying multiple rounds of germinal center reaction. We next generated monoclonal antibodies from IgE⁺ plasmablasts of NP. In the reactivity tests, we found that ~20% of expressed antibodies reacted with nasal resident bacteria such as *S. pyogenes* and *S. aureus*, while no clone was reactive to known inhalants including pollen and mites. As Th2 cells supposed to trigger not only IgE production but also local eosinophil infiltration and inflammation in ECRS, we examined T cell responses of patients against residential bacteria. Among the bacteria we tested, *S. pyogenes* induced activation of patient-derived blood CD4⁺ T cells with Th2 cytokine production, which was not observed in T cells from healthy donors. Collectively, we propose that nasal resident bacteria are one of the causative allergens of ECRS.

1101**Novel compounds that induce specifically class switching to IgA**

*Yamamoto, K.*¹, *Yonesu, K.*², *Kojima, H.*², *Okabe, T.*², *Shinkura, R.*¹

¹Nagahama Institute of Bio-Science and Technology, Immunology, Shiga, Japan, ²University of Tokyo, Drug Discovery Initiative, Tokyo, Japan

Allergic disorders, such as hay fever and asthma, afflict many people in developed countries. Allergies are thought to be a detrimental outcome of excessive immune response that acts against an innocuous antigen, allergen. IgE antibody is a central mediator of allergic responses. Therefore most of the current treatments for allergy target to inhibit IgE responses. However they are still palliative and not curative.

To suppress IgE production, we thought a new strategy that forced B cells to class-switch towards IgA instead of IgE. Upon antigen stimulation, IgM⁺ B cells can class switch to other isotypes such as IgG, IgE and IgA. However, the mechanism how B cells switch specifically to each isotype is not completely understood. Therefore we performed screening of chemical compounds which induced specifically class switching to IgA in mouse B cells. The chemical compounds (3,337 compounds) were provided by Drug Discovery Initiative of the University of Tokyo. As a result, we finally selected three candidate compounds which were structurally related. They induced IgA, but neither IgG1 nor IgE, production in mouse B cells in vitro.

1102**Plasmacytoid dendritic cell numbers predict individual interferon- α production more accurately than TLR7 expression**

*Murray, L.*¹, *Ferreira, M.*², *Upham, J.*^{1,3}

¹University of Queensland, Translational Research Institute, Woolloongabba, Australia, ²QIMR Berghofer Medical Research Institute, Herston, Australia, ³Princess Alexandra Hospital, Woolloongabba, Australia

People with asthma are more prone to have respiratory infections that spread to lower respiratory tract, which has been found to be due to impaired antiviral immune response, especially type I interferon. Plasmacytoid dendritic cells (pDC) and TLR7 are closely associated with interferon- α production. As TLR7 along with TLR8 have been associated with asthma, it is important to understand how TLR7/8 gene expression and gene variations affect cytokine production in response to viral activation.

Therefore, we stimulated PBMC from 150 people with and without asthma with rhinovirus-16 for 24h. RT-PCR from whole blood showed that the expression of pDC marker CD303 was the most significant predictor of interferon- α production ($r=0.559$, $p<.001$) ahead of TLR7 expression. TLR8 expression showed a negative effect on interferon- α production ($r=-0.328$, $p<.001$), which reflects its postulated role as a negative regulator of TLR7 function. No significant differences between asthma group and controls were observed, however women produced more interferon- α than men ($p>.05$).

Baseline TLR7 expression had seemingly no effect on interferon- α production, though study numbers are still small at this stage. Most of the variability in interferon- α production arose from variations in pDC numbers as demonstrated by CD303 expression. These results also highlight TLR8 involvement in interferon- α production, and we are currently examining genetic variations in TLR7/8. The future aim is to recruit a total of 300 people for genotyping TLR7/8 and to include more people with more severe asthma to distinguish whether type I interferon impairment is associated with disease severity.

1103**Dietary olive oil enhanced oral tolerance to attenuate allergic asthma responses***Chen, M.-L.¹, Yu, C.-J.¹, Yu, C.-H.²*¹*Chang Jung Christian University, Department of Nutrition and Health Sciences, Tainan, Taiwan, Republic of China, ²Chang Jung Christian University, Innovative Research Center of Medicine, Tainan, Taiwan, Republic of China*

It has been known that fatty acids modulate the responses of cells of the immune system. The monounsaturated fatty acid (MUFA)-rich diet has been shown to attenuate many chronic diseases, such as cardiovascular disease, diabetes, obesity, and asthma. However, the effects of dietary olive oil on T cell responses of allergic asthma are still unclear. Our previous study, the MUFA-rich diet increased the TGF- β 1 production by ConA-stimulated splenocytes from BALB/c mice. In this study, we like to study the effects of dietary MUFA on the regulatory T (Treg) cells and allergic immune responses. BALB/c mice were orally administered ovalbumin (OVA) in the initial of experiment process, then sensitized and challenged with OVA to induce allergic asthma. The mice were fed with AIN-76 diet that contains 5 or 20% olive oil during the experiment, individually. The results indicated that oral antigen significantly decreased bronchial airway hyper-responsiveness (AHR), which was increased in high fat diet group and olive oil treatment tends to attenuate the hyper-responsiveness. Furthermore, dietary olive oil was also significantly decreased Th2 cytokines and serum IgE levels. Furthermore, olive oil increased Foxp3 expression. These data suggested that the MUFA-rich diet did attenuate Th2 and AHR in allergic asthma and improved regulatory T cell expression, which associated with oral tolerance.

1104**Phenotyping of allergen-reactive CD8⁺T cells in IgE-mediated allergy***Samadi, N.¹, Kitzmüller, C.¹, Geyeregger, R.², Bohle, B.¹, Beatrice Jahn-Schmid, B.¹*¹*Medical University of Vienna, Department of Pathophysiology and Allergy Research, Vienna, Austria, ²Department of Clinical Cell Biology and FACS Core Unit, CCR, Vienna, Austria*

T cells play a main role in the induction and maintenance of IgE-mediated allergy. The function of CD4⁺ T cells in the pathophysiology of allergic disorders has been extensively investigated while the role of CD8⁺ T cells is still poorly understood and controversial. The aim of this project is to characterize allergen-specific CD8⁺ T cells in patients with different allergic manifestations (rhinoconjunctivitis, atopic dermatitis and atopic bronchial asthma). Different seasonal (birch pollen and grass pollen) and perennial allergens (cat dander and house dust mite) were included. PBMCs from allergic patients were stained with the proliferation dye eFluor 670 and incubated with allergen. Proliferating CD3⁺CD8⁺ cells were then assessed for the expression of differentiation markers (CD27, CD28, CD45RO, CXCR3, CRTh2, PD-1), homing markers (CCR4, CD62L, CD29b), intracellular cytokines (IL-4, IL-5, IL-13, IL-17, IL-22 and IFN- γ , TNF- α), and cytotoxic proteins (granzyme B and

perforin) by flow cytometry and compared to non-proliferating CD8⁺ cells. We found allergen-reactive CD8⁺ T cells in all allergic manifestations. Moreover, largest numbers were detected upon stimulation with house dust mite and grass pollen extracts. Proliferating cells contained higher numbers of cells producing IL-4, granzyme B and perforin than non-proliferating CD8⁺ T cells. In addition, a significantly higher expression of CD27, CD45RO, CD62L, and CD29b was detected in allergen-reactive CD8⁺ T cells indicating central memory T cells of mucosal origin. Thus, we could demonstrate allergen-reactive IL-4⁺ CD8⁺ T cells in different allergic manifestations which produce cytotoxic proteins. Their functional activity will be investigated in future experiments.

1105**Differential gene network analysis to identify asthma-associated therapeutic targets in house dust mite-specific T-helper memory responses***Troy, N.M.¹, Hollams, E.², Holt, P.G.², Bosco, A.²*¹*Telethon Kids Institute, The University of Western Australia, Perth, Australia, ²Telethon Kids Institute, Perth, Australia*

Background: Asthma is a heterogeneous disease characterized by airways inflammation, airways remodeling, and reversible airflow obstruction. The atopic form is thought to be driven in part, by allergen-induced Th2-associated inflammation. However, the vast majority of atopics do not develop asthma, suggesting underlying complexities in atopy pathogenesis, which cannot be fully explained via the classical Th1/Th2 paradigm.

Aim: The first aim was to test the hypothesis that variations in allergen-driven CD4 T-cell responses are associated with susceptibility to express an asthma phenotype. And secondly, to pinpoint novel therapeutic opportunities by mining gene expression data from HDM-stimulated CD4 T-cells.

Methods: The study population consisted of; House dust mite (HDM) sensitized atopics with asthma (n=22); HDM sensitized atopics without asthma (n=26); and HDM nonsensitized controls (n=24). PBMC were cultured in the presence or absence of HDM for 24 hours. CD4 T-cells were isolated and gene expression patterns profiled on microarrays.

Results: Differential network analysis identified a collection of asthma-associated genes in HDM-driven CD4 T-cell responses of sensitized atopics with asthma. The asthmatic response was characterized by upregulation of a Th2-associated gene network and was predicted to be driven by IL-2, IL-4, and TNF. This network was reconstructed to reveal a series of hub genes (highly connected) involved in inflammation. Finally, we identified putative negative regulators of asthmatic CD4 T-cell responses to HDM including genes, drugs and metabolites, which represent logical candidates for therapeutic intervention.

Conclusion: Differential network analysis of HDM-induced CD4 T-cell responses can reveal covert disease-associated genes and predict novel therapeutic targets.

1106**Immunological mechanisms of short-term pre-seasonal sublingual allergen immunotherapy tablet treatment for seasonal allergic rhinitis**

Varese, N.^{1,2}, Rolland, J.^{1,2}, Phan, T.^{1,2}, Deckert, K.², Hew, M.², O'Hehir, R.^{1,2}

¹Monash University, Immunology & Pathology, Melbourne, Australia, ²The Alfred Hospital and Monash University, Allergy, Immunology and Respiratory Medicine, Melbourne, Australia

Background: Oral sublingual tablets, Oralair[®], have been shown recently to be effective in treating allergic rhinitis. They provide a better tolerated option than conventional subcutaneous injection therapy. Oralair[®] tablets contain pollen extracts from 5 of the most common allergy-causing European grasses but include ryegrass, the major seasonal pollen for allergy in south-eastern Australia. A 7-month regimen is typical but patient adherence can be problematic. We performed a longitudinal study to investigate immunological changes after a 4-month pre-grass pollen season course of Oralair[®].

Study design: Patients with moderate to severe seasonal allergic rhinitis with or without mild stable asthma and with positive ryegrass pollen (RGP)-specific IgE were recruited. Oralair[®] treatment was used daily for 4 months prior to the grass pollen season. A cohort of clinically-matched patients was used as controls.

Results: Oralair[®]-treated patients showed clinical improvement based on visual analogue and symptom scores. Compared with baseline, serum RGP-specific IgG4 levels were increased after treatment. In vitro stimulation of peripheral blood mononuclear cells with RGP extract induced increased proportions of CD4+CD25+Foxp3+ and CD4+CD45RA-Foxp3+ Tregs after treatment, and CD4+CD45RA-CD27-CD25+ cell proportions were also increased. Higher levels of IFN- γ were present in RGP-stimulated culture supernatants after treatment. Control subjects showed no significant change in clinical symptoms or immunological parameters during the same time period.

Conclusions: Immunological and clinical benefits were observed following a shortened Oralair[®] treatment course, consistent with those for traditional sub-cutaneous allergen immunotherapy. Continuation of this short-course pre-seasonal regimen for another two years should provide patients with long lasting efficacy.

1107**Impact of hydrocarbon adsorbed on nanoparticles in house dust mite induced chronic asthma mouse model**

de Nadai, P., Ait-Yahia, S., Everaere, L., Balsamelli, J., Marquillies, P., Duez, C., Tsicopoulos, A.

Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U 1019 - UMR 8204 - CIL - Centre d'Infection et d'Immunité de Lille, CIL Team 13 Pulmonary Immunity, Lille cedex, France

Nanoparticles are a promising technology in many areas, however, little is known about the long-term impact of exposure to such materials, in particular in patients suffering from pulmonary diseases such as asthma. These particles are able to adsorb environmental pollutants such as polycyclic aromatic

hydrocarbons (PAH). The cytokine IL-22 is involved in skin repair and remodeling but also in epithelial-mesenchymal transition, a prerequisite for bronchial remodeling in chronic asthma. The Aryl hydrocarbon Receptor (AhR), a transcription factor binding PAHs, and involved in the production of IL-22, might represent a link between pollutants and airway remodeling in asthma.

We assessed the impact of nanoparticles adsorbed with the PAH Benzo(a)pyrene (B(a)P), on the production of IL-22 in a murine model of house dust mite (HDM) induced chronic asthma. We demonstrate that in WT mice, co-administration of a suboptimal dose of HDM and B(a)P-nanoparticles induced increase in IgE production, in broncho-alveolar lavage eosinophils and in Th2 type cytokines. Moreover, IL-22 was overexpressed whereas IL-17 was decreased. We observed an increase in mucus secretion but no difference in collagen deposition in lung sections. IL-22 KO mice were used to investigate the role of this cytokine, and showed a decrease in all parameters except for IgE production and a large increase in IL-17 expression.

In conclusion, in HDM chronic model, PAH nanoparticles are able to increase lung inflammation with low dose of allergen partially depending on IL-22 production.

1108**The house dust mite allergen Der p 13 is a fatty acid binding protein able to activate TLR2 signaling**

Satitsuksanoa, P., Jacquet, A.

Chulalongkorn University, Medicine, Bangkok, Thailand

Background: The House dust mite (HDM) allergen Der p 13 is a fatty acid binding protein which could participate to the initiation of allergic response through activation of innate immunity. We previously demonstrated that Der p 13 triggered IL-8 release by cultured airway epithelial cells. The goal of the present study was to characterize the signaling pathways activated by Der p 13 leading to such proinflammatory cytokine production.

Methods: Cultured human airway epithelial cells (BEAS_2B) were directly stimulated with different concentrations of rDer p 13 (1-20 μ g/ml) or Pam3Cys, a TLR2 ligand positive control under serum-free conditions. To investigate the activation of TLR2 signaling, the cells were pretreated with anti-hTLR2 blocking antibody or transfected with plasmid encoding dominant negative MyD88. The contribution of MAPK and NF- κ B in the Der p 13-induced cell activation was evaluated by cell pretreatment with specific MAPK or NF- κ B inhibitors.

Results: Preincubation of cells with blocking anti-human TLR2 mAb drastically reduced the Der p 13-induced IL-8 secretion from BEAS-2B cells. This TLR2-dependent cell activation was shown also to engage the MyD88 adaptor protein. The blockade of MAPK pathways through cell pretreatments with U0126, SB203580 or SP600125 (MEK1/2, p38, JNK MAPK inhibitors respectively reduced the IL-8 up-regulation caused by rDer p 13. Similar reduction of IL-8 release was observed with I κ B- α phosphorylation BAY-11-7082 and proteasome MG132 inhibitors.

Conclusions: Der p 13, through its ability to trigger TLR2-dependent innate immune signaling, must be considered as a potential contributor to the induction of the HDM allergic response.

1109**What data is sufficient and useful to define a protein as a cause of IgE mediated allergy?**

*Goodman, R., WHO/IUIS Allergen Nomenclature Subcommittee and the AllergenOnline.org expert panel
University of Nebraska--Lincoln, Food Allergy Research and Resource Program, Lincoln, United States*

Background: Identification of allergens in publications and sequence annotations are rapidly increasing, but often based on inadequate data. The route of exposure, types of proteins, abundance and complexity of the source materials present many challenges. Predicting proteins from DNA sequences may be incorrect. Identification and characterization of allergens are important for developing diagnostic and therapeutic reagents and for risk assessment of foods made from genetically modified organisms or novel ingredients. The WHO/IUIS Allergen Nomenclature Subcommittee (www.Allergen.org) and experts of AllergenOnline (www.allergenonline.org) suggestions:

Allergen characterization: Taxonomic source, tissue/organ; Amino acid sequence (N-terminal, LC-MS/MS or translated DNA with confirmation of a partial amino acid sequence); Post-translational modifications, glycosylation, disulfide-bridges; Abundance in the allergenic source; Test protein purity and source (purified natural or recombinant-source).

Demonstrated IgE binding: Sera from multiple relevant allergic human donors with history of exposure and reactions and from non-allergic donors; IgE binding tests (reducing, non-reducing or native immunoblot, ELISA), inhibition methods to demonstrate specificity, and characteristics of IgE detection reagents; Test IgE binding to pure protein and extract of source. Biological reactivity: In vivo challenges with pure protein are rarely performed (foods or inhalation), but skin prick tests are useful. Basophil activation or mediator release with surrogate or autologous cells, using a dose-response comparison of source and pure protein.

Allergen databases: Submit unpublished summary data to WHO/IUIS for an allergen name (e.g. Ara h 2) prior to publication. Publish a well described, controlled study and update WHO/IUIS. Publications are screened by AllergenOnline to update a risk assessment database.

1110**Implication of NOD-1 receptor in an experimental house dust mite-induced asthma model**

Ait Yahia, S.¹, Vorng, H.¹, Marquillies, P.¹, Duez, C.¹, Delacre, M.¹, Wallaert, B.^{1,2}, Chamaillard, M.¹, Tsiopoulos, A.^{1,2}

¹CILL - Center for Infection and Immunity of Lille INSERM U 1019 - CNRS UMR 8204, Lille, France, ²Clinique des Maladies Respiratoires, CHRU de Lille, Lille, France

Introduction: Allergic asthma is characterized by a Th2 adaptive immune response. Receptors of innate immunity, pattern-recognition receptor (PRR), have an important role in the induction and orientation of the immune response. Among these PRR, Nucleotid-binding oligomerization domain 1 (NOD1) is one of the receptors involved. NOD1, a sensor of Gram-negative peptidoglycan, has been shown to play a key role in

the induction of the systemic adaptive response by favoring a Th2 profile. Furthermore, polymorphisms of NOD1, have been associated to asthma. We demonstrated that NOD1 agonist displayed a pro Th2 adjuvant activity and contributed to the exacerbation of asthma in vivo. Moreover some allergens have been described as direct PRR activators. The aim of this study was to assess whether allergens can directly activate NOD1 receptor and may contribute to the development of increased immune response.

Methods: The role of NOD1 was investigated in the house dust mite (HDM)-induced asthma model, using Wild-Type and NOD1 deficient mice. Lung inflammation was assessed by determining total and differential cell counts in bronchoalveolar lavage (BAL), and by measuring Th2 cytokines and chemokines by ELISA in lung protein extracts. Total IgE levels were quantified in the serum.

Results: Our results show that HDM-sensitized/challenged NOD1 deficient mice exhibited less pulmonary inflammation when compared to the wild-type mice. Indeed eosinophilia and pulmonary Th2 cytokines were decreased in NOD1 deficient mice. Finally, the levels of total serum IgE were diminished in NOD1 deficient mice.

Conclusion: The preliminary results suggest that the allergen may directly activate NOD1 receptor.

1111**IgE sensitization to crab allergens due to inhalational exposure: identification of novel allergens**

Kamath, S.^{1,2}, Thomassen, M.³, Koeberl, M.², Nugraha, R.², Aasmoe, L.³, Bang, B.³, Lopata, A.^{1,2}

¹James Cook University, Australian Institute of Tropical Health and Medicine, Townsville, Australia, ²James Cook University, Centre for Biodiscovery and Molecular Development of Therapeutics, Townsville, Australia, ³University Hospital of North Norway, Tromsø, Norway

Occupational exposure and sensitization to food proteins and allergens is a serious health concern, and affects 4-36% of the workforce in the seafood industry. Although there have been previous attempts to identify and characterize air-borne food allergens, there is still a poor correlation between the inhalational allergen exposure on molecular level and clinical reactivity among the seafood processing workers. The aim of this study was to implement a combined proteomic- and allergenomic-based strategy to identify the major IgE-reactive proteins in crab processing factory among asthmatic or allergic crab workers and investigate the inhalational allergen load for major crab allergens.

Twenty crab-processing workers were recruited for this study with clinical asthma and/or positive skin prick test to crab extract. Air-borne allergen load was measured by sampling air from the personal breathing zones of the workers. IgE antibody reactivity to king and edible crab allergens were analysed using serum from worker by immunoblotting and mass spectrometric characterization of allergens.

90% of the tested crab workers showed strong IgE binding and recognition to crab proteins. Strong IgE reactivity was observed to crab tropomyosin and arginine kinase. In addition, IgE

binding to novel putative allergens were observed. The current study provides an insight into the role of major crab allergens, which maybe be the primary sensitizer in the development of allergy and asthma in the crab industry. The outcome of this study assists in better work safety for workers at risk of developing ingestion-induced food allergy and future development of immunotherapeutic strategies.

1112

Heterogeneity in IgE sensitization as well as T cell immunogenicity and cross-reactivity to major and minor grass pollen allergens

Gupta, S., Koed, G.K., Skovsgaard, J., Grauert, G., Lundegaard, C., Ipsen, H., Wurtzen, P.A.

ALK, Global Research-Immunology, Hørsholm, Denmark

Background: IgE mediated allergy to grass pollen has a high prevalence worldwide and various grass species are recognized by grass allergic patients in different geographical regions. The current study aimed to determine the frequency of T cell responses to individual allergens (group 1-5) from *Phleum pratense* (Phl p) and homologous allergens from other grass species and relate the T-cell responses to IgE reactivity.

Methods: Grass allergen-specific T-cell lines were established through stimulation of peripheral blood mononuclear cells (PBMC) from 51 Danish grass pollen allergic donors with natural purified grass allergens (group 1-5) in-vitro. T-cell responses were studied by fluorospot and proliferation assays. In parallel, serum IgE specific to the individual allergens was measured by ImmunoCAP and ISAC measurements. Cross-reactivity to related temperate and subtropical grasses was investigated for selected T-cell epitopes.

Results: T-cells from the majority of the donors reacted to Phl p 4 and 5, whereas responses to Phl p1, 2 and 3 were less frequent. This suggests that Phl p4 is a strong T cell immunogen and Phl p1 is less important for T-cell activation. In contrast, Phl p1 and 5 were recognized by IgE from more than 80% of the patients whereas less than 50% were IgE sensitized towards Phl p2 and 4. T-cell cross-reactivity differed considerably between allergens and epitopes but strong cross-reactive T-cell epitopes were found for most grass allergens.

Conclusion: IgE, T cell reactivity and cross-reactivity data will help us to understand the Immunogenicity of grass allergens towards developing novel vaccines for Allergen Immunotherapy.

1113

Expression of microRNA in mouse model of asthma treated by Jade-Screen Powder objective

Shen, C.-B.^{1,2}, Yu, L.¹, Ding, X.-Y.¹, Wang, S.¹

¹Shanghai TCM-Integrated Hospital, Pediatrics, Shanghai, China,

²Shanghai University of TCM, Shanghai, China

In order to investigate the effect of Jade-Screen Powder (JSP, Chinese traditional prescription) against asthma in mice by detect microRNA associated with modulated effects on helper T cells. Methods 40 Balb/c mice were randomized into control group, asthma model group, JSP treated group and

dexamethasone treated group. The mice model of allergic inflammation was established on both upper and lower airways by ovalbumin. The estimation of IL-13 (interleukin-13) and IL-17 in lung tissue homogenate was detected by ELISA and conventional pathological examination in lung was performed. The expressions of miR-146a, miR-146b, miR-210, miR-21a and miR126 in spleen tissue was analyzed by quantitative real time PCR. Results The expressions levels of IL-13 and IL-17 in JSP treated group were significantly lower than in asthma model group ($t=3.785$, $P=0.005$; $t=9.891$, $P=0.000$), and the expressions levels of IL-13 as well as IL-17 decreased in dexamethasone treated group than in asthma model group ($t=2.779$, $P=0.024$; $t=6.225$, $P=0.000$). The expression of miR-210 in JSP treated group was higher than in asthma model group (3.95 fold, $t=2.718$, $P=0.026$) and dexamethasone treated group (3.07 fold, $t=-3.962$, $P=0.012$). The expression of miR-126 in JSP treated group was higher than in asthma model group (2.15 fold, $t=2.405$, $P=0.043$), and dexamethasone upregulated the expression of miR-126 relative to asthma model group (4.48 fold, $t=-3.069$, $P=0.015$).

Conclusions: Th2 cells and Th17 cells participate in the pathogenesis of asthma and can be depressed by JSP. JSP is able to affect the helper T cells by regulate miR-210 and miR-126 to treat asthma.

1114

The effect of omega-3 on serum levels of Th1/Th2/Th9/Th17 and Th22 cytokines in patients with childhood asthma

Farjadian, S.¹, Moghtaderi, M.²

¹Shiraz University of Medical Sciences, Department of Immunology, Shiraz, Iran, Islamic Republic of, ²Shiraz University of Medical Sciences, Allergy Research Center, Shiraz, Iran, Islamic Republic of

There has been a considerable interest in the potential therapeutic value of dietary supplementation with omega-3 fatty acids in patients with asthma. To identify the effect of omega-3 fatty acids on pulmonary function and cytokines serum levels in asthma, 50 children with persistent asthma were scheduled to take a soft gel capsule including 180 mg EPA and 120 mg DHA daily for consecutive three months. Pulmonary function was evaluated by spirometry and serum levels of Th1, Th2, Th9, Th17 and Th22 cytokines were measured by flow cytometry using multiplex bead-based assay before and after the treatment. After treatment with omega-3, IL-17A and TNF- α levels decreased significantly. Peak expiratory flow (PEF) was also extensively improved in 32% of the patients. Omega-3 can be helpful in governing of inflammation in alternative therapy of asthma.

1115

IgE and non-IgE mediated reaction following anti-IgE therapy for severe asthma: a therapeutic dilemma

Ali, S.B.¹, Jodie, T.¹, Lucas, A.^{2,3}, Hollingsworth, P.N.¹, Lucas, M.^{1,4,5,6}

¹Sir Charles Gairdner Hospital, Immunology, Perth, Australia,

²Centre for Cell Therapy and Regenerative Medicine, University of Western Australia, School of Pathology and Laboratory Medicine,

Perth, Australia, ³Murdoch University, Institute of Immunology and Infectious Disease, Perth, Australia, ⁴University of Western

Australia, School of Medicine and Pharmacology, Perth, Australia, ⁵University of Western Australia, School of Pathology and Laboratory Medicine, Perth, Australia, ⁶Murdoch University, Institute of Immunology and Infectious Disease, Perth, Australia

Omalizumab (Xolair®) is a humanised monoclonal antibody against immunoglobulin E (IgE) for the treatment of allergic asthma and chronic urticaria. Xolair® contains polysorbates to enhance solubility (1) and can induce severe allergic reaction (anaphylaxis) in 0.1% of subjects (2).

We describe a 40-year old patient with severe asthma and frequent anaphylactic reactions in the community who commenced Xolair®. Thirty minutes after the first dose, she developed injection site ulceration, an urticarial rash and wheeze requiring adrenaline. Subsequent therapy (300mg subcutaneous; fortnightly) was given in intensive care unit with corticosteroid and anti-histamine premedication. Four months later, Xolair® treatment was ceased. Following this, she had 28 episodes of anaphylaxis in 16 months and multiple hospital admissions. Xolair® was reintroduced, each time accompanied with an anaphylactic reaction. Xolair® desensitisation was unsuccessful. The decrease in frequency of anaphylaxis in the community, hospital admissions and total serum IgE with satisfactory asthma control justifies current Xolair® continuation. Investigation of her anaphylactic episodes, demonstrated an acute (within 15 minutes) rise in platelets, lymphocytes, neutrophils and monocytes. Skin-prick testing (SPT) to Xolair® was initially negative but became positive later in the treatment, indicating an IgE-mediated reaction. SPT and injection site biopsy after 24-hours demonstrated a perivascular T-cell infiltrate. IFN γ ELISpot testing with Xolair® (10 μ g/mL) was also positive (>200 SFU/Million PMBC), indicating the presence of Xolair® specific T-cells.

This case points towards a pathophysiology involving both IgE and T-cell mediated immune responses. Further investigation to identify the cause and culprit (omalizumab or polysorbates in Xolair®) is underway.

1116

Mast cell-derived PGD₂ attenuates anaphylaxis via DP receptor

Nakamura, T.¹, Yamada, R.¹, Fujiwara, Y.¹, Fujii, W.², Hamabata, T.¹, Aritake, K.³, Urade, Y.³, Murata, T.¹

¹University of Tokyo, Graduate School of Agriculture and Life Science, Animal Radiology, Tokyo, Japan, ²University of Tokyo, Graduate School of Agriculture and Life Science, Applied Genetics, Tokyo, Japan, ³University of Tsukuba, International Institute for Integrative Sleep Medicine, Tsukuba, Japan

Objective: Activated mast cell releases several vaso-active mediators such as histamine and induce life threatening allergic reactions; anaphylaxis. Although mast cells strongly express hematopoietic-prostaglandin D synthase (H-PGDS), the role of prostaglandin D₂ (PGD₂) in anaphylaxis remains unknown.

Results: Mast cell activation by Compound 48/80 (C48/80) caused anaphylactic responses; vascular hyper-permeability and hypothermia in wild-type (WT) mice. Systemic H-PGDS deficiency exacerbated all of the C48/80-induced anaphylactic

responses. Serum histamine level and the effectiveness of the blockade of histamine H1 receptor were comparable in both lines of mice. Immunohistochemistry showed that mast cells strongly expressed H-PGDS in WT ear. Mast cell-specific H-PGDS deficiency (MCPT5Cre H-PGDS^{fl/fl}) exacerbated the C48/80-induced vascular hyper-permeability. Systemic PGD₂ receptor DP but not CRTH2 deficiency also exacerbates C48/80-induced hyper-permeability. Finally, DP receptor agonist abolished the C48/80-induced hyper-permeability and hypothermia in both WT and H-PGDS^{-/-}.

Conclusion: Mast cell-derived PGD₂ attenuated anaphylaxis by inhibiting vascular hyper-permeability through DP receptor activation.

1117

Dectin-1 plays an important role in HDM-induced allergic airway inflammation through the activation of CD11b⁺ dendritic cells

Ito, T.¹, Hirose, K.¹, Norimoto, A.¹, Tamachi, T.¹, Yokota, M.¹, Saku, A.¹, Takatori, H.¹, Saijo, S.², Iwakura, Y.³, Nakajima, H.¹

¹Chiba University, Department of Allergy and Clinical Immunology, Graduate School of Medicine, Chiba, Japan, ²Chiba University, Department of Molecular Immunology, Medical Mycology Research Center, Chiba, Japan, ³Tokyo University of Science, Research Institute for Biomedical Sciences, Chiba, Japan

It is well known that sensitization against fungi is closely associated with severity of asthma. Dectin-1 (gene symbol *Clec7a*), a C-type lectin receptor, recognizes not only a fungal cell wall component β -glucan but also some component(s) in house dust mite (HDM) extract. However, the roles of Dectin-1 in HDM-induced allergic airway inflammation remain unclear. In this study, by using Dectin-1-deficient (*Clec7a*^{-/-}) mice, we examined whether Dectin-1 is involved in HDM-induced allergic airway inflammation. We found that HDM-induced eosinophil and neutrophil recruitment into the airways was significantly attenuated in *Clec7a*^{-/-} mice as compared with that in wild-type (WT) mice. In addition, HDM-induced IL-5, IL-13, and IL-17 production from mediastinum lymph node (MLN) cells was reduced in HDM-sensitized *Clec7a*^{-/-} mice. Dectin-1 was expressed on CD11b⁺ dendritic cells (DCs), an essential DC subset for the development of allergic inflammation, but not on CD103⁺ DCs, plasmacytoid DCs, or lung epithelial cells. Transcriptome analysis revealed that the expression of chemokine/chemokine receptors including CCR7, which is indispensable for DC migration to draining lymph nodes, was decreased in *Clec7a*^{-/-} DCs. In accordance with these results, the number of HDM-labeled DCs in MLNs was significantly reduced in *Clec7a*^{-/-} mice as compared with that in WT mice. Taken together, these results suggest that Dectin-1 expressed on CD11b⁺ DCs senses some molecule(s) in HDM extract and plays a critical role in the induction of HDM-induced allergic airway inflammation by inducing the expression of chemokine/chemokine receptors in DCs.

1118

Annexin A1 (ANXA-1)-mimetic peptide controls the inflammatory and fibrotic effects induced by house dust mite (HDM) in mice*Ferreira, T.¹, Trentin, P.¹, Arantes, A.C.¹, Silva, T.¹, Castro, G.¹, Flower, R.², Perreti, M.², Martins, M.A.¹, Silva, P.¹*¹Oswaldo Cruz Foundation, Laboratory of Inflammation, Rio de Janeiro, Brazil, ²William Harvey Research Institute, Biochemical Pharmacology Department, London, United Kingdom

Endogenous glucocorticoid are critical on their potent anti-inflammatory activity, a response partially dependent on the release of pro-resolving mediators such as AnxA1. In many inflammatory and cellular settings, the anti-inflammatory activity of AnxA1 is reproduced by peptides derived from the N-terminal region of the protein, including the peptide Ac2-26. We investigated the therapeutic properties of peptide Ac2-26 on experimental asthma induced by HDM in mice. AnxA1 null and Balb/c mice were sensitized with intranasal instillation of HDM, every other day, during 3 weeks. In another experiments, Balb/c mice were treated with peptide Ac2-26 or budesonide, 1 h before antigen starting, on the week 2 of sensitization. 24h after the last challenge, lung function, inflammatory and fibrotic markers were measured. All the experimental procedures were approved by the Ethics Committee of Animal Use of FIOCRUZ (license LW57/14). HDM led to increased airways hyper-reactivity (AHR) to methacholine, eosinophil accumulation in lung tissue as well as an excessive deposition of extracellular matrix. Increased tissue generation of inflammatory cytokines was also detected. A clear exacerbation of these pathological changes was observed in AnxA1 null mice as compared to the controls. Ac2-26 peptide inhibited all the inflammatory parameters. Budesonide was less effective than the peptide. Taken together, we observed that AnxA1 null mice show an exacerbation of several aspects of asthma, indicating that AnxA1 plays a pivotal role in the features of severe asthma. In addition, peptide Ac2-26 protects against several pathological changes associated with asthma, suggesting a possible a therapeutic agent for severe asthma.

1119

New strategies of atopic dermatitis treatment in children*Slavyanskaya, T.¹, Derkach, V.²*¹'People' Friendship University of Russia, Allergology and Immunology, Moscow, Russian Federation, ²Pacific State Medical University, Vladivostok, Russian Federation

Targeted therapies in the management of atopic dermatitis in children is a topical issue. The determination of the immunopathogenic phenotype (IPF) in 300 children (Ch) 5-17 years with moderately severe AD during exacerbation has made it possible to discover an allergic form of AD with sensitization to HDMA in 94 Ch (SCORAD 44.2±2.46). It has been noted the prevalence of Th2-cells, phagocytic index/number falls down to 25.5±1.02% and 2.14±0.4 of microbial bodies respectively. Ch have received 3 different complex therapy program (CTP): 1CTP basic therapy (BT); 2CTP BT and subcutaneous immunotherapy (SCIT); 3CTP combined immunotherapy - CIT (BT+SCIT+

immunomodulator (IM), aimed at activation of phagocytes). Application of CIT contributed to IFN γ synthesis activation and reduction of level of IL-4 and IL-13 cytokines, stimulating specific IgE. The CIT has made it possible to conduct SCIT according to an accelerated schedule, increasing clinical effectiveness as compared 1CTP and 2CTP. In the 3CTP during 3 years, we have recorded a considerable decrease of the SCORAD, reduction of flare-ups, BT in the absence of AD manifestations and hospital care, improvement of quality of life, long-term treatment effect. Scientific research study confirms CIT clinical efficiency for children with AD. This study shows prospects for the use of CIT, indicated unmet needs. Inclusion of SCIT is reasonable only if AD is of allergic form. This study shows prospects for the use of CIT which joins SCIT and IM. A future strategy of treating Ch with AD should be based on application the individual targeted therapy.

1120

VISTA/PD-1H negatively regulates generation of Th2-mediated allergic responses*Ohno, T., Kondo, Y., Azuma, M.**Tokyo Medical and Dental University, Department of Molecular Immunology, Tokyo, Japan*

Objective: VISTA/PD-1H is an immune-checkpoint molecule of the CD28-B7 family. Unlike CTLA-4 and PD-1, VISTA is broadly and constitutively expressed on most of immune cells except for B cells and seems to function as a receptor on T cells and a ligand on APCs. Its counter receptor has not been identified yet. To investigate the roles of VISTA in Th2-mediated immune responses, we examined the effects of anti-VISTA mAb treatment in an OVA-induced allergic airway inflammation mouse model.

Methods and results: Anti-VISTA mAb (MIH63) was administered i.p. at the sensitization and airway hypersensitivity (AH) was assessed by methacholine-induced airway resistance after the continuous three day challenge. The anti-VISTA mAb treatment did not clearly affect AH responses. However, total cell numbers in BAL and spleens were significantly increased by the anti-VISTA mAb treatment. The proportion of macrophages, but not of eosinophils, in BAL was also increased. In addition, serum IgE and OVA-specific IgG1 and IgG2a were dramatically increased by the anti-VISTA mAb treatment. Production of IL-4 and IL-13 by OVA-stimulated CD4+ T cells from draining lymph nodes was also increased.

Discussion: Our results suggest that VISTA greatly contributes to inhibition of Th2 cell generation and Th2-mediated antibody production, but does not protect local airway tissue inflammation. We will further investigate the effects of anti-VISTA treatment at the challenge.

1121

Coronin-1 regulates the Fc ϵ RI-mediated mast cell functions*Oku, T., Ando, Y., Tsuji, T.**Hoshi University, Microbiology, Tokyo, Japan*

Coronin-1, a hematopoietic cell-specific actin-binding protein, is thought to be involved in immunological functions through

its interaction with actin filaments. We previously reported that 1) Coronin-1 is phosphorylated by protein kinase C (PKC) and that

2) Coronin-1 has two phosphorylation sites, Ser-2 and Ser/Thr-412 (Ser in mouse and rat, Thr in human).

In this study, we investigated roles of Coronin-1 in mast cell functions. First, we generated Coronin-1-knockdown RBL-2H3 cells by using lentiviral vectors. The stable knockdown of Coronin-1 rendered these cells less responsive to antigen cross-linking of FcεRI, resulting in decreased degranulation (β-hexosaminidase) and cytokine production (TNF-α). Next, we established hybridomas secreting antibodies specific for phospho-Ser-2 and phospho-Ser-412 of Coronin-1 to assess the phosphorylation of Coronin-1 during FcεRI-mediated activation of RBL-2H3 cells. Coronin-1 was found to be transiently phosphorylated at Ser-412 but not at Ser-2. The Ser-412 phosphorylation was inhibited by the pan-PKC inhibitor, chelerythrine, and by the PKCα/β-specific inhibitor, Gö6976. The treatment of RBL-2H3 cells with Gö6976 suppressed the β-hexosaminidase secretion and the TNF-α production. These results strongly suggest that Coronin-1 plays an important role in mast cell functions, and that the functions were regulated by its phosphorylation at Ser-412.

1122

TSLP mediated airway hyperresponsiveness via regulating the expression of epithelial NGF

Chen, Y.-L.

Graduate Institute of Clinical Medicine, College of Medicine, National Taiwan University, Taipei, Taiwan, Republic of China

Airway epithelium defends the invasion from microorganisms and regulates immune responses in allergic asthma. The nerve growth factor (NGF) and thymic stromal lymphopoietin (TSLP) would be produced from airway epithelium after the stimulations of allergens or proinflammatory mediators. They were highly expressed in bronchoalveolar lavage (BAL) fluid of asthmatics and have been suggested that these two cytokines are involved in allergic immune responses and probably in AHR. However, the correlation between these two cytokines was still unclear. In this study, we used RNA interference (RNAi), which can particularly silence target gene, in OVA-induced asthma model to reveal the functional roles of the specific cytokine in airway inflammation and AHR. The aim of this study was whether TSLP mediated AHR via NGF- neural pathway. Results were showed that the primary lung cells expressed TSLP receptors and was induced the expression of NGF by TSLP stimulation *in vitro*. In asthmatic model treated with lentivirus containing targeted shRNA, the degrees of AHR and airway inflammation were significantly reduced. The level of NGF in BAL fluids was significantly decreased. Furthermore, TSLP could regulate the expression of epithelial NGF. To compare the efficacy of two cytokines in airway inflammation, TSLP was a major cytokine in the recruitment of inflammatory cells and CD11c⁺ CD11b⁺ cells in airway. The finding showed a new role of TSLP in airway inflammation, and provided the consequence to TSLP therapy in allergic asthma.

1123

Study on fatty acids composition in breast milk and association with the occurrence of infant atopic dermatitis

Shen, Y.-J., Lin, B.-F.

National Taiwan University, Department of Biochemical Science and Technology, Taipei, Taiwan, Republic of China

Introduction: Atopic dermatitis (AD) is a pruritic skin inflammation disease that usually starts in early infancy. Recently, breast-feeding is considered to be good to infants. However, it is noted that some AD infants get better after stop breast feeding. We suspected that some nutrients in breast milk may be changed and affect AD. Because fatty acids are directly related to immune responses and easily affected by the diet, our aim is to investigate the association between fatty acids and infant AD.

Materials and methods: We collected breast milk samples and AD infants' condition from National Taiwan University Hospital. Breast samples were divided into control and AD group that from mothers who give birth to normal and AD infants. The fatty acid composition was expressed as percentage of total fatty acids detected with C8-C24 chain length and short chain fatty acids concentration by gas chromatography.

Results: The result shows that the percentage of docosahexaenoic acid (DHA) in breast milk samples from control group is greater than that from AD group (percentage of DHA 0.08±0.03% and 0.13±0.07% for control and AD group respectively; P< 0.1). We will report the complete analysis of fatty acid composition and allergic immune responses between control and AD group.

Conclusion: Our finding suggests that fatty acids composition in breast milk might affect infant allergic disease. These results may be used as a reference for mothers to adjust the diet with more beneficial fatty acids when breast-feeding to reduce the risk of infants suffering from allergic diseases.

1124

Subcutaneous allergen specific immunotherapy in patients with allergic rhinitis: efficacy, safety and predictors for clinical response

Ye, Y.-M.¹, Hur, G.-Y.², Kim, H.-A.¹, Shin, Y.-S.¹, Nahm, D.-H.¹, Park, H.-S.¹

¹Ajou University School of Medicine, Suwon, Korea, Republic of, ²Korea University College of Medicine, Seoul, Korea, Republic of

Background: Allergen specific immunotherapy is the only disease modifying treatment for allergic disease. We sought to evaluate the efficacy and safety of allergen specific subcutaneous immunotherapy (SCIT) in patients with allergic rhinitis (AR).

Methods: We reviewed medical records of 1,064 adult patients who were sensitized to HDM and/or pollens and have taken SCIT with Allergoid® from 2000 to 2012. Remission was defined as patients needed no medication for at least 1 year and without rhinitis symptoms.

Results: Of 1,064 patients with AR (mean age 33.8±13.5, 49.5% male, 48.9% severe AR, 49.1% HDM, 29.7% Rush), 374 (35.2%) achieved the remission of AR. Mean duration for achieving AR

remission was 7.56 ± 0.17 years. Cox regression analysis showed that old age (0.99, 0.98-0.99, $P=0.001$), severe AR (0.68, 0.54-0.85, $P=0.001$), and accompanied AA (0.74, 0.60-0.91, $P=0.005$) were significant determinants of poor response to SCIT. In total 232 (21.8%) patients had experienced adverse event (AE)s during their buildup phase, and particularly in patients starting SCIT with rush mode compared to those with conventional mode (39.2% vs 14.4%, $P < 0.001$). Cutaneous reactions (218 cases) including urticaria, angioedema and injection site swelling were the most frequent AE followed by respiratory symptoms (35 cases) and anaphylaxis (35 cases).

Conclusions: A retrospective cohort study covering 12 years of follow-up demonstrated that 35.2% of AR patients arrived at the remission with low occurrence of severe AEs with SCIT. Initial severity, age and accompanied asthma can impact on the clinical response to SCIT in patients with AR.

1125

Asthmatic airway inflammation is alleviated by adeno-associated viral vectors carrying CD39 in OVA-sensitized mice model

Huang, Y.-A., Wu, C.-J., Kuo, M.-L.

Chang Gung University / Graduate Institute of Biomedical Sciences, Taoyuan, Taiwan, Republic of China

Asthma is a chronic respiratory disease characterized by recurrently attacks of breathlessness and wheezing. Th2 response plays a crucial role in pathogenesis of asthma, resulting in eosinophilia and airway hyper-responsiveness (AHR). Extracellular ATP has been revealed as an important mediator and danger signal responding to cellular stress or tissue damage in acute and chronic inflammation. Accumulation of ATP in bronchoalveolar lavage fluid (BALF) of asthmatic subjects suggests that extracellular ATP and the downstream responses are involved in the pathogenesis of asthmatic airway inflammation. The concentration of extracellular ATP is regulated by CD39, an ecto-ATP/ADPase encoded by *entpd1*. Elevated ATP level in BALF of OVA-sensitized mice was compounded by the increased AHR, eosinophilia and IL-5 production as well as reduced expression of CD39. To lessen the level of extracellular ATP by increasing the expression of CD39, recombinant adeno-associated virus based CD39 expression vector (rAAV-CD39) was generated. rAAV-CD39 was administrated into the lungs of OVA-sensitized mice, and several cardinal features were analyzed. In rAAV-CD39 treated mice, expression level of CD39 was rescued, and ATP upregulation in BALF was restricted. AHR, eosinophilia and IL-5 produced by the draining lymph node cells were decreased by the administration of rAAV-CD39. These data indicated that the treatment of rAAV-CD39 attenuated the asthmatic airway inflammation and AHR. We considered that CD39 mediated reduction of extracellular ATP could be a potential approach for gene therapy on asthma.

1126

Adverse effects of prenatal wood smoke exposure on house dust mite-induced asthma

Jaffar, Z., Ferrini, M., Carvalho, S., Roberts, K.

University of Montana, Biomedical & Pharmaceutical Sciences, Missoula, United States

Globally, biomass burning (from woodstoves, wildfires and agricultural burns) contributes significantly to both indoor and outdoor wood smoke (WS) particulate matter ($PM_{2.5}$) exposures and associated adverse health effects. Although such exposures are associated with acute respiratory tract infections and are linked to childhood asthma, the adverse effects of prenatal WS exposure have not been adequately studied. Airborne allergens, including those of the house dust mite (HDM), are the most common trigger of asthma, particularly in children. Allergic asthma is a chronic bronchial inflammatory condition characterized by eosinophilic inflammation, airway hyperreactivity, mucus production and remodeling. To investigate possible linkage between WS exposure and asthma incidence, C57BL/6 offspring were exposed in utero to either WS (3 mg/m³ $PM_{2.5}$) or filtered air and the lung inflammatory response elicited by HDM allergen inhalation was compared. Prenatal WS exposure caused a pronounced increase in HDM allergen-induced airway inflammation, AHR and Th2 cytokine levels in the offspring compared to clean air. Moreover, in utero WS exposure resulted in a marked increase in peribronchial and perivascular inflammation and mucus production. Flow cytometric analysis revealed a marked elevation in the number of pulmonary dendritic cells and Siglec-F+ eosinophils in the offspring. The enhanced responsiveness was associated with airway cysteinyl leukotriene production. Collectively, these results demonstrate that prenatal WS exposure leads to exacerbation of allergic airway inflammation in the offspring and may identify future targets for prevention.

B Cells

1127

IL-10 producing B cells in breast cancer draining lymph nodes

Mehdipour, F., Talei, A., Razmkhah, M., Bagheri, M., Ghaderi, A.

Shiraz University of Medical Sciences, Shiraz Institute for Cancer Research, Shiraz, Iran, Islamic Republic of

Introduction: IL-10 producing (B10) B cells are mostly studied and characterized in autoimmune diseases while their phenotype and roles in solid malignancies are largely unknown. In the current study the frequency and phenotype of B10 cells in tumor draining lymph nodes (TDLNs) of breast cancer patients and its association with lymph node involvement were investigated.

Materials and methods: Mononuclear cells were isolated from 17 metastatic lymph nodes (MLNs) and 25 non metastatic lymph nodes (nMLNs). Lymphocytes and enriched B cells were stimulated in vitro and after surface and intracellular staining subjected to flow cytometry.

Results: The percentage of B10 cells did not show significant

changes in MLNs compared to nMLNs. However, the frequency of B10 cells significantly decreased in nMLNs of node positive compared to node negative patients ($P=0.001$). There were no significant correlations between the frequency of B10 cells and the percentages of IL-10+ or CD25+FoxP3+ regulatory T cells in TDLNs of breast cancer patients. Phenotypical characterization of B10 cells in TDLNs revealed that they were mostly enriched within CD24hiCD27+ memory pool and could have both switched (CD27+IgM-) and unswitched (CD27+IgM+) memory phenotype. Also most of B10 cells were CD5-, CD11c-, CD23- and CD43-.

Conclusion: IL-10 producing B cells could be detected in TDLNs of breast cancer patients mostly within the B cell memory pool. Also our study proposed a possible association between lymphatic involvement and the decrease in B10 cell frequency in nMLNs.

1128

Cytokine profile of B cells in breast cancer draining lymph nodes

Ghaderi, A., Mehdipour, F.

Shiraz University of Medical Sciences, Shiraz Institute for Cancer Research, Shiraz, Iran, Islamic Republic of

Introduction: B cells are generally considered as antibody producing cells and their role as cytokine producing cells is not well elucidated in solid malignancies. In this study, cytokine profile of B cells in tumor draining lymph nodes (TDLNs) of patients with breast cancer and its association with lymph node involvement were investigated.

Materials and methods: Mononuclear cells were isolated from 17 metastatic lymph nodes (MLNs) and 25 non metastatic lymph nodes (nMLNs). Enriched B cells were stimulated in vitro and after surface and intracellular staining subjected to flow cytometry.

Results: The percentages of IL-2, IFN- γ and TGF- β producing B cells were not significantly different in MLNs and nMLNs of breast cancer patients. The percentage of TNF- α producing B cells showed a non significant decrease in MLNs in comparison with nMLNs ($P=0.065$). However, the frequency of B cells with intermediate to high expression of TNF- α (TNFint/hi B cells) was significantly decreased in MLNs in comparison with nMLNs ($P=0.026$). The percentage of TNFint/hi B cells was also decreased in nMLNs of node positive compared to node negative patients ($P=0.034$). We also found a significant reverse correlation between the frequency of TNFint/hi B cells and the frequency of CD25+FoxP3+CD4+T cells in nMLNs ($R=-0.6$, $P=0.004$).

Conclusion: Our investigation revealed that invasion of the breast tumor to draining axillary lymph nodes had a negative impact on the B cell production of pro-inflammatory cytokine TNF- α even in nMLNs.

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Natural IgE production intrinsically requires CD1d expression on B cells

Kitoh, A.¹, Shibuya, R.¹, Kabashima, K.^{1,2,3}

¹*Kyoto University Graduate School of Medicine, Department of*

*Dermatology, Kyoto, Japan, ²Singapore Immunology Network (SIn), A*STAR, Biopolis, Singapore, ³PRESTO, Saitama, Japan*

Several studies have revealed antigen-independent effects of IgE antibodies on various mast cell functions in vitro. In addition, hapten-irrelevant IgE normally present in mice is required for sensitizing mice to contact hypersensitivity through mast cell modulation of dendritic cell migration and function, suggesting that naturally produced IgE plays an essential role in the maintenance of mast cell function in vivo. However, the mechanism by which IgE is naturally produced in vivo is unclear. We found that serum IgE levels were markedly decreased in CD1d-deficient mice compared to control wild-type BALB/c mice. In addition, amounts of IgE bound on skin and peritoneal mast cells were significantly lower in CD1d-deficient mice than in wild-type mice, suggesting that the defect of natural IgE production observed in CD1d-deficient mice was of physiological significance. Wild-type mice transplanted with bone marrow cells from CD1d-deficient mice showed significantly lower levels of serum IgE, compared to wild-type bone marrow cell-transplanted mice, suggesting that CD1d expression on hematopoietic cells is required for natural IgE production. To investigate the requirement of CD1d on B cells for their natural IgE production, we generated mixed bone marrow chimeric mice where wild-type B- or CD1d-deficient B-derived IgE could be distinguished and found that the IgE produced in these mice was largely produced from wild-type B cells. Taken together, our results suggest that CD1d expression on B cells is intrinsically required for natural IgE production in vivo.

1130

Memory B cell subset formation in acute and chronic viral disease

Pupovac, A., Good-Jacobson, K.

Infection and Immunity Program, Biomedicine Discovery Institute, Monash University, Department of Biochemistry and Molecular Biology, Clayton, Australia

Efficacious vaccine design is needed to tackle rapidly evolving pathogens. Memory B cell subsets provide the immune adaptability needed to challenge different pathogens. Human atypical memory B cells, originally characterised as "exhausted", are expanded in patients with chronic infectious disease. Currently, there is no animal model of atypical memory B cell formation. Therefore, little is known about the requirements for the formation of atypical memory in acute and chronic viral disease, and how the responses of atypical memory compare to that of classical memory in these disease states. To this end, we are utilising acute and chronic strains of lymphocytic choriomeningitis virus (LCMV) to understand memory B cell behaviour in vivo during chronic infections. Using an antigen-specific tetramer for LCMV to track antigen-specific B cells, our data indicates that while the chronic strain of the virus induced ongoing germinal centres (GC), very few of these cells were antigen-specific. In contrast to the GC B cell response after infection with the acute strain of the virus, the majority of B cells in chronic GCs were CXCR4+, suggesting an overrepresentation of dark zone B cells. Similar to human atypical memory B

cells, antigen-specific B cells *in vivo* had also modulated their expression of CXCR3, but only in the chronic strain was PD-1 upregulation evident. Identifying the requirements and functions of different memory B cell subsets in acute and chronic disease states will provide insight into the mechanisms driving B cell subset formation, which may allow for effective future vaccine development.

1131

A rare case of selective Ig-kappa light chain deficiency

Sadighi Akha, A.¹, Tschumper, R.², Mills, J.¹, Isham, C.¹, Viswanatha, D.¹, Snyder, M.¹, Murray, D.¹, Katzmann, J.¹, Jelinek, D.², Willrich, M.¹
¹Mayo Clinic, Laboratory Medicine and Pathology, Rochester, United States, ²Mayo Clinic, Immunology, Rochester, United States

Selective Ig-kappa and Ig-lambda deficiencies are extremely rare. So far, there have been 5 reported cases of Ig-kappa deficiency, 3 partial and 2 complete, the latter in two siblings also afflicted with cystic fibrosis. In 1985, sequencing the kappa constant region (*IGKC*) in one sibling identified a variant in each allele, p.Trp168Arg and p.Cys214Gly, that disrupted kappa chain folding.

Our case is a 73-year-old caucasian female suffering from peripheral neuropathy, but otherwise healthy and with no history of immunodeficiency. Immunofixation electrophoresis of her serum showed the total absence of Ig-kappa. Mass spectrometric analysis confirmed this finding and the polyclonal nature of the Ig-lambda repertoire. Flow-cytometric analysis showed no expression of Ig-kappa in the cytoplasm or surface of B cells. Other immunologic assessments only showed a mild decrease in her switched memory B cells; not atypical at her age. Sequencing *IGKC* showed she was homozygous for a missense mutation that interferes with kappa chain's folding.

The patient's 72-year-old brother, with no history of immunodeficiency, showed moderate B cell lymphopaenia, skewed kappa/lambda ratio in the serum, and decreased kappa/lambda expression in the cytoplasm and surface of B cells, but was otherwise normal. Sequencing *IGKC* demonstrated that he was heterozygous for the missense mutation identified in his sister.

These findings confirm that the complete absence of Ig-kappa is not incompatible with normal B cell development, and that it does not in itself manifest as an immunodeficiency. However, the presence of only one defective *IGKC* allele may have a quantitative effect on Ig-kappa expression.

1132

The functional response of B cells to antigenic stimulation during latent tuberculosis

Loxton, A.G., du Plessis, W., Chegou, N., Ronacher, K., Walzl, G.
 Stellenbosch University, Biomedical Sciences, Cape Town, South Africa

Mycobacterium tuberculosis (M.tb) remains a successful pathogen, causing tuberculosis disease numbers to constantly increase. Although great progress has been made in delineating the disease, the host-pathogen interaction is incompletely

described. B cells have shown to function as both effectors and regulators of immunity *via* non-humoral methods in both innate and adaptive immune settings. Here we assessed specific B cell functional interaction following stimulation with a broad range of antigens within the LTBI milieu. Our results indicate that B cells readily produce pro- and anti-inflammatory cytokines (including IL-1 β , IL-10, IL-17, IL-21 and TNF- α) in response to stimulation. TLR4 and TLR9 based stimulations achieved the greatest secreted cytokine-production response and BCG stimulation displayed a clear preference for inducing IL-1 β production. We also show that the cytokines produced by B cells are implicated strongly in cell-mediated communication and that plasma-memory B cells (CD19⁺CD27⁺CD138⁺) is the subset with the greatest contribution to cytokine production. Collectively our data provides insight into B cell responses, where they are implicated in and quantifies responses from specific B cell phenotypes. These findings warrant further functional B cell research with a focus on specific B cell phenotypes under conditions of active TB disease to further our knowledge about the contribution of various cell subsets which could have implications for future vaccine development or refined B cell orientated treatment in the health setting.

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The interplay between c-Myb and T-bet determines the fate of humoral response to influenza virus infection

Di Pietro, A.¹, Tempny, J.², Tarlinton, D.^{2,3,4}, Good-Jacobson, K.^{1,2,3}
¹Infection and Immunity Program, Biomedicine Discovery Institute, Monash University, Department of Biochemistry and Molecular Biology, Clayton, Australia, ²Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia, ³University of Melbourne, Department of Medical Biology, Melbourne, Australia, ⁴Monash University, Alfred Hospital, Department of Immunology, Prahran, Australia

Humoral and cellular immune responses are generated upon influenza virus infection of the respiratory tract, creating transitory germinal centres (GC) in peripheral lymphoid organs. B cells within the GC undergo affinity maturation, clonal selection of high-affinity variants and subsequent differentiation into antigen-specific antibody-secreting plasma cells and memory B cells. These populations help in clearing the infection and provide long-term immunity, however, the transcriptional programs that determine the fate of B cells during influenza infection are not fully elucidated. Here, we explore the intrinsic *in-vivo* activity of the transcription factor c-Myb in regulating humoral responses.

Our preliminary results denoted that the deletion of c-Myb in mature B cells of mice immunised with the haptenated protein NP-KLH triggers the expression of other transcription factors, such as T-bet. As a direct consequence, T-bet downstream target genes, normally expressed after influenza infection and suppressed during the NP-KLH response, were up-regulated, resulting in aberrant plasma cell differentiation and class-switching to the IgG2c isotype. Conversely, the deletion of T-bet in mature B cells reverted the phenotype to wild-type. Surprisingly, the aberrant response also occurred in c-Myb-deficient mice infected with HKx31 virus (H3N2), in which T-bet

expression is a normal component of the humoral response. ATAC-seq experiments also supported these observations, revealing a greater chromatin accessibility in the T-Bet locus in c-Myb-deleted GC B cells from infected mice, compared to wild-type GC B cells.

According to these results, c-Myb is fundamental for inhibiting dysregulation of the humoral response via suppression of aberrant levels of expression of T-bet.

1134

Molecular signals involved in human B cell migration into the retina

Stempel, A.J.¹, Bharadwaj, A.S.², Olivas, A.², Franzese, S.E.², Ashander, L.M.^{1,2}, Ma, Y.¹, Lie, S.¹, Appukuttan, B.¹, Smith, J.R.^{1,2}

¹Flinders University, Adelaide, Australia, ²Oregon Health Sciences University, Portland, United States

Purpose: Posterior uveitis is a blinding inflammatory disease that involves the retina. B cells have been implicated in disease pathogenesis, but trafficking of B cells to the retina remains unstudied and may present targets for more effective therapies. We investigated involvement of 3 molecules in B cell migration to retina: CXCL13, ICAM-1, and VCAM-1.

Methods: Neural retina and retinal pigment epithelium were dissected from human cadaver eyes (n=8), and CXCL13 expression was examined by RT-PCR and immunohistochemistry. Human B cells, isolated by negative selection from peripheral blood of healthy adults (n=7-13), were migrated through Bowden transwells populated with endothelial cells isolated from human retina by selection on CD31. Anti-ICAM-1, anti-VCAM-1, or negative control antibody was applied to upper chambers, and CXCL13 or medium alone was applied to lower chambers. Integrity of endothelial monolayers was confirmed by measurement of Texas Red-conjugated 70 kDa dextran diffusion.

Results: CXCL13 transcript was detected in both neural retina and retinal pigment epithelium. Immunohistochemistry identified CXCL13 protein in ganglion cells, photoreceptors and some vascular endothelial cells; human retinal endothelial isolates did not contain CXCL13 transcript suggesting endothelial expression was by transcytosis. Anti-ICAM-1 antibody significantly reduced B cell migration in comparison to control, in the presence and absence of CXCL13 ($p \leq 0.002$). Anti-VCAM-1 antibody reduced migration less significantly, and only when CXCL13 was absent ($p=0.02$).

Conclusions: Our results suggest that targeting ICAM-1 may be an effective treatment for patients with posterior uveitis. Future studies will evaluate the impact of ICAM-1 blockade on B cell migration under flow conditions.

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Control of germinal center and early memory B cell homeostasis by BAFFR

Lau, A.^{1,2}, Chan, T.^{1,2}, Brink, R.^{1,2}

¹Garvan Institute of Medical Research, Immunology Research Program, Sydney, Australia, ²University of New South Wales, St. Vincent's Clinical School, Sydney, Australia

The signalling duo BAFFR and its ligand, BAFF are required for naïve B cell development and survival. During T-dependent responses, germinal center (GC) B cells express high levels of BAFFR. The immunisation of intact BAFFR-deficient or BAFF-deficient mice leads to initiation of GC responses that fail to be sustained past 7-14 days and an absence of memory B cells. However, since these deficient mice have structural abnormalities in their secondary lymphoid organs, it remains unclear to what extent GC and memory B cells depend on cell-intrinsic expression of BAFFR.

To investigate this question, we performed adoptive transfer studies to examine the response of BAFFR-deficient hen egg lysozyme-specific (SW_{HEL}) B cells in normal intact recipient mice.

Immature BAFFR-deficient and BAFF-deficient B cells formed GC responses that underwent normal Ig class-switching and affinity maturation. In addition, the absence of T cell-derived BAFF had no detectable impact on selecting high affinity GC B cells. However, BAFFR-deficient SW_{HEL} responses failed to be sustained indicating that BAFF-BAFFR signalling plays an important role in mature GC B cell survival. Interestingly, we found that 20-30% of normal GC B cells expressed low levels of BAFFR. BAFFR expression did not correlate with either antigen affinity or the GC light and dark zones. We are currently trying to determine what role this population may play in regulating GC homeostasis. Finally, early GC-independent memory B cell numbers were found to depend on BAFFR expression, suggesting their distinct survival requirement from the BAFF-independent memory B cells that develop from GC precursors.

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ASCIZ and dynein light chain-1 are essential for the development of innate B1 cells

King, A.^{1,2}, Wong, D.¹, Li, L.^{1,2}, Heierhorst, J.^{1,2}

¹St Vincent's Institute of Medical Research, Fitzroy, Australia, ²The University of Melbourne, Department of Medicine, St Vincent's Hospital, Fitzroy, Australia

B1 cells play crucial roles in innate immunity by producing "natural" antibodies as a first line of defence against newly encountered pathogens. Defects in the development of B1 cells can lead to autoimmune disorders and leukaemia. In contrast to conventional B cells, the mechanisms regulating B1 cell development and maturation remain poorly understood.

Here we show that B1 cell development is severely impaired in conditional KO mice that lack dynein light chain1 (DYNLL1) or the DYNLL1-transcription factor ASCIZ in the B cell lineage. We found that these mice exhibit a profound reduction in B1 cells in the peritoneal cavity at 4 weeks of age, that is partially compensated with time. ASCIZ and DYNLL1 are also required for normal B cell development, but we found that B1 and B2 cell numbers are differentially affected by other mutations. Whereas MYC over-expression was synthetic lethal with Asciz- or Dynll1-deletions in immature B2 cells, it partially rescued the B1 cell deficiency in these mice. Conversely, knockout of the pro-apoptotic protein BIM rescued the B2 cell defect but had no effect on the B1 compartment.

Our data demonstrate that ASCIZ and DYNLL1 are essential for the development of B1 cells and highlight differential roles for MYC and BIM in the regulation of B1 and B2 cell populations.

1137

Overexpression of TACI leads to reduced B cell but increased plasma cell numbers*Garcillan, B., Lim, E.X., Fabienne, M.**The University of Melbourne, Microbiology and Immunology, Melbourne, Australia*

Transmembrane activator and CAML interactor (TACI) is a receptor for the TNF-like cytokines BAFF and APRIL that modulates B-cell function. BAFF signalling through TACI is not required for B cell development and survival but appears to negatively regulate B cell and controls B cell homeostasis. TACI signalling is also essential for T-independent antibody responses, isotype class switching and differentiation into plasma cells. In humans, mutations in TACI have been associated with common variable immunodeficiency, demonstrating the important role of this receptor in regulating B-cell function. TACI^{-/-} mice show B cell hyperplasia, absent T-independent immune responses and reduced IgA levels. Here, we characterized a novel transgenic mouse model where human TACI and GFP are expressed under the control of the mouse TACI promoter (hTACI-Tg). Our results show that human TACI fully functions in mice. We found the highest level of TACI expression in plasma cells, B1 and MZ B cells. In contrast to previously published data, we didn't find TACI expression in activated T cells. Overexpression of TACI in our mouse model reduced the peripheral B-cell compartment while it increased plasma cell numbers. Consistent with the important role of TACI in isotype switching and antibody production, we observed increased levels of serum IgA. Results with this new animal model allows us to finely track the fate and function of TACI-expressing B cells and also is the first animal model enabling targeting of human TACI *in vivo*.

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RAG2 participated in controlling Igk loci DNA demethylation

Wu, C., Zhao, X., Zhang, P., Li, S., Yu, X., Jiao, J., Dong, Y., Ji, Y.
Xi'an Jiaotong University Health Science Center, Key Laboratory of Environment and Genes Related to Diseases (Xi'an Jiaotong University), Ministry of Education of China, PR China, Department of Pathogenic Biology and Immunology, Xi'an, China

Antigen receptor genes are assembled by V(D)J recombination during lymphocyte development. RAG endonuclease and loci specific "accessible", containing histone modifications, DNA methylation and displacement and removal of nucleosomes contribute to control the process. In this study, we chose D708A-R1^{-/-}H, R1^{-/-}H and R2^{-/-}H transgene mice. D708A-R1^{-/-}H mice is a *Rag1*^{-/-} background containing an Asp708/Ala active site mutant RAG1 protein that interacts with RAG2 and binds DNA normally but lacks catalytic activity and then bred with mice harboring a functionally rearranged B1-8i *Igh* allele transgene to generate and exhibit developmental arrests at the pre-B, equivalent to those observed in *Rag1*^{-/-}B1-8i (R1^{-/-}H) and *Rag2*^{-/-}B1-8i (R2^{-/-}H). We isolated CD19⁺ pre-B cells from these mice and analyzed Igk loci DNA methylation status. We found that when RAG2 alone or D708ARAG1 and RAG2 exist, Igk loci were demethylated. But RAG1 alone

exist, Igk loci were methylated. Wild type RAG2 recruited into R2^{-/-}B cells, VJk recombination could be detected and Igk loci were demethylated. Mutations in RAG2's non-core region could increase the RAG activity, however Igk loci kept in DNA methylation status. We found that expression of Gadd45a mRNA was reduced in RAG2's non-core region comparing to wild RAG2. We propose that RAG2 has the abilities of limiting the excessive RAG activity and regulating accessibility of Igk loci.

Keywords: V(D)J recombination, B cell, Igk, DNA methylation, RAG2, Gadd45a

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Memory B cells develop from low-affinity clones in the germinal center light zone and are identified by CCR6 expression in mice and humans

Suan, D.^{1,2}, Krautler, N.¹, Maag, J.³, Butt, D.¹, Wood, K.¹, Hermes, J.¹, Priestley, D.¹, Statham, A.¹, Elliott, M.⁴, Marcel, D.³, Basten, A.¹, Tangye, S.¹, Brink, R.¹

¹Garvan Institute of Medical Research, Immunology, Darlinghurst, Australia, ²Westmead Hospital, Clinical Immunology and Allergy, Westmead, Australia, ³Garvan Institute of Medical Research, Genomics and Epigenetics Division, Darlinghurst, Australia, ⁴University of Sydney, Central Clinical School, Camperdown, Australia

Memory B cells (MBCs) and plasma cells (PCs) constitute the cellular output of germinal center (GC) responses that facilitate long-term humoral immunity and underpin vaccine efficacy. Whilst expression of the transcription factor BLIMP-1 is routinely used to identify cells undergoing PC differentiation, no such marker exists for the MBC lineage. It remains unclear, therefore, how MBC differentiation is integrated into the cyclic proliferation, somatic mutation and interzonal migration of GC B cells. Here we report that the chemokine receptor CCR6 uniquely marks MBC precursors in both mouse and human GCs. CCR6⁺ pre-MBCs are highly enriched within the GC light zone (LZ), are the most quiescent of all GC B cells and exhibit a gene expression signature indicative of MBC transition. Unlike PC precursors, pre-MBCs within the GC LZ possess low affinity for the immunising antigen, indicating a fundamental dichotomy in the processes that drive MBC and PC differentiation during GC B-cell responses.

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Selenoproteins regulate B cell receptor-mediated activation and development of B cells

Katkere, B., Markley, R., Shay, A., Prabhu, K.S., Kirimanjeshwara, G.
The Pennsylvania State University, Veterinary and Biomedical Sciences, University Park, United States

B cell development, activation and immunoglobulin affinity maturation are dependent on B cell receptor (BCR)-mediated signaling, which is sensitive to redox status of cells. Cellular redox status is regulated by several mechanisms including that

are controlled by selenoproteins, a class of proteins that contain amino acid selenocysteine at their catalytic sites. Although several selenoproteins are known to be expressed in developing and mature B cells, their precise role in B cell development and functions has not been explored. In order to bridge this gap in our understanding of B cell development and functions, we generated mice that lack expression of selenoproteins in B cells. BCR endocytosis, signaling, trafficking, and activation were significantly affected in selenoprotein-deficient B cells. Consistent with the role of BCR-mediated signaling in B cell development, mature B cell population was significantly (>40%) smaller in the absence of selenoproteins. Interestingly, BCR endocytosis was found to be regulated by selenoproteins with known redox regulatory functions (for example, Gpx1) while the downstream effects such as calcium signaling were regulated by selenoproteins that have redox-regulation independent functions (for example, Selk). These data suggest that several selenoproteins with overlapping and distinct functions may regulate BCR-mediated activation of B cells and their development. These studies provide the basis for discovery of potential targets for novel adjuvants and therapeutics.

1141

A lamprey monoclonal VLR antibody generated against multiple myeloma bone marrow aspirate specifically identifies plasma cells

Liu, Y.¹, Yu, C.², Chan, J.¹, Cooper, M.², Ehrhardt, G.¹

¹University of Toronto, Immunology, Toronto, Canada, ²Emory University School of Medicine, Atlanta, United States

Plasma cells are key effector cells to engage pathogenic challenges as well as contributors to autoimmune and malignant disorders. Only a limited number of plasma cell specific biomarkers have been identified thus far. The unique structural characteristics of the variable lymphocyte receptor (VLR) antibodies of the evolutionary distant sea lamprey prompted us to investigate whether VLR antibodies could be isolated that detect novel plasma cell antigens, which have not been recognized by conventional antibodies. Here we describe a monoclonal lamprey antibody, VLRB MM3, that was raised against primary multiple myeloma cells. VLRB MM3 recognizes a unique epitope of the CD38 ectoenzyme that is present on plasmablasts and plasma cells from healthy individuals and on most, but not all, multiple myelomas. Binding by the VLRB MM3 antibody coincides with CD38 dimerization and nicotinamide adenine dinucleotide (NAD) glycohydrolase activity. Our data demonstrate that the lamprey VLRB MM3 antibody is a unique reagent for the identification of plasmablasts and plasma cells with potential applications in the diagnosis and therapeutic intervention of plasma cell or autoimmune disorders. This study was supported by CCSRI grant 2012-701054 to GRAE and NIH grant 5U19AI096187-02 to GRAE and MDC.

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miR-338-5p regulates the biological function of B cells by targeting nuclear factor kappaB1

Xu, H., Ma, X., Song, D., Xue, D., He, X.

Third Affiliated Hospital of Soochow University, Urology Department, Changzhou, China

Objective: To investigate the effect of miR-338-5p on NF- κ B1 expression and the regulatory ability of IgG production by B cells.

Methods: Targeting verification assay of miR-338-5p on NF- κ B1 was conducted by dual-luciferase reporter assay. Purified CD20+ B cells were cultured with anti-IgM antibody, and/or recombinant human B cell activating factor (BAFF), after transfected with miR-338-5p agomiR, miR-338-5p antagomiR, NF- κ B1 siRNA (siNF- κ B1), and their corresponding negative control reagents. Real-time PCR and western blot assay were applied to determine NF- κ B1 (P105, P50) levels. Supernatant IgG concentration was detected by ELISA. Data were analyzed by SPSS 17.0 software, and $P < 0.05$ was considered to be significant.

Results: Compared with control groups, the hRluc/hLuc relative luciferase activity significantly increased in miR-338-5p mimic and NF- κ B1-3'-UTR Reporter co-transfection group; In the anti-IgM antibody and rhBAFF co-culture system, P105 and P50 levels in B cells, and IgG production by B cells transfected by miR-338-5p agomiR significantly increased, while those proteins' levels significantly decreased in miR-338-5p antagomiR transfection group. The effect of siNF- κ B1 was similar to those of miR-338-5p antagomiR. Correlation analysis results suggested that NF- κ B1 mRNA level was significantly positively correlated with IgG concentration.

Conclusions: miR-338-5p promoted IgG production by B cells through positively regulating NF- κ B1 expression, and indirectly regulating BAFF signal.

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Immune suppressive doses of ultraviolet radiation activate a unique subset of B cells that are required for sunlight protection of mice from EAE

Marshall, J.¹, Kok, L.F.¹, Marsh-Wakefield, F.^{1,2}, Gillis, C.^{1,2}, Halliday, G.^{1,2}, Byrne, S.^{1,2}

¹University of Sydney, Cellular Photoimmunology Group, Infectious Diseases and Immunology, Sydney, Australia, ²University of Sydney, Dermatology Research Laboratories, Sydney, Australia

The ultraviolet (UV) radiation contained in sunlight is a powerful and broad spectrum immune suppressant. Activation of regulatory B cells is a major way in which UV suppresses adaptive immune responses. Small doses of UV, equivalent to 10 minutes of summer sun for humans, are all that are required to activate a unique population of B cells within the skin draining lymph node of C57BL/6 mice. As determined by flow cytometry, MHC II^{hi} B220^{hi} UV-activated B cells expressed significantly higher levels of CD19, CD21/35, CD25, CD210 and CD268 as well as the co-stimulatory molecules CD80, CD86, CD274 and CD275. There was no difference in the expression of CD276. UV-activated B cells were CD1d^{low}CD5⁻ meaning

that they are a different population to B10 cells. Mice exposed to UV and immunised with MOG/CFA to induce experimental autoimmune encephalomyelitis (EAE) displayed significantly less demyelination and reduced infiltration of inflammatory cells into the spinal cord. Consequently, UV-exposed groups showed delayed EAE onset, reduced peak EAE score and significantly suppressed overall disease incidence and burden. UV-protection from EAE was dependent on the successful activation of lymph node B cells because UV could not protect mice from EAE who were pharmacologically depleted of B cells. Thus, maintenance of a pool of regulatory B cells in peripheral lymph nodes appears to be essential to prevent the activation of encephalitogenic cells that cause CNS-autoimmunity. Exposure to UV is one way in which this pool of EAE-protecting cells can be maintained.

1144

Does immunoglobulin class switching predominantly occur in germinal centers?

Roco, J.¹, Nefzger, C.², Mesin, L.³, Gonzalez, P.¹, Ellyard, J.¹, Polo, J.M.², Victoria, G.³, Toellner, K.⁴, Vinuesa, C.¹

¹John Curtin School of Medical Research, Australian National University, Department of Immunology and Infectious Disease, Canberra, Australia, ²Australian Regenerative Medicine Institute (ARMI), Monash University, Department of Anatomy and Developmental Biology, Melbourne, Australia, ³Whitehead Institute for Biomedical Research, Cambridge, United States, ⁴School of Immunity and Infection, Medical School IBR, University of Birmingham, Birmingham, United Kingdom

Class Switch Recombination (CSR) is an intrachromosomal DNA rearrangement by which mature B cells are able to express antibodies of different classes in response to antigen stimulation and co-stimulatory signals. Classical studies have identified germinal centers (GCs) as the main areas where CSR occurs. However one of the main caveats of these studies has been the inability to track rare antigen B-cell populations at early time points during the course of an immune response; therefore, most of the conclusions regarding CSR activation have been made after clonal expansion, when GCs are well formed. Thus, critical events that occur immediately after antigen-mediated B cell activation and prior to GC formation could have been missed. We have now taken advantage of mouse models in which we can track single antigen-specific B cells immediately after priming by T-cells, and prior to differentiation into GC or extrafollicular plasma cells, and then follow them during the course of GC reactions. To evaluate the timing and location of CSR *in vivo* we have analysed single antigen-specific B-cells at 12-24 hr intervals post-immunization. Without exception, $\gamma 1$ -, $\gamma 2b$ - and $\gamma 3$ -switch transcripts were expressed as early as 1.5 days after transfer but they were almost absent in GC B-cells. This rapid induction of switch transcripts reaches a peak between days 2.5 to 3.0 correlating with AID expression long before GC formation. Together, our data challenge the current dogma of isotype-switching occurring predominantly within GCs and suggest a model in which GCs play a regulatory role in this process.

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Systemic inflammation rapidly reorganizes mouse bone marrow B cells and their environment to alter repertoire development and drive early IgM responses

Moreau, J.^{1,2}, Paige, C.^{1,2}

¹University of Toronto, Immunology, Toronto, Canada, ²Princess Margaret Cancer Centre, Toronto, Canada

Maturation of B cells in the bone marrow is regulated by an organized tissue structure composed of local microenvironments, each providing a distinct cocktail of factors to the various stages of B cell development. As progenitors mature, they migrate between niches and towards the sinusoids. Systemic inflammation in mice disrupts B lymphopoiesis, however; it is unclear how B cell niches respond to these challenges and the impact on subsequent B cell fate remains unknown. In the current study, we report that injection of Freund's incomplete adjuvant induced a massive accumulation of mature B cells into the bone marrow. This response occurred rapidly (within hours) and was mediated by increased responsiveness to the chemokine CXCL12. B cell localization within the marrow itself was also altered with fewer cells in the sinusoids. Redistribution occurred concurrently with remodeling of supportive microenvironments, including a shift away from an early progenitor trophic environment in favour of more mature differentiation and antibody production. This was evidenced by reduced levels of IL-7, but increased expression of BAFF, MIF, IL-5, and IL-6, as well as, higher numbers of IgM secreting cells. Further, we observed expansion of a DX5⁺Thy1⁺ cell population previously shown to promote immature B cell survival and RAG expression. We identified these cells as basophils and noted reduced lambda light chain usage of immature B cells. These results demonstrate an exquisite sensitivity of the marrow to systemic inflammation and suggest that inflammatory signaling regulates B cell fate through control of B cell-niche interactions.

1146

NFATc1 supports imiquimod-induced skin inflammation by suppressing regulatory B cells

Muhammad, K.¹, Alrefai, H.¹, Rudolf, R.¹, Pham, D.¹, Patra, A.¹, Klein-Hessling, S.¹, Avots, A.¹, Tenzer, S.², Goebeler, M.³, Kerstan, A.³, Serfling, E.¹

¹University of Würzburg, Molecular Pathology, Würzburg, Germany, ²Johannes-Gutenberg-University, University Medical Center, Mainz, Germany, ³University Hospital Wuerzburg, Department of Dermatology, Würzburg, Germany

The epicutaneous application of aldera[®], a cream containing the TLR7 agonist imiquimod (IMQ), to mice induces skin inflammation that exhibits many aspects of human psoriasis. Mice depleted of B cells or IL-10-deficient B cells show more fulminant inflammation upon IMQ exposure indicating that IL-10 synthesized by B cells controls skin inflammation. Inactivation of NFATc1 in B cells suppresses IMQ-induced skin inflammation. Upon *in vivo* application, aldera[®] cream strongly affects the murine immune system leading to immunoglobulin heavy chain switch, to an increase in IL-10-producing B cells and

a defect in NFATc1 induction in B cells. In vitro studies also show that IMQ induces the proliferation of IL-10 producing regulatory B cells. The induction of IL-10 RNA by IMQ can be blocked by BCR signals that induce NFATc1. NFATc1 binds to HDAC1 and suppresses IL-10 expression that dampens TNF α and IL-17 production by T cells. Our data indicates a close link between NFATc1 and IL-10 expression in B cells during IMQ-mediated skin inflammation and suggest that the suppression of NFATc1 activity might be of benefit to treat human psoriasis.

1148

Prevalence, structure and putative mechanism for large genetic insertions in VDJ recombination

Koning, M.T.¹, van Bergen, C.A.M.¹, Trollmann, I.J.M.¹, Scherer, H.U.², van Attikum, H.³, Toes, R.E.M.², Kielbasa, S.M.⁴, Tijsterman, M.³, Veelken, H.¹

¹Leiden University Medical Center, Department of Hematology, Leiden, Netherlands, ²Leiden University Medical Center, Department of Rheumatology, Leiden, Netherlands, ³Leiden University Medical Center, Department of Human Genetics, Leiden, Netherlands, ⁴Leiden University Medical Center, Department of Medical Statistics and Bioinformatics, Leiden, Netherlands

Recently, large LAIR1 insertions at the V-D junction were described as a novel mechanism to generate antibodies against *P. falciparum* RIFIN antigens (Tan *et al.*, Nature 2016). We investigated presence, prevalence, and structure of such VDJ insertions in healthy donors.

We obtained >32,000 unique full-length VDJ sequences from 6 healthy donors by an unbiased template-switching anchored PCR and full-length single molecule PacBio NGS sequencing. Abnormally long sequences and junctions were searched for gene homologies by BLAST.

5 VDJ of the same donor, representing all sequences with a CDR3 region >150 bp, carried insertions exclusively located at VDJ junctions ($E \leq 10^{-37}$). RPLP0, ZNF316, and an inverted IGHV-IGHD sequence were insertions in unmutated IgM. The LAIR1 exon described by Tan *et al.* and an IGHV-IGHD intergenic region were inserted in IgG. Somatic hypermutation burden correlated strongly between the insertion and the IGHV segment ($r=0.9944$; $p < 0.001$). All insertions harboured cryptic RSS sites at both termini.

Insertions represent a rare but recurrent novel antibody diversification mechanism. Their presence in naïve B-cells, their exclusive positioning in VDJ junctions, and the universal presence of cryptic RSS suggest primary VDJ recombination as the generating event. Mechanistically, these insertions appear to represent transposons that are excised by the RAG complex at tandem cryptic RSS sites and are re-inserted in *trans* in analogy to immunoglobulin signal sequences during VDJ recombination. An AID-induced templated dsDNA break repair as suggested by Tan *et al.* appears unlikely. Certain loci (e.g. LAIR1) and certain individuals seem particularly prone to such insertions.

1149

Blimp-1 cooperates with Aiolos to regulate the survival of multiple myeloma cells through modulating the expression of apoptosis-related genes

Hung, K.-H.¹, Su, S.-T.¹, Chen, C.-Y.², Hsu, P.-H.³, Huang, S.-Y.⁴, Wu, W.-J.¹, Chen, M.-J.M.⁵, Chen, H.-Y.¹, Wu, P.-C.⁶, Lin, F.-R.¹, Tsai, M.-D.⁷, Lin, K.-I.¹

¹Academia Sinica, Genomics Research Center, Taipei, Taiwan, Republic of China, ²National Taiwan University, Department of Bio-Industrial Mechatronics Engineering, Taipei, Taiwan, Republic of China, ³National Taiwan Ocean University, Department of Life Science, Keelung, Taiwan, Republic of China, ⁴National Taiwan University Hospital, Department of Internal Medicine, Taipei, Taiwan, Republic of China, ⁵National Taiwan University and Academia Sinica, Genome and Systems Biology Degree Program, Taipei, Taiwan, Republic of China, ⁶National Taiwan University, Graduate Institute of Biomedical Electronics and Bioinformatics, Taipei, Taiwan, Republic of China, ⁷Academia Sinica, Institute of Biological Chemistry, Taipei, Taiwan, Republic of China

Transcriptional repressor Blimp-1 plays a role in the control of plasma cell differentiation, establishment of long-lived plasma cells, and maintenance of the survival of multiple myeloma (MM) through modulating gene expression. Hence, to understand the interactome of Blimp-1 and its molecular network facilitates us to realize the pathogenesis of MM. Toward this goal, we identified Aiolos, an anti-apoptotic transcription factor of MM cells, as a Blimp-1-interacting protein by mass spectrometry. To realize their mode of action, our results from ChIP-chip assay indicated that they co-bind to large number of genes, including apoptosis-related genes. Particularly, Blimp-1 and Aiolos sustain the survival of MM cells as well as normal plasma cells via binding to and suppressing the apoptosis-related genes. Previous reports showed that the new therapeutic effect of MM, lenalidomide, lies in the induction of the apoptosis of MM cells through promoting the proteasomal degradation of Aiolos. However, the relationship between Blimp-1 and the action of lenalidomide is still elusive. Here, we found that MM cells treated with lenalidomide caused ubiquitination and proteasomal degradation of Blimp-1. We also identified a new Blimp-1 direct target, *CUL4A*, a core component of multiple cullin-RING-based E3 ubiquitin-protein ligase complexes and is known to be involved in degradation of Aiolos after lenalidomide treatment in MM cells. Furthermore, the lenalidomide-induced apoptosis in MM cells was partially rescued after reintroduction of Blimp-1 or knockdown of *CUL4A*. Taken together, our study demonstrated the mechanisms by which Aiolos and Blimp-1 maintains plasma cell survival.

1150

Effect of *Mycobacterium tuberculosis* on B cell development and differentiation in BALB/C mice

Liu, W.¹, Wang, C.², Lv, Y.², Wang, T.², Wu, L.², Wu, Y.², Liu, Q.², Guo, L.², Bai, L.¹

¹Dali University, Department of Medical Immunology and Microbiology, Dali, China, ²Dali University, Department of Medical Microbiology and Immunology, Dali, China

Tuberculosis is a severe infectious disease that is harmful to human in China. Some data indicate that anti-tuberculosis immunity is mainly innate immunity and cell-mediated immunity, but recent studies have shown a protective effect of B cell responses in tuberculosis. However, it is unclear that infection of *Mycobacterium tuberculosis* (Mtb) influences B cell development and differentiation. In this project, the BALB/c mice were infected successfully with the dominant genotype of Mtb isolated from Yunnan and H37Ra by tail intravenous injection with 10^6 CFU in per mouse. The infectious models were confirmed by isolated bacteria from the lungs, livers and kidneys of infected mice. We collected the cell suspensions of the bone marrow and spleen from the infectious mice both in the early stage (4w after infected) and later period of the infection (8w after infected). Then the ratio of pro-B, pre-B, immature B, transitional B, mature B, activated B and memory B cells population were investigated by flow cytometry. The results show that Mtb infection promoted B cell maturation. The early stage B cells, including pro-B (B220⁺CD43⁺), immature B (B220⁺IgM⁺IgD⁻) and T1 B (B220⁺IgM^{hi}IgD^{low}) cells decreased and mature B (B220⁺IgM^{low}IgD^{hi}) cell increased. It also promoted B cell activation (B220⁺CD69⁺) and memory B cell (B220⁺CD27⁺) increase. The above change is the benefit to anti-tuberculosis infection. The results also show that the Mtb isolate and H37Ra are similar to the influence of B cell development and differentiation.

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Heat shock protein 70 is critically involved in CD19+CD24hiCD27+ B cells mediated suppression of Hashimoto thyroiditis

Wang, L.¹, Zha, B.², Chu, Y.³

¹Fudan University, Shanghai, China, ²Shanghai Fifth People's Hospital, Fudan University, Department of Endocrinology and Metabolism, Shanghai, China, ³Fudan University, Department of Immunology and Biotherapy Research Center, Shanghai, China

As we previously demonstrated, CD19+CD24hiCD27+ B Cells play a suppressive role in the peripheral blood of humans, with their numbers and function altered in autoimmune thyroid disease. Herein, we further reveal the mechanisms by which CD19+CD24hiCD27+ B Cells performed suppressive function in autoimmune thyroid disease. In this study, we show that the suppressive function of CD19+CD24hiCD27+ B Cells in human peripheral blood during Hashimoto thyroiditis (HT) is impaired, and the expression of intracellular heat Shock Protein 70 (Hsp70) which is highlighted by mRNA profiling decreased in CD19+CD24hiCD27+ B Cells. Moreover In vitro studies reveal that the induction of the expression of Hsp70 in HT patients' CD19+CD24hiCD27+ B Cells restored the suppressive function of this B cell subset, in contrast, inhibition of the expression of Hsp70 in healthy individuals' CD19+CD24hiCD27+ B Cells destroyed the suppressive function of this B cell subset. Furthermore, we show that the hsp70 related suppressive function of CD19+CD24hiCD27+ B Cells was interleukin(IL)-35 dependent and that silent the expression of IL-35 also

impaired the suppressive function of CD19+CD24hiCD27+ B Cells even when the intracellular hsp70 was highly expressed. These findings reveal a possible mechanism by which human regulatory B cells play suppressive role on autoimmune diseases.

1152

Exploring the role of c-Rel amplification and splicing in lymphomagenesis

Kober, M.^{1,2}, Kumar, D.^{2,3}, Schmidt-Suprian, M.^{1,2}

¹Technical University Munich, Hematology & Oncology, Munich, Germany, ²Max Planck Institute of Biochemistry, Molecular Medicine, Munich, Germany, ³Singapore Immunology Network (SigN), A*STAR, Singapore, Singapore

Constitutive activation of NF-κB transcription factors is a hallmark of various human lymphoid cancers. The vast majority of human lymphomas arise from B cells. This bias to the B cell lineage is thought to be due to DNA breaks, which occur during class-switch recombination and somatic hypermutation in germinal center B cells.

c-Rel is the only NF-κB family member which malignantly transforms lymphoid chicken cells in culture. Remarkably, the c-Rel gene locus is amplified in a high percentage of human B cell lymphomas, such as in Hodgkin lymphoma (HL), diffuse large B cell lymphoma (DLBCL) and primary mediastinal B cell lymphoma (PMBCL) (Barth et al., 2003; Gilmore et al., 2004; Weniger et al., 2007). In addition, a novel, notably active c-Rel splice variant has been detected in diffuse large B cell lymphoma patients, which was absent in healthy individuals (Leeman et al., 2008). Despite this evidence for an eminent involvement of c-Rel in human lymphomas, the role of c-Rel during lymphomagenesis remains unclear.

We generated novel mouse models to evaluate whether conditional c-Rel overexpression and aberrant splicing contribute to B cell lymphomagenesis *in vivo*. For this purpose, we used elaborate genetic tools based on BAC *cREL* gene locus modifications. We induced overexpression of c-Rel or GFP-c-Rel specifically in B cells (CD19Cre) or germinal center B cells (Cy1Cre). Initial results obtained from these mice will be discussed.

1153

Maintenance of IgG tetanus toxoid (TT) antibodies in humans is associated with TT-specific IgG⁺, but not IgD⁺IgM⁺, memory B cells

Tjiam, M.C.¹, Perera, R.², Fernandez, S.¹, French, M.A.^{1,3}

¹The University of Western Australia, School of Pathology and Laboratory Medicine, Perth, Australia, ²Health Department of Western Australia, Tuberculosis Control Program, Perth, Australia, ³Royal Perth Hospital and PathWest Laboratory Medicine, Department of Clinical Immunology, Perth, Australia

Immunoglobulin (Ig) G antibodies play a central role in vaccine-induced immunity against pathogens. Circulating IgG antibodies are secreted by plasma cells, however long-term vaccine-induced memory is retained by memory B cells, of which IgG⁺ and IgD⁺IgM⁺ are the major subsets. The contribution of these memory B cell subsets to the maintenance

of plasma IgG antibody levels in humans is unclear. We adapted a flow cytometry-based method to enumerate antigen-specific memory B cell subsets using tetanus toxoid (TT) as a model antigen. Plasma TT-specific IgG antibodies and TT-specific memory B cells were assayed in 4 healthy adults before and at weeks 1, 2, 3 and 4 after a booster vaccination with TT and in 30 adults with latent or active tuberculosis (TB) and unknown TT vaccination histories. B cells were enriched from PBMC via negative bead selection, stained with CD3-PerCP-Cy5.5, IgG-FITC, IgM-APC, CD20-APC-H7, CD27-V450, IgD-BV510 and a multimeric TT-PE probe, and assessed by flow cytometry. Plasma TT-specific IgG antibody levels were measured via ELISA. Both plasma TT-specific IgG antibody levels and TT⁺IgG⁺ memory B cell proportions increased following booster vaccination and were strongly correlated ($p=0.81$, $p<0.0001$). Proportions of TT⁺IgD⁺IgM⁺ memory B cells exhibited no change following booster vaccination. Similarly, in the adults with TB and unknown TT vaccination history, plasma TT-specific IgG antibody levels correlated with the proportions of TT⁺IgG⁺ ($p=0.70$, $p<0.0001$), but not TT⁺IgD⁺IgM⁺, memory B cells. These results suggest that TT⁺IgG⁺, but not TT⁺IgD⁺IgM⁺, memory B cells contribute to the maintenance of circulating TT-specific IgG antibody levels.

1154

IL-34-dependent differentiation of monocytic cell with B cell stimulating activity

Magari, M., Ogawa, S., Toya, Y., Hieda, K., Yamane, F., Kanayama, N., Tokumitsu, H., Ohmori, H.

Okayama University, Department of Medical Bioengineering, Okayama, Japan

Follicular dendritic cells (FDCs) play a critical role for generating high-affinity antibody in germinal center (GC). We recently found a novel class of CD11b⁺ monocytic cells named as FDMC (CXCR4⁺ CD115⁺ CD11c⁻ I-Ad), whose differentiation was dependent on CSF-1R signaling triggered by IL-34 produced from FDC line, FL-Y (*J. Leukoc. Biol.* 2014). IL-34 is a recently identified cytokine, which share a common receptor, colony stimulating factor (CSF)-1 receptor, with CSF-1. FDMC was induced from c-kit⁺ CD11b⁻ splenocytes. Herein, we examined the specific action of IL-34 underlying FDMC differentiation and immunological functions of FDMC for activating B cells. 1) The number of FDMC induced on a strep tagged-IL-34 transduced FL-Y was significantly increased, compared with that on an original FL-Y line. However, conditioned medium from FL-Y cells could not reproduce FDMC induction activity of FL-Y cells. In accordance, FDMC could not be induced when precursor cells were separated from FL-Y line with transwell membrane. These results suggest that membrane-bound molecule on FL-Y is involved in FDMC induction activity of FL-Y, and may modulate CSF-1R signaling. 2) FDMC strongly promoted the cell division and the expression of GC-associated markers (GL7, Fas) of anti-CD40-stimulated B cells *in vitro*. FDMC also induced somatic hypermutation at Ig gene in cultured B cells stimulated with anti-CD40 mAb. The frequency of SHM was significantly increased by the addition of anti-IgM plus IL-4/IL-21 *in vitro*. In the condition, proportion of annexin V⁺ B cells was significantly increased, suggesting that FDMC might regulate the differentiation of GC-phenotype B cells.

1155

Mcl-1 is critical for Lyn deficient plasma cells despite altered signalling pathways

Low, M.^{1,2}, Tarlinton, D.^{1,3}, Infantino, S.^{1,3}

¹Walter and Eliza Hall Institute of Medical Research, Immunology, Parkville, Australia, ²Monash Health, Department of Clinical Haematology, Clayton, Australia, ³Monash University, Department of Immunology, Prahran, Australia

Plasma cells (PC) are the effector cells for the humoral immune system producing antibodies with the aim of eliminating foreign pathogens. Despite the importance of PC, relatively little is known about the signalling pathways that control PC function and survival. Lyn, a member of the Src family kinases is known to play a role in PC signalling with an absence of Lyn leading to splenic plasmacytosis and a disease phenotype similar to human systemic lupus erythematosus. In PC, Lyn has a predominant role in negative signalling with an absence of Lyn leading to increased phosphorylation STAT3 and STAT5 from IL6 and IL3 respectively. We have compared Lyn deficient versus Lyn intact PC and have identified differing gene expression profiles by both RNA and protein levels. Some of these genes have roles in PC that are incompletely understood and include CD200, Ly6C & CD272 with functional analysis ongoing. Analysis of Lyn deleted PC revealed no difference in Mcl-1 protein levels, a key regulator of PC survival. Additionally, deletion of Mcl-1 in Lyn deficient mice leads to a loss of PC in spleen and bone marrow similar to wild type animals. These results show that Mcl-1 is still a critical survival molecule in Lyn deficient PC suggesting that the splenic plasmacytosis in Lyn deficiency is due to, as yet, undefined pathways. Characterisation of these pathways using our identified list of genes could lead to improved understanding of PC survival pathways and lead to novel therapies for diseases modulated by PC.

1156

B cell-intrinsic role of DOCK2 in T cell-dependent humoral immunity

Ushijima, M., Fukui, Y.

Kyushu University, Division of Immunogenetics, Medical Institute of Bioregulation, Fukuoka, Japan

The small GTPase Rac has been implicated in various cellular functions by acting downstream of multiple receptors including B cell receptor (BCR). However, its role in humoral immunity and the upstream regulator are poorly understood. DOCK2 is an atypical Rac activator predominantly expressed in hematopoietic cells. We found that DOCK2 is essential for BCR-mediated Rac activation and critically regulates immunological synapse formation. This finding led us to examine B cell-intrinsic role of DOCK2 in humoral immunity. For this purpose, we took two distinct approaches: one is adoptive transfer of B cells expressing antigen-specific BCR in a class-switchable manner, and the other is conditional KO mice lacking DOCK2 expression in B-cell specific manner. By analyzing adoptively transferred B cells and conditional KO mice, we show here that DOCK2-Rac axis plays a key role in antigen-specific IgG responses.

1157**Involvement of CD40 on immune cells in response to immune complexes**

Atsumi, K., Matsushima, M., Ogiso, H., Ochi, H., Kusatsugu, Y., Oyabu, S., Ogasawara, N., Takemura, K., Kawabe, T.
Nagoya University Graduate School of Medicine, Department of Pathophysiological Laboratory Sciences, Nagoya, Japan

Immune complex (IC), which is antibody-forming complex with its specific antigen, can cause immune responses by providing feedback. The immune responses against ICs by marginal zone B cells (MZBs), follicular dendritic cells (FDCs), and follicular B cells (FOBs) are seemed to be T cell-independent responses since GC formation and antibody production were induced in athymic nude mice by injection of ICs. CD40 is necessary for the generation of antibody responses to T cell-dependent antigens as well as for the development of GCs through the interaction with CD154 (CD40 ligand) on T cells. However, it is unclear whether immune responses against ICs are mediated by CD40-dependent mechanisms. In this study, we investigated the role of CD40 on immune responses against ICs, especially focused on the function of MZBs, FDCs, and FOBs.

CD40KO mice were not able to produce OVA-specific IgM and IgG as well as GC formation and B cell proliferation after IC immunization. IC localization in MZ and on FDCs after IC immunization were not affected even in the absence of CD40. However, the induction of FcγRIIB and VCAM-1 expression on FDCs were impaired in CD40KO mice after immunization with ICs even though ICs were trapped on FDCs. Although the expression of ICAM-1 seemed to be slightly increased by ICs in CD40KO mice, however, the extent of induction was quite low compared to that of WT mice.

These results suggested that CD40 would be important for activation of FDCs, but not for IC transporting, to provoke immune responses against ICs.

1158**Involvement of CD40 in the expression of Bcl6**

Ochi, H., Matsushima, M., Koderu, Y., Kusatsugu, Y., Atsumi, K., Oyabu, S., Ogasawara, N., Takemura, K., Kawabe, T.
Nagoya University Graduate School of Medicine, Department of Pathophysiological Laboratory Sciences, Nagoya, Japan

In immune response against T-dependent antigens, B cells are activated by the interaction with a cognate T cell and form germinal centers (GCs). Within GCs, B cells expressing high affinity antibodies are selected by the interaction with follicular dendritic cells and follicular helper T (Tfh) cells. Bcl6 is a transcriptional repressor required for GC formation. The expression of Bcl6 is restricted to the GC stage in mature B cells by transcriptional and posttranscriptional regulation. Moreover, Bcl6 expression in

T cells drives the formation of Tfh cells. CD40 is a molecule that belongs to the tumor necrosis factor-receptor family, and the engagement of CD40 and CD40 ligand plays an important role in T-dependent antibody responses. Our previous study reported that CD40 knock-out (KO) mice failed to form GCs by T-dependent antigens. In this study, we investigated the role

of CD40 on the expression of Bcl6 in GC-related lymphocytes, especially GC B cells and Tfh cells.

WT and CD40KO mice were immunized with 2×10^9 SRBC, and spleens were removed 3 or 6 days after immunization. We analyzed the expression of Bcl6 in B cells (B220⁺) and Tfh cells (CD4⁺CXCR5⁺PD-1⁺).

Although the expression levels of *Bcl6* mRNA in CD40KO B cells were comparable to WT B cells, the expression levels of Bcl6 protein was quite low in CD40KO mice. Moreover, Bcl6^{hi} cells in CD4⁺CXCR5⁺PD-1⁺ T cells were severely reduced in CD40KO mice. These results suggested that CD40 might contribute to the expression of Bcl6 in both B cells and Tfh cells.

1159**A new role for VpreB: an invariant surrogate antigen that selects Ig antigen binding sites**

Khass, M.¹, Blackburn, T.², Burrows, P.², Walter, M.², Capriotti, E.³, Schroeder, H.⁴

¹University of Alabama at Birmingham, Clinical Immunology and Rheumatology, Birmingham, United States, ²University of Alabama at Birmingham, Microbiology, Birmingham, United States, ³University of Düsseldorf, Department of Biology, Düsseldorf, Germany, ⁴University of Alabama at Birmingham, Medicine, Birmingham, United States

The preB cell receptor (preBCR) is formed by binding of VpreB and λ5 to μ heavy chain (μHC) and selects for μHCs ultimately capable of binding to conventional light chains. VpreB and λ5 also create a CDR-H3 sensing site that interacts with HC antigen binding sites. We examined preB cells from mice with altered CDR-H3 amino acid content. Reducing incorporation of tyrosine into CDR-H3 impaired preB cell development. Furthermore, Y101 and Y102 were enriched in living, but not apoptotic, preB cells. Both tyrosines interact extensively with the VpreB component of the CDR-H3 sensing site, yet also contact antigen in several antibody-antigen complexes. The VpreB CDR-H3 sensing site can thus select for specific CDR-H3 amino acids, potentially shaping the antigen specificity of the primary humoral response.

1160**SRSF1-3 has a role in nuclear localization of AID by regulating its nuclear export**

Kanayama, N., Kawaguchi, Y., Kawamoto, N., Nariki, H., Miyazaki, S., Yokoyama, K., Magari, M., Ohmori, H., Tokumitsu, H.
Okayama University Graduate School of Science and Technology, Department of Medical Bioengineering, Okayama, Japan

Activation induced cytidine deaminase (AID) is an essential factor for somatic hypermutation (SHM) and class switch recombination (CSR) on the immunoglobulin gene. We previously found that serine/arginine-rich protein splicing factor 1-3 (SRSF1-3) is an important factor in for SHM mechanism by using a chicken B cell line DT40, in which SHM occurs spontaneously⁽¹⁾. Here, we examined whether SRSF1-3 contributes to regulating subcellular localization of AID, by confocal microscopic analyses using AID-GFP and mCherry-SRSF1-3 fusion proteins. When AID-GFP was expressed in NIH3T3 cells, AID was observed predominantly in the cytoplasm. However, co-expression

of AID-GFP with mCherry-SRSF1-3 altered this predominant cytoplasmic localization into accumulation in the nucleus. Although C-terminus-truncated SRSF1-3 mutants showed various subcellular localizations, AID was colocalized with SRSF1-3 mutants. Thus, SRSF1-3 might be accompanied with AID in subcellular localization processes. In addition, whereas expression of a C-terminal nuclear export signal-deleted AID enhanced SHM in DT40 cells likely due to increased nuclear localization of the truncated AID, over-expression of SRSF1-3 with the mutant AID did not show any additional increase of SHM. On the other hand, when DT40 cells were treated with a proteasome inhibitor and a nuclear export inhibitor, AID could be observed in the nucleus both in the absence and presence of SRSF1-3, indicating that nuclear import of AID is intact in the SRSF1-3-deficient cells. Taken together, these results suggest that SRSF1-3 promotes nuclear localization of AID by regulating nuclear export of AID.

(1) Kanehiro et al. PNAS 109 (4), 1216-1221 (2012).

1161

TLR9-mediated B cells activation leads to production of brain-derived neurotrophic factor: possible neuroprotective role of B cells following infection

Lou, C.¹, Xu, J.-M.², Zhou, X.-F.¹, Hurtado, P.R.³

¹University of South Australia, School of Pharmacy and Medical Sciences, Adelaide, Australia, ²The Second Xiang-Ya Hospital of Central South University, Department of Anesthesiology, Changsh, China, ³Royal Adelaide Hospital, Renal Medicine, Adelaide, Australia

B cells constitutively express Toll-like receptor 9 (TLR-9), this receptors allows B cells to detect the presence of bacteria infection by recognizing unmethylated CpG sequences in bacteria-derived DNA. The engagement of TLR9 elicits strong activation signals, resulting in B cell proliferation and differentiation into plasma cells as well as the production of cytokines responsible for recruitment and activation of other immune cells and inflammation. Therefore, it would be expected that TLR9 activation should be associated with exacerbation of inflammatory neurodegenerative diseases such as multiple sclerosis, however, several studies has shown that TLR9 activation ameliorate disease progression. Our studies have shown that following TLR9 activation, B cells also produce brain-derived neurotrophic factor (BDNF), in a time and dose dependent manner. This effect only is observed with CpGB but not CpGA. BDNF is a neurotrophic factor best known for its ability to support neuron survival and to promote nerve regeneration. BDNF exerts its function by binding to Tropomyosin receptor kinase B (TrkB). Here, we propose that TLR9 activation of B cells during infections leads to the production of BDNF providing a neuroprotective safeguard against nerve damage that may result from the infection itself or the inflammatory immune response. Consequently, dysfunctions in B cells to produce, process or release BDNF following TLR9 signalling could contribute to the development and progression of neurodegenerative diseases.

1162

The role of NFκB1 in B cell homeostasis and function

Cornish, J.^{1,2}, de Valle, E.^{1,2}, Banerjee, A.³, Grigoriadis, G.³, Ramslund, P.^{2,4}, Gugasyan, R.^{1,2}

¹Monash University Central Clinical School, Immunology, Melbourne, Australia, ²Burnet Institute, Centre for Biomedical Research, Melbourne, Australia, ³Hudson Institute of Medical Research, Centre for Cancer Research, Melbourne, Australia, ⁴School of Science, RMIT University, Bundoora, Australia

NFκB1 (p50/p105) is a member of the NFκB family of transcription factors. Signalling through this pathway regulates numerous target genes, controlling cell survival, proliferation and inflammation. Polymorphisms in the human *Nfkb1* gene are associated with the development of autoimmune disease and haematopoietic malignancies. In a murine model, deletion of *Nfkb1* gives rise to an autoimmune disease in ageing mice, characterised by increased immunoglobulins, pathogenic auto-antibodies and a multi-organ immune cell infiltrate. Our analysis of peripheral B cells in both young and ageing *Nfkb1*^{-/-} mice revealed an increased number of follicular and germinal centre B cells, which coincided with the enhanced differentiation of follicular helper T cells. To our surprise, the *Nfkb1*^{-/-} GC B cells were phenotypically different and failed to form distinct GC structures in the spleen. However, the analysis of BM chimeric mice that consisted of an equal mix of wild-type and *Nfkb1*^{-/-} haematopoietic cells revealed distinct GC structures, suggesting that wild-type immune cells contribute to the formation of GC structures. Furthermore, the analysis of ageing *Nfkb1*^{-/-} mice revealed a striking overexpansion of plasma cells. This suggests that the absence of NFκB1 leads to altered terminal B cell differentiation. Collectively, these findings suggest that NFκB1 prevents the development of autoimmune disease in part by controlling peripheral B cell homeostasis and differentiation.

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Early IL-4-producing B cells regulate T helper cell dichotomy during cutaneous Leishmaniasis in BALB/c mice

Hurdajal, R., Revaz-Breton, M., Parihar, S.P., Govender, M., Brombacher, F.

International Centre for Genetic Engineering & Biotechnology, Cape Town Component and Institute of Infectious Diseases & Molecular Medicine, University of Cape Town, Immunology, Cape Town, South Africa

Immunity to the protozoan parasite *Leishmania major* is dependent on a polarised T helper 1 response whilst interleukin 4 receptor alpha (IL-4Rα)-driven Th2 immunity renders susceptibility to this parasite in BALB/c mice. B cells have also been implicated in disease progression but it is unclear if IL-4Rα-responsive B cells play a significant role. Here we investigated a role for IL-4Rα-responsive B cells in host immunity to cutaneous leishmaniasis. We used a novel BALB/c mouse lacking IL-4Rα expression on B cells (*mb1^{cre}IL-4Rα^{-lox}*), generated by gene targeting and site-specific recombination using the *cre/loxP* system under control of the *mb1* locus. Following subcutaneous infection in the footpad with *L. major* parasites, *mb1^{cre}IL-4Rα^{-lox}* BALB/c mice efficiently controlled cutaneous disease as shown

by significantly reduced footpad swelling and parasite burdens due to a shift to a protective CD4⁺ Th1 and type 1 immune response. In addition, IL-4R α -deficient B cells from resistant *mb1^{cre}IL-4R α ^{-lox}* BALB/c mice transcribed *ifn- γ* as early as day 1 after infection whilst transcription of *il-4* was barely induced in comparison to IL-4R α -responsive B cells from littermate control IL-4R α ^{-lox} BALB/c mice. Lastly, B cell-deficient (μ MT BALB/c) mice only slightly ameliorated fulminant cutaneous disease in comparison to *mb1^{cre}IL-4R α ^{-lox}* BALB/c mice during acute LV39 infection. Altogether our data indicate that while IL-4R α -unresponsive B cells are beneficial in cutaneous leishmaniasis and lead to host protective Th1 immunity in *mb1^{cre}IL-4R α ^{-lox}* BALB/c mice, early IL-4R α -responsive B cells producing IL-4 influence early T helper dichotomy towards detrimental Th2 responses, which leads to *L. major*-induced cutaneous leishmaniasis in BALB/c mice.

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Human memory B cells in clinically healthy gingiva

Mahanonda, R., Sa-Ard-Iam, N., Rerkyen, P., Subbalekha, K., Champaiboon, C.
Chulalongkorn University, Bangkok, Thailand

Large infiltrations of B cells, specifically plasma cells are the hallmark of periodontitis lesions. The role of the B cell response in periodontal health or disease has not been thoroughly studied. In this study, different B cell subsets were identified in human clinically healthy gingiva, gingivitis and periodontitis tissues. Flow cytometry analysis of gingival tissues showed very few CD19⁺CD27⁻CD38⁻ naïve B cells in all clinical groups. CD19⁺CD27⁺CD38⁻ memory B cells represented the majority in the B cell population in clinically healthy gingiva and gingivitis tissues, whereas CD19⁺CD27⁺CD38⁺ antibody secreting cells (ASCs) were the majority in periodontitis tissues. Unlike in gingival tissues, there were no differences in peripheral blood B cell subsets between the healthy or diseased stages. Peripheral blood-naïve B cells represented the majority followed by memory B cells and very few ASCs. Very little is known about memory B cells residing in human non-lymphoid tissues. The anatomical location of this specific population in histological sections was investigated. A cluster of memory B cells in clinically healthy gingiva was detected in the connective tissue subjacent to the apical region of the junctional epithelium. In this vicinity, blood vessels expressed peripheral node addressin (PNAd⁺) and ICAM-1⁺, which may be responsible for recruitment and an appropriate niche for memory B cells. Upon polyclonal B cell activation with TLR7/8 (R848) and IL-2, these healthy gingival tissue-memory B cells were functional and able to be differentiated *in vitro* into ASCs. This suggested a role of memory B cells in maintaining periodontal homeostasis.

1165

B lymphocyte single nucleotide polymorphisms and exomes in transient receptor potential ion channel and acetylcholine receptors in chronic fatigue syndrome

Johnston, S.¹, Marshall-Gradisnik, S.^{1,2}, Chacko, A.^{1,2}, Nguyen, T.^{1,2}, Smith, P.¹, Staines, D.¹

¹Menzies Health Institute Queensland, National Centre for

Neuroimmunology and Emerging Diseases, Parklands, Australia,
²Griffith University, School of Medical Science, Parklands, Australia

Background: Given the important roles in calcium (Ca²⁺) and acetylcholine (ACh) signaling in B cell activation and potential antibody development, the aim was to identify key SNPs and their genotypes from isolated B cells in Chronic Fatigue Syndrome (CFS).

Methods: 11 CFS patients (aged=31.82± 5.50years) defined according to the Fukuda criteria and 11 non-fatigued controls (aged=33.91±5.06 years) were included. Flow cytometric protocols were used to determine B cell purity, followed by SNP and genotype analysis from 21 TRP ion channel genes and 9 ACh receptor genes examined by iPLEX Gold assay. Exome analysis was conducted using Illumina HiSeq platform and SNP association and genotype was determined using ANOVA and PLINK analysis.

Results: Seventy-eight SNPs were associated with nicotinic and muscarinic ACh receptors in the CFS group: 35 were mAChM3, the remaining were nAChR delta, nAChR alpha 9, TRPV2, TRPM3, TRPM4, mAChRM2 and mAChRM5. Nine genotypes were identified from SNPs that were significant for TRPM3, TRPC6, mAChRM3, nAChR α 4 and nAChR β 1 located in intron and 3'untranslated regions in CFS compared with non-fatigued controls (OR 7.11-26.67). Two missense SNPs located on exome 1 for the subunit TRPP, and a synonymous SNP for TRPC3 were also significantly associated with CFS compared with non-fatigued controls.

Conclusions: We identified a number of SNPs and genotypes for TRP ion channels and AChRs from B cells in patients with CFS that may be involved in changes in B cell function and suggest a role for dysregulation of Ca²⁺ in the pathomechanism of CFS.

1166

A critical role for NK cells in exosome-induced CD8⁺ T cell cytotoxicity

Saunderson, S.C., McLellan, A.D.

University of Otago, Department of Microbiology & Immunology, Dunedin, New Zealand

We have previously shown that T cell help via CD40 and the IL-4 receptor induces exosome release from human and mouse B cells. Exosomes are lipid-bound vesicles derived from the endosomal pathway that play a role in cellular communication and immune function. Exosomes act as endogenous adjuvants to enhance T cell proliferative and endogenous cytotoxic lymphocyte (CTL) responses to exosome-associated antigens. However, the mechanisms of exosome-induced immunity are poorly understood. Intravenous immunisation of mice with ovalbumin incorporated into the exosome pathway resulted in significantly greater CTL responses, as compared to molar equivalents of soluble or latex bead-bound ovalbumin. Exosome immunisation greatly increased splenic cellularity, including ovalbumin-specific B cells. Antibody-mediated cell depletions showed that exosome-induced cytotoxicity was absolutely dependent on CD4⁺ T cells, CD8⁺ T cells and NK cells; the loss of any one of these subsets led to a complete loss of CTL activity. Furthermore, this NK cell involvement was independent

of IFN γ and perforin expression. Despite the high level of exosome capture within the marginal zone, and the known ability of MZ B cells to shuttle antigens into the white pulp, B cells were dispensable for exosome-induced cytotoxicity. While there was no clear role for B cells in exosome-induced cytotoxicity, preliminary data suggested a role for circulating immunoglobulin, which may implicate NK cell-expressed Fc and/or C3 receptors in the response to exosomes. We are currently utilising D(H)LMP2A mice that lack immunoglobulin, but retain B cells, to further investigate the role of circulating immunoglobulin in exosome-induced immune responses.

1167

CD28 regulates long lived plasma cell (LLPC) survival through Indoleamine 2,3-dioxygenase (IDO) mediated induction of autophagy in LLPCs

Maharaj, S., Nair, J., Utley, A., Carlson, L., Lee, K.

Roswell Park Cancer Institute, Immunology, Buffalo, United States

Long lived plasma cells (LLPCs) are essential for sustained antibody responses and protective humoral immunity. How these cells maintain longevity and a durable antibody response is largely dependent on the complex nature of the bone marrow microenvironment, in which they reside, and the pro survival factors produced in this niche. It has been published that CD28 signaling and autophagy are required for long lived plasma cell survival. Therefore we wanted to know if CD28 induces autophagy, which could contribute to LLPCs competitiveness within the bone marrow microenvironment. We have observed that there is no direct effect of CD28 signaling intrinsically on autophagy, however we have published that Indoleamine 2,3-dioxygenase (IDO) - which depletes tryptophan - is produced as a result of back signaling from CD28:CD80/86 interactions between plasma cells and bone marrow myeloid derived DCs. IDO is classically known to contribute to an immunosuppressive environment (specifically with respect to T cell activity). Paradoxically, we found that there are fewer LLPCs present in IDO KO mice in comparison to wild type mice. This leads us to a model where CD28, through back signaling to CD80/86 induces IDO production which leads to depletion of tryptophan, an essential amino acid, and this depletion then causes induction of autophagy a mechanism LLPCs use to compete and survive within the bone marrow microenvironment.

1168

Human B-1 and B-2 cells come from a Lin-CD34+CD38^{lo} progenitor population

Quach, T.D., Hopkins, T.J., Holodick, N.E., Rothstein, T.L.

Feinstein Institute for Medical Research, Manhasset, United States

B-1 cell population is an important bridge between innate and adaptive immunity owing in part to their natural antibody production. Murine B-1 and B-2 cells derive from two distinct progenitors. However, the origin of human B-1 and B-2 cells is unknown. To characterize the potential progenitors that delineate differentiation of human B-1 and B-2 cells, we separated cord blood (CB) and bone marrow Lin-CD34+

hematopoietic stem cells (HSC) into Lin-CD34+CD38^{lo} and Lin-CD34+CD38^{hi} populations. We found that only CD34+CD38^{lo} cells generate human CD19+ B cell engraftment after being transferred into immuno-deficient NOD.Cg-Prkdc^{scid} Il2rg^{tm1wjl}/SxJ neonates. Importantly, engrafted CD19+ B cells found in the spleen, bone marrow and peritoneal cavity of humanized mice display phenotypes of B-1 and B-2 cell subsets. Developmental marker analyses of engrafted splenic B-1 cells reveal that B-1 cells have a mature phenotype as evidenced by low-to-intermediate CD24 and CD38 expression levels. Repertoire analysis of engrafted B-1 cells shows a similar VDJ composition to fresh CB B-1 cells, including high usage of Vh4-34 sequences (8% versus 10%, respectively). Furthermore, in patients with hematologic malignancies undergoing HSC transplantation, human B-1-phenotype cells are found in the circulation as early as 8 weeks post-transplantation (and/or as soon as 5 CD19+ B cells are detected). Altogether, our data suggest that unlike mouse B-1 cells, human B-1 and B-2 cells come from the same HSC progenitor cell population, and more precisely from the Lin-CD34+CD38^{lo} cell population, and engrafted B-1 cells in humanized mice are comparable to native B-1 cells in term of Vh usage.

1169

B cells have a key role in aggravated atherosclerosis following myocardial infarction

Kyaw, T.^{1,2}, Loverland, P.¹, Kanellaki, P.¹, Cao, A.¹, Deswaerte, V.¹, Tipping, P.², Toh, B.-H.², Bobik, A.^{1,3}

¹*Baker IDI Heart and Diabetes Institute, Melbourne, Australia,*

²*Faculty of Medicine, Nursing and Health Sciences, Monash University, Centre for Inflammatory Diseases, Department of Medicine, Southern Clinical School, Melbourne, Australia,* ³*Faculty of Medicine, Nursing and Health Sciences, Monash University, Department of Immunology, Central Clinical School,, Melbourne, Australia*

The risk of re-infarction and death following Myocardial infarction (MI) is markedly increased in hyperlipidemic and atherosclerotic patients treated with conventional therapies. Recent mouse studies have shown that MI aggravates atherosclerosis and that B cells are activated following MI; but their role in post-MI aggravated atherosclerosis is unknown.

We found that that MI resulted in a 37% increase in aortic atherosclerosis, compared to sham-operated mice, confirming that MI aggravates atherosclerosis. Increased lesion B cells by 130% in MI-aggravated atherosclerosis suggest a possible role for atherogenic B cells in MI-aggravated atherosclerosis. B cell depletion in MI mice decreased atherosclerosis lesion size by 55% without affecting the extent of MI or cardiac function, compared to MI mice without B cell depletion. The reduced atherosclerosis was accompanied by decreased lipid and immune cell accumulation and reduced VCAM-1 and MCP-1 in atherosclerotic lesions. Serum IgG antibodies specific to oxidised lipid were reduced. A trend of decreased inflammatory TNF- α , IL-1 β and IL-6 mRNA levels was observed.

We then transferred B cells isolated from MI mice into high fat diet-fed mice and found that B cells from MI mice increased lesion size by 48% compared to vehicle control; in contrast, non-

MI B cells failed to increase atherosclerosis.

We conclude that, B cells are highly atherogenic following MI and contribute to accelerated progression of existing atherosclerosis. Our findings suggest B cell depletion in patients with MI as a potential therapeutic strategy to reduce the risk of re-infarction, especially within the first year following the acute event.

1170

Contact hypersensitivity enhanced in mice with B cell-specific-peroxisome proliferator-activated receptor-gamma deficiency

Wang, K.¹, Su, J.¹, Zhou, X.², Zeng, X.¹, Tao, L.¹, Bai, X.², Li, X.¹

¹School of Pharmaceutical Sciences, Southern Medical University, Guangzhou, China, ²School of Basic Medical Sciences, Southern Medical University, Guangzhou, China

Peroxisome proliferator-activated receptor γ (PPAR- γ) is a member of the nuclear hormone receptor superfamily that plays important roles not only in lipid metabolism but also in dampening inflammation by inhibiting pro-inflammatory cytokine production by dendritic cells and T cells. Recently we observed that mice with B cell-specific PPAR- γ -deficiency exhibited increased oxazolone-induced ear swelling, suggesting that the regulatory functions of B cells are dampened. The peripheral CD4⁺CD25⁺Foxp3⁺ regulatory T cell number and CD19⁺CD5⁺CD1d^{hi} regulatory B cell number were not obviously changed in this mice. While the IL-10 producing in splenic CD5⁺CD1d^{hi} regulatory B cells was reduced after activation. At the same time, deficiency of PPAR- γ didn't affect the CD86 expression in splenic CD5⁺CD1d^{hi} regulatory B cells after activation. These suggested the possible role of PPAR- γ involved in the inhibitory function of B cells by disturbing the activation of regulatory B cells.

1171

High-affinity IgM⁺ memory B cells are deficient in terminal differentiation potential to secrete IgM antibodies by restimulation with (4-hydroxy-3-nitrophenyl) acetyl-chicken γ -globulin

Tashiro, Y.^{1,2}, Murakami, A.³, Shimizu, T.⁴, Goitsuka, R.¹, Kishimoto, H.³, Azuma, T.²

¹Research Institute for Biomedical Sciences, Tokyo Univ of Science, Division of Development and Aging, Noda, Chiba, Japan,

²Antibody Technology Research Center, Co., Ltd., Noda, Chiba, Japan, ³University of Ryukyus, Department of Parasitology & Immunopathoetiology, Okinawa, Japan, ⁴Kochi Medical School, Department of Immunology, Kochi, Japan

IgM antibodies are thought to be secreted only by plasma cells differentiated from naive B cells but not by those generated from memory B cells throughout immune response. We found that high-affinity IgM⁺ memory B cells (MBCs) possessing somatic hypermutation (SHM⁺) are generated through germinal center (GC)-dependent pathway (GC-MBCs) in addition to SHM⁻ MBCs generated through GC-independent (non-GC) pathway (non-GC-MBCs). However, the V_H nucleotide

sequences of cells secreting anti-(4-hydroxy-3-nitrophenyl) acetyl (NP) IgM antibodies after secondary immunization had few SHMs, indicating that only non-GC-MBCs have the terminal differentiation potential, although GC-MBCs are maintained throughout immune response. Secondary IgM antibodies bound to several NP analogues, implying that IgM antibodies with broad cross reactivity might be selected at the stage of memory response to deal with reexposure of antigens with a wide range of structures similar to but changed from an original one.

1172

Plasma cell differentiation driven by antigen-independent signaling activity of the IgE B cell receptor

Yang, Z., Allen, C.D.C.

University of California, San Francisco (UCSF), Cardiovascular Research Institute, Department of Anatomy, and Sandler Asthma Basic Research Center, San Francisco, United States

Antigen-engaged B cells may undergo terminal differentiation into plasma cells (PC), the professional antibody-secreting cells, following activation by several extrinsic signals. However, the role of the isotype of the B cell receptor (BCR) in this process is unclear. It was previously reported that B cells expressing IgE, compared with IgG1, showed an increased propensity to differentiate into PCs. We sought to determine whether the BCR isotype has an intrinsic effect on PC differentiation. By ectopic expression of BCRs of different isotypes in primary B cells *in vitro*, we revealed that particular isotypes promoted PC differentiation to distinct extent in the absence of cognate antigen, whereas most isotypes mediated similar levels of PC differentiation in the presence of cognate antigen. B cells expressing IgE exhibited the highest propensity of PC differentiation in the absence of cognate antigen, correlating with *in vivo* observations of IgE⁺ B cells. The frequency of IgE⁺ PCs was reduced when B cells were treated with inhibitors of BCR signaling in the absence of antigen, further suggesting that the IgE BCR has antigen-independent signaling activity that can drive PC differentiation. By domain-swap experiments, we have determined contributions of different IgE domains in driving PC differentiation. In summary, we have identified that the IgE BCR drives antigen-independent PC differentiation, suggesting that the BCR isotype modulates cell fate in an intrinsic manner. Our study may allow a better understanding of the biology of IgE⁺ B cells and the BCR signals mediating PC differentiation.

Cell Trafficking

1173

Increased recruitment of human lymphocyte subsets in renal fibrosis and chronic kidney disease

Kassianos, A.^{1,2,3,4}, Lo, B.^{1,2,3}, Lindner, M.^{1,2,4}, Wang, X.^{1,2}, Beagley, K.³, Wilkinson, R.^{1,2,3,4}, Healy, H.^{1,2}

¹Pathology Queensland, Brisbane, Australia, ²Royal Brisbane and Women's Hospital, Brisbane, Australia, ³Queensland University of Technology, Brisbane, Australia, ⁴University of Queensland, Brisbane, Australia

Lymphocytes play pivotal roles in immune-mediated kidney diseases. However, the respective contributions of different lymphocyte subsets in diseased human kidneys are not certain, with previous studies limited by the methodology of immunohistochemistry in identifying infiltrating cells. In this study, we developed novel protocols for extracting renal lymphocytes from healthy kidney tissue and diseased biopsies with and without fibrosis. Lymphocyte subsets were identified, enumerated and phenotyped by twelve-colour flow cytometry. We detected significantly elevated numbers of total T cells (CD45⁺CD3⁺), consisting of increased T helper cells (CD3⁺CD4⁺), cytotoxic T cells (CD3⁺CD8⁺), γ/δ T cells (CD3⁺ γ/δ ⁺) and natural killer (NK)-T cells (CD3⁺CD19⁺), in diseased biopsies with interstitial fibrosis compared with diseased biopsies without fibrosis and healthy kidney tissue. Of CD3⁺ lymphoid cells, numbers of B cells (CD3⁺CD19⁺) and NK cells (CD3⁺CD56⁺), in particular CD56^{bright} NK cells, the major cytokine-producing NK subtype in human peripheral tissues, were also significantly elevated in fibrotic kidney tissue. The increased numbers of γ/δ T cells, NK-T and CD56^{bright} NK cells correlated significantly with loss of kidney function (based on eGFR levels). Furthermore, the expression of activation molecule CD69 on CD56^{bright} NK cells was significantly increased in fibrotic biopsies compared with non-fibrotic kidney tissue, indicative of a pathogenic phenotype. Collectively, our data show that lymphocyte subsets are differentially recruited into diseased human kidneys. The representation of specific lymphocyte subsets also correlates with the clinical severity of chronic kidney disease. Further identification and functional dissection of these lymphocyte subsets are necessary for the development of targeted anti-inflammatory treatment strategies.

1174

The atypical chemokine receptor ACKR4 regulates dermal chemokine bioavailability and dendritic cell egress

Bastow, C.¹, Bunting, M.², Kara, E.¹, McColl, S.¹, Comerford, I.¹

¹University of Adelaide, Molecular and Cellular Biology, Adelaide, Australia, ²Queensland Institute of Medical Research, Brisbane, Australia

Directional migration of cells toward chemokine gradients underpins a multitude of processes required for the initiation, differentiation and effector phases of adaptive immune responses. In skin, a rising gradient of immobilized CCL21 produced by lymphatic endothelial cells guides CCR7-dependent haptotaxis of mature dendritic cells into afferent lymphatic vessels, facilitating the priming of immune responses in draining lymph nodes. This CCL21 gradient must be intact to permit efficient dendritic cell homing, however the molecular mechanisms that maintain chemokine gradients *in vivo* are not well understood. Whilst it is widely thought that immobilized chemokine gradients are established and controlled by chemokine diffusion and distribution of chemokine binding sites, here we identify a novel role for the atypical chemokine receptor 4 (ACKR4) in maintaining the abundance of interstitial CCR7 ligands: CCL19 and CCL21. ACKR4-deficient mice displayed a greater abundance of dermal CCL19 and CCL21 that

was dependent on radio-resistant ACKR4 expression. Within the skin, ACKR4 mRNA and scavenging function was limited to dermal fibroblasts. Ligand hyperabundance in the absence of ACKR4 impeded CCR7-dependent homing of mature dendritic cells to lymphatic vessels, resulting in dendritic cell retention in the skin. These findings identify a novel mechanism that regulates immobilized chemokine availability in peripheral sites, consistent with the ligand scavenging role of atypical chemokine receptors, and emphasises the physiological importance of chemokine sequestration by ACKR4.

1175

Effector T helper cell recruitment to inflammatory sites via CCR2

Gregor, C., Kara, E., Comerford, I., McColl, S.

University of Adelaide, Department of Molecular and Cellular Biology, Adelaide, Australia

Migration of CD4⁺ T helper (Th) cells to extralymphoid sites of inflammation is pivotal for execution of their effector function. Differentiation of distinct Th cell subsets is coupled with induction of subset-specific and tissue-tropic chemokine receptors that drive their recruitment into diverse inflammatory lesions. However, increasing evidence indicates that trafficking of Th cell subsets to inflammatory sites can occur independently of subset-specific chemokine receptors. Thus, coordinated migration is likely to involve multiple integrated receptor inputs that are spatiotemporally regulated, but knowledge of this complexity in migration is limited. Recently, we demonstrated that homing of IL-23-driven pathogenic Th17 cells to the central nervous system was coordinated through the chemokine receptor CCR2 in experimental autoimmune encephalomyelitis, a murine model of the human autoimmune disease multiple sclerosis. Our more recent data indicates that functional CCR2 is induced on various effector Th cell subsets under disparate inflammatory settings. CCR2 ligands are not present at homeostasis, but are rapidly elicited by numerous cell types in response to pan-inflammatory stimuli including IL-1 and TNF. Here, we present evidence that CCR2 serves as a pan-inflammatory chemokine receptor driving Th cell trafficking to effector sites. Understanding how CCR2 functions in collaboration with subset-specific and tissue-tropic receptors to coordinate Th cell trafficking will inform the rational design of therapeutic strategies to intervene in this process in Th cell-driven pathology.

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Intravascular patrolling monocytes initiate CD4⁺ T cell-mediated inflammation in the glomerular microvasculature

Westhorpe, C.¹, Hall, P.¹, Li, A.¹, Finsterbusch, M.¹, Snelgrove, S.¹, Lo, C.², Kitching, A.R.^{1,3}, Hickey, M.¹

¹Monash University, Centre for Inflammatory Diseases, Clayton, Australia, ²Monash University, Monash Micro Imaging, Clayton, Australia, ³Monash Health, Departments of Nephrology and Paediatric Nephrology, Clayton, Australia

Autoimmune glomerulonephritis is a leading cause of end-stage renal failure. Effector CD4⁺ T cells can rapidly induce glomerular

inflammation in an antigen-dependent manner. However it is unclear how these cells recognise antigens within the glomerular microvasculature. The aim of this study was to investigate the mechanisms by which effector CD4+ T cells initiate glomerular inflammation. Intravital multiphoton microscopy of kidneys of MHCII-eGFP mice revealed that in the absence of inflammation, intraglomerular expression of MHCII was restricted to circulating leukocytes. Following transfer of activated OT-II (CD4+) T cells, both intravascular MHCII+ leukocytes and effector CD4+ T cells underwent periods of retention and migration in the glomerular capillaries and regularly interacted with each other. To induce antigen-dependent glomerular inflammation, the OT-II peptide pOVA₃₂₃₋₃₃₉ was deposited in glomeruli via conjugation to 8D1, a mAb specific for the glomerular basement membrane. Mice administered the 8D1/pOVA conjugate displayed increased retention of activated CD4+ T cells within 2 hours, indicating local antigen recognition. Of the circulating MHCII-expressing leukocytes, both B cells and monocytes underwent retention in the glomerular capillaries, although MHCII+ monocytes were retained in the glomerulus for significantly longer periods. Furthermore, while the absence of B cells did not affect CD4+ T cell-dependent inflammation, monocyte depletion significantly reduced this response. These data indicate that intravascular MHCII+ patrolling monocytes initiate CD4+ T cell responses in the glomerular microcirculation and this can result in glomerular inflammation.

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Investigating the role of invariant natural killer T cells in leukocyte recruitment following acute murine colitis

Shen, S.¹, Hickey, M.², Wong, C.²

¹Monash University, Medicine, Melbourne, Australia, ²Monash University, Melbourne, Australia

Inflammatory bowel disease (IBD) is a group of idiopathic, chronic, and relapsing inflammatory disease of the gut. The immunopathology of IBD involves decreased epithelial integrity, increased bacterial translocation, and elevated recruitment of neutrophils. However, it is still unknown what the precise roles of neutrophils and invariant natural killer T (iNKT) iNKT cells play in this disease. Therefore, this study aims to investigate the contributions of iNKT cells and neutrophils in a mouse model of dextran sodium sulphate (DSS)-induced colitis. Eight to ten weeks old male wildtype (WT) and *Ja18^{-/-}* were administered with 2% DSS in drinking water for a maximum of 7 days to induce acute colitis. Body weight, clinical and histopathological scores were assessed. Flow cytometry of immune cell populations were performed and intravital microscopy of the colon was conducted at different stages of disease to assess for neutrophil trafficking and leukocyte behaviour dynamics. Despite the reduced weight loss and improved clinical score in mice deficient of iNKT cells following DSS-induced colitis, we found no differences in colon length and histological score between DSS-treated WT and *Ja18^{-/-}* mice. There was a significant infiltration of neutrophils into the colon of WT mice at day 7 of DSS treatment, and this increase was exacerbated in *Ja18^{-/-}* mice. In contrast, the proportion of T cells was significantly reduced in DSS-treated *Ja18^{-/-}* mice compared to their wildtype counterparts. Therefore, our data

suggest iNKT cells play a modulatory role in the recruitment of leukocytes following a mouse model of acute colitis.

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GPR65 regulates neutrophil chemotaxis

McKenzie, C.¹, Tan, J.¹, Mason, L.¹, Mackay, C.¹, Macia, L.²

¹Monash University, Department of Biochemistry and Molecular Biology, Melbourne, Australia, ²University of Sydney, Charles Perkins Centre, Sydney, Australia

Inflammatory bowel disease (IBD) is a debilitating illness with a global incidence of approximately 4 million people. It is characterized by chronic gut inflammation resulting in severe diarrhoea and abdominal pain. This inflammation is due to the migration of activated neutrophils in the intestinal wall resulting in tissue damage. While the triggers of this inflammation are unknown, single-nucleotide polymorphisms in the G-protein coupled receptor 65 (GPR65) gene have been associated with IBD. GPR65 is expressed on neutrophils and is a proton-sensing receptor activated by low pH in sites of inflammation. The aim of this study was to determine the functional role of GPR65 in neutrophil migration and the pathogenesis of colitis. Colitis development was assessed in *Gpr65^{-/-}* mice versus wild-type mice treated with dextran sulphate sodium (DSS). Daily monitoring of diarrhoea and histological analysis of colonic sections demonstrated exacerbated colitis development in *Gpr65^{-/-}* mice as well as more of infiltration of neutrophils in the colon. An *in vitro* chemotaxis assay towards IL-8 demonstrated increased migration of *Gpr65^{-/-}* neutrophils under both basal and acidic conditions. To confirm the enhanced migratory phenotype of *Gpr65^{-/-}* neutrophils *in vivo*, we conducted a neutrophil-dependent KRN serum-transfer model of arthritis on *Gpr65^{-/-}* mice. Swelling, redness and neutrophil infiltration of the joints were increased in arthritic *Gpr65^{-/-}* mice. Taken together, our results suggest GPR65 regulates neutrophil chemotaxis. Single-nucleotide polymorphisms in *Gpr65* associated with IBD may therefore enhance neutrophil migration to the gut and promote IBD pathogenesis.

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The role of heparanase in NK cell migration and NK cell-mediated tumour surveillance

Putz, E.M.¹, Souza-Fonseca-Guimaraes, F.^{1,2}, Barkauskas, D.S.¹, Town, L.¹, Hulett, M.D.³, Smyth, M.J.¹

¹QIMR Berghofer Medical Research Institute, Immunology in Cancer and Infection, Herston, Australia, ²University of Queensland, School of Medicine, St. Lucia, Australia, ³La Trobe University, Department of Biochemistry, Melbourne, Australia

Natural Killer (NK) cells belong to the family of innate lymphoid cells, and they are known for their ability to spontaneously kill malignant cells. It is a long-standing paradigm that NK cells are highly efficient in the prevention of cancer metastasis; however they are less potent against primary tumours and are hardly found within a tumour mass. Heparanase is the only known mammalian glycosidase responsible for the cleavage of heparan sulfate proteoglycans, a major component of the extracellular

matrix. Heparanase is frequently upregulated in tumour cells and its expression correlates with metastatic potential. So far the impact of heparanase in NK cells has not been investigated. Our preliminary data show that the expression of heparanase is highly upregulated after NK cell activation *in vitro*. The NK cell-specific loss of heparanase in B6.*Hpse*^{fl/fl} NKp46-iCre/wt mice does not affect the development and maturation of NK cells. However, preliminary experiments show that NK cell invasion of matrigel plugs *in vivo* and the surveillance of B16F10 metastasis is impaired in the absence of heparanase. Further investigations will address the questions of (i) whether the loss of heparanase will have an impact on the surveillance of solid tumours and (ii) how the expression of heparanase could be driven to improve the NK cell infiltration of tumours. We hypothesize that triggering the expression of heparanase will allow NK cells to infiltrate and attack tumours. Augmenting the invasive potential of NK cells might constitute a promising novel way to improve and complement the surveillance of primary tumours.

1180

The *Leishmania* pathogenicity factors GP63 and LPG traffic in vesicular structures via a Sec22b-mediated pathway

Arango Duque, G.¹, Jardim, A.², Desjardins, M.³, Descoteaux, A.¹

¹INRS-Institut Armand-Frappier and Centre for Host-Parasite Interactions, Laval, Canada, ²Institute of Parasitology and Centre for Host-Parasite Interactions, McGill, Ste. Anne de Bellevue, Canada, ³Département de Microbiologie et Immunologie, IRCM, Université de Montréal, Montreal, Canada

Leishmania parasites conquer their hosts by sabotaging phagocytosis. Phagosomes become microbicidal via membrane exchanges that are mediated by soluble NSF attachment protein receptors (SNAREs), with organelles such as the ER-Golgi intermediate compartment (ERGIC). *Leishmania* uses pathogenicity factors such as the GP63 protease and lipophosphoglycan (LPG) to cleave host proteins involved in immunity and retard phagosome maturation, respectively. How GP63 and LPG traffic from the phagosome to the cytoplasm remained unknown. We hypothesized that these molecules are redistributed in the cytoplasm of infected cells in vesicles whose trafficking is mediated by host organelles. Using confocal microscopy, we demonstrated that GP63 and LPG were found in vesicles dispersed in the cytoplasm of infected phagocytes. Flotation assays showed that GP63 and LPG localize to low-density fractions containing vesicles, and to denser fractions enriched in ER/ERGIC proteins. We observed via immunofluorescence that these markers also colocalized with GP63 and LPG. Interestingly, Brefeldin A- and Monensin-mediated disruption of ER-Golgi transport hindered the redistribution of GP63 and LPG, as well as the cleavage of Syt XI, a GP63 substrate. This prompted us to study the role of the SNARE Sec22b - which regulates ER-Golgi transport - on the redistribution of these parasite molecules. In infected cells transduced with shRNA to Sec22b, the trafficking of GP63 and LPG was hampered. Moreover, this ensued in lessened cleavage of Syt XI. Together, our data revealed a novel mechanism by which an intracellular pathogen hijacks membrane fusion regulators in the ER/ERGIC to promote the trafficking of the pathogen's pathogenicity factors.

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Multifaceted regulation of immune responses by the atypical chemokine receptors ACKR4 and ACKR2

Comerford, I.¹, Bastow, C.¹, Kara, E.¹, Gregor, C.¹, Foeng, J.¹, Harata-Lee, Y.¹, Bunting, M.², McColl, S.¹

¹University of Adelaide, Molecular and Cellular Biology, Adelaide, Australia, ²Queensland Institute of Medical Research, Brisbane, Australia

Chemokines direct immune cell migration through cognate interactions with chemokine receptors expressed on distinct leukocyte subsets. Recently, members of a subfamily of atypical chemokine receptors that control chemokine distribution and bioavailability rather than mediate cell migration have been identified. We have used knock-out, knock-down and conditional transgenic approaches to explore the function of ACKR4 and ACKR2, two of the members of this subfamily of atypical chemokine receptors. Our data reveal various important roles for ACKR4 in many aspects of immune system function including immune system development and homeostasis, cell migration during inflammation, responses to infection, autoimmunity and in anti-tumour immunity. ACKR4 operates, at least in part, by removing the chemokine CCL21 from tissue micro-environments to establish optimal extracellular chemokine gradients *in vivo*, an effect that is mediated by its expression on tissue stromal cells. However, we also reveal that expression of ACKR4 or ACKR2 by lymphocytes can directly compete with classical signalling chemokine receptors expressed on the same cell to negatively regulate cell migration. Together these data point toward a complex system of regulation of the chemokine system exerted by atypical chemokine receptors that is relevant to numerous aspects of immune system function in a variety of pathological scenarios.

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CTL centrosomes dock with the immune synapse and direct lytic protein secretion during killing

Stinchcombe, J., Randzavola, L., Griffiths, G.

University of Cambridge, Cambridge Institute for Medical Research, Cambridge, United Kingdom

Cytotoxic T-Lymphocytes (CTL) kill by releasing granzymes and perforin which induce apoptosis. Release is controlled to maximise the 'lytic hit' and prevent healthy cell death. Lytic proteins are stored in lysosomes and released only on target contact, at the secretory domain of the immune synapse which forms at the contact site. We previously showed CTL centrosome polarisation directs lysosome to the contact site by aligning microtubules to run under secretory sites. Centrosome polarisation is unusual but found in ciliated cells. Haemopoietic cells do not make cilia. Therefore CTL could use similar mechanisms during killing.

We used high resolution TEM tomography and siRNA knockdown to investigate centrosome behaviour during killing. We find, like cilia, CTL centrosomes dock with membranes via mother centriole distal appendages. Docking occurs at specific sites at the immune synapse, often at membrane bumps. CTL can contain multiple centrosomes and duplicating centrioles,

but this does not affect centrosome docking. Unlike cilia, CTL centriole membrane association is transient and docked centrosomes retain the regulatory CP110/Cep97 complex and do not extend axonemes. CTL killing therefore resembles early but not late ciliogenesis. Failure to progress into ciliogenesis is probably important for killing multiple targets. Importantly, we find depletion of distal appendage protein Cep83 reduces CTL lysosome degranulation, supporting the role of centrosome docking in lysosome delivery.

CTL centrosome polarisation therefore provides fast directed delivery of the killing machinery towards a target, and rapid repolarisation allowing serial killing.

1183

The role of vascular adhesion protein (VAP)-1 in hepatic leukocyte recruitment during acute liver injury

Tickle, J., Shepherd, E., Adams, D., Weston, C., David Adams and Christopher Weston

University of Birmingham, Institute of Immunology and Immunotherapy, Birmingham, United Kingdom

Introduction: Vascular adhesion protein (VAP)-1 is expressed within the liver where it is thought to modulate inflammation and fibrosis. The catalytic activity of VAP-1 has been associated with immune trafficking. We sought to define the role of VAP-1 during acute inflammatory liver disease and to determine the importance of its enzymatic activity in this process.

Materials and methods: VAP-1 expression in patient tissue was assessed by immunohistochemistry. Catalytic activity was quantified by a novel Amplex UltraRed assay. Leukocyte adhesion to primary human hepatic sinusoidal endothelial cells or recombinant protein was assayed by flow-based assay and confocal microscopy. SSAO-KO mice, which possess a catalytically inactive form of VAP-1, were used to assess importance of VAP-1 in leukocyte recruitment during acute CCl₄ injury.

Results: Although the expression of VAP-1 was altered in patients with acute liver disease the enzyme had impaired catalytic activity. Adhesion assays demonstrated an important role for VAP-1 in neutrophil recruitment and the formation of neutrophil extracellular traps, suggesting that impairment of VAP-1 function may impact on disease severity. Acute CCl₄-induced hepatic injury in mice confirmed an important role for the enzyme activity of VAP-1 in leukocyte recruitment. Confocal microscopy of HSEC transfected with GFP-tagged VAP-1 protein (wild-type and catalytically inactive variant) revealed an intimate association between endothelial VAP-1 and leukocytes in an inflammatory environment.

Conclusions: Human VAP-1 is upregulated in acute inflammatory liver disease where it plays an important role in leukocyte extravasation. The enzymatic activity of VAP-1 is likely to play a key role in this process.

1184

CCR2 upregulated on T-cell populations in peripheral circulation among patients with osteoarthritis compared to bone marrow and to healthy control

Arkestål, K.¹, Enocson, A.², Linton, L.¹, Marits, P.¹, Eberhartsson, M.¹, Glise, H.¹, Andersson, J.¹, Winqvist, O.¹

¹Karolinska Institute, Translational Immunology unit (MED S), Stockholm, Sweden, ²Karolinska Institute and Karolinska University Hospital, Department of Clinical Sciences and Education, Section of Orthopaedics, Södersjukhuset, Stockholm, Sweden

A large portion of lymphocytes reside in bone marrow and early development of all leukocytes occurs in this locality. Although being the main primary lymphoid organ and although many questions on leukocyte maturation remain unanswered, relatively few studies have been conducted on bone marrow material if compared to peripheral blood. We have devised a protocol for extracting seemingly healthy bone marrow from patients undergoing orthopaedic surgery in large quantities without additional discomfort for the patients. Chemokine receptors reveal migratory characteristics of lymphoid cells and can also contribute in phenotyping cell populations into functional subcategories. This study show a difference in chemokine receptor 2 expression on T cell populations in peripheral blood among osteoarthritis patients compared with healthy controls which could prove useful as a screening tool for osteoarthritis patients. The difference in chemokine receptor 2 expression between peripheral circulation and bone marrow also suggest a mechanism for T-cell bone marrow egression.

1185

Tetraspanin CD53 regulates L-selectin shedding and promotes leukocyte transmigration

Yeung, L.^{1,2}, Demaria, M.², Wee, J.², Anderson, J.¹, Wright, M.D.², Hickey, M.J.¹

¹Monash University, Centre for Inflammatory Diseases, Melbourne, Australia, ²Monash University Central Clinical School, Immunology, Melbourne, Australia

Leukocyte recruitment requires the actions of adhesion molecules and their associated ligands. Tetraspanins are a superfamily of transmembrane proteins that promote the formation of tetraspanin-enriched microdomains (TEMs) in which molecules such as leukocyte integrins form associations with a range of other proteins. The leukocyte-expressed tetraspanin CD53 has recently been identified as a potential regulator of leukocyte recruitment. While the mechanism by which CD53 accomplishes this remains unknown, unpublished data suggest that it may occur through the regulation of L-selectin expression. Using mice deficient in CD53, it was observed that CD53-deficient T cells displayed an accelerated rate of L-selectin shedding following PMA activation, compared to wild type mice. Similarly, levels of L-selectin were reduced in neutrophils from CD53^{-/-} mice. Upon leukocyte activation, L-selectin is cleaved by the matrix metalloprotease (MMP) ADAM17, and congruent with this, the increased L-selectin shedding from CD53^{-/-} T cells could be partially rescued via treatment with the general MMP inhibitor GM6001. Furthermore, intravital microscopy analysis of chemokine-induced leukocyte recruitment in the cremaster

muscle demonstrated that transmigration of both neutrophils and monocytes was significantly inhibited in CD53^{-/-} mice. This observation was corroborated by confocal imaging of fixed cremaster muscles, where leukocytes of CD53^{-/-} mice were unable to fully transmigrate into surrounding tissues, instead, aggregating in the vascular lumen and within the vessel wall. This work indicates that the absence of CD53 results in impaired chemokine-induced leukocyte recruitment *in vivo*, and that this impairment may be a result of increased loss of L-selectin through uncontrolled cleavage by ADAM17.

1186

Tetraspanins CD37 and CD53 regulate successive stages of the inflammatory cascade

Wee, J.¹, Demaria, M.¹, Yeung, L.², Anderson, J.², Hammerling, G.³, Wright, M.¹, Hickey, M.²

¹Monash, Immunology, Melbourne, Australia, ²Monash, Centre for Inflammatory Diseases, Dept of Medicine, Clayton, Australia,

³German Cancer Research Center, Molecular Immunology, Heidelberg, Germany

Molecules that regulate leukocyte trafficking to sites of inflammation or homing to secondary lymphoid organs are crucial for an effective immune response. In particular adhesion molecules such as selectins and integrins play a critical role in mediating the molecular recognition and adhesion events that occur between leukocytes and the vascular endothelium in the classical inflammatory cascade. Here we will present evidence that CD37 and CD53 are two tetraspanins critical for the inflammatory cascade. Intravital microscopic analyses of CD37^{-/-} mice show an impairment in leukocyte (both neutrophils and monocytes) recruitment, as CD37 is critical for the β 2 integrin-mediated stable adhesion that occurs between leukocytes and the endothelium. CD37 is critical both for regulating β 2-integrin internalization, and also cytoskeletal-dependent processes such as neutrophil spreading and polarization. Similarly, leukocyte recruitment is also impaired in CD53^{-/-} mice; but, by contrast, CD53 has no discernable effect on integrin function but is critical for the function and expression of L-selectin. Consequently CD53^{-/-} leukocytes show no impairment in stable adhesion, but in both rolling along the endothelium, and in subsequent transmigration. Lymphocyte homing to peripheral lymph nodes is dramatically reduced in CD53^{-/-} mice, leading to a delay in adaptive immune responses. The role of CD37 and CD53 in leukocyte recruitment in inflammatory disorders will be discussed.

1187

Monocytes contribute to neutrophil-dependent kidney injury in acute glomerulonephritis

Finsterbusch, M.¹, Hall, P.¹, Li, A.¹, Kitching, R.A.^{1,2}, Hickey, M.J.¹

¹Monash University, Centre for Inflammatory Disease, Department of Medicine, Melbourne, Australia, ²Monash Health, Departments of Nephrology and Paediatric Nephrology, Clayton, Australia

Glomerulonephritis is a leading cause of end-stage renal failure, a condition characterised by injurious inflammation of glomeruli.

We recently showed that monocytes, similar to neutrophils, constitutively migrate within glomerular capillaries. The aim of this study was to investigate the contribution of monocytes to the pathology of acute glomerulonephritis. Using multiphoton and spinning disk confocal intravital microscopy, leukocyte behaviour was examined in the mouse kidney in a model of acute glomerulonephritis (using anti-glomerular basement membrane (GBM) antibodies). In this model, monocyte depletion reduced renal injury as assessed by albuminuria. Neutrophil recruitment, dwell time and the number of reactive oxygen species (ROS)-producing neutrophils were also diminished following monocyte depletion, suggesting a role for intercellular cross-talk between monocytes and neutrophils in mediating glomerular injury. Consistent with this, monocytes and neutrophils were seen to undergo cell-cell interactions in the glomerular microvasculature, and these interactions were prolonged during anti-GBM Ab-induced inflammation. Notably, neutrophils that underwent interactions with monocytes showed longer dwell times and were more likely to produce ROS relative to non-interacting neutrophils. In addition, renal monocytes, but not neutrophils, displayed TNF production upon inflammation as detected via flow cytometry, and TNF inhibition reduced neutrophil dwell time and ROS production, and renal injury. Together, our data indicate an important role for monocytes in mediating neutrophil recruitment, activation and triggering harmful neutrophil-dependent tissue damage. Moreover, evidence indicates that this response is associated with interactions between monocytes and neutrophils and promoted by monocyte-derived TNF.

1188

Plasmin and regulators of plasmin activity modulate the presentation and function of the homeostatic chemokine CCL21

Birch, N.P.^{1,2}, Lorenz, N.^{1,3}, Loef, E.J.^{1,3}, Kelch, I.D.^{1,3}, Verdon, D.J.^{1,3}, Black, M.M.¹, Middleditch, M.J.^{1,4}, Greenwood, D.R.^{1,3}, Graham, E.S.⁵, Brooks, A.E.S.^{1,3}, Dunbar, P.R.^{1,3}

¹University of Auckland, School of Biological Sciences, Auckland, New Zealand, ²University of Auckland, Centre for Brain Research, University of Auckland and Brain Research New Zealand, Rangahau Roro Aotearoa, Auckland, New Zealand, ³University of Auckland, Maurice Wilkins Centre for Molecular Biodiscovery, Auckland, New Zealand, ⁴University of Auckland, Auckland Science Analytical Services, Auckland, New Zealand, ⁵University of Auckland, School of Medical Sciences, Auckland, New Zealand

The homeostatic chemokine CCL21 playing a pivotal role in lymphocyte homing and compartment localization within the lymph node. CCL21's effects are modulated by its mode of presentation, with different cellular responses observed for surface-bound and soluble forms. In this study we report that the plasmin cleaves surface-bound CCL21 to release the C-terminal peptide responsible for CCL21 binding to cell surfaces and extracellular matrix, thereby generating the soluble form. Loss of this anchoring peptide enabled the chemotactic activity of CCL21 and reduced cell tethering. The plasmin-specific inhibitor α_2 -antiplasmin inhibits the migration of human T cells and DCs towards CCL21. We show that plasmin cleaves CCL21

in a concentration- and time-dependent manner to release the carboxyl terminus anchoring peptide. Plasmin is cleaved from the zymogen plasminogen by the enzyme tissue plasminogen activator (tPA). We found that tPA enhanced CCL21 processing through cleavage of plasminogen to plasmin. We also show that the tPA inhibitor neuroserpin blocked T cell and DC processing of CCL21 and partially inhibited cell migration. Finally, we show that plasmin reduced the tethering of T cells to DCs and this effect was blocked by α_2 -antiplasmin. These studies reveal a cellular mechanism where modified presentation of CCL21 by the plasmin system proteins plasmin, tPA, α_2 -antiplasmin and neuroserpin regulate CCL21 function.

1189

B-lymphocyte trafficking in human lymph nodes: CXCL13 does not derive from follicular dendritic cells

Park, S.M.^{1,2}, McIntosh, J.D.^{1,2}, Sheppard, H.M.^{1,2}, Brooks, A.E.S.^{1,2}, Chen, C.-J.J.¹, Cebon, J.³, Dunbar, P.R.^{1,2}

¹University of Auckland, School of Biological Sciences, Auckland, New Zealand, ²Maurice Wilkins Centre, Auckland, New Zealand,

³Olivia Newton-John Cancer Research Institute, Melbourne, Australia

During their transit through lymph nodes (LNs), B cells migrate through different microenvironments where they encounter antigen, become activated, and differentiate into plasma cells and memory B cells. Studies in mice have demonstrated that each stage of B cell trafficking is elegantly coordinated by migration cues from stromal cell populations, collectively ensuring efficient immune surveillance and generation of humoral responses. Compared with the extensive knowledge derived from murine models, regulation of B cell trafficking in human LNs remains relatively unexplored. We have examined the cells and molecules involved in controlling B cell trafficking in human LNs using 4-colour immunofluorescence microscopy, flow cytometry and *in vitro* functional assays. Our data reveal several important findings. Both the blood and lymphatic vasculature that enable ingress and egress of B cells are positioned very close to B cell follicles, potentially rendering trafficking through T cell zones unnecessary. Dynamic changes in CXCR5 expression support a crucial role for CXCL13 in driving B cell migration, but the main cell population producing CXCL13 in human LNs is not Follicular Dendritic Cells as reported in mice. Our results also point to an essential role of CD68+ antigen presenting cells (APCs) in maintaining a sphingosine 1-phosphate (S1P) gradient required for B cell egress from the LN. Collectively, these findings extend knowledge gained from murine models and highlight potentially unique features of B cell trafficking in human LNs.

1190

Trafficking of E7 specific CD8 T cells from the host to hyperproliferative graft

Jazayeri, S.D., Frazer, I.H.

University of Queensland, Diamantina Institute, Brisbane, Australia

Expression of Human Papilloma Virus (HPV) type 16 E7 in murine epidermis from a keratin 14 promoter causes epithelial

hyperplasia and chronic inflammation, characteristic of premalignant lesions associated with HPV infection in humans, and is characterised by an infiltrate of CD8+ve T cells. Further, E7-expressing epidermis is not rejected when transplanted onto immune competent mice. In this study we aimed to determine whether E7 specific CD8 T cells migrate to E7 transgenic grafts. To address this issue, ear skin of E7 transgenic and non transgenic control mice was transplanted to Nzeg-GFP mice, which have a normal T cell repertoire expressing green fluorescent protein (GFP), and E7 TCR269xNzeg-GFP mice, in which >10% of CD8 T cells are E7 peptide specific and express GFP. The number of migrated GFP positive T cells were determined post grafting in E7 transgenic and non transgenic grafts. Many more CD8 T cells migrated to E7 transgenic than to non transgenic grafts. However, there was no excess of graft recipient derived T cells in E7 transgenic grafts on E7 TCR transgenic mice when compared with E7 grafts on mice with a normal T cell repertoire. We conclude that E7 transgenic skin attracts T cells non specifically, and that there is no significant migration of E7 specific T cells to E7 transgenic skin grafts.

1191

Beneficial and detrimental effects of VLA-4 and LFA-1 blockade on T cell and monocyte infiltration of the CNS and clinical severity of Experimental Autoimmune Encephalitis

Issekutz, T.

Dalhousie University, Pediatrics, Halifax, Canada

Autoreactive T cell migration into the CNS is critical to induce disease in MS and EAE. Studies have shown that blocking VLA-4 inhibits T cell accumulation in the CNS and reduces clinical disease in animal models and in humans with MS. Anti-VLA-4 treatment of rats with EAE abolished spleen T cell infiltration into the CNS, and almost completely prevented CNS disease. Interestingly, VLA-4 blockade also prevented the migration of monocytes into the inflamed CNS despite the expression of LFA-1 and Mac-1 on these cells, and had no effect on infiltration in dermal inflammation which was highly dependent on CD11a and CD11b in these animals. Neutrophil migration to the CNS in EAE was minimal and not affected by VLA-4 or anti-CD18 mAbs which abolished neutrophil accumulation in dermal inflammation. Blockade of LFA-1 reduced T cell migration to the CNS by 40 - 50% but treatment with anti-LFA-1 significantly increased the clinical severity of EAE. Thus, monocytes appear to require VLA-4 for migration to the inflamed CNS in EAE and $\alpha 4$ integrin blockade in MS may have part of its beneficial effects through inhibiting monocyte infiltration. Blocking LFA-1 may inhibit T regulatory cell accumulation thereby increasing the severity of the CNS inflammation.

1192

S1P gradients position NK cells in lymph nodes and regulate the early IFN γ response to infection*Fang, V.¹, Ramos-Perez, W.D.¹, Chaluvadi, V.S.¹, Mendoza, A.¹, Chun, J.², Cammer, M.¹, Schwab, S.R.¹*¹*NYU School of Medicine, Skirball Institute of Biomolecular Medicine, New York, United States, ²The Scripps Research Institute, Department of Molecular and Cellular Neuroscience, Dorris Neuroscience Center, La Jolla, United States*

The lymph node periphery is an important site for many immune functions, from pathogen containment to T helper cell differentiation, yet we are only beginning to define the cues that position cells in this region. For example, NK cells sit near the medullary sinuses, poised to help sinus-lining macrophages after lymph-borne infection, yet the signals regulating NK cell localization are unknown. Here, using a sphingosine 1-phosphate (S1P) reporter, we find that cells sense higher levels of S1P in the medullary cords than the T zone, and the S1P transporter SPNS2 generates this gradient. SPNS2 on lymphatic endothelial cells, S1P receptor 5 on NK cells, and the chemokine receptor CXCR4 are all required for NK cell localization during homeostasis and rapid IFN γ production by NK cells after challenge. Our findings elucidate the spatial cues required for NK cell organization, and reveal a novel role of S1P in positioning cells within the medulla.

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Introduction to PlasmoSIP: an interactive online tool for exploring relevant structural, immunological and polymorphic features of *Plasmodium* proteomes*Guy, A.^{1,2}, Irani, V.^{1,3}, Ramsland, P.^{1,2,4}, Richards, J.^{1,3,5}*¹*Burnet Institute, Melbourne, Australia, ²Monash University, Department of Immunology, Melbourne, Australia, ³University of Melbourne, Department of Medicine, Melbourne, Australia, ⁴RMIT University, School of Science, Melbourne, Australia, ⁵Victorian Infectious Diseases Service, Royal Melbourne Hospital, Melbourne, Australia*

Malaria remains a significant global health burden. Development of a long-lasting, efficacious vaccine remains a major goal as part of a global push towards malaria eradication. The precise targets of protective immunity are still unknown, and there is a need for new tools to bring together existing computational and experimental data to assist in selection and design of vaccines. We will present a newly developed online tool (PlasmoSIP) for the visualisation of protein structural features, predicted immunological features, and known polymorphisms for proteins from various *Plasmodium* species including *P. falciparum* and *P. vivax*. This tool integrates a number of computational predictions, including predictors of protein disorder, MHC class I and class II binding peptides, predicted structural models and predicted B-cell epitopes. PlasmoSIP also utilises a number of experimental datasets, including data from multiple population genomics studies. Genomic markers of immune selection pressure are displayed in the context of

structural protein models alongside various physicochemical features. Within this package, we have also included an interactive 'proteome explorer', designed to enable users to view summary statistics for proteins within the proteome and then proceed to design custom filters to narrow down the selection of proteins for further analysis. Manipulation of data is both intuitive and responsive, with a particular focus on ease-of-use while maintaining a high level of functionality. This tool has utility in both the fields of vaccine design and biomarker selection.

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Cohort method for lymphocyte proliferation analysis: theory and applications*Kan, A.^{1,2}, Bryant, V.L.^{1,2}, Heinzl, S.^{1,2}, Lye, B.K.^{1,2}, Marchingo, J.M.^{1,2}, Slade, C.^{1,2,3}, Zhou, J.H.S.^{1,2}, Hodgkin, P.D.^{1,2}*¹*Walter and Eliza Hall Institute, Immunology, Parkville, Australia, ²The University of Melbourne, Department of Medical Biology, Parkville, Australia, ³Royal Melbourne Hospital, Department of Clinical Immunology and Allergy, Parkville, Australia*

Lymphocyte responses are dynamic processes whereby B and T cells undergo expansion and contraction triggered by pathogenic stimuli. Obtaining a quantitative understanding of response regulation poses a significant theoretical and experimental challenge for immunology. Flow cytometry coupled with division tracking dyes is a major method for measuring progression of lymphocyte responses. We have introduced a quantitative methodology for analysing proliferation assay results. This method is termed Cohort analysis, and it aims to estimate the core kinetic parameters that characterise cell division, death and the limit in the number of division rounds. This methodology has already proven insightful in recent work where it was used as an integral part of a proliferation assay [Hawkins et al., *Nature Communications* 2013 (doi:10.1038/ncomms3406); Marchingo et al., *Science* 2014 (doi:10.1126/science.1260044)].

Here we perform a meta-analysis of proliferation kinetics across a variety of stimulatory conditions. These include data from in vitro murine B cell activation via T-cell dependent and T-cell independent pathways, CD4 and CD8 T cell proliferation, as well as human lymphocyte responses. Our unified framework enables defining a "kinetic signature" that summarises the functional outcome for different responses. Next we introduce new developments in mathematical theory behind Cohort methodology. In particular, we explore stochastic properties of two of the variables that are used to quantify the responses: Mean Division Number and Division Destiny. Finally, we present open source software that implements Cohort, and we put forward the corresponding data format for proliferation assay results to facilitate data exchange and development of a global repository.

1195**Mathematical modelling of lymphocyte responses measured using time lapse microscopy***Kan, A.^{1,2}, Heinzel, S.^{1,2}, Marchingo, J.M.^{1,2}, Zhou, J.H.S.^{1,2}, Hodgkin, P.D.^{1,2}*¹Walter and Eliza Hall Institute, Immunology, Parkville, Australia,²The University of Melbourne, Department of Medical Biology, Parkville, Australia

Mathematical modelling plays an increasingly important role in studies of B and T lymphocyte responses. Based on accumulated experimental data, recent stochastic models of lymphocyte kinetics provided novel insights into response regulation, and allowed an accurate prediction of population progression over time. Such models are typically fitted and validated against data resulting from flow cytometry experiments. At the same time, time lapse microscopy coupled with single cell tracking offers an increased information resolution compared to bulk culture studies. Hence, parameter estimation and model selection based on microscopy data are significant yet still outstanding challenges to be addressed.

A statistical approach to these problems involves defining the likelihood of the data. Difficulties of defining such a likelihood include inter-dependency of individual cell measurements; lost or partially tracked cells; and age-dependency of clone proliferation process. Here we develop a framework to define a computationally tractable joint distribution function for measured single cell data that takes into account lineage relations. We show how the joint distribution can be decomposed, and how unobserved cells can be efficiently marginalised out. Moreover, we account for age-dependency by passing state variables between generations. Importantly, our method is based on biologically plausible assumptions supported by experimental evidence. We develop this framework for a generic lymphocyte response model, but also in application to a major proliferation model called Cyton. We successfully applied the proposed methodology to tracked movies showing lymphocyte responses, and thus demonstrated how our framework can release the potential of lymphocyte response modelling for microscopy data.

1196**An analysis of stochastic variation in T-cell pedigrees***Hicks, D.¹, Yassin, M.², Russell, S.²*¹Swinburne University of Technology, Physics and Astronomy, Hawthorn, Australia, ²Peter MacCallum Cancer Centre, Melbourne, Australia

The process by which T cells differentiate to form effector and memory cells continues to be an open question. To address this, time-lapse fluorescence microscopy has been used to track post-activation naive T cells and their progeny, recording the ancestral relationships over many generations and the phenotypic characteristics of individual cells. By organizing cells by their most recent common ancestors and examining the variation between and within groupings of descendants it has been possible to partition the observed variability. The resulting distribution of stochasticity shows which stages represent

dominant differentiation points and thus when commitment to a particular fate occurs. Improved understanding of this commitment process has potential implications for vaccination and cancer immunotherapeutic strategies.

1197**Determining the epigenetic mechanisms that underpin CD8+ T cell exhaustion during chronic infection***Piovesan, D., Olshansky, M., Mueller, S.N., Turner, S.J.**University of Melbourne, Peter Doherty Institute for Infection and Immunity, Microbiology and Immunology, Melbourne, Australia*

Functional exhaustion involves the hierarchical and progressive loss of CD8+ T cell effector functions due to persistent antigen stimulation during chronic infection with viruses such as HIV and hepatitis C. These changes are underscored by differences in the transcriptional profiles of exhausted versus functional T cells but the underlying molecular mechanisms regulating the transcriptional and functional differences observed in exhausted T cells is still not well understood. Since epigenetic mechanisms are known to be a key regulator of the transcriptional changes that accompany T cell differentiation, we hypothesize that qualitative differences between exhausted and functional T cells can be explained at the epigenetic level. To address this hypothesis, we used a murine lymphocytic choriomeningitis virus (LCMV) infection model with either the acute (Armstrong) or chronic (Clone-13) LCMV strain to generate functional and exhausted LCMV-specific CD8+ T cells for molecular comparisons. Using as few as 8×10^5 cells, we were able to optimize a low input ChIP-sequencing technique to compare genome-wide deposition of the active (H3K4me3) and repressive (H3K27me3) histone modification in antigen-specific effector (day 7) and memory/exhausted (day 28) CD8+ T cells. This novel approach enables the detection of regulatory DNA regions enriched for H3K4me3 or H3K27me3 to ultimately identify genes and pathways that are differentially regulated epigenetically in exhausted CD8+ T cells. They can then be selectively targeted via epigenetic modifiers or novel immunotherapies to rescue exhaustion and boost T cell immunity.

1198**Murine haematopoietic B-cell development during West Nile viral encephalitis***Cox, D., Ashhurst, T.M., King, N.J.**University of Sydney, Pathology, Sydney, Australia*

Central nervous system (CNS) infection with neurotropic West Nile virus (WNV), triggers infiltration of pathogenic bone marrow (BM)-derived monocytes, associated with enhanced myelopoiesis, with increased monocyte production, culminating in seizures and lethal immunopathology, all of which are absent in asymptomatic WNV-infected mice, which become immune. Antibodies are crucial to WNV immunity, but little is known about the haematopoietic B-cell response during WNV encephalitis. General B-cell progenitor depletion has been observed during systemic murine influenza virus infection and we showed a similar reduction in immature B cells in WNV

encephalitis, where virus infection is limited to the CNS. Detailed subset discrimination revealed that pro-, pre-, immature- and transitional-B-cells decreased during WNV encephalitis, however a population of mature B-cells was retained in the BM. Additionally, upregulation of the stem cell marker, SCA-1, was observed on B-cells, dendritic cells and monocytes, although not on BM neutrophils. This molecule is induced by interferons (IFN), suggesting a role in anti-viral haematopoiesis. Comparing symptomatic and asymptomatic mice, we found no significant difference in cell numbers in the BM. However, SCA-1 was upregulated on both B cells and cDC prior to the onset of symptoms, suggesting a role for these cells prior to the onset of symptomatic disease. Furthermore, SCA-1 was substantially upregulated on monocytes in lethal disease, but not in asymptomatic infected mice. This study provides an opportunity to better map and highlight disease-specific hematopoietic cell signaling mechanisms in inflammatory conditions, informing improved future targeted immunomodulatory therapies.

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CD40 signal strength regulates the rate of B cell differentiation to plasmablasts by altering the time to divide

Zhou, J.^{1,2}, Duffy, K.³, Kan, A.^{1,2}, Hawkins, E.^{1,2}, Markham, J.⁴, Dowling, M.^{1,2}, Hodgkin, P.^{1,2}

¹The Walter and Eliza Hall Institute of Medical Research, Immunology, Parkville, Australia, ²The University of Melbourne, Medical Biology, Melbourne, Australia, ³Hamilton Institute, Kildare, Ireland, ⁴Peter MacCallum Cancer Centre, Melbourne, Australia

B cells activated by helper T cells proliferate and rapidly generate a heterogeneous population of lymphoblasts and plasmablasts. Perhaps surprisingly, similar patterns of heterogeneity emerge when B cells are stimulated *in vitro* despite highly controlled conditions. Video-microscopy of single cell fates reveals that allocation of alternative fates is consistent with competition for different outcomes within single cells [Duffy *et al*, Science 2012]. By this model, complex population outcomes will arise with even small changes in times to each fate.

To explore how varying stimulation might control fate, we filmed cells under different conditions. Earlier flow cytometry data demonstrated that reducing CD40 stimulation had two effects on B cell fates: it slowed down B cell division, and produced a greater proportion of differentiated plasmablasts in each generation [Hawkins *et al*, Nat Comms 2014]. Modelling our filming data revealed that the competition hypothesis could explain these observations with a simple effect on division time alone: by slowing division, B cells gain more time to undergo differentiation before their next mitotic event. These data demonstrate how subtle changes to cell fate allocation can have greater, cumulative effects across the population. We are currently assessing how other cytokine dose effects operate to alter the population outcome, in particular IL-4 and IL-5.

By understanding how different components of the B cell response are controlled, we aim to build a better model of how these fates interact to form the overall outcome. Such a model has many potential applications in both immune deficiency diagnoses and immunotherapy.

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Effective diversity of T-cell repertoire

Xu, J.^{1,2}, Jo, J.^{1,2}

¹APCTP, Pohang, Korea, Republic of, ²POSTECH, Physics, Pohang, Korea, Republic of

To detect countless pathogen invaders in our life, we should equip with diverse immune receptors. Each immune cell expresses copies of one specific receptor. Therefore, one individual cannot have infinite diversity of receptors with a finite number of immune cells, but should have an effective repertoire. The immune system shapes the repertoire by filtering immature immune cells which express randomly-generated receptors. Thymus selects the thymocytes whose receptors have sufficient, but not too strong, interactions with self-peptides. The positive and negative selection prevents the maturation of negligible and autoimmune cells. We wonder why there is the certain diversity of T-cell repertoire, in order to understand the optimal coding of the immune system. Here we make use of the TCR-pMHC pairwise string model and T-cell activation model, to examine the risk of autoimmunity and immunodeficiency simultaneously. Through the models, important factors are discussed, i.e. T-cell repertoire diversity and the number of activated T cells required to initiate a response. It suggests that the thymic selection minimizes the risk of responding to safe self-peptides and ignoring dangerous nonself-peptides.

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Thorough screening the interacting proteins with tumor suppressor candidate 3 using NAPPA chip

Du, J.¹, Yu, X.², Yu, X.^{3,4}

¹Central Laboratory, Shandong Provincial Qianfoshan Hospital, Jinan, China, ²Department of Radiation Oncology, Shandong Provincial Qianfoshan Hospital, Jinan, China, ³National Center for Protein Sciences (Beijing), State Key Lab of Proteomics, Beijing Proteome Research Center, Beijing, China, ⁴Virginia G. Piper Center for Personalized Diagnostics, Biodesign Institute, Arizona State University, TEMPE, United States

Tumor suppressor candidate 3 (TUSC3) gene is located on chromosome region 8p22 and encodes the 34 kDa TUSC3 protein, which is a subunit of the oligosaccharyl transferase (OST) responsible for the N-glycosylation of proteins. Only recently was TUSC3 identified as a potential tumor suppressor gene when it was found deleted in a variety of human malignancies, including hepatocellular carcinoma. However, its thorough functions in liver cancer are still not clear. Nucleic Acid Protein Programmable Array (NAPPA), is a new type of protein chip on which containing about 20,000 disease-associated proteins and is adept at protein interaction. To probe the interacting proteins with TUSC3 in whole, we hybridized TUSC3 protein on NAPPA chip. Here, we present the first demonstration of using NAPPA protein microarrays to detect interacting proteins with TUSC3. These 28 proteins were screened using NAPPA and a 96-well protein ELISA (well-NAPPA). Four proteins (NF2, BIRC2, RTBND and BIRC8) were finally confirmed using Co-IP and intracellular localization in hepatocytes. However, it is need to be illuminated that the interactions play the vital roles in the development of liver cancer in the future. Our results will contribute to a

deeper understanding of carcinogenic mechanism of TUSC3 in hepatocellular carcinoma.

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Using correlative microscopy to understand dynamics of T cell signalling

Zibaei, K.¹, Chen, Y.^{1,2}, Yasin, M.², Ludford-Menting, M.², Russell, S.^{1,2}

¹Swinburne University of Technology, Centre for Micro-Photonics, Melbourne, Australia, ²Immune Signalling Laboratory, Peter MacCallum Cancer Centre, Melbourne, Australia

As the central players of adaptive immune responses, T cells and their responses to the external stimuli have been widely investigated. Time lapse microscopy provides a powerful method for understanding signalling, but quantitation of fluorescent microscopy remains a challenge.

Using combined microscopy methods (time lapse and super resolution microscopy) as well as new analysis methods, we can now draw a picture of the different events that contribute to the T cell activation and fate determination. In particular, we can quantify characteristics of how the T cell docks onto the Dendritic cell (DC), how this relates to T cells signals such as calcium flux and shedding of the surface protein, CD62L, and how this impacts upon subsequent formation of the synapse (as measured by confocal and super resolution microscopy), and on the progeny of that naive T cell. In this work I will present new findings regarding T cell signalling which has been achieved through our approach.

1203

A framework for digital RNA sequencing (dRNA-Seq) and its application for gene expression profiling of small number of immune-related cells

Matsumoto, Y.¹, Kryukov, K.², Ikawa, T.¹, Imanishi, T.², Shiroguchi, K.¹

¹RIKEN, IMS, Yokohama, Japan, ²Tokai University School of Medicine, Molecular Life Science, Isehara, Japan

Recently developed molecular barcoding techniques enable digital quantification of pre-amplified nucleic acid molecules genome-wide by massively parallel sequencing of amplicons. In the barcode sequence, random bases were widely used due to advantages including higher dynamic range and lower cost. However, the efficacy of random base barcodes for accurate quantification of nucleic acids has not been studied well. We established, by combination of experiments and computational analyses, criteria which ensure that the digital quantification of nucleic acid molecules using random base barcodes works. Through this process, we found that alterations in the original barcode sequences, likely from errors generated in amplification and/or sequencing, caused over-estimation of the number of molecules. Thus, we developed the following framework to remove the effect of these errors during downstream analysis: designing barcode sequences to

include error detection codes and developing original analysis software. We show that this framework provides accurate quantification of nucleic acid molecules; the measured number of molecules was highly consistent with the original number of molecules before amplification. These results enable us to propose parameters for barcode design and also sequencing depth required to accurately quantify nucleic acid molecules, which may become a standard guideline for the use of random base molecular barcodes. Using this framework, we developed dRNA-Seq which provides absolute quantification of RNA molecules genome-wide. We will present the gene expression profile of immunology-related cells during different stages of differentiation measured by dRNA-Seq from small number of cells.

1204

From healthy to transformed B cells - a model of how chemotherapeutic drugs act against lymphoma

Pham, K.¹, Zhou, J.H.S.¹, Kan, A.¹, Dowling, M.R.¹, Duffy, K.², Hodgkin, P.D.¹

¹The Walter and Eliza Hall Institute, Division of Immunology, Parkville, Australia, ²Hamilton Institute, Maynooth, Ireland

B lymphocytes balance division and death during a normal immune response. Homogeneous populations of naive B cells can generate significant heterogeneity upon activation that can be described by a competing cell fate model (Duffy K, *Science*, 2012; Hawkins ED, *Nat Comms*, 2014). We hypothesise that this same competition model could explain why mitosis inhibiting drugs that are commonly used in cancer chemotherapy, lead to the death of lymphoma cells. By blocking division, the underlying time to die is revealed and death is an inevitable consequence.

To explore this we exposed dividing B cell lymphoma lines (J558, NS.1) to cell cycle inhibiting or apoptosis promoting chemotherapeutic drugs and measured times to fate from a previous mitotic event using single cell microscopy. After examining >1000 single cell fate outcomes we find that these results are generally consistent with our hypothesis. Chemotherapeutic S-phase inhibitors etoposide and aphidicolin delay and inhibit division in the J558 cell line, and lead to a time to die that is unaffected by increasing concentrations. In contrast, these drugs simultaneously delay both division and death times in the NS.1 cell line.

Using mathematical modelling we can quantify the changes for each lymphoma cell on the frequency of division, time to divide and time to die in the population. Our model of therapeutic drug action opens up new possibilities for assessing drug efficacies or synergies. We are now using these results to predict the combined effects of multiple chemotherapeutic agents and explore effective combinations in B cell malignancies.

1205**Immunosequencing reveals diagnostic signatures of pathogen infection and HLA type in the T-cell receptor repertoire**

DeWitt, W.¹, Emerson, R.¹, Vignali, M.¹, House, S.¹, Desmarais, C.¹, Rieder, M.¹, Carlson, C.², Gravley, J.², Hansen, J.², Robins, H.²

¹*Adaptive Biotechnologies Corp, Seattle, United States, ²Fred Hutchinson Cancer Research Center, Seattle, United States*

We analyzed the T-cell response to cytomegalovirus (CMV) by sequencing rearranged T cell receptors (TCRs) from 650 subjects (294 CMV+, 356 CMV-). We assessed association with CMV serostatus for ~90 million unique TCRs, identifying 157 CMV-associated TCRs (FDR \approx 0.15). Training a classifier on these features, we predict CMV serostatus in a cross-validation procedure on the same cohort with a diagnostic odds ratio of 44. Testing on an independent cohort of 120 subjects with known CMV serostatus yielded a diagnostic odds ratio of 49.

HLA-restriction of each CMV-associated TCR was determined by enrichment of specific HLA alleles among subjects that carry each CMV-associated TCR. Of 157 CMV-associated TCRs, 61 were HLA-restricted at $p \leq 10^{-3}$, and none of these were significantly associated with multiple alleles in any locus. We find substantial concordance between our data and previously published CMV-specific TCRs. Most previously-reported CMV-specific TCRs are present in our data, however only 5/157 CMV-associated TCRs identified in this study have been previously reported. Of these, 4 were significantly HLA-restricted and confirmed previous findings.

We separately investigated association of TCRs with each HLA allele present in the cohort. Cross validation indicated accurate prediction for over a broad range of allele frequencies, demonstrating that HLA type can be inferred from immunosequencing data.

In summary, we demonstrate the validity of association studies using immunosequencing for detection of public T-cell responses to infection, and report that assessing the presence of associated TCRs can serve as a powerful diagnostic classifier.

1206**Changes in T cell receptor repertoire associated with combined variable immune-deficiency (CVID)**

Ismail, M.¹, Matjeka, T.¹, Husovsky, C.¹, Heather, J.¹, Best, K.^{1,2}, Burns, S.³, Chain, B.¹

¹*University College London, Division of Infection and Immunity, London, United Kingdom, ²UCL Centre for Mathematics, Physics and Engineering in the Life Sciences and Experimental Biology (CoMPLEX), London, United Kingdom, ³UCL Institute of Immunity and Transplantation, Department of Immunology, Royal Free Hospital, London, United Kingdom*

We have recently developed a reliable and economical amplification protocol that can be used to characterise the T cell receptor (TCR) repertoire using high-throughput sequencing, and a robust computational pipeline for quantitative downstream analysis. Introduction of barcodes, which label every cDNA molecule before amplification allow for correction of PCR bias, and PCR and sequencing error. The protocol can be

used to sequence TCR repertoires of diverse types of samples, including ex vivo collection of whole or FACS fractionated blood or tissue, or after in vitro culture and expansion.

We have recently used this protocol to better define the multiple levels of immune dysregulation which characterise chronic infection with HIV. In this study, we analyse the TCR sequences of a small number of individuals with CVID and develop metrics to characterise their TCR repertoire and compare it to a cohort of healthy controls. We detected a reduced number of TCRs shared between the CVID patients and healthy volunteers, suggesting that the CVID repertoires were abnormal. Changes were detected in the number of beta J gene deletions, the lengths of the CDR3 sequences and in V alpha and beta gene usage in CVID patients. Strikingly, the CVID patients showed a marked deficiency in alpha genes associated with mucosal associated invariant T (MAIT) cells.

Quantitative analysis of the TCR repertoire in a larger CVID cohort may give insights into the pathogenesis leading to immunodeficiency in these individuals, as well as providing additional information with which to stratify this heterogeneous group of immunodeficiencies.

1207**Patterns of single cell fate discoverable via novel multidimensional analyses and dynamic visualization**

Gao, J.¹, Crampin, E.², Naik, S.³

¹*The Walter and Eliza Hall Institute of Medical Research, Molecular Medicine, Melbourne, Australia, ²The University of Melbourne, Systems Biology Laboratory, Melbourne, Australia, ³WEHI, Melbourne, Australia*

Identifying the progeny of many single progenitor cells simultaneously can be achieved by tagging progenitors with unique heritable DNA barcodes, and allows inferences of lineage relationships. While this approach has shed new light on single cell fate heterogeneity, data interpretation remains a major challenge. In this study, we applied the t-Distributed Stochastic Neighbor Embedding (tSNE) algorithm with novel customizations to derive a refined classification of hematopoietic stem and progenitor cells. We further classify lymphoid-primed multipotent progenitors into discrete categories based on cell output. We also use "tSNE pie map movies" to visualize clonal dynamics of hematopoietic reconstitution in primates and identify novel developmental patterns, namely hematopoietic progenitors with early T cell and later granulocyte production. Augmenting tSNE visualizations of high-dimensional data can be widely applied across biology to explore data, perform systems-level analyses and generate novel hypotheses for subsequent validation.

1208

Interaction of aloe emodin with lipoxygenase towards the development of novel NSAIDs*Suresh, S.¹, Sabu, A.², Haridas, M.¹*¹Kannur University, Inter University Centre for Bioscience, Kannur, India, ²Kannur University, Department of Biotechnology and Microbiology, Kannur, India

LOXs are potential drug targets in the treatment of diseases such as asthma, atherosclerosis, cancer, and a variety of inflammatory conditions. It is assumed that blocking the arachidonic acid (AA) metabolism via COX inhibition by either traditional NSAIDs or selective COX-2 inhibitors could lead to the generation of pro-inflammatory leukotrienes and lipoxins via the LOX pathway partly accounting for the side effects seen with traditional NSAIDs and selective COX-2 inhibitors. To counter this, several LOX, PLA₂ inhibitors have been reported nowadays. Investigations on LOX inhibitory activity of *Cassia angustifolia* (Vahl.), elucidation of bioactive compound responsible for inhibition and their binding interactions were reported in this study. Results indicate that ethyl acetate extract of *Cassia angustifolia* (Vahl.) could inhibit LOX with 79 ± 0.002% of inhibition rate. Bioactive compound responsible for inhibition isolated via standard protocols and MS data revealed the presence of Aloe emodin (270.2 m/z) which was already reported from this plant. The binding potential of the compounds with LOX was determined using molecular docking studies. In order to corroborate the in silico results, enzyme kinetics and isothermal titration calorimetric analysis were performed. From the analysis, it was concluded that aloe emodin can act as competitive inhibitors to the enzyme with an IC₅₀ of 29.49 µM and may be used as anti-inflammatory agent. ITC results indicated the interaction of LOX with aloe emodin is endothermic in nature with a stoichiometry of n= 3 and binding free energy found to be -7.69 kcal/mol.

1209

A computational framework for simultaneous single-cell characterization of surface phenotype and gene expression profile of antigen specific lymphocytes*Rizzetto, S.¹, Elthala, A.¹, Rasoli Pirozyan, M.¹, Keoshkerian, E.¹, Brownlee, C.², Lloyd, A.¹, Bull, R.¹, Luciani, F.¹*¹University of New South Wales, Systems Medicine in Infectious Diseases, Sydney, Australia, ²University of New South Wales, Bio Resources Imaging Lab, Sydney, Australia

Upon recognition of an antigen, activated lymphocytes (both B and T cells) proliferate and differentiate, generating a heterogeneous progeny able to perform a vast array of functions. The phenotype of the initial naive repertoire, as well as the specificity between T and B cell receptors (TCR and BCR) for the antigen are critical components that determine the success of the immune response and establishment of protective immunity. Single cell technologies are now significantly improving the understanding of these highly dynamic and heterogeneous molecules. The rise of multi-omics approaches inevitably requires computational workflows to analyse and integrate large and multiple datasets together. We have developed a computational pipeline to link the cell

surface phenotype with the full transcriptome profile, including TCR and BCR. This model combines gene expression profile from scRNA-Seq with surface markers identified with flow cytometry index sorting on the same single cell. TCR and BCR were detected from scRNA-Seq reads using an updated version of the in-house developed tool to reconstruct VDJ sequence, called VDJPuzzle. This pipeline has been applied to Ag specific B cells and T cells derived from human peripheral blood mononuclear cells (PBMC) collected from a patient infected with Hepatitis C Virus and allowed us to identify surprisingly high heterogeneity. Our computational analysis contributes to the understanding of the evolution of lymphocytes during an immune response both at the population and molecular level providing insight into the interrelation and functioning of larger systems.

1210

AbDesigner3D*Saethang, T.¹, Payne, M.¹, Pisitkun, T.^{1,2}*¹Faculty of Medicine, Chulalongkorn University, Systems Biology Center, Research Affairs, Bangkok, Thailand, ²Epithelial Systems Biology Laboratory, NHLBI, National Institutes of Health, Bethesda, United States

Antibody-based techniques have been widely used in system biology-based experiments. The key of success for such techniques is the antibody-design procedure. AbDesigner, an online tool for the design of peptide-directed antibodies, was previously developed and implemented as the web application (helixweb.nih.gov/AbDesigner/). This software provides crucial information of target immunogen including immunogenicity (antigenicity) score, uniqueness (predictor of specificity), conservation (predictor of multispecies cross-reactivity), and protein features such as PTMs, domain architecture, site of sequence variation, and other regions or site of interest extracted from the corresponding Swiss-Prot record. AbDesigner successfully applied in antibodies productions that are human nephrin antibody, human podocin antibody, and human apolipoprotein L1 antibody. Although AbDesigner has been widely used in the community, it omits the 3D structure information of protein which is commonly used in studies of protein binding nowadays. In this study, AbDesigner3D was developed by integrating 3D structures information from PDB into the previous AbDesigner. New features that are accessible surface area and contact residues are also included in AbDesigner3D to enable users to optimize their target peptides with more confidence. In addition, interactive user interface was created to help user judge which region of peptide should be used as an epitope by displaying trade-offs between features of that region.

1211**The influence of nature, nurture and noise on humoral immunity**

Menzel, U., Greiff, V., Miho, E., Weber, C., Cook, S.C., Reddy, S.T. ETH Zürich, D-BSSE, Basel, Switzerland

The humoral immune system manages host protection and body maintenance by accessing an immensely diverse antibody repertoire; the exact balance between the forces that control repertoire formation and selection is however still unknown. Therefore, we set out to quantify the fraction of antibody repertoire diversity, which is *predetermined* by genetic composition (nature) or antigen challenge (nurture) or *unpredictable* due to stochastic variation (noise). In order to accomplish this, we performed high-throughput antibody repertoire sequencing throughout the entire progression of the B-lymphocyte lineage (pre B cells/PreBC, naïve follicular B cells/NFBC, memory plasma cells/BMPC). This resulted in a dataset comprising ≈ 100 samples and ≈ 600 million total sequence reads, originating from four C57BL/6 cohorts ($n=5$), (i) unimmunized, (ii-iv) prime-boost immunized with Ovalbumin/NP-hen egg lysozyme/Hepatitis B surface antigen). Leveraging a multi-scale linear regression approach, we found that the germline V gene usage of PreBC and NFBC was highly similar across all mice and was thus dominated by nature (germline correlation $R^2 \approx 90\%$) whereas it was predominantly stochastic in the BMPC population (noise $\approx 90\%$). On the antibody clonal level, we found antigen-specific and plasma cell-specific repertoire fragmentation into clonal clusters of reproducibly high CDR3 sequence similarity (nature and nurture $\approx 90\%$). Throughout B-cell development, we found unexpected and hitherto unreported high clonal overlap ($\approx 15\%$) among mice (exception: unimmunized BMPC). Extrapolation of the convergent repertoires allowed us to quantify the total murine naïve repertoire diversity to a size of $\approx 10^{14}$ clones. In conclusion, nature, nurture and noise dictate the structure and size of antibody repertoires in an ontogenetic fashion.

Emerging Technologies

1212**Immunomedia project: learning, lecturing and spreading Immunology**

Corell, A.¹, Martín Alonso, C.¹, Zarzuela, J.C.¹, Sanz, L.A.², Aragón, J.C.², Sempere, J.M.³, Hudrisier, D.⁴, Rodrigues Santos, P.⁵, Álvarez Álvarez, S.⁶, Arnaiz, V.⁶, Verdú Perez, M.J.⁷, Regueras Santos, L.M.⁷, de Castro Fernández, J.P.⁷, Martínez-Quiles, N.⁸, Reche Gallardo, P.A.⁸, Pascual García, S.³, Martínez Peinado, P.³, Regueiro, J.R.⁸

¹Universidad de Valladolid, Inmunología, Facultad de Medicina, Valladolid, Spain, ²Universidad de Valladolid, Servicio de Medios Audiovisuales, Valladolid, Spain, ³Universidad de Alicante, Biotecnología, Alicante, Spain, ⁴Université de Toulouse, UPS, IPBS, CNRS, Toulouse, France, ⁵Universidade do Coimbra, Instituto de Inmunologia, Coimbra, Portugal, ⁶Universidad de Valladolid, Lengua Española, Soria, Spain, ⁷Universidad de Valladolid, Teoría de la Señal y Comunicaciones e Ingeniería Telemática, Valladolid, Spain, ⁸Universidad Complutense de Madrid, Inmunología, Madrid, Spain

In the ICTs era, the universities have novel roles (using social media, designing multimedia materials among others). In this context, the Immunomedia project (with the participation of 5 Universities from Spain, France and Portugal) try to fill some needs and gaps in the lecturing of Immunology, mainly, in Biomedical degrees.

The project has been structured in 7 axis: first "Developing and disseminating high quality minivideos" on Immunology, under the name "Inmunepills" (youtube) with a high impact (>800.000 views) among Spanish-speaking universities; second "Collecting, selecting and tagging OAMs and other lecturing materials" ("Content Curation") onto boards which provide students and lecturers with useful information, verified and organized by modules. Among the used content curation tools, "Scoop.it" and Pinterest gave the greatest impacts; third "involving actively the students into their learning process" and collaborating among universities with the "University Journal of Immunology, Immunopathology and Immunotherapy": the students tweet relevant immunology news using #hashtags, which are tracked and generate a journal at "Paper.li"; fourth, "Opening the University to all the people" by developing multimedia materials in immunology and disseminating them on social media, patients associations and public places (video collection "Defences'Channel" at YouTube); fifth, universalising the materials by subtitling the Immunepills into Spanish (for deafs), English and French or dubbing them into Portuguese; sixth, developing and designing a gamification approach to assess learning success at different levels and with an interactive interface between students; and finally, developing an Immunomedia site to connect all the material, activities, lecturers and students.

1213**Releasable RapidSpheres™ enable immunomagnetic purification of highly viable and functional immune cells from complex tissues in less than 30 minutes**

Clarke, S.J.¹, Kokaji, A.I.¹, Kellerman, D.W.¹, Ewen, C.L.¹, Chambers, M.N.¹, Chan, M.¹, Woodside, S.M.¹, Eaves, A.C.^{1,2}, Thomas, T.E.¹
¹STEMCELL Technologies Inc., Vancouver, Canada, ²Terry Fox Laboratory, BC Cancer Agency, Vancouver, Canada

Understanding how the cells of the immune system communicate relies on the success of cell-based assays; however, it remains challenging and time-consuming to purify large numbers of high-quality cells from complex tissues. EasySep™ Release is a fast and easy cell isolation method that utilizes Releasable RapidSpheres™. This novel magnetic particle technology improves cell purities by reducing contaminants and allows for gentle particle removal to mitigate potential interference in downstream assays. The 29-minute protocol requires first labelling target cells with Releasable RapidSpheres™ and then separating the labelled cells using a hand-held magnet. Particle-free purified cells are obtained by applying a mild dissociation reagent followed by a final magnetic separation.

We tested EasySep™ Release by purifying T, B and NK cells from peripheral blood mononuclear cell samples containing 5 to 800 million cells. Cell purities were $99.7 \pm 0.1\%$ (SD, $n=17$) for CD3+ T cells, $99.4 \pm 0.4\%$ ($n=17$) for CD4+ cells, $98.8 \pm 0.5\%$ ($n=17$) for

CD8+ T cells, $98.6 \pm 0.7\%$ (n=6) for CD19+ cells and $95.6 \pm 1.7\%$ (n=6) for CD56+ cells. Isolated cells were confirmed particle-free, viable and functional. We obtained similar high performance using unprocessed leukapheresis samples of up to 5 billion cells, demonstrating excellent scalability and compatibility with more complex samples. Finally, we show how EasySep™ Release can be paired with commercially available antibodies or sequential separations to isolate almost any cell type, including those with a complex phenotype.

1214

Soluble biomarkers for pressure ulcers therapy monitoring - protein microarray technology view

Constantin, C.¹, Boda, D.², Căruntu, C.², Păunică-Panea, G.³, Constantin, V.D.³, Neagu, M.¹

¹Victor Babeş' National Institute of Pathology, Immunology, Bucharest, Romania, ²Carol Davila' University of Medicine and Pharmacy, Dermatology, Bucharest, Romania, ³Sf. Pantelimon' Emergency Hospital, Surgery Clinics, Bucharest, Romania

Proteomics is a tremendous domain with a real-time development bringing continuous updates in biological process serving elucidation in therapeutical approaches. The protein microarray technology gains a definite place in innovative attempts such as biomarkers discovery and validation, and elucidation of cellular pathways involved in various pathologies. Wound healing is a continuous challenge, latest 'omic data identifying cellular networks and molecular events which outlines markers for healing monitoring according with therapeutical endeavor. Thus, the aim of the study was to establish biomarkers panels in pressure ulcers of different pathogenesis reflecting the best evolution of wound healing in the presence of some innovative patches. Using a protein microarray platform 42 analytes were semi-quantitatively detected in serum obtained from patients suffering of pressure ulcers, harvested at different time points. Three types of patches (classic cotton; 84, 8% poly-2-hydroxyethylmetylacrilate based; collagen of animal origin based) were used in patient treatment and biological samples were harvested before, during and after therapy procedure. As controls were enrolled subjects with age and gender matched. All enrolled subjects given their written informed consent and the study had all the Ethical approvals. Several panels of markers were outlined defined by level of expression and protein family in every experimental group tested. Especially markers from chemokines family (e.g. ENA-78 or MDC) display different level of expression depending on experimental condition making protein microarray a very accessible and very actual tool for identification of useful (bio) markers for management of pressure ulcers.

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1215

Gallium (III) phthalocyanine for photodynamic therapy of epithelial type transformed cells - approaches on SHSY5Y cell line

Constantin, C.¹, Matei, C.N.², Tampa, M.Ş.², Lupu, A.-R.¹, Ion, R.-M.^{3,4}, Neagu, M.¹

¹Victor Babeş' National Institute of Pathology, Immunology, Bucharest, Romania, ²Carol Davila' University of Medicine and Pharmacy, Bucharest, Romania, ³The National Institute for Research & Development in Chemistry and Petrochemistry, Bucharest, Romania, ⁴Valahia' University, Târgovişte, Romania

Gallium (III) phthalocyanine is less investigated in comparison with other metallated phthalocyanines (e.g. with Zn²⁺ or Al³⁺) in experimental photodynamic therapy. Neuroblastoma is a solid tumor that comprises on its therapeutical list Ga (III), as such Ga(III) nitrate is clinically approved by FDA for the treatment of different cancers, but its administration is somewhat invasive. Therefore it is highly imperative to find new compounds, especially for localized and inaccessible tumors. Ga(III) as phthalocyanine chloride form represents a drug option combining its already acknowledged biomedical benefits with PDT procedure in order to induce cancer cells' death. Our study focuses on the dark toxicity testing of a Chloro-Gallium-phthalocyanine photosensitizer (Ga-Pc) upon neuroblastoma SHSY5Y cell line, and further on the efficacy of experimental PDT. Thus, evaluating Ga-Pc we have shown that cellular proliferation restrain a similar pattern for all incubations times designating a "protective" effect-like of Ga(III)Pc upon SHSY5Y cells. In addition, cellular impedance evaluations have shown that long time co-incubation of cells in Ga-Pc (over 125 h) does not significantly affect cell proliferation even at $\geq 20 \mu\text{g/mL}$ Ga-Pc. When irradiating neuroblastoma cells loaded with non-toxic concentration of Ga-Pc we have obtained a massive cell death and the remaining proliferative capacity of treated cells is due to a fraction that escapes the PDT protocol. This fraction, after 4 h post- PDT, drastically declines as well. Ga-Pc represents a potential photosensitizer that can display exploitable PDT properties in solid tumors.

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1216

Gliadin-containing, tolerogenic immune modifying nanoparticles (TIMP) reverse gluten-dependent enteropathy in a celiac disease mouse model

Freitag, T.¹, Podojil, J.R.², Shea, L.³, Miller, S.D.⁴, King, N.J.⁵, Getts, D.R.²

¹Helsinki University Hospital Laboratory (HUSLAB), Helsinki, Finland, ²Cour Pharmaceuticals Development Co, Chicago, United States, ³University of Michigan Medical School, Ann Arbor, United States, ⁴Northwestern University Feinberg School of Medicine, Chicago, United States, ⁵The University of Sydney, Sydney, Australia

In celiac disease, tolerance to gluten proteins from cereals is lost. Tolerogenic Immune Modifying Nanoparticles (TIMP) encapsulating autoreactive antigens induce peripheral, antigen-specific tolerance in numerous models of autoimmune disease. The identification of gliadins as primary epitopes in Celiac

Disease suggested that this approach may also be applied to the treatment of CD. As such, TIMP encapsulating full gliadin proteins (TIMP-GLIA) were developed and tested in rodents for their therapeutic potential.

The ability of TIMP-GLIA to inhibit antigen specific T cell responses was initially examined using Delayed Type Hypersensitivity (DTH). The intravenous infusion of TIMP-GLIA not only ameliorated the DTH response, by greater than 60%, but was associated with an antigen specific diminution of T cell proliferation and cytokine responses in gliadin immunized mice. Subsequent experiments tested the ability for TIMP-GLIA to reverse gliadin-induced enteropathy in a mouse model of celiac disease. TIMP-GLIA or TIMP-LYS (irrelevant antigen control containing lysosyme) were intravenously infused into Rag1-/- reconstituted with gliadin-sensitized memory T cells. TIMP-GLIA treatment resulted in the reversal of weight loss and reduced the severity of duodenitis, as determined by histology. This disease amelioration was further associated with a significant reduction in antigen specific TH1 responses. Observations in TIMP-GLIA treated animals were similar to mice maintained on a gluten-free diet, suggesting that this treatment was capable of restoring gliadin tolerance and supports the introduction of gluten into the diet of animals with gluten sensitivity.

1217

Molecular mimicry is bad for your heart: when T-cell attack the wrong target

Cole, D.K.¹, Raman, M.C.C.², Rizkallah, P.J.¹, Simmons Zoe Donnellan, R.², Donnellan, Z.², Dukes, J.², Bossi, G.², Le Provost, G.S.², Todorov, P.², Baston, E.², Hickman, E.², Mahon, T.², Hassan, N.², Vuidepot, A.², Sami, M.², Jakobsen, B.K.²

¹Division of Infection and Immunity, Cardiff University School of Medicine, Cardiff, United Kingdom, ²Immunocore Limited, Abingdon, Oxon, United Kingdom

Main body: Enhanced, modified T cell receptors (TCRs), are currently being developed for a wide range of cancer targets, included in a number of current clinical trials. However, there are potential dangers associated with mutating TCRs outside of the rigors of thymic selection. Indeed, we recently demonstrated that one of these reagents, intended to target the MAGE-A3 cancer antigen, displayed an unexpected cross-reactivity leading to fatal cardiac toxicity in 2 patients enrolled in phase I clinical trials. Here, we reveal the structural basis for lethal the toxicity against cardiac tissue of a MAGE-A3 specific TCRs and establish the need for a better understanding of the mechanisms that govern T cell cross-reactivity when developing engineered TCR cancer therapies.

1218

A biomimetic glyceride prodrug approach to promote the delivery of immune-modulating drugs to targets in the lymphatics and immune cells

Gracia, G., Han, S., Quach, T., Hu, L., Simpson, J.S., Trevaskis, N.L., Porter, C.J.
Monash Institute of Pharmaceutical Sciences, Monash University, Melbourne, Australia

Background: The lymphatics and associated immune cells have three main physiological functions: the preservation of fluid balance, dietary lipid absorption and regulation of immunity. Additionally, the lymphatics play key roles in modulating the pathogenesis of immune/inflammatory disease, allergy, cancer, metabolic disease, infection etc. Site-specific delivery of drugs to lymph-associated targets has the potential to enhance the efficacy and reduce the toxicity of treatments for these diseases.

Aim: To establish the potential to enhance drug delivery to the lymphatics by linking a model immunosuppressant drug, mycophenolic acid (MPA), to a glyceride backbone to form a biomimetic glyceride prodrug (2-MPA-TG) that integrates into the physiological process of dietary triglyceride absorption.

Methods: 2-MPA-TG vs. MPA were administered intestinally to rats or orally to dogs. Intestinal lymph fluid and lymph nodes at different sites in the body were collected at set time points. Lymphocytes were separated from lymph. Sample concentrations of drug and prodrug were analysed via LC-MS/MS.

Results: 2-MPA-TG efficiently incorporated into the dietary triglyceride absorption pathway, thereby markedly increasing intestinal lymph transport of MPA derivatives in rats (90-fold) and dogs (288-fold) and lymph lymphocyte concentrations of active MPA in dogs (19-fold) when compared to parent drug MPA. 2-MPA-TG also facilitated a significant increase in the accumulation of active MPA in local mesenteric lymph nodes, but not peripheral lymph nodes, in rats.

Conclusion: Biomimetic glyceride prodrugs increase drug exposure to lymphatic-associated targets, particularly in the mesentery, thereby representing a promising strategy to enhance the treatment of disease involving lymphatics such as immune/inflammatory disease.

1219

Identifying exosome binding and internalization in blood cell subsets by imaging flow cytometry

Pugsley, H., Friend, S., Probst, C., Morrissey, P.
Amnis part of Merck, Seattle, United States

Only recently has the importance of extracellular vesicles as key mediators of intercellular communication been appreciated. Extracellular vesicles are membrane derived structures that include exosomes, microvesicles and apoptotic bodies. Exosomes have been shown to transfer molecules between cells and have the potential to transfer signals between cells. Exosomes are released under normal physiological conditions; however, they are also believed to serve as mediators in the pathogenesis of neurological, vascular, hematological and autoimmune diseases as well as cancer. Quantifying and characterizing exosomes in a reproducible and reliable manner has been difficult due to their small size (50 - 100 nm in diameter). Exosomes analysis can be done using high-magnification microscopy however this technique has a very low throughput. Attempts to analyze exosomes using traditional flow cytometers has been hampered by the limit of detection of such small particles and low refractive index. To overcome these limitations we have employed multispectral imaging flow cytometry that has the advantage of combining

high throughput flow cytometry with higher sensitivity to small particles and the added benefit of imaging that can provide visual confirmation of particle integrity and characterization. In this study we use multispectral imaging flow cytometry to investigate the interaction of exosomes with white blood cells. Exosomes derived from different cell types will be investigated for their preferential interactions with blood cell subsets by combining immunophenotyping with morphological parameters to measure their binding and internalization.

1220

Detailed mapping and quantification of human T cell subset proteomes using tandem mass spectrometry and SWATH

Weerakoon, H.^{1,2,3}, Potriquet, J.¹, Wong, Y.^{1,4}, Haigh, O.¹, Miles, J.J.^{1,4}, Mulvenna, J.^{1,2}

¹QIMR Berghofer Medical Research Institute, Brisbane, Australia,

²University of Queensland, School of Biomedical Sciences, Brisbane, Australia, ³Rajarata University of Sri Lanka, Faculty of Medicine and Allied Sciences, Saliyapura, Sri Lanka, ⁴University of Queensland, School of Medicine, Brisbane, Australia

T cells are core regulators of innate and adaptive immune system function and are key players in the defence against foreign invaders and cancer. A detailed knowledge of the proteomes of human T cells is obligatory if we are to understand the function and behaviour of this important compartment and to clearly identify, sort and target unique lineages via distinctive cytosolic and plasma membrane markers. Detailed phenotyping can be achieved through mass spectrometry based proteomics due to the unmatched capability of this platform in identifying thousands of proteins in complex mixtures. Relative quantification of proteins across large number of samples can also be performed using a new label free quantification method termed SWATH (sequential window acquisition of all theoretical fragment masses). In this study, we generated a vast human T cell library (>4,000 proteins) using cell multiparametric sorting, OFFGEL electrophoresis and tandem mass spectrometry. We identified 22% of the protein library to be of plasma membrane origin. Using quantitative SWATH technology, we observed hundreds of proteins to be differentially expressed on defined T cell subsets, which were further differentially expressed during the course of cell activation. These data suggest that SWATH will likely be an important tool to both study protein expression patterns among different T cell lineages and uncover new immunological correlates of disease.

1221

3D visualisation and comprehensive analysis of the conduit network in entire murine lymph nodes

Kelch, I.D.^{1,2}, Bogle, G.^{1,3}, Sands, G.B.³, Phillips, A.R.J.^{1,2,4}, LeGrice, I.J.^{3,5}, Dunbar, P.R.^{1,2}

¹Maurice Wilkins Centre for Molecular Biodiscovery, The University of Auckland, Auckland, New Zealand, ²School of Biological Sciences, The University of Auckland, Auckland, New Zealand,

³Auckland Bioengineering Institute, The University of Auckland, Auckland, New Zealand, ⁴Faculty of Medical and Health Sciences, The University of Auckland, Department of Surgery, Auckland, New

Zealand, ⁵Faculty of Medical and Health Sciences, The University of Auckland, Department of Physiology, Auckland, New Zealand

A network of fibroblastic reticular cells forms a complex 3D mesh within lymph nodes (LNs) and constructs a conduit filtering system that distributes lymph-born immune stimulants and antigens within different cell compartments. Despite its crucial function in immune surveillance, the organisation of the labyrinthine conduit network and structural changes following immune challenge are still insufficiently defined. We developed innovative 3D imaging and computational techniques to produce high resolution images of entire murine lymph nodes, in order to explore their organisation at whole organ scale. Using the automated extended-volume confocal imaging system (EVIS), we generated unprecedented 3D images of the conduit network in a murine LN at 1 µm pixel resolution. The extensive imagery disclosed the precise arrangement of individual fibres within LN subcompartments, around high endothelial venules (HEVs), and revealed increasingly wider spacing within the T cell zone. We employed custom-designed computational tools to re-visualise these image data as a continuous 3D model and quantify topological features such as fibre diameters, lengths, and spacing. Our ongoing investigations are focussed on utilising this 3D map to compare fibre dimensions within functional LN subregions, as we have recently shown for the LN blood vasculature, and track structural changes after immune stimulation. The resulting data have the potential to advance current models of immune cell migration and improve our understanding of LN remodelling and its implications for LN functionality.

1222

Emerging technologies for the study of humoral mechanisms underlying human antibody responses

Ippolito, G.C.¹, Lavinder, J.J.¹, Lee, J.¹, Boutz, D.R.¹, Kouivaskaia, D.V.², Chromikova, V.³, Krammer, F.³, Chumakov, K.², Georgiou, G.¹

¹The University of Texas at Austin, Austin, United States, ²U. S. Food and Drug Administration, Center for Biologics Evaluation and Research, Silver Spring, United States, ³Icahn School of Medicine at Mount Sinai, New York City, United States

We have developed and patented technologies for the extensive molecular analysis of human antibody responses which allow for the direct comparison of protein-level serum antibody repertoires to DNA-level repertoires in circulating peripheral blood B cells. These techniques uncover completely new facets of the response to the inactivated poliovirus and seasonal influenza vaccines that have major implications for human health. To perform this analysis we capitalized on a series of interrelated, recently published technologies developed by the Georgiou lab including:

- an LC-MS/MS proteomic strategy for the quantitation and identification of the IgG antibody clonotypes that comprise the serum polyclonal repertoire to a given vaccine antigen;
- very high-throughput determination of natively paired VH and VL sequences in hundreds of thousands of B cells; and
- expression and functional characterization of representative serum antibodies.

In contrast to extensive prior studies by many labs, using small numbers of antibodies encoded by peripheral B cells and isolated by B-cell immortalization of single-cell cloning techniques—which have generally shown that B-cell receptors with very broad binding to different viral strains are generally quite rare—our data now show that broadly cross-reactive immunoglobulins are strikingly prevalent in human serum. When cloned and expressed as monoclonal antibodies, we document that top-ranking, highly abundant serum IgG clonotypes are enriched for cross-neutralizing and even cross-protective biologic activity.

1223

Harnessing human blood to examine bio-nano interactions at the cellular level

Glass, J.J.¹, Chen, L.², Crampin, E.³, Thurecht, K.², Mann, S.K.^{4,5}, Whittaker, M.R.⁶, Quinn, J.F.⁶, Davis, T.P.⁶, Such, G.K.⁵, Johnston, A.P.R.⁴, Kent, S.J.^{1,7}, De Rose, R.¹

¹ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, and Department of Microbiology and Immunology, The University of Melbourne, at the Peter Doherty Institute for Infection and Immunity, Melbourne, Australia, ²ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, and Australian Institute for Bioengineering and Nanotechnology, The University of Queensland, St Lucia, Australia, ³ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, and Systems Biology Laboratory, Melbourne School of Engineering, University of Melbourne, Parkville, Australia, ⁴ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, and Drug Delivery Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Australia, ⁵The University of Melbourne, Department of Chemistry, Parkville, Australia, ⁶ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Australia, ⁷Melbourne Sexual Health Centre and Department of Infectious Diseases, Alfred Health, Central Clinical School, Monash University, Melbourne, Australia

Aim: The capacity to predict interactions between nanomaterials and cells will benefit the rational design of novel nanomedicines. These fundamental relationships have not been adequately defined. By examining various nanoparticle systems with primary human blood cells, we are establishing principles of bio-nano interactions and aim to generate predictive relationships that can inform the next generation of nanomedicines.

Methods: Nanoparticles were generated with varying charge and surface chemistries, and incubated with primary human blood (cell association/uptake) or purified cells (immunoactivation) at 37°C and 4°C. Nanoparticle cell-association or activation markers were analysed by flow cytometry.

Results: We have determined that the properties of nanoparticles can significantly impact immunological outcomes, including immunoactivation and leukocyte uptake.

a. Hyperbranched polymers (HBPs) differentially activated primary human blood dendritic cells based on charge. HBP of 8 nm diameter were prepared and tested under endotoxin-free conditions. Cationic (+16 mV) HBPs activated myeloid but

not plasmacytoid dendritic cell populations while neutral and anionic (-15 mV) HBPs were not immunostimulatory.

b. Charge dictated HBP association with different immune cell subsets. All cell types associated with cationic HBPs, while at 37°C anionic HBPs preferentially associated with cells specialized for pathogen clearance and processing.

c. Terminal disulphide groups enhanced nanoparticle association with human blood components, particularly phagocytic cells.

d. Altering the chemical composition of nanoparticle surfaces can reduce phagocytic clearance. Brushed (vs. linear) poly(ethylene)glycol of same molecular weight enhanced phagocytic uptake.

Conclusion and future: By creating a nanoparticle 'characteristic matrix', we will apply mathematical modelling principles to generate a predictive model of bio-nano interactions.

1224

Standardization and quality control using control samples to minimize batch effects and improve reproducibility of mass cytometry (CyTOF) assays in human immunology studies

Kleinsteuber, K.^{1,2,3}, Corleis, B.¹, Rashidi, N.¹, Nchinda, N.^{1,2}, Lisanti, A.¹, Kwon, D.¹, Walker, B.^{1,2}

¹Ragon Institute of MGH, MIT and Harvard, Cambridge, United States, ²Howard Hughes Medical Institute, Chevy Chase, United States, ³Heinrich-Pette-Institute, Hamburg, Germany

Mass cytometry, a mass spectrometry based single cell technology, allows utilization of 35+ antibodies in a single sample and is a promising screening and surveillance tool in translational immunology. Despite availability of several analysis tools, a straightforward method for standardization and quality control within longitudinal mass cytometry studies is still lacking. We sought to develop a method to monitor batch effects and quality of individual samples in human immunology studies. Here, we demonstrate a protocol requiring only minimal changes to established staining protocols and propose a standardized analysis procedure allowing efficient quality control of mass cytometry experiments. Each sample is spiked with control PBMCs from a healthy donor, derived from a single large blood draw. Thus, all subsequent experiments are performed using the same control PBMCs. This method facilitates reproducible data analysis by:

1) performing quality control of each antibody in the panel, 2) monitoring batch effects, and 3) application of a robust gating strategy for patient samples. We demonstrate the utility of this method using two different experimental setups:

1) phenotyping of CD8⁺ T cells in HIV⁺ patients and 2) comparison of cytokine expression in bronchoalveolar lavage and peripheral blood samples from HIV-infected subjects. Control PBMCs within these experiments facilitated quality control based on positive populations for each antibody and exhibited potential substandard sample preparations. Semi-automated gating for each antibody using negative populations in the control PBMCs revealed a robust gating strategy and resulted in reproducible downstream analysis with diminished batch effects.

1225**Deep sequencing of T-cells specific for a mycobacterial glycolipid reveals shared T-cell receptor motifs with diagnostic potential in population-based studies**

DeWitt, W.¹, Quan, K.², Emerson, R.¹, Sherwood, A.¹, DeRosa, S.³, Finak, G.³, Gottardo, R.³, Day, C.⁴, Scriba, T.⁵, Robins, H.¹, Seshadri, C.²

¹Adaptive Biotechnologies Corp, Seattle, United States, ²University of Washington, Department of Medicine, Seattle, United States,

³Fred Hutchinson Cancer Research Center, Seattle, United States,

⁴Emory University, Atlanta, United States, ⁵University of Cape Town, Capetown, South Africa

The somatically rearranged complementarity determining region 3 (CDR3) of the T-cell receptor (TCR) α and β chains determine antigen-specificity of T cells, so CDR3 sequences are potential biomarkers of antigen-specific T-cell responses. Population-level convergence in TCRs binding a specific antigen presented by major histocompatibility complex (MHC) proteins is complicated by polymorphisms that modulate binding strategies across individuals with differing MHC haplotypes. We hypothesized that the non-polymorphic CD1 family of lipid antigen-presenting molecules would provide a structural constraint lacking in the MHC and facilitate population level convergence in CDR3 usage for a single lipid antigen. We used CD1b tetramers loaded with glucose monomycolate (GMM) to isolate GMM-specific T-cells from four subjects infected with *Mycobacterium tuberculosis* (*M.tb*). We used TCR immunosequencing to characterize the CDR3 sequences of *in-vitro* expanded and re-sorted tetramer-positive T-cells. We identified highly stereotyped TCR-a and TCR-b motifs present in all four donors, and clonal analysis support the co-expression of these motifs by a single T-cell. The TCR-b motif was largely absent in PBMC from 587 healthy bone marrow donors at low risk for *M.tb* infection in the USA. By contrast, we found significant enrichment in both the TCR-a ($p < 0.01$) and TCR-b ($p = 0.09$) motifs among active tuberculosis patients when compared to uninfected control subjects in South Africa. We confirmed the expansion of GMM-specific T-cells among tuberculosis patients using flow cytometry ($p = 0.001$). These data reveal the existence of an antigen-specific shared T-cell repertoire that could be used to develop molecular diagnostics for diseases such as tuberculosis.

1226**In vivo cellular barcoding using a novel Cre Lox system**

Miles, D.¹, Weber, T.², Dukes, M.³, Glaser, S.¹, Duffy, K.², Naik, S.¹

¹Walter and Eliza Hall Institute, Parkville, Australia, ²Hamilton Institute, Maynooth, Ireland, ³University of Strathclyde, Glasgow, United Kingdom

Cellular barcoding is a useful biotechnology tool that allows single cell lineage tracing in various biological systems by marking cells with unique DNA barcodes *ex vivo* using lentivirus, followed by transplantation in a live animal. Subsequent comparison of barcode profiles between progeny cell types establishes the lineage relationship between these cell types (e.g. shared barcodes indicate a 'common' progenitor, differing

barcodes indicates 'distinct' progenitors). The best characterised example of its utility is in the study of haematopoiesis. However, there are limitations to this current system including its relatively low barcode diversity (~2,500) and the inability to barcode cells in their living native environment (*in vivo*). To overcome these restrictions we have devised a barcoding method that relies upon the well-established Cre Lox system through use of multiple in-tandem barcodes flanked by alternating LoxP sites. By exploiting inherent sequence distance constraints during site-specific recombination we identify a cassette combination that allows maximal code diversity, greater than 10^{12} . Once constructed, we aim to use this tool to create stable barcodes to better understand haematopoiesis at the single cell level in a native *in vivo* context.

1227**Protective immunity against botulinum neurotoxin elicited by genetic immunization with PLGA nanoparticle formulated DNA vaccine**

Zeng, M.¹, Ruwona, T.², Xu, H.², Li, J.¹, Diaz-Arévalo, D.¹, Kumar, A.², Chen, Y.¹, Cui, Z.²

¹Texas Tech University Health Sciences Center El Paso, Center of Emphasis in Infectious Diseases, El Paso, United States, ²University of Texas at Austin, College of Pharmacy, Pharmaceutics Division, Austin, United States

Botulism is a rare but potentially life-threatening bacterial illness that is caused by botulinum neurotoxins (BoNTs) for which there is currently no approved vaccine. Recent efforts in developing vaccine candidates against botulism have been directed at the heavy chain fragment of BoNT, because antibodies against this region have been shown to prevent BoNT from binding to its receptor and thus to nerve cell surface, offering protection against BoNT intoxication. In the present study, it was shown that genetic immunization with plasmid DNA that encodes the 50 kDa C terminal fragment of the heavy chain of BoNT serotype C (i.e., BoNT/C-Hc50) and is carried by cationic poly (lactic-co-glycolic) acid (PLGA) nanoparticles induces stronger BoNT/C-specific antibody responses, as compared to immunization with the plasmid alone. Importantly, the antibodies have BoNT/C-neutralizing activity, protecting the immunized mice from a lethal dose of BoNT/C challenge. A PLGA nanoparticle formulated DNA vaccine encoding the Hc50 fragments of BoNT serotypes that cause human botulism may represent a viable vaccine candidate for protecting against botulinum neurotoxin intoxication.

1228**Identification of CD99 counter-receptor by pull-down method using dimeric CD99-human IgG Fc fusion proteins in combination with DTSSP crosslinking**

Takheaw, N.¹, Kasinrer, W.^{1,2}, Pata, S.^{1,2}

¹Division of Clinical Immunology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand, ²Biomedical Technology Research Center, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development

Agency at the Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand

CD99 is a human leukocyte surface molecule involving T cell activation, cell adhesion, cell migration and cell death. Its counter-receptor is, however, still a matter of controversy. In an attempt to identify CD99 counter-receptor, a pull-down assay was developed. The successful of the pull-down method is indeed depending on the bait protein structure. To obtain bait protein composed of a similar structure and post-translational modification to native CD99 protein, we produced secreted dimeric CD99-human IgG Fc fusion proteins in 293T cell line. The CD99-IgG Fc fusion proteins were, then, used to search for the CD99 ligand, in combination with 3,3'-dithiobissulfosuccinimidylpropionate (DTSSP) crosslinking, in various human cell lines by indirect immunofluorescence staining. Upon several cell lines tested, only THP-1 cell line showed positive staining with CD99 bait protein. To pull down CD99 counter-receptor, THP-1 cells were stained with CD99-IgG Fc fusion proteins, crosslinked with DTSSP and pulled down by protein G beads. By western blotting, the molecular weight of the pulled down protein was higher than the CD99 bait protein indicating the presence of CD99 counter-receptor in the pulled down material. In this study, we introduced here the pull-down method using a flexible-dimeric CD99 fusion protein in combination with DTSSP crosslinking for identification of its counter-receptor.

1229

Monoclonal mouse antibodies against PBMC subpopulations of New World camelids

Holzlohner, P.¹, Butze, M.¹, Hebel, N.¹, Weschke, D.¹, Schliebs, E.², Naumann, F.², Fünér, J.², Micheel, B.¹, Hanack, K.¹

¹University of Potsdam, Immunotechnology, Potsdam, Germany,

²Preclinics GmbH, Potsdam, Germany

The immune system of New and Old World camels is of special interest because it expresses besides the conventional four chain antibodies also heavy chain only antibodies. Furthermore camelids gain more and more significance as livestock. To investigate the immune system of camelids and to have a diagnostic tool, it would be advantageous to divide the different immune cell populations with the help of specific antibodies. The generation of immune cell-specific murine antibodies was performed by immunization of Balb/c mice with different camelid PBMC subclasses. For this llama blood was centrifuged and erythrocytes lysed with Geys Media. Different cell preparations were used for immunization. Monocytes were gained after a 2 hour plastic adhesion. Dendritic cells were generated from camelid monocytes after a 7 day treatment with human IL-4 and GM-CSF. In addition to that mice were immunized with total alpaca spleen or lymph nodes cell population, respectively. Monoclonal antibody-producing cells were generated by hybridoma technology. Immune cell-specific hybridomas were selected using a cell-based ELISA. Purified antibodies were used to stain different blood cell populations and investigate them via flow cytometry. Furthermore we used the generated monoclonal antibodies for magnetic cell sorting

of camelid PBMCs. We were able to generate monoclonal antibodies that could bind all populations of camelid PBMCs as well as antibodies that bind only to different populations of PBMCs. Due to their position and scattering in flow cytometry the cell populations could be related to camelid lymphocyte, granulocyte and monocyte populations.

1230

Whole organ imaging for analysis of immune responses in lymph nodes

Devi, S.^{1,2}, Hor, J.L.^{1,2}, Loi, J.K.^{1,2}, Heath, W.R.^{1,2}, Mueller, S.N.^{1,2}

¹The Peter Doherty Institute for Infection and Immunity, Department of Microbiology and Immunology, Parkville, Australia,

²The ARC Centre of Excellence in Advanced Molecular Imaging, University of Melbourne, Parkville, Australia

Interactions between immune cells are critical for the initiation and effector phases of immunity. To understand the cellular events involved in the priming of T cell responses to viral infection we have examined T cell interactions with dendritic cell (DC) subsets by intravital 2-photon microscopy. However, this method limits analysis to defined regions of the lymph nodes (LN). To examine and quantify interactions between T cells and DC in whole lymphoid organs, we utilised a novel clearing method that preserves fluorescent protein function and allows immunofluorescent antibody labelling *ex vivo* to identify multiple cell types concurrently. Using 2-photon microscopy on cleared LN we visualised the vascular and lymphatic networks, and remodeling after infection. We imaged CD4 and CD8 T cell clustering with migratory and resident DC after infection to determine regional differences in T cell subset activation by DC in whole LN. Thus, we describe a method to imaging whole cleared lymphoid organs *ex vivo* by 2-photon microscopy for quantitative spatial analysis of immune responses.

1231

Development and characterization of a novel cell-based biosensor for screening of inflammatory agents in bioaerosols

Khera, S.¹, Sharma, V.¹, Fejer, G.¹, Tyrrel, S.², Bennett, A.³, Jackson, S.¹

¹Plymouth University, Plymouth, United Kingdom, ²Cranfield University, Milton Keynes, United Kingdom, ³Public Health England, Porton Down, United Kingdom

Bioaerosol exposure has been associated with adverse effects on human health and airborne transmission of infections. The lack of technology to determine exposure-response relationship has hampered the assessment of health risks and development of regulatory frameworks associated with bioaerosol emissions. Cell-based biosensors have been recognized as potential leaders in the next generation of functional biosensing as they provide rapid and useful information on physiological responses to a variety of bioactive analytes. We have developed 2D and 3D cell cultures which show promise as cell-based biosensors in a variety of settings. Alveolar macrophages (AM) are the first line of defence against airborne environmental microbes. Recently, we have described a novel, continuously

growing, non-transformed, model of lung AMs (MPI cells). This robust system provides an excellent new model for AMs without restricted availability. We used MPI cells and bronchial epithelial cells to develop novel biosensors to characterize bioaerosol components (such as LPS) and their unique inflammatory signatures. Detection and characterization of responses to bioaerosol-derived microbial ligands was achieved through macrophage gene expression, including cytokine responses using Luminex multiplex platforms, RT-PCRs and proteomic analysis. The results from these experiments will provide the first direct model of human airway cell responses to LPS and other microbial molecules from bioaerosols. Moreover, understanding these cell responses will allow the future development of high throughput and potentially 'in-field' use of these cell-based biosensors.

1232

Immunobiosensor for measurement and characterization of soluble CD147 molecule

Laopajon, W.¹, Kampoun, T.¹, Takeaw, N.¹, Cheunsirikulchai, K.¹, Kasinrerak, W.², Pata, S.²

¹Faculty of Associated Medical Sciences, Chiang Mai University, Department of Medical Technology, Chiangmai, Thailand,

²Biomedical Technology Research Center, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency at the Faculty of Associated Medical Sciences, Chiang Mai University, Department of Medical Technology, Chiangmai, Thailand

Soluble CD147 (sCD147) is the shedding form of membrane bound CD147 which share at least two extracellular Immunoglobulin (Ig)-like domains and also has the ability to control cell functions. The evaluated expression of sCD147 is related to many diseases and can be observed in human body fluids. In this study, we developed the system to detect sCD147 by using anti-CD147 monoclonal antibody (mAb) which recognized the different domain epitopes of extracellular part of CD147 and BLItz biosensor. The single antibody system, anti-CD147-domain 1 mAb clone M6-1E9 showed the highest limit of detection (LOD) at concentration 890 ng/ml of CD147 recombinant protein (CD147Rg). For CD147 domain 2 detection, MEM6/6, anti-CD147 domain 2 mAb showed the LOD at 8.137 µg/ml. In sandwich system which can define the shedding structure of sCD147, a pair of primary mAb MEM6/3 (anti-CD147 domain 1) and secondary MEM6/6 antibodies showed the best LOD at concentration 66 ng/ml. In contrast, using MEM6/6 as primary mAb, M6-1E9 showed the good LOD at 1.093 µg/ml. All in all, the real time sCD147 detection systems were established which can be investigate the presence of sCD147 and also clarify the shedding structure. The generated strategies could be used as tools for better understanding CD147 associated- diseases, diagnosis and prognosis in the future.

1233

Performing systems immunology using next generation high-dimensional single-cell cytometry systems: bright lights and heavy metals

Ashhurst, T.^{1,2}, Lim, A.³, Duckett, L.⁴, Balderas, R.⁴, Fazekas de St Groth, B.^{2,5}, Smith, A.^{2,6}, King, N.^{1,2,6}

¹The University of Sydney, Pathology, Sydney, Australia, ²The University of Sydney and the Centenary Institute, The Ramaciotti Facility for Human Systems Biology (RFHSB), Sydney, Australia, ³BD Biosciences, Macquarie Park, NSW, Australia, ⁴BD Biosciences, San Jose, United States, ⁵The Centenary Institute, Camperdown, Australia, ⁶The University of Sydney and the Centenary Institute, Sydney Cytometry Facility, Sydney, Australia

The rise of mass cytometry has allowed for characterisation of the immune system in unprecedented detail, enabling the detection of more than 40 parameters simultaneously on single cells. Additionally, the comparative lack of spillover between reagents, when compared with fluorescence cytometry, has led to the development of assays capable of interrogating highly related cellular subsets. However, the development of new fluorescent dyes, and enhancement of acquisition electronics has led the way in the development of next generation fluorescence flow cytometers. Here we sought to employ these new cytometry techniques to move beyond bulk measurement of a system, and towards interrogating the complex interaction of large numbers of cells that define the behavior of the immune system. For mass cytometry, we utilised a CyTOF2/Helios (Fluidigm) platform. For fluorescence cytometry we equipped a 10-laser Becton Dickinson (BD) LSR-II special order research product (SORP) platform with a newly developed electronics system, increasing the number of detectable signals to ~30. We incorporated a range of traditional dyes into our panels, in addition to novel polymer dyes developed by Sirigen. Here we also describe the development of a novel optimisation program, combining the use of the existing cytometer setup and tracking (CS&T) program, with newer methods to generate optimised voltage target values for panels of up to 30-colours. Using both systems, we conducted comprehensive profiling of the murine haematopoietic system in the bone marrow during viral infection. Our investigation revealed cell cycle and signaling modifications that resulted in enhanced monopoietic outputs.

1234

Selelction of PBMC from whole blood with FABian[®] by using CD81+ marker

Stanar, K.¹, Stadler, H.¹, Gerdes, W.², Bergmann, S.², Kiene, M.-L.¹

¹IBA GmbH, Göttingen, Germany, ²IBA GmbH, Leipzig, Germany

Objective: Peripheral blood mononuclear cells (PBMCs) selections often apply methods that are time consuming and complex. Additionally, target cells are manipulated by diverse selection reagents. To cope with these disadvantages we applied the magnet-free, mild and fast T-CATCHM technology to the automatic system FABian[®]. The research was aimed at the isolation of PBMCs from whole blood in highest yields, purity and viability without using Ficoll.

Method: FABian® was equipped with a column containing an Agarose-Strep-Tactin® matrix and 11 tubes as carriers for the required reagents. After drawing up CD81-Fab-fragment into the column, whole blood was passed through the matrix. Only CD81+ cells bind to the Fab -Strep-agarose matrix. Unbound cells were washed out. Washing steps were followed by an elution with biotin resulting in the release of PBMCs.

Results: The average purity of the selected CD81+ cells was >90% with a viability of >90% and a yield >10x10⁶ CD81+ cells out of samples of 9ml whole blood. During washing steps only a loss of 6% of CD81+ cells was detected and led to the elimination of neutrophil and basophil granulocytes.

Conclusion: The isolation of PBMCs with the T-CATCHTM technology applied with FABian® presents a valid method to isolate non-activated, label-free authentic target cells in high purity, viability and yields. PBMCs isolated with FABian® far exceed the results of Ficoll-based isolations in purity and yield. With Fabian® various cell types can be selected from whole blood and buffy coats. Sequential isolations to select specific cells (eg. CD14 or CD19) are possible.

1235

Simultaneous flow cytometric measurement of attachment and phagocytic processes of phagocytes

Laopajon, W.¹, Takheaw, N.¹, Pata, S.^{1,2}, Kasinrerk, W.^{1,2}

¹Division of Clinical Immunology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiangmai, Thailand, ²Biomedical Technology Research Center, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency at the Faculty of Associated Medical Sciences, Chiang Mai University, Chiangmai, Thailand

Phagocytosis can be divided into three stages including attachment, internalization, and degradation of the microbes. The available phagocytosis assays, however, could not differentiate the stages of phagocytosis. In this study, the method for concurrent detection the attachment and engulfment of phagocytosis was developed by using latex beads coated with lipopolysaccharide (LPS), rabbit IgG, and carboxyfluorescein diacetate succinimidyl ester (CFSE). The generated CFSE-LPS-IgG coated latex beads were incubated with whole blood at 37°C for 1 hour. After incubation, the cells were stained with PE-Cy5.5 anti-rabbit IgG antibody. The phagocytosis was analyzed by flow cytometry. The results demonstrated that CFSE and IgG coated on beads could be detected by flow cytometer. The developed methods could be used for determination of attachment and phagocytic processes. In the presence of NaN₃ and NaF, phagocytosis inhibitors, inhibition of both the attachment and the engulfment was demonstrated. Under on-ice condition, the engulfment step was blocked, while the attachment process could still be observed. In summary, the established methods could be employed for the detection of both attachment and engulfment of phagocytes, simultaneously. This method is a valuable tool for the diagnosis of phagocytosis disorder as well as the study of the molecules involved in phagocytosis.

Genetics

1236

Interethnic variability in sickle cell disease pathophysiology is associated with polymorphisms of the endothelin-1 gene

Noble, J., Pirela, L., Thomas, B.

Rochester Institute of Technology, Biomedical Sciences, Rochester, United States

Sickle cell disease, a multisystem disorder arising from the mutation of the beta globin gene, and characterized by multiple complications has been shown to demonstrate extensive variability in disease severity among and between individuals, the variability highlighted by differing genetic haplotypes.

Reports of functional significance due to polymorphisms of endothelial nitric oxide synthase (eNOS) and endothelin-1 genes abound. The role of these polymorphisms in regulating disease complications among African Americans is presently unclear.

To deconvolute African American SCD, we examined the genetic diversity and haplotype frequency of eNOS and ET-1 polymorphisms in disease (n=331) and control (n=379) groups, utilizing a PCR-RFLP assay. We report that allelic and genotypic frequencies of eNOS polymorphic variants are not significantly different between cases and controls. eNOS homozygote mutants, shown to have clinical significance elsewhere, demonstrated no statistical significance in our study. Contrary to our African cases, the endothelin-1 homozygote mutant (5665T) showed significant difference between disease and control groups in genotypic (P=2.84E-12) and allelic frequencies (P=2.20E-16). Additionally, the most common haplotype in our study is the combination of T786C wild-type variant with mutant variants of 5665T (ET-1) and 298Asp (eNOS). The lack of difference in eNOS polymorphisms between African and American groups implies disease has retained its evolutionary origin.

The significance of this observation per disease severity or individual clinical outcomes and implications for advancing personalized care for sickle cell disease patients, utilizing pharmacogenomics tools based on ethnic population structure is discussed.

1237**Australian Autoinflammatory Diseases RegistrY (AADRY): a national approach to the genetic and immunological evaluation of patients with suspected autoinflammatory disease**

Moghaddas, F.^{1,2}, Allen, R.³, Ellis, J.⁴, Smart, J.⁵, Munro, J.³, Oshlack, A.⁶, Cox, A.^{7,8}, Ojaimi, S.⁹, Harrison, L.¹⁰, Piper, S.^{7,8}, Campbell, D.¹¹, Wong, M.¹¹, Vekic, D.¹², Woods, J.¹², Bryant, V.¹³, Cains, G.¹², Chan, D.¹⁴, Gillis, D.¹⁵, Gray, P.¹⁶, Hissaria, P.¹⁷, Akikusa, J.³, Gowdie, P.^{7,8}, Slade, C.¹⁰, Katelaris, C.¹⁸, Mehr, S.¹¹, Wicks, I.^{1,2}, Masters, S.^{1,2}

¹Walter and Eliza Hall Institute of Medical Research, Inflammation, Melbourne, Australia, ²The University of Melbourne, Department of Medical Biology, Melbourne, Australia, ³The Royal Children's Hospital, Paediatric Rheumatology, Melbourne, Australia, ⁴Murdoch Children's Research Institute, Population Health, Melbourne, Australia, ⁵The Royal Children's Hospital, Paediatric Allergy and Immunology, Melbourne, Australia, ⁶Murdoch Children's Research Institute, Bioinformatics, Melbourne, Australia, ⁷Monash Children's Hospital, Paediatric Rheumatology, Melbourne, Australia, ⁸Monash University, Clayton, Australia, ⁹Monash Children's Hospital, Paediatric Infectious Diseases, Melbourne, Australia, ¹⁰The Royal Melbourne Hospital, Immunology and Allergy, Melbourne, Australia, ¹¹The Children's Hospital at Westmead, Immunology, Westmead, Australia, ¹²Liverpool Hospital, Dermatology, Sydney, Australia, ¹³Walter and Eliza Hall Institute of Medical Research, Immunology, Melbourne, Australia, ¹⁴Women's and Children's Hospital, Immunology, Adelaide, Australia, ¹⁵Royal Prince Alfred Hospital, Immunology, Woolloongabba, Australia, ¹⁶Sydney Children's Hospital, Immunology, Sydney, Australia, ¹⁷Royal Adelaide Hospital, Immunology, Adelaide, Australia, ¹⁸Campbelltown Hospital, Immunology, Sydney, Australia

Traditionally defined as periodic fever syndromes lacking markers of autoimmunity such as high titre self reactive T cells and auto-antibodies, the spectrum of monogenic autoinflammatory diseases has broadened substantially in recent years. The clinical heterogeneity of newly described conditions such as STING associated vasculopathy with onset in infancy (SAVI) and Deficiency in ADA2 (DADA2) has forced reconsideration of traditional designations, with the common link between these disorders being inappropriate inflammation and cytokine dysregulation. These new diagnoses can be attributed to the introduction of whole exome sequencing (WES) into the diagnostic algorithm of patients with suspected, but currently genetically undefined, autoinflammatory diseases. We believe it's important to take a national approach to the diagnosis of autoinflammatory disorders given their rarity and introduce the establishment of AADRY, an Australian Autoinflammatory Diseases RegistrY. AADRY proposes to collect epidemiological and clinical data on patients with suspected and confirmed autoinflammatory diseases, and will perform whole exome sequencing in patients who are yet to be genetically characterised. We also aim to perform in vitro testing and harness CRISPR-Cas9 gene editing technology to determine the functional significance of novel variants and their potential contribution to clinical phenotype. We describe bioinformatic analyses and other results from the first 12 AADRY cases analysed, including one trio and one family of five.

1238**HLA class II protein stability and disease susceptibility**

Miyadera, H.^{1,2}, Ohashi, J.³, Tokunaga, K.²

¹National Center for Global Health and Medicine, Research Center for Hepatitis and Immunology, Ichikawa, Japan, ²University of Tokyo, Graduate School of Medicine, Bunkyo-ku, Japan, ³University of Tokyo, Graduate School of Science, Bunkyo-ku, Japan

Genes encoding the human leukocyte antigens (HLA) are associated strongly with susceptibility/protection to various immunological diseases; however, underlying mechanisms have not been fully explained. We hypothesized that allelic variation in the protein stability of HLA, in addition to variation in the peptide-binding spectrum, might contribute to disease susceptibility. We tested this hypothesis by analyzing the association between the stability of HLA-DQ and genetic risk for type 1 diabetes (T1D). As reported previously through the SDS-stability assay (Ettinger et al. *J Immunol* 1998), we found a correlation between HLA-DQ instability and susceptibility to T1D (Miyadera, et al. *J Clin Invest* 2015). Moreover, polymorphic variants that decrease the intrinsic stability of HLA-DQ were associated with T1D risk. We next estimated the stability of HLA-DR and -DP allele products through the cell-surface expression assay and analyzed their associations with various disorders, including T1D, narcolepsy, and chronic hepatitis B. In this presentation, we show the stability profiles for HLA-DR and -DP allele products and their relation with disease risk, and discuss potential mechanisms that might underlie genetic associations of HLA with diseases.

1239**ATG16L1 gene polymorphism associated with chronic hepatitis B virus infection in a Thai population**

Kimkong, I.^{1,2}, Tantithavorn, V.¹, Hirankarn, N.³, Tangkijvanich, P.⁴

¹Faculty of Science, Kasetsart University, Department of Microbiology, Bangkok, Thailand, ²Kasetsart University, Center for Advanced Studies in Tropical Natural Resources, National Research University, Bangkok, Thailand, ³Faculty of Medicine, Chulalongkorn University, Center of Excellence in Immunology and Immune Mediated Diseases, Department of Microbiology, Bangkok, Thailand, ⁴Faculty of Medicine, Chulalongkorn University, Research Unit of Hepatitis and Liver Cancer, Department of Biochemistry, Bangkok, Thailand

Hepatitis B virus (HBV) infection is a serious and life-threatening health problem. Approximately 350 million people are chronically infected with HBV and estimated 1 million deaths per year from HBV-related liver disease. Autophagy is an important pathway for host defense against viral infection. Various studies have reported the association of autophagy 16-like 1 (ATG16L1) polymorphisms with certain diseases. However, the association of this gene with chronic HBV infection is still not investigated. Therefore, we explored the effect of single nucleotide polymorphism (SNP) of ATG16L1 (rs2241880) in chronic HBV infection. We recruited 237 Thai patients with chronic HBV infection (110 with HCC and 127 without HCC), 124 recovered individuals and 139 healthy controls in this study. The rs2241880 polymorphism was analyzed by polymerase chain

reaction-restriction fragment length polymorphism (PCR-RFLP) method. The results showed that A allele of ATG16L1 rs2241880 was associated with an increased risk in chronic HBV infection as compared to healthy controls [OR (95% CI) = 1.50 (1.07-2.11), $P = 0.014$]. To test the effect of rs2241880 genotypes, we compared mRNA expression level of ATG16L1 among individuals containing different genotypes (AA, AG and GG). We found that ATG16L1 mRNA level of the subjects carrying AA and AG genotypes of rs2241880 was down-regulated when compared with GG genotype ($P = 0.018$; $P = 0.051$, respectively). Our study suggested that A allele of ATG16L1 rs2241880 may provide a risk effect in this disease.

1240

Replication of the reported deletion of the GLUT3 gene SLC2A3 on chromosome 12 conferring substantial protection against rheumatoid arthritis

Simpfendorfer, K.R., Kothari, H., Shih, A., Li, W., Armstead, B., Lee, A., Gregersen, P.K.

Feinstein Institute for Medical Research, Robert S. Boas Center for Genomics and Human Genetics, Manhasset, United States

The deletion of a 129kb segment on chromosome 12p13.31 is a low frequency copy number variant resulting in loss of the glucose transporter genes encoding GLUT3 and GLUT14. Recently, deletion of the 129kb segment at 12p13.31 was shown to be substantially protective for Rheumatoid Arthritis in Swedish, UK and US cohorts (Veal *et al* Human Mutation 2013). *SLC2A3* (encoding GLUT3) is most highly expressed in brain and human white blood cells, predominantly in granulocytes. Neutrophils are the predominant infiltrating cell type in inflamed RA joints, and neutrophil derived neutrophil extracellular traps (NETs) may be the primary source of antigen such as citrullinated proteins to which RA autoantibodies (anti-citrullinated protein antibodies, ACPA) arise. Given the principal role of neutrophils in RA, and the high expression of *SLC2A3* in neutrophils, we hypothesize that subjects lacking one copy of *SLC2A3* are protected against RA because their neutrophils are deprived of glucose. To replicate the association between 12p13.31 deletion and protection against RA we have designed a fast and reliable genotyping assay to screen a US cohort of RA cases and matching controls. This assay negates the requirement for the previously used lengthy and unreliable P1 PCR assay, or for SNP chip PennCNV analysis. If replication of the initial report is achieved, we will proceed with functional studies to investigate the association between loss of *SLC2A3* and pathogenic neutrophil phenotypes.

1241

Slc11a1 gene modulates immune-inflammation genes in macrophages during pristane-induced arthritis in mice

Correa, M.A.¹, Canhamero, T.¹, Borrego, A.¹, Katz, I.², Jensen, J.R.¹, Guerra, J.L.³, Cabrera, W.H.K.¹, Starobinas, N.¹, Gonçalves, J.F.¹, Ribeiro, O.G.¹, Ibañez, O.M.¹, De Franco, M.¹

¹Instituto Butantan, Sao Paulo, Brazil, ²Instituto Pasteur, Sao Paulo, Brazil, ³Universidade Anhembi Morumbi, Sao Paulo, Brazil

Pristane-induced arthritis (PIA) in AIRmax mice homozygous for *Slc11a1 R* and *S* allele was used in this study to characterize the role of *Slc11a1* gene on immune response. Previous reports showed that the presence of *Slc11a1 S* allele increased the incidence and severity PIA in AIRmaxSS, suggesting that this gene could interact with inflammatory loci to modulate PIA. AIRmaxSS macrophages demonstrated exacerbated cellular and gene expression profiles during PIA, with higher expression/production of H2O2, NO, IL-1 β , IL-6, TNF- α and several chemokines. On the other hand, *Slc11a1 R* allele could control the intensity of macrophage activation, restricting the arthritis development. Our data demonstrated the fine tune role of *Slc11a1* alleles modulating macrophage activation and consequently PIA susceptibility in these lines.

1242

The H2-Ab1 Class II gene regulates the level of susceptibility and immune responses in the mouse model of tuberculosis infection

Korotetskaya, M., Apt, A., Logunova, N.

Central Institute for Tuberculosis, Immunology, Moscow, Russian Federation

Tuberculosis (TB) pathogenesis is multistage, highly complex sequence of events, with many cell types involved. To identify genetic loci determining the spectrum of natural genetic resistance to *Mycobacterium tuberculosis* (MTB) three teams of researchers applied the forward genetic approach (from the phenotype to gene) for genome-wide association studies on the basis of different combinations of inbred mouse strains displaying resistant and susceptible phenotypes. Regardless of parental combinations, all three groups reported complex, polygenic control of host susceptibility/resistance to TB. Our group mapped three quantitative trait loci (QTL) on chromosomes 3, 9 and 17. The latter QTL was located within the mouse major histocompatibility complex (H2).

We have established a panel of congenic, MHC-recombinant mouse strains bearing differential small segments of chromosome 17 transferred from the TB-susceptible I/St (*H2^b*) strain onto the genetic background of TB-resistant C57BL/6 (B6) mice (*H2^b*). This allowed narrowing the QTL interval to 17Ch: 33, 77-34, 34 Mb, containing 36 protein-encoding genes. In two recombinant strains, B6.I.100 and B6.I.139, recombination breakpoints occurred in different sites of the *H2-Ab1* gene, providing polymorphic variations in the domain $\beta 1$ of the $A\beta$ -chain. These variations were sufficient to produce different TB-relevant phenotypes: the more susceptible B6.I-100 strain demonstrated shorter survival time, more rapid body weight loss, higher mycobacterial loads in the lungs and more severe lung histopathology compared to the more resistant B6.I.139 strain. Thus, we directly demonstrated for the first time that the classical *H2-Ab1* Class II gene is involved in TB control.

1243**An examination of single nucleotide polymorphisms in transient receptor potential ion channels and acetylcholine receptors in chronic fatigue syndrome patients**

Marshall-Gradisnik, S.^{1,2}, Chacko, A.^{1,2}, Johnston, S.^{1,2}, Smith, P.¹, Nilus, B.³, Staines, D.¹

¹*Menzies Health Institute Queensland, National Centre for Neuroimmunology and Emerging Diseases, Parklands, Australia,*

²*Griffith University, School of Medical Science, Parklands, Australia,*

³*University of Leuven, Leuven, Belgium*

Background: Chronic fatigue syndrome (CFS) is an unexplained disorder characterised by debilitating fatigue, neurocognitive, immune, gastrointestinal, cardiovascular and autonomic symptoms. Transient receptor potential (TRP) ion channels as well as Acetylcholine receptors (AChRs) have important roles in calcium and ACh signaling vital for physiological functioning. The purpose of this study was to examine their role in CFS.

Methods: The study comprised 115 CFS patients (age = 48.68±1.06 years) and 90 non-fatigued controls (age = 46.48±1.22 years). CFS patients were defined according to the Fukuda criteria for CFS. A total of 240 single nucleotide polymorphisms (SNPs) for 21 TRP genes and 464 SNPs for 9 AChR genes were examined via the Agena Biosciences iPLEX Gold assay. Statistical analysis was performed using the PLINK analysis software.

Results: Thirteen SNPs for TRPs and 17 SNPs for AChRs were significantly associated with CFS patients compared with the controls. The majority were associated with TRPM3, TRPC4, mAChRM3 and nAChRa10. Furthermore, 11 genotypes were identified from these SNPs that were significant in TRPM3 (rs12682832; rs11142508; rs3763619) and mAChM3R (rs12036141; rs589962; rs1072320; rs7543259; rs7520974; rs726169; rs6669810; rs6429157) in CFS patients compared with non-fatigued controls.

Conclusion: The findings of this pilot study suggest TRP ion channels and AChRs that are predominantly M3, are associated with CFS. SNP anomalies in TRP and AChR genes may contribute to the aetiology and pathomechanism of CFS.

1244**Comprehensive analysis of cytokine gene polymorphisms defines the association of IL-12 gene with ophthalmopathy in Korean children with autoimmune thyroid disease**

Jang, J.-P.¹, Cho, W.-K.², Baek, I.-C.¹, Choi, E.-J.³, Shin, D.-H.¹, Suh, B.-K.², Kim, T.-G.¹

¹*The Catholic University of Korea, Department of Microbiology, Seoul, Korea, Republic of,*

²*The Catholic University of Korea, Department of Pediatrics, College of Medicine, Seoul, Korea,*

³*The Catholic University of Korea, Hematopoietic Stem Cell Bank, College of Medicine, Seoul, Korea, Republic of*

In early onset autoimmune thyroid disease (AITD) showing a strong genetic tendency, cytokines have been suggested to play a critical role in the development of AITD. To directly compare the influences of several cytokine gene polymorphisms, 25 single nucleotide polymorphisms (SNPs) in 17 cytokine genes were analyzed on 104 Korean children with AITD [Hashimoto's

disease (HD) = 44, Graves disease (GD) = 60 (thyroid-associated ophthalmopathy (TAO) = 29, non-TAO = 31)] and 192 controls. Compared with healthy controls, any significant association with polymorphisms of cytokine genes was not found in HD and GD. Among GD patients, non-TAO group only showed significant associations with IL-12 C allele (rs3212227: A>C) (76.6% vs. 51.6%, OR = 0.3 [0.15-0.71], *P* = 0.007). Particularly, the frequency of IL-12 C allele was significantly lower in the non-TAO group than in the TAO group (82.8% vs. 51.6%, *P* = 0.018). Our comprehensive analysis of cytokine gene polymorphisms suggests that IL-12 gene may play impact on specific pathogenesis of ophthalmopathy in Korean children with AITD.

1245**Haplotype investigation of Killer cell immunoglobulin-like receptor genes from natural killer cells in chronic fatigue syndrome/myalgic encephalomyelitis patients**

Huth, T.^{1,2}, Brenu, E.^{1,2}, Staines, D.^{1,2}, Marshall-Gradisnik, S.^{1,2}

¹*National Centre for Neuroimmunology and Emerging Diseases, Menzies Health Institute Queensland, Gold Coast, Australia,*

²*School of Medical Sciences, Griffith University, Gold Coast, Australia*

Sixteen Killer cell immunoglobulin-like receptor (KIR) genes encoding inhibitory or activating receptors on Natural Killer (NK) cells have been characterised. KIRs are genetically diverse and can be classified as haplotype A or B. The predominance of inhibitory genes in haplotype A and activating genes in haplotype B suggests a distinct role of KIR haplotypes in governing effector functions of NK cells. Reduced NK cell cytotoxic activity has been consistently reported in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) patients and KIR haplotypes remain to be investigated. Therefore, the aim of this paper was to examine KIR haplotypes in CFS/ME patients. NK cells were isolated and deoxyribonucleic acid was extracted from CFS/ME patients and non-fatigued controls (NFC). KIR genotyping was performed on an Illumina MiSeq platform and the sequencing data was aligned to the Immuno Polymorphism-KIR Database to determine KIR haplotypes. A total of 20 CFS/ME patients and 20 non-fatigued controls (NFC) were included in this study. A lower frequency of the telomeric A/B motif (*p* < 0.05) was observed in CFS/ME patients compared to NFC. This pilot study is the first to report differences in the frequency of KIR on the telomeric A/B motif in CFS/ME patients. As the activity of NK cells is governed by the balance between activating and inhibitory signals, differences in the gene content profile of KIR haplotypes may create different activation thresholds for NK cells in CFS/ME patients. Further investigations are required to validate these findings in a larger cohort of CFS/ME patients.

1246

Association of CD40 functional genetic variants and CD40 mRNA expression with rheumatoid arthritis from western Mexico

Román Fernández, I.V.¹, Ávila Castillo, D.F.¹, Cerpa Cruz, S.², Gutiérrez Ureña, S.², de la Cruz Mosso, U.¹, Hernández Bello, J.¹, Muñoz Valle, J.F.¹

¹Universidad de Guadalajara, Instituto de Investigación en Ciencias Biomédicas (IICB), Guadalajara, Mexico, ²O.P.D. Hospital Civil de Guadalajara Fray Antonio Alcalde, Servicio de Reumatología, Guadalajara, Mexico

Rheumatoid arthritis (RA) is a multifactorial chronic inflammatory autoimmune disease. The *CD40* locus has been identified as a genetic susceptibility marker for RA. In RA, CD40 is involved in the production of autoantibodies, proinflammatory cytokines and other molecules that promotes joint destruction. The *CD40* single nucleotide polymorphisms (SNPs) rs1883832 and rs4810485 have been associated with RA and other autoimmune inflammatory diseases. Several studies suggest that both SNPs affect *CD40* mRNA and protein expression. The aim of this study was to investigate the association of these *CD40* SNPs with susceptibility to RA and their influence in *CD40* mRNA levels in western Mexican population. A total of 278 RA patients and 318 healthy subjects (HS) were included. Genomic DNA and total RNA were obtained from peripheral blood leukocytes. Genotyping was performed by PCR-RFLP method and mRNA levels were determined by real-time qPCR. Minor allele frequencies for rs1883832 and 4810485 were 18.5% and 18.9% in the RA group and 19.5% and 19.8% in HS, respectively; no significant differences were found. In addition, these genetic variants were not associated with the presence of autoantibodies or the clinical activity of the disease, when stratified by genotype. *CD40* mRNA expression was elevated in RA patients compared to HS (1.5 fold), however, no differences were found between mRNA expression and the different genotypes. These results suggest that the *CD40* SNPs rs1883832 and rs4810485 are not susceptibility markers for RA in western Mexican population; moreover, further studies are needed to clarify its role in *CD40* mRNA expression.

1247

Expression of Interferon Regulatory Factor-5 (IRF5) in asthmatic and non-asthmatic patients

Voon, Y.N.¹, Lim, K.C.¹, Wong, S.F.¹, Mak, J.W.¹, Lee, K.M.¹, Woo, K.²

¹International Medical University, Bukit Jalil, Malaysia, ²Gleneagles Medical Centre, Kuala Lumpur, Malaysia

Asthma is a very common inflammatory disease of the respiratory system. Asthma affects about 1 million people in the United States and 24% of children in the state of Selangor, Malaysia. It is postulated that an imbalance in the Th1 and Th2 immune responses may contribute to the pathophysiology of atopic asthma. IRF5 encodes key transcription factors in the production of Type 1 interferons and a number of inflammatory cytokines. Polymorphism in IRF-5 expression had been found to be associated with several autoimmune diseases such as systemic lupus erythematosus, rheumatoid

arthritis and systemic sclerosis. Interestingly, polymorphism in IRF5 expression had also been associated with atopic asthma in Dutch and English populations. However, the association between IRF5 polymorphism and asthma in the Asian population has yet to be established. Therefore, this study was conducted to determine the association of IRF5 gene polymorphisms (rs10954213/rs11770589 and rs2004640) and asthma in the Malaysian population. Genomic DNA was isolated from buccal cells and sequenced to identify IRF5 polymorphisms (rs10954213/rs11770589 and rs2004640). So far, 187 non-asthmatic samples and 152 asthmatic samples had been sequenced for the detection of IRF5 gene polymorphisms at rs10954213 and rs11770589, whereas 147 non-asthmatic samples and 150 asthmatic samples had been sequenced for the detection of IRF5 gene polymorphism at rs2004640. Results showed no association between IRF5 gene polymorphisms (rs10954213/rs11770589 and rs2004640) and asthma in the Malaysian population. Through this study, IRF5 polymorphisms (rs10954213/rs11770589 and rs2004640) may not be applicable biomarkers for the Malaysian population.

Lymphocyte Signalling

1248

FcγRIIB-independent mechanisms controlling membrane localization of the inhibitory phosphatase SHIP in human B cells

Pauls, S.¹, Ray, A.¹, Hou, S.¹, Vaughan, A.², Cragg, M.², Marshall, A.¹

¹University of Manitoba, Immunology, Winnipeg, Canada,

²University of Southampton, Southampton, United Kingdom

SHIP is an important regulator of immune cell signaling that functions to dephosphorylate the phosphoinositide PIP₃ at the plasma membrane and mediate protein-protein interactions. One established paradigm for SHIP activation involves its recruitment to the phosphoITIM motif of the inhibitory receptor FcγRIIB. While SHIP is essential for the inhibitory function of FcγRIIB, it also has critical modulating functions in signaling initiated from activating immunoreceptors such as B cell antigen receptor. Here we find that SHIP is indistinguishably recruited to the plasma membrane after BCR stimulation with or without FcγRIIB co-ligation in human cell lines and primary cells. Interestingly, fluorescence recovery after photobleaching analysis reveals differential mobility of SHIP-EGFP depending on the mode of stimulation, suggesting that while BCR and FcγRIIB can both recruit SHIP, this occurs via distinct molecular complexes. Mutagenesis of a SHIP-EGFP fusion protein reveals that the SHIP-SH2 domain is essential in both cases while the C-terminus is required for recruitment via BCR stimulation, but is less important with FcγRIIB co-ligation. Experiments with pharmacological inhibitors reveal that Syk activity is required for optimal stimulation-induced membrane localization of SHIP, while neither PI 3-kinase or Src kinase activity are essential. BCR-induced association of SHIP with binding partner Shc1 is dependent on Syk, as is tyrosine phosphorylation of both partners. Our results indicate that FcγRIIB is not uniquely able to promote membrane recruitment of SHIP, but rather modulates

its function via formation of distinct signaling complexes. Membrane recruitment of SHIP via Syk-dependant mechanisms may be an important factor modulating immunoreceptor signalling.

1249

Control of B cell activation and migration by PI 3-kinase: role of inositol polyphosphate 4-phosphatases

Hou, S., Li, H., Marshall, A.

University of Manitoba, Immunology, Winnipeg, Canada

Balanced activation of the PI3K pathway by antigen and other stimuli is required for effective humoral immunity while avoiding autoimmunity and lymphoproliferative disease. The critical regulatory phosphatase SHIP dephosphorylates the PIP3 generated by PI3K to produce a distinct molecule PI(3,4)P2. A number of key signaling molecules bind to PIP3, including the protein kinase Btk; however much less is known about the function of PI(3,4)P2. The inositol phosphatase INPP4A can specifically hydrolyze PI(3,4)P2; however the functions of this phosphatase in B cell activation are unknown. We hypothesized that INPP4A can provide a tool to deplete PI(3,4)P2 and determine its range of functions. Human B lymphocytes over-expressing active or phosphatase-dead INPP4A were generated, and the cells expressing active enzyme were found to generate lower levels of PI(3,4)P2 upon chemokine or BCR stimulation. Chemokine-induced migration responses were inhibited by active, but not inactive INPP4A. An assessment of BCR signaling was carried out that compared BCR-stimulated cells expressing active versus inactive INPP4A in a screen encompassing over 800 protein phosphorylation sites. The results indicate that active INPP4A can suppress phosphorylation of Akt and known Akt targets. We are currently assessing whether PDK1, a major kinase upstream of Akt which also directly binds PI(3,4)P2, is regulated by INPP4A. Interestingly, other kinases known to interact with PDK1 (PKC δ , RSK) or to be directly phosphorylated by PDK1 (PKC γ) showed decreased phosphorylation in cells expressing active INPP4A. We are currently testing the hypothesis that PDK1/Akt and Btk/PLC γ 2 signalosomes are differentially regulated by INPP4A.

1250

Interleukin-21 mediated STAT1 activation in the germinal centre response to T-dependent antigen

Jandl, C.^{1,2}, Loetsch, C.^{1,2}, Warren, J.¹, King, C.^{1,2}

¹Garvan Institute of Medical Research, Immunology Division, Sydney, Australia, ²St. Vincent's Clinical School, UNSW Australia, Department of Medicine, Sydney, Australia

Interleukin-21 (IL-21) signalling plays a fundamental role for the germinal centre (GC) reaction to T-dependent antigen supporting both the generation of high-affinity antibody producing GC B cells as well as T follicular helper (Tfh) cells. Although the importance of IL-21 mediated signalling for Tfh cells is widely appreciated, the signalling events downstream of the IL-21 receptor remain largely unknown. Here, we investigate the importance signal transducer and activator of transcription

(STAT) proteins activated by IL-21 receptor ligation on Tfh cells, with the help of mice harbouring a damaging point mutation within the cytoplasmic tail of the IL-21 receptor (*Il21r^{STAT}* mice). In response to IL-21, *Il21r^{STAT}* lymphocytes exhibited diminished STAT1 phosphorylation whereas STAT3 signalling remained intact. In response to T dependent antigen *Il21r^{STAT}* mice exhibited decreased GCs with a T cell intrinsic reduction in Tfh cells as well as a B cell extrinsic reduction in both GC B cell and IgG1+ class-switched B cells. The reduction of Tfh cells was more profound in *Il21r^{STAT}* mice than in *Il21r^{-/-}* mice, indicating a failure of IL-6 redundancy in the former. RNAseq analysis of *Il21r^{STAT}* CD4+ T cells exhibited a unique gene expression profile after *in vitro* stimulation with IL-21, with an anticipated decrease in the expression of STAT1 target genes, MAP kinase pathway genes as well as a profound increase in unique ubiquitination pathway genes that modulate the Jak-STAT pathways. Taken together, these findings indicate a hitherto unappreciated role for IL-21 mediated STAT1 signalling on T cells in the GC reaction.

1251

Search for endogenous molecules recognized by C-type lectin receptor Dectin-2

Mori, D.¹, Saijo, S.², Yamasaki, S.¹

¹Kyushu University, Medical Institute of Bioregulation, Division of Molecular Immunology, Fukuoka, Japan, ²Chiba University, Medical Mycology Research Center, Department of Molecular Immunology, Chiba, Japan

C-type lectin receptors (CLRs) recognize various ligands derived from both damaged-self and pathogens to trigger immune responses. It is known that CLRs recognize various pathogen-derived ligands, however only a few self-ligands for CLRs have been identified thus far. To search for novel endogenous CLR ligands, we co-cultured CLR-bearing reporter cells with various tissues and cells. We found that peritoneal exudate cells (PEC) potentially activated reporter cells expressing Dectin-2, whereas cells from other lymphoid organs, such as thymus, spleen, and lymph node did not activate the reporter cells. In addition, bone marrow derived dendritic cells (BMDCs) strongly activated the Dectin-2 expressing reporter cells. This activity was decreased when BMDCs were stimulated with LPS. Moreover, an excessive amount of mannose inhibited the reporter activity, implying the contribution of terminal mannose to the ligand recognition. BMDCs were also positively stained by Dectin-2-Ig fusion protein, suggesting that Dectin-2 recognizes glycosylated molecule(s) expressed on the surface of resting BMDCs. We therefore purified Dectin-2-Ig-binding protein using the plasma membrane fraction of BMDCs and identified gp68 as a Dectin-2 ligand. Indeed, Dectin-2 reporter cells were strongly activated when co-cultured with macrophage cell line overexpressing gp68. These results suggest that Dectin-2 may sense particular glycosylation status on the surface of myeloid cells to modulate immune responses.

1252**Binding of membrane lipids by the SH2 domain of ZAP70 is critical for T cell receptor signaling**

Jung, D.-J.¹, Park, M.-J.¹, Sheng, R.², Silkov, A.³, Wang, Z.-G.², Xin, Y.², Kim, H.², Thiagarajan-Rosenkranz, P.², Song, S.¹, Yoon, Y.², Nam, W.¹, Kim, I.⁴, Kim, E.¹, Lee, D.-G.¹, Chen, Y.², Singaram, I.², Jang, M.H.⁴, Hwang, C.-S.⁵, Honig, B.³, Ryu, S.⁵, Lorieau, J.², Kim, Y.-M.^{1,5}, Cho, W.²

¹Pohang University of Science and Technology, Division of Integrative Biosciences and Biotechnology, Pohang, Korea, Republic of, ²University of Illinois at Chicago, Department of Chemistry, Chicago, United States, ³Columbia University, Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biophysics, New York, United States, ⁴Institute for Basic Science, Academy of Immunology and Microbiology, Pohang, Korea, Republic of, ⁵Pohang University of Science and Technology, Department of Life Sciences, Pohang, Korea, Republic of

T cells are activated by antigen recognition of T cell receptors (TCRs). The TCR signaling pathway involves several cytosolic tyrosine kinases, phosphatases and adaptor molecules, many of which have Src-homology 2 (SH2) domains. SH2 domain is a prototypic protein-interaction domain that reads phosphotyrosine (pY) signals to link various tyrosine kinase substrates to downstream signaling molecules. Since most of the SH2 domain-containing proteins are thought to function near the plasma membrane, we purified 76 human SH2 domains to analyze the membrane lipid binding and found that the majority of them effectively bind the plasma membrane-mimetic vesicles with submicromolar affinities. These SH2 domains have alternative cationic patches for lipid binding separate from the pY-binding pockets and simultaneously interact with pY-peptides and membrane lipids independently of each other. Among the proteins expressed in T cells, we found that the C-terminal SH2 domain (cSH2) of ZAP70 strongly binds membrane lipids with high selectivity for PI(3,4,5)P₃. To analyze the role of lipid binding by cSH2 of ZAP70, we mutated specific residues involved in the lipid binding and reconstituted T cell lines lacking the endogenous ZAP70 with the mutants. We found that many aspects of the TCR signaling were significantly down-regulated in the cells expressing lipid binding-defective ZAP70. In contrast, cells expressing a gain-of-function mutant with a higher lipid binding affinity showed enhanced TCR signaling compared to the ones expressing wild type proteins. Our data demonstrate that the direct lipid binding by SH2 domains confers exquisite modulation of ZAP70 functions and TCR signaling.

1253**Nano-clustering of the T cell receptor (TCR) explains the high signaling efficacy in antigen experienced T cells**

Segal, G.¹, Gaus, K.^{1,2}

¹UNSW, School of Medical Sciences, Single Molecule Science, Sydney, Australia, ²UNSW, School of Medical Sciences, ARC Centre for Advanced Molecular Imaging and Australian Centre for NanoMedicine, Sydney, Australia

T cells scan and recognize antigens in a process that is highly specific and mediated by the interaction between a T cell

receptor (TCR) and an antigen-MHC complex. This process is tightly regulated by several molecular and cellular factors to avoid unwanted attack to self to occur. Our group had recently described a mechanism of immune regulation based on the pre-existence of TCR clusters. The presence of this dense TCRs cluster regulates the signaling efficacy and ultimately translates into effective immune responses. We hypothesize that an antigen experienced T cell will have physical differences to a naïve T cell at the cell membrane level. Using single molecule localization techniques combined with computational analysis we have determined the clustering of single TCRs and how these are triggered in different scenarios. By using memory T cells generated *in vivo* and comparing those with naïve T cells, we have established the topographic differences that partially explain why memory.

T cells are faster and more efficient in responding to antigen. This understanding allows us to engineer the TCR distribution in T cells and rearrange it to enhance their effector functions in strategies such as vaccination and immunotherapies.

1254**The role of Themis in T cell activation and effector function**

Fu, G.^{1,2}, Liu, C.¹, Tang, J.¹, Ma, L.¹, Jia, X.¹, Chen, X.¹, Liu, W.-H.¹

¹Xiamen University, School of Life Sciences, Xiamen, China,

²Xiamen University, State Key Laboratory of Cellular Stress Biology, Xiamen, China

Themis, a T cell specifically expressed protein, is required for proper T cell development. Previous studies using germline knockout or ENU-mutant mice from our lab and others showed that Themis is an adaptor molecule involved in TCR proximal signaling by binding to LAT signalosome and negatively regulating TCR signal strength. However, the physiological role of Themis in peripheral T cells is not clear and needs detailed investigation. By specifically deleting Themis gene in mature T cells using distal-Lck Cre mice, we found that Themis is required for mature T cell activation and T_{FH} differentiation. In primary response, upon OVA/Alum/LPS immunization, Themis conditional knockout mice showed impaired T_{FH} differentiation and slightly reduced antibody production. In contrast, in secondary response after NP-OVA/Alum immunization, antibody production was more dramatically reduced in Themis conditional knockout mice. Mechanistically, upon *in vitro* anti-CD3 and anti-CD28 stimulation, Themis deficient naïve CD4⁺ T cells showed sustained lower CD69 and CD44 expression. In addition, IFN γ and IL-2 production was reduced in Themis-deficient T cells when stimulated with PMA/Ionomycin *ex vivo*. Thus our findings demonstrated an essential role of Themis in T cell activation, T_{FH} differentiation, and memory response development. To further understand the function of Themis in T cell activation and helper T cell differentiation (such as T_H1, T_H2, T_H17 and Treg), we will explore the signaling pathways that Themis may be involved in mature T cells. Our study will provide new insights of how Themis functions in the context of immune responses and autoimmune diseases.

1255**Characterisation of the tissue distribution and function of the C-type lectin receptor CD302 in humans and mice**

Lo, T.H.^{1,2}, Fromm, P.^{1,2}, Verma, N.¹, Vu, A.¹, Kupresanin, F.¹, Kato, M.³, Clark, G.J.^{1,2}, Hart, D.N.J.^{1,2}, Silveira, P.A.^{1,2}

¹ANZAC Research Institute, Dendritic Cell Research, Sydney, Australia, ²University of Sydney, Sydney Medical School, Sydney, Australia, ³Mater Medical Research Institute - University of Queensland, Brisbane, Australia

The majority of C-type lectin receptor (CLR) family members are used by the immune system to recognise danger associated molecular patterns, but some contribute to physiological functions including adhesion, migration or glycoprotein turnover. We identified CD302, a CLR expressed by human myeloid phagocytes that co-localises to podosomes, suggesting a role in cellular adhesion or migration. Our study investigated the tissue expression of CD302 and its immunological functions. We characterised the transcriptional expression of CD302 in mouse and human tissues as well as immune cells using quantitative PCR. Mouse CD302 transcripts were highest in the liver, as in humans. Moderate expression of CD302 transcripts was also detected in mouse lungs, lymph nodes (LN) and spleen with myeloid cells expressing the highest levels. Interestingly, greater CD302 expression was detected in migratory compared to resident DC populations and M1 versus M2 macrophages. CD302 knockout (KO) mice generated for functional studies showed a partial deficiency in migratory DC proportion and number within the LN of CD302KO mice. *In vitro* studies showed that CD302KO and wild-type (WT) DC had equivalent capacity to prime T cells and migrate towards the lymphoid-homing chemokines, CCL19/CCL21. However, *in vivo*, fewer CD302KO migratory DC trafficked into draining LN after FITC skin painting and reduced OT-IT cell priming occurred after OVA immunisation. Our data indicates a specialist role for CD302 in DC and macrophage membrane functions, which contribute to the interactions required for DC migration to draining LN. This knowledge of CD302 function will be applied to future translational studies.

1256**Cis-interaction of TCRs and antigen/MHC class I complexes on CD8⁺ T cells causes their activation**

Kishi, H.¹, Jin, A.², Hiroshi Hamana, H.¹, Shitaoka, K.¹, Tajiri, K.¹, Kobayashi, E.¹, Kusano, S.³, Yokoyama, S.³, Ozawa, T.¹, Nagai, T.¹, Obata, T.⁴, Hatakeyama, S.¹, Horii, M.¹, Hu, Y.², Zhang, F.², Muraguchi, A.¹

¹University of Toyama, Toyama, Japan, ²Harbin Medical University, Harbin, China, ³RIKEN Structural Biology Laboratory, Yokohama, Japan, ⁴Toyama Industrial Technology Center, Takaoka, Japan

T-cells play an important role in defending against intracellular microbes and tumors. A central dogma in T-cell immunology is that T-cells are activated by the recognition of antigenic peptides presented by major histocompatibility complex (MHC) molecules on antigen-presenting cells or tumor cells by antigenic T-cell receptors (TCRs) (*trans*-interaction). Here, we demonstrate that TCRs and MHC-class I (MHC-I) molecules

can interact on a single CD8⁺ T-cell (*cis*-interaction), leading to the activation of that T-cell. We analyzed CD8⁺ T-cells at the single-cell level using microwell-array chips. We observed that antigenic peptide/MHC-I complexes (p/MHC-I) on a T-cell could bind a TCR on the same T-cell to induce cytokine secretion. In addition, CD28 costimulatory molecules, as well as adhesion molecules involved in forming the immunological synapse, play important roles in T-cell activation through *cis*-interaction of TCR and p/MHC-I. Furthermore antigenic proteins expressed internally in CD8⁺ T cells induced the differentiation of the T cells to express CD107a at the single cell level. Our results demonstrate that a TCR on a T-cell can interact with not only antigenic peptides presented on MHC-I molecules on antigen-presenting cells (*trans*-interaction) but also those on the same T-cell (*cis*-interaction), leading to their activation. Our findings indicate a new T-cell activation mechanism, which has significant implications for peptide-based immunotherapy of cancer patients as well as the onset of autoimmune diseases.

1257**Mesenchymal stem cells regulate AMPK/mTOR pathway and mRNA translation to potentiate T cell suppression via nitric oxide production**

Yoo, H.S.^{1,2}, Lee, G.^{1,3}, Na, K.^{1,3}, Jeon, M.-S.^{1,2,3}

¹Translational Research Center INHA University Hospital, Incheon, Korea, Republic of, ²INHA University School of Medicine, Department of Molecular Biomedicine, Incheon, Korea, Republic of, ³IRIMS, Incheon, Korea, Republic of

Mesenchymal stem cells (MSCs) are known to suppress T cell activation and proliferation. However, the molecular mechanisms how MSCs directly regulate the T cell signaling are not completely understood. We found that mouse MSCs inhibit protein expression of IL-2 receptor alpha (CD25), but not mRNA expression in activated T cells. In this study, we investigated to understand the mechanism how MSCs influences on the CD25 protein expression. We obtained the following results;

1) IL-2 production was significantly increased in MSC-treated T cell culture medium. Thus, in the presence of MSCs, initial TCR signaling is intact and the increased IL-2 detection was occurred by the decreased CD25 expression.

2) Nitric oxide (NO) produced by MSCs was involved in the decreased CD25 expression.

3) MSCs induced phosphorylation of AMPK and TSC2 and reduced phosphorylation of S6K and 4E-BP1 in T cells. Similar results were obtained when T cells were cultured with NO donor. Thus, NO produced by MSCs inhibit the AMPK/mTOR-mediated mRNA translation.

4) 4EGI-1 treatment, an inhibitor of translation, reduced the CD25 expression in T cells.

Taken together, NO produced by MSCs inhibit the CD25 translation through the regulation of AMPK/mTOR pathway to potentiate T cell suppression.

1258

Epigenetic regulator PRC2 modulates proliferative response in CD4⁺ T cells*Kong, L.^{1,2}, Marchingo, J.^{1,2}, Hodgkin, P.^{1,2}, Allan, R.^{1,2}, Heinzl, S.^{1,2}**¹The Walter and Eliza Hall Institute of Medical Research (WEHI), Parkville, Australia, ²The University of Melbourne, Department of Medical Biology, Parkville, Australia*

The Polycomb Repressive Complex 2 (PRC2) is an epigenetic silencing complex that acts by trimethylating H3K27. Loss of PRC2 in T cells leads to severe inhibition of T cell expansion during the primary in vivo immune response. However whether this is due to defects in activation, proliferation or survival is not well understood. To address this, we investigated the proliferative response of PRC2 deficient cells in vitro.

The expansion of T cells after activation is a tightly controlled process governed by division rates, number of divisions undergone before returning to quiescence (termed division destiny) and life span, which are controlled by both internal and external factors such as the type and strength of stimulation.

As expected, a dose-dependent increase in division destiny was observed in response to anti-CD28 stimulation in naïve CD4⁺ T cells from control mice. In contrast, naïve CD4⁺ T cells deficient for the PRC2 components (EZH2, the catalytic enzyme, or the scaffold proteins SUZ12 and EED) were able to expand after activation but showed a markedly defect in their proliferative capacity in response to CD28. Quantitative analysis revealed that division rate was unaffected, but PRC2 deficient cells underwent fewer divisions and died sooner than wild type cells. This defect was not due to CD28 cell surface receptor levels and could not be overcome by increasing CD28 stimulation strength, suggesting that PRC2 acts downstream of CD28 receptor.

Cumulatively, these results demonstrate that PRC2 modifies sensitivity to CD28 co-stimulation in naïve CD4 T cells, thereby affecting their proliferative capacity.

1259

The calpain-calpastatin system activity is necessary for human T cell proliferation and development*Mikosik, A.¹, Frąckowiak, J.E.¹, Bryl, E.², Dąca, A.², Henc, I.², Ruckemann-Dziurdzińska, K.², Fulop, T.³, Haponiuk, I.⁴, Witkowski, J.M.¹**¹Medical University of Gdańsk, Pathophysiology, Gdańsk, Poland,**²Medical University of Gdańsk, Pathology and Experimental Rheumatology, Gdańsk, Poland, ³University of Sherbrooke, Center for Ageing Research, Sherbrooke, Canada, ⁴Nicolaus Copernicus Hospital, Pediatric Cardiac Surgery, Gdańsk, Poland*

Ubiquitous, strictly Ca²⁺ - dependent neutral proteases called μ - and m-calpain, together with their endogenous inhibitor - calpastatin - form the calpain-calpastatin system (CCS) involved in physiological and pathological signal transduction, proliferation and apoptosis of many cell types. We have previously demonstrated its existence in both leukemic and normal human lymphocytes. However, the role of the CCS in the function and development of human T cells is poorly understood. Using multicolor flow cytometry to detect and quantify the CCS proteins, to measure calpains' activities in the

adult peripheral blood T cells and thymocytes obtained during cardio-surgical interventions in young children, and to quantify the cytokines released during in vitro cultures, we have shown that constitutive activity of these proteases is present in all peripheral T cells and that its inhibition greatly reduces their proliferation and cytokine production in vitro. This activity is paralleled by constitutive expression of genes for both calpains (CANP1 and CANP2) and for calpastatin (CAST) in the T cells, as shown by quantitative real time PCR. We have also seen strong expression of CCS components in all thymocytes, as well as high proteolytic activity of calpains in the CD4⁺CD8⁺ and CD4⁺CD8⁻, and much weaker in the CD4⁺CD8⁻ and CD4⁻CD8⁻ thymocytes. Together, these data suggest strong involvement of the CCS activity in the development of T cells and in the maintaining of readiness of peripheral T lymphocytes for proliferative and secretory response to antigenic challenges.

1260

Ubiquitination role in TCR signaling and costimulation via GITR*Muller, J.¹, Zhang, G.², Silva, H.M.², Neubert, T.², Dustin, M.³**¹New York University Langone Medical Center, Sackler Institute**/ Immunology, New York, United States, ²New York University Langone Medical Center, Skirball Institute of Biomolecular Medicine, New York, United States, ³Oxford University, Kennedy Institute of Rheumatology, Oxford, United Kingdom*

Essential steps in TCR sorting, signaling, and degradation, are mediated by ubiquitination of components in TCR microclusters. Signaling through the costimulatory family of TNF receptors (GITR, OX40, 4-1BB, etc.) utilize a series of ubiquitination events that regulate the signaling process. A number of substrates of ubiquitin ligase like Cbl-B, ITCH, and CIAP1/2 have been identified. However, a detailed analysis of the global pattern of ubiquitination from TCR signaling is lacking. To address this we have utilized SILAC labeling and proteomics to profile the dynamic changes in ubiquitination that result from TCR signaling alone or in combination with GITR signaling. We have identified 4,500 ubiquitination sites with 44 regulated by TCR or TCR and GITR signaling. Using bioinformatics, we have identified key ubiquitin ligases that are responsible for the dynamic ubiquitination observed in our screen and confirmed their function by knock down, IP and ubiquitination of targets by western blot. Moreover, we have used IP with linkage specific antibodies to identify the specificity of the chains on specific ubiquitinated signaling molecules. Our work demonstrates the application of ubiquitin proteomics to primary murine antigen specific T cells, cross talk between TCR and GITR signaling, and the ubiquitination events downstream of the TCR and GITR that regulate their signaling.

1261**Mechanisms controlling activation-induced chromatin decondensation and proliferative competence in peripheral T cells**

Eikenbusch, S., Shields, M., Swatzel, H., Ward, A., Bingham, K., Lee, M., Meredith, J., Mitchell, T., Rawlings, J.
Furman University, Greenville, United States

Antigen presentation to the T cell receptor (TCR) initiates multiple signaling cascades required for the activation and proliferation of peripheral T cells during an immune response. Clonal proliferation of activated cells is achieved, in part, due to the changes in chromatin status as a function of activation: naïve T cells possess a condensed chromatin that decondenses during T cell activation. Previously, we demonstrated that chromatin decondensation is required for the acquisition of competence to respond to cytokines such as IL-2, providing mechanism such that only activated T cells will proliferate during an immune response. Here, we analyze the signaling pathways downstream of the TCR for their role in chromatin decondensation and the acquisition of competence to respond to IL-2. Upon TCR stimulation, phosphatidylinositol 4,5-bisphosphate (PIP₂) is hydrolyzed into inositol triphosphate (IP₃) and diacylglycerol (DAG); the former induces calcium flux from the endoplasmic reticulum while the latter activates protein kinase C (PKC). We demonstrate that both IP₃ and DAG signaling are required for the proper initiation of chromatin decondensation. However, DAG, but not IP₃ signaling, is sufficient to make T cells competent to respond to IL-2. Our findings suggest that there may be multiple TCR-driven mechanisms leading to the decondensation of distinct domains chromatin during T cell activation.

1262**Novel functions of necroptotic and apoptotic pathways in T cell proliferative and survival signaling**

Zhang, J.
Thomas Jefferson University, Microbiology and Immunology,
Philadelphia, United States

Apoptotic cell death is executed by caspases, which help digest cellular molecules to minimize tissue damage. Recent studies reveal a new type of programmed cell death pathway, namely necroptosis, which is activated when caspase-mediated apoptosis is defective. Apoptosis plays an important role in homeostasis in the immune system. Mutations in the Fas receptor or its ligand, FasL, lead to autoimmune-lymphoproliferation (lpr) diseases characterized by lymphadenopathy, splenomegaly and autoantibody production. FADD binds to Fas, recruits and activates the initiator caspase 8. However, unlike Fas mutant mice which have no developmental defect, FADD knockout mice die during midgestation. This paradox has been resolved by recent studies including ours which reveal a novel function for FADD in suppressing necroptosis, while promoting apoptosis. Other proteins including Daxx have also been suggested to interact with Fas. Interestingly, deletion of Daxx also leads to early embryonic lethality. It is unclear whether necroptosis and/or apoptosis play a role in cell death in Daxx-deficient embryos. Moreover, the role of Daxx in Fas-induced cell death *in vivo*

remains a controversy. In the current study, the function of Daxx in relationship with necroptosis and apoptosis was analyzed, using novel animal models. Our data reveals an unexpected interplay between necroptosis and apoptosis in Daxx-mediated pathways, as well as a novel function of Daxx in primary T cells, which is unrelated to Fas signaling.

1263**Investigating the regulation of protein translation by kindlin-3 in chronic myeloid leukemia cells**

Qu, J., Tan, S.M.
Nanyang Technological University, School of Biological Sciences,
Singapore, Singapore

Kindlin-1, 2 and 3 are 4.1-ezrin-radixin-moesin-containing cytoplasmic proteins that regulate integrin-mediated cell-cell and cell-extracellular matrix attachments. Kindlin-3 is expressed in hematopoietic cells, platelets, osteoclasts, and endothelial cells. The importance of kindlin-3 is underscored by the bleeding and immune compromised disease Leukocyte Adhesion Deficiency III, which is attributed to mutations in kindlin-3. Kindlin-3 is well established to regulate immune cell adhesion, migration and signaling. Kindlin-3 stabilizes cell adhesion mediated by β 1, β 2 and β 3 integrins. Recent studies suggest a role of kindlin-3 in tumor progression. Herein, we showed that kindlin-3 via its interaction with the receptor for activated-C kinase (RACK1) regulates protein translation in human chronic myeloid leukemia cell line K562. Data suggest that kindlin-3 associates with the ribosome and RACK1 serves as the bridging molecule. We showed that silencing of kindlin-3 expression in K562 cells reduced c-Myc protein expression but not its mRNA expression. Consequently, there was a decrease in the proliferation rate of these cells *in vitro* and a significant reduction in tumor mass in xenograft experiments. Our data suggest that kindlin-3 may regulate protein translation indirectly via the integrin α 5 β 1 Akt-mTOR-p70S6K pathway and directly via its association with RACK-1-containing ribosome. These data also suggest the possibility of similar signaling conduits in other immune cells.

1264**A conserved T cell receptor transmembrane structure mediates transbilayer signalling**

Krishnan, L.^{1,2}, Park, S.³, Im, W.³, Call, M.^{1,2}, Call, M.^{1,2}
¹Walter & Eliza Hall Institute of Medical Research, Parkville, Australia, ²The University of Melbourne, Department of Medical Biology, Parkville, Australia, ³The University of Kansas, Department of Molecular Biosciences and Center for Computational Biology, Lawrence, United States

Signalling through the T cell receptor (TCR) is crucial for T cell development and functions during infection, and provides the basis for immune responses against tumour antigens. The TCR is an assembly of eight single-pass membrane proteins that are held together by an intra-membrane electrostatic network. Upon encountering peptide:MHC ligand presented on antigen-presenting cells, the $\alpha\beta$ TCR heterodimer transmits activating

signals through the constitutively associated CD3 $\epsilon\delta$, CD3 $\epsilon\gamma$ and ζ modules. The mechanism by which ligand binding to TCR $\alpha\beta$ is communicated to the CD3 dimers is unknown. We hypothesised that upon ligand engagement, conformational changes are transmitted through the transmembrane domains (TMDs) of the receptor complex to cause alteration in the cytoplasmic domains. To test this hypothesis, we combined intra-membrane cysteine-scanning, solution nuclear magnetic resonance (NMR) spectroscopy and computational modelling methods to interrogate the arrangement of interacting TMDs within the assembled, un-liganded receptor complex. Our results revealed a specific helix-helix interface between TCR α and TCR β TMDs governed by a novel polar interaction that is absolutely conserved across vertebrate evolution. We also found that similar interface is present and conserved in the $\gamma\delta$ TCR sequences, suggesting a fundamental role for this polar interaction. Using a cellular assay measuring IL-2 production upon TCR stimulation, we showed that mutations within the key contact residues resulted in impaired T cell signalling. Collectively, these findings provide evidence for receptor-intrinsic, conformationally-regulated transbilayer signalling during TCR triggering. Additionally, these findings highlight the role of intra-membrane polar interactions for membrane receptor assembly and functions.

1265

Centrosome and Golgi-associated proteins are novel substrates of protein kinase A in T-lymphocytes

Chalasani, M.L.S.¹, Ong, S.T.¹, Fazil, M.H.U.T.¹, Low, J.H.^{1,2}, Prasannan, P.¹, Kelleher, D.³, Verma, N.K.^{1,4}

¹Nanyang Technological University (NTU), Lee Kong Chian School of Medicine, Singapore, Singapore, ²Nanyang Technological University (NTU), School of Biological Sciences, Singapore, Singapore, ³University of British Columbia, Faculty of Medicine, Vancouver, Canada, ⁴Singapore Eye Research Institute, Singapore, Singapore

Background: Protein Kinase A (PKA) is a cAMP-dependent serine/threonine kinase with wide substrate specificity. PKA substrate phosphorylation is crucial in many cellular processes, including cell adhesion and migration. This study aims to determine the role of PKA and its substrates in LFA-1 integrin-induced T-cell migration.

Methods: Human T-cells were triggered to migrate via LFA-1 stimulation by incubating on the ICAM-1 ligand-coated plates. Cellular responses were determined using RNA interference-mediated gene knockdown, co-immunoprecipitation, Western-immunoblotting, confocal microscopy and High Content Analysis.

Results: Previous High Content Analysis showed an active involvement of PKA in T-cell migratory phenotypes. To further clarify the scope of PKA signalling cascades in T-cell migration, we performed immunoprecipitation-based assays focussed on the identification of PKA substrates. We identified CG-NAP, pericentrin and dynein as substrates for PKA phosphorylation. Pre-treatment of T-cells with forskolin, a potent activator of adenylyl cyclase that elevates cAMP-dependent PKA activity, increased the phosphorylation levels of pericentrin and

dynein (>2-fold). Co-immunoprecipitation of cellular lysates further confirmed a direct interaction of these proteins with PKARII α , which was found to be co-localized with pericentrin at centrosome and GM130 at the cis-Golgi. RNA interference-mediated knockdown of CG-NAP (>70% knockdown) in T-cells disrupted PKARII α /pericentrin/ α -tubulin co-localization at centrosome.

Conclusion: This study, for the first time, provides a mechanistic insight into the role of PKA in T-cell signalling. The identification of novel substrates for PKA at centrosomal and Golgi region may open a potential new avenue for fine-tuning of T-cell migration and the development of specific peptide inhibitors targeting T-cell-mediated immune regulations.

1266

Role of Mincle in the recognition of *Helicobacter pylori*

Nagata, M.¹, Doi, R.¹, Iwai, S.¹, Ishikawa, E.¹, Miyamoto, T.², Yamasaki, S.¹

¹Division of Molecular Immunology, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan, ²Department of Natural Products Chemistry, Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan

Helicobacter pylori (*H. pylori*) is a Gram-negative bacterial pathogen which causes gastrointestinal disorders, such as gastric ulcers and carcinoma. *H. pylori* possess various unique glycolipids that are thought to be beneficial for their survival in host. However, it is unclear whether these glycolipids are recognized as pathogen-associated molecular patterns (PAMPs) by innate immune receptors.

To investigate immunostimulatory activity of *H. pylori* lipids and identify the active constituents, we extracted lipophilic components and co-cultured with reporter cells expressing various pattern recognition receptors (PRRs). We found that macrophage-inducible C-type lectin (Mincle) recognizes the lipid extract of *H. pylori*. Subfractionation and further purification of this extract revealed that Mincle recognizes two particular fractions which gave single spots on thin-layer chromatography (TLC). Both glycolipids activated bone marrow-derived dendritic cells (BMDCs) to produce inflammatory cytokines, whereas these were abrogated in Mincle^{-/-} BMDCs. In addition, these glycolipids potently activated BMDCs to promote IL-17 production in CD4⁺ T cells from OT-II mice.

Finally, we investigated the role of Mincle against infection of *H. pylori* SS1. Mincle deficiency led to an increase of bacterial number in the stomach at 2 weeks after infection.

These results suggested that Mincle is involved in immune responses against *H. pylori* through the recognition of their characteristic glycolipids.

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K33-linked polyubiquitination of Zap70 by E3 ligase Nrdp1 controls activation of CD8⁺ T cells

Yang, M.¹, Chen, T.¹, Li, X.¹, Yu, Z.², Cao, X.^{1,2,3}

¹Second Military Medical University, Institute of Immunology and National Key Laboratory of Medical Immunology, Shanghai, China, ²Zhejiang University School of Medicine, Institute of Immunology,

Hangzhou, China, ³Chinese Academy of Medical Sciences, Institute of Basic Medical Sciences, Beijing, China

Nrdp1 (neuregulin receptor degrading protein 1, also known as RNF41, FLRF or RBCC) is a C3HC4 type ring-finger-containing E3 ligase implicated in the ubiquitination of various substrates and regulation of several pathological processes, such as cancer, Parkin's disease, ischemia-reperfusion injury and diabetes. Previously we find that Nrdp1 preferentially promotes the production of type I interferons triggered by Toll-like receptor response. Until now roles of Nrdp1 in regulating T cell activation or anergy have not been reported. Here we also find that Nrdp1 may play a role in T cell activation. We find that Nrdp1 is expressed highly in naïve CD8⁺ T cells than in naïve CD4⁺ T cells, which inspires us to investigate the roles of Nrdp1 in CD8⁺ T cell activation. By using CD8⁺ T cells derived from *Nrdp1*^{-/-} mice, we found that Nrdp1 deficiency promotes proliferation and cytokine production of CD8⁺ T cells, leading to enhanced antiviral and antitumor effector functions of CD8⁺ T cells. Further analysis of Nrdp1-interacting molecules and the signaling pathways indicate that Nrdp1 can interact with Zap70 and Sts1/2 (acidic phosphatase-like proteins), polyubiquitinate Zap70 and promote the dephosphorylation of Zap70 by Sts1/2. Our study indicates that Nrdp1 may be a Zap70-targeted E3 ligase that links Sts1/2 to activated/polyubiquitinated Zap70. Nrdp1-mediated inactivation of polyubiquitinated Zap70 through Sts1/2 may represent an important machinery for negative regulation of proximal TCR signaling.

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Dual regulation of T lymphocyte function in autoimmunity by O-GlcNAc glycosylation

Ramakrishnan, P., Tomalka, J., de Jesus, T.

Case Western Reserve University, School of Medicine, Department of Pathology, Cleveland, United States

T lymphocytes play a major role in autoimmunity. CD4⁺ T cells produce pro-autoimmune cytokines and promote the cytotoxic activity of CD8⁺ T cells, while T regulatory (T reg) cells act to suppress autoimmunity. Type 1 diabetes is an autoimmune disease where T cells destroy pancreatic beta cells leading to hyperglycemia and chronic inflammatory complications. Adverse pathological effects of hyperglycemia include posttranslational modification of proteins by the sugar N-acetyl glucosamine (GlcNAc) in a process called O-GlcNAcylation. We found that hyperglycemia induces O-GlcNAcylation of NF-kappaB protein c-Rel, which is a critical regulator of T cell function and T regulatory cell development. O-GlcNAcylation of c-Rel at serine residue 350 activates the transcription of c-Rel-dependent pro-autoimmune cytokines interleukin-2 (IL-2), interferon gamma (IFNG) and granulocyte macrophage colony-stimulating factor (GM-CSF). Our recent results show that c-Rel O-GlcNAcylation inhibits the expression of T regulatory cell specific transcription factor FOXP3. We explored the molecular mechanism and found that O-GlcNAcylation differentially regulates the DNA binding of c-Rel at the promoters of cytokines and FOXP3. Thus, c-Rel O-GlcNAcylation may serve as a key regulatory switch with dual, but reciprocal, roles in positively regulating autoimmune T cell

and negatively regulating immunosuppressive T regulatory cell functions. The net result of these two opposing effects may exacerbate autoimmunity in type 1 diabetes. Despite decades of research, drugs based on molecular targets to treat autoimmune diabetes have remained elusive. This study reveals c-Rel O-GlcNAcylation as a potential therapeutic target for autoimmune diabetes and a novel molecular mechanism regulating T lymphocyte function in autoimmunity.

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Rapid multiplex analysis of lipid raft signaling components with single lymphocyte resolution

Schatzmaier, P.¹, Göschl, L.², Ellmeier, W.², Huppa, J.B.¹, Stockinger, H.¹

¹Medical University of Vienna, Institute for Hygiene and Applied Immunology, Vienna, Austria, ²Medical University of Vienna, Institute of Immunology, Vienna, Austria

Lipid rafts, a distinct class of highly dynamic cell membrane microdomains, are integral to cell homeostasis, differentiation and signaling. Raft-association of important lymphocyte receptor signaling molecules - e.g. Src family kinases - and stimulation-induced variations in raft composition was demonstrated as well as a clear correlation of their raft-association with signaling function. However, biochemical quantitative analysis of lipid raft components involves laborious and time-consuming cell lysate fractionation via sucrose density gradient ultracentrifugation. Further, this method needs a large number of input cells and single cell information is completely lost. This complicates or even precludes the examination of rare cells, developmentally heterogeneous cell populations or weakly raft-associated factors. We established a fast and reliable method that is based on the low g centrifugation of cells through a detergent gradient, requiring little starting material and effort. Our widely applicable protocol enables multidimensional and sensitive flow cytometric quantitation of raft-associated proteins with single cell resolution. It allows easy and precise assessment of endogenously and ectopically expressed membrane components from a few cells in complex isolates as well as their dynamics due to cell differentiation, signaling and mutation. In conclusion, our approach is well suited to elucidate the role of lipid rafts in regulation of factors that govern proximal signaling thresholds of crucial leukocyte receptors, including the T cell antigen receptor. This work was supported by the Cell Communication in Health and Disease (CCHD) PhD Program.

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Low concentration of hydroxychloroquine potentiates TLR-9 signalling: potential implications for clinical practice

Hurtado, P.R.¹, Hurtado-Perez, E.², Peh, C.A.^{1,2}

¹Royal Adelaide Hospital, Renal Medicine, Adelaide, Australia, ²Adelaide University, Medicine, Adelaide, Australia

The antimalarial drug hydroxychloroquine (HCQ) is widely used for the treatment of a variety of systemic autoimmune diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). This effect seems to be mediated by its

ability to increase the pH in intracellular endosomes, inhibiting intracellular pathways involved in the pathogenesis RA and SLE such as antigen presentation, autophagy and signalling of endosome-bound toll-like receptors such as TLR-3, 7, 8 and 9. There are, however, a number of reports where the use of HCQ has also been associated with unexplained increase in disease activity. In this study we show that HCQ has a bimodal effect on its capacity to influence the immune response mediated by TLR-9 signalling. While HCQ strongly inhibits TLR signalling at pharmacological concentrations (1 to 10 μ M), at low concentrations (1-100 nM), HCQ exerts the opposite effect, potentiating the stimulatory effects of CpG, a TLR-9 ligand. The presence of HCQ at low concentration results in a significant increase in the expression of MHC class II and co-stimulatory molecules CD40 and CD86 by B cells as well as increase in *in vitro* antibody synthesis and cytokine production by B cells. This *in vitro* finding may be highly relevant to clinical practice, and stresses the importance of appropriate dosing as well as patient compliance.

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A novel two-ligand system to analyse signal integration by non-catalytic tyrosine-phosphorylated receptors

Paterson, R., Denham, E., Goyette, J., van der Merwe, P.A.

University of Oxford, Sir William Dunn School of Pathology, Oxford, United Kingdom

Appropriate immune responses require integration of a variety of activating, inhibitory and co-stimulatory signals from cell surface receptors. One important class of receptors are the non-catalytic tyrosine-phosphorylated receptors (NTRs), a group of over 100 receptors, such as PD-1, CTLA4, CD28, and the T cell antigen receptor (TCR). The mechanism through which NTR receptor-ligand engagement leads to signal initiation and integration is not well understood and may offer novel targets for disease treatments.

We are using a cellular system to analyse NTRs by manipulating their extra- and intra-cellular dimensions. We hypothesise that proximity of receptors is important for optimal signal integration and requires compatible receptor-ligand dimensions. We also hypothesise that intracellular domain length influences the effectiveness of inhibitory receptors such as PD-1.

To manipulate the dimensions of receptor-ligand interactions, CHO.K1 cells ("ligand cells") have been transfected with Spy-tag constructs of different lengths and either CD48 or HLA-A2 single chain dimer (SCD) constructs, also of different lengths. Spy-tag can be covalently coupled via Spy-catcher to Strep-Tactin[®], which binds the short peptide tag Strep-tagII[®]. CD48 and the HLA-A2 SCD presenting cognate peptide can be used to provide activating signals by binding to a 2B4-CD64 fusion protein and the 1G4 TCR, respectively, in THP-1 and Jurkat "receptor cells".

Initial experiments show that "ligand cells" are able to activate IL-8 production by THP-1 "receptor cells" expressing 2B4-CD64 and this is inhibited by engagement of Strep-tagII-tagged PD-1 with the Strep-Tactin ligand. These cells and assays are now being used to further analyse NTR signal integration.

1272

Completing the pro-survival BCL-2 family portrait: characterization of the A1 knockout mouse model

Schenk, R.^{1,2}, Tuzlak, S.^{1,3}, Vasanthakumar, A.¹, Preston, S.^{1,2}, Tai, L.¹, Strasser, A.^{1,2}, Herold, M.J.^{1,2}

¹Walter & Eliza Hall Institute, Melbourne, Australia, ²University of Melbourne, Medical Biology, Melbourne, Australia, ³University Innsbruck, Innsbruck, Austria

The BCL-2 family proteins are the regulators of the form of programmed cell death known as apoptosis. The family is subdivided into pro-survival and pro-apoptotic proteins, with the balance between the two groups determining whether a cell lives or dies. This apoptotic control is important in the context of disease, as defects in apoptosis are associated with cancer and autoimmunity. In particular, deregulation of the pro-survival group of proteins can see the survival of potentially harmful cells. There are five known pro-survival proteins: BCL-2, BCL-XL, BCL-W, MCL-1 and A1. Of these, A1 is poorly studied, but it appears to be specifically expressed in haematopoietic cells, with suggested roles in B cells, T cells and granulocytes. It has been difficult to generate an A1-deficient mouse model, as there are three functional A1 isoforms in the mouse (A1-a, -b and -d). However, our lab has successfully created a novel mouse model that lacks all functional A1 isoforms, and here I present the phenotypic characterisation of these mice. Overall, we observed no major differences in the haematopoietic compartment between A1-deficient and wild-type mice. Since A1 has been reported to be important upon antigenic challenge for B and T cell survival, we have started to challenge mice with different antigenic stimuli, such as influenza virus and LCMV. Initial results suggest that A1-deficient and wild-type cells respond similarly to viral infection. We are currently performing more in-depth analysis of A1-deficient mice challenged with different antigenic stimuli to further elucidate its role in the haematopoietic lineage.

1273

Inducible T cell kinase regulates late stages of CD8+ T lymphocyte effector function

Kapnick, S.¹, Ritter, A.¹, Stinchcombe, J.², Lippincott-Schwartz, J.¹, Griffiths, G.², Schwartzberg, P.¹

¹National Institutes of Health (NIH), Bethesda, United States,

²Cambridge Institute for Medical Research, Cambridge, United Kingdom

CD8+ cytotoxic T-lymphocytes (CTLs) are critical for killing virally infected cells, and defects in CTL responses can lead to secondary lymphoproliferative syndromes. Patients with mutations in Inducible T-cell Kinase (ITK), a kinase required for full activation of PLC γ 1 that serves as an amplifier of T-cell receptor (TCR) signaling, develop lymphoproliferative disease associated with high EBV viral loads. We found CTLs from ITK-deficient mice exhibit impaired killing of multiple targets, suggesting ITK-deficiency leads to global defects in cytolysis. Killing by CTLs occurs when TCR signaling triggers adherence to targets, centrosome polarization, and release of secretory granules inducing cytolysis of cells. Although early events

such as adhesion, actin ring formation, and cell polarization were intact in ITK-deficient murine CTLs, we found defects in degranulation, suggesting ITK may play an unappreciated role in the final stages of killing. Surprisingly, prolonged culture of ITK-deficient CTLs in IL-2 could rescue defects in killing and degranulation, similar to known effects of IL-2 on promoting NK cytotoxicity. Together these experiments provide clues to novel roles for ITK and TCR signaling in regulating late stages of CTL function that may contribute to reduced viral clearance in patients with mutations in ITK. To further understand what regulates cytotoxic granule release, we are also examining the role of actin in degranulation. Similar to the role of actin in regulating the initiation of secretion, we see that actin recovery can affect the termination of secretion. We are currently evaluating mechanisms that may control actin clearance and recovery at the synapse.

1274

Maintenance of microtubule architecture by an adaptor protein CG-NAP/AKAP450 is crucial for T-cell migration

Ong, S.T.¹, Chalasani, M.L.S.¹, Fazil, M.H.U.T.¹, Low, J.H.^{1,2}, Prasannan, P.¹, Wright, G.³, Kelleher, D.^{1,4}, Verma, N.K.^{1,5}

¹Nanyang Technological University, Lee Kong Chian School of Medicine, Singapore, Singapore, ²Nanyang Technological University, School of Biological Sciences, Singapore, Singapore,

³Institute of Medical Biology, A*STAR, Singapore, Singapore,

⁴University of British Columbia, Vancouver, Canada, ⁵Singapore Eye Research Institute, Singapore, Singapore

Background: Centrosome- and Golgi-localized protein kinase N-Associated Protein (CG-NAP/AKAP450) is an adaptor protein, which provides a platform for targeted positioning of multiple signalling molecules and their interactions that are critical for cellular functioning. Here, we investigated the mechanism of CG-NAP involvement in migrating T-cells.

Methods: Human primary and cultured T-cells were stimulated to migrate via the integrin LFA-1/ICAM-1 cross-linking. Standard molecular and imaging assays, including RNA interference-mediated gene knockdown, co-immunoprecipitation, Western immunoblotting, High Content Analysis, confocal and 3D Structured Illumination super-resolution Microscopy (3D-SIM) were performed.

Results: We observed that CG-NAP, a 450kDa protein, was predominantly expressed in activated primary T-cells as compared to monocytes, platelets, naïve CD4+ and CD8+ T-cells. CG-NAP was found to co-localize with γ -tubulin and pericentrin at the centrosome as well as CLASP2, GM130 and TGN46 at the Golgi. RNA interference-mediated silencing of CG-NAP (>70% knockdown) in T-cells caused Golgi fragmentation accompanied by ~2-fold decrease in the expression of GM130 and CLASP2 proteins. CG-NAP depletion significantly inhibited LFA-1/ICAM-1-induced T-cell migration and transwell chemotaxis towards the chemokine SDF-1 α . Western-immunoblotting and confocal microscopy showed that CG-NAP knockdown interfered with tubulin acetylation and detyrosination, which regulate microtubule dynamics in migrating T-cells. 3D-SIM revealed that microtubule arrays emanating from either the centrosome or the Golgi were disrupted in CG-NAP depleted T-cells.

Conclusion: CG-NAP plays a central role in regulating microtubule nucleation, maintaining Golgi architecture and acting as a downstream effector of LFA-1 signalling in migrating T-cells. This information enhances the fundamental understanding of T-cell migration mechanism implicated in adaptive immune responses.

1275

Nanoscale clustering of the T cell receptor regulates T cell signalling efficiency

Pageon, S.V., Tabarin, T., Yamamoto, Y., Gaus, K.

University of New South Wales, EMBL Australia Node in Single Molecule Science & ARC Centre of Excellence in Advanced Molecular Imaging, Sydney, Australia

T cells are crucial players in the adaptive immune system. When the T cell receptor (TCR) recognises a peptide ligand bound to the restricting major histocompatibility complex molecule (pMHC), it transmits a signal *via* the associated CD3 complex. The mechanism by which the extracellular antigen recognition event leads to intracellular phosphorylation remains unclear. Recent advances in imaging technologies have provided new tools for biologists to observe the organisation of receptors and signalling proteins at the membrane of immune cells at unprecedented resolution. Using single-molecule localisation microscopy techniques (SMLM) such as PALM and dSTORM, we show that the TCR is organised into nanometre-scale clusters in resting cells and that TCR engagement reorganises the TCR-CD3 complex into clusters of increased molecular density. By combining two-colour imaging with a degree-of-colocalisation (DoC) analysis, we determined where TCR molecules are being phosphorylated within a cell. Our data show that the TCR is preferentially phosphorylated, i.e. *triggered*, in clusters of higher TCR density. These dense clusters were also associated with downstream signalling proteins, suggesting that an intrinsic biological threshold, namely the molecular density within clusters, dictates signal initiation and amplification. Both pMHC dose and TCR-pMHC affinity determined the density of molecules in TCR-CD3 clusters, which in turn scaled with the overall level of receptor phosphorylation. Thus, we demonstrate that the formation of dense signalling-competent clusters is a process of antigen discrimination.

1276

T cell-intrinsic role of the cytosolic DNA-sensing for T cell function

Imanishi, T., Saito, T.

RIKEN Center for Integrative Medical Sciences (IMS), Yokohama, Japan

STING has a key role in detecting the cytosolic DNA in innate immune system, which results in the induction of type I interferon and interferon-stimulated genes (ISGs) to serve as the first line of host defense against infectious agents. However, the physiological role of the cytosolic DNA-sensing pathway in T cells remains unclear. Here we show that STING ligands induce the expression of type I interferon, ISGs and co-stimulation

to enhance IL-2 production in T cells upon TCR stimulation. Concomitantly, we found that stimulation by STING ligands strongly inhibits T cell growth. This STING-mediated growth arrest is partly dependent on IRF3, but not TBK1. These analyses demonstrate that cytosolic DNA-sensing in T cells induces both cytokine responses and growth arrest.

1277

Critical role of the DHR-1 domain in localization and function of DOCK8

Sakurai, T., Uruno, T., Fukui, Y.

Kyushu University, Division of Immunogenetics, Medical Institute of Bioregulation, Fukuoka, Japan

Cell migration involves membrane polarization and cytoskeletal dynamics, both of which are regulated by Rho family of GTPases, Rho, Rac, and Cdc42. These molecules act as molecular switches by cycling between GDP-bound inactive and GTP-bound active states, and stimulus-induced formation of the active form is mediated by guanine nucleotide exchange factors (GEFs). There are two distinct families of GEFs: Dbl-homology (DH) domain-containing proteins and DOCK proteins. Until recently, DH domain containing proteins have been considered to be the universal GEFs in eukaryotes. However, accumulating evidence indicates that the DOCK proteins act as major GEFs in varied biological settings. DOCK family proteins have two highly conserved domains, DHR-1 and DHR-2. We recently found that DOCK8 plays a key role in interstitial migration of dendritic cells. Although this function of DOCK8 critically depends on the DHR-2 domain mediating Cdc42 activation, the role of DHR-1 domain remains unknown. In this study, we will discuss the binding partner of DOCK8 DHR-1 and its physiological relevance to localization and function of DOCK8.

1278

Engagement of CD99 surface molecules inhibits T cell responses

Laopajon, W.¹, Pata, S.^{1,2}, Kasinrer, W.^{1,2}

¹Division of Clinical Immunology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiangmai, Thailand, ²Biomedical Technology Research Center, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency at the Faculty of Associated Medical Sciences, Chiang Mai University, Chiangmai, Thailand

CD99 is a broadly expressed transmembrane glycoprotein which has been described as a T cells- co-stimulator. We previously demonstrated that CD99 molecules were translocated into immunological synapse during T cell activation. However, the complete function of CD99 involving in T cell activation is still unclear. In this study, the role of CD99 on T cell responses was investigated. T cells proliferation, induced by anti-CD3 mAb, was inhibited by an anti-CD99 monoclonal antibody (mAb). The inhibition ability of anti-CD99 mAb was neutralized by CD99 recombinant proteins indicated that blocking of T cell proliferation was resulted from the engagement of CD99

molecules by anti-CD99 mAb. In addition, we found that engagement of CD99 before CD3 activation could not induce the T cell proliferation inhibition. This data suggested that stimulation of CD3 was required for induction of inhibition of T responses by CD99. The mechanism underlying this phenomenon was investigated. The expression of IL2 receptor (CD25) and IL2 was altered upon CD99 engagement. Our results illustrated that, during T cell activation, stimulation of CD3 molecules may provide a signal involving CD99 function. Subsequently, ligation of CD99 will generate T cell inhibitory signal. The generated inhibitory signal by CD99 molecules might be the negative feedback mechanism of T cell activation.

1279

Altered peptide ligands: qualitative or quantitative effects on signaling in monoclonal T cells?

Yazicioglu, Y.F.^{1,2}, Ellestad, K.K.^{2,3}, Anderson, C.C.^{2,3,4,5}

¹Gulhane Military Medical Faculty, Ankara, Turkey, ²Alberta Diabetes Institute, Edmonton, Canada, ³University of Alberta, Department of Medical Microbiology and Immunology, Edmonton, Canada, ⁴University of Alberta, Department of Surgery, Edmonton, Canada, ⁵Alberta Transplant Institute, Edmonton, Canada

Altered peptide ligands (APLs) are derivatives of antigenic peptides containing substitutions at residues involved in T cell receptor (TCR) contact or major histocompatibility complex (MHC) binding. While these substitutions can quantitatively affect T cell signaling following APL stimulation, the question remains whether APLs can also have qualitatively different TCR signaling effects. In this study, we employed the Marilyn (CD4+ anti-H-Y) TCR transgenic system and designed several H-Y-derived APLs bearing substitutions at MHC-II anchor or TCR contact residues. Marilyn PD-1^{-/-}

T cells lacking a co-inhibitor and thus thought to be biased toward enhanced TCR signaling were also included in the assays to examine threshold effects that could mediate qualitatively different responses to APLs. The proliferative response to different concentrations of native H-Y or these APLs was examined by both in vivo and in vitro proliferation assays using mixed Marilyn and Marilyn PD-1^{-/-} T cells. The ability of the APLs to bind MHC-II and potential for particular APLs unable to stimulate proliferation to inhibit T cell responses to native H-Y peptide were also assessed. We found that Marilyn and Marilyn PD-1^{-/-} T cells proliferated similarly in response to H-Y and all of the APLs tested in a dose dependent manner and in accordance with their predicted TCR and MHC affinity. Particular APLs could block the proliferative response of T cells to H-Y in co-culture. Overall our data suggest that APLs have primarily quantitatively different signaling effects on T cells, although some APLs may have utility in blocking the response to native epitopes.

1280

A negative regulatory role of SARM in TCR signaling

Tian, S.-H., Chen, Z.-L., Wang, Q.-L., Li, Y.

Sun Yat-Sen University, Guangzhou, China

The TIR domain containing adaptor SARM was first identified as

a negative regulator of TRIF-dependent TLR signaling in innate immunity. In this study, we described a novel function of SARM in response to TCR stimulation. Knockdown of SARM enhanced TCR-induced tyrosine phosphorylation and Lck protein level, which were reduced by overexpression of SARM. SARM-induced reduction of Lck protein level was partially lysosome-dependent. Upon TCR stimulation, Lck phosphorylated SARM and recruited it to the immunological synapse, which in turn promoted the degradation of activated Lck. Our results reveal a negative regulatory role of SARM in TCR signaling and provide a new insight into the mechanism of Lck regulation in T cells.

1281

Detection of immunomodulatory polysaccharides in food materials using the enzyme-fused innate immune receptors

Adachi, Y., Kasahara, K., Tetsui, J., Yamanaka, D., Ishibashi, K.-I., Ohno, N.

Tokyo University of Pharmacy and Life Sciences, School of Pharmacy, Laboratory for Immunopharmacology of Microbial Products, Tokyo, Japan

Beta-glucans from seaweed and fungi bind to innate immune receptor, dectin-1. Recently, the oral administration of a water soluble beta-glucan, laminarin, has been reported to modify the microbiota in gut and control the Treg function. To measure the content of beta-glucans in food materials, luciferase-based split enzyme immune assay specific for beta-glucans are prepared. In this paper, specificity and sensitivity of this new assay system will be reported.

A recombinant beta-glucan recognition protein (BGRP) from invertebrate was expressed as luciferase-fusion proteins, N-terminal and C-terminal fragments. The nLuc-BGRP and/or cLuc-BGRP were subjected to various polysaccharides, then substrate for luciferase was added and chemiluminescence was monitored.

To examine the assay specificity, various polysaccharides from bacteria, plants, algae, and fungi were tested. The positive enzyme activity was not observed with lipopolysaccharides, mannan, agarose, pectins, and cellulose. Whereas, 1,3-beta-glucans from mushrooms, yeast, and seaweeds showed significantly positive. The luciferase activity requires the both fragments in response to the beta-glucans, suggesting that cross-linking luciferase fragments, nLuc and cLuc, were induced by interaction of BGRP with 1,3-beta-glucans. The sensitivity of the Luc-BGRP split enzyme system was above the ng/mL order of beta-glucans. These results suggest that the split enzyme assay using BGRP and Luciferase fragments might be applicable to detect 1,3-beta-glucans in food ingredients.

1282

Spatiotemporal organisation of CD4 during T cell activation

Yamamoto, Y., Pigeon, S., Rossy, J., Gaus, K.

EMBL Australia Node in Single Molecule Science & ARC Centre of Excellence in Advanced Molecular Imaging, University of New South Wales, School of Medical Science, Sydney, Australia

CD4 is a co-receptor that plays an important role during initiation

of T cell receptor (TCR) activation by interacting with MHC class II molecules on the surface of antigen presenting cells (APCs). Past studies have shown that while CD4 is not required for TCR activation, it can significantly enhance the process by promoting associations with APCs, leading to increased cytokine secretion and cell proliferation.

While CD4 mediated enhancement of T cell responses is widely observed and a well-established phenomenon, the mechanisms that facilitate this enhancement remain poorly understood. In this study, we constructed supported lipid bilayers containing pMHC to mimic APC and found that OTII T cell activation only occurred robustly in the presence of CD4. Using this setup, we performed single-molecule localisation microscopy (PALM/dSTORM) to look at the nanoscale organisation of CD4 on the surface of activated T cells. The results revealed that upon TCR activation, the number of CD4 clusters observed on the cell surface is reduced and the CD4 clusters were denser than in non-activating conditions. These clusters of CD4 were also found to co-localise with some of the TCR clusters. Our data support a model in which CD4-mediated enhancement of T cell activation is dependent on TCR-complexed CD4. Further studies looking at the lateral organisation of CD4 in relation to other T cell signalling proteins would contribute to better understanding of how CD4 enhances T cell activation.

Therapeutic Antibodies

1283

Production of anti-MICA antibodies for targeting cancer therapy

Phumyen, A.¹, Chuangchot, N.^{2,3}, Jantasorn, S.³, Jumnainsong, A.^{3,4}, Leelayuwat, C.^{3,4,5,6}

¹Chulabhorn International College of Medicine, Thammasat University, Rangsit Campus, Pathum Thani, Thailand, ²Khon Kaen University, Biomedical Sciences Program, Graduate School, Khon Kaen, Thailand, ³Khon Kaen University, Centre for Research and Development of Medical Diagnostic Laboratories (CMDL), Faculty of Associated Medical Sciences, Khon Kaen, Thailand, ⁴Khon Kaen University, Department of Clinical Immunology and Transfusion Sciences, Faculty of Associated Medical Sciences, Khon Kaen, Thailand, ⁵Khon Kaen University, Research Cluster: Specific Health Problems of Greater Maekong Subregion (SHeP-GMS), Khon Kaen, Thailand, ⁶Khon Kaen University, Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen, Thailand

Major histocompatibility complex (MHC) class I chain-related (MIC) gene A (MICA) is NKG2D ligands able to stimulate immune activities of NKG2D-expressing effector cells. Abnormal cells such as tumor cells that express NKG2D ligands can be eliminated. Our previous study found that MICA antigens was over expressed in 85% of cholangiocarcinoma (CCA) lesion. So, the expression of MICA on tumor cells might be the target molecules for killing tumor cells. In this study, PCR-random mutagenesis and phage display technology was employed to produce the high binding activity of anti-MICA antibodies, doxorubicin conjugated phages displaying anti-MICA antibodies was developed for targeted delivery of anti cancer drug to MICA expressing cell lines. The drug-conjugated phages

were analyzed by flow cytometry. Our study found that DOX-phages were more efficient than free drug in killing cancer cell lines approximately 1.6-6 times on Hela cells, and CCA cell lines. To avoid the immunogenicity, we aim to produce soluble anti-MICA antibodies from our previous experiment and tag them on another nanoparticle such as liposome which contain anti-cancer drug. E.coli HB2151 was brought to produce soluble antibodies and the indirect ELISA method was employed to confirm their antigen-binding specificity against MICA antigens. We obtained the clones of soluble antibodies that exhibit high antigen-binding activity indicated by OD. These clones of antibodies can be further modified, the new delivery platforms may able to target selectively and show specificity to cancer cells over-expressing MICA proteins.

1284

Isolation and characterization of target antigen specific VHHs from alpaca synthetic VHH phage display libraries

Murakami, A.¹, Yoshida, M.², Muraoka, J.³, Tsukahara, N.¹, Azuma, T.², Nakayama, H.³, Kishimoto, H.¹

¹University of Ryukyus, Graduate School of Medicine, Okinawa, Japan, ²Antibody Engineering Research Center Inc., Chiba, Japan, ³Panasonic, Bioscience Technology, Kyoto, Japan

A single domain antibody is an antibody fragment consisting of a monomeric variable antibody domain. The first single domain antibodies were engineered from heavy chain antibodies found in camelids and those are called VHH (variable domain of heavy chain of heavy-chain antibody). The VHHs represent the smallest antibody that was proven of diagnostic and therapeutic usefulness. Because of their small size (~15KDa), VHHs can penetrate tissues faster than other antibodies, and break through the brain's blood barrier. VHHs also have higher stability to changes in temperature and chemical environments, so that these have the great potential of gastrointestinal stability and oral availability. Here we present two major strategies to construct large (>1 billion variants) alpaca synthetic VHH libraries. One of the strategies is replacing the heavy chain CDR3 region of alpaca VHH by a random sequence of seventeen amino acids. The other is mimicking the strategy of V(D)J recombination. After several cycles of panning with those libraries, antigen-specific VHHs against more than 10 antigens were obtained. (Target antigen: influenza hemagglutinin, norovirus capsid protein, human/mouse serum albumin, cannabinoids, etc.). These VHH libraries enable the generation of panels of high-affinity VHHs within a week.

1285

Comparison of an *in vitro* cytokine release model with *in vivo* serum cytokine responses in AML patients administered CSL362 in a Phase 1 study (CSLCT-AML-11-73)

Dyson, A.R.¹, Millar, C.¹, Hung, D.², Barnden, M.¹, Souravi, G.¹

¹CSL Limited, Clinical and Translational Science, Parkville, Australia, ²CSL Limited, Bioprocess Development, Parkville, Australia

Pharmaceutical manufacturers of therapeutic antibodies are requested by regulatory agencies to investigate the possible

release of cytokines *in vivo* directly related to the administration of the therapeutic antibodies, which may induce cytokine related infusion reactions in patients. The U.S. Food and Drug Administration (FDA) recommend, "*in vitro* assessments of cellular activation, including proliferation and cytokine release in human whole blood or peripheral blood mononuclear cells" prior to first-in-human (FIH) infusion. In this study, *in vitro* methods have been investigated to develop a model to evaluate the ability of CSL362, an anti-IL-3R α (anti-CD123) monoclonal antibody, to elicit cytokine responses that may mimic *in vivo* responses seen in patients. The *in vitro* model was able to detect cytokine release when whole blood from healthy volunteers (N=30) was exposed to CSL362. Thirteen cytokines/chemokines were evaluated and eleven of these (IL-6, IL-8, GM-CSF, IL-1 α , IL-1RA, IFN- γ , IL-12p70, IL-2, TNF- α , IL-12p40 and IL-1 β) were measured at concentrations >2-fold in some of the healthy volunteer blood samples exposed to CSL362 compared to buffer control. Acute myeloid leukemia (AML) patients administered CSL362 during a Phase 1 clinical study (CSLCT-AML-11-73) demonstrated transient elevation of seven (IL-6, IL-8, GM-CSF, IL-1RA, IFN- γ , IL-1 β and TNF- α) of the eleven cytokines/chemokines detected with the *in vitro* assay. This study demonstrates that *in vitro* models can be used to assess the potential of therapeutic antibodies' to elicit *in vivo* cytokine responses in humans.

1286

Bispecific antibodies

Gazumyan, A.

Rockefeller University, New York, United States

Cloning and expression of IgG genes from single B cell provided a powerful tool for the isolation and characterization of anti-Env antibodies with broad neutralizing activity.

BNabs (broadly neutralizing antibodies), that target four distinct epitopes, including the CD4-binding site, the V1V2-glycan region, the V3-glycan site, the gp41 membrane-proximal external region (MPER) were characterized.

In vitro neutralizing activities of double, triple and quadruple Ab combinations against a large panel of pseudo-viruses showed substantially improved the potency and the breadth compared to the corresponding single Abs.

Development of bispecific anti-HIV1 antibodies offers significant advantages over conventional monoclonal antibodies, as they combine the breadth and potency of two broadly neutralizing antibodies and overcome viral escape mechanisms associated with the use of single antibody.

We generated bispecific Abs by fusing two full-sized IgG antibodies via their C-termini utilizing sortase transpeptidation and click-chemistry, creating a covalently linked IgG antibody heterodimer. The neutralization by antibody dimer with bispecific activity that retains the activity and stability of the two original antibodies will be discussed.

1287**Composite molecules with EGF and its mutants as targeting elements and bundles of cytotoxic molecules or toll-like receptor agonists as effector elements**

Lin, C.-J.¹, Duh, Y.¹, Lin, C.Y.¹, Tian, W.-T.¹, Chen, J.-H.¹, Chu, H.-M.¹, Chang, T.-W.^{1,2}

¹Immwork Inc., Taipei, Taiwan, Republic of China, ²Genomics Research Center, Academia Sinica, Taipei, Taiwan, Republic of China

While anti-EGFR mAbs, such as Cetuximab, and anti-HER2/neu mAbs, such as trastuzumab, represent major advancement in the treatment of tumors expressing EGFR and HER2/neu, developing new drugs to increase response rates and decrease side effects in treating those tumors remains a major challenge. Herein, we report the development of a new class of pharmaceuticals with both targeting and effector moieties (T-E drugs), which adopt Fc-fusion, extended IgG, or/and multi-arm linker configurations. The targeting elements in those molecules are EGF or its EGF(S2W/D3V) mutants, which bind to multiple EGFR types. The effector elements are bundles of cytotoxic drugs or toll-like receptor (TLR) agonists, such as LPS and imiquimod. Each bundle carried 5 or more molecules and an Fc-fusion construct or IgG molecule with a C-terminal peptide linker and a cysteine residue extended from the CH3 domains was coupled with 2 of such bundles of drugs. In other T-E constructs, the scFv specific for PD-1 (CD279) or CD3 was fused to the C-termini of the Fc-fusion protein. Our results showed that the T-E drugs bound to EGFR- or ERBB3-overexpressed cancer cell lines in fluorescence flow cytometric assays. Furthermore, the T-E molecules with bundles of cytotoxic drugs or scFv against CD3 induced enhanced cytotoxicity of human epidermoid carcinoma cell line A431 and breast adenocarcinoma cell line MDA-MB-231 as compared to that of fusion protein with only targeting moiety. These results suggest that the T-E pharmaceuticals designed in this study may confer increased therapeutic efficacy and decreased side effects.

1288**Understanding the biology of the CD300f inhibitory receptor as a target for anti-AML antibodies**

Clark, G.^{1,2}, Gasirowski, R.¹, Kupresanin, F.¹, Mekkawy, A.¹, Hart, D.^{1,2}

¹ANZAC Research Institute, Sydney, Australia, ²University of Sydney, Sydney, Australia

Each year around 900 Australians will be affected by acute myeloid leukemia (AML) and, with currently available treatments, the majority will die from their disease. Therapeutic antibodies to immune cell surface molecules have revolutionised the treatment of many cancers but there is a need to develop human antibodies to more targets. CD300f belongs to the evolutionarily conserved CD300 family. It has a number of signalling motifs that both inhibit and activate cell functions. Human CD300f binds phosphatidylserine, an "eat-me" receptor inducing phagocytosis of dead cells. CD300f is expressed on myeloid lineage cells. Antibodies to CD300f prevent proliferation of AML cell lines in immunosuppressed mice. Our work has investigated CD300f as a potential anti leukemic target.

We showed by multiparameter flow cytometry that primary AML blasts from 78% (28/33) of patients and the CD34+CD38-leukaemia stem cell enriched fraction from 65% (13/20) of patients are CD300f+. We have used a panel of CD300f mAbs to demonstrate distinct epitopes on AML and healthy CD34+ cord blood (CB) cells. One mAb bound most AML samples and healthy CB Lin-CD34+CD38-CD45RA-CD90+ cells, whilst another bound only to monocytic AML samples and not healthy CB HSC. We found different CD300f protein isoforms on leukemic cell lines and identified differential expression of CD300f splice variants. These results indicate that isoforms of CD300f are differentially expressed across AML blasts and HSC. Only antibodies to key epitopes will be suitable for developing into AML therapeutic antibodies and these are now the focus of our program.

1289**Increment in drug loading on an antibody drug conjugate induces higher binding to the human Neonatal Fc receptor in acidic conditions *in vitro***

Brachet, G.¹, Respaud, R.², Arnoult, C.¹, Henriquet, C.³, Dhommée, C.¹, Viaud-Massuard, M.-C.¹, Heuzé-Vourc'h, N.⁴, Joubert, N.¹, Pugnère, M.³, Gouilleux-Gruart, V.¹

¹Université François Rabelais de Tours, CNRS, UMR 7292 Génétique, Immunothérapie, Chimie et Cancer, Tours cedex, France, ²CHRU de Tours, Pharmacie, Tours, France, ³Institut de Recherche en Cancérologie de Montpellier, INSERM, U 1194, Montpellier, France, ⁴Université François Rabelais de Tours, INSERM U 1100, Tours cedex, France

Antibody drug conjugates are an innovative class of biomedicine. They combine the high specificity of monoclonal antibodies to the power of cytotoxic drugs such as tubulin inhibitors, allowing specific delivery of the toxic payload and therefore fewer adverse effects. However, the technologies currently used for bioconjugation lead to highly heterogeneous solutions, composed of several species with different drug-to-antibody ratios. In the case of brentuximab vedotin, which is bioconjugated via the chemical reduction of cysteine residues involved in disulfide bonds, five main species are present inside the stock solution. These species have a drug-to-antibody ratio of zero, two, four, six or eight vedotin molecules per antibody. Monoclonal antibodies have an important half-life *in vivo*, notably thanks to neonatal Fc Receptor-mediated recycling. We investigated the influence of drug loading on the binding of the antibody to neonatal Fc Receptor (FcRn). We developed a hydrophobic interaction chromatography method for separating the different species of brentuximab vedotin. Physicochemical characterization showed that species with higher antibody-drug ratios tended to form more aggregates. We assessed the binding of these different species to FcRn in a cellular assay based on flow cytometry and surface plasmon resonance. FcRn binding assays showed that the most conjugated species, particularly those with saturated loading, interacted more strongly than unconjugated brentuximab vedotin with the FcRn in acidic conditions. However, affinity at neutral pH was negligible whatever the drug-to-antibody ratio, suggesting that the shorter half-life of these species is due to a factor other than modifications in the antibody-FcRn interaction.

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Immune signature profiles in cancer immunotherapy revealed by mass cytometric analysis

McGuire, H.^{1,2}, Asad, S.¹, Choi, C.¹, Smith, A.^{2,3}, McNeil, C.⁴, Hersey, P.⁵, Fazekas de St Groth, B.^{1,2}

¹Centenary Institute, T Cell Biology, Sydney, Australia, ²University of Sydney, Ramaciotti Facility for Human Systems Biology, Sydney, Australia, ³University of Sydney, Advanced Cytometry Facility, Sydney, Australia, ⁴Chris O'Brien Lifehouse, Sydney, Australia, ⁵Centenary Institute, Immuno Oncology, Sydney, Australia

The introduction of monoclonal antibodies that block immune checkpoint pathways represents a major advance in treatment of cancers such as melanoma and lung cancer. Check-point inhibitors targeting CTLA-4, PD-1 and PD-L1 can generate objective responses in patients with advanced disease that has proven refractory to treatment with chemo- and/or radio-therapy. However not all patients respond and we currently lack the ability to *predict* which individuals within a group of patients can be successfully treated. Further limitations include debilitating autoimmune-related side-effects in a subset of patients. Our previous lab findings of 'immune signatures' in autoimmune disease, combined with the essentially autoimmune nature of responses to autologous tumour, suggested that immune signature analysis could provide important insights into anti-tumour responses during checkpoint inhibitor therapy.

Utilising a highly innovative frontier technology; mass Cytometry with Time Of Flight analysis (CyTOF), we have investigated immune signatures in cancer patients both before and during treatment with checkpoint inhibitors. Using CyTOF we were able to quantitatively analyse 40 different surface and intracellular markers to profile each patient's immune system to an unprecedented resolution. We mapped progressive changes in circulating immune subset phenotypes and marker expression in metastatic melanoma patients following initiation of check-point inhibitor therapy. Our ultimate aim is to identify candidate immune signatures for prediction of response to cancer immunotherapy and likely severity of side-effects. We envisage that this strategy will facilitate selection of individually appropriate therapy options with minimal side effects across a broad range of diseases - the ultimate goal of personalised medicine.

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High levels of IgG antibodies against malondialdehyde modified collagen type IV predict coronary events

Vallejo, J., Dunér, P., Nilsson, J., Bengtsson, E.

Lund University, Department of Clinical Sciences, Malmö, Sweden

Objectives: In atherosclerosis, LDL particles are accumulated and oxidized in the vessel wall, which are considered as key events in the development of atherosclerotic lesions. During lipid oxidation, reactive adducts such as malondialdehyde (MDA) are formed and may leak out modifying surrounding extracellular matrix proteins, which could activate immune responses. Collagen type IV is the most abundant protein in the basement membranes and has an important role in the

maintenance of basement membrane integrity.

Approach and results: This study analyzed whether autoantibodies against MDA-modified collagen IV predict coronary events in a 15 years follow up study consisting of 795 subjects (385 individuals with coronary events and 410 age and gender matched controls) from Malmö Diet and Cancer study. IgG MDA-collagen IV antibodies were higher in individuals with coronary events compared to controls [0.7 (0.6 - 1.0) versus 0.6 (0.5 - 0.9); p= 0.0001]. Subjects from the highest tertile of MDA-collagen IV IgG, had an increased risk to develop coronary events [hazard ratio 1.561 (95 % confidence interval, 1.219 - 1.999); p for trend 0.0004]. This association remained significant after adjusting for Framingham risk factors and diabetes mellitus [HR 1.471 (1.121 - 1.930); p for trend 0.005]. Positive correlations between anti MDA-collagen IV IgG antibodies and plasma IL-6, MMP-10, MMP-12, cathepsin D and cystatin B were observed.

Conclusions: Individuals with higher levels of IgG MDA-collagen IV antibodies have an increased risk to develop coronary events. These antibodies were associated to proteases, which may reflect an increased degradation of the extracellular matrix.

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Cytokine-neutralizing therapeutic antibodies designed with tissue-targeting functions for increased delivery to diseased sites

Chen, J.-H.¹, Chu, H.-M.¹, Lin, C.Y.¹, Tian, W.-T.¹, Lin, C.-J.¹, Duh, Y.¹, Chang, T.W.^{1,2}

¹Immunwork Inc., Taipei, Taiwan, Republic of China, ²Academia Sinica, Genomics Research Center, Taipei, Taiwan, Republic of China

Therapeutics, such as adalimumab and etanercept specific for tumor necrosis factor- α (TNF- α), have been approved for treating rheumatoid arthritis, secukinumab specific for interleukin 17 (IL-17) for psoriasis, belimumab specific for B cell-activating factor (BAFF) for systemic lupus erythematosus (SLE), and denosumab specific for receptor activator of nuclear factor kappa-B ligand (RANKL) for osteoporosis and tumor metastases to bone. Those antibodies neutralize key cytokines and mediate therapeutic effects, but also cause a wide range of side effects, such as serious infections. We have engineered single-chain variable fragments (scFv) specific for extracellular matrix proteins, which are relatively abundant in the affected connective tissues, onto the respective therapeutic antibodies, so that they can be channeled to those tissues upon administration. For this purpose, adalimumab is fused *via* the CH3 domain with scFv against collagen type II, which is abundant in cartilage of the joints, secukinumab and belimumab with scFv against collagen type VII, a key component of anchoring fibrils between epidermis and dermis of the skin, and denosumab with scFv against osteonectin, which is uniquely prominent in bone matrix. Herein, we describe the generation and characterization of denosumab-scFv anti-osteonectin in an "extended IgG configuration". The bispecific molecule retained the binding and functional characteristics of denosumab, but with the additional activity to bind to osteonectin. In BALB/c mice, denosumab-

(scFv anti-osteonectin) showed a distribution pattern similar to an anti-osteonectin monoclonal antibody, reaching to bones at larger proportions than denosumab. These antibodies with tissue-targeting functions may have better therapeutic efficacy and fewer side effects.

1293

Interleukin-17 blockade in rheumatoid arthritis: a systematic review and meta-analysis

Shan, J.¹, Chen, Z.², Chen, X.³, Chen, W.¹, Jin, H.¹, Xu, Y.¹, Cheng, X.¹

¹Chengdu Medical College, Chengdu, China, ²Chengdu First People Hospital, Chengdu, China, ³Sichuan University, Chengdu, China

Background: The interleukin-17 (IL-17) plays an important role in the pathogenesis of rheumatoid arthritis (RA). Several IL-17 blockers, including the anti-IL-17A antibodies secukinumab and ixekizumab, and the anti-IL-17 receptor subunit A antibody brodalumab have been used in patients with RA. In this study, we aim to evaluate the efficacy and safety of the IL-17 blockers for the treatment of RA.

Methods: The Cochrane Central Register of Clinical Trials, MEDLINE, and EMBASE were searched for identification of relevant trials. All controlled trials comparing IL-17 blockers versus placebo were eligible for inclusion.

Main results: Six trials (a total of 1126 participants) were included. The trials were generally of low risk of bias. Follow-up ranged from 12 to 16 weeks. Meta-analysis showed that IL-17 blockade could significantly improve the ACR20 (RR = 1.36; 95% CI: 1.14-1.61), ACR50 (RR = 2.12; 95% CI: 1.42-3.15) and ACR70 (RR = 2.86; 95% CI: 1.29-6.37). A greater reduction in DAS28-CRP score from baseline was observed when secukinumab and ixekizumab used. There was no evidence of statistically significant differences in the overall AE incidence rate (RR = 1.05; 95% CI: 0.93-1.18) and infections (RR = 1.32; 95% CI: 0.98-1.77) between IL-17 blocker and placebo.

Conclusions: IL-17 blockade results in significant improvement in clinical signs and symptoms of RA, and there was no evidence of a difference in the rates of AE between groups, suggesting that IL-17 blocker may offer an alternative treatment for RA, especially among patients who with inadequate response to other treatments, including TNF blocker therapy.

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Synergistic therapeutic efficacy of Unituxin and PD-1 blockade in a murine neuroblastoma model

Hung, J.-T., Huang, J.-R., Yu, A.L.

Institute of Stem Cell & Translational Cancer Research, Taoyuan, Taiwan, Republic of China

The approval of Unituxin, a chimeric anti-GD2 antibody, for the treatment of high-risk neuroblastoma by US FDA and European Commission in 2015 marks the first new agent targeting a glycolipid molecule, thereby widening the net of potential pharmaceutical targets. Dr. Yu has led the development of ch14.18 from IND to the randomized phase III clinical trial, which showed a significant improvement in 2-year event-free survival from 46% with standard therapy to 66% with immunotherapy.

However, at least 1/3 of high-risk neuroblastoma patients still fail this immunotherapy. To improve the efficacy of anti-GD2, we evaluated its combination with anti-PD-1 antibody in a murine NXS2 neuroblastoma model, given the reported expression of PD-L1 in neuroblastoma. The suboptimal dosages of murine anti-GD2 mAb14G2a and anti-PD-1 mAbJ43 antibodies were first determined in mice bearing neuroblastoma NXS2 cells. At 31 days after tumor injection, the percentage of CD4+ cells and CD8+ cells in spleen was greater but the percentage of MDSCs was lower in mice treated with suboptimal dose of mAb14G2a than those treated with PBS control. Notably, the percentage of CD3+PD-1+ was higher in mice treated with mAb14G2a than control mice. Next, mice were treated with mAb14G2a, anti-PD-1 alone, or in combination, three days after tumor injection. The median survival of NXS2-bearing mice treated with combined anti-GD2/anti-PD-1 antibodies (40 days) was significantly longer than those treated with anti-GD2 (33 days) or anti-PD-1 (33 days) ($p < 0.001$). Our findings provide preclinical evidence for future clinical trials of Unituxin combined with anti-PD-1 in patients with neuroblastoma.

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Targeting Epstein-Barr virus transformed B lymphoblastoid cells using antibodies with TCR-like specificities

Lai, J.¹, Tan, W.J.^{1,2}, Too, C.T.¹, Choo, J.A.L.¹, Wong, L.H.², Mustafa, F.¹, Srinivasan, N.³, Lim, A.P.C.⁴, Zhong, Y.¹, Gascoigne, N.¹, Hanson, B.⁴, Chan, S.H.³, Chen, J.², Macary, P.¹

¹National University of Singapore, Department of Microbiology and Immunology, Singapore, Singapore, ²Infectious Disease Interdisciplinary Research Group, Singapore-MIT Alliance for Research and Technology (SMART), Singapore, Singapore,

³National University of Singapore, WHO Immunology and Training Research Centre, Singapore, Singapore, ⁴Defense Medical and Environmental Research Institute, Defence Science Organization National Laboratories, Singapore, Singapore

Epstein-Barr virus (EBV) is an oncovirus associated with several human malignancies including Hodgkin's and Burkitt's lymphomas, nasopharyngeal carcinoma as well as post-transplant lymphoma in immunosuppressed patients. While EBV displays restricted gene expression during different latency programmes, the viral gene products that are frequently detected in EBV-associated tumors include EBNA1, LMP1 and LMP2A. We showed here that anti-EBV T cell receptor-like monoclonal antibodies (TCR-like mAbs) E1, L1 and L2 bound to their respective HLA-A*0201-restricted EBV peptides EBNA1₅₆₂₋₅₇₀ (FMVFLQTHI), LMP1₁₂₅₋₁₃₃ (YLLEMLWRL) and LMP2A₄₂₆₋₄₃₄ (CLGGLTMV) with high affinities and specificities. These mAbs recognized endogenously presented targets on EBV B lymphoblastoid cell lines (BLCLs) but not to PBMCs from which they were derived. Furthermore, these mAbs displayed similar binding activities on several BLCLs despite inherent heterogeneities between different donor samples. A single weekly administration of the naked mAbs reduced splenomegaly, liver tumor spots and tumor burden in BLCL-engrafted immunodeficient mice. In particular, mice that were treated with the E1 mAb displayed delayed weight loss and significantly prolonged survival. *In vitro*, these TCR-like mAbs

induced the upregulation of surface phosphatidylserine and early apoptosis of BLCLs, thereby enhancing their Fc-dependent phagocytic uptake by macrophages. Taken together, these data demonstrate anti-EBV TCR-like mAbs as potential therapeutic candidates to treat EBV-associated diseases.

1296

Characterization of human CD83 expression on immune cells identifies a unique CD83+ T cell population

Ju, X.¹, Silveira, P.A.^{1,2}, Elgundi, Z.¹, Verma, N.¹, Alingcastre, R.¹, Hsu, W.-H.^{1,2}, Fromm, P.D.^{1,2}, Kupresanin, F.¹, Li, Z.^{1,2}, Lo, T.H.^{1,2}, Clark, G.J.^{1,2}, Hart, D.N.J.^{1,2}

¹ANZAC Research Institute, Dendritic Cell Research, Sydney, Australia, ²University of Sydney, Sydney Medical School, Sydney, Australia

CD83 is an important marker of activated dendritic cells (DC). We targeted CD83 using a rabbit polyclonal anti-CD83 antibody and a human anti-human CD83 monoclonal antibody (mAb), 3C12C, to deplete activated DC as a novel approach to immunosuppression. Anti-CD83 prevented graft versus host disease (GVHD) and preserved human T cell responses after transplantation in SCID mice. Because CD83 is also expressed on other immune cells, we undertook a comprehensive analysis of its expression and glycosylation pattern on various immune cell populations. There were distinct CD83 splice variants (full length CD83 plus splice variants CD83a, CD83b and CD83c) in different immune cells. CD83 glycosylation status also differed, with glycosylation required for expression on activated DC, whereas its expression on activated B cells and monocytes was resistant to de-glycosylation. Monocytes released more soluble CD83 than DC. Interestingly, different T cell stimuli (PHA, CD3/CD28 beads and mixed leukocyte reaction (MLR) culture) increased CD83 expression on T cells with different kinetics, underlining the distinct signaling pathways involved. Our investigations established that human T cell activation induced transient transcription and surface expression of CD83 on a specific responding T cell subpopulation, which co-express MHC class II and exhibit a memory phenotype. These distinct expression profiles of CD83 isoforms in different human immune cells are likely to contribute to several immune regulatory pathways.

1297

IPH4301, an antibody targeting MICA and MICB exhibits potent cytotoxic activity and immunomodulatory properties for the treatment of cancer

Morel, A., Viaud, N., Bonnafous, C., Trichard, S., Joulin-Giet, A., Grondin, G., Mizari, S., Cohen-Tannoudji, L., Morel, Y., Rossi, B., Patrel, C., Buffet, R., Gauthier, L., Wagtmann, N., Bléry, M.
Innate Pharma, Marseille, France

MICA, MICB, and ULPB1-6 are ligands for NKG2D, an activating receptor expressed on NK cells and subsets of T cells. As markers of cellular stress and tumorigenesis, MICA and MICB proteins are attractive targets for cancer therapy. MICA/B are also compelling targets for immunomodulation. Chronic exposure to MICA and -B cause internalization of NKG2D,

leading to desensitization of cytotoxic NK and T cells in cancer patients. Moreover, NKG2D ligand expression is induced on immunosuppressive macrophages in cancer patients raising the possibility that anti-MICA/B antibodies may be used to counter local immunosuppression by targeting myeloid derived suppressor cells.

We have identified and humanized an anti-MICA/B antibody, IPH4301, that binds to all allotypes of MICA and MICB. First, IPH4301 induces killing of MICA/B expressing tumor cells through antibody-dependent cell cytotoxicity and antibody-dependent cell phagocytosis measured towards MICA expressing cells. Second, IPH4301 blocks the binding of MICA/B to NKG2D. In tumor, NKG2D down-modulation is primarily induced by expression of MICA/B. By blocking the NKG2D-MICA/B interaction, IPH4301 effectively restored NKG2D expression *in vitro* on primary NK and T cells. Third, *In vitro* differentiated M2 macrophages, but not M1 macrophages, impaired the cytotoxic functions of autologous NK cells towards MICA expressing tumor cell lines. This suppression could be overcome by IPH4301, which triggered ADCC by these otherwise impaired NK cells.

Altogether, IPH4301 is a novel, first-in-class anti-MICA/B mAb with both cytotoxic and immunomodulatory properties. Ongoing work aims to perform regulatory toxicology studies and manufacture a clinical grade product for testing in a clinical trial.

1298

Lymphatic access of therapeutic proteins after IV administration is size-dependent and primarily occurs within the liver and mesentery

Yadav, P., McLeod, V., Johnston, A.P.R., Kaminskas, L.M., Trevaskis, N.L.
Monash University, MIPS, Drug Delivery, Disposition and Dynamics, Melbourne, Australia

Background: The lymphatic system is implicated in cancer metastasis, infectious disease and inflammatory disease. These diseases are typically treated with small molecule drugs that display limited lymphatic affinity. Therapeutic proteins may better target and treat lymphatic diseases since they are cleared from the interstitium via lymphatics. Recent studies demonstrate substantial lymph access of IV administered therapeutic proteins but the mechanism is unknown.

Aim: To determine the major sites and protein properties that facilitate lymph access of IV administered therapeutic proteins.

Methods: Cannulas were inserted into rat's jugular vein (IV injection), carotid artery (blood collection) and liver, intestine or thoracic lymph duct (lymph collection). Therapeutic proteins (PEGylated-interferons IFN-PEG12 (31kDa), IFN-PEG40 (60kDa) and trastuzumab (150kDa) were administered IV, plasma and lymph collected over time and protein concentrations quantified via ELISA.

Results: Thoracic lymph recovery was significantly greater for trastuzumab and IFN-PEG40 (4-7% dose) vs IFN-PEG12 (1-2% dose). Conversely, the thoracic lymph/plasma (L/P) concentration ratio, an indication of lymph transfer efficiency, was highest for IFN-PEG12 (90-100%) and similar for IFN-PEG40 and trastuzumab (15-30%). The lower lymph transport

of IFN-PEG12 reflected more rapid plasma clearance. For all proteins, lymph access primarily occurred in the liver (>40%) and mesentery (>40%) but lymph access mechanism differed in these tissues.

Conclusions: Lymph access of IV therapeutic proteins is enhanced for proteins with long circulation half-lives and that are small enough to readily transfer to lymph. Access predominantly occurs in the intestine and liver. Optimising therapeutic proteins represents a viable approach to target and treat lymphatic diseases.

1299

Idiopathic mast cell activation disorder: control of idiopathic and venom immunotherapy associated anaphylaxis with low dose Omalizumab

Berry, R.¹, Hollingsworth, P.¹, Lucas, M.^{1,2,3,4}

¹Sir Charles Gairdner Hospital, Department of Clinical Immunology, Perth, Australia, ²University of Western Australia, School of Medicine and Pharmacology, Perth, Australia, ³University of Western Australia, School of Pathology and Laboratory Medicine, Perth, Australia, ⁴Murdoch University, Institute for Immunology and Infectious Disease, Perth, Australia

Mast cell disorders (MCD) are classified as primary (mastocytosis and monoclonal mast cell activation syndrome; MMAS), secondary (known trigger and polyclonal) and idiopathic. MCD patients are at increased risk of anaphylaxis to hymenoptera stings and to its treatment with venom specific immunotherapy (VIT). Omalizumab, an anti-IgE antibody has been reported to permit safer VIT application and prevent anaphylaxis associated with VIT.

Here we present a patient who suffers from severe bee venom anaphylaxis, who was found to have a constant elevation of his mast cell tryptase (MCT; range 14.9-25.0 µg/L) and was initiated on bee VIT. Adrenaline auto-injectors were prescribed for anaphylaxis management. He suffered recurrent anaphylaxis to VIT, and remained symptomatic in the weeks between VIT injections with cognitive difficulty, mood disorder and hypotension to the extent of disability. His clinical symptoms and elevated MCT were reflective of a MCD, however clonality markers, bone marrow biopsy and FDG-PET revealed no evidence for primary MCD. VIT could not be continued and treatment with high dose anti-histamines and montelukast did not alter his symptoms. We initiated low dose Omalizumab treatment at 150mg SC monthly. This allowed for reintroduction of VIT and also led to a resolution of clinical symptoms. The patient was able to return to work while on bee VIT maintenance. His MCT remains stable at 20 µg/L.

This presents the first case reported in the literature where low dose Omalizumab has been a successful treatment in idiopathic non-clonal MCD with symptomatic improvement and prevention of anaphylaxis during bee VIT.

1300

Potent antitumor activity of IL-2-Fc fusion proteins through FcγR-dependent depletion of CD4⁺ CD25⁺ Tregs

Vazquez-Lombardi, R., Webster, K.E., Sprent, J., Christ, D.

Garvan Institute of Medical Research, Immunology Division, Sydney, Australia

Interleukin-2 (IL-2)-mediated expansion of cytotoxic CD8⁺T-cells and natural killer cells has been successfully used in cancer immunotherapy. However, clinical use of IL-2 is complicated by dose-limiting toxicities and inevitable expansion of CD4⁺ CD25⁺ regulatory T-cells (Tregs). Previous attempts to overcome these limitations have focused on maximization of cytotoxic cell expansion, as exemplified by IL-2/anti-IL-2 immune complexes and IL-2 'superagonist' proteins. Here, we report potent and selective activity of engineered fusion proteins consisting of IL-2 and the antibody Fc region. As expected, disruption of CD25 binding through mutation of the IL-2 component led to strong expansion of CD25⁻ cytotoxic subsets. Surprisingly, however, when tested for tumor rejection, such constructs were in fact less effective than wild-type IL-2-Fc protein. Further analyses revealed that efficacy was crucially dependent on selective depletion of Tregs through Fc-mediated immune effector functions. Our results reveal an unexpected mechanism of action of IL-2-Fc fusion proteins and provide important guidelines for the development of next-generation IL-2 immunotherapies.

1301

Low-dose doxorubicin reduces an immunosuppressive Ly6C^{lo} monocyte population to improve efficacy of anti-41BB monoclonal antibody against B cell lymphoma

McKee, S.J., Doff, B.L., Gandhi, M.K., Mattarollo, S.R.

University of Queensland Diamantina Institute, Translational Research Institute, Woolloongabba, Australia

Tumours promote the expansion of myeloid populations with immunosuppressive activity. This immunosuppressive microenvironment has a negative impact on the efficacy of therapeutic monoclonal antibodies that aim to stimulate anti-tumour lymphocyte populations. In a murine model of *c-myc* driven B cell lymphoma (Eµmyc) we have observed a significant expansion of a CD115⁺ Ly6C^{lo} monocyte population. Expansion of immunosuppressive monocytes was also observed in Hodgkin's lymphoma patient samples. These cells displayed immunosuppressive activity against effector T cell populations via high expression of PDL-1. Granulocytes and classical Ly6C^{hi} monocytes expressed significantly lower levels of PDL-1. Intravenous administration of low dose (1 mg/kg) soluble or liposomal doxorubicin into tumour bearing hosts was able to reduce the abundance of CD115⁺ Ly6C^{lo} PDL-1^{hi} monocytes without affecting T cell populations. Doxorubicin-mediated reduction of the immunosuppressive monocyte population improved the efficacy of therapeutic anti-41BB monoclonal antibody to stimulate T cells and correspondingly decreased PD-1 expression on CD8⁺ and CD4⁺ T cells. When used in combination, low dose doxorubicin and anti-41BB monoclonal antibody were able to significantly reduce the number of lymphoma cells and improve survival of lymphoma bearing

mice. Low dose doxorubicin is a promising pre-conditioning step to improve the efficacy of anti-41BB monoclonal antibody therapy against lymphoma via selective reduction of immunosuppressive myeloid cells.

1302

IL3R-alpha as a novel therapeutic target in systemic lupus erythematosus

Oon, S.^{1,2,3}, Huynh, H.⁴, Tai, T.Y.⁴, Ng, M.⁴, Monaghan, K.⁴, Biondo, M.⁴, Vairo, G.⁴, Maraskovsky, E.⁴, Nash, A.⁴, Wicks, I.^{1,2,3}, Wilson, N.⁴

¹The Walter and Eliza Hall Institute of Medical Research, Inflammation Division, Parkville, Australia, ²The Royal Melbourne Hospital, Department of Rheumatology, Parkville, Australia, ³The University of Melbourne, Department of Medical Biology, Parkville, Australia, ⁴CSL Limited, Parkville, Australia

There remains significant morbidity in SLE despite current therapeutics. The autoantibody-producing B cell has been a major therapeutic target as has, more recently, type I IFN, produced by pDCs in response to TLR7 and TLR9 activation by SLE immune complexes. We have developed a novel mAb targeting the IL3R-alpha, CSL362, and demonstrate here that it affects multiple pathogenic cell types and cytokines. Via antibody-dependent cell-mediated cytotoxicity, CSL362 potently and specifically depletes CD123^{high} pDCs and basophils. Through pDC depletion, TLR7-, TLR9- and SLE serum-stimulated IFN-alpha production, and IFN-inducible gene expression is markedly reduced. These effects were seen *in vitro* in a heterogeneous population of SLE donors and healthy controls, and confirmed *in vivo* in cynomolgus macaques. We also found that TLR7- and TLR9-induced plasmablast expansion was inhibited by CSL362. This depended upon decreasing not only IFN-alpha, but IL-6, production, through depleting pDCs. Importantly, we show that CSL362 more effectively inhibits IFN-inducible gene expression and plasmablast expansion than IFN-alpha blockade alone. This may be because pDC depletion by CSL362 reduces production of cytokines other than IFN-alpha, such as IL-6, and interferon types other than type I. Indeed, we found that CSL362 reduced production of type III IFN, a cytokine which is postulated to cause ongoing disease activity despite type I IFN blockade. The unique ability of CSL362 to affect two major contributors to SLE, and newer pathogenic targets such as the basophil, IL-3 and type III IFN, presents a strong rationale for its therapeutic evaluation in SLE.

1303

Establishment of a therapeutic anti-pan HLA-class II monoclonal antibody that directly induces lymphoma cell death via large pore formation

Matsuoka, S.¹, Ishii, Y.², Abe, M.¹, Ohtsuji, N.¹, Mizutani, N.³, Nakao, A.⁴, Yagita, H.⁵, Hino, O.¹

¹Juntendo University School of Medicine, Pathology and Oncology, Tokyo, Japan, ²RIKEN Center for Integrative Medical Sciences, Vaccine Design, Yokohama, Japan, ³Kyorin University of Medicine, Pathology, Tokyo, Japan, ⁴Department of Research Interdisciplinary Graduate School of Medicine and Engineering, Immunology, Yamanashi, Japan, ⁵Juntendo University School of

Medicine, Immunology, Tokyo, Japan

To develop a new therapeutic monoclonal Antibody (mAb) for Hodgkin lymphoma (HL), we immunized a BALB/c mouse with live HL cell lines, alternating between two HL cell lines. After hybridization, we screened the hybridoma clones by assessing direct cytotoxicity against a HL cell line not used for immunization. We developed this strategy for establishing mAb to reduce the risk of obtaining clonotypic mAb specific for single HL cell line. A newly established mouse anti-human mAb (4713) triggered cytoskeleton-dependent, but complement- and caspase-independent, cell death in HL cell lines, Burkitt lymphoma cell lines, and advanced adult T-cell leukemia cell lines. Intravenous injection of mAb 4713 in tumor-bearing SCID mice improved survival significantly. mAb 4713 was revealed to be a mouse anti-human pan-HLA class II mAb. Treatment with this mAb induced the formation of large pores on the surface of target lymphoma cells within 30 min. This finding suggests that the cell death process induced by this anti-pan HLA-class II mAb may involve the same death signals stimulated by a cytolytic anti-pan MHC class I mAb that also induces large pores formation. This multifaceted study supports the therapeutic potential of mAb 4713 for various forms of lymphoma.

1304

Targeting Sperm protein 17 for developing an immunotherapeutic treatment for ovarian cancer

Xiang, S.D.¹, Gao, Q.¹, Wilson, K.L.¹, Heyerick, A.², Stephens, A.³, Plebanski, M.¹

¹Monash University, Centra Clinical School, Department of Immunology and Pathology, Melbourne, Australia, ²PX Biosolutions Pty Ltd, South Melbourne, Australia, ³Hudson Institute of Medical Research, Ovarian Cancer Biomarker Laboratory, Clayton, Australia

Ovarian cancer (OC) is the leading cause of death from gynaecological malignancy. Immunotherapeutic strategies are considered less toxic and more specific than current treatments. Sperm protein (Sp17) is a protein aberrantly expressed in ovarian tumors and other cancers, but undetectable on normal tissues except on male spermatozoa, making it a highly specific target for treatment of OC. Our previous studies identified a specific region (hSp17₁₁₁₋₁₄₂) in the human Sp17 sequence which is highly immunogenic, induced high levels of antibodies and IFN-γ producing T cells both in C57BL/6 and in C57BL/6-HLA-A2.1 transgenic mice, when adjuvanted with CpG or nanoparticles. C57BL/6 mice immunised with CpG adjuvanted hSp17₁₁₁₋₁₄₂ significantly prolonged the life-span of the mice bearing the ovarian carcinoma ID8 cell line. We further mapped the immuno-dominant B and T cell epitope regions within the hSp17₁₁₁₋₁₄₂ and identified a single immuno-dominant B cell (134-142 aa) epitope and 2T helper 1 (Th1) cell epitopes (111-124 and 124-138 aa). Further exploring the therapeutic potential of the hSp17₁₁₁₋₁₄₂, our recent studies showed that anti-hSp17₁₁₁₋₁₄₂ serum antibody can directly kill the OC tumor cell lines derived from either mouse or human origins *in vitro*. Sp17 expression is indispensable for tumor survival *in vivo*, and highly co-localized with 2 markers (patent pending) which associate with the tumor escape and drug resistant. This provides an opportunity

to design an immunotherapeutic regiment targeting Sp17 and combing other immune based therapies/drugs to eliminate the “toughest” cancer cells which often resistant to chemotherapy, and offers a new and tremendous advantages.

1305

Targeting EBV positive cancers with affinity enhanced T cell receptors

Wong, Y.¹, Gostick, E.², Lomax, M.³, Vuidepot, A.³, Baston, E.³, Shingler, W.³, Hassan, N.³, Dukes, J.³, Smith, C.¹, Khanna, R.¹, Ladell, K.², Cole, D.K.², Sewell, A.K.², Burrows, S.R.¹, Jakobsen, B.³, Price, D.A.², Miles, J.J.¹

¹QIMR Berghofer Medical Research Institute, Immunology, Herston, Australia, ²Cardiff University School of Medicine, Infection and Immunity, Cardiff, United Kingdom, ³Immunocore Ltd, Abingdon, United Kingdom

Epstein-Barr virus (EBV) is associated with over 30 different diseases and malignancies. The pathogen is associated with over 300,000 cases of cancer annually and is thought to be involved in various pathologies affecting over one million people annually. Currently, no commercially available EBV specific therapy or vaccine exists. Using phage display directed evolution; we generated a panel of affinity enhanced T cell receptors (TCR) that targeted an EBV antigen from LMP2. We then generated Immune Mobilising Monoclonal TCRs Against Cancer (ImmTACs) by fusing an anti-CD3 effector payload to the constant region of the TCR. In soluble form, EBV ImmTACs were able to redirect T cell specificity *in vitro* in low picomolar concentrations against nasopharyngeal carcinoma, Burkitt's lymphoma, gastric carcinoma, diffuse large B-cell lymphoma and lymphoblastic cell lines. Using multiparametric flow cytometry, we observed EBV ImmTACs could redirect large numbers of polyfunctional CD8+ and CD4+ T cells against EBV positive cancers. We are currently testing these soluble therapeutics in humanised and tumor xenograft EBV mouse models to gauge safety and *in vivo* efficacy. EBV ImmTACs are first in class and represent a promising new “off-the-shelf” therapy against EBV associated cancers and could be a significant stride forward in treating the wide spectrum of diseases associated with this pathogen.

1306

Identification of the single immunodominant epitope of the native human CC chemokine receptor 6

Yssel, H.¹, Dorgham, K.¹, Dejou, C.², Piesse, C.³, Pène, J.⁴, Gorochov, G.⁵
¹Centre d'Immunologie et des Maladies Infectieuses, Inserm U 1135 Bât C.E.R.V.I., Paris, France, ²Institut de Recherche en Cancérologie, Montpellier, France, ³Sorbonne Universités, UPMC Univ Paris 06, IBPS-FR 3631, Service de Synthèse Peptidique, Paris, France, ⁴Institute for Regenerative Medicine and Biotherapy, Inserm U 1183, Montpellier, France, ⁵AP-HP, Groupement Hospitalier Pitié-Salpêtrière, Paris, France

Chemokines and their receptors play an important role in cell trafficking and recruitment. The CCR6 chemokine receptor, selectively expressed on leukocyte populations, has been

shown to play a deleterious role in the pathogenesis of various chronic inflammatory diseases and, as such, may constitute a prime target for the development of novel immunotherapeutic treatment. However, the development of strategies to modulate inflammation mediated by this chemokine receptor in human has been hampered by the lack of specific inhibitory small molecules. Moreover, several anti-human CCR6 (huCCR6) antibodies have been developed in different species, but to date none have proven function-blocking activity. Here, we report strategies to generate neutralizing mouse mAbs specific for huCCR6. Immunization of mice with peptides mimicking extracellular domains, potentially involved in CCR6 function, failed to induce Abs reactive with the native receptor. In contrast, although the use of NIH-3T3 cells expressing huCCR6 resulted in the isolation of mAbs specific for this receptor, they were not able to block the interaction between huCCR6 and huCCL20. Finally, the results show that all mouse mAbs generated by immunization with native huCCR6, will invariably recognize a unique, but non-neutralizing, immunodominant epitope in the first part of its N-terminal domain. All together, this work allows a better understanding of the impediments to generate specific inhibitory molecules against the huCCR6.

Transcription Factors

1308

Transcription factor availability rather than modulated enhancer availability regulates CD8⁺ T cell differentiation

Russ, B.¹, Olshansky, M.¹, Li, J.¹, Nguyen, M.¹, Chen, E.¹, Khoury, G.¹, Nussing, S.¹, Nguyen, T.¹, Olson, M.¹, Purcell, D.¹, Speed, T.², Rao, S.³, Turner, S.¹

¹The University of Melbourne at the Peter Doherty Institute for Infection and Immunity, Melbourne, Australia, ²Walter & Eliza Hall Institute, Melbourne, Australia, ³Canberra University, Canberra, Australia

The changes in phenotype and function that characterize the differentiation of naïve T cells to effector and memory states are underscored by large-scale, coordinated, and stable changes in transcription. In-turn, these changes are choreographed by the interplay between transcription factors (TFs) and epigenetic regulators that restructure the genome, ensuring lineage-appropriate gene expression. To better understand how differentiation state-specific transcriptional profiles arise and are maintained during lymphocyte differentiation, we have studied transcriptional enhancer (TE) usage within virus-specific naïve, effector and memory CD8⁺ T cells. TEs serve as binding sites for lineage specific TFs, and are thus key determinants of transcriptional regulation and cellular differentiation. Using ChIP-Seq to map TEs based on their chromatin state, we identified ~45,000 T cell TEs as putative regulators of virus-specific T cell differentiation; Strikingly, the majority of these are shared by naïve, effector and memory cells, implying that T cell differentiation is largely coordinated by differentiation state-specific TF binding, rather than *de novo* TE acquisition and loss. Indeed, combining RNA-seq data with genome-wide mapping of TFs, we show that many of the TEs that we have identified show an “active” chromatin signature within naïve T cells, and

prior to expression the TFs they bind. Finally, we identified a subset of TEs with a non-canonical enhancer chromatin signature that mark immune and other highly expressed genes. This unique class of TEs are strongly enriched for binding of key T cell TFs including BATF and IRF4 upon effector differentiation, suggesting they are critical determinants of T cell differentiation.

1309

Epigenetic control of Gata3 gene expression in T helper 2 cells via histone modification and DNA conformation

Tumes, D.^{1,2}, Onodera, A.², Papadopoulos, M.², Nakayama, T.²

¹South Australian Health and Medical Research Institute, Infection and Immunity Theme, Adelaide, Australia, ²Chiba University, Department of Immunology, Graduate School of Medicine, Chiba, Japan

Expression of the transcription factor Gata3 is both necessary and sufficient for differentiation of Type-2 cytokine-producing CD4⁺ T helper 2 (Th2) cells. IL-4 induces Gata3 expression by signalling through STAT6. We recently reported that loss of the polycomb repressive complex 2 protein Ezh2 results in increased sensitivity to IL-4-induced Th2 polarisation and spontaneous development of Type-2 inflammation *in vivo*, but how Ezh2 physically regulated Th2 cell differentiation remained unknown. This study aimed to determine if conformational changes to the Gata3 gene occur during Th2 differentiation and if Ezh2 regulates these changes. Using formaldehyde-assisted isolation of regulatory elements and next-generation sequencing (FAIRE-Seq), we found that IL-4-induced Th2 differentiation resulted in increased accessibility of a functional STAT6 binding site within the Gata3 gene. Interestingly, loss of Ezh2 function mimicked this effect, but in an IL-4-independent fashion, and this was accompanied by increased levels of the histone acetyltransferase CBP and accumulation of H3K27-acetylation. Using chromatin conformation capture (3C) we found that the Gata3 transcriptional start site (TSS) physically associated with this same STAT6 binding site within the nuclei of differentiated Th2 cells. We propose a new model of physical epigenetic regulation of Gata3 expression whereby Ezh2 regulates accessibility of the Gata3 gene to STAT6. STAT6 binding then results in a conformational change to the Gata3 gene that forms a transcriptional hub bringing the TSS into direct contact with the STAT6 binding site and initiating transcription. These findings will likely have implications for rational drug design for the treatment of Type-2 inflammatory diseases.

1310

Cathelicidin binds to transcriptional complexes in cancer cells

Pinheiro da Silva, F.¹, Munoz, M.², Martins de Lima, T.¹, Severino, P.³, Cerqueira César Machado, M.¹, Moraes Reis, E.⁴, Nakaya, H.², Irineu, V.¹

¹University of Sao Paulo, Emergency Medicine, Sao Paulo, Brazil,

²University of Sao Paulo, Pharmacology, Sao Paulo, Brazil,

³Hospital Albert Einstein, Sao Paulo, Brazil, ⁴University of Sao Paulo, Chemistry, Sao Paulo, Brazil

Antimicrobial peptides (AMPs) are key molecules of innate immunity, but the cellular mechanisms regulated by these molecules and how these pathways are activated remain poorly understood by the scientific community. Our group was the first to describe that LL-37, an antimicrobial peptide, migrates to the nucleus during systemic bacterial infections and was the first to suggest that, under certain conditions, this peptide could act as a transcription factor. We therefore decided to investigate whether such translocation occurs in other complex diseases, such as autoimmune diseases and cancer. A ChIP DNA was processed into a standard Illumina ChIP-Seq library and sequenced to generate >>2 million reads. Reads were aligned to the human genome (hg19), and after removal of duplicate reads, 4.6 million alignments were obtained. A signal map showing fragment densities along the genome was generated and visualized in the Integrated Genome Browser (IGB). The results show that the Cathelicidin ChIP-Seq data show a small number of strong to very strong peaks, fewer than in the positive data set shown, but clearly many more than in the negative Input data in the middle track. To further examine the specificity of these peaks, we analyzed their location in more detail and found that 85% of the 421 peaks are located in promoter regions, and the peak sequences are enriched in the binding sites for transcription factors NFYA and SP1. Thus, we conclude that Cathelicidin may participate in transcriptional complexes.

1311

Tumor suppressor WW domain-containing oxidoreductase regulates hematopoiesis

Lo, J.-Y.¹, Hsu, L.-J.^{2,3,4}

¹National Cheng Kung University, Basic Medical Sciences, Tainan, Taiwan, Republic of China, ²National Cheng Kung University Medical College, Medical Laboratory Science and Biotechnology, Tainan, Taiwan, Republic of China, ³National Cheng Kung University Medical College, Center of Infectious Disease and Signaling Research, Tainan, Taiwan, Republic of China, ⁴National Cheng Kung University Medical College, Research Center for Medical Laboratory Biotechnology, Tainan, Taiwan, Republic of China

Human *WWOX* gene resides in the common fragile site FRA16D on chromosome 16q23.3-24.1, and encodes a candidate tumor suppressor WW domain-containing oxidoreductase (designated *WWOX*, *FOR* or *WOX1*). Previous studies have suggested that *WOX1* mediates cell apoptosis upon stress responses. Downregulation of *WOX1* protein expression has been found in many types of human cancers. Strikingly, using our generated *Wwox* knockout murine models, significantly reduced hematopoietic cell numbers were determined in the periphery of *Wwox*^{-/-} mice as compared with wild-type and heterozygous littermates. A pronounced defect in multilineage hematopoiesis was observed in *Wwox*^{-/-} bone marrow. We determined that *WOX1* deficiency caused significantly reduced cell proliferation of both myeloid and lymphoid lineage precursors. We demonstrated that, compared with wild-type controls, *Wwox*^{-/-} lineage-negative bone marrow cells failed to differentiate into mature myeloid cells or lymphocytes *in vitro*

using soft agar colony formation assay or stromal coculture systems, respectively. Adoptive transfer experiments revealed similar results *in vivo*. These data indicate an intrinsic defect in *Wwox*^{-/-} progenitor cells during hematopoiesis. Interestingly, we determined significantly reduced expression of transcription factor FoxO1 in *Wwox*^{-/-} hematopoietic progenitor cells, suggesting that WOX1 may regulate hematopoiesis via controlling FoxO1 expression. Together, our findings unravel a novel function of tumor suppressor WOX1 as a crucial regulator of transcription factor FoxO1 in hematopoiesis.

1312

T-bet mediates rapid CD8+ T cell programming via interaction with regulatory genetic elements

Prier, J., Olshansky, M., Turner, S.

Peter Doherty Institute for Infection and Immunity, Department of Microbiology and Immunology, Melbourne, Australia

T-bet (encoded by *Tbx21*) is a transcription factor whose expression is essential for terminal CD8+ T cell effector function. While it has long been appreciated that T-bet expression is required for effective CD8+

T cell responses to infection, little is known about the timing and precise mechanism of action.

Using an Influenza A virus (IAV) infection model in mice, we demonstrate that while *Tbx21* deficient CD8+ T cells are able to undergo early events associated with T cell activation, including several rounds of cellular division, there is a failure to sustain CD8+ T cell lineage specific cytokine expression. This is further supported with whole transcriptome analysis at this early time point demonstrating that *Tbx21* deficient CD8+ T cells exhibit global dysregulation in early programming events. Importantly, the *Ifng* locus within *Tbx21* deficient IAV-specific CD8+T cells, exhibited a transcriptionally inactive chromatin signature compared to WT cells. Importantly, this chromatin signature correlated with an inability of *Tbx21*-deficient CD8+ T cells to activate "poised" *Ifng* enhancer elements, a step required for transcriptional activation of the *Ifng* locus.

In all, we propose a model whereby TCR stimulation of CD8+ T cells induces rapid induction of T-bet. This ultimately leads to correct CD8+ T cell programming and differentiation via T-bet binding to specific regulatory elements that then promote chromatin remodeling and transcriptional activation of key gene loci.

1313

Studying signal transduction pathways using multi-spectral imaging flow cytometry

Friend, S., Vaidyanathan, S., Kong, R., Pugsley, H., Morrissey, P.

Amnis - part of Merck, Seattle, United States

Signals from extracellular stimuli on the cell surface cascade along intracellular pathways to the nucleus to regulate genes that are critical for orchestrating the immune system. Immune responses against pathogens, self, tolerance and the normal development of immune cells all rely on signal transduction pathways. The translocation of NFκB into the nucleus is one

of the key steps in the activation of monocytes through LPS receptor engagement; translocation of NFAT is important for T cell activation and subsequent activation of the IL2 gene. Traditional methods to study the messengers that move from the cytoplasm into the nucleus include western blot analysis or staining for phosphorylated versions of proteins for flow cytometry as well as visual evaluation of location using microscopy. However, western blot does not provide a per-cell measurement, the phosphoprotein staining for flow cytometry analysis does not provide sub-cellular, localization and microscopy is low throughput. Multispectral imaging flow cytometry overcomes these obstacles by providing multiple spatially registered fluorescent images of every cell in flow, and unlike microscopy, is high throughput and not subject to operator interpretation. Simultaneous staining of cell surface markers along with intracellular staining of transcription factors and DNA allow the direct measurement of the transcription factor location in specifically identified cell subsets at the single cell level, but in numbers that are statistically robust. Data will be presented using the multi-spectral imaging flow cytometer to measure the translocation of NFκB, MAP Kinase, Stat 3 and NFAT in cell lines and subsets of peripheral blood mononuclear cells.

1314

Validating the contribution of microRNAs and lncRNAs to the molecular signature and plasticity of iTreg vs. nTreg in humans

Malatesta, K.¹, Sadlon, T.², Pederson, S.¹, Brown, C.¹, Barry, S.C.^{1,2}

¹Molecular Immunology, Robinson Research Institute, Adelaide, Australia, ²Women's and Children's Health Network, Gastroenterology, Adelaide, Australia

Regulatory T cells (Treg) play a key role in tolerance and immune homeostasis. Recent research has revealed that Treg generation, function and stability rely on gene networks regulated by FOXP3 and microRNAs. It has been suggested that these gene networks facilitate functional plasticity within T cell subsets, including peripheral Tregs (pTreg) and Tconv cells. Mouse models indicate that pTregs have an important physiological role in the gut and contribute to mucosal immunity, however a lack of discriminatory biomarkers hinders pTreg research in the human. FOXP3⁺ Tregs (iTregs) can be generated *in vitro* from CD25⁺ Tconv and are proposed to be closely related to pTreg in mouse. In order to discover the molecular basis for the induction of FOXP3 and the functional plasticity in the human, we have profiled microRNA expression in donor matched human tTreg, Tconv and iTregs and found that iTregs express a unique microRNA signature comprising both Treg and Tconv specific miRs. In contrast with nTreg, we find that microRNA-31 is elevated in iTreg compared with nTreg, and this inversely correlates with levels of FOXP3. Our FOXP3 ChIP data and bioinformatics suggest that miR31 is a putative target of FOXP3 and FOXP3 is a putative target of miR31. This may provide the rheostat for regulating plasticity in Treg subsets.

1315**FOXP3 tightly controls target gene expression using microRNAs in human regulatory T cells, and regulates Treg plasticity**

Brown, C.¹, Beyer, M.², Pederson, S.¹, Goodall, G.³, Schultze, J.², Sadlon, T.⁴, Barry, S.C.^{1,5}

¹Molecular Immunology, Robinson Research Institute, Adelaide, Australia, ²LIMES, University of Bonn, Bonn, Germany, ³Centre for Cancer Biology, University of South Australia and SA Pathology, Gene Regulation, Adelaide, Australia, ⁴Women's and Children's health Network, Gastroenterology, Adelaide, Australia, ⁵Women's and Children's Health Network, Gastroenterology, Adelaide, Australia

FOXP3 is essential for the formation and function of regulatory T cells (Tregs) as demonstrated by autoimmune disease in the scurfy mouse and in IPEX patients. However, little is known about the molecular basis of human FOXP3 function or the relationship between direct and indirect target genes in human Treg. A genome wide analysis for human FOXP3 target genes in natural regulatory cells using chromatin immunoprecipitation (ChIP) combined with gene expression profiling and micro RNA profiling identified 63 micro RNAs that are potential human FOXP3 target genes. A subset of these are up regulated in Treg compared with CD25- T-helper cells, and when we used shRNAi to ablate FOXP3, expression levels were also reduced for a subset of these miRs, suggesting regulation by FOXP3. We next identified putative miR target genes in our down regulated human Treg gene signature, and analysed their expression in FOXP3 ablated Treg. This identified a number of genes that are direct targets of the miRs that are dependent on FOXP3, and suggest that these target genes may be tightly regulated as part of the human Treg gene network. These targets include the chromatin remodelling gene SATB1, and this gene is tightly repressed to maintain Treg function (Beyer *et al* Nat Immunol 2011). We are now investigating the repression of ZEB2 by FOXP3 and miR 155 in human Treg as part of a transcription factor network. These results confirm the importance of miRs in human Treg and provide new insights into the mechanism of action of FOXP3.

1316**Methylation status and clinical significance of Bin1 in esophageal squamous cell cancer**

Jia, Y., Liu, L.

Hebei Medical University, Shijiazhuang, China

Objective: To detect the expression and methylation status of Bin1 and analyze their association with clinical characteristics of esophageal squamous cell cancer (ESCC) patients.

Methods: The expression of Bin1 mRNA and its methylation status in ESCC tissues and corresponding para-carcinoma normal tissues were detected with real-time PCR and methylation specific PCR (MSP).

Results: The methylation frequency of Bin1 in ESCC tissues (58.62%, 34/58) was significantly higher than that in the corresponding para-cancerous normal tissues (25.86%, 15/58) ($P < 0.01$), and it was closely correlated with TNM stage,

tumor invasion depth, tumor differentiation and lymph node metastasis status. Compared with para-carcinoma tissue specimens, carcinoma tissue specimens had significantly lower Bin1 mRNA abundance ($2^{-\Delta\Delta CT} = 0.78 \pm 0.05$, $P < 0.01$). The expression of Bin1 in ESCC tissues with Bin1 methylation was significantly lower than that in ESCC tissues without methylated Bin1 gene (0.68 ± 0.04 vs 0.85 ± 0.07 , $P < 0.05$).

Conclusion: In ESCC tissue, the expression of Bin1 is negatively correlated at mRNA level and its methylation, and the DNA methylation of its promoter is one of the possible mechanisms that leads to low expression or deletion of Bin1 in ESCC. The DNA methylation of Bin1 is closely associated with tumor invasion and lymph node metastasis of ESCC.

Vaccines

1317

Evaluation of DNA vaccines based on the BAB1_0267 and BAB1_0270 open reading frame of pathogenic *Brucella abortus* 2308 strain in a mice model

Oñate, A., Gómez, L., Alvarez, F.

Universidad de Concepción, Department of Microbiology, Laboratory of Molecular Immunology, Concepción, Chile

Brucella abortus is the etiological agent of brucellosis, a zoonosis affecting bovines and humans. This bacterium is a facultative intracellular pathogen capable to evade the immune response because it expresses several virulence factors. Some of these factors are codified in open reading frames (ORFs) present in the genomic island 3 (GI3). It was previously demonstrated that BAB1_0267 and BAB1_0270 ORFs of *B. abortus* 2308, located at GI3, play important roles in the intracellular survival and replication of this pathogen. Based on these reports, we used both ORFs to construct DNA vaccines, named pV267 and pV270, and to evaluate their immunogenicity in mice. Results showed that the immunization with pV267 significantly increased the production of IgM, IgG and IgG1 immunoglobulins, IFN- γ and the lymphoproliferative response of splenocytes. On the other hand, the pV270 DNA vaccine significantly increased the IgM, IgG and IgG2a immunoglobulins, IFN- γ , TNF α and IL-6, and the lymphoproliferative response of splenocytes. These results indicated that both vaccines increased the level of IFN- γ , a pivotal cytokine in the Th1 immune response, effective against intracellular pathogens. Nevertheless, although the immunization with either of these vaccines induced an immune response, none of them was able to confer significant protection against 10⁶ CFU of the pathogenic *B. abortus* 2308 strain, a higher dose than the described in the literature. Therefore, although pV267 and pV270 induced an immune response against the product codified by either of these ORFs, under the conditions of protection assayed, is insufficient to confer a significant protection against *B. abortus*.

1318

The specific antibody production against HER2 peptide CH401MAP in NOG-IL4-Tg mice

Miyamoto, A.¹, Katano, I.², Kikuchi, Y.¹, Tsuda, B.³, Tokuda, Y.³, Ito, M.², Habu, S.⁴, Kametani, Y.¹

¹Tokai University School of Medicine, Department of Immunology, Isehara, Japan, ²Central Institute for Experimental Animals, Kawasaki, Japan, ³Tokai University School of Medicine, Department of Breast and Endocrine Surgery, Isehara, Japan, ⁴Juntendo University School of Medicine, Department of Immunology, Tokyo, Japan

Background: We previously reported that a 20mer HER2 peptide CH401MAP induced specific IgG in immunized BALB/c mice. This peptide is predicted to bind to more than 90% of class- I HLA and 30-50% of class- II HLA of Japanese breast cancer patients.

Objective: To detect the specific antibody production against HER2 CH401MAP in humanized NOG-IL4-Tg mice.

Materials and methods: Human peripheral blood mononuclear cells (PBMCs) were transplanted into NOD/Shi-scid, IL-2gc-null(NOD/SCID/gc-null; NOG)-IL4-Tg mice (i.v.). These mice were predicted to have Th2 immuno-environment by constitutively producing human IL4 proteins. After stimulation with CH401MAP at Day 0 and Day 14, spleen cells and plasma were collected at Day 28. Human immune cells were analyzed by FACS analysis. Specific antibody was measured by ELISA.

Results: Almost all lymphocytes of transplanted mice had memory phenotype. B cell ratio was significantly increased and CD8+ T cell ratio was significantly decreased 28 days after the transplantation. CH401 MAP specific IgG level was increased in the immunized mouse plasma. Antibody productivity was not directly correlated with the binding affinity predicted by the algorithm (SYFPEITHI).

Conclusion: NOG-IL4-Tg mice maintained human Th cells and B cells for 4 weeks, and induced specific IgG production by CH401MAP immunization. This mouse system may become an effective tool for the screening of immune response of human immunity.

1319

Characterization of *L.tropica* secreted exosomes and development of exosome-based preventive vaccine against *L.tropica* induced cutaneous leishmaniasis

Gungor, B.¹, Ayanoglu, İ.C.¹, Tincer Konig, G.^{2,3}, Ozbel, Y.⁴, Ozbilgin, A.⁵, Girinkardesler, N.⁵, Ozensoy Toz, S.⁴, Gursel, I.², Gursel, M.¹

¹Middle East Technical University, Ankara, Turkey, ²Bilkent University, Ankara, Turkey, ³CRTD / DFG-Center for Regenerative Therapies, Dresden, Germany, ⁴Ege University, Izmir, Turkey, ⁵Celal Bayar University, Manisa, Turkey

Leishmaniasis is an infectious disease caused by *Leishmania* protozoa transmitted to mammalian hosts by infected sand flies. The World Health Organization considers leishmaniasis to be the second most serious tropical parasitic diseases after malaria. The absence of a good protective vaccine against leishmaniasis limits prevention options and enforces the use of highly toxic pentavalent antimonials for treatment. Herein, we aimed to develop a prototype preventive vaccine against *L.tropica* cutaneous infection in mice. For vaccine development, first purified, *Leishmania* antigen-rich small vesicles (exosomes) secreted from *L.tropica* amastigotes. Following their physical and biochemical characterization, the exosomes were combined with several different vaccine adjuvants or their combinations, including, cyclic-di-GMP, different classes of CpG ODNs, CpG nanorings and their immunoprotective activity were tested in a mouse model of cutaneous leishmaniasis. To monitor disease progression, lesion sizes were monitored for 10 months and parasite burdens in each group was predicted by real time PCR. *Leishmania* soluble antigen-specific serum IgG1 and IgG2a antibody titers were assessed by ELISA and Th1/Th2/Th17 cytokine profiles was analyzed by cytometric bead array shortly after initiation of infection. Our results show that Th1 type vaccine adjuvanted exosomes (particularly the combination of CpG ODN and cyclic-di-GMP) suppressed parasite burden, increased serum anti leishmania IgG2a antibody titers and enhanced the protective ability of the exosomes. In conclusion,

the exosome based vaccine approach tested herein plus the proposed adjuvants constitute a novel approach in design of vaccines against neglected parasitic diseases.

1320

Dendritic cell-targeting DNA vaccines elicited distinct immune responses directs by intracellular antigen trafficking

Lee, B.K., Chen, Z.W.

University of Hong Kong, Aids Institute, Dept. of Microbiology, Hong Kong, Hong Kong

Understanding antigen processing in dendritic cells (DCs) can improve HIV vaccine immunogenicity. Targeting antigen to DCs has been explored as novel strategy of HIV vaccine design, while few studies have investigated the influence of intracellular antigen processing on vaccine immunogenicity when various DC surface receptors are engaged. Here, we conduct parallel experiments to determine the immunogenicity and possible underlying mechanism of DC-targeting HIV vaccines via the native ligands of PD-1 and CTLA-4, respectively.

A panel of DNA fusion vaccines was constructed including soluble PD-1 (sPD1)-p24, soluble CTLA-4 (sCTLA-4)-p24, deletion mutant sΔPD1-p24, sΔCTLA4-p24 and p24 alone. BALB/c mice were immunized with each of these DNA vaccines at 100µg dose via intramuscular *in vivo* electroporation following a prime/2-boost regimen with 3-week intervals. Post vaccination, immunogenicity profiles were analyzed using ELISA and ELISpot. Confocal microscopy was used to investigate antigen trafficking to MHC-I or -II compartments in DC based on several endosomal markers.

DC-targeting HIV vaccines were significantly more potent than corresponding deletion mutant controls. sPD1-p24 is superior to CTLA4-p24 with following distinct immune responses:

(1) enhanced IgG2a (Th1) antibody responses,
(2) greater p24-specific CD8⁺ T cell responses besides increase in CD4⁺ T cells by IFN-γ ELISpots. Mechanistically, both sPD1-p24 and sCTLA4-p24 routed antigen to Lamp-1 endosomes for MHC-II presentation, but only sPD1-p24 routed to Rab14 endosomes for MHC-I cross-presentation.

PD-1 and CTLA-4 lead to distinct immunogenicity outcomes in our DC-targeting vaccines. Our findings provide evidence that the intracellular antigen processing in DC influences vaccine immunogenicity when different DC surface receptors are engaged.

1321

Optimization of Survivin synthetic vaccine for treatment of cancers

Maherzi, C.^{1,2}, Onodi, F.², Taboas, C.², Tran, T.², Ben Hamouda, N.³, Gey, A.³, Kerzerho, J.⁴, Maillere, B.⁵, Bouzidi, A.⁶, Tartour, E.^{2,3}, Tanchot, C.²

¹Université Paris Descartes (Paris V), Paris, France, ²Inserm U 970 Immunotherapy and Anti-Angiogenic Therapy in Oncology, Paris, France, ³Hôpital Européen Georges Pompidou, Service d'Immunologie Biologique, Paris, France, ⁴Vaxeal Research SAS, Genopoles Entreprises, Evry, France, ⁵Institute of Biology and

Technologies (Commissariat à l'Energie Atomique), Laboratory of Immunochemistry of Cellular Immune Response, Gif-Sur-Yvette, France, ⁶Vaxeal Holding SA, Vevey, Switzerland

A central aim of novel cancer therapies is the induction of effective anti-tumor immunity in cancer patients leading to elimination of tumors and long-lasting protection against relapses. In this regard, modern therapeutic vaccination has been shown to elicit tumor antigen specific T-cell immunity but showed only modest clinical effects as several factors negatively impact their therapeutic effect in cancer patients. In this project, we developed an innovative therapeutic cancer vaccine enabling to overcome limitations to current vaccine approaches. The vaccine is composed of 3 long synthetic peptides (LSP) derived from the Survivin antigen, an ubiquitous protein over expressed and playing vital functions in large number of human tumors. The therapeutic efficacy of the formulated vaccine candidate was evaluated during tumor rejection assays performed in BALB/c mice engrafted with various syngeneic tumor models expressing the human Survivin. Results clearly demonstrated the high therapeutic efficacy of the Survivin vaccine against various established tumor models, such as colorectal carcinoma (CT26), renal adenocarcinoma (Renca) and B cell lymphoma (A20) models. This was associated with the induction of intense and long-lasting Survivin specific T-cell responses not impaired by the presence of the tumor cells. Tumor rejection assays performed in CD8 or CD4 depleted mice, demonstrated that both CD8⁺ and to a lesser extent CD4⁺ T cells are involved in the high efficacy of Survivin vaccine. Together, these results constitute a relevant proof-of-concept to bring this vaccine as a first-generation product in human trial.

1322

Intranasal delivery of Enterovirus 71 vaccine with Zymosan and Chitosan as adjuvants to enhance mucosal and systemic immune responses

Chin, C.-L.¹, Lin, Y.-L.², Cheng, P.-Y.², Lin, S.-Y.¹, Yuan, H.-P.², Chiang, B.-L.^{1,2}

¹National Taiwan University, Immunology, Taipei, Taiwan, Republic of China, ²National Taiwan University Hospital, Medical Research, Taipei, Taiwan, Republic of China

To prevent or constrain viral infection at the site of entry, mucosal vaccine is a potent tool to induce IgA secretion for defense. Since innate receptor ligands could serve as strong adjuvants, two ligands as cell wall components of microbes are of our interests. Zymosan, a glucan in the cell wall of yeast, interacts with Toll-like receptor 2/6 heterodimer and C-type lectin receptor dectin-1 to induce inflammatory signaling pathways. Chitosan, a deacetylated chitin, takes charge of activating NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome. Thus, we aimed to study the potential immuno-inducibility of zymosan and chitosan as adjuvants in enterovirus 71 (EV71) mucosal vaccine. Bone-marrow derived dendritic cells (BMDCs) were targeted for the *ex vivo* screening assay, cytokine production being analyzed by ELISA. Naïve BALB/c mice were intranasally immunized with zymosan and/or chitosan at five-, eight-, and eleven-week-old to investigate the

in vivo effects of the vaccine and adjuvants. The EV71-specific IgA in nasal wash and feces were assayed, and the proliferation level, cytokine production, and numbers of IgA secreting cells of EV71 stimulated splenocytes were measured. The results showed IL-12 and IL-1 β production were induced upon treatment of BMDCs with zymosan and chitosan respectively. Furthermore, not only the level of EV71-specific IgA from nasal wash, feces, and splenocytes, but the proliferative responses and the production of IL-17 in groups with either of the adjuvants were higher than those of EV71 alone. Our data revealed the high immune-inducibility of zymosan and chitosan as adjuvants in EV71 mucosal vaccine.

1323

A streptococcal multiple-epitope recombinant protein protects mice against group A streptococcal infection

Kuo, C.F.¹, Hsieh, I.C.², Tsao, N.²

¹I-SHOU University, Department of Nursing, Kaohsiung, Taiwan, Republic of China, ²I-SHOU University, Department of Biological Science and Technology, Kaohsiung, Taiwan, Republic of China

Group A streptococcus (GAS) is an important human pathogen. Virulence factors such as streptococcal pyrogenic exotoxin B (SPE B), streptolysin S (SLS), M proteins, and streptococcal fibronectin-binding proteins (Sfb1), were known to involve the GAS evasion from host immune defense. In our previous study, we found that the C-terminal residues 345~398 of SPE B is a major binding site for human IgG and complement C3. Immunization of BALB/c mice using this C-terminal domain peptides was able to protect mice from M49 strain GAS-induced death but not significantly protected mice against invasive M1 strain GAS infection. To achieve the comprehensive protection, we further constructed and expressed a multiple-epitope recombinant protein FSBM, which contained four different epitopes including of the conserve segment of M protein, the immunogenic domain of Sfb1, the N-terminal segment of SLS, and the C-terminal domain of SPE B. Immunization of BALB/c mice with FSBM induced high titers of antibodies against Sfb1, SLS, SPE B and different serotypes of M proteins. *In vitro* experiments also indicated the opsonization activity and phagocytic killing of group A streptococci were enhanced by anti-FSBM antisera. The immunization of FSBM efficiently protected mice against lethal infection with different M-types of group A streptococci, even the invasive M1 strain GAS. These findings suggest that the recombinant antigen FSBM could be applied as a vaccine to protect group A streptococcal infection.

1324

Improving delivery system for malaria vaccines

Krastev, C., Walters, A., Milicic, A., Hill, A.

University of Oxford, Jenner Institute, Oxford, United Kingdom

Most commercial vaccines are administered in multiple doses with prime-boost intervals ranging from one to several months or years.

We aim to develop a novel vaccine delivery platform for single-injection prime-boost immunisation regime that would be

applicable to current and future vaccines requiring a prime-boost approach. By producing biodegradable polymeric microparticles into which the boost vaccine is incorporated and then released in a delayed time fashion, we could reduce the number of injected doses from multiple into a single immunisation where both un-encapsulated and encapsulated vaccine are administered simultaneously. To demonstrate this, we are using our leading malaria candidate vaccine with a heterologous prime-boost regime: chimpanzee adenoviral vector encoding the *Plasmodium falciparum* pre-erythrocytic thrombospondin related adhesion protein (ME-TRAP) followed by a boost using modified vaccinia Ankara (MVA) ME-TRAP virus. If successful, this technology would greatly improve immunisation coverage benefitting public health systems worldwide.

1325

Should we boost or supplement the *M. bovis* BCG antigen repertoire?

Aagaard, C., Thomsen, I., Kristiansen, E., Agger, E.M., Andersen, P.
Statens Serum Institut, Department of Infectious Immunology, Copenhagen, Denmark

Although the current vaccine for human use (*Mycobacterium bovis* (*M. bovis*) BCG) is effective in protecting infants against childhood forms of the disease, a more effective and longer lasting vaccine is needed for protection of adolescents and adults against pulmonary tuberculosis (TB).

The *M. tuberculosis* (*M.tb*) and *M. bovis* genomes are >99,95 % identical and share a large number of proteins and potential vaccine antigens. The majority of proteins expressed by *M. tuberculosis* are also correctly expressed by *M. bovis*. However, all *M bovis* BCG strains used for immunization are attenuated substrains of *M. bovis*. During the attenuation process *M bovis* BCG lost >100 genes, including genes encoding virulence factors and proteins involved in secretion through a type VII secretion system termed ESX-1. Because of the gene loss, a number of proteins are not expressed or secreted correctly by *M bovis* BCG and therefore dysfunctional. The ESX-1 secretion system secretes important virulence factors including well-known T cell antigens used for diagnosis of tuberculosis (ESAT-6, CFP10 and Rv3615).

In addition to ESX-1, *M.tb* has four additional type VII secretion systems (ESX-2 - ESX-5). The genes encoding these systems are present in *M. bovis* BCG and these secretion systems are therefore functional and secrete well-known antigens (TB10.4, Rv3619, Rv3620, and PPE42).

The functional difference between secretion systems that secretes highly expressed and protective antigens allows us to investigate if it is preferable to supplement *M. bovis* BCG with antigens or boost and modifying an *M. bovis* induced immune response.

1326**Tumor cell lysates as maturation stimulus and antigen source for therapeutic dendritic cells against gallbladder cancer**

Rojas-Sepúlveda, D.^{1,2}, Gleisner, M.A.^{1,2}, Pereda, C.^{1,2}, López, M.^{1,2}, Salazar-Onfray, F.^{1,2}

¹Universidad de Chile, Institute of Biomedical Sciences, Santiago, Chile, ²Millennium Institute on Immunology and Immunotherapy, Santiago, Chile

Introduction: Gallbladder cancer is a leading cause of death in women over 65 years in Chile and five-year survival rate is less than 10%. We have been able to produce therapeutic dendritic cells (DC) using a heat-shocked melanoma cell lysate named TRIMEL. Sixty percent of patients treated with these DCs showed a delayed type hypersensitivity reaction against TRIMEL that correlated with a three-fold prolonged patient survival. Here, we investigated the effect of tumor lysates derived from gallbladder cancer cell (GCC) lines on immature DCs.

Material and methods: DCs were stimulated with different individual (8) or mixed lysates (8) from GCC lines and analyzed by flow cytometry for MHC-I, MHC-II, CD80, CD83, CD86, CCR7, CD1d. DCs supernatants were analyzed by flow cytometry for IL-1 β , IL-8, IL-10, IL-12p70, TNF- α . Finally, allogeneic CD3+ T cells were co-cultured with DCs and analyzed by flow cytometry for CD4, CD8, and cytokine release.

Results: All GCC lysates induced DCs maturation, increasing expression of MHC-I and II, CD80, CD83, CD86, CCR7, CD1d and the release of IL-1 β , IL-10 and IL-12p70. GCC lysate mix number 2 and 5 achieved the highest maturation of DCs indicating that diverse maturation factors may be present in different cell lines. CD3+ T cells co-cultured with DCs increased the percentage of CD4+ IFN γ + and CD8+ IFN γ + T cells.

Discussion: Based on these results, GCC lysates induces DCs maturation and activation capacity and may be considered in future new treatments.

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1327**Self-adjuvanting glycolipopeptide cancer vaccines**

McDonald, D.M.^{1,2}, Corcilus, L.², Byrne, S.N.¹, Payne, R.J.²

¹The University of Sydney, Infectious Diseases and Immunology, Sydney, Australia, ²The University of Sydney, School of Chemistry, Sydney, Australia

Mucin 1 (MUC1) is a transmembrane glycoprotein that is highly overexpressed in a range of epithelial tumours. During cancer development, aberrant expression of glycosyltransferases leads to expression of tumour-associated carbohydrate antigens (TACAs), which are absent from healthy cells. For this reason, MUC1 has been ranked 2nd/75 of the most promising cancer antigens.

We have designed and synthesised novel cancer vaccine candidates comprising lipopeptide adjuvants covalently attached to MUC1 glycopeptides. These vaccines were

constructed in an iterative manner, conjugating MUC1 peptides glycosylated with different TACAs to lipopeptide adjuvants to generate the fully synthetic, self-adjuvanting vaccines. When injected into C57/BL6 mice, these vaccines induced high titres of class-switched, MUC1-specific antibodies. Surprisingly, we could detect no *in vivo* CTL response to MUC1-expressing targets nor could we detect any IFN γ or IL-4-producing CD4+ T cells. These vaccine candidates induced robust humoral immune responses despite the lack of observed CD8+ and CD4+ T cell responses.

In order to increase T cell recognition of the MUC1 vaccines, we considered the effect of the position and identity of MUC1 glycosylation. Subsequently, we synthesised a series of vaccines bearing a range of TACAs at just two sites within the MUC1 VNTR sequence, which generated MUC1-specific antibodies as well as giving rise to MUC1-specific *in vivo* cytotoxicity. The investigation of these vaccines in tumour-bearing mice will be discussed.

This work demonstrates that targeting tumour-associated changes in post-translational protein modification with self-adjuvanting vaccines is a viable route towards cancer vaccination, giving rise to potent humoral and cell-mediated immune responses.

1328**Pneumococcal *pep27* mutant immunization stimulates cytokine secretion and confers long-term immunity with a wide range of protection, including against non-typeable strains**

Kim, G.-L., Seon, S.-H., Rhee, D.-K.

Sungkyunkwan University, Pharmacy, Su-Won, Korea, Republic of

Streptococcus pneumoniae is comprised of more than 90 serotypes and is the major causative agent of pneumonia, which results in over 1 million deaths worldwide every year. However, currently available injectable vaccines can protect against only 13-23 serotypes, and none of them protect against initial colonization in the nasopharynx. Thus, development of a vaccine conveying broader protection at the colonization stage is required. This study examined whether the $\Delta pep27$ mutant could provide protection at the nasopharynx against a broad range of serotypes. $\Delta pep27$ immunization stimulated secretion of IL-4, IL-10, TNF- α , and INF- γ in the lung, and significantly increased secretory-IgA levels in bronchoalveolar lavage fluid. Colonization and opsonophagocytosis assays demonstrated that $\Delta pep27$ immunization could protect against many heterologous infections, including non-typeable strains, at the nasopharynx, and prompted efficient killing of heterologous strains, suggesting that $\Delta pep27$ immunization provides a wide range of cross-protection. Furthermore, $\Delta pep27$ immunization significantly increased both the survival rate and the level of IgG 3 months post-immunization, demonstrating long-lasting immunity. Thus, $\Delta pep27$ could serve as a highly feasible mucosal vaccine once it is further developed into a non-transformable strain.

1329**Investigation of specific immunological memory in prostate cancer patients vaccinated with the li-key modified HER-2/neu peptide AE37**

Anastasopoulou, E., Voutsas, I., Baxevanis, C., Perez, S. St Savas Cancer Hospital, Cancer Immunology and Immunotherapy Center, Athens, Greece

Introduction: Cancer vaccines seem to be a promising therapeutic approach in cancer, aiming at prevention of recurrence through the generation of immunological memory. Therefore, investigating specific-antitumor responses along with immunological memory is essential in the context of vaccine evaluation. We have previously reported that prostate cancer patients, vaccinated the modified li-key hybrid of the HER-2/neu (HER2) native peptide AE36 (HER2(776-790)), developed specific immune responses that could be detected even 3 years after the last inoculation. Here, we aim to investigate the potential of AE37 vaccine to induce long-lasting antigen-specific T cells. **Materials and methods:** Using a MHC class II tetramer (AE37/DR11), we assessed DRB1*11/AE37(LRMKGVGSPYVSRLLGICL) specific CD4+ T-cells in peptide-stimulated cultures of pre- and post-vaccination PBMCs samples of DRB1*11+ prostate cancer patients. Phenotypic characterization of antigen-specific CD4+ T-cells was performed with multicolor flow-cytometry. In order to ascertain the *in vivo* generation of antigen-specific memory, we assessed antigen specificity in sorted memory cell subsets, using Fluorescent-Activated Cell Sorting.

Results: AE37 vaccine induced HER2-specific memory CD4+ T-cells in all analyzed prostate cancer patients. Most CD4+ T-cells acquired an effector memory phenotype (CD45RA-CCR7-CD28+) upon *in vitro* AE37 stimulation. HER2 specific T-cells could be detected in all sorted memory subsets. Interestingly, stem cell memory T cells (Tscm) were generated *in vitro* in sorted naïve CD4+ T-cells, indicating the potential of AE37 vaccine to induce Tscm, a finding that merits further investigation.

Conclusions: Our data clearly demonstrate that the AE37 vaccine can induce long-lasting HER2 specific CD4+ T-cells, possibly including Tscm, in AE37-vaccinated prostate cancer patients.

1330**Evaluation of the adjuvant effect of gold nanocages *in vitro***

Yavuz, E.^{1,2}, Sakalak, H.³, Cavusoglu, H.^{1,3}, Uyar, P.^{1,4}, Yavuz, M.S.^{1,3}, Bagriacik, E.U.²

¹Selcuk University, Advanced Technology Research and Application Center, Konya, Turkey, ²Gazi University Medical School, Immunology, Ankara, Turkey, ³Selcuk University, Metallurgy and Materials Science Engineering, Konya, Turkey, ⁴Selcuk University, Biotechnology, Konya, Turkey

Hepatitis B is a potentially life-threatening disease caused by the hepatitis B virus (HBV). Unfortunately, despite the ongoing vaccine campaigns HBV infection is not completely managed. In order to increase the immune modulation capacities of Hepatitis B vaccines different adjuvant systems have been studied. Especially nanoparticle-based adjuvants are being widely investigated. Biocompatible and bioinert gold

nanoparticles have been commonly used *in vitro* and *in vivo* biological research. Recently, gold nanocages (AuNCs), a special design with ultra thin porous walls and hollow interiors, have shown ample potential and have a promising future in the fields of cancer diagnostics and treatment. Our goal is to use AuNCs as an adjuvant in Hepatitis B model *in vitro*.

In this project, synthesized and characterized Au nanocages are used. Following the adsorption of HBsAg protein onto Au nanocages, adsorption efficiency of AuNCs is investigated. The uptake of HBsAg-AuNC by macrophages and its colocalization within the cell are analyzed by flow cytometry and confocal microscopy. The *in vitro* effect of the internalization of only AuNCs or HBsAg-AuNC on macrophage activation, antigen presentation and the cytokine profile are analyzed by flow cytometry and ELISA techniques. Here we aim to study the immunomodulation properties of the porous gold nanoparticles as an adjuvant *in vitro*.

1331**Successive intramuscular boosting with IFN-alpha protects *Mycobacterium bovis* BCG- vaccinated mice against *M. lepraemurium* infection**

Guerrero M, G.G.¹, Rangel-Moreno, J.², Islas-Trujillo, S.³, Rojas-Espinosa, O.³

¹Universidad Autonoma de Zacatecas, Unidad Academica de Ciencias Biologicas, Zacatecas, Mexico, ²University of Rochester Medical Center, Department of Medicine, Division of Allergy, Immunology and Rheumatology, Rochester, United States, ³Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Immunology, Distrito Federal, Mexico

Leprosy caused by *Mycobacterium leprae*, primarily affects the skin and peripheral nerves. As a human infectious disease, is still a significant health and economic burden on developing countries. Although multidrug therapy is reducing the number of active cases to approximately 0.5 million, the number of cases per year is not declining. Therefore, alternative host-directed strategies should be addressed to improve treatment efficacy and outcome. In this work, using murine leprosy as a model, -a very similar granulomatous skin lesion to human leprosy-, we have found that successive IFN-alpha boosting protects BCG-vaccinated mice against *M. lepraemurium* infection. Not difference in the seric isotype and all IgG subclasses measured, neither in the Th1 or Th2 type cytokine production was seen. However, an enhanced iNOS/NO production in BCG-vaccinated/ *i.m.* IFN-alpha boosted mice was observed. The data provided in this study suggest a promising use for IFN-alpha boosting as a new prophylactic alternative to be explored in human leprosy by targeting host innate cell response.

1332**Vaccination enhances the ability of ovine macrophage to kill *Mycobacterium avium* subspecies *paratuberculosis* in vitro**

Pooley, H., de Silva, K., Purdie, A., Begg, D., Whittington, R., Plain, K.
The University of Sydney, School of Life and Environmental Sciences, Faculty of Veterinary Science, Camden, Australia

This work examines whether clinical outcomes in ruminant vaccine trials can be correlated with the *in vitro* ability of peripheral blood mononuclear cells (PBMCs) to kill bacteria. *In vitro* killing ability was examined with adherent monocytes infected with *Mycobacterium avium* subspecies *paratuberculosis* (MAP) and co-cultured with autologous lymphocytes. The viability of intracellular MAP was determined with a novel method using the relationship between the rate of MAP growth and the concentration of live MAP at the start of culture. A pilot study was carried out on cells from Gudair® vaccinated MAP exposed diseased (histopathological lesions with a score of Perez type 3) and non-diseased (no histopathological lesions) sheep as well as vaccinated and non-vaccinated unexposed control sheep. The MAP exposed sheep were orally exposed to MAP 12 months prior to the collection of cells for the *in vitro* assay. Two sheep were used for each vaccinated treatment group and four in the non-vaccinated controls. Variations in the killing ability of PBMCs from non-vaccinated control animals were found. In contrast, vaccination decreased variability between animals and increased *in vitro* killing of MAP. However we were unable to differentiate between diseased and non-diseased vaccinates, so further testing of a larger number of animals is planned. In conclusion the assay has proven to be a useful tool for examining the host response to vaccination. Use of the assay to test earlier infection time points would improve our understanding of the effects of vaccination on the ability of PBMCs to kill MAP.

1333**Impact of early life exposure to ionizing radiation on influenza vaccine response in an elderly Japanese cohort**

Lynch, H.¹, Hayashi, T.², Geyer, S.³, Yoshida, K.², Furudoi, K.², Sasaki, K.², Morishita, Y.², Nagamura, H.², Maki, M.², Hu, Y.², Hayashi, I.⁴, Kyoizumi, S.², Kusunoki, Y.², Ohishi, W.⁵, Fujiwara, S.⁶, Shterev, I.¹, Nikolich-Zugich, J.⁷, Murasko, D.⁸, Sempowski, G.¹, Nakachi, K.²

¹Duke University, Duke Human Vaccine Institute, Durham, United States, ²Radiation Effects Research Foundation, Department of Radiobiology/Molecular Epidemiology, Hiroshima, Japan, ³University of South Florida, Department of Pediatrics Health Informatics Institute, Tampa, United States, ⁴Hiroshima University Faculty of Dentistry, Central Research Laboratory, Hiroshima, Japan, ⁵Radiation Effects Research Foundation, Department of Clinical Studies, Hiroshima, Japan, ⁶Hiroshima Atomic Bomb Casualty Council, Hiroshima, Japan, ⁷University of Arizona College of Medicine, Department of Immunobiology and the Arizona Center on Aging, Tucson, United States, ⁸Drexel University, Department of Biology, College of Arts and Sciences, Philadelphia, United States

Negative effects of age and acute radiation exposure on vaccine response are well characterized, but compounding late radiation

effects and increased age have not been investigated. The Adult Health Study (AHS; Hiroshima, Japan) consists of a unique study population of atomic bomb survivors who are all aged 65 years or more. The hypothesis that early radiation exposure exacerbates age-associated decreased immune response to influenza vaccination was tested in 292 AHS subjects over two influenza vaccination seasons. Humoral response to each of three vaccine antigens and immune cell function were assessed using hemagglutination inhibition antibody titers and *in vitro* stimulated PBMC cytokine production, respectively. Impact of sex, age, and exposure dose on antigen-specific humoral response were evaluated. Although complete vaccine failure was more likely in females than in males, sex was not found to have a consistent and significant impact on strain-specific flu vaccine response in the elderly population. Advanced age did not have a consistent nor significant impact on strain-specific response in this unique elderly population. Interestingly, subjects exposed to 1 Gy or higher dose were more likely to seroconvert to two antigens than lower dose exposure, but the effect was not consistent in both study years. No cytokine profiles were consistently associated with sex, age, or dose effects on response to all three antigens, although some cytokines were associated with response to individual antigens. Data from this unique study suggest overall constitutional and immunological "robustness" of individuals who survived more than 65 years following high-dose radiation exposure.

1334**A novel mucosal vaccine targeting Peyer's patch M cells induces protective antigen-specific IgA responses**

Shima, H.¹, Watanabe, T.², Fukuda, S.³, Fukuoka, S.-I.⁴, Ohara, O.², Ohno, H.²

¹Showa Pharmaceutical University, Machida, Japan, ²RIKEN Center for Integrative Medical Sciences (IMS), Yokohama, Japan, ³Keio University, Tsuruoka, Japan, ⁴Aoyama Gakuen University, Kanagawa, Japan

Mucosal vaccines can induce mucosal immunity, including antigen-specific secretory IgA production, to protect from invasion by pathogens and to neutralize toxins at mucosal surfaces. We established an effective antigen-delivering fusion protein, anti-GP2-SA, as a mucosal vaccine. The anti-GP2-SA consists of streptavidin (SA) fused to the antigen-binding fragment region from a mono clonal antibody against glycoprotein 2 (GP2), an antigen-uptake receptor specifically expressed on M cells. Anti-GP2-SA targets antigen-sampling M cells in the follicle-associated epithelium covering Peyer's patches. Immunofluorescence showed that anti-GP2-SA specifically bound to M cells. Orally administered biotinylated ovalbumin peptide (bOVA) conjugated with anti-GP2-SA more efficiently induced OVA-specific fecal IgA secretion compared with bOVA alone or bOVA conjugated with SA. Furthermore, mice immunized by oral administration of the biotinylated *Salmonella enterica* serovar Typhimurium (S. Typhimurium) lysate conjugated with anti-GP2-SA were significantly better protected from subsequent infection by virulent S. Typhimurium than mice treated with the bacterial lysate alone or conjugated with SA. These results suggest

that anti-GP2-SA-based M-cell-targeting vaccines are a novel strategy for inducing efficient mucosal immunity.

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Immunological involvement of airway mucociliary function in the claudin-4-targeting pneumococcal nasal vaccine

Suzuki, H.¹, Nagatake, T.¹, Nasu, A.¹, Ikegami, K.², Setou, M.², Kiyono, H.³, Yagi, K.⁴, Kondoh, M.⁴, Kunisawa, J.^{1,3}

¹National Institute of Biomedical Innovation, Health and Nutrition, Laboratory of Vaccine Materials, Ibaraki, Japan, ²Hamamatsu University School of Medicine, Department of Cell Biology and Anatomy, Hamamatsu, Japan, ³Institute of Medical Sciences, University of Tokyo, Division of Mucosal Immunology, Department of Microbiology and Immunology/International Research and Development Center for Mucosal Vaccines, Minato-ku, Japan, ⁴Graduate School of Pharmaceutical Sciences, Osaka University, Laboratory of Bio-Functional Molecular Chemistry, Suita, Japan

Mucosal vaccine requires efficient vaccine delivery system to mucosal tissues including mucosa-associated lymphoid tissues. We previously reported that C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE) targeted claudin-4 which is highly expressed on nasopharynx-associated lymphoid tissue (NALT) epithelium, and thus C-CPE is an effective as a nasal antigen delivery system for respiratory vaccine antigen. In this study, we aimed to elucidate the immunological role of airway ciliary function on C-CPE mediated vaccine antigen delivery using mice lacking tubulin tyrosine ligase-like family, member 1 (Ttll1). Ttll1 KO mice show the accumulation of mucus in nasal cavity due to the impaired airway ciliary motility. When Ttll1 KO mice were nasally immunized with C-CPE fused with pneumococcal surface protein A (PspA-C-CPE), insufficient binding of PspA-C-CPE to NALT epithelium was noted due to the mucus accumulation in the nasal cavity, although Ttll1 KO mice showed normal expression of claudin-4 on NALT epithelium. As a result, Ttll1 KO mice showed the decreased levels of nasal PspA-specific IgA responses against nasally immunized PspA-C-CPE. In those mice, the numbers of germinal center B cells and follicular helper T cells were decreased in the NALT, suggesting the impaired germinal center formation. These results collectively indicate that accumulation of nasal mucus caused by impaired airway mucociliary function is an impedimental factor in the claudin-4-targeting pneumococcal nasal vaccine.

1336

Development of a synthetic virus for enterovirus A71 vaccine candidate

Wang, J.-R.^{1,2,3}, Cheng, C.-K.¹, Huang, S.-W.²

¹National Cheng Kung University, Medical Laboratory Science and Biotechnology, Tainan, Taiwan, Republic of China, ²National Cheng Kung University, Center of Infectious Disease and Signaling Research, Tainan, Taiwan, Republic of China, ³National Health Research Institutes, National Institute of Infectious Diseases and Vaccinology, Tainan, Taiwan, Republic of China

Human enterovirus A71 (EV-A71) has become an emergent infectious disease worldwide most notably in Asia. EV-A71

infection occasionally causes neurological diseases with pulmonary edema, which is fatal for children. Based on phylogenetic analysis of VP1 protein coding sequences, most EV-A71 isolates are classified into genotypes A, B and C, while some isolates belong to the recently discovered genotypes D, E, and F. Synthetic viruses have been applied to produce vaccine seed viruses for influenza virus and others. To produce a synthetic virus of EV-A71, we used 4643-TW-98 (genotype C2) as the template (r4643), de novo synthesized the capsid protein genome of genotype C4 and introduced into the cDNA clone of r4643 to produce reverse genetics virus (r4643-C4VP). We then compared the viral properties of this r4643-C4VP with parental r4643 and native 4643 virus. The r4643-C4VP showed relatively smaller plaque morphology than native 4643, but the virus-induced cytopathic effect was similar to both r4643 and native 4643. Synthetic reverse genetics viruses and native virus showed no significant difference in growth rate in both RD and Vero cell lines. In addition, mouse anti-r4643 and anti-r4643-C4VP sera showed similar neutralizing antibody titers against both r4643-C4VP and r4643 viruses, suggesting that both viruses had similar antigenicity. Furthermore, both antisera had similar neutralization activity to different genotypes of EV-A71, including genotypes B2, B4, B5, C2, and C4. Collectively, these results prove that the synthetic virus could be applied to EV-A71 vaccine seed candidate which will contribute to next generation vaccine development.

1337

SCV platform: a novel replication-incompetent vaccinia viral vaccine vector system

Hayball, J.¹, Liu, L.¹, Cooper, T.¹, Eldi, P.¹, Howley, P.²

¹University of South Australia, Pharmacy and Medical Sciences, Adelaide, Australia, ²Sementis Ltd, Melbourne, Australia

Vaccinia virus is known for its use as the vaccine for smallpox which led to the first human disease to be eradicated. Vaccinia is now one of the most promising live viral vector systems for antigen delivery. However, severe site reactions and the potential for life threatening complications significantly limit its usage. To address these limitations a novel vaccine delivery system based on Vaccinia virus has been engineered that maintains the best qualities of the parent vector. This system consists of a replication-defective Vaccinia virus (SCV) and a co-developed rescue cell line for virus production. To render SCV completely attenuated, an essential viral assembly gene was deleted. Virus production was rescued by genetically engineering CHO cells to express the essential genes. In vitro and in vivo mice studies demonstrated that the SCV vector was completely attenuated and has an increased safety profile compared to its parental Vaccinia. A single immunization (10^6 pfu) conferred long-lived protection against mousepox infection showing SCV retained the ability to evoke superior immune responses. Multiple foreign genes could be inserted and a model ovalbumin antigen, elicited antigen-specific cellular and humoral immune responses. In summary, SCV is a safe and effective vaccine platform capable of delivering a payload of antigens for multivalent vaccine development. The SCV vector system has the potential to be tailored to an array

of prophylactic or therapeutic vaccine applications requiring antigen-driven humoral and cell-mediated immune responses.

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Poly(amino acid)/squalene nanocomplex (PA/S-NCs) as efficient vaccine adjuvants for immune activity enhancement

Lim, J.-W.¹, Kim, H.-O.¹, Na, W.², Yeom, M.², Yun, D.¹, Kim, J.¹, Park, G.¹, Chun, H.¹, Song, D.², Haam, S.¹

¹Yonsei University, Chemical and Biomolecular Engineering, Seoul, Korea, Republic of, ²Korea University College of Pharmacy, Department of Pharmacy, Sejong, Korea, Republic of

As the outbreak of infectious disease is getting more frequent and devastated year by year, the advent of more efficient anti-infectious disease vaccines is greatly expected. Therefore, the development of better vaccine adjuvant accompanied with vaccine to boosting immune response for enhancing immunogenicity has been greatly required. Herein, we address nanocomplex of amphiphilic grafted poly(amino acid) and hydrophobic squalene (PA/S-NC) as a strongly potent vaccine adjuvant against influenza virus. The results showed that these nanocomplex performed proper loading efficiency and stability. More importantly, intramuscular co-administration of PA/S-NCs and an H1N1 pandemic strain (CA04) produced antibodies and cytokines in a conditional mouse model. Furthermore, The PA/S-NC/CA04 immunization prevented disease symptoms and protected mouse model against lethal infection with H1N1 influenza virus. Consequently, our PA/S-NCs could be one of great potent vaccine adjuvant for enhancing vaccine-induced immune responses.

1339

A murine cytomegalovirus vaccine vector protects against murine gamma herpesvirus 68

Stevenson, P.G.^{1,2,3,4,5}, Yunis, J.⁶

¹School of Chemistry and Molecular Biosciences, University of Queensland, Viral Immunology Research Laboratory, St Lucia, Australia, ²Australian Infectious Diseases Research Centre, University of Queensland, Brisbane, Australia, ³Centre for Children's Health Research, South Brisbane, Australia, ⁴National Institutes of Health (NIH), Bethesda, United States, ⁵Australian Research Council, Canberra, Australia, ⁶School of Chemistry and Molecular Biosciences, University of Queensland, St Lucia, Australia

EBV, a human gamma herpesvirus, causes Burkitt's lymphoma and nasopharyngeal carcinoma in susceptible hosts. Currently, there are no effective vaccines. Vaccine design is hindered by species specificity. Murine gamma herpesvirus 68 (MHV-68) provides a model to develop new vaccines. Mice infected with MHV-68 develop B cell lymphoproliferation, which we aim to abolish using a CD4+

T cell-based vaccine. The rationale is that CD4+ T cells have proved essential for long-term virus control, and a lack of CD4+ T cells in humans is associated with a high frequency of EBV disease.

Cytomegaloviruses (CMV) are of interest as possible live-attenuated vector vaccines to elicit protection against

persistent viruses. BALB/c and C57BL/6 mice were vaccinated intraperitoneally with murine CMV (MCMV WT) or a recombinant expressing membrane bound ovalbumin (MCMV mOVA) from the viral genome as a model CD4+ T cell antigen in the BALB/c strain and a model CD8+ T cell antigen in the C57BL/6 strain. MCMV WT and MCMV mOVA vaccinated mice were challenged intranasally with recombinant MHV-68 expressing membrane bound ovalbumin (MHV-68 mOVA). MCMV mOVA vaccinated C57BL/6 mice were protected from lytic infection in the nose and lung compared to MCMV WT while in BALB/c mice, virus titers were reduced. MCMV mOVA vaccinated C57BL/6 mice had no detectable latent virus in the lymph nodes or spleens while in BALB/c mice, lymph node and spleen titers were comparable to MCMV WT.

Results show vaccine induced CD4+ T cells reduce lytic infection while CD8+ T cells control lytic and latent infection.

1340

Modified liposomes for the delivery of cancer vaccines to the cytosol of antigen presenting cells through the proton sponge effect

Lee, K., Gamble, A., Hook, S.

University of Otago, School of Pharmacy, Dunedin, New Zealand

Despite our advances in cancer therapies, chemotherapy remains the main mode of treatment. Chemotherapy results in adverse effects due to off-target activity of drugs, and efficacy remains poor in some types of tumours. To alleviate these issues, alternative methods of treatment such as immunotherapies, are being actively researched. One subset of immunotherapies include therapeutic cancer vaccines, which may be utilised to mobilise a cytotoxic T lymphocyte (CTL) response against cancerous cells. Unfortunately, the response rate remains poor, prompting the need for alternatives.

One hurdle to an effective vaccine is the process of cross presentation in antigen presenting cells. However, cross presentation is still poorly understood, and limited methods are available to improve cross presentation. Delivery of tumour antigen and adjuvants to the cytosol of antigen presenting cells would allow the antigen to be processed by the proteasome and presented on MHC-I as if it were an endogenous antigen. Polyethyleneamine (PEI) has been used successfully for transfection studies, as it has been used to deliver genetic material into the cytosol of cells. To achieve this, PEI uses the 'proton sponge effect' to release contents of endosomes into the cytosol.

Currently, we are exploring the possibility of delivering ovalbumin (a model protein) complexed with PEI to exploit the proton sponge effect. To aid this process, the complexes are encapsulated in liposomes to protect the protein and improve phagocytic uptake. Lastly, the liposomes are embedded with artificial cyclodextrin channels to allow the flow of ions across an otherwise impenetrable membrane.

1341**Targeting of hemagglutinin to MHCII increases antibody responses against highly pathogenic avian influenza (H7N1) in mice**

Andersen, K.¹, Zhou, F.², Cox, R.J.², Grødeland, G.¹, Bogen, B.¹

¹K.G. Jebsen Center for Influenza Vaccine Research, University of Oslo and Oslo University Hospital-Rikshospitalet, Department of Immunology, Oslo, Norway, ²K.G. Jebsen Centre for Influenza Vaccine Research, Influenza Centre, The Gade Institute, University of Bergen, Bergen, Norway

Targeting of influenza hemagglutinin (HA) to major histocompatibility complex class II (MHCII) molecules on antigen presenting cells (APCs) have previously been shown to raise quick and robust antibody responses against H1N1 influenza in mice (Grødeland et al., J. Imm., 2013.). Here, we have used the same strategy to develop a vaccine against H7N1 influenza, which is currently regarded as a potential pandemic strain. The vaccine was delivered as DNA, a format that allows rapid production and deployment in the event of a pandemic emergence.

HA from H7N1(A/chicken/Italy/13474/1999) was inserted into a homodimeric vaccine format, where hemagglutinin and MHCII-specific targeting units are bivalently displayed. A single DNA vaccination in mice demonstrated that MHCII-targeting significantly increased antibodies and T-cell responses against recombinant HA from the H7N1 virus. The antibodies showed neutralizing activity against heterotypic H7 strains of avian influenza.

MHCII-targeting is crucial for the enhanced immune responses observed after DNA immunization. In order to investigate the mechanism behind this effect, we have tracked naïve idiotype-specific B- and T-cells in vitro or in vivo after MHCII-targeted (or non-targeted) vaccine delivery. At low doses, MHCII-targeted vaccines rapidly activated proliferation of idiotype specific B- and T-cells, in contrast to non-targeted controls.

In conclusion, we have demonstrated that targeting of HA to MHCII molecules increases the induced immune responses against H7N1 influenza, and are continuing experiments to investigate the mechanism behind the associated formation of long-lived memory cells.

1342**Iron oxide nanoparticles as vaccine carriers and immune responses against covalently bound protein**

Powles, L.¹, Wilson, K.², Xiang, S.D.², Selomulya, C.¹, Plebanski, M.²

¹Monash University, Department of Chemical Engineering, Clayton, Australia, ²Monash University, Department of Immunology, Melbourne, Australia

It has been demonstrated previously that polystyrene nanoparticles are able to enhance both antibody mediated and cellular immune responses against covalently conjugated protein and peptide. This response depends strongly on the size of the nanoparticles with a hydrodynamic size of 50 nm promoting the strongest responses. As they are not biodegradable, the future applicability of these particles as vaccine vectors is limited and therefore biodegradable

nanoparticles are being investigated as alternatives.

The biodegradation rate of these particles has been suggested to influence the immune response developed. To test this, two iron oxide nanoparticles stabilized with the polysaccharide pullulan are compared as potential antigen delivery vehicles. While the size of the particles, 40-60 nm is similar, their cores are composed of different iron oxide phases. The first is non-magnetic with limited crystallinity while the second is well ordered magnetite/maghemite. These differences manifest themselves in the stability and degradation profiles of the nanoparticles with the magnetically ordered particles exhibiting greater stability in a range of media along with a lower degradation rate as measured by the release of iron *in vitro*. The particles have been successfully conjugated to protein and ongoing work analyzing their capacity to enhance both antibody based and cellular immune responses, as well as their interactions with antigen presenting cells will be presented. The studies outcomes will aid in the design of future nanoparticulate vaccine vectors.

1343**Intranasal administration with a NanoStat™-MOMP vaccine reduces the incidence of oviduct pathology in *Chlamydia*-infected mice**

Trim, L.¹, Fattom, A.², Bitko, V.², Beagley, K.¹

¹Institute of Health and Biomedical Innovation, Brisbane, Australia,

²Nanobio Corporation, Ann Arbor, United States

Vaccination against *Chlamydia trachomatis* is recognized to be the most likely means of reducing the incidence of pelvic inflammatory disease, ectopic pregnancy and infertility in women. Using the mouse model of chlamydial infection, the potential of NanoStat™, an oil-in-water nanoemulsion adjuvant, in combination with the chlamydial major outer membrane protein (MOMP) was investigated. Female BALB/c mice were vaccinated intranasally and then challenged with *C. muridarum* to determine vaccine effectiveness against infection and pathology. Intranasal immunization resulted in high MOMP-specific IgG and IgA antibody titers in serum and vaginal lavage as well as strong splenocyte proliferation as determined by CFSE proliferation. Splenocytes from immunized mice secreted high levels of IFN γ , TNF- α and IL-17A, which are essential for effective immune responses to chlamydial infection. Mice that were immunized with NanoStat™ and 20 μ g MOMP cleared infection faster and had 80% reduced incidence of hydrosalpinx (oviduct occlusion), an indicator of inflammatory pathology, compared to PBS immunized controls. These results demonstrate the effectiveness of NanoStat™ as an intranasal adjuvant targeting infection and pathology associated with genital chlamydial infections.

1344**Genetic background influences the humoral responses to vaccines**

Nouri-Shirazi, M., Zeng, M., Nourishirazi, E., Abu-Nuwar, E.
 Florida Atlantic University, Charles E. Schmidt College of Medicine,
 Boca Raton, United States

Background: The assessment of TLR agonists as promising candidate adjuvants for the induction of effective Th1 immunity continues to rely on the use of inbred mouse. However, the genetic variation among commonly used inbred mice may influence the efficacy of candidate adjuvants and bias the study conclusion. Indeed, we have found significant differences in the recruitment, phenotypes and cytokines production of DCs and NK cells and the generation of effector T cell in lymphoid tissues of two genetically non-identical mouse strains immunized with a protein based vaccine formulated with TLR agonists.

Objective: In this study, we further evaluated the nature and amplitude of humoral immune responses of B6 and BALB/c strains immunized with protein antigen ovalbumin plus Alum, TLR3, TLR4, TLR7/8, or TLR9 agonists.

Results: Our data suggest that compared to the Alum, all other adjuvants induced more effectively the recruitment of B cells and production of OVA-specific antibodies in both strains. We also observed significant differences between two strains. BALB/c strain recruited less B cells but produced significantly higher amounts of IgG2a in response to any of the TLR agonists. In addition, both strains produced similar amounts of IgG1 and IgG2b in response to TLR agonist except for MPLA which induced significantly more of IgG2b production in B6 strain.

Conclusion: Thus, our data suggests that genetic background should be taken into consideration when evaluating and comparing the activities of adjuvants for use in new prophylactic and therapeutic vaccines.

1345**Autoreactive potential of cross-reactive influenza antibodies**

Pillai, M.R., Chang, T.-C., Thomas, P.G., McGargill, M.A.
 St Jude Children's Research Hospital, Memphis, United States

A universal influenza vaccine that protects against multiple strains of influenza virus would be highly beneficial. However, the immune mechanisms that mediate protection to the conserved portions of influenza viruses are not completely understood. Antibodies specific for influenza virus epitopes common to multiple strains have been identified in humans; however, these cross-reactive antibodies are extremely rare despite multiple influenza vaccinations and infections. One possibility is that antibodies cross-reactive against multiple strains of influenza are also autoreactive to self-proteins, and therefore may be deleted during the course of the immune response. Thus, we wanted to examine whether cross-reactive influenza antibodies had a higher potential to be autoreactive. We recently demonstrated that treatment of mice with a low dose of the immunosuppressive drug, rapamycin, during vaccination with a H3N2 strain provided protection from subsequent lethal infections with H5N1, H7N9 and H1N1 strains. This enhanced protection was due to an altered

antibody repertoire with an increase in cross-reactive influenza-specific antibodies. Utilizing this approach, we generated cross-reactive antibodies to influenza, and tested these antibodies for reactivity against a panel of self-antigens that are commonly targeted in autoimmune disorders. Our results indicate that cross-reactive influenza antibodies have a higher potential to also be cross-reactive against specific self-antigens. Further, we are testing if transfer of these cross-reactive antibodies increases autoimmune inflammation.

1346**Cross-protection studies in mice immunized with iron-regulated *Pasteurella multocida* serotype B:3,4 vaccine**

Odugbo, M.
 National Veterinary Research Institute, Bacterial Vaccine
 Department, Vom, Nigeria

Fowl cholera (FC) and Haemorrhagic septicaemia (HS) are specific economic diseases of avian and bovine species caused by certain serotypes of *Pasteurella multocida*. The FC causing serotypes include A:1, A:3, A:4 and A:5 and HS causing serotypes include B:1, B:2, B:3,4 and E:2. The immunological relationship between some of the common strains associated with FC and HS, and a prototype *P. multocida* vaccine strain B:3,4 was evaluated using active mouse protection test. The cross-protective efficacy of the *P. multocida* serotype B:3,4 grown under iron-regulated condition, formalin-inactivated and adjuvanted with sodium alginate was examined in the mouse model by challenging the vaccinates with standardized virulent *P. multocida* serotypes A:1, A:4, B:2, B:3,4 and E:2. With the exception of serotype E:2, cross-protection with the prototype vaccine was observed against the challenge serotypes; and homologous protection was observed against serotype B:3,4 challenge strain. All serotypes invariably produced death of the unvaccinated control mice. Whole cell bacteria proteins of the *P. multocida* B:3,4 vaccine and the challenge serotypes were analyzed by SDS-PAGE and compared. The separation showed more than 12 clearly visible protein bands ranging from 26 to 100 kDa molecular weights. On the basis of stain intensity, the major protein bands occurred between the 30 kDa and 40 kDa of the strains. From this limited study the prospect of a single-strain vaccine that is cost-effective and capable of inducing cross-protection and eliminating antigenic competition may be feasible for use in both poultry and cattle. Further research may be of value toward vaccine development.

1347**Vaccine-induced protection against murine tuberculosis is associated with the development of antigen-specific IFN γ -IL-17+ CD4+ T-cells**

Bhattacharyya, N., Counoupas, C., Feng, C.G.
 The University of Sydney, Infectious Diseases and Immunology,
 Camperdown, Australia

One third of the world's population is infected with *Mycobacterium tuberculosis* (*M.tb*), the pathogen that causes tuberculosis (TB). Although the current TB vaccine *M. bovis*

Bacillus Calmette-Guérin (BCG) is effective at protection against childhood TB, its protective efficacy wanes over time. Therefore, there is an urgent need to develop a new generation of TB vaccines. Whilst some recent vaccine candidates have been shown to be effective in protecting against *M.tb* infection in mice, the correlates of protection remain elusive. To this end, we have developed an adoptive transfer approach using transgenic CD4⁺ T-cells specific for the *M.tb* antigen Early Secretory Antigenic Target 6 (ESAT-6) to characterise the antigen specific immune response generated by novel vaccine candidates. Immunisation of mice with ESAT-6 with the dimethyldioctadecylammonium liposome/monophosphoryl lipid-A (DDA/MPL) adjuvant has been shown to elicit protection against *M.tb*. We demonstrate that the MPL/DDA/ESAT-6 induced protection is associated with the generation of a cell population with a CXCR3⁺CCR6⁺ phenotype in the draining lymph nodes. In addition, the antigen-specific CD4⁺ T-cells co-express both T-bet and ROR γ t and produce both IFN γ and IL-17A. Together, these data highlight that a protective CD4⁺ T-cell response is associated with a Th1 and Th17 signature. This may have important implications on the design and screening method of future TB vaccine strategies.

1348

Lipovaxin, a versatile chelating liposomal vaccine platform for surface-loading of recombinant antigens to generate self-adjuncting immune-stimulatory particles for improved immune responses

MacLennan, N., Price, J., Rusden, A., Gosling, K., Atmosukarto, I. Lipotek Pty Ltd, Canberra, Australia

Lipotek have developed a liposome platform that utilises chelating lipid-based immune-stimulatory nanoparticles for the co-delivery of recombinant antigens and immune potentiating agents. Antigens are attached to the surface of Lipovaxin liposomes by way of the commonly used poly-histidine tag engineered in the recombinant protein, which anchors the protein to a proprietary chelating lipid included in the liposome bilayer. As a result, antigens are all anchored in the same orientation and are displayed as highly ordered array on the surface of the liposomes, a conformation that mimics the display of surface antigens on viral particles. Chelating liposomes can be prepared with various immune-stimulatory properties, by including TLR ligands to achieve disease-relevant vaccine design.

This approach has been tested successfully with a number of antigens, including the model antigen OVA, a fusion of the tuberculosis antigen fusion ESAT6-Ag85C and the malaria antigen MSP2. In combination these studies have confirmed that particle delivered-surface anchored antigens are effectively drained to the lymph node resulting in greater antigen presentation. Furthermore we have demonstrated that the immune-stimulatory content of the particles increases internalisation by antigen presenting cells, the maturation of dendritic cells and the secretion of a range of cytokines. In mouse models humoral and CD4 and CD8 T cell responses have been induced successfully. The data presented will highlight the use of the Lipovaxin as a versatile vaccine platform.

1349

Developing an influenza vaccine using lipid nanoparticle-encapsulated nucleoside-modified mRNA

Pardi, N.¹, Naradikian, M.S.¹, Parkhouse, K.², Hensley, S.E.², Cancro, M.P.¹, Weissman, D.¹

¹University of Pennsylvania, Department of Medicine, Philadelphia, United States, ²Wistar Institute, Philadelphia, United States

Despite significant progress in influenza research, no effective universal vaccine against the virus has been developed, thus requiring yearly revaccination. mRNA has recently emerged as a potential therapeutic agent that effectively generates potent vaccine responses.

We have generated a new vaccine platform using *in vitro* transcribed mRNAs that were optimized for higher levels and extended translation by incorporation of selected UTRs, modified nucleosides, 5' cap, 3' poly(A)-tail, and other modifications and were FPLC-purified to remove dsRNA contaminants. mRNAs were encapsulated into lipid nanoparticles (LNPs) that have recently proved to be safe and efficient tools for *in vivo* nucleic acid delivery. Mice were intradermally vaccinated with a single injection of low dose PR8 hemagglutinin (HA) encoding mRNA-LNPs. At various times post immunization, animals were bled or sacrificed and antigen-specific T and B cell immune responses were evaluated.

Potent antigen-specific CD4⁺ T cells where half of the response was T follicular helper cells was generated. Increased numbers of germinal center and memory B cells was associated with high titers of neutralizing antibodies. Most interestingly, over a third of B cell responses directed at the stalk, which is conserved across groups.

Our results demonstrate that antigen-encoding nucleoside modified mRNA in LNPs induces highly potent and broad influenza-specific immune responses and has great potential for vaccination against infectious diseases.

1350

Immunogenicity of *Mycobacterium tuberculosis* antigens immobilized on bacterially derived polyester beads

Rubio Reyes, P.¹, Parlane, N.A.², Wedlock, N.², Rehm, B.H.A.^{1,3,4}

¹Institute of Fundamental Sciences, Massey University, Palmerston North, New Zealand, ²AgResearch, Hopkirk Research Institute, Palmerston North, New Zealand, ³MacDiarmid Institute for Advanced Materials and Nanotechnology, Wellington, New Zealand, ⁴Polybatics Ltd, Palmerston North, New Zealand

Traditional approaches to vaccine development have failed to identify better vaccines to replace or supplement BCG for the control of tuberculosis (TB). This study evaluates the immunogenicity of bacterially derived polyester beads displaying the reverse vaccinology antigens Rv1626, Rv2032 or Rv1789 from *Mycobacterium tuberculosis*. The beads were formulated in the adjuvant dimethyl dioctadecyl ammonium bromide and used to immunize groups of C57BL/6 mice. Antibody responses were evaluated by Enzyme-Linked Immuno Sorbent Assay (ELISA). Specificity of IgG antibody responses was assessed by immunoblotting cell lysates prepared from the vaccine production strain with antisera from the vaccinated

mice. Mice vaccinated with beads displaying Rv1626 had significantly higher IgG1 responses compared to all the other vaccinated groups ($p < 0.05$). Immunoblotting of antisera from these mice indicated that antibody responses were Rv1626 antigen-specific as there was no detectable immune response to the polyester component of the vaccine. Cytokine responses were evaluated by cytometric bead array (CBA); not showing statistical differences between the beads vaccinated groups. Overall this study suggests that selected TB antigens, derived from a reverse vaccinology approach to antigen identification can be displayed on nano-polyester beads to produce specific immune responses potentially relevant to the prevention of disease.

1351

Assessment of a novel mutated PAP-derived vaccine for the treatment of Prostate Cancer

Le Vu, P., Vadakekolathu, J., Pockley, A.G., Rees, R.C., McArdle, S.E.B. John Van Geest Cancer Research Centre, Nottingham Trent University, School of Science and Technology, Nottingham, United Kingdom

Prostate cancer is the second most frequent cancer in men. Patients are treated with local therapies such as prostatectomy or radiation therapy. However, treatment fails in 1 in 3 cases, thereby leading to an advanced-stage of metastatic disease. The standard treatment for such disease is androgen deprivation therapy, however most patients will develop resistance and progress to metastatic castration-resistant prostate cancer (mCRPC). At this stage, the only treatment has been the chemotherapeutic drug docetaxel, which confers an increased median overall survival benefit. Immunotherapy has demonstrated to be capable of reducing and even curing cancer in some patients. Sipuleucel-T vaccine, which was FDA approved in 2010, provides an additional therapeutic option for mCRPC. Sipuleucel-T has been shown to prolong the overall survival by 4 months, highlighting the feasibility of such an approach. Our laboratory has shown that a PAP-derived vaccine could induce PAP-specific T-cells responses and reduce tumour growth in established heterotopic tumour model of prostate cancer. We have since elongated and mutated the PAP-peptide sequence and shown its ability to induce a stronger immune response. The elongated mutated 42mer PAP-derived sequence was shown to increase the peptides specific Interferon γ response towards the wild-type PAP-derived peptides. In order for any given vaccine to work successfully, the delivery system and the adjuvants needs to be considered in addition to the immunogenicity of the antigen of interest. Different adjuvant strategies and delivery systems were used to boost this response and the results of these experiments will be presented at the conference.

1352

A nanoparticle based Sp17 peptide vaccine exposes immunodominant and species cross-reactive B cell epitopes

Gao, Q.¹, Xiang, S.D.¹, Wilson, K.L.¹, Heyerick, A.², Stephens, A.³, Plebanski, M.¹

¹Monash University, Immunology, Melbourne, Australia, ²PX Biosolutions Pty Ltd, Melbourne, Australia, ³Hudson Institute of Medical Research, Melbourne, Australia

Cancer testis antigen sperm protein antigen 17(Sp17), an immunogenic antigen with highly aberrant expression in ovarian cancer lesions, is a promising antigen candidate for developing ovarian cancer vaccines. Herein we describe the nanovaccine formulation human Sp17(hSp17) sequence derived peptides conjugated to delivery nanoparticles and the new properties of immune responses induced by these nanovaccines. Mapping the T cell and antibody epitopes using such nanovaccine show that the primary T and B cell immunodominant region within Sp17 was within amino acids(aa)111-142, which is the same as mapped by vaccine formulation of hSp17 peptides mixed with conventional proinflammatory CpG adjuvant. However, this nanovaccine formulation can change the dominant antibody isotype from IgG2a to IgG1 and the fine specificity of the B cell epitopes within hSp17111-142, from an immuno-dominant region 134-142 aa for CpG, to region 121-138 aa. Associated with this change in specificity was a substantial increase in antibody crossreactivity between mouse and human Sp17. In addition, unpublished data show the potentially therapeutic antibodies induced by the nanovaccine can be long lasting. These results indicate conjugation of antigen to nanoparticles can have major effects on fine antigen specificity, which could be beneficially used to increase the cross-reactivity of antibody responses.

1353

Ex vivo evaluation of Sendai virus vectors for delivery of CMV IE-1 and pp65

*Kiener, R.¹, Schwegler, C.¹, Wiegand, M.², Asbach, B.¹, Wagner, R.¹
¹University of Regensburg, Molecular Microbiology (Virology), Regensburg, Germany, ²AmVac Research GmbH, Martinsried, Germany*

Human Cytomegalovirus (CMV) remains a major health problem during pregnancy and in immunocompromised patients. For the delivery of CMV IE-1 and pp65 antigens we compared the novel murine Sendai Virus (SeV) platform to the well-established modified Vaccinia Ankara (MVA) vectors in a human *ex vivo* model with regard to their suitability as future vaccine candidates.

BHK-21, Vero cells and monocyte-derived Dendritic Cells (mdDCs) were infected and antigen expression was assessed via Western Blot or intracellular staining and flow cytometry. Maturation of mdDCs was investigated by staining cells with antibodies against CD80/CD86/HLA-DR. Proinflammatory cytokines in cell culture supernatant were quantified in a bead-based multiplex assay. Restimulation of T cell clones after co-culture with infected mdDCs was assessed by intracellular IFN γ staining and flow cytometry.

All viruses induced expression of IE-1 or pp65 in permissive cell lines. In mdDCs, moderate antigen expression was detectable for MVA. Depending on vector modifications, varying amounts of transgene expression were observed for the Sendai vectors at comparable MOIs. None of the tested vectors were capable

of inducing maturation of mdDCs under the applied conditions and MVA infection led to downregulation of HLA-DR and CD86. Depending on the viral vector and vector modifications, varying cytokine profiles were detected. In the restimulation of T cell lines by infected mdDCs, SeV vectors were superior to MVA.

SeV vectors constitute a promising new vector platform since they induce secretion of relevant inflammatory cytokines after infection of mdDCs and show a high capacity for T cell restimulation despite little antigen expression.

1354

Immunogenicity of M13 phage vaccine displaying N-terminal region of amyloid beta peptide: comparison of M13 phage vaccine expressed as g3p fusion and g8p fusion

Miyahara, R.¹, Shimotsu, A.¹, Matsushita, Y.¹, Shinozaki, T.¹, Tsukada, M.¹, Hashimoto, M.¹, Sugimura, K.², Hashiguchi, S.¹

¹Kagoshima University, Graduate School of Science and Engineering, Kagoshima, Japan, ²Kagoshima University, Graduate School of Medical and Dental Sciences, Kagoshima, Japan

Antibodies against amyloid- β peptide (A β) can reduce amyloid deposits and are considered as a potential therapeutic approach for Alzheimer's disease. We have recently shown that M13 phage stimulate an innate immune response and induce a strong primary IgG response in mice without any inflammatory adjuvant materials. Even a single immunization with 10^{11} pfu of phage induced a long-lasting antibody response. To investigate the potential of M13 phage as a vaccine carrier for A β peptide, the sequences of 1-15 region of A β were genetically linked to the N-terminus of M13 gene 3 protein or gene 8 protein, that correspond to A β -g3p phage and A β -g8p phage, respectively. When C57BL/6 mice were immunized subcutaneously with 10^{11} pfu of A β -g3p phage in PBS solution, anti-A β IgG response was induced in two weeks after the secondary immunization. In the case of A β -g8p phage, anti-A β IgG response was induced during a primary response. Anti-A β antibody titer was comparable in the two mice groups. We also observed that A β -g8p phage induced IgG class switch in athymic (nu/nu) BALB/c mice, indicating that there are different immunological mechanisms of phage vaccine between g3p fusion and g8p fusion. Considering safety and habitual presence of an M13 phage, A β 1-15-displaying M13 phage may be promising as a safe AD vaccine.

1355

Generation of recombinant LC3 of *Entamoeba histolytica* as candidate vaccine for intestinal amoebiasis

Martínez-Hernández, S.-L.¹, Cervantes-García, D.¹, Montes de Oca-Luna, R.², Loera-Arias, M.D.J.², Ascacio-Martínez, J.A.I.³, Ventura-Juárez, J.¹

¹Universidad Autónoma de Aguascalientes, Morfología, Aguascalientes, Mexico, ²Universidad Autónoma de Nuevo León, Histología, Monterrey, Mexico, ³Universidad Autónoma de Nuevo León, Bioquímica y Medicina Molecular, Monterrey, Mexico

Entamoeba histolytica (Eh) is a protozoan parasite that causes dysentery and amoebic liver abscess. In Mexico, approximately 8% of the population had suffered amoebiasis. The development

of recombinant proteins is a prophylactic alternative used for generation of safe vaccines against this parasite. In this project we are developing a set of recombinant fusion proteins to generate an effective immune response. The LC3 coding region (52 kDa) of the Gal-GalNAc heavy subunit was codon optimized. This LC3 gen fragment was fused to the exotoxin A gene sequence from *Pseudomonas aeruginosa* deleted in domain III (PEDIII) and to a KDEL sequence. Both Open Read Frames (ORFs) were synthesized by the GenScript company. These constructs PEDIII-LC3-KDEL, PEDIII-LC3, LC3-KDEL and LC3 were subcloned into the expression vector pET6xHN, and used for transformation of *Escherichia coli* BI21 (DE3). After purification of recombinant proteins, immunization tests in male golden hamsters (*Mesocricetus auratus*) will be performed. All animals will be challenged antigenically with *Eh* trophozoites (strain HM-1: IMSS virulent phase V). From the synthesized sequences we derivate the ORFs PEDIII-LC3-KDEL3 (2375 bp), PEDIII-LC3 (2336 bp), LC3-KDEL3 (1232 bp) and LC3 (1193 pb) proteins. These ORFs were subcloned into the expression vector pET6xHN, and characterized by PCR, restriction analyses and sequencing. *Escherichia coli* BI21 (DE3) clones carrying the recombinant plasmids were obtained by electroporation. These clones will be used for the production of the recombinant proteins. Four recombinant plasmids for the expression of LC3 antigen fused or no to PEDIII and/or KDEL were generated.

1356

Gamma-irradiated influenza A virus vaccine: irradiation conditions & vaccine efficacy

David, S., Paton, J., McColl, S., Alsharifi, M.

University of Adelaide, Department of Biological Sciences, Adelaide, Australia

Gamma-irradiation has been used experimentally to inactivate viruses for vaccine purposes, and the cross-protective efficacy of gamma-irradiated Influenza A virus vaccine (Gamma-FLU) has been illustrated previously. While sterility of irradiated vaccines can be achieved using low gamma-radiation doses, 50kGy is recommended to sterilise highly pathogenic viruses such as Avian H5N1. Considering the possible inclusion of highly pathogenic Influenza strains in future clinical development of Gamma-FLU, the irradiation dose required for vaccine inactivation may be increased. Here we examined the effect of different irradiation conditions, including high irradiation doses, on the overall structure of Influenza A virus, the possible damage to surface proteins, and the immunogenicity of Gamma-FLU. Our data illustrate that irradiation at freezing temperatures (using dry ice) is associated with reduced damage to viral structure compared to irradiation at room temperature. Importantly, intranasal vaccination with dry-ice irradiated vaccines at 25kGy (low dose) and 50kGy (high dose) provided 100% protection against lethal Influenza challenge. In addition, use of comparably low vaccine doses shows vaccination with 50kGy Gamma-FLU is associated with reduced virus neutralisation compared to 25kGy Gamma-FLU, despite the induction of equivalent antibody titres. Our data also illustrates this reduction in vaccine efficacy due to use of high radiation doses can be overcome by increasing vaccine dose. Overall, our data highlights the immunogenicity of

50kGy-irradiated materials, which may allow the future clinical development of Gamma-FLU based on highly pathogenic Influenza A viruses.

1357

IgM and IgG dominant LipL32 peptides revealed by leptospirosis patient's sera and therapeutic monoclonal antibodies

Pissawong, T.¹, Maneewatcharangsri, S.², Rithisunthorn, N.², Soonthornworasiri, N.³, Reamtong, O.², Kalambaheti, T.⁴, Adisakwattana, P.⁵, Chaisri, U.⁶, Doungchawee, G.⁷

¹Thammasat University, Chulabhorn International College of Medicine, Pathumthani, Thailand, ²Mahidol University, Department of Molecular Tropical Medicine and Genetic, Faculty of Tropical Medicine, Bangkok, Thailand, ³Mahidol University, Department of Tropical Hygiene, Faculty of Tropical Medicine, Bangkok, Thailand, ⁴Mahidol University, Department of Microbiology and Immunology, Faculty of Tropical Medicine, Bangkok, Thailand, ⁵Mahidol University, Department of Helminthology, Faculty of Tropical Medicine, Bangkok, Thailand, ⁶Mahidol University, Department of Tropical Pathology, Faculty of Tropical Medicine, Bangkok, Thailand, ⁷Mahidol University, Department of Pathobiology, Faculty of Science, Bangkok, Thailand

Leptospirosis is regarded as a globally neglected zoonosis frequently found in tropical countries affects both human and animals. LipL32, an immunodominant outer membrane protein of pathogenic *Leptospira* spp. has been used as diagnostic biomarker, vaccine candidate for a broad spectrum vaccine, and therapeutic target in passive immunotherapy for leptospirosis. This study, LipL32 dominance peptides in middle (LipL32₉₃₋₁₈₄) and carboxy-terminal (LipL32₁₇₁₋₂₇₂) domains have been investigated during acute and convalescence phases of infections by using confirmed leptospirosis patient's paired sera collected from acute febrile illness patients in Thailand (MAT titer \geq 1:400, total no.=58). Equally IgM reactivities specific to middle and C-terminal domains (0.22 \pm 0.11 and 0.21 \pm 0.14) have been investigated. Antigenic specificity of pool acute sera revealed IgM dominance peptides of LipL32₁₄₈₋₁₈₄ and LipL32₁₇₁₋₂₁₄ with OD ELISA at 0.28 \pm 0.17 and 0.28 \pm 0.24, respectively. IgG reactivities of convalescent sera to LipL32₉₃₋₁₈₄ were significant higher than LipL32₁₇₁₋₂₇₂ (0.39 \pm 0.25 and 0.26 \pm 0.09 OD ELISA, respectively) and IgG dominance LipL32 peptide was mapped to LipL32₉₃₋₁₄₇ in middle domain of LipL32 (0.53 \pm 0.26 OD ELISA). IgG dominance of two therapeutic epitopes were located at LipL32₂₄₃₋₂₅₃ of C-terminal b-turn and amphipathic a-helix and LipL32₁₂₂₋₁₃₀ at surface-exposed loop in middle domain of LipL32 structure and have been shown less immunodominant (< 30%) in epitope-blocking ELISA. From this research, LipL32₁₄₈₋₂₁₄ spanning two domains and LipL32₉₃₋₁₄₇ in middle LipL32 are major IgM and IgG antibody responses, respectively during *Leptospira* infections. The IgM/IgG dominant LipL32 peptides revealed by patient's sera and therapeutic antibodies could be further perspectives for LipL32 immunodominance peptide-based serodiagnosis and LipL32 epitope-based vaccine.

1358

Investigating the effect of multiple dosing of an RHDV VLP-based immunotherapeutic vaccine for melanoma

Sadrolodabai, Y.¹, Ward, V.², Young, S.¹

¹University of Otago, Department of Pathology, Dunedin, New Zealand, ²University of Otago, Department of Microbiology and Immunology, Dunedin, New Zealand

Metastatic melanoma has a poor prognosis and continues to be a challenging disease to treat, but recent immunotherapeutic approaches have demonstrated promising results. We have developed a cancer vaccine from virus-like particles (VLPs) derived from *Rabbit hemorrhagic disease virus* (RHDV) which acts as a highly immunogenic scaffold to deliver tumour-associated antigens to the immune system. Previous work has shown that one dose of RHDV VLP carrying gp100₂₅₋₃₃ can specifically activate a potent anti-tumour response and trigger the formation of immunological memory against gp100₂₅₋₃₃ to prevent tumour recurrence. Here we investigated what effect multiple dosing of RHDV VLP coupled to gp100₂₅₋₃₃ would have on the anti-tumour response. *In vivo* cytotoxicity assays carried out in mice showed that administering 3 doses of the vaccine did enhance the specific lysis of target cells compared to a single dose. This observation was also seen in a tumour trial with subcutaneous B16 melanomas. Mice that received 3 doses of the vaccine had improved overall survival and remained tumour free following rechallenge. Administering 6 doses of the vaccine did not significantly enhance the anti-tumour immune response above that achieved by administering 3 doses. An antibody response against the VLP capsid protein was detectable in all vaccinated mice, with mice that received more doses of vaccine producing more antibody. A VLP uptake assay identified that anti-VLP antibodies can enhance the early uptake of VLP by dendritic cells, but whether this has an effect on the anti-tumour immune response is unclear.

1359

Ex vivo generation of mature antigen presenting cells from inflammatory monocytes by oligomannose-coated liposomes

Matsuoka, Y.¹, Kawauchi, Y.¹, Kawauchi, K.², Kojima, N.¹

¹Tokai University, Applied Biochemistry, Hiratsuka, Japan, ²Tokyo Women's Medical University, Medical Center East, Department of Medicine, Tokyo, Japan

Oligomannose-coated liposomes (OMLs), in which the antigens are entrapped, have been shown to serve as effective antigen delivery vehicles and as a novel adjuvant to induce antigen-specific cellular immune response. Here, we demonstrate that inflammatory monocytes in PBMC can differentiate into mature professional antigen presenting cells *ex vivo* in response to uptake of OMLs.

When PBMC obtained from C57BL6 mice was co-cultured with OMLs in the presence mouse serum, OMLs were preferentially and rapidly incorporated into CD11b⁺Ly6C⁺ murine inflammatory monocytes. Interestingly, the expression of CD86, CCR7, CD83, and MHC class II on murine inflammatory monocytes was significantly enhanced within 24 h after OML

uptake. In addition, OML-ingesting inflammatory monocytes can activate T cells from OT-1 mice.

OMLs were also preferentially incorporated into human monocyte-derived DCs *ex vivo*, and in response to OML uptake, expression of CD86 and HLA-DR was upregulated. When, human PBMC were directly co-cultured with OMLs in the presence of human serum, CD14⁺ monocytes significantly ingested OMLs within 1 hour. Furthermore, during 5 day *ex vivo* culture with OMLs, the expression of HLA-DR and CD86 was significantly enhanced, and the expression of CD206, CD209, and CD83, which are not expressed on CD14⁺ monocytes, was induced on OML-ingesting CD11b⁺CD14⁺ cells.

Above results suggest that inflammatory monocytes in PBMC is differentiated into mature APCs, which may be distinguished from monocyte-derived DCs, in response to *ex vivo* OML uptake in both mouse and human models. Thus, use of OMLs may provide a new approach for *ex vivo* professional APC generation.

45 Minute Oral

16:45:00 - 17:30:00

Inflammation

T cell subsets in regulation of autoimmunity and anti-tumor immunity

Kuchroo, V.K.

Evergrande Center for Immunologic Diseases, Harvard Medical School and Brigham and Women's Hospital

Recently a subset of interleukin (IL)-17-producing T cells (T_H17) distinct from T_H1 or T_H2 cells was described and shown to have a crucial role in the induction of autoimmune tissue injury. Accumulating data suggests that there are three distinct steps in Th17 differentiation: Induction, Amplification and Stabilization mediated by distinct cytokines and loss of any of the cytokines (TGF- β , IL-6, IL-21 or IL-23) in the pathway results in a defect in generation of Th17. However not all Th17 cells are pathogenic and induce autoimmunity, IL-23 is a key cytokine that induces pathogenicity in Th17 cells (Lee et al., 2012). Using expression profiling at very high temporal resolution, novel computational algorithms and innovative nano-wire based "knock-down" approaches, we have developed a regulatory network that governs the development of Th17 cells. In addition to high-density temporal microarray analysis, we have performed single-cell RNA-seq of Th17 cells in order to characterize cellular heterogeneity, identify subpopulations, functional states and learn how gene expression variation affects Th17 effector functions. We have identified novel regulators of Th17 cells both *in vivo* and *in vitro* that do not affect Th17 differentiation but affect pathogenic vs. non-pathogenic functional states of Th17 cells. Some of the genes that are expressed in non-pathogenic Th17 cells are also utilized by CD8⁺ T cells to induce T cell exhaustion. Deletion of some of these novel genes cooperate with known "check-point inhibitors to suppress anti-tumor immunity.

Antibody

Broadly neutralizing antibodies and a new mechanism of diversification

Lanzavecchia, A.

Institute for Research in Biomedicine

We use cell culture-based high-throughput methods to interrogate human memory B cell and plasma cell repertoires and to isolate antibodies selected on the basis of their neutralizing potency and breadth. Relevant examples are antibodies that neutralize all influenza A viruses or even four paramyxoviruses. By targeting conserved structures, these broadly neutralizing antibodies are less prone to select escape mutants and are promising candidates for prophylaxis and therapy of infections, as well as tools for vaccine design. The value of a target-agnostic approach to vaccine design is illustrated by our discovery of extremely potent antibodies that neutralize

human cytomegalovirus, which led to the identification of their viral ligand, a pentameric complex that was then produced and tested as an effective vaccine. By reconstructing the genealogy trees of specific B cell clones, we investigate the role of somatic mutations in affinity maturation and in generation of antibody variants with broader or different specificity. Somatic mutations can also generate autoantibodies, as found in patients with pemphigus and autoimmune pulmonary alveolar proteinosis. Recently, while searching for antibodies that broadly react with malaria variant antigens, we discovered a new mechanism of antibody diversification, which relies on the interchromosomal transposition of genomic DNA sequences into rearranging T cell subsets in regulation of autoimmunity and anti-tumor immunity

Stimulation of camel polyclonal antibody against human T cell immunoglobulin and Mucin-3

Homayouni, V.

Isfahan University of Medical Sciences, Isfahan, Iran, Islamic Republic of

Background: T cell Immunoglobulin and Mucin (TIM)-3 is a type I transmembrane glycoprotein and a member of the TIM family which is identified as a receptor. This receptor expresses on T helper type 1 (Th1) cells and binds to galectin-9 (Gal9). This interaction induces an inhibitory signal, resulting in apoptosis of Th1 cells and cytotoxic CD8 T cells in vitro. Antibody therapy for immune checkpoint blockade has achieved promising results for several types of malignant tumors. Therefore, this immunomodulatory molecule may be used as a novel target for clinical purposes. The production of camel heavy chain antibodies (HCAbs) against TIM-3-expressing cell line was reported in this study.

Methods: A pre-synthesized human TIM-3cDNA was inserted into pcDNA3.1 plasmid and the new construct was transfected in HEK cell. TIM-3 expression was confirmed by qRT-PCR and flow cytometry methods. A 6 months old camel was immunized with the lysate prepared from rTIM-3 expressing HEK cells 4 times. Then, the anti-TIM-3 antibody level was evaluated using ELISA method.

Results and conclusion: TIM-3 was successfully cloned in HEK cells and more than 88% of the cells expressed TIM-3. Hence, using HEK cells was produced a readily obtainable source of TIM-3. It was used for camel immunization. High level of anti-TIM-3 antibody was detected in its serum after the final injection. This antibody may be useful for future diagnostic or therapeutic approaches.

B cell depletion compromises CD8+ T cell response in murine *T. cruzi* infection

Fiocca Vernengo, F., Beccaria, C.G., Araujo Furlán, C., Tosello Boari, J., Gorosito Serrán, M., Montes, C., Acosta Rodriguez, E.V., Gruppi, A.
School of Chemistry, National University of Córdoba, Córdoba, Argentina

Considering CD8+T cells play a major role in *T. cruzi* protective immunity, the signals that promote the generation and maintenance of CD8+T cell responses needs to be identified. Many reports highlight the role of B cells in promoting cellular immunity; however, if B cells affect CD8 responses remain uncharacterized. To assess B-cell function in promoting CD8 responses in Chagas disease, C57BL/6 mice were intraperitoneally injected with anti-CD20, to deplete B cells, or with control antibodies. Eight days after treatment, mice were infected with 5000 trypomastigotes. Flow cytometric analysis using tetramer staining revealed that at day 14 post infection, depleted mice had a lower frequency of parasite-specific CD8+T cells in spleen, blood and liver compared to control mice ($p=0,0003$, $0,0353$ and $0,0447$ respectively). Interestingly, B-cell deficient mice showed higher percentages of naïve CD8+T cells (CD44-CD62L+) and lower frequency of effector memory CD8+T cells (CD44+CD62L-). CD8+T cells from B-cell depleted mice presented a Memory Precursor Effectors Cells phenotype unlike CD8+T cells from B-cell sufficient mice who exhibit a Short Lived Effectors Cells phenotype. Also, we found that depleted mice had lower frequencies of IFN- γ - or TNF-producing CD8+T cells and displayed lower apoptosis and proliferation rate. Unexpectedly CD8+T cells from B-cell depleted mice overexpressed molecules associated to activation/migration than control mice. These results identify

functional defects in CD8+T cells generated in B cell-depleted mice, highlighting B cell influence on CD8+T cell response. Furthermore, our study urges caution during B-cell depletion therapies due to its possible side effects on the immunity against infections.

Transgenic expression of N-acetylglucosaminyltransferase V in T cells accelerates autoimmune diabetes in NOD mice by enhancing pathogenicity of CD8 T cells

Chien, M.-W.¹, Khoo, K.-H.², Sytwu, H.-K.¹

¹National Defense Medical Center, Department and Graduate Institute of Microbiology and Immunology, Taipei, Taiwan, Republic of China, ²Academia Sinica, Institute of Biological Chemistry, Taipei, Taiwan, Republic of China

β 1,6 N-acetylglucosaminyltransferase V (Mgat5) is a glycosyltransferase that increases N-glycan branching by transferring N-acetylglucosamine (GlcNAc) from UDP-GlcNAc to hydroxyl group on carbon 6 of the α 1,6-linked mannose of N-glycans. Previous studies have shown that deficiency of Mgat5 in mice decreased the T cell activation thresholds and exacerbated the incidence of experimental autoimmune encephalomyelitis (EAE). The non-obese diabetic (NOD) mice, a widely used animal model for human type 1 diabetes (T1D), developed spontaneous autoimmune diabetes and mainly caused by T cell-dependent destruction of pancreatic β -cells. Recently, NOD mouse is reported as an EAE susceptible strain since less N-glycan branching expressed on T cells. However, the relationship between Mgat5-mediated N-glycan branching and diabetogenic processing on T cells in NOD mice is still unknown. Here, we observed that Mgat5 transgenic T cells expressed higher level of N-glycan branching and had attenuated proliferation potential compared to control littermate T cells in NOD mice. Unexpectedly, a significantly higher diabetic incidence was found in both Mgat5 transgenic NOD mice and Mgat5/NY8.3 doubly transgenic mice that express a highly pathogenic transgenic TCR on CD8 T cells. Further, the higher diabetic incidence was also found in NOD/SCID recipients received adoptive transfer of Mgat5-overexpressed CD8 T cells, indicating that Mgat5 overexpression enhances the pathogenic properties of CD8 T cells in an antigen-specific manner. Taken together, our results suggest that upregulated Mgat5-mediated N-glycan branching on CD8 T cells contributes to autoimmune diabetes in NOD mice.

Features of phenotypic structure and functional activity of cord blood immune cells depending a gestational age

Lyapunov, V.¹, Chistyakova, G.¹, Bychkova, S.², Remizova, I.¹, Chereshev, V.³, Ustyantseva, L.²

¹Mother and Child Care Research Institute in Russian Public Health Ministry, Immunology and Clinical Microbiology, Ekaterinburg, Russian Federation, ²Mother and Child Care Research Institute in Russian Public Health Ministry, Pathology of Premature Infants, Ekaterinburg, Russian Federation, ³Institute of Immunology and Physiology UBRAS, Ekaterinburg, Russian Federation

In order to identify genotypic characteristics of the composition and functional activity of immune cells of umbilical cord blood (UCB) as a function of gestational age (GA), studied 102 UCB samples of babies born at 25-28, 29-32 and 33-36 weeks of GA and 31 UCB samples from comparison group (37-41 weeks GA). By the method of flow

cytometry was determined the number of CD3, CD19, CD4, CD8, CD16/56 cells; the level of expression of activation markers on the monocytes and lymphocytes (HLA- DR/CD14, CD71/CD14, CD25/CD4, CD95/CD3); adhesion receptor (CD11b). All premature babies with leukopenia in the background found an increase in the relative number of lymphocytes and decreased ability to absorb opsonized bacteria by granulocytes. Defined a reduction in the percentage of HLA-DR+/CD14+-cells, increasing the number of CD3+CD95+-population in group of children with a GA 25-28 weeks. Decrease at the level of NK-cells in group infants less than 32 weeks of GA was observed. It was determined a reduced percentage of CD3+CD11b+ cells in the 29-32; 33-36 weeks of GA. At the 33-36 weeks of the GA found a reduction in the number CD3+CD95+ lymphocytes and increased expression of the CD25+CD4+ lymphocytes.

Overall, the findings suggest about the features of the immune system, specific to a particular GA preterm infants. A number of the investigated parameters give the opportunity to talk about the partial achievement of the immunological response preparedness for a potential antigen that dictates the need for further research aimed at understanding the functional activity of immune cells.

The importance of serum visfatin levels in Behcet's disease patients

Ozbalkan, Z.¹, Enecik, M.E.¹, Keskin, G.², Karaaslan, Y.¹

¹Ankara Numune Education and Research Hospital, Rheumatology Department, Ankara, Turkey, ²Ankara University School of Medicine, Immunology Department, Ankara, Turkey

Objectives: Adipose tissue is an active endocrine organ and releases adipokines. Visfatin is one of them. Visfatin is related to TNF- α and IL-6, IL-1 beta, co-stimulators like CD40, CD54, CD 80 and endothelial ICAM-1 and ICAM-2. We aimed to search the relation among levels of serum visfatin in BD activity.

Materials and methods: 60BD patients (30 in active state and 30 in remission) diagnosed according to criteria of WGIBD and 20 healthy subjects as controls were involved in to the study. Serum visfatin levels were compared in between three groups.

Results: Visfatin levels were significantly higher in both group of patients compared to the healthy control group (both $p < 0,001$). Serum visfatin levels in active state patients were higher than those in inactive state ($p < 0,001$). The same way in all cases statistically significant correlation between visfatin and CRP ($p < 0,001$) and visfatin and ESR ($p < 0,01$). According to the symptoms of the patients in the active state, patients with genital aphthous ulcers had higher serum visfatin levels than the active patients without genital aphthous ulcers ($p < 0,001$).

Conclusion: Serum visfatin levels in BD patients with active and inactive states are higher than the healthy control group. That could be concluded as visfatin as a proinflammatory cytokines have a role in chronic inflammatory reactions and sustains the cellular expression of the inflammatory cytokines in BD.

Kinetic analysis of type 2 innate lymphoid cells in tissues from IL-25- and IL-33-induced murine models of asthma

Li, Y.¹, Lv, Z.², Wang, J.³, Chi, Y.³, Huang, P.², Corrigan, C.J.⁴, Huang, K.¹, Wang, W.², Ying, S.²

¹Beijing Chao-Yang Hospital, Capital Medical University & Beijing

Institute of Respiratory Medicine, Department of Respiratory and Critical Care Medicine, Beijing, China, ²Capital Medical University, Department of Immunology, School of Basic Medical Sciences, Beijing, China, ³Capital Medical University, Department of Laboratory Animal Sciences, Beijing, China, ⁴King's College London, MRC & Asthma UK Centre in Allergic Mechanisms of Asthma, Division of Asthma, Allergy & Lung Biology, London, United Kingdom

Background: Type-2 innate lymphoid cells (ILC2) play important role in the pathogenesis of asthma through producing large amounts of Th2-type cytokines. The mechanisms of ILC2 chemotaxis into lungs, however, are still unclear.

Methods: To answer these, BALB/c mice were intranasally changed with murine IL-25 or IL-33, while OVA and saline challenged mice were used as positive and negative controls. Bone marrow, spleen, thymus, mesenteric lymph nodes, mediastinal lymph nodes, lung tissue and bronchoalveolar lavage fluid (BALF) were collected from the mice at experimental acute, subacute, chronic and convalescent phases. Flow cytometry, *in vitro* chemotaxis assay and small animal *in vivo* imaging were used to measure the distribution and abundance and chemotaxis of ILC2 *in vitro* and *in vivo*.

Results: Both IL-25 and IL-33 alone induced accumulation of ILC2 into mediastinal lymph nodes, lung tissue and BALF, but with different kinetics. The peak of IL-25 induced ILC2 accumulation into lung tissue and BALF was earlier than that of the IL-33- induced mice. In quantity, the numbers of ILC2 in BALF, lung tissue and mediastinal lymph nodes were significantly higher in IL-33-induced mice compared with IL-25-induced mice. *In vitro* IL-33, but not IL-25 directly induced ILC2 chemotaxis. Small animal *in vivo* imaging further confirmed that single intranasally inhalation of IL-33 was sufficient to induce accumulation of injected ILC2 through tail vein into the lungs.

Conclusion: The data suggest that airways epithelium-derived IL-25 or IL-33 can induce accumulation of ILC2 to the lungs and that IL-33 has direct effect on ILC2 chemotaxis.

Immune responses to oral polio vaccine (OPV) in patients with X-linked agammaglobulinemia (XLA)

Wu, Y.¹, Mao, H.W.², Chan, S.M.¹, Lam, K.T.¹, Tu, W.¹, Lau, Y.L.¹

¹University of Hong Kong, Paediatrics and Adolescent Medicine, Hong Kong, Hong Kong, ²HKU-ShenZhen Hospital, ShenZhen, China

Background: XLA is characterized by impaired antibody response resulting from Bruton's tyrosine kinase (BTK) mutation. Unlike other primary immunodeficiency with no predisposition to extraordinary risk of viral infection, XLA renders a unique susceptibility to enteroviruses infection including poliovirus. OPV can induce paralytic poliomyelitis in XLA patients. Of great public concern is that the attenuated OPV can persist and revert to a virulent form in these patients, which renders a high risk of reintroducing virulent poliovirus into general population. It is known that BTK is involved in toll-like receptor 3 (TLR3) signaling, and TLR3-mediated type I IFN responses play critical roles in host protection against poliovirus. However, whether XLA patients have impaired type I IFN responses to OPV are not clear.

Hypothesis: BTK mutations in XLA impair TLR3-mediated type I IFN response, leading to the unique susceptibility to poliovirus in XLA patients.

Method: To investigate the response of XLA patients to OPV, monocyte derived macrophages (MDM) from XLA patients and healthy controls were infected with OPV Sabin strain 1 (OPV1). Further to examine the role of BTK in OPV infection, MDM from healthy controls were infected with OPV1 in the presence or absence BTK inhibitor.

Result: IFN- α production was measured by ELISA. OPV virus replication and IFN-inducible gene expressions including Mx1, Mx2, OAS1 were detected by qPCR. Results were compared between XLA patients and healthy controls

Conclusion: This study investigated the immune response of XLA patients to OPV and helped to reveal the role of BTK to control OPV infection.

Human placenta-derived multipotent cells (PDMCs) protect against *Klebsiella pneumoniae*- induced pneumonia by enhancing polymorphonuclear granulocytes (PMN) functions

Wang, L.T.^{1,2}, Chao, Y.Y.², Lee, W.¹, Huang, L.Y.³, Liu, K.J.⁴, Siu, L.K.³, Yen, B.L.²

¹Graduate Institute of Life Sciences, National Defense Medical Center, Taipei, Taiwan, Republic of China, ²Institute of Cellular & System Medicine, National Health Research Institutes (NHRI), Miaoli County, Taiwan, Republic of China, ³National Institute of Infectious Diseases and Vaccinology, NHRI, Miaoli County, Taiwan, Republic of China, ⁴National Institute of Cancer Research, NHRI, Miaoli County, Taiwan, Republic of China

Human mesenchymal stem cells (MSCs) are multilineage somatic progenitors with strong immunomodulatory properties which have been well-demonstrated for T lymphocytes and dendritic cells/macrophages. However, interactions with neutrophils (polymorphonuclear granulocytes or PMNs)—the most abundant population of human leukocytes—are less well understood. Therefore, we investigated the interactions of human placenta-derived multipotent cells (PDMCs), a population of fetal-stage MSCs, with PMNs through *in vitro* studies and a mouse model of *Klebsiella pneumoniae* (KP)-induced pneumonia. KP is a highly virulent gram-negative bacterium and a leading cause of community- and hospital-acquired infections, especially pneumonia. We found that after co-culture with PDMCs, PMN expression of CD11b—a marker of activation—as well as phagocytosis of FITC- labeled KP was significantly increased. Moreover, PMN oxidative metabolism was significantly increased as well, which manifested as increased anti-bacterial activity and improved killing of KP. To ascertain the therapeutic efficacy of PDMCs on bacterial infections, we infected wild type mice with KP by intratracheal inoculation with subsequent intravenous administration of PDMCs. Surprisingly, while injection of PDMCs reduced the influx of PMNs in KP-infected lung tissue, respiratory burst activity was simultaneously enhanced. PDMC treatment also decreased bacterial counts both locally in lung tissue and systemically in the bloodstream. Most importantly, administration of PDMCs significantly increased survival rates in this mouse model of KP pneumonia. Taken together, we found that PDMCs enhance PMN functions *in vitro* and *in vivo* towards KP without increasing overall lung inflammatory damage. Our data strongly implicate a possible therapeutic role for PDMCs towards gram-negative bacterial infections.

PTEN ameliorates autoimmune arthritis through down-regulating STAT3 activation with reciprocal balance of Th17 and Treg

Lee, S.H.¹, Byun, J.-K.¹, Park, J.-S.¹, Jhun, J.Y.¹, Jung, K.², Seo, H.-B.¹, Moon, Y.-M.¹, Park, S.-H.¹, Kim, H.-Y.¹, Cho, M.-L.¹

¹The Catholic University of Korea, Seoul, Korea, Republic of, ²Impact Biotech, Seoul, Korea, Republic of

PTEN is a tyrosine phosphatase that conducts a significant function in an inhibition of STAT3 activation. Recently, inactivation of STAT3 has been demonstrated as a therapeutic candidate for autoimmune arthritis. The expression of PTEN is controlled by p53 which regulates autoimmune arthritis through modulating balance between Th17 and Treg. We hypothesized that PTEN regulated by p53 may reduce CIA severity and inflammatory response via inhibition of STAT3 activation. Here, we reveal that PTEN ameliorates experimental autoimmune arthritis by reducing STAT3 activity and Th17 differentiation. Systemic infusion of PTEN overexpression downregulated CIA severity. Additionally, PTEN overexpression decreased activation of T cells and modulated reciprocal differentiation of Th17 and Treg cells. We observed that p53 deficiency downregulating PTEN expression induces the activation of STAT3. Loss of p53 exacerbates autoimmune arthritis and dysregulated the population of Th17 and Treg. These data suggest that induction of the STAT3-modulatory activity of PTEN may be a therapeutic target for rheumatoid arthritis therapy.

Notch and TCR signaling modulate the effector functions of human gamma delta T cells

Bhat, S., Chiplunkar, S.

ACTREC (Tata Memorial Centre), Chiplunkar Lab, Mumbai, India

Gamma Delta ($\gamma\delta$) T cells account for 5-10% of CD3⁺ peripheral blood T cells. $\gamma\delta$ T cells are activated by phosphoantigens, isopentenyl pyrophosphate (IPP) or 4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) which are intermediates of mevalonate/Rohmer pathway. Notch proteins play an important role in cell fate decisions, T cell development and are evolutionary conserved. The present study focuses on understanding how Notch signaling modulates the effector functions of $\gamma\delta$ T cells. Notch receptors (Notch1 and Notch2) were found to be dominantly expressed on $\gamma\delta$ T cells along with Notch ligands Delta- like ligand 1 and Jagged 1. Knockdown of Notch1 and Notch2 genes in $\gamma\delta$ T cells using small interfering RNA inhibited their antitumor cytotoxic potential. A marked decrease in cytotoxic effector function of $\gamma\delta$ T cells was observed with concomitant decrease in the expression of effector molecules like perforin and granzyme B. Altered expression of key transcriptional factors Eomes and Tbet was observed upon inhibition of notch signaling. This further leads to significant changes in the histone modifications, H3K9 acetylation and H3K9 methylation on the promoter region of different effector genes. Inhibition of Notch signaling decreased the expression of IL-2 receptor subunits (CD25, CD122 and CD132) and downstream signaling molecules like pAKT, NF-B and pSTAT5. Further, inhibition of notch signaling in $\gamma\delta$ T cells caused G1-G0 cell cycle arrest. In conclusion, our study demonstrates that notch signaling is indispensable for anti-tumor effector functions of $\gamma\delta$ T cells.

Evaluation of the adjuvant effect of gold nanocages *in vitro*

Yavuz, E.^{1,2}, Sakalak, H.³, Cavusoglu, H.^{1,3}, Uyar, P.^{1,4}, Yavuz, M.S.^{1,3}, Bagriacik, E.U.²

¹Selcuk University, Advanced Technology Research and Application Center, Konya, Turkey, ²Gazi University Medical School, Immunology, Ankara, Turkey, ³Selcuk University, Metallurgy and Materials Science Engineering, Konya, Turkey, ⁴Selcuk University, Biotechnology, Konya, Turkey

Hepatitis B is a potentially life-threatening disease caused by the hepatitis B virus (HBV). Unfortunately, despite the ongoing vaccine campaigns HBV infection is not completely managed. In order to increase the immune modulation capacities of Hepatitis B vaccines different adjuvant systems have been studied. Especially nanoparticle-based adjuvants are being widely investigated. Biocompatible and bioinert gold nanoparticles have been commonly used *in vitro* and *in vivo* biological research. Recently, gold nanocages (AuNCs), a special design with ultra thin porous walls and hollow interiors, have shown ample potential and have a promising future in the fields of cancer diagnostics and treatment. Our goal is to use AuNCs as an adjuvant in Hepatitis B model *in vitro*.

In this project, synthesized and characterized Au nanocages are used. Following the adsorption of HBsAg protein onto Au nanocages, adsorption efficiency of AuNCs is investigated. The uptake of HBsAg-AuNC by macrophages and its colocalization within the cell are analyzed by flow cytometry and confocal microscopy. The *in vitro* effect of the internalization of only AuNCs or HBsAg-AuNC on macrophage activation, antigen presentation and the cytokine profile are analyzed by flow cytometry and ELISA techniques. Here we aim to study the immunomodulation properties of the porous gold nanoparticles as an adjuvant *in vitro*.

IL-15 activated NK cells overcome DC maturation defects induced by head and neck cancer microenvironment

Upreti, D., Zhang, M., Kung, S.K.P.

University of Manitoba, Winnipeg, Canada

Head and neck squamous cell carcinoma (HNSCC) patients have the lowest 5-year disease-free survival rate. There is need to develop novel therapeutics of HNSCC. Natural Killer (NK) cells play key roles in innate immunity against infections and transformed cells. Through interactions with dendritic cell (DC), NK cells can shape also subsequently induced adaptive T-cell immunity.

Here we used an immunocompetent mouse model (AT-84) to evaluate anti-tumor potential of IL-15 activated NK cells. A direct injection of NK cells at tumor site significantly suppressed AT-84 tumor growth *in vivo*. It induced also protective memory responses against a secondary AT-84 challenge. AT-84 tumor cell was relatively resistant to IL-15 activated NK cells killing *in vitro*, suggesting that direct killing of AT-84 is unlikely the major underlying mechanism. We therefore hypothesized that IL-15 activated NK cells promoted anti-tumor activities via NK-DC crosstalk. Using bone marrow derived DC cultures; we observed that conditioned medium of AT-84 impaired DC maturation induced by TLR ligands *in vitro*. These DC were impaired in inducing T cell activations *in vitro*. Addition of IL-15 NK cells to the AT-84-exposed DC overcame the DC maturation and functional defects *in vitro*. These *in vitro* data was further corroborated by *in vivo* data obtained from the tumor

infiltrating dendritic cells. Collectively, our data demonstrated that IL-15 activated NK cells were able to reverse the immunosuppressed DCs to an immunostimulatory state that correlated well with tumor regressions. It supported future development of IL-15 NK-based immunotherapy of HNSCC.

Plasticity in CD1d-lipid antigen recognition by non-canonical NKT cells

Almeida, C.F.^{1,2}, Sundararaj, S.^{3,4}, Le Nours, J.^{3,4}, Patel, O.³, Cao, B.⁵, Pellicci, D.G.^{1,2}, Williams, S.⁵, Rossjohn, J.^{3,4,6}, Uldrich, A.P.^{1,2}, Godfrey, D.I.^{1,2}

¹The University of Melbourne and The Peter Doherty Institute for Infection and Immunity, Department of Microbiology and Immunology, Melbourne, Australia, ²Australian Research Council Centre of Excellence in Advanced Molecular Imaging, The University of Melbourne, Melbourne, Australia, ³School of Biomedical Sciences, Monash University, Department of Biochemistry and Molecular Biology, Melbourne, Australia, ⁴Australian Research Council Centre of Excellence in Advanced Molecular Imaging, Monash University, Monash, Australia, ⁵School of Chemistry, Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Melbourne, Australia, ⁶Institute of Infection and Immunity, Cardiff University, School of Medicine, Heath Park, Cardiff, United Kingdom

Natural Killer T (NKT) cells are specialised lymphocytes that recognise lipid antigens presented by the MHC Class I-like molecule CD1d. Following activation, they rapidly secrete a broad range of immunoregulatory cytokines that can influence other mediators of immune responses and therefore they represent a promising therapeutic target for cancer and other diseases. The most extensively studied are type 1 NKT cells, which recognise a derivative of a marine sponge glycolipid α -galactosylceramide (α -GalCer), express a semi-invariant T cell receptor (TCR) and have a well-established role in the immune system. Much less is known about type 2 NKT cells, which do not recognise α -GalCer and express a diverse TCR repertoire. Using a panel of CD1d mutants, we reveal that different type 2 NKT cell hybridomas can adopt multiple ways to interact with CD1d, and furthermore, we identify a new population of type 2 NKT cells that specifically recognises the microbial derived lipid-antigen α -glucuronosyl-diacylglycerol (α -GlcA-DAG). Single cell sequencing of CD1d- α -GlcA-DAG tetramer⁺ cells reveals a polyclonal TCR repertoire distinct from type 1 NKT cells. Collectively, our results suggest that type 2 NKT cells express highly diverse TCRs and rely on mechanisms different to type 1 NKT cells to recognise distinct antigens. The knowledge obtained from these studies increases the scope of antigens recognized by NKT cells and provides valuable insight in how these cells can be manipulated for therapeutic purposes.

Mucosal route of immunotherapy with transgenic rice seeds expressing hypoallergenic whole T cell epitopes of Cryj1 and Cryj2 - Investigation in murine model of cedar pollinosis

Kawauchi, H.¹, Aoi, N.¹, Yamada, T.², Takagi, H.³, Takaiwa, F.³

¹Shimane University, Faculty of Medicine, Otorhinolaryngology, Izumo, Japan, ²Shimane University, Center for Integrated Research in Science, Department of Experimental Animals, Izumo, Japan, ³National Institute of Agrobiological Sciences, Ministry of Agriculture, Tsukuba, Japan

For the last decade, we have been investigating the therapeutic

effect of mucosal route of administration of transgenic rice (Tg- rice) seeds, which contain T-cell epitopes of Cryj1 and Cryj2, on murine allergic rhinitis models and reported its clinical efficacy to actually attenuate nasal symptoms. However, its mechanism remains to be further investigated and adverse events of this therapeutic approach should be very least with more sophisticated manners.

Results: Therefore, we have examined the effect of natural feeding with protein bodies (PB) of transgenic rice seeds expressing hypoallergenic whole T cell epitopes of Cryj1 and Cryj2 (PB-Tg rice), in comparison with whole Tg-rice, in a murine model of cedar pollinosis. The numbers of sneezing after final intranasal challenge in mice naturally fed with PB Tg-rice powder were significantly decreased in a dose dependent manner, with less doses, in comparison with those of whole Tg-rice powder. Histopathological findings correspondingly demonstrated that the number of eosinophils infiltrating into nasal mucosa decreased and the damage of epithelial cells was less found in each group of mice. Sublingual route of administration is also effective to attenuate nasal symptoms.

Conclusion: Protein body fraction of Tg-rice more efficiently downregulated nasal symptom in murine model of cedar pollinosis with natural feeding or sublingual administration. These result implicates that intake of protein body form of Tg-rice can be more promising strategy and material to be utilized for mucosal route of immunotherapy to attenuate nasal symptoms of patients with cedar pollinosis.

Enhancement of NK cell-mediated cytotoxicity during chronic virus infection

Ha, S.-J., Oh, J.

Yonsei University, Department of Biochemistry, Seoul, Korea, Republic of

NK cells are critical for the clearance of parasites, virus-infected cells and tumors. Recently, several groups reported that NK cells could control virus-specific T cell responses at early stage of chronic infection. However, the physiological role of NK cell in a host infected with chronic virus has not been extensively studied. To identify the role of NK cell in chronic virus infection was studied in the mice persistently infected with LCMV-CL13. In this study, we found that terminally differentiated CD27^{low}KLRG1⁺CD11b^{high} NK cells were more abundant in the chronically infected mice than in naive mice. Furthermore, the NK cells from CL13-infected mice were shown to express higher level of an activating receptor, NKG2D and lower levels of the inhibitory receptors, NKG2A and Ly49C/I than those from naive mice. Such an integrative signal seemed to enhance NK cell activity, elevating expressions of CD69 and Granzyme B. At the functional level, NK cells in the CL13-infected mice display increased *ex vivo* IFN- γ production and increased *in vitro* cytotoxicity. Indeed, the CL13-infected mice, but not naive mice, dramatically delayed tumor formation when various tumor cells such as TC-1 lung adenocarcinoma and B16F10 melanoma were inoculated. The significant decrease of tumor promotion in CL13-infected mice was abrogated by depletion of NK cells, suggesting NK cell-mediated cytolytic activity for tumors. Currently, we are investigating which molecular are able to empower NK cells with enhanced cytolytic activity. Discovery of the key regulator for the enhanced activity of NK cell could be a potent cancer drug.

CD169 identifies an anti-tumor macrophage subpopulation in human hepatocellular carcinoma

Wu, Y., Zhang, Y., Zheng, L.

Sun Yat-sen University, Guangzhou, China

Macrophages are a major component of most solid tumors and can exert both anti- and pro-tumorigenic functions. Although the immunosuppressive/pro-tumor roles of macrophages have been widely examined, significantly less is known about macrophage subpopulations that have potential anti-tumor properties in humans. In the present study, a population of CD169⁺ macrophages with relatively high expression levels of HLA-DR and CD86 was identified in human hepatocellular carcinoma tissues. The frequency of CD169-expressing macrophages within cancer nests was significantly lower compared with that found in paired non-tumor areas. *In vitro* experiments revealed that in the presence of anti-CD3 stimulation, CD169⁺ macrophages could significantly enhance the proliferation, cytotoxicity, and cytokine production capacity of autologous CD8⁺ T cells in a CD169 molecule-dependent manner. Autocrine TGF- β produced by tumor-stimulated macrophages was involved in the down-regulation of CD169 expression on these cells. Moreover, CD169⁺ macrophage accumulation in tumor tissues was negatively associated with disease progression and predicted favorable survival in hepatocellular carcinoma patients, which was in contrast to the trend observed for total CD68⁺ macrophages. Therefore, CD169 may act as a costimulatory molecule for cytotoxic T cell activation, and could define a population of tumor-infiltrating macrophages with potential anti-tumor properties in human hepatocellular carcinoma tissues.

All the authors declare no competing financial interests and concur with the submission.

Combination of TLR2 and TLR3 ligands enhances maintenance of CD4 T cells and production of class-switched antibody

Ha, S.-J.¹, Lee, B.R.¹, Jeong, S.K.², Ahn, B.C.², Shin, S.J.³, Yum, J.S.²

¹Yonsei University, Department of Biochemistry, Seoul, Korea, Republic of, ²CHA Vaccine Institute, R&D Center, Seongnam-si, Korea, Republic of, ³Institute for Immunology and Immunological Diseases, Brain Korea 21 PLUS Project for Medical Science, Yonsei University College of Medicine, Department of Microbiology, Seoul, Korea, Republic of

The efficacy of the vaccines in numerous studies including priming or boosting regimens for prophylactic purpose as well as its therapeutic application depends on the type of adjuvant. Thus, a better understanding on the combination of different type adjuvants is essential for optimal immune responses. Here, we investigated the mechanistic actions of a combined adjuvant Pam3csk4 and PolyI:C (hereafter referred to as L-pampo) to improve the adjuvanted-vaccine efficacy. Notably, an administration of ovalbumin (OVA) with L-pampo induced the enhanced production of antibody, correlated to reciprocal expansions of germinal center B cells and multi-functional CD4T cells concomitantly producing IFN- γ and TNF- α in an antigen-specific manner after tertiary immunization with OVA. To dissect the precise mechanism for the synergy by TLR2 and TLR3 ligands in L-pampo, we subdivided courses of the immune responses after primary immunization into three different phases. During 24 hrs after immunization, unlike PolyI:C alone, the levels of type I IFN in serum and spleen selectively decreased

along with an remarkably attenuated IRF3 signaling by L-pampo administration. However, at an effector phase, L-pampo and Pam3 comparably induced expansion of CXCR5⁺PD-1⁺ follicular helper T cells. Interestingly, during the memory phase, L-pampo showed prominent maintenance of antigen-specific CD4 T cells together with a high level of antibody titers when comparing to Poly:I:C or pam3 alone. Collectively, these findings suggest that combination of TLR2 and TLR3 ligands modulates cytokine production and prolong CD4 T cells that may lead to prominent antibody response and expansion of multi-functional CD4 T cells upon boosting.

Adjuvant therapy of chronic uroinfections in colonised patients

Vaskova, S.^{1,2}, Blazickova, S.^{2,3}

¹Laboratoria Piestany, Dept. of Clinical Microbiology, Piestany, Slovakia, ²Trnavian University, Faculty of Health Science and Social Work, Trnava, Slovakia, ³Laboratoria Piestany, Dept. of Clinical Immunology, Piestany, Slovakia

Introduction: Urinary tract infections represent the most commonly acquired bacterial infections. Identical bacterial species permanently isolated from urine of patients with chronic uroinfections are supposed to colonize distal segment of genitourinary tract and to form biofilm on mucous surface. Biofilms are the well-organized microbial communities and decrease the susceptibility to the host immune system and to the antimicrobial agents. Therefore, therapy of chronic infections should be different than therapy of acute ones.

Aim: To compare antibiotic therapy of chronic uroinfections with adjuvant immunomodulatory therapy in colonised patients.

Material and methods: 36 patients with monobacterial chronic uroinfections and bacterial species permanently isolated from urine that produce biofilm *in vitro*. Patients were classed into two groups: group A - patients treated with antibiotics (n = 29), group B - patients treated with antibiotics and adjuvant immunomodulatory therapy (n = 7). Inflammatory and blood elements parameters were analysed in the patients.

Results: We did not observe statistically significant differences between clinical benefits in patients with antibiotic and adjuvant immunomodulatory therapy. Clinical benefit after antibiotic treatment was observed in 9 patients (31 %); clinical benefit after adjuvant immunomodulatory therapy was observed in 4 (57 %) patients. We did not see statistically significant changes in other laboratory parameters.

Conclusion: The present study is a pilot study. Adjuvant therapy seems to have benefit for patients with chronic uroinfections. We would like to focus on analyse changes in immune system in colonised patients with chronic uroinfections and in patients with acute uroinfections.

Keywords: chronic uroinfections, biofilm, immunomodulatory therapy

Strategies for the *in silico* selection of immunogenic epitopes using non-model organisms: use case with *Histoplasma capsulatum*

Rubio C., M.^{1,2}, Cano Restrepo, L.E.^{1,3}, Ochoa Deossa, R.A.⁴

¹Corporación para Investigaciones Biológicas (CIB), Micología Médica Experimental, Medellín, Colombia, ²Universidad de Antioquia, Instituto

de Biología, Medellín, Colombia, ³Universidad de Antioquia, Escuela de Microbiología, Medellín, Colombia, ⁴Universidad de Antioquia, SIU-PECET-CIEMTO, Medellín, Colombia

The dimorphic fungal pathogen *Histoplasma capsulatum* (*Hc*) causes respiratory and systemic diseases. The generation of a protective immune response to the pathogenic fungus *Hc* is critically dependent on the interaction between antigen-reactive T cells and macrophages. Recently, new findings are available about the proteome and secretome of this fungus. Nevertheless, there is a huge gap in knowledge regarding interactions between constituent molecules of the fungus and the host during infection. Therefore, the evaluation of new immunological interfaces will serve us to deepen in the pathogenesis of Histoplasmosis, and then propose possible molecular targets for vaccine or diagnostic tests. Approaches based on bioinformatics tools have contributed to know more about the immunogenicity generated by CD8T cells, optimizing and directing experimental efforts to elucidate the problem. According to the principles of reverse vaccinology, we propose a pipeline that involves: i) search for molecular patterns from immunogenic/antigenic proteins experimentally proven; ii) find the patterns on the complete set of annotated proteins to find unique epitopes; iii) identify whether the proteins are secreted and predict the feasibility of being recognized by CD8T cells. Beta-lactamase, aryl-alcohol-dehydrogenase and GARP complex component are potential new targets in *Hc* related with the adaptive immune response. Functionalities such as the union to penicillin, catalysis of aromatic alcohol and protein transport between Golgi, endosomal, and vacuolar compartments are associated to these targets respectively. The computational protocol was applied and a set of epitopes predicted for further experimental validation. Colciencias grant 221356933526.

Regulation of inflammasome during clinical sepsis: a PCR array study

Esquerdo, K.F.¹, Shama, N.K.¹, Brunialti, M.K.C.¹, Machado, F.R.², Silva, E.³, Rigato, O.⁴, Salomao, R.¹

¹Disciplina de Infectologia, Escola Paulista de Medicina, Hospital São Paulo, Universidade Federal de São Paulo - UNIFESP, Medicine, Sao Paulo, Brazil, ²Disciplina de Anestesiologia, Escola Paulista de Medicina, Hospital São Paulo, Universidade Federal de São Paulo - UNIFESP, Medicine, Sao Paulo, Brazil, ³Hospital Israelita Albert Einstein, Sao Paulo, Brazil, ⁴Hospital Sírio Libanês, Sao Paulo, Brazil

Sepsis is a systemic inflammatory response triggered by an infection. Nod like receptor family (NLR) is involved in this process through inflammasome oligomerization. To study the regulation of inflammasome under sepsis, we analyzed patients (survival n=19, non-survival n=8) at admission and after 7 days of follow-up (survival n=13, non-survival n=5) with healthy volunteers (n=11). In brief, mononuclear cells were separated, cDNA synthesized from isolated RNA from cells and analyzed by real time PCR array (35 genes). Genes were considered differentially modulated when the expression was higher than ± 1.5 and $p \leq 0.05$. The resulted data was processed through Ingenuity Pathway Analysis for functional analysis and interactions. The gene expression study enables us to identify alteration in 8 genes at admission and 7 genes after day 7 in survival, 16 genes at admission and 11 genes after day 7 in non-survival.

Pyroptosis, production of reactive oxygen species and synthesis of nitric oxide, and inflammatory response were activated in survival patients at D0 and D7. Furthermore, bacterial infection inhibition was found in survivals. Interestingly, similar functions were also identified in non-survivals but differences were the activation or inhibition z scores. These results highlight the activation of inflammasome during sepsis, with a different degree of modulation in survivors and non-survivors. Further validation of some genes at protein level is under process.

AS01-adjuvanted vaccine induces a transient innate immune response in humans

Smolen, K.¹, Herve, C.², Brouwer, M.¹, Taton, M.¹, Auquier, P.², Fissette, L.², Marchant, A.¹, Van Belle, P.², Burny, W.², Didierlaurent, A.², Willems, F.²
¹ULB, Institute for Medical Immunology, Gosselies, Belgium, ²GSK, Rixensart, Belgium

AS01 is an Adjuvant System containing 3-O-desacyl-4'-monophosphoryl lipid A (MPL), *Quillaja saponaria* Molina, fraction 21(QS-21) and liposome. AS01 has been selected for the clinical development of several candidate vaccines including the malaria and varicella zoster vaccine. AS01 promotes both humoral and cellular responses. Yet, little is known about the immunological mechanisms in humans by which AS01 induces effective immune responses. Given the fundamental role of the innate immune system in controlling the adaptive immune responses, we have conducted a phase II randomized, single centre, single-blind study (NCT01777295) to characterize the kinetics of the early immune response to immunization with an AS01-adjuvanted vaccine.

60 HBV-naive adults (18-45 years old) received 2 doses of Hepatitis B virus surface antigen (HbsAg) adjuvanted with AS01B (50µg MPL, 50µg QS-21) or aluminium hydroxide at days 0 and 30. All subjects received a saline placebo (baseline) 30 days prior vaccine administration. Whole blood samples were collected at defined time points (0h to 7 days) before and after placebo, dose 1 and dose 2.

Immunization with AS01B-adjuvanted vaccine leads to an early and transient inflammatory response in blood, characterized by changes in some cytokines, innate cell number and activation. Cellular variations were specifically reflected in monocyte subsets-particularly intermediate monocytes- and NK cells, indicative of the stimulation of the innate immune system. Although restricted to HbsAg and a naive population, identification of these early signatures may provide new insights into the mechanisms of AS01-adjuvanted vaccine induced protective immune response.

Analysis of CTLA-4 gene polymorphisms (-319 C/T, +49 A/G, CT60 G/A) in primary Sjögren's syndrome

Carrillo-Ballesteros, F.J.¹, Muñoz-Valle, J.F.¹, Palafox-Sánchez, C.A.¹, Valle, Y.¹, López-Villalobos, E.F.¹, Badial-Hernández, M.F.¹, Hernández-Martínez, M.A.¹, Rodríguez-Machuca, V.U.¹, Vázquez-Villamar, M.¹, Orozco-Barocio, G.², Oregon-Romero, E.¹
¹Universidad de Guadalajara, Instituto de Investigación en Ciencias Biomédicas (IICB), Guadalajara, Mexico, ²Hospital General de Occidente, SSJ., Zapopan, Mexico

Introduction: Primary Sjögren's syndrome (pSS) is an autoimmune disease affecting salivary and lacrimal glands. It is characterized by a progressive lymphocytic infiltration, mainly T cells. Cytotoxic

T-Lymphocyte Antigen 4 (CTLA-4) plays a critical role in the prevention of autoimmune pathology by a critical inhibitory role in T cell responses resulting in diminished proliferation and cytokine production. Polymorphic sites located at -319C/T, +49A/G and CT60G/A in the *CTLA-4* gene have been associated with elevated CTLA-4 serum levels (sCTLA-4) and autoimmunity.

Objective: Determine the frequency of *CTLA-4* polymorphism in pSS.

Methodology: The study included 111 Mexican mestizo patients with pSS, classified according to the American-European Consensus criteria. As control group, 122 clinically healthy subjects were included. The polymorphisms were genotyped by PCR/RFLP technique. Statistical analysis was carried out with *Pasw statistics 18* and *EmHapFre* softwares.

Results: Polymorphisms were in Hardy-Weinberg equilibrium and showed moderate linkage disequilibrium (50%, $p=0.009$). The most frequent haplotypes were: CAG(34%), CAA(26%), CGG(18%), and CGA(17%). Patients that carried CAA haplotype showed high sCTLA-4 levels, lymphocytic infiltrated and autoantibody production without statistic significance. In addition, high levels of sCTLA-4 were observed in pSS patients carriers of -319CT genotype ($p=0.018$ vs CC) and +49AA genotype ($p=0.013$ vs GG).

Conclusion: Our results suggest that *CTLA-4* polymorphisms -319 and +49 could have a role for the development of pSS. However, the low associations of sCTLA-4 with clinical parameters suggest that the potential effect of *CTLA-4* polymorphisms needs to be investigated in subsequent studies in Mexican patients in order to elucidate it.

Simple and effective tumour immunotherapy using intratumoural complete Freund's adjuvant

Fahrer, A.¹, Carroll, C.¹, Andrew, E.¹, Orange, M.², Allavena, R.³

¹The Australian National University, Research School of Biology, Acton, Australia, ²Arlesheim Klinik, Onkologie, Arlesheim, Switzerland, ³University of Queensland, School of Veterinary Science, Gatton, Australia

We demonstrate that intra-tumoural injection of Complete Freund's Adjuvant can result in a potent anti-tumour immune response. In order to test the efficacy of this treatment, we have initiated pre-clinical trials in three species: mice, dogs and horses. Efficacy has been demonstrated in a range of solid tumours, including mastocytoma, mammary tumours and melanoma. Complete tumour regressions have been observed in all three species. Evidence of systemic immune responses (regression of non-injected metastases) have also been observed. We characterise the immune cells infiltrating mouse tumours after treatment. Finally we provide case reports on the treatment of four human cancer patients; suffering from lung cancer, metastatic osteosarcoma, or breast cancer, with one patient having a partial response to treatment with intra-tumoural Complete Freund's Adjuvant.

Taken together, our data demonstrate that our treatment has major anti-tumour effects in a proportion of treated animals, and is safe for use in human cancer patients. Further trials in human cancer patients are therefore strongly warranted. Our novel treatment provides a simple and inexpensive cancer immunotherapy, applicable to a wide range of solid tumours, and of potential benefit to cancer patients around the world, including patients from developing countries.

Preclinical efficacy of Periostin short interfering RNA in pulmonary fibrosis

Hinne, J.¹, D'Alessandro-Gabazza, C.N.¹, Toda, M.¹, Yasuma, T.², Kentaro, F.³, Nishihama, K.², Kobayashi, T.³, Etsuko, H.¹, Gabazza, E.¹, Yazunori, Y.⁴

¹Mie University School of Medicine, Department of Immunology, Tsu, Japan, ²Mie University School of Medicine, Department of Diabetes and Metabolism, Tsu, Japan, ³Mie University School of Medicine, Department Pulmonary and Critical Care Medicine, Tsu, Japan, ⁴Aqua Company, Tsu, Japan

Idiopathic Pulmonary Fibrosis (IPF) is a fatal disease with a mortality rate of 3 years after diagnosis. While pathogenetic mechanisms are incompletely understood, the currently accepted paradigm proposes that injury to the alveolar epithelium is followed by a burst of pro-inflammatory and fibroproliferative mediators that invoke responses associated with normal tissue repair. Recent research in this area has focused on treatment regimen which seems to improve lung function but none has so far been found to increase survival.

Periostin which is a recently characterized matricellular protein belonging to the fasciclin 1 family has been shown to be elevated in IPF patients and in bleomycin (BLM) induced lung fibrosis.

In this study we evaluated the effect of periostin siRNA on BLM-induced lung fibrosis.

The results indicate that periostin is significantly increased on day 3 after BLM treatment and reduced on days 7 and 14 but elevated on day 21. There was increased infiltration of inflammatory cells in the scrambled siRNA treated group as compared to periostin siRNA treated group. The levels of periostin, TGF-beta 1 and collagen were significantly reduced in periostin siRNA treated group as compared to scrambled siRNA treated group. Fibrosis was also significantly reduced as measured by the Ashcroft score. The overall survival was also improved in the periostin siRNA treated group as compared to the control groups. These results show that blockade of periostin with siRNA may be an effective treatment option for the management of pulmonary fibrosis.

The involvement of IL-17A and IL-17F in chronic pulmonary mycobacterial infection

Umemura, M.¹, Fukui, M.¹, Tamura, T.², Nakae, S.³, Iwakura, Y.⁴, Matsuzaki, G.¹

¹Tropical Biosphere Research Center, University of the Ryukyus, Okinawa, Japan, ²Leprosy Research Center, National Institute of Infectious Diseases, Tokyo, Japan, ³Institute of Medical Science, University of Tokyo, Tokyo, Japan, ⁴Research Institute for Biomedical Science, Tokyo University of Science, Chiba, Japan

The interleukin (IL)-17 cytokine family is comprised of six members, namely IL-17A to IL-17F. Recent studies using cytokine- and receptor-deficient mice showed that IL-17A and IL-17F were required for responses to extracellular bacterium *K. pneumoniae* in the lungs and *C. rodentium* in the colon, respectively. However, the involvement of IL-17A and IL-17F in protective immunity was well not clearly demonstrated in mycobacterial infected lung. In this study, we analyzed role of IL-17A and IL-17F in host defense against chronically infected *M. tuberculosis* using IL-17A and IL-17F KO mice. The IL-17A KO mice showed significantly decreased survival rates compared with the wild-type (WT) mice during 250-day observation period. In

contrast, survival rate of the IL-17F KO mice were similar to that of the WT mice. Bacterial burdens of various organs of the IL-17F KO mice on the day 250 were nearly the same as that in the WT mice. In the infected lungs, the IL-17A KO mice produced less IFN- γ , TNF and IL-6 in comparison to those from the WT mice, while cytokine production of the IL-17F KO mice were similar to that of the WT mice. This result indicated that the generation of Th1 cells was impaired in the IL-17A KO mice but not in the IL-17F KO mice infected with *M. tuberculosis*. These data strongly support the notion that the lack of IL-17F neither suppress nor enhances protective immunity in the lung after mycobacterial infection.

Expression of catalytically inactive Lyn tyrosine kinase limits inflammation and autoimmune disease

Maxwell, M.¹, Kong, A.¹, Tsantikos, E.¹, Tarlinton, D.^{1,2}, Hibbs, M.¹

¹Monash University, Dept. of Immunology and Pathology, Melbourne, Australia, ²Walter & Eliza Hall Institute, Melbourne, Australia

Systemic lupus erythematosus (SLE) is characterized by circulating IgG antibodies reactive with nuclear antigens, immune complex deposition in tissues including skin, brain and kidneys with commensurate activation of the complement cascade and myeloid cell activation. Activation of the innate arm of the immune system results in the production of pro-inflammatory cytokines, exacerbating inflammation and the adaptive immune response. The combination of inflammation and antibody causes significant and widespread tissue damage. Lyn-deficient mice with their hyper-responsive B lymphocytes and myeloid cells develop autoimmunity with parallels to SLE, and require both inflammatory factors and B cells to switch the disease to a pathogenic state. To dissect whether the kinase activity of Lyn is critical for maintenance of self-tolerance, we have generated mice carrying a kinase-dead mutation of Lyn. We show that Lyn^{KD/KD} mice share many of the B cell characteristics of Lyn-deficient mice including B cell developmental defects, hyper-responsiveness and enhanced signaling, and they are autoreactive. Unlike older Lyn-deficient animals, aged Lyn^{KD/KD} mice do not develop splenomegaly or an expanded myeloid compartment, yet Lyn^{KD/KD} myeloid cells are intrinsically hyper-responsive to growth factor. Dampened inflammation is not sufficient to drive T cell activation in Lyn^{KD/KD} mice, and while the mice have circulating self-reactive IgG, the levels do not escalate with age and consequently, they have mild renal disease and markedly enhanced survival. These data demonstrate that expression of an inactive form of Lyn is sufficient to restrain development of autoimmune disease through effects on the inflammatory component of the disease.

In vitro generation of tolerogenic DCs using an inhibitor for glycogen synthase kinase 3 beta

Ha, S.-J., Yoo, S.B.

Yonsei University, Department of Biochemistry, Seoul, Korea, Republic of

Although tolerogenic dendritic cells (tolDCs) has come into the spotlight as a promising treatment for autoimmune disease and in transplantation, the optimal protocol for generating tolDCs and their clear identity have not been absolutely defined. Here, using bone marrow-derived DCs (BMDCs), we investigated the effect of various reagents such as cytokines, organic molecules, small chemicals,

and pathogen-derived factors on DCs to find out which reagent can efficiently induce tolDCs. BMDCs were stimulated with those factors and the expressions of co-stimulatory, co-inhibitory, and MHC molecules on BMDCs were observed. In addition, we investigated cytokine profiles of BMDCs by ELISA to further estimate tolerogenic potential of BMDCs. Notably, treatment of inflammatory cytokine, IFN- γ , up-regulated the expression of inhibitory molecules (PD-L1 and PD-L2) on DCs. Another interesting finding was that treatment with one of the inhibitors for glycogen synthase kinase 3 beta (GSK-3 β) slightly up-regulated PD-L1.

Moreover, upon stimulation with lipopolysaccharide (LPS), the GSK-3 β inhibitor-treated DCs showed significantly increased IL-10 production. Finally, we performed functional assay using OT-I and OT-II cells to test the abilities for antigen uptake, processing and T cell priming of induced tolDCs. Through our investigation, we could take another step for searching tolerogenic agent and establishing protocols for generating tolDCs, which can be further applied into clinical trials where immune suppression is needed.

Histamine contributes to the resistance against tick-blood-feeding during the re-infestation

Ohta, T.¹, Yoshikawa, S.¹, Ishiwata, K.², Yamaji, K.², Okayama, N.¹, Yamanishi, Y.¹, Kanuka, H.², Watanabe, N.³, Ohtsu, H.⁴, Karasuyama, H.¹

¹Tokyo Medical and Dental University, Department of Immune Regulation, Tokyo, Japan, ²Jikei University School of Medicine, Department of Tropical Medicine, Tokyo, Japan, ³Jikei University School of Medicine, Department of Allergology, Tokyo, Japan, ⁴Tohoku University, Department of Engineering, Miyagi, Japan

Ticks transmit a variety of pathogens from reservoir host to humans and animals during the tick-blood-feeding, leading to tick-borne infections such as Lyme disease. Some studies have reported that tick-infested animals acquired resistance against tick-blood-feeding and furthermore prevent transmission of pathogens from tick to animals. We previously reported that basophils infiltrate around the tick-bite-sites during the re-infestation but not primary infestation, and basophil-depletion before re-infestation can abolish the resistance against tick-blood-feeding. Moreover, adoptive transfer experiments elucidated the importance of Ig-Fc receptors on basophils for the resistance against tick-blood-feeding. These findings suggested that basophil-activation through the Ig-Fc receptors are important for the resistance against tick-blood-feeding during the re-infestation. Basophils are well known for their release inflammatory molecules such as histamine and protease from their secretory granules in response to IgE/antigen stimulation. We examined whether histamine is involved in resistance against tick-blood-feeding using histamine (*HDC*)-deficient mice. We confirmed that immune reactions such as the levels of tick antigen reactive IgE, basophil-infiltration and their-activation in tick-feeding-site were comparable between wild type and *HDC*-deficient mice during the re-infestation. However, *HDC*-deficient mice failed to develop resistance against tick-blood-feeding. These findings implied that histamine released by basophils upon IgE plus tick antigens is important for the resistance to tick-blood-feeding during the re-infestation.

Blood gene expression profiles from distinct outcomes of infection with *Leishmania infantum*

Gardinassi, L.G.¹, Rocha Garcia, G.¹, Costa Silva, V.², Costa, C.H.N.², de Miranda Santos, I.K.F.¹

¹University of Sao Paulo - Ribeirao Preto Medical School, Biochemistry and Immunology, Ribeirao Preto, Brazil, ²Federal University of Piaui / Natan Portella Tropical Diseases Institute, Teresina, Brazil

Visceral leishmaniasis (VL) can be lethal if left untreated; however, the majority of human infections with the etiological agent *Leishmania infantum*, are asymptomatic. Using Bead Chip microarray technology (Illumina), we investigated the profiles of gene expression from blood of VL patients, patients that received therapy, asymptomatic individuals and controls. Our data demonstrate that each group included in the study presents a unique transcriptional signature. By employing computational analysis based on diverse functional methods as differential gene expression, gene set enrichment analysis, weighted gene co-expression network analysis and immune cell deconvolution, our findings support that VL patients present transcriptional profiles associated to an activation of T lymphocytes via MHC class I. Of interest, VL patients also presented negative associations with transcriptional modules related to cell adhesion and chemotaxis, monocytes, neutrophils and B cells. Treated patients presented with a mixed transcriptional profile, sharing several features of untreated patients or healthy individuals. This group of study presented a positive regulation of cell cycle and proliferation of T cells, as well as transcriptional activity correlated with a modulation of cell adhesion and chemotaxis and the Notch signaling pathway. Asymptomatic and uninfected individuals present similar patterns of gene expression. Nevertheless, asymptomatic individuals present subtle particularities, such as a strong association with type I interferon response. We conclude that VL patients present dysfunctions on several compartments of the immune response and suggest that an efficient global regulation of the immune response is associated with protection to development of clinical symptoms.

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A multi-color Natural Killer-cell mediated cytotoxicity detection of tumor cells using fluorescence and direct cell imaging

Sundararaman, S., Roen, D., Karulin, A., Caspell, R., Lehmann, P.

Cellular Technology Ltd, Shaker Heights, United States

The most essential role of effector immune cells such as CD8+ cells and Natural Killer (NK) cells is to identify and lyse target cells. NK Cell- and Antibody Dependent (ADCC) Cell - Cytotoxicity has traditionally been assessed by the release of radioactive Chromium from target cells following lysis. These assays are laborious and require substantial quantities of patient blood to detect minor changes in cell lysis. We have previously shown that we can achieve similar results as the Chromium Release Assay by the direct imaging of fluorescently labelled target cells and visualizing the loss of fluorescence signal upon cytolytic activity. We showed that when performed in a 96-well plate, the % of target cell lysis is inversely proportional to the number of effector cells in each well. In order to further reduce the amount of cell material required and detect the effect of NK cells on different target cell lines we have now developed a multi-color

cytotoxicity detection assay. We stained three different cancer cell lines (one of which had intact MHC receptors) with three different dyes and incubated them in the same well with Effector to Target ratios that match one cell line per well. The results obtained show that we can simultaneously detect the cytolytic effect of NK cells on three different target cell types using only a third of the effector cells as previously required. Also, the data show that control target cells with MHC receptors are not susceptible to NK killing.

Four color T- and B cell ELISPOT assays for simultaneous detection of analytes

Hanson, J., Caspell, R., Sundararaman, S., Karulin, A., Lehmann, P. Cellular Technology Ltd, Shaker Heights, United States

ELISPOT assays are a key research tool for enumerating antigen-specific T and B cells in PBMC. As both T and B cells occur in major classes, immune monitoring has to be concerned with identifying these as well. So far, ELISPOT assays have been primarily done single or double color. We report the development of four color T and B cell ELISPOT assays. We show that the four color assay has the same sensitivity for detecting individual cells secreting analytes as the respective single color assays, and that the four fluorescent colors can be discerned unambiguously, without overlap. Cells secreting any of the four analytes can therefore be identified unambiguously in an automated fashion, without the need for compensation. Cells co-expressing analytes can be identified by superimposing the individual colors. Studying B cells and T cells experimentally has permitted us to verify the accuracy of co-expression measurements. Each B cell secretes only one type of Ig class/subclass. T cells, in contrast, frequently coexpress cytokines. Serial dilution experiments showed that for T cells the numbers of co-expressors linearly decreased with the numbers of cells plated. For B cells, no coexpressors were found.

A positive control for the detection of functional CD4 T cells in human PBMC - CPI protein pool

Schiller, A., Ansari, T., Li, R., Horvath, K., Sundararaman, S., Lehmann, P. Cellular Technology Ltd, Shaker Heights, United States

Testing of PBMC for immune monitoring purposes requires verification of the functionality. This is of particular concern when the PBMC have been shipped or stored for prolonged periods of time. The CEF peptide pool has become the gold standard for testing CD8 cell functionality. A positive control for establishing CD4 cell quality, that also requires intact antigen processing and presentation functionality, is so far lacking. Protein antigens from infectious/environmental organisms have been selected that are ubiquitous. Of an initial selection of 12 antigenic systems, (Varicella, Influenza, Parainfluenza, Mumps, Cytomegalovirus, Streptococcus, Mycoplasma, Lactobacillus, Neisseria, Candida, Rubella, and Measles) three were selected because they A) elicited CD4 cells exclusively, and B) elicited recall responses in the majority of human donors. These were inactivated Cytomegalo, Parainfluenza, and Influenza virions. While individually none of the three antigens triggered recall responses in all of the donors, the pool of these three antigens did. In 100 of 100 human donors (of different demographics) tested so far, the CPI (Cytomegalo, Parainfluenza, and Influenza viruses) protein

pool triggered a positive IFN- γ recall response by CD4 cells. Therefore, the CPI protein pool is suitable as a positive control for CD4 functionality.

Direct detection of T and B memory lymphocytes reveals HCMV exposure that serum antibodies fail to identify

Caspell, R.¹, Terlutter, F.¹, Nowacki, T.², Li, R.¹, Sundararaman, S.¹, Lehmann, P.¹

¹Cellular Technology Ltd, Shaker Heights, United States,

²University hospital of Münster, Munster, Germany

It is essential to identify donors who have not been infected with HCMV in order to avoid transmission to human recipients of transfusion products or of organs. In addition, since HCMV infection has been linked to the pathogenesis of various diseases, it is important to unambiguously establish who has, and who has not been infected. In the present study, we tested the reliability of seronegativity as an indicator for the lack of HCMV exposure of human subjects. Sixty six HCMV seronegative individuals have been identified and their PBMC were tested in ELISPOT assays for the presence of HCMV-specific CD4, CD8 T - and B - memory lymphocytes. Fifty seven percent of the HCMV seronegative subjects displayed CD4 and CD8 T cell memory in addition to HCMV specific memory B cells providing three independent lines of evidence for having developed immunity to HCMV. Fifteen percent of the 66 seronegative donors possessed CD4 and CD8 memory cells to HCMV, however, in the absence of memory B cells, and 16% had CD4 memory cells in isolation. Only 12% of the seronegative donors showed neither T- nor B- cell memory to HCMV qualifying as immunologically naive to the virus. The data suggest that measurements of serum antibodies frequently fail to reveal HCMV exposure of humans, which may be better identified by direct detection of HCMV-specific memory lymphocytes.

Maximizing odds for detecting a positive T cell response by ELISPOT

Roen, D., Karulin, A., Sundararaman, S., Lehmann, P. Cellular Technology Ltd, Shaker Heights, United States

It has been a matter of debate to determine the best cut offs in ELISPOT assay analysis for the unambiguous identification of a positive T cell response. To address this issue we carried out experiments on HLA-A2-0201 positive human subjects who had been infected with HCMV. However, we selected donors whose PBMC, when tested at 100,000 cells per well and challenged with the HLA-A2-0201-restricted HCMV peptide, pp65(495-503) did not display spot counts over medium background. Increasing the number of PBMC plated per well resulted in higher positive to negative count ratio, lower relative experimental error (CV), and higher power for detecting pp65-induced positive responses without causing false positive results from HCMV negative subjects. This decrease of CV and increase in the power of the test was directly proportional to the numbers PBMC plated. We tested the PBMC for pp65 reactivity in 96 replicate wells to establish the distributional properties of these low frequency ELISPOTs. The results were analyzed using diagnostic Q-Q plots and Shapiro-Wilk normality tests which showed that the spot counts in replicate wells follow Normal distribution. We showed that parametric statistics, such as Student *t*-Test can be used and provide

induced proinflammatory signaling, monovalent or divalent targeting with either IgA, IgG, IVIg or with Fab/F(ab')₂ of anti-FcR antibodies can trigger inhibitory signals towards a whole array of cellular functions such as phagocytosis, exocytosis and TLR- or cytokine-mediated responses. At the molecular level, ITAMi is defined by weak phosphorylation of distal ITAM tyrosines, transient recruitment of Syk followed by stable recruitment of the phosphatase SHP-1. This inhibitory effect renders anergic myeloid cells despite of heterologous receptor engagement. *In vivo* ITAMi induction by antibodies or Ig prevents and treats several inflammatory disorders in mice such as nephritis or arthritis. Moreover, *ex-vivo* monovalent or divalent targeting of FcR on synovial cells from untreated rheumatoid arthritis patients reversed the spontaneous ITAMa to an ITAMi anti-inflammatory configuration. Shifting the constitutive ITAMa signals observed in patients to an ITAMi signaling could thus reverse cell activation by inducing a myeloid tolerance state, providing ground for novel treatment options in inflammatory diseases.

Recurrent herpes virus infection: treatment with dendritic cells vaccines

Leplina, O., Tyrinova, T., Starostina, N., Ostanin, A., Chernykh, E.
Institute of Fundamental and Clinical Immunology, Novosibirsk, Russian Federation

Chronic recurrent infections caused by herpes simplex virus (HSV) types 1 and 2 are a serious medical and social challenge. Induction of an antigen-specific immune response using dendritic cell (DCs) vaccines, seems to be promising approach for the treatment of HSV infections. 29 patients (14-with labial and 15 - with genital herpes) have been enrolled in this study. DCs were generated in the presence of GM-CSF and IFN- α , loaded with recombinant viral proteins (HSV1gD or HSV2gD) and were utilized as 2 courses of vaccinations conducted during 9 months. Immunotherapy with DCs did not induce serious side effects and was accompanied by two-fold decrease in the relapses rate and increase in the length of remission during the 9 months of treatment. There was a further reduction in the frequency of relapses and a threefold increase in the duration of remissions during the subsequent 6-month follow up. The clinical effect during the treatment and subsequent 6 months follow up was noted in both labial and genital herpes and was associated with the induction of antigen specific proliferative response. Analysis of long-term results based on a survey of patients with followed up more than 24 months has shown that the beneficial effect of immunotherapy manifested as reduction in the relapse rate was maintained in 77.8% of the respondents. At this time, antigen specific proliferative response was maintained in 66.7% of patients. The data obtained suggests that dendritic cell vaccines may be a promising new approach to the treatment of recurrent herpes.

Elucidating the oncogene-driven regulatory T cell responses during melanoma tumorigenesis

Shabaneh, T.B., Steinberg, S.M., Zhang, P., Turk, M.J.
Geisel School of Medicine at Dartmouth, Lebanon, NH, United States

Regulatory T cells (Tregs) are critical mediators of tumor immune suppression. While Tregs are found in established tumors, little is

known about the kinetics and dynamics of Treg accumulation and the factors promoting it during oncogene-driven tumorigenesis. The present studies characterize Treg response kinetics and conversion dynamics during early tumor development in a model of autochthonous, tamoxifen-inducible *Braf*^{F600E} *Pten*^{-/-} melanoma. While microscopic skin dysplasia appeared 16 days following tumor induction, FoxP3⁺ Treg frequency and absolute numbers accumulated by day 26, coinciding with locally-invasive neoplasm development. *Braf*^{F600E} inhibition with PLX4720 prevented Treg accumulation, suggesting that oncogenic *Braf* controls this process. Following adoptive transfer of CD4⁺ T cells specific to TRP-1, antigen-specific FoxP3⁺ Tregs preferentially accumulated in tumor-induced skin and draining lymph nodes (dLNs), as compared to tumor-free counterparts. In contrast, depleting Tregs prior to transfer abrogated the TRP-1-specific Treg response, suggesting a predominant role for natural Tregs.

Furthermore, we observed an increase in chemokine (C-C motif) ligand 17 (Ccl17) and Ccl2 prior to the Treg influx to tumor-induced skin. Oncogenic *Braf* regulated expression of Ccl17 and Ccl2, both of which mediated Treg migration *in vitro*. We are currently investigating the putative role of Ccl17/2-Ccr4 chemotactic axis in Treg recruitment to sites of *Braf*-driven tumorigenesis *in vivo*.

Raf kinase inhibitor protein mediates intestinal epithelial cell apoptosis and promotes IBDs in humans and mice

Lin, W., Fasheng Su, Chunmei Ma, Xiaojian Wang
Zhejiang University, Institute of Immunology, Hangzhou, China

Objective: Raf kinase inhibitor protein (RKIP) appears to control cancer cell metastasis and its expression in colonic tissue is related to colonic cancer development. We sought to identify the roles of RKIP in maintaining homeostasis of gastrointestinal tract. **Design:** The expression of RKIP was determined by immunohistochemistry and western-blot analysis. RKIP knock-out (KO) and wild-type (WT) mice were administered dextran sulfate sodium (DSS) or 2, 4, 6-trinitrobenzenesulfonic acid (TNBS) to induce experimental colitis, and the mice were assessed based on colitis symptoms and biochemical approaches. The mechanism was analyzed using immunoprecipitation and pull-down experiments.

Results: The RKIP expression is positively correlated with the severity of inflammatory bowel disease (IBD). RKIP deficiency protects mice from dextran sulfate sodium (DSS) - or 2, 4, 6-trinitrobenzenesulfonic acid (TNBS)-induced colitis and accelerated recovery from colitis. RKIP deficiency inhibits DSS-induced infiltration of acute-phase immune cells and reduces production of proinflammatory cytokines and chemokines in colon. RKIP deficiency inhibits DSS- or TNBS-induced colonic epithelial barrier damage and intestinal epithelial cell (IEC) apoptosis. RKIP deficiency also inhibits TNF- α induced IEC apoptosis and colitis.

Mechanistically, RKIP enhances the induction of P53-upregulated modulator of apoptosis (PUMA) by interacting with TGF- β -activated kinase 1 (TAK1) and promoting TAK1-mediated NF- κ B activation. This is supported by the observation that TAK1 activation is positively correlated with the expression of RKIP in human clinical samples and the development of IBD. **Conclusions:** RKIP contributes to colitis development by promoting inflammation and mediating IEC apoptosis and might represent a therapeutic target of IBD.

LBP3 enhanced antitumor immune responses partly via releasing the brakes of immunosuppression in the tumor microenvironment of H22 tumor-bearing mice

Deng, X.L.^{1,2}, Hu, M.H.¹, Wang, Y.Y.¹, Ma, F.L.¹, Luo, X.², Zhou, L.²

¹*Infinitus Chinese Herbal Immunity Research Centre, Guangzhou, China,*

²*Guangzhou University of Chinese Medicine, Guangzhou, China*

Recent studies have shown that targeting of immunosuppressive cells and immune checkpoints in the tumor microenvironment is a promising approach to enhance the efficacy for cancer immunotherapy. Polysaccharide from *Lycium barbarum* LBP inhibits tumor growth in vivo at least partly via improving antitumor immunity. However, the mechanisms of how LBP mounts an effective antitumor immune response are not well understood. In the present study, the modulation of a polysaccharide fraction from LBP (LBP3) on the immune system in H22 tumor-bearing mice was investigated. The results showed that LBP3 could effectively inhibit the solid tumor growth of H22 tumor-bearing mice. Thymus indexes, the numbers/percentages of T cells in peripheral blood and tumor tissues, killing activities of natural killer (NK) cells, cytotoxic T lymphocytes (CTL) and peritoneal macrophages against H22 cells were also improved in three LBP3 treated groups. Besides, LBP3 treatment could decrease the secretion of TGF- β , IL-6, IL-2 and IL-10 levels in serum. The percentages of regulatory T cells (Tregs) in tumor-draining lymph nodes (TDLN) and tumor tissues were also significantly decreases in the all LBP3 treated groups. Furthermore, LBP3 treatment could effectively down regulate the PD-1 expression on T cells in TDLN and tumor tissues. Taken together, these findings indicate that LBP3 enhanced antitumor immune responses partly via releasing the brakes of immunosuppression in the tumor microenvironment of H22 tumor-bearing mice.

All the authors contributed equally to this manuscript).

Interaction between breast cancer cells and microglia in the microenvironment of brain metastasis

Foo, S.L.^{1,2}, Lim, H.K.L.^{1,2}

¹*NUS Graduate School for Integrative Sciences and Engineering, National University of Singapore, Singapore, Singapore,* ²*Inflammation and Cancer Laboratory, Immunology Programme, Yong Loo Lin School of Medicine, National University of Singapore, Physiology, Singapore, Singapore*

The occurrence of breast cancer metastases is preferential to certain organs, and one of it being the brain. As the brain microenvironment is continuously monitored by microglia- a major component of the brain immune system, invasion of metastatic breast cancer cells to the brain should stimulate an immune response in the microglial cells. In this study, we use an in vitro model comprising of microglia and metastatic breast cancer cell lines to investigate microglial reactions to metastatic breast cancer cells, particularly in modulating tumour cell proliferation, cell invasion, immune suppression, and microenvironment modification. In contrast to macrophages, co-cultures and insert-cultures of microglia and breast cancer cells demonstrated that microglia are activated by tumor cell-oriented factors, resulting in priming or sensitization of microglia to adopt an M1 (pro-inflammatory), rather than M2 (anti-inflammatory) phenotype. It is also observed that there are chemoattractant factors secreted by breast cancer cells that induced a significant chemotactic

activity for microglial cells in vitro, and the recruited microglia have further been found to reduce cancer cells growth. The findings of the metastasis-antagonizing role of microglia suggest that there are possibly differences between tumour associated peripheral macrophages and brain intrinsic microglia in terms of their responses towards breast cancer cells and that differences may also exist in microglial reactions toward primary breast cancer cells as compared to cancer cells that have metastasized to the brain. Therefore, it would be of interest to investigate the functional aspects of microglia in invasion, dormancy, and relapse of brain metastatic breast cancer.

Correlation between the metabolome and binder of ST2 receptor in chronic periodontics in elderly

Borges, A.¹, Carvalho, M.², Venturini, G.³, Vieira, C.⁴, Paulino, T.⁵, Botelho

Miguel, C.⁶, Oliveira, C.⁶, Pereira, A.³, Binivignat, O.⁷, Rodrigues, W.^{4,7,8}

¹*Faculdade Mineirense - Fama, Odontology, Mineiros-GO, Brazil,*

²*Faculdade Mineirense - FAMA, Odontology, Mineiros, Brazil,* ³*Heart*

Institute (InCor)/Univ of Sao Paulo Med Sch, São Paulo, Brazil, ⁴*Federal*

University of Uberlandia, Uberlandia, Brazil, ⁵*Federal University of*

Goiás Triangulo Mineiro - CEFORES, Uberaba, Brazil, ⁶*Federal University*

of Triangulo Mineiro, Uberaba, Brazil, ⁷*Faculdade Mineirense - Fama,*

Mineiros-GO, Brazil, ⁸*Federal University of Goiás Triangulo Mineiro,*

Uberaba, Brazil

Introduction: High relationship of senescence with the emergence and/or compromise of disease in oral cavity is a triggering factor of disease in this age group including chronic periodontal disease (CPD). Some markers have been associated with disease prognosis, as well as systemic diseases, such as ST2 binder.

Objectives: Evaluate the correlation between metabolites and ST2 receptor binder in crevicular fluid in the elderly. **Methods:** All procedures were approved by the Ethics Committee in Research of the Federal University of Triangulo Mineiro, under number: 017430/2014. Twenty individuals were selected after applying the inclusion and exclusion criteria. They were divided into 2 groups, CPD (N = 10 - Clinical evaluation + probing depth higher or equal to 3 mm, and at least presence of marginal bleeding at a site) without disease - Control (n = 10). To the assessment of metabolites was performed the metabolome (triplicate - GC/MS Agilent -7890B GC; 5977A MS). The ELISA was performed for the detection and quantification of IL-33 (R&D Systems). The Prism software was used for statistical evaluation.

Results: Were identified 969 metabolites, of which 64 were selected to analyze. After analysis 5 metabolic (2,3 dihydroxypropyl icosanoate, glycerol, serine, 5-aminovaleric acid e putrescine) obtained a ratio CPD/Control greater than 2. The elevation was accompanied of increase of the ST2-binder, where was found a positive correlation.

Conclusion/discussion: The data point to a relationship of metabolic activity inducible IL-33/ST2 in CPD in the elderly.

T-bet expressing B cells are required for the development of autoimmunity

Kira Rubtsova^{1,2}, Anatoly V. Rubtsov^{1,2}, John W. Kappler^{1,2,3,5} and Philippa Marrack^{1,2,4,5}

¹ Howard Hughes Medical Institute, Denver, CO, 80206.

² Department of Immunology, National Jewish Health and University of Colorado Health Sciences Center, Denver, CO 80206, USA.

⁴ Department of Pharmacology, University of Colorado School of Medicine, Aurora, CO 80045, USA.

⁵ Department of Medicine, University of Colorado School of Medicine, Aurora, CO 80045, USA.

⁶ Department of Biochemistry and Molecular Genetics, University of Colorado Health Sciences Center, Aurora, CO 80045, USA.

Autoimmune diseases are common, affecting about 8% of the population of the USA. Since there is no cure for autoimmunity, it is extremely important to study the mechanisms that trigger these diseases. The majority of autoimmune diseases affect predominantly females more than males, indicating a strong gender bias.

We have recently identified a subset of B cells that accumulates in aged female mice and can be characterized by the expression of CD11c and the transcription factor, T-bet. A related subset is found in autoimmune mice, appearing at the onset of the disease. T-bet⁺ B cells obtained from autoimmune mice produce high levels of auto-antibodies upon stimulation. Expression of T-bet in B cells is necessary and sufficient for the appearance of these cells.

These data led us to hypothesize that T-bet expressing B cells are major precursors of autoantibody producing B cells during the autoimmune response. Therefore ablation of this subset should lead to the ablation of or significant delay in the onset of autoimmunity. In this study we explored the role of T-bet expressing B cells in the appearance of autoantibodies in SLE1,2,3 mice, a model of lupus. We have generated mice that lack

T-bet expression only in B cells by crossing (SLE1,2,3 x T-bet^{fllox/fllox} x CD19^{CRE}) animals. We followed the appearance of autoantibodies in these mice, comparing them to (SLE1,2,3 x T-bet^{fllox/fllox}) littermate controls. Our data indicate that kidney function and survival is significantly improved in (SLE1,2,3 x T-bet^{fllox/fllox} x CD19^{CRE}) mice. At the same time the titers of autoantibodies were dramatically decreased in the (SLE1,2,3 x T-bet^{fllox/fllox} x CD19^{CRE}) mice when compared to littermate controls. Analysis of the T and B cell compartments revealed a reduction of germinal center B cells, pre-plasmablasts, activated/memory T cells and IFN γ producing T cells. These data indicate that T-bet expression in B cells plays an important role during the onset of lupus-like

autoimmunity. Moreover, T-bet expression in B cells was not required for the development of germinal centers and production of antibodies in response to vaccination, suggesting that T-bet in B cells can serve as a novel target for the treatment of autoimmunity without dramatic impact on the other aspects of immune system.

In conclusion our data suggest that T-bet expression in B cells is required for the generation of autoimmune responses, appearance of pathogenic autoantibodies and destruction of kidney function. Therefore conditional deletion of T-bet from B cells leads to the overall improvement in autoimmune-prone mice.

A high throughput method to characterize monoclonal antibodies and select native antigen specific IgG hybridoma cells

Haolin Liu¹, Janice White¹, Frances Crawford^{1,2}, Gongyi Zhang¹, Philippa Marrack^{1,2} and John W. Kappler^{1,2}

¹ Department of Biomedical Research, National Jewish Health, Denver, CO 80206 and

² Howard Hughes Medical Institute

B cell hybridomas are an important source of monoclonal antibodies. However, the traditional method of using antigen-coated ELISA plate to detect antibody secretion and using limiting dilution to select hybridoma cells is time and labor consuming. Moreover, antigen coated ELISA can't distinguish antibodies against the native and denatured antigen. To solve these problems, we developed a high throughput method to characterize mouse IgG antibodies using Surface Plasmon Resonance (SPR) technology. This assay rapidly determines their sub-isotypes, whether they bind native antigen and their approximate affinities for the antigen with only microliters of hybridoma cell culture supernatant. It could be used to find out pairs of antibodies that can be used as coating and detecting antibodies for commercial ELISA kit and blocking antibodies of virus entry into host. Meanwhile, we found that mouse hybridomas secreting IgG antibodies also have membrane form IgG expression. Based on this surface IgG, we isolated rare γ 2a isotype switched variants from a γ 2b antibody secreting hybridoma cell line. We also used fluorescent antigen to single cell sort native antigen binding hybridoma cells from bulk fusion mixture thus saving time and labor.

