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Poster Abstracts

BSI Congress 2013

Antigen Presentation of MHC Class I and MHC Class I-Like Molecules

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Immunization with a conserved rhinovirus capsid protein generates cross-serotype protective immune responses

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Human rhinovirus (RV) infections are the principle cause of common colds, precipitating asthma and chronic obstructive pulmonary disease exacerbations. There is no vaccine for RV which is largely due to the existence of ~150 serotypes/strains. We hypothesised that highly conserved regions of the RV polyprotein, when used as an immunogen, would generate broadly cross-reactive protective immunity to RV.

We used a bioinformatic approach to define highly conserved areas of the RV proteome, produced recombinant proteins and tested their usefulness as candidate immunogens for a broadly cross-reactive vaccine using a mouse infection model. C57/BL6 mice were immunised subcutaneously with the RV immunogen prior to intranasal challenge with RV. At specific time points following live RV challenge, serum and bronchoalveolar lavage antibody responses were determined by western blot, ELISA and *in vitro* neutralisation assay. RV load in the lungs was determined by quantitative RT-PCR.

Regions of the VP0 (VP4+VP2) capsid protein were identified as having high homology across RVs. Immunization with a recombinant VP0 combined with a Th1 promoting adjuvant induced systemic, antigen specific, cross-serotype, humoral and cellular immune responses. Similar cross-reactive responses were observed in the lungs of immunized mice after infection with heterologous RV strains. Immunization enhanced the generation of heterosubtypic neutralizing antibodies and caused more rapid virus clearance.

Conserved domains of the RV capsid are immunogenic in mice and induce cross-reactive immune responses that neutralise RV *in vitro* and are protective *in vivo*. This approach has identified candidates for the continued development of a broadly reactive subunit RV vaccine.

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Increased cytokine milieu, IgA, intraepithelial lymphocytes and CD103⁺ mature DC in the human right colon compared with its left paired counterpart

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Background: Human intestinal dendritic cells (DC), the most potent antigen presenting cells, control the type and location of immune responses. CD103 identifies a subset of regulatory DC that maintains immune homeostasis in the gut. Immune compartmentalization is not usually considered in intestinal immune studies. Since human left and right colon have different embryological origins, physiology and gene expression profiles we propose that the immune system, including DC properties, is compartmentalized though its length.

Material and methods: Paired colonic biopsies from ascending and descending colon were obtained from healthy human controls. Cytokine milieu was assessed in culture supernatants and intraepithelial (IEL) and lamina propria (LP) cells characterized by flow cytometry and electron microscopy. Paired statistics were applied in at least eight independent comparisons.

Results: The cytokine milieu in the right colon had higher levels of leptin, Th17 related cytokines (IL-6, IL-22, IL-23, IL-27) and total IgA. There were more IEL (CD45⁺CD3⁺CD103⁺) in the right colon. Within them, there were fewer TCRγδ⁺ and more TCRαβ⁺CD8⁺ in the right colon. Electron microscopy revealed higher densities of plasma cells and DC in the right colon. The latter was also confirmed by flow cytometry and DC identified as HLA-DR⁺CD3⁺CD14⁺CD16⁺CD19⁺CD34⁺. LP DC from the right colon had decreased expression of innate immunity receptors (TLR2/4), gut homing markers (CCR9/β7) and were more mature (increased CD40, CD80, CD86 and decreased dextran-phagocytic capacity and ILT-3 expression) than their left counterparts. Right colonic DC had increased IL-12 and decreased IL-10 intracellular ongoing production. Right colon DC also had higher stimulatory capacity for CD4⁺CD45RA⁺ allogeneic T-cells with decreased homing (β7) and cytokine (IFNγ) imprinting capacity on responding T-cells. Cx3CR1 was virtually absent in colonic DC but expressed on macrophages. Tolerogenic CD103⁺DC were restricted to β7⁺DC and were mainly found in CD11c⁺DC. Despite CD103⁺DC did not constitute the main DC type in any compartment they were decreased in the right colon in agreement with the increased maturation and stimulatory capacity of right colon DC.

Conclusion: The human right colon is more immunologically active than its paired left counterpart including increased levels of cytokines, adipoikines, IgA, B cells, IEL and CD103⁺ mature DC. Immune compartmentalization should therefore be considered in immune studies though the length of the human gut.

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Two non-classical MHC class II DM beta chain genes in the chicken differ in their regulatory and structural features and are differentially expressed in tissues

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Although smaller, simpler and rearranged, the chicken major histocompatibility complex (MHC) contains most of the core antigen presentation genes found in the mammalian MHC. Strong associations of chicken MHC haplotypes with resistance and susceptibility to pathogens and inactivated vaccines are attributed to single dominantly expressed MHC class I (BF) and class II (BL) molecules. The single dominant class I is the result of co-evolution with the closely linked antigen processing genes TAP and tapasin, but the situation for class II is less clear. To facilitate investigation of whether single dominant class II expression could result from similar co-evolution with associated antigen presentation molecules, we characterised the chicken class II DM region. We show that chickens have three DM genes in their MHC, a single alpha chain gene DMA, and, unusually, two beta chain genes DMB1 and DMB2, of which DMB2 is dominantly expressed. The chicken DM genes encode proteins with high structural and sequence homology to those described in mammals. However, the two beta chains differ substantially at the amino acid level and several features of DMB1 suggest an unusual function. We find DMB1 and DMB2 differentially expressed in tissues at the RNA and protein level, with DMB1 predominantly expressed in intestinal epithelial cells, suggestive of a role in gut immunity. In pursuing the functional properties of the DMs and their interaction with the classical class II molecules we aim to enhance our understanding of chicken MHC associations with immune responses and, more generally, illuminate mechanisms underlying class II evolution.

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Allele-independent protein turnover enables codominant expression of HLA class Ia alleles in human APCs

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Background and aims: MHC class I (MHCI) heavy chains (HC) are expressed from three loci in the MHC (HLA-A, -B, and -C in humans), whose extensive polymorphism maps to the antigen-binding groove, diversifying the bound peptide repertoire. Maternally and paternally derived MHCI alleles are codominantly expressed, but how this is achieved at the protein level is poorly understood. Here, we have examined the effect of polymorphism on the turnover rates of MHCI molecules.

Methods: Human antigen-presenting cell (APC) lines with functional MHCI peptide loading pathways (EBV-B cells and the myeloid cell line, KG-1), as well as monocyte-derived DCs (MoDCs), were labeled biosynthetically with heavy water ($^2\text{H}_2\text{O}$). Folded MHCI molecules were immunoprecipitated with the W6/32 mAb, and tryptic digests analysed by mass spectrometry. MHCI-derived peptides were assigned to specific alleles and isotopes, and turnover rates quantified by ^2H incorporation, after correcting for cell growth.

Results: MHCI turnover half-lives ranged from undetectable to a few hours, depending on cell type (MoDCs > KG-1 cells > EBV-B

cells), activation state (slow-down post LPS in MoDCs), donor variation, and MHCI isotype (somewhat faster turnover for HLA-C). However, in all settings, the turnover half-lives of alleles of the same isotype were similar. This included the HLA-B27 alleles associated with spondyloarthritides.

Conclusions: MHCI protein turnover rates in APCs with functional peptide loading pathways are largely allele-independent. This may be an important feature enabling the normal function and codominant expression of MHCI alleles. B27 misfolding, previously implicated in the pathogenesis of spondyloarthropathy, arises despite normal turnover kinetics of folded B27 molecules. Lastly, this is one of very few studies to date using stable isotope/mass spectrometric techniques to assess the effect of structural polymorphisms on protein turnover. In this regard, MHCI structural diversity provides a useful test bed for refining analytical techniques in kinetic proteomics.

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Peptide presentation on infected cells is required for the inflation of CMV specific CD8 T-cells

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Defined cytomegalovirus (CMV) antigens induce CD8 T-cell responses, which contract upon the clearance of lytic infection and assume a central memory (CM) phenotype (CD62L⁺CD127⁺), while others induce an inflation of CD62L⁺CD127⁻ effector memory (EM) CD8 cells, whose count remains permanently elevated. Therefore, CMV-specific EM T-cells dominate the memory compartment of seropositive hosts and present the most vigorous immune response known in clinical medicine. The mechanisms underlying this exceptionally strong immune response have not been clearly defined.

Using a murine model of infection with mouse CMV (MCMV) recombinants expressing the K(b) restricted peptide SSIEFARL at the C-term of the M45 gene, and the D(b) restricted peptide HGIRNASFI in its middle, we observed that the CD8 response to SSIEFARL was sustained and cells were EM, whereas HGIRNASFI induced an immunodominant response at day 7 post infection, but it contracted to very low levels by day 28 post infection. To test if this discrepancy was due to differences between peptide sequences, or in their localization within the M45 gene, we abrogated presentation of the HGIRNASFI peptide from its native site and inserted its copy at the 3' end of the M45 gene, thus generating the MCMV^{Cterm} recombinant. The CD8 response to HGIRNASFI differed drastically upon infection with MCMV^{Cterm} or the wild-type (wt) MCMV. The initial response was eightfold stronger when HGIRNASFI was present at the 3' end of the gene and almost 40-fold stronger by day 60 post infection. Interestingly, ~40% of HGIRNASFI-specific CD8 T-cells retained the EM phenotype in MCMV^{Cterm} infection, whereas they switched over time to the CM phenotype in mice infected with wt MCMV. Since the same peptide was expressed from the same gene, we assumed that the difference in the size and quality of the immune response had to depend on the antigenic processing and surface presentation on infected cells. Co-cultivation of MCMV infected cells with a HGIRNASFI-specific cytotoxic T-cell line (CTL) revealed that wt MCMV hardly induced any CTL response, while MCMV^{C-term} induced vigorous IFN γ secretion from the CTLs.

Therefore, our data argue that the sustained immunogenicity of an antigenic CMV peptide decisively depends on its location within a protein and its availability for processing and MHC-presentation in the infected cell. This information may bear significant relevance for the rational design of CMV-based vaccine vectors.

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Naturally occurring ERAP1 haplotypes encode functionally distinct alleles with fine substrate specificity

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Endoplasmic Reticulum Aminopeptidase (ERAP1) performs the key final step in the processing of N-terminally extended peptides for stable presentation on MHC I, influencing the magnitude and specificity of CD8⁺ T cell responses. Recently, single nucleotide polymorphisms (SNPs) within ERAP1 have been associated with the onset of a number of autoimmune diseases, as well as being a predictor of disease progression and clinical outcome in cervical carcinoma. However, it is unclear whether these SNPs act independently or in specific combinations to form haplotypes. Furthermore, the effect of individual SNP and SNP combinations on the ability to generate optimal antigenic peptides is poorly understood.

To elucidate whether ERAP1 SNPs form specific haplotype combinations, we screened the ERAP1 sequences from 20 individuals, identifying multiple combinations of SNPs that form nine distinct haplotypes within the population. Using a model peptide substrate with N-terminal extensions, we assessed trimming function of these haplotype encoded alleles, categorising trimming activity into three functional classes:

- 1 efficient,
- 2 hypoactive and
- 3 hyperactive trimmers.

Further fine mapping of the individual amino acid trimming capacity of each ERAP1 allele identifies distinct profiles that correlate with the SNPs they possess, suggesting that each ERAP1 allele may have a range of trimming activities depending on the amino acid requiring processing.

These results demonstrate the highly polymorphic nature of ERAP1 within the population, and reveals that the repertoire of peptides displayed at the cell surface depends on the combination of both MHC I and ERAP1 expressed within an individual. This therefore has important implications for predisposition and ability to combat disease and also in cancer vaccine efficacy.

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Lymph borne CD8 α ⁺ DCs are uniquely able to cross-prime CD8⁺ T cells with antigen acquired from intestinal epithelial cells

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Background and aims: Cross-presentation, the presentation of exogenous antigen on MHCI, is crucial for priming of CD8⁺ T cells and the generation of immunity to intracellular pathogens- particularly viruses. In the intestine, various populations of mononuclear phagocytes have been shown to cross-present soluble antigens *in vitro*. However, it is not clear which of these cells is responsible for transport and cross-presentation of cellular antigen *in vivo*.

Methods: To examine cross-presentation of intestinal cellular antigen, we made use of IFABP-tOVA 232-4 mice, which express ovalbumin exclusively in small intestinal epithelial cells (IECs). In order to test which migrating antigen-presenting cells were capable of

cross-presentation in this system we isolated dendritic cells (DCs) from the mesenteric lymph nodes (MLNs) and intestinal lymph of 232-4 mice. DC subsets were purified by FACS and cocultured with ovalbumin-specific OT-I CD8⁺ T cells. In order to assess the ability of lymph DCs (LDCs) to induce proliferation of CD8⁺ T cells *in vivo*, we developed a surgical technique, which allows sorted LDC subsets to be injected into the subcapsular sinus of the MLN of recipient mice that had also received OT-I cells.

Results: Among LDCs only the CD103⁺ CD11b⁻ CD8 α ⁺ subset of DCs were able to cross-present IEC-expressed ovalbumin to OT-I CD8⁺ T cells *in vitro*. Similarly, in the MLN, cross-presentation of IEC ovalbumin was limited to the CD11c⁺ MHCII^{hi} CD8 α ⁺ migrating DCs, but absent from all other DC populations, including the resident CD11c⁺ MHCII^{int} CD8 α ^{hi} DCs. Crucially, subcapsular MLN transfer of sorted CD8 α ⁺ LDCs but not other LDC subsets was sufficient to induce proliferation of ovalbumin specific CD8⁺ T cells *in vivo*. Finally, CD8 α ⁺ LDCs from 232-4 mice dosed with R848, a TLR7 agonist and analogue of viral infection, were uniquely able to prime the production of IFN- γ from dividing CD8⁺ T cells.

Conclusion: Migrating CD8 α ⁺ intestinal DCs are indispensable for cross-presentation of cellular antigen and, in conditions of inflammation, the induction of effector CD8⁺ T cell differentiation. They may represent an important target for the development of therapies for viral infection or tumours.

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Towards rational iNKT based immunotherapies in human autoimmune disease

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Background: Invariant Natural Killer T cells (iNKT) are key players of immunological tolerance and restoring the iNKT repertoire in spontaneous and induced mouse models prevents autoimmune inflammation. However, translation to human clinical trials has been disappointing, indicating the need for a better understanding of the human iNKT repertoire. Using novel molecular tools, we have recently described a new subset classification based on the iNKT cells' clonally distributed T-cell receptor (TCR) binding strength (affinity) to their restriction element, i.e. the non-polymorphic lipid-binding protein CD1d. Here we have compared the iNKT repertoire in early Rheumatoid Arthritis (RA) with age-/gender-matched healthy controls.

Methods: Using a series of novel molecular assays we conducted a cross-sectional cohort study on the quality of the iNKT repertoire and CD1d antigen presentation function of monocytes in 100 early RA patients versus 54 age and gender matched healthy subjects.

Results: Similar to previous published reports, iNKT cells in RA were significantly ($P < 0.01$) reduced in peripheral blood *ex vivo*. Independent of iNKT frequency, analysis of the clonal iNKT repertoire revealed a significant qualitative shift that was based on iNKT receptor affinity for CD1d. Furthermore, iNKT clones in RA secreted more Th1 and less Th2 cytokines in response to CD1d, independently of CD1d antigen presentation.

Conclusion: The observed changes in the quality of the iNKT repertoire that are evident already in the early stages of RA indicate that iNKT cell subsets defined by different TCR affinities exert different immunological functions during autoimmune inflammation. These repertoire changes have to be taken into consideration when designing future iNKT based therapies in human autoimmune disease.

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Qualitative difference in the iNKT repertoire of people with recent onset type 1 diabetes mellitus**A. S. Tocheva, S. Mansour, R. I. G. Holt & S. Gadola***Southampton General Hospital, University of Southampton, Southampton, UK*

Background and aims: iNKT cells are a highly conserved class of potent regulatory T-cells that recognise lipid antigens in the context of CD1d. iNKT defects are strongly associated with autoimmune inflammation in various mouse models, including type 1 diabetes in NOD mice, and restoration of a functional iNKT repertoire effectively prevents autoimmunity in NOD and other autoimmune prone mouse strains. In humans, the iNKT repertoire is heterogeneous with regard to the T-cell receptor (TCR) affinity of individual clones for CD1d, which translates into significant functional differences between 'high-' and 'low-affinity' TCR-bearing iNKT clones.

Here we aimed to compare the iNKT repertoire in people with early stage type 1 Diabetes Mellitus (T1DM) and age-matched

healthy controls, in relation to the clonal expression of 'high-' and 'low-affinity' TCRs.

Methods: The iNKT repertoire was examined in 10 people with early (≤ 3 years) T1DM and 10 age- and gender-matched healthy controls. We employed different lipid-antigen loaded recombinant CD1d-tetramers to compare the composition of the iNKT repertoire in people with T1DM and healthy controls. In addition, we compared the cytokine release from iNKT clones with 'high-' and 'low-affinity' TCR between the two groups.

Results: The proportion of high-affinity iNKT clones is significantly reduced in people with T1DM compared to healthy age-matched controls ($P = 0.0009$). Furthermore, the high-affinity iNKT clones from people with T1DM are less responsive to antigenic stimulation and have a selective loss of T_H2 cytokine production compared to healthy controls.

Conclusions: The iNKT repertoire in early stage T1DM is skewed towards lower-affinity iNKT cells and these changes are associated with diminished secretion of regulatory cytokines. These results should be useful to inform future iNKT cell-targeting therapies in human autoimmune diseases.

Autoimmunity

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BACH2 represses effector programmes to stabilize T_{reg}-mediated immune homeostasis

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Through their functional diversification, distinct lineages of CD4⁺ T cells play key roles in either driving or constraining immune-mediated pathology. Transcription factors are critical in the generation of cellular diversity, and negative regulators antagonistic to alternate fates often act in conjunction with positive regulators to stabilize lineage commitment. Genetic polymorphisms within a single locus encoding the transcription factor *BACH2* are associated with numerous autoimmune and allergic diseases including asthma, Crohn's disease, coeliac disease, vitiligo, multiple sclerosis and type 1 diabetes. While these associations point to a shared mechanism underlying susceptibility to diverse immune-mediated diseases, a function for Bach2 in the maintenance of immune homeostasis had not been established. We have found that Bach2 plays a broad role in maintaining immune homeostasis, by stabilizing T_{reg}-mediated immunoregulatory capacity while repressing the differentiation programmes of multiple effector lineages in CD4⁺ T cells. Bach2 was required for efficient formation of regulatory (T_{reg}) cells and consequently for suppression of lethal inflammation in a manner that was T_{reg} cell dependent. Assessment of the genome-wide function of Bach2, however, revealed that it represses genes associated with effector cell differentiation. Consequently, its absence during T_{reg} polarization resulted in inappropriate diversion to effector lineages. In addition, Bach2 constrained full effector differentiation within Th1, Th2 and Th17 cell lineages. These findings identify Bach2 as a key regulator of CD4⁺ T-cell differentiation that prevents inflammatory disease by controlling the balance between tolerance and immunity.

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Omalizumab-conditioned rush allergen immunotherapy: method and results

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Background and aims: One of the primary obstacles to extending the benefits of allergen immunotherapy to the wider population is the time-cost involved in a large number of physician office visits during the traditional dose-escalation schedules for subcutaneous allergen immunotherapy, which often entail one or more visits per week for much of the first year.

Methods: A case series of patients treated with omalizumab for 3 months using once-monthly injections, followed by a single day of rush antigen dosing schedule successfully raised all patients to therapeutic maintenance dose delivery of antigen, and enabled them to transition immediately to a once-monthly maintenance allergen injection dosing schedule.

Results: The details of the patient responses during the rush day of therapy will be presented, along with details of the antigens used, and therapeutic outcomes. This approach has been utilized for both inhalant allergy of the respiratory tract as well as for Hymenoptera venom-induced anaphylaxis immunotherapy. No serious treatment-induced allergic reactions have been encountered.

Conclusions: Using monoclonal anti-IgE pre-treatment for rush immunotherapy makes it possible for patients to start allergen immunotherapy any time of year, enjoy immediate improvement in allergic symptoms, and never have to make a physician office visit more often than once per month. This makes subcutaneous allergen immunotherapy much more feasible for a wider spectrum of the allergic population, and is safe and effective.

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Autoimmune paraneoplastic choreoathetosis and limbic encephalitis in patient with small cell lung cancer; case report

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Background: Paraneoplastic neurologic syndrome (PNS) is a very uncommon presentation of cancer, occurring in <1/10,000 patients diagnosed with a malignancy. Anti-neural antibodies have been associated with PNS, suggesting that this condition may reflect immune mechanisms.

Methods: We report a rare case of paraneoplastic neurologic syndrome associated with small cell lung cancer presented with choreoathetosis, limbic encephalitis, and ataxia. A 75-year old patient presented with unexplained behavioural and neurological symptoms for 8 months, on the basis of the clinical history, examination and neuroimaging, paraneoplastic syndrome was considered a likely diagnosis and cancer was suspected and appropriate investigations were initiated.

Results: CT chest and histopathology confirmed diagnosis of small cell lung cancer and serum samples were sent to look for onco-neuronal antibodies. Both Anti-Hu and Anti-CV2/CRMP-5 were detected while all other autoantibodies tested (anti-amphiphysin, anti-Yo, anti-Ri, anti-Ma1, anti-Ma2 and anti-NMDA receptor antibodies) were negative.

Conclusion: Very few patients presenting with chorea as a paraneoplastic symptom have been reported, paraneoplastic chorea can therefore be considered a 'very rare entity' since it represents a minimal fraction of a rarely occurring disorder. Although many neuronal antibodies have been associated with PNS including Hu, CV2/CRMP5, Ma1, Ma2, anti-Yo, anti-Ri, and amphiphysin, there has been no definitive proof of any specific cause-effect relationship between the presence of antibodies and the development of clinical syndrome. Further research is required to improve our understanding of the exact pathophysiology of PNS and to classify immunological subsets; this may allow more specific targeting of treatments.

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Memory B cell subsets in type 1 diabetes

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Type 1 diabetes (T1D) is an autoimmune disease where T cells damage the islet beta cells but B cells also play an important role. B cell depletion using anti-CD20 treatment delayed loss of islet beta cells in newly diagnosed patients. Furthermore, the presence of autoantibodies provides the earliest detectable marker that autoimmunity is present. Little is known about the B cells that produce the autoantibodies. The aim of our study was to examine the phenotype and function of memory B cells from T1D patients <2 years from diagnosis and healthy control subjects.

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation and B cells were stained with surface

antibodies. Using flow cytometry, we showed that patients with T1D have more naive IgD+CD27- B cells. Corresponding to this, there are proportionally fewer IgD-CD27+ memory cells in the PBMC population compared with the healthy control subjects.

The isolated PBMCs were stimulated for 7 days with polyclonal stimuli CpG oligonucleotide, pokeweed mitogen and *S. aureus* Cowan strain to activate and induce the differentiation of memory B cells. B cells were stained with surface antibodies. We observed that IgD was downregulated and the IgD-CD27++ subset increased in all subjects. The IgD+ CD27+ subset disappeared and the IgD+ CD27-subset was reduced giving rise to CD27^{int} and CD27^{hi} subsets. Preliminary analysis indicates that proportions of these subsets differ between patients and healthy control subjects.

In summary, our data suggest that there are differences in responses of memory B cell subsets in patients with T1D and healthy control subjects. These may form the basis for development of a new biomarker for monitoring progression of disease or effect of treatment.

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Neo-self antigen presentation by keratinocytes under inflammatory conditions can modulate systemic CD4 T cell responses

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Background and aims: Type and strength of an immune response can be influenced by the local environment of a given tissue. Therefore, we intended to investigate the contribution of tissue cells to self-antigen presentation.

Methods: We generated a transgenic mouse in which an immunodominant peptide of myelin basic protein (MBP) covalently linked to a MHC class II β -chain is expressed specifically in keratinocytes of hair follicles. As there is no endogenous MHC class II expression under homeostatic conditions, the transgenic β -chain construct cannot pair with the endogenous MHC class II α -chain and is therefore not present at the cell surface. However, MBP presentation should occur only upon skin inflammation, when MHC class II expression is induced.

Results: Acute skin inflammation, induced by a single application of a contact sensitizer, resulted in presentation of MBP-MHC class II complexes exclusively on keratinocytes and activation of MBP specific CD4 T cells in the skin but not in draining lymph nodes or spleen. These activated, auto-reactive CD4 T cells could induce experimental autoimmune encephalomyelitis (EAE) following administration of pertussis toxin. Furthermore, persistent MBP presentation by keratinocytes upon chronic skin inflammation reduced the severity of EAE symptoms in mice which were immunized with MBP peptide in CFA. Moreover, Dickkopf-3 was found to contribute to the down-modulation of auto-reactive CD4 T cells during chronic inflammation.

Conclusion: Neo-self antigen presentation by keratinocytes under inflammatory conditions has the potential to modulate auto-reactive CD4 T cell responses in a distant organ.

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Defective crosstalk between regulatory B cells and plasmacytoid dendritic cells contributes to the pathogenesis of systemic lupus erythematosus

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Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by heterogeneous cellular abnormalities contributing to the disease pathogenesis. Overproduction of interferon alpha (IFN α) is a hallmark of SLE, however, up to date it remains unclear why the production of this cytokine is dysregulated in patients with SLE. In this study, we investigate the outcome of interaction between plasmacytoid dendritic cells (pDCs), the primary producers of IFN α , and regulatory B cells (Bregs), the contributors to the maintenance of tolerance, in patients with SLE compared to healthy individuals.

Here, we show a novel feedback mechanism by which pDCs support IL-10 production by B cells in healthy individuals, but fail to exhibit similar functions in SLE patients. These IL-10-producing Bregs, in turn, efficiently restrain pDC activation, maturation and cytokine production in healthy individuals. We further demonstrate that this mechanism is impaired in SLE patients, resulting in hyperactivation of pDCs and subsequent overproduction of IFN α . IL-10 partially suppresses pDC activation and function, suggesting there are additional mechanisms involved. In order to dissect these other mechanisms of the pDC-Breg interaction, we combine extensive surface screening of 332 markers along with imagestream cytometry analysis of pDCs and Bregs from healthy individuals and SLE patients. Of note, we show that SLE patients treated with rituximab (B cell depletion) therapy possess newly repopulated Bregs with recovered IL-10 producing capacity and 'normalized' pDCs.

This study therefore suggests that skewing of the pDC-Breg interaction in patients with SLE contributes to the disease pathogenesis.

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Defects in cholesterol metabolism drive immune cell dysfunction in patients with systemic lupus erythematosus (SLE)

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Patients with SLE are characterized by dyslipidaemia and elevated membrane cholesterol that contribute to defective lymphocyte function. Liver X Receptor β (LXR β) is a master regulator of lipid metabolism that controls cellular cholesterol along with proliferation, differentiation and inflammatory signalling. This indicates that T cell functional capacity may be regulated by mechanisms controlling cholesterol homeostasis.

The response of CD4⁺T cells from 20 healthy donors and 50 SLE patients to LXR β stimulation was assessed by quantitative PCR, Western blotting, flow cytometry and confocal microscopy.

TCR and oxysterol (GW3965) stimulation significantly upregulated LXR β in healthy and SLE T-cells. However, TCR stimulation increased and GW3965-stimulation reduced membrane cholesterol levels. Interestingly, TCR-induced cholesterol upregulation was inhibited by co-stimulation with GW3965. In addition GW3965-treated T cells from healthy donors were unable to form stable immune synapses (IS). This was accompanied by significant internalization and redistribution of CD3 away from the IS, reduced phosphorylation of TCR-zeta and decreased proliferation. In contrast, GW3965 stimulation of SLE CD4⁺T cells had no effect on IS formation and proliferation suggesting a defect in LXR β -mediated signaling pathways.

LXR β and its target genes ncp1/2, srebp2 and LDLR, were significantly elevated in SLE CD4⁺T cells, suggesting an increase in LXR β

stimulation. Furthermore, increased LXR β and membrane cholesterol expression were induced in healthy CD4⁺T cells upon their culture with SLE serum. Hence defects in LXR β signalling in SLE may be induced by extrinsic factors.

Abnormal cholesterol metabolism in patients could therefore exacerbate the loss of immune tolerance, an integral factor in SLE pathogenesis.

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The biological role of *C5orf30* in rheumatoid arthritis pathogenesis

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Background: A recent genome wide association study identified the variant rs26232 in the first exon of an uncharacterized gene *C5orf30* as a rheumatoid arthritis (RA) susceptibility variant₁. In addition, we have found this variant to be associated with severity of radiological joint damage suggesting a role in tissue breakdown₂. To date there is no biological data on *C5orf30* and neither the gene or protein show homology to any known functional sequences. However, *C5orf30* is highly conserved in chimpanzee, dog, cow, mouse, chicken, and zebrafish (orthologs).

Methods: Real time PCR and western blotting was used to examine *C5orf30* transcript and protein levels in fibroblast-like synovial cells (FLS stimulated with TNF & hypoxia) and peripheral blood leukocytes isolated from RA patients and healthy individuals. Immunohistochemistry on synovial samples was used to determine expression of *C5orf30* including co-localisation using antibodies to macrophages (CD68), fibroblasts (5B5), T (CD3) & B (CD19) cells. To investigate *C5orf30* function knockdown was performed in rheumatoid synovial FLS *in vitro*.

Results: Expression of *C5orf30* was detected at lower levels (5.2-fold) in peripheral blood leukocytes of RA patients compared to healthy controls (117 patients versus 102 controls, $P = 0.00052$). *C5orf30* was expressed in FLS and was found to be up-regulated by hypoxia (eightfold) and down-regulated by TNF (0.5-fold). Confocal microscopy revealed *C5orf30* to be strongly expressed in both the nuclear and cytoplasmic compartment of RA synovial lining cells including macrophages and fibroblasts but was undetectable in osteoarthritis or control synovium. *C5orf30* siRNA knockdown increased significantly FLS invasion into matrigel ($n = 5$ $P = 0.01$) and migration into a scratch-wound assay ($n = 5$ $P = 0.02$) compared to FLS transfected with a non-targeting control siRNA.

Conclusions: *C5orf30* is expressed in synoviocytes but not in circulating PBMC obtained from RA patients, suggesting that *C5orf30* is expressed in a tissue-specific manner. *C5orf30* knockdown increased FLS migration into matrigel suggesting *C5orf30* is negatively regulating cellular invasion. We are currently assessing the gene signature arising from *C5orf30* siRNA knockdown in FLS and mass spectrometry experiments are currently being performed to determine the three-dimensional structure of *C5orf30* and its potential protein binding partners.

References:

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IL-17+CD8⁺ T-cells are enriched in the joints of patients with psoriatic arthritis and correlate with markers of disease activity and joint damage progression

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Introduction: Psoriatic arthritis (PsA) and rheumatoid arthritis (RA) are chronic inflammatory joint diseases associated with pain and disability. Whilst RA is strongly associated with HLA class II, PsA is HLA class I associated, suggesting a role for CD8⁺ T-cells. To date, very little is known regarding the contribution made by CD8⁺ T-cells to the cytokine environment in the PsA joint. Since IL-17 and Th17 cells are considered to be important mediators of synovial inflammation in RA and skin inflammation in psoriasis, we hypothesized that IL-17 producing CD8⁺ T-cells are increased in the PsA joint and contribute to the disease process.

Methods: Mononuclear cells from paired synovial fluid (SF) and peripheral blood (PB) from patients with PsA or RA were stimulated *ex vivo* and CD4⁺ T-cells examined by flow cytometry for cytokine expression, cytotoxic markers, gamma-delta T cell or mucosal associated invariant T (MAIT) cell frequencies. Clinical measures of arthritis activity (CRP, ESR, DAS28) and Power Doppler Ultrasound (PDUS) score of the aspirated knee were recorded.

Results: IL-17+CD3⁺ T-cells were increased in both PsA SF ($P = 0.0004$, $n = 21$) and RA SF ($P = 0.001$, $n = 14$) compared to paired PB. Within the CD3⁺ T-cell compartment, both IL-17+CD4[−] and IL-17+CD4⁺ T-cells were significantly enhanced in PsA SF versus PB ($P = 0.0003$ and $P = 0.002$ respectively), whereas in RA only IL-17+CD4⁺ T-cells were increased in SF versus PB ($P = 0.008$). IL-17+CD4⁺ T-cells were predominately CD8⁺ and CD161⁺, with only a small percentage characterised as MAIT or $\gamma\delta$ ⁺ T-cells ($n = 4$). Additionally, IL-17+CD8⁺ T-cells lacked expression of typical cytotoxic markers ($n = 4$). PsA SF IL-17+CD4[−] T-cells positively correlated with CRP ($r = 0.52$, $P = 0.01$), ESR ($r = 0.59$, $P = 0.004$) and DAS28 ($r = 0.52$, $P = 0.01$) and were increased in erosive disease ($P < 0.05$). Moreover, the frequency of IL-17+CD4[−] T-cells positively correlated with PDUS, a marker for active synovitis ($r = 0.49$, $P = 0.04$).

Conclusions: Our data show, for the first time, that the PsA joint, but not the RA joint, is enriched for IL-17+CD8⁺ T-cells, which correlate with disease activity measures and are associated with erosive status. Our data suggest that IL-17+CD8⁺ T cells are a hitherto unrecognised cell population in PsA that may represent a functionally important mechanism in the pathogenesis of this disease.

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SLE patients with atherosclerosis are characterised by a distinct iNKT cell phenotype and altered CD1d-mediated lipid antigen presentation

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Background: Patients with systemic lupus erythematosus (SLE) have an increased risk of atherosclerosis compared to the general population, irrespective of traditional risk factors. We investigated whether this was attributable to differences in the phenotype and function of invariant Natural Killer T cells (iNKT), which promote atherosclerosis by recognising and responding to lipid antigens presented by CD1d, yet have been shown to be deficient in SLE patients.

Methods: Peripheral blood was obtained from 20 healthy donors and 50 SLE patients assessed for carotid and femoral atherosclerotic plaque by ultrasound scan. Phenotyping of iNKT cells and CD1d⁺ antigen presenting cells was performed by flow cytometry, confocal microscopy and ImageStream cytometry.

Results: The frequency of iNKT cells in SLE patients with plaque was maintained at healthy levels compared to a significant deficiency in non-plaque SLE patients. Following adjustment for disease activity, iNKT cells from plaque-positive patients were predominantly CD4 positive and had a distinct phenotype characterised by increased expression of CD69 and CCR6 and elevated IL-4 and IL-10 production.

We hypothesised that this plaque-associated iNKT cell phenotype could be driven by differences in their activation by CD1d⁺ lipid-antigen presenting cells. CD1d expression was significantly decreased on B cells in all SLE patients, which was coupled with increased lipid raft expression only in B cells from plaque-negative SLE patients. In addition, increased recruitment of CD1d to lipid rafts in B cells from plaque-positive patients was observed by confocal microscopy and ImageStream cytometry.

The differential iNKT cell phenotype and CD1d distribution identified in plaque-positive versus plaque-negative patients was recapitulated in cells from healthy donors by culture with SLE patient serum. Furthermore, serum from plaque-positive patients induced an altered pattern of iNKT cell expansion and cytokine production compared to plaque-negative patients and healthy controls. This was accompanied by more stable interactions with monocytes but not B cells.

Conclusion: These findings support a differential role for iNKT cells in the immunopathogenesis of SLE in patients with and without atherosclerosis that could be maintained by dyslipidaemia. iNKT cell phenotype could represent a novel biomarker to predict atherosclerosis in SLE.

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Inflammatory arthritis around the clock

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Background: The circadian clock is an internal timing mechanism which enables organisms to tune their physiology to the 24h environment. In mammals, the central clock, located within the hypothalamic suprachiasmatic nucleus, is the master oscillator synchronising numerous peripheral clocks located in organs and cells throughout the body. Many cellular components of the immune system have been attributed with autonomous clocks, including macrophages, T cells, mast cells and natural killer cells. These endogenous oscillators imprint 24 h rhythms on immune cell functions thereby imparting circadian rhythms onto immune responses. Inflammatory diseases, such as rheumatoid arthritis and asthma, show circadian variation in their symptoms, which is likely due to clock regulation of the underlying pathology. This work investigates circadian variation in disease markers in a murine model of inflammatory arthritis.

Methods: Male DBA/1 mice were immunised with an intra-dermal injection of bovine collagen in complete Freund's adjuvant in the middle of the light (rest) phase (Zeitgeber time (ZT)6). Twenty-one days later, a booster was administered. Paw inflammation and swelling was assessed from day 18. In mice with established chronic inflammation, serial samples of blood were taken at two opposing times of day [lights on (ZT0) and lights off (ZT12)] and analysed by BioPlex assay for 23 pro-inflammatory cytokines/chemokines. At the end of the study joints were collected at 4 circadian time points, and

RNA and protein extracted for analysis of cytokine/chemokine transcription and production.

Results: Mice with chronic inflammation in the limbs showed robust circadian variation in disease markers. Levels of a number of circulating cytokines and chemokines (e.g. Interleukin 6, Interleukin 1 β and CXCL1) were significantly higher at ZT0 (start of the light phase) versus ZT12 (start of the dark phase). This correlated with a significantly increased local transcription and protein levels of these pro-inflammatory markers. In keeping, the degree of paw swelling was significantly higher during the light phase.

Conclusions: This study clearly demonstrates circadian variation in disease markers in collagen induced arthritis, and models the diurnal variation reported by patients suffering from rheumatoid arthritis. A key goal now is to map how the clock mediates these inflammatory pathways with the potential of identifying key therapeutic nodal points.

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Gut-microbiota induced IL-1 β and IL-6 control the differentiation of regulatory B cells

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Regulatory B cells (Bregs) help to restrain immune-driven diseases by inhibiting the expansion of pathogenic T cells. However, much remains to be understood regarding the molecular and cellular processes that govern the differentiation of these cells. Here we report that the constituents of the microbiome promote Breg differentiation in the spleen and mesenteric lymph nodes (MLN). Alteration imposed on the gut-microbiome by antibiotics treatment, or by changes in the sterility of housing conditions, caused a deficiency in the number and functional capacity of Bregs. Furthermore we show that IL-1 β and IL-6 are only produced following arthritis development in mice housed in a conventional unit (CNV), and that in combination these cytokines are pivotal in the induction of Bregs. Disruption of IL-1R1 or IL-6R exclusively on B cells in CNV-mice exacerbated arthritis and reduced IL-10 production by splenic B cells. Finally we report that transitional-2 marginal zone precursor cells (T2-MZPs), thought to reside exclusively in the spleen, are found in the MLN of inflamed mice and differentiated into IL-10 producing Bregs *in situ* in response to local stimuli. Taken together, these data show that the host-symbiont relationship in the gut is important in the induction and function of Bregs.

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Epigenetic control of colonic inflammation via the methyl-binding protein Mbd2

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Introduction: Methyl-CpG binding protein domain protein-2 (Mbd2) is a transcriptional co-repressor that binds to methylated DNA. Mbd2 can recruit a nucleosome remodelling complex which contains chromatin remodelling and histone deacetylase properties.

Mbd2 deficient mice are viable and fertile. However, they display a dysregulated immune phenotype with an aberrant T cell cytokine response, decreased intestinal tumorigenesis, and increased susceptibility to intestinal helminth infection. However, the impact of Mbd2 deficiency on innate immune cell function and the immunological phenotype in the GI tract is currently unknown.

Aim: To assess the impact of Mbd2 deficiency on CD11c+ Dendritic Cells (DCs) *in vitro* and in a murine model of GI tract inflammation.

Methods: *Mbd2*^{-/-} mice were produced as described previously. Single cell suspension of colon lamina propria (LP) were isolated as previously described from *Mbd2*^{-/-} mice or wildtype (WT) littermate controls. CD11c+ DCs were generated by 10 days GM-CSF culture from *Mbd2*^{-/-} or WT bone marrow as previously described and assessed by FACS. CD11c^{Cre}*Mbd2*^{Flox} and WT mice were given 2% Dextran Sulphate Sodium (DSS) in drinking water for 7–10 days and assessed for weight loss, symptom score, colon histology score and mRNA expression of selected cytokines.

Results: *Mbd2*^{-/-} mice displayed a decreased number and proportion of CD11b⁺CD11c⁺F4/80⁻ LP DCs compared to WT, in steady state. All other macrophage and DC subsets were equivalent in all parameters measured. *Mbd2*^{-/-} GM-CSF CD11c+ DCs presented significantly reduced expression of CD40, CD80 and CD86 activation markers *in vitro*. CD11c^{Cre}*Mbd2*^{Flox} mice were more resistant to DSS colitis with significantly reduced weight loss, symptom score, colon histology score and IFN- γ and IL-17 as assessed by colon mRNA.

Conclusion: These data reveal for the first time that epigenetic processes can regulate CD11c+ cell activation *in vitro* and that CD11c+ cell expression of Mbd2 is of critical importance in orchestration of DSS colitis *in vivo*. They also identify methyl-binding proteins and/or genes that they regulate as exciting new targets for therapeutic modulation of GI inflammation.

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Uptake and presentation of myelin basic protein by normal B cells is dependent on complement and complement receptor 2

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Background and aims: B-cell depletion improves certain autoimmune diseases, including multiple sclerosis. On the other hand, a role for regulatory B cells in maintenance of peripheral tolerance has been suggested. The detrimental and protective functions of B cells are likely to be linked to their antigen-presenting capacity. Our aim was to analyse the ability of normal B cells to take up myelin basic protein (MBP) and present MBP-derived peptides, and to assess the influence of complement on the process. Moreover, we aimed to characterise the phenotypic profile of the MBP-presenting B cells.

Methods: Peripheral blood mononuclear cells (PBMC) were purified from healthy donors with the HLA-DR2 haplotype. The monoclonal antibody MK16, recognising MBP85-99 in the context of HLA-DRB1*1501, was used as a probe for antigen-presentation by B cells. To inhibit complement, serum was heat inactivated or added EDTA/sodium polyanethole sulphonate. To block CR2 or CR1 PBMC were incubated with polyclonal anti-human CR2 sheep IgG or anti-human CR1 rabbit IgG. FACS analysis was performed using various surface markers to characterise antigen presenting B cells (CD19, CD14, CD5, CD24, CD27, IgM, CD80 and CD86).

Results: We observed that a subset of B cells were capable of binding MBP ($2.6 \pm 3.8\%$) and presenting MBP85-99 ($3.7 \pm 5.3\%$) in

serum-free media. In presence of serum, however, the bulk of B cells took up MBP and presented the peptide. The promoting effect of serum was abrogated by complement inactivation by heat treatment, EDTA or sodium polyanethole sulphonate. Blockade of complement receptor 2 (CR2, CD21) led to significantly reduced binding of MBP to B cells ($46.0 \pm 14.5\%$ inhibition, $P < 0.001$), while CR1 blockade had no effect. B cells presenting relatively large amounts of MBP85-99 in the presence of serum were enriched with CD27+CD5- and CD27+CD5+ cells. Although the co-stimulatory molecules CD80 and CD86 were expressed constitutively on the bulk of B cells, CD86 expression was significantly increased on B cells presenting high amounts of MBP85-99.

Conclusion: We conclude that under physiological conditions, the bulk of B cells take up MBP and present MBP-derived peptides in a complement-dependent manner involving CR2, accompanied by increased expression of CD86, the primary co-stimulatory molecule for Tregs. We propose that B cells, irrespective of their specificity, thus are active in modulation of autoimmune responses.

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Fine mapping and expression of a locus overlapping three types of inflammatory arthritis

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Background and aims: In a recent study using the Immunochip array, the *RUNX1* region was strongly associated with rheumatoid arthritis (RA $P = 5 \times 10^{-10}$), juvenile idiopathic arthritis (JIA $P = 1 \times 10^{-8}$) and psoriatic arthritis (PsA $P = 6 \times 10^{-4}$). In each disease the SNP rs9979383 confers disease protection with odds ratios (OR) of 0.8–0.9. This SNP maps 290 kb upstream of *RUNX1*, indicating that it may be involved in regulation of gene expression. *RUNX1* encodes a transcription factor (TF) which has been shown to interact with the regulatory T cell marker *FOXP3*. Unlike many loci on the Immunochip, variation in the *RUNX1* region is not well covered, therefore further fine mapping is required.

Methods: Forty-two common (MAF > 0.05) tag SNPs ($r^2 > 0.9$) from the Utah residents (CEPH) with Northern and Western European ancestry (CEU) 1000 genomes July 2010 release were selected for fine mapping. 2255 RA cases and 1877 healthy controls from the United Kingdom Rheumatoid Arthritis Genetics Group (UKRAG) were genotyped using the Sequenom iPLEX MassARRAY platform. SNP/samples which reached >90% success rate were analysed. Association testing was performed using PLINK v.1.07 and functional annotation performed using ASSIMILATOR. To determine if rs9979383 represents an expression quantitative trait locus (eQTL), Taqman genotyping and gene expression assays were performed in 75 healthy controls from the national repository healthy volunteers study. *RUNX1* expression was normalised to two endogenous controls (*ACTNB* and *GAPDH*) and linear regression performed in STATA v.11.2.

Results: In the UKRAG cohort, association with rs9979383 was replicated ($P = 0.02$, OR = 0.9, CI 95% 0.82–0.98) with an OR identical to the previous RA study. Functional annotation of rs9979383 indicates that it lies within a region of open chromatin with TF binding potential, making it an ideal candidate for gene expression studies.

In whole blood, *RUNX1* expression was not correlated with genotype at rs9979383 in healthy controls ($P = 0.92$) indicating that rs9979383 may alter expression of another gene or that this eQTL may only be present at a cell specific level.

Conclusion: The results indicate that the *RUNX1* association is localised to rs9979383 in RA but the association requires confirmation in independent JIA and PsA cohorts. Although no significant eQTL was observed in whole blood in healthy controls, cell specific whole transcriptome studies in CD4⁺ T cells are currently underway to determine whether this SNP is an eQTL. Combined with the fine mapping this will inform further investigations of the role of the *RUNX1* region in the common pathogenesis of inflammatory arthritis.

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Characterisation of 'ex-Th17' cells in the context of autoimmunity

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Th17 cells play an important pathogenic role in autoimmune diseases. However Th17 cells are unstable, particularly under inflammatory conditions where they can lose their characteristic expression of IL-17 and ROR γ t and gain expression of IFN- γ and Tbet, thus resembling Th1-like cells. We investigated the characteristics of these 'ex-Th17' cells compared to Th17 and Th1 cells in order to determine whether they retain characteristics of Th17 cells, or are equivalent to Th1 cells. It has been shown that Th17 cells have stem cell like characteristics, and exhibit enhanced survival. In addition Th17 cells have been shown to express HIF1 α which directs the cellular response to hypoxia.

CD4 T cells were stained with a panel of cell surface markers and sorted into Th1 (CD161- CXCR3+CCR4-CCR6-), Th17 (CD161+ CXCR3-CCR4+CCR6+) and ex-Th17 (CD161+, CXCR3+CCR4-CCR6-) populations. Cell populations were stimulated and cultured, either as single cell clones or cell lines and then stained for intracellular expression of HIF-1 α , β -catenin and the anti-apoptotic protein Bcl-2.

In comparison to Th1 cells, both Th17 and exTh17 cell lines and clones expressed higher levels of HIF-1 α , β -catenin and Bcl-2. Increased expression of HIF-1 α and Bcl-2 suggests that Th17 and ex-Th17 cells may be better able to survive the hypoxic conditions in inflamed tissues, while higher β -catenin expression indicates that ex-Th17 cells retain the stem cell like characteristics of Th17 cells. These data indicate that although ex-Th17 cells have lost the ability to express IL-17, they retain certain characteristics of Th17 cells which may confer pathogenicity in the context of autoimmunity.

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Lymphocytes from the inflamed joint of Juvenile Idiopathic Arthritis patients express reduced levels of CD73 and have a functional defect in adenosine production

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Background and aims: The nucleotidases CD39 and CD73 are responsible for the catabolism of pro-inflammatory ATP to AMP, and the consequent dephosphorylation of this nucleotide to regulate adenosine. CD39 protein has previously been observed to

be elevated on JIA (Juvenile Idiopathic Arthritis) SFMC (synovial fluid mononuclear cells) with a correspondent increase in ATPase activity, while CD73 has been found decreased on JIA SFMC, potentially affecting the cells ability to synthesize suppressive adenosine.

To test the hypothesis that JIA SFMC have a reduced ability to synthesize adenosine.

Methods: Samples (unsorted or sorted CD8+/CD19+) from 32 patients with JIA, 30 healthy adult and 5 age-matched controls were tested by flow cytometry and HPLC (high performance liquid chromatography). PBMC were stimulated with CpG or anti-CD3mAb and anti-CD28mAb. Data were compared by Mann-Whitney (2-tailed) and are expressed as medians; analysis was performed using Prism (v5.03, Graphpad).

Results: We have previously described that JIA SFMC lymphocytes express decreased levels of CD73 compared to JIA patients and control PBMC, with the most marked difference on CD8+ and CD19+ cells. Both total SFMC and CD8+ SFMC showed a decreased ability to breakdown AMP. It was confirmed that B cells are the only cell type to co-express CD39 and CD73 and were therefore able to generate adenosine from ATP. As B cells do not express the Adenosine deaminase-CD26 complex they cannot breakdown adenosine to inosine. Despite increased levels of CD39 on CD19+ SFMC, levels of coexpression of CD39 and CD73 were decreased on CD19+ SFMC (49%) as compared to B cells from JIA PBMC (64%, $P = 0.0007$) and PBMC from age matched (56%, $P = 0.3$) and adult controls (71%, $P < 0.0001$). To test whether the reduced CD73 expression found in the joint is related to the stimulatory phenotype of SFMC, this was modelled *in vitro* via stimulation of B cells via TLR9 or T cells via TCR stimulation: stimulation led to CD73 down-regulation and reduced AMPase activity compared to unstimulated PBMC.

Conclusions: Decreased expression levels of CD73 on lymphocyte SFMC corresponded to decreased AMPase activity, the same situation as for stimulated PBMC, suggesting that JIA SFMC are less able to synthesize adenosine as a consequence of their activated status. These data suggest a functional defect within the joint of the production of anti-inflammatory adenosine.

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Autoimmunity to the alpha 3 chain of type IV collagen is triggered by 'autoantigen complementarity'

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Background and aims: 'Autoantigen complementarity' is a theory proposing that the initiator of an autoimmune response is not necessarily the autoantigen or its molecular mimic, but may instead be a peptide that is 'antisense/complementary' to the autoantigen. We investigated whether such complementary proteins play a role in the immunopathogenesis of autoimmune glomerulonephritis.

Methods: Experimental autoimmune glomerulonephritis, a model of anti-glomerular basement membrane (GBM) disease, can be induced in Wistar Kyoto (WKY) rats by immunization with the $\alpha 3$ chain of type IV collagen. In this study, WKY rats were immunized with a complementary $\alpha 3$ peptide (c- $\alpha 3$ -Gly) comprised of amino acids that 'complement' the well characterized epitope on $\alpha 3(IV)NC1$, pCol (24–38).

Results: Within 8 weeks post-immunization, these animals developed crescentic glomerulonephritis, similar to pCol(24–38)-immu-

nized rats, while animals immunized with scrambled peptide were normal. Anti-idiotypic antibodies to epitopes from c- α 3-Gly-immunized animals were shown to be specific for α 3 protein, binding in a region containing sense pCol(24–38) sequence. Interestingly, anti-complementary α 3 antibodies were identified in sera from patients with anti-GBM disease, suggesting a role for 'autoantigen complementarity' in immunopathogenesis of the human disease.

Conclusions: This work supports the idea that autoimmune glomerulonephritis can be initiated through an immune response against a peptide that is anti-sense or complementary to the autoantigen. The implications of this discovery may be far reaching, and other autoimmune diseases could be due to responses to these once unsuspected 'complementary' antigens.

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Accelerated turnover of MHC class II molecules in NOD mice is developmentally and environmentally regulated *in vivo* and dispensable for autoimmunity

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Background and aims: The H2-A^{B7} (A^{B7}) MHC class II (MHCII) allele is required for type 1 diabetes (T1D) in NOD mice. A^{B7} not only has a unique peptide binding profile, but has been reported to exhibit biochemical defects, including accelerated protein turnover. Such defects have been proposed to impair antigen presentation, and thus self tolerance. Here, we report the first measurements of murine MHCII protein synthesis and turnover *in vivo*.

Methods: NOD mice and BALB/c controls were labeled continuously with heavy water (²H₂O), and splenic B cells and DCs were isolated. MHCII molecules were immunoprecipitated and digested with trypsin. Digests were analysed by liquid chromatography/mass spectrometry (LC/MS) to quantify the fraction of newly synthesized MHCII molecules, and thus turnover.

Results: MHCII turnover was faster in DCs than B cells, varying slightly between mouse strains. Some A^{B7} molecules exhibited accelerated turnover in B cells from young, but not older, prediabetic female NOD mice. This acceleration was not detected in a second NOD colony with a high incidence of T1D. Turnover rates of A^{B7} and A^d were indistinguishable in (NOD × BALB/c) F1 mice.

Conclusions: Accelerated MHCII turnover may occur in NOD mice, but reflects environmental and developmental regulation, rather than a structural deficit of the A^{B7} allele. Moreover, this phenotype wanes before onset of overt T1D and is dispensable for development of autoimmune diabetes. Our observations highlight the importance of *in vivo* studies in understanding the role of protein turnover in genotype/phenotype relationships and offer a novel approach for addressing this fundamental research challenge.

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The pattern of expression of chemokines receptor and apoptosis markers on peripheral blood lymphocytes of patients with rheumatoid arthritis

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Introduction: Rheumatoid arthritis (RA) is a chronic autoimmune disease with persistent inflammation of several synovial joints, which results in progressive tissue destruction of cartilage and bone.

Aim: To investigate the expression of some chemokines receptors (CCR5, CX3CR1) as inflammatory and (CCR7) as naive T cell markers together with the expression of apoptosis marker (CD95) in blood samples of patients with RA. The levels of IL-6, TNF- α and IL-17 cytokines were also measured in their plasma samples.

Methods: A total of 40 patients with RA (34 female and six male; with age range 21–68 years) were included. Twenty healthy volunteers (16 female and four male) matched for age (range 21–34 years) were served as controls. Disease activity score (DAS28, CRP-ESR) system was assessed and active disease was defined as DAS $\geq$ 3.2. According to DAS-28 25 (62.5%) were classified with active RA and inactive RA in 15 (37.5%) RA patients. The chemokines receptors expression were analyzed by flow cytometry on the cell surface of peripheral blood lymphocytes and the cytokines levels were measured by enzyme-linked immune-sorbent assay (ELISA).

Results: The plasma concentrations of IL-6, IL-17 and TNF- α were significantly higher in patients than controls. Flow cytometry analysis showed significant increases in the expression levels of CCR7 and CCR5 on CD95⁺ T-cells in both active and inactive RA groups compared to healthy control group. On the other hand, the expression of CX3CR1 on T-cells was found significantly lower in both viable and apoptotic cells regardless to the disease activity score. Additionally, positive correlations were detected between CCR7 with IL-17 and TNF- α ($r = 640$ and 524 respectively) and a negative correlation between CX3CR1 and IL-6 ($r = -526$).

Conclusions: These results may shed light on the roles of chemokine receptors in pathogenesis of RA and could contribute to the development of new diagnostic and therapeutic strategies.

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Molecular mimicry between human endogenous retrovirus HERV-K10 and IgG1Fc: a possible mechanism in the pathogenesis of rheumatoid arthritis?

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Human endogenous retroviruses (HERVs) constitute 8% of the human genome and represent previous 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, a number of these so called 'fossil viruses' have retained intact open reading frames within *gag*, *pol* and *env* genes, can be activated, and produce retroviral particles and products.

HERV-K10 is one such virus that has been linked with rheumatoid arthritis (RA). Preliminary bioinformatic analysis of HERV-K10 by our group 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), we observed significant IgG antibody reactivity ($P < 0.024$) to this epi-

tope in RA patients as compared to disease controls and healthy subjects. Subsequent amino acid sequence alignment of MAG1 revealed a homologous region (GKEYK) within the CH2 domain of IgG1Fc, a key autoantigen in RA. This finding prompted us to question whether an antibody raised to MAG1 would also react with IgG1Fc. On developing a rabbit polyclonal antibody (termed PAb-MAG1), we have found reactivity to HERV-K10 viral peptide MAG1 and IgG1Fc proteins in ELISA. Validation in Western blotting also confirmed the recognition of IgG1Fc products of appropriate molecular weight. These results confirmed immune reactivity to both virus and autoantigen thus highlighting the potential for molecular mimicry between HERV-K10 Gag matrix and IgG1Fc. Intriguingly, we also have shown that the same epitope targeted by PAbMAG1 on IgG1Fc is targeted by rheumatoid factor antibodies. Consequently HERV-K10 could trigger and/or augment reactivity to IgG1Fc in RA patients and provide a mechanism which contributes to the pathogenesis of this autoimmune disease.

This notion is supported by our observation of IgG antibodies to peptide MAG1 that is suggestive of a persistent antigen-driven immune response to HERV-K10 in RA. Finally our results raise the possibility of HERV-K10 peptide or antibody as potential therapeutic blocking agents of rheumatoid factors.

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Lymphoid cell aggregations in experimental autoimmune uveoretinitis

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Background/introduction/context/objective: Experimental autoimmune uveoretinitis (EAU) is a Th1/Th17-mediated murine model of the chronic human non-infectious ocular disease uveitis. Previous work from our group has established that during clinical resolution of disease a significant population of lymphocytes remains within the retina. We investigated whether this lymphoid infiltrate within the retina during EAU undergoes a process of structural organisation as has been shown to occur in the lymphoid infiltrate in other organs affected by chronic autoimmune disease, where organised tertiary lymphoid tissue has been observed.

Material and methods: C57BL/6 mice were immunised subcutaneously with RBP₁₋₂₀ uveitogenic peptide emulsified with complete Freund's adjuvant and pertussis toxin intraperitoneally to induce EAU. The clinical course of EAU was monitored by topical endoscopic fundal imaging. At a range of time points mice were sacrificed. Retinas were dissected from 1 eye of each mouse and homogenised, mRNA isolated and cDNA generated. Optimised primer sets for lymphoid chemokines CXCL13, CCL19 and CCL21 and podoplanin (a cell surface marker specific to Th17 cells) were used to measure increases in mRNA relative to the housekeeping gene GAPDH. The contralateral eye from each mouse was snap-frozen and semi-thin sections obtained for immunofluorescent staining for CD3 and B220.

Results: An increase in expression of lymphoid chemokines CXCL13 and CCL21 in later stages of disease was found. Podoplanin expression was upregulated at day 35 post-immunisation but not at earlier or later timepoints. Aggregations of CD3-positive cells and smaller number of B220-positive cells were found both within the retina and in the subretinal space. Lymphoid aggregations were seen in 34% of all eyes examined (11 out of 32 eyes), and increased to 62.5% of eyes (five out of eight eyes) at the later stage of EAU (day 40 post immunisation or later). The presence of lymphoid aggregates did not correlate with the severity of EAU as measured by a clinical grading score.

Conclusion and discussion: We have demonstrated the upregulation of lymphoid chemokines and the focal aggregation of lymphoid cells with the retina in EAU. These findings are consistent with the lymphoid cell infiltrate within the retina in EAU undergoing a process of structural organisation.

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Unexpected role of IL-4R α in regulating local tissue-resident stromal cells to support tertiary lymphoid structure formation at site of inflammation

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Background: Tertiary lymphoid structures (TLS) are a hallmark of various autoimmune diseases. Phenotypical and functional changes associated with the acquisition of lymphoid-like features have been described in the stroma during TLS and cancer and suggested to drive formation and maintenance of the pathological lesions. To date the mechanisms responsible for the regulation of these changes are not known. IL-4R signalling has been recently described to regulate, upon injury, mesenchyme differentiation in muscle or adipose tissue and we hypothesise that the same signalling might play a role in the transition from normal to lymphoid like/pathological mesenchyme.

Methods: To test our hypothesis we used an inducible mouse model of TLS formation by retrograde cannulation of the salivary glands with a replication deficient adenovirus, able to induce TLS similar to those observed in Sjogren's syndrome and Rheumatoid arthritis. Salivary glands of C57BL/6 mice wild type and knockout mice (IL-4R α ^{-/-}, IL-4^{-/-}, IL-13^{-/-}) were cannulated and sacrificed at different time points post cannulation (p.c.). Immunofluorescence, flow cytometry and RT-PCR were used to evaluate the dynamic of acquisition of lymphoid features and stromal cell activation.

Results: In our mouse model of TLS formation, we demonstrated that in absence of IL-4R mediated signalling (IL-4R^{-/-} mice) stromal cell activation does not occur and absence of lymphoid stromal cell markers is associated with abrogated chemokine expression and defective TLS formation. Similar phenotype was observed in IL-13^{-/-} mice but not IL-4^{-/-} mice where normal induction of TLS occurs. Investigation of IL-13^{GFP} and IL-4^{GFP} reporter mice as well as WT mice showed a high level of IL-13 but not IL-4 expression in the salivary gland at early time-points after TLS induction, thus suggesting that IL-13 more than IL-4 is responsible for stromal cell transition to a lymphoid phenotype. Interestingly, investigation of bone-marrow chimeric mice showed that the source of IL-13 was not only haemopoietic cells but both epithelial cells and other stromal cells were chiefly responsible for producing IL-13 in response to adenovirus infection. Interestingly, administration of recombinant IL-13 in absence of virus was sufficient to cause stromal cell activation in absence of viral insult.

Conclusions: All together these results suggest that activation of quiescent tissue-resident stromal cells is mediated by engagement of IL-4R on resident stroma and strongly suggests the need for therapeutic approach aimed to stroma to achieve disease control. Therapeutic intervention aimed to block this signalling in autoimmune conditions is currently under development.

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The functional role of genetic variants at the *THEMIS*/*PTPRK* locus in multiple sclerosis

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Background and aims: Genome wide association studies (GWAS) have identified a number of loci containing genetic variants associated with susceptibility to multiple sclerosis (MS). The locus containing the genes *THEMIS* and *PTPRK* on chromosome 6q has been identified as one such region. *THEMIS* and *PTPRK* have been linked to thymocyte development and peripheral T-cell functions in mice and humans – both of which have been identified as defective in MS. The aim of this study was to look at the functional mechanisms underlying the associated SNP in the *THEMIS*/*PTPRK* locus. This knowledge will contribute to our understanding of the mechanisms of this disease and may identify a pathway that could be targeted for the therapeutic management of MS.

Methods: PBMCs were isolated from 32 individuals recruited through the Cambridge BioResource based on their genotype at the most associated SNP identified in the GWAS study. To study the effects of genotype on phenotype, the proportion of CD4⁺ and CD8⁺ cells with a naive, central memory (CM), effector memory (EM), effector memory RA (TEMRA) and regulatory phenotype was determined by flow cytometry. Genotypic effects on expression of *THEMIS*, *PTPRK* and *RP11-103C16.2* (an antisense gene to *PTPRK*) were studied on magnetically separated CD19⁺, CD4⁺ and CD8⁺ cells by quantitative PCR using the Taqman 7900 Sequence Detection System. Thymic activity was measured indirectly using the sjTRECs/ml assay on whole blood leukocytes, and by the sjTRECs/ μ g DNA assay to differentially investigate the naivety of CD4⁺ and CD8⁺ cells in peripheral blood.

Results: In the analysis correlating genotype with gene expression, no significant results were identified for any of the three genes investigated; however there was a trend towards increased expression of *THEMIS* in CD8⁺ cells with the risk homozygous genotype. A trend towards decreased memory cells within the CD8⁺ compartment was also identified in those with the risk homozygous genotype. No trends were identified in the analyses correlating genotype with sjTRECs/ml and sjTRECs/ μ g DNA.

Conclusions: The results obtained exclude a large effect of the risk variant on T-cell development and phenotype. However, due to the sample size, firm conclusions cannot be drawn regarding more moderate effects. In addition, as yet we have not investigated the effects of genotype on cell function. Future work includes expanding the sample size of the study, and performing functional assays – including studying the role and kinetics of *THEMIS* and *PTPRK* expression upon T-cell activation.

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Characterising the role of lipoprotein-associated phospholipase A₂ (Lp-PLA₂) in experimental autoimmune uveoretinitis (EAU)

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Background and aims: Macrophage activation can be regulated via hydrolysis of oxidised low density lipoproteins (oxLDL) by Lp-PLA₂. This produces lysophosphatidylcholine and non-esterified fatty acids that up-regulate expression of chemokines and adhesion molecules, induce macrophage migration and promote pro-inflammatory cyto-

kine release. Inhibition of this enzyme may therefore perturb macrophage function and attenuate inflammation. We utilised Lp-PLA₂ knockout (KO) mice to determine the effect of Lp-PLA₂ depletion in autoimmune retinal inflammation, employing a murine model of autoimmune uveitis, EAU, in which macrophages are central to progression and expression of disease.

Methods: C57BL/6 Lp-PLA₂ KO mice, heterozygotes (HET) and wild type (WT) controls were immunized to induce EAU by subcutaneous injection of RBP-3 (IRBP) 1–20 peptide in Complete Freund's Adjuvant, plus intra-peritoneal (IP) injection of heat inactivated pertussis toxin. Clinical disease was monitored by Topical Endoscopic Fundus imaging (TEFI). Mice were sacrificed on day 26 post immunisation, infiltrating leukocytes were quantified by flow cytometry and histological disease severity was assessed.

Results: Lp-PLA₂ KO mice showed substantially fewer infiltrating CD45⁺ cells compared to both WT and HET controls, which correlated with a lower disease score by TEFI and histology. There were a greater proportion of CD11b⁺Ly6G⁺ neutrophils in the retinas of the KO mice. Oxidised phospholipid (oxPL) staining was positively correlated with infiltrating CD45⁺ cells and increased disease severity, rather than Lp-PLA₂ depletion and subsequent substrate accumulation.

Conclusions: Lp-PLA₂ KO mice, immunised to induce EAU experienced decreased macrophage infiltration and less clinical disease. Together we infer from these findings a mechanism of reduced migration and infiltration of cells as well as suppressed leukocyte activation.

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Two's company, three's a crowd: a model for cross-linking of receptor trimers by ligand trimers as triggers for signalling in the TNF/TNFR superfamily

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Background and aims: The tumor necrosis factor (TNF) family of cytokines is involved in orchestrating immunological processes. Their effects are mediated by binding to the cell surface members of the TNF-receptor super-family (TNFRSF). They thus induce the activation of intracellular signaling pathways. Evidence indicates that members of the TNFRSF including TNF itself, function as trimers that bind to three copies of the corresponding receptors. The TNFRSF members contain cysteine-rich domains (CRD) in their extracellular, ligand-binding regions. There is evidence to prove that the membrane distal CRD-1 forms homologous interactions in the absence of ligand to form receptor complexes. The CRD-1 is therefore termed a pre-ligand assembly domain (PLAD). The MBTI model is of profound importance as a means to understand the architecture of how PLADs interact with each other and how they bind TNF- α . Here, we have utilised the MBTI model to make an informed decision to mutate four residues that are identified to be critical to PLAD interaction.

Methods: We used the MultiSite Gateway 3-fragment cloning technology to produce five expression clones. The expression clones included full-length constructs of the wild type (WT) and four mutants (K19A, T31A, D49A and D51A). The expression clones were transfected into the SK-HEp1 cell line. We used flow cytometry to generate the expression profiles of the transfected cells, and we then proceeded to investigate the ability of the mutants to bind TNF- α .

Results: We found that both WT and mutants are expressed on the cell surface and observed that K19A and D51A could not bind the latter when compared to the wild type protein. T31A and D49A could clearly bind TNF- α but not to the same extent as that of the

wild type (low level of binding). From our results it can be inferred that these mutations prevented the TNFR1 protein from self-associating and in turn, from carrying out PLAD interactions to form trimers. However, the T31A and D49A mutations do not seem to affect the overall three-dimensional structure of the receptor greatly.

Conclusions: In conclusion, our results reinforce the possibility that a preferential homotrimer formation of PLAD in the absence of a ligand is favoured.

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Murine models of colonic inflammatory bowel disease: a new look at immunological and pathological phenotypes

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Inflammatory bowel disease represents a spectrum of chronic gastrointestinal diseases, which are increasing in global prevalence and which generate significant morbidity. The study of experimental models of intestinal inflammation has aided recent advances in understanding the pathology and immunology of Crohn's disease (CD) and ulcerative colitis (UC). However, reverse translation from human disease to experimental models is essential to investigate genetic and biological mechanisms central to disease pathogenesis. The more advanced and tractable an animal model is with respect to phenotype, gene expression, histopathology and the immune response, the better suited it is for translational study. Although many mouse models of colitis have been developed, more rigorous phenotyping is required to take full advantage of the resource. Here we take a new look at colitic mouse models, comparing what is known with specific pathological and immunological hallmarks of colonic CD and UC. This highlights the strengths and weaknesses of each colitis model and should lead to a more focused study of each for translational investigation.

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Pathogenicity of gut-derived Th17 cells

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In steady-state conditions the intestine contains a significant number of Th17 cells. Here they play a major role in maintaining homeostasis, such as promoting IgA production and reinforcing barrier function. During pathogenic immune responses in the gut and other tissues, Th17 lymphocytes are found to increase in number and in some cases undergo switching towards a Th1-type phenotype. Therapeutic interventions targeted at pathogenic IL-17 producing cells in inflammatory disorders may also have significant detrimental effects due to the interference of beneficial Th17-mediated effects in the intestine. This raises the question of what are the differences between gut-resident Th17 cells and *de novo* pathogenic IL-17A-producing T cells seen during immunopathology. To address this issue, we make combined use of IL-17 reporter mice and adoptive transfer models and induce inflammatory responses (bacterial infection or induction of autoimmune EAE) to delineate the contributions of gut resident or *de novo* induced Th17 cells. Current and future work will concentrate on revealing the molecular differences between intestine-resident Th17 cells and Th17 lymphocytes generated after immunological challenge.

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Understanding the T cell mechanisms of human immune thrombocytopenia

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Human primary immune thrombocytopenia (ITP) is a rare blood disorder defined by low platelet counts but normal bone marrow. It is an autoimmune condition with antibodies detected against several platelet surface antigens. The main immune mechanisms are thought to be destruction of platelets and megakaryocytes by either T cell-mediated or auto-antibody-mediated processes. Ineffective thrombopoiesis has also been observed.

There is a dearth of T cell studies in the ITP literature. Thus, this study aims to characterise T cell fitness, frequencies, and function in ITP. A cross-section of ITP patients ($n = 20$) and matched controls ($n = 20$) will be recruited from the Royal London Hospital's ITP patient registry, the largest of its kind in Europe.

We will analyse the frequencies of CD8⁽⁺⁾ CTLs, CD4⁽⁺⁾ effector T cells, and CD4⁽⁺⁾ regulatory T cells, in the peripheral blood of patients. The pro- and anti-inflammatory potential of T cell subsets will be assessed; for example by intracellular cytokine staining for IFN γ , IL-17A, IL-4, IL-10 and Granzyme B. Cytotoxic potential will also be investigated.

An improved understanding of the T cell mechanisms in ITP is fundamental for the development of new therapies and better clinical management of the disease.

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Peripheral fibroblastic reticular cells: an important source of local chemoattractants during autoimmunity?

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Chronic inflammation is a specific feature of many diseases including autoimmune disorders, persistent infections and chronic graft rejections. It has been previously reported that structures similar to secondary lymphoid organs, with organized lymphoid stromal cell network, can be found in chronically inflamed tissues. These tertiary lymphoid organs (TLO) are believed to promote local immune response development resulting in disease progression. Type 1 diabetes is an autoimmune disease characterized by on-going, chronic Th1 response in the islets of Langerhans. TLO is a prominent feature of islet inflammation in multiple mouse models of type 1 diabetes. Importantly, treatment with LT β R-Ig fusion protein, which has been shown to disrupt TLO, prevents disease development and can even reverse established inflammation in the islets. These findings indicate that LT β R-dependent lymphoid stromal cells, essential for TLO, may be important in creating inflammatory milieu facilitating disease progression. However, little is known about the lymphoid stromal cell composition of TLO in the islets and their specific roles in type 1 diabetes development.

Here we show that islets of pre-diabetic non-obese diabetic mice have increased numbers of fibroblastic reticular cells (FRC), normally found in T cell zone of secondary lymphoid organs. FRC numbers strongly correlate with infiltration severity and are virtually absent in islets of mice that do not develop islet infiltrates. Furthermore, islet FRC are the principal producers of Th1 cell-attracting chemokine Cxcl9 and homeostatic chemokine Ccl19, which receptor Ccr7 is widely expressed on naïve T cells. IFN γ was necessary and sufficient for inducing Cxcl9 and Ccl19 expression by islet FRC. Suppression of

IFN γ by islet antigen-specific Tregs *in vivo* led to a decrease in Cxcl9 and Ccl19 expression, reduction of T cell recruitment, and prevention of progression to overt diabetes. Therefore, it is possible that islet FRC can attract T cells from the circulation and subsequently orchestrate their migration and organization in the islets, similarly to what can be observed in the secondary lymphoid organs, facilitating cell interactions and disease progression. Consequently, our results suggest that FRC are central to the perpetuation of chronic inflammation in the islets and intercepting the cross talk between T cells and FRC may be an effective strategy to halt progressive tissue destruction in type 1 diabetes and other autoimmune disorders.

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Ankylosing Spondylitis patients have an increased proportion of CD16⁺ mononuclear cells able to induce CCR6 on CD4⁺ T cells

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Background and aims: Ankylosing Spondylitis (AS) is a chronic systemic inflammatory disease primarily affecting the axial skeleton. The genetic association between AS and the MHC class I gene HLA-B27 was discovered 40 years ago, however the pathogenic role of this molecule remains elusive. IL-17 secreting cells appear to be important in driving pathology both in AS patients and in the HLA-B27 transgenic (B27-TG) rat SpA model. Dendritic cells (DCs) activate naïve T cells and maintain the balance between activation and suppression of the immune response. If affected by HLA-B27, DCs are therefore likely to contribute to the T cell-mediated aspects of AS pathogenesis. Studies in our laboratory, using B27-TG rats, have uncovered deficiencies in tolerogenic DC populations that may contribute to disease development through IL-17 promotion. We therefore aimed to characterise the functions of DC populations in AS patients, to understand the role of DCs in AS pathogenesis.

Methods: T cell and DC subsets isolated from AS patients and age-matched healthy controls were analysed using multiparameter flow cytometry. DC subsets purified by flow cytometric sorting, were co-cultured with allogeneic T cells; T cell proliferation was measured by CFSE dilution and chemokine expression by flow cytometry. Plasma cytokines were measured by ELISA. Using linear regression and the Kruskal Wallis Spearman correlative test with a Dunn multiple comparisons post test, correlative analysis was performed using clinical and immunological patient parameters.

Results: Compared to age and sex-matched healthy controls, blood from AS patients contained increased proportions of CD4⁺ CCR6⁺ activated T cells. Additionally, AS patients contained a significantly higher ratio of pro-inflammatory (CD16⁺ CD14⁺ CD11c⁺) mononuclear cells compared to CD1c⁺ DCs, and higher concentrations of IL-23. In co-culture experiments, these CD16⁺ DCs induce CCR6 expression on responding naïve T cells. The frequency of circulating CCR6⁺ and CCR9⁺ CD4⁺ T cells in AS patients increased with disease severity and inflammation.

Conclusions: CCR6 expressing T cells are associated with a Th17 phenotype. Our data indicate that along with increased systemic levels of IL-23 in AS patients, a shift in circulating myeloid populations towards the CCR6-inducing CD16⁺ population may contribute to the induction of T cell-mediated pathogenesis. Furthermore, we have identified a potential role for CCR6⁺ and CCR9⁺ T cells in disease pathogenesis.

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Profiling autoantibodies in COPD using protein microarrays technique

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Background: Chronic obstructive pulmonary disease consists of a combination of two phenotypes of the disease: chronic bronchitis and emphysema, both of which can lead to the main feature of this disease; airflow obstruction. COPD is the third leading cause of mortality in the world. In recent studies, high levels of circulating autoantibodies have been detected in patients with COPD; suggest that the lung disorder may have an autoimmune component.

Objective: Evaluation of the autoimmunity associated with COPD by measuring the levels of IgG autoantibodies in the COPD patients. **Methods:** To achieve the project aim, we have employed antigen microarray. Before applying this technique, quality control experiments have been used to optimize the procedure such as testing different slides coated surfaces, selecting the best blocking buffers and amplifying the signal intensities.

Results: The obtained results demonstrated that the combinations of Aminosilane slide with I block buffer; Epoxysilane slide with PVA buffer and aldehyde slide with mix of both buffers gave the highest signal intensities and the lowest background. In addition to the signal intensities the morphology of the spots was even (circular and regular) across the whole print.

The signal intensity was highly amplified using Genisphere-CY5 40 at a dilution of 1:3200 with very low background.

Conclusions and future work: Protein microarray techniques have pivotal future roles in diagnosis of COPD as well as new options of therapy in the disease. The optimized procedure will be applied on samples of COPD patients and healthy controls.

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Tissue resident CD8 T cell regulation of the retinal microenvironment during chronic experimental autoimmune uveitis (EAU)

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Using CD4 T cell mediated Experimental Autoimmune Uveitis (EAU) as a model for human intraocular inflammation we have established that effector memory CD8 T cells (T_{EM}) accumulate in the secondary progressive phase of EAU. It remains unclear whether they contribute to the persistence of disease. Since CD8 T cells of an effector memory phenotype become resident in tissues following viral infection our purpose was to interrogate the function of autoreactive retinal CD8 T cells within target tissue.

C57BL/6J mice were immunised with RBP-3 (IRBP [Interphotoreceptor Retinol Binding Protein]) 1–20 peptide to induce EAU. Disease progression was measured by Topical Endoscopic Fundal Imaging (TEFI) and CD8 T cell retinal infiltrate and phenotype assessed by flow cytometry. Cytokine production and cytotoxic potential was measured by intracellular cytokine staining. Treatment with FTY720 was used to determine the tissue half-life of retinal CD8 T cells. Anti-PD-1 monoclonal antibody was used for local *in vivo* PD-1 blockade. Monoclonal antibodies YTS 169.4 and 156.7 directed against CD8 were used for *in vivo* depletion of CD8 T cells.

Resident memory signatures CD69 and CD103 were upregulated compared to splenic counterparts, however only a subset of retinal T_{EM} expressed CD103. Treatment with FTY720 decreased CD4 and

CD8 T cells in the blood while in the retina the number of CD8 T cells remained whilst numbers of CD4 T cells declined substantially. Retinal T_{EM} expressed increased levels of PD-1, correlating with a progressive lack of effector function. Local blockade of PD-1 in one eye provoked an increase in inflammation and increased cellular infiltrate, compared to isotype administration in the contralateral eye. PD-1 blockade increased CD103 expression on retinal T_{EM}. Depleting CD8⁺ cells from the retina resulted in an increase in cellular infiltrate, specifically CD4 T cells and CD11b macrophages.

In conclusion, effector memory CD8 T cells during autoimmune retinal inflammation are comparable in phenotype and function to tissue resident CD8 T cells observed after infection in other tissues. The residency results in chronic antigen stimulation in the retina, which in turn illuminates a phenotype of T cell exhaustion. Upregulation of PD-1 on retinal T_{EM} may provide a negative signal by which these cells regulate the local environment.

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Glucocorticoid receptor trafficking is independent of T helper cell phenotype

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Glucocorticoids remain the primary treatment for a variety of autoimmune diseases, however up to 30% of individuals fail to achieve disease control at tolerable doses and are thus classified as steroid refractory (SR). We have observed that CD4⁺ T cells from SR uveitis patients are biased to express IL-17 following TCR engagement, which is consistent with the SR Th17 phenotype reported in other disease groups. The purpose of this study was to establish whether this disparity in steroid responsiveness between T helper cell subsets is due to impaired glucocorticoid receptor expression or trafficking.

In humans, IL-17 producing CD4⁺ T cells are primarily derived from the effector memory population identified by expression of CD45RO and CCR6. We sorted (BD Influx) CD4⁺ CCR6⁺ and CD4⁺CCR6⁻ cells from the PBMCs of normal volunteers and cultured for 2 weeks in Th17 and Th0 (unpolarised) conditions respectively. Intracellular interleukin (IL)-17 and interferon gamma (IFN- γ) expression was quantified by flow cytometry (BD LSR II) after 24 h exposure to the synthetic glucocorticoid dexamethasone (Dex). The nuclear translocation of GR (anti-GR mAb, Santa Cruz 3D5) was quantified after 30 min of exposure to Dex using laser scanning confocal microscopy of paraformaldehyde fixed cells. We used Velocity 6.2 (Perkin Elmer) to analyse full depth z-stack images and quantified translocation (nuclear density) as total red (GR) divided by nucleus volume (DAPI). Matched cell samples were stored in RNAlater and the relative expression of GR isoforms was quantified when normalised to GAPDH (Applied Biosystems).

We found that exposure to Dex significantly increased the nuclear density (translocation) of the GR in both Th0 and Th17 cells when compared to untreated control cells. Further, there was no difference between the cell types in their overall ability to translocate the GR. The expression of both total GR and the GR β isoform did not differ between Th0 and Th17 cells, and was not affected by exposure to Dex.

We have shown previously that human Th17 cells maintain their phenotype and proliferation in the face of steroid treatment. Here we have investigated two potential cellular mechanisms that may confer this glucocorticoid resistance using *in vitro* derived Th17 and

Th0 cells and have shown that neither impaired GR translocation nor altered GR isoform expression explains the phenotype observed clinically.

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A novel role for CD248 in controlling the immune response to antigenic challenge

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Background and aims: Chronic autoimmune diseases such as rheumatoid arthritis (RA) are amongst the most debilitating conditions in the developed world. Autoimmune diseases are associated with aberrant humoral responses taking place in secondary and tertiary lymphoid organ germinal centres and results in production of antibodies to non-pathogenic self-antigens. A thorough understanding of how the breakdown of tolerance occurs remains elusive. CD248 (otherwise known as tumour endothelial marker-1 or endosialin) a well-known pericyte marker has been shown to be upregulated in the synovium of RA patients and it has been observed to be involved in lymph node remodelling following immunization.

Methods: The response to NP-CgG immunisation of CD248KO mice was analysed by confocal microscopy, flow cytometry, and quantitative real-time PCR.

Results: I present evidence that CD248 has a role in regulating the formation of follicular dendritic cell (FDC) networks following immunisation, and in the absence of CD248, these networks do not accurately form.

Conclusions: This aberrant formation may function in controlling the ability of FDC to control B cell maturation and produce high affinity antibodies, thus resulting in inability to accurately respond to immune challenges and possible tolerance breakdown as observed in RA.

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Profiling of natural autoantibodies in relation to chronic obstructive pulmonary disease (COPD)

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The aim of this study is to investigate natural autoantibodies in relation to chronic obstructive pulmonary disease (COPD). Natural autoantibodies are antibodies found in circulation of healthy individuals and react with one or more auto-antigens. They are encoded by genes that lack extensive somatic mutations and affinity maturation. However, under certain circumstances, they may undergo somatic mutation and have potential to be associated with generation of pathological associated autoantibodies. One potential target of these autoantibodies is extra cellular matrix such as elastin peptides and these have been shown to be involved in pathogenesis of COPD. COPD is a complex condition consisting of chronic bronchitis, respiratory bronchiolitis and emphysema and is characterized by progression airflow limitation, which is not fully reversible. In addition COPD is accepted as inflammatory disorder with evidence of circulating autoantibodies against a variety of targets. The aim of this study is to examine why natural autoantibodies in some individuals may shift from a physiological role to pathological autoantibodies in relation to COPD. We have developed assays to measure antigen-specific antibody responses in

serum and to examine cross-reactivity. The long term aim is to identify diagnostic markers and therapeutic targets in relation to COPD.

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Plasmacytoid dendritic cells recognise apoptotic cell-derived DNA complexes and promote immune regulation via IL-10

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Background and aims: The current paradigm for the pathogenesis of systemic lupus erythematosus (SLE) suggests that self-tolerance to apoptotic cells (AC) is breached following defective uptake, leading to TLR9 stimulation of plasmacytoid dendritic cells (pDC), which secrete interferon (IFN)- α , and B cells secreting high titre anti-nuclear autoantibodies. Conversely, in health TLR9 is essential for the induction of regulatory B cells in response to DNA-complexes expressed by AC. A regulatory role for TLR9 has also been demonstrated in murine autoimmune diseases, where mice deficient in TLR9 have more severe lupus-like disease. We wanted to gain a better understanding of how pDC respond to AC in health, as this may help to determine why immune regulation breaks down in autoimmune diseases, such as SLE.

Methods: Splenic pDC were purified from wild type and TLR9-deficient mice. pDC were activated with synthetic TLR7 and TLR9 ligands and were co-cultured with apoptotic thymocytes generated by UVB-irradiation or with freeze-thawed necrotic cells. Interactions with the receptor for advanced glycation endproducts (RAGE) were blocked using the antagonist Box A from high mobility group box 1 (HMGB1). Cytokine secretion was analysed by ELISA and flow cytometry.

Results: IL-10 protein concentration was enhanced in supernatants when pDC interacted with whole AC, but not necrotic cells. AC-mediated cytokine production was switched to IFN- α when pDC were primed with the TLR9 ligand CpG Type A, a viral mimic. Enzymatic digestion of AC surface nucleic acids revealed DNA was important for enhancing the responses. TLR9 was required to maintain IL-10 secretion for up to 1 week. Blocking RAGE on pDC inhibited both IL-10 and IFN- α protein production. Furthermore, CD4⁺ T cells were induced to secrete IL-10 by pDC-AC co-culture. Removal of contaminating UV-irradiated antigen-presenting cells (APC) from the AC feed inhibited the IL-10 production, suggesting that pDC require a second APC to licence the secretion of IL-10.

Conclusions: The results suggest that in health, pDC respond to DNA complexes expressed on whole AC by promoting immune regulation. However, the regulatory response produced by pDC interactions with AC may be lost in the context of viral infection. Crosstalk with other APC, such as conventional DC and B cells, and signalling through RAGE may influence the regulatory response promoted by pDC.

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Regulatory T cells in autoimmune disease: can estrogens break tolerance?

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Autoimmunity is characterised by a loss of immune tolerance to self-antigens and by the activation of both auto-reactive cells and persistent production of pathogenic auto-antibodies. These immune responses to self-antigens lead to chronic and often debilitating disease. While the exact pathogenic mechanisms for many autoimmune diseases remain to be elucidated, often their onset is linked to genetic susceptibility. However, recent studies have suggested that dysregulation of regulatory T cells (Tregs) is one of the major factors conferring risk factor for the development of these diseases. Furthermore, it is known that autoimmunity occurs more frequently in women than in men and in some diseases the flares are associated with certain phases of the menstrual cycle. These observations suggest a mechanism by which estrogens affect the disease activity. Antagonistic and cooperative interactions between estrogens and the vitamin A metabolite, retinoic acid, have been also reported. *In vivo* adaptive Tregs (iTregs) are generated throughout the course of the immune response and retinoic acid in combination with TGF- β can convert naive CD4⁺ T cells into Tregs and stabilise the expression of Treg master gene, FOXP3. The hypothesis tested in this study was that the dysfunctional cross-talk between retinoic acid and estrogens could impair the expression of genes controlling both Tregs generation and fate. To this aim iTregs have been generated *in vitro* in the presence of TGF- β , ATRA and different concentration of β -estradiol. The results showed that although both proliferation and phenotype of these cells are not influenced by the presence of the hormone, the suppressive ability was vigorously affected. This result leads to the analysis of different pathways implicated in Treg functions and whether microRNAs could play a key role. The expression of miR10a, which has been previously described highly expressed in iTregs and induced by retinoic acid, was significantly decreased by the presence of β -estradiol. This inhibition could be the cause of reduced Treg function. Altogether the results obtained so far in this study provide mechanistic insight into the cross-talk between sexual hormones and iTreg generation and function.

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Immunoregulation during a Multiple sclerosis relapse: effects of corticotherapy

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Background: Multiple sclerosis (MS) is a chronic inflammatory disease resulting in demyelinating lesions in the central nervous system. MS has multifactorial components involving a genetic predisposition and environmental factors. Although high dose intravenous methylprednisolone (ivMP) is commonly used to treat acute relapses, its mechanism of action is only partly known. The proinflammatory events leading to MS relapses have been largely investigated. Here, we focus our work on the regulatory responses activated during the relapse.

Material and methods: Peripheral Blood Mononuclear Cells (PBMC) were collected from relapsing MS patients before ivMP treatment on day 1 and on day 5. Regulatory T cells (Treg) frequency was assessed by methylation-specific qPCR of the first intron of FOXP3. CD4⁺ T

cells were isolated by magnetic cell separation in a subgroup of relapsing patients and healthy controls, and stimulated *in vitro* by the artificial polyclonal reagent Cytostim (Miltenyi Biotech). Cytokines were analyzed by qPCR *ex vivo* and after stimulation. CD39 expression was analyzed *ex vivo* by flow cytometry in CD4⁺, CD8⁺, CD19⁺, NKP46⁺ cells as well as in CD4⁺Foxp3⁺CD25^{hi} Treg.

Results and discussion: Treg frequency is similar between MS patients and healthy controls, and is not increased by ivMP. We show an *ex vivo* increased expression of CD39 and IL-22, a cytokine with possible protective effects, during an acute relapse. Exposure to ivMP had no effect on IL-22 expression. Conversely, the expression of CD39 is further reinforced by ivMP treatment. CD39 is an ectoenzyme that cleaves ATP to AMP. As ATP has numerous proinflammatory effects, its degradation by CD39 has anti-inflammatory influence. The expression of CD39 is not increased on Treg but on a subset of cells negative for B, T and natural killer (NK) cell surface markers. Further studies will be conducted in order to characterize these cells, possibly innate lymphoid cells.

All together, these data suggest that anti-inflammatory responses are activated independently of Treg by the organism when a MS relapse occurs. IvMP treatment might strengthen these mechanisms but does not increase Treg frequency.

We are currently studying the expression of the aryl hydrocarbon receptor (AHR). This transcription factor is a crucial link between environmental factors and host immune function and plays a critical role in IL-22 and CD39 production. Preliminary experiments carried out in our laboratory show that ivMP increases AHR mRNA expression. Identifying therapeutic targets to promote anti-inflammatory pathways is of great interest for the treatment of relapsing MS.

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Profiling autoimmunity markers in autoimmune diseases using antigen microarray

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There is a growing recognition for the need to stratify patients with systemic autoimmune diseases, such as rheumatoid arthritis, for early detection and improved differential diagnostics. This requires the development of high throughput technologies that allow rapid measurement of multiple specific proteins in a single small sample, at low cost.

The aim of this study is to develop an antigen microarray to support the simultaneous interrogation of patient samples for multiple autoantibodies against common autoantigens associated with rheumatoid arthritis and other systemic autoimmune diseases. The capabilities of protein microarrays make them ideally suited to high-content and high throughput processing to profile disease biomarkers in support of enhanced patient stratification and personalized therapeutic intervention.

Optimization of the microarray process is key to the reproducibility of the techniques. Over 40 target autoantigens and controls have been incorporated on a single small microarray, and blocking and signal enhancement technologies have been resolved to provide a reproducible assay, requiring just one well of a microtitre plate per patient sample. The array system has been validated using a range of samples. Application of the technique to clinical samples from a broad range of rheumatology samples is ongoing.

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hBD3 exacerbates the MDA5-mediated type I interferon response to the viral mimic PolyI:C both *in vitro* and *in vivo*, but suppresses TLR3 and RIG-I responses

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Double stranded RNA (dsRNA) is a pathogen associated molecular pattern indicative of viral pathogens and recognised by the endosomal receptor TLR3 and the cytosolic receptors RIG-I and MDA5. PolyI:C is a synthetic dsRNA that is recognised primarily by both TLR3 and MDA5. hBD3 is a β -defensin known as an antimicrobial peptide and can also suppress both Myd88-dependent and independent TLR-4 mediated LPS response. We show here that in primary bone marrow derived macrophages the polyI:C-induced production of several proinflammatory cytokines (including TNF- α and IL-6 but not CXCL10) and the type I interferon response are significantly increased in the presence of the β -defensin hBD3. This effect is also observed *in vivo* with a significant increase in serum levels of cytokines following injection of hBD3 and PolyI:C compared to PolyI:C alone. Peritoneal injection of PolyI:C into transgenic mice that express hBD3 also show an exacerbated serum cytokine production in comparison to wildtype mice. The polyI:C enhanced effect is conserved in primary macrophages from TICAM1 (TRIF) knockout mice that only signal through the cytoplasmic receptors because they are deficient in TLR3 signalling. The effect is mitigated however in BMDM from mice that can only signal through TLR3 due to deletion of IPS-1, the co-adaptor for RIG-I and MDA5. Recent work suggests that hBD3 like LL-37 allows self DNA and the CpG mimic to enter pDC more effectively which results in increased type I interferon signalling through TLR9. We show here that increased cytoplasmic entry achieved by using lipofection does not increase the PolyI:C-induced IFN- β production in BMDM from wildtype mice or those from TRIF null mice where signalling is only through MDA5 and so it is unlikely that this explains the effect that we see. However, in contrast, the induced cytokine and interferon response achieved when using a specific ligand for RIG-I signalling (which requires a transfection reagent) is significantly reduced in the presence of hBD3. We conclude that the antimicrobial peptide hBD3 can both suppress and augment RNA induced innate immune responses. *DEFB103* the gene for hBD3 is on a highly copy number variable locus in human and its effect on MDA5 signalling may have phenotypic consequence in autoimmune disease.

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Deciphering the gene expression signatures associated with the pathogenesis of rheumatoid arthritis – a pivotal step towards personalised medicine

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Background and aims: Rheumatoid Arthritis (RA) is a chronic autoimmune disease characterized by articular damage, synovitis and other related systemic complications in brain, lungs and vasculature. The synovitis in RA patients is characterized by a rapid influx and proliferation of immune cells leading to excessive cytokine and chemokine production, tissue destruction and other co-morbidities. In this study, we have deduced various genomic signatures and bio-

logical pathways associated with RA using systems immunological methods.

Methods: Raw microarray expression (Affymetrix CEL) files in the public domain derived from studies on RA patients were downloaded from the Gene Expression Omnibus (GEO) and analysed by Genespring GX 12.5 software (Agilent, USA). The significantly expressed genes were selected by a standard cut-off at twofold increased expression compared with the normal controls. These differentially expressed genes were then classified based on Gene Ontology (GO) such as the expression of immune cell receptors, regulation of inflammatory response, cytokines and chemokines etc., Moreover, the differentially expressed genes were investigated by Ingenuity Pathway Analysis (IPA) software for the identification of canonical and novel pathways implicated in RA.

Results: The genomic signatures identified clearly showed the imbalance in proinflammatory and anti-inflammatory cytokines and chemokines in RA synovia that enhance the destruction of adjacent cartilages and sub-chondral bone erosions, ligaments and the laxity of tendons in RA.

Conclusions: The systems immunological analysis of high throughput RA microarray data is necessary to understand various disease promoting gene signatures and help to efficiently devise personalised therapeutic strategies to control the disease and successfully prevent relapse and attain remission in RA patients.

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Generation of human antigen-specific regulatory T cells by MHC-class I restricted T cell receptor gene transfer

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The adoptive transfer of regulatory T cells (Treg) as a therapy in autoimmune diseases has been well demonstrated in mouse models of disease. In the case of Type 1 diabetes, this therapy could potentially prevent or dampen down the auto-reactive immune response to the islets of the pancreas, a process in which CD8⁺ T cells play a pivotal role.

However, this therapy is limited to the number of antigen-specific Treg that can be isolated and expanded from patients. To overcome this, lentiviral gene transfer technology can be utilised to engineer whole populations of Treg to express any T cell receptor (TCR) of choice, re-directing their antigen specificity. Furthermore, by generating Treg whose TCR is MHC class I restricted and islet antigen specific, these cells can be targeted directly to the site of the inflammation and similarly against the CD8⁺ T cells that elicit a large proportion of islet cell damage.

TCR genes from the HLA-A2+ 1E6 pre-proinsulin specific and 868 GAG specific clone have previously been isolated and cloned into clinical grade lentiviral vectors. Upon introduction of the 868 TCR into Treg, transduced cells can successfully re-direct their antigen specificity and become reactive to the cognate GAG peptide. These re-directed Treg also prove to suppress antigen specific responses upon activation with cognate peptide. In contrast to this, the 1E6 TCR, which reacts to the pre-proinsulin peptide with ~1000-fold lower affinity than the 868 TCR, is unable to re-direct the antigen specificity of Treg in response to its cognate peptide. However, when 1E6 transduced Treg are activated with a heteroclitic peptide for which the 1E6 TCR has greater affinity, transduced Treg can successfully elicit suppressive activity.

These data demonstrate that a MHC class I restricted TCR with abundantly low affinity for its cognate peptide is unable to re-direct the antigen specificity of a CD4⁺ Treg without the presence of the

CD8 co-receptor. Therefore, in order to generate clinically relevant MHC class I restricted islet antigen specific Treg using the 1E6 TCR transduced Treg will require further manipulations, such as the introduction of the CD8 co-receptor. Thus, the selection of autoimmune MHC class I restricted TCRs for Treg therapy may prove more complex due to their naturally low affinity for cognate antigen.

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Danger signalling in sterile inflammation

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Background and aims: Sterile inflammation in the absence of infection is thought to be an underlying initiator/driver of chronic obstructive pulmonary disease (COPD), systemic lupus erythematosus (SLE) and other inflammatory disorders. The largest risk factor for COPD is smoking, which causes tissue damage and leads to necrotic cell death in the lung. These necrotic cells release 'danger-associated molecular patterns' (DAMPs), normally sequestered intracellularly, thereby signalling their death. Innate immune cells recognise these DAMPs and respond in a pro-inflammatory fashion. We aim to understand and model human sterile inflammation in COPD *in vitro*.

Methods: We have established an assay whereby human cells are necrosed with cigarette smoke extract and applied to primary human monocyte-derived macrophages or specific reporter cell lines, and the responses measured.

Results: Several cell lines were necrosed and assayed, and a human bronchial epithelial cell line (BEAS-2B) provoked the largest inflammatory response as measured by multiplex cytokine analysis. Assays with TLR and other reporter cell lines, together with antagonists of known and hypothesized danger receptors and DAMPs, uncovered an important role for TLR4 sensing of an as-yet-unidentified DAMP. All cultures tested negative for microbial content, and further assays determined that the potentially novel DAMP is pre-formed, cell-associated, and signals in an MD2-dependent manner.

Conclusions: Future work will aim to further characterise other DAMP(s) and danger receptor(s) that could be involved, as well as to identify the TLR4 DAMP. We will then examine their involvement in COPD and SLE using *in vivo* models and clinical samples.

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Immune responses to heat shock during Behçet disease

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Background and aims: Behçet disease is a chronic systemic inflammatory affection with an uncertain etiology. HSPs have been implicated in disease pathogenicity. In this study, we investigated the effect of heat shock on immune responses during Behçet disease.

Methods: Peripheral blood was collected from 42 patients with Behçet disease and 16 healthy subjects. Samples were incubated at 40° for 1 h. After incubation at 37° in humidified atmosphere with CO₂ (5%) during 36 h, plasmas were harvested and NO and urea were assessed in culture supernatants by modified Griess and Berthe-

lot methods respectively. Results were analyzed by ANOVA. Antibodies against self-cutaneous biopsies or bovine ocular structures were investigated by immunohistochemistry.

Results: Our results showed the presence of plasma self-reactivity against the cutaneous biopsies in all patients but not in control subjects. This reactivity was more important during active stages. Furthermore, heat shock induced a qualitative and quantitative modification in plasma reactivity. Plasma reactivity was more heterogeneous against bovine ocular structures. However, heat treated ocular tissues showed a higher reactivity than untreated tissues. Otherwise, without induction, nitric oxide levels were significantly increased in patients during active stage by comparison to control subjects ($P < 0.05$) while urea didn't show any difference ($P = 0.57$). Heat shock induced significant modifications in both nitric oxide and urea concentrations ($P < 0.01$). However, this effect was different depending on disease activity and the clinical manifestations ($P < 0.001$).

Conclusions: Our results showed the implication of heat shock in the antibodies production and the modulation of nitric oxide and urea production during Behçet disease. These results suggests that heat stress and/or induction of HSPs are implicated in the immune disorders encountered during the disease.

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Investigating the role of T-follicular helper cells in experimental atherosclerosis

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Background and aims: Cardiovascular Disease (CVD) is now the biggest cause of mortality worldwide, with atherosclerosis accounting for the majority of deaths in CVD cohorts. Multiple cells of immune decent accumulate within atherosclerotic arteries, with many being pro-atherogenic in nature. These cells drive the progression of intra-arterial plaques via cell-cell interactions and the secretion of mediators. Of these cells, T-cells and B-cells are of crucial importance for plaque formation and progression. Using the hypercholesterolemic apolipoprotein E deficient ($\text{apoE}^{-/-}$) mouse model, we investigated the role of T-follicular Helper (Tfh) cells in experimental atherosclerosis. We have investigated Tfh cell, germinal centre (GC) B-cell and antibody profiles during atherosclerosis formation and development.

Methods: We have used flow cytometric analysis of lymphoid organs to investigate changes in T-cell and B-cells subsets in $\text{apoE}^{-/-}$ and wild type (WT) mice over time. These animals were fed either standard chow diet or high fat diet (HFD) for 1, 2, 4, 8 or 12 weeks. ELISAs were used to determine whether the presence of Tfh cells and GC B-cells correlated with anti-malondialdehyde (MDA)-low density lipoprotein (LDL) antibody levels.

Results: The percentages of Tfh cells in the peripheral lymphoid organs of $\text{apoE}^{-/-}$ and WT mice fed chow diet remained consistently low at all time points. The percentage of Tfh cells significantly increased in HFD fed $\text{apoE}^{-/-}$ mice at early time points, in comparison to control mice (1 and 2 weeks). The number of Tfh cells then decreased at 4 and 8 weeks, and then reached the peak following 12 weeks of HFD. The percentage of GC B-cells peaked in both chow fed and HFD fed $\text{apoE}^{-/-}$ mice after 4 weeks of diet and coincided with the onset of plaque formation. The peak observed in GC B-cell number was followed by increased anti-Ox-LDL IgG2c titres, which peaked at 8 weeks. No differences in anti-MDA-LDL IgM or IgG1 titres were found.

Conclusions: Our results demonstrate that increased Tfh cell, GC B-cell and anti-MDA-LDL antibody levels correlate with atherosclerosis formation and progression. These results provide an interesting insight into the potential significance of Tfh cell modulation in atherosclerosis.

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Meta-analysis of AIRE regulated gene expression microarray data reveals a network of transcriptional interactions mediated by intermediate nodes

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The monogenic disorder Autoimmune Polyglandular Syndrome 1 is characterised by hypoparathyroidism, Addison's disease and chronic mucocutaneous candidiasis and is caused by mutations in the AIRE (Autoimmune regulator) gene. AIRE encodes for a transcription factor and is primarily expressed in the thymus where it regulates the promiscuous expression of tissue restricted antigens. Consequently AIRE has an important role in the process of negative selection in central tolerance.

AIRE regulated gene expression has predominantly been explored using mouse models, and has not been determined in a human thymic model. We therefore generated a recombinant AIRE expression variant of the TEC 1A3 human thymic epithelial cell line, using the Flp-In system of site-specific recombination. Microarray analysis revealed 512 genes to be differentially expression as a result of AIRE expression ($P < 0.05$, FDR $< 5\%$), which we validated by Q-PCR.

We then carried out a meta-analysis to determine a list of genes showing conserved AIRE/Aire regulated expression across different model systems using the repertoire of AIRE-regulated genes in our data set with those determined in four different data sets from Aire knockout mice of different genetic backgrounds, including C57BL/6, BALB/c and NOD. We identified 447 genes that were differentially expressed in our TEC 1A3 model system and at least three of the four mouse data sets. We subsequently performed *in silico* analysis of these conserved genes using EdgeExpress DB and revealed a large network of transcriptional interactions with a number of transcription factors, such as E2F6, IRF7 and STAT1, each having over 30 interactions with other AIRE/Aire regulated genes. This suggests that AIRE may operate via intermediate transcription factors to regulate the expression of its full repertoire of downstream genes.

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Induction of regulatory T cells by products of helminth parasite *Fasciola hepatica* as a preventative vaccine for autoimmune diseases

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Regulatory T (Treg) cells play a fundamental role in suppressing excessive inflammatory responses to pathogens and in maintaining peripheral tolerance to self-antigens. A breakdown in self tolerance or defect in Treg cells can result in uncontrolled effector T cell responses to self antigens and the development of autoimmune disorders. Conversely, approaches that enhance the generation of auto-antigen-specific Treg cells have potential for the prevention or treatment of autoimmune disease. The induction of Treg cells is enhanced by anti-inflammatory cytokines, with IL-10 and IL-27 promoting the generation of IL-10 secreting Tr1-type Treg cells and TGF- β with retinoic acid inducing peripheral conversion of naïve T cells into conventional CD4⁺CD25⁺Foxp3⁺ Tregs. Certain immunomodulatory molecules form bacteria or helminth parasite can modulate dendritic cells (DCs) to promote induction of Treg cells *in vivo*.

Finally, parasitic infections in humans are associated with lower incidence of allergy and autoimmune diseases. Previously, we have found that excretory-secretory (ES) products from helminth *Fasciola hepatica* have potential to attenuate experimental autoimmune encephalomyelitis (EAE). The aim of this study was to examine the capacity of ES products and soluble extract (SE) obtained from whole liver fluke *F. hepatica* to act as adjuvants to promote the induction of Treg cells against self antigen and thereby attenuate the clinical course of an autoimmune disease in a mouse model. Treatment of mice with a low molecular weight fraction of ES increased the frequency of Foxp3⁺ Treg cells, whereas SE enhanced frequency of IL-10-secreting and TGF- β -secreting T cells. Furthermore, vaccination of mice with neuronal self-antigen MOG and SE as an adjuvant before induction of EAE resulted in delayed onset of symptoms and attenuated the course of the disease. Flow cytometry analysis showed that immunization with MOG and SE significantly decreased the infiltration of pathogenic IL-17-producing T cells into the spinal cords and brains of mice following induction of EAE. These findings demonstrate the strong potential of using Treg-inducing vaccine approach to prevent T-cell mediated autoimmune diseases.

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Generation of human islet antigen-specific regulatory T cells for the treatment of type 1 diabetes

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Adoptive cell therapy has been applied to a number of disease settings where the transfer of regulatory T cell populations has proven effective at suppressing autoimmune disease in animal models. Therefore, this treatment approach shows potential for the control

of the autoimmune destruction of insulin producing islets in Type 1 Diabetes (T1D).

The transfer of large numbers of polyclonal natural Tregs (nTregs) has been shown to control disease in the NOD mouse model for T1D. However, the transfer of auto-antigen specific nTregs provides a considerably more efficacious suppression of disease; this in itself presents a problem for the transfer of this therapy to the human setting as nTregs are a rare subset of T cells and therefore, producing a large enough population with the desired specificity proves problematic.

In order to overcome the low frequency of nTregs with the desired specificity, the antigen reactivity of T cells can be re-directed via transfer of T cell receptor (TCR) genes using lentiviral vectors. Proof of principle work has shown the viability of this technique, primarily in the setting of cancer therapy. To apply TCR gene transfer to T1D the aim is to create lentiviral vectors encoding TCRs specific for auto-antigens involved in disease in order to deliver these genes to patient's nTregs.

The TCR sequences of auto-antigen specific Treg clones specific for epitopes of IA-2 and proinsulin, along with a control T-effector clone specific for an epitope of hemagglutinin, were determined and the alpha and beta chains of each assembled in to lentiviral vectors. Lentivirus was then produced for the three TCRs using 293T cells and used to transduce CD4 positive TCR negative and CD4 positive TCR positive Jurkats.

Upon transduction of TCR negative Jurkats with lentivirus, each TCR can be detected at the cell surface. Recent work has also found that in TCR + Jurkats the hemagglutinin TCR can be expressed and responds to its cognate peptide plus MHC complex. Therefore, this ectopically expressed TCR is able to compete, in Jurkats, with the endogenous TCR for signalling machinery without any optimisation of the TCR.

In summary, TCRs from three T cell clones have been successfully cloned and assembled into lentiviral vectors. In the case of the hemagglutinin specific TCR it has been shown that this TCR can be triggered in Jurkats by its cognate peptide and that this ectopically expressed TCR can signal in the presence of an endogenous TCR.

B Cells: Bridging the Innate and Adaptive Immune Systems

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Numerical and functional deficits in CD19⁺CD24^{hi}CD38^{hi} regulatory B cells in patients with coronary atherosclerosis: a novel immune defect in atherosclerosis

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Aim: T and B lymphocytes, the main cellular effectors of adaptive immunity, are deeply involved in the chronic inflammatory process that drives atherosclerosis. As the main source of antibodies, B cells were originally thought to be anti-atherogenic via production of neutralising antibodies against oxidised low density lipoprotein (LDL). Recent studies that depleted B lymphocytes in animal models of atherosclerosis have unravelled pro-atherogenic effects of conventional mature B2 B cells and protective roles for the innate B1a B-cell subset. Recently, B cells with regulatory function (Bregs) have also been identified in both animals and humans, but their role in atherosclerosis is currently unknown. Impairment of Bregs has been implicated in the pathogenesis of autoimmune disorders, but whether the same is true in atherosclerosis is yet to be determined. Our aim was to examine Bregs in patients with coronary atherosclerosis.

Methods: Bregs were defined as CD19⁺CD24^{hi}CD38^{hi} B cells. The percentage and absolute number of Bregs was quantified in the circulation of patients with myocardial infarction (MI, $n = 60$) and stable angina (SA, $n = 40$), as well as in healthy subjects ($n = 30$) using flow cytometry. The production of interleukin-10 (IL-10) by mature, memory and Breg cells was quantified by intracellular staining and flow cytometry.

Results: We found a marked reduction in the percentage and absolute number of Bregs in patients with coronary atherosclerosis (MI and SA) compared to healthy subjects. No differences were noted in the percentage and absolute number of total B cells, mature and memory B cells in the study groups, suggesting a specific numerical deficit in Bregs in patients with coronary atherosclerosis. Furthermore, Bregs from patients with coronary atherosclerosis produced significantly less IL-10 in response to CpG and CD40L compared to Bregs from healthy subjects, while the production of IL-10 from mature and memory B cells was not impaired. We are now characterising the molecular mechanisms that underlie the defects in Bregs in patients with coronary atherosclerosis.

Conclusion: Our data show for the first time that patients with coronary atherosclerosis harbour marked numerical and functional deficits in Breg cells that may tip the balance in favour of pro-inflammatory B and T lymphocytes. A better understanding of these defects may reveal novel targets for future therapies to quench the inflammatory process that drives atherosclerosis.

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Effect of T follicular helper cells on regulation of mucosal immunity to influenza haemagglutinin by CpG-DNA

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Background: Stimulation of the innate immune system is known to be important in the initiation and regulation of adaptive immunity.

Therefore, inclusion of some immunological adjuvants such as Toll-like receptor ligands, which trigger early innate responses to enhance the adaptive responses, is crucial to vaccine effectiveness. T follicular helper cells (T_{FH}) have been shown to be critical in germinal centre function and in the regulation of adaptive immunity. The aim of this study is to investigate whether and how CpG-DNA, a TLR-9 ligand, regulates mucosal immunity to influenza haemagglutinin through T_{FH} cells.

Methods: Mononuclear cells (MNC) were isolated from adenotonsillar tissue collected from children and adults and co-cultured with influenza antigens with or without CpG-DNA. B cell antibody production in tonsillar cells was analysed by ELISA. T_{FH} cells and effect of CpG-DNA on their function were analysed by flow cytometry and intracellular cytokine staining.

Results: Our results demonstrate that T_{FH} in tonsillar MNC express high levels of IL-4, IL-10 and IL-21 which are important for B cell antibody production. Stimulation of adenotonsillar MNC by CpG-DNA significantly increased the number of T_{FH} cells and that was correlated with the antigen-specific antibody production to haemagglutinin (HA) of seasonal influenza virus in adenotonsillar cells.

Conclusion: CpG-DNA promotes T_{FH} cells in nasal-associated lymphoid tissue which correlates with the enhancement of influenza HA-specific antibody production. Understanding the mechanisms by which TLR ligands regulate adaptive immunity through T_{FH} cells may lead to novel vaccines against respiratory infections.

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A novel pro-inflammatory B cell population infiltrating the rheumatoid synovium can be identified by expression of FcRL4+

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Background and aims: The importance of the B cell lineage in rheumatoid arthritis (RA) pathogenesis is highlighted by the clinical effectiveness of rituximab. Potential mechanisms by which B cells drive disease processes in RA include the ability to produce autoantibodies, act as antigen-presenting cells, and secrete cytokines. Our recent findings suggest a pro-inflammatory and destructive role for B cells via production of cytokines including RANKL, TNF- α and IL-6. A memory B cell subset normally resident in epithelial niches which is characterised by expression of the inhibitory receptor FcRL4 has previously been described to express RANKL. As we made the observation that B cells in the RA synovium produce RANKL, we sought to determine whether these RANKL-expressing cells belonged to the FcRL4⁺ B cell subset.

Methods: Synovial fluid, peripheral blood and synovial tissue samples were obtained from patients fulfilling 1987 ACR criteria for RA. Mononuclear cells isolated from synovial fluid and peripheral blood were stained with antibodies against CD19, CD20, CD27, IgD, CD11c, RANKL, FcRL4, CD95, CD21, CD80 and CD86, and analysed by flow cytometry. Mononuclear synovial fluid cells were stained with antibodies against CD19 and FcRL4 and sorted using a Mo-Flo[™] cell sorter. Taqman[™] microfluidic cards were used to detect mRNA expression of 48 cytokines by real-time PCR in these samples. Immunofluorescence staining was performed on frozen synovial tissue sections with antibodies against CD20, RANKL and FcRL4.

Results: FcRL4-expressing B cells, comprising 4–22% of the B cell population, were detected in the synovial fluid at a significantly higher frequency compared to matched peripheral blood ($n = 12$, $P = 0.0004$). RANKL was significantly enriched in the FcRL4⁺ B cell population compared to the FcRL4⁻ population ($P = 0.006$). FcRL4⁺ B cells were predominantly switched IgD-CD27⁺ memory B cells, expressing significantly higher levels of CD11c, CD20, CD95, CD80

and CD86, and lower CD21 levels than the FcRL4⁻ population. FcRL4⁺ B cells expressing RANKL were also present in synovial tissue ($n = 4$). At the mRNA level, sorted FcRL4⁺ B cells were found to express significantly higher levels of both RANKL and TNF compared to the FcRL4⁻ fraction ($n = 9$).

Conclusions: We have identified a novel subset of B cells in the RA synovium which is characterised by expression of FcRL4 and has not previously been described in RA or any other chronic inflammatory joint condition. FcRL4⁺ B cells produce RANKL and express high levels of TNF mRNA, indicating a destructive, pro-inflammatory role for this B cell subpopulation in RA pathogenesis.

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TLR7/8 agonists and type I IFN induce the differentiation of human IL-10-producing B cells into antibody-producing B cells via the TLR-MyD88-STAT3 pathway

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Introduction: IL-10-producing B cells function mainly by secreting IL-10. However, currently, the regulation of IL-10 production by IL-10-producing B cells and the fate of these cells are not completely understood. In addition to CD40-CD40L ligation and BCR triggering, increasing evidence has indicated the importance of TLR and type I IFN on the biology of B cells.

Aim: To understand:

- 1 the effect of TLR agonists and IFN α on IL-10 production by B cells;
- 2 the signalling pathways involved in the IL-10 production; and
- 3 the fate of B10 cells.

Methods and results: Here, we report that TLR7/8 signalling, but not CD40-CD40L ligation nor BCR triggering, was required for the induction of IL-10 production by human CD19⁺ B cells. Interestingly, IFN α greatly enhanced R848-induced IL-10 production. Stimulation with R848 and IFN α enhanced the phosphorylation of STAT3 in B cells and blocking the activation of STAT3 nearly completely inhibited R848-induced IL-10, but not IL-6, production, showing that TLR7/8 agonists induce IL-10 production by CD19⁺ B cells via the phosphorylation of STAT3. We and others have previously shown that the TLR-MyD88-STAT3 pathway governed antibody production, but not IL-6 production, by B cells upon TLR stimulation. This suggests that the TLR-MyD88-STAT3 pathway is responsible for both IL-10 and antibody production in B cells. Interestingly, upon R848 and IFN α stimulation, the level of IL-10 produced peaked on day 2–4 and decreased afterwards, whereas antibody production appeared from day 4. Therefore, we hypothesized that regulatory B cells differentiate into antibody-producing B cells upon TLR and IFN α stimulation. Indeed, FACS sorted IL-10⁺ B cells produced relatively high levels of antibody, whereas antibody was barely produced by the IL-10⁻ B cells. In line with this, CD38⁺CD27⁺ plasmablasts or plasma cells were only observed in the IL-10⁺, but not in the IL-10⁻, population.

Conclusions: We show in this study that the TLR-MyD88-STAT3 pathway is required for the induction of IL-10 by B10 cells and IFN α increased TLR-induced IL-10 production via enhancing the activity of the TLR-MyD88-STAT3 pathway in B cells. The TLR-MyD88-STAT3 pathway governs both IL-10 and antibody production in B cells. Upon stimulation, regulatory B cells differentiate into antibody producing B cells. Thereby, regulatory B cells limit inflammation and immune responses by the transient production of IL-10, and may facilitate clearance of antigens through the capacity of generating antibody-producing B cells.

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TLR7-elicited regulatory B cells use IL-10 to suppress airway inflammation through induction of CD4⁺FoxP3⁺ T cells

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Traditionally, the role of B cells in disease was thought to be restricted to that of antibody production. Recently, the ability of B cells to negatively regulate cellular immune responses and inflammation has been described and the concept of regulatory B cells (Breg) proposed. A variety of cytokines produced by Breg subsets have been reported, with IL-10 being the most studied (B10 cells). Concurrently, it has emerged that helminths are potent modulators of the immune system using a diverse range of mechanisms, one of which is the generation of B10 cells. These helminth-induced B10s have been shown have suppressive activity and ameliorate inflammation in several murine models of inflammation.

Using, microarray technology we have identified genetic characteristics of *Schistosoma mansoni*-elicited Breg that produce IL-10. With respect to innate activation of these cells, toll-like receptor 7 (*Tlr7*) was significantly upregulated. Ligands of TLR7 both *in vitro* and *in vivo* stimulated the generation of B10s – with TLR7-elicited B10s displaying a similar phenotype to helminth-elicited B10 cells. In a mouse model of allergic lung inflammation the use of either TLR7 ligands to induce B10 cells *in vivo* or the adoptive transfer of *in vitro* generated B10 cells both reduced airway inflammation and improved lung function. We further investigated if TLR7-elicited B cells suppress inflammation via the expansion of T regulatory cell responses in an IL-10 mediated mechanism. The study of helminth-immune interactions can yield novel means of generating regulatory B cells and, furthermore, provide new insights into their functions in inflammation.

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The role of the p110 δ subunit of PI3K in the development of regulatory B cells

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The p110 δ catalytic subunit of PI3K is highly expressed in haematopoietic cells, and has been shown to be an essential, non-redundant regulator of B cell development and activation. Mice lacking p110 δ have reduced numbers of B1 and marginal zone B cells, and are defective in B cell antigen receptor signalling. It is thought that the p110 δ catalytic isoform of PI3K is also important for the development of regulatory B cells and in the molecular regulation of cytokine production. Regulatory B cells have recently been recognised as an important component of the immune system, and have been shown to have protective roles in animal models of autoimmune disease by suppressing excessive inflammatory responses through the production of the anti-inflammatory cytokine IL-10. A subset of B cells, which have a rare CD1d^{hi}CD5⁺CD19^{hi} phenotype, have been designated B10 cells, and are responsible for most IL-10 production by B cells. In order to study the role of PI3K in regulatory B cell

development, tissue specific gene targeting has been used to generate mice in which p110 δ is deleted in early B cell development. These mice lack peritoneal B1 cells, and marginal zone cells, and are deficient in the regulatory B10 cell subset. Despite this, there was no difference between p110 $\delta^{\text{fl/fl}}$ mb1^{cre/+} mice and p110 $\delta^{\text{fl/fl}}$ control mice in the inflammatory model contact hypersensitivity.

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The role of hedgehog signalling in B cell development

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Background and aims: Morphogens are signalling molecules that determine cell fate and organogenesis. They diffuse from a localised source forming a concentration gradient. Hedgehog protein is a morphogen that is essential for the determination of cell fate in a wide variety of tissues in different species. There are three homologues of hedgehog protein, Sonic, Indian and Desert hedgehog protein. Hedgehog proteins signal via a similar pathway involving two receptors, Patched and Smoothened. Hedgehog signalling has been shown to be crucial for T cell development but the role it plays in B cell development is still unclear. The aim of this study is to further characterise the molecular events that occur downstream of Hedgehog signalling in B cell development.

Method: Purified mouse B cells were stimulated with antiCD40/IgM and cultured with recombinant Sonic Hedgehog protein (rShh) for 18 and 40 h before analysis for expression of cell-surface markers for activation, differentiation and survival.

Results: At 18 h, co-culture with rShh enhances activation of B cells together with their enhanced survival resulting in the appearance of increased numbers of B220+CD23+CD25- B-cells. At 40 h, co-culture with rShh enhances differentiation of B cells along a B220+CD25+CD27+CD23- lineage. The B-cells are also found to be dying more in the presence of rShh as judged by annexin staining. Additionally, antibody production in the presence of rShh is increased at 18 h compared to stimulated control and at 40 h there is no further increase in antibody production in the presence of rShh thus suggesting a negative regulatory effect of Hh signalling at this time point. This negative regulatory effect of hedgehog protein has not been reported before.

In order to determine the effect of absence of Hh protein in this system, B cells were isolated from desert knockout mice and analysed as previously described. B cells from Dhh-/- mice were found to be spontaneously activated as judged by CD23 expression compared to littermate controls. At 40 h, B-cells isolated from Dhh-/- mice were found to express enhanced levels of CD23 and less CD25 and CD27 compared to littermate control.

Conclusion: These data indicate that hedgehog signalling may exert both a positive and negative regulatory effect on B cell development at different time points. It is possible that the enhanced numbers of CD25+ B cells appearing in the presence of rShh are either regulatory B cells or memory B cells. The molecular events underpinning these observed effects are yet to be determined.

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Corticosteroid withdrawal normalises the proportion of transitional B cells but does not significantly change gene expression in renal transplant recipients

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Background and aims: Following renal transplantation all patients receive mandatory immunosuppression therapy. However, the use of these pharmaceuticals long term has been linked with increased mortality and morbidity and despite their use, acute rejection is still occurring. Thus, even after 30 years experience with current immunosuppressants, the necessary level of therapy each patient needs is still largely unknown and thus controlled with blood levels or patient weight, which we know is inaccurate. In addition, the discovery of a subset of patients who maintained good graft function in the absence of immunosuppressants allowed for the characterisation of these 'operationally tolerant' patients, who showed differential gene expression and B lymphocyte expansion. These findings led to the development of a signature of tolerance to potentially identify those patients that would be safely maintained with therapy adjustments.

We aimed to study the effect of immunosuppressants on the expression of this signature of tolerance. An available clinical model was used to focus on the effect of corticosteroids, whereby we analysed the results from a set of patients undergoing corticosteroid withdrawal. We aimed to demonstrate that corticosteroid withdrawal would not alter the expression of the signature of tolerance.

Methods: Forty-two renal transplant recipients from the GAMBIT study (REC no: 09/H0713/12) were studied prospectively on a programme of corticosteroid withdrawal. Flow Cytometry was used to assess lymphocyte populations and RT-PCR was used to assess the gene expression associated with the tolerant state in isolated PBMCs from a limited number of patients.

Results: Percentages of Transitional B cells in peripheral blood were significantly increased in patients who successfully underwent corticosteroid withdrawal. However, only one out of 12 of the genes included in the signature of tolerance was significantly different in a group of the same patients.

Conclusion: This is the first evidence that levels of corticosteroid dose uniquely affect transitional B cells and also α -1,2 mannosidase gene expression, both important biomarkers in the characterisation of tolerance. Therefore it can be concluded that for the full clinical utility of the biomarkers of tolerance to be realised, and translation to the clinic achieved, patients might need to be stratified according to their corticosteroid dose. Therefore, a larger study, including more patients should be conducted in which patients are categorised according to their corticosteroid dose, and standardised in regard to the other immunosuppressants they are receiving, to cement the findings in this study.

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IL-21 synergistically enhances TLR-MyD88-STAT3 pathway but not the conventional TLR-MyD88-NF- κ B pathway in human B cells, resulted in increased antibody production

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Introduction: Both IL-21 and TLR signaling are important regulators of B cells responses and the combination of IL-21 and TLR stimulation results in an increased antibody production by B cells. However, it is not clear yet how IL-21 interacts with TLR signaling in B cells.

Materials and methods: To understand the interplay between IL-21 and TLR signaling in human B cells, we isolated human total CD19-positive B cells from buffycoats and stimulated these cells with different TLR agonists in the presence or absence of IL-21. The interaction between IL-21 and TLR signaling was studied by analyzing antibody and cytokine production as well as the activation of TLR downstream signaling pathway using flow cytometry and immunoblotting.

Results: We show that IL-21 enhances TLR-induced IgG production by human B cells while no effect on TLR-induced IL-6 production. Intriguingly, stimulation of B cells with IL-21 and TLR7/8 or TLR9 agonists synergistically increases of the phosphorylation of STAT3 in human B cells while the activation of NF- κ B is not affected. These results indicate that the enhanced TLR-induced IgG production following IL-21 stimulation is explained by the modulation of the TLR-MyD88-STAT3 pathway, but not the conventional TLR-MyD88-NF- κ B pathway. Interestingly, like IL-21, IL-10 in combination with TLR signaling also enhances phosphorylation of STAT3 in B cells, allowing optimal IgG production.

Conclusion: IL-21 synergistically enhances the TLR-MyD88-STAT3 pathway but not the conventional TLR-MyD88-NF- κ B pathway explaining the selective effects on antibody production without increasing cytokine-production of human B cells.

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Immunoglobulins drive terminal maturation of splenic dendritic cells

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Nature and physiological status of antigen-presenting cells, such as dendritic cells DCs, are decisive for the immune reactions elicited. Multiple factors and cell interactions have been described that affect maturation of DCs. Here, we show that DCs arising in the absence of immunoglobulins (Ig) *in vivo* are impaired in cross-presentation of soluble antigen. This deficiency was due to aberrant cellular targeting of antigen to lysosomes and its rapid degradation. Function of DCs could be restored by transfer of Ig irrespective of antigen specificity and isotype. Modulation of cross-presentation by Ig was inhibited by coapplication of mannan and, thus, likely to be mediated by C-type lectin receptors. This unexpected dependency of splenic DCs on Ig to cross-present antigen provides insights into the interplay between cellular and humoral immunity and the immunomodulatory capacity of Ig.

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Investigation of the effects of cigarette smoke on immunoglobulin levels in saliva and serum, in relation to COPD

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Cigarette smoke has many damaging effects on the body. These include inflammatory damage to the lungs, which can lead to the development of chronic obstructive pulmonary disease (COPD). Chronic obstructive pulmonary disease can be defined as the permanent damage the airways of the lung. It is estimated that it affects approximately three millions people in UK. Although not all smokers are susceptible to COPD, it is predominately caused by smoking.

The immunological effects of cigarette smoke are being investigated and provide essential insight into mechanisms of smoking related disease. The aim of this study is to determine whether the levels of immunoglobulin (Ig) isotypes are different in the serum and saliva specimens of non-smoking individuals and smoking individuals (with or without COPD). Examination of serum and saliva will provide information on the effects of cigarette smoke systemically and in the upper respiratory tract, respectively.

In order to determine Ig isotype levels, enzyme-linked immunosorbent assays (ELISAs) have been established and calibrated for determining sample concentrations of IgM, IgG, IgA and IgD.

If cigarette smoke is found to influence the serum and salivary levels of Ig isotypes, then the mechanisms underlying these effects will be investigated.

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Aged B cells outperform young B cells in old and as well young immunological environments

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Immunosenescence is a well known phenomenon of old age. Our ability to produce adequate responses to vaccination declines dramatically as we enter our 70 s. How B cells are affected by aging remains unclear. Different B cell subsets have been shown to increase as we age and are also associated with autoimmunity. In addition mRNA stability has been shown to decrease. Here we examine the contributions of the splenic environment compared to that of the intrinsic effects of aging on B cells. Using young (~8 weeks) and old (>18 months) host and donor mice, in conjunction with NP-binding B cells from quasi monoclonal mice (QM, specific for the hapten NP) we investigated the response to a T cell independent antigen: NP-Ficoll. In the presence of high frequencies of QM B cells this antigen induces extrafollicular plasma cell differentiation and short lived germinal centres. In both the young and old hosts B cells from old donors produced more antibody and longer lived germinal centres than their young counterparts. This was associated with an increase in serum IgM and higher Aicda expression in germinal centre B cells. Our data also show that the aged environment is repressive to immune responses of young B cells pointing to both cell intrinsic effects of aging as well as effects of the changing milieu.

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A novel role for the atypical chemoattractant receptor CCRL2 in the B cell antibody response

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Background and aims: The chemerin receptor CCRL2 is a member of the atypical chemoattractant receptor (ACR) family. ACRs do not signal upon ligand binding, but instead act to regulate the response to their ligands. We aim to dissect the role of CCRL2 in the antibody response.

Methods: WT and CCRL2 KO mice are immunised with specified antigen and the antibody response analysed by ELISA, flow cytometry and histology. To dissect which cells influence the phenotypes seen, we use irradiation bone marrow chimeras, B cell receptor transgenic mice and *in vitro* stimulation of cells.

Results: Compared to wild type mice there is no difference in the amount of antibody produced upon primary immunisation of CCRL2 deficient with the thymus-dependent antigen NP-CGG. However, in response to the thymus-independent antigen NP-Ficol CCRL2 deficiency leads to increased antibody levels in the blood and increased plasma cell numbers in the spleen.

Although CCRL2 has previously been shown to be expressed by endothelium, irradiation bone marrow chimeras indicate that CCRL2 deficiency in haematopoietic cells is responsible for the observed phenotype. Furthermore, transfer of CCRL2 KO NP-specific B cells into WT hosts results in increased antibody levels compared to transfer of their WT counterparts. Interestingly, CCRL2 KO B cells undergo increased proliferation *in vitro* upon stimulation with IL-4 and LPS.

Conclusions: Altogether our results suggest that CCRL2 expression plays a role on B cells, and deficiency of CCRL2 can lead to increased B cell responses. This indicates a new role of CCRL2 in the regulation of the plasma cell response.

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NF- κ B and cMyc cooperate to induce B-cell transformation and plasma cell neoplasia

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Mature B-cell derived cancers remains a challenge in medicine. Activating NF- κ B mutations and increased cMyc expression are recurrent events in the ABC-subgroup of Diffuse Large B-cell Lymphoma (ABC-DLBCL), and in Multiple Myeloma (MM). However, it unknown whether NF- κ B signalling and cMyc cooperate to induce B-cell transformation and are at the origin of ABC-BLCL and/or MM.

To test this hypothesis we used conditional targeted mutagenesis in the mouse, to express a constitutively active IKK2 molecule and/or cMyc in CD19pos B-cells through tamoxifen-induced Cre-mediated elimination of a STOP-cassette. This genetic system is unique in two ways: first, transgene activation occurs in a small fraction of CD19pos B-cells (3-5%) through transient Cre expression, mimicking the sporadic nature of oncogenic events; second, due to incomplete Cre-mediated recombination in individual cells, the CD19+B-

cell population is constituted by IKK2capos/MYCpos, IKK2capos/MYCneg and IKK2caneg/MYCpos expressing cells, providing all possible 'oncogene' combinations in the same mouse at the same time.

While the fraction of IKK2capos/MYCneg and IKK2caneg/MYCpos B-cells respectively remained constant or decayed over time, the fraction of IKK2capos/MYCpos B-cells increased and eventually, 100% of the mice succumbed to a plasma cell neoplasia derived from this population.

Thus, using a conditional targeted mutagenesis system that recapitulates the sporadic nature of oncogenic events, we demonstrate that NF- κ B cooperates with cMyc to induce B-cell transformation and MM in the mouse. These results suggest that this oncogene combination represents a primary event in MM rather than ABC-DLBCL.

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Investigating the role of B cells in periodontitis

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Epidemiological evidence suggests periodontitis (PD) is more common in rheumatoid arthritis (RA) patients. B cells play key roles in the pathogenesis of both diseases, including production of antibodies and induction of osteoclastogenesis by expression of receptor activator of nuclear factor kappa-B ligand (RANKL). Therefore, investigating B cells in PD could be useful for developing specific B cell targeted therapies with applications in PD and RA.

Samples of gingival tissue from eleven patients with periodontitis were collected during periodontal surgery. B1a cells (CD19+CD5+) which are more likely to become autoreactive, and plasma cells (CD138+) producing IgA or IgG were quantified by immunohistochemistry. B cell subsets were investigated in a murine model of PD induced by oral infection with *Porphyromonas gingivalis*. Six weeks post infection, alveolar bone loss is clearly evident around the teeth. The proportions of mature B cell subsets (B2 marginal zone (MZ), B2 follicular (FO), B1a and B1b) were determined in gingiva, draining lymph node (dLN), peripheral blood and peritoneal fluid by flow cytometry. B cell phenotype with respect to RANKL expression was also investigated.

Large numbers of B cells and plasma cells were found in human PD patient gingiva, but CD19+CD5+ B1a cells constituted <1% of the total number B cells. In the blood and peritoneal fluid of mice with PD compared with controls, there was a small increase in B2 MZ cells (CD19+CD43-CD23-), a decrease in B2 FO cells (CD19+CD43-CD23+) and no difference in the proportions of B1a cells. In the gingiva of mice with PD, there was a trend towards increased numbers of B1a (CD19+CD43+CD5+), B1b (CD19+CD43+CD5-) and B2 MZ cells and a decrease in B2 FO cells, compared with control mice. In the dLN of animals with PD, B1a and B1b cells expressed significantly more RANKL than B2 MZ and B2 FO cells ($P < 0.01$, One-Way ANOVA). A similar pattern was observed in gingival B cell RANKL expression, which was highest in B1a cells.

In conclusion, the proportion of B1a cells was not significantly altered in the gingiva of human PD patients, or systemically in mouse models of PD. However, B1a cells express more RANKL than other mature B cell subsets in the gingiva and dLN of mice with PD. It remains to be determined whether this particular feature of B1a cells plays an important role in the disease process.

Cancer Therapy through Immune Modulation

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A comparison of anti-CD20 chimeric antigen receptors in targeting B-cell malignancies

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Chimeric antigen receptors (CARs) are constructs typically encompassing an antibody single chain variable fragment (scFv) linked via a spacer region to a transmembrane domain allowing surface expression on effector cells. The intracellular region includes activation domains of co-stimulatory molecules to induce effector cell function. Previous studies have shown CARs can elicit potent anti-tumour responses against a variety of B cell malignancies. However, the mechanisms of CAR function and factors determining efficacy remain largely unknown.

Anti-human CD20 scFvs were generated by PCR, and purified using an anti-HA column. These comprised heavy and light variable chains derived from the antibodies Leu16, rituximab and B-HH2. CARs were generated using these domains, along with an IgG-Fc spacer and CD4 transmembrane region. Three signalling regions were included in the intracellular region; the CD3 ζ chain and the activation domains of CD28 and 4-1BB. The CARs were expressed transiently on 293F HEK cells and shown to bind CD20 successfully. These interactions have been demonstrated to be specific using a blocking assay. A difference in binding efficiency was observed between the various CARs. Retroviral transduction of the CAR constructs into a T cell reporter line has demonstrated activation upon CD20-CAR interaction *in vitro*. Activated splenocytes have also been transduced with the CAR constructs and these will be transferred into a humanCD20 transgenic mouse model to investigate their ability to mediate B-cell depletion *in vivo* and compare the different CARs. CAR-T-cell proliferation and *in vivo* survival will also be evaluated.

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Combined TLR and CD40 targeting in tumour therapy

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Background: Monoclonal antibody (mAb) treatments for cancer are expensive as generally the whole system must be flooded with costly mAb. We discovered fortuitously that a combination of CD40mAb (clone 10C8) with the TLR-4 agonist MPLA eliminates B cell lymphoma in a therapeutic setting, without antigen, at a low (10 μ g) dose of mAb. This study aims to determine whether this is a property general to CD40mAb of different isotypes and whether other TLR agonists would be equally effective as a combination therapy for the treatment of B cell lymphoma.

Materials and methods: BALB/c mice were inoculated subcutaneously on the right flank with 10^5 A20 cells (murine B cell lymphoma) on day 0 and thereafter mice were treated with 10 μ g CD40mAb alone or in combination with 50 μ g poly I:C (TLR-3), 10 μ g MPLA or PHADTM – the synthetic form of MPLA (TLR-4), and 50 μ g CpG (TLR-9) on days 1 and 8 after tumour challenge. Tumour size was measured every 3 days to daily and survival monitored for a period of 60 days.

Results: Three clones of CD40mAb (1C10, 10C8 and 4F11) prevented A20 tumour cell growth to the same extent. Combination therapy of CD40mAb with the TLR-9 agonist CpG was more effective than the TLR-4 agonists MPLA and PHADTM or the TLR-3 agonist Poly I:C. CD40mAb and CpG combination therapy injected on the same flank as the tumour site resulted in higher survival than when injected on the opposite flank.

Discussion: The fact that all CD40mAb clones tested were equally effective at preventing tumour growth implies that the effect of CD40mAb is not dependent on epitope or subclass. However, the proximity of the injection site to the tumour is of importance for effective tumour prevention in this model, as combination therapy given on the same flank as the tumour site resulted in a better outcome. Ongoing studies aim to determine the mechanism of action of this combination therapy through the *in vivo* depletion of CD4 and CD8 T cells using antibodies and of macrophages using chlodronate liposome therapy prior to tumour inoculation.

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Molecular mechanisms of endothelial cytoprotection by CD31-mediated signals

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Background: CD31 is a member of the immunoglobulin gene superfamily expressed by leukocytes and endothelial cells (ECs). CD31 can engage in homophilic interactions both in EC:EC junctions and in EC:leukocyte interactions, thus regulating leukocyte trans-endothelial migration. Recent studies have demonstrated a wider range of functions for this versatile molecule including participation in maintenance of adherent junction integrity and permeability, regulation of catenin localization and other transcriptional activities. CD31 signals can protect the endothelium from pro-apoptotic stimuli, such as those mediated by inflammatory cytokines, including TNF-alpha, although the molecular mechanism is still largely unknown.

Results: Here we show that CD31 functions prominently in inhibiting apoptosis induced by TNF-alpha and T cell induced cytotoxicity but has no significant effect on cell lysis induced by complement fixing antibodies. CD31-afforded protection is dependent on the activation of AKT and ERK signalling pathways. In addition, CD31 engagement was found to be associated with increased levels of c-Flar (CASP8 and FADD-like apoptosis regulator). These data suggest the possibility that the pro-survival activities of CD31 are mediated via the c-Flar-dependent activation of Akt. Furthermore, amino acid substitutions within CD31 cytoplasmic ITIM at 663 and 686 positions completely abolished CD31-mediated cytoprotection.

Conclusion: Taken together, these results implicate a CD31-mediated protection of the endothelium from cell death induced by pro-inflammatory stimuli via the triggering of AKT and Erk pathways. Isoforms of CD31 containing point mutations in the ITIM motifs (Y663 and Y686) and the recruitment of SHP1/2 to these domains have been showed to be important in these pro-survival signals.

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Tumour-infiltrating T-helpers and regulatory-cells in patients with breast cancer from Iraq

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Introduction: The contributions of Th1 and Th17 involvement in breast tumour progression are the subject of intense investigation in Iraq.

Aim: To investigate the tumour-infiltrating inflammatory cells in breast tumours, focusing on transcription factors defining effector (Th1 and Th17) and T-regs (Foxp3), using an antigen retrieval immunohistochemical method on archived breast tumour tissue (before treatment), and compared it with benign tumour condition.

Patients and methods: Fifteen paraffin-embedded breast tissue were obtained from patients with malignant tumour (Invasive forms). Fifteen control breast tissue were obtained from patients with benign breast tumour. Antigen retrieval from deparaffinized breast tissue was obtained through the combined use of high temperature and appropriate buffers. Expression of T-bet, GATA3, IL-17 and Foxp3, markers of Th1, Th2, Th17 and Treg cells, was determined using a previously defined semi-quantitative immunological scoring system using a polymeric conjugate system (EnVision, Dako). Expression of CD4, CD8 and CD68 was also determined. Intra-tumour lymphocytic infiltrate was assessed semi-quantitatively.

Results: Prominent CD4, CD8, CD68, T-bet, Gata 3, Foxp3 and IL-17 positivity were present in ducts, lobules and connective tissue of the breast tissue of both malignant and benign forms. However, T-bet, CD-4, CD-8, CD68, IL -17 and *FOXP-3* positive cells except GATA-3 were significantly higher in patients with malignant tumours than in benign forms ($P < 0.01$). *FOXP3*, Th1 and Th-17 staining of mild to moderate severity were present in malignant group in both invasive ductal and lobular carcinoma. Th1 staining cells in malignant tumour are significantly correlated with Th17 and CD68 staining cells ($P < 0.02$).

Conclusion: The expression of T-bet, Th17, GATA-3, Foxp3 and CD68 indicates that acquired and innate immunity is involved in both conditions of breast tumour. The higher expression of Th1, Th17, Foxp3 and macrophages in patients with malignant tumour suggests that they are involved in progression of tumour.

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Induction of immunogenic cell death by anti-CD20 antibodies

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Background and aims: The anti-CD20 monoclonal antibody (mAb) rituximab has significantly improved the outcome for patients with B-cell malignancies and has led to the development of a number of other anti-CD20 mAb. We have recently characterised the anti-CD20 mAb GA101 (Obinutuzumab) as a type II anti-CD20 mAb which induces direct Programmed Cell Death (PCD) through a novel non-apoptotic pathway involving actin reorganisation, lysosomal permeabilisation and ROS generation. We investigated whether this PCD was immunogenic and compared it to tumour cell death initiated by the type I rituximab via complement dependent cytotoxicity.

Methods: Human B-lymphoma cell lines including Raji and Daudi were incubated with GA101 or rituximab with and without human serum and supernatants (s/n) analysed for presence of HMGB1, heat shock proteins and ATP. Human immature dendritic cells were cultured for 48 h in the presence of supernatant taken from GA101 treated Raji cells and DC were phenotyped for upregulation of CD83 and CD86. GA101 s/n matured DC were cultured with allogeneic T-cells in a 5 days MLR to measure T-cell proliferation.

Results: PCD induced by type II GA101 led to the release of the damage associated molecular pattern molecules (DAMPs) HSP60, HSP90, HMGB1 and ATP from lymphoma cell lines. DAMP release occurred as a result of the PCD pathway as shown by dependence on actin reorganisation and ROS generation. No DAMP release was seen following culture of rituximab with tumour cells, most likely

due to the lack of PCD induction by rituximab. However, rituximab was able to induce cell death via complement dependent cytotoxicity which was associated with the release of HMGB1 through an actin-independent pathway. Importantly, supernatant from GA101 treated Raji cells was able to induce dendritic cell maturation and subsequent T-cell proliferation.

Conclusions: Despite the successes of anti-CD20 mAbs in the clinic, the precise mechanism of action in patients is unclear. Generation of a cellular immune response post anti-CD20 mAb therapy is an important strategy for improving responses. We have demonstrated that the novel cell death induced by type II CD20 mAbs such as GA101 is a form of immunogenic cell death typified by the release of DAMPs such as HMGB1, HSP90 and ATP. Importantly this cell death can induce dendritic cell maturation and T-cell proliferation and as such may enhance the generation of T-cell responses post antibody therapy.

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Head and neck cancers: human papilloma virus and tumour angiogenesis

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Background and aims: Head and neck squamous cell cancers (HNSCC) are aggressive tumours and survival rates remain poor in spite of advances in surgical and oncologic approaches. Recently, the oncogenic human papilloma virus (HPV) has been associated with 60–70% of oro-pharyngeal cancers. HPV-positive HNSCC are known to carry a better prognosis than HPV-negative tumours. However the reasons behind this are not understood. The aim of our work was to dissect the impact of HPV on angiogenesis in HNSCC with the long-term goal to identify novel therapeutic targets.

Methods: We analysed 18 newly diagnosed HNSCC patients and controls. Patients with inflammatory disorders that could potentially alter the immune status were excluded from analysis. HPV tumour status was ascertained by p16 staining. Eleven pro- and anti-angiogenic factors were quantified in sera using a multiplex assay. In addition, angiogenic factors were analysed in the tumour tissue using immunohistochemistry.

Results: We found that circulating levels of anti-angiogenic factor endostatin was higher in all HNSCC patients compared to healthy subjects. No difference in endostatin was noted between HPV-positive and negative patients. Notably, we detected higher levels of the proangiogenic factors angiopoietin-1 and vascular endothelial growth factor (VEGF) in the circulation of HPV-negative patients compared to patients with HPV-positive tumours. We next analysed these angiogenic factors in the tumour tissue. VEGF was expressed by tumour cells as well as stromal cells. There was no difference in VEGF expression between HPV-positive and negative tumours *in situ*. Interestingly, angiopoietin-1 was differentially expressed in HPV-negative and HPV-positive tumours: whilst in HPV-positive tumours angiopoietin-1 was only expressed in tumour cells, in HPV-negative tumours angiopoietin-1 was found both in tumour and stromal cells. Another interesting finding was that macrophages in HNSCC had an M2 'alternatively activated' phenotype and appeared to be the major source of endostatin in the stroma.

Conclusions: Our novel results indicate that angiogenic factors are differentially expressed in the circulation and tumour tissue in patients with HPV-positive and HPV-negative HNSCC. We found a

marked difference in circulating levels and pattern of *in situ* expression of pro-angiogenic factors VEGF and angiopoietin-1, which could potentially confer a more aggressive phenotype to HPV-negative tumours. In particular, we identify stromal cells (i.e. M2 macrophages) as an important source of angiogenic factors. Better understanding of the mechanisms by which HPV induces changes in tumour stroma may reveal novel therapeutic strategies to tackle these aggressive cancers.

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Extracellular ATP promotes immunosuppressive mesenchymal stromal cells (MSCs) by enhancing their kynurenine production and increasing their metabolic activity: impact of necrosis on immunosuppressive MSCs

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Background and aims: Mesenchymal stromal cells (MSCs) are known to act as immunosuppressive cells, partially due to the expression of the enzyme indoleamine dioxygenase (IDO) which converts tryptophan to kynurenine. Decreased concentration of tryptophan and increased kynurenine, both inhibit lymphocyte proliferation and promote a shift from Th1 to Th2 immune response. Accumulation of MSCs within tumor tissue is associated with tumor progression and poor prognosis.

Necrotic cell death with subsequent release of damage associated molecular patterns (DAMPs) is a characteristic feature of advanced solid tumors. ATP is a DAMP family member as it leaks out of the cytoplasm when cell membrane integrity is perturbed. Hence, unphysiologically increased concentrations of ATP is found around stressed and necrotic (tumor) tissue.

We have already shown that DAMPs act as chemoattractants on MSCs also promoting their proliferation. We now sought to determine if DAMPs have any impact on immunosuppressive capacities of MSCs with specific regard to IDO-activity.

Methods: To *in vitro* expand MSCs, heparinized bone marrow from healthy donors was cultured for 14 days in alpha-MEM supplemented with 10% platelet lysate. Fibroblastoid-shaped adherent cells with capacity for chondrogenic, osteogenic, and lipogenic differentiation and positive for CD73, CD90, CD 105 and HLA-A, B, C and negative for CD34, CD3, CD45 were referred to as MSCs. In the presence of ATP at concentrations between 62 and 2000 μ M, MSCs were cultured for 4 days in DMEM containing 10% human serum and 100 μ g tryptophan/ml. Supernatants were tested for kynurenine in a colorimetric assay, as described previously. Metabolism was assessed by measuring WST-1 cleavage. MSC proliferation was measured by using CyQuant detecting DNA-content.

Results: Following *in vitro* stimulation of MSCs with ATP, we demonstrate an increased kynurenine concentration within the supernatant, in a dose depended manner. Of note, ATP intensely enhanced MSC metabolism without having any influence on their proliferation.

Conclusions: We characterized ATP as a DAMP family member responsible for necrosis-induced immunomodulation. Given the increased concentration of DAMPs within tumor tissue and the fact that necrotic material/DAMPs can act as chemottractants to MSCs, our results have implications for therapeutic strategies targeting the tumor microenvironment.

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Increased levels of myeloid derived suppressor cells in pancreatic adenocarcinoma patients

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Introduction: Pancreatic adenocarcinoma remains one of the deadliest human cancers with poor prognosis as it often presents at an advanced stage. While the role of immune suppressive cells in pre-clinical studies has helped to generate immunotherapeutic agents, these cells remain under investigated in pancreatic cancer (PC). Myeloid derived suppressor cells (MDSC) are a heterogeneous group of immature myeloid cells that negatively regulate the immune responses during tumour progression, inflammation, and infection. The aim of this study was to characterise the different subsets of MDSCs and evaluate their levels and functions in the circulation and tissue of PC patients.

Methods: Flow cytometric staining was performed on peripheral blood samples of 24 PC patients, 12 patients with chronic pancreatitis (CP) and 16 age-matched healthy donors. Tumour ($n = 7$) and benign ($n = 7$) pancreatic tissue samples were also examined for comparative analysis.

Results: A significant increase in the frequency of circulating and tumour infiltrating granulocytic (Lin-HLA-DR-CD33+CD11b+CD15+), but not monocytic (Lin-HLA-DR-CD14+) MDSC was observed in PC when compared with healthy donors and matched benign pancreatic tissues. The circulating MDSCs from PC patients express arginase 1 (ARG 1), which represent the functional state of these cells. MDSC levels, in peripheral blood, showed no association with PC stage or pre-operative levels of tumour markers.

Conclusions: Our findings provide a first characterisation of the phenotype of different subsets of peripheral and local MDSCs in PC patients and suggest that the frequency and contribution of these cells are predominantly granulocytic. This information demonstrates that MDSCs have a role in pancreatic cancer and it may, if incorporated into future large validation studies, help to evolve new immunotherapeutic strategies via inhibiting and eliminating MDSCs in PC.

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The role of antigen-presenting cell subsets in directing anti-lymphoma immune responses to radiation induced tumour cell death in combination with anti-CD40 monoclonal antibody

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Background and aims: Radiation therapy (RT) is administered to around 50% of all cancer patients making it one of the most important treatment modalities for cancer. Recent evidence demonstrates that in addition to the direct cytoreductive effect, RT-induced tumour cell death can also be immunogenic. We have previously demonstrated that the immune response to RT-induced tumour cell death can be substantially enhanced by combination with α CD40 mAb therapy through the generation of tumour antigen specific

CD8⁺ cytotoxic T lymphocytes (CTL). However, it remains unknown how the different sub-sets of antigen presenting cells (APC) frequently found to infiltrate established tumour impact on the priming of these tumour specific CD8⁺ CTL following therapy and whether anti-tumour responses can be improved by manipulation of these tumour-resident APC populations.

Methods: Mice were inoculated subcutaneously with either B (A20 and BCL1) or T cell (EL4 and EG7) lymphoma cell lines. Dendritic cells (DC) depletion was achieved using the CD11c-Diphtheria Toxin Receptor (DTR) transgenic mice. B cell (for non-B cell lymphomas) and macrophage depletion was achieved by administration of α CD20 mAb or clodronate encapsulated liposomes respectively. Radiotherapy was delivered either alone or in combination with a single dose of α CD40 mAb when tumours were established and measured at least 100 mm³.

Results: Combination therapy involving RT and α CD40 mAb therapy leads to improved survival in syngeneic models of B and T cell lymphoma through the activity of CD8⁺ CTL. Our data demonstrate that whilst the depletion of macrophages or B cells did not impact on the survival of mice with established B or T cell lymphoma following combination therapy, therapeutic efficacy was completely abrogated by the depletion of DC. In addition, the depletion of DC enhanced the growth of distant metastases outside of the radiation field in a model of T cell lymphoma demonstrating their critical role in systemic tumour control.

Conclusions: Our findings demonstrate that intra-tumoural macrophages or B cells do not impact the generation of tumour reactive CTL following combination treatment with radiotherapy and an agonistic α CD40 mAb and that the induction of anti-tumour immune responses is dependent on the priming of CD8⁺ T cells by DC.

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Contribution of heparan sulphate binding domain of chemokine CCL21 to trans-endothelial migration of breast cancer cells

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Lymph node metastasis constitutes a key event in Breast Cancer progression. Chemokines are small proteins, which can promote metastatic spread by inducing cancer cell migration and invasion. Chemokine function is dependant upon their binding to both cell surface heparan sulphate (HS) molecules and to their specific receptor. Our group has demonstrated a significant increase in chemokine receptor CCR7 expression in cancerous breast epithelia compared to healthy controls. This study is designed to test the hypothesis that a non-HS binding forms of chemokine CCL21 can disrupt the normal response to CCL21, therefore reducing the metastasis of CCR7-expressing cancer cells.

Truncated CCL21 chemokine (Δ 98-134 C-terminal basic extension), was synthesised to investigate a possible linkage between chemokine binding capacity and cell activation. Wild type (WT) and mutant-CCL21 were tested for their ability to stimulate a dose-dependent increase in intracellular-free calcium in peripheral blood mononuclear cell (PBMC) and breast cancer epithelial cells MDA-MB-231. Mutant-CCL21 at concentrations 50 and 100 nM showed potential to mobilise Ca²⁺ at levels similar to that produced by WT-CCL21.

A series of experiments was performed to determine how deletion of the HS-binding site altered the ability of CCL21 to stimulate chemotaxis within a concentration gradient generated by free solute diffusion. PBMC stimulated to migrate by wild-type CCL21 was not significantly different from that stimulated by mutant ($P > 0.05$). Similar results were observed in assays using MDA-MB-231 cells.

A further series of experiments was performed to compare the potential of WT and mutant-CCL21 to stimulate the migration of cells across endothelium. In contrast to results for trans-filter migration, it was found that the non HS-binding mutant stimulated no increased in transendothelial cell migration above the background at each of the tested concentrations, 10, 30 and 50 nM respectively ($P > 0.05$). However, WT-CCL21 stimulated significant increased PBMC migration at each of the tested concentration (all $P < 0.001$). Similar results were observed in assays using MDA-MB-231.

Furthermore, the effect of heparin on chemotactic properties of WT and mutant- CCL21 was examined. Interestingly, heparin (250 μ g/ml) completely inhibit the chemotaxis mediated by WT-CCL21 (50 nM; $P < 0.001$), whereas it did not inhibit the chemotaxis at concentrations 100, 250 & 500 μ g/ml in response to mutant CCL21 (50 nM; $P > 0.05$). Similar assay will be performed using MDA-MB-231 cells.

Work is ongoing to characterise the biophysical properties of mutant-CCL21 and determine its potential role for a therapeutic blockade of the migration of breast cancer cells *in-vivo*.

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Characterization of adhesion molecule and chemokine expression by tumour endothelium isolated from colorectal cancer

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A higher density of tumour infiltrating T-cells within colorectal cancer (CRC) stroma is reported to strongly correlate with increased patient survival. Currently, mechanisms that modulate T-cell proliferation and differentiation are targeted as potential anticancer therapeutics, however, key data regarding regulation of T-cell trafficking in CRC immune-environment are lacking. We show data characterizing the tumour specific phenotype regarding adhesion markers of colorectal tumour endothelium.

Endothelial cells (EC) isolated from colorectal cancer and matched normal colon (NC) specimen using anti-CD31 immunomagnetic and fluorescent assisted cell sorting were analysed for expression of adhesion markers with RT-QPCR and flow cytometry. Proteome profiling arrays identified chemokines and static adhesion assays blocking adhesion molecules were used.

Cytometric analysis and immunocytochemistry confirmed expression of standard endothelial markers (CD31/VWF) and Tumour endothelial marker-8 expression in tumour ECs validated a tumour phenotype. RT-PCR revealed increased gene expression of ICAM-1, VCAM-1 and E-Selectin in all tumours (up to 10.96-, 23.43- and 2.28-fold increase respectively, $n = 4$). MadCAM-1 gene expression was elevated in some samples and down regulated in others. We found no correlation with stage. We observed a trend between increased gene expression of ICAM-1 and VCAM-1 and right-sided tumours and lymphovascular invasion. Blockade of the MadCAM-1/ α 4 β 7 axis in CRC and not NC, significantly inhibits CD3⁺ T cell adhesion. Soluble chemokine RANTES/CCL5 was found in cultured tumour endothelium supernatant and was absent in normal endothelial supernatant.

Our data shows that endothelium within colorectal cancer express molecules and chemokines typically required for lymphocyte trafficking. Of note, ICAM-1, VCAM-1 and E-Selectin expression at a genome and protein level was elevated in all tumour endothelium

although the heterogeneity of MAdCAM-1 expression supporting the paradigm that down-regulation of expressed adhesion markers by tumour endothelium can reduce the number of infiltrating T cells and may lead to immunotherapeutic targets in CRC.

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Effects of concomitant therapies on gd T cell responses to zoledronate in patients with advanced prostate cancer

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Gamma delta (gd) T cells have attracted interest as a biological tool for cancer therapy owing to demonstrated tumour cytotoxic potential *in vitro*, and their correlation with improved outcome in patients with malignancies. They are also not MHC-restricted, offering opportunities beyond individual-specific therapies. Indeed, clinical trials with bisphosphonate-activated gd T-cell-based immunotherapy are ongoing. However any clinical adoption of gd immunotherapy will need to be in the context of other therapies ('standard-of-care'). The influence of concomitant therapies such as chemotherapy on the effector function of gd T cells remains largely unexplored. We developed an immunomonitoring protocol to determine the gd T cell response to zoledronate (ZOL) in the context of hormonal therapy with or without docetaxel chemotherapy in patients with advanced prostate cancer. Blood samples were collected prior to ZOL and chemotherapy and 6 days later, with a second collection cycle after 4 weeks. Phenotypic and functional analyses were performed on purified peripheral blood mononuclear cells from each of the four successive collection points.

Many patients receiving ZOL show a markedly low percentage of gd T cells. Moreover, the percentage of NKG2D⁺ gd T cells is significantly reduced in both treatment groups, although the majority of patients experienced a conspicuous boost in this compartment after ZOL treatment. However, this 'bounce-back' effect was lost in patients who have received ZOL long term. Thus, ZOL modulates gd T cell phenotypes *in vivo* but repeated long term ZOL treatment could lead to exhaustion of this immune compartment. This observation could have important clinical implications with regards to the duration of bisphosphonate treatment, which currently is undetermined. Furthermore, it the form of an interventional clinical trial, we are now assessing whether adjuvant IL-2 therapy can prolong the activation potential of gd T cells in the context of ZOL, offering a simple immune-enhancement potential that might usefully be adopted for particular cohorts of patients.

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Using zinc finger nucleases to improve CD8⁺ T cell function for cancer immunotherapy

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Clinical trials in cancer patients utilising adoptive T cell therapy have given encouraging results. However, this therapy does not work routinely for all patients leaving considerable scope for improvement such as the use of genetically manipulated T cells.

Mouse studies, from our group and others, have shown that the transfer of CD8⁺ T cells deficient in a negatively regulating protein

tyrosine phosphatase (PTP), termed SHP-1, are superior to wild-type cells at killing tumour cells. Furthermore, *in vitro* studies have shown that SHP-1 deficient T cells form more conjugates with antigen presenting cells, divide more readily in response to T cell receptor triggering and are more readily activated. It is presently unclear, whether a SHP-1 deficiency would have similar effects in human T cells and whether SHP-1 deficient human T cells could provide improved immunotherapy.

Owing to the lack of natural human SHP-1 knockout T cells, we have generated SHP-1 deficient human T cells using SHP-1 gene specific zinc finger nucleases (ZFNs).

A pair of SHP-1 specific ZFNs was delivered to T cells by infection with two recombinant lentiviruses each encoding for a separate SHP-1 specific ZFN. Mutations in the SHP-1 gene target site were confirmed by a DNA mismatch assay (Cel-I), T cells cloned, SHP-1 protein deficiency confirmed by Western blotting and the mutated loci sequenced. The SHP-1 deficient cells have been analysed for functional responses, such as T cell receptor down regulation in response to peptide pulsed antigen presenting cells. A SHP-1 deficient Molt3 leukemic cell line showed a significant increase in T cell activation when compared to wild-type and mock infected cells indicating that SHP-1 deficiency in human T cells also leads to a more profound T cell activation due to the lack of negative regulation by this PTP.

Additional leukaemic cell lines and tumour-specific CD8⁺ T cell clones are currently being genetically manipulated and examined for functional differences such as conjugate formation, cytokine production and target cell killing.

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A preliminary study of the effect of BET bromodomain inhibition on interleukin-6 secretion in multiple myeloma

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Background: Multiple myeloma (MM) is a malignant and progressive neoplasm of plasma cells, which is characterised by destructive lytic bone lesions, plasma cell infiltration in the bone marrow and monoclonal protein in the serum or urine. Overproduction of interleukin-6 (IL-6), a pleiotropic cytokine, is believed to play a key role in the pathogenesis of MM. Consequently, attention has focused on targeting IL-6 as a novel therapeutic strategy for MM. Bromodomains are a diverse family of protein interaction modules, which are the principal readers of acetylation marks on histones and have a vital role in transcriptional regulation. Recent studies have demonstrated that inhibitors of the bromodomain and extraterminal (BET) family of proteins suppress the expression of several pro-inflammatory cytokines including IL-6. The aim of this study is to investigate the effect of JQ1, a highly potent small molecule bromodomain inhibitor, on human MM cells and IL-6 secretion.

Methods: After obtaining informed consent, primary MM cells were isolated from venous blood of a patient with refractory MM. To explore the effect on IL-6 secretion, primary MM cells were pre-treated with various concentrations of JQ1 then stimulated with lipopolysaccharide (LPS) and an IL-6 ELISA was performed. The effect on cell viability was measured using the WST-1 colorimetric assay.

Results: Pre-treatment of LPS stimulated primary MM cells with JQ1 led to a dose-dependent suppression of IL-6 secretion. In addition, JQ1 exposure caused a time-dependent decrease in primary MM cell viability.

Conclusion: Treatment with JQ1 not only suppresses secretion of IL-6 in MM cells but is also cytotoxic. These findings suggest that it is likely that bromodomain inhibitors will emerge as a new class of immunomodulatory drugs for the treatment of cancer.

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MAGE-specific T cells are elevated in the peripheral blood of testicular germ cell tumour patients**H. Pearce***, **P. Hutton†**, **R. Viney†**, **P. Patel†**, **E. Porfiri†**, **S. Chaudhri†**, **N. S. Deshmukh†** & **P. Moss*†**

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Background and aims: A large lymphocytic infiltrate is observed within testicular germ cell tumours (TGCTs). This is particularly apparent in seminomatous tumours, in which a variety of Cancer Testis Antigens (CTAg)s are expressed. Here, we examine the frequency, function, and kinetics of MAGE-specific T cell responses in TGCT patients.

Methods: Peripheral blood lymphocytes were isolated from heparinised whole blood from patients with testicular cancer prior to, and at intervals following adjuvant chemotherapy. The frequency and kinetics of MAGEA1, A3 and A4-specific T cell responses were determined by MHC-Dextramer analysis, and by the use of overlapping peptides in an IFN γ ELISPOT assay. Functional analysis was performed on MAGE-specific T cell clones.

Results and conclusion: MAGEA1, A3, and A4 responses were detected in a significant proportion of patients with seminomatous tumours. In the vast majority of these patients, multiple MAGE responses were observed. Interestingly, only MAGEA3-specific T cells were detected in patients with non-seminomatous disease. Furthermore, detailed analysis revealed a mixture of both CD4⁺ and CD8⁺ T cell responses. The frequency of MAGE-specific T cells in responding patients was as high as 0.25% of total PBMC. These inflated populations had diminished by up to 95% following chemotherapy. MAGE-specific T cell clones isolated prior to chemotherapy secreted inflammatory cytokines and demonstrated cytotoxic potential against tumour cells presenting endogenously processed antigen. Our data suggests MAGE-specific T cells are recognising cognate antigen produced by the tumour, and subsequently undergoing clonal expansion leading to their increased frequency in peripheral blood.

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ps20 expression inhibits tumour growth and regulates immunity within the prostate**O. J. Hickman***, **C. Galustian***, **A. Vyakarnam†** & **P. Dasgupta***

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prostate stromal 20(ps20) encoded by the gene WFDC1, is a 24 kDa secreted factor containing the highly conserved whey-acidic protein domain, normally associated with modulating innate-immune responses at mucosal membranes. Ps20 has been shown to exhibit immuno-modulatory and growth inhibitory properties and is highly expressed in the prostate-stroma, as well as the brain, eyes, cardiovascular system and in CD4 T cells, though its expression is frequently lost in tumours. While its physiological function remains largely uncharacterized, ps20 has been shown to be permissivity factor in HIV spread between CD4 T cells, and to be associated with reduced murine hepatitis virus titers through modulation of neutrophil chemo-attractant chemokines and an increased neutrophil response.

We investigated the immune-suppressive and anti-tumorigenic properties of ps20 by over-expression of two WFDC1 cDNAs, full-length (FL), and exon 3 truncated (TR), in ps20 negative prostate-stromal WPMY-1 cells. Conditioned media from ps20 expressing WPMY-1 cells potently inhibits growth of PC-3, DU145 and LNCaP prostate cancer cell lines and halts the proliferation of CD3/28 acti-

vated CD4 and CD8 T cells. These functions are retained following depletion of ps20 from the conditioned media suggesting involvement of downstream factors.

We performed an unbiased microarray on EV, ps20^{FL} and ps20^{TR} WPMY-1 cells identifying numerous upregulated cytokines, chemokines and growth factors. T cell chemo-attractive CCL5, CXCL1, CCL2 were upregulated in ps20 expressing cells, along with the growth inhibitory molecules SerpinF1 and COX-2, as were a host of innate-immune associated cellular factors.

We hypothesize therefore that through regulation of a host of lymphotactic and growth-inhibitory paracrine effectors prostate-associated ps20 regulates the infiltration and expansion of T lymphocytes via modulation of the inflammatory milieu in a manner similar to mesenchymal-stromal cells. Further, that ps20 expression in healthy prostate-stroma is a key mediator of tissue homeostasis through induction of an immune-surveillance phenotype and anti-proliferative environment, thereby acting to restrict neoplastic growth.

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The presence of pre-operative carcinoembryonic antigen (CEA)-specific Th1-type cells is associated with early recurrence of colorectal cancer following resection**M. Scurr***, **C. Brown***, **G. Betts†**, **R. Hills‡**, **A. Gallimore*** & **A. Godkin*§**

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Colorectal cancer (CRC) is the third most common malignancy in the UK. After surgical resection, the primary tumour is graded using TNM staging to predict patient prognosis and direct future treatment. An increasing body of evidence now suggests that anti-tumour IFN-g-producing T cells perform a crucial role in limiting disease progression; thus we sought to assess the utility of pre-operatively measured anti-tumour T cell responses in predicting 5-year outcome in 76 CRC patients (TNM stage I-III) who had undergone a resection with curative intent.

T cells derived from pre-operative blood samples were subjected to *ex vivo* IFN-g ELISpot assays, measuring responses to the up-regulated tumour auto-antigen CEA, the tumour-specific oncofoetal antigen 5T4 and two control re-call antigens. The influence of immune regulation was also recorded by repeating the experiments after *in vitro* depletion of regulatory T cells.

Although subjects with stage III-graded tumours had an increased likelihood of recurrence, the most significant risk factor during the 5-year follow-up was the presence of pre-operative CEA-specific T cell responses; the negative influence of these responses was independent of the TNM stage (log rank test $P = 0.0008$, hazard ratio 5.00, 95% CI 1.96–12.77). Pre-operative immune responses to other antigens did not reflect outcome, although 5T4-specific responses improve overall survival in the subgroup of patients responding to CEA.

Given the significance of anti-CEA Th1 responses in predicting tumour recurrence irrespective of tumour stage, this information could improve prognostication and help re-direct adjuvant treatment strategies. These results also suggest caution in the use of auto-antigens such as CEA in future cancer immunotherapies for CRC.

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The immunomodulatory drug Lenalidomide alters the threshold for NK cell activation and augments NK cell effector functions

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The immunomodulatory drug Lenalidomide is widely used for the treatment of multiple myeloma. Although there is some evidence that the drug can influence both the phenotype and function of Natural Killer (NK) cells, little is known about its effects on specific NK cell effector functions. For example, it has not yet been established whether the effects of Lenalidomide on NK cells are direct or indirect, e.g. through augmenting cytokine secretion from other cells. Here, we show that Lenalidomide augments NK cell-mediated cytotoxicity and also increases the secretion of IFN- γ from primary human NK cells in conjugate with NK-sensitive target cells. Treatment with Lenalidomide resulted in a twofold increase in the proportion of primary NK cells producing IFN- γ , and a threefold increase in the amount of IFN- γ produced per cell, in response to ligation of specific activating receptors, including CD16 and NKG2D. This establishes that Lenalidomide acts downstream of specific activating receptor signalling. In addition, in the presence of Lenalidomide, NK cells were activated by lower concentrations of MICA used to coat slides, indicating that Lenalidomide alters the threshold for NK cell activation. Taken together, these results show that Lenalidomide can directly influence the NK cell immune response. We are now investigating the molecular basis by which Lenalidomide has this effect on NK cells.

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Immunotherapy: identification of 5T4 epitopes for individual colorectal cancer patients

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Anti-tumour effector Th1 CD4⁺ T cells (Teff) dominated by IFN- γ production are considered beneficial in patients. Regulatory CD4⁺ Foxp3⁺ CD25^{high} T cells (Tregs) control host auto-reactive immune responses, and may inhibit anti-tumour immune responses. The use of a broad repertoire of T cell receptors argues for antigen specificity in Tregs as well as Teff. The aim of this project was to compare the range of target epitopes in a tumour oncofetal antigen (5T4) for each type of T cell in HLA-typed subjects. In order to achieve this, we initially measured T cell responses using IFN- γ ELISpot assays against overlapping peptides covering the whole 5T4 protein. We obtained a map highlighting 5T4 peptides capable of being presented by 6 HLA -DR types most common in the local population ($n = 42$). Anti- HLA DR antibodies confirmed the restriction of 11 candidate peptides. *In silico* peptide prediction program suggested these peptides bound to the relevant HLA-DR molecules. To evaluate the impact of Tregs on Teff responses, CD25 depletion was performed with magnetic beads before peptide stimulation. The Teff response increased after depletion to three out of 11 HLA-DR restricted epitopes measured in three subjects, suggesting dual recognition of some, but importantly not all, epitopes by Teff and Tregs. The detailed characterisation of these T cell responses is now planned using in-house MHC class II tetramers based on these mapped epitopes.

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Investigating the role of high endothelial venules in colorectal cancer progression

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Colorectal cancer (CRC) is one of the leading malignancies causing death in the Western world. Despite continuous efforts to treat the condition, the tumour still reappears in 40–50% of the patients posterior to tumour excision. A prolonged patient survival, better response to therapy and lack of tumour recurrence is associated with a higher cytotoxic and memory T cell density, thus indicating that the population of tumour-infiltrating lymphocytes (TILs) can determine tumour fate. High endothelial venules (HEV), present in secondary lymphoid organs (SLO), are specialised post-capillary venules expressing peripheral node addressins (PNAds), ligands for L-selectin expressed on naïve T cells. Expression of PNAds on HEV mediates infiltration of naïve and central memory T cells from the blood into the SLO. Thus, HEV may also serve as a facultative lymphocyte route of entry into the tumour site, possibly facilitating the immune response. Limited studies to date have demonstrated an association of tumour HEV with a better patient outcome in breast cancer and melanoma. In this study, we investigated whether HEV neogenesis occurs during progression of colorectal cancer. Immunohistochemical staining has revealed that CRC is associated with development of organised lymphoid follicles in the submucosa, which are often associated with newly formed HEV. Interestingly and in contrast to previous reports (breast cancer and melanoma) HEV were rarely found within the tumours. The presence of HEV in the submucosa was however associated with the frequency of T cells at the invasive margins of the tumours. The significance of these findings in terms of disease progression and prognosis is under investigation.

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ICAM-1 mediated regulation of tumour specific CD8⁺ T cell responses

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Introduction: Tumour infiltrating CD8⁺ T cell are essential in controlling and preventing tumour growth. Priming naïve CD8⁺ T cells by tumour antigens to become effector Cytotoxic T-Lymphocytes (CTL), occurs either as a consequence of direct or indirect antigen presentation by metastatic tumour cells or dendritic cells respectively. However, despite the activation of large numbers of tumour specific CTL their subsequent inability to control tumour growth often results from the immune suppression that is created within the tumour microenvironment. Several mechanisms of tumour-mediated immune suppression have been shown which can vary from one tumour to another. We have shown that elevated levels of prostaglandin (PG) E₂ secretion within tumours can directly suppress anti-tumour CTL responses through the increased level of intracellular cyclic Adenosine monophosphate (cAMP) within CD8⁺ T cells, which in turn, reduces T cell proliferation as well as IFN- γ production and prevents the up regulation of ICAM-1. ICAM-1 provides potent alternative co-stimulatory signal via the binding to LFA-1 expressed by naïve CD8⁺ T cells that is crucial for CD8⁺ T cell activation in the absence of the classical stimulation (CD80/CD86) on most cancer cells.

Material and methods: Murine renal carcinoma cells (Renca) which over-express cyclooxygenase 2, resulting in increased levels of PGE₂

production, were further transfected with a recombinant plasmid containing full length murine ICAM-1. Using Renca specific TcR transgenic CD8⁺ T cell, experiments were carried out to assess the anti-tumour CTL response to these Renca cells *in vitro* and *in vivo*.

Results: ICAM-1 is a potent alternative costimulatory molecule for naïve CD8⁺ T cell activation. Overexpression of ICAM-1 by Renca cells can counteract the immune suppressive effect that PGE₂ has on CD8⁺ T cell responses *in vitro* and can restore productive activation of CTL responses *in vivo* which can prevent tumour growth.

Conclusion: Immunotherapeutic strategies that target ICAM-1 interactions with LFA-1 on tumour-specific CD8⁺ T cells could counteract tumour mediated immune-suppression and control tumour growth.

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T cell trafficking mediated by high endothelial venules (HEV) facilitates tumour control after regulatory T cell depletion

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CD4⁺Foxp3⁺ regulatory T cells (Tregs) inhibit antigen-specific T cell activation. Tregs thereby prevent autoimmunity and limit excessive inflammatory responses. Tregs are found in tumours where they may contribute to immunosuppression by limiting tumour-specific T cell activation. Selective Treg depletion in order to initiate an effector T cell response to tumour antigens holds promise as a potential cancer immunotherapy.

We use a mouse model of carcinogen-induced tumorigenesis within which *de novo* fibrosarcomas are induced by injection of the carcinogen methylcholanthrene (MCA), to examine the impact of Treg depletion on tumour progression. Our data shows that therapeutic depletion of Tregs can result in significantly reduced tumour growth. Furthermore, data have demonstrated a highly significant association between tumour growth rate and extent of entry of tumour-infiltrating T cells (TIL) into tumours. Following this observation, some tumours were found to contain high endothelial venules (HEV). HEV are specialized post capillary venules normally found in secondary lymphoid tissues which facilitate extravasation of naïve and central memory T cells from blood to lymphoid organs. When TIL^{hi} and TIL^{lo} tumours were compared, we observed an absolute concordance between TIL^{hi} tumours and the presence of HEV. Critically, HEV were not observed in tumours from Treg-replete mouse indicating that in this model Treg depletion is an essential pre-requisite to HEV development. These data support the hypothesis that Tregs control development of HEV in peripheral sites.

We are now investigating signaling factors involved in HEV neogenesis in tumours via a series of *in vivo* cytokine blockade and cell depletion assays. Thus far, our data indicate that blockade of TNF α signalling prevents control of tumour growth after Treg depletion. Experiments are now in progress to determine whether this is due to a role for TNF α in promoting HEV neogenesis or whether TNF α has a direct anti-tumour effect. Data generated from this project could provide rationale for the modification of cancer immunotherapies to incorporate means through which access of efficacious antigen-specific T cells to the tumour mass can be improved.

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HEV formation in murine tumours is not dependent on presence of tertiary lymphoid organs

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Immune evasion by tumours limits anti-tumour immune responses but the mechanisms involved are not fully understood. Regulatory T cells (Tregs) protect the body from excessive inflammation and autoimmunity but they can also dampen anti-tumour immune responses. Activation of tumour-specific T cells can be facilitated by the depletion of Tregs. However, failure of activated lymphocytes to infiltrate the tumour remains a problem and there is no significant association between T cell activation and the rate of tumour growth. In contrast, there is a highly significant association between extent of T cell infiltration and tumour growth rate. We examined the effect of Treg depletion on intra-tumoural T cell infiltration in a mouse model of carcinogen-induced tumours. We found that the tumours from Treg depleted mice fell into two groups – those with high T cell numbers and those with low T cell numbers. Tumours were analysed for the presence of high endothelial venules (HEVs), blood vessels normally found in secondary lymphoid tissue, which are specialised for lymphocyte recruitment. It was observed that increased numbers of CD4⁺ and CD8⁺ T cells, but not B cells, were found only in HEV⁺ tumours. HEVs were only observed in tumours from Treg-depleted mice.

As HEVs have been found in tertiary lymphoid organs (TLO) we addressed whether presence of HEVs in the murine tumours was linked to TLO formation. Therefore, the tumours were immunohistochemically stained for HEVs, T cells, B cells and follicular dendritic cells (FDC) to look for TLOs. The vast majority of HEV⁺ tumours showed no evidence of TLOs.

These results suggest TLO formation is not necessary for HEV formation. Further experiments are needed to determine the mechanism behind HEV formation and the role of Tregs in this process as this information could provide a new strategy for tumour immunotherapy.

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The effect of retinoic acid on regulatory and effector T cells in anti-tumour immunity

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Tumour eradication by the immune system is largely dependent on pro-inflammatory signals and effector cell infiltration into the tumour. However, tumours can overcome effector immune responses, for example by the secretion of immunosuppressive factors like TGF- β or the recruitment of regulatory cells. TGF- β has been shown to induce the conversion of naïve CD4⁺ T cells into regulatory T (Treg) cells and this is enhanced by retinoic acid (RA), a Vitamin A metabolite. In the present study we investigated the capacity of a RA receptor-alpha antagonist (RARI) to block the induction of Treg cells and thus to enhance anti-tumour immunity. We found that RA and TGF- β induced conversion of naïve CD4⁺ T cells into Treg cells could be blocked by RARI. TGF- β in combination with RA also converted committed CD4⁺ T cells into Treg cells, decreasing the effector T cell population and IFN- γ production *in vitro*. Furthermore, RARI suppressed the secretion of the anti-inflammatory cytokine IL-10 and enhanced IL-12 production from TLR-activated dendritic cells (DCs). Using a B16 melanoma murine tumour model, we found that RARI enhanced the therapeutic effi-

cacy of a TLR-activated antigen-pulsed DC vaccine, significantly attenuating tumour growth and enhancing survival. Treatment of DCs with RARi *in vitro* prior to transfer in to mice with tumours reduced the frequency of tumour infiltrating Foxp3⁺ and IL-10⁺ Treg cells, while enhancing IFN- γ -secreting CD4⁺ and CD8⁺ T cells. In addition, RA has been shown by others to target tumour cells directly, promoting differentiation and inhibiting proliferation. We are currently investigating the effect of direct therapy with RA on tumour growth and the development of effector versus regulatory cells when administered at different stages of tumour progression. We hypothesize that RA given at an early stage will promote Treg cells and enhance tumour growth, whereas late RA treatment will promote effector T cell responses and impair tumour cell proliferation. Our findings demonstrate that RARi enhances the efficacy of a tumour vaccine but that RA may also have potential as a combination immunotherapy against tumours.

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The impact of antigenic modulation on anti CD20 effector mechanisms

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Background: Rituximab is a B cell deleting anti-CD20 monoclonal antibody (mAb) used in the treatment of diseases ranging from leukaemia and lymphoma to rheumatoid arthritis. There are three major effector mechanisms by which CD20 mAb are proposed to work, complement dependent cytotoxicity (CDC), antibody dependent cellular cytotoxicity (ADCC) and antibody dependent cellular phagocytosis (ADCP). Following treatment, antigen antibody complexes can be lost from the cell surface, potentially resulting in a diminished therapeutic response and patient relapse. Two hypotheses have been put forward to explain this phenomenon: the 'shaving' reaction involving an Fc γ Receptor (Fc γ R) dependent, physical removal of antibody: CD20 complexes by saturated/exhausted effector cells; and 'antigenic modulation' whereby internalisation of these complexes occurs on the target cell. Here we have employed a range of *in vitro* assays to address the impact of these processes on mAb effector function.

Material and methods: Fc γ R dependent effector functions and CDC were measured following initial or prolonged incubation with antibody and were correlated with the level of modulation using established assays. To investigate the shaving reaction macrophages were 'fed' latex beads at various concentrations, fluorescently conjugated anti-CD20 opsonised B cells were then used to determine levels of modulation and 'shaving'.

Results: We found that over 6 h isolated B cells opsonised with Rituximab showed a decrease in the level of surface antibody and this correlated with decreased CDC, ADCP and ADCC. This was independent of effector interaction as B cells were incubated with antibody prior to introduction to effector cells or complement. ADCP mediated by Fc γ Rs, of which 'shaving' and modulation are both reliant, is emerging as a major mechanism of action. The introduction of B cells to latex filled macrophages resulted in reduced phagocytosis as bead load increased, although B cell: macrophage interaction was maintained. As macrophage bead load increased the level of shaving and modulation decreased, in contrast to what has previously been suggested.

Conclusion: We conclude that antigenic modulation can impact on all effector mechanisms investigated and that 'shaving' of antigen antibody complexes from the cell surface does occur but only in the context of on-going phagocytosis by non-saturated macrophages and, at least *in vitro*, this is not the rate limiting factor for this mechanism. Rather target intrinsic antigenic modulation appears more important and there is now the need to examine whether these results can be confirmed *in vivo*.

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The serotonin receptor antagonist ondansetron shows antiproliferative properties which are associated with a down-regulation of interleukin 1 gene expression in acute lymphoblastic leukaemia cells

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Introduction and background: Ondansetron is a serotonin (hydroxytryptamine, 5-HT) receptor-3 (5-HT₃) antagonist, used as anti-emetic agent in the prophylactic treatment of chemotherapy induced nausea and vomiting (CINV) in children with acute lymphoblastic leukaemia (ALL). It has previously been reported that ondansetron shows antiproliferative effects on ALL cells *in vitro*, further associated with increases in the production of interferon-gamma (IFN γ) and nitric oxides (NO), as well as with an overexpression of the inducible NO-synthase (iNOS). Since the release of IFN γ and NO, also known as reactive nitrogen intermediates (RNI), are mostly related to the production of pro-inflammatory cytokines, it was of further interest to analyse the effects of ondansetron on the expression of interleukin 1 alpha (IL-1 α) in ALL cells.

Material and methods: The B-cell precursor leukaemia cell line REH, derived from a patient with ALL at first relapse, was used in all the performed experiments. REH cells in the logarithmic growth phase were cultured at cell concentrations of $1-2 \times 10^4$ cells/ml. Dot-Blot mRNA *in situ* hybridizations were performed with aid of an high specific gene probe for human IL-1 α , by using a commercially available non-radioactive DIG-labelling and detection Kit.

Results: After 72 h incubation in standard conditions, reproducible amounts of IL-1 α mRNA were systematically detected and further quantified in all the tested cell pellets, either in the presence or in the absence of 5–50 μ M ondansetron. Interestingly, the analysed REH cells showed reproducible and significant decreases in the expression of IL-1 α mRNA after 72 h incubation with 5–50 μ M ondansetron, either in the presence (34–43%), or in the absence (41–51%) of 10 μ M exogenous 5-HT. Moreover, the observed decreases of IL-1 α mRNA were further found to correlate with the previously detected antiproliferative effects of ondansetron in the ALL cell line REH.

Discussion and conclusions: Taken together, the 5-HT₃ antagonist ondansetron was found to differently modulate the expression of pro-inflammatory markers, such as IL-1 α (downregulation), or IFN γ , NO/RNI and iNOS (upregulation) in ALL-cells *in vitro*. These results may represent further beneficial effects of ondansetron as a selective anti- or pro-inflammatory anti-leukaemic compound, and further support its actual use in the current therapy of CINV in ALL patients.

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TLR2 inhibition with OPN-305 increases the efficacy of gemcitabine in a murine model of pancreatic cancer

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Pancreatic adenocarcinoma was the fourth leading cause of death from cancer in 2010. The 5-year survival rate is 6%. Survival of 6–11 months is typical for untreated patients with locally advanced disease, but is <6 months in untreated patients presenting with metastatic disease. In this study, we investigated the efficacy of OPN-305, an IgG4 humanised anti-TLR2 antibody, in combination with gemcitabine in a spontaneous pancreatic cancer model. Tumour size was assessed by ultrasound prior to treatment. Gemcitabine or OPN-305 alone showed a modest increase on overall survival (OS) compared with no treatment. However, when OPN-305 and gemcitabine were combined, there was a significantly greater increase in OS ($P < 0.01$). This correlated with a decrease in metastases, proliferation index and number of circulating tumour cells. QPCR analysis of tumour tissue revealed that combination therapy led to increased Th1, CD8a and NK1.1 markers, and decreased Th2, neutrophil and monocyte markers. There was a decrease in IL-6 and IL-1 β expression and a normalisation of gemcitabine-induced TNF- α expression following TLR2 inhibition. Transwell experiments showed that cell lines derived from KPC mice secreted a soluble factor that decreased CD80 expression on macrophages. This effect was reversed by TLR2 inhibition. Fluorescent microscopy on pancreatic lesions also showed that combination therapy led to a preservation of ductal morphology, this correlated with the decrease in metastases. In conclusion, inhibition of TLR2 by OPN-305, in combination with a first line standard of care significantly increased OS in this model and represents a novel potential therapeutic for the treatment of pancreatic cancer.

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The unique profile of tumour-primed natural killer cells shows loss of activating receptor expression and pro-inflammatory cytokine secretion upon activation

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Background: Natural killer (NK) cells play an important role in host protection against tumours. Co-engagement of multiple NK cell activating receptors is a requirement for NK cell-mediated natural cytotoxicity against tumour targets. Work by our group defined this co-stimulation into two discrete stages; the first 'priming' signal can be

delivered by an activating cytokine or an appropriate tumour target cell, to achieve NK cell adhesion, conjugate formation and granule polarization. The second 'triggering' signal results in NK cell granule exocytosis and NK cell-mediated killing of targets. We have previously shown that CTV-1, an NK-resistant leukemic cell line, is capable of priming resting NK cells to lyse previously resistant targets, partially through ligation of CD15 ligand by CD2 on NK cells. Here, the activation profiles of tumour-primed NK cells (TpNKs) are defined, and the tumour-priming signal as delivered by CTV-, is further dissected.

Methods: Human NK cells were isolated from peripheral blood mononuclear cells (PBMCs) of normal healthy donors by magnetic cell sorting for CD56⁺ cells. Resting NK cells (2×10^5) were washed twice and mixed with CTV-1 (4×10^5), IL-2 (200 IU/ml), IL-7, -12, -15 or 21 (10 ng/ml) in 200 μ l of complete medium. Cells were incubated for the indicated time at 37°, 5% CO₂. Thereafter, immunophenotyping and 8-colour flowcytometry (MACS Quant) were carried out or supernatants were collected and stored at -80° pending measurement. The concentrations of cytokines were quantified by a multiplex immunoassay (Luminex 100 IS; Invitrogen). Blockade studies involved pre-treatment of NK cells with saturating concentrations of antibodies against indicated receptors. Data analysis was performed using GraphPad software.

Results: Uniquely, TpNKs showed significant ($P < 0.05$) downregulation of important NK cell activating receptors including CD16, NKG2D, NKp46, DNAM-1, ICAM-1 and 2B4 ($n = 7$). Differential NK cell secretory responses were also observed by TpNKs, which produced a pro-inflammatory cytokine profile characterized by MIP-1 α , MIP-1 β , RANTES, TNF- α and IFN- γ , with accelerated kinetics ($n = 4$). Blockade studies showed that receptor cooperation between CD2, NKG2D and LFA-1 is crucial for the delivery of the tumour-priming signal as delivered by CTV-1. Results suggest that the ligand expression profile of CTV-1, might hold the code for optimal synergy among NK cell activating receptors for enhanced effector functions against tumours.

Conclusion: Tumour-priming of NK cells is different from cytokine-priming as evidenced by unique NK cell surface expression patterns and secretory profiles. Further defining tumour-specific responses of NK cells, can enhance NK cell-based immunotherapeutic strategies for cancer.

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Synergistic targeting of breast cancer stem cells by human $\gamma\delta$ T cells and cytotoxic CD8⁺ T cells

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Background and aims: Breast cancer stem cells (bCSCs) are considered as the principal cause of disease recurrence, distant metastases, and mortality in breast cancer patients. The inherent resistance of these cells to existing therapies has largely hampered the development of effective treatments for patients with advanced breast cancer. Our research aims at establishing novel immunotherapy approaches efficiently targeting bCSCs by harnessing $\gamma\delta$ T cells as non-MHC-restricted killer cells and simultaneously as potent APCs to induce tumour-specific CD8 T cell responses.

Methods: By the use of a ras-transformed human mammary epithelial cell line (HMLER), an experimental model allowing reliable distinction of CSCs and non-CSCs was setup based on a range of phenotypical, morphological and functional criteria. Preferential killing of CSCs and non-CSCs by $\gamma\delta$ T cells and CD8 T cells was assayed in co-cultures of two target cell populations that had been prelabelled with different fluorescent membrane dyes. In order to provide proof-of-principle for CSC-targeting immunotherapies by cytotoxic CD8 T cells, viral model T-cell epitopes such as FluM1

and CMVpp65 were used as surrogates for yet-to-be-discovered bCSC-associated antigens.

Results: Stable sublines with characteristics of CSCs and non-CSCs were generated from HMLER cell line as confirmed (i) by their distinct expression profiles of CD24, CD44 and GD2, (ii) by their mesenchymal-like and epithelial-like characteristics and (iii) by their differential growth patterns in mammosphere culture. Both CSCs and non-CSCs were found to be resistant to cytotoxicity by human $\gamma\delta$ T cells but could be sensitized by inhibition of farnesyl diphosphate synthase (FPPS) through pretreatment with zoledronate or FPPS-targeting shRNA, resulting in efficient activation of $\gamma\delta$ T cells and upregulation of APC markers including MHC class I and class II molecules as well as CD80 and CD86. CSCs expressing FluM1 or CMVpp65 exhibited stronger resistance to antigen-specific CD8⁺ T cell cytotoxicity as compared to their non-CSC counterparts, which may be in part due to down-regulation of MHC class I and ICAM-1 expression. Of note, pretreatment of FluM1-or CMVpp65-expressing CSCs and non-CSCs with gd T cell conditioned supernatant significantly increased their surface expression of MHC class I and ICAM-1 and their susceptibility to CD8⁺ T cell-mediated killing.

Conclusions: Our findings identify a powerful synergism between MHC-restricted and non-MHC-restricted human T cells in the effective eradication of both CSCs and non-CSCs. These findings also suggest that patients receiving CD8⁺ T cell based immunotherapies may benefit from co-administration of zoledronate and/or co-transfer of $\gamma\delta$ T cells.

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Carbamylation of immunoglobulin abrogates the classical pathway of complement activation

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Background: The carbamylation of positively charged lysine residues to neutral homocitrulline occurs primarily under inflammatory conditions through myeloperoxidase-dependent cyanate formation. This study was undertaken to analyse the pattern of IgG₁ carbamylation and the effects that this modification has on the ability to trigger complement activation via the classical pathway.

Material and method: Human myeloma IgG₁ was carbamylated by incubation with 0.1 M KCNO. The complement-activation capacity of the modified antibodies was thereafter investigated by measuring the deposition of C1q, C4b and C3b in normal human serum. The pattern and extent of the carbamylated samples was employed by mass spectrometry of IgG₁ samples carbamylated *in vitro* and immunoglobulins purified from synovial fluid from patients with rheumatic inflammatory joint disease.

Results: In the current study, we found that selected lysine residues in IgG₁ were rapidly modified after exposure to KCNO. Interestingly, these modifications were specifically limited to Lys residues within the hinge region and N-terminal fragment of the CH2 domain. A complement activation assay combined with mass spectrometry data showed high correlation between carbamylation of lysine residues K222, K246, K322, K326 and K334 and proper binding of C1q to human IgG₁ and subsequent complement activation. We were able to confirm our *in vitro* findings using synovial fluid from rheumatoid arthritis patients.

Conclusion: Our data suggest that carbamylation has a profound impact on the complement activating ability of IgG₁ and reveals a pivotal role for previously uncharacterised lysine residues in that process.

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Cloning, expression and characterisation of the globular head regions of mouse C1q

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The classical pathway of complement system, activated by immune complexes, involves binding of globular heads of C1q to the Fc

regions of aggregated IgG or IgM. At the C-terminal region of C1q, is globular head region (gC1q) where each globular head is composed of one A (ghA), one B (ghB) and one C (ghC) chain. In order to understand modularity of gC1q region of mouse C1q and to generate useful reagents for *in vivo* studies, we have expressed these recombinant individual heads of mouse C1q in *Escherichia coli* as soluble proteins linked to maltose-binding protein (MBP). We have examined their interaction with heat-aggregated mouse IgG and IgG isotypes, mouse C-reactive protein (CRP), pentraxin-3 (PTX-3) and prion peptide (PrP). These fusion proteins (MBP-ghA, -ghB, and -ghC) were found to bind differentially to mouse IgG, IgG isotypes, PTX3, CRP and PrP. Both MBP-ghA and MBP-ghB also inhibited C1q-dependent hemolysis of IgG sensitized sheep erythrocytes. The expression and functional characterisation of individual mouse gC1q domains has allowed to examine specificity and selectivity of individual globular heads of mouse C1q to known ligands. Production of mouse gC1q domains will be helpful in antibody mapping of anti-C1q autoantibodies recognising gC1q region.

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Roles of innate immune molecules in pre-term birth

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Pre-term birth (birth at <37 completed weeks of gestation) is a major cause of perinatal mortality and morbidity. Emerging evidence implicates surfactant proteins SP-A and SP-D, two important regulators of homeostasis and innate immunity, in the pathogenesis of pre-term labour. In particular, they have been implicated in modulation of labour through the regulation of prostaglandins. Preliminary data has shown that there's a difference in the expression of SP-A and SP-D in human myometria (uterine tissue) from women with term and pre-term labour, and also that treatment of human myometrium cells with 10 and 20 µg/ml of rhSP-D after 12 and 24 h leads to increased mRNA expression (twofold or more) of oxytocin receptor and connexin 43 compared to control, which are key contractile genes that take part in parturition. In this study we will also (i) use inflammatory cytokines to mimic a preterm environment *in vitro* and study the subsequent regulation by SP-A, SP-D and C1q (a innate immune molecule of the classical complement pathway) and finally (ii) examine the modulation of immune cells in amnion, chorion and decidua by SP-A, SP-D and C1q. This study will provide a novel insight into the molecular mechanisms of preterm labour at uterine level, and provide exciting avenues towards new therapeutic strategies.

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Misleading serology in HAE: are we misclassifying some cases?

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Background: HAE 1 has reduced C1 inhibitor level (C1INH) function (C1INHf) and complement C4. HAE 2 has reduced C1INHf and C4 but normal/increased C1INH. Mutational analysis may be helpful if C1INH deficiency likely but serology uncertain. We present three patients from the same family with an identical HAE 2 mutation who could be serologically misclassified.

Methods: A 22 year-old lady with HAE had symptoms for 5 years. Her tests showed marginally reduced C1INH and C1INHf with low C4 repeatedly, when well. One of two brothers and her father have

angioedema and/or intermittent abdominal symptoms. One of our HAE patients was related to the index case (paternal great uncle). Serial C1INH, C1NHF and C4 in father and great uncle were similarly unremarkable when well irrespective of treatment, but C4 levels were low. C1INH was neither as high as in typical HAE 2 nor as low as in typical HAE 1.

Results: Three family members (index case, father and paternal great uncle) had a heterozygous 'S438P/p.Ser460 Pro exchange' mutation previously associated with HAE type 2. Only C1NHF reliably differentiated affected individuals.

Discussion: This mutation has previously been associated with a HAE 2 serological picture. Serological diagnosis of HAE is more uncertain than is generally recognised. Individuals with suspected HAE should be managed in Specialist Centres. Some HAE cases may potentially be hidden in serologically unremarkable patients, or misclassified, if changes in C4 are not checked in an attack.

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***N*-acetylmannosamine (ManNAc) supplementation of drinking water does not protect mice from nephrotoxic nephritis or complement-mediated renal injury**

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Background and aims: Sialic acids have many important roles in human biology. Mice with a homozygous knock in mutation in the gene for a key enzyme in the sialic acid biosynthetic pathway (GNE/MNK) unexpectedly developed severe proteinuric renal injury and podocytopathy. The lethal phenotype could be rescued by oral supplementation of pregnant mice with *N*-acetylmannosamine (ManNAc), a sialic acid precursor. Loss of binding of complement factor H to sialic acid residues is also important in the pathogenesis of atypical haemolytic uremic syndrome (aHUS), a complement-mediated renal disease. Free sialic acid has been reported to regulate the complement cascade.

We hypothesised that supplementing drinking water with ManNAc would reduce the severity of injury seen in a complement-mediated mouse model of aHUS induced by accelerated nephrotoxic nephritis (aHUS/NTN) by increasing glomerular endothelial sialylation. We also investigated effects of ManNAc in wild type (WT) mice given NTN.

Methods: The drinking water of WT mice and mice susceptible to aHUS was supplemented with ManNAc 1 mg/ml for 1 week prior to the induction of NTN. ManNAc water was continued until termination of experiment. There were four groups of $n = 8$ mice in each experiment: i) WT or aHUS ii) WT or aHUS + ManNAc iii) WT or aHUS + NTN iv) WT or aHUS +NTN + ManNAc. Experiments were terminated when 70–80% of mice in each experiment had 3+ haematuria by dipstick measurement. This was D11 for WT/NTN mice and D3 for aHUS/NTN mice. Sialic acid staining in the kidney was measured by fluorescent lectins. Renal histology, plasma urea, glomerular C3 and CD31 expression were measured in all groups.

Results: Supplementation with ManNAc significantly increased levels of $\alpha 2$ -3 and $\alpha 2$ -6 linked sialic acid in the kidney ($P < 0.001$) in WT mice. This was significantly reduced following NTN ($P < 0.05$) but to a lesser extent in mice supplemented with ManNAc. Renal histological injury and plasma urea levels were significantly increased in all groups receiving NTN compared to baseline. ManNAc supplementation was not protective. C3 and CD31 glomerular staining were reduced in mice which received ManNAc.

Conclusions: ManNAc supplementation of drinking water does not protect WT mice from NTN or from aHUS/NTN. There was a trend towards increased severity of injury in ManNAc treated groups. Reduced CD31 staining intensity in glomerular capillary vessels in mice who received ManNAc supplementation may indicate vessel injury/dropout.

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Direct visualisation of HLA-B27 forms in cell lines and Spondyloarthropathy tissues

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Background: The strong association of the human leukocyte antigen HLA-B27 (B27) with the Spondyloarthropathies (SpA), particularly with Ankylosing Spondylitis (AS), was discovered more than four decades ago, yet the role of B27 plays in disease remains unclear. Our research group discovered that B27 free heavy chains (B27 HC) can form dimers (B27₂) and/or non-classical B27 molecules (NC-B27), which can bind innate immune receptors in non-conventional way, compared with other MHC class I molecules. We therefore investigated the pathogenic role for these forms of B27 in AS.

Methods: We used a novel HD6 antibody, generated against B27₂, alongside the conventionally used HC10 and ME1 antibodies to examine surface expression of NC-B27 in AS patient and healthy control primary cells and transduced cell lines using flow cytometry, confocal microscopy and immunoprecipitation techniques.

Results: Confocal microscopy experiments revealed that NC-B27 can form clusters at the cell surface of B27-transduced cells. Immunoprecipitation data demonstrated that NC-B27 are present at the surface of the B27-positive cells more abundantly in a higher molecular weight form (approximately 80 kDa) compared with the monomeric form (approximately 45 kDa). Moreover, low pH treatment of cells can further induce both forms of B27. HD6 did not stain monocyte-derived dendritic cells (moDCs) from AS patients or healthy controls, irrespective of moDC maturation state. However, LPS treated AS moDCs expressed higher levels of HC10-reactive molecules than those from healthy controls. HD6 and HC10 staining can be induced in AS moDCs by low pH treatment.

Conclusions: Confocal microscopy data brought additional insight into the segregation patterns of classical and non-classical B27 molecules at the cell surface. We showed by flow cytometry and immunoprecipitation that HC-B27 expression on AS moDCs is significantly increased compared with healthy controls. Importantly, we showed that low pH treatment can induce HD6 and HC10 staining on the B27 positive cells.

Conservation and Divergence of Mammalian Immune Systems

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Discovery and characterisation of bovine CD16⁺⁺CD14⁻ cells: a novel myeloid cell population in peripheral blood elevated in Johne's disease

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In humans and mice, CD16 and CD14 distinguish myeloid subpopulations which play distinct roles in the response to pathogens. In cattle, we have identified four distinct peripheral blood populations based on CD16 and CD14 expression and cytokine profiles following LPS stimulation. CD14⁻CD16⁺ cells expressed the NK marker Nkp46 and had low responses to LPS. Based on differential cytokine expression, we have demonstrated that the CD16⁻CD14⁺ and CD16⁺CD14⁺ populations are similar to the human/mouse classical and non-classical monocytes respectively. In contrast, the CD16⁺⁺CD14⁻ population has a highly pro-inflammatory profile, expresses high levels of MHC-II and co-stimulatory molecules and is highly phagocytic, characteristics similar to Slan DCs, a human DC subset.

Slan DCs are increased in patients with inflammatory disorders such as Crohn's disease. In cattle, *Mycobacterium avium paratuberculosis* (MAP) infection results in the chronic gut inflammatory condition Johne's disease (JD) which has similar aetiology and pathology to Crohn's disease. Disease control of JD, characterised by an extended subclinical period is hampered by the poor performance of current tests to detect subclinical cases. An increased proportion of the CD16⁺⁺CD14⁻ subset was found in cattle diagnosed with both clinical and subclinical JD. We hypothesise that CD16⁺⁺CD14⁻ cells are a specialised subset of blood DCs associated with MAP infection and disease progression. Further investigations on the function and involvement of this novel myeloid population with JD could lead to the development of an early diagnostic test for the disease.

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Comparison of the innate responses of cattle and sheep cells to *Chlamydia abortus* infection

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Objective: *Chlamydia abortus* is an obligate intracellular bacterium that is a major cause of abortion in sheep worldwide but is rarely linked to abortion in cattle. The reasons for this differential pathogenesis are unknown. We are investigating the innate responses of ovine and bovine cells to chlamydial infection to address this. Pattern recognition receptors (PRR) such as TLR2/4 that activate NF-κB-mediated cytokine production and NOD-like receptors (NLRs) that activate the inflammasome complex are known to recognise chlamydial infection in human and mouse cells. Production of inflammasome dependant IL-1β has been implicated in both protection and pathology, we therefore hypothesize that early innate immune interactions determine the outcome of *C. abortus* infection in ruminants.

Methods: Cattle and sheep dendritic cells (DC) were generated from CD14⁺ve peripheral blood mononuclear cells using IL-4 and GM-CSF. Oro-nasal turbinate cells were derived post-mortem. Cells were

infected with *C. abortus* or exposed to UV-killed organisms and then analysed for PRR and cytokine/chemokine expression.

Results: Both cattle and sheep DC and turbinate cells expressed TLR2, TLR4, NOD1 and NLRP3. Infection of DCs resulted in early IL-1β production whereas turbinate cells produced no IL-1β but did produce CXCL8 late into the chlamydial developmental cycle. This cytokine/chemokine profile was not observed in cells exposed to UV-killed organisms. CXCL8 expression was higher in sheep than cattle turbinates.

Conclusions: Ruminant DC and turbinate cells exhibit different responses to *C. abortus* infection despite similar PRR expression. Cattle and sheep cells do not appear to differ qualitatively in their responses but do differ quantitatively.

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Hybrid immunoglobulin light chain IgG4 molecules in normal human serum

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Background and aims: Human IgG4 molecules are dynamic and have been shown to exchange half molecules to become bi-specific antibodies in a process termed Fab-arm exchange. Bi-specific molecules cannot cross-link antigen nor elicit lymphoid cell activation. It has been proposed that this mechanism may dampen-down unnecessary inflammatory responses. The aim of this work was to discover the extent of polyclonal IgG4 Fab-arm-exchange and whether this process can result in molecules with both kappa and lambda light chains.

Methods: Polyclonal IgG4 was purified from pooled or individual donor batches of human serum and sequentially fractionated into monomeric IgG4κ, IgG4λ and IgG4κ/λ. These were analysed by ELISA, SDS-PAGE, immunoblotting, and MALDI-mass spectrometry.

Results: Polyclonal IgG4 purified from normal serum contained a substantial portion that could not be fractionated by light chain specificity. Size exclusion chromatography showed that this material was principally composed of immunoglobulin monomers (150 kDa) and not aggregates. Analysis of these monomers by ELISA and western blotting suggested the presence of both kappa and lambda light chains on the same IgG4 molecule. Analytical antibodies different to those used during the purification process were employed to rule out idiotypic false positives. MALDI-Mass spectrometry showed that polyclonal light chain hybrid IgG4κ/λ had an average molecular weight approximately half-way between that of IgG4κ and IgG4λ. Reduction with DTT released both kappa and lambda light chains from only the former. The analytical techniques clearly indicated that IgG4 proteins containing different light chains on the same molecule were present. Based on the molecular weight these molecules were formed of 2 IgG4 heavy chains plus 1 kappa and 1 lambda light chain. Polyclonal IgG (IgG4-depleted) was purified and similarly fractionated according to light-chain specificity. No evidence of hybrid IgG κ/λ antibodies was observed. This would rule out the possibility that the hybrid light-chain immunoglobulins had been generated *in vitro*, and suggests that they are restricted to the IgG4 subclass. A total of 5 different human sera were analytically fractionated. Compositional analysis indicated that about 30% of purified polyclonal IgG4 was of the hybrid κ/λ form.

Conclusions: These results have unambiguously demonstrated that hybrid asymmetrical IgG4κ/λ antibodies compose a substantial portion of IgG4 from normal (non-immunised) human serum. This is a logical outcome from the process of 'Fab-arm' exchange *in vivo*.

Comparative evolutionary analysis of IL6 in lagomorphs

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Background and aims: Interleukin 6 (IL6), also known as interferon beta 2, is a class-I helical cytokine with a broad spectrum of biological activities in humoral and cellular defense. This class of cytokines has a gene structure conserved throughout vertebrates, with five coding exons.

IL6 is involved in the immune response against rabbit hemorrhagic disease virus that causes a highly fatal disease in the European rabbit. Previously, IL6 from European rabbit samples belonging to the subspecies *Oryctolagus cuniculus cuniculus*, was shown to differ from the other mammals by extending for further 27 amino acids. This difference results from a mutation in the typical stop codon into a glutamate encoding codon. However, in other leporids (*Sylvilagus* spp. and *Lepus* spp.) that diverged from European rabbit approximately 12 million years ago this mutation was also not present.

The purpose of this study was to confirm the mutation of the stop codon in other lagomorph specimens: *Oryctolagus cuniculus algirus*, *Brachylagus idahoensis*, *Sylvilagus bachmanii*, *Lepus europaeus* and *Ochotona princeps*.

Methods: The IL6 gene was PCR-amplified and sequenced for the five lagomorph species. The obtained sequences were translated and compared with other mammalian IL6 sequences retrieved from public databases (GenBank, Ensembl and Uniprot).

A maximum-likelihood (ML) tree was inferred in MEGA5 with the following options: HKY+G model, 500 bootstrap replicates and partial deletion to gaps/missing data treatment.

Results: We confirmed the presence of the mutated stop codon in both *O. c. cuniculus* and *O. c. algirus*. In agreement with previous reports, we found that the stop codon is not mutated in *S. bachmanii* and *L. europaeus*. We further extended this observation to the leporid *B. idahoensis* and ochotonid *O. princeps*.

In rabbits, sequence translation of IL6 continues into the exonic sequence and stops in the next STOP codon (81 nucleotides downstream). Typically, the IL6 protein has five cysteine residues that might be important to establish disulfide bonds. In rabbit, the 27 amino acid extension has four more cysteine residues.

The inferred phylogeny for the IL6 gene is in agreement with what has been accepted for the mammals and lagomorphs.

Conclusions: Our results indicate that in the ancestral of the *Oryctolagus* genus, (approximately 2 million years ago), a single mutation at exon 5 occurred that made IL6 longer than for the other mammals. Biological implications of this extension remain to be assessed but the occurrence of the 4 extra cysteine residues might suggest some functional relevance.

Myb-independent macrophages: a family of cells that develops with their tissue of residence and is involved in its homeostasis

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Background and aims: In most metazoan, all tissues contain phagocytes 'in residence', generally termed 'macrophages' in vertebrates. In contrast to myeloid cells produced continuously by the bone marrow, tissue resident macrophages develop during embryogenesis together with their tissue of residence, and persist in adulthood, independently of hematopoietic stem cells and the transcription factor Myb. They therefore represent an independent lineage from blood monocytes, dendritic cells, and monocyte/macrophages that are recruited to tissues during inflammation.

Methods: We reinvestigated the origin and developmental relationship between different tissue resident myeloid populations using complementary fate mapping strategies based on Cre/Lox systems. Fate mapping of HSC-derived cells was performed using Flt3-Cre mice whereas HSC- and Myb-independent macrophages were pulse labeled during embryonic development using tamoxifen in Csf1r-mer iCre mice.

Results and conclusions: Tissue resident macrophages functions are yet to be completely defined. They all share the ability to scavenge toxic compounds, lipids, microorganisms, dead cells and contribute to tissue remodeling, via phagocytosis and the production of growth factors. In contrast the production of inflammatory mediators seems more associated with bone marrow derived cells. Tissue resident macrophages and bone marrow derived myeloid cells thus differ in developmental origin and functions. A genetic and molecular dissection of resident macrophages functions will reveal their roles in tissue metabolism and the maintenance of homeostasis independently of the extravasation of inflammatory leucocytes, and in the control of the recruitment of bone marrow derived cells in overt inflammation.

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Immune escape strategies of a recently emerged contagious cancer, Devil Facial Tumour Disease

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Background and aims: In rare cases cancer cells do not die within a single individual, but successfully pass between individuals becoming a contagious cancer derived from a single neoplastic cell. Devil Facial Tumour Disease (DFTD) is one such contagious cancer that has emerged in the Tasmanian devil, a carnivorous marsupial endemic to the island of Tasmania. Despite an efficient immune system DFTD does not elicit a protective immune response from host devils, resulting in 100% mortality of affected animals and rapid decline of the species. The emergence of DFTD provides an opportunity to understand the immunological basis for the transmission of a tumour in a wild population.

Results: We have shown that DFTD cells down-regulate cell surface MHC class I molecules both *in vitro* and *in vivo*. The loss of class I molecules is not due to structural mutations, but to epigenetic down-regulation of genes essential for antigen processing, including β_2 -microglobulin (β_2m), the Transporters associated with Antigen

Processing (TAPs), MHC class II A (MHCIIA) and DMB. MHC class I molecules can be restored to the surface of DFTD cells *in vitro* using recombinant devil IFN- γ , which is associated with up-regulation of the MHC class II transactivator (CIITA), a key transcription factor with de-acetylase activity. Further, expression of MHC class I molecules by DFTD cells can occur *in vivo* during lymphocyte infiltration. We have examined the response of DFTD cells to IFN- γ using transcriptomics and find that wildtype DFTD cells predominantly express transcripts for a non-classical MHC class I gene, while IFN- γ treated cells express transcripts for classical MHC class I and class II genes, indicating that wildtype DFTD cells are differentially regulating their MHC class I.

Conclusions: The loss of MHC molecules from the surface of DFTD cells provides a molecular basis for how DFTD can pass between individuals without being targeted by cytotoxic T cells. Further, we suggest that down-regulation of MHC molecules using regulatory mechanisms allows evolvability of transmissible cancers and could affect the evolutionary trajectory of DFTD as well as its interaction with the host immune system. Finally, a potential whole-cell vaccine strategy in the form of IFN- β treated MHC positive DFTD cells is now generating preliminary results and may help preserve this vulnerable species in the wild.

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Association of human beta defensin-1 (DEFB1) polymorphisms and *in vivo* protein expression: first population-based analysis

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Background and aims: Single nucleotide polymorphisms (SNP) of the gene encoding for human beta defensin 1 (hBD1) are associated with increased risk of vertical HIV transmission (rs1799946) and reduced risk of periodontal disease (a predictor of poor obstetric outcome, rs1047031). This study aims to explore the relationship between DEFB1 genotype and serum hBD1 expression in pregnant women.

There are conflicting reports from *in vitro* studies on the effect(s) of hBD1 SNPs on protein expression. This is the first *in vivo* study to describe the relationship between DEFB1 genotype and hBD1 protein expression.

Methods: This is a retrospective case control study. Genomic DNA and serum were extracted from blood collected from women at 11–13 weeks' gestation attending King's College Hospital March 2006–September 2010. The SNPs rs1799946 (5'UTR) and rs1047031 (3' UTR) were genotyped by KASP assay (Kompetitive Allele Specific PCR, LGC). Serum hBD1 concentration was determined by ELISA. Data were analysed using the Kruskal–Wallis and Mann–Whitney–U-Test.

Results: DNA and serum samples were available for 292 women. Genotyping was successful in 98% (rs1047031) and 96% (rs1799946) cases. Data were assessed initially in three groups (ancestral allele homozygotes, heterozygotes, and SNP homozygotes). SNP homozygotes

(rs1047031) had a lower median serum hBD1 concentration than those heterozygous or homozygous for the ancestral allele (3321 pg/ml, IQR 2497 pg/ml versus 5824 pg/ml, IQR 3857 pg/ml; $P = 0.0013$). SNP homozygotes and heterozygotes (rs1799946) had a higher median serum hBD1 concentration than those carrying only the ancestral allele (5955 pg/ml, IQR 3925 pg/ml versus 5477 pg/ml, IQR 2951 pg/ml; $P = 0.0241$).

Conclusion: The DEFB1 SNPs rs1799946 and rs1047031 have a clear relationship with hBD1 protein expression *in vivo* rs1799946 is associated with higher serum concentrations of hBD1 in the first trimester while rs1047031 genotype mediated lower serum concentrations of hBD1 in the first trimester.

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IGHE genetic diversity in european rabbit and hares (leporidae)

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Aims: The European rabbit (*Oryctolagus cuniculus*) has long been an important model for immunological studies. Despite this, studies of rabbit immunoglobulin genetic diversity have focused on the Ig gamma isotype disregarding other Ig isotypes. In fact, the information available for rabbit IgE is extremely scarce with only two sequences available in public databases. In this study we sequenced the complete IGHE exons for the two European rabbit subspecies as well as for four species of another leporid genus, *Lepus*.

Methods: The four IgE exons, CH1, CH2, CH3 and CH4, were PCR amplified and sequenced for wild European rabbits belonging to the subspecies *Oryctolagus cuniculus cuniculus* and *O. C. algirus* and for four extant *Lepus* species: *L. americanus*, *L. californicus*, *L. europaeus* and *L. granatensis*. The primers were designed on European rabbit IGHE available sequences. The location on the IgE structure of the observed polymorphic positions was analyzed by mapping the residues onto the human IgE crystal structure.

Results: A total of 71 variable nucleotide positions were observed, 35 of which, corresponding to 12 aminoacid modifications, tell apart the European rabbit and hares. The European rabbit IgE showed 10 SNP that translate to two amino acid modifications. Within hares 31 SNP were observed, that translate to 13 aminoacid changes. Specific amino acids were detected for *O. c. algirus* and for each of the four studied *Lepus* species. The greatest nucleotide variability is found on the CH2 domain and the greatest amino acidic variability is found on the CH4 domain. The majority of the observed polymorphic positions occupy fairly exposed positions on the IgE protein.

Conclusions: IGHE shows a great sequence homology in the studied European rabbit and *Lepus* species. Nonetheless, specific residues were observed for the European rabbit *algirus* subspecies as well as for the four analysed *Lepus* species. These are distributed along the four IgE domains. The exposed position occupied by these residues suggests that these may be important for the interaction with effector molecules such as the IgE-Fc receptors.

Innate Immune Defence Mechanisms in the Lung

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The effect soluble products from *Pseudomonas aeruginosa* on immune cells

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Pseudomonas aeruginosa is an opportunistic pathogen that represents a major burden to the healthcare system as it accounts for a vast number of hospital-acquired infections and is a particular threat in Cystic Fibrosis (CF) patients. This bacterium expresses an arsenal of exoproducts that play a fundamental role in the invasion of the damaged physical barriers of the host and abrogate immune responses which aid the resistance and persistence of *P. aeruginosa* infections. The aim of this study is to elucidate the role of *P. aeruginosa* secretions in the modulation of the immune system and determine the key factors and their specific impact on immune cells such as T helper cells and monocytes. Our results to date indicate inhibitory effects of *P. aeruginosa* supernatants on T cell proliferation in response to *Staphylococcus* Enterotoxin B (SEB), but not anti-CD3 and anti-CD28 stimulation in peripheral blood mononuclear cells cultures. This effect appears to be mediated by the action of *P. aeruginosa* products on monocytes, a precursor of antigen presenting cells. Indeed PA supernatants exert cytotoxic effects on purified monocytes that do not lead to increased LDH release. Future work will involve the characterisation of the cytotoxic agent and the type of cell death triggered on monocytes. Understanding the key factors and signalling events triggered in monocytes upon stimulation with *P. aeruginosa* secreted products may lead to the design of better therapeutic interventions for infections caused by this organism.

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A tale with a STING: pivotal pathways in innate immune cells that control immunity to DNA

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Indoleamine 2,3 dioxygenase (IDO) is an inducible, intracellular enzyme that catabolizes compounds containing indole rings such as the essential amino acid tryptophan (Trp) to produce kynurenes (Kyn). Both Trp depletion and Kyn production mediate potent effects on immune cells that suppress T cell immunity and activate Foxp3-regulatory T cells (Tregs) that promote local tolerance. IDO is induced by interferons (IFNs) at sites of inflammation and IDO catabolizes tryptophan to regulate immune responses. IDO is induced by local malignant cell growth and some pathogen infections to create formidable local barriers to effective immunotherapy in patients with cancer and chronic infections. Thus, IDO inhibitors offer a novel strategy to enhance effective clinical responses in these hypo-immune syndromes. Discrete populations of dendritic cells (DCs) in mice and humans can express IDO to activate and stabilize the regulatory phenotype of Tregs. The IDO-specific inhibitor 1-methyl-[D]-tryptophan (1MT) synergizes with anti-tumor vaccines and with chemo-radiation therapy in mouse models of skin and brain tumors, respectively, to enhance anti-tumor responses. Moreover, Murine leukemia virus (MuLV) induces progressive increase in IDO activity suggesting

that MuLV exploits the IDO pathway to promote viral persistence, which also increases the risk of leukemia. The mechanisms by which malignancies and infected cells exploit the IDO pathway to suppress anti-tumor and anti-pathogen immunity will be discussed in relation to adaptive immunity and innate immune responses to DNA and dying cells.

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Comparison of the anti-inflammatory interactions of the beta-2-adrenoreceptor agonists salbutamol, formoterol and indacaterol with human neutrophils *in vitro*

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Background/introduction/context/objective: Beta-2-adrenoreceptor agonists (β_2 -agonists) which are used primarily as bronchodilators in the therapy of bronchial asthma and chronic obstructive pulmonary disease also possess secondary anti-inflammatory properties. The clinical relevance of these, however, remains uncertain, possibly due to differences in molecular structure and agonist activity. To address this issue, we have compared the anti-inflammatory activities of 3 different categories of commonly used β_2 -agonists, viz. salbutamol (short-acting), formoterol (long-acting) and indacaterol (ultra-long-acting) at concentrations of 1–100 nM with human blood neutrophils *in vitro*.

Materials and methods: Neutrophils were activated with either N-formyl-L-methionyl-L-leucyl-L-phenylalanine or platelet-activating factor in the absence and presence of the β_2 -agonists followed by measurement of the generation of reactive oxygen species and leukotriene B₄, release of elastase, and expression of the β_2 -integrin, CR3, using a combination of chemiluminescence, ELISA, colourimetric, and flow cytometric procedures. These were correlated with alterations in the concentrations of intracellular cyclic-3'-5'-adenosine monophosphate (cAMP) and cytosolic Ca²⁺.

Results: At the concentrations tested, formoterol and indacaterol caused statistically significant ($P < 0.05$ at 10 nM) equivalent, dose-related inhibition of all of the pro-inflammatory activities tested, while salbutamol was much less effective ($P < 0.05$ at 100 nM and higher). β_2 -agonist-mediated suppression of neutrophil reactivity was accompanied by elevations in intracellular cAMP and accelerated clearance of Ca²⁺ from the cytosol of activated neutrophils.

Conclusion/discussion: These findings demonstrate that: (i) the anti-inflammatory interactions of β_2 -agonists with neutrophils are associated with cAMP-mediated clearance of Ca²⁺ from the cytosol of activated cells; and (ii) meaningful anti-inflammatory activity is not a common property of these agents.

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B cell differentiation factor BAFF and chemokine CXCL13 are expressed in a murine model of human respiratory syncytial virus infection

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Background: Production of antibody in the lungs is an essential defense mechanism against respiratory pathogens. However, little is known about the mechanisms of local B cell activation in airway mucosa. Previously, we have demonstrated expression of the BAFF in human RSV infection (McNamara et al 2013 Thorax).

To better understand this process we examined BAFF expression in a murine model of RSV infection and measured expression of the CXCL13, CCL19 and CCL21 which could influence lymphocyte recruitment.

Methods: We measured BAFF, CXCL13, CCL19 and CCL21 expression in homogenised lung tissue from control mice at day 0 and mice challenged with RSV (A2 strain) or control UV-treated RSV at days 1, 2, 4 or 7 post infection ($n = 3$) by ELISA. Cytokine mRNA and RSV N gene expression were measured by Taqman PCR.

Results: BAFF was elevated significantly post RSV infection at day 1 (mean 1092 pg/ml, $P = 0.07$), day 2 (1157 pg/ml, $P = 0.01$) and day 7 (2941 pg/ml, $P = 0.0001$) in comparison to UV treated RSV control on day 1 (421 pg/ml), day 2 (370 pg/ml) and day 7 (393 pg/ml). BAFF mRNA expression was similarly increased on day 1 (fold increase 2, $P = 0.03$) and day 7 (1.7, $P = 0.02$).

CXCL13 protein was increased post RSV infection at day 1 (mean 762 pg/ml, $P = 0.01$), day 2 (612 pg/ml, $P = 0.02$), and day 7 (388 pg/ml, $P = 0.007$) in comparison to control day 1 (374 pg/ml), day 2 (278 pg/ml) and day 7 (249 pg/ml). CCL19 or CCL21 levels were not increased.

Conclusion: RSV infection results in up-regulated BAFF and CXCL13 expression, consistent with a role for CXCL13 in recruiting B cells and BAFF in promoting airway B cell differentiation.

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The role of Pellino1 in modulating signalling pathways controlling the inflammatory response to viruses in airway epithelial cells

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Introduction: Exposure to viral pathogens can exacerbate airway inflammation and tissue destruction generated by recruited neutrophils, culminating in altered epithelial cell responses in diseases such as asthma and Chronic Obstructive Pulmonary Disease (COPD). Pellino1 is an E3 ubiquitin ligase that has been associated with the regulation of viral signalling pathways activated via TLR3, modulating both interferon production and activation of NF- κ B. Our previous work has identified Pellino1 as a potential target to enable the downregulation of neutrophilic inflammation whilst retaining antiviral immunity by selectively regulating the NF- κ B signalling branch of the TLR3 signalling pathway.

Objectives: To investigate the physiological role of Pellino1 and its regulation in human primary bronchial epithelial cells (HBEPs) in response to the viral mimic, Poly(I:C).

Methodology: HBEPs underwent Pellino1 knockdown using targeted siRNA, followed by stimulation with Poly(I:C). Changes in IL-8, IFN β and Pellino1 mRNA levels were measured by qPCR whilst cytokine release, specifically IL-8 and RANTES, was measured using ELISA. Regulation of signalling molecule protein expression was measured using western blot. Caspase-8 activity was measured by luminescent assay.

Findings: Pellino1 knockdown led to a reduction in Poly(I:C) induced IL-8, IL-1 α and β gene expression and release of IL-8. The cytokines IL-1 and IL-8 play critical roles in airway inflammation and neutrophil recruitment respectively. Pellino-1 knockdown also inhibited phosphorylation of p38 MAP Kinase and the NF- κ B transcription regulatory units, IKK α and IKK β . There was also a reduction in the total and processed forms of non-canonical NF- κ B p100/p52. RIP1, a Pellino1 binding partner, is a negative regulator of non-canonical NF- κ B and plays a role in apoptosis by activating Caspase-8. Importantly, Pellino1 knockdown resulted in increased caspase-8 activity. These data support a role for Pellino1 in regulating both the canonical and non-canonical NF- κ B pathways in HBEPs.

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Activation of annexin A1-FPR2 axis promotes resolution of house dust mite induced airway hyper-reactivity

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Allergic asthma is a chronic inflammatory disease of the conducting airways. Exacerbations in allergic asthmatics are induced by inhalation of an innocuous protein that promotes an aberrant immune response. Current therapy has focused on the initiation of inflammation but less is understood about the mechanisms that control the regulation of allergen induced mucosal injury. Indeed, deficiencies in endogenous pro-resolving mechanisms may contribute to the persistence of inflammation in the lung. One such endogenous molecule is Annexin A1, a 37 kDa peptide with potent anti-inflammatory and pro-resolving properties. It exerts these effects primarily through FPR2. However, expression of Annexin A1 and FPR2 in the pulmonary allergic inflammatory environment is not well described. Using a mouse model of house dust mite (HDM) induced allergic airways disease (AAD) we investigated the role of Annexin A1. BALB/c mice receive intranasal administration of HDM three times a week for 3 weeks. We established in a naive mouse that Annexin A1 is highly expressed in alveolar macrophages and, at lower levels, in airway epithelium and polymorphonuclear cells. The receptor FPR2 displayed a similar distribution. Following HDM treatment, Annexin A1 mRNA transcripts were increased eight-fold and numbers of Annexin A1+ cells in the cellular infiltrate were significantly elevated. Interestingly, expression of Annexin A1 remained significantly higher during the resolution phase of inflammation. To investigate the functional importance of Annexin A1 in AAD, we exposed Annexin A1 knock-out mice to inhaled HDM. Mice deficient in Annexin A1 exhibited an exacerbated allergic inflammatory phenotype, with increased airway hyper reactivity (AHR) and a five-fold increase in airway eosinophilia. Th2 immunity was also elevated: Th2 lymphocytes increased three-fold [7.45×10^3 cells/ml versus 2.57×10^3 cells/ml, $P < 0.05$] IL-13 was up regulated [33.9 pg/ml versus 18.4 pg/ml, $P < 0.05$] and levels of the leukotriene LTB4 increased 2-fold. To corroborate the importance of Annexin A1 in our model we treated allergic mice with an Annexin A1 mimetic for

the final week of HDM exposure. These mice showed ameliorated AHR and a 35% decrease in lung tissue inflammation. Conversely, the use of an FPR2 antagonist WRW4 heightened AHR and promoted a 41% increase in total lung inflammation [166.0×10^5 cells/ml versus 117.3×10^5 cells/ml, $P < 0.05$]. These data indicate that Annexin A1 and FPR2 are critical regulators of the pulmonary mucosal response following HDM challenge. Targeting Annexin A1 and its receptor FPR2 may provide a novel target to treat airway hyper-reactivity in allergic individuals.

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The antimicrobial activity of the cationic host defence peptide, LL-37, is inhibited by exposure to nanomaterials

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Background and aims: Cationic host defence peptides (CHDP; also known as antimicrobial peptides) are key components of the immune system, with broad spectrum antibacterial, antiviral and immunomodulatory activities. The human cathelicidin, hCAP18/LL-37, has been shown to be upregulated at sites of infection and inflammation and has significant immunomodulatory activity. Several studies have shown that LL-37 has pluripotent roles within the immune system and that dysregulation or dysfunction of peptide activity could have significant implications for health. The importance of this peptide is evidenced by human patient groups and mouse models that are deficient in neutrophil cathelicidin being susceptible to severe infections of the lung, skin, gut and urinary tract.

Increasing usage of nanomaterials has provoked a growing concern that exposure to nano-sized particles could result in immune dysfunction. Studies have shown them to be more toxic than their larger bulk counterparts in cell and animal models, and it has been shown that nanomaterials can adsorb proteins to their surface resulting in structural and functional changes. The lungs remain the most vulnerable organ for nanoparticle exposure due to their large surface area and continuous contact with inhaled materials. As such, we aim to identify if interactions can occur between nanomaterials and the innate immune peptide LL-37, and to determine whether or not these interactions could translate to functional changes in the peptide, resulting in immune dysfunction as a result of nanomaterial exposure.

Methods: LL-37 peptide and carbon black nanoparticles were incubated together for 1 and 4 h at 37°. Bicinchonnic acid (BCA) protein assay, SDS-PAGE, ELISA and MALDI-TOF mass spectrometry were used to analyse peptide-particle interactions. Antimicrobial assays using *Staphylococcus aureus* (NCIB 6571) and *Escherichia coli* (NCIB 86) were used to assess antimicrobial function of the peptide following nanomaterial exposure.

Results: Our results suggest that there is an interaction occurring between the peptide and nanomaterials, resulting in reduced detectable LL-37. ELISA and SDS-PAGE analysis suggest a loss of intact peptide from solution in the presence of nanoparticles. Furthermore, the antimicrobial function of the peptide was significantly inhibited when exposed to the nanoparticles at all time points studied.

Conclusion: We hypothesise that the α -helical structure of LL-37 is disrupted due to interactions with the surface of the nanomaterials, potentially resulting in a loss of antimicrobial and immunomodulatory function. This has significant health implications as exposure to nanomaterials could translate to increased susceptibility to respiratory infections in vulnerable patient populations.

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Changes in inflammatory cytokine production by airway epithelial cells in response to mild acidification and LPS stimulation under acidic conditions

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Background: Reflux-aspiration is common in children with severe neurodisability (ND) and can lead to increased risk of recurrent lower respiratory tract infections and cause pulmonary fibrosis. The current consensus suggests damage is caused by chronic micro-aspiration which leads to mild but persistent airway acidification. Our group has previously shown presence of inflammatory cytokines in BAL fluid from children with severe ND.

Aim: To investigate how mildly acidic conditions affect inflammatory cytokine (IL-6) production in airway epithelial cells (AECs) and the response of AECs to bacterial challenge [using lipopolysaccharide (LPS)] under mildly acidic conditions.

Methods: BEAS2B cells were cultured to 70% confluence in 24 well plates in BEGM (Lonza).

BEGM media pH was adjusted using 1 M hydrochloric acid (Normal = pH7.4).

Cells were incubated in acidic media at increasing increments of acidity for 4, 8, 16 h before media was changed to pH 7.4 media in which cells were incubated for a further 24 h.

For LPS experiments cells were rested for 1 h in pH 7.4 media following incubation in acidic media. After 1 h resting the cells were stimulated with *Escherichia coli* LPS (5 μ g/ml) for 24 h in pH 7.4 media.

AEC IL-6 mRNA expression was measured by RT-PCR (Taqman). Secreted protein (cell supernatant) concentration was measured by ELISA (R&D).

Results: Mildly acidic conditions increase IL-6 mRNA expression, with corresponding protein production, in AECs at all time-points. AECs produce less IL-6 protein in response to LPS following incubations at low pH, however, there appears to be little or no change in corresponding IL-6 mRNA expression.

Conclusions: Mild acidification of AECs induced inflammatory cytokine production. Incubation of AECs at low pH does not appear to alter response to LPS stimulation. It is possible that reduction in IL-6 protein expression, following LPS challenge, may be due to cell cytotoxicity, however, experiments are on-going to elicit the mechanism.

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Investigating the mechanism of interaction between neutrophils and virus in Respiratory Syncytial Virus (RSV) disease

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Background: Neutrophils are the most numerous cell in the airway during Respiratory Syncytial Virus (RSV) bronchiolitis. We have previously found neutrophils in bronchoalveolar lavage (BAL) from infants with RSV disease contain RSV proteins. I hypothesise that neutrophils may play a vital role in viral clearance in the airway. My aim is to understand the mechanism of interaction between the neutrophil and virus by using *in vitro* modelling.

Methods: Highly purified neutrophils from whole blood of healthy adult donors were incubated with A2 strain RSV in the presence of GM-CSF, used as a neutrophil pro-survival factor. Samples were taken at 1, 2, 4, and 20 h for quantitative PCR of RSV N gene, FACS analysis of RSV F, G and N protein and cytopins for immunocytochemistry.

Results: Interaction of RSV and neutrophils was replicated *in vitro*. Neutrophils were positive for RSV F, G and N proteins by FACS analysis, and immunocytochemistry using a monoclonal antibody showed RSV in the cytoplasm of the neutrophil. Quantitative PCR revealed a 1000–2000 fold increase in RSV N gene expression compared to the L 32 housekeeping gene, maximal between 2 and 4 h with some inter-donor variability. Expression reduced in all experiments by 20 h. Uptake was enhanced by autologous serum, with a strong dose response. Uptake is 5-fold higher when comparing 0.1% serum with 10% serum ($P < 0.05$). Although these results suggested phagocytosis as a mechanism of viral entry the use of a phagocytosis inhibitor, cytochalasin D, did not reduce uptake, nor did a monoclonal antibody to RSV, Palivizumab, enhance uptake.

Conclusion: Phagocytosis of complement-opsonized bacteria, assisted by antibodies, is a well-documented role for the neutrophil. A similar process could be involved in viral clearance as part of the immune response to viral invasion. However, experiments to date do not appear to support this hypothesis and experiments are on going to investigate what alternative mechanism might be involved in viral uptake by the neutrophil.

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Contribution of alkyl-quinolone quorum sensing to the interaction of *Pseudomonas aeruginosa* with lung epithelial cells

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Lung mucosal immunity is at the frontline in the combat against bacterial infection. The signaling molecules from *Pseudomonas aeruginosa* (Pa), alkyl-quinolone quorum-sensing molecules (PQS), coordinate the expression of virulent factors and are present in the lung in patients with cystic fibrosis indicating a role in the pathogenesis of Pa infection. Indeed, previous studies indicated that PQS can suppress the activation of murine macrophages and epithelial cells. However, how the cross-talk between the airway barrier and Pa shapes the innate immune recognition of this pathogen and the role of PQS in this process remain unknown. In this study, an *in vitro* infection model has been established to investigate the contribution of alkyl-quinolone QS to the initial recognition of Pa by human airway epithelium. This was achieved by infecting human differentiated bronchial-epithelial cells with PAO1 (Wt) and its isogenic PQS-deficient mutant strain. Unexpectedly, although the expression of virulence factors, pyocyanin and rhamnolipids were highly reduced in the PQS-deficient mutant, no differences between the Wt and mutant strains were detected regarding bacterial growth, cellular invasion, amount of bacteria across the epithelial barrier, cytotoxicity and the induction of proinflammatory cytokines. Both strains induce transcripts for GM-CSF, TNF- α , IL-6, IL-8 and IL-17c and largely remained cell-associated. Taken together, our findings indicated that lung epithelium alone might not be able to discern the impact of PQS on Pa infection and the collaboration of professional innate immune cells could be required to identify its contribution to Pa virulence.

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Epigenetic control of Th2 induction by dendritic cells via the methyl-CpG binding protein Mbd2

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Background and aims: Dendritic cells (DCs) play a central role in Th2 priming against allergens and helminths, yet the mechanism by which they direct Th2 polarization is poorly understood. Methyl-CpG binding domain protein-2 (Mbd2), which links CpG methylation to repressive chromatin structure, has been shown to play a dominant role in regulating T cell cytokine production. However, it is currently unknown whether Mbd2 is also important for the function of innate cells such as DCs.

Methods: We first performed mRNA microarray and H3K9/14 acetylation ChIPseq analysis on *Mbd2*^{-/-} bone marrow derived DCs to determine if Mbd2 controls DC gene expression. To determine the ability of Mbd2 in governing the ability of DCs to polarize T cells, we generated mice with deletion of Mbd2 in CD11c+ cells (*Mbd2*^{ΔDC}) and challenged them with heat killed *Salmonella* or eggs from the parasitic helminth *Schistosoma mansoni*. This approach was complemented by using *Mbd2*^{-/-} DCs to assess whether Mbd2 controlled their ability to prime Th2 responses *in vitro* and *in vivo*. WT or *Mbd2*^{-/-} DCs were co-cultured with cytokine reporter T cells *in vitro*, or exposed to schistosome egg Ag (SEA) or house dust mite (HDM) and transferred s.c. or intranasally into naive recipient mice. Readouts included Th2 development in the draining LN, and HDM challenge lung inflammation.

Results: Despite *Mbd2*^{-/-} DCs showing unimpaired development, a large number of genes across several key pathways that related to DC function displayed altered levels of mRNA expression and H3K9/K14 acetylation. *In vitro* assays demonstrated that *Mbd2* controlled key aspects of DC function, and several dysregulated genes have been previously shown to be required for DC-mediated Th2 induction. *Mbd2*^{ΔDC} mice displayed impaired Th2 development following *S. mansoni* egg injection, but in-tact Th1/Th17 development in response to *Salmonella*. Further, *Mbd2*^{-/-} DCs were dramatically less able than WT DCs to promote Th2 polarization *in vitro* and exhibited severely impaired induction of helminth Th2 responses. Finally, Mbd2 was key for regulating DC ability to initiate HDM allergic airway inflammation *in vivo*.

Conclusions: These data demonstrate that epigenetic regulation of DCs, via the action of Mbd2, can be critical for Th2 induction and development against either helminths or allergens, *in vitro* and *in vivo*. Ongoing work is investigating the genes targeted by Mbd2, with the aim being identification of specific mechanisms employed by DCs that are fundamental for Th2 promotion.

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Phosphoinositide 3-kinase (PI3K) inhibition modulates responses to rhinovirus by mechanisms that are predominantly independent of autophagy

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Background and aim: Human rhinoviruses (HRV) are a major cause of exacerbations of airway diseases, such as asthma and chronic

obstructive pulmonary disease (COPD). The control of HRV infection may include signalling via PI3K, and the class III PI3K-dependent pathway, autophagy. We therefore aimed to investigate the extent to which the responses of airway epithelial cells to HRV were dependent upon these pathways.

Methods: The bronchial epithelial cell line BEAS-2B cells were infected with RV-1B or RV-16 (minor or major group HRV, respectively) at MOI approximately 3, or stimulated with poly(I:C). Transient knockdown of autophagy proteins was performed using siRNA. Pharmacological inhibitors of PI3Ks and mammalian target of rapamycin (mTOR) were used accordingly. Cytokine release was measured by ELISA, protein expression by western blot, and intracellular viral RNA and IFN β mRNA levels were quantified by qPCR. Fluorescently labelled LC3 puncta were examined using fluorescence microscopy.

Results: HRV infection did not increase autophagic flux in BEAS-2B cells. Knockdown of the autophagy regulators Beclin-1 or Atg7 inhibited autophagosome formation. However, the knockdown had no or minimal effects on HRV-induced production of the pro-inflammatory cytokine CXCL8 and the antiviral cytokine CCL5, and viral replication. Similarly, siRNA targeting of the autophagy protein LC3, or the autophagy-specific class III PI3K (Vps34), also caused no impairment of cytokine release. The PI3K inhibitor 3-MA, commonly used to inhibit autophagy, reduced HRV-induced cytokine generation. However, comparison to a general PI3K inhibitor, LY294002, and a panel of class I-selective PI3K inhibitors revealed that several PI3Ks cooperatively regulated the cytokine responses to HRV. Subsequent studies demonstrated that 3-MA, LY294002 or PI-103 (a pan class I PI3K inhibitor) also impeded viral replication. To explore whether the inhibitory effect of the above PI3K inhibitors on HRV-induced cytokine production was due to a reduction in the viral dsRNA replicates responsible for triggering this response, further studies were conducted using a synthetic dsRNA mimic, poly(I:C). 3-MA, LY294002 or PI-103 also inhibited cytokine generation induced by poly(I:C), indicating that the mechanisms of action of these PI3K inhibitors on cytokine generation were independent of viral replication. Interestingly, inhibition of mTOR, a negative regulator of autophagy, by rapamycin also resulted in reduced HRV- or poly(I:C)-induced cytokine production.

Conclusion: Our data demonstrate that class I PI3Ks and mTOR, but not class III PI3K or the autophagy proteins Beclin-1, Atg7 and LC3, are involved in induction of inflammatory responses to HRV infection.

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Cytokines produced by alveolar macrophages after Respiratory Syncytial Virus (RSV) infection

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Alveolar macrophages (AMs) in the lung are, together with epithelial cells, the first cell types to encounter microbes or particles that reach the lower airways. AMs account for 95% of airspace leukocytes and they are crucial for clearing particles, destroying microbes and initiating immune responses to clear invaders. AMs are long-lived leukocytes with a life span of at least 10 weeks. They are thought to derive from blood monocytes and it is suggested the lung environment is crucial for their development. AMs can perform the tasks usually associated with macrophages such as phagocytosis, pathogen destruction and cytokine and chemokine production. However, the position of AMs in the alveolus, exposing them to air, makes them unique compared to other macrophages. AMs are responsible for initiating innate immune responses to various infections such as Respiratory Syncytial Virus (RSV). RSV is one of the most important

pathogenic infections of childhood and the primary cause of bronchiolitis, the most common cause of hospitalisation in children <1 year of age. It is currently not clear which cytokines and chemokines that are produced by AMs during RSV infection. We are therefore investigating which inflammatory mediators that AMs produce after *ex vivo* exposure to RSV. This study highlights the importance of AMs in the early events after RSV infection.

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The effect of cigarette smoke extract on dendritic cells generated *in vitro* as a model to study COPD

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Chronic obstructive pulmonary disease (COPD) is an airway disorder characterized by airway obstruction that is not fully reversible. The main cause for the development of COPD is cigarette smoking, however not all smokers are susceptible. Immunological response has a major role in the development of this disease. One of the interesting cells that could have an important role in the development of COPD is the dendritic cell (DC). These cells are considered as orchestrators between innate and adaptive immunity and are professional antigen presenting cells. Their role in the development of COPD still not fully understood. In this study, our aim is to establish a non-animal model that could be used to study the effect of cigarette smoke on DCs.

Immature DCs were generated from positively selected CD14⁺ monocytes that were cultured in the presence of IL-4 and GM-CSF and stimulated with either LPS or Poly I:C. In addition, the effect of CSE and nicotine was assessed by protein expression level for DCs cell surface markers using flow cytometry and cytokine secretion using antibody microarray. Moreover, the genetic profile of the cells treated with 1% CSE and 3% CSE has been screened using transcriptomic analysis.

Our results indicate that both CSE and nicotine don't have any effect on the expression of the chosen screened proteins. On the other hand, the gene profile of DCs stimulated with CSE revealed alterations in a number of genes that could be a key to some interesting proteins that may have relation to COPD.

In conclusion, we have established an *in vitro* system to look at the effect of cigarette smoke on DCs that would be suitable to examine COPD patients' samples.

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PHD inhibition induces a distinct type of murine and human M2-like macrophage and enhances murine lung fibrosis

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Background: Pulmonary fibrosis is a common consequence of lung inflammation. Macrophages play a role in lung homeostasis and alternatively activated (M2) macrophages have been associated with lung fibrosis. Prolyl hydroxylases (PHD) are the main oxygen sensors and regulators of hypoxia inducible factors (HIF). The PHD/HIF pathway is known to play a role in tissue inflammation and fibrosis, but its role in macrophage polarisation is not fully understood.

Methods: We employed a combination of pharmacological (PHD inhibitors DMOG and FG41) and genetic (HIF and PHD-null) tools. The bleomycin lung fibrosis model was used to define the effect of

PHD inhibition on murine lung fibrosis and clinical samples from IPF patients were used to study the effect on human macrophages.

Results: *In vitro* in murine bone marrow derived macrophages (BMDM) PHD inhibition augmented IL-4 induced M2 polarisation generating a distinct type of 'augmented M2-like' macrophage in which M2 polarisation was enhanced but Fizz-1 induction selectively attenuated. In the bleomycin model, PHD inhibition (through DMOG administration) worsened lung fibrosis when induced during the fibrotic phase of the model. Enhanced lung fibrosis was associated with augmented M2-like polarisation. Mechanistically, the 'augmented M2-like' polarisation was maintained in HIF-1A or HIF-2A deficient macrophages. PHD-3 deficient macrophages had normal M2 polarisation.

In human macrophage cultures, PHD inhibition led to a distinct M2-like macrophage in which the M2 markers MRC1 and ALOX15 were unchanged but CCL18, an M2 marker associated with IPF disease progression, was selectively inhibited. CCL18 studies in bronchoalveolar lavage fluid (BALF) from IPF patients showed that CCL18 is elevated in the lungs of patients with IPF, but high levels did not correlate with disease severity or disease progression.

Conclusion: We have shown that pharmacological PHD inhibition promotes a distinct murine and human M2-like macrophage and worsens lung fibrosis when the drug is administered during the fibrotic phase of the bleomycin model. In human macrophages, PHD inhibition attenuated CCL18 production, an 'M2' chemokine reported to be a biomarker in IPF progression. However, our data did not support a role for CCL18 in disease progression.

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Phenotype and function of pulmonary macrophages after Respiratory Syncytial Virus (RSV) infection

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The hallmark of pathology in respiratory syncytial virus (RSV) infection is thought to be due to excess inflammation and often. Therefore, understanding and managing inflammation in RSV disease is of great importance. RSV infection is followed by activation and recruitment of innate cells into the airway and lungs which then mediates the release of an array of cytokines and chemokines. Amongst the cells that are involved in the innate response of RSV immunity are the recruited inflammatory monocytes and resident alveolar macrophages. Studies of recruited inflammatory monocytes in murine model of influenza infection demonstrated the role of these cells in the contribution to the pathology of the disease.

We hypothesized that inflammatory monocytes recruited from the bloodstream into the lung and not resident alveolar macrophages contributes to inflammation during the innate stage of RSV immunity. In our model, we aim to characterise the recruited recruited monocytes and the resident alveolar macrophages. We saw recruitment of inflammatory monocytes in the airway and lung at day 2 post RSV infection. Frequency of these cells started to decrease both in the airway and lungs at day 4 post infection suggesting their possible role early in RSV infection.

We then deplete blood monocytes via intravenous injection of clodronate liposomes in RSV infection. We found that intravenous injection of clodronate liposomes reduced the number of recruited monocytes both in the airway and lung at days 4 and 7 compared RSV infection only. In murine model of RSV, pathology is measured via weight loss. Administration of clodronate liposomes altered RSV pathology. Depleting recruited monocytes demonstrated a significant

protection against RSV in terms of weight loss. Although there was an increase in NK cell number in the airway at days 4 and 7 post infections with clodronate liposomes treatment, we saw no significant changes in CD4⁺ and CD8⁺ T cell percentage and number. Our data suggest that depletion of recruited monocytes altered CD4⁺ and CD8⁺ T cell function in RSV infection.

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Interferon-a/b receptor signalling is amplifying early pro-inflammatory cytokine production in the lung during Respiratory Syncytial Virus (RSV) infection

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Respiratory Syncytial Virus (RSV) is a major cause of bronchiolitis and subsequent hospitalization of infants. Severe bronchiolitis is associated with polymorphisms in several innate immune response genes, in particular many that control the interferon (IFN) system. Type I IFNs are produced very early upon direct recognition of virus and are crucial for both inducing anti-viral defences and for directing immune responses. Signalling through the IFN-a/b receptor (IFNAR) induces more than 300 IFN stimulated genes, including genes important for limiting viral replication and for further production of type I IFNs. The mechanisms underpinning how these cytokines regulate host resistance and immunopathology early during RSV infection is poorly understood. Using IFNAR1 knockout (IFNAR1^{-/-}) mice we found that the lack of IFN signalling increased viral load and pathology (weight loss) during RSV infection. In addition, the production of all IFNs and, surprisingly, proinflammatory cytokines and chemokines was dependent on signalling through the IFNAR. Furthermore, low levels of proinflammatory cytokines were detected in the lungs of IFNAR1^{-/-} mice after TLR agonist stimulation and recombinant IFN-a alone was shown to potentiate the production of inflammatory mediators in the lungs of wildtype mice. Thus, IFNs and IFN-receptor signalling are crucial in amplifying the early cytokine production in the lung and are therefore involved in shaping the immune response important for infections and inflammatory disease.

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Accumulation of airway hyaluronic acid after influenza infection: consequences for innate immunity

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Background and aims: Severe respiratory viral infections are often clinically associated with bacterial complications. The regulation of innate immunity has previously been linked to altered susceptibility, e.g. expression of the negative regulator CD200R on alveolar macrophages. Hyaluronan accumulates in the airway in a number of inflammatory disease models, initiating and perpetuating inflammation through TLR-4. Hyaluronan also self-limits macrophage inflammatory responses; interactions with CD44 induce upregulation of the TLR negative regulators A20 and IRAK-M. This work describes the altered hyaluronan landscape in the airways following viral infection, the effect this has on alveolar macrophages and the consequences for subsequent bacterial complications.

Methods: Airway hyaluronan content in the airways of female BL/6 mice was determined by ELISA following infection with PR8 H1N1 influenza. Hyaluronan metabolism enzyme expression was determined in whole lung RNA extracts by qRT-PCR. Alveolar macrophages were isolated and treated with hyaluronan prior to stimulation with LPS, then cytokine production quantified by ELISA. Alternatively, mice were treated *in vivo* with hyaluronan, then airway macrophages isolated and stimulated with LPS or LTA. Finally, mice were treated with hyaluronan and then infected with *S. pneumoniae*, and bacteraemia determined.

Results: Airway hyaluronan content increased >1000-fold following influenza infection, and was associated with upregulated synthase expression in the lung. These changes persisted following resolution of infection, resulting in a long-lived alteration to hyaluronan homeostasis in the airway. Inflammatory HA-protein complexes were also observed in the airways during influenza-induced inflammation. Histological staining of lung sections for hyaluronan revealed a loss of localisation of hyaluronan around the larger airways and dissemination into the alveolar space, along with more frequent HA-CD44 interactions. MIP-2 and histamine-induced oedema resulted in only a modest increase in airway hyaluronan, suggesting that influenza-associated inflammation drives *de novo* hyaluronan synthesis. Incubation of alveolar macrophages with hyaluronan either *in vivo* or *ex vivo* resulted in desensitisation to stimulation with the bacterial TLR agonists LPS and LTA. This modulation of the threshold of macrophage activation by hyaluronan translated into increased susceptibility to bacterial infection *in vivo*.

Conclusions: Influenza induces hyaluronan synthase expression and results in an altered hyaluronan homeostasis long after resolution of infection. Macrophage-hyaluronan interactions restrict the ability of alveolar macrophages to respond to the TLR-ligands, whilst *in vivo* hyaluronan treatment limits the ability of mice to clear bacterial infection. The mechanisms that regulate hyaluronan synthesis and the subsequent cross-tolerance to TLR-ligands will provide novel therapeutic targets for the management of secondary bacterial complications.

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Alveolar macrophages modulate early immune responses to Respiratory Syncytial Virus (RSV) infection through MAVS signalling and type I interferons

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Human respiratory syncytial virus (RSV) is the leading cause of severe lower respiratory tract infections in infants as well as in the elderly and immunocompromised individuals. Type I interferons (IFNs) are crucial for inducing an antiviral response, protecting from viral spread and inducing appropriate immune responses. To identify the cellular source of type I IFNs following RSV infection in mice we utilized a mouse strain in which green fluorescent protein (GFP) is expressed under the control of the *Ifna6* promoter. Upon intranasal RSV infection, GFP signal could only be detected in alveolar macrophages (AMs). Neither different populations of dendritic cells (DCs; CD103⁺, CD11b⁺ DCs or pDCs) nor epithelial cells or lung interstitial macrophages were a source of IFN- α 6 (as detected by GFP). We also investigated the role of pattern recognition receptors in the detection of RSV and subsequent type I IFN production. A deficiency in MAVS, the adaptor protein for cytosolic RIG-I-like receptors (RLRs), led to a complete loss in IFN- α production from

AMs. Furthermore, mice lacking MAVS displayed increased pathology, increased viral load and diminished production of other cytokines and chemokines. In addition, a marked deficiency in the recruitment of monocyte-derived DCs (moDCs) and inflammatory monocytes (infMO) was observed. We further showed that the monocyte recruitment was dependent on the production of IFN- α during RSV infection, as recombinant IFN- α given to MAVS KO mice 6 h post infection, restored moDCs and infMO recruitment into the lungs. Moreover, we could further prove the role of AMs in monocyte recruitment by adoptively transferring wildtype AMs intranasally into MAVS KO mice prior to RSV infection. In conclusion, AMs are the major source of IFN- α during RSV infection and this production is dependent on cytosolic RLRs. The IFN- α production is crucial for the production of other early cytokine and chemokines that are necessary for the recruitment of infMO and moDCs to the site of infection and also for dampening pathology.

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Mast cell-*Bordetella pertussis* interaction is mediated by macrophage galactose-type lectin and mannose receptor

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C-type lectin receptors (CLRs) play an important role in the activation of immune cells by bacterial pathogens. Mast cells are crucial in the development of immunity against *Bordetella pertussis* although the mechanism of interaction is not fully understood. In this study two CLRs, macrophage galactose-type lectin (MGL) and mannose receptor (MR) were detected for the first time on the surface of mast cells. The involvement of MR and MGL in the stimulation of mast cells by the heat inactivated *B. pertussis* antigen (BP) was investigated by the use of blocking antibodies and specific carbohydrate ligands. The cell wall lipooligosaccharide (LOS) of BP was also isolated to explore its role in this interaction. Mast cells stimulated with heat-inactivated BP or BP LOS induced cytokine (TNF- α , IL-6 and IFN- γ) secretion, which could be suppressed by blocking of MR or MGL. Spleen tyrosine kinase (Syk) was found to be involved in the CLR-mediated activation of mast cells by BP. Bacterial recognition by immune cells has been predominantly attributed to TLRs, in this study the novel role of CLRs in BP-mast cell interaction is highlighted.

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Human respiratory syncytial virus viroporin SH: a viral recognition pathway used by the host to signal inflammasome activation

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Respiratory syncytial virus (RSV) remains the leading cause of serious viral bronchiolitis and pneumonia in infants and young children throughout the world. The burden of disease is significant, with 70% of all infants being infected with RSV within the first year of their life. 40% of those children discharged from hospital have recurrent, repeated respiratory symptoms and wheezing for at least 10 years. The infection is also an important illness in the elderly and immunocompromised individuals. Ongoing symptoms relate to continued lung inflammation. One cytokine that is associated with RSV infection is IL-1 β , but the mechanism of activation remain unclear. In the current study, we set out to decipher the molecular mechanisms

of RSV-induced inflammasome activation. Using deletion mutants of the virus and measuring IL-1 β secretion, as well as caspase 1 expression via western blotting, we demonstrate that the NLRP3 inflammasome is activated through the small hydrophobic (SH) RSV viroporin which induces membrane permeability to ions or small molecules. Confocal microscopy revealed that during virus infection, SH seems to accumulate within lipid rafts in the Golgi compartments. Upon RSV infection, SH gets localised in the cell membranes and intracellular organelle membranes, and then induces permeability by disrupting membrane architecture, thus leading us to believe that formation of viral ion channels in lipid bilayers of cells is a viral recognition pathway used by the host to signal inflammasome activation.

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Rhinovirus-induced calcium flux triggers NLRP3 and NLRC5 activation in bronchial cells

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Human Rhinoviruses have been linked with underlying lung disorders such as asthma and chronic obstructive pulmonary disease in children and adults. However the mechanism of virus induced airway inflammation is poorly understood. In this study, using virus deletion mutants as well as silencing for NLRs, we show that the rhinovirus ion channel protein 2B triggers NLRP3 and NLRC5 inflammasome activation and IL1 β secretion in bronchial cells. 2B protein targets the Endoplasmic reticulum and Golgi and induces Ca²⁺ reduction in these organelles thus disturbing the intracellular calcium homeostasis. The NLRP3 and NLRC5 act in a cooperative manner during the inflammasome assembly by sensing intracellular Ca²⁺ fluxes and trigger IL1 β secretion. These results reveal for the first time that human rhinovirus infection in primary bronchial cells triggers inflammasome activation.

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Abnormal CFTR affects the IL-17 pathway in cystic fibrosis

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Background and aims: Cystic fibrosis (CF) is a hereditary disease caused by loss / impairment of a cellular chloride channel known as cystic fibrosis transmembrane conductance regulator (CFTR). CF patients are extremely susceptible to chronic respiratory infections caused by extracellular pathogens, but the reasons for this are still unknown. Our aim was to investigate CF patient susceptibility to chronic respiratory infections and assess the contribution of CFTR dysfunction to this.

Methods: CF patient and healthy control peripheral blood mononuclear cells (PBMCs) were stimulated with phytohaemagglutinin, staphylococcal enterotoxin B, or bacterial lysates. For CFTR inhibition assays, healthy PBMCs were pre-treated with CFTR inhibitors, GlyH-101 or CFTR_{inh}-172, before stimulation. On Day 6/7 cytokines in supernatants were measured and T helper cell subsets analysed by flow cytometry.

Results: CF PBMCs produced significantly less IL-17A than healthy control PBMCs upon *in vitro* stimulation (2–7-fold less, $P \leq 0.05$).

A similar impairment in IL-17A occurred in healthy PBMCs upon pharmacological inhibition of CFTR (1.5 to 4-fold reduction after GlyH-101 treatment, $P \leq 0.05$). IFN- γ production (which is also attenuated in CF patients) was not affected by CFTR inhibition in healthy PBMCs, suggesting that CFTR function is intrinsically linked to the induction of an IL-17 response. Further, preliminary results indicate that CFTR inhibition in healthy PBMCs led to a decrease in the percentage of Th17 cells, but not Th1 cells, after stimulation.

Conclusions: IL-17 has been postulated as a therapeutic target in CF because of its importance in immunity against extracellular pathogens and its potential role in mediating pulmonary destruction. Studies comparing IL-17A and IL-17F in CF versus control pulmonary samples have hypothesised that overproduction of IL-17 may drive chronic lung inflammation in CF. These studies, as in the case of our investigation using CF PBMCs, cannot distinguish between primary defects caused by CFTR deficiency and immune adaptation to the chronic inflammation characteristic of CF. Our results using CFTR inhibitors are the first to indicate that CFTR itself could play a role in the induction of IL-17-mediated inflammation. In contrast, our data also suggest that the reduction in IFN- γ production observed in CF patients is likely due to an adaptation to recurrent infection. It is essential to understand the precise role of the CFTR / IL-17 axis in CF respiratory disease as this knowledge can direct the development of therapeutic approaches aimed at eradicating chronic respiratory infections whilst controlling inflammation-mediated tissue damage.

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TAM receptors and their role in the regulation of lung innate immunity

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Background: The role of TAM receptors are well characterised in autoimmunity, developmental biology and cancer biology. Their role in infectious diseases is now being elucidated. The goal of this study is to characterise TAM receptor expression profiles of various innate immune components at homeostasis as well as in various infection/inflammation models. The hypothesis of this study is that up-regulation of TAM receptors, AXL and MerTK, are responsible for the desensitisation of airway macrophages to bacteria following influenza infection. Furthermore, I hypothesise that TAM receptors may be involved in limiting innate immune activation at homeostasis.

Methods: Using a murine model, we characterised TAM receptor expression on various innate immune populations following viral (influenza A) and bacterial (*Streptococcus pneumoniae*) infections. We also investigated the role of AXL in influenza and bacteria single/co-infection models using AXL^{-/-} mice.

Results: AXL and MerTK are differentially expressed by innate populations in the respiratory system depending on their location. Alveolar macrophages and infiltrating monocytes significantly up-regulate TAM receptor expressions during the influenza infection. Lack of AXL significantly potentiates innate immunity by up-regulating the release of inflammatory cytokines *in vitro*.

Conclusion: Up-regulation of AXL following a viral infection may explain the exacerbation of secondary bacterial complications as its up-regulation limits the innate inflammatory responses.

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A spoonful of sugar helps the medicine go down... In the most delightful way! (Sialic-acid nanoparticles induce potent anti-inflammatory effects through targeted activation of Siglec-E)

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Sialic acid-binding Immunoglobulin-like lectins (Siglecs) are cell-surface inhibitory receptors found predominantly on haematopoietic cells, which when cross-linked can inhibit pro-inflammatory signalling.

Here we describe a surface functionalized nanoparticle that potentially down-regulates endotoxin-induced pro-inflammatory cytokine release through the targeting of macrophage inflammatory responses.

We demonstrate that poly (lactide-co-glycolide) acid nanoparticles functionalized with disialic acid, di(a,2→8) N-acetylneuraminic acid, specifically bind to Siglec-E, which is up-regulated on macrophages on lipopolysaccharide (LPS) stimulation. By depletion of macrophages, we have shown that the nanoparticle therapeutic was primarily targeted to those cells. This Siglec-E dependent binding of the nanoparticle induced a potent anti-inflammatory effect, blocking LPS-induced production of both interleukin-6 (IL-6) and tumor necrosis factor- α (TNF α) in a tyrosine specific phosphatase 2 (SHP-2) dependent manner.

Notably, we have discovered a novel IL-10 dependent pathway leading to upregulation of Siglec-E, which is enhanced by these nanoparticles. Indeed, the nanoparticles are ineffective in IL-10 knockout mice, which lack Siglec-E.

We found that these therapeutic and anti-inflammatory effects are conserved *in vivo* using both the intra-peritoneal model of LPS-induced endotoxic shock and the caecal ligation and puncture model of polymicrobial sepsis. In these models, nanoparticle treatment significantly increased survival in mice over controls in the LPS shock model (100% versus 12.5% survival at 40 h, $P = 0.009$) and in the caecal ligation and puncture model (90% versus 0% survival at day 9, $P < 0.0001$).

We have shown similar efficacy in human monocyte-derived macrophages which clearly demonstrates the potent and importantly, the immediate immuno-suppressive properties of these sialic acid-conjugated nanoparticles and their potentially beneficial use as anti-inflammatory therapeutic modalities in the treatment of acute inflammatory conditions such as sepsis, ALI and ARDS.

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The role of epithelial cells in the immune response during respiratory tract infections

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The airway epithelium is the first line of defence against viral respiratory pathogens, acting both as a physical barrier as well as in the detection of the virus, and subsequent induction of the host immune response. Respiratory syncytial virus (RSV) is the most important cause of acute lower viral respiratory tract infections in infants, and influenza A viruses (IAVs) are significant pathogens of seasonal, endem-

mic and pandemic respiratory infections. The characteristics of RSV and IAV infection and spread in the host lung are not well characterised, however epithelial cells are thought to be the first cells of infection. In spite of this assumption, there is very little data that address this *in vivo*. In addition, the contribution of epithelial cells in the interferon and cytokine production *in vivo* has not been established. Using a dispase-agarose based method of lung epithelial cell isolation we have been able to identify alveolar epithelial cells using flow cytometry. We are optimising a method to obtain a pure population of epithelial cells from mouse lung using magnetic-activated cell sorting (MACS) and fluorescence-activated cell sorting (FACS) in order to perform gene expression analysis and identify the inflammatory mediators such as interferons, cytokines and chemokines produced by epithelial cells *in vivo* during respiratory infection. We have preliminary data suggesting an upregulation of several inflammatory mediators in epithelial cells early after RSV infection *in vivo*. This confirms a role for epithelial cells in the initial stages of RSV infection *in vivo*. Furthermore, the results from this study will help future development of therapeutics and vaccines against respiratory viral infections.

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Dissecting the role of sphingosine kinase isozymes in mast cells: significance to anaphylaxis and allergic airway inflammation

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Background and aims: anaphylaxis is caused by an acute, potentially lethal immune reaction primarily induced by the elevated immunoglobulin-E (IgE) antibodies that signal through the high affinity Fc ϵ RI receptor to release inflammatory mediators from immune cells such as mast cells. Similarly, airway inflammation is driven by increased mast cell activation and Th2 cytokines/chemokines. The sphingosine kinases (SphK1 & SphK2) have been investigated as potential therapeutic target for the treatment of allergic anaphylaxis, refractory asthma and chronic airway inflammation. The isoenzymes, SphK1 and SphK2, generate the pleiotropic sphingolipid mediator called as sphingosine-1-phosphate (S1P).

Methods: Using Ingenuity Pathway Analysis (IPA) software, we have constructed the biological networks initiated through Fc ϵ RI receptor in mast cells and the intracellular molecular activation networks regulated by SphK1 and SphK2 during the activation process in allergic responses. In our preliminary *in vivo* studies, we have specifically silenced *SPHK1* or *SPHK2* *in vivo*, using specific siRNAs. We have used both Passive Cutaneous Anaphylaxis (PCA) and Passive Systemic Anaphylaxis (PSA) models to study the isoform specific *in vivo* role of SphKs which has not been well-characterized.

Results: Our *in silico* analysis using IPA has precisely shown that various pro-inflammatory cytokines and chemokines are triggered through the S1P/SphK signalling in immune cells. Besides, our study demonstrates that SphK1, but not SphK2, is indeed required for most of the effector responses triggered during anaphylaxis in mice models.

Conclusions: Hence, dissecting the S1P/SphK1 signalling in mast cells could provide novel cues for the effective treatment and management of an extensive range of allergic disorders in a typical clinical setting.

Innate Lymphoid Cells

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Significant role of the activating NK cell receptor NKG2D in cancer-linked to inflammation

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There is increasing evidence linking chronic inflammation to cancer progression. Yet, the cellular and molecular mechanisms involved in this process are poorly understood and whether natural killer (NK) cells and NK receptors play a role is still unknown.

NKG2D is a key activating NK receptor expressed on effectors of the innate and adaptive immune system including NK, NKT, $\gamma\delta$ T cells and activated CD8+T cells. NKG2D participates in the anti-tumour and anti-viral functions of these cells upon recognition of specific ligands generally expressed on transformed and infected cells but absent from normal tissues. Recent studies have identified these ligands on normal tissues in the context of autoimmune diseases suggest a role of NKG2D and its ligands in inflammatory disorders.

Here we investigated the relevance of NKG2D in chronic inflammation linked to liver damage and tumorigenesis using a well-described model of chemically-induced hepatocellular carcinoma (HCC). NKG2D-deficient mice and control littermates were treated with the carcinogen diethyl-nitrosamine to induce persistent liver injury and consequent HCC. Our data show that NKG2D favors tumor progression. Specifically, when compared to wild-type mice (WT), NKG2D-deficient mice (KO) showed a better survival, a lower liver/body weight ratio and lower ALT level, associated with liver damage.

In addition, we observed differences in the distribution of NKG2D-expressing cells in the tumor infiltrate when comparing WT and KO mice. CD8+T cells but not NK cells significantly accumulated in WT tumor indicating that NKG2D favors the recruitment and/or expansion of CD8+T cells in damaged liver. On the contrary, NKT cells were significantly reduced in the tumor environment of WT mice demonstrating a role for NKG2D in regulating the inflammatory mediators present in the tumor environment.

Together, these findings unravel a new contribution for NKG2D in cancer, as a tumor promoter, and suggest to carefully select the type of tumors which may benefit from therapeutic approaches designed at increasing NKG2D ligand expression.

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Human V γ 9V δ 2 T cells can selectively promote TH1 or TH2 responses via interactions with dendritic cells or B cells *in vitro*

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The V γ 9V δ 2 subset of human $\gamma\delta$ T cells can induce maturation of dendritic cells (DC) into antigen-presenting cells (APC) and B cells into antibody-secreting plasma cells. Since B cells are capable of presenting antigens to T cells, we investigated if V γ 9V δ 2 T cells can influence antigen presentation by these cells. V γ 9V δ 2 T cells, B cells and monocytes were isolated from human blood. V γ 9V δ 2 T cells were expanded by phosphoantigen stimulation and monocytes were induced to differentiate into immature DC. V γ 9V δ 2 T cells were co-cultured with equal numbers of DC or B cells. Expression of APC markers, production of cytokines and antibodies, and stimulation of T cells by the DC or B cells was then measured using flow cytometry and ELISA. V γ 9V δ 2 T cells induced expression of CD86 and HLA-DR and the secretion of IL-6, TNF- α and IFN- γ by DC. They augmented the ability of DC to stimulate proliferation and IFN- γ production by antigen-specific and alloreactive T cells. In contrast,

V γ 9V δ 2 T cells induced CD86, HLA-DR and CD40 expression by B cells and they promoted the release of IL-6, TNF- α , IL-4 and IgG, IgA and IgM by B cells. B cells matured with V γ 9V δ 2 T cells stimulated proliferation but not cytokine secretion by conventional T cells. These data suggest that V γ 9V δ 2 T cells can induce maturation of DC and B cells into APCs, but while they prime DC to stimulate T_H1 responses, they prime B cells to stimulate T_H2 responses.

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GATA3 expressing innate lymphoid cells are an important early source of Th2 cytokines driving allergen induced inflammation and AHR

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Background and aims: Allergic asthma is a T helper (Th)2 cytokine driven chronic inflammatory disease. Several cell types contribute to disease pathology including eosinophils and Th2 cells. Recently, a novel lymphocyte population termed innate lymphoid cells (ILCs) has been suggested as an alternative source of Th2 cytokines and a potential driver of pathology. Administration of allergen or the innate cytokine IL-33 has been shown to induce IL-13+ILCs. However, the relative contribution of ILCs and Th2 cells to the initiation of allergic disease is not well understood. We aimed to investigate T cells and ILCs as important early producers of IL-13 and assess their relative importance in driving AHR and allergic inflammation.

Methods: Adult balb/c mice (6–8 weeks) were given either house dust mite extract (HDM), alternaria (ALT) or recombinant cytokine (IL-33, IL-25 or TSLP) intranasally three times over a period of 1 week. Airway responsiveness was determined by direct measurements of resistance and compliance in anaesthetised and tracheostomised mice 18 h after the 3rd dose of allergen or cytokine. The lymphocyte populations in the lung, bronchoalveolar lavage and mediastinal lymph nodes were compared to control (PBS) treated mice.

Results: Using both the HDM and ALT models of acute allergic airways disease we show that a population of IL-13 secreting ILCs (Lin-CD45+ICOS+) was induced in bronchoalveolar lavage, lung and mediastinal lymph nodes compared to control treated mice. An expansion in the IL-13+ILC population was also observed in mice administered intra-nasal recombinant IL-33 or IL-25 but not TSLP. In both the allergen and IL-33 treated mice an expanded IL-13+ILC population correlated with a significant increase in AHR compared to control mice.

We further characterised the allergen induced ILCs showing all IL-13+ILCs co-expressed the Th2-associated transcription factor GATA-3. The innate cytokine IL-33 is implicated in the generation of the IL-13+ILC population as these cells could not be induced in mice lacking functional IL-33 receptors (T1ST2). Although the frequency of IL-13+Th2 cells (CD4+CD3+) increased in the lungs after HDM, ALT or IL-33 administration, this cell population represented a significantly lower proportion of the total lymphocytic IL-13 producing cell population compared with ILCs.

Conclusions: In conclusion, the predominant early lymphocytic source of IL-13 in the lung and lung draining lymph nodes following allergen exposure are ILCs suggesting these cells likely play a key role in the generation of asthma pathology and AHR.

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Circulating mucosal-associated invariant T cells are depleted in patients with common variable immunodeficiency

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Background and aims: Common Variable Immunodeficiency (CVID) is the most commonly diagnosed primary immunodeficiency. It is clinically heterogeneous but always characterized by abnormally low immunoglobulin titres and recurrent infections in patients. Since T cell help is required for B cell differentiation and immunoglobulin production, we investigated if circulating conventional and innate T cell and lymphocyte subsets are numerically or phenotypically altered in CVID patients alongside B cell subsets.

Methods: Blood was obtained from 28 healthy controls and 21 CVID patients attending St. James's Hospital, Dublin. Using flow cytometry we determined the frequencies and absolute numbers of plasmablasts, naive and transitional B cells, unswitched and switched memory B cells, CD4⁺, CD8⁺ and CD4⁺ CD8⁺ T cells, natural killer (NK) cells, $\gamma\delta$ T cell subsets (V δ 1, V δ 2 and V δ 3), invariant Natural Killer T (iNKT) cells and Mucosal-Associated Invariant T (MAIT) cells. Clinical data were also collected from patient records.

Results: B cell maturation was skewed in CVID patients, with significantly increased frequencies of naive B cells ($P < 0.005$) and decreased frequencies and absolute numbers of switched memory B cells and plasmablasts (all $P < 0.0001$). NK cells and $\gamma\delta$ T cell subsets were normal. iNKT and MAIT cells were significantly depleted in CVID patients ($P < 0.0001$ and $P < 0.01$, respectively). MAIT cells also exhibited an atypical CD4/CD8 distribution, and their frequencies were particularly low in patients with CVID and autoimmune complications ($P < 0.005$).

Conclusions: These data show that circulating MAIT and iNKT cells are depleted in patients with CVID. iNKT cells can influence B cell differentiation and antibody production. MAIT cells require B cells for their expansion and could, like iNKT cells, affect B cell maturation and function. Their reduction in patients with autoimmune complications moreover suggests that they have regulatory functions in peripheral tissues.

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ROR γ ⁺ innate lymphoid cells form a unique microenvironment within the mesenteric lymph node

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Whilst significant advances have been made in understanding the biology of innate lymphoid cells (ILCs), much has focused on the gut or more recently within non-lymphoid tissue. Whilst the role of lymphoid tissue inducer cells in the development of lymph nodes is well established, the subsequent roles of ILCs within these tissues in the adult are not clear. Analysis of individual lymph nodes revealed significant differences in the composition of ILCs between peripheral lymph nodes (e.g. inguinal, brachial) and the mesenteric lymph nodes. Within peripheral lymph nodes, the majority of ROR γ ⁺ cells are $\gamma\delta$ ⁺ T cells and $\alpha\beta$ ⁺ T cells, with ROR γ ⁺ ILCs a rare population. In contrast, within mesenteric lymph nodes, ROR γ ⁺ ILCs make up a significant part of the ROR γ expressing population, with $\gamma\delta$ ⁺ T cells largely absent. Strikingly, many of these ROR γ -expressing cells reside within the same area of the lymph node. In mesenteric lymph nodes,

unlike peripheral ones, this domain is populated almost entirely by ROR γ ⁺ ILCs, forming a unique microenvironment. The consequences of this on immune responses in the lymph node are currently being investigated, with effects on adaptive and also innate cells analysed. We hypothesise that the ROR γ ⁺ ILCs in this region provide local cytokine and costimulatory signals to other immune cells. Trafficking experiments using KAEDE mice indicate that the higher frequency of ROR γ ⁺ ILCs in the mesenteric lymph node reflects continuous tracking from the gut, suggesting a role in the homeostasis of responses to antigens in the gut.

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Investigating the role of ROR γ ⁺ innate lymphoid cells in regulatory T cell survival

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Background and aims: A greater understanding of regulatory T cell (Treg) survival and the emerging concept of regulatory memory would have far reaching therapeutic implications. Using confocal microscopy we have observed co-localisation of some FoxP3⁺ Tregs with ROR γ ⁺ Innate Lymphoid Cells (ILCs) in secondary lymphoid tissue, particularly in the mesenteric lymph node. This ILC population expresses Tumour Necrosis Factor superfamily members, such as OX40L and CD30L, which are important in T cell homeostasis. OX40 has also been implicated in Treg survival. ROR γ ⁺ ILCs also express MHC class II molecules in the absence of CD80 and CD86, the classic co-stimulatory molecules for driving T cell expansion. Therefore we have sought to investigate whether ROR γ ⁺ ILCs are important for the survival of Tregs.

Methods: To track antigen-specific Tregs, we have used immunisation with 2W1S peptide to expand an endogenous, polyclonal population of antigen-specific CD4 T cells, which can then be identified using tetramers. Although tracking antigen specific Tregs is technically challenging, we have found that an agonistic anti-TNFRSF25 antibody enhances numbers of antigen-specific Tregs.

Results: We detected significantly fewer 2W1S⁺ Tregs in ROR γ ^{-/-} mice, which lack ROR γ ⁺ ILCs. Interestingly we have found that TNFRSF25 is expressed by both ROR γ ⁺ ILCs and Tregs.

Conclusions: Following these observations, current studies are investigating whether ROR γ ⁺ ILCs directly support antigen-specific Tregs and if so, by what mechanism.

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Contrasting patterns of natural killer and T cell reconstitution in the first 4 weeks following allogeneic haemopoietic stem cell transplantation

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Background and aims: Allogeneic haemopoietic stem cell transplantation (HSCT) is used as a treatment for high-risk or relapsed haematological malignancies. Effective lymphocyte reconstitution is an important factor in determining risk of infection and overall outcome following HSCT. However very little is currently known about lymphocyte reconstitution in the first 4 weeks immediately following HSCT.

Methods: Peripheral blood lymphocytes were isolated from heparinized whole blood from patients undergoing HSCT with samples taken at various time points: pre-transplant and day 7, day 14 and 4 weeks following HSCT. Lymphocytes were analysed using flow cytometry to assess lymphocyte subsets and proliferation by Ki-67 positivity. Cell counts were measured using Trucount tubes (BD Biosciences). Sorted NK cells were purified and analysed by microsatellite assay for chimerism analysis.

Results: Immediately pre-HSCT, patients are lymphopenic with mean peripheral blood lymphocyte counts of 1300 cells/ μ l (SD $\pm$ 800). This represents both their underlying disease process and previous chemoradiotherapy. Lymphocyte numbers drop rapidly to 96 cells/ μ l (SD $\pm$ 59) by day 7 following HSCT, gradually increasing to 150 cells/ μ l (SD $\pm$ 79) by day 14 and 330 cells/ μ l (SD $\pm$ 200) by week 4.

There are striking differences in the pattern of reconstitution between lymphocyte subsets during this time period. T cells comprise the majority of peripheral blood lymphocytes pre-transplant with a mean of 870 cells/ μ l (SD $\pm$ 710) but numbers drop to 20 cells/ μ l (SD $\pm$ 15) at 4 weeks following SCT. In contrast, NK cells have a pre-transplant level of 118 cells/ μ l (SD $\pm$ 102), which drops to 25 cells/ μ l ($\pm$ 28) and 40 cells/ μ l ($\pm$ 58) at days 7 and 14 respectively. However, by week 4 NK cell numbers have reconstituted to levels exceeding those seen pre-transplant –160 cells/ μ l ($\pm$ 120).

NK cells identified at day 14 following HSCT stain strongly for Ki-67 (>95%; n = 6) indicating rapid proliferation of these cells. This contrasts with the low Ki-67 staining seen in healthy donor NK cells (<5%; n = 4). In addition, chimerism analysis of NK cells at day 14 have confirmed full donor chimerism at this very early time point.

Conclusions: NK cell numbers recover quickly following HSCT, contrasting with the small numbers of T cells identified at matched time points. These cells are proliferating rapidly, originate from the donor stem cells infused and do not represent residual patient NK cells.

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Reciprocal interactions control the development of invariant NKT-cells and RANK-dependent medullary thymic microenvironments

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The thymus recruits blood-borne lymphoid progenitors and provides specialized cortical and medullary microenvironments that guide their differentiation into multiple lineages. While positive selection in the thymic cortex generates conventional $\alpha\beta$ T-cells expressing a diverse T-cell receptor repertoire recognizing antigens bound to self-MHC, the thymic medulla controls production of additional T-cell subsets including CD4⁺Foxp3⁺ regulatory T-cells and invariant V γ 5⁺ dendritic epidermal $\gamma\delta$ T-cells. In turn, thymus medulla formation requires RANKL expression by both innate and adaptive immune cells, highlighting the complex interactions controlling its development and function. Invariant Natural Killer T (iNKT) cells, a specialized subset of $\alpha\beta$ T-cells linked to the regulation of both innate and adaptive immune responses, are also generated within the thymus. While initiation of iNKT-cell development requires recognition of the non-classical MHC class I-like molecule CD1d on CD4⁺8⁺ progenitors in the thymic cortex, the role of the thymic medulla in iNKT-cell development, as well as the potential impact of these cells on medulla development, are unclear.

Here, we show that iNKT-cells express RANKL during early stages in their intrathymic development, and trigger Aire⁺ medullary thy-

mic epithelial cell (mTEC) development, thereby contributing to thymus medulla formation. Furthermore, we show that mTEC are required during later stages of iNKT-cell development, and can be partly replaced by soluble IL15/IL15Ra complexes, correlating with their expression of genes required for IL15 trans-presentation.

Collectively, our findings reveal a novel role for iNKT-cells in thymus medulla development, and further highlight the importance of the thymus medulla in the development of non-conventional T-cell subsets.

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NAD levels modulate macrophage subset responses

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It has been determined that intracellular levels of NAD have a critical role in modulating a wide range of cell signalling pathways. Among these, regulation of gene expression by the NAD-consuming enzymes has been shown to have an important role in modulation of the expression and secretion of the pro-inflammatory cytokine, TNF- α . In order to investigate potential roles of NAD (NAMPT, salvage and tryptophan catabolism) pathways in determining macrophage effector subset responses, we have investigated NAD levels and their relationship to cytokine production using THP-1 cell line-derived M1 (pro-inflammatory) and M2 (anti-inflammatory) macrophages via differentiation by PMA and vitamin D₃, respectively. Results show that NAD levels differ markedly between M1 and M2 macrophages with M1s having much higher basal levels. Upon stimulation with LPS, NAD levels increase dramatically in M1s but not in M2s. Coupled with this NAD profile, secretion of TNF- α parallels LPS-induced NAD levels in M1s but not M2s. This data compares to the published association of intracellular NAD levels and cytokine production in THP-1 monocytes, suggesting NAD as a key mediator of TNF- α synthesis. In conclusion, M1 and M2 macrophage subsets display differential production and utilization of NAD in response to LPS activation, with downstream effects on pro-inflammatory cytokine production. These findings open the possibility of pharmacological modulation of NAD synthesis as a way of selective modulation of macrophage subset responses.

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Human invariant natural killer T cells control multiple B cell functions *in vitro*

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Human B cells express CD1d and therefore are likely to present glycolipid antigens to invariant natural killer T (iNKT) cells. We investigated if B cells could present the iNKT cell agonist glycolipid, α -galactosylceramide (α -GC) to expanded autologous iNKT cells *in vitro* and if α -GC-stimulated iNKT cells could reciprocally activate the B cells. Since iNKT cells comprise functionally-distinct subsets based on CD4 and CD8 α expression, we analysed the effects of co-culturing sorted CD4⁺, CD8 α ⁺ and CD4-CD8 α ⁻ double negative (DN) iNKT cells with B cells. B cells were capable of presenting α -GC to CD4⁺ and DN (and to a lesser degree CD8 α ⁺) iNKT cells resulting in IFN- γ , TNF- α , IL-4, IL-5 and IL-13 secretion by iNKT cells. However B cells were 10-200-fold less efficient than DC at stimulating the production of these cytokines by iNKT cells. Reciprocally, all subsets of iNKT cells could induce antibody production by autologous B cells *in vitro*, but interestingly, α -GC was not

required. Culture of B cells with CD4⁺ or DN iNKT cells resulted in the expansions of cells with regulatory B (BREG; CD1dhiCD5⁺, CD24hiCD38hi) phenotypes and CD4⁺ iNKT cells promoted IL-10 production by some B cells. These BREG phenotypes were induced without the need for α -GC. These results suggest that human iNKT cells can differentially control adaptive immune responses, depending on whether or not stimulatory glycolipid ligands are present. In the presence of α -GC, human B cells can weakly activate iNKT cells, resulting in Th1 and Th2 cytokine secretion. In the absence of α -GC, B cells do not stimulate cytokine secretion by iNKT cells, but iNKT cell subsets can induce antibody production and promote differentiation of B cells into IL-10-producing BREG cells.

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The role of invariant natural killer T cells in recovery from thymic damage

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While the role of the thymus in generating T cells is well-established, the reciprocal crosstalk between developing thymocytes and thymic epithelial cells (TEC) is still being elucidated. The formation of the thymus medulla requires interactions between conventional CD4⁺ $\alpha\beta$ T cells, V γ 5⁺ dendritic epidermal T cells, or invariant natural killer T cells (iNKT cells), and TEC, wherein the developing thymocytes provide TNF family signaling to drive maturation of medullary TEC (mTEC). In addition, innate lymphoid cells (ILC) expressing IL-22, though dispensable for thymic development, appear to significantly contribute to thymic recovery after insult. Unique among thymocytes, iNKT cells are selected on lipid antigens presented by CD1d molecules on CD4⁺CD8⁺ thymocyte progenitors, moving from CD44⁺ early stage 0–1 and mature CD44⁺ stage 2–3 cells. Unlike most thymocyte subsets, which move into the periphery upon completing development, a population of mature CXCR3⁺ iNKT cells remains as long-term thymic residents. Mature iNKT cells are heterogeneous and can be further subdivided based on their expression of cell surface and secreted molecules, including CD4, IL-17RB, IL-4, IL-13, and IL-22, which are implicated in conflicting inflammatory and suppressive roles in peripheral tissues. We aim to more fully characterize the repertoire of phenotypically distinct recirculating and resident thymic iNKT cells and understand their potential roles in adult thymus homeostasis and recovery from insult, which are as yet unknown. In order to characterize the repertoire of thymic iNKT cells and their function in thymus biology, we have utilized flow cytometry and quantitative PCR in iNKT and TEC populations from embryonic and adult wildtype and genetically altered mice. We have investigated the role of iNKT cells in the steady state thymus, as well as following cytoinductive damage. The heterogeneous thymic-resident iNKT population appears to contain a subset that acts on TEC, which is particularly important for recovery from thymic damage. In addition to their role in thymic medullary development, iNKT cells may aid in thymic rejuvenation following injury.

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Vaccine-induced activation of natural killer cells is restricted to the CD57⁺ subset

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Introduction: Natural killer (NK) cells contribute to the effector phase of vaccine-induced adaptive immune responses, secreting cytokines and killing infected cells. The proportion of responding NK cells varies between individuals and by vaccine, suggesting that functionally discrete subsets of NK cells with different activation requirements may be involved. Here, we have used the components of the DTwP vaccine- diphtheria toxoid, tetanus toxoid, and whole cell pertussis- to characterise the NK cell subsets involved in restimulation responses.

Methods: Adult volunteers were recruited from among staff and student donors at LSHTM. PBMCs were cultured with vaccine antigen, with or without a sub-optimal low dose concentration of accessory cytokines (IL-12, IL-18), in the presence or absence of IL-2 blockade. Functional characteristics of NK cells and NK cell subsets (CD56^{bright} and CD56^{dim}CD57⁺, CD56^{dim}CD57^{intermediate(int)} and CD56^{dim}CD57⁺) were measured by flow cytometry. Responses of these NK cell subsets were similarly measured to receptor crosslinking and a high dose of accessory cytokines. *Ex vivo* expression of receptors for these cytokines, IL-12R β 2 and IL-18R α , was also compared between subsets.

Results: *In vitro* stimulation of PBMC with tetanus toxoid, diphtheria toxoid, or whole cell pertussis induced CD25 expression and IFN- γ production in previously vaccinated individuals. Mechanistic studies with tetanus toxoid demonstrated that NK responsiveness was associated with the presence of antigen-specific IL-2⁺ CD4⁺ T cells and NK cell responses were abrogated by IL-2 blockade. Functional analysis of discrete NK cell subsets revealed that increasing CD57 expression correlated with a gradual loss of responsiveness to exogenous cytokines. Accordingly, NK cells making IFN- γ in response to tetanus toxoid, diphtheria toxoid or whole cell pertussis were predominantly CD57⁺ while highly differentiated CD57⁺ NK cells responded very poorly. CD25⁺IFN γ ⁺ responses were the most robust against whole cell pertussis, with responses to tetanus toxoid being particularly low frequency. IL-12R β 2 and IL-18R α expression decreases with increased expression of CD57, perhaps partially explaining the differential responses. Backgating all CD25⁺IFN γ ⁺ cells in response to pertussis stimulation, showed that while CD56^{bright} NK cells are robust responders, their contribution to the CD25⁺IFN γ ⁺ response is minimal as compared to CD56^{dim}CD57⁺ NK cells, which represent a much larger subset.

Conclusions: Given that CD57⁺ NK cells accumulate with increasing age, particularly in individuals with human cytomegalovirus infection, these findings have implications for the ability of NK cells to contribute to effector response after vaccination and their contribution to vaccine-induced immunity in older individuals.

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NK cell phenotype defines the balance of functional responses in malaria responders

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Background and aims: Natural killer (NK) cells are important initial producers of gamma interferon (IFN γ) when incubated with malaria parasites *in vitro*, but may also be capable of killing infected

erythrocytes (iRBCs) by direct cell contact, as indicated by upregulation of CD107a/LAMP-1 on the NK cell surface and the formation of 'rosettes' between iRBCs and NK cells. However, cellular immune responses between individuals are highly heterogeneous both in terms of cytokine responses and cytotoxic activity. Here, we have looked at variation in these individual responses by IFN γ production and CD107a expression, with a view to looking for evidence of 'rosetting' and formation of the immune synapse.

Methods: Fresh PBMCs were obtained from 21 healthy, malaria-naïve donors at LSHTM. PBMCs were cultured with MACS-isolated *P. falciparum* schizonts, strain 3D7, for 18 h and stained for functional markers of NK cells (IFN γ +, CD25+, CD107a+) and NK cell subsets analysed by flow cytometry.

Results: Co-culture of fresh PBMCs with *P. falciparum*-infected erythrocytes induced both IFN γ production and CD107a/LAMP-1 surface expression within NK cell subsets. Donors appeared to be capable of both responses overall, although individual NK cells tended to only respond by increasing either IFN γ or surface CD107a. In responsive donors, immature NK cells (CD57–) contributed to the response by preferentially producing IFN γ compared to mature NK cells (CD57+), which may indicate a change in function from cytokine production to cytotoxicity with increased NK cell maturity. Upregulation of CD107a in response to direct contact with iRBCs has previously been controversial, and these data show that the variation between the responsiveness of donors as well as the distribution of immature versus mature NK subsets within donors may account for this inconsistency. We also found a slight correlation between NK cell IFN γ responses to iRBCs and high concentrations of IL-12 and IL-18 (HDC), which may indicate that the capability of donors to induce NK cell responses differs regardless of the type of stimulus.

Conclusions: The variation in NK cell response between donors may account for the differences in reported responses to malaria parasites, and certain NK subsets may be responsible for the cytokine-producing versus cytotoxic responses. Individuals identified from this work as preferentially favouring cytotoxic responses will be used in further work to look at immune synapse formation.

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Investigating the role of innate lymphoid cells in adaptive immune responses

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With their expanding range of functions and emerging importance in a range of disease states, an increased understanding of the role of innate lymphoid cells (ILCs) following immunisation promises to provide a variety of new targets for the manipulation of immune responses. Although well characterised in the gut, comparatively less is known of their function in secondary lymphoid tissue, important sites for the generation of adaptive immune responses. Using immunofluorescence microscopy to stain for the critical transcription factors, we have located clusters of GATA-3+ and ROR γ + ILCs co-localising within lymph node tissue. Consistently we detect these cells at the interface of the B cell and T cell areas, particularly within the interfollicular regions of lymph nodes. These sites are thought to be critical microenvironments for interactions between responding adaptive immune cells during an immune response. Whilst ILCs are rare populations within lymph nodes, clustering of these cells may enable different ILC populations to influence other immune cells through their local cytokine production. Using draining lymph node immunisation models, combined with tracking antigen specific B

and T cells, we aim to characterise the behaviour of ILCs within a responding lymph node. We observe co-localisation of activated antigen specific lymphocytes with ILCs at early time points following immunisation. We hypothesise that here these cells receive signals from ILCs, and aim to determine the consequence of this on the progression of an adaptive immune response using models where ROR γ + ILCs are depleted. Preliminary experiments suggest that whilst published data from our group shows that ROR γ + ILCs support the survival of memory CD4+ T cells in secondary lymphoid tissue, they are not essential for initial CD4+ T cell expansion. We observe that loss of ROR γ + ILCs results in increased numbers of GATA-3+ ILCs within these niche locations, and investigate whether the resulting altered ILC cytokine environment affects the type of immunity generated.

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Increase of NKp44+ group 3 innate lymphoid cells (ILC3) in psoriasis

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Innate lymphoid cells (ILC) are a recently characterized population of immune cells with emerging key roles in tissue immunity. However, their function in human tissue homeostasis and disease remains to be completely clarified. In this study we investigated the role of ILC in human skin from healthy individuals and from the inflammatory skin disease psoriasis. We found that a considerable number of IL-17A and IL-22 producing cells in skin and blood of healthy individuals and psoriasis patients are CD3 negative innate lymphocytes. Detailed immunophenotyping of human ILC subsets showed a significant increase in the frequency of circulating NKp44+ ILC3 in blood of psoriasis patients compared to healthy individuals or atopic dermatitis patients. Circulating NKp44+ ILC3 expressed cutaneous lymphocyte-associated antigen indicating their potential for skin homing. Further analysis of skin tissue revealed a significantly increased frequency of total ILC in skin compared to blood. Interestingly the frequency of NKp44+ ILC3 was significantly increased in non-lesional psoriatic skin compared to normal skin, indicating a potential role of such cells in starting the psoriatic disease progression. Moreover the clinical response to anti-TNF therapy in one patient followed during treatment showed a close association with the frequency of circulating NKp44+ ILC3. Our data suggest a potential role for NKp44+ ILC3 in psoriasis pathogenesis.

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The role of eosinophils in perivascular adipose tissue

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Eosinophils are innate immune cells present in the mesenteric perivascular adipose tissue during steady state conditions. Classically the function of eosinophils has been associated with tissue destruction via release of cytotoxic granule contents however, recent evidence

has demonstrated a more diverse role for eosinophils. Interestingly, adipose eosinophils regulate metabolic homeostasis through maintenance of adipose alternatively activated macrophages. To further elucidate the function of eosinophils in adipose tissue we investigated the role of eosinophils in perivascular adipose tissue (PVAT). The release of adipokines from PVAT plays an important role in reducing tone in a variety of vascular beds and this anti-contractile effect is lost in obesity, which may contribute to hypertension. We investigated the role of eosinophils in maintaining normal PVAT function and artery constriction using wire myography on eosinophil deficient mice (Δ dblGATA) and littermate control wildtype (WT) mice.

In WT mice healthy PVAT exerts an anti-contractile effect on arteries in the mesenteric bed. However, in Δ dblGATA mice, despite normal contractility of arteries, the anti-contractile effect of the PVAT was lost. The anti-contractile effect of WT PVAT was mediated via a soluble factor that could restore anti-contractile function in the absence of eosinophils. Importantly, the anti-contractile effect of PVAT in Δ dblGATA mice could be rescued both *in vivo* and *in vitro* through the reconstitution of the mice or PVAT respectively with eosinophils. The altered PVAT function did not appear to be mediated by alternatively activated macrophages, although the effect is dependent on IL-4 and adiponectin. As well as alterations in PVAT anti-contractile effects the loss of eosinophils in Δ dblGATA mice had an important effect on blood pressure with elevated systolic blood pressure observed in the Δ dblGATA mice compared to littermate WT control mice.

Our data demonstrates an important and novel role for eosinophils in mediating the anti-contractile effect of PVAT and an elevation in blood pressure in mice lacking eosinophils. The pathway by which the effect(s) is being mediated is yet to be fully elucidated but IL-4 and adiponectin both play key roles.

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Human ILC2s selectively produce PGD₂ in response to IL-33

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Asthma is understood to be a Th2 mediated condition where the associated cytokines (IL-4, IL-5 and IL-13) and lipid mediators such as PGD₂ are central to disease pathogenesis. PGD₂ is understood to play a role in asthma through type-2 cell recruitment and activation, via its receptor CRTh2 expressed by basophils, Th2 cells and type-2 innate lymphoid cells (ILC2s). ILC2s have been shown to be crucial for effective type-2 immunity in several mouse models and to require IL-25 and IL-33 for their expansion and function. ILC2s have been shown to secrete type 2 cytokines, in particular IL-13 and IL-5, which were required for effective helminth eradication. ILC2s have also been identified in the human lung and nasal polyps and may play a role in allergic disease.

We sought to develop methods for the isolation, phenotyping and culture of humans ILC2s from peripheral blood and to assess gene activation and mediator production in response to IL-25 and IL-33 treatment *in vitro*. ILC2s were analysed and sorted using flow cytometry and cultured for up to 14 days in the presence of IL-25 and IL-33. Time course experiments were performed to assess the kinetics of gene activation and mediator production from these cells using flow cytometry, real time PCR, microarray analysis and ELISA.

Our results demonstrate human ILC2s to have a similar phenotype to their murine equivalent and to also proliferate in response to IL2/7/25 and 33 *in vitro*. We found IL-33 to significantly upregulate

IL-5 and IL-13 gene and protein expression. Microarray and real-time PCR analyses showed that IL-33, but not IL-25, induced expression of the enzymes required for PGD₂ synthesis, cPLA2, COX2 and prostaglandin D2 synthase. Measurement of PGD₂ levels by ELISA showed that ILC2s express high levels in response to IL-33. Furthermore, we found that IL-33 treatment down regulated CRTh2 expression by ILC2s, indicating that these cells may respond to PGD₂ in an autocrine fashion. Collectively these results show that human ILC2s produce PGD₂ in response to IL-33 and that ILC2s may recruit other CRTh2⁺ cell types to the site of inflammation via expression of PGD₂.

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Innate lymphoid cell 'memory' to bacterial infection

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The innate and adaptive immune systems have traditionally been segregated into separate compartments. The innate immune system being defined as short lived cell populations which rapidly respond in a non-specific manner where secondary exposure to the pathogen results in an identical response to that initiated at the first exposure. In contrast, the T and B cells of the adaptive immune response respond more slowly in a highly antigen specific manner. Clonal expansion of antigen specific effector cells resulting in a pool of long-lived memory cells which respond far more efficiently to re-encounter with the same pathogen. However, these definitions of innate and adaptive have been re-assessed in light of evidence demonstrating 'memory' responses in NK cells against viral pathogens, although bacterial memory responses have yet to be identified. The population of innate lymphoid cells (ILCs) has recently been expanded beyond the classical NK cells. A diverse range of subsets of ILCs which parallel the subsets and functions of previously defined Helper T cells (Th) subsets. ILCs have been shown to play a substantive role in limiting the expansion of microorganisms. However, the potential of ILCs to develop into memory cells emulating classical NK cells has not yet been investigated. Potential 'memory' ILCs (from C57/BL6 mice immunised 7 days previously with heat-killed *Pseudomonas aeruginosa*) or naive (sham immunisation) were isolated from the spleen by magnetic separation and transferred to unexposed hosts concurrently with *Pseudomonas aeruginosa* infection. The transfer of these 'memory' ILCs resulted in a significant reduction in bacterial CFUs in the peritoneal fluid ($P = 0.006$) and prevented systemic spread compared to naive ILC transfer. To assess if this was simply due to bystander activation of the innate lymphocytes, memory was induced by immunisation with either heat-killed *Staphylococcus aureus* or *P. aeruginosa*. Whilst the *P. aeruginosa* memory cells were able to significantly reduce a *P. aeruginosa* infection, *S. aureus* induced memory cells were not. This demonstrates the pathogen specificity of innate lymphoid cell memory response. The protective effect was demonstrated to be IL-17 dependent, indicating a role for ILC3 cells. Further flow cytometric characterisation of these cells was carried out and it was shown that both NK cells and ILC3s were present. This is the first demonstration that ILCs can confer a 'memory' response against a bacterial pathogen; these highly novel findings offer insight into an unexplored area of ILC role in response to bacterial infection.

Metabolic Control of Immunity in Health and Disease

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Altered functions and adhesive capacity of neutrophils in diabetes with poor glycemic control

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Neutrophils were evidenced to be associated with atherosclerosis, but their roles in diabetes-related atherosclerosis remain unknown. This study aimed to investigate the effect of hyperglycemia and advanced glycation end product (AGE) on the phenotypes and biological functions of neutrophils. Type 2 diabetic patients were divided into two groups according to the level of HbA1c, good glycemic control ($\text{HbA1c} \leq 7\%$) and poor glycemic control ($\text{HbA1c} > 9.0\%$) and neutrophils were isolated from peripheral venous blood of the patients. *In vitro* effects of high glucose and AGE on neutrophils were studied in the cells derived from normal subjects under exposure to different conditions; 5 mM glucose, 25 mM glucose, 100 $\mu\text{g}/\text{ml}$ BSA and AGE-BSA. The cells were then determined for basal and PMA-stimulated production of reactive oxygen species (ROS), expression of CD11b and CD66b, release of myeloperoxidase (MPO), cell migration and adhesion to endothelial layer. In diabetic subjects, cells from well controlled patients produced significantly higher basal and PMA-stimulated ROS. Poorly controlled patients showed significantly increased expression of CD11b, CD66b and MPO production. After incubation with AGE-BSA *in vitro*, the release of MPO was significantly increased after PMA stimulation in comparison to unmodified BSA. High glucose incubation significantly enhanced neutrophil migration towards IL-8 and neutrophil adherence to endothelial cells. From this study, the results showed activated status of neutrophils in diabetic patients and increased adhesive capacity of neutrophils in hyperglycemic condition *in vitro*. These altered functions may lead to the development and progression of atherosclerosis in diabetes.

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Mitochondrial morphological change during dendritic cell maturation

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Growing evidence shows that mitochondria play an important role in innate immune responses. Mitochondrial tethering to endoplasmic reticulum according to increased fusion of mitochondrial network regulates mitochondrial antiviral signaling protein. In addition, mitochondrial fusion/fission events are closely related with various cellular events, such as programmed cell death, cell differentiation and aging. However, the relationship between mitochondrial fusion/fission events and acquired immune response is still unclear. Dendritic cells, professional antigen presenting cells, are essential for an acquired immune responses including activation of T cells. In this study, we investigated the characteristic features of mitochondria in dendritic cells during the activation. As a result, mitochondrial fusion/fission events in mouse bone marrow-derived dendritic cells

were confirmed by the observation using laser scanning microscopy. Here, we report the precise relations of mitochondrial fusion/fission with activation of dendritic cells.

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Soluble CTLA-4 is a marker of inflammation in elderly people and correlates with cardiovascular disease risk factors especially in females

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Cardiovascular disease (CVD) refers to any disease that affects the cardiovascular system, principally cardiac disease, vascular diseases of the brain and kidney, and peripheral arterial disease and is the leading cause of deaths worldwide. One of the studies dedicated to cardiovascular disease is the Whitehall II study which investigates the importance of various factors in the development of CVD in healthy population. By using this study, our aim was to find any immunological parameters that can be used to predict the development of CVD in healthy populations. The cohort that we used in this study consisted of 68 people, 56 females and 12 males aged from 55 until 75 years old. Our data shows that soluble CTLA-4 (sCTLA-4) can be used as a marker of inflammation in elderly people. This novel isoform of CTLA-4 is produced by alternative mRNA splicing and importantly not by proteolytic digestion or shedding of membrane-bound CTLA-4. Data from the Whitehall II study cohort revealed positive associations between sCTLA-4 and circulating cytokine levels ($n = 68$). sCTLA-4 correlated with both pro-inflammatory IL-1 β ($R = 0.254$, $P = 0.037$), IL-6 ($R = 0.447$, $P < 0.0001$), TNF- α ($R = 0.729$, $P < 0.0001$) and IFN- γ ($R = 0.496$, $P < 0.0001$) and anti-inflammatory cytokines IL-10 ($R = 0.27$, $P = 0.026$). All associations with clinical/biochemical parameters were not statistically significant. Therefore, we further analysed the cohort based on sex and age differences. The cohort was divided into three groups; male (55–64 years), female (55–64 years) and female (65–75 years). No associations were found between sCTLA-4 and any parameters in the male group ($n = 12$) but sCTLA-4 correlated with several parameters in the female groups. sCTLA-4 positively correlated with coronary artery calcification ($R = 0.346$, $P = 0.031$) while it negatively correlated with total cholesterol level ($R = -0.42$, $P = 0.008$) and Low Density Lipoprotein ($R = -0.356$, $P = 0.026$) in females aged 55–64 years old ($n = 39$). However, analysis from the older female group (65–75 years, $n = 17$) demonstrated correlations of sCTLA-4 with High Density Lipoprotein ($R = 0.568$, $P = 0.017$) and triglyceride level ($R = -0.514$, $P = 0.025$). These associations indicate sCTLA-4 secretion and regulation can be related with lipid metabolism. However, sCTLA-4 associations with lipid parameters gave a mixed picture about its role in CVD development; therefore a larger cohort is needed to confirm these associations.

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The effect of obesity-induced metabolic stress on T cell migration

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Purpose and objective: Chronic low-grade inflammation is a key feature of many cardiovascular metabolic diseases associated with obesity. T cells have been shown to infiltrate obese adipose tissue, where pro-inflammatory immune responses are preponderant over immunomodulatory and immunosuppressive responses and as such drive disease progression. The regulation of energy metabolism is crucial for many aspects of T cell-mediated immunity including activation and proliferation, and more recently has also been shown to affect T cell migration. However how changes in metabolic status orchestrate pathological T cell trafficking is still unknown.

Results: Activated T cells from obese mice show no difference in their activation state as measured by CD44 positivity or their proliferation rate, but do show increased migration to the pro-inflammatory cytokine IP-10 *in vitro* and increased recruitment in the inflammatory sites *in vivo*. In syngeneic recipient mice, activated T cells from obese mice left the bloodstream at a faster rate than T cells from lean mice and were recruited more efficiently to the peritoneal cavity in a model of zymosan-induced peritonitis. This phenotype was independent of whether recipient mice were obese or lean. Activated T cell from obese mice showed an increase in their effector memory population which is dependent on functional PI3K-signalling.

Conclusions: Obesity causes a metabolic imprinting of T cells switching them towards a memory effector phenotype, which leads to an increased ability of T cells to migrate towards inflammatory sites. Aberrant T cell migration could favour the establishment of chronic inflammation in obesity.

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Expansion of arginase-expressing myeloid-derived suppressor cells in hepatotropic viral infections

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Background and aims: Previous work from our group has pointed to a role for nutrient deprivation in driving a global T cell defect in chronic hepatitis B infection (CHB), indicating that the conditionally essential amino acid L-arginine is depleted and arginase-1 activity is increased in the HBV-infected liver (Das, *JEM* 2008). In this study we postulated that myeloid-derived suppressor cells (MDSC), a population known to expand at sites of chronic antigenic stimulation, may contribute to the arginase-rich suppressive milieu in CHB.

Methods: Using 11-colour flow cytometry and image stream technology, we analysed the circulating frequency of granulocytic MDSC (gMDSC) and their expression of putative regulatory mediators in patients with CHB ($n = 80$) and healthy controls ($n = 52$). Patient and healthy control serum L-arginine levels were measured by tandem high-pressure liquid chromatography mass spectrometry.

Results: Circulating gMDSC were expanded approximately 10-fold in CHB compared to healthy controls ($P < 0.001$). This increase in gMDSC occurred in all patients regardless of disease activity, but expansion was significantly further increased in those patients without biochemical evidence of liver damage ($ALT < 401$ U/l). Analysis of the patient cohort according to clinical stage showed that the most striking expansion was evident in patients in the 'immunotolerant' phase of disease characterized by extremely high viral replication in the absence of liver damage. A striking further enrichment of gMDSC was found in the intrahepatic compartment, the site of viral replication. Another hepatotropic viral infection, HCV, likewise resulted in MDSC expansion in the circulation that was further amplified in the liver compartment, whereas CMV serostatus did not affect MDSC frequencies. One key function of gMDSC is their production of suppressive regulators, including arginase-1 which depletes the amino acid L-arginine required for T cell effector function; we were able to demonstrate strong expression of this enzyme by the majority of gMDSC by flow cytometry and image stream analysis. A functional role for arginase-1 was corroborated by the fact that depletion of serum L-arginine concentrations was most striking in the patient group without liver inflammation, who had the largest gMDSC expansions. Preliminary sorting experiments have confirmed the capacity of MDSC to suppress bystander T cell function in an arginase-dependent manner.

Conclusions: Our data therefore show that gMDSC accumulate in hepatotropic viral infections and have the potential to suppress global T cell function through nutrient deprivation, thereby contributing to the down-regulation of liver inflammation.

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Insights into the role of inflammation in dystroglycanopathies - analysing the effect of alpha dystroglycan glycosylation on immune function

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The dystroglycanopathies are a sub-group of muscular dystrophies with a wide clinical phenotype ranging from the severe Walker Walburg Syndrome and Muscle Eye Brain Disease which are associated with structural eye and brain involvement in addition to the muscular dystrophy, to milder forms of limb girdle muscular dystrophy. Central to the disease process is a defect in alpha dystroglycan (α DG) glycosylation which perturbs binding to extracellular matrix ligands such as laminin and perlecan and leads to basement membrane defects. However, α DG is also expressed by cells of the immune system and inflammatory infiltrates into the muscle are common in dystroglycanopathy patients. The aim of this investigation was to determine whether a disruption of α DG glycosylation alters the immune system and so contributes to the pathogenesis of disease. In order to do this Large^{myd} dystrophic mice, which express hypo glycosylated α DG and Large^{myd}LV5, which express hyperglycosylated α DG but are no longer dystrophic, were used to analyse the steady state of the thymus, spleen and lymph nodes in 12 and 20 weeks old mice. Our preliminary results suggest glycosylation of α DG is important in the control of memory and naive pools of CD4 and CD8 T cells. We also detected altered activation states of B cells in the spleens of mice with either hypo or hyperglycosylated α DG. In addition T cell development and the nature of α DG's capacity to bind laminin in primary and secondary lymphoid organs was evaluated along with characterisation of the inflammatory infiltrate in both the Large^{myd} and Large^{myd}LV5 mice. This work confirms a role for α DG glycosylation in the immune system, and furthermore investigates whether there is any detrimental effect on the immune system by inducing hyperglycosylation of α DG which is currently being considered as a therapeutic approach.

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Characterizing human *Burkholderia pseudomallei* infections using untargeted metabolomics

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Metabolomics is a discipline that allows comprehensive profiling of the small biochemical molecules, the metabolites, in a living organism. Because the impairment of metabolic homeostasis is common in critical illnesses, metabolomics is increasingly integrated in studies of pathophysiological processes in disease, and in the discovery of disease biomarkers. *Melioidosis* is an infectious disease caused by the bacterium *Burkholderia pseudomallei*, with septicemia being the most common clinical presentation amongst melioidosis patients. Here, we investigated the interaction between *B. pseudomallei* and human host cells during infection using an untargeted metabolomics approach. First, we characterized the *Burkholderia* intracellular metabolome in *in vitro* culture. We determined the metabolic profile of different *Burkholderia* spp. strains, including *B. pseudomallei* NCTC13177, *B. pseudomallei* K96243, *B. pseudomallei* NCTC10276, *B. thailandensis* E264, and *B. thailandensis* 49639 at *in vitro* bacterial growth phases of late stationary phase for the first time. We subsequently profiled the metabolic changes that occur in human macrophage cell line U937 during *in vitro* infection with *B. pseudomallei* K96243. We will discuss how metabolomics characterization studies may help to understand the interaction between *B. pseudomallei* and human host cell metabolism, and to discover candidate biomarkers for melioidosis.

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Novel insights in translational control of myeloid hematopoietic cell function through maintaining mTOR phosphorylation

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Background and aims: Mammalian myeloid cells are crucial effectors of host innate immune defence against pathogens. Immune responses of these cells require adaptation to signalling stress through the hypoxia-inducible factor 1 (HIF-1) transcription complex and associated pathways. Upon adaptation to inflammatory stress myeloid cells activate the mammalian target of rapamycin (mTOR), initially by phosphorylating its S2448 residue. This leads to activation of the mTOR pathway which induces *de novo* translation of vital signalling proteins. On the other hand, phosphorylation of the T2446 residue of mTOR leads to its inactivation and could be a cause of myeloid cell death. However, the molecular mechanisms underlying the maintenance of mTOR phosphorylation remain unclear. We therefore aimed to provide additional insight into understanding this concept.

Methods: THP-1 human myeloid leukaemia cells were used for the experiments. Six week old CD1 male mice (25 ± 2.5 g) were also involved following approval by the Institutional Animal Committee and handled in accordance with the Helsinki Declaration. Western

blot analysis, ELISA, quantitative real-time PCR, in-cell Western and luminometric/colorimetric assays were used for analysis.

Results: We demonstrate for the first time that inactivation of HIF-1, by silencing its inducible alpha subunit, significantly decreases mTOR S2448 phosphorylation caused by ligand-dependent activation of human myeloid leukaemia cells. This occurs due to activation of adenylate kinase leading to an increase in the intracellular AMP levels. AMP triggers activation of AMP-dependent kinase which causes phosphorylation of mTOR on the T2446 residue thus preventing phosphorylation of S2448. On the other hand, inhibition of xanthine oxidase (XOD), which converts hypoxanthine into xanthine and further into the uric acid, also leads to a similar effect. In the latter case, lack of XOD-dependent AMP degradation leads to an increase in AMP kinase activity thus leading to mTOR phosphorylation on T2446. The results were partly confirmed using *in vivo* models.

Conclusions: These results indicate that mTOR-mediated translational control during myeloid cell responses depends on successful activation of the HIF-1 transcription complex and balanced purine catabolism.

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Highly specific targeting of human myeloid hematopoietic cells using gold nanoparticle-based conjugates

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Background and aims: Application of nanomaterials in immunological research and translational medicine has become increasingly promising during last 10 years. Currently there is a wide range of biomedical applications for nanomaterials. However, it remains extremely problematic to generate biocompatible nanomaterials which are able to specifically recognise immune cells. Development of these technologies is one of the primary focuses of Nanobiotechnology and Immunology since this type of technology is of potential importance for targeting non-adherent blood cells responsible for host immune defence. We therefore explored the opportunity of using functionalised gold nanoparticles to target leukocytes.

Methods: Functionalised gold nanoconjugates were produced from citrate-stabilised gold nanoparticles. Nanomaterials contained both immune receptor ligands (toll-like receptor 4 or CD203c) and pharmacological signal transduction inhibitors. THP-1 human myeloid leukaemia cells and primary human basophils were used in the experiments. Western blot, fluorescent/colorimetric tests, in-cell assays and microscopy were used for analysis.

Results: We demonstrated a new approach for highly specific targeting of viable human leukocytes of myeloid lineage by functionalised gold nanoconjugates. Using this technology we managed to deliver pharmacological inhibitors (rapamycin and ascomycin) into the target cells where they were able to induce the necessary functional effects.

Conclusions: This approach demonstrates the possibilities of using nanoconjugates for drug delivery and immune therapy.

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The contribution of Foxp3 and TGF β to the iTreg proteome**D. Howie & H. Waldmann***Sir William Dunn School of Pathology, Oxford University, Oxford, UK*

Given the current state of knowledge and uncertainty on how Treg deliver suppression, there is clear value in generating information of proteins whose levels selectively change in Treg. We conducted a comparative quantitative proteomics study to compare the contribution of TGF β and the transcription factor Foxp3 to the Treg proteome. This study complements our previously established SAGE and microarray databases of regulatory T-cell populations in comparison with other CD4 T-cell subsets. To measure the relative importance of Foxp3 versus TGF β to the Treg proteome we used two cell models. First, we used a conditional Foxp3 T cell line expressing a GFP-tagged tamoxifen responsive Foxp3 construct *cFoxp3tg.GFP* which translocates into the nucleus only in the presence of 4'hydroxy

tamoxifen. Second, we used highly purified TGF β -induced iTreg from Foxp3 reporter mice expressing human CD2 under control of the Foxp3 promoter. Using SILAC and heavy dimethyl labelling we quantified differences between nuclear and cytoplasmic Foxp3-containing T cell proteomes and Foxp3 positive iTreg and Foxp3 negative activated CD4 T cells. We used Thermo Q Exactive Orbitrap mass spectrometers coupled to UHPLC 50 cm long nano-columns with 8 h gradients along with a streamlined minimal sample loss methodology important for dealing with low cell numbers. Analysis of the data using geneset enrichment analysis and Metacore analysis has highlighted significant differences between the proteomes of T cells with conditional or induced Foxp3 and T cells which lack Foxp3. Our data suggest that Foxp3 drives a significant shift in the respiratory capacity of T cells by upregulating several members of the mitochondrial electron transport chain. The significance of these changes are discussed.

Modulation of host response by infection

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Significant increase in numbers and functional activity of CD56+ T cells in healthy CMV sero-positive subjects

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Human T cells expressing CD56 are capable of tumour cell lysis following activation with IL-2 but their role in viral immunity has been less well studied. Proportions of CD56+ T cells were found to be highly significantly increased in CMV seropositive compared to seronegative healthy subjects ($9.1 \pm 1.5\%$ versus $3.7 \pm 1.0\%$; $P < 0.005$). Proportions of CD56+ T cells expressing CD28, CD62L, CD127, CD161 and CCR7 were significantly lower in CMV+ than CMV- subjects but those expressing CD4, CD8, CD45RO, CD57, CD58, CD94 and NKG2C were significantly increased ($P < 0.05$), some having the phenotype of T_{EM} cells. Functionally, cells from CMV+ subjects expressed higher levels of IFN γ and TNF α than those from CMV- subjects following stimulation with CMV antigens. More CD56+ T cells were induced to express CD107a and to proliferate following stimulation with CMV antigenin CMV+ than CMV- subjects. Using different Class I HLA Pentamers (HLA-A2, HLA-B7 and HLA-B8), it was found that CD56+ T cells represented a substantial proportion of the antigen-specific CD8+ T cells in CMV+ subjects of several different HLA types. In view of the link between CD56 expression and T cell cytotoxic function, this strongly implicates CD56+ T cells as being a major component of the cytotoxic T cell response to CMV in healthy carriers.

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The Mycobacterium tuberculosis protein ESAT-6 induces inflammasome activation and apoptosis in monocytes

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ESAT-6 is a secreted protein of *Mycobacterium tuberculosis* (Mtb) important for virulence and immunity and a putative vaccine candidate. The function of ESAT-6 is not fully understood, however, its ability to cause macrophage cell death is thought to contribute to the tissue damage and inflammation characteristic of Mtb infection. Previously published reports indicate that recombinant ESAT-6 causes apoptosis of macrophage cell lines. The aim of this study was to investigate the susceptibility of primary human macrophages to ESAT-6-mediated cell death and characterise the cell death mechanism.

Human alveolar macrophages were obtained by bronchoalveolar lavage. Monocytes purified from buffy coats were used immediately, or differentiated to macrophages (MDMs) or dendritic cells (DCs). THP-1 cells were differentiated with PMA for 72 h. Cells were stimulated with recombinant ESAT-6 (1–10 $\mu\text{g/ml}$), and viability was determined by propidium iodide staining. Caspase and inflammasome activation were assessed by western blotting and ELISA.

In agreement with previous reports, ESAT-6 treatment induced caspase-independent apoptosis of THP-1 macrophages. Surprisingly, alveolar macrophages treated with ESAT-6 remained viable for up to 72 h. We next compared the response of monocytes, MDMs and DCs to ESAT-6. Similar to alveolar macrophages, MDMs and DCs did not undergo cell death following exposure to ESAT-6. In con-

trast, freshly isolated monocytes from the same donors succumbed to ESAT-6-mediated apoptosis within 24 h, with nuclear fragmentation and activation of caspase 3. Susceptibility to ESAT-6-mediated apoptosis correlated with inflammasome activity and monocyte apoptosis was abrogated by the NALP3 inhibitor glyburide.

Infiltrating monocytes play an important role in shaping innate and adaptive immune responses to mycobacteria by secreting cytokines at the site of infection and differentiating into macrophages and DCs. Mtb may exploit the resistance of macrophages and DCs to ESAT-6-mediated apoptosis to establish a niche while simultaneously eliminating newly recruited monocytes, limiting the numbers available to differentiate into professional antigen presenting cells in the granuloma. The mode of cell death induced by ESAT-6 (apoptosis accompanied by the secretion of pro-inflammatory cytokines) may also influence adaptive immunity and have implications for vaccine design.

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T cells from intraperitoneally infected donors control migration of toxoplasma to the brain

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Introduction: *Toxoplasma gondii* is a widely spread protozoan parasite that cause fatal infection in immunocompromised host. Recently we have shown that mice infected intraperitoneally (ip) with RH strain of toxoplasma had less parasites in their brain compared to orally infected. In this study we analyze the role of T cells in control of toxoplasma dissemination to the brain.

Materials and methods: T cells from ip infected A/Snell mice were transferred to intact recipients which later received CFSE-labeled bone marrow derived dendritic cells (DC) infected with tachyzoites. The presence of infected DC or free tachyzoites in different organs was assessed 24 h post transfer by microscope.

Results: Interestingly, while we observed CFSE labeled DC as well as free tachyzoites released from DC in spleens and lymph nodes of controls there were no CFSE labeled DC in the brain of these mice. However free CFSE labeled tachyzoites were found in their brain in abundance. In contrast, mice that received T cells from ip infected mice had neither CFSE labeled tachyzoites nor DC in their brain. *In vitro* studies of infected DC migration confirmed our *in vivo* observation that specific T cells are able to control the spread of infected DC. T cells from per os infected mice were not able to control the spread of infected DC. The mechanisms of T cells control of dissemination are currently under the study.

Conclusion: Our results define the role of T cells in control of toxoplasma infected DC migration to the target organ.

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Suppressor of cytokine signalling three regulated intestinal epithelial cell turnover: impact on microbial resistance

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Background: Intestinal epithelial cell (IEC) turnover has been implicated in the expulsion of and resistance to intestinal pathogens, including the murine whipworm *Trichuris muris*. Failure to initiate a transient increase in IEC turnover can lead to chronic infection. Over-expression of SOCS3 *in vitro* leads to decreased proliferation and reciprocally, lack of epithelial SOCS3 promotes proliferation and

tumour growth in intestinal injury and cancer models. SOCS3 may have an important role in regulating the magnitude and/or duration of IEC turnover and consequently resistance to pathogens. This study aims to examine the ability of SOCS3 to regulate IEC turnover in an *in vivo* murine model of *T. muris* infection.

Methods: SOCS3 expression was examined in the intestine of *T. muris* resistant or susceptible mice by immunohistochemistry and real-time PCR. Mice with and without IEC SOCS3 conditional knock-down were infected with *T. muris* and intestinal tissue examined at 3, 14, 21 and 35 days after infection. Crypt depth was measured and cell proliferation rates determined using an EdU assay. Outcome of infection was assessed at 35 days.

Results: SOCS3 mRNA was increased in the intestine of mice susceptible to *T. muris* infection compared to resistant mice both prior to infection and at 14 days following infection. IEC SOCS3 protein was increased in susceptible mice at 15 and 21 days post-infection. This led to the hypothesis that increased SOCS3 blocks IEC turnover and a reduction in IEC SOCS3 expression may promote IEC proliferation and expulsion of parasites. Examination of tissue from IEC SOCS3 knockout mice, 3 days post infection, showed little difference in crypt depth or inflammation but suggested an increase in cell turnover, i.e. proliferation. It was too early to determine the impact of lack of IEC SOCS3 on helminth expulsion but this will be determined from later time points.

Conclusion: During the early stages of helminth infection SOCS3 may be blocking proliferation as suggested by data from susceptible mice and preliminary knockout experiments. Lack of IEC SOCS3 may promote resistance to *T. muris* infection.

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Dectin-1 is required for normal CD4⁺ T-cell function in the murine GI tract during systemic candidiasis

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Candida albicans is a medically important human fungal pathogen causing both mucosal and systemic infections. Optimal CD4⁺ T-cell responses during infection are critical to the development of mucosal immunity, thus our understanding of these cells' behaviour during infection is important in developing future treatments, such as adjunctive immunotherapies. Dectin-1, an essential component of protective anti-fungal responses, has been shown to be important in innate and adaptive immunity to *C. albicans*. We have recently shown that the gastrointestinal (GI) tract is more heavily infected in Dectin-1 deficient animals during systemic candidiasis. We therefore sought to understand how Dectin-1 may influence CD4⁺ T-cell responses during murine systemic candidiasis, with a focus on trafficking into the GI tract. Using T-cell receptor transgenic animals to track antigen-specific CD4⁺ T-cells *in vivo*, we show that Dectin-1 plays an influential role on CD4⁺ T-cell survival and proliferation in the murine gut during systemic infection. Our data highlights an important relationship between innate recognition and the induction of adaptive anti-fungal immunity.

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TGF β and T regulatory cells mediate nasopharyngeal carriage of *Streptococcus pneumoniae*

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Rationale: Nasopharyngeal carriage of *Streptococcus pneumoniae* is a prerequisite for invasive disease but the majority of carriage episodes in adults are asymptomatic and self-resolving. The interactions determining the development of carriage versus invasive disease are poorly understood but will influence the effectiveness of vaccines or therapeutics that disrupt nasal colonization.

Objectives: We sought to elucidate the immunological mechanisms underlying non-invasive pneumococcal nasopharyngeal carriage.

Methods: Pneumococcal interactions with human nasopharyngeal and bronchial fibroblasts and epithelial cells were investigated *in vitro*. A murine model of nasopharyngeal colonization and an experimental human pneumococcal challenge model were utilized to characterize immune responses in the upper airways during carriage.

Results: We describe the previously unknown immunological basis of non-invasive carriage and highlight mechanisms whose perturbation may lead to invasive disease. We identify the induction of active TGF β 1 by *S. pneumoniae* in human host cells and highlight the key role for TGF β 1 and T regulatory cells in establishment and maintenance of nasopharyngeal carriage in both mice and humans. We identify the ability of pneumococci to drive TGF β 1 production from nasopharyngeal cells *in vivo* and show that an immune tolerance profile, characterized by elevated TGF β 1 and high nasopharyngeal T regulatory cell numbers is crucial for prolonged carriage of pneumococci.

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***Mycobacterium bovis* growth in bovine monocyte-derived macrophages is inhibited by siRNA silencing of IL10 and c-MAF**

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Background: Bovine tuberculosis, caused by *Mycobacterium bovis*, is an increasing problem in the UK. Inhaled *M. bovis* bacilli are phagocytosed by alveolar macrophages, where the intracellular pathogen employs a range of mechanisms to prevent detection and promote survival. The macrophage response is key to determining the outcome of infection, either pathogen clearance or persistence. However, the host-pathogen interactions involved are not fully understood.

Materials and methods: The functional significance of several candidates; interleukin 10 (IL10), suppressor of cytokine signaling 3 (SOCS3), Mediterranean Fever (MEFV), v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog (c-MAF) and the microRNA miR-147, was investigated by siRNA-induced gene silencing in bovine monocyte-derived macrophages (bMDM) and infection with the attenuated *M. bovis* strain BCG.

Results: Treatment of bMDM with the gene specific siRNAs or scrambled control did not affect BCG uptake. Intracellular mycobacteria or DNA was extracted from bMDM 7 days post infection for mycobacteria quantification. Reduced numbers of BCG were isolated from bMDM treated with siRNA targeting IL10 and c-MAF. This result was confirmed by qPCR analysis of BCG genome copy number. Interestingly, the bovine genome copy number was increased in

IL10 siRNA treated cells, suggesting that cell proliferation was occurring. Further analysis revealed proliferation of a low granularity myeloid cell population in IL10 siRNA treated cells infected with BCG.

Conclusion: IL10 is rapidly up-regulated by bMDM in response to *M. bovis* infection and appears to be an important regulator of early infection. c-MAF regulates IL10 expression, which may explain how it affects *M. bovis* growth.

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Distributions of CD4 T lymphocyte epitopes on Epstein-Barr virus latent antigens correlate with binding affinities to class II HLA

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Background and aims: Epstein-Barr virus (EBV) infects >90% of humans, and is associated with certain immune-mediated diseases. After initial infection, the virus establishes a latent infection in B-lymphocytes, during which six nuclear antigens (EBNAs 1, 2, 3A, 3B, 3C, and -LP), two membrane proteins (LMP 1 and 2) may be expressed. EBNA1 is most widely expressed (latency patterns 1, 2 and 3), followed by LMPs 1 and 2 (latency 2 and 3). Both acute and latent viral infections stimulate humoral and cellular adaptive immune responses, but despite this, the virus is never cleared. EBNA1 has been shown to stimulate relatively few CD4 responses, which localise to its C-terminal portion. LMP1 stimulates numerous CD4 responses in its N-terminal portion, but few in its C-terminal portion. LMP2 has not been epitope mapped in detail. Here we epitope map LMP2 and investigate whether the skewed distributions of latent antigen epitopes might be explained by class II HLA binding.

Methods: Epitope mapping was performed by incubation of PBMC with peptides from LMP2; cellular proliferation was measured by thymidine incorporation and cytokine secretion by cELISAs. Class II HLA affinities for the four commonest European HLA alleles were assessed by the Concensus and NAlign algorithms,

<http://tools.immuneepitope.org>. MHC-peptide binding and rate assays were performed by the ProImmune REVEAL™ system for DRA*01:01;DRB1*01:01 and DRA*01:01;DRB1*15:01.

Results: Epitope mapping for LMP2, showed frequent IL-10 eliciting helper epitopes across most of its sequence. Distributions of helper epitopes for all EBV latent proteins correlated with predicted affinities for common class II HLA molecules, with areas of strikingly infrequent epitopes concentrated in frequently expressed latent antigens, in contrast with more randomly distributed affinities for other latent and lytic EBV proteins. To determine whether domains of high affinities have evolved to bind specific HLA motifs, viral sequences were randomly permuted and affinities retested. Observed patterns were similar to native, implicating amino acid composition as the main determinants of binding. Furthermore, observed patterns of epitope distribution correlated well with hydrophobicity. Predicted affinities for LMP1 were confirmed using binding assays for HLA DRB1*01:01 and *15:01.

Conclusions: These analyses highlight the importance of class II HLA binding affinities in determining immune responses to latent infection with EBV and raise the possibility that latent viral sequences are subject to selective pressure with respect to binding affinities for class II HLA molecules.

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Trichuris muris infection is associated with exacerbated intestinal tumours

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Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the gastrointestinal tract affecting around 2 million people in Europe and the USA. A new and novel approach to treatment of IBD has been the administration of helminths, such as *Trichuris suis*. IBD patients, however, bear an increased risk of developing colorectal cancer with IBD ranking as one of the top three high risk factors in intestinal neoplasia. *T. muris* is a natural gut-dwelling parasitic nematode of mice. Inbred strains of mouse can be resistant or susceptible to infection, but importantly, all strains of mouse mount an immune response against this parasite. Interestingly, infection of cancer-model mice with chronic *T. muris* infection leads to an increase in tumour numbers in the intestine and an increase in tumour development.

APC^{min/+} and Fabp1Cre:APC^{15/+} mice, that develop spontaneous tumours in the intestine, were administered a low dose *T. muris* infection, thus ensuring a chronic infection. Tumours were assessed at d18 and d42 post-infection and associated immune responses analysed by ELISA, CBA and worm burden analysis.

APC^{min/+} mice were assessed at d42pi. At this time point, infected animals had significantly more tumours than non-infected animals throughout the intestine i.e. tumour enhancement was not restricted to the site of infection. Infected mice additionally had a higher number of smaller tumours suggesting that *T. muris* infection was initiating tumour development. Enhanced proliferation and apoptosis associated with *T. muris* infection was only evident in the caecum of infected mice suggesting no causative relationship with tumour development. Fabp1Cre:APC^{15/+} mice were also found to develop more tumours in association with *T. muris* infection at both d18 and d42 p.i. Infection was associated with a Th1 phenotype as expected. Again, enhanced tumour formation was seen throughout the intestine rather than restricted to the site of infection demonstrating the systemic effects of *T. muris* infection.

Chronic *T. muris* infection increases the number of tumours in two different cancer-model mice. Though more work is needed to dissect out the cause of the enhanced tumour genesis, this study highlights that Trichuriasis is not a benign infection and is associated with considerable host damage, an important consideration when these helminths are used as immunotherapy agents.

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Pathogen-specific local immune fingerprints in acute disease: towards point-of-care diagnosis of bacterial infection

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Background and aims: Effective management of patients with suspected infections is hampered by the poor performance of current diagnostics and hence inadequate or delayed implementation of specific treatment. Standard microbiological identification of the causative pathogen is inefficient and slow, and failure to isolate organisms in the face of clear clinical indication of infection is a frequent occurrence. Moreover, neither microbiological culture nor molecular methods (PCR, mass spectrometry) discriminate between pathogens causing disease, asymptomatic carriage and contaminants. Early patient management thus remains largely empirical, which may lead

to unnecessary and inappropriate treatment, chronic or recurrent infection, and increased morbidity and mortality. Oversubscription of antibiotics ultimately drives multidrug resistance, one of major global health threats identified by the WHO. Stratified approaches and rapid and accurate point-of-care methods are needed that direct effective therapy, improve outcome and reduce costs for the NHS. Despite this unmet need the physiological/pathophysiological events driving local and systemic inflammatory responses to specific pathogens remain poorly characterised.

Methods: The immune system is capable of a rapid, sensitive and specific detection of a broad spectrum of microbes, which has been optimised over millions of years of evolution. A patient's early immune response is therefore likely to provide better insight into the true nature and severity of microbial infections than any conventional test. To exemplify this we characterised the local immune responses of peritoneal dialysis (PD) patients during episodes of acute PD-associated peritonitis using multi-colour flow cytometry and multiplex ELISA, and defined the immunological signatures in relation to microbiological culture results and clinical outcomes using ROC analyses and multivariate statistical approaches.

Results: We provide evidence that unique local 'immune fingerprints' robustly discriminate between culture-negative, Gram+ and Gram- episodes of infection and even predict eventual outcome, on the first day of presentation with acute peritonitis. Those humoral and cellular parameters most promising for the definition of disease-specific immune fingerprints include the local levels of IL-1 β , IL-10, IL-22, TNF- α and CXCL10, as well as the relative proportion of peritoneal neutrophils, monocytes/macrophages and $\gamma\delta$ T cells.

Conclusions: Our data indicate the real possibility that a 'fingerprint'-based point-of-care test can be developed for primary and secondary care use, or even as self-administered home test. We are now studying the molecular and cellular mechanisms underlying these unique responses and addressing whether and how pathogen-specific 'immune fingerprints' can be exploited in the clinic to facilitate targeted prescription and improved patient management.

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TH17 related cytokine response with pneumococcal pilus proteins in human nasopharynx associated lymphoid tissues

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Background: *Streptococcus pneumoniae* remains a major cause of infection in humans, despite the availability of polysaccharide vaccines for more than three decades. The pneumococcal polysaccharide vaccines are not effective in young children and the conjugate vaccines are expensive with narrower serotype coverage. One of the major focuses in current research is to develop protein vaccines. Pneumococcal pilus plays an important role in virulence. We have recently shown that pilus proteins induce strong antibody responses. However, it is not known whether they induce CD4+ T cell response, especially TH17 response.

Aims and objectives: To investigate whether pneumococcal pilus antigens activate human TH17 cells, and whether there is any association between TH17 activation in human nasopharynx-associated lymphoid tissue (NALT) and pneumococcal carriage.

Methodology: Bacterial culture of nasal swabs was performed for pneumococcal carriage. Adenotonsillar cells were co-cultured with pneumococcal concentrated culture supernatants (CCS) derived from wild-type or pilus RrgA or RrgB-deficient strains, followed by analysis of CD4+ T cell activation and intracellular cytokine staining by flow-cytometry; and measurement of *in vitro* cytokine production in cell culture supernatants by ELISA.

Results and Conclusion: Our results suggest that pneumococcal pilus proteins RrgA and RrgB proteins are capable of activating CD4+ T cells including TH17 cells in human nasopharynx-associated lymphoid tissue (NALT) and peripheral blood mononuclear cells. *In vitro* production of TH-17 cytokines (IL-17A, IL-17F and IL-22) in cell culture supernatants after stimulation with pneumococcal CCS containing both of these pilus proteins (TIGR4wt) was significantly higher than that of isogenic (RrgA^{-/-} and RrgB^{-/-}) mutants. The TH17 responses appeared to be higher in pneumococcal carriage negative than in carriage positive children.

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Comparison of kinetics of cytokines (IL-6 and CINC-1) in juvenile versus mature rats after inflammatory stimulation

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The aim of this study was to be cleared the kinetics of interleukin-6 (IL-6) and cytokine-induced neutrophil chemoattractant (CINC-1) in juvenile and mature rats. Furthermore, the kinetics of α_2 -macroglobulin (α_2 M), typical acute phase protein in rats, was compared. A total of 15 Sprague-Dawley rats (3, 7 and 11 weeks of age) were used in this study. Turpentine oil was intramuscularly injected at 0.2 ml/kg body weight to be induced inflammation. Blood was collected by ventricular puncture before turpentine oil injection, and 6, 12, 24, 48, 72 and 96 h after injection under anesthesia with pentobarbital. The serum concentrations of IL-6, CINC-1 and α_2 M were measured by ELISA. The maximum concentration of CINC-1 in 3-week-old rats was observed 6 h after turpentine oil injection. The serum concentrations of IL-6 and CINC-1 increased more quickly in juvenile rats than in mature rats after inflammatory stimulation. The concentrations of IL-6 and CINC-1 in juvenile rats were significantly higher than in mature rats. Then, only the concentrations of α_2 M were not observed significantly difference at 6 h after inflammatory stimulation between juvenile and mature rats. No significant difference was observed for the area under the concentration versus time curve (AUC) of IL-6, but that of CINC-1 in 3-week-old rats was significantly lower than that in 7- or 11-week-old rats. These results suggested that the synthesis of IL-6 and CINC-1 did not differ in an age-dependent manner. However, the synthesis of α_2 M increased in an age-dependent manner and the AUC for α_2 M was significantly different among 3, 7 and 11 weeks of ages. These results suggest the synthesis of IL-6 and CINC-1 were not difference between in juvenile and mature rats, however, the synthesis of α_2 M was increased in mature compared to juvenile rats.

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Respiratory syncytial virus (RSV) infection during early life suppresses antibody responses by the induction of interferon gamma

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Respiratory syncytial virus (RSV) infects most children in the first year of life and is a major single cause of hospitalization in infants and young children. There is no effective vaccine, and antibody generated by primary neonatal infection is poorly protective against reinfection even with antigenically homologous viral strains, suggesting inhibition of the host response by the virus. Studying the immunological basis of these observations in neonatal mice, we found that antibody responses to infection were low and unaffected by CD4 depletion, in contrast with adult mice, which had stronger CD4 dependent antibody responses. NK cell depletion or co-depletion of CD4⁺ and CD8⁺ cells during neonatal RSV infection caused a striking increase in anti-RSV antibody titer. Neonatal RSV infection lead to the induction of IFN- γ in NK and T cells and blocking IFN- γ enhanced RSV specific antibody responses in neonates. In addition, infection with a recombinant RSV engineered to produce IFN- γ reduced antibody titer, confirming that virally induced IFN- γ modulates the antibody response after neonatal infection. Strikingly when neonatal mice were immunized with tetanus toxoid during RSV infection, the antibody titer in response to the vaccine was reduced, potentially by the same effect. These unexpected findings show that the induction of a strong cellular immune response by viral infection may limit antibody responses in early life and that pathogens which induce IFN- γ secreting cells during the neonatal period might be less protective than those that do not.

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Cell-matrix interactions regulate the immune response to Mycobacterium tuberculosis

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Background and aims: A central tenet of tuberculosis (TB) pathology is that caseation of TB granuloma leads to matrix degradation and pulmonary cavitation, resulting in Mycobacterium tuberculosis (Mtb) transmission and disease progression. Lung destruction must involve activity of Matrix metalloproteinases (MMPs) which are emerging as key proteases contributing to lung tissue pathology in TB. MMP-1 is the dominant collagenase in human pulmonary TB; however mice do not express an orthologue of human MMP-1 in the lung. The precise relationship between the formation of caseous necrosis and lung matrix destruction has not been systematically examined.

Methods: We studied lung matrix integrity in human TB granulomas by Masson's Trichrome, Sirius red and Elastin van Gieson stains. We investigated the effect of transgenic expression of human MMP-1 and -9 in the mouse on TB pathology after intranasal infection with the Indo-oceanic Mtb strain. In cellular models, PBMCs were infected with Mtb in the presence of type I collagen and cell survival was assessed.

Results: In human granulomas, extracellular matrix destruction and caseous necrosis co-localise, with loss of collagen fibres in areas of caseous necrosis. In the mouse model, wild type (WT) mice showed typical histological appearance of TB infection with areas of macrophages infiltration but the alveolar wall structure remained intact. Transgenic expression of human MMP-1 caused collagen destruction and caseous necrosis without modulating the release of cytokines or altering bacterial growth, whereas MMP-9 transgenic expression did not, suggesting that the initial event in caseation is collagen breakdown. Consistent with this hypothesis, in cell culture collagen improves survival of Mtb-infected cells, assessed by LDH release and Propidium iodide staining.

Conclusions: We re-examined the sequence of events underlying TB immunopathology and demonstrated that collagen destruction is a critical initial event. This results in rather than arises from caseous necrosis. Together, these data demonstrate that the extracellular matrix regulates the host-pathogen interaction in TB.

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The long-term effect of neonatal respiratory syncytial virus (RSV) infection on the response to adult allergen challenge

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Respiratory syncytial virus (RSV) infection is a major cause of bronchiolitis in young children, resulting in around 600,000 hospitalisations annually in the USA. Developing RSV bronchiolitis during a 'critical window' as an infant is associated with a long-term increased risk of wheezing, allergy and asthma, persisting to early adulthood. In a murine model of RSV infection, the pulmonary inflammatory response to RSV is exacerbated in adults if the mouse has first been 'primed' with RSV as a neonate. This effect also occurs if the adult challenge is an unrelated infection such as influenza virus, suggesting it is not solely due to an adaptive memory response.

We aimed to investigate the innate component of RSV's neonatal priming effect in a clinically relevant model. We hypothesised that a neonatal RSV infection would alter the airway cytokine and cellular responses to an adult challenge with house dust mite (HDM) extract, a common allergen and potent stimulus of innate immunity.

Neonatal BALB/c mice received intranasal RSV A2, or HEp2 cell supernatant as a control. As adults they were challenged intranasally with HDM or PBS. Four hours later, bronchoalveolar lavage (BAL) fluid was taken for leukocyte counts, and RT-PCR performed on lung tissue for cytokine and chemokine expression.

The HDM challenge resulted in rapid airway neutrophilia and increased expression of inflammatory cytokines including CCL2, IL-6, IL-1 α and neutrophil chemoattractants. When mice had been neonatally primed with an RSV infection, expression of these genes was significantly increased. HDM-induced BAL neutrophilia was also significantly augmented both in terms of percentages (64% versus 42%) and numbers (5.1×10^4 versus 2.3×10^4 cells). This indicates that a neonatal RSV infection can have a long-term 'innate imprinting' effect resulting in exacerbated responses to the unrelated antigens found in HDM.

Our findings model the clinical observation that infants hospitalised with RSV bronchiolitis are more likely to experience pulmonary

immune dysregulation later in life, supporting the hypothesis that early life environmental exposures can have a persisting influence on the health status of an individual.

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Novel type three secretion system inhibitor enhances innate resistance and antibody response to salmonella

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Introduction: The rise of antibiotic resistance in the wide range of bacterial species facilitated the interest to look for novel strategies of anti-infective therapy efficient for the evolved drug resistant strains. Disruption of innate resistance and adaptive immune response as a result of antibiotic (ab) treatment is another problem that may lead to chronic infection and inflammation. In contrast virulence inhibitors including type three secretion system inhibitors (TTSSi) may help to overcome drug resistance and presumably enhance protective immunity. However, the benefits of TTSS inhibitors treatment on immune response were not fully assessed. In this study we compare the effects of novel TTSSi (CL55) and ab treatment on early innate reactions and specific antibody response in mice infected with salmonella.

Materials and methods: A/JsnYCit (A/Sn) mice were infected intraperitoneally with 10^3 CFU of *S. enteridis* serovar Typhimurium, and immediately treated with either TTSS inhibitor TTSS (CL55) (5 days, 50 mg/kg) or ampicillin (20 mg/kg). The recruitment of neutrophils, macrophages, dendritic cells, B cells and NK cells and expression of activation markers on macrophages was assessed by flow cytometry. The levels and isotypes of specific antibodies were evaluated by ELISA.

Results: No decrease in neutrophil recruitment at day 1 post infection (PI) and no late raise in recruited neutrophils at day 7 PI was found in TTSSi treated compared to ab treated group. Higher level of inflammatory macrophages was observed in ab treated group compared to TTSSi treated group while more recruited phagocytes were found in TTSSi treated group. Higher level of B cells was observed in TTSSi treated at day 1 PI compared to ab treated group. The level of NK cells was increased in TTSSi treated group at days 3–8 PI. TTSSi did not affect MHC II and CD40 expression on macrophages while ab treatment decreased the expression of these molecules. In contrast to ab treatment TTSSi did not decrease specific IgG2a antibodies, while the decrease in IgG1 antibodies was less pronounced in this group.

Conclusions: Overall in contrast to antibiotic treatment our TTSS inhibitor enhanced innate reactions and antibody response to salmonella.

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Switching adjuvants from alum to CpG improves the efficacy of the acellular pertussis vaccine

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The incidence of whooping cough caused by *Bordetella pertussis* is increasing in many developed countries, despite extensive vaccine

coverage. The acellular pertussis vaccine (Pa), which is the vaccine currently in use in most of these countries, consists of a number of *B. pertussis* antigens adsorbed to alum as the adjuvant. We have previously shown that Th1 cells play a crucial role in the clearance of *B. pertussis* from the respiratory tract during a primary infection. However, the current alum-adjuvanted Pa promotes both Th2 and Th17 responses. Interestingly, the Th17 cells confer protection induced by the Pa vaccine, whereas protection with the more efficacious whole cell pertussis vaccine (Pw) is mediated by Th1 cells. We have found that replacing alum with a Th1-promoting adjuvant, the Toll-like receptor agonist CpG, enhanced the efficacy of the vaccine by promoting the induction of Th1 cells. Our study showed that mice immunized with detoxified pertussis toxin and FHA in combination with CpG as the adjuvant (Ag + CpG) had significantly lower bacterial load in the lungs post *B. pertussis* challenge when compared with mice immunized with the same antigens adsorbed to alum. Importantly, the efficacy of the Ag + CpG vaccine was also comparable with that observed with Pw. We are currently assessing the effect of adding CpG to the efficacy of Pa already formulated with alum and comparing memory responses following immunization with Pa formulated with alum or CpG as the adjuvant.

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Induction of Th17 in nasopharynx-associated lymphoid tissue by pneumococcus and the effect of IL-21 and IL-15

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Background: Th17 cells have been suggested to be crucial in bacterial clearance from the host although those cells have a negative role in autoimmune diseases. It has been proved that IL17 provides protection from intracellular pathogens such as mycobacterium but IL17 can participate in tissue damage, particularly in intracellular infection. In this study, we have studied the induction of Th17 cells in children by pneumococcus in human nasal-associated lymphoid tissue (NALT) especially in the presence of exogenous cytokines that favour Th17 cells' environment.

Methods: Adenotonsillar mononuclear cells (MNC) were isolated from children undergoing adenotonsilectomy. The cells (CD45R0-depleted MNCs) were then stimulated for 7 days with or without concentrated pneumococcal culture supernatant (CCS) derived from a type II pneumococcus D39 followed by intercellular staining of IL17. The culture supernatant was collected on day 7 to measure the level of IL-17 production in adenotonsillar MNC by ELISA Assay.

Results and conclusions: Our results reveal that different pneumococcal stimulants can induce a high proportion of Th17 and a considerable production of IL17 from CD45R0- depleted MNCs in the presence of different combinations of Th17-favoured cytokines. The favourite combination for Th17 condition was IL21+IL1-β+TGF-β and it is considered the greatest one in inducing Th17. However, we have demonstrated that IL21 + IL15 together amplified the induction of IL17 in stimulated cells with pneumoCCS compared to IL21 alone and IL21+IL1-β+TGF-β. It is suggested that the development and balance of Th17 cells and Foxp3+Treg in the local mucosal immune tissues play an important role in modulating the specific immunity against pneumococcal carriage in humans.

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Investigating macrophage proliferation: IL-4 and CSF1 in conflict or cooperation?L. H. Jones*, G. D. Thomas[†], S. J. Jenkins[‡], D. A. Ruckerl*, S. M. Duncan*, D. J. Gow[§], D. A. Hume[§] & J. E. Allen**Institute of Immunology and Infection Research, University of Edinburgh, Edinburgh, UK, [†]La Jolla Institute for Allergy and Immunology, San Diego, CA, USA, [‡]Centre for Inflammation Research, University of Edinburgh, Edinburgh, UK, [§]The Roslin Institute, University of Edinburgh, Edinburgh, UK

Over the last 2 years, a paradigm shift has occurred in macrophage biology. Monocytes are no longer considered to be a major contributor to the tissue resident pool of macrophages in the steady state. Contributing to this paradigm shift was the finding that the archetypal Th2 cytokine IL-4 can drive local macrophage proliferation, both in tissues and body cavities, in response to nematode infection. In the steady state, the prototypical macrophage growth factor CSF1 is known to be required for macrophage proliferation but IL-4 is not involved. CSF1 also plays a role in the recruitment of monocytes and neutrophils in inflammatory settings but no inflammatory recruitment occurs in response to IL-4. In the context of nematode infection, there exists both IL-4R α dependent and CSF1-dependent macrophage proliferation. CSF1 is capable of inducing low-level proliferation of macrophages *in vitro*, in contrast to the limited ability of IL-4 to do so. Using gene targets identified by microarray and RNAseq analysis we are beginning to understand the disparity between the actions of IL-4 and CSF1. *In vitro* treatment of macrophages and *in vivo* delivery of IL-4, CSF1 or both cytokines together are uncovering the similarities and differences in their modes of action. Furthermore we are beginning to unravel the complex regulation of macrophage activation that exists in infection settings where both IL-4 and CSF1 are prevalent.

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Innate defences at mucosal barriers: the anti-parasitic potential of the purified gastric mucin MUC5AC

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Background and aims: The epithelium of the gastrointestinal tract is protected from pathogens by the gel-like extracellular matrix, mucus. The primary components of mucus are the large O-linked glycoproteins called mucins, and it is their interaction with each other, which generates the mesh-like structure of mucus. Mucins are synthesised and secreted by goblet cells, which are interspersed within the epithelium. The classic Th2 immune response to parasitic infection in the intestine can regulate mucin production and secretion by goblet cells and this ultimately affects the properties of the mucus barrier. It is well established that the physical barrier provided by the mucin network is an important aspect of innate immune defence. However, preliminary work from our laboratory suggests a more direct role for mucins in combating parasitic infection. We have recently demonstrated that expression of the mucin Muc5ac, normally expressed in non-intestinal mucosa, is highly up-regulated in the intestine following infection with the murine homologue of *Trichuris trichiura*, *T. muris*. Moreover, genetic deletion of Muc5ac leads to complete susceptibility to infection in an otherwise resistant mouse strain, indicating Muc5ac plays a major role in protection against this parasite. We now have preliminary data to suggest that Muc5ac is acting directly as an anti-parasitic molecule.

Methods: In order to evaluate the effect of Muc5ac on the viability of *T. muris*, both adult worms and larval stages were incubated *ex vivo*

with purified Muc5ac. A number of biological indicators of viability were assessed following incubation. This included video capture of worm movement, assessment of metabolism by cytochrome C oxidase staining and determining fecundity of adult female worms.

Results: Muc5ac affects the motility of larval *T. muris*. Reduced motility was observed in worms cultured in the presence of Muc5ac, when compared with those cultured in media alone. The effect on worm motility was at some life cycle stages comparable to the reduction observed in anti-helminthic drug-treated worms.

Conclusions: The barrier function of mucins in protection of the host is well accepted as an important aspect of innate defence. However, our data suggests that mucins have a much more direct role in combating parasites and are an important part of the coordinated immune response to infection, with Muc5ac being an essential anti-parasitic effector molecule.

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***Nippostrongylus brasiliensis* hookworm infection, Th2 activation and onset of chronic obstructive pulmonary disease**

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Background and aims: The rodent hookworm *Nippostrongylus brasiliensis* is widely used as a model for strong Th2 induction and as a model for human hookworm infection. During infection development the larvae move from the skin to reside in the lungs, then passage through the airways to be swallowed and develop into egg-laying adults in the intestine. The lung stage is very brief, but cause dramatic bleeding and destruction. The lung heals rapidly and by gross morphology appears similar to uninfected lungs within 2 weeks. However, the lungs of infected animals develop progressive emphysema with continuous microbleeding. Interestingly, human hookworm infection is a risk factor for emphysema development. Previous studies have suggested that the rapid repair of the lungs is Th2-immune mediated and involves IL-4R α -dependent alternative macrophage activation, whereas emphysema development was found to be independent of IL-4R α in BALB/c mice. Our aim was to further elucidate the acute repair response and subsequent emphysema in different mouse strains as well as mice deficient in RELM- α , an IL-4R α induced protein associated with tissue repair and fibrosis.

Methods: Infected mice were analysed on immune status and pathology by histology, FACS, protein and mRNA. Parasite burden was assessed by egg and worm counts, mRNA and antigen levels.

Results: As expected *N. brasiliensis* infection in both C57BL/6 and BALB/c mice led to pulmonary immune response activation, with early neutrophilia, followed by alternative macrophage activation, Th2 cytokine production, damage repair and subsequent tissue deregulation and disruption. In long-term experiments (≤ 60 days) pulmonary pathology was dependent on host genetic background, with BALB/c mice showing a severe emphysema-like pathology along with increased Th2 cytokines and antibody production compared to C57BL/6 mice. However, C57BL/6 mice still developed marked emphysematous pathology and this was not altered in IL-4R α deficient mice, as previously reported on the BALB/c background. IL-4R α deficient C57BL/6 mice also did not expel the adult worms even at 60 days post infection. In contrast to previously published data, experiments in the C57BL/6 RELM α deficient mice suggest that this protein contributes to the IL-4R α dependent differences in parasite recovery as mice lacking RELM α were less capable of expelling the adult worms, and had higher egg burdens than WT controls. RELM α deficient mice also exhibited lower

Th2 cytokines and antibody production but substantially increased Eosinophilia.

Conclusions: We are using this highly tractable model to understand the relationship between Th2-linked pulmonary immunology, lung repair emphysematous development and anti-worm effector mechanisms.

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Th2 cell intrinsic tolerance determines susceptibility to helminth infection via the PD-1/PD-L2 pathway

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The suppression of protective Type 2 immunity is a principal factor driving the chronicity of helminth infections, and has been attributed to a range of Th2 cell-extrinsic immune-regulators. However, the intrinsic fate of parasite-specific Th2 cells within a chronic immune down-regulatory environment, and the resultant impact such fate changes may have on host resistance is largely unknown. We used IL-4gfp reporter mice to demonstrate that during chronic helminth infection with the filarial nematode *Litomosoides sigmodontis*, CD4⁺ Th2 cells are conditioned towards an intrinsically hypo-responsive phenotype representing a novel form of T cell anergy or exhaustion. This Th2 cell-intrinsic hypo-responsiveness was characterised by a loss of functional ability to proliferate and produce the cytokines IL-4, IL-5 and IL-2. It was a key element determining susceptibility to *L. sigmodontis* infection, and could be reversed *in vivo* by blockade of PD-1 resulting in long-term recovery of Th2 cell functional quality and enhanced resistance. Contrasting with T cell exhaustion in Type 1 settings, the control of Th2 cell-intrinsic hypo-responsiveness by PD-1 was mediated through PD-L2, and not PD-L1. Thus, intrinsic changes in Th2 cell quality leading to a functionally hypo-responsive phenotype play a key role in determining susceptibility to filarial infection. The therapeutic manipulation of Th2 cell quality provides a novel avenue for promoting resistance to helminths, or for tolerising pathogenic Th2 cells for the treatment of Th2-mediated allergies and fibrosis.

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Comparison of the effects of *Clostridium difficile* toxins A and B in peripheral blood monocytes and intestinal macrophages

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Background and aims: *Clostridium difficile* associated colitis is mediated by secreted toxins A and B and results in recruitment of immune cells to the intestinal mucosa. The aim of our study was to compare the effects of *C. difficile* toxins A and B on peripheral blood monocytes (PBM) and intestinal macrophages (IM).

Methods: Varying concentrations of the purified toxins were incubated with either human intestinal lamina propria cells or washed whole blood cells at 37° for 1h. Reductions in forward scatter (indicative of subsequent cell death) were analysed by flow cytometry, using expression of CD14 and HLA-DR (together with cell size) to identify the cells of interest. The obtained median forward scatter

values were expressed as a% of the relevant control (cells incubated without toxin) and the means (SEM) were calculated.

Results: At all three concentrations studied (2.5, 5.0 and 10.0 µg/ml), PBM and IM were significantly ($P < 0.05$) more sensitive to the effects of toxin B than to toxin A [e.g. at 10 µg/ml: PBM – 49.8 (7.2)% versus 92.9(2.0)%, $P = 0.019$; IM – 81.8(1.6)% versus 96.8 (1.2)%, $P = 0.008$]. CD14+ve PBM were found to be more sensitive to toxin B than HLA-DR+ve PBM [e.g. at 5 µg/ml, 57.8(3.7)% versus 69.4(5.4)%; $P = 0.003$]. HLA-DR+ve PBM were more sensitive to toxin B than HLA-DR+ve IM [e.g. at 5 µg/ml, 69.4(5.4)% versus 83.3(2.6)%; $P = 0.008$]. In addition, CD14+ve IM were more sensitive to toxin B than CD14-ve IM [significant at 5 µg/ml, 74.6(6.8)% versus 95.8(4.0)%; $P = 0.042$].

Conclusions: This study showed significantly greater effects of *Clostridium difficile* toxin B than toxin A in both peripheral blood monocytes and intestinal macrophages. Of the two cell types, monocytes were more sensitive to the effects of toxin B than macrophages. Furthermore our study suggests that CD14 expression may increase the sensitivity of monocytes and macrophages to the effects of toxin B.

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The role of dendritic cells in inducing Th2 responses to schistosoma mansoni eggs

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Background and aims: *Schistosoma mansoni* eggs (SME), which are the main cause of Schistosomiasis, provoke a strong Th2 immune response that can lead to granulomatous reactions and fibrosis in the affected organs. Recently, CD11c depletion has been shown to severely disrupt Th2 immune responses against *Schistosoma mansoni*, suggesting that antigen presenting cells expressing CD11c mediate this immune response. Dendritic cells (DCs) are the most likely candidates as they migrate from epithelia, where they encounter antigens, to the draining lymph nodes where they activate naive T-cells. However, the precise role of DCs in inducing immune responses against SME has not been studied.

Methods: SME were injected into the subserosal layer of the small intestine of C57BL/6 male mice, at laparotomy. Five days later, mesenteric lymph nodes (MLN) were harvested and single cell suspensions were prepared. In restimulation experiments, cells were then cultured with Schistosoma egg antigen and culture supernatants were collected for analysis by ELISA. To characterize migrating DC populations, MLN cells were analysed by flow cytometry.

Results: We confirm a Th2 response against SME in a physiologically relevant *in vivo* mouse model with intestinal migratory dendritic cells as likely mediators. When SME are injected into the subserosal layer of the small intestine they provoke a Th2 response in the draining MLNs. This response, identified by the release of characteristic cytokines from MLN T-cells restimulated with SEA *in vitro*, was only observed in MLNs from egg injected animals. The proportions of the four intestinal dendritic cell subsets, identified by their expression of CD103 and CD11b, do not change between egg and PBS injected animals.

Conclusions: Our results indicate that an antigen-specific immune response is induced against SME, and that this does not require a change in the proportions of the migrating DC subsets. We plan to use this system to dissect the contributions of each of the migratory intestinal DC populations to the anti-SME immune response. This knowledge will contribute to a better understanding of how the

immune response against *Schistosoma mansoni* eggs is mediated. Information that can be applied not only against battling chronic Schistosomiasis but may also be applied to Th2 driven responses against other parasites or Th2 allergic responses.

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***Leishmania mexicana* modulates dendritic cell migration towards draining lymph nodes**

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Following infection, migration of dendritic cells (DCs) is important for the activation of an adaptive immune response. This migration is driven by CCR7 expression by DCs, which facilitates migration towards CCL19-expressing lymphatic vessels and the draining lymph node. Therefore, manipulation of chemokine and chemokine receptor expression by pathogens presents a potential opportunity for evasion of protective immunity. *Leishmania mexicana* is a protozoan parasite that establishes chronic, non-healing skin lesions, suggesting a lack of effective adaptive immune response, although the mechanism of immune evasion remains unclear.

Here we have examined the impact of *L. mexicana* on DC activation and migration. DCs co-cultured with *L. mexicana* fail to upregulate CCR7 expression and are refractory to LPS-stimulation. Using time-lapse imaging we show a concomitant failure of these cells to migrate towards CCL19 *in vitro*, and DCs exposed to *L. mexicana* fail to successfully infiltrate lymphatic vessels upon transfer into naïve mice. Similarly, infection with *L. mexicana* *in vivo* is associated with a failure of MHC-II-expressing cells to migrate towards lymphatic vessels. Finally, we investigate the potential mechanism by which the parasite influences DC migration, with evidence suggesting that the parasite virulence factor cysteine protease B (CPB) plays an important role.

Our data demonstrate that *L. mexicana* can impair DC migration and that reduced expression of CCR7 plays a key role in this process. Additionally, parasites lacking CPB fail to suppress DC migration, hinting that this virulence factor may play a key role in the immune-evasion of *L. mexicana*.

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Phosphatidylserine-containing liposomes: modulators of rhinovirus induced inflammatory responses

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Background: Human rhinoviral infections are major contributors to the healthcare burden associated with acute exacerbations of asthma. We, and others have recently demonstrated that rhinovirus (RV)-induced inflammatory responses are mediated by multiple signalling mechanisms, such as IL-1/MyD88 (1) and TLR3/RIGI (2). We have also previously published work showing that TLR signalling is effec-

tively inhibited by phosphatidylserine-containing liposomes (SAPS), through the disruption of membrane microdomains (3). Evidence has also suggested that membrane microdomains may influence infections with RV. In this study, we explored the ability of SAPS to modulate responses to the natural viral pathogens, RV-1B and RV-16.

Method: The immortalized bronchial epithelial cell line, BEAS-2B or primary bronchial epithelial cells were infected with RV-1B or RV-16 at a TCID₅₀/ml of 1×10^7 for 1 h. Immediately following infection, various concentrations of SAPS were added and changes in cytokine release were measured at 24 h. SAPS remained present throughout. Type I and III interferon (IFN) expression and rates of viral replication were measured by quantitative PCR. Virus quantification was also performed using a viral CPE assay, and IFN signalling was measured by western blot. Liposome stability was characterised and intracellular trafficking of fluorescently labelled SAPS in BEAS-2B cells was investigated using confocal microscopy. For *in vivo* studies, female wt Balb/c mice were pre-treated with SAPS for 2 h prior to infection with RV as previously described and changes in BAL cell number, BAL cytokine production and viral replication were quantified (4).

Results: Characterisation of SAPS liposomes by mass spectrometry showed no obvious signs of oxidation over the time period tested, and liposome size remained constant. Preliminary confocal studies revealed that SAPS was rapidly internalised within the cell and was found to associate with intracellular compartments such as the early endosome and golgi. Viral infected BEAS-2B cells co-incubated with SAPS, showed notably impaired responses to RV as assessed by release of CXCL8 and CCL5. SAPS also reduced RV-induced IFN β production and STAT-1 phosphorylation, without significantly influencing viral replication rates. Modest increases in viral particle production were only observed at 48 and 72 h time points. Suppression of viral-induced cytokine production was also observed in primary bronchial epithelial cells and pilot *in vivo* studies showed that SAPS results in reduced KC production at 24 h post viral infection, and this was associated with reduced neutrophil numbers within the BAL fluid.

Conclusion: Our data demonstrates a potential means of modulating inflammatory responses induced by human rhinovirus.

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A key role for CD11c+ cells in maintenance of the Th2 response during helminth infection

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Background and aims: Many pathogens survive for prolonged periods, exposing their host to ongoing antigen stimulation. Although it is clear that CD11c+ dendritic cells are both sufficient and necessary to initiate Th2 polarisation against helminths, the role of CD11c+ cells in maintenance of CD4+ T cell responses is poorly understood. This study aimed to clarify the role of CD11c+ antigen presenting cells in maintenance of the CD4+ T cell response during murine infection with the parasitic helminth *Schistosoma mansoni*.

Methods: We used a murine model that allows both inducible depletion of diphtheria toxin receptor expressing CD11c+ cells (following administration of diphtheria toxin - DTx), and identification of cells that express IL-4 mRNA (an F1 cross of B6-CD11c.DOG and Balb/c-4get mice). Animals were infected with approximately 40 or 80 *S. mansoni* cercariae. DTx was administered between day 42 and 51

of infection and immunology, parasitemia and pathology assessed by multi-colour flow cytometry, ELISA, liver enzyme assays, egg counts and histology.

Results: At a lower infection intensity (approximately 40 cercariae) CD11c depletion led to a reduction in the percentage and number of CD4⁺ T cells in the liver, which is a key effector site in *S. mansoni* infection. This was associated with a significant decrease in the Th2 response, characterised by the loss of IL-4 mRNA expression and protein production. At higher intensity infection (approximately 80 cercariae) CD11c depletion induced a striking impairment of the hepatic CD4⁺ T cell response, with significant reductions in IL-4, IL-5, IL-10 and IL-13, and also IFN- γ . CD11c depletion at high, but not low, dose infection was also associated with weight loss and an overall increase in severity of pathology. In contrast to the liver, there was comparatively little impact of CD11c depletion on the Th2 response in the MLN.

Conclusions: Surprisingly, these data suggest that CD11c⁺ cells are important for maintenance of the Th2 response at effector sites during helminth infection, possibly through recruitment, retention or reactivation of CD4⁺ T cells.

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Immunological silence and chronic *Trichuris muris* infection

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Background and aims: Soil transmitted helminth infections present a major public health problem. *Trichuris muris*, a gastro intestinal nematode, is used as a mouse model of the human parasite, *T. trichiura*. During chronic *T. muris* infection, the host immune response is well characterised with high levels of the Th1 cytokine IFN- γ being a dominant feature. *Trichuris muris* is in intimate contact with the murine host, with the excretory secretory products (ES) being the first host parasite interaction by bathing the intestinal niche where the parasite resides. The major component of ES is a 43kDa protein (43) of unknown function. qRT-PCR revealed the greatest increase in expression of 43 occurred at the immunomodulatory, L3 stage. This study examined the murine immune response to native ES, purified 43 (P43), recombinant, baculovirus derived 43 (B43) or ES with 43 removed (termed flow through, FT). A role for the 43 in metal binding was also examined.

Method: The 43kDa ES protein has a naturally occurring C-terminal histidine tail, which allows purification of P43 using nickel beads. Serum and cellular responses were compared to ES, P43, B43 and FT. Antigen processing and presentation were investigated using bone marrow derived dendritic cells and the ability of the various antigens to drive FoxP3⁺ Tregs was also analysed. Metal binding by P43 was examined using ICP-MS and its effects on metal availability determined by culturing live worms with a strain of *Salmonella typhimurium* containing a metal-responsive reporter gene (*lacZ*).

Results: Serum responses from *T. muris* infected resistant and susceptible mice showed little antibody recognition of P43 and B43 whereas there was a strong antibody response to FT. *In vitro* re-stimulation assays using resistant BALB/c mice also confirmed that there was little immune recognition of P43 and B43 and that Th2 cytokine production was due to ES proteins in the FT. β -galactosidase assays revealed live worms can affect metal levels when cultured with *S. typhimurium* and P43 was shown to be a metal-binding protein using ICP-MS.

Conclusion: The 43kDa ES protein is surprisingly non-immunogenic but may still perform an important role in immune homeostasis. The availability of iron in the intestine can be influenced by the host microflora and our results also suggest that *T. muris* can affect metal

levels. This may affect the interplay between the parasite, the host microflora and the immune response.

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Mechanisms of impaired polymorphonuclear neutrophil functions in diabetes mellitus in response to *Burkholderia pseudomallei*

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Diabetes mellitus (DM) is a major risk factor for melioidosis, an infectious disease caused by *B. pseudomallei* infection. Recent evidence from our group illustrates that polymorphonuclear neutrophils (PMNs) from DM patients exhibited decreased functions in response to *B. pseudomallei* when compared with healthy subjects. In this study, we aimed to investigate mechanisms of impaired PMN functions in DM subjects in terms of phagocytosis, bacterial killing, and migration. The inhibition of phagocytosis by cytochalasin D (Cyt-D) showed that Cyt-D could decrease phagocytosis of PMNs only from healthy but not in DM subjects. Moreover, inhibition of oxidative burst by chloroquine (CQ) on PMNs showed that CQ could enhance intracellular survival of *B. pseudomallei* in PMNs of both healthy and DM subjects. Taken together; these data suggest that PMNs from DM impaired their phagocytosis activity, but not killing via oxidative burst. Furthermore, the migration capacity of PMNs from DM in response to IL-8 was impaired as compared with that of healthy subjects. Additionally, the reduced chemokine, IL-8, production was observed in PMNs from DM in response to *B. pseudomallei* compared with healthy-PMNs. These findings indicate that PMNs from DM shows impairment in phagocytosis, migration capacity in response to IL-8, and cytokine production to *B. pseudomallei* infection. These may contribute to the host susceptibility to *B. pseudomallei* infection in DM patients.

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Herpesvirus modulation of immunological synapses

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Gammaherpesviruses, such as Kaposi's Sarcoma-associated Herpesvirus (KSHV) and Epstein-Barr Virus (EBV), establish persistent infections that can be associated with lymphoproliferative disease. The Murid Herpesvirus 4 (MuHV-4) is used as a mouse model to study gammaherpesvirus pathogenesis. A critical determinant of persistence is the ability to establish latent infection in memory B cells. To accomplish that, virus-infected B cells go through a germinal centre reaction. T cell help is essential for this process and it is still unknown whether the virus is capable of modulating the B cell - T cell immunological synapse (IS) for its own benefit. The MuHV-4 M2 protein is a good candidate for this hypothetical modulation since it is expressed during the latency phase and it has been shown to assemble multiprotein complexes with B cell signalling proteins (Vav1, NCK1, PLC γ 2, SHP2, p85 α). Thus, we investigated the impact of M2 on the formation of B-T IS.

Expression of M2 in B cells lead to upregulation of adhesion and cell activation molecules involved in the B-T IS. We observed that upon IS formation M2 polarised to the contact zone. Moreover, the host cell microtubule organising centre was closer to the synapse under the influence of M2. Using a system in which the T cells have TCR specificity for the ovalbumin peptide (OVAp) we addressed B-T conjugation. We showed by flow cytometry and confocal microscopy that M2 expression in B cells promoted conjugation with CD4⁺ T cells, even in the absence of peptide. Accordingly, expression of M2 lead to an increase in the number of T cells activated. However, this increase did not correlate with an increase in the magnitude of each individual T cell response. We also demonstrated *in vitro* that M2-expressing B cells successfully compete for T cell conjugation when mixed with control B cells.

Taken together, these results indicate that M2 promotes B-T conjugation, independently of specific antigenic recognition, which may confer a competitive advantage to the infected B cell in entry and maintenance within the germinal centre reaction.

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Identification of a hybrid macrophage polarisation state following recovery from LPS tolerance

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Background and aims: Lipopolysaccharide (LPS) tolerance is an essential immune-homeostatic response towards repeated exposure to LPS which prevents excessive inflammatory responses. LPS tolerance induces a state of altered responsiveness in macrophages resulting in repression of pro-inflammatory gene expression and increased expression of factors which mediate the resolution of inflammation.

Methods: In this study we analysed the transcriptional plasticity of macrophages following LPS tolerance using genome-wide transcriptional profiling.

Results: We demonstrate that LPS tolerance is a transient state and that the expression of pro-inflammatory genes is restored to levels comparable to the acute response to LPS. However, following recovery from LPS tolerance a number of genes remained locked in a tolerable state including IL33, CD86, IL10 and NFIL3. Furthermore, we identify a number of genes uniquely induced following recovery from LPS tolerance. Thus, macrophage adopt a unique transcriptional profile following recovery from LPS tolerance and have a distinct expression pattern of regulators of antigen presentation, antiviral responses and transcription factors.

Conclusions: Our data suggests that recovery from LPS tolerance leads to a hybrid macrophage activation state which is pro-inflammatory and microbicidal in nature but which possesses a regulatory anti-inflammatory profile distinct from that of LPS tolerant and LPS activated (M1) macrophages.

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Prolonged vaginal shedding and upper genital tract pathology in DBA/2 mice infected with clinically important serovar D of *C. trachomatis*

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Introduction: *Chlamydia trachomatis* is a widely spread urogenital infection that induces a range of severe inflammatory consequences including ectopic pregnancy and infertility. Development of novel

anti-bacterial and immunotherapy is highly desirable. However as the majority of mouse strains are resistant to *Chlamydia trachomatis* serovars pathogenic for humans that creates serious limitations in extrapolation of results obtained with *Chlamydia muridarum* commonly used in mouse models. In this study we developed a novel model of *C. trachomatis* serovar D intravaginal infection in susceptible DBA/2 mice.

Methods: Female DBA/2 and BALB/c mice received 33 mg/kg of covinan (proligestone) and 7 days later were infected with 10⁶ IFU of *C. trachomatis* serovar D. Chlamydia shedding was analysed in chlamydia infection assay *in vitro*. Real time PCR was used to detect Chlamydia DNA in ovaries and uterines. Pathology of upper genital tract was assessed visually and by H&E staining. ELISA and *in vitro* neutralization assay were used to detect Chlamydia specific antibodies.

Results: We found that while virtually no chlamydia vaginal shedding was observed in BALB/c mice starting from day 3 post infection (PI), moderate level of bacteria shedding (5 × 10²) was still observed at day 17 PI in DBA/2 mice. Chlamydial DNA was consistently detected in ovary and uterine tissues of DBA/2 mice at day 25 PI. Visual examination of uterine horns in DBA/2 mice revealed the presence of hemorrhages and moderate hydrosalpinx and H&E staining of ovaries revealed polymorphonuclear leucocytes infiltration. Chlamydia specific antibodies of IgG2a, IgG1 and IgA isotypes were detected in serum of infected DBA/2 mice 2 weeks PI. Neutralization assay revealed the presence of neutralizing antibodies in serum of DBA/2 mice.

Conclusion: Our results confirmed the susceptibility of mice of DBA/2 strain to clinically important *C. trachomatis* serovar D which alongside with other reported traits of this strain such as misbalanced iron metabolism and susceptibility to pregnancy disorders make them attractive model for chlamydia intravaginal infection.

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Suboptimal TCR-ligand interactions determine host colonization in gammaherpesvirus infection

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Host colonization by lymphotropic gammaherpesviruses depends critically on the expansion of viral genomes in germinal centre (GC) B cells. Yet virus driven lymphoproliferation is regulated by host cytotoxic CD8⁺ T cell (CTL) responses and lymphoproliferative disease readily emerges with immunodeficiency. Thus, a balance must exist that allows host colonization still lymphoproliferation control. Here we have looked at this balance by assessing the *in vivo* impact of T cell receptor (TCR) avidity in the host control of a gammaherpesvirus infection. Using Murid herpesvirus-4 (MuHV-4) infection of mice our lab has shown before that CTL control of virus driven lymphoproliferation in GCs can depend on a single MHC-class I-restricted epitope encoded by a latency-associated viral protein, designated M2. In this work we demonstrate that presentation of the ovalbumin (OVA) H-2K^b epitope, a high-affinity TCR ligand, within the M2 context, generates a functional CTL response that prevents viral expansion in the GCs. MuHV-4 recombinants were constructed expressing OVA altered peptide ligands (APLs) in the M2 C' terminus. OVA APLs were selected in order to maintain binding to the H-2K^b molecule but vary the functional avidities towards the OVA₂₅₇-specific TCR transgenic (OT-I) cells. Our experimental approach involved the generation of a monoclonal OVA-specific TCR transgenic animal model with a polyclonal CD4⁺ T cell

repertoire. This was achieved by co-transferring Ly5.1⁺ Rag^{-/-} OT-I cells from Ly5.1⁺ Rag^{-/-} OT-I mice and CD4⁺ T cells from C57BL/6 mice into Ly5.2⁺ TCRalpha^{-/-} mice. Infection with MuHV-4 recombinants expressing OVA APLs demonstrated that the strength of the TCR ligation dictated the magnitude and the activation profile of the generated CD8⁺ T cell responses. Strong TCR stimulation prevented gammaherpesvirus latency amplification in GC B cells. In contrast suboptimal TCR stimulation allowed viral expansion and persistent infection. Thus, our data demonstrate that suboptimal TCR-ligand interactions determine host colonization in gammaherpesvirus infection.

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Cytokine profiles of primary human dendritic cells following Hazara virus infection

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Hazara virus (HAZV) is a tick-borne virus and is a member of the same serogroup as the clinically important Crimean-Congo Haemorrhagic Fever virus (CCHFV) species that are endemic in Africa and the Middle East. CCHFV are the causative agents of fatal haemorrhagic fever in humans with increased incidences being reported in Turkey and Iran. HAZV is not known to cause disease in humans and does not require high level containment facilities. It is an antigenically-relevant surrogate for pathogenic CCHFV. Recent work has shown that similar pathologies and morbidities are achieved for both HAZV and CCHFV in type I interferon receptor knockout mouse models. In this work, we report the cytokine/chemokine profiles of immature and mature dendritic cells following infection of HAZV to gain a better understanding of the potential mechanisms of pathogenesis. It also provides an opportunity for assessing the effects of host responses and their potential role in immunomodulation.

Immature and mature monocyte-derived dendritic cells (Mo-DC) were prepared from human peripheral blood mononuclear cells (PBMCs) using GM-CSF and IL-4. The phenotype of Mo-DCs were confirmed by flow cytometry. Immature/mature Mo-DCs were infected with HAZV at multiplicity of infection ratios of 0.1, 1.0 and 5.0. Cytokine profiles were assayed by using a Novex[®] Cytokine Human Magnetic 25-Plex Panel kit at 6 and 72 h post-infection using the on a Luminex[®] platform. Non-infected cells for each cell type were assayed in parallel. Seven cytokines were showed statistically significant increases in immature DCs for Eotaxin, GM-CSF, MIP-1 β , MCP-1, IL-7, IP-10, IFN- α and IL-8. For mature DCs, only GM-CSF, MCP-1, IFN- α and IP-10 showed statistically significant increases in secretion.

These results will help to elucidate the differences between infection with a non-pathogenic virus (HAZV) versus that with a severe human infectious pathogen (CCHFV) to determine the contribution of the immune response to disease susceptibility in humans.

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MHC II tetramer analysis of CD4⁺ T cell responses to primary and persistent Epstein-Barr virus (EBV) infection

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Virus-specific CD4⁺ T cells are key orchestrators of host responses to viral infection yet, compared to their CD8⁺ T cell counterparts, remain poorly characterised at the single cell level in humans. Here we use MHC II tetramers to carry out the first concerted analysis of a human CD4⁺ T cell response to viral infection using the ubiquitous gammaherpesvirus Epstein-Barr virus (EBV). The virus' association with the clinically recognisable syndrome of infectious mononucleosis (IM) in primary infection has provided a unique opportunity to follow the evolution of the CD4⁺ T cell response from primary to persistent infection. Nine MHC II:epitope peptide tetramers representing a range of EBV latent and lytic cycle proteins were used in multicolour flow cytometry to visualise the evolution of the CD4⁺ T cell response. Our donor cohort included 21 IM donors undergoing primary infection, of which 11 gave further donations through to convalescence, and 10 EBV sero-positive donors with no history of IM. We find that in primary infection, MHC II tetramer-positive CD4⁺ T cells specific for EBV antigens were expanded to frequencies that reached 10-fold above those seen during viral persistence. Furthermore, the diverse range of epitopes targeted by the highly activated CD4⁺ T cell response was large enough to impact on the phenotype of the overall CD4⁺ population. Responses were rapidly culled during convalescence to values typical of life-long virus carriage, where most tetramer-staining cells display conventional memory markers but some, unexpectedly, revert to a naive-like phenotype. Interestingly, CD4⁺ T cell responses to one EBV protein, EBNA1, are greatly delayed in IM patients, and we have shown that this correlates to a similar delay in development of IgG antibody responses to the same protein. We suggest from an *in vitro* system, that this may be explained by differences in antigen supply. Current experiments are expanding MHC II tetramer analyses to study the cytokine profiles of EBV-specific MHC II tetramer-positive cells in health and disease.

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Anti-bacterial and immunopotentiative activity of novel CPAF protease inhibitor

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Introduction: Chlamydial protease-like activity factor (CPAF) is an important chlamydia virulence factor that cleaves a range of immunologically significant targets including transcription factors RFX5 and USF1 required for the expression of MHC, the pro-apoptotic factors Bim and Puma, p65/RelA, nectin1 and CD1d. In the same time it was described as a highly immunogenic antigen able to elicit protective immunity *in vivo*. This suggests that inhibitors of CPAF protease activity may provide not only specific anti-bacterial therapy but also boost protective immunity to this pathogen. In this study

we have analyzed a range of novel bioinformatically predicted CPAF inhibitors for their anti-chlamydial activity and ability to abrogate CPAF mediated RFX5 degradation. The ability of two inhibitors to restore bone marrow dendritic cells maturation impaired due to CPAF protease activity was also assessed.

Materials and methods: Identification of novel small-molecule CPAF inhibitors was performed by structure based virtual screening of 2071038 chemical compounds against CPAF crystal structure. Selected compounds were assessed for anti-chlamydial activity in *Chlamydia trachomatis* *in vitro* assay. GST-tagged rCPAF was expressed in pGEX-4T vector, grown in *E. coli* and purified. rCPAF enzymatic activity and its inhibition were estimated in western blotting by degradation of its substrate -eukaryotic factor RFX5. Bone marrow dendritic cells maturation in the presence of LPS, CPAF and its inhibitors was estimated by expression of MHC, CD40 and CD86.

Results: We found that 10 of 30 non-toxic for eukaryotic cells compounds with predicted CPAF inhibitory activity had dose-dependent inhibitory effect on intracellular development of *Chlamydia trachomatis*. We also found that selected inhibitors blocked rCPAF proteolytic activity that was assessed by inhibition of RFX5 degradation. Additionally the ability of two inhibitors with both proteolytic and anti-chlamydial activity to restore bone marrow dendritic cells maturation in the presence of CPAF was evaluated. Indeed one of these inhibitors was able to restore MHC, CD40 and CD86 upregulation in response to LPS that was blocked in the presence of rCPAF. The precise mechanisms of CPAF inhibitor activity and *in vivo* protective potential of inhibitor treated rCPAF loaded dendritic cells with restored expression of MHC and costimulatory molecules are currently under investigation.

Conclusions: Our results confirm that at least one of CPAF inhibitors under the study possessed both specific anti-bacterial activity *in vitro*, abrogated the consequences of CPAF proteolytic activity on immunologically significant targets such as RFX5 and MHC and restored dendritic cells maturation in response to LPS.

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Impact of SIV infection on pDC-associated IFN- α production in African green monkeys, a non-human primate model of protection against AIDS

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Background and aims: Chronic immune activation during HIV infection in human and simian immunodeficiency virus (SIV) infection in macaques (MAC) is considered the cause of AIDS. In natural hosts of SIV, such as African green monkeys (AGM), T cell activation and inflammation is resolved by the end of acute infection, and AIDS does not develop. In this work, we sought to investigate the role of plasmacytoid dendritic cells (pDC) in the different inflammatory profiles between pathogenic and non-pathogenic SIV infection.

Methods: We collected blood and lymph nodes from 4 to 10 animals per species at 2–4 timepoints before and 4–8 timepoints after SIV infection. We stimulated mononuclear cells using SIV-infected cells, seven distinct SIV isolates and HSV *in vitro*. We used A151, a TLR7-, and G-ODN, a TLR9-antagonist, to inhibit the TLR7 and/or TLR9 pathway. Bioactive type I interferon (IFN-I) levels were mea-

sured by the VSV/MDBK assay. Using flow cytometry we identified IFN α + cells and measured pDC counts and pDC surface markers.

Results: We found that pDC are the primary source of IFN-I in response to SIV in AGM. AGM responded *in vitro* to free SIV particles, non-infectious SIV and SIV-infected cells by producing normal levels of IFN-I. We discovered that the NF- κ B pathway is functional in AGM, as they produced TNF- α and IL-6 after stimulation. The levels of cytokines produced varied among SIV isolates. During acute SIV-infection, AGM pDC rapidly lose the capacity to make IFN-I in response to SIV stimulation. This reduced response is not due to a general anergy of the pDCs, as AGM displayed an increased response to stimulation with HSV after SIV infection. We further studied this virus-specific response, finding that SIVagm is sensed mainly through TLR7, as already reported for HIV and SIVmac. However, AGM pDC did not fully mature *in vitro* upon SIV stimulation. Moreover, AGM pDC did not display detectable surface levels of CD4, hinting another mechanism of viral endocytosis could play a role in this model.

Conclusions: We show that AGM make a robust inflammatory response to SIV *in vitro*, showing the resolution of inflammation is not due to a constitutive SIV sensing mechanism defect. While the capacity to respond to SIV is rapidly blunted after *in vivo* infection, AGM pDC remain reactive to HSV, an unrelated virus. Natural hosts of SIV thus display perfect virus-host equilibrium.

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Effect of *Porphyromonas gingivalis* outer membrane vesicles on gingipain-mediated detachment of cultured oral epithelial cells and immune responses

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Introduction: *Porphyromonas gingivalis* is a major etiological agent of periodontal diseases and the outer membrane vesicles (OMVs) contain virulence factors such as LPS and gingipains. Recently, the robust mucosal immunogenicity of *P. gingivalis* OMVs was shown in a mouse model (Nakao R. *et al.* 2011. PLoS One). However, the role of *P. gingivalis* OMVs in the immunopathology and immunogenicity during development of periodontal diseases in human has remained obscure.

Materials and methods: OMVs were prepared from a culture of *P. gingivalis* ATCC 33277 at early stationary phase by a standard method using a combination of ultracentrifugation and membrane filtration. Patient serum samples with *P. gingivalis* seroconversion were used for ELISA and Western blots. To investigate the effect of OMVs on host cells, we assayed their ability to cause detachment of oral squamous epithelial cell lines.

Results: Firstly, we found that sera from periodontitis patients had significantly stronger reactivity against the OMV-producing wild type 33,277 strain than the isogenic OMV-depleted strain. These findings prompted us to investigate the immunopathological role of OMVs as a vehicle of antigens and virulence factors. OMVs were found to be highly antigenic, as absorption of patient sera with OMVs greatly reduced reactivity with whole cells of *P. gingivalis*. LC-MS/MS analysis of OMVs revealed multiple forms of gingipains and several gingipain-related proteins. Western blots of OMVs using patient sera

revealed a conserved immunoreactive antigen profile resembling the profile of OMV antigens that were recognized by gingipain antiserum, suggesting a potential role of OMV-associated gingipains in triggering antibody-mediated immune responses to *P. gingivalis* infection. OMVs also caused detachment of oral epithelial cells, which was gingipain-dependent.

Discussion: These data suggest that gingipain-laden OMVs may contribute to tissue destruction in periodontal diseases by serving as a vehicle for the antigens and active proteases.

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Promastigote secretory gel reduces inflammatory cytokine production in an *in vitro* skin cell model of *Leishmania* infection

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Background: Following transmission of *Leishmania* parasites from the sand fly vector, establishment of an infection requires the parasite to evade innate and adaptive immune response of the mammalian host. *Leishmania* are able to ensure their survival and propagation within the mammalian host by altering key signalling pathways that trigger adaptive immunity. Recent evidence has highlighted the importance of the environment at the site of inoculation, including the immune response of localised keratinocyte cells in the progression of Leishmaniasis. *Leishmania* derived extracellular proteins impact on the host immune response. In particular, promastigote secretory gel (PSG), a gel-like structure composed of filamentous proteophosphoglycan (fPPG) actively egested by the sand fly, has an established functional role in the transmission of *Leishmania*, and exacerbates *Leishmania* infection in mice.

Methods: To explore the localised effects of PSG on the initiation of an immune response to *Leishmania mexicana* (*L. mexicana*) infection an *in vitro* model utilising HaCaT keratinocytes was used. Interleukin (IL)-6, IL-10 and tumour necrosis factor (TNF- α) mRNA was assessed using qRT-PCR.

Results: Increase production of keratinocyte-derived T helper cell 1 (Th1) associated inflammatory cytokines, IL-6 and TNF- α mRNA, was observed in response to lysed *L. mexicana* promastigotes. Addition of PSG to the cell culture system reduced the induction of IL-6 and TNF- α in response to *L. mexicana* and flagellin (TLR5 ligand).

Conclusions: PSG exerts an immunomodulatory effect at the site of inoculation that may promote *Leishmania* survival; facilitating infection. PSG may modulate the induction of an immune response by targeting TLR signalling pathways. Therefore, PSG may present itself as a novel prophylactic target for the treatment of leishmaniasis.

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Dengue and other flavivirus specific T cell specific responses and antibody responses in past severe and non-apparent dengue viral infections in Sri Lanka

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Background and aims: Infection with the dengue virus (DENV) causes severe clinical disease and may be fatal in some individuals but results in mild or asymptomatic infection in the majority of individuals. Therefore, we set out to determine correlates of a DENV-specific protective immune response.

Methods: Of 1035 healthy individuals aged 5–80 years were recruited from the community and serum was tested for the presence of DENV and Japanese encephalitis virus (JEV) antibodies. Using DENV-NS3 peptides, JEV live vaccine and non DENV peptides ('FEC') *ex vivo* IFN γ ELISpot assays were done in 183 individuals with past non-apparent and 58 past severe DENV infection and 44 seronegative volunteers.

Results: 933/1035 were seropositive for DENV and 73(7.05%) had a past severe dengue infection. DENV-NS3 specific responses were seen in 141/182(77.47%) of those with past non-apparent DENV infections and in 47/55(85.45%) of those with past severe dengue infections. *Ex vivo* NS3 responses in those with non-apparent DENV infections (mean 399.5 SFU/1 million PBMCs) and severe (mean 294.6SFU/1 million PBMCs) were not significantly different ($P = 0.7120$) JEV responses were also seen in 36/55 (65.45%) of those with past severe DENV infections and 120/182 (65.93%) with past non-apparent DENV infections. *Ex vivo* JEL responses in those with non-apparent DENV infections (mean 258.3 SFU/1 million PBMCs) and severe (mean 145.3 SFU/1 million PBMCs) were not significantly different ($P = 0.1028$). The granzyme B levels in elispot supernatant were also not significant between the two groups for NS3, JEL and non-dengue peptides.

253/988(25.6%) of DENV-seropositive individuals had anti-JEV antibodies, while all of the DENV seronegative individuals were also seronegative for anti JEV antibodies. Individuals with a past severe DENV infection were more likely to have JEV specific antibodies when compared to those with asymptomatic dengue infection which was significantly different ($P = 0.028$) (Odds Ratio 2.16, CI: 1.07–4.35).

Conclusions: Our data suggest that the IFN gamma NS3-specific T cell response does not associate with severe disease. The presence of JE antibodies is likely to be due to the presence of highly cross reactive dengue antibodies.

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Streptococcal superantigens: recent development in the equine species

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Superantigens are potent toxins that bypass the conventional mechanism of MHC-restricted antigen presentation and may interfere with the development of a protective immune response. Superantigens bind to MHC class II molecules and T-cell receptors (TCR) outside the peptide groove, causing unspecific lymphocyte activation, with subsequent proliferation and overzealous pro-inflammatory cytokine synthesis. Superantigens are expressed mostly by Staphylococcal and Streptococcal bacteria and are associated with several diseases in humans, such as toxic shock syndrome and necrotising fasciitis. In recent years, increasing amounts of evidence suggest superantigens are key factors for *Streptococcus equi* (*S. equi*) and *Streptococcus zooepidemicus* (*S. zooepidemicus*), 2 of the most prevalent horse pathogens.

Streptococcus equi is a host-restricted equid pathogen that causes a disease characterised by the abscessation of the lymph nodes in the head and neck. *Streptococcus equi* produces four superantigens (SeeH, SeeI, SeeL and SeeM) that share homology with the mitogenic toxins of *Streptococcus pyogenes*. *Streptococcus equi* superantigens activity has now been well characterised *in vitro*, but their role *in vivo* remains to be defined. An allelic replacement mutant of *S. equi* strain 4047 with deletion of the superantigen genes was generated (*S. equi* DHILM). Deletion of seeI, seeL and seeM completely abrogated the mitogenic activity of the strain 4047 culture supernatant in equine T cells. The *S. equi* DHILM strain was used to experimentally infect a group of seven ponies. Clinical signs of diseases were

recorded and compared with a control group of seven ponies experimentally infected with the wild type *S. equi* strain. PBMC proliferation and cytokines synthesis (IFN gamma, TNF alpha, IL-10 and IL-4) were also measured after infection. Preliminary results will be presented and discussed here. The acquisition of superantigen genes by *S. equi* probably represents a key step in the evolution of *S. equi* from an ancestral *S. zooepidemicus* strain.

The *S. zooepidemicus* population is very diverse, able to infect numerous animal species, including horses and humans. A significant proportion of *S. zooepidemicus* strains produce superantigens (szeF, szeN, szeP and szeQ), which have been recently discovered. In the horse species, their presence is significantly associated with lymph-node abscessation ($P = 0.007$), and dissociated from cases of uterine infection/abortion ($P = 0.003$). We believe that *S. zooepidemicus* superantigens are of importance to both animal and human health and therefore their activity requires to be further characterised. Using sequence analysis and site directed mutagenesis, the TCR-binding site of SzeQ has been investigated and results will be presented and discussed.

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Neutrophils deliver TRAIL mediated protection from acute cytomegalovirus infection

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Pathogenic herpesviruses including cytomegalovirus target numerous host organs during infection, but the immunological mechanisms that afford antiviral protection in different tissue microenvironments require a better understanding. Utilizing the murine cytomegalovirus (MCMV) model, we demonstrate that neutrophils are critical antiviral effector cells that restrict early viral replication and associated pathogenesis in immune-competent and immune-deficient hosts. Depletion of Ly6G⁺ neutrophils exacerbated virus-induced weight loss and elevated virus load in a tissue-restricted manner. MCMV-elicited neutrophil responses were orchestrated by the induction of the neutrophil-attractant chemokine CXCL1 in MCMV-infected peripheral organs. By employing a novel *in vitro* assay, we demonstrate that neutrophils directly inhibit cytomegalovirus infection. The antiviral properties exhibited by neutrophils *in vitro* are dependent upon production of the TNF family member, TRAIL. These data highlight a previously unappreciated and critical role for neutrophils in countering cytomegalovirus infection.

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The regulation of intestinal epithelial cell turnover and its impact on life- and healthspan in *Drosophila melanogaster*

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Background: Intestinal epithelial cells (IEC) play an important role as a barrier from infection and damage and are replaced through intestinal stem cell (ISC) division. Renewal and repair of the intestinal epithelium must be carefully regulated, as dysregulation may lead to diseases including barrier impairment. The JAK-STAT pathway regulates ISC proliferation and differentiation and is needed to

maintain gut homeostasis. JAK-STAT regulation of IEC turnover is highly conserved from human to *Drosophila melanogaster*. Suppressors of Cytokine Signalling (SOCS) are negative regulators of the JAK-STAT pathway.

Methods: We used the UAS/GAL4 system in a *Drosophila* model to produce an ISC-specific knockdown of SOCS36E - a homologue of mammalian SOCS5, which regulates intestinal JAK-STAT signalling. We assessed the impact of SOCS36E on the regulation of ISC proliferation through anti-phospho-histone H3 immunolabelling, both in the steady state and following infection with *Erwinia carotovora carotovora 15* (Ecc15). Functional health was assessed using negative geotaxis and exploratory walking assays analysing several parameters of walking behaviour. Life span was also monitored.

Results: Knockdown of SOCS36E led to increased basal ISC proliferation as well as sustained proliferation following Ecc15 infection. SOCS36E knockdown significantly decreased median and maximum lifespans of female flies, compared with controls ($P < 0.001$). Knockdown accelerated the onset of age-associated declines in all walking parameters in infected male flies when compared with uninfected and infected controls ($P < 0.01$). Neither knockdown nor infection affected the age-associated decline in negative geotaxis assay performance.

Conclusions: Although increased IEC turnover could lead to more rapid clearance of Ecc15, continued loss of JAK-STAT regulation, through SOCS36E knockdown, had a negative effect on functional health and lifespan in male and female flies respectively. This could be due to dysplasia or intestinal degeneration caused by overproliferation of ISC.

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The PI3K pathway negatively regulates inflammatory tissue destruction in tuberculosis

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Pulmonary Tuberculosis (TB) is characterized by lung extracellular matrix (ECM) destruction and cavitation. The architectural framework of the lung is resistant to proteolytic degradation despite external insults. Matrix metalloproteinases (MMPs), especially the collagenase MMP-1, can cleave types I, II and III fibrillar collagens of the lung ECM at neutral pH, and MMP-1 drives immunopathology in TB. Although the pathways driving inflammation in TB are well understood, relatively little is known about regulatory mechanisms that suppress pathology. We investigate the Phosphoinositide 3-kinase (PI3K) pathway and demonstrate a novel negative regulatory role that limits tissue destruction. TB up-regulates gene expression and secretion of MMP-1 in primary human monocyte derived macrophages (MDMs) and THP-1 cells. LY294002, a pan-PI3K inhibitor, significantly augmented MMP-1 secretion and gene expression in Mycobacterium tuberculosis (Mtb)-infected MDMs. IC87114, a PI3Kδ selective inhibitor, similarly increased MMP-1. Pharmacological inhibition of PTEN, a PI3K phosphatase that leads to accumulation of PI (3,4,5)P3, suppressed MMP-1. Interestingly, AKT inhibition suppressed MMP-1 secretion, suggesting that the PI3K regulatory mechanism is AKT independent. Pre-treatment of MDMs with Rapamycin, a specific inhibitor of mTORC1, also augmented the MMP-1 secretion compared to the levels driven by Mtb infection. Taken together, Mtb induces MMP-1 secretion in THP-1 cells and in primary macrophages and the PI3K pathway limits excessive production of such tissue damaging proteases, independently of AKT activity. PI3K inhibitors are entering clinical trials, but conversely may accentuate tissue damage in inflammatory conditions.

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Hepatitis C triggers inflammation via inflammasome dependent activation of intrahepatic T-cells

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Background: Hepatitis C virus (HCV) is a single stranded RNA virus that is hepatotropic. Cytotoxic T cell responses play a role in clearing HCV, and HCV specific responses are found in those patients who do clear the virus, but are difficult to detect in patients who develop chronic disease. A population of CD8⁺ T cells (MAIT cells) expressing the C-type lectin receptor CD161 are enriched in the liver of patients with HCV representing up to 50% of CD8⁺ T cells. Intra-hepatic inflammasome activity and local production of IL-18 in response to HCV may, along with other cytokines (e.g. IL-12) lead to local activation of MAIT cells and interferon gamma (IFN-γ) production. This would provide a mechanism for recruitment of the adaptive immune system to the liver in HCV infection and may form the basis of an effective immune response, capable of clearing the virus.

Methods: Liver biopsy specimens were obtained from patients with HCV infection ($n = 55$) or NASH ($n = 6$) at S. Bortolo Hospital and histology scored using the Ishak system. Control samples were normal adjacent tissue from 6 uninfected volunteers undergoing liver resection for other reasons (Proteogenex, CA, USA). Ethical approval was obtained, and all patients provided written informed consent. RNA was extracted and quantitative PCR used to determine relative expression of genes of interest. Immunostaining was performed on explanted liver tissue from patients with HCV. *In vitro* experiments were performed using genotype 2a HCV strain J6CF-JFH1, and a variety of sources of macrophages; including the monocyte cell line THP-1 cells, monocyte derived macrophages and macrophages generated from induced pluripotent stem cells. *In vitro* experiments were analysed using IL-18 ELISA and flow cytometry staining.

Results: In this study, a series of *ex vivo* experiments (i) demonstrated that IL-18 is present in the livers of patients with HCV, and (ii) identified the likely cellular source as liver resident macrophages.

To further define the mechanism of IL-18 production in the context of HCV activation, a series of *in vitro* co-culture experiments were performed using HCV activated macrophages along with MAIT cells. These experiments showed that HCV activated macrophages induced the release of IFN-γ from intrahepatic MAIT cells in an IL-18 dependent, TCR-independent manner.

Conclusion: These experiments demonstrate that HCV-induced inflammasome-derived IL-18 from hepatic monocytes can trigger MAIT cells. Within an inflamed liver this process may contribute to local inflammation, including production of antiviral cytokines.

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Studying the humoral immunogenicity of a novel CCHF vaccine

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Crimean-Congo Haemorrhagic Fever (CCHF) is the most widespread tick-borne human viral disease. The virus is endemic in the Balkans, Asia and Africa. The wide distribution of CCHF virus, the potential for person-to-person transmission, the severity of the disease and the high fatality rate make CCHF a major public health concern. In October 2012 a CCHF case was imported to the UK from Afghanistan. There is no licensed vaccine or effective treatment

against CCHF available on the market. Mice deficient in the type I interferon signalling pathway (e.g. strain A129) are the only small animal models available for *in vivo* study of CCHF. Modified Vaccinia virus Ankara (MVA) is an attenuated poxvirus with a proven safety record. Despite its growth deficiency in most mammalian cells, MVA is able to promote high-level expression of recombinant genes. Its potent immunogenicity elicits cellular and humoral immunity, and avoids the requirement for an adjuvant to be co-administered. MVA can be inexpensively manufactured to GMP and has an established regulatory package for development as an Investigational New Drug. A viral-vectored vaccine based on recombinant MVA expressing the major CCHF glycoproteins Gn and Gc was constructed. Three different strains of mice, including A129, were vaccinated in a homologous prime-boost regimen, and antibody responses to CCHF proteins analysed by Western Blot. Method optimisation was required due to severe haemolysis of several samples. The novel CCHF vaccine candidate induced a CCHF-specific humoral response in mice with various genetic backgrounds, including those with an abrogated type I interferon response. Mass spectrometry of specific bands will be performed to learn which glycoprotein subunit is immunogenic. Future work will include testing these antibodies for *in vitro* neutralisation or protective efficacy and the development of an ELISA test to measure anti-subunit Gn antibodies.

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M2a macrophages are necessary and sufficient to mediate eosinophil-dependent immunity to filarial helminth infection

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Eosinophils are effector cells in the immune control of tissue dwelling helminths. Whilst eosinophil responses are induced by Th2 adaptive immunity, it is not known how eosinophils are instructed to home from the blood to target migratory stages of parasites.

Here we provide evidence from an experimental model of filarial infection (*Brugia malayi* mouse model) that eosinophils are a crucial component of the anti-filarial response that limits establishment of infectious larvae. *B. malayi* infectious larvae also induce M2 activation (alternative activation) of tissue resident macrophages, which is further pronounced following vaccination with heat-killed larvae. Absence of a functional interleukin 4 receptor α chain (IL-4Rα) leads to failure of M2a activation, impaired eosinophil recruitment and susceptibility to *B. malayi* establishment to the adult phase. To test the functional relevance of M2a development in the eosinophil larvicidal response, we undertook targeted depletion of macrophages by clodronate liposome (CL) treatment. CL-treatment rendered mice highly susceptible to infection with associated impaired eosinophil recruitment. Add-back of purified M2a into CL-treated WT mice restored both M2a expansion and eosinophil influx. Th2 responses were intact in CL-treated WT mice, suggesting that M2a development directly regulated eosinophil recruitment at the infection site. Consistent with this, an increase in CCL11 transcripts from immune cells derived from the infection site of WT but not IL-4Rα^{-/-} mice was apparent. We therefore tested the direct role of M2a in eosinophil regulation and parasite killing by adoptively transferring purified WT M2a into susceptible severe combined immune-deficient mice (SCID). WT M2a SCID recipients induced a rapid eosinophil response and killing of infectious larvae. In a complementary approach, supplying an exogenous source of IL-4 to condition resident macrophages toward an M2a phenotype at the point of infec-

tion rendered SCID mice more resistant to larval establishment. Thus we conclude that M2a conditioning via IL-4Ra is both necessary and sufficient in the absence of additional adaptive immune activation to induce resistance to filarial infection via larvicidal eosinophil recruitment.

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IL33 exacerbates periodontal disease

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Background: Periodontal disease is a bone-destructive inflammatory disease, associated with a pathogenic shift in the oral biofilm and dysregulation of the immune response. Efficient control of the biofilm is mediated by a predominantly Th1 response. In contrast, the chronic periodontitis lesion is dominated by a B cell and plasma cell infiltrate and associated polyclonal B cell activation. This ineffective immune response, with bystander damage, leads to destruction of the connective tissues and alveolar bone loss (ABL). This tissue destruction provides a source of extracellular IL33, a cytokine termed an 'alarmin' due to its role in signalling cell damage through binding its receptor ST2, expressed on immune cells, including Th2 and B cells. This study aimed to investigate the role of IL33 in periodontal disease.

Methods: Gene expression of IL33 and ST2 were quantified in the gingival tissue of 12 healthy controls and 17 patients with chronic periodontitis. BALB/c and ST2^{-/-} mice were infected with the periodontitis-associated pathogen *Porphyromonas gingivalis* or carrier (sham) only. Exogenous IL33 or PBS was administered intra-peritoneally to BALB/c mice to investigate the effects of over-expression of IL-33.

Results: IL33 and ST2 mRNA expression were significantly up-regulated in tissue from patients with chronic periodontitis compared with healthy controls ($P < 0.05$). Similarly, infection with *P. gingivalis* resulted in increased IL33 mRNA expression in mouse gingival tissue ($P < 0.05$). *P. gingivalis*-infected animals treated with IL33 had significantly increased ABL relative to *P. gingivalis*-infected controls (-0.39 mm versus -0.13 mm, $P < 0.001$). ST2^{-/-} animals were protected from ABL relative to controls (0.02 mm versus -0.19 mm, $P < 0.05$). IL33-treatment was associated with a reduction in Th1 and Th17 polarisation in the draining lymph nodes (DLNs) and an increase in secretion of Th2 cytokines *ex vivo*. There were no apparent differences in T cell polarisation in the ST2^{-/-} animals compared with wild type controls. Flow cytometry analysis of B cell and T cell RANKL expression in DLNs and gingival tissues of IL33-treated, *P. gingivalis*-infected animals at 1-week post-infection revealed a significant increase in the proportion and number of CD3+ and CD19+ cells expressing RANKL.

Conclusions: These data suggest that elevated IL-33 exacerbates ABL. This is associated with an increased Th2-mediated humoral response and increased RANKL expression. Thus, we propose that increased expression of IL33 during periodontal disease perpetuates the Th2-mediated humoral response associated with chronic periodontitis and ABL.

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Critical role of IL-6, IL-17 and IL-23 in the control of the experimental *Paracoccidioides brasiliensis* infection

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Introduction: *Paracoccidioides brasiliensis* (Pb), a thermally dimorphic fungus, is the causative agent of paracoccidioidomycosis (PCM), one of the most frequent systemic mycosis that affects the rural population in Latin America. Th17 cells are an arm of the immune system that enhances host protection against several infections, including mycosis. To better understand the mechanisms which are involved in resistance to *P. brasiliensis* infection we evaluated the role of IL-6, IL-17 and IL-23 during the PCM experimental. **Methods and results:** At first, we realized intravenous infection in C57BL/6 (WT) mice with 1×10^6 yeast forms of Pb18, a highly virulent Pb strain. At 7 days post infection (dpi), we measured by ELISA the IL-6, IL-17 and IL-23 secretion in the lung of mice, which was statistically higher compared to uninfected mice. Subsequently we infected IL-6^{-/-}, IL-17R^{-/-} and IL-23^{-/-} mice under the same conditions mentioned above and we verified that the infection induced increased colony forming units (CFU) recoveries from the lung, liver and spleen of knockouts mice compared with WT mice. Using silver staining, we proved that the absence of IL-6, IL-17R and IL-23 impaired the control of the fungal replication, due the increased amount of yeast observed at lung from knockouts mice in relation to WT mice. Histopathological analysis showed that IL-6 and IL-17 contribute to compact granulomas formation due to adequate production of reticulin fibers. By immunohistochemistry, we verified that the deficiency of IL-6 or IL-17R was accompanied of disorganized CD4⁺ T cell infiltration at lung. The absence of IL-6, IL-17R or IL-23 resulted in lower production of IFN- γ and IL-10 compared with WT lung at 15 and 30 dpi. Additionally, the frequency of CD3⁺CD4⁺IL-17⁺ cells, as well as IL-17 production, was decreased in IL-6^{-/-} or IL-23^{-/-} mice when compared with WT mice at 15 and 30 dpi. This was associated with an impaired neutrophils and macrophages lung recruitment analyzed by flow cytometry in lung from IL-6^{-/-}, IL-17R^{-/-} and IL-23^{-/-} mice in relation to WT mice.

Conclusion: Taken together, these results demonstrate that IL-6, IL-17 and IL-23 contribute to control of experimental Pb-infection through an efficient granulomatous organization.

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Fasciola hepatica tegumental antigens modulate mast cells function by impairing their ability to drive Th1 immune responses

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The parasitic worm, *Fasciola hepatica* induces strong Th2 and T-regulatory (Treg) immune responses while simultaneously suppressing Th1 driven immune responses to bystander microbial infections. It also prevents the initiation of Th1 mediated autoimmune disorders in mice through the suppression of Th17 and Th1 immune responses and this can be mimicked by parasite derived molecules. We have isolated *F. hepatica* tegumental coat antigen (FhTeg) and demonstrated its suppressive effect *in vivo* by directly targeting dendritic cells impairing their ability to drive Th1 responses. Mast cells are critical in promoting

Th1 protective immunity during bacterial infection and driving Th1-mediated pathology in autoimmune diseases. We have previously shown that FhTeg induces mast cells numbers *in vivo* while *in vitro* FhTeg stimulated mast cells fail to promote Th2/Treg immune responses. Here we show that FhTeg inhibits the ability of mast cells to drive Th1 immune response by suppressing cytokine secretion (TNF- α , IL-6, IFN- γ and IL-10) and ICAM1 expression in mast cells. This inhibition also suppresses mast cells ability to promote Th1 immune responses in CD4⁺ T-cells and a role for ICAM1 was demonstrated in this process. FhTeg suppresses the activation of transcription factors (MAPK and NF- κ B) in the TLR signalling pathway while simultaneously promoting SOCS3 expression a negative regulator of the TLR signalling. This could explain the decrease in cytokine production and ICAM1 expression. We conclude that FhTeg targets mast cells inhibiting their ability to drive Th1 immune responses highlighting the important role of mast cells in the bystander effects of helminths.

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Targeting HIV-1 where it hurts

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Virus diversity and escape from immune responses are the biggest challenges to the development of an effective vaccine against HIV-1. Such vaccine may have to induce both broadly neutralizing antibodies and effective T cells. We aim to optimize induction of the T cell responses and hypothesized that only T cells targeting conserved regions of the HIV-1 proteome can efficiently control infection. Here, we describe the first-ever administration of conserved immunogen vaccines delivered by using heterologous prime-boost regimens of DNA, simian adenovirus and modified virus Ankara to uninfected low-risk UK volunteers. The vaccine induced high levels of CD8⁺ T cells that recognized virus-infected autologous CD4⁺ cells and inhibited HIV-1 replication in tissue culture. The virus inhibition was mediated by both Gag- and Pol- specific effector CD8⁺ T cells targeting epitopes, which are typically subdominant in natural HIV-1 infection. These results provide proof of concept for using vaccines focusing T cells at conserved epitopes.

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Determining the presence of classical and alternatively activated macrophages during a *Francisella tularensis* infection

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Francisella tularensis is a Gram-negative intracellular bacterium that has the ability to multiply within numerous different cell types, in particular the macrophage. Macrophage phenotype/activation can determine whether the infection is cleared or the host succumbs to the disease. Previously published data has suggested that *F. tularensis* LVS actively induces the alternative phenotype as a survival mechanism. In these studies we show that this is not the case for the fully virulent strain of *F. tularensis*, SCHU-S4 using an intranasal mouse model of infection. Immuno-histochemistry demonstrates that iNOS positive macrophages (classical) are present at 72 h post-infection and remain high, while arginase positive cells (alternative) appear later and remain low. This continued presence of iNOS positive

macrophages undoubtedly contributes to the cytokine storm and the tissue pathology seen during inhalation challenges of *F. tularensis*. Therapeutics that target this macrophage switching may be a suitable strategy to increase host survival following infection.

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Immunomodulation of epithelial cells by oral biofilms

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Background: Periodontal disease is the result of chronic inflammation mediated by the host immune response to bacterial biofilm (plaque) on the root surface, leading to destruction of supporting structures of the teeth and ultimately tooth loss. In health, commensal oral biofilms form around the gum margin without notable disruption of the tooth supporting tissues. However, in periodontal disease, a destructive immune response is associated with a shift in the microbial community from a predominantly Gram-positive, aerobic biofilm, to a microbial community dominated by anaerobic, Gram-negative species.

Objectives: To investigate the mechanisms by which oral biofilms alter epithelial cell viability, cytokine and chemokine production in co-culture with *in vitro* formed model biofilms, representing 'healthy', 'intermediate' or 'disease-associated' biofilms.

Materials and methods: Biofilms were grown *in vitro* over 3–7 days, with sequential washing then addition of different bacterial species under either aerobic or anaerobic culture conditions. The 'healthy' biofilm included *Streptococcus mitis*, *Streptococcus intermedius* and *Streptococcus oralis*; the 'intermediate' biofilm additionally included *Veillonella dispar*, *Actinomyces naeslundii*, *Fusobacterium nucleatum* and *Fusobacterium nucleatum* spp. *Vincentii*; and the 'disease-associated' biofilm included further addition of *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Aggregatibacter actinomycetemcomitans*. OKF6-TERT2 oral epithelial cells were stimulated with each of the different biofilms for 4 h. Epithelial cell viability was assessed by Alamarblue. Changes in mRNA and protein expression of multiple chemokines and cytokines were assessed by quantitative PCR and Luminex[®].

Results: Significant differences in the epithelial response to 'healthy', 'intermediate' and 'disease-associated' biofilms were observed at both protein and mRNA level. Epithelial cell co-culture with 'disease-associated' and 'intermediate' biofilms resulted in increased levels of TNF α , CXCL1, and CSF-2 gene expression in epithelial cells compared with co-culture with 'healthy' biofilms. IL-8 protein release increased ten fold between 'healthy' and 'disease-associated' biofilm co-culture. A significant 20% increase in epithelial cell death was observed following co-culture with 'disease-associated' biofilms compared with the media control. These results demonstrate that different microbial biofilms may modulate the epithelial cell response to biofilms and influence disease pathogenesis.

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HIV-1 virus protein U activates the inflammasome**C. Ward, K. Triantafilou & M. Triantafilou***Institute of Infection and Immunity, School of Medicine, Cardiff University, Cardiff, UK*

HIV-1 patterns, such as nucleic acids and envelope glycoproteins have been implicated in the activation of pattern recognition receptors (PRRs) such as Toll-like receptors, providing "signal 1" for inflammasome activation. Precise HIV-1 PAMPs and DAMPs activating "signal 2" via Nod-like receptors (NLRs) are currently unknown. Little is known about PRR-mediated recognition of HIV-1 Virus Protein U (Vpu), an accessory protein and viroporin involved in immune evasion and viral release. Previous studies have demonstrated that other viroporins may activate the inflammasome via dysregulation of intracellular cation homeostasis as a sign of damage (DAMP), and that Vpu displays cation channel activity. In this study, human monocytes or vaginal epithelial cells were transfected with a plasmid encoding HIV-1 Vpu. Immunoblot for caspase-1 activation and HEK reporter assays for IL-1 β demonstrated upstream activation of the NLR system in HIV-1 Vpu expressing cells compared to isotype controls. Confocal microscopy demonstrated activation of NLRP3 in HIV-1 Vpu-expressing cells. Calcium or proton channel blockade, as well as ROS inhibition removed caspase-1 activation and IL-1 β secretion in Vpu-expressing cells, compared to positive controls. It was shown that HIV-1 Vpu activates the inflammasome likely via dysregulation of intracellular cation homeostasis and ROS generation.

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Further characterisation of established lines of CD4⁺ $\alpha\beta$ -TCR transgenic mice specific for *T. muris***M. Moradi & R. K. Grencis***Department of Immunology, FLS, University of Manchester, Manchester, UK*

Background and aim: The availability of clonal populations of specific T cells has greatly facilitated the study of the immune responses *in* and *ex vivo*. TCR Tg technology is widely applied in different fields of human /mouse disease, and offers the prospect of modelling diseases to gain a better understanding of the outcome of disease and as an aid to investigate the mechanisms of susceptibility and resistance to disease. The aim of our work was to generate an $\alpha\beta$ T cell receptor transgenic mouse (TCR Tg) specific for *T. muris*. Two mouse lines of *Trichuris muris* specific CD4⁺ TCR transgenic mice have been generated on a BALB/c background. The two lines selected are C3.19 and E10.17. Both have been backcrossed onto BALB/c RAG2^{-/-}. Several sets of experiments set up to assess the *in vivo* behaviour of Tg cells.

Method: Straightforward infection of *T. muris* specific C3.19 or E10.17 TCR transgenic mice. Mice were euthanized at several time points post infection to assess worm burdens and MLNs and spleens by flow cytometry for efficiency of adoptive cell transfer (and cytokine array).

Adoptive transferred between 2–3 $\times 10^6$ CD4⁺ T cells (40–50% Tg cells) of C3.19 and E10.17 mice cells into Rag^{-/-} BALB/c recipient mice have been set up, to test and compare *in vivo* behaviour of naïve monoclonal CD4⁺ T cells (specific) with naïve polyclonal CD4⁺ T cell (non-specific).

Results: Both lines contain elevated numbers of *T. muris* specific CD4⁺ T cells. Cells from non-infected C3.19 and E10.17 mice respond to *T. muris* antigens *in vitro*, proliferate and produce an array of cytokines. Proliferation appears to be at least as equivalent to that seen in well established CD4⁺ TCR transgenic mice such as

OT-II. C3.19 or E10.17 TCR transgenic mice expel both a high dose and low dose challenge *T. muris* infection in an accelerated manner in comparison to non transgenic BALB/c mice and congenic Rag^{-/-} BALB/c recipient as control mice.

Conclusion: Data suggests that cells in both C3.19 and E10.17 TCR transgenic mice recognize *T. muris in vivo* leading to a functional protective immune response. We hope our TCR Tg mice; will be crucial in advancing our understanding of the interplay between immune system and *T. muris*.

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Acute influenza A pathogenesis is ameliorated in a murine model of latent gammaherpesvirus infection**G. Hardisty, M. Quigg Nicol, Y. Ligertwood, K. Bryson, J. Hopkins & B. M. Dutia***The Roslin Institute, University of Edinburgh, Edinburgh, UK*

Background: Human populations are infected with multiple herpesviruses that persist in a latent form within the host for the lifetime of the individual. In a murine model of latent gammaherpesvirus infection, systemic immune responses are skewed towards a pro-inflammatory Th1 phenotype, with elevated levels of circulating IFN- γ and TNF- α cytokines. These are attributed with suppressing reactivation of the latent virus infection but have the potential to play a role in the outcome of other viral infections. The aim of the current project is to understand how the immune responses to latent infection impact the pathogenesis of a concurrent acute viral infection. Influenza A virus (IAV) causes significant morbidity and mortality through seasonal transmission and pandemics as seen in the H1N1 2009 outbreak. Fatality in young, healthy individuals is often associated with overwhelming immune responses, cytokine storms and considerable inflammatory immunopathology. Understanding how co-infections and/or on-going immune responses can alter the outcome of influenza virus infections is critical for development of novel strategies for prevention and treatment of severe infections.

Methods: BALB/c mice were infected with murine gammaherpesvirus 68 (MHV-68) and upon establishment of latency were challenged intranasally with influenza A/WSN/33 H1N1 virus. 2, 4 and 6 days after influenza virus infection, lungs, bronchoalveolar lavage and spleens were harvested for analysis. Viral loads were measured by qPCR and plaque assay, composition of immune cells in bronchoalveolar lavage by flow cytometry and inflammatory cytokine profile in the lungs by qPCR and ELISA.

Results: Intranasal infection of mice harbouring existing latent herpesvirus infection with influenza virus resulted in lower peak influenza viral titres in the lung when compared to mice infected with influenza virus alone. Latently infected mice had lower levels of IFN- γ in lung homogenates throughout the course of acute influenza virus infection, compared to influenza virus only controls. There were also differences in lung immune cell populations in bronchoalveolar lavage between groups. MHV-68 viral loads in the spleen were unaltered by influenza infection suggesting the protective effect was not a result of reactivation.

Conclusions: These results provide evidence that a latent gammaherpesvirus infection impacts on the host immune response to a heterologous respiratory viral infection, such as influenza A, and has implications for understanding the pathogenesis of acute respiratory virus infections.

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Modulation of neutrophil apoptosis by *Staphylococcus aureus* facilitates intracellular survival

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Increasing antibiotic resistance by *S. aureus* necessitates development of an effective vaccine, a challenge complicated by the immense capacity of *S. aureus* for immune evasion. Neutrophils are regarded as the first line in host defence, however *S. aureus* can also manipulate neutrophil responses and evidence suggests that intracellular survival can occur within neutrophils to facilitate dissemination. The naturally short lifespan of neutrophils however seems a logical deterrent to intracellular survival.

Studies have demonstrated the propensity for T-cell derived cytokines (IFN γ , IL-17) to regulate neutrophil recruitment during *S. aureus* infections, the effects of these cytokines on neutrophil apoptosis however is unknown. Here, we investigate if *S. aureus* can manipulate neutrophil apoptosis, either directly, or indirectly through engagement of host T-cell cytokines, which in-turn influence neutrophil apoptosis.

Using a murine model of *S. aureus* surgical wound infection we investigated neutrophil apoptosis in IFN $\gamma^{-/-}$, IL17 $^{-/-}$ and WT mice using TUNEL staining (detects fragmented DNA). On day 7 IFN $\gamma^{-/-}$ mice had increased levels of neutrophil apoptosis compared to WT mice which corresponded with lower bacterial burdens. In contrast IL17 $^{-/-}$ mice exhibited lower neutrophil apoptosis compared to WT mice but increased bacterial burden. These results suggest that inhibition of neutrophil apoptosis provides a niche for bacteria to survive intracellularly. Neutrophils were also isolated from the peripheral blood of healthy donors and treated with cytokines (100 ng) for 2 h. IFN γ treatment decreased cleavage of pro-Caspase 3 to its active form, supporting the notion that IFN γ inhibits apoptosis. To investigate the direct modulation of neutrophil apoptosis by *S. aureus* strains, neutrophils were cultured with/without *S. aureus* (MOI 10). *Staphylococcus aureus* significantly decreased cleavage of pro-Caspase 3 to its active form, suggesting that *S. aureus* may also be downregulating the apoptosis pathway directly.

Our results demonstrate the capacity of *S. aureus* to manipulate neutrophil apoptosis both directly and indirectly in order to facilitate a niche for intracellular survival.

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Interleukin-10 plays opposing roles during *Staphylococcus aureus* nasal colonisation and infection

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In contrast to its invasive infectious potential, *S. aureus* is a common coloniser of the anterior nares. Consequently, the host immune system is exposed to this organism on a continuous basis, however the influence that this exposure has on host immunity has not been well documented. Gut commensal organisms and other colonising organisms of the respiratory tract e.g. *S. pneumoniae* have evolved mechanisms to induce regulatory immune responses to facilitate their survival in the host. The ability of *S. aureus* to drive regulatory T-cell responses in the context of colonisation or infection remains to be established.

Interleukin (IL)-10 is an important immuno-regulatory cytokine that controls excessive inflammatory responses and regulates differentiation/proliferation of immune cells. IL-10 has previously been shown to play a protective role during systemic bacterial infection

through its ability to control inflammatory cytokines and associated organ damage.

Here we investigate the role played by IL-10 during *S. aureus* systemic infection and nasal colonisation.

IL-10 $^{-/-}$ mice were intranasally inoculated with spontaneous streptomycin-resistant *S. aureus* Newman (2×10^8 CFU/nose). IL-10 $^{-/-}$ mice displayed decreased nasal colonisation on days 3 and 10 compared to wild-type (WT) mice. This reduced colonisation was associated with increased levels of neutrophil recruitment, IFN- γ and IL-17 production in the cervical lymph nodes. To investigate the role of IL-10 during systemic *S. aureus* infection, IL-10 $^{-/-}$ mice were challenged by intra-peritoneal injection of *S. aureus* (5×10^8). At 24hrs post infection, IL-10 $^{-/-}$ mice were hypothermic and demonstrated increased mortality compared to WT but this was not associated with increases in bacterial burden. Increased pathology in the IL-10 $^{-/-}$ mice was due to excessive pro-inflammatory cytokine responses both at the site of infection and systemically.

Our results demonstrate that IL-10 plays a protective role during infection by controlling excessive inflammatory responses, however the induction of IL-10 during colonisation facilitates bacterial survival through a similar mechanism of dampening local pro-inflammatory responses.

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Immunoreactive antigens recognized by sera of mice intranasally immunized with outer membrane vesicles of *Porphyromonas gingivalis*

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Introduction: Gram-negative bacteria produce spherical microstructural bodies called outer membrane vesicles (OMVs). *Porphyromonas gingivalis* OMVs carry a wide range of virulence factors such as LPS and gingipains. Recently, we have reported that *P. gingivalis* OMVs elicited strong mucosal immune responses in a mouse model. Therefore, *P. gingivalis* OMVs are a feasible immunogen for use as a periodontal disease vaccine. In this study, we aimed to identify immunoreactive antigens profile in serum of mouse intranasally immunized with OMVs of *P. gingivalis*.

Materials and methods: OMVs were isolated from a culture of *P. gingivalis* at early stationary phase using a combination of membrane filtration and ultracentrifugation. In animal experiments, BALB/c mice were intranasally immunized with 1 μ g of OMVs and 10 μ g of polyribonucleosinic polyribocytidylic acid on day 0 and week 3. At two weeks after the second immunization, mouse sera were collected and used for ELISA and Western blots. Two-dimensional PAGE (2D-PAGE) using the OMVs was also performed by a standard method. Protein spots on a 2D-PAGE gel were identified by MALDI-TOF mass spectrometry and peptide mass fingerprinting.

Results: Whole-cell ELISA using mouse sera revealed that intranasal immunization with OMVs strongly induced *P. gingivalis*-specific IgG antibodies in the sera. The immunoreactive antigen profiles of OMVs on SDS-PAGE gels were examined by Western blots using seven sera of mice immunized with OMVs and four sera of mice immunized with PBS. The Western blot results of immunized mouse sera demonstrated that relatively sharp three bands appeared at 40-, 43-, and 46-kDa. Mass spectrometry analysis of spots on a 2D-PAGE gel showed the presence of several proteins in OMV preparation, as follows: minor fimbrial protein, MfaI; hypothetical protein, PGN_0477; hypothetical protein, PGN_1808; lysine-specific gingipain, Kgp; receptor antigenB, SusD; major fimbrial protein, FimA; and immunoreactive 42-kDa antigen PG33.

Discussion and conclusion: We could identify several major OMV proteins on the 2D-PAGE gel. We suggest that major fimbrial protein FimA may be immunodominant proteins in our model mice that are intranasally immunized with OMVs.

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Different subtypes of S-FLU pseudotyped influenza vaccines protect against homologous and heterologous viruses

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Influenza virus causes widespread respiratory disease and is particularly severe when new subtypes occur to which the general population has little immunity. In the last few years two novel subtypes of influenza have emerged in the human population and caused a worldwide pandemic in the case of H1N1 pdm2009 and a localized outbreak in the case of H7N9. Subunit vaccines to influenza are effective but are strain specific, time consuming and expensive to produce, so novel cross-protective vaccines to this virus are needed. Haemagglutinin Signal Sequence Deleted Influenza (S-FLU) is a non-replicating attenuated form of influenza virus that has the potential to be used as a vaccine against seasonal and pandemic strains of influenza. In S-FLU the haemagglutinin (HA) gene is replaced such that the vRNA no longer encodes a full length HA protein and so production of viable virus from infected cells is blocked. However if the S-FLU is grown in cells transfected with HA the release of pseudotyped virus can occur. Since the packaging MDCK line can be transfected with any HA, any strain of virus can be produced as long as the HA is compatible with the NA encoded by the viral genome. We have produced forms of S-FLU with HA from A/PR/8/34 (H1), A/Eng/195/2009 (pandemic H1), A/VN/1203/2004 (H5 with a modified cleavage site) and also the newly emergent A/Anhui/1/2013 H7 HA. Vaccination of mice with S-FLU in its different forms led to protection against otherwise lethal doses of heterotypic or homotypic strains of virus A/PR/8/34 (H1) or X31 (H3). This protection was also associated with the induction of strain specific HA antibody detected by ELISA and neutralization assays, and strong cross-reactive NP specific CD8⁺ T cell responses. This S-FLU system offers a rapid protective response to novel pandemic and seasonal influenza.

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Interesting phenotypes found as part of the infection challenge in the Wellcome Trust Sanger Institute's Mouse Genetics Programme

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As part of the Wellcome Trust Sanger Institute's Mouse Genetics Programme all mutant mouse lines generated in this high throughput programme are challenged with *Salmonella* Typhimurium an intracellular pathogen which induces a systemic disease and *Citrobacter rodentium* a natural mouse pathogen which forms attaching and effacing lesions on the surface of the gastrointestinal lumen (details of the challenges can be found on the poster "Phenotyping of knockout mice using bacterial pathogens as part of the Wellcome Trust Sanger Institute's Mouse Genetics Programme"). To date we have identified 72 genes which contribute to controlling the susceptibility to bacterial infection out over 250 knockout mice lines phenotyped so far. These phenotypes include hits in novel genes as well as gene of known function and range from severe phenotypes, were the

mice succumb to infection rapidly, too more subtle phenotypes. Here we would like to show a selection of the data generated as part of this programme and details of how to freely access all the data and resources from the programme as a whole.

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A central role for type I IFN in Th2 induction by dendritic cells

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We have shown that dendritic cells (DCs) are critical for induction of Th2 immunity against the medically important parasitic helminth *Schistosoma mansoni*. However, relatively little is known about how DCs become activated and function in response to *S. mansoni* or other helminths. Murine bone marrow cultured with Flt3-L differentiates into DC subsets thought to be representative of populations found *in vivo*, particularly in lymphoid organs. We have used FLDCs to assess how Flt3-L-dependent DCs respond to soluble egg Ag (SEA, a potent Th2-inducing Ag) from *S. mansoni*. Unexpectedly, we have identified an SEA-specific Type I IFN signature in FLDCs, as well as in splenic DCs following SEA injection. Although Type I IFNs are primarily associated with anti-viral immunity and Th1/17 responses, their role in Th2 settings is currently unknown. SEA-pulsed IFNAR-deficient FLDCs, lacking the Type I IFN receptor and thus unable to respond to IFN-alpha or beta, displayed a dramatically impaired ability to induce Th2 cytokines following adoptive transfer into naïve wildtype recipient mice. This suggests a novel role for Type I IFN responsiveness for Flt3-L-dependent DCs to efficiently generate optimal Th2 immunity against helminths such as *S. mansoni*. Despite this striking phenotype *in vivo*, and impairment in SEA-specific activation, IFNAR-deficient FLDCs capably stimulated T cell proliferation and Th2 polarisation *in vitro*. This dichotomy strongly suggests a role for Type I IFN responsiveness in effective DC trafficking to the draining lymph node *in vivo*, following exposure to SEA.

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Phenotyping of knockout mice using a range of immunological agents as part of the Wellcome Trust Sanger Institute's Mouse Genetics Programme

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The Wellcome Trust Sanger Institute's Mouse Genetics Programme is running an extensive sequence/phenotype driven mutagenesis programme as part of international efforts aimed at mutating over 95% of known mouse genes in embryonic stem cells. The knockout mice, resources and data generated in this programme are then freely available to the scientific community. All mutant mouse lines generated in this high throughput programme are examined using a common battery of phenotyping tools. At the Sanger Institute a component of this phenotyping programme involves challenging the knockout mice with selected immunological agents. We are currently using five standard models of infection - *Salmonella* Typhimurium an intracellular pathogen which induces a systemic disease in mice

similar to that of human typhoid fever. *Citrobacter rodentium* a natural mouse pathogen which forms attaching and effacing lesions on the surface of the lumen to colonise the host gastrointestinal tract in a similar way to enterohaemorrhagic *E. coli* (EPEC). Influenza a viral infection affecting the lung. Dextran Sodium Sulphate (DSS) a model of IBD which looks at the regeneration of the gut and challenge via normal flora. *Trichuris muris* a parasitic infection driving a Th2 type response in the gut. We feel that these models will uncover defects in many of the immune pathways.

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Characterization of IL-10-producing CD4⁺ T cells in the large intestine following *Helicobacter hepaticus* infection

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We have previously shown that IL-10 KO mice develop large intestinal inflammation when infected with *Helicobacter hepaticus* (*Hh*). Conversely, WT mice infected with *Hh* do not develop intestinal inflammation, but mount a disease-protective IL-10 response to the bacterium. Using a *Hh*/RAG KO transfer model of colitis, we further demonstrated that the IL-10⁺ cells responsible for protection against *Hh*-induced inflammation belong to the CD45RB^{low} CD4⁺ T cells isolated from mesenteric lymph nodes (MLN) of *Hh*-infected, but not uninfected WT mice, and that they prevent colitis through their secretion of IL-10. To characterize the IL-10-producing CD4⁺ T cells at the site of *Hh* colonisation (i.e. the cecum and colon), we isolated large intestinal lamina propria cells from uninfected and 2-week *Hh*-infected WT mice, stimulated them with PMA/ionomycin, and stained for surface markers, cytokines, and the transcription factor FoxP3. Although, as expected, WT mice did not develop colitis following *Hh* infection, the total number of intestinal CD4⁺ T cells in these animals was twice that of uninfected controls when examined at 2 weeks post bacterial inoculation. As would be expected from a non-inflammatory setting, there was no significant difference in the proportion of CD4⁺ T cells producing IFN- γ or IL-17A between the two groups. In contrast, there was a significant increase in the percentage of CD4⁺ T cells producing IL-10 in the *Hh*-infected mice ($7.8 \pm 1.5\%$ compared to $3.2 \pm 0.8\%$ in uninfected mice), corresponding to a 5-fold increase in the total number of CD4⁺ T cells capable of secreting IL-10 in these hosts. Phenotypically, the majority of the IL-10-producing CD4⁺ T cells in both uninfected and *Hh*-infected animals expressed FoxP3 (approximately 55% of the IL-10⁺ CD4⁺ T cells being FoxP3⁺CD25⁺ and approximately 18% being FoxP3⁺CD25⁻). These data suggest that *Hh* infection does not induce a specific expansion in IL-10⁺FoxP3⁺ cells over IL-10⁺FoxP3⁻ cells, but that both these populations expand to a similar extent. When examining co-expression of other cytokines, a small proportion of the IL-10⁺ cells in uninfected mice secreted IL-17A (approximately 7% of the IL-10⁺ CD4⁺ T cells). Interestingly, following *Hh* infection the IL-10⁺IL-17A⁺ subset comprised approximately 17% of the total IL-10⁺ CD4⁺ T cells, indicating that this subset expanded to a greater extent than the IL-10⁺IL-17A⁻ cells in response to *Hh* challenge. The signals that drive the expansion of IL-10⁺IL-17A⁺ cells and the role of this population in disease protection remains to be elucidated.

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***P. acnes* induces the natural killer group 2 member D (NKG2D) system in the stomach mucosa**

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The gastrointestinal tract is the largest reservoir of commensal bacteria in the human body. Several gastrointestinal disorders have been attributed to dysbiosis, a condition wherein the composition of the endogenous microbiota is disturbed. Lymphocytic gastritis (LyG), a special form of chronic gastritis, has so far an unclear etiology. Indeed, it is believed that *Helicobacter pylori* triggers disease, although in about 1/3 of LyG cases *H. pylori* cannot be detected. The hallmark of LyG disease is infiltration of the stomach epithelium with CD8 positive (cytotoxic) lymphocytes leading to tissue damage. LyG is also often associated with celiac disease (CD), but the exact pathophysiologic link between CD and LyG remains unknown. Recently, we identified *Propionibacterium acnes* as a possible cause of LyG. *Propionibacterium acnes* produces propionic acid as its main fermentation product, which has been shown to induce the natural killer group 2 member D (NKG2D) system in epithelial cells. The NKG2D system is responsible for the recruitment of CD8 positive intraepithelial lymphocytes. The aim of the study was to prove the concept if *P. acnes* can induce the NKG2D system in LyG. Human upper gastrointestinal (GI) biopsies, including stomach (corpus) and duodenum specimens were obtained from patients suffering from LyG, *H. pylori*- gastritis, CD or healthy controls. By means of qRT-PCR we identified transcripts corresponding to the NKG2D receptor and its ligand MICA significantly up-regulated in LyG compared to *H. pylori*-associated gastritis and healthy controls. Moreover, we infected the human gastric epithelial cell lines AGS and KATO III with human *P. acnes* GI isolates and also treated them with different amounts of propionic acid. Both strategies increased expression of MICA and ULBP3 ligands, underscoring the link of *P. acnes*, propionic acid and the NKG2D system in LyG.

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Serum ficolins enhance phagocytosis and killing of *Aspergillus fumigatus* leading to a modulation of the pro-inflammatory cytokine response

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Invasive aspergillosis is a devastating systemic fungal infection, prevalent in developed countries. The main route of infection is via inhalation of ubiquitous airborne *Aspergillus fumigatus* spores which penetrate deep into the alveoli and germinate to form tissue invading hyphae. Immuno-competent patients can effectively remove inhaled spores but patients with compromised immunity, such as leukaemia, transplant and neutropenic patients are at high risk of infection with an associated mortality rate of up to 90%.

The body can generate a robust immune response following *A. fumigatus* challenge, one which is dominated by alveolar macrophages, neutrophils, complement and pattern recognition proteins, such as the collectin mannose-binding lectin (MBL) and the homologous ficolin family. Humans possess two serum ficolins, L-ficolin and H-ficolin. Conversely, rodent serum only contains the orthologue of L-ficolin, termed ficolin-A. Ficolins are capable of binding to *A. fumigatus* and facilitating their phagocytosis by type II alveolar epithelium, consequently modulating an increase in IL-8 secretion. However, the interactions between ficolin opsonised spores and host leukocytes are poorly understood. Therefore, we utilised both L-ficolin and ficolin-A and investigated their abilities to enhance phagocytosis and killing of *A. fumigatus* spores by lung epithelial cells (A549 cells), human

monocyte-derived macrophages (MDM) and neutrophils. In addition, we investigated whether ficolin opsonisation could modulate the inflammatory cytokine profile.

Here we observed that both L-ficolin and ficolin-A opsonisation were capable of enhancing phagocytosis, reducing hyphal germination and decreasing viability of *A. fumigatus* following incubation with MDM and neutrophils. Incubation of ficolin opsonised spores with A549 cells did not lead to hyphal inhibition or an increase in killing. Opsonisation by ficolin-A and L-ficolin led to an increased secretion of IL-8 from A549 cells but other inflammatory cytokines were left unaffected. Opsonisation by both ficolins led to an anti-inflammatory response resulting in a decrease in the secretion of IL-8, IL-1 β , IL-6, IL-10 and TNF- α from MDM and IL-8, IL-1 β , IL-6 and TNF- α from neutrophils. Interestingly, both ficolins alone were capable of inducing an increase in constitutive cytokine secretion.

We conclude that serum L-ficolin and its rat orthologue ficolin-A play an important role in the early recognition and removal of *A. fumigatus* conidia, leading to reduced fungal viability and hyphal inhibition. Additionally, these effects were observed in combination with an anti-inflammatory response providing a novel insight in to the functions of ficolins within innate immunity. However, *in vivo* studies are still a necessity.

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Characterisation of porcine monocytes, macrophages and dendritic cells

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Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) infects cells of the myeloid lineage, namely monocytes, macrophages and dendritic cells. Fundamental to understanding such interactions is the full characterisation of porcine myeloid cells and their responses to environmental mediators which may relate to virus success.

Porcine monocytes were isolated from peripheral blood and treated with a panel of cytokines and activating factors. Monocyte derived macrophages (MoM ϕ s) were differentiated using M-CSF and stimulated with activators to achieve classical (LPS and IFN- γ) or alternative activation (IL-4) as described for human macrophages. Monocyte derived dendritic cells (MoDCs) were differentiated using GM-CSF and IL-4 and activated with a maturation cocktail containing LPS, IFN- γ , IL-1 β , IL-6, TNF α and PGE₂. Following initial results with monocytes showing up-regulation of CD163 and CD169, the putative receptors for PRRSV, the effects of dexamethasone and IL-10 were also assessed on porcine MoM ϕ s and MoDCs. Porcine myeloid cell types and their subsets were characterised *in vitro* by phenotypic and functional analysis. Additionally, gene expression analysis was also applied to MoM ϕ using a porcine pan-genome microarray to allow a more detailed comparison with their human counterparts.

Porcine monocytes are able to proliferate in response to M-CSF, and several factors including dexamethasone and IL-10 significantly modulate the expression of CD163 and CD169. In MoM ϕ s, dexamethasone and IL-10 resulted in two distinct activation states of MoM ϕ s which were not consistent with the proposed deactivation. Classical and alternative activation of porcine MoM ϕ s showed some similar characteristics with human macrophages, but were not identical with regard to functional markers such as CD206, CD163 and CD209. Porcine MoDCs were similar to human DCs upon maturation,

albeit the expression of CD83 on immature DCs before maturation suggests that this marker is variable between species, as recently described in the horse.

In summary, porcine myeloid cells displayed a high degree of heterogeneity in response to inflammatory mediators, some of which were correlated with CD163 and CD169 and hence providing some insight into PRRSV pathogenicity. Their characterisation revealed that porcine myeloid cells do not completely mirror their human counterparts, in particular with regard to macrophages, but further studies will be required to resolve the heterogeneity across myeloid cells and the comparison across mammalian species.

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Phenotype and function of epitope-specific CD8⁺ T cells in experimental human infection with respiratory syncytial virus

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Background: Respiratory Syncytial Virus (RSV) causes severe respiratory disease in some infants, immunocompromised patients and the elderly. Despite intensive research, therapy of RSV disease remains supportive, and no vaccine is yet available. RSV is relatively stable antigenically; immunity from natural infection reduces the risk of severe lower respiratory tract disease but does not prevent recurrent infection. In animal models T cells are essential for viral clearance but it is not known how they contribute in man. We therefore used human experimental infection to examine adaptive immunity in RSV, particularly RSV-specific CD8⁺ cells responses.

Methods: Twenty-eight healthy adult volunteers were inoculated with M37 strain RSV and quarantined for 10 days. Nasal lavage samples were taken daily for viral load determination and symptom diaries were kept. Whole blood samples taken at baseline, day 7, 10, 14 and 28 post-infection were used for FACS analysis of CD8⁺ T cells. All subjects were HLA typed and RSV-specific CD8⁺ cells were tracked using MHC class I/peptide tetramers. Phenotypic analysis of epitope-specific CD8⁺ T cells was achieved by co-staining with antibodies for markers of activation/proliferation, homing and cytotoxicity.

Results: Virus was recovered from nasal samples of 16/28 (57%) of subjects, demonstrating that infection had occurred. Expansion of antigen-specific CD8⁺ T cells was identified in the blood in infected volunteers only. RSV-specific CD8⁺ T cells were rare prior to challenge: antigen-specific cells against an immunodominant HLA-A1-restricted epitope made up, on average, only 0.008% of CD8⁺ T cells. Following infection, these cells expanded and altered their phenotype. Antigen-specific cells peaked at day 10, at which time the vast majority expressed CD38 and Ki67 (markers of activation and proliferation). These cells were mainly effector memory (CD45RA⁻ CCR7⁻) with a few of central memory (CD45RA⁻ CCR7⁺) phenotype. On activation, over 60% of RSV-specific tetramer⁺ cells became perforin⁺ and granzyme B⁺, indicating their cytotoxic capacity, and altered their expression of homing markers such as CD62L to enable their migration to infected tissues.

Conclusion: Our data indicates that RSV-specific CD8⁺ cells are present at extremely low frequencies in the peripheral blood of healthy individuals. Despite a proliferative burst and acquisition of markers of effector function during infection challenge, epitope-specific CD8⁺ T cells contract rapidly following viral clearance and their numbers are not maintained long-term. These findings are important in understanding immunity to RSV in man, and potentially to the development of vaccines.

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The response of migrating intestinal dendritic cells to oral *Salmonella* infection

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Background and aims: Priming of immune responses is controlled by specific APCs, such as dendritic cells (DC). The recent literature contains contradictory data regarding the functions, and even the identification of the APCs in the intestine, which induce responses after infection with *Salmonella typhimurium* (STM). In this project we have developed tools to unambiguously identify intestinal CD11c⁺ APC populations, and have begun to examine the roles of DCs and CD11c⁺ macrophages in T cell priming after oral infection of antibiotic-treated mice with STM. By identifying the precise roles of specific APCs, it may be possible to devise new “rational” immunological adjuvants to enhance the efficacy of future vaccines.

Methods: Before infection with wild-type STM, C57BL/6 or CX3CR1-GFP mice were treated with an antibiotic cocktail for at least 14 days, then rested for 48 h. Mononuclear phagocyte populations in the intestine and mesenteric lymph nodes were analysed by flow cytometry. On occasion, lymph was collected from mesenteric lymphadenectomised mice by thoracic duct cannulation. STM infection levels in tissues and stool were analysed by colony counting.

Results: DCs and macrophages in the intestine both express CD11c and MHC class II. While macrophages have previously been identified by their expression of high levels of CX3CR1, we have also identified DC populations, migrating in steady state intestinal lymph, which also express CX3CR1, albeit at lower levels than intestinal macrophages. After treatment with antibiotics, required to enable establishment of an STM infection, no differences are observed in the DC and macrophage populations in the draining MLN. However, STM infection appears to change the populations of DCs and macrophages in the MLN. We now aim to use this system to investigate the effects of STM infection on DC migration *in vivo*.

Conclusions: Oral infection with STM drives strong systemic immune responses, and is reported to cause specific changes in the myeloid cells that migrate from the intestine. We have developed a unique set of tools to investigate these responses, in detail, and have begun to establish the functions of carefully-defined macrophage and DC subsets in response to STM.

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Human dendritic cells produce thymic stromal lymphopoietin in response to pattern recognition receptor ligation and this secretion is augmented by endoplasmic reticulum stress

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Thymic stromal lymphopoietin (TSLP) is a cytokine that has an important role in inducing Th2 cell differentiation and has also been implicated as a pathogenic factor in the development of inflammatory arthritis. We have previously shown that human monocyte-derived dendritic cells (DCs) secrete significant quantities of TSLP. The precise stimuli which induce TSLP secretion in DC and the role of this autocrine production of TSLP are poorly understood.

Our aim was to identify the key signals that induce and modulate TSLP induction.

DC were differentiated from peripheral blood monocytes, purified using positive magnetic selection, by stimulation with IL-4 and GM-CSF. FACS analysis of MoDC phenotype showed them to be CD1c⁺ CD11b^{Hi} CD11c^{Hi} CD14^{Lo} CD86^{Lo} and HLA-DR^{Hi}. Transcriptional analysis was assayed by qRT-PCR following RNA extraction and

quantified relative to HPRT; cytokine secretion was assessed by ELISA of cell supernatants.

Heat killed Gram-negative bacteria including *S. typhi*, *P. aeruginosa* and *E. coli* stimulated TSLP secretion. To further examine the PRR requirements for TSLP expression we utilised the pattern recognition receptor (PRR) agonists, Peptidoglycan (TLR2 and NOD2) ultra pure LPS (TLR4) and particulate forms of β -1,3 glucan (dectin 1). These agonists stimulated TSLP mRNA transcription and protein secretion by DC with the most potent effects induced by β -1,3 glucan (475 pg/ml SEM $\pm$ 200).

TSLP secretion induced by β -1,3 glucan was dependent on the dectin 1 adaptor molecule Syk. NF- κ B and p38/MAPK were shown to be essential signalling molecules for this response. Addition of IL-1 receptor antagonist substantially blocked TSLP secretion. Although TNF α was previously shown to enhance TSLP by synovial fibroblasts, neutralisation of TNF α did not reduce TSLP secretion by DC. Dectin 1 stimulation alone was sufficient to activate ER stress as demonstrated by eIF2 α phosphorylation and XBP-1 splicing. Inhibitors for the ER stress signalling molecules IRE-1 α and PERK reduced TSLP secretion, suggesting that ER stress signals contribute significantly to induction of TSLP.

In conclusion, we show that TSLP secretion by DC is induced following a complex integration of signals from PRRs, ER stress pathways and cytokine receptors. The autocrine production of TSLP by DC, has the potential to contribute important modulatory signals in the context of inflammation where these pathways are engaged.

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CD4 Virus + T cell subsets in experimental human infection with respiratory syncytial

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Introduction: Respiratory syncytial virus (RSV) causes infantile bronchiolitis, common colds in healthy adults and significant morbidity and mortality in the elderly and immunocompromised. Despite limited viral diversity, natural antibody responses are only partially protective and repeated infections occur throughout life. There is currently no safe and effective vaccine. In animal models, CD4⁺ T cells are important in viral clearance but may also cause lung injury, and although they are crucial in coordinating adaptive immunity, their role in human RSV infection is largely unknown. In particular, there are little data regarding more recently described subsets such as follicular helper T cells (T_{fh}), which are known to be especially important in supporting antibody production in lymph node follicles.

Methods: We infected 52 healthy adult volunteers with the M37 strain of RSV, achieving a 63.5% infection rate (by viral PCR detection). Blood samples were collected at days 0, 7, 10, 14 and 28. The peak of viral shedding occurred at days 7 and 8. Infected patients suffered mild/moderate common cold symptoms (sneezing, cough, nasal discharge and sore throat) and viral shedding correlated with symptom scores. Multi-colour FACS showed that polyclonal CD4⁺ T cell activation and proliferation peaked at day 10. On days 0, 10 and 28 post-inoculation, we stimulated PBMCs *in vitro* to study Th1, Th2 and Th17 profiles. Infection was associated with a peak of IFN- γ production on day 10, but there were no significant changes in Th2 or Th17 cytokines. In addition, we examined cells with a T_{fh}-like phenotype in the blood. These also peaked at day 10 and were characterised by a minimum two-fold increase in the frequency of expression of ICOS (an activation marker of T_{fh} cells) before returning to baseline by day 28.

Conclusion: Experimental RSV infection leads to short-lived, self-limiting respiratory tract infection with mild symptoms. This results

in a Th1-biased response in cells of the peripheral blood as well as activation of T_H-like cells that are specialised in promoting antibody production. Further analysis of the specificity and functionality of CD4⁺ T cells from the local tissue and peripheral blood may assist in the development of effective vaccines and novel therapeutics for RSV disease.

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***Yersinia pestis* V antigen modulates pattern recognition receptors**

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Sepsis, the syndrome that results from overproduction of cytokines, is an often-fatal response of the immune system against microbial pathogens. The molecular mechanisms that have been designed to protect the host from invading pathogens are responsible for the cause of damage and injury.

Despite the growing understanding of the events occurring in the disorder, this has not translated to the clinic. In our search for such inhibitors, we have recently found, *Yersinia pestis* V-antigen (LcrV). LcrV is a multifunctional protein and has been shown to exhibit immunomodulatory features. We have used it in *in vitro* studies and it has been shown to be able to downregulate pattern recognition receptors, such as TLR4 and CD14 and to inhibit LPS-induced inflammatory responses. Recently, we investigated whether LcrV is able to inhibit LPS responses *in vivo*. We employed a mouse model of sepsis in order to test the efficiency of LcrV to inhibit cytokine secretion in response to LPS as well as to determine whether it can improve the mortality rate. Our results show that LcrV is able to dampen innate immune responses even if administered up to 6 h after LPS challenge, therefore suggesting that LcrV is a potent modulator of the innate immune response.

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Repeated parasite exposure induces resistance to experimental cerebral malaria by modifying brain inflammatory signatures

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Malaria remains a significant health problem, resulting in 655,000 deaths annually. The vast majority of these deaths are due to a complication termed cerebral malaria (CM), which is characterised by damage to the central nervous system (CNS). There is epidemiological evidence that the age associated susceptibility to CM, where young children are susceptible to the condition but older children are, in general, resistant, is due to the evolution of the anti-parasitic immune response following repeated parasite exposure.

An experimental cerebral malaria (ECM) model in which susceptible C57BL/6 mice are infected with *Plasmodium berghei* ANKA has been found to produce a syndrome with similar symptoms and CNS

pathology as that seen in humans with CM. CD8⁺ T-cells have been identified as key mediators of CNS pathology within this model.

In this project we established a model of repeated *P. berghei* ANKA infection and drug cure to induce resistance to the development of ECM. Three rounds of infection and drug cure were sufficient to induce resistance to the development of ECM during a subsequent fourth infection. Microarray analysis was performed on the brains of ECM susceptible and exposure-induced ECM resistant mice. Complimentary immunophenotyping of the CD8⁺ T-cell population within the brain and spleen was carried out by flow cytometry. Different gene signatures were observed in the ECM susceptible and exposure-induced ECM resistant mice. CD8⁺ T-cells from exposure-induced ECM resistant mice had a less activated phenotype than those from ECM susceptible mice.

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Modifications of the redox intracellular environment during the Human Immunodeficiency Virus type 1 (HIV-1) infection associated with the Src kinase activation

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Human Immunodeficiency Virus (HIV) and other structurally simple retroviruses developed, as a defense mechanism, numerous replication strategies to escape from apoptotic mechanism triggered by host cells. HIV maintains a persistent infection by stimulating intracellular (host) production of cytokines and of reactive oxygen and nitrogen species (ROS and RNS) (1). Levels of ROS and RNS and of their antioxidant counterparts determine the redox environment. Alterations on the redox environment are sensed by raising levels of the antioxidant enzyme Cu/Zn and Mn Superoxide dismutase (SOD). Such alterations are potentially associated with the regulation of HIV-replication signaling pathways (2).

In the present study we used isolated human CD4 T lymphocytes submitted to a protocol of *in vitro* infection with purified HIV viral particles. Levels of the antioxidant enzymes, Cu/Zn and Glutathione peroxidase (GPx), Glutathione reductase (GR), and of the tri-peptide Glutathione (GSH) were measured in infected and non-infected CD4 T lymphocytes. There was an increase on the activities of the antioxidant enzymes followed by a decrease on intracellular GSH levels. In addition we followed the temporal pattern of activation of Src kinase. Src is a cytoplasmic protein tyrosine kinase which has been shown to play an important role in HIV-1 replication (3).

Our findings suggest a possible relationship between the alterations in intracellular redox environment and the activation pattern of Src kinase in HIV-infected human CD4 T lymphocytes. These results also emphasize the importance of the intracellular redox environment in regulating the Src-mediated signaling pathway associated with HIV infection.

Molecular basis and prevention of alloreactivity

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HLA binding preferences influence the differential selection of epitopes that induce either effector or regulatory CD4⁺ T cell responses in human disease

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Background and aims: Human CD4⁺ T cell responses to the RhD protein have been well-characterised and provide a unique opportunity to identify the factors determining effector and regulatory cell induction in immune-mediated disease. The RhD protein is targeted as a foreign blood group in haemolytic disease of the newborn (HDN), and as an autoantigen in autoimmune haemolytic anaemia (AIHA). The aim of this work was to determine how binding affinity for HLA class II molecules guides epitope selection for specific T helper (Th) and T regulatory (Treg) cells.

Methods: RhD epitopes that elicit either Th or Treg responses were mapped in RhD-alloimmunised donors, and in AIHA patients, selecting individuals expressing the HLA-DRB1*1501 allele positively associated with both conditions. The affinities for HLA-DR15 of 15-mer peptide sequences spanning the sequence of the RhD protein were determined by predictive algorithms and a competitive binding assay.

Results: Th and Treg cells from each donor group were specific for peptides with different HLA binding characteristics. In the RhD-alloimmunised donors, high affinity for HLA-DR15 molecules was exhibited by peptides that stimulated Th cells, but not by sequences recognised by Treg cells. In contrast, intermediate or low affinity peptides typically elicited Th responses in AIHA patients, with Treg cells specific for strong binders.

Conclusions: The efficiency with which peptides are presented by restricting class II molecules determines whether Th or Treg cells are induced, with opposing effects when derived from foreign or self antigen. These results suggest novel approaches to peptide therapy.

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Recipient CD207⁺CD11c⁺ cell negatively regulates corneal allograft survival in early postoperative period

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Langerhans cell (LC)s, expressed MHC class II, CD205, CD11c, and langerin (CD207), are professional antigen-presenting cells involved in induction of immunity and maintenance of tolerance. As they are found on the surface of specialized organs, including cornea, LCs may play an important role in corneal allograft. The purpose of this study is to determine the role of CD207⁺CD11c⁺ cells and investigate the local tissue factors for trafficking of these cells. Standard protocols for murine orthotopic corneal transplantation were used as described previously. Briefly, donor center corneas (2-mm diameter) were excised from C57BL/6 mice and B6.CgTg (CAG-mRFP1)1F1Hadj/J mice. Then, the donor cornea was sutured on to the recipient graft beds prepared by excising a 2.0 mm site in the central cornea of BALB/c mice for allogeneic and B6 mice for syngeneic graft. Simultaneously, some

BALB/c mice received syngeneic (BALB/c) grafts to control for the non-allospecific effects of surgery. All grafts were evaluated using slit lamp biomicroscopy at 3-day intervals for 2 weeks and then weekly intervals thereafter for 8 weeks. Flow cytometry analysis showed that CD207⁺CD11c⁺ cells were significantly increased in rejecter cornea and draining lymph node (LN). Most CD207⁺CD11c⁺ cells were found to infiltrate into the corneal stromal area, not the epithelium, in allograft rejecter. To find the specific chemokines that facilitate LG migration, flow cytometry for CD207⁺CD11c⁺ cell by chemokine inhibitor treatment was performed. Graft survival and CD207⁺CD11c⁺ cell infiltration was significantly improved by inhibition of CCL2, CCL4, and CCL11, which were also found to regulate the migration of CD207⁺CD11c⁺. To enhance corneal allograft survival, targeting LC migratory chemokine receptors and their ligands may be a new strategy for modulating immunity in corneal transplantation.

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Kidney transplantation: chronic molecular signatures in acute kidney injury

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Background and aims: Almost 50% of kidney allografts have interstitial fibrosis and tubular atrophy/chronic rejection (IFTA) after 10-years. Innate inflammatory mechanisms produce perioperative oxidative stress (OS), which might induce chronic tubular epithelial cell (TEC) injury. If OS produces chronic/deep senescence, the associated secretome may predispose allografts to IFTA. Micro RNAs (miRNA) are stable in urine and might provide a minimally invasive resource for the prediction of allograft outcome.

To determine whether OS induces deep senescence or fibrosis/epithelial to mesenchymal transition (EMT) in TECs. To examine the feasibility of miRNA isolation from 1 ml urine. To assess whether the miRNA profile in TECs after OS or urine from transplant patients is indicative of TEC pathology.

Methods: HKC8 TEC were treated with 600 μ M H₂O₂ for 2hrs to induce OS; the transcriptome was analysed after 8 and 24 h and proteome after 48 and 72 h.

miRNA was extracted from urine ($n = 5$) using the Norgen Bio-tek Urine Extraction kit.

Results: OS induced transcript upregulation of TGF- β 2 (2–6-fold, $P < 0.01$), β 6-integrin (2–10-fold, $P < 0.001$) at 8 h; and p21 (6–17-fold, $P < 0.0001$) at 24 h. Upregulation of mesenchymal proteins vimentin and S100A4 and vimentin ($P < 0.05$) were seen after 72 h. Reduction of epithelial proteins cytokeratin-19 and zonula occludens-1 was seen after 48 and 72 h. No pattern was seen in miR-21, miR-34a and miR-146a expression in the TEC model or patient urine.

Conclusions: OS upregulates profibrotic genes and produces EMT in kidney TECs. OS also induces TEC senescence, due to the time points observed it is unknown if this is chronic. miRNA was successfully extracted from the aqueous phase of urine. Results support the possibility of performing a larger study of analyzing miRNA in urine to allow patient stratification for individualized treatment.

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The specificity of alloantibody in Bovine Neonatal Pancytopenia (BNP)

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Background and aims: Bovine Neonatal Pancytopenia (BNP) is a haemorrhagic disease of calves characterised by profound depletion of leukocytes and thrombocytes. The disease is mediated by alloantibodies transferred in colostrum, and has been linked epidemiologically to vaccination of the dams of affected calves with a particular vaccine (Pregsure), which incorporates a novel adjuvant. It has been suggested that alloantibodies are directed against MHC class I molecules, and are induced by contaminant bovine cellular material from MDBK cells used to grow virus for the vaccine.

Methods: A complement mediated cytotoxicity assay was used to assess antibody reactivity of BNP-dam sera. Specificity of alloreactivity was investigated using cell lines of defined MHC genotypes and transfected lines expressing individual MHC I alleles, including the MHC I alleles from the MDBK cell line. A mutated line lacking expression of classical MHC I alleles was also assessed.

Results: Cows vaccinated with Pregsure were shown to have significantly higher levels of cytotoxic serum alloantibody than both unvaccinated controls and cows vaccinated with an alternative product

containing a different adjuvant. Furthermore, among cows vaccinated with Pregsure, BNP-dams had significantly higher levels of cytotoxic antibodies than cows producing clinically unaffected calves. This pattern of reactivity was the same with transfected mouse lines expressing the MDBK-MHC I alleles, although sera generally reacted more strongly with bovine cellular targets than the transfected murine cells. Individual BNP-dams showed heterogeneity in titres and specificities of alloantibody. This appeared to change little with sequential parturitions, suggesting that the influence of the calf in the generation of BNP alloantibody is minimal. However, this variable pattern of cytotoxicity did not show complete correlation with MHC I haplotype. Furthermore, several BNP-sera retained strong reactivity with a bovine cell line in which expression of the classical MHC I alleles had been deleted.

Conclusions: The results of analyses of sera from cows vaccinated with Pregsure and other inactivated vaccines are consistent with Pregsure being implicated in the pathogenesis of BNP, and with the involvement of contaminant bovine cellular material used in vaccine production in induction of the alloantibodies. BNP alloantibody shows strong reactivity with classical MHC I molecules, suggesting that this is a target antigen in BNP. However the alloantibody also appears to have additional antigenic targets and these are currently being explored.

Neuroimmunology – Immune/Nerve Cell Interactions

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Influenza A virus infection contributes to Parkinson's disease by driving pathological CD4⁺ T cell responses in the brain

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Background: The mechanism of dopaminergic neuronal cell death in Parkinson's disease remains a mystery. Epidemiological and experimental based evidence links Parkinson's disease with Influenza A virus infection. However, influenza virus induces an acute infection, whereas Parkinson's disease is a chronic condition, suggesting that the virus has an indirect mode of action. An immune mediated mechanism may link the infection to the chronic neurological disease, with studies implicating CD4⁺ T cells in the neurodegeneration. We propose that the initial viral insult in the substantia nigra induces an inflammatory response in the CNS, resulting in the release of damaged self antigen that can be processed and recognized by CD4⁺ T cells, leading directly or indirectly to neurodegeneration in the substantia nigra.

Methods: A murine model was established by intranasally inoculating C57BL/6 mice with the neurotropic H1N1 A/WSN/33 influenza strain. Following infection, mice were terminally perfused and the brains harvested and examined for the presence of the virus, T cell infiltrate and dopaminergic neuronal loss by immunohistochemistry. Cells from enzymatically digested brain tissue were phenotypically characterised by flow cytometry. Proliferation assays were performed to assess the reactivity of CD4⁺ T cells isolated from the CNS following infection and alpha synuclein, a protein central to the pathology in Parkinson's disease, was assessed as a potential autoantigen.

Results: Our analysis shows that A/WSN/33 influenza virus enters the brain and induces CD4⁺ T cell infiltration. CD4⁺ T cells isolated from the infected brain tissue are autoreactive to alpha synuclein, and adoptively transferred alpha synuclein reactive CD4⁺ T cells migrate to the CNS. The interactions of these CD4⁺ T cells with dopaminergic neurons and microglia are currently under investigation.

Conclusion: Identifying immune mediated dopaminergic neuronal loss would radically change therapeutic approaches in Parkinson's disease, and may thus provide new targets to prevent the disease or preserve the quality of life of the patients. We thus need to further examine the role of immunopathology in Parkinson's disease.

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Neuroinflammatory and behavioural changes induced by a systemic bacterial infection

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Systemic bacterial infections are a common cause of morbidity and mortality in the elderly, and in particular those with a neurodegenerative disease, but the mechanism underlying these observations remain unclear. Experimental models of neurodegeneration have shown that LPS-induced systemic inflammation increases neuronal damage, a process that is believed to be mediated by activation of primed microglia. The effects of a systemic bacterial infection on the innate immune cells of healthy and diseased brain are less well described: thus we investigated the acute and long term effects of an

experimentally induced Salmonella infection on microglia. We predict that systemic infection will result in a 'primed' microglial phenotype. Mice with or without a pre-existing neurodegenerative disease, prion disease, were infected with *S. typhimurium*. Behaviour of mice was assessed using the Porsolt's forced swim test and burrowing assay. Microglial priming was assessed by immunohistochemistry, quantitative PCR and LPS restimulation. Salmonella infected mice showed a long lasting activation of innate immune cells of the brain, characterised by MHCII expression on the brain endothelium and a microglia hyper-responsiveness when restimulated by LPS. *S. typhimurium* infected mice with neurodegeneration showed a robust infiltration of T cells into the brain parenchyma, while T cell infiltration was very low in mice without neurodegeneration. Mice with pre-existing neurodegeneration showed an exaggerated neuroinflammatory response when infected with *S. typhimurium*. At the peak of the peripheral immune activation, *S. typhimurium* infected mice showed depressive behaviours that coincided with increased IDO expression in the brain in mice with and without pre-existing neurodegeneration. Conversely, we show exaggerated burrowing deficits in prion mice when animals are infected at early disease stages, whereas NBH control mice with a systemic infection did not show any changes in burrowing. Our study shows that systemic infection with non-neurotropic *S. typhimurium* has long-term consequences for homeostasis in the brain, due to activation of the cerebral vasculature and priming of microglia. The prolonged and enhanced brain cytokine production in infected mice may have a detrimental effect on neuronal function and may lead to progression of pre-existing neurodegenerative disease.

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Toxin-mediated FoxP3⁺ cell depletion: Impact on remyelination?

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Background: CD4⁺ T cells have been identified as potential regulators of remyelination in the central nervous system, a crucial regenerative process in demyelinating diseases such as Multiple Sclerosis. This study aimed to identify the effect of FoxP3⁺ regulatory T cells (Treg) on remyelination using transgenic FoxP3-DTR mice in which FoxP3-expressing cells can be selectively depleted by diphtheria toxin (DT) administration.

Methods: To induce demyelination, lysolecithin was injected into the spinal cord white matter of FoxP3-DTR and C57BL/6 control mice. Mice were injected daily with DT to deplete FoxP3-expressing cells prior to and following demyelination. Spinal cord lesions were analysed by immunohistochemistry and oligodendrocyte lineage cells were quantified.

Results: Foxp3-depletion reduced both oligodendrocyte precursor cells and mature myelin-producing oligodendrocytes in remyelinating lesions compared to controls suggesting a beneficial role of FoxP3⁺ Treg cells in remyelination.

Unexpectedly however, all DT-treated mice, including C57BL/6 wildtype mice developed bilateral paralysis, scoring up to 4.5 on an experimental autoimmune encephalomyelitis (EAE) scale. The shape and size of lesions did not correlate with clinical signs of paralysis suggesting that lesion cellular composition or alterations beyond lesion boundaries may underlie neurological impairment. Interestingly, mice that underwent sham surgery (no lysolecithin) but received DT did not develop paralysis demonstrating that lysolecithin-induced damage was required for DT-induced paralysis.

Conclusion: Further studies are required to determine the cellular cause of paralysis in DT-treated mice however these studies reveal unexpected pathogenic effects of DT in otherwise DT-resistant mice.

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Changes in extracellular matrix chondroitin sulphate proteoglycans in multiple sclerosis white matter lesions: a role for ADAMTS-9

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Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) characterised by discrete acute and chronic lesions of inflammation and demyelination, predominantly located in CNS white matter (WM). Extracellular matrix (ECM) changes have been reported in the CNS of MS post mortem brain tissue due to both the release and activation of matrix metalloproteinases (MMPs) as well as increased synthesis of ECM components. To elucidate the potential pathophysiological role of A Disintegrin And Metalloproteinase with Thrombospondin motif (ADAMTS) enzymes in the ECM changes in MS, we have investigated expression of CNS ECM chondroitin sulphate proteoglycans (CSPGs) in normal and MS white matter by dual label immunohistochemistry (IHC) using antibodies which specifically recognise intact and ADAMTS-cleaved aggrecan and versican, two major components of the ECM in the CNS. Frozen blocks (80) of brain tissue were obtained from the UK MS Society Tissue Bank, Imperial College London. Blocks were classified by routine histology using haematoxylin and eosin (H&E) and oil red O (ORO) stains for inflammation and demyelination respectively, and by IHC using antibodies against HLA-DR, to assess macrophage activation and myelin oligodendrocyte (MOG) for evidence of demyelination. In active MS lesions, CSPGs aggrecan and versican expression was increased in areas of macrophage activation and decreased myelin, evidenced by loss of MOG staining, compared with normal appearing white matter. Immunostaining for aggrecan neopeptides was particularly strong at the interface between myelinated and demyelinated areas at the plaque border, suggesting active enzymatic cleavage of aggrecan. Microglia were specifically stained for versican neopeptide expression. The ECM proteins demonstrated a fibrous distribution within the white matter. Preliminary studies have found ADAMTS-9 expression in WM of MS cases. In summary, this study provides evidence that changes in CSPGs are associated with microglial activation and demyelination in WM lesions. Future work will assess the functional consequence of these changes in the ECM using *in vitro* neurite outgrowth assays. (Funding provided by the Libyan Government to Esadawi Abuneeza).

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Neuro-immune *in vitro* study model of primary cultures of human differentiated neuronal and astrocytic cells from adult brain tissue co-cultured with their autologous leucocytes

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Neuronal culture models present important tools in understanding neurophysiology and the mechanisms governing neurotoxicity and

neuroprotection. This is a report of primary cultures of viable and differentiated neuronal and astrocytic cells from human adult brain maintained *in vitro*. Primary human adult neuronal-astrocytic cultures were established from human adult cortex and cerebellum tissue obtained from 29 adult neurosurgical patients. Differentiated phenotypes of neuron-like cells and astrocytes in culture were characterized by immunofluorescence and flow cytometry analyses of MAP2a&b, Tau and GFAP cytoskeletal markers. Flow cytometry analyses of cellular viability and mRNA expression studies of neuronal interaction mediator N-cadherin determined that neuronal and astrocytic cells remained viable and neurotransmission competent throughout culture duration (14–21 days *in vitro*). During their *in vitro* lifespan, neuron-like cells and astrocytes retained their differentiated properties and plasticity and regenerated neurite projections and inter-cellular communication network.

Neuro-immune cellular interactions were studied in a system of co-cultures between neuronal-astrocytic primary cultures and their autologous lymphocytes and leucocytes obtained from patient peripheral blood samples. Subpopulations of CD3-positive T-cells, CD19-positive B-lymphocytes and CD14-labeled monocytes were characterized and their viability and HLA-DR antigen expression were studied by flow cytometry in neuro-immune co-cultures. In response to their autologous neuronal-astrocytic cells, B-cells remained largely unaffected while T-lymphocyte populations gradually declined. Also, inherent activation status of monocyte populations associated with neurotoxicity responses in a sample-dependent fashion. Our evidence portrays cause-and-effect responses between autologous neuronal-astrocytic and inflammatory populations that diverge between individuals and are suggestive of both neurodegenerative and immunosuppressive processes.

In conclusion, we present an optimized culture system for generating primary neuronal-astrocytic cultures from human adult brain tissues. Furthermore, we present a neuro-immune co-culture system between neuronal-astrocytic cells and their autologous leucocytes for studying neuronal and immune cellular responses to their direct interaction and the cellular events contributing to neuroinflammatory processes.

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Can FTY720 attenuate infection induced changes in the brain in an Alzheimer's disease model?

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Evidence from this laboratory has indicated that blood brain barrier permeability increases with age in wildtype mice, and also in transgenic mice which overexpress amyloid precursor protein (APP) and presenilin 1 (PS1; APP/PS1 mice), a commonly-used animal model of Alzheimer's disease (AD). This is accompanied by increased infiltration of peripheral immune cells, including T cells, which may contribute to age-related inflammatory changes in the brain. Recent studies have suggested that infectious agents can induce inflammatory changes in the brain, and may be a risk factor for the progression of AD. The objectives of this study were to assess the effect of infection on infiltrating immune cells in the brain of APP/PS1, compared with wildtype, mice and to determine whether treating APP/PS1 mice with FTY720 can prevent infection-induced changes by retaining T cells in the lymph node.

Wildtype and APP/PS1 mice (10 months old) were infected with the respiratory pathogen *Bordetella pertussis*. The bacteria persist in the lungs of infected mice for around 5 weeks. The brains of these animals were assessed for infiltrating IFN- γ - and IL-17-producing T

cells and NK cells 8 weeks post-infection. In a separate series of experiments, wildtype mice were infected with *B. pertussis* and a group of mice were chronically treated with FTY720 at 0.3 mg/kg for the duration of the experiment. Control- and FTY720-treated *B. pertussis*-infected mice were culled at a number of time points throughout the experiment and lungs processed for analysis of bacterial load and infiltrating immune cells in the lung.

The data showed that infection resulted in a significant enhancement of the absolute number of CD3⁺ T cells in the brains of APP/PS1 mice. Interestingly the absolute number of IL-17⁺CD4⁺ T cells was significantly increased in both wildtype and APP/PS1 mice infected with *B. pertussis* in comparison with non-infected controls. There was also significantly greater absolute numbers of IFN- γ or IL-17 secreting CD8⁺ T cells in infected compared with non-infected APP/PS1 mice.

The data indicate that a respiratory infection enhances T cell migration into the brain, and that APP/PS1 mice were more susceptible to the effects of infection than wildtype mice. The experiments with FTY720 will initially determine whether chronic treatment with this drug will have an effect on the ability of the host to resolve *B. pertussis* infection, before being used as a method to attenuate the infection induced changes observed in APP/PS1 mice.

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Is CGRP involved in the gut immune response to the helminth *Trichuris muris* infection in BALB/c and AKR mice?

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Calcitonin gene related peptide (CGRP) has previously been linked to immune modulatory functions *in vitro*. Here we examined the expression and release of CGRP in BALB/c and AKR mice during helminth infection *Trichuris muris*. We administered either CGRP or its receptor antagonist and a chemical vanilloid blocker to assess CGRP role. Background levels of CGRP expression in nerve fibres within the caecum were significantly lower in AKR compared to BALB/c. On infection, BALB/c showed immediate and rapid decrease in tissue CGRP expression which correlated with worm expulsion and little pathology. Conversely, AKR showed an increase in tissue CGRP expression, which correlated with increased pathology, and persistent chronic infection. We administered CGRP and its receptor antagonist hCGRP8-37 *in vivo* during a *T. muris* infection, between days 14 and 21 p.i. in both AKR and BALB/c mice. BALB/c mice responded with an increase in muscle hypertrophy in the colon and caecum at all-time points demonstrating an active response to this ligand. Furthermore, BALB/c mice showed trends to increase T helper 2 (TH2) cytokines IL4, IL13 and TNF α . By contrast, AKR mice did not demonstrate any significant changes in response to CGRP. Conversely, hCGRP 8-37 treatment increased muscle depth in AKR mice at day 35 p.i., but not in BALB/c. CGRP has been found to induce both TH1 and TH2 related cytokines *in vitro*. To investigate the critical role CGRP had during a *T. muris* infection in BALB/c, its release and functionality were blocked during *T. muris* infection by administering both the CGRP receptor antagonist hCGRP8-37, and the transient receptor potential vanilloid 1 (TRPV1) receptor antagonist capsazepine. BALB/c mice treated with hCGRP 8-37 and capsazepine had retained worms on day 19 p.i. and showed increased muscle hypertrophy and crypt length in comparison to vehicle control treated mice. Additionally, blocking CGRP release and functionality resulted in a decreasing trend in TH2 cytokines IL4, IL13 and TNF α . Furthermore, CGRP was administered in AKR mice to examine if CGRP was essential for the development of a TH2 immune response to *T. muris*. CGRP reduced the worm bur-

den at day 35 p.i., however, with no significant change in the histology or cytokine levels between treated groups and controls.

This study suggests that CGRP is important in early development of TH2 immune response to *T. muris* in BALB/c mice but not necessarily in AKR. Additionally, These findings indicate that TRPV1 insensitivity in AKR is also mirrored by an apparently poor response to CGRP.

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The role of pattern recognition receptors in amyloid-beta-induced inflammation

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Alzheimer's disease (AD) is a neurodegenerative disorder and the most common cause of dementia in the elderly. Patients show a gradual onset and progression of memory loss and other cognitive deficits. It is widely accepted that the extracellular accumulation of amyloid β (Ab) in senile plaques is a principal event in the pathogenesis of Alzheimer's disease, but the cellular events leading to plaque-induced neuronal dysfunction are still unclear. Genetic factors have been suggested to either cause or predispose to AD including mutation in β -amyloid precursor protein (APP), which is the protein that generates Ab by proteolytic processing, mutation in presenilins 1 and 2 as well as polymorphism to apolipoprotein A and Complement receptor 1 (CR1). In addition to genetic factors, studies have suggested that inflammatory and immunological processes are not just mere bystanders but that microglia and invading bone marrow derived mononuclear phagocytes are central in the initiation and progression of Alzheimer's disease. It is our hypothesis that deposition of Ab peptide can activate the innate immune system via pattern recognition receptors (PRRs), including complement, and evoke Alzheimer's pathology. Deposition of Ab in the brain might activate microglia, initiating a pro-inflammatory cascade that results in the release of potentially cytotoxic molecules, such as cytokines, complement, proteases and other acute phase proteins, ultimately causing neurodegeneration. In the current study, we focused on the role of the innate immunity system of the brain in the initiation and the propagation of inflammatory process in AD and the interplay between Toll like receptors (TLRs) and the complement system. We examined in detail the significance of Ab peptide as danger-associated molecular patterns (DAMPs) in the activation of a wide array of pattern recognition receptors (PRRs) in astrocytes, such as Toll-like and NOD-like receptors along with the complement system. Our results suggest that Ab can activate TLRs, as well as NALPs, such as NALP3, and complement receptors and regulators, such as CD59. This is the first study to investigate how the collaboration between different PRRs orchestrates the triggering of the brain inflammation observed in AD.

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Acute *Toxoplasma gondii* infection in Alzheimer's disease**R. Montacute, H. Hopkins, J. Curran, S. Cruickshank & S. Allan***Faculty of Life Sciences, University of Manchester, Manchester, UK*

Infection is known as a risk factor for Alzheimer's disease (AD), and can worsen symptoms in established disease. *Toxoplasma gondii* is a protozoan parasite which infects around a third of the world's population. However, very few studies have investigated the effects of *T. gondii* infection on AD development or progression. In this study therefore our aim was to establish the effect of acute *T. gondii* infection in the triple transgenic AD mouse (3 × Tg-AD). Young (2–3 months of age) 3 × Tg-AD mice were infected with *T. gondii* and culled at 5, 7 and 14 days post infection (PI). Aged (11 months of age) 3 × Tg-AD mice were infected and culled 11 days PI. The 3 × Tg-AD mice were more susceptible to infection than the non-transgenic controls, showing increased weight loss and sickness behaviour. 3 × Tg-AD mice of both ages had severe liver pathology with tissue necrosis and infiltration of inflammatory foci, responses which were present but less severe in non-transgenic mice. Systemic

inflammatory responses were raised in both young and aged 3 × Tg-AD mice. Serum from aged 3 × Tg-AD mice 11 days PI had elevated levels of proinflammatory cytokine IFN- γ (500 fold), as well as significant increases in levels of proinflammatory cytokines IL-6 and IL-1 β and the chemokines CXCL1 and CCL2. Serum from young 3 × Tg-AD 12 days PI had elevated levels of IFN- γ (800 fold), TNF (30 fold) and IL-6 (12 fold). Anti-inflammatory responses were reduced in young 3 × Tg-AD mice, with levels of IL-10 in serum 10 fold lower at day 5 PI. Soluble tachyzoite antigen (STAg) stimulation of splenocytes to detect T cell specific responses to *T. gondii* infection showed increases in proinflammatory cytokine production. Aged 3 × Tg-AD mice had increased levels of IFN- γ (500 fold), IL-6 (30 fold) and IL- β (4 fold) and increases in chemokines CXCL-1 (200 fold) and CCL-2 (10 fold). Young 3 × Tg-AD mice following STAg stimulation had increased levels of IL-1 β , 15 fold higher than in non-Tg mice at 5 days PI. These data suggest that in 3 × TgAD mice an enhanced proinflammatory response results in greater susceptibility to infection. Whether this contributes to changes in AD-like pathology and behaviour remains to be determined, and is the subject of ongoing studies.

Quantitative Approaches to Immunology: New Experimental Challenges

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User versus software-dependent variability of ELISPOT counts obtained from ten different laboratories

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Introduction: There are a defined number of T cells specific for any given antigen within a PBMC pool. However, an accurate and reproducible method to measure this response, between different laboratories has been controversial. In ELISPOT assays, antigen-induced spots show a broad spectrum of sizes and densities over variable background. Therefore, even for experienced investigators using ELISPOT analysis software, it is difficult to judge the minimal spot size/density to be counted, and the gates for upper threshold to distinguish single cell-derived spots versus clusters.

Methods: We studied PBMC plated in serial dilutions, with 24 replicates per dilution, to establish the distributional properties of IFN- γ ELISPOTS elicited by antigens with defined HLA-Class I or Class II restriction. We also sent ELISPOT plates, and image files of such, to 10 different laboratories for independent counting. The plates were machine counted by each laboratory relying on the subjective counting parameters assessment by different investigators, and by the statistics-based auto-gating method in conjunction with auto-threshold algorithm.

Results: Both these CD8- or CD4 cell-derived spots were found to closely follow log-normal distribution. These log-normal distributional properties of ELISPOTS permit to set the lower and upper gates for counting by means of statistics, automatically, with a 95% confidence interval.

Conclusions: While counts based on judgment call of the investigator showed considerable variability, the counts obtained in the laboratories by the statistic gating method were comparable to each other and thus establishes the prospect of harmonizing ELISPOT counting.

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An examination of monocyte adenosine metabolism in common variable immunodeficiency

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Background: Granulomatous masses form in a variety of idiopathic inflammatory diseases, including sarcoid, Crohn's disease and common variable immunodeficiency (CVID). 10 to 20% of CVID patients form granuloma which can further reducing life expectancy by some 15 years.

Methods: The activity of adenosine forming exoenzymes CD39 and CD73 on the external surface of CVID monocytes and T cells were

investigated and related to activation and monocyte fusion *in vitro*. Peripheral blood mononuclear cells were cultured with IL4 and GM-CSF and adenosine metabolism investigated with antibodies and specific inhibitors of adenosine receptors.

Results: Multinucleate cell formation of CVID individuals exceeded that of normal cells by over 2 fold although expression of MHC II DR, CD80 and CD86 was similar. Both CVID and normal giant cells were highly biochemically active and phagocytic, engulfing similar quantities of yeast and fluorescent beads. Adenosine metabolism in CVID monocytes and T cells was disturbed although receptor function was normal, indicating a possibility that adenosine metabolism may be connected with immunosuppression in CVID.

Conclusions: The lower augmentation of CVID monocyte fusion index by inflammatory reagents suggests that CVID monocytes are already partly activated *in vivo*. The disturbance of expression of CD73 and the adenosine A2A receptor on activated T effector cells is related to cell activation and may be involved in the dysfunction of regulatory T cells in CVID.

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Quantitative assessment of regulatory T cell numbers in human tissue through a novel immunohistochemical approach

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Background and Aims: CD4(+) CD25(high) FOXP3(+) regulatory T cells (Tregs) are key mediators of immunity, which orchestrate and maintain immunological tolerance. Their quantitative assessment is of vast medical interest since different diseases such as type 1 diabetes, multiple sclerosis and inflammatory bowel disease (IBD) have been linked to changes in Treg numbers. Yet, a tool for the quantitative and depictive investigation of Tregs in the affected tissue is still missing.

In this study we established a novel immunohistochemistry of Tregs for quantification and for assessment of the localization of Tregs *in situ*, allowing staining of three markers in one section.

Methods: Biopsies in 15 healthy subjects (mean age 25.5 ± 4.2 years, $6/9$ f/m) and in 18 patients diagnosed either ulcerative colitis or Crohn's disease (mean age 42.0 years ± 13.6 , $11/7$ f/m) were collected from colon or ileum and formalin-fixed paraffin-embedded. Sections were deparaffinised, followed by antigen retrieval through HIER (heat induced epitope retrieval). Single-stainings with specific primary antibodies for the following three markers of Tregs were carried out using fluorescence-labelled secondary antibodies: CD4 (cell surface), CD25 (cell surface) and FoxP3 (nucleus). Subsequently, the three markers were combined into a triple staining in one section.

Results: In healthy subjects numbers of regulatory T cells varied from 0.0 to 4.1 percent Tregs in CD4+ cells (mean $1.2\% \pm 0.3$ SEM), whereas in patients with chronic inflammatory bowel disease the counts ranged from 1.5 to 17.8 percent Tregs in CD4+ cells (mean $8.7\% \pm 1.1$ SEM). In ulcerative colitis patients we observed a relationship between disease activity scores and percentage of Tregs in CD4+ cells. These results clearly demonstrate higher Treg counts in chronically inflamed intestine compared to healthy tissue.

Conclusions: This new immunohistochemical method allows quantification of Tregs in paraffin-embedded human biopsies. Frequency of Tregs is substantially lower in healthy controls compared to IBD patients. After validation against Treg quantification using FACS, this new method should allow further insight in the role of Tregs in autoimmune diseases in humans.

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Generation of an open-source library of mouse knockout immunophenotyping data

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The Infection and Immunity Immunophenotyping (3i) consortium has been formed to conduct a high-throughput immunological phenotyping of approximately 800 knockout mouse lines generated by the Wellcome Trust Sanger Institute (WTSI) over the next 5 years. The project aims to build an encyclopedia of vertebrate gene function and to make fundamental discoveries relating to human health. Funded by the Wellcome Trust and led by our team at King's College London, the consortium includes partners from WTSI, Imperial College, and the Universities of Oxford, Cambridge, and Manchester.

The phenotyping has two components, an observational screen and a challenge screen. In the observational screen the immune cell compartments of different organs will be analysed in order to identify genes regulating the immune system at steady state. The challenge screen will look at responses to chemical stress and to viral and bacterial infections that collectively mimic major aspects of human exposure to the environment and that therefore permit the likely identification of genes that regulate the human immune response under challenge.

The project will employ and further refine state-of-the-art multi-colour flow cytometry and fluorescent microscopy and explore cutting-edge tools for automated data analysis and dissemination. All generated data will be open source and available to the scientific community whose involvement will be encouraged by the programme's outreach component.

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Quantitative approaches to immunology: new experimental challenges Dissecting the transcriptional signature of pre-TCR signalling in developing T lymphocytes using genome-wide transcriptional modelling

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Background and Aims: T-lymphocytes develop along a well characterized programme whereby CD4-CD8- double negative (DN) cells give rise to the double positive cells which undergo selection to form self-MHC restricted and self-tolerant CD4 or CD8 single positive T-cells. The DN population can be subdivided into CD44+CD25-(DN1), CD44+CD25+(DN2), CD44-CD25+(DN3) and CD44-CD25-(DN4) cells. TCR- β chain expression together with pre-T α and CD3 molecules to form a pre-TCR complex represents a critical check point in T-cell development. Extensive work over the past few decades has helped in identifying few genes important in this process, however, our understanding of the temporal regulation of the genome-wide molecular events underpinning this developmental programme remains very restricted and is the focus of this study.

Methods: To dissect the gene networks underlying the pre-TCR we tracked transcriptomes of the developing thymocytes using time course microarrays during this transition. We utilize embryonic thymuses from Rag1^{-/-} mice to perform foetal thymic organ cultures

(FTOCs) and stimulate them with anti-CD3 to model the pre-TCR signal transduction.

Results: We show by canonical correspondence analysis that thymocytes in anti-CD3 treated Rag1^{-/-} FTOCs acquired transcriptomic profiles of DN4 or DP populations and mimicked the response to pre-TCR signal transduction at the molecular level. We observe that expression and degradation profiles are heterogeneous among both the up and down-regulated transcripts. Exploration of the effects of degradation on network modelling revealed that the targets of the same transcriptional activity could generate very different expression profiles and vice-versa. Traditional system approaches based solely on transcript expression levels are inefficient in dissecting these dynamic network activities downstream of such a stimuli-driven complex response. We used a pre-validated strategy dubbed genome-wide transcriptional modelling (GWTM) based on the principle that the gene expression observed at a particular moment for a particular gene is the difference of the synthesis and degradation of its mRNA. This allowed us to group genes based on their respective transcriptional activities and describe at least five distinct transcriptional profiles initiated by the pre-TCR signal.

Conclusions: Our work provides a novel perspective in understanding this phase of development and in decoding the transcriptional signature of pre-TCR signal by temporally integrating all the molecular mediators under the pre-TCR into defined transcriptional groups. In the process we reveal previously unknown regulatory factors that are crucial for this phase of development and will be used in functional studies of T-cell development.

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High-throughput multicolor flow cytometry phenotyping to identify novel mutations regulating mouse immunobiology

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The Infection and Immunity Immunophenotyping (3i) consortium has been formed to conduct systematic high-throughput immunological analysis of >800 gene knockout mouse lines generated by the Wellcome Trust Sanger Institute (WTSI). One aspect of the project is the high-dimensional flow cytometry analysis of multiple lymphoid tissues. The flow cytometry screen is aimed at identifying novel genes involved in both immune development and homeostasis.

Spleens and mesenteric lymph nodes are analyzed using three separate 12-colour antibody panels designed to investigate T/NK-cell, B-cell and myeloid cell phenotypes. In addition, a 12-colour panel is dedicated to assessing haematopoietic development in the bone marrow. Each panel is comprised of a combination of lineage-specific markers and activation markers allowing enumeration of specific subsets within the tissues; simultaneously providing information on the activation status of these cells.

Owing to the size and complexity of the data set produced, identification of immunological phenotypes will be performed using state-of-the-art automated gating/cluster analysis. This approach serves to streamline the data analysis and assist in identifying novel immunological phenotypes. Ultimately, all data will be published to an open access online repository. In this manner the larger community's investigations of immune cell regulation are provided with two key resources: the identification of novel regulatory mechanisms and novel highthroughput approaches to large scale integrative data analysis.

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Large scale human T cell epitope mapping of *Burkholderia pseudomallei*

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Melioidosis is an infectious tropical disease caused by Gram-negative bacillus, *Burkholderia pseudomallei*. The pathogen has been classified as a Category B (Tier 1) and bio-threat agent, there are no vaccines available. Previous studies by our group and the others have identified interferon-gamma as an essential immune component for host survival to the infection. Our previous study has also used *B. pseudomallei* protein microarray to identify 40 immunogenic proteins of this microorganism recognized by seropositive healthy and recovered melioidosis individuals living in the endemic area of *B. pseudomallei*. Here we have extended the study to map human T cell epitopes of these proteins from *B. pseudomallei* by overlapping peptides of the whole proteins (average 35 peptides/library/protein) and assayed for human T cell responses of various *B. pseudomallei* infected groups by ELISPOT. The peptide libraries from candidate proteins were screened for production of interferon-gamma by human peripheral blood T cells. The analysis of peptide epitopes was evaluated in the context of human HLA typing. These experiments offer the chance of generalizing information on recognized epitopes from the HLA class I and class II alleles commonly represented in the Thai population to a broader panel of alleles relevant across many ethnic groups. This study is part of the large scale epitope discovery and expected to reveal between 500–1000 confirmed T cell epitopes for submission to Immune Epitope Database by the year 2014. The information is essential to modify algorithms for human T cell epitope prediction and provide a more accurate tool for effective vaccine development.

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Does antigen excess exist in IgG4 subclass assays?

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IgG4 is a minor component of the immunoglobulin G class with uncertain functions. It has an inherent instability due to inert disulphide bond structures. IgG4-related disease has recently been recognised as a fibroinflammatory condition which can affect virtually all organ systems. Elevated levels of IgG4 subclass in serum is used to aid the diagnosis of IgG4-related disease. These patients need to be correctly identified therefore it is important that IgG Subclass assays are able to detect high levels of IgG4. This experiment was designed to assess the ability of methods currently in use to produce the correct result and not a false result within the normal range due to antigen excess.

A single patient donor sample expected to contain a high level of IgG4 was distributed by UK NEQAS for Immunology, Immunochemistry & Allergy to all participants within the IgG Subclasses

scheme. Participants tested the sample using various methods and the results were analysed.

The majority of methods were able to correctly detect the high IgG4 concentration. However, some laboratories reported results which fell within the normal range. This could possibly indicate an antigen excess problem which will be investigated in future distributions. Furthermore, there appears to be method related differences in the calibration with respect to IgG and an absence of an international standard.

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Development of IgE microarray assays for classification of allergic individuals

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Allergic (hypersensitivity) reactions are pathological immune reactions against common, harmless environmental proteins (allergens). These reactions are raised by mechanisms of innate and adaptive immunity, which are mediated by a variety of inflammatory soluble mediators and immune cells. IgE antibodies play critical role in the immediate phase reaction of allergic immune responses. Cross linking of allergen-specific IgE to its receptors on effector cells leads to release of a complex immune cascade and signal pathways, which may cause local or systemic symptoms within minutes. Therefore, this kind of allergy is referred to as immediate or type I allergic reactions. The primary goal of this project is to profile specific IgE antibody levels in serum for the diagnosis and prognosis of allergic patients by microarray technology. This technique (multi-allergen assay) is being developed containing allergens used for skin prick testing, which will be printed onto glass slides and screened simultaneously with serum samples from allergic patients. This assay will enable the measurement of IgE reactivity against many individual allergens in a single step, thus requiring small volume of serum and reducing costs.

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Production and characterisation of novel cell lines expressing mutant TNFR1 using TALENS technology

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TNF receptor associated periodic syndrome (TRAPS) is caused by mutations in the TNF receptor-1 (TNFR1) gene. This leads to misfolding of the mutated TNFR1 protein and ligand-independent signalling resulting in activation of inflammatory pathways. Many aspects of these pathological processes remain to be elucidated. Furthermore, the consequences of mutant TNFR1 expression may vary between cell types, thereby giving rise to the variety and heterogeneity of clinical phenotypes. Detailed *in vitro* investigation of this disease therefore requires a range of cell types expressing mutant TNFR1. This can be partly achieved using TRAPS patient-derived cell lines and conventionally-transfected cell lines; however, this is not possible for all relevant cell types. We are therefore using TALEN technology to facilitate the production of novel TNFR1 mutant cell lines. TALEN is a novel class of an engineered restriction enzyme that is generally created by fusing a TAL effector-DNA domain, which is derived from plant pathogenic bacteria such as *Xanthomonas*, to a DNA cleavage domain. The DNA-binding domain of TALEN contains a highly conserved of identical repeat units from 33 to

35 amino acids with an exception at region 12 and 13. To date, we have produced the building blocks for TALEN production using Golden Gate Cloning technology. These are being used to produce cell lines expressing TRAPS-associated TNFR1.

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Development of microarray assays for immunological measurements

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The increasing interest in comprehensive analysis of immune responses using small sample size has led to a drive for microarray platform development. Here we briefly outline the development of numerous microarray technologies for measurement of intracellular signalling pathways, cellular markers and (auto) antibodies, with spe-

cific focus on cytokine markers in serum and supernatants. These microarray based assays can be used as direct replacements for existing techniques such as Western Blot and ELISA and provide improvements in quantification and dynamic range. Protein based microarrays have the added advantages of requiring reduced sample input, are compatible with high throughput processing, and can provide increased quality control, replication and reduced cost. The methodologies are amenable to both signal amplification and fluorescence-based multiplexing to further enhance comprehensive data collection.

Here we present data for the development of cytokine microarrays, outlining the steps required quality control. The selection of printing and blocking buffers is examined and the use of amplification for detection of low abundant cytokines is discussed.

In conclusion, microarray assays can be optimised for measurement of a variety of immunological parameters to enable fast, reproducible data generation.

Regulation of T Cell function

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The core is not enough: modulation of CD4 T-cell mediated immunity

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Introduction: CD4 T-cells direct immune responses against pathogens, cancer and can cause autoimmunity. However, how CD4 T-cell responses are governed by the interaction between the membrane bound T-cell receptor (TCR) and short peptides presented at the cell surface by major histocompatibility complex molecules class II (pMHC-II) is not well established. MHC-II presented peptides are of a variable length (10–25 m) and consist of a core 9mer (reflecting the MHC-II binding motif) and peptide flanking residues (PFRs) that can extend from both the N- and C-terminus of the binding groove. The full role of PFRs during CD4 T-cell immunity is unknown.

Materials and Methods: Peptide derived from the HLA-DR*0101 influenza haemagglutinin epitope, HA305-320 (DR1-HA), with altered PFRs were designed using a combinatorial approach and tested biophysically, and using pMHC-II tetramers. CD4 T-cells were clonotyped after stimulation with modified peptides. DR1-HA reactive CD4 T-cells were sorted *ex vivo* from DR1 healthy volunteers that had been inoculated with the seasonal flu vaccine.

Results: We have shown that pMHC-II restricted TCRs bind with weaker affinity compared to pMHC-I restricted TCRs (*Journal of Immunology*, 2007), limiting the experimental/clinical use of pMHC-II tetramers. We have recently shown that modifying peptide flanking regions can enhance TCR-pMHC-II binding affinity and modify CD4 T-cell responses (*Nature Communications*, 2012). Our unpublished data shows how these modifications can be used for developing enhanced pMHC-II tetramers.

Conclusions: PFR have an underappreciated role during CD4 T-cell mediated immunity. Altered PFRs can enhance TCR binding with implications for CD4 T-cell diagnostics and vaccine development.

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Modulation of human T helper cell responses by biological therapeutics

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Background: IL-17+CD4+ T cells (Th17 cells) have been implicated in the pathology of several immune-mediated inflammatory diseases. Recent publications have demonstrated that these typically pro-inflammatory effector T cells can acquire anti-inflammatory potential characterised by the expression and production of interleukin-10 (IL-10). Recent data from our laboratory show that TNF inhibitors (TNFi) induce expression of IL-10 in human IL-17+CD4+ T cells *in vitro* and *in vivo*. The aim of this work was to examine whether blockade of other pro-inflammatory cytokines (IL-1, IL-6) or CD80/CD86 costimulation have similar effects and to examine the role of IL-10 in the induction of IL-10+IL-17+ CD4+ T cells.

Material and Methods: CD4+CD45RO+ T cells and CD14+ monocytes were isolated from peripheral blood from healthy volunteers and co-cultured for 3 days in the presence of anti-CD3 mAb. Co-cultures were performed in the absence or presence of adalimumab (anti-TNF α), tocilizumab (anti-IL-6R) or abatacept (CTLA-4-Ig); blocking antibodies to IL-1R1, or IL-10 and IL-10R; or the cytokines

TNF- α or IL-10. Cells were then stimulated with PMA and ionomycin in the presence of GolgiStop and assessed for intracellular cytokine expression by flow cytometry.

Results: Our results show that TNFi addition induces IL-10 expression in IL-17+CD4+ T cells and conversely, that TNF- α addition suppresses IL-10 expression in these cells. IL-1R1 blockade is also capable of increasing IL-10 expression in IL-17+CD4+ T cells, whilst neither tocilizumab nor abatacept have this effect. We demonstrate that IL-1 β addition can overcome TNFi-mediated IL-10 induction. By blocking IL-10 and IL-10R we show that IL-10 signalling is required for TNFi-mediated induction of IL-10 expression in IL-17+CD4+ T cells.

Discussion: Our data demonstrate that blockade of either TNF- α or IL-1 β , but not IL-6 signalling, induces IL-10 expression in IL-17+CD4+ T cells. TNF- α inhibition was shown to operate via an IL-10-dependent mechanism. Elucidating the mechanisms involved in the regulation of IL-10 responses in typically pro-inflammatory T helper cells has important implications for the development of future therapeutic strategies.

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Increased expression of T cell recruiting chemokines in the colonic mucosa of microscopic colitis patients

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Microscopic colitis (MC), comprising collagenous colitis (CC) and lymphocytic colitis (LC), is a common cause of chronic diarrhea. In this study we compared gene expression of chemokines and their receptors in colon biopsies from MC patients in active disease or in histological remission (CC/LC-HR) with controls by qRT-PCR. The Th1-associated chemokines CCL2/MCP-1, MIP-1 β /CCL4, MIG/CXCL9, IP10/CXCL10, I-TAC/CXCL11, the Th2-associated chemokine MDC/CCL22, the Th17-associated chemokine IL-8/CXCL8 and cytotoxic T cell-associated fractalkine/CX3CL1 expressions were increased in both active CC and LC patients compared to controls. The Th1/Th2-associated chemokine RANTES/CCL5 expression increased only in LC patients whereas the Th1-associated chemokine MIP-1 α /CCL3 expression increased in CC patients only compared to controls. Both CC and LC patients had increased receptor expressions (*ligand(s) in parenthesis*): CCR3 (CCL5,7), CCR4 (CCL5,22), CXCR1 and 2 (CXCL8), whilst, CX3CR1 (CX3CL1) and CCR2 (CCL2,7) expressions were increased only in CC patients. CC-HR and LC-HR patients showed increased expression of CCR3, CXCR2 and CX3CR1, whereas CCL22, CXCL9, CXCL11, CX3CL1, CCR4, and CXCR1 expressions were increased in LC-HR patients compared to controls. Contrary, CCL2, 3, 22, CXCL8, CCR2, 4, 5, CX3CL1, CXCL9, 10, 11 and CXCR3 expressions decreased in CC-HR patients compared to CC patients. This study shows similar mucosal expression of T cell recruiting chemokines in CC and LC but altered expressions in different disease stages. These findings are important for elucidation of MC immunopathogenesis and identification of potential therapeutic candidates.

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Increased expression of immunosuppressive receptors in T-cell subsets in poor prognosis chronic lymphocytic leukaemia

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We previously showed that an inversion of the CD4:CD8 ratio in CLL patients (CLL^{IR}) is associated with poor clinical outcome and the preferential expansion of highly differentiated CD8⁺ T-cells. In order to explore this further we evaluated the expression of immunosuppressive receptors in T-cell subsets in a cohort of 56 CLL patients and 21 age-matched controls. We hypothesized that CLL^{IR} patients would have an increased percentage of highly differentiated CD8⁺ T-cells co-expressing PD-1, TIM-3 and LAG-3. Contrary to our prediction, we found no evidence for increased expression of LAG-3 or PD-1 in CD8⁺ T-cells, although we did observe significant increases in CD8⁺TIM3⁺ T-cells within the EM ($P = 0.037$) and EMRA subsets ($P = 0.079$). In contrast, we observed more pronounced effects among the CD4⁺ T-cells in CLL^{IR} with an overall increase in PD-1⁺ ($P < 0.0001$) and TIM-3⁺ ($P = 0.0057$), and LAG-3⁺ T-cells ($P = 0.0305$) within the CD4⁺ EM subset. Furthermore, PD-1 expression was positively correlated with TIM-3, LAG-3 and a highly differentiated CD4⁺CD57⁺CD27⁺ phenotype; none of these associations were apparent in CD8⁺ T-cells. Overall these findings suggest that CLL^{IR} patients have an increased percentage of CD4⁺ T-cells with an "exhaustion" phenotype, whereas their CD8⁺ T-cells appear to have an "activation" phenotype. The functional and clinical significance of these findings are under investigation.

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Analysis of epitope-specificity and T cell receptor usage in CD8+ T-cell responses against *Theileria parva* in MHC I homozygous and heterozygous individuals

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Diversity of TCR in epitope-specific populations can have profound implications for the functional capacity of CD8⁺ T-cell responses. For example, narrow TCR repertoires in immunodominant epitope-specific responses have been associated with 'escape mutations' in HIV that contribute to the loss of immunological protection. In previous work we have demonstrated that 3 epitopes from the Tp2 antigen of *Theileria parva* (Tp2₉₈₋₁₀₆, Tp2₄₉₋₅₉ and Tp2₅₀₋₅₉) were presented by the N*01201 MHC I allele of BoLA-A10 homozygous cattle. In this study we use pMHC I-tetramers and an unbiased template-switch anchored RT-PCR technique to examine the frequency and TCR diversity of these epitope-specific responses in a range of BoLA-A10 homozygous and heterozygous animals. In 5 *T. parva* immunized A10-homozygous animals, the Tp2₄₉₋₅₉ epitope was found to be highly dominant (30–50% of CD8 T cells), while smaller percentages of cells specific for Tp2₅₀₋₅₉ (2–7%) and Tp2₉₈₋₁₀₆ (1–4%) were present. By contrast, in 6 BoLA-A10 heterozygous animals (3 pairs of MHC I-haploidentical animals), CD8 T cells specific for the Tp2₄₉₋₅₉ and Tp2₅₀₋₅₉ epitopes were not detected. In each MHC I haploidentical pair only 1 individual had a detectable Tp2₉₈₋₁₀₆-specific response (10–50%). The different profiles of antigen specificities indicate that different naïve epitope-specific T cells are selected for expansion and differentiation during infection of homozygous and heterozygous animals. Through analysis of TCR β chain (TRB) usages, a completely conserved TCR β chain rearrangement was identified within the Tp2₅₀₋₅₉-specific CD8 T cell populations of all 5 BoLA-A10 homozygous animals. However, in three BoLA-A10 heterozygous animals, Tp2₉₈₋₁₀₆-specific CD8 T cells

showed varies TRB diversities with the preference of V β 13.5 usages, but no identical CDR3 sequences. These results provide some explanations for the diverse antigen responses in BoLA-A10 homozygous and heterozygous animals. It also provides some useful materials for *in vitro* generation of naïve antigen-specific T cells for studies of induction of CD8 T cell responses against *Theileria parva*.

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Combination peptide immunotherapy suppresses antibody and helper T cell responses to the RhD protein in HLA-transgenic mice

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Background and Aims: The RhD blood group is the major target for maternal alloantibodies in hemolytic disease of the newborn (HDN). If alloimmunisation occurs, it cannot currently be reversed, but manipulating T helper (Th) responses offers a novel strategy for treating affected women. We have previously mapped peptides containing immunodominant Th epitopes from the RhD protein and the purpose of the work was to develop and test these as a product for suppression of established anti-D responses.

Methods: The physico-chemical properties of four immunodominant RhD peptides and a panel of analogues were defined. A selected version of each sequence with improved solubility was combined in mixture RhDP_{mix}, which was tested for suppressive ability in HLA-DR transgenic mice previously immunised with RhD protein.

Results: It was confirmed that each of the peptides in RhDP_{mix} retained recognition by human Th cells. A single dose of RhDP_{mix} given intranasally ($P = 0.008$) or subcutaneously ($P = 0.043$) rapidly inhibited the ongoing antibody response in RhD-immunised HLA-DR15 transgenic mice. This was accompanied by reduced Th responsiveness to the RhD protein and recruitment of suppressive cells with a FoxP3⁺ Treg phenotype.

Summary: The results identify a tolerogenic peptide product with a defined mechanism of action and a simple dosing regimen, suitable for translation to human trials as the first specific treatment for women at risk of HDN due to existing anti-D antibodies.

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Unraveling the regulation of IL-10 expression in Th17 cells: a role for Aiolos

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Background: TNF inhibitors have revolutionised the treatment of inflammatory diseases and have been used to treat over 4 million patients worldwide. CD4⁺ T cells producing the pro-inflammatory cytokine IL-17 (Th17 cells) are key contributors to the pathogenesis of many human inflammatory diseases, however it is currently unknown how these cells are affected at the molecular and

functional level by TNF inhibitors. Here we demonstrate that TNF inhibitor treatment has a profound effect on Th17 cells by inducing a regulatory IL-10 pathway, which may contribute to the efficacy of this widely used therapeutic.

Methods: Cytokine analysis was conducted immediately *ex vivo* using peripheral blood mononuclear cells from either healthy controls or patients with rheumatoid arthritis. Additionally, co-cultures of CD4⁺ T cells and CD14⁺ monocytes were set up, using cells from healthy controls, in the presence of anti-CD3 mAb and TNF inhibitors commonly used in clinical practice (adalimumab, infliximab, etanercept or certolizumab). Following culture, cells were analysed by cytokine staining or cells were sorted for further analysis via qPCR, confocal microscopy and ChIP.

Results: We demonstrate that TNF inhibitor drugs increase the percentage of Th17 cells (IL-17⁺ CD4⁺ T cells) *in vitro* and *in vivo* whilst also driving expression of the anti-inflammatory cytokine IL-10. Mechanistically, we show that induction of IL-10 expression by Th17 cells is Treg/Foxp3 independent, requires IL-10 and is overcome by the pro-inflammatory cytokine IL-1 β . TNF inhibitor exposed Th17 cells were found to be molecularly and functionally distinct, with a unique gene signature characterised by expression of *IL10* and the transcription factor *IKZF3* (encoding Aiolos). We show that Aiolos binds conserved enhancers in the *IL10* locus in Th17 cells and that Aiolos overexpression is sufficient to drive *IL10* expression in primary CD4⁺ T cells.

Conclusion: Together, these data demonstrate that TNF- α blockade induces a regulatory IL-10 pathway in Th17 cells and suggest that this process is regulated by the transcription factor Aiolos. Our data reveal not only a novel mode of action of TNF inhibitor drugs but also suggest an important role for the transcription factor Aiolos in the attenuation of Th17 cell pathogenicity.

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The role of Interleukin -1 signaling in the immune defense and for the development of T helper cells lineage

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In this project we will investigate the role of IL-1 signaling in haematopoietic cells. For this, we will use genetically engineered mice harbouring a conditional IL1- RI ^{Δ / Δ} gene. Breeding regimens underway to create mice whereby the IL-1 receptor has been inactivated specifically in haematopoietic cells IL1- RI ^{Δ / Δ} Vav Cre⁺. These mice will then be challenged by parasites like *T. muris*. Following infection, the T helper cell response will be measured using a variety of standard approaches.

According to the genotyping PCR we found that the conditional mice mutant IL1- RI ^{Δ / Δ} Vav Cre⁺ was obtained successfully. Moreover, the inactivation of the IL1- RI gene in Hematopoietic stem cell was confirmed by DNA sequencing for IL1-RI delta allele.

Ribbon diagram of IL-1 β bound to the ectodomains of IL-1RI showed that the IL1- RI ^{Δ / Δ} Vav Cre⁺ loss the binding site of IL-1 α and /or IL-1 β . This study showed that both stimulus LPS or IL-1 β induce the expression of IL1- RI in IL1-RI ^{Δ / Δ} Vav Cre⁻ mice not IL1- RI ^{Δ / Δ} Vav Cre⁺ mice using Western blot. In addition, the stimulation of IL1-RI with LPS or IL-1 β cause an increase in the concentration of IL6 and MCP-1 in IL1-RI ^{Δ / Δ} Vav Cre⁻ compare with IL1- RI ^{Δ / Δ} Vav Cre⁺ using ELISA assay.

According to these result the IL1-RI gene has been inactivated was confirmed *in vitro* and the mice ready for the infection experiment.

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Agonistic OX40 antibodies drive expansion of T effector cells at the expense of follicular T helper cells and germinal centres

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Background: Signals through OX40:OX40L are important in the survival of CD4 T cells in a primary immune response through the action of molecules such as Bcl-2 and survivin and this pathway has been identified as a therapeutic target. It is unknown however, how and if they contribute to CD4 memory T cell survival and expansion. Provision of OX40L by ROR γ ⁺ innate lymphoid cells has been proposed as an explanation for the impaired survival of CD4 memory T cells in ROR γ ^{-/-} mice.

Materials and Methods: To dissect this under physiological conditions we used blocking or agonistic antibodies to temporally control signalling through OX40. Mice were immunised with *Listeria monocytogenes* expressing the immunogenic peptide 2W1S and antigen specific T cells were tracked using MHC class II tetramers.

Results: Blocking or agonistic antibodies given for 4 weeks post formation of antigen specific memory cells in WT mice did not affect CD4 memory T cell survival. Post secondary challenge blockade only modestly impaired expansion however agonistic antibodies resulted in a significant increase in number and skewed the memory compartment towards T effector cells. In a primary response agonistic antibodies administered during the short window of OX40 expression drove a vast increase in T-bet⁺ effector cells, which was carried through to the memory compartment. However, this caused a complete loss of follicular T helper cells (TFH) and germinal centres. Using Rag1-CreTbx21 fl/flRosa26-tdRFP mice we show that intrinsic expression of T-bet is not required to drive this effector T cell phenotype.

Discussion: These data indicate that T-bet⁺ effector CD4 T cells develop from OX40⁺ progenitors and their expansion is at the expense of TFH precursors when driven by agonistic OX40 antibodies. Temporal provision of signalling through OX40 is imperative in shaping the resulting effector and memory cell pool and has implications for the administration of agonistic antibodies in the clinic.

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Effect of iron chelation on T cell responses in experimental arthritis

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Background: Rheumatoid Arthritis (RA) is a systemic auto-immune disorder affecting 1% of the UK population. It is characterised by chronic inflammation, mediated by proliferation of pro-inflammatory, autoreactive CD4⁺ T cells. Our approach is based on the observation that chronic inflammation leads to sequestration of iron within synovial cells, leading to increased cell proliferation and tissue damage. Previously, a clinically approved iron chelator, Desferrioxamine (DFO), was used in a clinical trial for RA, but the trial was halted due to toxicity, although RA symptoms did improve. We are investigating a novel class of iron chelators which have a greater specificity for iron.

Methods: Our laboratory has tested these chelators in a mouse model of experimental arthritis using C57/BL 6 mice primed with Type II Collagen. In-vitro experiments were also conducted on stimulated human CD4⁺ T cells. Proliferation was measured by ³H thymidine incorporation and cytokine production was measured by

ELISA. Western blot and Micro-array analysis were used to determine the mechanism underlying the chelator's effect.

Results: Results show that the chelators were able to significantly reduce disease symptoms both prophylactically and therapeutically in the murine model and in *in-vitro* experiments, with the most prominent effect being on CD4 + T cell proliferation. A comparison with non-chelating, structurally related compounds confirm that this is an iron dependent mechanism.

Furthermore, *in-vitro* experiments on antigen stimulated human CD4 + T cells, confirm that the chelators reduce T cell proliferation by more than 50% and IFN γ production by 30%. Extensive experiments have demonstrated that the chelators exert their effects on CD4 + T cells rather than APC. Experiments investigating possible mechanisms of action of the chelators indicate that they may be exerting their effect on the IL-2 pathway as exogenous IL-2 was able to completely overcome the suppression of proliferation by the chelator, validating its involvement in the mechanism. Preliminary micro-array results also show the genes most significantly modulated by iron chelation are those encoding for cell cycle regulators and inhibitors of DNA replication, hence preventing cell proliferation, as indicated by our *in-vitro* data.

Conclusion: In conclusion, our study demonstrates that intracellular iron has the ability to modulate inflammatory responses and indicates that the use of novel iron chelators could prove to have therapeutic potential for RA and diseases driven by excessive T cell proliferation.

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A systems model for STAT complex formation describes the mechanism of T cell phenotype switching

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Background: Signal transducers and activators of transcription (STATs) are key molecular determinants of the T cell fate and effector function. As an altered balance of T cell phenotypes and cytokine secretion contributes to numerous disease states, including autoimmunity, cancer and chronic infection, STATs represent viable therapeutic targets in these settings. However, it is still unclear how exactly the same STAT proteins regulate the development of different T cell phenotype and their plasticity during changes in extracellular conditions.

Methods: A systems model has been developed for intracellular STAT dimerization and the dimerization-mediated T-cell phenotype formation. We applied our model predictions to the experimentally observed IFN- γ to IL-10 switching that self-regulates human Th1 responses and observed qualitative agreement with the experimental data. Although the predictive power of the model has been explored in the IFN- γ to IL-10 switching example in the context of rheumatoid arthritis, the implications of the proposed principle are broader than this example.

Results: The model predicts that the underlying intracellular STAT dimerization balance is a major contributor to the T cell phenotype switching. The underlying molecular mechanism for the switching is the relative redistribution of STAT homo- and heterodimer complexes due to the STAT completion effects.

Conclusions: In this project we derived a new systems biology model for IL-10 and IFN- γ production regulation via the JAK-STAT pathways. The model predicts that the competitive STAT binding reactions can cause the same cells to induce different cytokine responses. The model integrates known properties of the cytokine-induced STAT

pathways and is able to link the changes in STAT signalling circuits with the phenotype switching effects.

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Loss of the TGF β -activating integrin $\alpha\beta$ 8 on dendritic cells protects mice from chronic intestinal parasitic infection via control of type 2 immunity

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Background and Aims: Chronic intestinal parasite infection is a major global health problem, but mechanisms that promote chronicity are poorly understood. TGF β plays a central role in regulating T-cell responses in the intestine, yet the role and regulation of TGF β during responses to intestinal infections remain unclear. Here we describe a novel cellular and molecular pathway involved in the development of chronic intestinal parasite infection.

Methods: Immunity to a chronic infection with the murine intestinal parasite *Trichuris muris*, a model for prevalent human whipworm infection, was assessed after antibody mediated blockade of TGF β , in mice lacking the TGF β -activating integrin $\alpha\beta$ 8 specifically on DCs (*Itgb8* (CD11c-Cre)) and DERE mice, which allow specific ablation of Foxp3 + T regulatory cells.

Results: We show that, early during development of chronic infection with the intestinal parasite *T. muris*, TGF β signalling in CD4+ T-cells is induced and that antibody-mediated inhibition of TGF β function results in protection from infection. Mechanistically, we find that enhanced TGF β signalling in CD4+ T-cells during infection involves expression of the TGF β -activating integrin $\alpha\beta$ 8 by dendritic cells (DCs), which we have previously shown is highly expressed by a subset of DCs in the intestine. Importantly, mice lacking integrin $\alpha\beta$ 8 on DCs were completely resistant to chronic infection with *T. muris*, indicating an important functional role for integrin $\alpha\beta$ 8-mediated TGF β activation in promoting chronic infection. Protection from infection was dependent on CD4+ T-cells, but appeared independent of Foxp3+ Tregs. Instead, mice lacking integrin $\alpha\beta$ 8 expression on DCs displayed an early increase in production of the protective type 2 cytokine IL-13 by CD4 + T-cells, and inhibition of this increase by crossing mice to IL-4 knockout mice restored parasite infection.

Conclusions: Taken together, these data indicate that lack of the TGF β -activating integrin $\alpha\beta$ 8 on DCs results in a heightened Th2 immune response during *T. muris* infection, culminating in parasite expulsion. Our results therefore provide novel insights into how type 2 immunity is controlled in the intestine, and may help contribute to development of new therapies aimed at promoting expulsion of gut helminths.

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Optical tweezers: manipulating and quantifying immune cell interactions to understand the importance of L-arginine on T-cell function

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Background: The initial interaction between a naïve T-cell and dendritic cell (DC) is critical for determining effector function and evidence suggests that either the strength or duration of contact influence T-cell activity. Other reports indicate that depletion of nutrients, such as L-arginine, regulate T-cell signalling and activation. Here we aim to quantify interaction forces between T-cells and dendritic cells and determine the requirement for L-arginine in maintaining cell contact.

Methods: To quantify interaction forces between T-cells and DCs, we used a modified optical trapping system with novel beam geometry to directly manipulate and control a T-cell whilst ensuring cell viability was maintained. Ovalbumin-specific T-cells were brought into contact with antigen-pulsed DCs and the strength of the trap required to subsequently separate the cells determined. By performing such experiments in the presence or absence of L-arginine, we investigated its role in regulating T-cell responses.

Results: Using optical tweezers, we quantify the forces associated with the first moments of antigen-recognition by live CD4⁺ T-cells. We demonstrate an increase in the strength of interaction from 3.3pN (± 1.39 pN) in the absence of antigen, to 8.5pN (± 5.73 pN) upon antigen recognition. Finally we show that in the absence of L-arginine, despite T-cells failing to proliferate and produce cytokines, there is a significantly increased interaction force between T-cells and DCs, irrespective of antigen recognition.

Conclusion: Optical tweezers are the ideal tool for investigating immune cell interactions; controlling contact time and quantifying interaction forces at various stages of interaction. As evidence of this, our results suggest a hitherto uncharacterised role for L-arginine in T-cell activation and its potential for therapeutic targeting.

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Exhaustive characterization of the T-cell functionality after systemic inflammation/sepsis

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Background/Introduction/Context/Objective: Features of immune suppression are often found in chronically critically ill (CCI) patients in the aftermath of systemic inflammation or sepsis. In later stages of sepsis it is generally assumed that T-cells are severely compromised or paralyzed in their ability to fight infections. The molecular mechanisms which effects and impacts the various T-cell compartments after a systemic inflammation (SIRS)/sepsis are so far inadequately understood. Thus, it is not known if a systemic inflammation affects T-cells systemically on a per-cell-basis or if rather the T-lymphocyte repertoire is impacted in a clonal or sto-

chastic fashion. Our project aims to characterize the functionality of T-lymphocytes and the molecular mechanisms responsible for any detected abnormalities of T-cells at post-acute stages of sepsis. In particular we want to decrypt how the systemic, burst-like acute release of cytokines (cytokine storm) affects T-cells.

Material and Methods: Different mouse models for systemic inflammation/SIRS (CpG), endotoxemia/SIRS (LPS) or *bona fide* sepsis (peritoneal injection of human faeces, PCI) are used to delineate the individual contributions of the systemic cytokine release versus bacteraemia. For a more profound picture of the T-cell efficiency after a systemic inflammation T-lymphocytes are analyzed at different time points after the insult. To challenge and test the function of the CD4⁺/CD8⁺ T-lymphocytes we pursue 2 approaches: a) exhaustive characterization of splenic and peripheral T-cells *ex vivo*; b) a 2-hit *in vivo* model with LCMV infection. We compare and complete our results with data from T-cells purified out of whole blood samples from CCI-patients and healthy controls.

Results: In contrast to the anticipated disturbance of T-cell function we did not observe severe impairment of individual T-cell responses at all time points examined. This is assessed on the ability of splenic and peripheral T-cells to respond to cognate antigen stimulation or a wide range of TCR/co-receptor triggers at the level of TCR signalling, activation marker expression (CD25, CD69), proliferation and cytokine release. However, SIRS and sepsis trigger a rapid and clear decrease in leukocyte cellularity accompanied by a significant loss of T-lymphocytes.

Conclusion and Discussion: Therefore, SIRS and sepsis do not apparently affect the function of CD4⁺/CD8⁺ T-lymphocytes on a per-cell-basis. It appears more likely that SIRS compromises adaptive immunity by decreasing T cell numbers and/or changing the repertoire composition of the T cell pool at the initial, acute phase of the systemic inflammation.

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Deciphering the functional role of Butyrophilin-like genes in mucosal immunity

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More than 50% of our immune system is located in the gastro-intestinal tract. The intestinal epithelium, which forms an interface between the organism and environment, harbors intraepithelial T lymphocytes (IEL) which comprise a mixture of conventional $\alpha\beta$ T cells, and unconventional T cells such as $\gamma\delta$ T cells or $\alpha\beta$ T cells expressing the CD8 $\alpha\alpha$ homodimer. Hence, the intestinal epithelium and the epithelial resident IEL represent an important barrier in the prevention of infection against orally acquired pathogens and thus, are critical effector components of mucosal immunity. Although the cross-talk between the intestinal epithelial cells and the IEL is considered as important for regulating immune responses in the intestinal mucosa, few molecular conduits of this have been identified. It was recently shown that the new immunoregulatory Butyrophilin-like (Btl) molecules not only can suppress T cell activation in the periphery, but can also regulate T cell mediated local immune responses by modulating intestinal epithelial cell - IEL interactions (Arnett *et al.* J. Immunol., 2007; Bas *et al.* PNAS, 2011). To further examine the capacity of Btl-family members to regulate immune responses in the intestinal tissue, we have raised antisera against Btl4 and Btl6; two Btl genes expressed primarily in the intestine, and have developed a series of transfected cells that display Btl4 and Btl6 on the surface. The results of ongoing experiments will be presented. To this point, our data delineates the protein expression

pattern of Btl4 and Btl6, characterizes their distinct expression requirements and identifies a novel heteromeric complex in the intestinal mucosal epithelium. Altogether, our data further supports the notion that Btl genes are novel regulators of tissue-specific immune responses.

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Antigen-specific iTreg cells require both IL-10 and CTLA-4 to control autoimmune disease

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Thymic generation of Foxp3⁺ T regulatory (Treg) cells upon the recognition of self antigen forms an important mechanism in the control of autoimmune disease. In recent years, subsets of peripherally induced Treg cells, including IL-10-secreting cells and Foxp3⁺ peripheral Treg (pTreg) cells, have been shown to develop upon recognition of extra-thymic antigen, thus providing protection against undesired immune responses to innocuous foreign antigen as well as self antigens not present in the thymus. Foxp3 expression can be induced in naive CD4⁺ T cells *in vitro* by activation of these cells in the presence of Interleukin (IL)-2 and Transforming Growth Factor-beta (TGF-beta). Transfer of *in vitro* induced Treg cells (known as iTreg cells to differentiate them from the *in vivo* generated pTreg cells) has been suggested as a therapeutic target for tolerance induction in various conditions, including transplant rejection and autoimmune disease. Questions remain, however, about the optimal method of iTreg cell generation, requirements for antigen-specificity, as well as iTreg cell functionality and stability *in vivo*.

In this study, using TCR-transgenic Tg4 mice, where CD4⁺ T cells recognise a Myelin Basic Protein peptide (MBP Acl-9), or non-transgenic B10.PL mice, we have generated iTreg cells at very high purity (typically 90–95%) using either anti-CD3 and anti-CD28 antibody or specific peptide to activate the cells. We demonstrate that antigen-specific Tg4 iTreg cells, but not polyclonal B10.PL iTreg cells, can protect effectively against the induction of experimental autoimmune encephalomyelitis (EAE) using specific peptide in Complete Freund's adjuvant in Tg4 mice. These antigen-specific Tg4 iTreg cells, generated using either antibody or antigen, retain their suppressive Foxp3⁺ phenotype after disease induction, even after several weeks.

The negative co-stimulatory molecule CTLA-4 and immunosuppressive cytokine IL-10 have both been associated with the functionality of Treg cell subsets. CTLA-4-deficient naive CD4⁺ T cells demonstrate a reduced ability to differentiate into iTreg cells, whereas IL-10 deficiency does not affect iTreg cell differentiation. Both CTLA-4 and IL-10, however, are of pivotal importance for iTreg cell-mediated immune suppression as iTreg cells deficient in either molecule not only fail to protect against the induction of EAE, but aggravate disease progression.

In conclusion, *in vitro* generated iTreg cells specific for self-antigen can protect against autoimmune disease and require both IL-10 and CTLA-4 for their function.

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Diacylglycerol kinase alpha transcriptional upregulation by TGFβ limits TCR signals and favors Foxp3 expression during peripheral induction of regulatory T cells

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Background and Aims: Regulatory T (Treg) cells maintain tolerance by suppressing heightened immune responses, and contribute to tumor immune evasion. Treg lineage commitment requires upregulation of the transcription factor Foxp3 during thymic development in natural (nTreg) or in the periphery in induced (iTreg) cells. The conversion into iTregs needs of suboptimal antigenic stimulation and the presence of TGFβ. Peripheral Foxp3 upregulation by naive CD4⁺CD25⁻ T cells requires suboptimal T cell receptor (TCR) triggering and is mediated by the cytokine TGFβ. Diacylglycerol kinase α (DGKα) phosphorylates the diacylglycerol (DAG) generated during TCR triggering and acts as a negative regulator of TCR signals by limiting DAG-mediated activation of the Ras/ERK axis. DGKα is transcriptionally repressed during activation of naive CD4 T cells so we decided to investigate its regulation during iTreg induction.

Methods: We studied the transcriptional regulation of DGKα during *ex vivo* TGFβ-mediated differentiation of iTreg cells. We used DGKα-deficient mice, to investigate a causal relationship between DGKα expression and TGFβ-mediated iTreg induction. Finally, we made use of a tumor rejection model to test the DGKα contribution to TGFβ functions *in vivo*.

Results: We demonstrate that TGFβ-stimulation of naive CD4⁺CD25⁻ T cells correlates with enhanced transcription of DGKα. Analysis of DGKα-deficient mice showed that this upregulation limits TCR signaling, to allow TGFβ-mediated Foxp3 expression. DGKα transcriptional regulation downstream the TCR is thus critical for Thelper versus T regulatory differentiation in the periphery. DGKα deficiency did not affect other TGFβ functions such as CD25 upregulation or IL2 suppression. Finally, we demonstrate *in vivo* resistance to TGFβ activity by showing that DGKα^{-/-} mice show impaired intratumor induction of CD4⁺CD25⁺Foxp3⁺ cells whereas Foxp3⁺CD4 population than remained normal in the periphery.

Conclusions: We identify transcriptional control of DGKα expression as an essential component of the mechanisms that regulate peripheral Foxp3 triggering and suggest that local intratumor generation of Foxp3⁺ T cell represents a significant population that is likely to differ from nTreg cells in its contribution to tumor rejection.

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Increased IL-23 receptor expression is observed on KIR3DL2+ CD4+ T cells in Ankylosing Spondylitis and correlates with IL-23R polymorphisms

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Background: T helper 17 (Th17) cells are a subset of pro-inflammatory CD4⁺ T cells implicated in numerous inflammatory arthritides including the Spondyloarthritides (SpAs). Ankylosing Spondylitis (AS), the commonest spondyloarthropathy, has genetic associations both with HLA-B27 and with IL-23 receptor (IL-23R) single nucleotide polymorphisms (SNPs). Having previously reported that KIR3DL2+ CD4⁺ T cells are expanded in the peripheral blood of individuals with AS the aim of this study was to further characterize KIR3DL2+ CD4⁺ T cells and to correlate IL-23R expression on KIR3DL2+ CD4⁺ T cells to IL-23R genotype.

Methods: The frequency and expression level of Th17 markers IL-23R, IL-1R and CCR6 on peripheral blood KIR3DL2+ CD4+ T cells was investigated by flow cytometry and confirmed by qRT-PCR. IL-23R expression on paired peripheral blood and synovial fluid samples from SpA patients was investigated by flow cytometry. Cytokine production by anti-CD2/3/28-stimulated FACS-sorted KIR3DL2+ and KIR3DL2- CD4+ T cells was investigated using multiplex bead analysis. Immunochip GWAS and ENCODE data were integrated to predict functional SNPs in the IL-23R secondary region of association.

Results: KIR3DL2+ CD4+ T cells were enriched for expression of the Th17 phenotypic markers IL23R, CCR6 and IL-1R, compared to KIR3DL2- CD4+ T cells. IL-23R expression levels were also increased on KIR3DL2+ CD4+ T cells compared to KIR3DL2- CD4+ T cells from AS patients. SNPs in three (non-coding) putative regulatory regions (PRRs) of IL-23R were identified (PRR1-3). The presence of the AS risk-associated allele at PRR1 correlated with increased expression of IL-23R on KIR3DL2+ CD4+ T cells. KIR3DL2+ CD4+ T cells accounted for the majority of peripheral blood CD4+ T cell IL-23R expression in AS patients, and this was significantly greater compared to HLA-B27-healthy controls. KIR3DL2+ CD4+ T cells from AS patients produced significantly more IL-17 than KIR3DL2- CD4+ T cells. IL-17 production significantly increased in the presence rIL-23 and rIL-1. Furthermore, IL-23R expression was increased on KIR3DL2+ CD4+ T cells from SpA synovial fluid samples compared to match peripheral blood samples when available.

Discussion: KIR3DL2+ CD4 Th17 cells are expanded in patients with Spondyloarthritis. These cells constitute the majority of peripheral blood CD4 T cell IL-23R expression and produce increased levels of IL-17, which is further increased by the presence of Th17 cytokines. Correlation of IL-23R expression on KIR3DL2+ CD4+ T cells with putative regulatory SNPs suggest genetic and epigenetic control of IL-23R expression may contribute to the pathogenesis of AS.

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Nck proteins modulate differentiation and effector functions of T cells

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Background and Aims: Nck proteins are cytoplasmic signaling adaptors mediating diverse cellular functions such as actin rearrangement and enhancing T cell receptor signaling. In T cells, the SH2 domain and the first SH3 domain interact with SLP-76 and CD3ε, which are both key components of T cell receptor (TCR) signaling, respectively. With T cell-specific Nck knock-out (Nck.T^{-/-}) murine model, Nck proteins have been proved to be essential for T cell selection in thymus and responsiveness in periphery. However, the contribution of Nck proteins in T cell differentiation and effector functions remains undecided. Thus, our aim is to make it further clarified.

Methods: Here we work on Nck.T^{-/-} murine system. Animals were challenged with T cell-dependent antigens and then accessed for their serum antibody levels by ELISA and germinal center cell populations in spleen by flow cytometry. Histological sections were analyzed for

germinal center structure in spleen. Experimental autoimmune encephalomyelitis (EAE) model was induced by MOG₃₅₋₅₅ peptides and followed for disease scores. Cells infiltrated into central nerve system were isolated and analyzed by flow cytometry.

Results: Disrupted germinal center (GC) formation and impaired production of antibodies were found after immunizing the Nck.T^{-/-} mice with T cell-dependent antigens. Both total and GC-associated follicular helper T cell (T_{fh}) populations are decreased in Nck.T^{-/-} mice. In line with the results above, Th2/T_{fh}-related cytokines such as IL-4, IL-10, and IL-21 are all decreased in Nck.T^{-/-} model. Moreover, Nck.T^{-/-} T cells are found to be more susceptible to apoptotic death. These can be attributed to decreased level of Akt phosphorylation found in Nck.T^{-/-} model. On the other hand, in the experimental autoimmune encephalomyelitis (EAE) model the disease was ameliorated and delayed in Nck.T^{-/-} mice. Reduced infiltration of CD4⁺CD183^{hi}CD196^{hi} T cells into central nerve system was found.

Conclusions: Taking the results together, both T cell-dependent humoral and cellular immunity were affected after loss of Nck proteins in T cells. Our data are compatible with the idea that Nck proteins not only participate in thymic selection or periphery responsiveness but also in the differentiation and effector functions of T cells, especially in Th2, Th17, and T_{fh}-dependent responses.

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A single nucleotide polymorphism in an immunoreceptor tyrosine based switch motif in Ly9 (CD229, SLAMF3) alters SH2 domain binding and T cell activation

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Ly9 (CD229, SLAMF3) belongs to the SLAM family receptors, which signal through immunoreceptor tyrosine based switch motifs (ITSMs), so called as they exhibit overlapping specificities for activating and inhibitory SH2 domain containing proteins. A genome-wide linkage study of UK and Canadian SLE families identified an association of a non-synonymous single nucleotide polymorphism (SNP) within an ITSM of Ly9, which might account for the observed distorted T cell population in patients with systemic lupus erythematosus (SLE) (Graham et al., 2008). The SLE associated ITSM sequence (TMVYQV) is found in 80% of the population and differs from the "protective" allele by a single amino acid (TVYVQV). To examine whether this subtle difference in sequence affected interactions with intracellular SH2 containing proteins, we measured the affinity of the adaptors SAP and EAT-2 for phosphopeptides representing the two Ly9-ITSM variants. The more commonly expressed M-containing ITSM reported to be associated with SLE susceptibility bound SAP and EAT-2 with two to three fold higher affinities than the rarer V-variant.

As Ly9 is expressed in T cells, we are developing assays to test the functional consequences of these biochemical differences in Jurkat cells. Knockdown of endogenous Ly9 using shRNA revealed an activating effect of Ly9 consistent with it signalling through recruitment of SAP to ITSMs in its cytoplasmic region (Simarro et al., 2004). To compare the effects of the two variants we used a bicistronic vector which knocks down endogenous Ly9 and expressed the two variants at high levels. We have developed a single cell assay based on CD69 expression and costimulation with Ly9 antibodies to analyse differences between the two protein isoforms. Preliminary data indicate the protective allele is less activating and more inhibitory. As disease has been correlated with higher surface expression of Ly9 (Chatterjee et al., 2012) it is important to distinguish between effects based on receptor levels and signalling. We

are currently testing the specificity of the two ITSM variants for inhibitory SH2 domain containing proteins and optimising the functional assays.

By distinguishing between the effects of the two Ly9 variants we shall gain new insights into the influence of this receptor in immune regulation and its association with autoimmune diseases such as SLE.

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Tolerance induction in memory CD4 T cells requires two rounds of antigen specific activation

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Background and Aims: A major goal for immunotherapy is to tolerate the immune cells that coordinate tissue damage in autoimmune and alloantigen responses. CD4 T cells play a central role in many of these conditions and improved antigen specific regulation or removal of these cells could revolutionise current treatments. A confounding factor is that little is known about whether and how tolerance is induced in memory CD4 T cells.

Methods: We have used MHC class II tetramers to track and analyse a population of endogenous antigen specific memory CD4 T cells exposed to soluble peptide in the absence of adjuvant in wild-type mice. By combining these analyses with *in vivo* functional readouts of T cell tolerance, we have defined the extent of tolerance induction in memory CD4 T cells exposed to tolerogenic signals.

Results: Memory CD4 T cells activated by antigen alone proliferated and re-entered the memory pool, apparently unperturbed by the incomplete activation signals. Upon further restimulation *in vivo*, CD4 memory T cells exposed to tolerance signals proliferated, provided help to primary responding B cells, and migrated to inflamed sites, suggesting that they had not been tolerised. However, these reactivated memory cells failed to survive. The reduction in T cell number was marked by their low expression of the anti-apoptotic molecule, Bcl2, and increased expression of activated caspase molecules which are involved in apoptosis. Consequently, these cells failed to sustain a delayed-type hypersensitivity response. Moreover, following two separate exposures to soluble antigen no T cell recall response and no helper activity for B cells could be detected.

Conclusion: These results suggest that the induction of tolerance in memory CD4 T cells is possible but that at least two rounds of antigen specific T cell activation are required to delete and thereby permanently remove the CD4 T cells. In addition, the data demonstrate that CD4 T cells can discriminate between activation and survival signals, pathways that are usually mediated by similar intracellular signalling pathways.

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The role of NF- κ B signalling in T cell homeostasis and function

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Inhibitor of NF- κ B kinase 1 (IKK1) and 2 (IKK2) forms part of the functional IKK complex, necessary for NF- κ B signalling. NF- κ B is vital for survival, development and function of multiple cell types in the body. The aim of this study was to determine the function of NF- κ B in thymic development and the maintenance of the peripheral T cell compartment.

For this, we are using the Cre-lox system in order to delete one or both of Ikk1 and Ikk2 genes. Cre recombinase activity was either under the control of a developmentally regulated promoter specific to the B and/or T cell lineage or was temporally regulated by virtue of a tamoxifen inducible Cre in fully developed adults.

Developmental deletion of Ikk1 and Ikk2 in the thymus revealed that NF- κ B signalling was redundant for early stages of thymic development and selection, but was instead critical for late stage maturation of single positive thymocytes. Ikk1 Ikk2 double deficient mice had a severe reduction in mature single positive thymocyte numbers and scarcely any peripheral T cells. Members of the TNF receptor super family (Tnfrsf) are potent activators of NF- κ B. We therefore tested the hypothesis that Tnfrsf signalling could be regulating developing thymocytes. We therefore assessed the role of TNF. *In vivo* blockade of TNF in the IKK1/2 deficient animals resulted in an almost complete rescue of thymocyte development. Cell culture experiments revealed that IKK1/2 deficient thymocytes were specifically susceptible to TNF induced cell death. TNF receptor (TNFR1) was identified as the key surface receptor and Tnfrsf1 IKK1/2 triple deficient mice had normal thymic development. Surprisingly, however, peripheral T cell numbers were only rescued minimally. Analysing cell phenotype revealed that peripheral T cells lacked expression of IL-7R α that is critical for the survival and homeostasis of naïve T cells. Our preliminary evidence suggests a role for members of the TNF superfamily in controlling expression of IL-7R α . Therefore, our data reveal multiple roles of NF- κ B signalling in T cell development and homeostasis.

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Chemical allergen-induced perturbation of the mouse lymph node DNA methylome

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There is increasing evidence that epigenetic regulation of gene expression plays a pivotal role in the orchestration of immune responses and may determine the vigour, quality and/or longevity of such responses. It has been demonstrated previously that chemical allergens can be broadly divided into two categories; contact allergens (such as dinitrochlorobenzene; DNCB), that cause type 1/type 17 polarized responses and respiratory allergens (such as trimellitic anhydride, TMA) that induce a preferential type 2 response. Such polarization occurs upon repeated (13 day) topical exposure of mice. In order to explore the regulation and maintenance of such responses at the molecular level, the genome wide pattern of DNA methylation following treatment with the reference allergens DNCB and TMA was characterized. Mice ($n = 5$ per group) were exposed to DNCB, TMA or vehicle control for 13 days, and draining auricular lymph nodes excised. DNA was extracted, sonicated and processed for methylated DNA immunoprecipitation (MeDIP) followed by hybridization to a high resolution DNA promoter array representing 28,000 probe ranges, covering promoter regions and known CpG islands. Changes in DNA methylation profiles for allergen-activated tissues were compared with the vehicle-treated control samples, with a cut off of $P < 0.01$. Selected differentially methylated regions (DMR) were technically validated using qPCR and demonstrated similar patterns of methylation to the genome-wide assay. More DMR were recorded for DNCB than TMA (6319 versus 2178), with approximately half of the TMA DMR common to DNCB. Direct comparisons between DNCB and TMA revealed 268 DMR.

This pattern remained when analysis was limited to DMR associated with promoter regions; 781 genes were uniquely associated with DNCB induced DMR but only 140 genes were unique to TMA DMR. The DMR associated with gene promoters unique to either DNCB or TMA were generally hypomethylated whereas DMR common to both allergens were evenly split between hypo- and hyper-methylation. These data demonstrate chemical allergen exposure results in characteristic patterns of DNA methylation indicative of epigenetic regulation of the allergic response. Furthermore, both DNCB and TMA are associated with unique activating epigenetic marks which may prove to be epigenetic biomarkers of both chemical and drug induced allergy.

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The differential requirement of aryl hydrocarbon receptor activation for Th17 and Tc17 development

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The aryl hydrocarbon receptor (AhR) has been shown to play an important role in the polarization of T helper (Th) cell subsets; specifically it is required for optimal Th17 cell activation. Th17 cells provide immunity against extracellular pathogens and are implicated in autoimmune and allergic diseases. An analogous subset of T cytotoxic (Tc) cells, designated Tc17, has also been identified and is thought to be the main effector cell in many diseases, however, their dependence upon the AhR is relatively unknown. In the current investigations the role of the AhR in cytokine production by Th17 and Tc17 cells has been compared. Tc (CD8 +) and Th (CD4 +) cells were isolated by negative selection from the peripheral lymph nodes of naive mice and polarized with plate-bound anti-CD3 and anti-CD28 antibodies and appropriate cytokine cocktails (Th1/Tc1: interleukin [IL]-12; Th17/Tc17: IL-6, transforming growth factor- β and IL-1 β). Cell differentiation was assessed as a function of mRNA (RT-PCR) and protein (ELISA and flow cytometry) expression for interferon (IFN)- γ and for key IL-17 cytokines. Th17 cells displayed a type 17 profile exclusively, demonstrated by relatively low IFN- γ and high IL-17 production. In the presence of a selective AhR antagonist (CH-223191; 3 μ M), Th17 cell activity was reduced significantly. Addition of the natural AhR agonist 6-formylindolo[3,2-b]carbazole (FICZ; 300 nM) markedly enhanced the frequency of Th17 cells as well as levels of IL-17 and IL-22 production. In contrast, the Tc17 cells polarized into 3 distinct subsets: those producing either IL-17 or IFN- γ alone and those producing both cytokines. The addition of the AhR antagonist had only a limited impact on the frequency of IL-17 expressing CD8 + cells, unlike the significant decrease recorded for Th17 cells. Exposing Tc17 cells to exogenous FICZ did not enhance the frequency of IL-17 producing cells (of any phenotype) or the amount of IL-17 produced. Interestingly FICZ supplementation induced relatively low levels of IL-22 by Tc17 compared with Th17 cells. This lack of response to exogenous FICZ is likely to be related to the observation that although Tc17 cells express baseline AhR, unlike Th17 cells, there is no marked upregulation during polarization. These data demonstrate that the optimal development of Th17 cells is much more dependent upon AhR activation than is the development of Tc17 cells, thus endogenous AhR ligands play a much greater role in driving Th17 cell responses *in vitro*.

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T-cell trafficking during inflammation is regulated by a novel PEPtide Inhibitor of Trans-Endothelial Migration, a pathway defective in chronic disease

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T-cells are recruited from the blood into extra-vascular tissues during acute inflammation. However, in chronic inflammatory diseases, an inappropriate accumulation of T-cells in the diseased tissue contributes to pathogenesis. Very little is known about the mechanisms by which T-cell trafficking is regulated during inflammation. Here we describe a unique immune regulatory peptide that imposes a tonic inhibition of T-cell trafficking during inflammation. PEPtide Inhibitor of Trans-Endothelial Migration (PEPITEM) introduces a new paradigm into the pathways that regulate the inflammatory response. We present evidence that this new pathway is compromised in chronic inflammatory diseases. We propose that loss of this regulatory pathway makes the immune system 'leaky', allowing inappropriate access of T-cells to vulnerable tissues.

Lymphocyte trafficking was assessed *in vitro* using videomicroscopy on TNF- α /IFN- γ activated endothelial cells (EC) and lymphocytes isolated from healthy donors or patients with chronic inflammatory diseases. *In vivo*, lymphocyte recruitment was assessed in a model of zymosan-driven peritoneal inflammation. PEPITEM was identified using mass spectrometry.

Our studies began with an interest in adiponectin, an anti-inflammatory adipose tissue-derived cytokine. Using an *in vitro* migration assay, we observed that the migration of human lymphocytes was dose-dependently blocked by adiponectin with an EC50 = 37.2 nM. Adiponectin achieves its effects on T-cell migration by the induction of a novel mediator released from B-cells. Thus, the effect of adiponectin was lost when B cells are absent, but could be regained by the addition of supernatants from adiponectin stimulated B-cells. Interestingly, the B-cell derived product did not act directly on T-cells; rather, it stimulated EC to release the lipid mediator sphingosine-1-phosphate, which in turn inhibited the migration of T-cells. We used mass spectrometry to isolate a B-cell derived peptide, corresponding uniquely in the human genome to a proteolytic excision product of the 14.3.3 $\zeta\delta$ protein. Synthetic PEPITEM could also effectively inhibit T-cell migration with an EC50 = 18.6pM. In zymosan-induced peritonitis in the mouse, T-cell recruitment was significantly increased in BABL/C mouse strain lacking B cells (Jh^{-/-}) when compared to wild-type animals. This excess of T-cell recruitment was ameliorated by intravenous treatment with PEPITEM. Importantly, lymphocytes isolated from patients with chronic inflammatory disease (type-1-diabetes) were released from the inhibitory effects of adiponectin, but this regulatory pathway could be re-established by the addition of exogenous PEPITEM. We believe that PEPITEM and its associated pathway may have therapeutic efficacy in a number of disease scenarios.

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Glycogen synthase kinase-3 regulates IL-10 production in Th1 cells**E. V. Hill, C. M. Oakley, T. H. S. Ng, B. R. Burton & D. C. Wraith***Cellular and Molecular Medicine, University of Bristol, Bristol, UK*

The ubiquitous serine/ threonine kinase glycogen synthase kinase-3 (GSK3) is a point of convergence of several signaling pathways. In the immune system GSK3 has been shown to have an important role in the balance of production of pro- and anti-inflammatory cytokines. GSK3 inhibition has been shown to have an anti-inflammatory effect in several disease models and in monocytes, macrophages and human memory T cells has been shown to increase production of the anti-inflammatory cytokine IL-10. We have used inhibitors of GSK3 to examine its role in the production of IL-10 by CD4⁺ T cells from myelin basic protein (MBP)-specific TCR transgenic (Tg4) mice. Treatment with GSK3 inhibitors of naive cells from Tg4 mice does not affect their production of IL-10. However, in differentiated Th1 cells, culture in the presence of the GSK3 inhibitors CHIR99021, SB216763 or SB627772 leads to a dramatic increase in the production of IL-10. These GSK3 inhibitor treated cells caused less severe disease than control treated cells in adoptive transfer experiments where experimental autoimmune encephalomyelitis (EAE) was induced. Inhibition of GSK3 in Th1 cells can therefore enhance the differentiation of IL10-Tregs and redirect the fate of pathogenic Th1 cells to a regulatory cell type. These findings are particularly important in the context of Th1-mediated autoimmune disease where it may be possible to reprogram disease-causing cells by GSK3 inhibition to become regulatory, disease-suppressing cells.

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Hedgehog signalling and T-cell development**C. I. Lau & T. Crompton***Department of Immunobiology, Institute of Child Health University College London, London, UK*

T cell development is tightly regulated and takes place in the thymus, thus the thymic microenvironment is crucial to the process. The hedgehog (Hh) family of secreted proteins and their signalling pathway are involved in patterning and development in many tissues during mammalian embryogenesis and in T cell development in the thymus. This study showed the role of downstream mediators of Hh signalling in the regulation of thymocyte development and differentiation.

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A positive and negative role for human β -defensin 2 in the development of human regulatory T cells**D. Chen, S. Outram, J. George & D. Rowley***School of Health, Sports & Bioscience, University of East London, London, UK*

Background and Aims: Human β -defensins (hBDs) are a group of cationic peptides capable of directly killing a wide range of microbial pathogens. Additional to this direct microbial killing, defensins have also been shown to be capable of modulating both the innate and adaptive immune responses. The aims of this study are to further elucidate the means by which hBD-2 may regulate the human CD4⁺ T cell response.

Methods: Human PBMCs were isolated from healthy donors by using the Ficoll-Hypaque density centrifugation method. The iso-

lated cells were seeded and stimulated with soluble anti-CD3 and anti-CD28 antibodies in the presence or absence of 1, 3, 10 μ g/ml hBD-2. Expression of CD4 and CD25 at the cell surface together with Foxp3 within the cell were analysed by flow cytometry at 18 and 42 hrs following the initial stimulus. Proliferation of CD4⁺ T cells was measured by staining PBMCs prior to culture with CFSE and then analysed as before at 72 and 96 hrs post initial stimulus.

Results: In this study, we demonstrate that co-culture of human PBMCs with hBD-2 together with anti-CD3 and anti-CD28 causes an up-regulation in numbers of CD4⁺CD25^{high} T cells at 18 h and these numbers remain elevated following 40 h incubation. Analysis of expression of the specific marker for Tregs, Foxp3, also reveals an up-regulation of expression of this transcription factor in the CD4⁺CD25^{high} T cells. However, interestingly, we demonstrate that expression of Foxp3 is significantly reduced following 40 hrs incubation with hBD-2 (10 μ g/ml) in these CD4⁺CD25^{high} T cells. We would hypothesize that following 40 h incubation there might be a loss of immunosuppressive activity of Tregs induced by hBD-2 in these cultures. Consistent with this idea we demonstrate that analysis of CFSE stained PBMCs revealed that proliferation of CD4⁺ T cells *in vitro* is enhanced after 72 h and 96 hrs in the presence of hBD-2 (10 μ g/ml) suggesting that any negative control of proliferation of these CD4⁺ T cells has been partially lost.

Conclusion: These results suggest that hBD-2 may play both a positive and negative role in the development of human Tregs by regulating the expression of Foxp3. The initial positive regulatory effect is characterised by an up-regulation in numbers of CD4⁺CD25^{high} Tregs that also express elevated levels of Foxp3. The later negative regulatory effect is characterised by a loss of Foxp3 in these Tregs which may lead to a limited ability to the negative control of the proliferation of CD4⁺ effector T cells.

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RIP2 kinase inhibition reduces inflammatory cytokine production in *ex vivo*-cultured inflammatory bowel disease biopsies**A. Vossenkamper^{*}, B. Votta[†], P. Biancheri^{*}, L. Casillas[†], B. Desai[†], K. Foley[†], P. Gough[†], D. Lipshutz[†], M. Reilly[†], J. Bertin[†] & T. T. MacDonald^{*}***^{*}Barts and the London School of Medicine and Dentistry, London, UK,**[†]GSK, Collegeville, PA, USA*

Background and Aims: The loss of epithelial barrier integrity is a common feature of inflammatory bowel disease (IBD). Disrupted barrier function allows bacteria to penetrate from the gut lumen, driving inflammation through activation of host pattern recognition receptors (PRRs). Although it is unclear which PRRs are most important in this process, accumulating evidence points to a role for the cytoplasmic PRRs NOD1 and NOD2, which signal through RIP2 kinase to activated NF- κ B.

Methods: We utilized the highly potent and selective RIP2 inhibitor GSK214 to examine the function of RIP2 in spontaneous cytokine production by *ex vivo*-cultured inflamed mucosal biopsies isolated from Crohn's disease and ulcerative colitis patients. The inhibitor was added to the culture medium and explants were cultured for 24 h. Cytokine levels were determined by ELISA.

Results: Treatment with GSK214 potentially inhibited production of IL-1b, IL-6 and TNF- α in explant cultures of inflamed mucosa. Approximately 70% of patient cultures responded to GSK214 in a dose-dependent fashion, which was similar to the response rate observed for the corticosteroid prednisolone. Data are also presented on the effect of GSK214 on activation of RIP2 as measured by Ser176 autophosphorylation.

Conclusion: Our results highlight the importance of RIP2 kinase in promoting intestinal inflammation, and suggest that RIP2 inhibitors may have therapeutic potential in the treatment of IBD.

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Innate immune cell CD45 regulates lymphopenia-induced T cell proliferation

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There is a drive to maintain a constant number of T cells. When the numbers are low peripheral T cells divide by lymphopenia-induced proliferation (LIP). Factors known to be involved in this expansion include the cytokines IL-7 and IL-15, and MHC engagement. LIP occurs in neonates and after a reduction in T cell numbers due to infection or immunosuppressive drug treatment. This phenomenon also occurs experimentally when T cells are transferred into lymphopenic mice. LIP is associated with autoimmunity and it is required for some mouse models of autoimmune disease.

The leukocyte specific tyrosine phosphatase, CD45, is required in T cells for optimal T cell receptor signalling and mice lacking this protein have severely reduced peripheral T cell numbers. We identified a skew in the peripheral CD4:CD8 T cell ratio in CD45 deficient (CD45KO) mice which was opposite to the ratio of CD4:CD8 single positive populations in the thymus. Upon further investigation we found that CD45KO mice were defective in their ability to support T cell LIP and the CD4:CD8 skew observed in CD45KO mice was the result of the peripheral expansion of CD8 T cells being more severely impaired than that of CD4 T cells. To confirm that the defect was due to the lack of CD45 in innate immune cells we adoptively transferred CD45-positive T cells into lymphopenic RAG1-deficient or RAG1 and CD45 double deficient mice (45RAGKO) and showed that LIP was defective in the CD45 deficient hosts. We also again saw a larger affect in CD8 T cells than CD4s. The LIP deficiency in 45RAGKO mice was partially rescued by the transfer of wild type, but not CD45-deficient CD11c⁺ cells, suggesting a role for CD45, in these cells in promoting T cell LIP. The inability of CD45KO CD11c⁺ cells to rescue T cell LIP was shown to be independent of antigen presentation and co-stimulatory molecule expression as these cells were competent at stimulating cognate antigen-induced T cell proliferation both *in vitro* and *in vivo*. We found that IL-7 made by the CD45-negative stromal cells was reduced in the secondary lymphoid organs of 45RAGKO mice, identifying a novel regulatory pathway between CD45-positive innate immune cells and IL-7-producing stromal cells. Therefore, innate immune cell CD45 regulates T cell LIP by at least two mechanisms; firstly, by a signal from CD11c⁺ cells and secondly, indirectly, by the induction of IL-7 production by stromal cells.

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Differential expression of the transcripts encoding epidermal growth factor receptor and its ligands by CD4⁺ T helper subsets in response to T cell receptor engagement

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Amphiregulin (AREG) belongs to a family of seven ligands for the epidermal growth factor receptor (EGFR). Its role in T cells remains

unclear and expression of the EGFR and other EGFR ligands by these cells has not been investigated.

CD4⁺ T cells isolated from AREG^{-/-} and AREG^{+/+} mice showed similar proliferative responses and susceptibility to apoptosis, suggesting that AREG is not necessary for these key functions of unpolarised cells. A role in polarised T cells was therefore explored.

Quantitative reverse transcriptase-PCR analysis was undertaken on T cell receptor (TCR)-stimulated unpolarised, Th1, Th2, Th17 and induced regulatory T cell (Treg) populations over 48 h. Transcript abundance for epigen, ephreclin and TGF- α was below detection levels, while relative expression of mRNA encoding EGF remained unchanged in all populations. In contrast, mRNA encoding heparin-binding EGF-like growth factor (HB-EGF) and EGFR was induced by TCR engagement, the former particularly in Th1 and the latter particularly in Th2 cells.

A rapid, transient peak of AREG transcript expression was induced upon TCR engagement of the unpolarised CD4⁺ T cells and each polarised subset except Th2 cells, which showed a gradual, sustained increase. Furthermore, both AREG and HB-EGF transcripts were more abundant in thymic Tregs than conventional memory CD4⁺ T cells. Ability to differentiate to any of the Th subsets was not influenced by a lack of AREG, based on expression of canonical transcription factors.

On-going experiments are exploring the function of AREG and the EGFR in Th2 cells and Tregs, distinguishing between transcriptional and non-transcriptional activities.

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Co-inhibitory receptor LAG-3 positive CD8 T cells produce immunosuppressive TGF- β in chronic HBV

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CD8 effector T cells are pivotal in clearing infection with hepatitis B virus (HBV). However, HBV-specific CD8 T cells show impaired functionality in patients with chronic HBV (CHB). We are here using CHB as a model infection to study the auto-regulatory function of CD8 T cells. Lymphocyte activation gene-3 (LAG-3) is a co-inhibitory receptor which has been found to be expressed on functionally exhausted, but also on regulatory T cells. We find that CD8 T cells in CHB have increased expression of LAG-3 compared to those from healthy individuals. LAG-3⁺ CD8 T cells in CHB are largely PD-1 negative; therefore they seem to be distinct from the classical phenotype of functionally exhausted cells. We find that LAG-3⁺ CD8 T cells produce the immune suppressive cytokine TGF- β directly *ex-vivo*. *Ex-vivo* TGF- β production is significantly elevated in chronic HBV compared to healthy individuals and correlates with the frequency of LAG-3 positive cells, strongly suggesting an immune regulatory function of this subset. Furthermore, the majority of LAG-3 positive cells co-express CD49b, a phenotype recently described to define a subset of human CD4⁺ regulatory T cells (Gagliani et al. 2013). In CHB the LAG-3 CD49b double positive subset is also enriched in the rare CD8⁺ FoxP3⁺ T cells. Of note, the expression of LAG-3 on CD8 T cells is age dependent. In healthy individuals below the age of 40 expression is generally low but increases after the age of 40 to levels comparable with those in CHB. In contrast LAG-3 levels are significantly increased even in young CHB patients. Our findings suggest that LAG-3⁺ CD8 T cells have immune regulatory function. Since the frequency of these cells increases with age, LAG-3 might be a marker of age related immune senescence, an immunological state that is likely hastened by CHB.

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The presence of CD8+CCR7+ T-cells at day 14 post allogeneic stem cell transplant predisposes patients to acute graft versus host disease

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Background and Aims: Stem cell transplant (SCT) is an accepted strategy for the treatment of high risk haematological disease and involves replacement of the recipient's immune system with that of an HLA matched individual. T-cell reconstitution post-SCT is critical in induction of both the graft versus leukaemia effect (GVL), and graft versus host disease (GVHD), and thus in determining the outcome of SCT. However, as thymic output is limited in adult leukaemia patients, thymic-independent pathways, primarily homeostatic proliferation, will be key to the shaping of the adaptive immune response. Here we aimed to assess the dynamics and mechanisms of T cell reconstitution in the first 14 days post transplant assessing the impact of reconstitution on subsequent development of aGVHD.

Methods: T cells at day 14 were isolated from peripheral blood and from stem cell donation bags and characterised by multicolour flow cytometry for phenotype, Ki-67 positivity and/or Vbeta TCR repertoire. MACS sorted T cells were analysed by micro-satellite analysis for the percentage of donor and recipient T cells and patient serum analysed by luminex for the presence of homeostatic cytokines at days 2, 7 and 14 post SCT.

Results: Strikingly in a cohort of 51 patients the presence of CD8+CCR7+ T cell populations at week 2 post SCT was associated with development of classical acute GVHD with logistic regression models revealing a 90% sensitivity and 85% specificity. At 14 days post SCT patient T cells were of 100% donor origin and were undergoing extremely high rates of proliferation (>90%). Serum levels of IL-7 were inversely correlated with CD8 T cell numbers indicating that homeostatic mechanisms support this rapid proliferation. However Vbeta repertoire analysis also revealed a restricted T cell repertoire at day 14 when compared to the stem cell product, especially in patients who went on to develop aGVHD, indicating that both homeostatic and antigen-specific forces early post SCT contribute to development of aGVHD.

Conclusions: To our knowledge, this is the first work to characterise specific CD8 T-cell subpopulations associated with subsequent development of aGVHD at day 14 post SCT. Analysis of these populations may allow identification of patients at risk of aGVHD development providing a window of opportunity for treatment of patients prior to disease onset.

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Alloantigen reactive human regulatory T cells, expanded using the 'optimal' antigen presenting cell: implications for clinical application

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Transfer of human regulatory T cells (Tregs) has become an attractive therapeutic alternative to improve the long-term outcome in transplantation and thus reduce the side effects of conventional immunosuppressive drugs. Clinical protocols, using 'polyclonal'

Tregs (PC-Tregs) have shown to prevent graft-versus-host-disease after bone marrow transplantation. There are currently no trials of Treg therapy in the context of solid organ transplantation and murine studies in this setting have implicated antigen-specific Tregs to be more potent than PC-Treg. In this regard, we have previously demonstrated the superiority of murine and human Treg lines with specificity for the graft compared to polyspecific Tregs, in protection against graft damage, using both murine heart transplant and humanised-mouse-model of skin-allograft transplantation.

Our previous protocol for the expansion of Tregs with direct allo-specificity involved the use human myeloid CD1c⁺ (BDCA-1) dendritic cells (DCs) and cell sorting of Tregs based on expression of activation markers after 3 days of mixed lymphocyte reaction (MLR). More recently, however, we have established a method to activate and expand *in vitro* donor B cells using K562 cells, expressing CD40 ligand, to stimulate the expansion of alloantigen-specific, donor-reactive, human Tregs (drTregs). The expanded Tregs were highly donor-alloantigen reactive and were stable *in vitro*. The functional superiority of the drTregs over PC-Tregs *in vivo* was shown in a humanised mouse model of human skin transplantation.

Extending our recent findings that drTregs can be expanded *ex vivo* with allogeneic CD40L activated B cells, we are currently comparing the potency of CD40L activated B cells with DCs with the aim of generating the most 'superior' Treg for clinical application; one which is phenotypically stable in culture and has the greatest antigen specific suppressive function. Moreover, with the emergence of studies delineating the phenotype and function of the various B cell subsets, we have further broadened our study to investigate the importance of these different B cell subsets on the generation of allo-antigen specific Tregs.

Altogether the identification of the 'optimal' APC will inform current protocols for the manufacture of alloantigen specific Tregs which is timely in view of trials of Treg therapy being planned in solid organ transplantation.

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Phenotypical characterization of circulating helper T cells show an imbalance between Treg cells and CCR4+ CCR9+ cells in active perennial allergic conjunctivitis patients

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Background and Aims: Allergic conjunctivitis (AC) is one of the most common eye disorders in ophthalmology. In mice models, it has been suggested that control of allergic conjunctivitis is a delicate balance between Tregs and inflammatory migrating effector cells. Our aim was to evaluate the frequency of Tregs and the frequency of homing receptors expressing cells in peripheral blood mononuclear cells (PBMC) from patients with perennial allergic conjunctivitis (PAC).

Methods: PBMC were obtained from patients with PAC and healthy-controls (HC), and analyses of phenotypic markers on CD4+ T cells were performed by flow cytometry.

Results: CD4+ CD25+ cells were 15-times more frequent in PBMC from patients than HC, the vast majority of these CD4+ CD25+ cells were FOXP3-, and most of CD4+ T cells were CCR4+ and CCR9+ cells. Upon allergen-stimulation, no significant changes were observed in frequency of Treg; however an increased frequency of, CD4+ CCR4+ CCR9+ cells, CD4+ CD103+ cells, and CD4+ CD108+ cells, with increased IL-6 and IL-8 secretion were observed.

Conclusions: These findings suggest an immune dysregulation in PAC, characterized by diminished frequency of Tregs, and increased frequency of circulating activated CD4⁺ T cells; upon allergen-stimulation, these cells were expressing cell-surface molecules related to mucosa homing and were able to trigger an inflammatory microenvironment.

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Randomized, double-blind trial of topical methylprednisolone combined with oral dialyzable leukocyte extract in patients with moderate atopic dermatitis

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Background and Aims: To investigate efficacy and safety of Dialyzed Leukocyte Extracts (DLE) as adjuvant therapy for moderate atopic dermatitis (AD).

Design: Double blind, placebo-controlled, randomized, clinical single-centre trial. NCT01902836.

Methods: Fifty-eight paediatric-patients with moderate AD were enrolled and randomized in two groups: (i) Conventional treatment (CT) + DLE: Cetirizine (0.25 mg/kg), once daily/4 weeks, Chlorpheniramine (0.35 mg/Kg), daily divided in 3 doses/4 weeks; and topical Methylprednisolone 0.1%, twice daily/10 days, then once daily/10 days, and ending every 48 h/10 days; plus oral DLE (2 mg/5 ml), daily/5 days, then every 72 h to complete 1 month. (ii) CT + placebo, same dosage and administration. Main outcome measures: Severity of skin lesions evaluated with SCORAD-Index, and immunophenotypical changes at day 14, and at end of treatment.

Results: A significant clinical improvement was observed since day 14 with both, CT and CT + DLE therapy, no significant differences in the main clinical outcome measures were found among groups; however a diminished frequency of CD8⁺ CD103⁺ cells, and increased frequency of CLA⁺ cells, was observed in CT+ placebo-group.

Conclusions: Adjuvant therapy with DLE was safe and well-tolerated. Despite both groups of patients significant improved after treatment, individuals treated with DLE preserved cell subsets related to skin immunological regulation, and avoided systemic expansion of cells related to skin inflammation.

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Peripheral CD4⁺ T cell survival is dependent upon GIMAP1

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The capacity of the immune system to respond efficiently to new antigens depends upon a continuous source of naive CD4⁺ T cells. Such cells exit from the thymus and join the recirculating T-cell pool where they persist for many months. This long lifespan is balanced by tight homeostatic control of lymphocyte numbers in order to maintain a healthy immune system.

GIMAPs (GTPases of the Immunity Associated Proteins) are a family of small GTPases related to septins and dynamins that are predominantly expressed in lymphoid tissue, suggesting an important immunological function. We have previously shown that a conditional knockout of family member *Gimap1* results in a dramatic loss of both T and B lymphocytes in the periphery, despite near normal development of these cells in primary lymphoid organs (Saunders *et al.* Blood 2010). This could be due to the defective maturation of lymphocytes and/or the failure of mature lymphocytes to survive upon reaching the periphery. In order to understand the role that GIMAP1 plays in peripheral lymphocyte survival, we have generated a mouse line: *Gimap1*^{fllox/flox}; *ER*^{T2}*Cre* (GIMERT1), in which *Gimap1* can be knocked-out inducibly by the synthetic oestrogen, 4-hydroxytamoxifen (4-OHT).

Using an *in vitro* model of CD4⁺ T cell survival, we show that peripheral CD4⁺ T cells require continued expression of GIMAP1 for their survival. Following addition of 4-OHT, purified GIMERT1 CD4⁺ T cells cultured in IL-7 lost GIMAP1 expression after 5 days and died between days 7 and 10 of culture. Cell death was associated with Annexin V binding, activation of Caspase 3, and loss of mitochondrial membrane potential - all indicators of apoptosis. Additionally, we find that cells also require GIMAP1 expression for their survival during activation-induced proliferation. Again, death of activated CD4⁺ T cells in the absence of GIMAP1 is associated with markers of apoptosis.

These data show that GIMAP1 is required for the survival of both resting and activated peripheral CD4⁺ T cells and places GIMAP1 as a critical regulator of peripheral CD4⁺ T cell function.

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Expression and regulation of the TGF- β -activating integrin $\alpha_v\beta_8$ on human dendritic cells

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Background and Aims: Induction of regulatory T cells (Tregs) is necessary in the gut to suppress immune responses to commensal bacteria and innocuous antigen. In the mouse gut, expression of the integrin $\alpha_v\beta_8$ on intestinal dendritic cells (DC) is important for TGF- β activation, Treg induction, and protection from colitis. Modulation of integrin $\alpha_v\beta_8$ expression might therefore be important in the pathogenesis of human inflammatory bowel disease.

Previous work from our lab has shown that murine $\alpha_v\beta_8$ is highly expressed on intestinal CD103⁺ DC compared to other intestinal mononuclear phagocytes and splenic CD103⁺ DC. This was shown to be required for *in vitro* Treg induction by CD103⁺ intestinal DCs, and maintenance of *in vivo* immune homeostasis. However the pathways that control integrin $\alpha_v\beta_8$ expression are currently unknown and the role of integrin $\alpha_v\beta_8$ in human immunology is poorly understood.

Materials and Methods: Resected intestinal samples were digested and analysed by flow cytometry to analyse the expression of integrin $\alpha_v\beta_8$ in the human immune system. To investigate how $\alpha_v\beta_8$ expression is regulated, human monocyte-derived dendritic cells (MoDC) were screened with a panel of gut-associated molecules and expression levels of $\alpha_v\beta_8$ and maturation markers were analysed.

Results: Mutually exclusive CD64⁺, CD1c⁺ and CD141⁺ mononuclear phagocyte (MNP) populations were found in the HLADR^{hi} gate of human colonic lamina propria. Each of these expressed different levels of integrin $\alpha_v\beta_8$, hinting at functional differences between the subsets. Treatment of MoDC with LPS was found to

cause upregulation of integrin $\alpha_v\beta_8$ expression, concurrent with expression of maturation markers CD80 and CD86.

Conclusions: Induction of integrin $\alpha_v\beta_8$ on MoDC suggests that recognition of the microbiota may influence the high $\alpha_v\beta_8$ expression on intestinal DC. Since $\alpha_v\beta_8$ is able to activate latent TGF- β , this pathway might be an important link between microbial colonisation and development of TH17/Treg responses in the gut. The observation of enriched $\alpha_v\beta_8$ expression on certain MNP subsets in the human gut may implicate those populations in control of TGF- β dependant immune responses.

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***Skint1* encodes a marker of epithelial normality sensed by intraepithelial T cells**

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Background and Aims: Many T lymphocytes reside constitutively within tissues where they are activated by environmental changes, either through their T cell receptors (TCRs), or *via* up-regulation of stress-induced ligands for innate receptors. By contrast, there is almost no evidence that T cells sense normality and are held in check by it. This critical question was investigated using an experimental system permitting T cells to be easily observed.

Dendritic epidermal T cells (DETC) are innate-like intraepithelial lymphocytes residing in murine epidermis. Most DETC express an identical V γ 5V δ 1 TCR. A recent study demonstrated sustained TCR-dependent, stress-sensitive, steady-state interactions between DETCs and neighbouring epithelial cells. However, neither the function of these interactions, nor their epithelial determinants were elucidated.

Skint1 is an Ig super-family gene expressed in thymic epithelium that is critical for DETC development. Since it is also expressed by keratinocytes, we investigated whether *skint1* is the epithelial regulator of steady-state interactions between keratinocytes and neighbouring T cells, and the functional implications.

Method: Confocal microscopy was used to quantify the number of DETC-keratinocyte interactions in strains of FVB mice differentially expressing *skint1* and/or the V γ 5V δ 1 TCR. Epidermal *skint1* was measured by qPCR, as was epidermal *IL-13* expression for the assessment of DETC responsiveness.

Results: Compared to wild type (WT) FVB mice, there were fewer DETC-keratinocyte interactions in mice lacking either V γ 5V δ 1 or WT *skint1*. This reduction in the frequency of interactions mirrored the effect of TPA application.

Hypothesising that *skint1* might communicate the status of the epithelium, we found that its expression was down-regulated by TPA and UVB. Moreover, transgenic mice expressing a *skint1* allele that could not be down-regulated, maintained their DETC-keratinocyte interactions even after TPA application.

The reduced interactions of V γ 5V δ 1⁺ DETCs with keratinocytes in mice lacking WT *skint1* correlated with enhanced up-regulation of *IL-13* in response to UVB irradiation compared to WT FVB mice. In *skint1* transgenic mice in which DETC-keratinocyte interactions were more resistant to stress, DETCs showed an impaired *IL-13* up-regulation in response to UVB irradiation.

Conclusions: *Skint1* expression by epithelial cells communicates normality to neighbouring T cells through the formation of contact foci that constrain T cell activation. Upon stress, *skint1* is down-regulated, permitting contact disruption and rapid T cell responsiveness. Hence T cells can display an added level of regulation that is functionally similar to missing-self regulation of NK cells. The results also emphasise the importance of epithelial cells in controlling T cell responses.

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IL-27 receptor signalling restricts the formation of pathogenic, terminally differentiated Th1 cells during malaria infection by repressing IL12 dependent signals

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The IL-27R, WSX-1, is required to limit IFN γ production by effector CD4⁺T cells in a number of different inflammatory conditions but the molecular basis of WSX-1 mediated regulation of Th1 responses *in vivo* during infection has not been investigated in detail. In this study we demonstrate that WSX-1 signalling suppresses the development of pathogenic, terminally differentiated (KLRG-1⁺) Th1 cells during malaria infection and establishes a restrictive threshold to constrain the emergent Th1 response. Importantly, we show that WSX-1 regulates cell-intrinsic responsiveness to IL-12 and IL-2, but the fate of the effector CD4⁺T cell pool during malaria infection is controlled primarily through IL-12 dependent signals. Finally we show that WSX-1 regulates Th1 cell terminal differentiation during malaria infection through IL-10 and Foxp3 independent mechanisms; the kinetics and magnitude of the Th1 response, and the degree of Th1 cell terminal differentiation, were comparable in WT, IL10-R1^{-/-} and IL10^{-/-} mice and the numbers and phenotype of Foxp3⁺ cells were largely unaltered in WSX-1^{-/-} mice during infection. As expected, depletion of Foxp3⁺ cells did not enhance Th1 cell polarisation or terminal differentiation during malaria infection. Our results significantly expand our understanding of how IL-27 regulates Th1 responses *in vivo* during inflammatory conditions and establishes WSX-1 as a critical and nonredundant regulator of the emergent Th1 effector response during malaria infection.

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IL-10 suppression in HCMV infection results in enhanced CD8⁺ T-cell mounted response to disease

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Human Cytomegalovirus (HCMV) is a common ubiquitous β -herpes virus. In healthy individuals HCMV infection is asymptomatic; however in immune-compromised individuals such as post-transplant recipient patients, HCMV infection is more deleterious to the host due to inability to control viral replication. CD8⁺ T-cells are essential for the immune control of viral infections and CMV-specific CD8⁺ T-cells account for a median of 10.2% of the total circulating T-cell repertoire in CMV seropositive individuals. Indeed CD8⁺ T-cell immunotherapy has been shown to be effective at controlling CMV disease when treated by adoptive transfer therapy. Recent advances in T-cell culture techniques have enabled rapid generation of large numbers of cells for such therapeutic strategies, and this approach also affords the opportunity to optimize the function

and, perhaps, clonal repertoire of these cells. IL-10 is a crucial regulator of inflammation during an immune response and can exert a suppressive effect on immune cells. The importance of IL-10 in HCMV infection is implied by the evolutionary acquisition by this virus of a functional IL-10 homologue (vIL-10). Importantly, we have demonstrated in a murine model of cytomegalovirus infection that IL-10 dramatically inhibits the accumulation and antiviral function of CMV specific CD8⁺ T-cells *in-vivo*. Herein, we sought to investigate whether IL-10 impinges on HCMV-specific CD8⁺ T cells. Data presented here suggests that blocking the immune-suppressive effect of IL-10 preferentially allows for the expansion of HCMV specific CD8⁺ T-cells directly *ex-vivo*. In the absence of IL-10 receptor (IL-10R) signalling, HCMV specific CD8⁺ T-cells are more functional as measured by IFN- γ and exhibit unique phenotypic profiles. These results suggest that manipulation IL-10R signalling may improve the antiviral function of virus-specific T cells during *in-vitro* expansion, and imply that mammalian and/or vIL-10 may impinge on HCMV-specific CD8 T cell function *in-vivo*.

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A small molecule agonist of the chemokine receptor CXCR3 prevents experimental graft-versus-host disease

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Background: The chemokine receptor CXCR3, expressed by activated T cells, is crucial to the pathogenesis of graft-versus-host disease (GVHD). GVHD targets the skin and liver and affects 50% of patients following allogeneic haematopoietic stem cell transplantation. Therapeutic blockade of chemokine receptors such as CXCR3 has proven difficult to achieve clinically using conventional antagonist approaches. This study was designed to determine whether a new desensitisation approach using a CXCR3 agonist could modulate GVHD. **Material and Methods:** Tissue-infiltrating T cells and histopathology scores were quantified using an *in vitro* human GVHD skin explant model together with electrochemiluminescent measurement of cytokine production. A humanised murine model of GVHD was used to determine the efficacy of the agonist to modulate *in vivo* liver inflammation. To define a mechanism for the effect of the agonist *in vitro* and *in vivo* transendothelial migration assays were performed using the natural chemokine mixture from inflamed human skin or activated liver cells. Affinity activation of the $\alpha 4\beta 1$ integrin was assessed by flow cytometry using a VCAM-1.Fc fusion protein.

Results: The CXCR3 agonist reduced the migration of alloantigen specific T cells into the skin explant resulting in a significant decrease in production of IFN γ , IL-6 and IL-1 β ($P < 0.01$, $n = 5$), as well as a decrease in histopathological damage, graded according to the Lerner scale ($P < 0.05$, $n = 5$, mean GVHD score 3.3 versus 1.8). The agonist also significantly reduced liver inflammation in the humanised murine GVHD model ($P < 0.05$, $n = 16$, 55 ± 8 versus 29 ± 4 T cells per field).

The chemokine mixture in conditioned media from inflamed human skin and activated biliary epithelial cells was found to contain more than six different chemokines. Transendothelial migration of CXCR3⁺ activated T cells towards each chemokine mixture was reduced by 70% by the agonist ($P < 0.01$) whereas migration of CXCR3⁻ T reg was not inhibited. Similarly, *in vivo* migration of human T cells towards liver conditioned media was reduced by 52% in the presence of the agonist ($P < 0.05$, $n = 20$). Finally, flow cyto-

metric analysis of the $\alpha 4\beta 1$ integrin showed that the agonist prevented normal affinity activation following exposure to a range of chemokines.

Conclusion and Discussion: Together these data highlight CXCR3 agonism as a potentially powerful and novel anti-inflammatory therapy for T cell mediated diseases such as GVHD.

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T cell receptor signal strength regulates the development of Th17 cells

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Background: T helper-17 (Th17) cells are implicated in the pathogenesis of rheumatoid arthritis (RA) and other autoimmune conditions. A greater understanding of the mechanism that regulates their development is vital for therapies designed to inhibit dysregulated responses without comprising host immunity to pathogens. Our aim was to assess how the potency of signalling through the T-cell Receptor (TCR) influences Th17 responses.

Methods: TCR transgenic CD4⁺ T-cells recognizing the RA candidate autoantigen aggrecan provide an ideal model to modulate signal strength by changes to either peptide affinity or density. To assess the *in vitro* Th17 response, naive transgenic T-cells were co-cultured with syngeneic bone marrow-derived dendritic cells (DC) presenting the cognate aggrecan peptide (GRVRVNSAY) at different densities or presenting altered peptide ligands (APL) of varying affinities for the transgenic TCR. After 5 days of culture, T-cells were restimulated with DC presenting a high density of cognate peptide. Production of interleukin (IL)-17, IL-4, IL-22, Interferon (IFN)- γ and Granulocyte-macrophage colony-stimulating factor (GM-CSF) was measured by ELISA and Intracellular Cytokine Staining (ICS). Immunisation with either a high or low dose of cognate peptide into the footpad allowed for the assessment of the *in vivo* T-cell response following restimulation of lymph nodes cells.

Results: Our data demonstrated that both changes to peptide affinity and density were capable of regulating Th17 development. Surprisingly IL-17 production was enhanced by low cognate peptide densities or by low affinity APL, whereas IFN- γ and IL-4 production was promoted by high antigen doses of the cognate peptide. Production of IL-22 and GM-CSF associated with the pathogenic Th17 phenotype, were found to be similarly regulated; both increased with a reduced level of TCR signalling. Neutralisation of IL-2 using a monoclonal antibody enhanced IL-17 production at a high peptide density. This suggested a mechanism whereby low signal strength via the TCR resulted in reduced IL-2 mediated suppression of IL-17 producing cells. The recall response following *in vivo* peptide immunisation demonstrated an increase in the IL-17:IFN γ ratio at a lower peptide density.

Conclusion: Our results support the hypothesis that a lower strength signal via the TCR favours development of Th17 cells. Further understanding of the mechanisms that regulate Th17 responses may lead to specific targeting of this pathogenic subset whilst preserving immunity to infectious agents, an advance with potentially major therapeutic benefits.

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Fate mapping of IL-22 in gut inflammation**H. Ahlfors, P. Morrison, Y. Li, J. Biro, A. Potocnik & B. Stockinger***National Institute for Medical Research, London, UK*

Background and Aims: Interleukin 22 (IL-22) is a cytokine that regulates tissue homeostasis at barrier surfaces. Several cell types producing IL-22 has been identified, but their stability and relationship to other subsets is largely unknown. We therefore generated a fate reporter mouse that would allow not only the identification of IL-22 producing cells but also their fate mapping *in vivo*.

Methods: To trace cells expressing IL-22, a sequence encoding Cre recombinase was cloned into the *Il22* locus (*Il22^{Cre}*). To visualize Cre activity, the *Il22^{Cre}* mice were then crossed with reporter mice expressing enhanced yellow fluorescence protein (eYFP) under the control of the endogenous Rosa26 promoter (*R26R^{eYFP}*). In *Il22^{Cre}R26R^{eYFP}* mice, the fluorescent reporter permanently labels cells that have switched on *Il22* expression regardless of the present production status of this cytokine.

Results: Using this fate reporter mouse strain we show that IL22 expression is restricted to immune cells. In gut the main producers are innate lymphoid cells (ILC) in gut whereas in skin or lung IL22 is mainly expressed by gdT cells in an unchallenged mouse. In a model of imiquimod induced psoriasiform skin inflammation we observed a substantial accumulation of IL22-eYFP+ cells, demonstrating the functionality of our reporter *in vivo*. Infecting the IL22 fate reporter mice with *C. rodentium* demonstrated the plasticity of CD4+ T cells, which was not observed in ILCs.

Conclusions: Further studies are aimed at investigating the role of IL-22-producing cells in infectious, inflammatory and autoimmune diseases and to delineate their relationship with other cytokine producing cell subsets, notably innate lymphoid cells.

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Cell-intrinsic and extrinsic factors influence CTL efficiency**T. Hogan*, U. Kadolsky†, S. Tung*, A. Yates† & B. Seddon***

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Quantifying the rate at which individual cytotoxic T lymphocytes (CTL) can survey and kill target cells is important for deriving ground-up estimates of critical CTL densities required for the control of infections. Estimates of this quantity have been derived from *in vivo* killing assays, using the relative survival of target and control cells exposed to spleen-resident CTL. However, we expect there to be significant heterogeneity in this process, both from the spatial organisation of the spleen and from the population of cells used as targets. Are all CTL capable of participating? Are some target cells more difficult to kill than others? And what impact is made by peptide turnover rates or expression levels? To address these questions, we revisited the splenocyte killing assay using transgenic F5 CTL specific for the NP366-374 influenza epitope. Using flow cytometry and quantitative imaging, we demonstrate that targets which differ in both their MHC expression levels and migration patterns within the spleen are killed at different rates by the same population of CTL. Decreasing the level of peptide on the target cells resulted in progressively less efficient CTL activity in both *in vivo* and *in vitro* assay systems. Taken together, these data demonstrate that CTL efficiency is sensitive to both peptide availability and target cell type. Furthermore, mathematical models of CTL killing allow us to place bounds on the rate at which single CTL can survey and kill targets over a range of peptide expression levels. Our analysis demonstrates how

population-average estimates of immune response parameters can be dissected to account for both spatial and cellular heterogeneity.

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Large-scale monitoring of human immune responses to H1N1 'flu vaccination

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The immune system is central to maintaining human health yet when dysregulated plays a key role in diseases such as multiple sclerosis, Type 1 diabetes and cancer. Mostly from animal studies, fundamental immunological molecules, cells, and pathways have been identified and characterised. However, we remain ignorant of how these are integrated in the human response to infection or vaccination. Challenging this can aid the design of vaccines and immunoregulatory treatments, and improve our understanding of immunopathologies. We therefore used the H1N1 (N.B swine flu rather than seasonal flu) vaccine to study how the immune system responds to a specific stimulus.

Longitudinal molecular, cellular, and functional immune-monitoring was undertaken in 186 healthy volunteer UK residents, either side of a richly adjuvanted H1N1 vaccine, with blood sampling at days -7 and day 0 (pre-vaccination); day +1; day +7; day +14; day +63. The data reflect gene expression profiling in peripheral blood mononuclear cells, multiplex detection of serum cytokines and chemokines, and cell phenotyping, as reconciled with initial clinical phenotype (adverse events [AE] versus asymptomatic) and downstream outcome (serological responder versus non-responder).

The results illustrate the practicality of immune-monitoring, and its capacity to provide new knowledge concerning the stability and dynamics of human immune responses. They reveal: [a] highly significant changes in serum inflammatory markers within 24 h in synchrony with innate immune gene profiles that diverge according to symptoms; [b] a B cell-rich response by day +7; [c] specific genes associated with AE, including those not anticipated *a priori*. The study has also identified candidate signatures predicting adequate antibody responses. Thus, the intrinsic content of this study and its comparison with similar studies conducted elsewhere, can close in on a consensus of the human response to vaccination and general immune function, thereby allowing for the identification of key components of immunodeficiency and immunopathology.

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Induction of T regulatory cells by streptococcus pneumonia**A. Rider***, A. Mubarak*, S. Derbyshire†, P. McNamara†, A. Kadioglu* & Q. Zhang*

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Background: *Streptococcus pneumonia* (*pneumococcus*) is an important human pathogen that causes a number of serious diseases including pneumonia and septicaemia. According to the World Health Organisation, pneumococcal diseases cause around 1.6 million deaths every year worldwide. Th17 and Treg cells have been suggested to play an important role in modulating pneumococcal clearance and carriage in nasopharynx. Th17 cells may mediate the clearance of pneumococcus by the recruitment of phagocytes and Treg cells may act to inhibit the inflammatory response mediated by other effector cells including Th17 cell response. Studies have shown that Foxp3+ Treg cells present in the nasopharynx may have a potent inhibitory effect on effector CD4+ T cell proliferation and could contribute to the persistence of carriage seen in young children. It has been shown in a murine study that a polysaccharide with pneumolysin induces Treg.

Aims: To study what pneumococcal component(s) induce Foxp3+ Treg cells in humans.

Materials and Methods: Mononuclear cells (MNC) from adenotonsillar tissues and peripheral blood mononuclear cells (PBMC) were isolated from children undergoing adenotonsilectomy. Depletion of memory and effector T cells from MNC was performed using CD45RO and CD25 microbeads and MACS cell sorting. Numbers of Treg and Th17 cells were analysed by intracellular staining of Foxp3 and IL-17 following stimulation by concentrated pneumococcal culture supernatant (CCS) derived from a wild type D39 strain, its isogenic mutant lacking pneumolysin (Ply^{-/-}) or by type 3 pneumococcal polysaccharide plus a pneumolysin toxoid (W433F). Induction of Treg cells and cytokine responses were measured using flowcytometry analysis and immunoassay.

Results: Stimulation of memory T cell-depleted adenotonsillar MNC or PBMC with pneumococcal CCS induced an increase in numbers of Foxp3+ Treg compared to unstimulated controls, and Ply^{-/-}CCS appeared to induce less Treg compared to wild-type CCS. Stimulation with type 3 polysaccharide (T3P) and pneumolysin toxoid (W433F) induced a more marked increase in numbers of Foxp3+ Treg than the control samples.

Discussion: Stimulation of adenotonsillar MNC and PBMC by a combination of T3P + W433F induced a significant increase in Foxp3+ Treg cells. Further work is ongoing to study whether different types of polysaccharide in combination with pneumolysin or other proteins could also induce Treg cells.

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Targeting HIV gag to the human endocytic receptor DEC-205 elicits Th1 T cell responses in mice with reconstituted human immune system components**C. S. K. Leung***, M.-A. Rochat†, M. Maurer‡, A. Raykova*, A. Lippmann*, R. Speck† & C. Münz*

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HIV displays limited tropism. Mice, reconstituted with a surrogate human immune system, can be used to study HIV pathogenesis and to test potential vaccines and therapeutic strategies against HIV infection. Targeting HIV antigens to the dendritic cell receptor, DEC-205, has been shown to elicit specific immune response in mice. However, the therapeutic potential of this vaccine strategy has to be tested in a model that recapitulates the human immune responses to the virus and allows challenging these with HIV infection. Here, we used HLA-A2 transgenic NOD-*scid* $\gamma_c^{-/-}$ (NSG) mice reconstituted with human immune system components (hu-NSG) to test the *in vivo* efficacy of this vaccine. We demonstrated that vaccination with α DEC-205-HIV gag p24 antibodies with the Toll-like receptor 3 agonist poly ICLC as an adjuvant primed p24-specific IFN γ -secreting T cells with low frequency. These p24-specific IFN γ -secreting T cells were cloned and expanded *ex vivo*. Most of the expanded p24-specific T cells were CD4⁺ T cells. One of these T cell clones was characterized in details. This p24-specific T cell clone is specific for the epitope PVGEIYKRWIILGLN, corresponding to HIV gag protein amino acid sequence 257–271, and restricted via DPB1*0301. It has a functional avidity of 700 nM, measured as the peptide concentration required to achieve 50% maximum recognition. Spectratyping indicated the clone expresses T cell receptor β -chain variable region 7. In addition, this p24-specific T cell clone recognizes processed p24 after DEC-205 targeting. Interestingly, adoptively transfer of this p24-specific CD4⁺ T cell clone into hu-NSG mice, followed by HIV challenge, resulted in lower viral load compared to mock treated mice. These studies shed light on DEC-205 targeting as a vaccination approach against HIV and the possibly protective value of HIV-specific CD4⁺ T cell responses.

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The role of pTalpha isoforms in alpha beta T cell development**C. L. A. Grandjean, J. Mahtani-Patching, D. J. Pang, S. Williams & D. J. Pennington**

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Mature $\alpha\beta$ T cells, that express a heterodimeric $\alpha\beta$ T cell receptor (TCR $\alpha\beta$), develop in the thymus from a common lymphoid progenitor and are indispensable components of a healthy immune system, displaying a range of functions (being cytotoxic, regulatory, or providing “help”) and sites of migration (to tissues such as gut versus secondary lymphoid organs). Early in thymic development, in order to successfully traverse an important developmental checkpoint, immature thymocytes up-regulate a pre-TCR complex that is composed of a rearranged TCR β chain, the invariant pT α chain, and various CD3 signalling molecules. The availability of two alternatively-spliced pT α isoforms (referred as pT α^a and pT α^b) provide the cell with the choice between two distinct preTCR complexes (preTCR^a and preTCR^b). However, the truncated form; pT α^b , has been frequently omitted from studies or described as non-functional despite

previous work suggesting the opposite. Our recent work has demonstrated that despite lacking an extracellular Ig-loop that was previously implicated in pre-TCR signalling, pT α^b could successfully drive early T cell development. Subsequently, BAC-transgenic animals expressing only full-length pT α (pT α^a) were generated and are currently being used to ascertain a role for both pT α isoforms. Preliminary data suggest that pT α^a alone is sufficient for the development of conventional $\alpha\beta$ T cells (CD8 $^+$ and CD4 $^+$ T cells), but does not seem to rescue a particular population of gut T cells referred to as unconventional TCR $\alpha\beta^{(+)}$ CD8 $\alpha\alpha^{(+)}$ intraepithelial lymphocytes (IELs). Furthermore, the data reveal a hitherto unrecognized role for pT α in the positive selection of $\alpha\beta$ T cells at the CD4 $^+$ CD8 $^+$ "DP" stage.

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Relative APC numbers regulate T-cell CD28-costimulation requirements

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Introduction: As the predominant source of T-cell costimulation, CD28-signaling promotes the generation of effector T-cell populations through effects upon proliferation, survival and differentiation. These contributions to T-cell activation are prevented by Cytotoxic T-Lymphocyte Antigen (CTLA)-4, which shares affinity for the CD28 ligands CD80 and CD86. Inhibition of CD28 signaling by CTLA-4 is a mechanism of regulatory T-cell biology and a target for the therapeutic modulation of pathological T-cell responses by CTLA-4Ig (abatacept). Variable patient responses to abatacept treatment in Rheumatoid Arthritis (RA) patient cohorts imply differential CD28-costimulation contributions to T-cell activation, however the mechanisms underlying this are not fully understood.

Methods: CD4 $^+$ CD25 $^-$ T-cells were isolated from PBMCs derived from healthy donors and were cultured for 5 days in the presence of anti-CD3 or Toxic Shock Syndrome Toxin (TSST)-1 as T-cell receptor (TCR) stimulus and either CD80/CD86 transfected CHO cells or allogeneic human monocyte derived Dendritic cells (DC) as sources of costimulation. The impact of CTLA-4Ig on these stimulations was identified in terms of effects upon proliferation of T-cell responders.

Results: Whilst potentially inhibiting T-cell activation driven by anti-CD3 and CHO-CD80/86 cells CTLA-4Ig had a limited impact upon T-cell proliferation stimulated by soluble anti-CD3 in conjunction with DCs. This apparent CD28-independent T-cell activation was found to be associated with the strength of TCR stimulation occurring in association with anti-CD3/DC-driven T-cell activation because stimulation with decreased anti-CD3 concentrations was strongly inhibited by CTLA-4Ig. Additionally, CTLA-4Ig inhibited T-cell activation more robustly in the presence of lower DC:T-cell ratios. When stimulating V β 2 TCR $^+$ T cells with TSST-1 we again found increased inhibition of T-cell activation by CTLA-4Ig in the presence of both decreasing superantigen concentration and relative DC numbers. Decreasing TSST-1 concentrations and lower DC:T-cell ratios were both associated with reduced TCR downregulation by activated T-cells which is indicative of decreased TCR stimulus strength.

Conclusions: These data suggest that relative APC numbers can modulate the strength of TCR stimulation which has a subsequent impact upon CD28-requirements for T-cell activation.

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The role of *P. gingivalis* in the regulation of Th17/IL17 responses in chronic periodontitis

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Objective: Periodontitis is caused by the interaction of pathogens such as *Porphyromonas gingivalis* (*P. gingivalis*) in the plaque biofilm with the host immune response. The pathogenic mechanisms of chronic periodontitis and rheumatoid arthritis may be similar as both diseases involve chronic inflammation resulting in tissue damage and bone destruction. The Th17/IL-17 pathway has been implicated in the pathogenesis of rheumatoid arthritis, but its role in the pathogenesis of periodontitis is not fully understood. The aims of this study were:

(1) to investigate the presence of IL-17 producing cells in inflamed gingival tissue;

(2) to investigate whether *P. gingivalis* promotes a Th17/IL-17 response through the activation of monocytes *in vitro*; and

(3) to compare the Th17/IL-17 response between healthy controls and patients with periodontitis.

Methods: Gingival tissue cells were isolated from periodontal surgery samples from patients with periodontitis through collagenase digestion. Cells were stimulated *ex vivo* with PMA and ionomycin for 3 h in the presence of GolgiStop prior to intracellular staining. For *in vitro* studies, bulk CD4 $^+$ T cells (0.5×10^6 cells) and CD14 $^+$ monocytes were isolated from PBMC of healthy donors, and in some cases periodontitis patients, and co-cultured at a 1:1 ratio in the presence of soluble anti-CD3 mAb with or without heat-killed *P. gingivalis* (hk-Pg). IL-17, IFN- γ , IL-1 β , IL-6 and TNF- α production in culture supernatants were measured by ELISA. The expression of activation markers on the surface of monocytes after stimulation with hk-Pg was detected by flow cytometry.

Results: IL-17 producing T cells are present in gingival tissue cells from periodontitis, and these cells are predominantly CD4 $^+$ T cells. In co-cultures of monocytes and CD4 $^+$ T cells from healthy donors, addition of hk-Pg induced IL-17 production in a dose- and time-dependent manner. Hk-Pg-stimulation of monocytes resulted in a statistically significant increase in the expression of activation markers (CD40, CD54 and HLA-DR) as well as the production of TNF- α , IL-1 β and IL-6 when compared to monocytes cultured with medium. Preliminary data further suggest that hk-Pg induces a higher IL-17 response in anti-CD3 stimulated co-cultures of monocytes and CD4 $^+$ T cells from patients with periodontitis ($n = 4$) versus healthy controls ($n = 3$).

Conclusions: IL-17 producing CD4 $^+$ T cells are present in gingival tissue from periodontitis lesions. Furthermore, the results suggest that *P. gingivalis* can activate monocytes resulting in subsequent induction of IL-17 responses in human CD4 $^+$ T cells.

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Decreased infiltration and altered activation status of T cells occurs during the carcinogenic process from Barrett's oesophagus to oesophageal adenocarcinoma

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Background and Aims: Barrett's oesophagus (BO) is a premalignant lesion linking gastro-oesophageal reflux disease (GORD) and oesophageal adenocarcinoma (OAC), known to have a chronic inflammatory component and is associated with an increased risk of developing OAC. An important role for T-cells in oesophageal

inflammation has recently been identified. It is therefore important to delineate the role of T cells at each disease stage in order to identify potential immunotherapeutic targets. The purpose of this study was to examine the T-cell profile in normal, BO and OAC tissue using *ex-vivo* patient samples and to examine factors released into the tissue microenvironment which may influence T-cell activation and possibly drive disease progression.

Methods: Biopsies were obtained from BO and OAC patients undergoing endoscopy at the BO surveillance clinic at St. James's Hospital over an 8 month period. Biopsies were enzymatically digested and T-cells were immunophenotyped by flow cytometry. Expression of CD4, CD8, CD45RO, CD45RA, CD62L and CD69 was examined. Further biopsies were cultured for 24 h to generate tissue conditioned media (TCM). Levels of IL-2, IL-8, IL-12p70, IL-1 β , GM-CSF, IFN- γ , IL-6, IL-10, TNF- α and IL-4 in TCM were measured using multiplex MSD ELISAs.

Results: The proportion of infiltrating CD4⁺ and CD8⁺ T-cells was found to significantly decrease from normal tissue to BO ($P < 0.05$) and OAC tissue. Expression of CD45RA and CD45RO significantly decreased from normal to OAC on both CD4⁺ ($P < 0.05$) and CD8⁺ cells. A similar but non-significant decrease was observed in CD69 expression. Increased percentages of CD8⁺CD62L⁺ cells were also observed from normal to BO and OAC. Levels of IL-6 were found to significantly ($P < 0.05$) higher in BO TCM compared to normal TCM. IL-6, GM-CSF, IL-8, IL-1 β , TNF- α and IL-10, were significantly increased from BO to OAC ($P < 0.05$). Levels of IL-12p70, IFN- γ and IL-2 were significantly increased in OAC TCM compared to control ($P < 0.05$).

Conclusion: These results suggest that T-cell recruitment into diseased tissue may be inhibited and the T cells that are present within the oesophageal tissue display a diminished activation phenotype as the disease progresses. In addition, a significant increase in IL-6 levels in BO TCM suggests an inflammatory response, whereas in the OAC TCM there is an increase in both the anti-inflammatory cytokine IL-10 and pro-inflammatory cytokines IL-1 β , IL-8 and TNF- α , suggesting a mixed T cell profile within the tumour tissue.

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Gli2-mediated transcription attenuates CD4⁺ T-cell activation and IL-2 responsiveness

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Background: T-cell activation requires the integration of signals transduced by the T-cell receptor, co-stimulatory molecules and cytokine receptors. However, the role of other microenvironmental cues in the subtle modulation of T-cell responses is not fully understood. Different tissues present diverse and dynamic cellular niches, which may provide distinct tissue-derived signals to local immune cells. Hedgehog (Hh) proteins are secreted inter-cellular signalling molecules, essential for morphogenesis during embryonic development. Hh signalling is also important in post-natal cellular homeostasis, malignancy, tissue repair, thymocyte development and in modulating T-cell activation.

Methods: Here we used transgenic models where gene transcription by the Hh-responsive transcription factor Gli2, is constitutively activated (Gli2A) or repressed (Gli2R) in T-cells, to identify novel Hh target genes in lymphocytes, and to investigate the mechanisms that link Hh and T-cell signalling in the regulation of cellular function.

Results: We show that Gli2-mediated transcription in CD4⁺ cells altered the expression of multiple genes involved in cell metabolism,

signalling, differentiation, and the control of T-cell transcription. Functionally, Gli-driven transcription influenced the expression of molecules required for T-cell signalling, activation, proliferation, cell death and the induction of and response to IL-2. Active Gli2-mediated transcription in T-cells impaired the immediate-response calcium flux and reduced the DNA binding capacity of key T-cell transcription factors upon TCR ligation. These data provide a mechanism for the role of Gli2 in attenuating T-cell activation.

Conclusions: This study has importance for understanding the regulation of T-cell function in tissues where Hh proteins, or other molecules that activate Gli-dependent transcription, are upregulated, including during inflammation, tissue damage and repair, or in tumour microenvironments.

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Investigating expanded CD8⁺ T-cell populations in chronic lymphocytic leukaemia (CLL) patients with an inverted CD4:CD8 ratio

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Although Chronic Lymphocytic Leukaemia (CLL) is an accumulation of malignant B-cells, it is also associated with immunological impairment of T-cells. We have previously shown CLL patients with inverted CD4:CD8 ratios (CLL^{IR}) are associated with an increased tumour load and poorer prognosis. In addition, we observed an expansion of CD8⁺ EMRA T-cells resulting in an overall skewing towards a more differentiated CD57⁺CD27⁺CD28⁺ phenotype. Although we hypothesised that the expansion was that of a senescent, potentially 'terminally differentiated' population, we set out to characterise the activation status of this expanded population. We compared several markers for activation and differentiation in 22 normal CD4:CD8 ratio patients (CLL^{NR}) and 13 CLL^{IR} patients. Consistent with our previous findings, we demonstrated a skewing towards the CD8⁺ EMRA subset in CLL^{IR} patients and a significant increase of EMRA CD57⁺ T-cell frequency ($P = 0.0338$) compared to CLL^{NR} patients (44.2% versus 28.7%). However, we also observed an increase in EMRA CD57⁺ cells expressing the activation marker CD38 ($P = 0.0026$). CD57⁺ cells in CLL^{IR} patients also demonstrated increased co-expression of KLRG-1 with CD38 ($P = 0.0043$). These results show that CD57⁺ EMRA CD8⁺ T-cells within the CLL^{IR} cohort have a complex profile co-expressing markers associated with senescence and poor proliferation (KLRG-1) and markers associated with activation (CD38). We will continue to analyse these CD8⁺ subpopulations, including examining HCMV serostatus to exclude CMV infection as a confounding factor. Based on these results, we will isolate phenotypically distinct subpopulations from the expanded CD8⁺ T-cell pool to investigate their replicative history and role in clinical prognosis.

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CD14⁺⁺CD16⁻ monocytes polarise memory T cells to a Th17 phenotype which is refractory to glucocorticoid treatment**A. D. Dhanda^{*,†}, B. Liu[‡], P. Lait^{*}, L. Schwetz-Bowers^{*}, P. L. Collins[†], A. D. Dick^{*,§,¶}, R. Nussenblatt[‡] & R. W. Lee^{*,¶}**^{*}School of Clinical Sciences, University of Bristol, Bristol, UK,[†]Department of Liver Medicine, University Hospitals Bristol NHSFoundation Trust, Bristol, UK, [‡]Laboratory of Immunology, National Eye Institute, National Institutes of Health, Bethesda, MD, USA,[§]Academic Unit of Ophthalmology, University Hospitals Bristol NHSFoundation Trust, Bristol, UK, [¶]Inflammation and Immunotherapy

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Monocytes either as antigen presenting cells or by interacting with T cells in tissue sites regulate T cell proliferation and polarisation, in turn modulating inflammation. Glucocorticoids are widely used to treat inflammatory conditions but a proportion of patients are refractory to therapy. In addition to a number of described mechanisms of glucocorticoid resistance, the influence of glucocorticoids on monocyte subset manipulation of T cell phenotype may answer why, in particular, Th17 cells are reportedly refractory to glucocorticoids in inflammatory disease.

To investigate this, PBMCs from 14 healthy volunteers were sorted by FACS into CD14⁺⁺CD16⁻ and CD14⁺⁺CD16⁺ monocytes and memory CD4⁺ T cells. Each subset was cultured for 5 days with CFSE labelled memory T cells in the presence or absence of Dexamethasone (Dex) 10⁻⁶M. T cell proliferation was determined by the proportion of CFSE^{low} cells and their intracellular interleukin (IL)-17 and interferon gamma (IFN γ) expression was quantified by flow cytometry. We found that CD14⁺⁺CD16⁻ monocytes drove significantly greater memory T cell proliferation (38% versus 29%; $P = 0.009$), more IL-17 expression (13% versus 9%; $P = 0.0004$) but similar IFN γ production (26% versus 29%), compared to the CD14⁺⁺CD16⁺ subset. T cell proliferation and IFN γ production were less suppressible by Dex when cultured with CD14⁺⁺CD16⁻ monocytes than the CD14⁺⁺CD16⁺ monocyte subset (5% versus 28%, $P = 0.03$ and 33% versus 62%, $P = 0.04$ respectively). IL-17 expression was not suppressed by Dex in any co-culture.

To ensure that it was the monocyte subset that was the mediator of T cell glucocorticoid sensitivity separate cultures were conducted in which each monocyte subset was pre-treated with Dex for 24 h and then washed thoroughly prior to addition of autologous memory T cells. After 5 days of co-culture we again found suppression of T cell proliferation and IFN γ production remained significantly lower in cultures with CD14⁺⁺CD16⁻ compared to CD14⁺⁺CD16⁺ monocytes (37% versus 59%, $P = 0.04$ and 20% versus 54%, $P = 0.04$ respectively).

In conclusion, CD14⁺⁺CD16⁻ monocytes drive a proliferating Th17 phenotype and overall, glucocorticoid treatment of CD14⁺⁺CD16⁻ monocytes was ineffective in suppressing T cell responses. The data display further mechanisms through which we observe clinical glucocorticoid resistance.

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Defining the signaling mechanisms of Lymphocyte Activation Gene-3 (LAG-3) in T cells**N. Alhumeed, T. Thaventhiran, L. Al-Huseini, H. X. Aw Yeang & J. Sathish**

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Lymphocyte activation gene-3 (LAG-3) is a molecular marker of immune inhibition expressed on activated T cells to induce high level of T-cell inhibition upon interaction with MHC class II molecules at the DC- T cell interjunction as means to modulate T cell homeostasis, proliferation and activation. LAG-3 reportedly functions by inhibiting CD3/TCR signaling and TCR-induced Ca²⁺ - fluxes, possibly through the intracytoplasmic association with LAP. The exact mechanism of action is unknown. Biopharmaceuticals based on LAG-3 immune control are showing promise in ongoing clinical trials. For a better understanding of LAG-3 induced immune regulation, there is a need for experimental system/s to identify key signaling events downstream of LAG-3. We have used a transgenic F5-TCR mouse model to optimise an experimental system to study LAG-3 with an objective to define the components of the TCR & co-stimulatory receptor signalling pathways that are intersected by LAG-3, to define the components of the IL-2/IL-2R signalling pathway that are intersected by LAG-3, and to investigate the role of LAP in LAG-3 function. Using this T cell model, we present data on phosphorylation of key TCR signalling molecules influenced by LAG-3 engagement.

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CD161⁺ plastic TH17-type cells exhibit enhanced polyfunctionality, reduced susceptibility to regulation by treg cells and are highly enriched in the RA joint**S. A. Basdeo^{*,†}, B. Moran^{*}, J. McCormick[‡], K. H. Mills^{*}, D. J. Veale[‡], U. Fearon[‡] & J. M. Fletcher^{*,†}**^{*}School of Biochemistry & Immunology, Trinity College, Dublin,Ireland, [†]School of Medicine, Trinity College, Dublin, Ireland,[‡]Department of Rheumatology, St. Vincent's University Hospital,

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Background and Aims: Regulatory T (Treg) cells play a crucial role in maintaining tolerance and preventing autoimmunity. Th1 and Th17 cells have been implicated in the pathogenesis of autoimmune diseases such as rheumatoid arthritis (RA). Th17 cells originate from CD161⁺ Th cells and retain expression of CD161 throughout their lifespan. An emerging literature indicates that Th17 cells exhibit functional plasticity; becoming IFN- γ -producing Th1-like 'ex-Th17' or 'non-classical Th1' cells. We sought to identify differences between Th1, Th17 and 'ex-Th17' cells in terms of their effector functions and susceptibility to regulation by Treg cells. We aimed to characterise the expression of CD161 on CD4⁺ T-cells in the RA joint and assess CD4⁺CD161⁺ cytokine production to ascertain if CD161⁺Th17 cells are plastic ex-Th17-type cells in the context of autoimmune inflammation.

Results: Our data indicates that CD161⁺ Th cells exhibit increased polyfunctionality in terms of cytokine expression compared with CD161⁻ Th cells. Furthermore, 'ex-Th17' cells were more polyfunctional than Th1 and Th17 cells, thus, making them potentially more pathogenic in the context of autoimmunity. We assessed Treg-mediated suppression of proliferation and cytokine production using a flow-cytometry suppression assay. The data illustrates that effector CD161⁺ cell proliferation and cytokine production are significantly less suppressed by Treg cells compared with the suppression of effector CD161⁻ cells. Moreover, when CD161⁺ Th cells are sorted into

Th17 and ex-Th17 cells, the data indicates that 'ex-Th17' cells, but not Th17 cells, are resistant to suppression by Treg cells. These findings have relevance in the context of autoimmunity since we observed increased frequencies of polyfunctional, IFN- γ -producing CD161⁺ ex-Th17-type cells in the synovial fluid of RA patients. Synovial fibroblasts cultured with CD161⁺ Th cell supernatants showed enhanced production of IL-8 and increased expression of RANKL (responsible for bone degradation) and ICAM (an adhesion molecule which promotes trafficking into the site of inflammation) compared with CD161⁻ Th cell supernatants.

Conclusion: This data suggests that plastic ex-Th17 CD161⁺ cells play a key role in propagating inflammation in RA joints by secreting pro-inflammatory cytokines which signal to immune cells in the synovium and to surrounding fibroblasts. These plastic CD161⁺ cells are less susceptible to suppression by Treg cells and could, therefore, evade regulation in the RA joint. We show that ex-Th17/non-classical Th1-type cells are functionally different to Th1 cells as illustrated by increased polyfunctionality and effects on fibroblasts. Collectively, this data demonstrates that CD161⁺ Th cells are a potential target for immunomodulatory therapy.

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Characterisation of IL-27-induced IL-10⁺ CD4⁺ T cells

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Background and Aims: IL-27 is an important anti-inflammatory cytokine that induces IL-10 expression in CD4⁺ T cells and suppresses autoimmune inflammation. We aimed to determine the subtype of IL-27-polarised IL-10⁺ CD4⁺ T cells, as the current literature suggests such cells could be Th1, Tr1 or inducible Treg cells.

Methods: Immunomagnetically purified CD4⁺ cells from wildtype and 10BiT mice, which express Thy1.1 as a reporter molecule of IL-10 expression, were activated with anti-CD3/anti-CD28 and polarised with IL-27. T cells were characterised by transcription factor and cytokine expression profile using flow cytometry, qPCR analysis of mRNA expression and ELISA. Transcription factor and cytokine expression levels were compared to activated CD4⁺ control cells.

Results: Characterisation of IL-27-induced IL-10⁺ CD4⁺ T cells showed high expression of T-bet and IFN- γ , markers characteristic of Th1 cells. Furthermore IL-27-induced IL-10⁺ CD4⁺ T cells had low expression of Foxp3, which is a marker of Treg cells and low expression of Tr1 cell markers c-maf, IL-5 and IL-21.

Conclusions: Our results infer that IL-27-induced IL-10⁺ CD4⁺ T cells generated *in vitro* are Th1 cells, and not iTreg or Tr1 cells. These data suggest that IL-27 may polarise inflammatory T cells to an anti-inflammatory phenotype, potentially having implications in resolution of inflammation.

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Intrathymic migration into the medulla is required for Foxp3⁺ regulatory but not conventional CD4⁺ thymocyte development

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T-cell development in the thymus is under the control of anatomically and functionally distinct thymic compartments. The thymic medulla represents a specialized microenvironment that contains CD4⁺ and CD8⁺ single positive (SP) thymocytes generated as a

result of positive selection in the thymic cortex. Successful positive selection initiates the intrathymic re-localization of newly selected CD4⁺ and CD8⁺ SP thymocytes into medullary regions, a process that has been linked with chemotactic migration. The medulla is thought to not only mediate negative selection of potentially autoreactive cells, but also foster the continued maturation of CD4⁺ and CD8⁺ SP thymocytes. Such roles for the thymic medulla correlate with a medullary residency time for SP thymocytes of up to 14 days, and the reported blockade in CD4⁺ SP thymocytes evident in RelB-deficient mice that lack medullary thymic epithelial cells (mTEC). Yet, the mechanisms the medulla utilises to control later stage SP thymocyte differentiation remain unclear.

Here we have re-examined the importance of mTEC at stages in $\alpha\beta$ T-cell development post-positive selection, whilst exploring the chemokine receptors that drive thymocyte migration into this specialized region. We show that mTEC are not required for the continued development of newly selected CD4⁺CD69⁺ SP thymocytes towards naive conventional CD4⁺ T-cells. In contrast, FoxP3⁺ regulatory T-cells and their CD25⁺FoxP3⁺ progenitors are mTEC dependent. In addition we show that while CCR4 acts as a marker for early stages in conventional CD4⁺ SP development and CD25⁺FoxP3⁺ progenitors, this chemokine receptor is dispensable for both medullary accumulation and intrathymic development of both lineages.

Collectively these findings highlight differences in the developmental requirements of conventional and regulatory CD4⁺ T-cells, whilst ruling out a role for CCR4 and its ligands in cortex to medulla migration.

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Interleukin-8 (CXCL8) production is the major effector potential of T cells in human neonates

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Background and Aims: Given their precipitous encounter with the environment, newborns might be assumed to possess abundant immuno-protective mechanisms. It thus seems paradoxical that newborn infants display grossly impaired T-helper (Th) 1 and Th17 responses, which ordinarily promote anti-bacterial, anti-fungal and anti-viral immunity. Although, they show some skewing toward Th2 and tolerogenic functions that may limit inflammatory damage to developing tissues, this still does not account for most T cells. An opportunity to further study T cell function in newborn infants arose in a cohort of preterm infants recruited into a study of intestinal permeability whose blood was sampled weekly for 4 weeks.

Methods: PBMC or purified CD4 T cells isolated from preterm infants, term infants or adult controls were activated with PMA/Ionomycin or anti-CD3/CD28 with/without TLR stimulation and cytokine production assessed by intracellular staining. CXCL8 production was also assessed by immunohistology of paraffin embedded sections of neonatal intestine.

Results: As has been established, there was marked impairment in neonatal versus adult IFN- γ production by TCR $\alpha\beta^+$ CD4 $^+$ T cells in response to polyclonal stimulation, and there was likewise negligible production of IL-17A. By contrast, up to 50% of TCR $\alpha\beta^+$ CD4 $^+$ T cells of preterm infants (average >27% [$n = 28$]) showed the production of intracellular CXCL8. CXCL8 production was also provoked by antigen receptor engagement; was enhanced by Toll-like receptor signalling; and was clearly separable from those few T cells producing Th1, Th2, and Th17 cytokines. Its production was associated with infection and associated pathology *in vivo*. It was strikingly apparent in preterm infants; declined with post-natal age relative to the emergence of conventional Th subsets; and an equivalent response was not evident in mice.

Conclusions: This study identifies the production of CXCL8, commonly regarded as a non-T cell-derived cytokine, as the major T cell effector function in human newborn infants, with potential to activate anti-microbial neutrophils and gd T cells. Thus CXCL8-producing T cells are a signature of the human newborn infant with the potential to couple adaptive responsiveness to innate immunity. This provides a new perspective on immuno-protection, immuno-pathology, and immuno-intervention in very early post-natal life.

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Dendritic cell-expressed common cytokine receptor γ -chain is essential for effective IL-15 transpresentation to CD4 $^+$ T cells at the immunological synapse

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IL-15Ra-mediated transpresentation of IL-15 is important for NK and CD8 $^+$ T cell activation by dendritic cells (DC). Here we show that IL-15 transpresentation is also required for optimal antigen-induced CD4 $^+$ T cell activation. Unexpectedly, we find that DC expression of the common cytokine receptor gamma chain (γ c) is critical for effective IL-15 transpresentation to CD4 $^+$ T cells. Using high resolution imaging in combination with a planar lipid bilayer system that mimics the T cell surface, we observe that following MHCII ligation, γ c is recruited in DC to the contact interface, and promotes IL-15Ra colocalization with engaged MHCII. These data reveal a novel mechanism for recruitment of DC IL-15/IL-15Ra complexes at the immunological synapse (IS), leading to CD4 $^+$ T cell costimulation through localized IL-15 transpresentation that is coordinated with antigen-recognition. Our findings demonstrate that DC-expressed γ c actively assembles a microenvironment for cytokine signaling at the IS that is required for full T-cell activation, highlighting the importance of molecular events on the DC side of the IS during T-cell priming. We propose a model of CD4 $^+$ T-cell activation by DC in which transpresentation of IL-15 and peptide-loaded MHCII are coupled at the IS to allow spatial coordination of TCR engagement with co-stimulation through the JAK/STAT signaling pathway.

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IL-4 induces IL-10 in Th1 cells

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In autoimmune diseases, such as multiple sclerosis (MS), an inappropriate immune response is mounted against self-antigens. In Tg4 mice, experimental autoimmune encephalomyelitis (EAE), a murine model of MS, can be induced by administration of an acetylated nine-residue peptide of myelin basic protein (Ac1-9) in combination with adjuvant. Tg4 mice express a transgenic T-cell receptor specific for the Ac1-9 peptide. Resistance to EAE induction can be achieved by repeated administration of soluble MBP Ac1-9. Disease is driven by CD4 $^+$ Th1 cells; the tolerance mechanism causes differentiation of these cells *in vivo* to cells that no longer express IFN- γ or IL-2 but express IL-10 and are anergic.

In vitro experiments suggest that IL-4 plays a role in the induction of IL-10 seen over multiple rounds of stimulation of polarized Th1 cells. Restimulating Th1 cells in the presence of exogenous IL-4 enhances the induction of IL-10 secretion. On the other hand, blocking IL-4 in Th1 restimulated cultures, with a monoclonal antibody (11.B.11), inhibits IL-10 induction. Ongoing experiments are looking at the mechanisms through which IL-4 exerts its influence on Th1 cells and causes the secretion of IL-10. The induction of IL-10 seems to correlate with the expression of the transcription factor, c-maf. The effect of Th2 derived IL-4 on Th1 cells is also being investigated using *in vitro* co-cultures with results confirming that Th2 derived IL-4 causes induction of IL-10. Future work will explore the role of IL-4 *in vivo* in the induction of IL-10 from Th1 cells in the context of peptide induced tolerance. Together these investigations will give us a further understanding into the role of IL-4 in the context of induced tolerance to EAE.

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Manufacturing of highly specific and functional T cells for adoptive immunotherapy from G-CSF-mobilised donors based on activation-dependent CD137 expression

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Cytomegalovirus (CMV) reactivation remains an important risk after hematopoietic stem cell transplantation, which can be effectively controlled through adoptive transfer of donor derived CMV-specific T-cells (CMV-T). CMV-T are usually obtained from donor PBMCs collected prior to G-CSF mobilisation. Despite previous studies showed impaired T-cell function following G-CSF mobilisation, recent publications suggest G-CSF-primed PBMCs retain anti-viral function and are a suitable starting material for CMV-T manufacturing. Our objective was to assess the feasibility of generating CMV-T from G-CSF-mobilised donors using the activation marker CD137 in comparison with conventional non-primed PBMCs.

We isolated and expanded CMV-T from G-CSF-mobilised ($n = 6$) and non-mobilised ($n = 5$) donors based on CD137 expression following stimulation with a CMVpp65 peptide pool (PepTivator-CMVpp65). CD137 $^+$ cells were sorted by magnetic enrichment, isolated CMV-T were expanded in short term culture and functional assays were performed following re-challenge with CMVpp65. Expanded cells were analysed for surface marker expression and IFN- γ /IL-2/IL-10/IL-4/IL-5/TNF- α /Granzyme B synthesis by intracellular cytokine staining and cytometric-bead-array. Cytotoxic activity was assessed using CMVpp65-loaded PHA-blasts (calcein-AM assay) and proliferation by CFSE-staining after 5-day culture with

CMVpp65-loaded PBMCs. CTL specificity was determined by Pentamer/Streptamer staining in HLA-A*0201 donors.

With G-CSF-mobilised donors, mean yield following CD137+ selection was $27.12\% \pm 7.70$ and mean purity in the positive fraction $68.32\% \pm 14.45$, consisting of CD4+ ($44.07\% \pm 19.07$) and CD8+ ($24.25\% \pm 15.74$) T-cells. CD137 expression was lost during expansion and analysis of memory phenotype showed CMV-T were mainly central- and effector-memory T-cells. Interestingly, they had significantly higher central-memory ($P = 0.0453$) and lower effector-memory ($P = 0.0497$) T-cell phenotype compared to non-mobilised CMV-T.

Following CMVpp65 re-challenge, mean CD137+ expression in expanded CMV-T raised to $55.64\% \pm 17.59$ (G-CSF-mobilised) or $57.07\% \pm 21.95$ (non-mobilised). Expanded CMV-T from G-CSF-mobilised donors secreted high levels of IFN- γ /Granzyme B, low levels of IL-2/TNF- α and no IL-4/IL-5/IL-10, successfully lysed CMV-loaded PHA-blasts ($60.41\% \pm 18.13$ in 20:1 effector:responder ratio) and proliferated after CMV-loaded PBMC addition (mean $66.43\% \pm 2.44$). CMV-T CTLs expressed the TCR specific for the immunodominant peptide pp65₄₉₅₋₅₀₃, analysed with Pentamer ($81.60\% \pm 10.75$ Pentamer + CD8+) and Streptamer ($82.75\% \pm 9.69$ Streptamer + CD8 +) HLA-multimers. No significant differences were observed between G-CSF-mobilised and non-mobilised samples.

In conclusion, we successfully manufactured highly specific, functional and cytotoxic CMV-T from G-CSF-mobilised donor PBMCs. Their anti-viral function was equivalent to non-mobilised CMV-T and memory phenotype would suggest their long term maintenance following adoptive transfer. We confirm that the use of an aliquot from G-CSF-mobilised donor samples is suitable for the manufacturing of CMV cellular therapies and thereby abrogates the need for successive donations and ensures the availability for patients with unrelated donors.

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Maternal T cell function is modulated by progesterone - a potential role in recurrent miscarriage therapy

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Background and Aims: Recurrent miscarriage (RM), the consecutive loss of three or more pregnancies, occurs in 1% of couples. In the majority of cases there is no identifiable cause but a maternal immunological response to the allogenic fetus has been implicated. Progesterone (P4) is currently being trialled as a therapeutic agent in women with RM and is suggested to have immunomodulatory properties. We have investigated the immunomodulatory effect of progesterone on maternal CD4 and CD8 T-cells. In addition we have studied the maternal CD8 T cell immune response to fetal antigens in women with RM.

Methods: PBMCs from healthy maternal donors were isolated from whole blood and stimulated with PHA and treated with either control, 1 μ M or 10 μ M P4 for 48 h at 37°, 5% CO₂. Multi-colour flow-cytometry was used to assess the effects of treatment on the cytokine profile and proliferation of T cells. HLA-peptide dextramers were used to identify fetal specific (HY) antigen specific T cells.

Results: The effect of P4 at a range of concentrations on maternal T cells following stimulation by PHA was studied. IFN γ and TNF α production by both CD4 and CD8 T cells was significantly reduced by P4 at 10 mmolar whereas IL4 was significantly increased. In addition,

P4 significantly reduced both the poly functional cytokine production by maternal CD4 and CD8 T cells and cell proliferation.

Fetal (HY) antigen specific CD8 T cell responses were detected in 40% (8/20) of women with RM. This is the first identification of fetal (HY) specific T cells in women with RM. Using fetal antigen specific T-cell clones it was also demonstrated that P4 significantly reduced IFN γ release in response to fetal antigen (Median 36% reduction, $P \leq 0.001$).

Conclusion: P4 can modulate both the cytokine profile and the proliferation of maternal T cells. Fetal specific CD8 T cells can be detected in women with a history of recurrent miscarriage and P4 has an immunomodulatory effect on HY-specific cells. Further experiments are investigating the cellular mechanism of action of P4. A greater understanding of P4 action may aid selection of RM patients who would benefit from P4 therapy and the rational design of better therapeutic strategies.

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Expression and function of atypical chemokine receptor CCRL1 in the thymus

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Thymus colonisation and thymocyte positioning are mediated by interactions of CCR7 and CCR9 and their ligands CCL19/CCL21 and CCL25, respectively. These chemokines also interact with the atypical receptor CCRL1, which is expressed in the thymus and may impact on chemokine availability and function. We used CCRL1-eGFP and CCRL1^{-/-} mice to map thymic expression and analyse the function of CCRL1. CCRL1 is expressed by thymic epithelial cells (TECs) and primarily by CD40 MHC-II^{low} immature cortical TECs. We also show CCRL1 expression by the cells surrounding vessels at the corticomedullary junction and by podoplanin⁺ cells within the subcapsular zone (SCZ). CCRL1^{-/-} mice have a reduction in CD25⁺ DN thymocytes in the SCZ compared to littermate controls, but no overt changes in thymic function. Thymus cellularity is unaffected in CCRL1^{-/-} mice in both embryo and adult, suggesting that CCRL1 is not essential for thymus colonisation. Moreover all thymocyte subsets are comparable in WT and CCRL1^{-/-} mice. These findings conflict with the published results, by Bunting *et al.* and we discuss possible reasons for such discrepancy. We suggest that CCRL1 expression within the cortex and SCZ influences the migration of CD25⁺ DN thymocytes to the SCZ – a phenomenon apparently not essential for T cell development.

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Colonisation with 'complex' microbiota reduces Tregs in the neonatal intestinal mucosa

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Background and Aims: It has been suggested that differences in early life microbial colonisation may be associated with variable predisposition to allergic autoimmune and inflammatory diseases.

The exact underlying mechanisms are difficult to study in human infants, however, the similarities in physiology and nutritional requirements between pigs and humans suggest that piglets can be good models to elucidate the pathways involved.

We hypothesise that this observation may be partly due to effects of microbiota on the mucosal CD4⁺CD25⁺FoxP3⁺ cells, known as regulatory T cells (Tregs).

We have previously shown that transfer of piglets into a specific pathogen free (SPF) facility and antibiotic treatment significantly reduces Tregs, in comparison to piglets that stayed in the farm. To further investigate the microbiota association with Tregs, we used specific microbial inoculation allowing the elimination of other environmental influences.

Methods: To address the effect of different gut microbiota in the regulation of immune responses we have used fluorescence immunohistology to investigate CD4⁺ CD25⁺ FoxP3⁺ regulatory T cells in the intestinal lamina propria of 21 day old piglets. A total of 24 piglets were born with a Caesarean section and these were transferred in an SPF facility. All piglets received colostrum the first 24 h after birth and were inoculated with a combination of the following microbes: *Parabacteroides* sp. (ASF519), *Lactobacillus amylovorus* DSM 16698T and *Clostridium glycolicum* on days 1, 2 and 3 after birth. Half of the piglets were additionally inoculated with faeces from an unrelated sow on days 3 and 4 after birth. This group was termed as the 'complex' microbiota group.

Results: The group that was inoculated with faeces ('complex' microbiota group) had significantly fewer Tregs in intestinal mucosa compared to the group that was inoculated with the 3 microbes alone. There was no significant difference between the two groups in terms of CD4 T cell numbers.

Conclusions: In this and previous experiments we have shown that different gut colonisation in early life results in differences in Tregs. In this experiment 'complex' microbiota colonisation resulted in reduction of Treg cells without affecting the overall CD4 T cell population. This suggests that the complex microbiota have shifted the CD4 T cell population towards a less regulatory phenotype. Our results provide further evidence and a possible explanation for the link between microbiota in early life and hypersensitivities.

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Assessing chemokine profiles and corresponding T cell subsets in the liver and visceral adipose tissue of obesity-associated cancer patients

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Background and Aims: Oesophageal adenocarcinoma (OAC) is increasing rapidly in western countries with a 5-year survival rate of approximately 15%. OAC has the strongest association with obesity providing a clinically relevant model with which to study the role of CD4⁺ and CD8⁺ T cells in adipose tissue and liver inflammation. We have previously shown that the omentum, part of visceral adipose tissue (VAT) depot, is a rich source of activated pro-inflammatory T cells. We propose that such cells are trafficked to and sequestered within the VAT and contribute to obesity-associated inflammation.

Methods: T cell memory phenotype and chemokine receptor expression was assessed in the blood, liver and omentum of obese and non-obese OAC patients using surface and intracellular flow cytometry. Chemokine levels in the serum and conditioned media from VAT and liver was assessed by MSD multiplex ELISAs.

Results: Significantly higher proportions of effector/memory T cells were observed in the omentum when compared to blood ($P < 0.05$). The proportions of CD8⁺ T cells producing TNF- α , IFN- γ and IL-10 in the omentum were also significantly elevated when compared to blood ($P < 0.05$).

Significantly higher levels of IL-8 ($P < 0.001$), IP-10 ($P < 0.05$), fractalkine ($P < 0.05$), MIP-1 α ($P < 0.01$) and MIP-3 α ($P < 0.05$) were observed in the omentum compared to blood and liver. Furthermore, TARC, ITAC and RANTES were significantly lower in the liver and omentum of these patients compared to blood ($P < 0.001$).

IP-10 was significantly higher in the omentum from obese OAC patients compared to non-obese ($P < 0.05$), while RANTES was significantly higher in the blood of obese OAC patients compared to non-obese ($P < 0.05$).

Differential expression of chemokine receptors was also observed on CD4⁺ and CD8⁺ in the blood, omentum and liver, with substantially elevated expression of CCR5 in the omentum and liver compared to matched blood.

Conclusions: The altered expression of T cell chemoattractant chemokines by the omentum and liver is likely responsible for the increased infiltration and sequestration of inflammatory effector memory T cells observed within the omentum and liver of these cancer patients. Once sequestered within the omentum and liver these inflammatory T cells are likely to exacerbate pathological systemic inflammation associated with cancer development and progression and potentially hinder any ongoing anti-tumour immune response.

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Marked age-related rise of a novel CMV-induced regulatory T cell subset is associated with higher blood pressure

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Cytomegalovirus (CMV) infection has been associated with immunosenescence and increased vascular pathology in older age. Following CMV infection, the vascular endothelium becomes target for CMV-specific CD8 T-cells. So far, it has remained unexplored whether CMV-induced adaptive Tregs (iTreg) may be involved in this process by suppressing immunity and limiting tissue damage caused by expanded CMV-specific effector T cells.

We have identified CMV-specific iTreg in peripheral blood of CMV-infected people, using a novel ex-vivo assay combining classic Treg markers, CD25 and CD39, alongside the activation marker CD134. CMV-specific iTreg share antigen specificity with conventional CD4 T-cells and can suppress antigen-specific and non-specific proliferative responses.

Compared to young controls (20–35 year-old), CMV-specific iTregs significantly increase in CMV-infected healthy older people (60–85 year-old), particularly women. The frequencies of iTregs and CD8 effector T cells specific for CMV correlate with each other and both are significantly correlated with increased blood pressure.

These observations support a contribution of CMV to increased vascular stiffness in older life and suggest that CMV-induced iTregs may have a damage-control role against vascular pathology.

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The role of Hedgehog signaling in $\gamma\delta$ T cells**K. Mengrelis & T. Crompton***Department of Immunobiology, Institute of Child Health, University College London, London, UK*

Background: Although $\alpha\beta$ and $\gamma\delta$ T cells derive from common multipotential thymic progenitors, the molecular mechanisms that regulate $\gamma\delta$ T cell lineage commitment are not well understood. Hedgehog signaling pathway plays a role in the regulation of $\alpha\beta$ T cell development, selection, activation and function whereas its contribution to $\gamma\delta$ T cells is largely unknown. Here, we examine how Hh signaling affects $\gamma\delta$ T differentiation.

Aims and Methods: We use Shh+/-, Ihh+/- and Dhh -/- mice as well as GFP-Gli reporters and transgenic models where Hh-dependent gene transcription is constitutively active (Gli2A) or repressed (Gli2R) in T cells to examine the role of Hh signaling in the differentiation and distribution of thymic and peripheral $\gamma\delta$ T cells.

Results: We show that Dhh and Shh negatively regulate $\gamma\delta$ T cell differentiation. Furthermore, Gli2A transgenic mice show decreased $\gamma\delta$ T cell numbers in the lymph nodes whereas an opposite effect is seen in the thymus.

Conclusions: This study is important for elucidating the role of Hh signaling pathway in $\gamma\delta$ T cell development.

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The role of autophagy protein Atg16L1 for intestinal T cell homeostasis**A. M. Kabat, J. Pott & K. Maloy***Sir William Dunn School of Pathology, University of Oxford, Oxford, UK*

Inflammatory bowel diseases (IBD) are chronic debilitating inflammatory disorders of the gastrointestinal tract. IBD are thought to arise due to a breakdown in intestinal immune homeostasis. Despite years of extensive research the aetiology of IBD is not fully understood. Polymorphisms in autophagy genes, such as *ATG16L1* or *IRGM*, were recently associated with susceptibility to IBD; however the mechanisms responsible for this correlation remain unclear. Autophagy is a homeostatic process of recycling intracellular components in response to starvation or stress. It is also involved in clearance of defective organelles and defence against intracellular pathogens. Furthermore, autophagy is important for the activation and survival of T cells. To elucidate how the autophagy pathway contributes to T cell homeostasis in the gastrointestinal tract we utilized mice wherein the autophagy protein Atg16L1 was selectively ablated in T cells. At steady state mice with *Atg16L1*-deficient T cells harboured significantly reduced frequencies of CD4⁺ and CD8⁺ T cells in the periphery, despite normal thymocyte development. Furthermore, these mice exhibited a decrease of Foxp3⁺ regulatory T cells and effector/memory T cells, which was most pronounced in the gastrointestinal tract. In a T cell transfer model of colitis *Atg16L1*-deficient CD4⁺ T cells did not drive intestinal inflammation. These cells failed to reconstitute lymphopenic host due to an intrinsic defect in proliferation. Future work will further focus on elucidating the functional consequences of the observed alterations in the intestinal T cell populations.

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Human IL-10^{high} CD49b⁺ LAG-3⁺ CD226⁺ Tr1 cells are differentiated from a sub-population of naïve CD4⁺ T cells following CD55 co-stimulation**R. Sutavani, L. Durrant & I. Spendlove***Department of Academic Clinical Oncology, University of Nottingham, Nottingham, UK*

Background and Aims: Co-stimulatory molecules other than CD28 are known to induce differentiation of different T cell subsets. However, the exact role of these alternate co-stimulatory molecules in shaping T cell responses remains to be elucidated. We have previously assessed CD55 co-stimulation of human CD4⁺ T cells demonstrating activation and IL-10 production. We hypothesised that CD55 co-stimulation induced Tr1 cells.

Methods: Naïve CD4⁺ cells, isolated from PBMCs of healthy donors, were stimulated with plate bound anti-CD3 and anti-CD55/CD28 mAbs. Cell proliferation was assessed by [³H] thymidine uptake assay. Secreted cytokines were assessed in culture supernatants by ELISA. The cells were assessed for surface marker expression by flow cytometry and for cytokine secretion by the cytokine capture assays, following the recommended protocols.

Results: Here, we show that CD55 co-stimulation of naïve CD4⁺ T cells results in the differentiation of a discrete population of IL-10^{high} IFN- γ ⁻ IL-4⁻ CD49b⁺ LAG-3⁺ CD226⁺ Tr1 cells. During primary and secondary stimulations, CD55 is as effective as CD28 in driving cellular activation and proliferation of naïve CD4⁺ cells. But, unlike CD28, that induces an IFN- γ ^{high} IL-10^{low} IL-4⁻ Th1 phenotype, CD55 induces an IL-10^{high} IFN- γ ⁻ IL-4⁻ Tr1 phenotype. On secondary stimulation these cells maintain their Tr1 phenotype and are able to inhibit T cell proliferation in an IL-10 dependent manner. Analysis of the cells for IL-10 and IFN- γ secretion revealed 2 discrete populations - an IL-10 single positive population and an IFN- γ single positive population. The IL-10 single positive cells represent ~1–5% of the culture. IL-10 secretion peaks at day 3 of primary stimulation and is maintained on re-stimulation with CD55. The remaining cells have a Th1-like phenotype but are prevented from secreting IFN- γ by the Tr1 cells. Enriched Tr1 cells maintain the Tr1 cytokine profile, IL-10 dependent suppression and the expression of recently identified Tr1 markers CD49b, LAG-3 and CD226.

Conclusion: We have identified CD55 as a preferential co-stimulator of Tr1 cells. Next question - do these 1–5% Tr1 cells originate from a discrete sub-population of naïve CD4⁺ cells?

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Hedgehog signaling regulates the development of thymic stroma**J. I. Saldana, H. Sahni, A. L. Furmanski & T. Crompton***Department of Immunobiology Institute of Child Health, University College London, London, UK*

Background and Aims: The thymus provides a specialised microenvironment that supports the development of $\alpha\beta$ T cells by providing the necessary signals required for their survival, differentiation and expansion. The thymic microenvironment is compartmentalised into cortical and medullary regions formed of Thymic epithelial cells (TEC) among other cells. TEC support the development of CD4⁺ T cell progenitors into mature, self MHC-restricted and self-tolerant CD4⁺ and CD8⁺ thymocytes and the mechanisms underlying T cell development have been extensively studied. However, the signals that instruct TEC development are not as clear.

Hedgehog (Hh) proteins are secreted signaling molecules that act as morphogens and are essential for embryonic development. Hh signalling plays an important role in T cell differentiation in the thymus; however, to date nothing is known about the involvement of

the pathway in the development of either cortical (cTEC) or medullary (mTEC) epithelial cells.

Methods: We performed *ex vivo* and *in vitro* assays using Hh knock out and transgenic mice to assess TEC at different stages of development.

Results: We show that Sonic hedgehog regulates cTEC and mTEC differentiation as well as their potential to drive thymocyte selection. In addition Hh signalling could also influence the balance of CD205⁺ epithelial progenitors during earlier stages of TEC development.

Conclusions: This study illustrates how a morphogen can contribute to the development of the thymic stroma and provides new data that could be used to inform the design of strategies to improve thymic function in conditions of immunodeficiency as well as other diseases.

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Redox control of T cell receptor mediated T cell activation

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Proteins at the surface of leukocytes contain many conserved disulfide bonds. These bonds serve three purposes, some are to stabilise protein structure in the harsh extracellular environment, some form the active site of reductase and isomerase enzymes and some are extremely labile and when broken alter the structure and/or function of the protein that contains them. When T cells are activated, the level of free thiols on their surface increases as a function of time, suggesting that labile disulfide bonds are being reduced as a result of activation. Previously we developed a proteomics based screen to identify the proteins that contained these redox labile disulfide bonds, and in some cases the actual disulfide bond that is labile. The T cell receptor complex was among 87 proteins identified on a murine T cell hybridoma that contained redox labile disulfide bonds. Here we report the functional consequences of reducing disulfide bonds in the TCR/CD3 complex which results in an inhibition of TCR mediated activation. The cells ability to react to other stimuli (CD3e, PMA/ionomycin) is not inhibited nor is the TCR-Peptide MHC interaction significantly weakened, suggesting signal transduction across the plasma membrane after MHC engagement is inhibited.

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Major contribution of gamma delta T cells to IL-17A production and ovarian cancer cell growth *in vivo*

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A significant body of evidence implicates gamma delta T cells in tumour immunosurveillance. The anti-tumour function of gamma delta cells stems from their potent cytotoxicity and their capacity to produce high levels of IFN-gamma. However, gamma delta cells were also recently implicated in promoting tumour growth, possibly as a result of their ability to secrete IL-17A. Considering the ultimate goal of manipulating gamma delta cells for cancer treatment, we aim to further dissect the pro-tumour mechanisms of gamma delta cells. We use a transplantable ID8 ovarian cancer cell line, that grows slower upon IL-17A neutralization. Our data show that gamma delta cells infiltrate ID8 tumour foci and are a major source of IL-17A in the tumour microenvironment. Furthermore, gamma delta cell-deficient mice (TCRdelta^{-/-}) show reduced ID8 tumour burden, which strongly suggests that the pro-tumour role of IL-17A is mediated by

gamma delta cells. A significant fraction of gamma delta cells is proliferating *in situ* and accounts for a 2.3-fold increase in gamma delta cell numbers in the peritoneal cavity, 6 weeks after ID8 cell injection. The majority of these gamma delta cells display a Vgamma1- Vgamma4-phenotype, indicating they belong to the Vgamma 6 + subset which is naturally enriched in the peritoneal cavity. Interestingly, both IFN-gamma+ and IL-17A+ gamma delta cells accumulate in the tumour microenvironment, potentially suggesting a dominance of the pro-tumour effects of IL-17A over the anti-tumour functions of IFN-gamma. We are currently generating TCRdelta^{-/-} IL-17A^{-/-} mice to further assess the contribution of gamma delta cells to the IL-17A-mediated tumour growth in this ovarian cancer model.

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A requirement for PI3K in $\gamma\delta$ T cell development

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Background and Aims: Gamma delta ($\gamma\delta$) T cells play important roles in immune responses against pathogens and tumours which seem to strongly correlate with the secretion of cytokines, such as IFN γ and IL-17A. Recent evidence suggests that $\gamma\delta$ cells are predominantly pre-committed to certain effector fates during thymic development; T cell receptor (TCR)-agonists favouring the development of IFN γ -producing $\gamma\delta$ cells (that are variously identified by CD27, CD122 and NK1.1), whereas the absence of ligand interactions was suggested to appear to generate IL-17A-producing $\gamma\delta$ cells (identified as CD27^{-/-}, but expressing CCR6). The aim of this study was to determine clarify the role of the TCRgd and TCRgd signalling in the development of $\gamma\delta$ T cell subsets and in the adoption of effector fates in the thymus.

Methods: Integrating all the markers described above including CD24 and CD25, we used flow cytometry to establish a gating strategy to identify IFN γ -secreting and IL-17A-secreting in the lymph nodes and thymus of C57BL/6 (BL/6) mice. Subsequently, we sorted various $\gamma\delta$ subsets and put them into different culture systems including fetal thymic organ cultures (FTOC) as well as on to OP9-DL1 stromal cell line to analyse $\gamma\delta$ cell development and the adoption of different effector functions under different conditions. These conditions included inducing TCR signalling by cross-linking with an activating antibody or in a ligand-independent manner by generating a TCR that was incapable of binding a ligand.

Results: Our results demonstrate that at least four distinct subsets of $\gamma\delta$ cells can be identified in both the thymus and periphery of various strains mice. These subsets display distinct cytokine-secreting and proliferative potential. Our data describe precursor/product relationships between thymic $\gamma\delta$ cell subsets and establishes a $\gamma\delta$ cell developmental sequence. Moreover, we find that TCR $\gamma\delta$ signalling induced by cross-linking with an activating antibody, or in a ligand-independent manner, does not favour the development of IL-17A-secreting CD27^{-/-} $\gamma\delta$ T cells. Our results suggest that the effector fate of $\gamma\delta$ subsets is determined by the quality, rather than quantity, of TCR $\gamma\delta$ signalling during T cell development. We also present data that identifies a requirement for phosphoinositide 3-kinase (PI3K) p110 δ in the development of IL-17A secreting $\gamma\delta$ cells. These findings suggest similarities between $\gamma\delta$ T cell and B cell development.

Conclusions: We conclude that effector fate of $\gamma\delta$ subsets is not likely to correlate with ligand-dependent versus ligand-independent signalling but instead appears to correlate with quantitative and/or qualitative differences in TCR signalling.

RNA Regulation of Development and Activation in the Immune System

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Interferon gene expression signature in rheumatoid arthritis neutrophils predicts response to anti-TNF therapy

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Background: Neutrophils are central to the initiation, progression and resolution of inflammatory diseases. The aim of this study was to use RNA-Seq to identify molecular biomarkers which may predict response to anti-TNF therapy in rheumatoid arthritis (RA).

Methods: RNA was isolated from healthy and RA neutrophils obtained pre- and 12-weeks post-anti-TNF therapy. RNA was sequenced by Illumina HiSeq 2000. Reads were mapped to the human genome using Tophat. Expression analysis was carried out using edgeR (5% false discovery rate). Signalling pathway analysis was carried out using Ingenuity software (IPA).

Results: IPA predicted activation of interferon (IFN) signalling in RA neutrophils, specifically that expression of 178 'IFN-response' genes was regulated by IFN- α , IFN- β or IFN- γ ($P < 0.01$). IPA also predicted activation of STAT transcription factors in RA neutrophils ($P < 0.01$), which was confirmed by Western blotting. Heterogeneous expression of IFN-response genes meant that patients could be categorised as 'IFN-high' or 'IFN-low'. IFN-high patients achieved a better response to anti-TNF therapy (Δ ADAS28, $P = 0.03$). Expression of IFN-response genes predicted a good response (EULAR criteria) to anti-TNF using ROC-curve analysis (AUC 0.76). Anti-TNF therapy caused an up-regulation of IFN-response genes in non-responders.

Conclusions: RA neutrophils had a gene expression signature indicating activation *in vivo* by interferons. The IFN signature was more evident in patients who achieved a good response to anti-TNF therapy, and was significantly up-regulated in anti-TNF non-responders by anti-TNF therapy. Expression of IFN genes may be a useful predictive marker of good response to anti-TNF therapy in RA patients.

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Gene expression profiling of cytokine stimulated neutrophils *in vitro*

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Background: Neutrophil priming is an important pre-requisite to the effective clearance of invading pathogens. Priming also precedes inappropriate neutrophil activation, which occurs in inflammatory diseases such as rheumatoid arthritis, asthma and COPD. Neutrophils significantly up-regulate discrete sets of genes in response to different priming agents. We characterised the gene expression profiles of neutrophils following priming with a range of cytokines associated with inflammatory disease, using RNA-Seq.

Methods: Neutrophil RNA was isolated following 1 h stimulation with (or without) GM-CSF (5 ng/ml), TNF- α (10 ng/ml), IL-6 (10 ng/ml), IL-8 (100 ng/ml), G-CSF (10 ng/ml), IFN- γ (100U/ml) and IL-1 β (10 ng/ml). RNA was sequenced on the Illumina HiSeq 2000 platform and mapped to the human genome using tophat2. Gene expression analysis was achieved using cufflinks and cuffdiff (applying a 5% false discovery rate). Signalling pathway analysis was performed using Ingenuity (IPA).

Results: The number of genes with significant changes in the level of expression after priming by each cytokine were: GM-CSF (110),

TNF- α (82), IL-8 (10), G-CSF (191), IFN- γ (209) and IL-1B (29) (FDR < 0.05). Pathway analysis predicted differential regulation of key inflammatory pathways and activation of cytokine-specific transcription factors: TNF- α , and IL-1B activated NF- κ B; GM-CSF and IL-8 activated NUPR1 whilst G-CSF and IFN- γ activated STATs ($P < 0.01$).

Conclusions: Genes relating to different aspects of neutrophil activation are significantly regulated in response to different priming stimuli. The nature of the priming agent is therefore important in determining both the genes that are expressed and ultimately the phenotype of the resulting primed neutrophil.

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The role of non-coding antisense transcription in V(D)J recombination

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Background: V(D)J recombination is the crucial mechanism that generates the diverse immunoglobulin repertoire by recombining a variable (V), diversity (D) and joining (J) gene segments together on the immunoglobulin heavy chain (Igh) locus. This mechanism is catalyzed by the recombinase activating complex (Rag). Differential accessibility of Rag to the gene segments ensures that Igh recombination is restricted to the B lymphoid lineage and is an ordered process.

Non-coding antisense transcription extends across the distal V regions of the Igh locus prior to V-to-DJ recombination (Bolland *et al.*, 2004). We propose that the process of antisense transcription recruits distal V genes to a transcription factory containing the intronic enhancer. This may juxtapose the distal V gene segments to Rag and the previous recombined DJ segment, thus promoting V-to-DJ recombination.

Method: To understand the role of antisense transcription, a mouse model was generated where antisense transcription was ablated by inserting a transcription stop cassette, containing 4xpolyA sites followed by 12 lac operators, downstream of the V antisense promoter within the J606 V gene family.

Results: J606 antisense transcription is successfully reduced in our mouse model without causing a defect in B cell development. Reducing J606 antisense transcription also results in reduced recombination of downstream V genes. Using 3C to probe locus conformation, preliminary data suggests that regions in the locus that recombine less frequently interact less frequently with the Igh intronic enhancer.

Conclusion: Our findings suggest that the process of antisense transcription influences the immunoglobulin repertoire by recruiting distal V genes into close proximity to the previously recombined DJ segment.

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Do deregulated miRNAs in sarcoidosis target key pathways towards progressing disease?

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MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression, mainly by inducing mRNA degradation. Aberrantly

expressed miRNAs have been reported in lung tissue and peripheral blood mononuclear cells obtained from sarcoidosis patients. Yet, there is limited information about the miRNA profile in bronchoalveolar lavage cells and their targets.

Thus, we aimed to investigate miRNA expression of 25 candidate miRNAs (*miR-125*, *-126*, *-146a*, *-148a*, *-150*, *-155*, *-186*, *-199a*, *-202*, *-204*, *-206*, *-212*, *-214*, *-21*, *-222*, *-223*, *-24*, *-25*, *-302c*, *-381*, *-424*, *-503*, *-885-5p*, *let-7c*, *let-7d*) in unseparated bronchoalveolar (BAL) cells obtained from 77 sarcoidosis patients and 14 control subjects using quantitative RT-PCR. Furthermore, we compared miRNA expression profiles in patients with remitting and progressing sarcoidosis. Various computer algorithms to predict potential miRNA targets have been applied; special emphasis has been given to key regulatory pathways linked to sarcoidosis activity, such as IL-23/Th17, CXCR3, TGF β and WNT pathways.

We detected down-regulated *miR-202* and *miR-204* and up-regulated *miR-146a* and *miR-150* expression in patients with pulmonary sarcoidosis when compared to control subjects. Subanalysis of expression profiles in clinical phenotypes showed lower number of *miR-204*, *miR-155* and *let-7c* transcripts in patients with remitting sarcoidosis in comparison to those with progressing disease. We are currently applying sequence analysis to identify putative miRNA binding sites in target mRNA of regulatory molecules involved in sarcoidosis-related pathways as well as in those linked to progressing disease.

Our study revealed deregulated expression of *miR-202*, *miR-204*, *miR-146a* and *miR-150* in unseparated BAL cells obtained from sarcoidosis patients when compared to controls. In addition, we detected lower number of *miR-204*, *miR-155* and *let-7c* transcripts in patients with remitting sarcoidosis in comparison to those with progressing disease. Sequence analysis to identify the interaction between miRNAs and potential target mRNAs of molecules involved in the pathogenesis of sarcoidosis is ongoing.

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The role of miR-31 in human CD4⁺ T cell differentiation

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MicroRNAs (miRNAs) are small endogenous RNAs that play a major role in cellular differentiation by post-transcriptional suppression of protein coding genes through complementary base-pairing to mRNA. Disruption of the miRNA machinery, as well as loss or deregulation of certain individual miRNAs, severely impairs immune development and responses leading to disorders such as autoimmunity and cancer. This is particularly relevant to Th2-driven diseases such as asthma, where the CD4⁺ Th2 cell subset and associated cytokines IL-4, IL-5 and IL-13 are central to disease onset and exacerbation leading for instance to IgE production. Although T-cell polarization is well characterized, the role of miRNAs in T-cell differentiation is still unclear. We have previously described an *in vitro* model of human T-helper cell differentiation that results in highly polarized Th1/Th2 cells. Using microarray-based miRNA profiling of human Th1/Th2 subsets we have found that miR-31 is selectively expressed in Th2 cells (Cousins *et al.* unpublished data). Using lentiviral technology, we have developed a miR-31 overexpression and knockdown protocol in human naïve T-cells and have validated this using quantitative real-time RT-PCR. Furthermore, we demonstrate via multicolor flow cytometric analysis that miR-31 promotes Th2-type lineage determination under neutral conditions by hindering IFN- γ production and increasing IL-13 levels. We propose that miR-31 may regulate human T-helper cell lineage commitment promoting Th2 cell differentiation.

Skin Immunity: Malfunction and Manipulation

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Roasting enhances peanut allergic sensitisation across mucosal and cutaneous routes

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Background: Peanut sensitisation has emerged as the major food allergy in the West, in sharp contrast with Eastern Asia where peanut intake is equally common but its allergic sensitisation ranks low. High temperature roasting of peanuts, extensively used by the Western food industry, exerts oxidative stress on peanut proteins and has been hypothesised to contribute to their enhanced allergenicity. Further, peanut oil commonly extracted from roasted peanuts is found in cosmetic products and cutaneous route of sensitisation has been postulated to play a role in peanut allergy. To date, however, no experimental evidence has directly addressed these hypotheses, which the present work set as its objective.

Material and Methods: Endotoxin-free peanut extracts were prepared from raw and in-house or commercially roasted peanuts in sterile PBS, and were used in adjuvant-free subcutaneous, epicutaneous, and gastrointestinal administrations. Peanut antigen-specific antibodies were monitored by ELISA and mesenteric lymph node T cell responses were assayed by thymidine incorporation and cytokine release. IgE functionality was tested using RBL cells, and basophilic infiltration of lamina propria was measured by flow cytometric analysis. Fluoresceinated or unlabelled Ara h1, purified from raw or roasted peanuts, were used in antigen presenting cell stimulation and binding studies.

Results: Extracts from roasted but not raw peanuts sensitised mice after subcutaneous administration, demonstrated by peanut-specific IgE and robust gut-associated Th2 responses upon peanut feeding. Similarly, direct gastrointestinal gavage of roasted but not raw peanuts primed for IgG1 and functional IgE responses. Further, in a murine model of atopic dermatitis-like food sensitisation, roasted peanut extracts applied to calcipotriol-induced skin lesions resulted in higher allergic sensitisation than raw antigens, characterised by gut-associated Th2 cytokines and eosinophilic infiltration of the lamina propria post-feeding. SC and EC peanut sensitisation resulted in more robust IgE and gut Th2 responses upon feeding than GI sensitisation. Roasted peanut extracts did not trigger signalling through conventional pro-inflammatory pattern recognition receptors, but bound avidly to dendritic cells via association with advanced glycosylated end product receptors.

Conclusions: We conclude that roasting increases the intrinsic immunogenicity of peanut antigens, potentially through the generation of oxidation-related ligands that target them to innate pattern recognition receptors, driving allergic sensitisation. This together with exposure via an efficient cutaneous sensitisation route carries important implications for peanut allergy in the West.

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T cell participation in autoreactivity to NC16a epitopes in bullous pemphigoid

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Introduction: Bullous pemphigoid is a blistering skin disease characterized by autoantibodies directed against the NC16a domain of bullous pemphigoid 180.

Aim: To characterize and map the fine specificity of T cell responses to NC16a.

Methods: PBMC from a total of 28 bullous pemphigoid patients and 14 matched controls were tested for proliferative and cytokine responses to recombinant NC16a and a complete panel of 21 overlapping peptides spanning this region of BP180.

Results: Proliferative responses to NC16A and the peptide panel in the patients with active disease were similar in frequency and magnitude to those in healthy donors, and included late responses typical of naive cells in about 60% of each group. IL-4 responses were slightly stronger for 6 peptides in patients than controls. Factor analysis identified factors that separate responses to the peptide panel discretely into IL-4 (Th2), IFN γ (Th1) and IL-10 or TGF β (Treg). Factors segregating IL-4 responses from TGF β were predicted by whether a patient or control, while factors separating IL-4 versus IL-10 were predicted by active blistering or remission. Finally, we confirmed a significant upregulation of IgE responses to BP180 in the patients with pemphigoid.

Conclusion: There are complex patterns of T cell phenotype and fine autoreactive specificity in responses to NC16A, found in patients and normal controls. However, specific IL-4 and IgE responses show the strongest associations with pemphigoid, suggesting an important contribution of Th2 cells to pathogenesis.

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The role of the common gamma chain in keratinocytes

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Severe combined immunodeficiency (SCID) is a group of genetic disorders characterised by defects of cellular and humoral immunity especially by the absence of functional T lymphocytes which leads to life-threatening infections. Currently, the only curative therapies are bone marrow transplantation (BMT) and gene therapy (GT). However, even after successful BMT or GT a significant number of patients develop chronic severe human papilloma virus (HPV) infections of the skin. Patients suffering from this susceptibility have mutations in either common gamma chain cytokine receptor subunit (also known as IL2RG) or Janus kinase-3 (Jak-3), but not mutations leading to other forms of SCID suggesting that IL2RG/Jak-3 signalling plays a role in anti-HPV immunity. As keratinocytes are not replaced by BMT or genetically corrected by GT and HPV infection is limited to these cells, we hypothesize that residual keratinocyte common gamma chain deficiency could result in persistent HPV susceptibility.

A role for IL2RG is implied by the fact that stimulation of keratinocytes with IL-15 which needs the common gamma chain to signal results in proinflammatory responses, leading to leukocyte recruitment. However, few papers have shown the expression or functionality of IL2RG in keratinocytes. We, therefore, first confirmed the

expression of IL2RG in primary keratinocytes and in NIKS (Normal Immortal KeratinocyteS, a human keratinocyte cell line) by reverse transcription PCR (RT-PCR) and flowcytometry. We then stimulated NIKS with IL-2 and results showed an increased phosphorylation of STAT5 in challenged cells compared to unchallenged cells.

We further examined the expression of IL2RG co-receptors including IL-2RB, IL-4R, IL-7RA, IL-9R and IL-15RA in primary keratinocytes and NIKS by RT-PCR, FACS and Western blot. All co-receptors were expressed in keratinocytes in both mRNA and protein levels.

These results indicate that the common gamma chain is expressed and functional on keratinocytes. In addition, not only IL2RG but also its co-receptors are expressed and functional on keratinocytes, suggesting that IL2RG signalling may play an important role in skin immunity mediated by these receptors.

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Electric fields – novel modulator of macrophage migration and phagocytosis

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Background and Aims: Understanding the signals and factors that control wound repair is important for devising new therapies to accelerate efficient healing in chronic wounds. Small electrical fields (EF) occur naturally at wounds where they enhance healing of damaged tissue. Moreover, synthetic devices based on EFs decrease infection and augment healing in persistent wounds but the mechanisms for this are unknown. To date the focus has been on defining how EFs control re-epithelialisation for wound closure, and effects on immune cells have been largely overlooked. Macrophages play important roles in wound healing by clearing apoptotic cells and debris, secreting pro-healing mediators and controlling infection. The aims of this study were to determine whether physiological EFs influence macrophage functions such as migration and phagocytosis and to explore the underlying cellular mechanism.

Methods: Human monocyte-derived macrophages were seeded into custom built chambers and exposed to physiological strength (50–450 mV/mm) direct current EFs for 2 h. Macrophage migration was assessed from serial time-lapse images recorded during the application of the EFs. Changes in calcium levels were measured using Fluo4-AM esters and time-lapse imaging. Phagocytic uptake of apoptotic neutrophils, carboxylate beads or *Candida albicans* was compared with or without 2 h EF exposure.

Results: Time-lapse microscopy revealed EF-polarised macrophage migration directed predominantly anodally at EFs as small as ~50 mV/mm, peaking around ~300 mV/mm. Although the directional migration was significantly different from macrophages with no EF, the overall speed of migration and distance migrated were not altered. EFs did not affect cell viability, or induce apoptosis. Importantly, phagocytic uptake of carboxylate beads or *Candida albicans* was consistently and significantly up-regulated in response to 150 mV/mm EFs, which approximate those measured at human skin wounds. Confocal microscopy showed substantial polarisation of actin to the anode-facing leading edge of macrophages after application of EFs. Intra-cellular calcium levels increased when macrophages were exposed to 150 mV/mm EFs. Preliminary experiments indicate that EF-induced PI3Kinase activity was important for enhanced migration and phagocytosis. Thus our results provide important new insights into how physiological EFs act as strong directional cues for macrophage migration *in vitro* and also how importantly they stimulate phagocytic activity.

Conclusions: These findings suggest a novel and additional mechanism by which EF-devices used in medical settings enhance wound

resolution and limit infection and suggest that EF exposure might be exploited to modulate other macrophage-mediated diseases.

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IL-1: intracellular regulation by degradation in dendritic cells

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Interleukin (IL)-1 α (IL-1 α) and IL-1 β are key players in the innate immune system. The expression of these cytokines by dendritic cells (DC) is integral to the development of pro-inflammatory responses. These cytokines are not secreted via the classical secretory pathway. Instead, 2 independent processes are required in DC; an initial signal to induce up-regulation of the precursor pro-IL-1, and a second signal to drive cleavage and consequent secretion of the bioactive molecule. It is postulated that the requirement for 2 signals allows for greater control of IL-1 production. In these investigations, we have sought to characterize the intracellular regulation of IL-1 in mouse bone marrow DC (BMDC). DC were cultured in GM-CSF for 8 days to generate an immature BMDC population. Cells were stimulated with lipopolysaccharide (LPS; 0.1 μ g/ml) and IL-1 α and IL-1 β content of supernatants (secreted cytokine) and cell lysates (intracellular cytokine) were analysed by ELISA. The impact of cyclohexamide, the proteasome inhibitor MG132, and the autophagy inhibitor 3-methyladenine on IL-1 production was examined. Immunoprecipitation and Western blotting were performed to examine the role of ubiquitination. Treatment with LPS stimulated a marked increase in intracellular IL-1, that peaked at ~8 hr and declined rapidly thereafter, without concomitant detection of secreted product. It was concluded, therefore, that IL-1 α and IL-1 β products were rapidly degraded following stimulation of DC with LPS.

Examination of the kinetics of the response using a 1 hr pulse of cyclohexamide (10 μ g/ml) to inhibit *de novo* translation revealed that there was a 4-h lag before IL-1 degradation was detected. Treatment with MG132 (10 μ M), but not with 3-methyladenine (10 mM), significantly inhibited degradation of IL-1 without impacting upon cell viability or cytokine expression. Furthermore, following inhibition of degradation using MG132, Western blotting of IL-1 β immunoprecipitates from cell lysates with anti-ubiquitin detection antibodies revealed an accumulation of ubiquitinated cytokine. In summary, these data indicate that IL-1 α and IL-1 β conform to the ubiquitin-proteasome paradigm of protein degradation and that this process is initiated as a consequence of DC activation in the absence of secretion. It is postulated, therefore, that regulation of IL-1 proteasomal degradation may serve to control the vigour of IL-1 secretion, and ultimately may influence the potency of pro-inflammatory responses.

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Local concentration of interleukin-1 β determines cytokine requirement for Langerhans' cell migration

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Langerhans' cells (LC) are epidermal dendritic cells. Upon antigen/allergen recognition they migrate from the epidermis to the local lymph node where they initiate and regulate immune responses. We have shown previously that LC migration induced by topical exposure to the contact allergen oxazolone requires two independent cytokine signals delivered by tumour necrosis factor (TNF)- α and

interleukin (IL)-1 β . In contrast, however, another contact allergen 2,4-dinitrochlorobenzene (DNCB) provokes migration independently of TNF- α signalling. Here we have investigated whether TNF- α -independent LC migration induced by DNCB is a function of the local availability of IL-1 β .

Mice (BALB/c strain) were treated topically on the dorsum of each ear with lactoferrin (an iron-binding glycoprotein that inhibits TNF- α production in the skin) in cream (or vehicle [cream] alone). After 2 h, mice received intradermal injections of either 50 ng or 300 ng IL-1 β at the same site. Control (naïve) mice were untreated. Mice were terminated 4 h later, ears were removed and the epidermis isolated and LC frequency determined by fluorescence microscopy. In parallel investigations, IL-1 β mRNA expression profiles in the skin were examined by quantitative real time PCR 6 h after treatment on the dorsum of the ear with either 0.5% oxazolone or with 1% DNCB.

Both doses of IL-1 β induced significant LC migration in the vehicle control mice compared with resting LC frequencies in tissue derived from naïve mice. As observed previously, the 20–30% of LC available for rapid mobilisation were stimulated to migrate. However, pre-treatment with lactoferrin, inhibited LC mobilisation provoked by administration of 50 ng of IL-1 β , with LC frequencies identical to those of naïve controls recorded. In contrast, 300 ng IL-1 β was able to induce LC migration, despite lactoferrin pre-treatment. Topical treatment with 1% DNCB provoked a very vigorous increase in IL-1 β expression compared with application of 0.5% oxazolone; concentrations of the allergens that in each case induce optimal LC migration. Taken together these data demonstrate that under conditions where local IL-1 β concentrations are very high, LC migration is independent of TNF- α , in contrast to the prevailing view that all stimuli that provoke LC migration (allergens, irritants, UVB and trauma) are dependent upon the local availability of this cytokine. These data also highlight that skin sensitising chemicals may act through subtly different mechanisms to provoke optimal LC migration.

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Prostaglandin E induces expression of the skin homing receptor CCR8 by human naïve T cells through EP4 and cAMP

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The localisation of memory T cells to human skin is essential for long-term immune surveillance and the maintenance of barrier integrity. We recently reported that the chemokine receptor CCR8 is highly expressed by skin memory T cells and that its expression is regulated by skin-specific factor(s) derived from epidermal keratinocytes. Further investigation revealed that the keratinocyte-derived CCR8-inducing factor(s) are soluble, independent of vitamins A and D, and insensitive to heat or enzymatic digestion suggesting that the factor(s) are not proteins. Consistent with this finding, the CCR8 inducing capacity of the epidermal factor(s) could be replicated by culturing newly activated naïve T cells with either prostaglandin E1, E2 or E3 signalling primarily through EP4 receptor-mediated activation of cAMP. These findings are consistent with a previous report suggesting a role for PGE2 in promoting the differentiation of a skin homing phenotype indirectly by suppressing the gut-imprinting capacity of dendritic cells. Together these data suggest that epidermis-derived PGE, produced at nanomolar levels in skin cultures, promote the imprinting of a skin homing phenotype in activated human T cells.

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Differential requirement for CCR4 in the maintenance but not establishment of the invariant V γ 5⁺ dendritic epidermal T-cell pool

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Thymocytes expressing the invariant V γ 5 $\gamma\delta$ T-cell receptor represent progenitors of dendritic epidermal T-cells (DETC) that play an important immune surveillance role in the skin. In contrast to the bulk of $\alpha\beta$ T-cell development, V γ 5⁺ DETC progenitor development occurs exclusively in fetal thymus. Whilst $\alpha\beta$ T-cell development is known to require chemokine receptor mediated migration through distinct thymus regions, culminating in medullary entry and thymic egress, the importance and control of intrathymic migration for DETC progenitors is unclear. However, we recently revealed a link between V γ 5⁺ DETC progenitor development and medullary thymic epithelial cells expressing Aire, a known regulator of thymic chemokine expression, demonstrating that normal V γ 5⁺ DETC progenitor development requires regulated intramedullary positioning. Here we investigate the role of chemokines and their receptors during intrathymic V γ 5⁺ DETC progenitor development and establishment of the DETC pool in the skin. We report that thymic medullary accumulation of V γ 5⁺ DETC progenitors is a G-protein coupled receptor dependent process. However, this process occurs independently of Aire's influences on intrathymic chemokines, and in the absence of CCR4 and CCR7 expression by DETC progenitors. In contrast, analysis of epidermal $\gamma\delta$ T-cells at neonatal and adult stages in CCR4^{-/-} mice reveals that reduced numbers of DETC deficiencies in adult epidermis are not a consequence of diminished intrathymic embryonic development, nor deficiencies in initial epidermal seeding in the neonate. Collectively, our data reveal differences in the chemokine receptor requirements for intrathymic migration of ab and invariant $\gamma\delta$ T-cells, and highlight a differential role for CCR4 in the maintenance, but not initial seeding, of DETC in the epidermis.

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Defining the lymphoid stress surveillance response in human skin

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Background and Aims: The skin contains a large number of tissue-resident immune cells increasingly acknowledged as having key roles in maintaining tissue integrity and excluding infection. One mechanism of such immune activation is lymphoid stress surveillance (LSS), whereby lymphocytes are rapidly activated by stress ligands expressed by tissue cells following cellular stress and that engage the NKG2D receptor. We lack a clear picture of whether this capability exists in human skin, which is fundamental to understanding our interactions with environmental agents. To investigate this we have characterised the human skin-resident lymphocyte compartment, and assayed the cells' functional responsiveness, with a view to ascertaining if certain T cells have the capacity to respond rapidly to tissue stress.

Methods: Normal adult human skin was obtained as discarded material after cutaneous surgery. Protocols were established to isolate and characterise lymphocytes by either (i) tissue disaggregation or (ii) three-dimensional explant culture with/without cytokine supplementation. Skin-resident lymphocytes were characterized by flow cytometry, including cell-surface receptor expression and cytokine production following *in vitro* activation.

Results: Following *ex vivo* isolation distinct skin-resident lymphocyte subsets were identified, including the 'unconventional' lymphocytes $\gamma\delta$ T cells and NK cells. Explant culture greatly increased lymphocyte yield, enabling detailed characterisation. In addition, culture conditions significantly amplified the $\gamma\delta$ T cell compartment. Skin-resident lymphocytes displayed skin-homing surface markers, a memory phenotype and high levels of the activation marker CD69. Lymphocytes differed in expression of co-stimulatory receptors, with CD8⁺ T cells, $\gamma\delta$ T cells and NK cells expressing NKG2D. Skin-resident T cells also constitutively expressed the inhibitory receptor PD-1. Upon *in vitro* activation, skin-resident lymphocytes readily produced cytokines and displayed distinct cytokine-producing profiles, with the response dependent on the strength of TCR-mediated activation. Skin-derived NKG2D⁺ lymphocytes had clear potential to produce both TNF α and IFN γ ; however, the molecular pathways leading to these cytokines differed with implications for clinical immunosuppression.

Conclusions: Skin-resident lymphocytes, including 'unconventional' populations, could be isolated from healthy human skin. Consistent with published data, cultured skin-resident $\alpha\beta$ T cells maintain a 'tissue-resident memory' (T_{RM}) phenotype. In addition there is the novel observation that skin $\gamma\delta$ T cell and NK cells have a T_{RM} phenotype. Distinct NKG2D⁺ lymphocytes were identified, which showed variable expression of additional activatory or inhibitory co-receptors, and were functionally competent with distinct cytokine-producing subsets. This system provides a platform to further characterise human skin-resident lymphocytes and their potential to be directly activated by surrounding tissue dysregulation.

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Functional relevance of innate type 2 immune signals for skin repair

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Recruitment and activation of immune cells are characteristic features of tissue repair. It is not completely understood to which extent type 2 cytokines are beneficial or disadvantageous for tissue remodeling and homeostasis. Moreover, the exact mechanism how immune cells interact with tissue resident cells is not clear. There is emerging evidence that IL-4R alpha mediated macrophage activation (M2) might be critical in the resolution of inflammation upon tissue damage and restoration of tissue function. To explore the role of type 2 cytokines for macrophage activation in tissue repair, we used myeloid cell restricted IL-4R alpha deficient mice and analyzed the healing response in skin. During the healing response, myeloid cell restricted IL-4R alpha deficient mice displayed a disturbed M1/M2 balance, with a shift towards a prolonged pro-inflammatory M1 and attenuated M2 activation. Dysregulated macrophage activation was associated with delayed wound closure and impaired granulation tissue formation, suggesting that type 2 cytokines are critical to develop a functional granulation tissue and to restore tissue integrity. More-

over, unexpectedly, myeloid cell restricted IL-4R alpha deficient mice showed an altered collagen fibril assembly and less pro-fibrotic collagen cross-links, suggesting that M2 macrophages have a significant impact on ECM formation in fibrosis. Generally IL-4/IL-13 have been described as pro-fibrotic, but their impact on macrophages activation in this condition is poorly defined. We hypothesise that macrophage activation controls the extracellular matrix architecture through a crosstalk with fibroblasts at the wound site. Accordingly, we will present detailed data revealing the function of IL-4/IL-13 signaling in inflammation, repair and fibrosis. Our results provide a direct link between innate type 2 immunity and effective restoration of tissue integrity and homeostasis.

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Mechanisms by which pathogens induce cytokines and cell death in human and mouse epithelial cells

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Background: Atopic dermatitis (AD) is a multi-factorial chronic relapsing inflammatory skin disease, where *Staphylococcus aureus* is a key trigger of disease flares. The exact mechanism by which *Staphylococcus aureus* triggers the inflammatory response in skin epithelium is still unclear. Previous work in this laboratory demonstrated that *Staphylococcus aureus* does not induce its pro-inflammatory responses via TLR. Recently it has been shown that *Staphylococcus aureus* produces extracellular vesicles (EVs) which induce inflammatory damage in the skin. The working hypothesis is that it is these EVs that lead to the skin inflammation in atopic dermatitis.

Methods: The effects of live and killed *Staphylococcus aureus* were assessed on 3T3- fibroblasts in terms of both their cytotoxic effect using microscopy and flow cytometry, as well as TSLP, IL-33 and TNF- α production measured by ELISA. The possible role of secreted virulence factors were then studied using a trans-well system.

Results: Live, but not killed *Staphylococcus aureus* induced Th2-promoting cytokines TSLP, IL-33 and TNF- α by 3T3-fibroblasts. Direct contact with live bacteria was not essential, as similar inflammatory responses were observed using a trans-well system.

Conclusions and Future Work: The results support the working hypothesis that factors secreted by live *Staphylococcus aureus* are important in inducing inflammation. Future work will involve characterising the virulence factors in terms of their heat stability, structure and protease activity. Their effect on other cell lines from mice (NC eczema mouse, MSM control mice), as well as skin fibroblasts and keratinocytes from children with and without atopic dermatitis will also be studied. Experiments will also be designed to track their interaction with the target cell by use of immune-fluorescent staining.

Stromal and Epithelial Regulation of Immune Homeostasis at Mucosal Surfaces

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Gain-of-function STAT1 mutation decreases STAT3-inducible gene transcription in patients with Chronic Mucocutaneous Candidiasis (CMC)

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Introduction: STAT3 activation triggers transcription of interleukin (IL)-17 which is crucial for mounting protective immune responses against fungi. Mutations affecting STAT3/IL-17 pathway resulting in selective susceptibility to *Candida* (fungal) infection characteristic of Chronic Mucocutaneous Candidiasis (CMC) have been reported. In CMC we reported defective Th17 responses (Ng *et al*, JACI 2010) and identified an underlying gain-of-function (GOF) STAT1 (rather than STAT3) mutation (van de Veerdonk *et al* NEJM 2011), leading to hyper-phosphorylation of STAT1 (Smeekens *et al* PLoSOne 2011). How this affects STAT3 or leads to decreased IL-17 production is not known.

Objective: To assess how GOF-STAT1 mutations affect STAT3 activation, DNA-binding and gene expression following cytokines stimulation.

Methods: PBMCs and EBV-transformed cell lines were stimulated with IL-23/IL-6/IL-21/IL-27/IFN α /IFN γ . Activation and binding of STAT1 and STAT3 was measured by Western blotting (WB), electrophoretic-mobility-shift-assay (EMSA) using the STAT-consensus binding element (hSIE) and TransAm STAT3 kit. STAT3-inducible gene (ROR γ , IL-17, IL-22, IL-10, c-Fos, SOCS3) and STAT1-inducible gene (CXCL10, IRF1) expression was assessed by qRT-PCR.

Results: GOF-STAT1 mutations lead to hyper-phosphorylation of STAT1 and prolonged dephosphorylation. These mutations do not affect STAT3 phosphorylation, nuclear accumulation and DNA-binding, but decrease STAT3-induced gene expression. Decreased STAT3-induced gene transcription could be rescued by restoring acetylation with histone deacetylase inhibitor (HDACi) or inhibiting STAT1 phosphorylation with fludarabine (a STAT1 inhibitor).

Conclusions: We report for the first time GOF-STAT1 mutations decrease STAT3-induced gene transcription, which can be restored by promoting acetylation and inhibiting STAT1 activation. These findings may explain decreased IL-17 production and susceptibility to fungal infections in CMC.

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IL-1/TLR signaling inhibitors in microscopic and ulcerative colitis: Immunopathogenic markers of active disease and remission

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Microscopic colitis (MC), comprising collagenous colitis (CC) and lymphocytic colitis (LC), is a common cause of chronic diarrhea. The etiology of MC is unknown but thought to involve an abnormal immune response to luminal agents. Innate immune recognition of

microbial products via Toll-like receptors results in increased expression of inflammatory genes, which must be tightly controlled to ensure sufficient clearance of pathogens but simultaneously avoid extensive tissue damage. We compared expressions of IRAK-2, IRAK-M, IL-37 and microRNAs (miR) -146a, -155 and -21 in colon biopsies of CC, LC and UC patients in active disease or in remission with non-inflamed controls by qRT-PCR. IRAK-M expression was increased in LC patients with active disease in histopathological remission (LC-HR; $P = 0.02$) and UC ($P = 0.01$) patients, but no differences in IRAK-2 expression were detected compared to controls. miR-146a, miR-155 and miR-21 expressions were increased in LC-HR ($P = 0.04$, 0.07 , and 0.004) and UC ($P = 0.02$, 0.04 and 0.03) patients. UC patients with active disease had significantly enhanced expression of miR-146a and miR-21 expressions compared to UC-R ($P = 0.01$ and 0.04). Likewise, active CC patients showed significantly increased expression of miR-155 ($P = 0.003$) and miR-21 ($P = 0.006$). IL-37 expression was decreased in both CC ($P = 0.03$) and LC ($P = 0.04$) patients with a similar trend in UC patients, albeit not statistically significant, whilst it was increased in UC remission (UC-R) patients compared to controls ($P = 0.02$) and active UC ($P = 0.001$). The identification of differentially expressed miRNA, IL-37 and IRAK-M indicate different pathophysiologic mechanisms in various disease stages in LC, CC and UC.

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Peyer's patch dendritic cells constitutively migrate to the mesenteric lymph node in a CCR7 and S1P dependent manner

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Background and Aims: Dendritic cells (DCs) are fundamental in controlling intestinal immune responses by migrating to the mesenteric lymph node (MLN) and priming gut-tropic effector or regulatory T cells. Furthermore, DCs direct the immune response in Peyer's patches (PP). Nevertheless, it is unclear if they contribute to an MLN mediated immune response by migrating and presenting PP derived antigen.

Methods: In order to determine if DCs are able to migrate from the PPs to the MLN two novel methods were used. First, Kaede mice were used. These mice ubiquitously express the photoconvertible Kaede protein that irreversibly changes its fluorescent emissions from green to red following exposure to violet light. During laparotomy, all visible PPs were exposed to violet light, and the presence of Kaede-red DCs in the MLN was assessed 24 h later. Second, FITC injections were performed to examine the molecular mechanisms that control the migration of DCs to the MLN. A specific volume of FITC was injected directly into PPs using a microinjector. This enabled labelling of cells within the PP without labelling the surrounding lamina propria. The presence of FITC⁺ DCs was then investigated in the MLN.

Results: Here, we provide evidence that a novel population of DCs migrate from PPs to the MLN. First, after photoconversion of PPs in fluorescent transgenic Kaede mice, converted DCs were found in the MLN- indicating that a population of DCs migrates from the PP to the MLN. This population expands in response to the DC specific growth factor, Flt3 ligand. Second, we use a novel injection technique to label cells within a PP with FITC, FITC⁺ DCs were then found in the MLN. Significantly fewer DCs migrated from the PP to the MLN in CCR7^{-/-} mice and in mice pre-treated with FTY720, but were present in SIP3-deficient mice.

Conclusions: We have identified a previously unreported population of DCs that migrate from the PP to the MLN by a CCR7 and S1P dependent mechanism. These cells may play an important role in driving immune responses in the MLN and their manipulation could lead to significant advances in increasing the efficacy of oral vaccines.

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An *in vitro* model for analysis of the impact of the colonic milieu in collagenous colitis patients on peripheral T lymphocyte activation and differentiation

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Soluble factors released by mucosal cells contribute to immune homeostasis in the gut. We investigated the role of soluble factors from the intestinal mucosa of collagenous colitis (CC) patients in regulation of effector T cells using a system that mimics the *in vivo* exposure of newly recruited peripheral T cells to soluble factors from the colonic milieu of normal individuals and inflamed CC patient mucosa.

Denuded colonic biopsies (DNB) and isolated lamina propria mononuclear cells (LPMCs) from CC patients and non-inflamed controls were cultured and conditioned medium (CM) were collected. Peripheral blood CD4⁺ T cells from healthy donors were polyclonally activated in the absence or presence of CM, and proliferation and cytokine secretion was analysed.

Peripheral CD4⁺ T cells exposed to colonic CM demonstrated reduced proliferation. This inhibition was reduced with DNB-CM derived from CC patients compared to non-inflamed controls. In contrast, LPMC-CM from non-inflamed controls inhibited T-cell proliferation less than LPMC-CM from CC patients.

Both DNB-CM and LPMC-CM from CC patients induced increased production of IFN- γ , IL-17A, IL-6, TNF- α , IL-4 and IL-10 from peripheral CD4⁺ T cells compared to non-inflamed controls.

Our preliminary data indicate reduced inhibition of proliferation as well as increased production of both inflammatory and anti-inflammatory cytokines by peripheral CD4⁺ T cells in the presence of mucosa-derived soluble factors from CC patients compared to controls. This model can be valuable in evaluating the effect(s) of existing and new drugs on T cell differentiation in the intestinal mucosa.

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Constitutive Type I Interferon, via STAT1 activation, promotes regulatory T cell function in the healthy human intestine, but not in inflammatory bowel disease

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Background: Control of T-cell reactivity with the human intestinal mucosa is poorly understood. Type I Interferon (T1IFN) signals via the JAK/STAT pathway, particularly STAT1, and has been shown to support Treg function in mouse models of colitis. T1IFN has been used as a treatment in Inflammatory Bowel Disease (IBD) with some success. We therefore hypothesised that constitutive T1IFN had a regulatory role in human intestinal T cells.

Methods: Endoscopic biopsies or resection specimens were frozen for immunohistochemistry (IHC) or cultured in the presence of neutralising anti-IFN β or isotype-matched control antibody. Cells were harvested, stimulated with anti-CD3/CD28 antibodies and analysed for cytokine production by intracellular staining and by multiplex ELISA of culture supernatants. Phosphorylated STAT1 was measured by flow cytometry with or without prior T1IFN stimulation. Frozen sections of colonic mucosa were stained with anti-IFN β and analysed using fluorescent IHC. Finally, CD3⁺ T-cells were FACS sorted and expression of Interferon Stimulated Genes (ISGs) determined by quantitative real-time PCR.

Results: IFN β was detected in the lamina propria of both control and IBD tissue and ISGs MXA and 250AS) were expressed by intestinal T cells sorted from tissue. *In vitro*, IFN β neutralisation reduced the frequency of pSTAT1⁺ intestinal T cells ($n = 6$, $P = 0.05$) and, in healthy controls, decreased the proportion of IL10-producing intestinal T cells ($n = 8$, $P = 0.01$). There was a trend for more IFN γ -producers ($P = 0.059$) and IFN γ concentrations in supernatants were significantly increased ($n = 10$, $P = 0.016$). In IBD, intestinal T cells were more responsive to IFN β *in vitro*, as assessed by ISG induction, ($n = 10$ for patients and controls, $P < 0.03$) and constitutive pSTAT1 was increased in T-cells isolated from non-inflamed mucosa of IBD patients compared with controls ($n = 30$ IBD, 16 control, $P = 0.03$). In contrast to control tissue, neutralisation of IFN β in non-inflamed IBD samples led to a generalised increase in cytokine production, with a significant increase in the frequency of T cells producing all cytokines examined (IL-10, IFN γ , IL-17 and TNF α , $n = 10$).

Discussion: T1IFN is present in the mucosa of the human intestine and in health may have a regulatory role by selectively supporting T cell production of IL-10. There is increased responsiveness of the T1IFN pathway in T cells from non-inflamed tissue from IBD patients (increased pSTAT1 and ISGs), associated with a generalised suppression of cytokine production. Thus, immunoregulatory effects of intestinal T1IFN are context dependent and may modulate inflammation in healthy and dysregulated IBD tissue by distinct mechanisms.

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Alterations in gut microbiota composition in tissues and feces of *Gzi2*^{-/-} mice with colitis in parallel with enhanced cytokine secretion

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A key factor in the pathogenesis of Inflammatory Bowel Diseases (IBD) is an altered gut microbiota composition. We applied non-culture techniques to analyze the microbiota of a *Gai2*-deficient mouse model of spontaneous colitis and their wild type (WT) littermates in colonic and caecal tissues and feces. Colitis was scored histologically, and was correlated to changes in both the gut microbiota and cytokine gene expression. Terminal Restriction Fragment Length Polymorphism (T-RFLP) targeting eubacteria, and qPCR of Bacteroides, Lactobacillus, segmented filamentous bacteria (SFB) and the *Clostridium leptum* group were utilized. Initial T-RFLP analyses indicate that the composition of the gut microbiota vary between WT and *Gai2*^{-/-} mice in different stages of colitis. Based on a short database constructed from fragment analyses of 16S rRNA genes

from several bacteria, we identified some phylotypes from the different mice. Our data indicate that *Bacteroides* dominate the microbiota in the colon of WT and *Gai2*^{-/-} mice with low colitis scores. In contrast, unknown phylotypes characterize the composition of the colon in *Gai2*^{-/-} mice with medium or high colitis scores. qPCR results indicate that *Bacteroides* were significantly enhanced in feces from *Gai2*^{-/-} mice with mild colitis, but reduced in colonic tissue from *Gai2*^{-/-} mice, both compared to WT mice. In the *C. leptum* group a significantly reduced amount was found in feces from *Gai2*^{-/-} mice with mild colitis compared to WT mice. The amount of *Lactobacilli* was significantly increased in caecal tissue from *Gai2*^{-/-} mice with mild colitis, but was significantly reduced in feces of the same mice compared to WT mice. Similarly, *Lactobacilli* were significantly reduced in colons of *Gai2*^{-/-} mice with severe colitis compared to WT mice. SFB were reduced in caecal tissues of *Gai2*^{-/-} mice with mild colitis, whereas mice with severe colitis had significantly increased amounts of SFB in feces.

The changes in microbiota composition were paralleled by significantly enhanced mRNA levels of IL-17 and IFN- γ in the same mice in colons and caeca of *Gai2*^{-/-} mice with increasing severity of colitis, compared to WT mice. IL-27 was significantly increased in colons of *Gai2*^{-/-} mice with moderate-severe colitis and in caeca of mice with moderate colitis. In contrast, IL-10 mRNA levels were significantly reduced in caeca of *Gai2*^{-/-} mice with severe colitis, but were unchanged in colonic tissues. TGF- β mRNA levels were not affected by the genotype nor the colitis score.

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Compartment specific tolerance and immunity in the human gut; properties and functions of dendritic cells and macrophages in the colon versus the ileum

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Background: Gut dendritic cells (DC) and macrophages (M Φ) mediate intestinal immune tolerance, and are central to maintaining the balance between immunogenicity against invading pathogens and tolerance to the commensal microbiota. DC drive primary T-cell responses and imprint homing properties on T-cells. Distinguishing DC from M Φ is problematic and information regarding antigen-presenting cells (APC) in the human gut is scarce. Furthermore, the majority of murine data refers to the ileum or organized lymphoid tissues. We aimed to compare human gut DC and M Φ within the colon and ileum.

Methods: Human DC and M Φ were isolated from colonic and ileal biopsies, and characterized by flow cytometry, immunohistochemistry and electron microscopy. DC generated T-cell responses in a mixed-leucocyte reaction.

Results: Intestinal DC were CD103⁺CX3CR1⁺CCR7⁺ whilst M Φ were CD103⁺CX3CR1⁺CCR7⁻. Compared to DC, M Φ expressed higher levels of activation marker CD40 and Toll-like receptors 2/4, were more phagocytic and produced more IL-1 β . Furthermore, M Φ were more responsive to LPS than DC; LPS induced greater induction of pro-inflammatory cytokine production in M Φ (IL-12, TNF α and IL-1 β). Upon comparison of colon versus ileum, ileal DC and M Φ expressed more CD40 than their colonic counterparts, with enhanced production of IL-12/TNF α /IL-1 β , but exhibited a lower unsaturated lipid content. Both ileal and colonic DC imprinted gut-homing marker β 7 on stimulated T-cells, but ileal DC displayed an enhanced ability to stimulate T-cells and imprint small-bowel hom-

ing marker CCR9 (these CCR9⁺ T-cells were negative for skin-homing marker CCR4). Colonic DC imprinted more CCR4. Numbers of "tolerogenic" CD103⁺CCR7⁺ DC were increased in the colon; whilst DC in all cases generated IL-10-producing and T-bet expressing T-cells, colonic DC generated more FoxP3⁺CD25⁺IL-10-producing- and IL-4-producing T-cells whilst ileal DC favoured production of IFN γ -producing T-cells.

Discussion: We demonstrate for the first time, specific, functional differences between human colonic and ileal APC. Homeostatic properties of gut DC and M Φ , alongside DC ability to polarize tissue-specific T-cell responses, are dependent on their anatomical location within the gut. The generation of gut-homing, IL-10 and T-bet expressing T-cells supports mouse studies demonstrating a gut-specific, regulatory role for intestinal DC. Colonic DC ability to generate regulatory T-cell or Th2 type responses, in contrast to ileal DC favouring Th1 polarization, may represent an evolutionary adaptation to the increased bacterial load in the colon. This study highlights the importance of regarding the ileum and the colon as separate entities, each comprised of APC with distinct roles in gut homeostasis and imprinting.

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Expression of cryptdins from primary mouse small intestinal epithelial cultures isolated from mice that lack beta defensin 14 is increased upon stimulation

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A primary mouse small intestinal epithelial cell culture system was established in the laboratory using the methodology developed by Hans Clever et al. using a combination of growth factors and using matrigel as a scaffold, long term culture were established. The intestinal cells form 'organoids' which contain crypts that bud from a central cyst structure. The organoids were imaged by electron microscopy and were found to display typical small intestinal morphology, such as microvilli, goblet cells and Paneth cells.

Murine beta defensin 14 (mBD14) is a homologue of human beta defensin 3 (hBD3), for which a role in inflammatory bowel disease has been suggested. However mice that lack mBD14 do not have an increase susceptibility to DSS induced colitis. The mBD14^{-/-} mice did however display a significantly increased expression of the a-defensin cryptdin 4 compared with wildtype mice.

We utilised the organoid culture system to compare the expression of defensins in organoids extracted from WT mice and mice that lack mBD14. In agreement with the *in-vivo* model, cryptdin 4 was more highly expressed in organoids from mBD14^{-/-} mice compared with WT mice. Upon stimulation with TNF α or IFN γ expression of cryptdin 1 and cryptdin 4 increased in the organoids from mBD14^{-/-} mice but not from WT mice.

These results suggest that redundancy between mBD14 and cryptdin 4 may be a reason that no altered phenotype was observed in the DSS colitis model in the mBD14^{-/-} mice. Results from the organoid culture compliment the results obtained *in-vivo*.

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Gut resident macrophages require constant replenishment from circulating monocytes and fail to self-maintain locally throughout adult life

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Background and Aims: The origin of tissue macrophages (mφ) has been an area of intense debate in recent years. Although traditionally thought to derive from blood monocytes, several recent publications have proposed that all tissue mφ arise from yolk-sac (YS) and/or foetal liver precursors, with little or no involvement of blood monocytes. During development, these precursors seed tissues before the onset of bone marrow (BM) haematopoiesis and then maintain local mφ via self-renewal *in situ*. However this concept has never been studied in the intestine, which contains the largest population of tissue mφ in the body. Here we have investigated the relative contribution of YS and adult BM derived precursors to the intestinal mφ pool and explored whether the presence of the microbiota influences intestinal mφ.

Methods: CX3CR1-GFP reporter mice were used for phenotypic analysis of mφ in mouse colon. For adoptive transfer studies, Ly6C^{hi} BM monocytes from CX3CR1-GFP mice were transferred into CCR2^{-/-} mice. For parabiosis studies, congenic mice were surgically joined and analysed after 11 weeks. For BM chimeric studies, CD45.1/CD45.2 were lethally irradiated, reconstituted with WT and CCR2^{-/-} BM at 50:50 ratio and analysed after 8 wks.

Results: Using a combination of immunophenotyping, lineage tracing, BM chimeras, parabiosis and adoptive transfer of BM precursors, we demonstrate that the adult intestinal mφ pool is entirely dependent on CCR2 dependent replenishment by circulating Ly6C^{hi} monocytes. Mφ with the phenotype of YS mφ are present in the neonatal intestine, but by adulthood these are almost completely diluted out by BM-derived mφ. Although *in situ* proliferation of colonic mφ occurs during the first 2 weeks of life, adult colonic mφ do not divide *in situ* and the expansion of mφ after weaning is due to a influx of monocytes which is then maintained at low levels throughout adult life. After arrival in the mucosa, the Ly6C^{hi} monocytes differentiate locally into highly phagocytic, IL10-producing CX3CR1^{hi} mφ, which are hyporesponsive to TLR stimulation. As germ-free mice have significantly fewer mucosal monocytes and mφ, the recruitment of monocytes and their subsequent differentiation into mature mφ is clearly influenced by the microbiota.

Conclusions: We have shown definitively that unlike other tissue mφ, those resident in the gut wall require constant replenishment by circulating Ly6C^{hi} monocytes and this is driven partly by the microbiota. These results emphasise the intestine's need for constant monitoring by flexible myeloid cells and indicate that individual tissues require unique mechanisms of mφ replenishment.

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Four subsets of dendritic cells, with distinct functional profiles exist independently of macrophages in the intestine and develop from a single precursor

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Background: Although mononuclear phagocytes (MPs) such as dendritic cells (DCs) and macrophages (mφs) are central for immune responses in the gut, the nature and functions of the individual cell

types has been contentious, largely due to the use of non-specific and overlapping markers such as CD11c and MHCII. Distinction between DCs and mφs is crucial, as they play very different roles in intestinal immune responses. While DCs are essential for the induction of immunity and tolerance by sampling antigen, migrating to the mesenteric lymph nodes (MLN) and presenting the antigen to naive T cells, mφs are sessile and act as local scavengers or mediators of inflammation. Here we have developed more rigorous methods for characterising intestinal MPs, which reveal crucial differences between the populations.

Methods: DCs and mφs from mouse small intestinal lamina propria (SI LP) were analysed by multi-parameter flow cytometry, q-PCR and by assessing their ability to prime naive T cells *in vitro*. Their ontogeny was determined using KO mice and adoptive transfer of BM-derived progenitors. MPs in intestinal lymph were collected by thoracic duct cannulation after mesenteric lymphadenectomy.

Results: By defining DC populations based on their ability to migrate in lymph and lack of expression of the mφ markers CD64 and F4/80, we can identify four distinct subsets of intestinal DCs based on their expression of CD103 and CD11b. All these DC subsets are distinct from mφs, being *zbtb46*⁺, dependent on Flt3L and lacking phagocytic activity. In addition they arise from the common pre-DC precursor via a process of local differentiation and *in situ* proliferation, while intestinal mφs are monocyte-derived and do not proliferate *in situ*. Although all the DC subsets can prime naive CD4⁺ T cells *in vitro*, they have distinct functional roles, with the CD103⁺ subsets being more efficient at driving FoxP3⁺ Treg, while CD103⁺CD11b⁺ DCs induce Th17 cells preferentially. Mφs lack these T cell priming abilities. Importantly, we can identify analogous populations in human intestine based on the expression of CD103 and SIRPα expression.

Conclusions: Four populations of migratory DCs exist in the steady state intestinal LP which are distinct from tissue resident mφs and which have distinct functional profiles. By demonstrating the need for precise gating strategies, these studies help explain some of the controversies in the field and highlight how definition of individual DC subsets may provide important insights into intestinal immune function and human disease.

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Purinergic P2X7 receptors signalling initiates inflammation against *Toxoplasma gondii* infection

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Purinergic P2X7 receptor (P2X7R), an adenosine triphosphate-gated receptor, is widely distributed in various body systems and has been reported to regulate immune cell functions. However, whether P2X7R is necessary to initiate gut inflammation is unknown. Thus, the aim of this study was to clarify the role of P2X7R in the initiation and development of small intestinal inflammation using a physiological model of ileitis- *Toxoplasma gondii* infection. We found that both P2X7R-knockout (KO) and the control littermate mice had similar body-weight loss during infection but P2X7RKO mice developed more severe pathology of small intestine with less CD11c⁺ cell infiltration. Compared with control mice, there was a significant decrease in the early mucosal infiltration of CD103⁺CD11b⁺ dendritic cells (DCs) but not CD103⁺CD11b⁺DCs into the epithelial layer and lamina propria of P2X7RKO mice. One possibility for the reduction in DC recruitment in the absence of P2X7R is a lack of epithelial responsiveness and indeed we observed reduced levels of epithelial CCL5 and CCL20 in P2X7RKO mice in response to infection. Further studies looked at the resultant adaptive immune response. Strikingly, fewer IFN-γ⁺ CD4⁺ T cells and declined

serum IFN- γ levels were noted in P2X7RKO mice, which suggested a weakened Th1 immunity against infection. Current work is addressing parasite load in these mice. In conclusion, P2X7R signalling is necessary for early recruitment of antigen presenting cells in the small intestine and may influence the induction of inflammation as well as the subsequent adaptive immune response.

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The role of the novel IL-1 family member IL-37 in Inflammatory Bowel Disease

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Inflammatory Bowel Disease (IBD) is characterized by an imbalance between the effector and regulatory arms of intestinal immunity, with a prominent inflammatory cytokine profile. Emerging evidence suggests that IL-1 family members play a critical role in the maintenance of gut homeostasis. Previous studies highlight IL-1 α , IL-1 β and IL-18 as pathogenic in the development of IBD. In contrast, IL-37, a novel member of the IL-1 family has been shown to have an anti-inflammatory function. A recent study employing humanized transgenic mouse model demonstrated a protective role of IL-37 in murine colitis. However the role of this cytokine in human disease and its mechanism of action remain unclear. In an effort to characterize the role of IL-37 in IBD, we examined the levels of expression of IL-37 in rectal biopsies from pediatric patients with ulcerative colitis (UC) and Crohn's Disease (CD). We found that IL-37 expression was significantly lower in IBD patients when compared to healthy control patients, particularly in children with CD. To investigate the significance of these findings we examined signals which regulate IL-37 expression in the colonic mucosa. Aberrant NOD2 signaling is strongly associated with CD and interestingly stimulation of colonic epithelial cells with a NOD2 agonist (MDP) enhanced IL-37 expression while ligands for TLR2 (Pam₃Csk4) and TLR4 (LPS) had no effect. High expression levels of pathogenic T cell derived cytokines IFN- γ , IL-17 and IL-22 were found in the colonic mucosa of IBD patients and the profound involvement of these cytokines in driving pathology in IBD prompted us to investigate their effect on the expression of IL-37. We have demonstrated that the expression of IL-37 is regulated by IFN- γ but not by IL-17 or IL-22. Treatment with IFN- γ significantly inhibits endogenous and MDP-induced IL-37 in colonic epithelial cells. In order to more closely investigate the mechanism involved in IL-37 induced immunosuppression we have employed over-expression system to demonstrate that IL-37 significantly reduces production of IL-6 and TNF- α in response to TLR or NLR stimulation. This anti-inflammatory effect is associated with reduced NF- κ B activation in IL-37 transfected cells.

These observations indicate a novel role for IL-37 in the homeostasis of the intestinal mucosa which is altered in IBD. Further investigation of signaling pathways involved in regulation of IL-37 activity may help develop novel therapeutic strategies for treatment of IBD.

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Epithelial autophagy dampens chronic intestinal inflammation

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Autophagy is a fundamental cellular process required for cytosolic degradation and nutrient recycling in response to starvation or stress

and is also involved in clearance of defective organelles, intracellular pathogens and protein aggregates. Recent genome-wide association studies (GWAS) linked polymorphisms in the autophagy genes *ATG16L1* and *IRGM* with susceptibility to inflammatory bowel disease (IBD). The aetiology of these chronic intestinal inflammatory disorders are poorly understood but thought to arise from an inappropriate immune response towards the microflora.

The mechanisms through which alterations in autophagy may influence chronic intestinal inflammation remain unclear. We hypothesise that autophagy proteins regulate intestinal homeostasis through distinct processes within different cell types of the intestinal mucosa. To address this experimentally, we analysed tissue-specific Atg16L1 deficient mice in a *Helicobacter hepaticus* driven model of chronic colitis.

Here we present that mice lacking Atg16L1 in the intestinal epithelium (Atg16L1^{Avillin}) are more susceptible to development of chronic colitis. However, despite severely aggravated histopathology in Atg16L1^{Avillin} mice, we did not observe an elevated adaptive immune response in the lamina propria. Furthermore, mediators of inflammation, such as the proinflammatory cytokines IL1 β , TNF and IFN γ were only elevated at early time points in Atg16L1^{Avillin} mice but not during the chronic phase of the disease. These findings suggest that an epithelial cell intrinsic circuit, regulated by the Crohn's disease susceptibility gene *ATG16L1*, prevents chronic intestinal inflammation.

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Leukocyte accumulation following bacterial peritoneal challenge is dependent on Death Receptor 3

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Background and Aims: Death Receptor 3 is the closest family relative to TNFR1 and has an established role in autoimmune and inflammatory disease, as well as immunity to bacterial and viral pathogens, through driving the development of effector T cell responses. Here, we investigated the function of DR3 during the early stages of bacterial challenge-induced peritoneal inflammation.

Methods: Early events in inflammation were investigated in *Staphylococcus epidermidis* supernatant (SES)-induced peritonitis (SESP) in mice genetically deficient in the DR3 gene (DR3^{ko}) and their DR3^{wt} littermates. Histochemistry was used to quantitate DR3 expression in the peritoneal cavity. Multiparametric flow cytometry was used to scrutinise early leukocyte infiltration in SESP. Chemokine protein and mRNA levels were determined in peritoneal lavage by ELISA and in the peritoneal membrane by quantitative RT-PCR, respectively.

Results: DR3 expression was detected in the peritoneal membrane. DR3^{ko} mice showed significantly reduced numbers of multiple myeloid and lymphoid subsets following challenge with SES. This was associated with significantly lower levels of a range of chemokines likely derived from both stromal and immune cells.

Conclusion: In addition to its function as an enhancer of effector T cell function, DR3 acts early in the immune response, regulating leukocyte accumulation into locally inflamed peritoneal tissue through the production of multiple, select chemokines.

The Ageing Immune System: Consequences and Interventions

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The role of SIRP α in regulation of dendritic cell homeostasis in the intestine

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Background: SIRP α transmits a negative signal after ligation by its ubiquitously expressed ligand CD47. Although SIRP α is expressed by most dendritic cells (DC) and macrophages, we have shown recently that mice with a non-signalling mutation in SIRP α have a selective defect in CD103⁺CD11b⁺ DC in the intestine, together with reduced numbers of Th17 cells in steady state mucosa. These results indicate that SIRP α may play an important and very specific role in controlling immune responses by regulating DC homeostasis. However the mechanisms underlying this effect are unknown and surprisingly, we have found that the defects in SIRP α mutant mice resolve with age. Here we have investigated how SIRP α impacts on the intestinal immune system under different conditions and have explored how its effects change with age.

Methods: DC subsets were examined in the small intestinal lamina propria (SILP) and mesenteric lymph node (MLN) of SIRP α and wild type mice from birth until 11 months of age, using flow cytometry and Q-PCR, while Th17, Th1 and FoxP3⁺ T cells were enumerated by intracellular cytokine staining. To induce systemic tolerance, mice were fed ovalbumin, while active immunity was induced by oral infection with *Citrobacter rodentium*, or by induction of DSS colitis.

Results: We show that SIRP α mutant mice have defects in CD103⁺CD11b⁺ DC in LP and MLN from weaning, but that these are resolved by 11 months of age. This is mirrored by a defect in Th17 cells in LP, whereas the numbers of Th1 and FoxP3⁺ T cells are normal throughout. SIRP α mutant mice show no signs of illness as they age, and young animals develop systemic tolerance normally after feeding OVA. They also have normal susceptibility to DSS colitis, but show reduced clearance of *C. rodentium* and reduced Th17 cell generation during infection.

Conclusions: SIRP α is important for the development and function of CD103⁺CD11b⁺ DC induced Th17 cell responses in the intestine and this renders them susceptible to bacterial infection. However other aspects of immune function are normal and the selective defects in DC and T cells resolve during later adult life. These results suggest that SIRP α is particularly critical for responses to intestinal bacteria and that compensatory mechanisms can be recruited after prolonged exposure to the normal microbiota. It will now be important to explore if the effects of ageing reflect specific changes in the composition of microbial populations in the intestine.

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The impact of antigen processing on CD8⁺ T-cell memory inflation

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Background and Aims: "Memory Inflation" was first reported in murine cytomegalovirus (MCMV) infection, but is also seen in a range of other virus infections. Certain epitope-specific CD8⁺ T-cell responses were noted to gradually increase over time, and be maintained as 'effector memory' populations in blood and tissues. This parallels the huge expansion of CD8⁺ T-cells seen in those infected with human CMV (HCMV), especially in the elderly, where such

exaggerated responses are thought relevant to immune senescence. In CMV positive individuals there is a reduced naive T cell frequency and diversity compared to seronegative individuals, with earlier progression of immune senescence. This has been shown to have an impact on the individual's ability to respond to other infections, and has been associated with increased mortality in murine studies and more recently in humans.

We are able to mirror memory inflation very accurately using an Adenoviral model based on a non-replicating vector encoding LacZ, within which one epitope induces memory inflation and the other conventional memory responses. We aimed to use this model to define the rules governing epitope-specific memory inflation.

Methods: We initially used an *in vivo* model, inoculated intravenously into C57BL/6 (B6) mice, comprising a recombinant non-replicative Adenovirus expressing beta-galactosidase under a CMV promoter (Ad-LacZ). Two responses (an inflationary "D8V", and a non-inflationary "I8V" response) were tracked using MHC class I peptide tetramers and intracellular cytokine staining (ICS). We tested the effect of varying:

- (i) the promoter
- (ii) antigen processing, using B6 and LMP7^{-/-} mice and novel minigene constructs
- (iii) and the route of inoculation.

Results: Ad-LacZ produces robust memory inflation, equivalent to that of CMV. The effect is not dependent on a CMV promoter. LMP7^{-/-} mice immunised with Ad-LacZ show independence of the inflating epitope to the immunoproteasome, but strong dependence of the non-inflating epitope, I8V. Critically, inoculation into B6 mice of a minigene construct (Ad-I8V) rescues inflation. This was seen using different routes of administration.

Conclusion: Ad-LacZ (a non-replicating vector) is a good model of memory inflation, regardless of the route of infection and the transgene promoter. Inflating epitopes are associated with immunoproteasome independence, potentially suggesting presentation on non-classical antigen presenting cells. We can, however, induce inflation to a "non-inflating" epitope by removing the requirement for processing. These data have implications for the design of vaccines, as well as CMV associated immune senescence.

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Cell surface markers of stress and senescence that signal phagocytosis – differential loss of α -2,3 and α -2,6 linked sialic acids from erythrocytes

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Introduction: Human erythrocytes typically live for 120 days and senescent cells are efficiently removed by macrophages at 3 million per second in an immunologically silent process. Loss of sialic acids on erythrocytes has been proposed to signal senescence and to promote clearance.

Methods: A discontinuous Percoll fractionation technique was adapted to minimise centrifugation stress on erythrocytes, while separating senescent erythrocytes on the basis of their increased density. Briefly, decreasing percentages of Percoll were layered gently on top of red blood cells generating seven fractions of erythrocytes.

Expression of α -2,3 linked and α -2,6 linked sialic acids on RBC was determined from binding of the respective lectins MAL and SNA using flow-cytometry and Western blotting.

To remove N-linked sugars, erythrocytes were incubated with PNGase enzyme (New England Biolabs) overnight at 37°.

Results: The densest erythrocyte fractions were confirmed to contain senescent cells, since they were more prone to haemolysis by hypo-

tonic stress and showed greater surface deposition of complement C3 and IgG. All erythrocyte fractions exhibited consistent α -2,3 linked sialic acid expression, but, in contrast, α -2,6 linked sialic acids were lost from the densest 1% of cells. PNGase treatment of fresh red blood cells to remove N-linked sugars mimicked the sialic acid profile of senescent erythrocytes and modulated the response of macrophages.

Damaged (Heat, calcium ionophore, CuSO₄, osmotic shock, glucose deprivation) red blood cells showed a different lectin binding profile, with significant loss of α -2,3 linked sialic acids especially noticeable.

Discussions: The results suggest a model whereby loss of α -2,3 linked sialic acids is a reporter of stress, while predominant α -2,6 sialic acids loss signals natural aging. These differential signals may help to elicit appropriate responses by macrophages.

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Impact of age on the function of CD57 defined NK cell subsets

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Introduction: The acquisition of CD57 on human peripheral blood NK cells has recently been associated with changes in the ability of these cells to respond to receptor mediated or cytokine driven signals. Furthermore, NKG2C+ NK cells, which expand in association with human cytomegalovirus (HCMV) infection, are known to express high levels of CD57. We therefore compared NK cell phenotype and activation in a cross sectional study to assess the influence of age on the phenotype and functional potential of CD57 defined NK cell subsets.

Methods: Individuals were recruited from the villages of Keneba and Manduar (The Gambia, W.Africa), according to age-stratified groups. Subjects included in the study include children (1–2, 3–5; 5–9; 10–12 and 13–15 years) and adults (16–19 years, 20–25 years, 26–40 years and over 40 years of age). $n = 20$ per group. Changes in the phenotypic characteristics of NK cells and NK cell subsets (CD56^{bright} and CD56^{dim}CD57, CD56^{dim}CD57^{intermediate} and CD56^{dim}CD57⁺) with age were analysed by flow cytometry and functional characteristics of these cell subsets was measured in response to target cells, receptor crosslinking and cytokines.

Results: NK cells expressing high CD57 increased in frequency with age whilst the proportion of cells with intermediate expression remained stable, consistent with this being a transient population, alongside a concomitant reduction in CD57⁻ cells. Increases in the frequency of CD57⁺ cells and in CD57 MFI were largely attributed to an expansion of NKG2C+ cells before the age of 9. Increases in CD57⁺ cells were associated with progressive loss NK cell responses to IL-12 and IL-18 (CD107a and CD25 and IFN- γ expression), whilst the ability to respond to receptor cross-linking or target cells by degranulation or CD25 expression remained stable with age. HCMV seroprevalence was high (98%); only four infants were seronegative. Whilst NK cells from these infants responded better to cytokines than age matched controls, there was no overall association between HCMV exposure or serum IgG titre and altered NK cell function across the cohort.

Conclusions: Increasing representation of CD57⁺ NK cells with age impacts on overall NK cell functional capacity. As the majority of individuals are exposed to HCMV by the age of 2, HCMV associated remodelling of NK cell functional phenotype may occur early and it

is likely that other factors contribute to the functional differentiation of NK cells in this cohort. This work is funded by the Medical Research Council - UK (G1000808).

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Identifying thymic epithelial progenitors for thymus regeneration to combat thymic atrophy

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The thymus provides a specialized microenvironment for the generation of self-tolerant T cells. Histologically the thymus is divided into two specialized regions: cortex and medulla. Both regions are composed of thymic epithelial cells (TECs). Blood-borne lymphoid progenitors enter the thymus via the cortical-medullary junction and migrate into the cortex. Cortical thymic epithelial cells (cTECs), which are classified by the markers Keratin-8, ERTR4, CD205 and Ly51, select thymocytes that express a functional T cell receptor (TCR). Then, developing thymocytes migrate from cortex to medulla to undergo negative selection. Negative selection is driven by medullary thymic epithelial cells (mTECs), which are defined by the markers Keratin-5, Ulex Europaeus Agglutinin-1, ERTR5 and MTS10. The thymic medulla removes potentially auto-reactive thymocytes to prevent autoimmune diseases. With advancing age, T cell output from the thymus decreases due to a loss of thymic epithelium and consequently it causes an increased risk of infection and a higher chance of autoimmune diseases in the elderly. Several studies have tried to harness thymic epithelial cells and their progenitors to overcome thymic atrophy, but with limited success. To combat thymic atrophy, we have studied the phenotype of bipotent TEC progenitors and TEC developmental pathways in early embryos. While current models indicate the developmental pathway for each lineage is completely separate from each other, our data suggest that there is a link between cTEC and mTEC development from progenitors in terms of phenotypical characterization and functional responses to lineage-specific stimulation. Specifically, we find that Aire+ mTEC can develop from progenitors expressing the cTEC marker CD205, and CD205+ progenitors acquire the expression of mTEC regulator Receptor Activator of NF- κ B (RANK) before losing their cTEC phenotype. Collectively, our data help clarify potential cellular targets for the re-establishment of functional thymus tissue.

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Memory deflation: impact of CMV on antiviral CD8 T cell memory pools

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Background and Aims: Many vaccination strategies aim to generate strong and long-lived CD8 T cell memory responses. In particular, non-replicating recombinant adenoviruses (rAd) have shown promise as novel vaccination constructs as they are able to generate strong memory CD8 T cell responses. However, most studies follow the CD8 T cell response after immunization in 'clean' uninfected mice. In real world situations many individuals harbour or may become infected with life-long persistent infections with the potential to interfere with the generation of new CD8 T cell responses.

Cytomegaloviruses (CMVs) are prevalent in both human and murine populations. During persistent murine CMV (MCMV) infection, the phenomenon of CD8(+) T cell memory inflation occurs, which is characterized by the accumulation of high-frequency, functional Ag-specific CD8(+) T cell pools with an effector-memory phenotype and enrichment in peripheral organs. We have recently

characterized an epitope in beta-galactosidase (termed β gal96) on a rAd vector (rAd-lacZ) that also invokes memory inflation in C57BL/6 mice. We therefore investigated the impact of CMV infection on rAd-driven T cell memory and vice versa.

Methods and Results: To address how rAd – generated responses develop in a background of persistent infection, we immunized mice persistently infected with MCMV with rAd-lacZ and measured the size of the β gal96 effector memory population generated. We found that pre-existing MCMV did not interfere with the generation of β gal96 CD8 T cell effector memory responses. Also, the generation of the large effector memory CD8 T cell population to β gal96 did not affect the size of the pre-existing MCMV-specific inflated CD8 T cell populations. By contrast, when mice previously immunized with rAd-lacZ were subsequently infected with MCMV, we observed a dramatic reduction in the size of the β gal96 population in blood, liver, lungs and spleen (e.g. in blood: from 7.34% to 1.06%, $P = 0.008$). The generation of inflated memory responses to MCMV was unaffected.

Conclusions: Our results indicate that while immunization with rAd against a background of persistent MCMV infection does not affect the magnitude and longevity of anti-rAd CD8 T cell effector memory responses, the acquisition of an MCMV infection post rAd immunization may be detrimental to the survival of the CD8 T cell effector memory response and thus the quality of protection afforded by rAd-based vaccines. Our data also suggest there is immunologic space for multiple inflated T cell responses but indicate a clear 'deflating' impact of primary CMV infection on existing memory pools.

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Defects in Fas, Bim, Bax render CD4⁺CD28^{null} T cells resistant to apoptosis and explain their accumulation in patients with coronary atherosclerosis

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Aim: Atherosclerosis is now widely recognized as a disease with an underlying immune deregulation. Patients with coronary atherosclerosis that develop myocardial infarction (MI) have an expansion of a unique subset of T lymphocytes, the CD4⁺CD28^{null} (CD28^{null}) T cells, characterized by the lack of the CD28 co-stimulatory receptor. These cells have a pro-inflammatory and cell-lytic phenotype. Patients harbouring high numbers of CD28^{null} T have increased risk of recurrent severe acute coronary events (i.e. MI) and unfavourable prognosis. Why CD28^{null} T cells accumulate preferentially in patients with MI compared to patients with stable angina (SA) and healthy individuals is currently unknown. T cell homeostasis is maintained by elimination of unwanted T cells via apoptotic cell death. We hypothesized that apoptosis pathways that mediate elimination of T cells are dysregulated in CD28^{null} T cells in patients with MI. Our aim was to investigate molecules involved in apoptosis regulation in CD28^{null} T cells in patients with coronary atherosclerosis (MI and SA).

Methods: Levels of pro-apoptotic (i.e. Fas, FasL, Bim and Bax) and anti-apoptotic (Bcl-2, Bcl-xL) proteins were measured in patients with MI ($n = 25$) and SA patients ($n = 18$) using flow-cytometry. Apoptosis sensitivity of CD28^{null} T cells to the Fas-ligating antibody CH11 and C2-ceramide was measured with annexin-V and 7-AAD.

Results: Pro-apoptotic molecules Fas, Bim and Bax were dramatically reduced on CD28^{null} T cells in patients with MI, whilst anti-apoptotic molecules Bcl-2 and Bcl-xL were similar in CD28^{null} and CD28⁺ T cells. Notably, CD28^{null} T cells in patients with MI showed significantly lower Bim and Bax levels compared to CD28^{null} T cells in SA patients. We found that CD28^{null} T cells in MI patients were

resistant to apoptosis induction via Fas-ligation or ceramide. Furthermore, we show that proteasomal inhibition restores apoptosis sensitivity of CD28^{null} T cells in patients with MI.

Conclusion: We show that CD28^{null} T cells in patients with MI harbour marked defects in molecules that regulate T cell apoptosis, which tips the balance in favour of anti-apoptotic signals and endows these cells with resistance to apoptosis. Furthermore, we propose a novel mechanism that implicates the proteasome as a key regulator of apoptosis sensitivity of CD28^{null} T cells in patients with MI. A better understanding of the molecular switches that control apoptosis sensitivity of CD28^{null} T cells may reveal novel strategies for targeted elimination of these T cells in patients with coronary atherosclerosis.

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Seven-colour flow-FISH assessment of telomere length within CD8⁺ and antigen-specific T cell populations

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Introduction: Techniques which assess telomere length provide information on the replicative history of a cell, and the remaining proliferative potential that the cell may have. Such analysis is useful when assessing T cell immunity in older age as the T cell pool is maintained by homeostatic proliferation of memory cells. Here we describe a 7-colour flow- fluorescent in-situ hybridisation (FISH) technique which allows analysis of telomere length within CD8⁺ and antigen-specific T cell subsets.

Methods and Results: To determine the telomere lengths of the cells in kbps, a standard curve was constructed using nine PBMC samples with a known telomere length, as determined by southern blot analysis of telomeric restriction fragment. Flow-FISH was performed and the samples run on a BD LSRII simultaneously with Quantum Cy5 MESF Beads. The Quantum Cy5 Beads standardise the Cy5 telomere probe fluorescent from the sample by converting the signal into molecules of equivalent soluble fluorochrome unit (MESF) values which were then used to construct the standard curve ($R^2 = 0.9189$, $P < .0001$). Conversion into MESF values allows accurate calculation of kbps from flow-FISH samples over time. Using this technique combined with CMV-specific tetramers, we assess the telomere length of different subsets within the total CD8⁺ and CMV-specific T cell populations as defined by CD45RA and CD27 expression; naive (CD27⁺CD45RA⁺), central memory (CM, CD27⁺CD45RA⁺), effector memory (EM, CD27⁻CD45RA⁺) and EMRA (CD27⁻CD45RA⁺) cells. Surprisingly we found that the telomere length for EMRA CD8⁺ T cells was either similar to or longer than both CM and EM cells in the young (< 40 years old) and in the old (>65 years old) (all $P < 0.05$). Similar results were gained when using CD28/CD45RA defined T cell subset analysis and within the CMV-specific CD8⁺ T cell compartment (all $P < 0.05$).

Conclusion: With the increased availability of heat stable antibody conjugates it is now possible to perform up to 7 colour flow-FISH. This facilitates the telomere analysis of multiple populations within one sample without the requirement of first sorting the specific cells. It is also possible to assess antigen-specific telomere length by combining the flow-FISH technique with tetramer technology. Using this technique we have found, somewhat surprisingly, that end-stage CD45RA re-expressing memory T cells have relatively long telomeres compared to other memory T cell subsets. This suggests that the senescence features of these cells are governed by a telomere-independent mechanism.

The Dynamics of Immune Cell Recognition and Signalling

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The differential roles of TNF- α receptor 1 and TNF- α receptor 2 in influencing the balance of pro- and anti-inflammatory cytokine output from monocytes

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Background and Objectives: TNF- α blocking strategies are employed in chronic inflammatory diseases such as rheumatoid arthritis, but not all patients respond and there is evidence of pro-inflammatory events in some patients on treatment. Monocytes are the principal producer of TNF- α and the anti-inflammatory cytokine IL-10. This study aimed to clarify the effects of TNF- α signalling through its two receptors, on inflammatory output from monocytes.

Methods: Monocytes from 19 healthy controls were stimulated with lipopolysaccharide (LPS) with or without monoclonal antibodies against TNF- α , TNF- α receptor 1 (TNFR1) or TNF- α receptor 2 (TNFR2). Supernatants/cell pellets were harvested for protein and mRNA quantification using ELISAs and qPCR. Flow cytometry quantified the cell surface expression of TNFR1 and TNFR2.

Results: Autocrine binding of TNF- α up-regulated expression of pro-inflammatory cytokines (IL-1 β , IL-8, IL-6) through TNFR1. In contrast, TNFR2 ligation up-regulated the anti-inflammatory cytokine, IL-10. TNFR2 was the predominant receptor in the basal state and bound the majority of soluble TNF- α secreted by monocytes, effectively clearing the cytokine from the pericellular environment. (All *P* values < 0.05.)

Conclusion: Current TNF- α blocking therapy, whilst preventing pro-inflammatory signalling via the ubiquitous TNFR1 may also inadvertently prevent beneficial IL-10 production via TNFR2 activation on leukocytes. Our data support selective TNFR1 blockade in treatment of TNF- α driven disease, switching off pro-inflammatory signalling whilst leaving TNFR2 available to remove soluble TNF- α from the pericellular environment and to up-regulate IL-10.

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Maximal activation for any given TCR/pMHC interaction is afforded at a defined pMHC/CD8 strength

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Introduction: CD8⁺ T-cells recognise abnormal cells for deletion via small peptide fragments presented on MHC molecules at the cell surface. T-cells recognise foreign or self pMHC via their unique $\alpha\beta$ TCR; a single TCR may recognise up to a million ligands. The TCR/pMHC interaction affinity can vary appreciably, with cancer ligands being typified by a weaker affinity interaction than those presented in viral challenge. The CD8 coreceptor is quintessential for induction of CD8⁺ T-cell activation when the presented ligand is characterised by a weaker affinity. CD8 has multiple roles in CD8⁺ T-cell activation; one of these is allowing the TCR to focus and fine-tune between different ligands. As a result, the TCR can switch between the plethora of ligands which it is able to recognise. The pMHC/CD8 interaction is characterised by extremely weak affinities;

altering these kinetics allows for variation in peptide ligand recognition.

Methods: Using tetramer technology, I have examined how pMHC recognition is affected when pMHC/CD8 is altered. I have also examined T-cell activation when the pMHC/CD8 interaction strength is altered.

Results: TCR recognition of pMHC at the cell surface increases as the strength of the pMHC/CD8 interaction is increased as measured by tetramer staining. However, maximal T-cell activation for a given TCR/pMHC interaction is seen at a pMHC/CD8 interaction strength, which varies conversely with ligand affinity.

Conclusions: Data demonstrates that the strength of the pMHC/CD8 interaction that affords a maximal response varies for different TCR/pMHC affinities. This has direct relevance for the optimisation of adoptive T-cell therapies.

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Storage-related changes on the surface of red blood cells

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Background and Aims: Several changes to the surface of red blood cells (RBCs) have been proposed to result from senescence and to occur during storage for transfusion. These changes allow recognition of RBCs by macrophages, targeting them for clearance from the circulation. However, the molecular basis of recognition remains unclear. We compared changes in the expression of candidate senescence markers CD47 and CD147, phosphatidyl serine (PS) exposure, and the levels of IgG antibody on RBCs as they age *in vitro* and *in vivo*.

Methods: We developed a murine model of RBC storage under conditions analogous to those used by the blood transfusion service, and a density-based method to fractionate RBC on the basis of age. Flow cytometric analyses determined levels of CD47, CD147 and PS and enzyme-linked antiglobulin tests were used to identify IgG bound to stored and fractionated RBCs.

Results: Levels of the putative anti-phagocytic ligands CD47 and CD147 on RBCs progressively reduced with storage, and a concurrent increase in exposure of the pro-phagocytic apoptotic marker PS occurred in some samples. Despite levels of IgG bound by stored RBC remaining low, there was evidence of an increase.

Conclusion: Results are consistent with a model whereby changes between anti- and pro-phagocytic signals determine senescent RBC clearance, exemplified by reduced CD47 and CD147, and increased PS expression. Although, age-related changes to the RBC anion channel band 3 have been suggested to allow increased IgG binding, this is a relatively weak effect. These findings are relevant to understanding adverse effects during transfusion.

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Identification of committed mast cell progenitors in mouse blood

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Background and Aims: Mast cell progenitors migrate from the bone marrow to the peripheral organs, in which they mature. Although committed mast cell progenitors in adult blood have been postulated, they have not been found in mice or humans. The aim of this study was to find out whether committed mast cell progenitors are

present in adult mouse blood and if these cells differ between mouse strains.

Methods: Single cells of different populations were isolated using gradient centrifugation and two consecutive fluorescence-activated cells sorts. The isolated cells were cultured in a cytokine cocktail, which allowed differentiation into all myeloid lineages. The lineage commitment of the cultured cells was evaluated after 4 and 14 days.

Results: A population of lineage⁻ c-kit^{hi} ST2⁺ integrin $\beta 7^{\text{hi}}$ CD16/32^{hi} cells, constituting 0.0045% of the blood mononuclear cells, exclusively gave rise to mast cells after culture. Furthermore, the fraction of Fc ϵ RI⁺ cells among the lineage⁻ c-kit^{hi} ST2⁺ integrin $\beta 7^{\text{hi}}$ CD16/32^{hi} progenitors was higher in Th2-prone BALB/c than Th1-prone C57BL/6 mice.

Conclusions: We have identified committed mast cell progenitors in murine peripheral blood. These cells were more mature in Th2 than Th1 prone mice based on the expression of Fc ϵ RI.

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Characterization of memory CD4⁺ T cell responses to the dog allergen Can f 4 reveals a dominant epitope region

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Background and Aims: The recently characterized dog lipocalin allergen Can f 4 has turned out to be an important dog allergen in terms of IgE reactivity. However, the human T cell reactivity to Can f 4 has not been examined previously. The objective of this study was to characterize memory CD4⁺ T cell responses to Can f 4 in allergic and nonallergic individuals and to identify dominant T cell epitopes of Can f 4 suitable for peptide-based allergen immunotherapy.

Methods: Allergen-specific CD4⁺CD45RO⁺ memory T cell lines from allergic and healthy subjects were established with recombinant Can f 4 allergen and stimulated with 48 overlapping 16-m Can f 4 peptides. The proliferation and phenotype, as determined by the cytokine production and surface marker expression of the T cells, were analyzed.

Results: We observed a higher frequency and functional avidity of Can f 4 specific memory CD4⁺ T cells in the peripheral blood of allergic subjects. Moreover, we identified several epitope regions recognized by Can f 4-specific T cells in allergic subjects. The epitope region localized between the amino acids 43–67 of Can f 4 was recognized as immunodominant, as the T cell lines a majority of allergic donors with diverse HLA backgrounds recognized at least two overlapping peptides covering this region.

Conclusion: Can f 4-specific memory CD4⁺ T cells of allergic subjects differ functionally from those of nonallergic subjects and recognize a dominant epitope within Can f 4. A peptide containing the immunodominant epitope (p43-67) is a potential candidate for peptide-based immunotherapy of dog-sensitized subjects.

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Sphingosine 1-phosphate in ischemia-reperfusion injury: effects on trans-endothelial migration of neutrophils

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Sphingosine 1-phosphate (S1P), a bioactive lipid mediator and ligand of 5 G-protein coupled receptors, is involved in many cellular

processes including cell survival and proliferation, lymphocyte migration, and endothelial barrier function. Several studies report protective effects of S1P during ischemia-reperfusion injury (IRI) of different organs, such as myocardial, renal or intestinal IRI. Neutrophils are major mediators of IRI. The neutrophil - endothelium interaction and neutrophil trans-endothelial migration could be the targets of therapeutic approaches to IRI. The aim of this project is to assess whether S1P, or candidate drug analogues, can protect against inflammation during IRI by affecting neutrophil trans-endothelial migration, either by acting on neutrophils directly or indirectly through the endothelial cells.

The direct effects of S1P on isolated human neutrophils from healthy volunteers were assessed. Western blotting experiments showed that S1P signalling in neutrophils induces phosphorylation of ERK1/2, and enhances IL-8 induced phosphorylation. However, in chemotaxis assays, S1P pre-treated primary human neutrophils showed no altered migration towards IL-8 in comparison to untreated neutrophils. Moreover, in an *in vitro* flow-based adhesion assay, S1P pre-treatment did not have a significant effect on IL-8 induced neutrophil adhesion to VCAM-1 and ICAM-1.

Next, the indirect effects of S1P were assessed, using endothelial cells. When endothelial cell lines HMEC-1 and Ea.hy926 were treated with S1P and S1P receptor agonists, IL-8 production was induced, and adhesion molecules VCAM-1 and ICAM-1 were upregulated, as was measured by ELISA, RT-qPCR and flow cytometry. However, although S1P treated Ea.hy926 allowed more neutrophils to migrate during an *in vitro* trans-endothelial migration assay towards IL-8, compared to untreated cells, S1P treatment of HMEC-1 inhibited neutrophil trans-endothelial migration towards IL-8. Currently, experiments with primary HUVEC endothelial cells are being performed for comparison. Different S1P receptor expression profiles might explain the conflicting results.

In conclusion, functional assays indicated no direct effect of S1P on neutrophil migration, although S1P receptor signalling in neutrophils can activate MAPK/ERK signalling pathways and enhance IL-8 signalling. However, S1P can affect neutrophil migration indirectly, either by inducing IL-8 and adhesion molecules expression by endothelial cells, or by acting on endothelial cells to inhibit trans-endothelial migration of neutrophils. Experiments to assess effects on neutrophil adhesion to endothelium under flow conditions are currently under way. In the future, *in vivo* experiments in a mouse model will be performed to validate the *in vitro* results.

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Neutrophil recruitment and function in response to Alum adjuvant

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Background: New and improved adjuvants are essential for the development of novel vaccines. However, we have a very limited understanding of how adjuvants work *in vivo*, even for adjuvants in widespread clinical use, such as aluminum hydroxide (alum). Histological analysis has revealed that neutrophils comprise a significant part of the cellular inflammatory response to alum immunisation. Neutrophils are known to affect key cells involved in the adaptive immune response such as DCs, B cells and T cells, therefore we aimed to characterise the processes of neutrophil recruitment in response to alum and the role of these cells in alum adjuvant activity.

Materials and Methods: BALB/c mice were immunised with ovalbumin/alum (OVA/alum) and chemokine and chemokine receptor expression at the site of injection was determined using Taqman®

Low Density Arrays. Neutrophil recruitment to injection sites in response to OVA/alum was determined using flow cytometry and visualised with multi-photon microscopy. Neutrophils were depleted using the monoclonal antibody NIMP-R14 and specific T cell responses characterised by adoptive transfer of OVA TcR transgenic (DO11.10) x IL-4-GFP reporter T-cells (DO11.10x4get) into congenic recipients. B-cell antibody production was determined by ELISA and T-cell cytokine production was assessed using Luminex®.

Results: OVA/alum injection was found to induce rapid expression of several inflammatory chemokines, including CXCL1, CXCL2, CCL5 and the chemokine receptor CCR5 within 1–2 h of administration. Consistent with this expression pattern, *ex vivo* phenotyping revealed a significant influx of neutrophils 1–2 h following OVA/alum injection. This contrasted with the slower accumulation of cells in response to the microbial stimulus, LPS. Neutrophil depletion reduced the magnitude of the antigen specific T cell response following OVA/alum immunisation and also resulted in skewing of the T cell response from a characteristic Th2- towards a Th1- type response.

Conclusion: Overall our results demonstrate that neutrophils are rapidly recruited to the site of alum immunisation where they play an important role in directing the magnitude and phenotype of the evolving T cell response in the lymph node. Understanding how adjuvant induced inflammatory responses at the injection site influence changes in the downstream adaptive immune response will facilitate improved vaccine design.

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Role of the T cell receptor germline complementarity determining regions

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The bias of $\alpha\beta$ T cells for MHC ligands is not well understood. The germline complementarity determining regions (CDRs) of the TCR have been proposed to provide hardwired specificity for MHC ligands (1). Equally, the CD4 and CD8 co-receptors contribute to ligand restriction by co-localizing Lck with the TCR only when MHC ligands are engaged (2). To determine the importance of hardwired, germ-line encoded bias, the TCR complementarity determining regions were extensively diversified *in vivo* using V(D)J recombination (3). We show that engagement with MHC ligands during thymocyte selection and peripheral T-cell activation imposes remarkably little constraint over TCR structure. Such versatility is more consistent with an opportunist, rather than a predetermined, mode of interface formation. This hypothesis was experimentally confirmed by expressing hybrid TCRs containing immunoglobulin or TCR- γ chain germ-line CDRs which directed positive selection. This work has now been extended by making transgenic mouse lines expressing TCR beta with germline CDR1 & 2 replaced with glycine/serine linkers. Surprisingly, such TCRs engage MHC ligands during thymocyte development more effectively than those with CDR1 & 2 in germline configuration. These data suggest that the germline CDR structures function to moderate rather than promote engagement of TCR with MHCpeptide ligands.

(1) Yin, L. et al. *Immunol Rev.* (2012) 250: 49.

(2) Van Laethem, F. et al. *Trends Immunol.* (2012) 33: 437.

(3) Holland, S. J. et al. *Proc Natl Acad Sci U S A.* (2012) 109: E3111.

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The adjuvant potential of All-trans Retinoic Acid (ATRA) in humans

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Background and Aims: Diarrhoeal disease due to intestinal infection is one of the most important causes of morbidity and mortality in children in the tropics and there are few efficacious vaccines for prevention. Oral vaccines elicit diminished immune responses as low as 35% for Rotarix in Malawi. Recent animal studies demonstrated that ATRA up regulated CC chemokine receptor 9 (CCR9) and integrin $\alpha4\beta7$ increasing their gut tropism. It also up-regulated the IgA transport molecule polymeric Ig receptor (pIgR). We have recently demonstrated ATRA as an oral adjuvant in human participants. Adjuvants enhance immunity to vaccines by a variety of mechanisms. Here we look at the mechanisms of ATRA during oral vaccination.

Methods: Adult male volunteers aged between 20 and 57 years were randomized and vaccinated in the presence or absence of ATRA. Blood samples ($n = 28$) were collected and evaluated for effect of ATRA on expression of CCR9 and integrin $\alpha4\beta7$ using Flow cytometry. Jejunal biopsies were also collected from another group of volunteers ($n = 13$) and analyzed for changes in mRNA expression of pIgR using qRT-PCR.

Results: 43% of participants showed an up-regulation in both CCR9 and $\alpha4\beta7$ while 25% showed a down-regulation. We demonstrated a correlation in expression of CCR9 and $\alpha4\beta7$ in presence of ATRA and vaccine. Changes in mRNA expression of pIgR were varied some showing a huge fold increase while others had virtually no change.

Conclusion: The data suggest that ATRA has an effect on the expression of CCR9 and integrin $\alpha4\beta7$ and a greater response is seen in the presence of a vaccine. ATRA given on its own showed no change on pIgR.

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Macrophage c-Myc expression level predicts the risk of atherosclerosis and regulates disease progression

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Background: Macrophages acquire specialized phenotypes in response to signals from the local microenvironment that polarize them towards a specific activation state. In atherosclerosis, it has been suggested that different stages in disease progression are associated with presence of distinct macrophage subtypes. The prevalence of alternatively-activated macrophages (AAMs or M2) in early atherosclerotic lesions is proposed to be an anti-inflammatory and reparative mechanism that limits disease progression in the initial stages, while the accumulation of classically-activated macrophages (CAMs or M1) in the damaged vessel wall contributes to plaque expansion. Expression/activation of the transcription factor c-Myc has recently been linked to acquisition of an M2 phenotype.

Hypothesis: The contribution of c-Myc to many hematological malignancies and tumors is dependent on its level of expression. We

hypothesized that the level of c-Myc expression in resting peritoneal macrophages from apolipoprotein E-knockout mice (apoE-KO) fed standard chow (steady-state conditions) would predict the ability to mount an M2 response and the likely atherosclerosis burden in response to a high-cholesterol diet.

Methods and Results: Analysis of peritoneal macrophages isolated from apoE-KO mice under steady-state conditions revealed a normal (Gaussian) distribution of c-Myc mRNA expression, with most animals close to the mean but some animals with very low or very high c-Myc levels. The animals were subsequently fed a high-cholesterol diet for two months. Fat-fed mice that expressed the lowest levels of c-Myc in resting macrophages developed larger atherosclerotic plaques (by en face Oil Red O staining) than animals that expressed average levels, and animals expressing the highest levels of c-Myc developed the smallest lesions.

To investigate the role of c-Myc expression in the development of an anti-inflammatory macrophage response, we generated mice with macrophage-specific c-Myc deficiency by crossing c-Myc^{fl/fl} mice with *LysM^{cre/+}* apoE-KO mice, yielding c-Myc^{fl/fl} *LysM^{cre/+}* apoE-KO mice doubly deficient for c-Myc and apoE (Mq-c-Myc apoE-DKO mice) and control c-Myc^{fl/fl} apoE-KO littermates with intact c-Myc. We find that, compared with similarly treated control littermates, fat-fed Mq-c-Myc apoE-DKO mice mount a stronger inflammatory response characterized by higher levels of IL-6-producing Ly6C^{high} monocytes, Cxcl1-related neutrophilia, fibrin deposition (and even death) that results in greater atherosclerosis burden.

Conclusions: c-Myc mRNA expression level in peritoneal macrophages correlates inversely with atherosclerosis development in apoE-KO mice. Moreover, macrophage-specific c-Myc depletion significantly increases inflammation and atherosclerosis burden. These results identify c-Myc expression in macrophages as a predictor of atherosclerosis risk and suggest that increasing macrophage c-Myc expression has the potential to reduce atherosclerosis progression.

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Interaction of *Mycobacterium leprae* with Toll-like receptor-4 and the effect of BCG vaccination in macrophage response to *Mycobacterium leprae*

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Background and Aims: Toll-like receptor (TLR)-4 belongs to a family of pattern-recognition receptors that bind to microbial ligands. TLR4 is the only TLR which uses both MyD88- and TRIF-dependent signaling pathways. A role of TLR4 in the pathogenesis of several mycobacterial infections has been suggested. We hypothesized that *Mycobacterium leprae* binds to TLR4 leading to signal transduction and that TLR4 expression is modulated by *M. leprae*.

Methods: HEK-Blue 293 cells expressing TLR4 were stimulated with increasing concentrations of irradiated *M. leprae*. Peripheral blood mononuclear cells (PBMCs) of BCG and non-BCG vaccinated healthy volunteers were isolated. PBMCs were cultured in medium containing Macrophage colony stimulating factor (M-CSF). Attached macrophages were isolated after differentiation and were incubated overnight with increasing doses of *M. leprae* with or without neutralizing antibody for TLR4. ELISAs for pro-inflammatory cytokines were performed in the collected supernatants. RNA extraction, cDNA synthesis and Real-time PCR for TLR4-related genes were performed in *M. leprae* stimulated macrophages. Staining for macrophage markers CD14, CD16, CD68 and for TLR4 was performed by multicolor flow cytometry. The Median Fluorescence Intensity (MFI) for TLR4 expression was compared between BCG-vaccinated subjects and non-BCG.

Results: Stimulation of human macrophages with *M. leprae* led to TNF- α , IL-6 and CXCL10 cytokine production through the TLR4. Incubation of macrophages with *M. leprae* led to a modulation of several MyD88- and TRIF-dependent genes. In macrophages from BCG-vaccinated healthy volunteers the dose-response curve of % change of TLR4 MFI with increasing concentrations of *M. leprae* showed gradual down-regulation of the receptor. On the other hand, the % change of TLR4 MFI in non-BCG vaccinated healthy volunteers showed up-regulation of the receptor.

Conclusions: *Mycobacterium leprae* binds to TLR4 leading to signal transduction and cytokine production through both MyD88- and TRIF-dependent signaling pathways. TLR4 expression in macrophages is modulated after incubation with *M. leprae* in both BCG and non-BCG donors but in an opposite manner. BCG vaccination is normally expected to have an effect only on acquired immunity to mycobacteria but surprisingly here we showed that it affects innate immune cells as well by shifting the macrophage TLR4 response to *M. leprae*. This is a model for analysing TLR4 response *in vitro*. Further work on leprosy patients will validate these results.

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How do the lymphocyte receptors CD5 and CD6 regulate activation and inhibition of immune responses?

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CD5 and CD6 are co-receptors belonging to the scavenger receptor cysteine-rich superfamily that have both been shown to have activating and inhibitory function in T cells. Both receptors are implicated in diseases such as leukaemia and multiple sclerosis, but the exact niche of CD5 and CD6 in the immune response is not well understood. CD5 and CD6 contain a conserved serine and/or tyrosine phosphorylated SDSL motif that was shown to have activating and inhibitory effects in CD5. The goal of this project is to understand regulation by CD5 and CD6 at a molecular level by focusing on interactions with the SDSL motif.

Pulldowns with a serine and tyrosine phosphorylated CD5-derived SDSL peptide and subsequent tandem mass spectrometry analysis revealed potential interactions with activating and inhibitory SH2 domain containing T cell signalling proteins. A connection to the cytoskeleton via the ERM proteins ezrin and moesin was also identified. The interaction with ERM proteins was confirmed as direct by surface plasmon resonance and is dependent on tyrosine and serine phosphorylation of the SDSL motif.

Association of CD5 and CD6 SDSL directly or indirectly with activating and inhibitory signalling proteins could be an explanation for the varying effects of these receptors. In addition, the interactions with ERM proteins that are known to be important for the formation of the immunological synapse suggest a mechanism for the involvement of CD5 and CD6 in synapse formation. For a comprehensive analysis of the role of CD5 and CD6, future plans include examining functional effects of intracellular interactions, the localisation of CD5 and CD6 and the effects of engaging the extracellular regions of CD5 and CD6 during T cell activation.

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Activated bone marrow-derived mast cells from BALB/c mice exhibit LPS-specific effects on IL-9 production

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Mast cells express different TLR including TLR4, which recognizes lipopolysaccharide (LPS), a cell wall component from Gram-negative bacteria. Our previous work demonstrated that TLR4-induced signaling leads to the enhanced expression of inflammatory cytokines by IgE-activated mast cells, including IL-9, at least partly by boosting the activation of NF- κ B (Stassen et al., J. Immunol. 2001, 166:4391).

However, our knowledge about the regulation of IL-9 expression in mast cells is still scarce and deserves further investigation. Based on our unpublished observation that BMMC derived from C57BL/6 mice are low responders to LPS compared to BALB/c regarding the production of IL-9, we performed comparative analyses of BMMC from both strains to get further insight into the underlying mechanisms of IL-9 expression.

Thus, we generated BMMC derived from C57BL/6 and BALB/c mice. The output of cytokine production *in vitro* was assayed by ELISA. In qRT-PCR the expression of transcription factors, cytokines and other members of the LPS downstream signaling pathway were analyzed. Luciferase reporter gene assays specific for NF- κ B were performed.

In accord with our previously published work, LPS strongly promotes the production of IL-9 in IgE-activated BMMC derived from BALB/c mice. However, whereas the IL-9 production induced solely by IgE receptor signaling is comparable in BMMC derived from either BALB/c or C57BL/6, the latter only marginally respond to LPS as a cosignal for producing IL-9. Yet, the expression of IL-6, induced by IgE receptor signals in absence or presence of LPS, is comparable in BMMC derived from both strains. Production of IL-9 strongly depends on IL-1, and IL-1 mRNA expression induced by IgE and LPS is indeed lower in BMMC from C57BL/6, but IL-9 expression cannot be rescued by adding exogenous IL-1. To further explore the impact of IL-1 in regulating the IL-9 signaling cascade, we used the IL-1 receptor antagonist anakinra. BALB/c-derived BMMC express lower levels of IL-9 when anakinra is present, whereas IRF-4 and IL-18 mRNA expression is unimpaired.

Reporter gene assays revealed a stronger NF- κ B activation in BALB/c BMMC along with higher c-Rel mRNA levels in response to IgE and LPS. Furthermore, under these conditions, the expression of the transcription factor IRF-4 in BALB/c-derived BMMC is also increased. Although the underlying mechanisms are still partly obscure, higher activity of NF- κ B and expression of IRF-4 in BMMC from BALB/c likely explain stronger expression of IL-9.

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The effect of serum on mPr3 and CD177 expression on neutrophils

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Background: Proteinase 3 (Pr3) is a serine protease that is stored primarily in azurophilic granules and secretory vesicles in neutrophils. The high affinity Pr3 receptor, CD177, is expressed on a subset of neutrophils (ranging from 0–100%), but usually only half of the circulating neutrophils express CD177 in most normal individuals. As a serine proteinase, Pr3 is controlled by a variety of inhibitors, including alpha-1-antitrypsin (AAT), which is present in serum at 1.5–3.5 g/l. We investigated if the surface expression of Pr3 and

CD177 was affected by stimulation of neutrophils in the presence or absence of serum.

Methods: Neutrophil cells were isolated from healthy donors. The cells counted and stimulated with fMLP only or cytochalasin B followed by addition of fMLP, and compared to unstimulated controls, in the presence or absence of 100% autologous serum. The expression of Pr3, CD177, MMP-8 and MMP-9 was analysed by flow cytometry.

Results: The expression of membrane bound Pr3 (mPr3) by neutrophils was still detectable in the presence of serum however, the expression was reduced by 6-fold on both mPr3^{low} and mPr3^{high} cells in comparison to cells incubated in the absence of serum. No increase was observed in mPr3 expression following stimulation with fMLP in either the presence or absence of serum. In comparison, stimulation with cytochalasin B combined with fMLP resulted in a 9-fold increase ($P < 0.0001$) in the Pr3^{high} cells compared with unstimulated cells in the absence of serum. This increase was only 3-fold ($P < 0.05$) in the presence of serum.

Conclusion: Despite the ability of purified AAT to inhibit Pr3 binding to CD177, significant surface Pr3 was still found on the surface of CD177-positive neutrophils when stimulated in the presence of 100% autologous serum, but only following maximal stimulation of neutrophils.

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A new form of global leucopenia associated with mutation in the moesin gene

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In 5 young patients (1.3–15 years) from four different families, we observed a profound lymphopenia (181–660 CD3⁺/ μ l; 7–14 CD19⁺/ μ l) and low neutrophils count (0.2–1.4. 10^{-3} / μ l). Among the T lymphocytes, the naive compartment was particularly low and T cell proliferation *in vitro* is decreased as compared to the controls. All patients have a hypogammaglobulinemia and a poor response to vaccinal Ag. During childhood, most of them developed severe varicella, pneumopathies and recurrent pulmonary infections.

Using exome sequencing analysis, we have identified in all patients a unique missense mutation in the moesin gene (Xq11.1), introducing an amino acid change into the highly conserved FERM domain of the protein (p.R171W). Moesin is a member of the ERM protein family which link plasma membrane proteins with actin-based cytoskeletons and are implicated in various cellular functions such as survival, adhesion, migration and activation. This mutation is associated with a low protein expression especially in T cells. Upon *in vitro* activation, proliferation (as measured by CFSE analysis and cell cycle checkpoints analysis) and survival capacity of patient's T cells were impaired despite a expression of activation markers like CD25 and CD69. In addition, we observed enlarged synapses in patient's blasts.

We reported here, for the first time, the association of a defect in an ERM protein and a primary immune deficiency.

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Gly601 residue of human SARM is critical for interaction with other TLR adaptor proteins

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Background and Aims: Toll-like receptor (TLR) signalling is mediated by cytoplasmic adaptor proteins via Toll/interleukin-1 receptor (TIR) domains. Sterile α - and armadillo-motif-containing protein (SARM) is the fifth TLR adaptor protein identified in humans and has been described as a negative regulator of the innate immune response. The aim of this study is to investigate whether SARM interacts with other TLR adaptor proteins directly and identify amino acid residues critical for interaction.

Methods: The TIR-domains of human SARM and the other four TLR adaptor proteins, MyD88, MAL, TRIF and TRAM were expressed in *E. coli* and purified by affinity chromatography. GST pull-down assays were used to probe interactions between SARM-TIR and the other TLR adaptor proteins. Binding was assessed by Western blot analysis. Site-directed mutagenesis of SARM-TIR was used to generate a G601A mutant protein.

Results: Pull-down interaction assays indicate that affinity purified SARM-TIR binds to all other four TLR adaptor proteins under the conditions used, while alanine substitution of the Gly601 residue results in a complete loss of binding.

Conclusions: Results presented in this study indicate that the TIR domain of human SARM is able to interact with multiple TLR adaptor proteins and that the Gly601 amino acid residue located in the SARM BB-loop is critical for binding.

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The expression of Pr3 and CD177 on blood and salivary neutrophils

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Background: Peripheral blood neutrophils (bPMNs) and salivary neutrophils (sPMNs) are important cells that play an essential role in immunity and inflammation. In the oral cavity sPMNs have a major role against invading oral microorganisms with high numbers of sPMNs constantly migrate from the blood stream through the gingival crevice into the oral cavity. Their main function is to protect the oral environment from pathogens and prevent infection. Neutrophils contain several different proteases which are thought to play a role in aiding in transendothelial cell migration and are implicated in antimicrobial defence. The aim of this study was to compare intracellular and surface proteinase levels between bPMNs and sPMNs.

Methods: bPMNs and sPMNs were collected from healthy donors. The cells counted and stimulated with cytochalasin B followed by addition of fMLP, and compared to unstimulated controls. The expression of proteinase 3 (Pr3), CD177, metalloproteinase-8 (MMP-8), and MMP-9 were analysed by flow cytometry.

Results: The results showed that the percentages of surface Pr3 and CD177 expression on unstimulated bPMNs ranged from 0 to 100% in a given individual, but unstimulated sPMNs expressed only positive populations of CD177 and Pr3. The surface Pr3 expression on sPMNs was significantly higher than that on bPMN ($P = 0.001$). Moreover, Stimulation of bPMN and sPMNs from matched donors showed an 11-fold increase in surface Pr3 expression on bPMN, but no significant change in expression on sPMNs compared to unstimulated cells. The levels of intracellular Pr3 in sPMNs were detectable

however, they were significantly lower ($P = 0.0002$) than levels in bPMNs. The levels of MMP-8 and MMP-9 in sPMNs were almost negligible, indicating that most of these proteinases are released either through transmigration or due to contact with oral microbes. The intracellular level of Pr3 was significantly higher than the levels of MMP-8 and MMP-9 in sPMNs ($P < 0.0001$).

Conclusion: Migrated salivary neutrophils have released almost all of their MMP-8 and MMP-9 prior to collection and measurement. Only CD177-positive PMNs were found in the saliva, despite being bimodal in the periphery, and CD177 bound Pr3 on the surface of these. While intracellular levels of MMP-8 and MMP-9 were completely depleted in sPMN, detectable levels of intracellular Pr3 were still present, although they couldn't be mobilised by further cell stimulation.

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The duration of LPS priming determines the phenotype of macrophage, but not T cell P2X₇ responses

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Background: Both macrophages and dendritic cells are known to express functional P2X₇ receptors, and their stimulation results in a number of biologically important effects including antigen presentation, apoptotic and/or necrotic cell death, the release of IL-1 β , cell fusion and the release of microparticles. However, some confusion remains in the literature about the precise method of release of IL-1 β and about the nature of the response of these cells to P2X₇ stimulation. We explored the influence of LPS priming on the phenotype of the response of P2X₇-stimulated macrophages, as well as their profile of cytokine release.

Materials and Methods: Resident peritoneal macrophages were harvested from C57BL/6 mice by lavage with DMEM/10% FCS; prior injection with 4% thioglycollate was not required. Macrophages were allowed to adhere for 1-2 h, then washed, and maintained in DMEM-F12/10% FCS/penicillin/streptomycin at 37° and in 5% CO₂. Macrophages were analysed following 24-72 h of culture. T cells were obtained by dissection of peritoneal lymph nodes and were maintained in DMEM-F12/5% FCS, and were analysed by flow cytometry within 1 h of collection.

Results: Whereas T cells responded promptly to BzATP stimulation, wild-type macrophages were unresponsive by all criteria measured in the absence of LPS priming, including flow cytometric measures (changes in cell size, Ca²⁺ uptake and phosphatidylserine translocation), and morphologically on real-time imaging. Analysis with increasing durations of priming with LPS indicated a change in morphology towards a blebbing phenotype with release of microparticles and subsequent cell death upon BzATP stimulation.

Conclusion: The phenotype of macrophage response to P2X₇ stimulation is dependent on the duration of LPS priming. The majority of macrophages demonstrate PS translocation within 15 min of stimulation with BzATP for all durations of LPS priming, although there is a trend towards a greater proportion of cells translocating PS at higher durations of LPS priming. The percentage of cells undergoing blebbing is, however, different between the different durations of LPS priming, as are the rates of cell death. This provides new insight into the different mechanisms underlying P2X₇-induced IL-1 β release in murine macrophages.

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The phenotype of dendritic cell, but not macrophage, P2X₇ responses is independent of the duration of LPS priming

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Background: Both macrophages and dendritic cells are known to express functional P2X₇ receptors, and their stimulation results in a number of biologically important effects including antigen presentation, apoptotic and/or necrotic cell death, the release of IL-1 β , cell fusion and the release of microparticles. However, some confusion remains in the literature about the precise method of release of IL-1 β and about the nature of the response of these cells to P2X₇ stimulation. We explored the influence of LPS priming on the phenotype of the response of P2X₇-stimulated macrophages and dendritic cells, as well as their profile of cytokine release.

Materials and Methods: Resident peritoneal macrophages were harvested from C57BL/6 mice by lavage with DMEM/10% FCS. Macrophages were allowed to adhere for 1–2 h, then washed, and maintained in DMEM-F12/10% FCS/penicillin/streptomycin at 37° and in 5% CO₂. Macrophages were analysed following 24–72 hrs of culture. Dendritic cells were cultured from bone marrow cells. Cells were plated in 6 well plates at a density of 3×10^6 cells per well in cell culture medium (RPMI:10% FCS) supplemented with 2% GM-CSF and 5 ng/ml IL-4. Cells were harvested on day 7–10 and were plated onto either 6-well plates at a density of 3×10^6 cells per well, or onto glass-bottomed Petri dishes. They were allowed to adhere for 1–2 h, then washed, and maintained in supplemented cell culture medium.

Results: Macrophages and dendritic cells showed different response patterns to varying durations of LPS priming followed by P2X₇ stimulation. Both cell types required LPS priming for P2X₇ responses to occur, but whereas the characteristics of that response were dependent on the duration of priming in macrophages, they were independent of the duration of priming in dendritic cells. This observation held true for both morphological changes and IL-1 β release patterns. Microparticle release was detected in both macrophages and dendritic cells.

Conclusion: Both macrophages and dendritic cells require LPS priming for responses to P2X₇ stimulation to occur. However, the phenotype of that response is dependent on the duration of priming in macrophages, but not in dendritic cells. This provides new insight into the different mechanisms underlying P2X₇-induced activity in macrophages and dendritic cells, and may be important in P2X₇ functions such as antigen presentation.

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Nanoscale artificial immune synapses can be used to controllably activate T and NK cells

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As a technology to investigate T cell - antigen presenting cell interactions, we have created spatially structured surfaces that mimic nanoscale features of the immunological synapse. Although it is well-

known that TCR-ligand engagement plays a key role in activation of T cells, there is some controversy over the minimum stimulation required to induce signalling through the TCR leading to activation in T cells. Super resolution imaging studies have previously identified signalling clusters on the submicron scale, and structurally important features have been described on micro- and nano- length-scales. To investigate the effect of such structures in controlling signalling, we have prepared biomimetic surfaces that present antibodies and antibody fragments with nanoscale spacing using nanopatterned gold arrays. By controlling the nanoarray structure, and the biological ligands bound to the surface, we investigate how the spatial distribution of selected stimulatory ligands affects cell activation and downstream signalling. Using the technique of diblock copolymer micelle nanolithography, arrays of gold nanoparticles with spacings ranging from 25–104 nm have been formed. The nanoarrays have been successfully functionalized with biologically complex antibody F(ab')₂ fragments, bound through the di-sulphide hinge region to the gold nanoparticle.

We show that biomimetic nanoarrays functionalised with anti-CD3 ϵ fragments engage with human CD4⁺ T cells to form an “artificial immune synapse” and demonstrate that the level of T cell signalling can be controlled by varying the spacing of anti-CD3 ϵ anchoring points on the nano-scale. Specifically, we assess CD4⁺ engagement with these biomimetic nanopatterns, showing that the degree of signalling decreases with increasing spacing of anti-CD3 ϵ ligands. Parallel studies of NK cell stimulation by CD16-binding nanoarrays showed intriguingly similar results. These results indicate that immune cell activation can be driven by the spatial organization of receptor-ligand engagement on a sub-100 nm scale. The elucidation of these signalling and activation requirements will help mechanistic understanding of the activation process, and direct immunotherapy towards new nanoscale targets.

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Comprehensive study of intracellular signalling pathways and cellular homeostasis in the presence of TRAPS-associated mutation

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Tumour necrosis factor receptor associated periodic syndrome (TRAPS) is a hereditary autoinflammatory periodic fever syndrome. It is associated with autosomal dominant variants in the *TNFRSF1A* gene, which encodes the TNFR1. The missense (R92Q) polymorphism is the most common variant associated with TRAPS within the Caucasian population. The aim of this study is to comprehensively examine: the intracellular signalling pathways that are affected by the presence of the R92Q variant; the influence of R92Q on signalling homeostasis in the cell; and the cellular response to inflammatory stimuli in the presence of R92Q-TNFR1 in comparison to wild-type (WT) cells.

The endothelial cell line (SK-Hep-1) was stably transfected with full-length WT or R92Q *TNFRSF1A*. The cells were tested for expression of TNFR1 and TLRs. Reverse-phase protein microarray and antibody microarray were applied to examine different signalling molecules and inflammatory cytokines after stimulating the cells with diverse inflammatory stimuli.

The results demonstrated that a wide range of inflammation-associated pathways and secreted inflammatory cytokines were dysregulated in SK-Hep-1 cells expressing the R92Q-TNFR1 variant. These cells also showed altered responses post-stimulation with TLR-ligands and microbial extracts in comparison to SK-Hep-1 expressing the WT-TNFR1. Interestingly, the data showed that R92Q-

TNFR1 expression appears to lead to constitutive up-regulation of a number of signalling pathways, irrespective of stimulation with sources of PAMPs.

In conclusion, the inflammatory signalling pathways activated by TRAPS-associated mutation (R92Q) have been elucidated, showing up-regulation in the mutant more than WT cells. This finding will be useful for future therapeutic treatment using specific inhibitors in TRAPS patients.

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MHC class I and class II antigen processing pathways in *Theileria parva* infected lymphocytes

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Objectives: Infection of cattle with the tick-borne protozoan *Theileria parva* induces strong CD4⁺ and CD8⁺ T cell responses, which both directly recognise parasitized lymphoblasts. Recently, we have identified several *T. parva* antigens and their respective epitopes recognised by parasite-specific CD4 T cells, including some antigens also recognised by CD8 T cells. The aim of this study was to investigate and compare the pathways involved in antigen processing within parasitised cells for recognition by specific CD4 and CD8 T cells.

Methods: The study used parasitized cell lines and T cells from animals of defined MHC types, which had been immunised with *T. parva* and shown to generate CD4 and CD8 T cells specific for defined parasite antigens. Parasite antigen-specific CD4 and CD8 T cell lines were employed and antigen recognition by T cells was assayed by measuring IFN γ production. *T. parva*-infected cells and APC were incubated with various inhibitors to investigate the involvement of different steps in the antigen processing pathways.

Results and Conclusions: CD4 and CD8 T cell clones specific for the same *T. parva* antigen (Tp9) were generated and assayed on the same parasitised cell line expressing the appropriate MHC restriction elements (DRB3*01101 and MHC1 N*02301 respectively). Most of the specific T cell clones produced IFN γ in response to antigen recognition. Inhibitors of proteasomal activity substantially reduced recognition of parasitized cells by both CD4 and CD8 T cells. Inhibitors of autophagy had no effect on recognition by either T cell subsets. Inhibitors of endosomal activity had no effect on CD8 T cell recognition and resulted in only a minor reduction in CD4 T cell recognition. Further experiments are underway to analyse responses to other antigens and compare processing of the same antigens presented in the form of recombinant proteins.

The results to date indicate that a proteasome-dependent pathway is being utilised in *Theileria*-infected cells to process antigen for recognition by both CD4 and CD8 T cells.

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Structures of human CD6 and its ligand, CD166, reveal insights into a unique interaction

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Activation and differentiation of T lymphocytes depends on a complex set of finely tuned interactions, not only between the T-cell receptor (TCR) and peptide-MHC complexes, but also between numerous accessory molecules including CD6. CD6 is a type I membrane-anchored glycoprotein found on the surface of T-cells. It contains three extracellular scavenger receptor cysteine-rich (SRCR) domains and a long cytoplasmic domain with multiple signal trans-

duction motifs. CD6 binds CD166, also a type I membrane-anchored glycoprotein that contains five extracellular immunoglobulin (Ig)-like domains. The interaction between CD6 and CD166 is the only reported example of an interaction between an Ig-like domain and a SRCR domain. Blocking interactions between these two proteins perturbs antigen-specific T cell responses.

We present the individual X-ray crystal structures of the three extracellular SRCR domains of human CD6 and the two extracellular, membrane distal immunoglobulin (Ig) domains of human CD166. The CD6 structure is the first structure of multiple SRCR domains in tandem and reveals a kink in the domain organization.

Previously published mutagenesis experiments targeting the ligand binding domains, CD6 domain 3 and CD166 domain 1, have been mapped onto the structures, identifying a potential binding interface that is compatible with the calculated electrostatic potentials in these regions. The CD6 structure also allows the documented single nucleotide polymorphism (SNPs) to be mapped, revealing the precise location of two SNPs that are associated with multiple sclerosis (MS) progression. These are predominantly buried, consistent with their mutation disrupting domain structure and consequently expression level.

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Comprehensive intracellular inflammatory pathways study in TRAPS patients

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Background and Aims: Mutations in TNFRSF1A can result in the autosomal dominant TNF receptor-associated periodic syndrome (TRAPS): a complex and heterogeneous systemic autoinflammatory disorder. Misfolding, intracellular aggregation and ligand-independent signalling by mutant TNFR1 play central roles in disease pathophysiology.

This work was conducted to study the intracellular signalling pathway activation elicited by mutant TNFR1.

Methods: To understand the complexity of intracellular signalling pathway perturbation in TRAPS, a prototypic mutant TNFR1 (C33Y), or wild-type TNFR1 (WT), were expressed at near physiological levels in an SK-Hep-1 cell model system. TNFR1-associated signalling pathway intermediates were examined under a range of conditions, employing reverse-phase protein microarray. Peripheral blood mononuclear cells (PBMC) from C33Y TRAPS patients and matched healthy controls were similarly examined.

Results: In comparison to cells expressing WT TNFR1 alone, expression of C33Y-TNFR1 in SK-Hep-1 cells and TRAPS patients' PBMCs revealed a subtle up-regulation of a wide spectrum of signalling intermediates and their phosphorylated forms. These were associated with a proinflammatory/ anti-apoptotic phenotype, including NF- κ B, p38, MEK/ERK and JNK MAP kinase pathways, Phosphoinositide 3 kinase, STAT3, JAK2/c-Src, Gsk-3 β and transcription factors (including ATF, Elk, Jun). Increased activated Jak2/STAT3 may contribute to an "IL6 amplifier" positive feedback loop that promotes and sustains a proinflammatory state.

Conclusions: The study thus reveals the pleiotropic effect of a TRAPS-associated mutant form of TNFR1 on multiple inflammatory signalling pathways.

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Small molecule analogues of an immunomodulatory helminth product provide a novel approach to dissecting dendritic cell signal transduction pathways

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Background: ES-62 is a phosphorylcholine (PC) containing glycoprotein actively secreted by the rodent filarial nematode *Acanthocheiloneema viteae* during parasitism of its host. ES-62 is a key immunomodulator during filarial infection and is known to target multiple cell types including T and B lymphocytes, antigen presenting cells - macrophages and dendritic cells- and mast cells to generate an overall anti-inflammatory immunological phenotype. The unusual post-translational modification of addition of PC appears to be responsible for many of the immunomodulatory properties of this molecule as PC conjugated to ovalbumin can mimic ES-62 action. In mouse models of autoimmune diseases such as collagen-induced arthritis (CIA) and asthma ES-62 has been shown to have a therapeutic and preventative effect and as a result has raised the possibility of ES-62 being a potential drug candidate. However, such a large and hence immunogenic molecule is not an ideal drug. Therefore the focus has now moved to small molecule analogues (SMAs) of ES-62 that are based around its active PC moiety.

Methods: To identify SMAs that mimic the action of ES-62 an initial screening system was set up. Bone marrow derived dendritic cells were pre-incubated overnight with SMAs before LPS stimulation and the cytokine response was measured by ELISA. Cytokine RNA levels were also determined by RT-PCR.

Results: SMAs were selected based on their ability to down-regulate the LPS-induced cytokine response of dendritic cells in *in vitro* inflammation assays. In particular, compounds S3, S5, S6 and S9 are potent modulators of inflammatory cytokines IL-6 and TNF- α at both protein and RNA levels.

Conclusions: Certain SMAs show a selective inhibitory effect on production of inflammatory cytokines that mimic the action of ES-62, and so could be used to dissect the key signalling pathways responsible for the inflammatory phenotypes of DCs.

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CD44-dependent hepatic stellate cell dependent induction of myeloid derived suppressor cells from peripheral blood monocytes

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Myeloid derived suppressor cells (MDSCs) are a heterogeneous population of cells associated with the suppression of immunity. It is thought that MDSC develop due to soluble factors. However,

whether cell specific contact is required remains unclear. The liver is known for its immune regulatory functions. Here, we investigated the capacity of human hepatic stellate cells (HSCs) to transform peripheral blood monocytes into MDSCs. Mature peripheral blood monocytes co-cultured with HSCs down-regulated HLA-DR and developed a phenotypic profile similar to CD14⁺HLA-DR^{-low} MDSCs. Only activated but not freshly isolated HSCs were capable of inducing CD14⁺HLA-DR^{-low} cells. Such CD14⁺HLA-DR^{-low} monocyte-derived MDSCs suppressed T-cell proliferation in an arginase-dependent fashion. HSC-induced development of CD14⁺HLA-DR^{-low} monocyte-derived MDSCs was not mediated by soluble factors, but depended on physical interaction and was mediated by HSC-expressed CD44. Thus, activated human HSCs can convert mature peripheral monocytes into MDSCs. As HSCs are activated during chronic inflammation the subsequent local induction of MDSCs may prevent excessive liver injury due to inflammation. HSC-induced MDSCs functionally and phenotypically resemble those isolated from liver cancer patients. Thus, our data suggest that local generation of MDSCs by liver-resident HSCs may contribute to immune suppression during inflammation and cancer in the liver.

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Exploring the mechanism of T-cell receptor-mediated decision making

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The ability of the T-cell antigen receptor (TCR) triggering apparatus to measure the duration of TCR binding to peptide-MHC is believed to be essential for discriminating between antigens presented by pathogens versus 'self'. This temporal thresholding of ligand engagement should also filter out spurious receptor phosphorylation that can result from the reciprocal activities of kinases (such as Lck and ZAP70) and phosphatases on the TCR, which are in a dynamic equilibrium in the quiescent state. Although this model of kinetic proofreading is entirely plausible, there is no obvious mechanism for how a temporal filter in TCR triggering might be realised at the molecular level.

One potential way to control TCR signalling could be the requirement for ZAP70 translocation and subsequent activation at the plasma membrane. In support of this, we found that artificially recruiting ZAP70 to the plasma membrane in T cells is sufficient to drive activation in the absence of any direct TCR stimulation. The recruited ZAP70 still had an absolute requirement for receptor phosphorylation, however, suggesting that the forced relocation of ZAP70 was subverting the normal proofreading steps in the discrimination pathway. To directly visualise this kinetic 'lag' in signalling, we engineered a TCR complex where ligand binding and signalling are uncoupled but can be initiated through light stimulation. As predicted, we found a clear delay in ZAP70 recruitment to the plasma membrane at the onset of signalling.

We expected that this temporal filtering of signalling should be evident in the initial kinetics of TCR triggering. We used our previously characterised reconstituted receptor system to quantify the flux of signalling away from the TCR. We observed distinct phases in the triggering kinetics, which correlated with the sequential activation of Lck and ZAP70 kinases and provided further support for ZAP70 having a critical role in defining a threshold for cell activation.

Full ZAP70 kinase activity requires phosphorylation at Tyr493 and we found that this modification is primarily mediated by ZAP70 itself. As is also the case for Lck, trans-phosphorylation creates non-linearity in the reaction pathway and we believe this provides a mechanistic basis for the switch-like behaviour of ZAP70 activity that defines the selectivity filter of receptor triggering.

Overall, our results suggest a molecular basis for ligand discrimination by the TCR in cell activation, which may also extend to selection in the thymus.

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Herpes simplex virus 2-induced activation in vaginal cells involves toll like receptors 2 & 9 and DNA-sensors DAI & IFI16

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Genital HSV infection is the most frequent urogenital ulcer disease in the world. Herpes Simplex Virus type 2 (HSV2) is one of the most common sexually transmitted pathogens and can establish life-long latency infection. Innate immunity is the first line of defence during both primary and recurrent genital herpes infections. However the pathway by which HSV2 triggers the innate immune system in the urogenital system has not as yet been fully elucidated. In this study we aim to determine which pattern recognition receptors (PRRs) recognise HSV2 in primary vaginal epithelial cells. Once we decipher the receptors involved, we aim to target them in order to immunomodulate innate responses as a prophylactic or therapeutic intervention for early HSV2 infection. In order to determine which PRRs are involved receptor silencing as well as confocal microscopy were utilised. For immunomodulation, PRR agonists were utilised to induce a strong, local response to limit the infection and employed two quantitative methods, flow cytometry and plaque assays, to determine their effect on HSV2 replication. Our results show that HSV2 is detected by a plethora of PRRs in order to limit host infection. HSV2 is detected by TLR2 as well as DNA sensors TLR9, DNA-dependent activator of IFN regulatory factors (DAI) and to a lesser extent IFI16 which trigger cytokine secretion to protect the host. Using PRR agonists, such as lipoproteins, CpG DNA, as well as cyclic dinucleotides we could significantly limit HSV2 replication. Use of agonists that target and activate these PRRs appeared to be effective in preventing primary HSV2 infection in vaginal cells and could provide new insights in defence against HSV2 urogenital infections.

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Integrating experimental, mathematical and computational approaches to lymphoid tissue formation and function

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Secondary and tertiary lymphoid tissues develop through complex interactions between large numbers of phenotypically distinct cells. As an emergent phenomenon, understanding this process through

reductionist experimentation alone presents a significant challenge. To determine minimum requirements for the induction of lymphoid tissue, we developed an integrated approach to modelling lymphoid tissue development and function incorporating mathematical, computational, *in vivo* and *in vitro* models. Using an agent based approach in which cells are represented as dynamic agents in space and time, emergent behaviour can be encapsulated, allowing testing of predictions and generation of novel hypotheses. We integrated an agent-based simulation of tertiary lymphoid tissue formation with several numerical (ODE and PDEs) models to capture chemokine sequestration, receptor internalization, and molecular diffusion. To generate realistic stromal networks, a stochastic L-system algorithm was developed, with the network then utilised within a cellular automaton simulation. Our model is multi-scale, capturing cell-cell interactions and also modelling receptor decay, recycling and synthesis on an intracellular level.

Our *in silico* model was developed using a data-driven approach, integrating results from mouse models and *in vitro* assays. Our unique and versatile simulation visualization enables rapid prediction testing and hypothesis generation. Our model predicts that chemokine CXCL13 is sufficient for B-cell entry into B-cell follicles, despite receptor CXCR5 saturation on B cells at the follicle boundary. The creation of local gradients through CXCR5 internalization permits B-cells to enter the densely packed follicle. By tracking individual expression of particular molecules on cells over time, we can perform experiments that are currently impossible with wet-lab experimental models. Through this approach, CXCR5 expression on B-cells within a follicle was found to oscillate as a sinusoidal function, an emergent result arising from a combination of a stochastic ODE and cellular interactions within the agent-based simulation. This oscillatory expression enables B cells to maximize displacement within the follicle (low expression) and prevent exit from the follicle through CXCR5 up-regulation as the B cell reaches the boundary (high expression). Several experiments have been planned to find evidence to support these hypotheses *in vivo*.

We further plan to model lymph node organogenesis, to determine differential requirements of various lymphoid tissues under both normal and autoimmune conditions. Tertiary lymphoid tissue is likely to have an important impact in autoimmune disease and cancer; it is hoped that our simulations will provide a detailed understanding of their role in the autoimmune response and how this may be modulated therapeutically.