QRW ASI-NZ Immunology Abstracts
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I1: T cell therapies for Hematologic Malignancies: Beyond CARs

Catherine M. Bollard.

Children's National and The George Washington University.

T cells play a major role in the GVL effect that confers cure in patients who are incurable by chemotherapy and "small-molecule" drugs. However, the GVL effect is limited, especially in patients with advanced disease and those with aggressive malignancies. Adoptive T-cell therapy strategies have arisen out of the need to improve GVL. The first attempts to boost GVL used donor lymphocyte infusions (DLI). Since then, the field has diversified and the underlying biology of the interactions between T cells and tumor cells has become better understood. Here, we address the current developments in allogeneic T-cell therapy using a non-gene-transfer approach to direct specificity and describe ways in which similar strategies can be applied in the autologous setting. Hence, this presentation will focus on the recent advances in adoptive T-cell immunotherapies, not only for patients after hematopoietic stem cell transplantation, but also in the autologous setting using T cells early in the disease process for the treatment of the highest-risk patients with leukemias and lymphomas. The particular emphasis is to highlight the role of T-cell therapies for hematologic malignancies using a non-gene-transfer approach to direct specificity, including the clinical use of Tcell therapies for EBV-associated lymphomas and strategies for targeting nonviral lymphoma- and leukemia-associated antigens.

I2: Functional outcomes of fine tuning the expression of PD-L1 and PD-1 proteins to the melanoma immune response

Storm Taurere[1], Yale Michaels[2], Daniel Verdon[1], Ashleigh Gaines[1], Tudor Fulga[2], Rod Dunbar[1], Hilary Sheppard[1]

[1]School of Biological Sciences, University of Auckland, NZ, [2]Weatherall Institute of Molecular Medicine, Radcliffe Department of Medicine, University of Oxford, UK.

Immune checkpoint inhibitors have proved effective in clinical trials targeting a number of cancers including melanoma. A key example is the blocking antibody pembrolizmab (marketed as Keytruda™) which targets the inhibitory PD-L1/PD-1 pathway. It is proving effective in the clinic with responsive patients showing durable results. One predictor of patient response is PD-L1 positivity within the tumour. However some studies have shown that patients which score negative for PD-L1 expression can have durable responses and others with high PD-L1 levels do not respond at all [1]. Both PD-L1 and PD-1 expression levels can be dynamic which can confound these types of studies. Therefore questions still remain regarding the levels of PD-L1 within the tumour and the effectiveness of blocking antibodies such as Keytruda. We aim to answer some of these questions using an *in vitro* system in which we can fine-tune expression of both PD-L1 on melanoma cells and PD-1 on melanoma specific T cell clones. Our collaborators in Oxford University, UK have engineered a system to fine-tune mammalian gene expression based on engineered, synthetic microRNA response elements that can be appended downstream of any gene of interest [2]. Here the microRNA silencing-mediated fine-tuners (miSFITs) have been engineered into the 3' UTRs of both human PD-L1 and PD-1 to generate panels of lentivirus constructs which produce graded expression of these proteins. We will present our preliminary results assessing the functional outcomes of varying expression of both of these key proteins on the immune response to melanoma. We will also discuss the progress of our recent foray into using CRISPR/Cas9 ribonucleic proteins to edit endogenous genes in primary human T cells and primary human keratinocytes.

1. Daud AI, Wolchok JD, Robert C, Hwu WJ, Weber JS, Ribas A, Hodi FS, Joshua AM, Kefford R, Hersey P, et al.: P*rogrammed Death-Ligand 1 Expression and Response to the Anti-Programmed Death 1 Antibody Pembrolizumab in Melanoma*. *J Clin Oncol* 2016, 34:4102-4109.
2. Michaels Y, Mike B. Barnkob, Hector Barbosa, Toni A. Baeumler, Mary K. Thompson, Violaine Andre, Huw Colin-York, Marco Fritzsche, Uzi Gileadi, Hilary M. Sheppard, David D.J.H.F. Knapp, Thomas A. Milne, Vincenzo Cerundolo, Tudor A. Fulga: *Precise tuning of gene expression levels in mammalian cells*. *Submitted* 2018.

I3: Characterising dysfunctional T cells in colorectal cancer

Leman, J.[1], McCall, J.[2], Ward-Hartstonge, K.A.[1], Norton, S.[1], Munro, F.[2], McGuire, H.[3], Kemp, R.A.[1]

[1]Department of Microbiology and Immunology, University of Otago, Dunedin, New Zealand, [2]Department of Surgical Sciences, University of Otago, Dunedin, New Zealand, [3]Ramaciotti Facility, Human Systems Biology, University of Sydney, Sydney, Australia

Exposure to chronic antigen stimulation, in contexts such as chronic viral infection or in cancer, promotes a tolerant state of reduced functionality in T cells. This dysfunctional state is characterised by reduced proliferation and production of cytokines, and the upregulation of multiple inhibitory receptors. Immunotherapies which target these inhibitory receptors to reduce negative regulation of T cells in the tumour, which boosts the anti-tumour immune response. These immunotherapies have varied efficacy in treating colorectal cancer. The T cell infiltrate into colorectal tumours is strongly correlated with disease-free survival, and so immunotherapies targeting T cells have the potential to improve outcomes for patients. It is likely that these treatments will have to be targeted to a patient's specific type of cancer, and even particular immune response. Targeting patients' immune responses with specific immunotherapies will be the next step in improving the efficacy of these treatments.

Dysfunctional populations have classically been characterised by the expression of inhibitory receptors, but these receptors are also upregulated during T cell activation. To discern activated from dysfunctional cells, we are combining high-dimensional single cell analyses with an assay for transposon-accessible chromatin (ATAC-seq) epigenetic analyses to characterise dysfunctional populations. We have used a 40-parameter mass cytometry panel to define T cell populations within the tumours of CRC patients undergoing surgery. We have found a putatively dysfunctional population of CD4 T cells which is enriched in the tumour compared to adjacent non-tumour bowel (n=13). This CD4 population express high levels of PD-1, TIM-3, CTLA-4 and BLIMP-1. To confirm dysfunctionality, we will compare the chromatin accessibility profile of this population to cells which have been made dysfunctional *in vitro*, through chronic stimulation. Ultimately this will help to improve targeting of immunotherapies to the subsets of dysfunctional cells found in colorectal tumours.

I4: Detection and Quantification of Programmed Cell Death Protein-1 (PD-1) Receptor Occupancy by Single-Dose Nivolumab in Chronic Hepatitis B Patients.

Daniel Verdon[1], Anna E. Brooks[1,4], Anuj Gaggar[2], Jacky Woo[2], Mani Subramanian[2], Christian Schwabe[3], Edward J. Gane[3], Rod Dunbar [1,4].

[1]School of Biological Sciences, University of Auckland, NZ. [2]Gilead Sciences, Foster City, CA, USA. [3]Auckland Clinical Studies, Auckland, NZ. [4]Maurice Wilkins Centre for Molecular Biodiscovery, Auckland, NZ.

A dysfunctional T-cell response to Hepatitis B Virus (HBV) antigens is characteristic of chronic HBV infection (CHB) and limits durable viral elimination and control. T-cell exhaustion is a major pathway mediating impaired T-cell responses *in situ.* The PD-1:PD-L1 axis of T-cell inhibition is a well-validated therapeutic target in several malignancies, and blockade of PD-1 or PD-L1 can normalise HBV-specific-T-cell activity *in vitro[1].*

In this Phase I study (GS-US-330-1938) patients received a single dose of nivolumab at 0.1 mg/kg (n = 2) or 0.3 mg/kg (n = 22). Pan-T-cell- and subset-specific PD-1 expression patterns and PD-1 receptor occupancy (RO) by nivolumab were investigated using 16- and 9-parameter flow cytometric assays, respectively, up to 24 weeks following nivolumab administration.

PD-1 expression was restricted to non-naïve T-cells. PD-1 expression frequency was highest on $CD4^+$ and $CD8^+CD45RA^-CCR7^-T_{EM}$, and lowest on $CD4^+CD45RA^-CCR7^+$ T_{CM} and $CD8^+CD45RA^+CCR7^-$ T_{EMRA}. In all patients, RO was maintained at maximal levels for $\geq$42 days, with a subset of patients exhibiting prolonged occupancy to $\geq$85 days. Mean Pan-T-cell RO across 0.1 and 0.3 mg/kg cohorts days 7-42 post-infusion was 72.75% (95% CI 73.64, 76.85). No significant differences in maximal RO (*p=0.839)* or in RO duration were observed between 0.1 and 0.3 mg/kg cohorts. RO was subset-dependent and was most pronounced on pan-$CD45RA^-CCR7^-$ T_{EM} (mean RO 81.7%). Accurate quantification of RO on PBMC was highly temperature-sensitive, due to rapid PD-1 internalisation and *de novo* expression.

5 of 22 patients exhibited >0.5log_{10} decline in serum Surface Antigen (HBSAg) during this study, with one patient exhibiting a sustained and complete HBSAg loss and ongoing viral elimination. Single-dose nivolumab treatment at up to 0.3mg/kg resulted in sustained PD-1 occupancy for a minimum period of 42 days post-infusion, and in complete HBsAg clearance in one patient, providing proof-of-principle evidence that checkpoint-blockade inhibition can be therapeutically effective in CHB.

1. Fisicaro, P., et al., *Antiviral intrahepatic T-cell responses can be restored by blocking programmed death-1 pathway in chronic hepatitis B.* Gastroenterology, 2010. **138**(2): p. 682-93, 693 e1-4.

I5: A combination of chimeric switch-receptor T cells targeting both PD-1 and CTLA-4 suppresses tumor growth.

Peng Li[1], Xinru Wei[1], Le Qin[1], Zhenfeng Zhang[2], Xuchao Zhang[3], Robert Weinkove[4].

[1]Guangzhou Institutes of Biomedicine and Health, Guangzhou, Guangdong, China. [2]The Second Affiliated Hospital of Guangzhou Medical University, Guangzhou, China. [3]Guangdong General Hospital, Guangzhou, China. [4]Malaghan Institute of Medical Research, Wellington, NZ.

Chimeric antigen receptor (CAR)-T cells have been successfully used to treat B-cell malignancies; however, significant obstacles exist for the treatment of solid tumors. One major challenge is the immuno-suppressive effects of PD-L1 in tumors on T cells, and by CD80 and CD86 on myeloid-derived immune-suppressing cells, which are ligands for CTLA-4 on T cells. Dual blockade of PD-1 and CTLA-4 with monoclonal antibodies has shown promising anti-tumor effects in clinical studies. Here, we describe a novel strategy taking the advantage of these suppressive molecules on T cells using the two switch-receptor system. We first made cPD-1, which contained the truncated extracellular domain of human PD-1, the co-stimulatory domains of 4-1BB and TLR2, and the CD3z signaling domain. The cPD-1 T cells specifically lysed PD-L1$^+$ tumor cells and had enhanced cytokine secretion in vitro, and in patient-derived xenograft (PDX) models suppressed the growth of cells of lung cancer, gastric cancer, and osteosarcoma. Remarkably, the cPD-1 T cells also prevented metastasis in a gastric cancer PDX model. Because in PDX models CD80/CD86 are expressed in murine myeloid cells rather than human tumor cells, we designed cmCtla-4 targeting murine myeloid cells that were abundant in tumors. cmCtla-4 was composed of the truncated extracellular domain of murine Ctla4 and human CD28, TLR2, and CD3z signalling domains. In a gastric cancer PDX model, T cells expressing cmCtla4 significantly slowed tumor growth. In combination, the cPD-1 and cmCtla4 T cells synergistically induced superior anti-tumor effects in a lung cancer PDX model. Taken together, these findings reveal an approach of using converted immune checkpoint receptors to target both tumor cells and the tumor microenvironment, and indicate the potential clinical benefits of these chimeric switch-receptor T cells for cancer treatment.

Keywords: CAR-T, tumor microenvironment, solid tumor, PD-1, CTLA-4, PDX.

I6: Tumour cell-derived proteases contribute to antigen processing and enhance cross-presentation

Shields, N.J.[1], Young, K.A.[1], Jackson, C.[2], Young, S.L.[1]

[1]Department of Pathology, Dunedin School of Medicine, University of Otago, Dunedin, NZ.
[2]Department of Medicine, Dunedin School of Medicine, University of Otago, Dunedin, NZ.

Tumour lysates can serve clinical applications for immunotherapy, including vaccination and as a platform for the expansion for tumour-specific T-cells for use in adoptive cell transfer. Recently, immunogenic cell death (ICD) in tumour cells has proven crucial for the generation of robust anti-tumour immune responses, suggesting that the induction of ICD may improve the efficacy of tumour lysate-based immunotherapies. Here, we examined the immunogenicity of tumour lysates prepared from tumour cells exposed to increasing levels of heat (37°C, 42°C and 56°C), using the MC38-OVA/OT-I system.

Molecular correlates of ICD were assessed during the course of heat-treatment and in tumour lysate preparations. Notably, lethal heat-treatment (56°C) induced primary necrosis with concomitant caspase activation, calreticulin exposure, HMGB1 release and HSP upregulation. All tumour lysates promoted maturation of bone marrow-derived dendritic cells (BMDC) and elicited protective anti-tumour immunity in vaccinated mice. However, only tumour lysates prepared from lethal heat-treated tumour cells promoted cross-presentation in BMDC to elicit antigen-specific T cell activation *ex vivo*. Marked protease activity was observed in lethal heat-treated tumour cells, which were capable of processing antigen and binding resultant peptides. Using a broad protease inhibitor, inhibition of this protease activity markedly reduced cross-presentation. Selective inhibition of calpains, but not caspases, cathepsins or the proteasome, reduced antigen processing by heat-killed tumour cells.

Together, this demonstrates that tumour-derived proteases contribute to antigen processing and enhance cross-presentation. Our data suggest that in addition to DAMP release, antigenic processing by proteases in dying tumour cells can influence immunogenic cell death.

I7: Immune response to oral therapeutic vaccination against colorectal cancer

Naciute, M.[1], Niemi, G.[2], Gibson, B.[1], Kemp, R.[2], Hook, S.[1]

[1]School of Pharmacy, University of Otago, Dunedin, NZ, [2]Department of Microbiology and Immunology, University of Otago, Dunedin, NZ.

Colorectal cancer (CRC) is one of the most diagnosed cancers worldwide and the incidence rate of this cancer is increasing. Therapeutic oral vaccination might be beneficial for treatment of CRC because of potential to develop specific, local anti-tumor responses with a patient-friendly treatment. However, orally delivered vaccines have to be resistant to the harsh environment in the gastrointestinal tract as well as being capable of releasing drugs at the appropriate site. Two vaccine formulations, lipid particles (liposomes) and a lipid emulsion were developed, characterised and optimised to provide protection to the vaccine components. Liposomes were manufactured using microfluidics as this process can be easily transferred to a clinical setting.

Liposomes and lipid emulsion vaccines were administered orally to tumor-free BALB/c mice on 4 consecutive days, following be a boost 2 weeks later (again given orally over 4 consecutive days) and local and systemic immune responses examined. The vaccine formulations contained the CT26 peptide tumor antigen, a peptide construct combining CD4 T cell epitopes from tetanus and diphtheria toxins, and Pam2Cys as an adjuvant. Finally CT26 tumor cells were injected surgically into the caecum of mice, followed by treatment with vaccine 6-9 days later. Tumor size was measured 16-19 days post-surgery.

Vaccination using lipid emulsions induced detectable systemic T cell immune responses and both formulations were able to slow the growth of tumor cells. Future work will investigate the immune response generated in greater detail and the combination of vaccination and immunotherapy.

I8: Investigation of combination treatment of an aromatase inhibitor and anti-inflammatory treatment in a model of oestrogen receptor positive breast cancer

Hazlett, Jody [1]; Niemi, Ginny [2]; Kemp, Roslyn [2]; and Dunbier, Anita [1].

[1] University of Otago, Biochemistry Department, New Zealand
[2] University of Otago, Microbiology and Immunology Department, New Zealand

Oestrogen receptor (ER+) positive breast cancer is a leading cause of death in women worldwide. Current treatments involve anti-oestrogen drugs, which work well initially but the tumours frequently develop resistance which emerges in the metastatic setting. The immune system plays an important role in the development of resistance in multiple cancer types, however there is a lack of suitable immunocompetent mouse models to study this for ER+ breast cancer. We have developed a model of aromatase inhibitor treatment in ER+ breast cancer in an immunocompetent mouse allowing investigation into the relationship between anti-oestrogen treatment and tumour infiltrating lymphocytes (TIL's). We have successfully demonstrated that SSM3 cells, derived from a spontaneous mammary tumour from a *STAT1* knockout mouse, can be established in naive mice, and that these tumours respond to the anti-oestrogen treatment letrozole.

After establishment of tumours, mice were treated for 25 days with either letrozole alone, celecoxib alone, vehicle or the combination of the two drugs. We examined the immune cell infiltrate of these tumours using flow cytometry and determined that combination of a non-steroidal anti-inflammatory drug, celecoxib, with letrozole resulted in decreased numbers of $CD11b^+$ cells compared to vehicle treated tumours. Preliminary IHC data suggests that celecoxib, either alone or in combination with letrozole can reduce the level of CD3 infiltration in the tumours. Working within recommended guidelines for assessing stromal TIL's in H&E sections, we determined that the percentage of TIL's increased in all treatment groups. We observed a significant decrease in tumours treated with both celecoxib and letrozole compared to vehicle treated tumours. Further experiments will investigate Ki67, and immune cell markers such as CD19 and CD11 to further ascertain how these treatments alter the immune cell composition within the tumours.

I9: Effect of ascorbate on tumour associated macrophage phenotype *ex vivo*.

Ang, A.[1], Currie M.[1], Dachs, G.[1]

[1]Department of Pathology, University of Otago, Christchurch, NZ.

There is increasing evidence for the beneficial role of ascorbate in cancer treatment. However, the push for ascorbate supplementation is slow at gaining traction despite lower circulating ascorbate status in cancer patients. A better understanding of ascorbate-mediated anti-tumour effects could strengthen the case for more clinical studies investigating ascorbate as an adjunct therapy. Currently, only ascorbate-mediated cancer cell toxicity or tumour characteristics have been considered, and not the effect of ascorbate on immune cells. Circulating leukocytes have among the highest intracellular ascorbate levels, and this drops in cancer patients. Therefore, we aim to investigate the effect of ascorbate on immune cells in the context of the tumour-microenvironment.

Here, we studied the effect of ascorbate supplementation in an *ex-vivo* model of tumour associated macrophages/monocytes. Primary murine bone marrow monocytes were isolated and grown with Lewis Lung carcinoma cell conditioned-media (LLCM) (40% v/v) for 6 days with or without ascorbate supplementation (500 µM). Then, overnight monocyte conditioned-media was harvested for ELISA measurements. Additionally, monocytes were stimulated for 24h with tumour microenvironment stimuli such as hypoxia (1% O_2) or crude tumour cell lysate (to mimic alarmins from necrotic tumour cells).

After 7 days of culture in LLCM, monocytes attained a spindled shape associated with an M2 phenotype. M2 proteins, VEGF and TGF-β, accumulated in media of LLCM differentiated monocytes. Ascorbate supplemented monocytes were less spindled and had lower VEGF and TGF-β secretion. Hypoxia increased VEGF and TGF-β secretion, and this too decreased with ascorbate supplementation. Tumour cell lysate stimulation did not alter VEGF or TGF-β levels, but increased IL-6 secretion. VEGF and TGF-β levels decreased and IL-6 levels increased with ascorbate supplementation.

These results suggest that increasing ascorbate levels in monocytes may dampen the pro-tumour monocyte phenotype. Further studies will determine whether ascorbate would affect the function of these cells.

I10: Refining multiplex immunohistochemistry to quantify in situ immune infiltrate: improving survival of colorectal cancer patients

Janet L Rhodes[1], John L McCall[2], Sharon T Pattison[1], Kirsten A Ward-Hartstonge[1], Sarah K Sandford[1], Fran M Munro[2], Mik A Black[3], Roslyn A Kemp[1].
Department of Microbiology and Immunology, University of Otago[1], Dunedin, New Zealand; Department of Surgical Sciences, University of Otago[2], Dunedin, New Zealand; Department of Biochemistry, University of Otago[3], Dunedin, New Zealand

Colorectal cancer (CRC) is the second highest cause of cancer mortality in New Zealand. CRC is often treated with surgery alone, however some patients also require chemotherapy to optimise their chance of long term survival. With current staging techniques, 25% of early stage CRC patients suffer recurrence. Improving the accuracy of prognosis will help to direct appropriately personalised therapy and likely improve survival in that group of patients with early stage CRC.
The immune response to CRC has recently been accepted as a clinical prognostic parameter. Quantification of tumour infiltrating CD3+ and CD8+ T cells has been demonstrated to more accurately stage patients. Attempts to further characterise these cell populations have yielded indeterminate results. For example, quantifying T regulatory cell infiltrate (identified with the additional marker FOXP3) of CRC tumours has been shown to be beneficial in one study, but had nil effect in another. These conflicting results are likely to be a feature of incomplete characterisation of a heterogeneous and plastic population. This is demonstrated when additional markers are added to analysis. We demonstrated no prognostic gains by quantifying CD3+, CD8+, and FOXP3+ T regulatory cell infiltrate in our cohort of 32 NZ CRCs. However, we used the addition of BLIMP-1+ to delineate T regulatory cell populations into effector and non-effector T reg groups. This demonstrated improved prognostic accuracy.
Additional markers are also being explored. A 2016 preliminary study suggested that quantifying neutrophil infiltrate of CRC is also likely to be of prognostic assistance. We are incorporating the neutrophil markers CD66b and CD11b into our multiplex analysis.
Automated processes for measuring multiple immune markers in situ, in a single tissue section are yet to be refined. We are optimising automation of fluorescence immunohistochemistry, imaging and analysis for our multiplex analysis. We will process our optimised, automated multiplex on our tissue bank of >600 colorectal cancer specimens. This work will validate existing results, further refine prognosis, improve treatment selection, and thereby likely improve survival for patients with CRC.

I11: TotalSeq™ Simultaneous Proteomics and Transcriptomics - The Future of Single Cell Analysis

Friend, K.[1] Yeung, B.[2] Cornett, A.[2] Tam, M.[2] Nazor,K.[2] Cornell, A.[2] Swarbrick, A.[3] Al Eryani, G.[3] Junankar, S.[3] Stoeckius, M.[4]

[1]Biolegend National Sales Manager, Australia. [2]Biolegend, San Diego. [3]Garvan Institute, Sydney. [4]New York Genome Centre, New York.

Whilst high-throughput single cell RNA-seq techniques have transformed our understanding of complex cell populations, these do not allow for additional phenotypic information such as protein levels of cell-surface molecules. A recent publication by Stoeckius et al. described the use of antibodies coupled with oligonucleotides to simultaneously quantify proteins and RNA at the single cell level. The method, termed Cellular Indexing of Transcriptomes and Epitopes by sequencing (CITE-seq), combines highly multiplexed protein marker detection with unbiased transcriptome profiling for thousands of single cells and is compatible with current scRNA-seq platforms such as Drop-seq and 10X Genomics. BioLegend has an exclusive license to this technology, and reagents under the brand name TotalSeq are now available to facilitate CITE-seq and other similar workflows for simultaneous detection of RNA and protein at the single cell level.

I12: Investigating post-stroke immune responses in lean and obese mice

Silke Neumann[1], Emma Gowing[2], Sarah Young[1], Andrew Clarkson[2]

[1] Department of Pathology, Dunedin School of Medicine, University of Otago, Dunedin, New Zealand
[2] Department of Anatomy, University of Otago, Dunedin, New Zealand

Objective
Obese and diabetic people are significantly more likely to suffer a stroke, with poor prognosis for recovery. A hallmark of many pathological conditions including both obesity and stroke is chronic inflammation, which impairs recovery. However, the causal link between inflammation and recovery, and why obese and diabetic patients exhibit worse outcomes following a stroke, is not known. In this study, we aimed to delineate changes in post-stroke immune responses by studying myeloid and lymphoid cell populations in obese and lean mice.

Methods
Lean C57BL/6J mice and obese POUND mice were subjected to either focal stroke or sham surgery using the photothrombosis model of motor cortex stroke. Peripheral blood was analysed for pro- and anti-inflammatory cytokines post-stroke. Frequencies of immune cell populations were assessed in the bone marrow, spleen, blood and brain either pre- or 7 and 28 days post-stroke. Functional behavioural assessments (grid-walking and cylinder tasks) were carried out one week prior to surgery and then subsequently one- and four weeks post-stroke. Infarct sizes were assessed using cresyl violet staining.

Results and Conclusions
We observed that obese mice were more severely affected by the stroke, with increased mortality rates and increased levels of interleukin-6 (IL-6) in the blood as compared to lean littermate controls. However, surviving obese mice had similar stroke volumes and showed no significant differences in functional behavioural tests in comparison to lean mice. Interestingly, obese mice had higher levels of immature myeloid cells ($CD11b^+$ $Ly6G^+/Ly6C^+$) in the bone marrow, spleen and blood pre-stroke as compared to lean mice. These cells are known to suppress inflammatory responses and may represent a compensatory mechanism that dampens excessive inflammation post-stroke. We are currently investigating if these cells migrate to the brain pre- or post-stroke to delineate their role in stroke recovery.

I13: Novel investigation of Crohn's disease T cell phenotypes and functionality in an *in vitro* human colonoid model

Angus, HCK.[1], Aluzaite, K.[2], Butt, GA.[3], Schultz, M.[2], Kemp, RA[1]

[1]Department of Microbiology and Immunology; [2]Department of Medicine; [3]Department of Physiology, University of Otago, Dunedin, New Zealand

Crohn's disease (CD) is a type of inflammatory bowel disease caused by excessive and uncontrolled inflammation. There is no current consensus on how CD forms; however, four susceptibility criteria are required for disease: a microbial presence, an immune response, an environmental trigger, and genetic susceptibility. Current treatments include tissue resection and administration of immunosuppressants; however, these are often ineffective and do not accurately account for patient specific disease.

Organoids are miniature three-dimensional organs derived from stem cells from patient intestinal biopsies. Grown *in vitro*, organoids provide a sustainable self-renewing source of patient tissue which is critical for developing patient specific prognostic testing. We have successfully developed two-dimensional monolayers, derived from organoids, on transwell inserts which emulate *in vivo* intestinal-barrier arrangement.

Intestinal T lymphocytes have long been implicated in onset of CD. Using flow cytometry, we have identified Th17 (IL-17+), Regulatory (CD25hiFOXP3+), and Effector T cells (IL-2+IFNγ) from intestinal tissue and peripheral blood. To determine whether CD T lymphocytes respond differently to commensal bacteria than control T lymphocytes, patient immune cells were cocultured with commensals *Facalibacterium prausnitzii* and *Bacteroides fragilis*. Coculture with healthy PBMCs significantly reduced effector and Th17 populations and increased regulatory T cell frequencies. Acquisition of CD data for comparison is currently underway.

We aim to introduce controlled immune cell populations to the monolayer model system, as well as an eventual microbial influence. This model will provide data on cell intrinsic differences between CD and control immune cells and their effect on patient derived epithelia. We will use a combination of histological staining, Legendplex, and flow cytometry to monitor epithelial and immune responses to model conditions. This model could lead to a high-throughput prognostic testing assay using patient specific intestinal epithelium and immune cells.

I14: Novel agonists to explore the function of mucosal associated invariant T (MAIT) cells as cellular adjuvants

Joshua Lange[1], Olivier Gasser[1], Regan Anderson[2], Gavin Painter[2], Ian Hermans[1]

[1]Malaghan Institute of Medical Research, Wellington, New Zealand. [2]Ferrier Research Institute, Victoria University of Wellington, Wellington, New Zealand.

Invariant natural killer T (iNKT) cells act as cellular adjuvants by providing co-stimulation to antigen presenting cells (APCs) upon recognition of the glycolipid α-galactosylceramide (αGalCer) in the context of CD1d. Thus, when αGalCer is co-administered with soluble peptide antigens, iNKT cells are able to license APCs, thereby enhancing the immunogenic presentation of peptide:MHC complexes to CD8+ T cells, leading to increased antigen-specific immunity. However, in mice, iNKT cells represent ~1% of all circulating T cells while in humans iNKT cells are of a much lower frequency (~0.01 - 0.1% of all circulating T cells). Whether this significant discrepancy between mouse and human iNKT cell frequency will limit their capacity to act as cellular adjuvants in clinical applications is unknown.

Another innate-like T cell population, mucosal associated invariant T (MAIT) cells, has recently been recently described to play key roles in both sterile and non-sterile pathologies. Indeed, MAIT cells can rapidly exert effector functions upon TCR dependent recognition of riboflavin metabolites, presented in the context of MR1, or in a TCR independent manner via cytokines. Importantly, and in contrast to iNKT cells, they represent an abundant population of circulating human T cells (1-5%). Using novel chemistry, we have generated synthetic MAIT cell agonists to determine whether, similarly to iNKT cells, MAIT cells can be harnessed as immunotherapeutic adjuvants.

I15: Immune priming: Is it a major driver for rheumatic fever?

Lorenz, N.[1,2], Ho, T.[1], Baker, M.[2,3], Smeesters, P.[4], Moreland, N.[1,2], the Rheumatic Fever Risk Factors Study Group

[1] School of Medical Sciences, The University of Auckland, Auckland, New Zealand; [2] Maurice Wilkins Centre for Molecular Biodiscovery, University of Auckland, Auckland, New Zealand; [3] University of Otago, Wellington, New Zealand; [4] Universite´ Libre de Bruxelles, Brussels, Belgium

Acute rheumatic fever (ARF) is a serious autoinflammatory condition that develops after Group A Streptococcus (GAS) infection in susceptible children. The rates of ARF in Maori and Pacific children in New Zealand are amongst the highest in the world but drivers for the disease are poorly understood. Repeat exposures to GAS are thought to “prime” the immune system for an autoimmune reaction. However, it is not known if children who develop ARF experience more GAS exposures compared to those who do not, or if an underlying susceptibility is the main driver for disease development. The aim of this study is to answer this fundamental question by exploiting serum memory and the fact that humoral responses to GAS can persist for decades. Our laboratory has developed a methodology that allows retrospective mapping of GAS exposures in patient sera based on the emm-type of previously infecting strains. Emm sequence typing is the most widely used epidemiological typing tool for defining GAS strain diversity. We have generated a unique panel of 50-mer peptides matching the hypervariable region of the GAS M-protein, which corresponds to the ¬emm-type coding region of the protein. Our panel covers >90% of strain emm-types prevalent in New Zealand. This panel is now being used in immunoassays with human sera to measure M-type specific antibody responses, and in turn, determine the number of GAS exposures an individual has experienced. Initial results indicate that ARF patients have experienced multiple GAS exposures prior to developing the disease. By comparing the number of exposures in each ARF case with that of a highly matched control (collected as part of the recently completed nation-wide Rheumatic Fever Risk Factors study) this work will provide insight into the role of immune priming in the pathogenesis of ARF.

I16: Is the immune system of naïve mice affected by cow's milk?

Shirley, S.[1, 2], Kemp, R.[1], Cakebread, J.[2], Hodgkinson, A.[2]

[1]Department of Microbiology and Immunology, University of Otago, Dunedin, NZ, [2]Food and Biobased Products Group, AgResearch Ruakura, Hamilton, NZ.

Consumption of raw cow's milk in childhood has been associated with a decreased incidence of allergy, when compared to consumption of heat-treated milk [1]. Additionally, raw cow's milk was found to prevent asthma development in a murine model [2]. Abundant immune-related components within cow's milk have led to the proposal of numerous mechanisms by which raw cow milk may prevent allergy development [3, 4]. However, a clear understanding of the direct effects of raw milk on the immune system is still to be uncovered. We hypothesised that a protective mechanism of raw milk may be evident in naïve animals and these effects might be due to an augmentation of the naïve immune system.

We examined the effects on mouse splenocyte populations after feeding water, raw milk or processed milk for 7 and 28 days. Using flow cytometry, T cell numbers were assessed, as well as their effector/memory status based on CD44 expression. Further, the numbers of B cells, granulocytes, and monocytes were determined. It was found that $CD44^{Hi}$ effector/memory T cell numbers were increased in both raw milk and processed milk fed animals at day 28. Augmentations were also observed in B cell and granulocyte counts at day 7 and day 28. Interestingly, MHC-II high granulocyte counts were higher in mice fed milk compared with mice fed water. Taken together these results show that feeding milks can directly affect the immune cell profile of the spleen. Moreover, these effects do not appear to be specific to raw milk.

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I17: The effect of the probiotic *Streptococcus salivarius* (BLIS) K12 on human systemic immune responses

Laws, G.[1], Hale, J.[2], McConnell, M.[1], Kemp, R.[1]

[1]Department of Microbiology and Immunology, University of Otago, [1]Blis Technologies, Dunedin, NZ.

Emerging research indicates that probiotics can beneficially stimulate the immune system. Streptococcus salivarius K12 is an oral probiotic known to protect against oral pathogenic bacteria in humans. Preliminary studies have found increased levels of salivary IFN- twenty-four hours after consumption of this probiotic. All studies of immune responses to S. salivarius K12 have focused on the localised area of the mouth. Systemic immune responses to K12 have not been investigated. The aim of this study is to identify the acute systemic immune response to S. salivarius K12.

A preliminary clinical trial of a probiotic containing Lactobacillus acidophilus NCFM and Bifidobacterium lactis Bi-07 was conducted to establish appropriate methods to measure immune responses in human blood. Blood samples were gathered from participants, who then consumed the probiotic. Twenty-four hours later a secondary blood sample was taken and analysed to identify immune changes. These methods were repeated in a pilot trial of S. salivarius K12 with a further blood sample gathered at seven days post consumption.

Samples were analysed simultaneously using multi-parametric flow cytometry, to quantify immune cell frequency changes, and by a LEGENDPlex assay of human inflammatory cytokines to determine cell function. Consumption of L. acidophilus and B. lactis had no effect on immune cell frequencies or function after twenty-four hours; however, consumption of S. salivarius K12 resulted in an increase in the production of inflammatory cytokines including MCP-1 (monocyte chemo-attractant protein 1). These preliminary results form the basis for a larger, double-blinded, placebo-controlled clinical trial (ongoing) of systemic immune effects of S. salivarius K12. The information gathered in this study will help us understand the effect of probiotics on our immune system and their potential role in health and disease.

I18: Depletion of Langerin/CD207+ cells accelerated wound healing.

Rajesh, A[1], Gabriella Stuart[2], Nicola Real[2], Jenny Ahn, Allison Tschirley[1], Merilyn Hibma[1] and Lyn Wise[2].

[1]Department of of Pathology, University of Otago, Dunedin School of Medicine, NZ,[2]Department of Pharmacology and Toxicology, University of Otago, NZ.

Langerhans cells (LCs) are epidermal immune cells that play an important role in skin immunity via antigen presentation and maintaining tolerance. Previous research has hinted the involvement of LCs during wound healing, but their specific role and importance is yet to be explored. We observed that depletion of LCs in transgenic Langerin DTR mice resulted in 20% accelerated wound closure. Histological analysis was carried out to compare the differences between the control and depleted groups. Martius Scarlet Blue (MSB) staining revealed that the faster wound regression in the depleted group was due to increased neo-epidermal at days 3 and 6-post wounding. Immunofluoresence staining showed that there was an increase in proliferation of cells in the neo-epidermis. In addition to this, there was alos a two-fold increase in the granulation tissue area in the depleted group at day 6- post wounding, which could also be promoting faster healing. The accelerated granulation tissue formation in the depleted group is mediated by increased vasculature at day 6. This study suggests that LC could be involved in suppressing the processes involved in the proliferative phase of wound healing.

I19: Immunoregulatory Effects of Vitamin D and its Mechanism of Action in $CD4^+$ T Cells

Reuben McGregor, Estefania-Nova Lamperti, Paul Lavendar, Audrey Kelly, Claudia Kemper, Giovanna Lombardi, Behdad Afzali

Background:
$CD4^+$ T cell effector responses are crucial for clearance of pathogens but need to be controlled in order to avoid ensuing inflammatory disease. In this regard, Vitamin D is a well characterised immunomodulatory hormone, shown to have direct regulatory effects on $CD4^+$ T cells, inducing IL-10 and inhibiting pro-inflammatory cytokines, such as IFNγ and IL-17. The mechanisms underlying these Vitamin D effects on $CD4^+$ T cells remain ill-defined. As Vitamin D, bound to the vitamin D receptor (VDR) has histone modifying capacity, we hypothesised that this would be a major mechanism underlying it's immunoregulatory actions on $CD4^+$ T cells.

Methods:
We use a combination of high throughput assays; phospho-array, targeted RNA-seq and ChIP-seq, validated through cytometric bead array, western blot, flow cytometry and image stream to interrogate the effects of Vitamin D treatment on T cell biology.

Results:
Here we show that Vitamin d is crucial in inducing a human $CD4^+$ T cell contraction like phase, repressing pro-inflammatory cytokine production, and inducing a unique T regulatory phenotype, which includes IL-6 production. We show IL-10 production in these cells is dependent on a novel IL-6 signalling loop, for which Vitamin d is vital. We present evidence suggesting this signalling loop can function in an entirely self-contained fashion, including the enzymatic activation of vitamin D. Finally, we describe how Vitamin D regulates this phenotype largely through enhancer formation inducing H3K27 acetylation, and suggest that this is achieved directly through VDR binding and co-receptor recruitment.

Conclusion:
Ultimately, we have identified a novel Vitamin D mediated mechanism, which acts to counteract potentially deleterious $CD4^+$ T cell activation.

I20: CD28 co-stimulation delivered in trans can effectively initiate T cell activation.

Didsbury, A.J. [1,2], Verdon, D. [1,2], Sheppard, H. [1,2], Dunbar, P.R. [1,2], and Brooks, A.E.S. [1,2]

[1] School of Biological Sciences, The University of Auckland, Auckland, NZ, [2] The Maurice Wilkins Centre, Auckland University, Auckland, NZ

T cells are white blood cells that drive many types of immune responses within the body. In order to respond appropriately, a T cell must recognise both foreign antigen and also receive a second signal, mediated by an independent co-receptor. To date, intracellular signals following T cell stimulation and co-stimulation have been poorly studied. One reason is that the techniques used to manipulate T cells in culture introduce factors that can confound subsequent analysis. In addition, it is difficult to separate the synergistic effect of the two signals, preventing effective measurement of the individual contributions. Recent advances in culture methods allow the growth of T cells without the need to add stimulating cytokines, and furthermore, allow the delivery of each signal individually. Exploitation of these techniques has enabled us to separate and examine the key signals involved in T cell co-stimulation.

Using a combination of flow cytometry, impedance assays (xCELLigence), and bead-based immunochemistry assays, we are able to show that stimulation through the CD28 receptor produces a signal that is 'stored' within the cell for up to 6 hours, and results in the phosphorylation of key signaling proteins including NFκB and JNK(1/2). When CD28 is delivered in trans to a TCR stimulus, this signal is able to be integrated, resulting in an increase in proliferation and effector function.

Determining the differences between these signals in an *ex vivo* context has implications for approaches to immune therapy, including vaccines and adoptive cell therapy for both cancer and infectious disease.

I21: T-Cell Distribution in Melanoma from Exercising Mice

Buss, Linda[1], Currie, Margaret[1], Ang, Abel[1], Hock, Barry[2] and Dachs, Gabi[1]

[1]Mackenzie Cancer Research Group and [2]Haematology Research Group, Department of Pathology and Biomedical Science, University of Otago, Christchurch, NZ

Exercise improves survival in some cancers, but it is unclear whether this is true for melanoma. Mechanistically, rodent studies using breast and prostate tumour models have shown increased tumour perfusion and reduced hypoxia with exercise, and a mouse model of colorectal cancer has demonstrated increased CD8 gene expression with exercise. It has been shown that T-cells avoid regions of hypoxia (in a mouse model of fibrosarcoma), but it is unknown whether this changes with exercise. Mitochondrial content in skeletal muscle may be directly associated with exercise capacity, suggesting that a measure of mitochondrial content such as cytochrome c oxidase subunit 4 (COX-IV) expression could act as an exercise biomarker.

We aimed to investigate the effect of exercise on tumour growth, the tumour microenvironment and T-cell distribution/infiltrate in melanoma, and to validate muscular COX-IV expression as a biomarker of exercise.

Female C57BL/6 mice were randomly assigned to exercise or sedentary control groups following subcutaneous implantation of B16-F10 melanoma into the right flank. Exercising mice were provided with wheels to allow for voluntary running. When tumours reached maximum size, mice were injected with the hypoxia marker pimonidazole and the perfusion marker Hoechst 33342 before euthanasia and tissue harvest.

COX-IV expression was significantly increased in muscle of exercising mice ($p=0.04$). We observed no difference in tumour growth in exercising compared with sedentary mice. A significantly higher T-cell density was observed around perfused vessels compared with hypoxic areas ($p=0.005$) or inter-capillary space ($p=0.005$). T-cell distribution was unchanged in tumours from exercising compared with sedentary mice, but mice with high muscular COX-IV expression tended to have higher T-cell infiltrate than those with low COX-IV expression ($p=0.06$).

Our data indicate that tumour-infiltrating T-cells preferentially cluster around perfused vessels. In addition, a higher exercise capacity (measured by muscular COX-IV) may improve T-cell infiltrate into the tumour.

I22: Identifying Autoantibody Targets as New Biomarkers for Acute Rheumatic Fever using High Content Protein Arrays

Mei Lin Tay[1], Paulina Hanson-Manful[1], Jeremy M Raynes[1,2], Reuben McGregor[1], Nicole J Moreland[1,2] and the Rheumatic Fever Risk Factors Study Group

[1]Department of Molecular Medicine, University of Auckland, Auckland, New Zealand, [2]Maurice Wilkins Centre, Auckland, New Zealand.

Acute rheumatic fever (ARF) and associated rheumatic heart disease (RHD) are serious sequelae of Group A Streptococcus (GAS) infection. Rates of ARF/RHD remain unacceptably high in Maori and Pacific children in New Zealand. Specific diagnostic tests for ARF are lacking, with diagnosis instead relying on a set of clinical criteria. This presents a major hurdle in disease control efforts, with an accurate diagnosis requiring a series of assessments over a period of days. Our aim is to identify novel human targets of auto-antibodies in patients with ARF that can serve as biomarkers for diagnosis. Initially, sera from a limited number of patients with ARF, together with matched controls, were run on high content protein arrays (Protoarray, 9000 autoantigens, n=10; HuProt Array, 16,000 autoantigens; n=18). This identified 34 potential autoantigens for which the antibody binding signal was higher in ARF sera compared with controls. Pathway analysis found many of these autoantigens were linked to cardiac function and immune response networks (Qiagen Ingenuity® Pathway Analysis). Further testing of these autoantigens against a large ARF sample cohort (n=150; matched case-controls) in a focused protein array format has identified several autoantigens with biomarker potential. These will be validated in ELISA and bead-based immunoassays as a route to assessing the utility of the proteins in clinical diagnosis of ARF.

I23: Evaluation of a novel multi-Staphylococcal Superantigen like (SSL) fusion vaccine for Staphylococcus aureus

Janlin Chan, Ries Langley, Fiona Radcliff, Fiona Clow, John Fraser.

Department of Molecular Medicine and Pathology, University of Auckland, Auckland, New Zealand

Staphylococcus aureus is a major opportunistic pathogen common to nosocomial and community settings. The emergence of multi-drug resistant S. aureus has exacerbated the demand for new treatments and placed S. aureus on the WHO's list of high priority pathogens for research into new therapies. Currently New Zealand has the highest incidence of S. aureus infection in developed countries, with indigenous populations such as Māori and Pacific island people at the most susceptible. With the dearth of new antibiotics, prophylaxis in the form of vaccination is essential to curtail the rise of resistant strains. Staphylococcal superantigen-like proteins (SSLs) are a group of Staphylococcus aureus virulence factors with immune dampening and evasion functions crucial to S. aureus survival during infection. Past studies of SSLs have found the fourteen members ssl cluster to be conserved in every S. aureus strain. SSL3, SSL7 and SSL11 are representative members that target different branches of the host immune system. Evidence suggests that SSLs are immunogenic and vaccination of mice with SSLs could result in attenuation of disease.

Wildtype and attenuated SSL7, SSL3 and SSL11 were produced as recombinant poly-SSLs fusion proteins. Both poly-SSLs were characterised in functional assays and it was confirmed that attenuated SSL7-3-11 possesses reduced functionality compared to wild-type SSL7-3-11. Activities of both fusion proteins are comparable to the individual wild-type and attenuated SSL7 and SSL11. An assay for SSL3 functionality assay which looks at suppression of pro-inflammatory cytokines in THP-1 cells by the poly-SSLs is currently underway. Production of a soluble attenuated fusion SSL protein has been proven to be feasible and fusing SSLs has no apparent effect on the function of the protein. The potential of the recombinant protein as a vaccine for limiting disseminated staphylococcal disease will be further evaluated using a mouse model, with immunogenicity assessed using in-vivo assays.

I24: Functional Analysis of Streptococcus Virulence Factors using a Zebrafish Infection Model

Soh, K.Y.[1], Loh, J.[1,2] , Hall, C.[1], Proft, T.[1,2]

[1]Department of Molecular Medicine and Pathology, School of Medical Sciences, The University of Auckland, NZ, [2]Maurice Wilkins Centre for Molecular Biodiscovery, NZ.

Streptococcus pyogenes (Group A Streptococcus, GAS) causes a variety of diseases in humans ranging from pharyngitis and impetigo to more severe invasive diseases including cellulitis, necrotising fasciitis and toxic shock. Investigation of GAS pathomechanisms are hindered by the lack of suitable animal infection models, because GAS is an exclusive human pathogen. We have previously characterised two novel GAS virulence factors, *Streptococcus pyogenes* nuclease A (SpnA) and streptococcal 5'-nucleotidase A (S5nA). SpnA and S5nA have shown to be involved in immune escape by cleaving DNA and generating adenosine and deoxyadenosine. Orthologues of both these virulence factors have been identified in the related species *Streptococcus iniae*, a major fish pathogen. Biochemical analysis of recombinant form of SpnA and S5nA from *S. iniae* (rSpnAi and rS5nAi) showed that both proteins have very similar functions *in vitro* to rSpnA and rS5nA from GAS, making them true orthologues. We now aim to characterise the mechanisms of these two important GAS virulence factors *in vivo* by investigating their orthologues SpnAi and S5nAi by using a zebrafish infection model. Deletion mutants of *spnAi* and *s5nAi* genes in *S. iniae* have been generated by allelic replacement. Injection of 100 cfu wild-type *S. iniae* into the hindbrain of zebrafish larvae induced a lethal infection, while *S. iniae* Δ*SpnAi* and Δ*S5nAi* gene deletion mutants displayed greater than 80% survival at 24 h post-infection. This suggests that SpnAi and S5nAi contribute to the virulence of *S. iniae in vivo*. Further investigation into the mechanisms of these two enzymes will be carried out in the zebrafish infection model, which may be used to infer the *in vivo* functions of their GAS orthologues in the human host.

I25: Dissection of cellular niches by multi-dimensional tissue imaging

Yury Goltsev, Nikolay Samusik, Julia Kennedy-Darling and Garry Nolan.

Department of Microbiology and Immunology, Stanford University, Stanford, CA, United States

Survival depends on ability of a host to reorganize tissues in response to pathogens be it a foreign agent or a malignant cell. In the latter case the contextual modulation of immune reaction is especially pronounced as suggested by recent account of immunomodulatory factors expressed by cancer cells. In which context does immune response succeed and when does it fail? What local combination of cells does it take to achieve tumor clearance. Answering these questions needs a technique able to resolve multiplicity of cell types and their interactions in samples with preserved tissue architecture.

We have developed a highly multiplexed cytometric imaging approach, termed CODetection by indEXing (CODEX). CODEX iteratively visualizes antibody binding events using DNA barcodes, and either polymerization- or exchange- based indexing procedure. An accompanying unique set of algorithms successfully identifies individual cells in crowded environments and allows accurate quantification of expression by means of a special approach for positional spill compensation.

We used CODEX to characterize a variety of tissues in norm and disease (e.g. the dynamics of splenic architecture in the course of spontaneous autoimmunity in MRL/lpr model). A special set of paradigms for quantifying pairwise and combinatorial cell-to-cell interactions was established. Numerous foundational principles of tissue architecture (e.g. the prevailing homotypic adhesion) were observed in the data. Extensive and previously uncharacterized interaction dynamics in the healthy versus diseased state was observed. In addition we found quantified a ubiquiotous profound impact of the cellular neighborhood on the expression of protein receptors on immune cells. Finally multidimensional staining data provided a rich source for training and testing the diagnostic performance of unsupervised machine learning techniques such as convolutional neural networks.

Altogether the fidelity of multiplexed spatial cytometry by CODEX demonstrated in our study allows for quantitative systemic characterization of tissue architecture in normal and clinically aberrant samples.

I26: BCG vaccination induces ILC expansion and alters phagocyte dynamics early after mycobacterial challenge

Ryder B.M.[1], Steigler P.[2], Wiles S.[3], Kirman J.R.[1]

[1]Department of Microbiology, School of Biomedical Sciences, University of Otago, Dunedin, NZ,
[2]South African Tuberculosis Vaccine Initiative, University of Cape Town, Cape Town, South Africa
[3]Bioluminescent Superbugs Lab, Department of Molecular Medicine and Pathology, University of Auckland, Auckland, NZ.

Tuberculosis (TB) remains a primary driver of infectious disease burden worldwide. While the current TB vaccine – attenuated Mycobacterium bovis bacille Calmette-Guérin (BCG) – effectively protects children against disseminated TB infection, protection afforded against adult pulmonary infection varies between 0% and 80%. Efforts to develop more efficacious vaccines are hampered by an incomplete understanding of how BCG enhances immune responses to mycobacterial infection.

Innate immune cells have recently received increased recognition for their capacity to exhibit memory-like enhanced recall responses during infection, including innate lymphoid cells (ILCs). Our lab previously demonstrated that BCG vaccination induces accumulation of and interferon-gamma production by ILCs in the lungs of C57BL/6 mice, with the strongest enhancement occurring after intranasal BCG administration. In the present study, we have compared ILC abundance and spontaneous cytokine production after infectious challenge between vaccinated and unvaccinated mice, revealing specific expansion of a T-bet+RORγt+ ILC population in the lungs of vaccinated mice in response to challenge. Changes in phagocyte abundance and uptake of mycobacteria during the first two weeks of infection were also observed between mice previously BCG-vaccinated or unvaccinated, to determine how BCG vaccination influences recruitment and accumulation of phagocyte populations that are critical for early control of mycobacterial infection..

I27: PilVax: a novel peptide carrier for the development of vaccines against tuberculosis

Blanchett, S.[1], Loh, J.[1,3] Kirman, J. [2,3] Proft T.[1,3]

[1]Department of Molecular Medicine and Pathology, University of Auckland, Auckland, NZ, [2]Department of Microbiology, University of Otago, Dunedin, NZ, [3]Maurice Wilkins Centre, NZ

PilVax is a peptide delivery strategy for the generation of highly specific mucosal immune responses. The food-grade bacterium *Lactococcus lactis* is used to express selected peptides engineered within the Group A Streptococcal pilus, allowing for peptide amplification, stabilization, and enhanced immunogenicity.

The present study aims to demonstrate the suitability of PilVax for the generation of novel peptide vaccines against tuberculosis. Selected peptides (B cell and T cell epitopes), derived from tuberculosis vaccine targets ESAT6 and Ag85B, were genetically engineered into loop regions of the pilus backbone subunit and expressed in *L. lactis.* Western blots confirmed pilus formation on *L. lactis*.

A significant number of Ag85B peptide specific CD4+ T cells were detected when mice were immunised intranasally with an Ag85B PilVax construct, at levels similar to when mice were immunised with BCG. However no CD4+ T cells specific for ESAT peptides were detected. This is despite a significant amount of IL-2 and TNFa being detected in lung T cells from mice immunised with a PilVax ESAT peptide construct following stimulation with cognate peptide. We are currently also testing the antibody response to PilVax constructs expressing these same Ag85B and ESAT6 peptides. These results confirm at least one PilVax construct is able to elicit a similar CD4+ T cell response to a BCG vaccination, which will be tested further for protection.

I28: Improving mucosal immune responses generated by TeeVax, a T-antigen-based vaccine for the prevention of rheumatic fever.

Loh, J.M.S[1,2], Khemlani, A.H. [1], Proft, T.[1,2]

[1]Department of Molecular Medicine and Pathology, School of Medical Sciences, University of Auckland, NZ, [2]Maurice Wilkins Centre for Molecular Biodiscovery, NZ.

Rheumatic fever is a serious illness that can cause permanent heart damage leading to rheumatic heart disease. Progression to this disease is thought to be caused by repeated, untreated Group A Streptococcal (GAS) skin and throat infections. The GAS pilus is involved in bacterial adhesion and colonisation at these mucosal sites, making it an attractive target for a vaccine to prevent rheumatic fever.

We have a vaccine under development that is composed of domains from 18 different T-antigens (the major protein component of the GAS pilus) called TeeVax. TeeVax is expected to provide close to 100% coverage of all GAS strains found in NZ. Parenteral immunisation in mice and rabbits have shown to generate high systemic IgG responses against all T-antigens. However, there is early indication that high IgA levels at the respiratory mucosa may be what is required to provide protection from bacterial colonisation.

Current work compares the antibody responses generated by the immunisation of TeeVax with various adjuvants including cholera toxin B, *Lactococcus lactis*, alum, and Addavax™. We also investigate the use of nasal delivery to improve mucosal immune responses with hopes to improve the ability of our vaccine to prevent bacterial infection at common entry points to the body, thereby halting progression to disease.

I29: Modulation of CX3CR1 Expression on T cells *in vitro* by common gamma-chain cytokine exposure.

Gaines, A.K.[1], Verdon, D.J.[1], Blenkiron, C.[1], Print, C.[1], Dunbar, R.[1,2]

[1]University of Auckland, Auckland, NZ, [2]Maurice Wilkins Centre for Molecular Biodiscovery, University of Auckland, Auckland, New Zealand.

The chemokine CX3CL1/fractalkine, acts as both a chemoattractant and as an adhesion molecule aiding leukocyte arrest. Tumour-derived CX3CL1 has been shown to induce intratumoural trafficking of CX3CR1+ T cells [1] and the adoptive transfer of T cells engineered to ectopically express the fractalkine receptor CX3CR1 has been demonstrated to inhibit tumour growth [2]. We have previously shown that cells expanded in the presence of interleukin(s) (IL)-7/-21 retained a less differentiated naïve/T_{SCM} phenotype ($CD95^+CD45RA^+CD28^+CD62L^+CCR7^+IL\text{-}7Ra^+$), while those expanded with IL-2 or IL-15 displayed an effector/T_{EM} phenotype ($CD95^+CD28^-CD62L^-CCR7^-IL\text{-}7Ra^-$) [3]. To further investigate phenotypic differences between polarised clonal populations, we profiled differential gene expression patterns using pre- and post-expansion clonal populations.

Expression of CX3CR1 was highly polarised, demonstrating greater expression following expansion in IL-7/-21 relative to the original clonal population and a paired IL-2 expanded population. As CX3CR1 expression had previously been associated with a T_{EM} phenotype, a 12-parameter flow cytometric panel was used to profile CX3CR1 and T cell memory/differentiation marker expression by T cells within PBMC. CX3CR1 expression directly *ex vivo* was highest on ($CD45RO^+CCR7^-$)-T_{EM} and ($CD45RA^+CCR7^-$)-T_{EMRA} with negligible expression by naïve ($CD45RA^+CCR7^+$) and T_{CM} ($CD45RO^+CCR7^+$) populations, suggesting differential regulation of CX3CR1 *in vitro* and *in vivo*.

To assess the effect of signaling through the IL-2Rβ(CD122;shared by IL-2 and IL-15) we investigated: CX3CR1 expression on $CD8^+$ T cell clones differentially expanded in IL-7, IL-2 and IL-15; and CX3CR1 expression on polyclonal naïve T cells which had been expanded in titrated concentrations of IL-15. CX3CR1 expression was only retained in clonal T cells cultured in IL-7, and CX3CR1 expression on expanded naïve T cells was inversely correlated with IL-15 dose, suggesting that prolonged CD122 signalling inhibits CX3CR1 expression. The ability to modulate CX3CR1 expression *in vitro* may enable the production of T cells for both exhibiting a minimally differentiated phenotype and greater tumour-homing potential for immunotherapeutic applications.

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I30: PilVax – a Novel Peptide Delivery Platform for the Development of Mucosal Vaccines

Tsai, C. J.[1, 2], Loh, J. M.[1, 2], Proft, T. K.[1, 2]

[1]School of Medical Sciences, University of Auckland, Auckland, NZ, [2]Maurice Wilkins Centre for Molecular Biodiscovery, Auckland, NZ

Vaccines remain the most cost-effective and feasible means of infectious disease control in the community. Well-defined synthetic vaccines based on individual peptides are specific and safe. However, peptide antigens are usually poorly immunogenic and sensitive to proteolytic degradation, thus require costly conjugation to carrier proteins and administration with potentially toxic adjuvants. Lactic acid bacteria have become promising vehicle for delivering active molecules to mucosal sites. We have developed PilVax, a novel peptide delivery platform that allows the presentation of a stabilised and highly amplified peptide as part of the group A streptococcus (GAS) serotype M1 pilus structure (PilM1) on the surface of the food-grade bacterium *Lactococcus lactis*. Pili (*sing.* Pilus) are hair-like protrusions from the bacterial cell surface. GAS pili are mainly formed by multiple copies of the highly immunogenic, covalently-linked backbone pilin (Spy0128). We identified several surface-accessible, structurally-flexible loop regions within Spy0128 that can be replaced with the model peptide $OVA_{324-339}$. The modified pilus structure was expressed on the surface of *L. lactis* by a plasmid harbouring a strong, constitutive promotor. Pili assembly and peptide display were analysed by western blot and flow cytometry using specific antibodies. Intranasal immunisation of PilVax generated strong mucosal and systemic antibody responses in mice. We have further show that it is possible to insert more than one peptide into the same integration site, and peptide epitopes can be incorporated into structurally similar but antigenically different pilus structure. PilVax also provides benefits such as higher safety and lower production and transportation costs. Furthermore, the needle-free mucosal administration route is an additional advantage for the use in developing countries, where efficacious vaccines are most needed.

I31: The presence of mitochondrial DNA controls nuclear immune response gene expression in a breast tumour model

Berridge, M.V.[1], Eccles, D.[1], Grasso, C.[1], Lamiable, O.[1], Herst, P.[1, 2], McConnell, M.J.[3]

[1] Malaghan Institute of Medical Research, Wellington, NZ, [2] Department of Radiation Therapy, University of Otago Wellington, NZ, [3]School of Biological Sciences, Victoria University of Wellington, NZ.

Mitochondrial DNA (mtDNA), a circular double-stranded chromosome, contains 13 protein-encoding genes that are essential for mitochondrial respiration and for cell survival. Cancer cells without mtDNA are usually auxotrophic for uridine and often need pyruvate supplementation. We have developed mouse tumour cell lines devoid of mtDNA (ρ^o cells) and found that tumour development by these cells occurs only after a long lag period during which time injected cells obtain a complete mitochondrial genome by acquisition of whole mitochondria from the host mouse and respiration is restored[1, 2]. This novel physiological phenomenon has been unequivocally demonstrated in two metastatic mouse tumour models, B16 melanoma and 4T1 breast carcinoma, and in GL261 glioblastoma cells using mtDNA polymorphisms between the tumour cell lines and mouse strains from which they were derived. We have used 4T1 and 4T1ρ^o cells to investigate the effect of mtDNA loss on the expression of nuclear genes using MinION long-read cDNA sequencing. First we established that 4T1ρ^o cells did not express mitochondrial transcripts. Next we looked at differential nuclear gene expression between 4T1 and 4T1ρ^o cells. Transcripts of the most highly expressed genes were 5 times more abundant in 4T1ρ^o than in 4T1 cells. In contrast, of the 9 genes with highest transcript abundance in 4T1 cells, 8 were related to immune response or oxidant stress and therefore controlled directly or indirectly by the presence of mtDNA. In contrast, none of the top xx genes with highest abundance in 4T1ρ^o cells were associated with immune responses or oxidant stress. Our results confirmed functional expression of *Tslp* in 4T1 cells, and show that *Tslp*, *Ccl2, Ccl7* and several *Gst* (glutathione S-transferase) gene transcripts are regulated by the presence of mtDNA. We conclude that respiration competent mitochondria are key players in controlling immune responses and oxidant stress in breast cancer.

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I32: Altering the Mevalonate pathway to enhance CD8+ T cell responses

Burn, O[1,3]., Authier-Hall, A[1]., Brooks, C[1]., Anderson, R[2]., Marshall, A[2]., Painter, G[2]., Weinkove, R[1]., Hermans,I[1].

[1]Malaghan Institute of Medical Research., [2]Ferrier Research Institute, Victoria University of Wellington., [3]University of Otago.

Abstract embargoed at this time.

I33: Differential regulation of MR1- and cytokine stimulated MAIT cells

Lamichhane, R.[1], de la Harpe, S.[2], Harrop, T.[3], Vernall, A.[2], Tyndall, J.[2], Dearden, P.[3], Kirman, J.[1], Ussher, J.E.[1]

[1]Department of Microbiology and Immunology, University of Otago, [2] School of Pharmacy, University of Otago, [3]Department of Biochemistry, University of Otago

MAIT cells are innate-like T-cell subset which express a semi-invariant T-cell receptor (Vα7.2-Jα12/20/33) and recognize antigen presented by the evolutionary conserved MHC class I related protein, MR1. Recently, the ligand for MR1 was identified as unstable pyrimidines derived from a metabolic precursor of bacterial riboflavin biosynthesis pathway, 5-amino-6-D-ribitylaminouracil (5-A-RU)[1,2]. Concurrently, MAIT cells have been shown to recognize and kill cells infected with riboflavin producing bacteria[3, 4]. MAIT cells are the highest IL18 receptor expressing CD8+ T cell populations and also express IL12 receptor; they can be activated via their cytokine receptors by IL-12 and IL-18[5]. However, it is unclear how the effector function of human MAIT cells differ in response to different stimuli targeting the two independent modes of activation. In this study, we investigated the effector functions of MAIT cells to *E. coli,* 5-A-RU and cytokines (IL-12+IL-18). We observed rapid activation of human MAIT cells and significant cytokine production against all stimuli, although the magnitude and proportion of cytokines produced differed with the mode of activation involved. Differences in timing of maximal activation were seen with different stimuli, both at the protein and transcriptomic levels. To explore the effector functions of activated MAIT cells and further outline the differences between the two modes of activation, RNA sequencing was performed on MAIT cells stimulated with *E. coli*, 5-A-RU or IL-12+IL-18. Differences in MAIT cell response were observed at both transcriptome and protein level in cytokine and chemokine production and the expression profile of cytotoxic molecules, transcription factors and other regulatory molecules. Overall, this suggests that MAIT cells and their effector functions differ with the mode of activation.

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I34: To bind or not to bind: A kinase-independent role of Protein kinase R in Anthrax lethal-toxin treated macrophages.

Patel, D.[1], Schenone, M.[2], Clatworthy, A.[2,3], Hett E.C.[2,3], LeGros, G.[1],Collier, R.J.[4], Hung, D.[2,3].

[1]Malaghan Institute of Medical Research, Wellington, NZ, [2]Broad Institute, MA, USA, [3]Massachusetts General Hospital, MA, USA, [4]Department of Microbiology and Immunobiology, Harvard Medical School, MA, USA.

Even across the barriers of time microbes have been our companions through danger. A bond that has helped us grow a thick skin through life's bit-rich pains. Natures very own i-game designed for us to thrive alongside each other to build the foundations of what we are today, survivors.
Over the last thirty-seven years I have been deciphering the code to how we communicate. From the very first warm embrace as a newborn to the relentless noise of a foreign world, and unforgiving silence of an ageing body and soul. Searching for the earliest signals to be etched into our memories that give us, living beings, immunity. I have found my insight into the immune system through the most unusual of sources: the Earths microbial monsters; bacterial toxins that make intimate contact with our cells to give birth to their very own immune response.
My toxin of choice? The remarkable tripartite transformer, Anthrax. A super-scrum bumblebee structure that primes a pore through the macrophage cell barrier to activate the Nlrp1 inflammasome. Regulated by a novel kinase-independent process of Protein kinase R (Eukaryotic Translation Initiation Factor 2 Alpha Kinase 2)[1], the cell itself embarks on a transformation on the fringes of death that spans the cell surface, mitochondria and nucleus.
My work on the host response to Anthrax toxin has helped me see that boundaries are not set in stone. Boundaries are adaptable. Open to a Christmas invasion of gifts and hot tea to see in the new year.
"*Sometimes its the…*monsters…*that no one imagines anything of who do the things that no one can imagine"*, Alan Turing.

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I35: Assessing the utility of SpnA, a novel Group A *Streptococcus* antigen, to improve clinical streptococcal serology

Alana L. Whitcombe, Paulina Hanson-Manful, Thomas Proft and Nicole J. Moreland

School of Medical Sciences and Maurice Wilkins Centre, University of Auckland, Auckland, New Zealand

Acute rheumatic fever (ARF) and post-streptococcal glomerulonephritis are serious immune sequelae that develop several weeks after a Group A *Streptococcus* (GAS) infection. Evidence of prior infection with GAS is crucial to the diagnosis of these diseases and is provided from serological tests. Current tests measure anti-streptolysin-O (ASO) and anti-DNaseB (ADB) antibodies. Despite being widely used, these tests have limitations including a slow rate of decay in antibody response that can increase false positives in settings where GAS is endemic, and incompatible methodology that requires the two assays to be run in parallel. In this work, the utility of a novel GAS antigen, SpnA, was assessed in a multiplex bead-based immunoassay. Recombinant streptolysin-O, DNaseB and SpnA were conjugated to magnetic beads and a triplex assay was developed to measure serum antibody binding. Sera from patients with ARF collected in the Waikato and in Auckland were used for initial testing. Further sera was obtained from patients with ARF and matched healthy controls as part of the recently completed nation-wide study of rheumatic fever risk factors, as well as sera from community laboratories across Auckland. The ability of the antigens to detect a previous GAS exposure for ARF diagnosis was assessed using the 80th centile of the healthy control group as a cut-off (upper limit of normal). Using these experimentally determined cut-offs the sensitivity of anti-SpnA was compared to ASO and ADB. SpnA was found to have favourable immunokinetics for streptococcal serology, and the combination of SpnA with ASO and ADB in a multiplex assay should improve the efficiency and accuracy of streptococcal serology for clinical diagnosis.

I36: Neutrophils suppress mucosal associated invariant T cells

Schneider, M.[1], Hannaway, R.F.[1], Lamichhane R.[1], Ussher, J.E.[1]

[1]Department of Microbiology and Immunology, University of Otago, Dunedin, NZ.

Mucosal associated invariant T (MAIT) cells are abundant innate-like lymphocytes which are rapidly activated in response to bacterial and fungal infections. Upon activation MAIT cells produce cytokines, including INFγ and TNFα, and upregulate cell surface markers CD69 and CD137 (4-1BB). Neutrophils are crucial for the early immune response and have been shown to suppress the activation of innate-like lymphocytes, such as γδ T cells and iNKT cells. Here, we investigated the influence of neutrophils on MAIT cell activation. MAIT cells, monocytes, and neutrophils were isolated from the blood of healthy donors. Cells were stimulated with fixed bacteria, and their activation assessed by flow cytometry. We show that neutrophils supressed the activation of MAIT cells, both in co-cultures with monocytes and in PBMCs after stimulation with fixed bacteria. The production of effector cytokines INFγ and TNFα, as well as the upregulation of cell surface markers CD69 and CD137 (4-1BB), was inhibited. Therefore, neutrophils suppress the activation of MAIT cells and may play an important role in the regulation of the innate immune response to extracellular bacteria through the inhibition of innate-like lymphocytes. Investigations into the mechanism of suppression are ongoing with results to be presented at the conference.

I37: Human monocyte-derived dendritic cells respond chemokinetically to full-length CCL21, in contrast to the chemotactic responses to CCL19 and CCL21 that is C-terminally truncated by plasmin, as shown by live-cell microscopy.

Evert J. Loef [1 2] , Rod Dunbar [1 2] , Nigel P. Birch [1 3]

[1]School of Biological Science, University of Auckland, Auckland, New Zealand. [2]Maurice Wilkins Centre for Molecular Biodiscovery, University of Auckland, Auckland, New Zealand. [3]Centre for Brain Research and Brain Research New Zealand; Rangahau Roro Aotearoa, University of Auckland, Auckland, New Zealand

Immune function depends on the ability of immune cells to respond to migratory signals. We have shown that incubation of CCL21 with plasmin results in a C-terminally truncated form of CCL21, which has been reported to behave more like CCL19.
While migration induced by chemokines is often assumed to be directional (chemotaxis), end-point migration assays commonly used in the literature are confounded by the detection of increased cell migration that lacks directionality (chemokinesis).
To distinguish between chemotaxis and chemokinesis, we developed a live-cell video microscopy assay to monitor cell migration under agar. We placed chemokine in two separate wells in medium containing agar to generate different gradients of CCL19, CCL21 alone, or CCL21 with plasmin in the surrounding agar, and simultaneously live-imaged how these competing gradients influenced the migration of monocyte-derived human dendritic cells (moDCs). This allowed careful comparison of migratory responses to different types of chemokine signal.
The gradients generated in our assay system, imaged with a fluorescent marker, lasted several hours. Over that time-period moDCs migrated chemotactically in the direction of a CCL19 gradient. In contrast, under the same gradient of CCL21, moDCs showed increased exploratory chemokinesis, but only minor directional bias. When plasmin was added, the chemotactic response of moDCs to CCL21 increased, as seen by an increase in track straightness and displacement toward the CCL21 gradient. Furthermore, we showed that human T-cells can bind plasminogen and subsequently convert it into plasmin when activated. When these T-cells were added to CCL21, it also resulted in an increase in chemotaxis of the responding moDCs, which was inhibited by the plasmin specific inhibitor α2-antiplasmin.
These specific motility patterns suggest a concept that immune cells, use strategies to optimize their response between environmental cues, enabling exploration of new information and efficient scanning for target cells in peripheral tissues.

I38: Immunologic considerations of a surgical mouse model of colorectal cancer

Niemi, GR[1], Naciute, M[2], Cockerill, S[3], Kemp, RA[1].

[1]Department of Microbiology and Immunology, [2]School of Pharmacy, [3]Animal Welfare Office, University of Otago, Dunedin, New Zealand

Mouse models are used to study the immune response to cancer, allowing the testing of new therapies in the context of a whole organism. The tumour microenvironment in colorectal cancer (CRC) has a large impact on the quantity and quality of the anti-tumour immune response; however, many mouse models of CRC do not accurately model the tumour microenvironment. Orthotopic models of CRC, where the tumour grows in closest approximation to the site of human disease (bowel), can be modelled in mice using an intracaecal (IC) injection of CRC tumour cells (CT26 colon carcinoma). To inject the tumour cells, the caecum must be surgically exposed to access the subserosa. There are many aspects of this surgical process which can affect the immune response. Mice are exposed to anaesthetic drugs, have the abdomen open and exposed to the air, and require analgesics for surgery-related pain, all factors known to modulate local and systemic immunity.

We have optimised an IC mouse model to reduce immunological effects and to promote animal welfare. Key optimisation steps included: a switch from injectable to inhalant anaesthesia (to reduce anaesthesia exposure), performing the surgery in sterile conditions (to reduce the chance of contamination during surgery), and pre- and post-operative support (analagesics, antibiotics, fluid replacement, heat maintenance).

The frequency of immune cells in response to IC surgery was measured in the tumour, spleen and lymph nodes. Dendritic cells (CD11c+), macrophages (F480+ and CD11b+), T cells, (CD4+, CD8+, and CD4+ T regulatory), and B cells (CD19+) were quantified via flow cytometry.

Future work will involve treating mice with an immunomodulatory agent (chitosan gel cancer vaccine) before tumour challenge. This work will help identify potentially protective immune cell populations, which can be analysed in human CRC patients to determine a protective immune signature.

I39: Individual and cocktails of TLR ligands influence cytokine secretion by human skin explants

du Mez, E.[1], Johnson, R.[4,5], Kho, D.[4,5], Feisst, V.[1,2], Locke, M.[3], McIntosh, J.[1,2], Brooks, A.[1,2], Didsbury, A.[1,2], Graham, S.[4,5], Angel, C.[1,2]

[1]School of Biological Sciences, University of Auckland, Auckland, New Zealand, [2]Maurice Wilkins Centre, University of Auckland, Auckland, New Zealand, [3]Department of Surgery, South Auckland Clinical Campus, Middlemore Hospital, Auckland, New Zealand, [4]Centre for Brain Research, School of Medical Sciences, Faculty of Medical and Health Sciences, University of Auckland, Auckland, New Zealand, [5]Department of Pharmacology and Clinical Pharmacology, School of Medical Sciences, Faculty of Medical and Health Sciences, University of Auckland, Auckland, New Zealand

Antigen presenting cells (APCs) are the sentinel cells of the immune system and are found at sites susceptible to pathogen infiltration, including the skin. APCs detect invading pathogens via Toll-like receptors (TLR), enabling APCs to stimulate an appropriate immune response to distinct pathogens. We can exploit this mechanism by including synthetic TLR ligands in vaccines to augment an immune response against vaccine antigens. Currently, the TLR3 ligand Poly I:C and/or the TLR7/8 ligand R848 are being tested in clinical trials as potential cancer vaccine adjuvants, however, the effect of intradermal stimulation with these ligands individually or combinatorically on cutaneous APC subsets and other cells in human skin is not well characterised. We designed a human skin explant model which was intradermally injected with Poly I:C, R848, or a combination of these TLR ligands. Following a 48 hr culture period, the phenotype and maturation status of the APCs which migrated out of the skin and the concentration of cytokines secreted by cells in the explants were assessed. We found that R848 augmented the secretion of IL-1β, IL-10, IL-12/IL-23p40, IP-10, MIP-1α, MIP-1β, MIG, TNF, GM-CSF, MCP-1, and VEGF, while Poly I:C slightly increased IL-6 and IL-8 only. Combinatorically, Poly I:C and R848 increased IFN-γ above that of explants receiving Poly I:C or R848 alone. Poly I:C and/or R848 had no observable effect on the phenotype or maturation status of APCs that had migrated from the skin explants after intradermal injection with these ligands. These data suggest intradermal injection of Poly I:C and/or R848 modulates cytokine secretion by cells in human skin explants. Further work is required to elucidate the effect of these TLR ligands on APC subsets in human skin.

I40: Characterising T cell responses in an orthotopic intracaecal mouse model of colorectal cancer.

Gaskarth, D. A[1], Niemi, G. R[1], Highton, A[1], Hook, S[2] and Kemp, R. A[1]

[1] Department of Microbiology and Immunology, [2] School of Pharmacy, University of Otago, Dunedin, New Zealand

Tumour-infiltrating CD4+ and CD8+ T cells have a variety of functions within cancer and have been shown to be important determinants of patient outcome. Specifically, the number of CD4+ and CD8+ effector cells, which produce IFN-γ, is strongly associated with tumour regression. Research into CD4+ and CD8+ populations within human colorectal tumours remains limited, including the abundance of intra-tumoral memory T cells, which have been shown to prevent tumour relapse in mouse models. CD4+ regulatory T cells (Tregs) both support and supress tumour growth in colorectal cancer, indicating that they also require further investigation. Phenotypic and functional characterisation of T cell responses within mouse models of colorectal cancer can enable us to identify markers of good prognosis which can be targeted to improve patient outcome in humans.
In this study, an intracaecal mouse model of colorectal cancer (CT26 colon adenocarcinoma) will be used to characterise the phenotypes and functions of systemic and tumour-infiltrating T cells. CD4+ and CD8+ T cell phenotypes will be characterized by the production of IFN-γ and IL-2, indicative of effector function, the proliferation marker Ki67, and CD69, an activation marker. Memory subsets of these populations will also be identified via CD44 and CD122 expression. Finally, Tregs will be identified via expression of Foxp3 (a transcription factor), CD25 (IL-2α receptor) and production of suppressive cytokines IL-10 and TGF-β. Ultimately, characterisation of T cell responses in this model will provide information on how to better design immunotherapies to manipulate the tumour environment in favour of responses associated with positive patient outcomes.

I41: Potential for vaccination strategies targeting MAIT cells demonstrated by protection against lethal Legionella infection in mice.

Alexandra J Corbett[1], Huimeng Wang[1], Criselle D'Souza[1,2], Xin Yi Lim[1], Lyudmila Kostenko[1], Troi J Pediongco[1], Sidonia BG Eckle[1], Bronwyn S Meehan[1], Mai Shi[1], Nancy Wang[1], Jeffrey YW Mak[3,4], David P Fairlie[3,4,] Lars Kjer-Nielsen[1], Richard A, Strugnell[1], James McCluskey[1], and Timothy SC Hinks[1,5], Zhenjun Chen[1]

1. Department of Microbiology and Immunology, Peter Doherty Institute for Infection and Immunity, University of Melbourne, Parkville, Victoria 3000, Australia.
2. Centre for Animal Biotechnology, Faculty of Veterinary and Agricultural Sciences, University of Melbourne, Parkville, Victoria 3000, Australia.
3. Division of Chemistry and Structural Biology, Institute for Molecular Bioscience, The University of Queensland, Brisbane, Queensland 4067, Australia.
4. Australian Research Council Centre of Excellence in Advanced Molecular Imaging, The University of Queensland, Brisbane, Queensland 4067, Australia.
5. Respiratory Medicine Unit, Nuffield Department of Medicine Experimental Medicine, University of Oxford, OX3 9DU, Oxfordshire, UK

Mucosal-associated invariant T (MAIT) cells are a subset of T cells defined by semi-invariant TCR and restriction to MHC-related protein-1 (MR1). MAIT cells recognize riboflavin (vitamin B2)-derived metabolites and can provide immune protection against some bacteria. However, the mechanisms of MAIT cells' *in vivo* antibacterial properties are not well understood.

Legionella spp. can cause severe respiratory disease in humans, particularly in young, elderly or immune compromised individuals. In Australia and New Zealand *Legionella longbeachae* is most prevalent, whereas *L. pneumophila* is more common worldwide.

Using a mouse infection model of *Legionella longbeachae* infection, we found that MAIT cells accumulated in the lungs following infection. These MAIT cells, which produced IFNγ, GM-CSF and IL-17, contributed to the clearance of bacteria in C57BL/6 mice. In immune deficient mice, the role of MAIT cells in reducing bacterial load became even more important, and in severely immune deficient RAG2$^{-/-}$γc$^{-/-}$ mice, transferred MAIT cells were capable of protecting mice from lethal *L. longbeachae* infection.

Excitingly, the protective effect of MAIT cells on clearance of *L. longbeachae* could be increased by prior vaccination with synthetic Ag + TLR agonists or prior bacterial infection.

Thus, we have demonstrated an important role for MAIT cells in protecting against a respiratory pathogen and the potential for MAIT cell targeting in vaccination. Since MAIT cells recognise antigens that are conserved across many bacteria and yeast, these findings are potentially relevant to a number of human pathogens.

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