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Abstracts

Oral presentations

Peripheral NK cell dysregulation in Recurrent Pregnancy Loss (RPL)

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Background:
Recurrent pregnancy loss (RPL) affects ~1% of couples, causing significant physical and psychological harm. Despite its multifactorial aetiology, for ~50% of couples no cause is identified. Whether peripheral immune markers, in particular NK and T cells, are significantly altered in RPL remains contentious. Our principal objective was to perform detailed phenotypic and functional analysis of peripheral NK cells in RPL, accurately identifying potential diagnostic biomarkers.
Methods:
Peripheral blood samples were obtained pre-conception from women with RPL (N=100) (â‰¥2 losses), and controls (N=80). Lymphocyte subpopulation levels were examined by flow cytometry. Detailed NK cell phenotyping was performed by mass cytometry. An NK cell cytotoxicity assay using K562 target cells was also performed. Cytolytic molecule secretion by PBMCs was assessed by ELISA. Potential biomarkers were identified via computational statistical modelling.
Results:
Elevated NK cell prevalence and a primarily cytotoxic CD56dimCD16+ NK subset shift was measured in women with RPL, accompanied by decreased T cell levels ($p<0.05$). An increased presence of NK cell subsets lacking inhibitory receptor expression was detected ($p<0.05$), however total cytotoxicity was unaltered. Although NK cell degranulation appeared elevated in RPL, PBMC-mediated secretion of granzyme A, B, and perforin was reduced ($p<0.05$). NK/T cell ratio emerged as a potentially valuable diagnostic biomarker (AUC=0.8).
Conclusions:
Our findings highlight significant NK cell dysregulation in RPL. Our comprehensive analysis validated current tests, including NK and T cell level examination. Further investigation into local immune dysregulation and its relationship with systemic activity is supported.

Neuroimmunology of cryptococcal meningitis: exploring the roles of CNS-localized myeloid cells in T-cell responses

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The central nervous system (CNS) has long been regarded as an immune-privileged site characterized by the lack of antigen-presenting cells and classical lymphatic drainage system. However, CNS is populated with tissue-resident macrophages which has essential roles in antimicrobial defense. Growth of studies also exhibit the recruitment of other myeloid cells such as dendritic cells and macrophages to the brain during infection. However, their exact function in the CNS is still required to be explored. In particular, how these antigen-presenting cells are involved in the recruitment and activation of CD4 T cells within the CNS is only partially understood in the context of CNS infection. In order to answer this question, we study cryptococcal meningitis, which is

a lethal fungal neurological infection and dependent on CD4 T cells for protection, as a model CNS infection. First, we generated a novel T-cell receptor (TCR) transgenic mouse model (CnTII-Tocky) that reports Nr4a3-dependent TCR signaling activity and cytokine production of Cryptococcus-specific CD4 T-cells during in vivo cryptococcal infection, enabling us to correlate fungal burden, TCR signaling dynamic and cytokine production in the same animal. Using these mice, we show that fungal-specific CD4 T cells only migrate into the brain and meninges when fungal brain burden is near lethal levels. Brain infiltrating CD4 T cells had received TCR-mediated signals, undergone several rounds of cell division and produced IFNg. To determine which myeloid cells may be delivering the TCR activation signals, we used FACS and single cell RNA sequencing to characterize the antigen-presenting cells within the fungal-infected CNS. We show that the frequency of both classical dendritic cells and macrophages were elevated in the brain during infection. There were also several populations of macrophages that we identified by single cell RNAseq, which expressed several components of the immunoproteasome and upregulated antigen-presenting in response to fungal infection. Microglia, on the other hand, has no change in cell numbers and did not significantly upregulate these pathways. Our ongoing work aims to investigate which of these DC/macrophage populations control fungal-specific CD4 T cell responses in the CNS during cryptococcal meningitis.

The rapid loss of crucial NK effector functions upon tumour infiltration debilitates anti-tumour responses

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Tumour immuno-evasion is facilitated by dysfunctional effector cells, however why lymphocytes become defective within the TME remains to be completely understood. CD8+ T-cells become exhausted from chronic antigen exposure, whereas alterations to other effector cells, NK cells, are yet to be fully elucidated. NK cells target and kill cancer cells through perforin and granzyme secretion, however, crucially NK-DC crosstalk supports T-cell responses, recruiting cDC1s through XCL1 and CCL5, and priming intra-tumoural DCs via IFN-γ and TNF-α. We established tumour models using kaede mice, whereby temporal labelling of the entire tumour immune compartment from native 'kaede green+' fluorescence to 'kaede red+' enables newly entering and retained cells within the tumour to be distinguished. To reveal how NK cells change in tumours overtime, we performed scRNA-seq on 'kaede green+' and 'kaede red+' lymphocytes in MC38 tumours 24- and 72-hours post photoconversion. This identified 3 distinct NK clusters; CD11b+CD49a- newly infiltrating 'NK-1', CD11b-CD49a- 'NK-2', and CD11b-CD49a+ resident-like 'NK-3' cells which accumulated overtime, and strikingly appeared dysfunctional at the transcriptomic level compared to the newly infiltrating population. Using flowcytometry we further validated observations in multiple models, confirming a universal loss of NK activity including CCL5, IFN-γ. Interestingly, although IL-15:IL-15Rα treatment partially restored NK functions, IL-15:IL-15Rα promoted expression of inhibitory checkpoints (NKG2A, KLRG1) suggestive of further negative feedback mechanisms constraining NK activation. Collectively our data reveals rapid loss of NK cell function within tumours, provides a unique temporal characterisation of NK cells adapting to the TME, and investigates how potential therapeutic interventions impact this process.

Synovial tissue zonation underpins specific disease pathotypes in rheumatoid arthritis

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The synovial membrane, a thin mesenchymal tissue composed mainly of fibroblasts, undergoes significant remodelling in response to inflammation in rheumatoid arthritis (RA). This process results in the formation of a highly organised and pathogenic sub-lining tissue, as a result of endothelial derived NOTCH-3 signalling that drives the expansion and differentiation of fibroblasts into immune effector subtypes. We aimed to test the hypothesis that specific synovial fibroblast subtypes are responsible for the construction of microenvironmental tissue niches that support the pathogenic behaviour of infiltrating inflammatory cells in RA.

We performed spatial transcriptomics (using 10x Genomics Visium) on synovial tissue biopsies from patients with RA and osteoarthritis (OA), to define spatial tissue biology. Our analysis revealed that active RA was associated with the formation of sub-synovial tissue niches that are specific to disease pathotype. These niches are defined by specifically primed fibroblast subsets that support the localisation, organisation and pathogenic behaviour of specific immune cells and determine their localisation within the tissue. In lymphocyte rich tissues for example we observed the construction of peri-vascular T cell niches with fibroblasts that are primed to respond to T cells and support their retention in the synovium.

We propose that specific fibroblast subsets are responsible for the construction that support the pathogenic behaviour of infiltrating inflammatory cells as a result of niche dependent signalling. Targeting the mechanisms underlying the formation of these specific tissue niches may represent a novel therapeutic avenue in RA.

The Thymus Synchronises CD4 and CD8 Thymocyte Development That Fixes the CD4:CD8 Peripheral T-cell Ratio

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CD4 and CD8 single-positive (SP4 and SP8) $\alpha\beta$T-cells are well defined subsets of lymphocytes, with clear differences in their phenotype, role, and location in peripheral tissues. Despite this, the mechanisms that control the intrathymic development and ultimately determine the relative frequencies of CD4 and CD8 T-cells in the periphery are unclear.

Using flow cytometric analysis in Rag2GFP mice, where cellular age is determined by GFP levels, we have analysed the development of SP4 and SP8 lineages to show that throughout their thymic development, SP8 represent significantly older cells than SP4 owing to a lag in the transition between DP and the most immature SP8 thymocytes. Using intravenous anti-CD45 antibody injection, we are also able to identify cells in the perivascular space (PVS) of the thymus that are undergoing thymic egress. From this, we determine that the final stages of SP8 development mirror that of SP4, with isolation of thymocytes in the PVS showing that conventional SP8 follow the same organised 'conveyor belt' route as SP4, with the oldest cells preferentially exiting the thymus. Finally, we show that the CD4:CD8 ratio that exists in the periphery is established early in thymic T-cell development.

Together these data demonstrate that whilst SP4 and SP8 transition through comparable developmental stages, differences exist in their kinetics, with SP4 transiting through developmental faster than SP8. Additionally, our finding that the CD4:CD8 ratio is fixed early in SP development argues for an important role in thymic selection and not post-selection maturation, in controlled peripheral T-cell availability.

Fungal growth in microglia promotes microbial adaptation and infection of the central nervous system

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Cryptococcus neoformans is the major causative agent of fungal meningitis, affecting more than million people and causing ~150,000 death each year. Microglia are CNS-resident macrophages that become infected with C. neoformans. There is evidence for both protective and detrimental roles for microglia, with in vitro studies indicating that microglia kill fungi while others have indicated that microglia can host intracellularly-replicating fungi. To definitively determine whether microglia contribute to protection against this fungal infection we used several microglia depletion strategies, including transgenic mice (Cx3cr1-CreER-iDTR, Sall1Cre x CSF1Rflox) and the CSF1R inhibitor PLX5622, and examined the impact of microglia loss on control of infection. We found that microglia-depleted mice had reduced brain fungal burden, that was replicated in several depletion models and different infection routes. Using fluorescent fungal strains and a mutant that is defective in macrophage intracellular replication, we show that microglia are an essential growth niche for the fungus and enable optimal infection of the brain. We hypothesised that the fungus accesses restricted nutrients from microglia, such as copper, to adapt to growth conditions within the brain. We tagged the fungal copper transporter 4 (CTR4) with GFP to track expression by the fungus when interacting with microglia. We found that C. neoformans had stronger upregulation of copper-importing machinery within microglia compared to outside microglia, thus explaining the fungal dependence on these glial cells for early growth in the brain. Our work has identified one of the few examples of microglia acting to promote infection and microbial escape, which may help in the future identification of potential therapeutic targets for this life-threatening fungal infection.

TNF-α drives naïve CD4+ T cell metabolic reprogramming upon activation

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Upon activation, T cells undergo substantial metabolic reprogramming to support their effector functions, largely driven by T cell receptor and CD28 signalling. Whether inflammatory cytokines further amplify this process is not well understood but could have implications in chronic inflammatory disease. TNF-α has been previously identified to act as a co-stimulatory signal in T cells, increasing proliferation and cytokine production. However, whether TNF α controls T cell metabolism has not been interrogated.

Here, purified naïve human CD4+ T cells were activated in the presence of TNF-α, or a neutralising anti-TNF-α antibody/relevant isotype control. Whilst increased exogenous TNF-α demonstrated little effect on T cell metabolism or function, blocking TNF-α signalling caused a reduction in the activation of naïve CD4+ T cells, alongside significant decreases in their rates of mitochondrial oxygen consumption and lactate production. Consistently, alterations in glucose metabolism in anti-TNF-α-treated cells were observed by stable isotope-based tracing, and pathway analysis of RNA-sequencing data identified a failure to upregulate key genes involved in oxidative phosphorylation. Interrogation of downstream signalling pathways identified that TNF-α drives these metabolic changes in naïve T cells through the PI3K/Akt/mTOR pathway, with the metabolic effects of TNF-α blockade blunted by Akt inhibition. Analysis of T cell differentiation under anti TNF α conditions highlighted a role for both TNF-α and Akt signalling in driving inflammatory Th1 and Th17 cell metabolism and function.

This work provides novel insight into the role of inflammatory cytokines in regulating immune cell metabolism and may aid future developments of anti-TNF-α biologics in the treatment of inflammatory disease.

Combined single cell profiling technologies reveal novel modulation of Wnt signalling in synovial fibroblasts during the resolution phase of inflammatory arthritis

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Immune effector fibroblasts in the synovial membrane contribute to disease pathology by producing inflammatory mediators that drive the recruitment and retention of inflammatory cells within the joint. Resolution of joint inflammation requires the suppression of these pathogenic phenotypes, but the mechanism by which this is achieved is currently unknown.
In order to determine the disease mechanisms that drive a switch from a pro-inflammatory to a pro-resolving state in synovial fibroblasts during the resolution of inflammatory arthritis, we induced arthritis in mice using the serum transfer induced arthritis model, and harvested synovial tissue for combined single cell RNA ATAC sequencing.
We observed that Wnt signalling pathway members, including Wnt5a, were enriched in osteoblasts and distinct populations of Wnt responsive synovial fibroblasts. These fibroblasts upregulate Ryk (Wnt5a receptor) at the peak of inflammation and Cxcl5, whose gene contains T cell factor (Tcf) binding motifs in its promoter region - transcription factors that regulate Wnt target genes.
Combined scRNA-Seq and scATAC-Seq showed that during the resolution phase of joint inflammation, fibroblasts display a Wnt inhibitory signature with increased expression of Wif1 and Dkk3. Furthermore, we identify an enhancer of Axin2 (negative regulator of Wnt signalling) where greater chromatin accessibility is maintained once joint inflammation has fully resolved.
Overall, these findings suggest a novel mechanism, whereby Wnt signalling drives an inflammatory gene expression program in pathogenic fibroblasts, while resolution of joint inflammation is driven by a switch to negative regulation of Wnt signalling in fibroblasts that adopt a pro-resolving phenotype.

Nr4a1-timer of cell kinetics and activity (Tocky) reporter mice: A tool to exploring temporal T- and B-cell response to TCR engagement

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Nr4a receptors are activated by T cell receptor (TCR) signalling and play key roles in T cell differentiation. Having recently identified that Nr4a1/Nur77-GFP and Nr4a3-Timer of cell kinetics and activity (Tocky) reporter mice are differentially sensitive to T cell receptor signal strength and duration (Jennings et al., 2020), we developed Nr4a1-Tocky mice. Whilst Nr4a1-GFP reporter mice have been a useful tool to study Treg development, in addition to addressing B cell activation to antigen presence, temporal analysis is hindered by the persistence of GFP expression following antigen encounter. Nr4a1-Tocky reporter mice used here circumnavigate this as the half-life of the Timer Blue protein is 4 h (Bending et al., 2018), allowing for a sensitive readout over a much shorter time period. Utilising this novel reporter system, we aim to further explore the differential sensitivity between Nr4a1 and Nr4a3 receptors.
In this project, we have explored the basal expression of Nr4a1 and Nr4a3 using Nr4a1- and Nr4a3-Tocky mice, respectively, across several tissues (including liver, colon, spleen, lymph node and thymus). Following this, we explored the differential sensitivity of Nr4a1 and Nr4a3 receptors across T cells and B cells in response to a range of different concentrations and durations of stimuli, showing that Nr4a1 receptor is more sensitive to TCR activation and that the introduction of the Nr4a1-Tocky model provides a sensitive model for exploring temporal response to TCR engagement.

B cell activation and autoimmunity in a mouse model of B cell anergy

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B cell anergy is a key tolerance mechanism that prevents activation of self-reactive B cells. We have generated a mouse strain where the IgM B cell receptor (BCR) cytoplasmic tail has been replaced with the IgG1 cytoplasmic tail, resulting in chimeric IgMg1 BCR with enhanced signalling. During B cell development in IgMg1 mice, stronger negative selection results in phenotype that resembles B cell anergy, with reduced BCR expression and reduced BCR-stimulated calcium flux. Unlike most B cell anergy models, IgMg1 mice express a polyclonal repertoire of BCRs. IgMg1 mice have an impaired T cell-independent response, but normal T cell-dependent response consistent with the role of T cell help in activation of anergic B cells. Aged IgMg1 mice developed spontaneous germinal centres and plasma cells and developed tissue-specific autoantibodies more frequently than wildtype (WT) mice, indicating a breach of anergy and tolerance with ageing. To experimentally induce autoantibodies in young IgMg1 mice, they were immunised with conserved antigen. They responded comparably with WT mice, showing that self-reactive IgMg1 B cells are not inherently prone to breaching tolerance, and immune microenvironment changes with ageing may be important for the increased autoantibodies in aged IgMg1 mice. T follicular helper (Tfh) cells are increased in IgMg1 mice following immunisation, so we hypothesise that IgMg1 B cells may have increased antigen presentation and activation of Tfh cells. Nr4a3 reporter mice will be used to assess T cell receptor signalling and B-T cell interactions following immunisation.

Force application to the T cell receptor controls T cell activation

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Whilst T cell receptor (TCR) signalling pathways are increasingly dissected, the mechanism by which the TCR triggers is still not fully understood. Evidence suggests that mechanical forces play a role in this process, where forces acting upon the TCR have been shown to induce a TCR calcium signal. However, investigation has failed to address how force application to the TCR influences T cell activation and the phenotype of activated T cells.

Here, we apply force to the TCR using antibody coated superparamagnetic particles, which transfer a force to the TCR when subjected to external magnetic fields. Monitoring distal CD4+ T cell activation events shows that force application can increase T cell activation marker expression, cell proliferation and cytokine production. These findings correlate with downregulation of the TCR itself, where force application serves to induce a population of TCRlo, activation marker positive CD4+ T cells. Pre-treatment with the Src kinase inhibitor PP2 prevents the induction of these responses, indicating TCR specific activation. Interestingly, use of the actin polymerisation inhibitor Latrunculin A also prevents the establishment of TCRlo activation marker positive CD4+ T cells, indicating a potential force induced receptor migration mechanism, the end point of which is TCR internalisation.
Finally, where surface CD3 of the particle is sufficient to induce activation without external force, force application serves to downregulate activation marker expression and drive the expression of CTLA-4 and Tigit, highlighting a platform where force regulated TCR signal strength modulates the global T cell response and ultimately T cell fate.

Poster presentations

P.01 **CCR7 directs medulla-dependent development of effector gd T-cells in the murine thymus**

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gd T-cells are the first T-cell population to be made in the thymus and provide important innate-like protection at epithelial-rich sites. Like ab T-cells, gd T-cells are dependent on signals from the intrathymic microenvironments for their development. However, gd T-cells differ from their ab counterparts in that they acquire effector functions during their development within the thymus instead of relying on additional signals in the periphery. Whilst the development of gd T-cells in early life is well characterised, it is less clear how the adult thymus supports gd T-cell development. It has been shown that CCR7 plays a role somehow in gd T-cell development/egress, yet the details of this process remain unknown. To address this, we used RAGGFP and CD24 to identify newly produced gd T-cells in the adult thymus, which we can categorise into naïve, intermediate effector-committed and IFNγ+ effector cells. We found that both CD73+ and CD73+IFNγ+ cells were significantly reduced in medulla-deficient Relb-/- thymus, and a block of CCR7-dependent cortex-medulla migration in Ccl21-/- mice resulted in a similar phenotype. In bone marrow transplant (BMT) recipient mice, the thymic medulla fails to recovery, resulting in defective gd T-cell development. We found that effector gd T-cell development was also impaired in BMT recipient mice, further highlighting the importance of the medulla in gd T-cell development. Together these findings highlight a novel role for the thymic medulla in adult mice for the development of newly produced effector gd T-cells, a process which fails to recover in BMT and is dependent on CCL21.

P.02 **Cytokeratin-19 Labels A Population Of Multi-potent mTEC Progenitors In The Embryonic Thymus**

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Thymic epithelial cells (TEC) regulate the development and selection of self-tolerant T-cells. While cortical TEC (cTEC) support early stages of T-cell development and mediate positive selection of CD4+CD8+ thymocytes, anatomically distinct medullary TEC (mTEC) impose central tolerance by facilitating the deletion of self-reactive thymocytes during negative selection. Recent studies have highlighted the complex heterogeneity which exists within the mTEC compartment at a single cell level. Despite these descriptive analyses, the developmental origins of such mTEC heterogeneity remains poorly understood. To address this, and based on its ability to selectively mark hepatic progenitors, we have examined cytokeratin-19 (K19) as a potential novel marker within TEC lineages. Specifically, we have mapped TEC expression of K19 during embryonic thymus development, and performed lineage tracing studies using tamoxifen induced K19-Cre to determine the contribution of embryonic K19+ TEC to the postnatal mTEC pool. Ontogeny analysis shows that during early development, K19+ cells are positioned within cortical and medullary areas, and that they lack expression of markers associated with maturation e.g. Aire. Importantly, despite labelling cells within both cortex and medulla, fate-mapping experimentsdemonstrate that K19-expressing cells are mTEC restricted. Moreover, we show that K19+ TEC are upstream of RANK+ mTEC precursors, and give rise to multiple mature mTEC subsets, including Aire+ cells, CCL21+ cells and Tuft Cells. To conclude, we have identified K19 as a novel marker of mTEC restricted multi-potent progenitors within the embryonic thymus. Futher fate mapping studies aim to address the long-term contribution of such embryonic mTEC progneitors to the postnatal thymus.

P.03 **Vaccines against SARS-CoV-2 are effective among healthcare workers: a systemic review and meta-analysis**

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Introduction: Health care workers (HCWs) have been at increased risk of infection during the SARS-CoV-2 pandemic and as essential workers have been prioritised for vaccination. Due to increased exposure HCW are considered a predictor of what might happen in the general population, particularly working age adults. There have been multiple HCW studies which can be usefully summarised to understand the benefits of vaccination in this 'at risk' cohort.
Methods: A systematic review and meta-analysis of observational trials of vaccine effectiveness (VE) in HCWs using individual participant data was conducted. Studies which defined breakthrough infection as a positive SARS-CoV-2 test 14 days after complete vaccination with an MHRA approved COVID-19 vaccine were pooled to calculate VE against infection, symptomatic infection, and hospitalisation. VE over time was assessed.
Results: 10 studies met criteria for pooling. Participants were predominantly female and working age. VE against infection, symptomatic infection, and infection requiring hospitalisation were 93% (95% CI 79% – 98%; $p < 0.0001$), 93% (95% CI 70% – 98%; $p < 0.0001$), and 94% (95% CI 85% – 98%; $p < 0.0001$). Waning protection against infection was reported by three studies, although protection against hospitalisation for severe infection persists.
Conclusions: Vaccination is protective against infection, symptomatic infection, and hospitalisation. Waning protection is reported but await more mature studies to understand durability more clearly. The UK SIREN study which monitors HCW longitudinally and the VIBRANT study examining vaccine breakthrough will enable identification of risk factors and predictors of breakthrough infection to inform national vaccine strategy.

P.04 **Eosinophils are an essential element of a type 2 immune axis that controls thymus regeneration**

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Therapeutic interventions used for cancer treatment provoke thymus damage and limit the recovery of protective immunity. Here, we show that eosinophils are an essential part of an intrathymic type 2 immune network that enables thymus recovery after ablative therapy. Within hours of damage, the thymus undergoes CCR3-dependent colonization by peripheral eosinophils, which reestablishes the epithelial microenvironments that control thymopoiesis. Eosinophil regulation of thymus regeneration occurs via the concerted action of NKT cells that trigger CCL11 production via IL4 receptor signaling in thymic stroma, and ILC2 that represent an intrathymic source of IL5, a cytokine that therapeutically boosts thymus regeneration after damage. Collectively, our findings identify an intrathymic network composed of multiple innate immune cells that restores thymus function during reestablishment of the adaptive immune system.

P.05 **Characterising migration of memory B cells emerging from germinal centres**

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Antigen activated B-cells upregulate CCR7, migrate to T-cell areas, after receiving T-cell help they form germinal centres (GCs). Memory B-cells (mBCs) are thought to arise from GC B-cells with intermediate affinity BCRs. We showed that GC-derived mBCs in the lymph node (LN) migrate towards the subcapsular sinus (SCS), driven by S1PR1. mBCs in the SCS may egress LNs and enter distant tissues. They may also recycle to follicles to reenter GCs following local CCL19/21 gradients maintained by ACKR4. We explore the role of S1PR1 for emigration from reactive LNs (drLN) and whether CCR7 ligands are involved in entry into distant lymphoid tissues.
Mice were immunized with NP-CGG or NP-KLH, and drLN and lymphoid tissues were examined at the peak of the response. Light-sheet imaging of S1PR2CreERT2 Rosa26fl-Stp-fl-dTomato drLNs revealed mBCs emerging from GC (BEM) and migrating towards SCS. BEM were in proximity with antigen-carrying SCS macrophages. S1PR1-blockade revealed its crucial role in BEM egress from LNs. Examining lymphoid tissues of CCL19-deficient mice revealed that mBC entry into the spleen is CCL19-dependent. RNA-sequencing and flow cytometry of BEM revealed differential gene expression between GC B-cells and BEM.
We distinguish BEM from GC B-cells in drLN and show that BEM interact with SCS antigen-carrying macrophages before recycling to the GC. This may facilitate responses to antigenic variants during an ongoing immune response by moving variant-specific mBCs back into the GC. We show that BEM egress from the drLN is S1PR1-dependent and the entry into other lymphoid tissues is CCR7 ligand-dependent.

P.06 **PD1 pathway blockade augments T cell receptor signal strength within CD4+ Tumor Infiltrating Lymphocytes**

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The mechanisms underlying responses among patients to immune checkpoint blockade (ICB) therapy remain unclear. There is thus a clear clinical need to generate treatment monitoring biomarkers that associate with patient benefit. We recently developed a new metric, called “TCR.strong”, that represents five genes (IRF8, TNFRSF4 (OX40), STAT4, TNIP3, ICOS) that are specifically upregulated in response to PD1 pathway blockade in CD4+ T cells. The TCR.strong metric correlates with survival outcomes when applied at the bulk tumor biopsy level, however the extent to which this metric is driven by responses from CD8+ versus CD4+ T cells (or potentially other cells) remains to be determined. To further probe the cellular changes in response to PD1 pathway blockade, we utilised the MC38 pre-clinical cancer model. Nr4a3-Tocky Ifng-YFP Il10-GFP mice were inoculated in the flanks with MC38 cells and treated with anti-PD-L1 antibody and phenotypic changes in CD4+ and CD8+ Tumor infiltrating lymphcoytes (TILs) analysed. Anti-PD-L1 treatment drove significant increases in OX40 and ICOS on CD4+ TILs, whilst CD8+ TILs only showed an increase in ICOS expression. CD4+ TILs also displayed a significant increase in the proportion expressing Il10-GFP in anti-PD-L1 treated tumors. Collectively these data suggest that the TCR.strong metric captures the strengthened self/ neo-antigen driven TCR signalling within the CD4+ T cell subset that results early on from anti-PD-L1 immunotherapy.

P.07 **CXCL12 Identifies Heterogeneity In Cortical Epithelial Cells**

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Thymic epithelial cells can be sub-divided into medullary and cortical epithelial cells (mTEC, and cTEC), each playing a vital role in the development of $\alpha\beta$T cells. More specifically CD4-CD8- T cells and CD4+CD8+ T cells can be found in the cortex and it's the constant interactions with cTEC through both β selection and positive selection that allows a normal program of development.
Whilst mTEC are extremely well characterised, less is known about cTEC and their heterogeneity. The CXCL12-CXCR4 axis has been shown to play a role in the positioning of CD4+CD8+ T cells within the cortex, and CXCL12 expression is widespread in cTEC.
Using the CXCL12dsRed reporter mice, in which all cells that express CXCL12 are dsRed+ we show in the neonate the majority of cTEC express CXCL12dsRed. CXCL12dsRed- cTEC begin to emerge 1 week postnatally and reaches a plateau at 10 weeks.
Previous studies have shown CXCL12 is a Foxn1 dependant gene. Utilising bulk RNA sequencing we found that CXCL12dsRed- cTEC have reduced expression of Foxn1 and other Foxn1 dependant genes when compared to CXCL12DsRed+ cTEC.
We show that β selection influences cTEC heterogeneity with reduced appearance of CXCL12dsRed- cTEC compared to WT in $Rag2^{-/-}$x CXCL12dsRed mice.
Collectively we identified cTEC heterogeneity, and have identified a subset of cTEC that lack the expression of Foxn1 and other key known functional genes in cTEC. We have also shown that the appearance of CXCL12dsRed- cTEC could be in apart due to DN3a thymocytes as seen by the reduction in $Rag2^{-/-}$x CXCL12dsRed mice.

P.08 **Immunomodulatory effects of Salmonella, E. coli and Lactobacillus- treated tumour organoids**

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Development of bacterial modification, along with knowledge of bacterial functionality, brought with it a resurgence of research into bacterial cancer therapy (BCT). Salmonella enterica typhimurium (STm), Escherichia coli Nissle 1917 (EcN) and Lactobacillus Casei (L.c) are all widely studied and demonstrated to produce cytotoxic effects in cancer models. Whilst beneficial, it has been shown T cells are not essential to produce these effects in models using STm. Our lab aims to understand why T cells are redundant for BCT, with the intent to reverse this and improve therapeutic methods. Therefore, using TCR reporter mice (Nr4a3-Tocky-Ifn-γ -YFP), we modelled soluble signalling between splenic T cells and colorectal cancer-derived organoids. STm and EcN-infected organoids induce disruption of TCR signalling within T cells, whilst maintaining a high level of cellular activation indicated by CD69 expression. Additionally, both STm and EcN lowered production of IFN-γ through the TCR, instead inducing alternative activation routes to produce IFN-γ. In contrast, L.c exhibited a more generalised inhibition of T cell activation demonstrated by a significant reduction in CD69 expression upon activation. TCR signalling was also disturbed, indicated by an increase of arrested TCR signalling, as seen with STm and EcN, accompanied by a larger population with an absence of TCR signalling, an effect exaggerated in CD8+ cells. This work demonstrates a shared ability of bacteria to elicit an interruption of T cell activation, with specific targeting of TCR signalling. Broadening our understanding of these interactions will enable us to optimise BCT for use in tandem with existing therapies.

P.09 **Cross reactivity of serological response to SARS-CoV-2 vaccination with viral variants of concern detected by lateral flow assay**

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Introduction: As it is unknown whether antibodies produced in response to vaccination with Astra Zeneca ChAdOx1 nCoV-19 (AZ) and the Pfizer-Biontech COVID-19 (PFZ) or wildtype infection/natural infection (NI) will be able to recognise variants of concern (VOC). This study evaluated the serological response of vaccinated and NI cohorts in relation to four VOC: UK/Alpha, South Africa/Beta, Brazil/Gamma and Indian/Delta.
Materials and Methods: We used AbC-19™ rapid IgG LFT (Abingdon Health) lateral flow assays (LFAs) specific for the Wuhan and four VOC to determine the serological response in three cohorts: PFZ vaccinated (n =20, median 47 [20-80] years), AZ vaccinated (n =20, median 72 [61-81] years) and NI (n =10, median 49 [28-60] years). SARS-CoV-2 spike protein specific antibody status was confirmed by ELISA before analysis on LFAs. Each LFA was scored using a visual scorecard by three individuals to provide semi-quantitation.
Results: The majority of the PFZ, AZ and NI cohorts showed pan cross-reactivity against the VOC (100%, 91% and 70% respectively). Serum positive by AbC-19™ LFT (Wuhan) were also positive against the different VOC LFTs, albeit at lower levels. The PFZ group reacted strongly against each VOC and the mean scores were higher than both the AZ and NI groups ($p < 0.0001$).
Conclusion: Antibodies in each cohort successfully interacted with the SARS-CoV-2 spike protein found on the four VOCs. Antibody levels were greatest in the PFZ vaccination cohort and lowest in the NI cohort. Age differences may account for the observed differences in PFZ and AZ vaccine cohorts. This study provides evidence of the practical utility of LFAs in immunosurveillance, which can be particularly useful in low-to-middle income countries (LMICs).

P.10 **Is the serum Ig subclass response post SARS-CoV-2 vaccination affected by prior SARS-CoV-2 infection?**

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Introduction: SARS-CoV-2 vaccination has been given irrespective of prior immune status. Prior infection can lead to an increase in humoral immune response to SARS-CoV-2 vaccination. However, differences in the immunoglobulin (Ig)subclass have not been explored extensively. Here we have a cohort of HCWs and measure their IgG subclass response to the SARS-CoV-2 Spike protein (SP), prior to and following vaccination with the of Pfizer/BioNTech COVID-19 vaccine (PFZ).
Materials and Methods: Serum was taken at baseline and 4 weeks post-2nd dose of PFZ vaccination from 91 adults (>18yrs) (N=48 infection naïve; N=43 prior infection). Samples were applied to ELISA plates coated with SP before being probed with monoclonal antibodies specific to IgG subclasses antibodies, IgG1-IgG4.
Results: Post vaccination, there were significant increases in response to SP seen across all the IgG subclasses, with IgG1 & IgG3 subclasses giving the greatest response. This includes those with prior infection, replicating what has been shown with total IgG response. Across all the IgG subclass there was no, or trivial difference seen between post vaccination groups, regardless of prior immune status.
Conclusions: We have developed assays to measure the relative subclass SP response and have shown a predominantly IgG1 and IgG3 subclass response following vaccination or infection. IgG1 and IgG3 are required to activate the classical complement cascade resulting in pathogen removal. This study suggests that prior exposure preceding vaccination has no effect on the subsequent IgG subclass response to SP. Conclusions: We have developed assays to measure the relative subclass SP

response and have shown a predominantly IgG1 and IgG3 subclass response following vaccination or infection. IgG1 and IgG3 are required to activate the classical complement cascade resulting in pathogen removal. This study suggests that prior exposure preceding vaccination has no effect on the subsequent IgG subclass response to SP.

P.11 **A cell biology imaging analysis pipeline to characterise SARS-CoV-2 variants**

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Background. SARS-CoV-2 variants of concern have global distribution and differences in transmissibility, tissue pathogenesis, disease severity and sensitivity to vaccines and treatments. There is little information linking genomic mutations in variants to differences in cell biology profiles of infected cells, mainly due to restricted access to high containment facilities required for live virus propagation. We developed a high content, high resolution imaging analysis pipeline to characterise existing and emerging variants and to assess their impact on therapeutic interventions.
Methods. We compared Wuhan, Alpha and Delta infection of A549-ACE2 lung epithelial cells. We measured fluorescence intensity and cellular distribution of spike, nucleocapsid protein and dsRNA, and quantified the number and area of syncytia in the presence or absence of neutralising antibodies (nAbs).
Results. Variants were more fusogenic than original Wuhan strain (Wuhan < Alpha < Delta). We also noted that there was reduced spike fluorescence intensity in Alpha-infected cells compared to Wuhan and Delta, particularly on the cell membrane. The Delta variant had the lowest membrane nucleocapsid protein expression. This correlated with a drop in anti-N antibody responses during the Delta wave of the pandemic in a cohort of 416 convalescent adults. Prophylactic nAbs applied at the same time as the virus, were effective against all variants, however therapeutic nAbs applied 24hr after viral infection did not prevent syncytia formation in Delta infected cells.
Conclusions. Quantification of cell biology features of viral infection reveals differences between variants that can have clinical implications for diagnostics (eg nucleoprotein detection), prophylactic and therapeutic antibodies.

P.12 **Phosphoantigen-based prodrugs (ProPAgens) as potent stimulators of Vγ9Vδ2 T cell activation and anti-tumour cytotoxicity**

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Human γδ T cells expressing Vγ9Vδ2 T cell receptors are capable of sensing small-molecule phosphoantigens (PAgs), such as intracellular isopentenyl pyrophosphate (IPP) and microbial (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), in immunity towards tumours and microbes, respectively. Zoledronate (ZOL) is an aminobisphosphonate used clinically and preclinically as stimulator of Vγ9Vδ2 lymphocytes to promote expansion of these cells and cytotoxicity towards ZOL-sensitised tumour cells. However, ZOL is a highly hydrophilic compound, meaning that its membrane permeability is limited, and it relies heavily on the activity of intracellular pathways, which may vary between different cell types. Therefore, in this work, novel PAg-based prodrugs (ProPAgens) were designed and investigated for their ability to sensitise a range of tumour targets, as well as to activate Vγ9Vδ2 T cells in vitro in comparison to ZOL. Briefly, to assess Vγ9Vδ2 T cell activation, peripheral blood mononuclear cells were isolated from healthy donor blood and incubated with ZOL and different ProPAgens overnight. In addition, for assessment of cytotoxicity towards different tumours, several tumour cell targets were treated with ProPAgens and ZOL, and co-cultured with ZOL-expanded Vγ9Vδ2 T cells from healthy individuals. Our work shows that the

ProPAgens are not only able to potently activate the Vγ9Vδ2 T cells, but also that these compounds can sensitize tumour cells and induce stronger Vγ9Vδ2 T cell cytotoxicity at concentrations thousands of fold lower than ZOL. These findings establish the ProPAgens as ideal alternatives to the currently used, much less potent ZOL, as well as open doors for further investigations as novel immunotherapeutic agents.

P.13 **A comparison of serum and saliva antiviral antibody levels following SARS-CoV-2 infection and vaccination in healthcare workers**

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Background: Vaccination is the most effective strategy to prevent severe disease from SARS-CoV-2 infection. However, the capacity of widely used intramuscular vaccines to induce mucosal immunity is unknown.
Materials and Methods: Serum and saliva was taken at baseline (Spring 2020) and 4 weeks post-2nd Pfizer (PFZ) vaccination from 91 adults (>18yrs) (N=48 infection naïve; N=43 prior infection (PI)) and in a subset of participants post-3rd PFZ (2021). Samples were applied to ELISA plates coated with SARS-CoV-2 Spike and probed with IgG, IgA, and IgM.
Results: There were detectable serum IgG anti-spike levels in all participants following 2 and 3 doses of PFZ and were significantly higher in individuals with prior SARS-CoV-2 infection. Serum IgA levels were detectable in the majority of naïve and PI with higher levels in the PI cohort following 2 & 3 doses of PFZ. Saliva levels of IgG anti spike were detectable in over 75% of naïve and PI following 2 doses of PFZ and above 90% in both naïve and PI following a third dose of PFZ. Saliva levels of IgA anti-spike were detectable in less than 50% of naïve and PI with no difference in levels post 2 doses of PFZ and in less than 30% in both naive and PI with no difference in levels post 3 doses of PFZ.
Conclusions: Our study demonstrates that both the serum and salivary antibody repertoire contains both IgG and IgA following infection and vaccination and salivary anti-spike IgG seropositivity increased following each vaccine dose with salivary IgA declining by the third dose.

P.14 **Hypoxia suppresses TCR signalling, activation and effector function of CD8+ T cells**

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Microenvironmental hypoxia may drive immunosuppression and disease progression in cancer. Here, we aim to understand precisely how hypoxia influences CD8+ T cell anti-tumour capacity through analysis of key immune functions, cellular metabolism and signalling pathways. To do so, CD8+ T cells from human peripheral blood are pre-conditioned overnight in either normoxic (21% oxygen) or hypoxic (1% or 0.3% oxygen) conditions prior to anti-CD3/anti-CD28 stimulation and analysis. As early as 8 hours, IFN-gamma secretion by hypoxic CD8+ T cells is reduced and effects are sustained to 72 hours. Conversely, TNF-alpha secretion and cytotoxicity (granzyme-B release and CD107a externalisation) do not change, indicating specific rather than generalised effects. IFN-gamma suppression was not associated with nutrient restriction, since glucose concentrations in hypoxic culture media were not decreased but rather increased in hypoxic vs. normoxic cultures, potentially indicative of poorer activation. In agreement with this, expression of CD25 was also reduced in hypoxia, whilst CD69 increased. To further probe for defects in T cell activation we employed the Nr4a3-Tocky reporter system, which identified substantially delayed and suppressed activation of T cells in hypoxic conditions. Consistently, in human T cells we observed decreased phosphorylation of T cell receptor (TCR)-signalling proteins, nuclear NFAT translocation and

expression of TCR-induced genes. Taken together the data indicate that hypoxia impairs T cell activation and certain downstream effector functions through direct effects on signalling pathways. Further work is needed to underpin the mechanisms driving this and define their relevance for CD8+ T cell suppression in the tumour microenvironment.

P.15 **Imaging inflammation and bleeding in the fungal-infected CNS**

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Cryptococcal meningitis (CM) is a severe fungal infection causing over 100,000 deaths per year in immunosuppressed individuals, with surviving patients often developing long-term neurological impairment. The meninges are the main site of cryptococcal infection in humans, yet our understanding of the immune response and resulting inflammation at this site has not been extensively studied. This is in part due to tools and techniques to study this tissue only recently becoming established. We have developed imaging protocols using immunofluorescence staining of meningeal whole mounts to examine the inflamed meninges following fungal infection in our in vivo cryptococcal meningitis mouse model. Our initial observations reveal increased bleeding and loss of vascular integrity that correlates with severity and time of infection. In line with that, we found platelets accumulate in the meninges during infection and formed clots in the tissue as well as coat the inner surfaces of blood vessels. Moreover, we observed inflammatory lesions within the infected meninges, consisting of fungi, immune cells, and platelets. Our ongoing work is attempting to unravel the functional relevance of these lesions and the role of platelets in the anti-fungal immune response within the meninges. This includes understanding how what is happening in the meninges relates to the pathology in the brain parenchyma, which may provide insights into how the fungus overcomes the blood-brain barrier. Our imaging studies have revealed the dynamic behaviour of platelets interacting with immune cells and fungi, leading to novel insights into how these blood cells participate in immune responses.

P.16 **Bacterial cancer therapy induces patient-specific responses in human colorectal tumour organoids**

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Treatment options for colorectal cancer (CRC) remain limited, and advances in immunotherapies are effective in a very small proportion of CRC patients. Attenuated Salmonella typhimurium (STmΔaroA) specifically home to and colonise tumours. This specificity for tumour tissue makes bacterial cancer therapy (BCT) an attractive prospect, but the therapeutic mechanism remains unclear. Previously, we have shown oral delivery of STmΔaroA significantly reduced tumour burden and size in two autochthonous models of intestinal cancer. We observed altered transcriptomic and metabolomic profiles of the tumours, via a direct impact on the tumour epithelium. Currently, we are investigating response of colorectal cancer patient-derived organoids (PDOs) to treatment with STmΔaroA. We have identified that the bacteria preferentially invade proliferating cells in PDOs, recapitulating what we have seen in mouse models. Key genes affected in the mouse models, namely reduced proliferative markers and increased epithelial phenotype were similarly changed in a subset of PDOs following STmΔaroA infection. We then used single-cell RNA sequencing to interrogate differences between PDOs before and after STmΔaroA treatment. Analysis suggests transcriptional differences between patients before treatment, possibly leading to the observed differential responses to BCT. Of interest, we have also identified patient-specific transcriptional changes in epithelial populations after treatment, with the suggestion that BCT can activate different cell death pathways in different patients. Our current hypothesis is that preferential invasion of fast-dividing cancer stem cells leads to cell death effectively diminishing the stem cell pool within the tumour. Future work will delineate the activation of cell death pathways in greater detail and investigate generation of immunogenic responses.

P.17 **Characterising B-cell derived extracellular vesicles and their role in T-cell trafficking**

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Background: Leukocyte recruitment is an essential step in the immune response. We have identified a B-cell derived immunoregulatory peptide (PEPITEM) that limits T-cell migration into inflamed tissues. PEPITEM is a cleavage product of the 14-3-3ζ protein which has been reported in B-cell extracellular vesicle (EV) cargo.
Aims: We hypothesise 14-3-3ζ is secreted from B-cells via EVs following adiponectin stimulation, which is cleaved in the extracellular milieu to produce PEPITEM.
Methods: B-cells isolated from healthy volunteers were treated with or without adiponectin (10μg/mL). EVs from immortalised Raji-cells (Raji-EV) were investigated as a model for B-EV in the PEPITEM pathway. Nanoparticle tracking analysis was used to investigate size and concentration of B-EV and Raji-EV under resting conditions and following adiponectin-stimulation. Immunoaffinity capture using the ExoView platform was used to isolate and phenotype B-cell derived exosomes.
Results: Nanoparticle tracking analysis identified B-EV with a mean size of 273nm (100-150nm mode) and Raji-EV with a mean size of 216nm (100-150nm mode). Concentration of vesicles was similar in adiponectin stimulated B-cells (2.85x109 /ml) compared to the unstimulated control (2.14x109 /ml). ExoView analysis identified 14.3.3ζ+ and CD19+ B-EV but not Raji-EV.
Conclusion: Our results have shown resting and adiponectin-stimulated B-cells secrete B-EV with similar size and concentration. Raji-EV are not suitable models for the PEPITEM pathway as they do not contain 14.3.3ζ. Future work aims to characterise vesicle cargo following adiponectin stimulation. Our results offer a novel understanding of the biochemistry of the PEPITEM pathway, which is required to take full advantage of its translational potential.

P.18 **Interplay between NAIPs and prostaglandins in colonic infection, inflammation and cancer**

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Maintenance of intestinal integrity is dependent on crosstalk between epithelial, stromal and immune cells and the gut microbiota. Nod-like receptor (NLR) apoptosis inhibitory proteins (NAIPs) activate the NLRC4 inflammasome upon recognition of gram-negative bacteria, leading to pyroptosis/apoptosis, intestinal epithelial cell expulsion and release of IL-1β, IL-18 and prostaglandin E2 (PGE2). NAIPs also appear to have homeostatic roles within the intestinal epithelium, as our group has previously shown that NAIPs suppress colonic tumourigenesis but enhance colonic inflammation. Eicosanoids, such as PGE2, have a well-established but complex role in gut maintenance, colorectal cancer and colitis. We aimed to further understand how epithelial NAIPs impact the immune compartment and understand the role of PGE2 in this axis. Using co-culture models of colonic organoids and mouse splenocytes as well as ex vivo analysis of the IEL compartment. We have identified altered basal levels of prostaglandins (PGF2α) and IL-15/IL-15R complex and concomitant alterations in IEL subsets as well as activation-induced IFNγ production in CD4 T cells when NAIPs are lacking in the intestinal compartment. Our ongoing studies aim to further evaluate the mechanisms and impact on mucosal immunity in response to inflammatory challenge.

P.19 **Improving B cell responses to self in cancer**

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Specifically targeting cancer-expressed antigens is a major challenge, mainly due to immune responses to self-antigens being inhibited through immunological tolerance. We developed an active vaccination protocol that efficiently induces specific polyclonal autoantibodies targeting Robo4, a self-antigen expressed selectively on tumour vascular endothelium but not healthy vasculature, resulting in reduced tumour growth in mice. This project aims to optimise the vaccine-induced antibody response to Robo4 in tumours and understand how the vaccine breaches immune tolerance in cancer.

We plan to use the non-toxic fragment C of tetanus toxoid (FrC) as a carrier protein, because there is widespread pre-existing memory to this antigen, guaranteeing efficient recruitment of T cell help. By linking the extracellular domain of Robo4 to FrC, Robo4-specific self-reactive B cells should recruit T cell help from carrier-specific memory T helper cells.

Preliminary results, using keyhole limpet hemocyanin (KLH) as carrier to prime mice, 4-hydroxy-nitrophenyl-KLH (NP-KLH) could efficiently induce the production of high titer and high affinity of NP specific antibodies.

Robo4, FrC and Robo4-FrC proteins were expressed and purified from the culture medium. The same protocol as before, FrC as a priming carrier and a secondary immunisation with soluble Robo4-FrC also generates Robo4-specific IgG1 response. Following the same injection protocol, along with MC38 tumour cells transplant in the second injection, showed production of Robo4-specific antibodies in some mice and although there was no difference in tumour growth, we detect a trend showing less Robo4 expression in the vessels and higher number of B cells in Robo4-FrC vaccinated mice by comparing to controls. This vaccination protocol is under optimization.

Keywords: Cancer, Immunotherapy, Robo4, vaccination