Fuelling the immune response III: UK immunometabolism 2024 Abstracts

Selected short talks

Ectopic GLUT5 expression enables CAR and TCR T Cells to utilise fructose as carbon source, fuelling functional efficacy in vitro and in vivo

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Introduction:
Cellular immunotherapy is standard of care for many malignancies, however resistance can occur, impacting clinical outcomes. Metabolic competition is one mechanism of resistance. Glucose, the main source of cellular carbon, is limited in the tumour microenvironment (TME) due to poor vascularization and competition with tumour cells. We aimed to overcome this limitation by equipping T-cells with the capability to use fructose as an alternative carbon source (ACS) to glucose.

Methods:
Chimeric antigen receptor (CAR) T-cells specific for prostate specific membrane antigen (PSMA) – 4P28ζ - were transduced with GLUT5 (GLUT5_4P28ζ). A truncated CAR (PTr) was used as control. Effector function was tested in vitro in co-culture with PSMA expressing PC3-LN3 cells. HER-2 CAR and gp100 peptide-HLA specific TCR constructs were also tested. To interrogate anti-tumour function in vivo, PC3-LN3-PSMA xenografts were established in NOD SCID Gamma mice. After 9 days, 1x106 4P28ζ, 4PTr or GLUT5_4P28ζ transduced CAR T-cells were intravenously administered followed by daily 300mg/kg of fructose.

Results:
TME-like glucose levels limit the effector function of CAR T-cells. GLUT5 expression allowed CAR T-cells to use fructose in a glucose-depleted environment, preserving proliferative and cytotoxic capacities in vitro. Similar results were obtained with GLUT5_HER-2 CAR and GLUT5_gp100-specific TCR transduced T-cells. In vivo, GLUT5_4P28ζ CAR T-cells led to significant delay in xenograft progression and survival prolongation compared to control.

Conclusion:
Fructose can act as an ACS for T-cells expressing GLUT5 and modified with CAR or TCRs. This simple manipulation is highly compatible with clinical development approaches to optimise cell therapy outcomes.

Demanding energy in T cells: RNA cap regulation of ribosomes

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The rapid proliferation, differentiation and cytokine secretion following T cell activation creates a massive energy demand. Proteomes are reshaped to create differentiated lineages, cells undergo rapid proliferation, and millions of copies of effector proteins are produced; all requiring rapid and sustained protein production. Protein synthesis is the most energy demanding process in the cell and therefore ribosome biogenesis must be carefully controlled to match demand. We report that upregulation of the RNA capping enzymes following T cell activation co-ordinates mRNA production and processing with ribosome biogenesis and translation. Regulation of the RNA capping enzymes has critical roles in the response to infection and tumours. Targeting the RNA capping enzymes provides therapeutic opportunities to direct T cell function.

Long-chain fatty acid uptake from the bone marrow microenvironment suppresses T cell function in Multiple Myeloma

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Multiple Myeloma (MM) is a plasma B cell malignancy that develops in the bone marrow (BM) and is preceded by the asymptomatic condition monoclonal gammopathy of undetermined significance (MGUS). Despite observations that T cell function is dysregulated in MM, checkpoint blockade therapies have not been successful, indicating that alternative mechanisms operate in the BM microenvironment to suppress T cell activity. Here, we assessed T cell phenotype, function and metabolism in matched peripheral blood (PB) and BM samples from healthy aged-matched donors and across the spectrum of MM disease progression. We identify that BM CD8+ T cell abundance and cytokine expression decrease with MM development, and furthermore, that CD8+ T cell function is consistently impaired within BM samples compared to matched PB, confirming suppression within the BM microenvironment. These changes were accompanied by decreased mitochondrial mass and an elevated capacity of T cells to take up long-chain fatty acids. In vitro approaches confirmed that uptake of BM lipids reduces BM CD8+ T cell mitochondrial mass and supresses cytokine expression, which were decreased by culture in autologous BM plasma but rescued by lipid removal. Preliminary work suggests that cytotoxicity against MM cell lines using a BCMA-CD3 bispecific antibody is also decreased in the presence of MM plasma and is restored by lipid removal. This work gives new insight into the pathology of MM disease and suggests new ways to restore T cell function of MM bone marrow.

IL-2 stimulates Glutaminolysis to Enable de novo Pyrimidine Synthesis in Natural Killer Cells

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Natural Killer (NK) cells are innate lymphocytes that are key to intrinsic cancer immunosurveillance and an important target for cancer immunotherapy. Understanding basic human NK cell metabolism

provides opportunities for optimising adoptive cell therapies. With this in mind, we investigated glutamine metabolism in human NK cells given that (i) glutamine is a key amino acid that supports many cell functions (ii) many tumour types are glutamine addicted depriving NK cells of this nutrient in the TME cells and (iii) data from murine NK cells has suggested that glutamine metabolism is not a key fuel for the TCA cycle and oxidative phosphorylation.

We undertook U13C-glutamine tracing experiments by Liquid Chromatography Mass Spectrometry (LCMS) analysis, of NK cells stimulated with IL2 for 18 hours. It was clear that glutamine was taken up by NK cells (most of the glutamine was labelled) and that this increased further upon IL-2 stimulation. Metabolite labelling analysis identified that IL-2 increased the conversion of glutamine to glutamate, allowing for anaplerotic flux into the TCA cycle. The fate of the glutamine derived carbons diverged at oxaloacetate (OAA): some continued around the TCA cycle while others were exported, converted to aspartate and subsequently used for pyrimidine synthesis. The data indicated that glutamine is a key nutrient taken up by human NK cells, and that IL2 drives glutaminolysis. Subsequent glutamate is used to support the TCA cycle, generating energy and providing intermediates for de novo pyrimidine synthesis.

PhD Bright Sparks: short talk – selected abstracts

Pentose Phosphate Pathway derived NO orchestrates macrophage immunometabolic responses; a target for Mycobacterium tuberculosis immune evasion

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Glycolysis has emerged as key to macrophage responses against Mtb infection. Work within our group has demonstrated Mtb mediated deceleration of macrophage bioenergetic metabolism, a process dependent on upregulation of anti-inflammatory microRNA-21 and subsequent targeting of glycolysis. Through multiple means of metabolic analysis, I've further characterised this immuno-evasive phenomenon as a bacterial strategy to promote PPP derived NO and subsequently target TCA cycle metabolism.

NO is an endogenous gas produced by iNOS and proposed to have direct bactericidal activity. However, this model is poorly understood and moreover dismissed in the context of Mtb infection of macrophages, with traditional dogma suggesting an inability of human macrophages to generate substantial NO in response to infection. Recent evidence challenging the bactericidal capacity of NO necessitates a more comprehensive view of processes influenced by NO, including orchestration of metabolic reprogramming.

Here, I have demonstrated during infection of human and murine macrophages, Mtb represses host glycolysis and reroutes metabolism towards induction of a cyclic-PPP, promoting NO accumulation. Supplementation and sequestration of cellular NO reveals this Mtb mediated metabolic manipulation as positive feedback, further promoting PPP-cycling in addition to IRG1 mediated accumulation of TCA cycle derived itaconate. This results in reduced secretion of anti-mycobacterial cytokine IL-1β, thus enhancing Mtb survival. Supplementation of itaconate and use of irg1-/- murine models reveals the ability of this metabolite to promote iNOS expression, again highlighting positive feedback hinging on NO-generation. Furthermore, I've linked this NO-driven PPP activity to increased accumulation of cellular lipids, promoting a permissive environment for bacterial replication.

Inducing an Oxidized Redox-Balance Leads to Stress Adaptive Responses and Improves CD8+ T Cell Function

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Metabolic rewiring plays an important role in cellular adaptations to nutrient availability by attuning metabolic programs to support function of CD8+ T cells. This metabolic rewiring supports the enhanced cytokine production and anti-tumour function of CD8+ TE cells in vivo, and was previously associated with enhanced glucose allocation to anabolic metabolic pathways such as the pentose phosphate pathway (PPP). Although it is suggestive that enhanced anabolic metabolism of PPP increased anti-tumour function in CD8+ TE cells, it remains unclear how the oxidative PPP is related to the metabolic attunement and to T cell immunity. Manipulating PPP activity in TE cells revealed that both glucose restriction and increased PPP activity with menadione led to elevated mitochondrial reactive oxygen species (ROS) and heightened IFNg production. Glucose-restricted TE cells exhibited enhanced function through mitochondrial complex III-derived ROS (mtCIII-ROS), observed both in vitro and in vivo. Human tumor-infiltrating lymphocytes demonstrated improved cytokine production with induced redox imbalance. Alterations in PPP activity in CD8+ TE cells, in humans and mice, significantly increased Ifng and Prf1 transcripts upon T-cell receptor restimulation. Moreover, blocking mtCIII-ROS in the absence of PPP activity reduced transcription factors Atf4 and Nrf2 expression, linked to cellular adaptation and redox balance. Our data suggests that the redox imbalance caused by mtCIII-ROS mediates cellular adaptive responses which ultimately benefit anti-tumour function of CD8+ T cells.

The influence of co-stimulatory domains on the metabolic regulation of chimeric antigen receptor (CAR) T-cells

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Chimeric antigen receptor (CAR) T-cell therapy has shown remarkable clinical success in treating B-cell leukaemias and lymphomas. The two main clinically approved CAR-constructs, termed ‘second generation CARs’, deliver both an activation signal akin to the T-cell receptor (CD3ζ) and a co-stimulation signal via CD28 or 4-1BB; these drive two distinct T-cell functional and metabolic profiles. Unfortunately, the treatment of solid tumours with second generation CAR-T therapy has not been equally successful. The challenging tumour microenvironment (TME), lacking in nutrients and oxygen, drives T-cell exhaustion, characterised by a loss of metabolic fitness and mitochondrial health. To probe CAR-T cell resilience to metabolic challenge modelling the TME in-vitro, we have been using the MEMIC (Metabolic Microenvironment Chamber), a culture system which creates an ischemic gradient of nutrient depletion and hypoxia. Using this system, we tested the impact of co-stimulation on metabolic resilience in both second-generation CAR-T cells and novel parallel-CAR-T

cells (pCAR). pCAR efficiently delivers synergistic CD28 and 4-1BB co-stimulation via two separate receptors, one with the CD3ζ and CD28 signalling domains and one with the 4-1BB signalling domain alone. We find that pCAR T-cells have an increased anti-tumour activity compared with conventional CAR-T in nutrient depletion and hypoxia. pCAR T-cells showed significantly higher expression of key nutrient transporters, enhanced metabolic activity and increased resistance to T-cell exhaustion. Taken together we show that delivery of efficient synergistic CD28 and 4-1BB co-stimulation signalling in pCAR T-cells leads to increased metabolic fitness, resulting in superior T-cell function compared to single-domain co-stimulation.

Asparagine availability controls B cell homeostasis

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Germinal centre (GC) B cells proliferate at some of the highest rates of any mammalian cell, yet the metabolic processes which enable this are poorly understood. We performed integrated metabolomic and transcriptomic profiling of GC B cells, and found that metabolism of the non-essential amino acid asparagine (Asn) was highly upregulated. Asn was conditionally essential to B cells, and its synthetic enzyme, asparagine synthetase (ASNS) was upregulated following their activation, particularly more markedly in the absence of Asn, through the integrated stress response sensor general control non-derepressible 2 (GCN2). When Asns is deleted B cell survival and proliferation in low Asn conditions were strongly impaired, and removal of environmental Asn by asparaginase or dietary restriction markedly compromised the GC reaction, impairing affinity maturation and the humoral response to influenza infection. Using stable isotope tracing, we found that metabolic adaptation to the absence of Asn requires ASNS, and that oxidative phosphorylation, mitochondrial homeostasis, and synthesis of nucleotides was particularly sensitive to Asn deprivation. Altogether, we reveal that Asn metabolism acts as a key regulator of B cell function and GC homeostasis.

Exploring the metabolic programs of human γδ T cells across lymphoid tissues

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γδ T cells have emerged as significant players in immunotherapy for cancer due to their cytotoxic capabilities and non-MHC restricted recognition of antigens. Despite their therapeutic potential, our understanding of their cellular biology remains vastly incomplete, prompting a pressing need to elucidate the mechanisms underlying their activities.

A major challenge in the field of γδ T cells has been the limited translatability of findings between mouse and human models due to their distinct biological features. While mouse models have been instrumental in investigating the functional roles of γδ T cells and the metabolic requirements upon challenge, there is still a major gap in our understanding of human γδ T cell metabolism.

By using cutting-edge single-cell RNA sequencing and high dimensional immunophenotyping, we have successfully mapped different subsets of γδ T cells in different healthy human lymphoid tissues. This has provided insights into their metabolic requirements during immunosurveillance activities, revealing correlations between their functional characteristics and metabolic pathways. Intriguingly, our study has unveiled correlations between the functional attributes of γδ T cells and the utilization of specific metabolic pathways, facilitated by the targeting of nutrient transporters and metabolic enzymes. This enables us to determine the energy sources during the activation of the cells. Coupled with our preliminary findings regarding distribution patterns and functional phenotypes disparity between lymphoid tissue clusters and PBMCs, our research underscore the importance of this research to further investigating the metabolism of human γδ T cells and better understand their roles in immunity.

Characterisation of pulmonary macrophage immunometabolism in type 2 settings

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Current understanding of macrophage immunometabolism is centred around in vitro experiments using bone marrow derived macrophages. However, these cultures fail to capture the complexity and heterogeneity of macrophage phenotypes found in vivo. It is therefore unsurprising that our understanding of macrophages has historically been over simplified. We now know that the tissue environment governs macrophage phenotype and function, with this often linked to cellular metabolism. However, there is a need to improve our fundamental understanding of the metabolic pathways that govern tissue macrophages in vivo, particularly in type 2 settings, where macrophages produce biomolecules such as Relmα and Ym1 that define 'alternative' activation. In the lung, macrophage production of such molecules is likely controlled by the metabolically unique pulmonary environment. To gain a deeper understanding of the relationship between metabolism and pulmonary macrophage phenotype and function in vivo, we have utilised several type 2 systems: intranasal delivery of recombinant IL-4 complex; infection with the lung migrating helminth Schistosoma mansoni; and allergic airway inflammation induced by intranasal challenge with live Aspergillus fumigatus fungal spores. We have identified how murine lung macrophage subsets adopt distinct activation phenotypes during type 2 inflammation in vivo that link to their metabolic profiles. Further, we have used metabolic inhibitors to delineate the metabolic pathways required for murine pulmonary macrophage activation in response to type 2 stimuli ex vivo. Our data demonstrate a clear connection between metabolism and murine pulmonary macrophage phenotype and function during type 2 inflammation, revealing novel and fundamental differences to bone marrow macrophages.

PostDocs Bright Sparks: short talk – selected abstracts

Nicotinamide Riboside Kinase 1 augments cytoplasmic NAD/H upon CD4+ T cell activation, controlling NADP/H synthesis, reactive oxygen species abundance, inflammatory function and survival

Victora Stavrou, Myah Ali, Nancy Gudgeon, Emma Bishop, Taylor Fulton-Ward, Rebecca Mann, Adam Boufersaoui, Bryan Marzullo, Silke Heising, Gareth Lavery and Sarah Dimeloe

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CD4+ T cell function is underpinned by metabolic reprogramming upon activation. Increased glycolysis provides biosynthetic precursors for clonal expansion and promotes cytokine expression. In parallel, elevated mitochondrial oxidative phosphorylation (OXPHOS) generates heightened ATP and reactive oxygen species (ROS). ROS disseminate and signal, promoting T cell differentiation, but must be mitigated to prevent oxidative damage. Nicotinamide adenine dinucleotide (NAD/H) is an essential redox cofactor for glycolysis and mitochondrial OXPHOS. It is also phosphorylated to NADP/H, which regulates ROS levels. NAD/H abundance increases in line with CD4+ T cell metabolism upon activation, but synthetic pathways are not fully characterised.

In this study, we interrogated expression and activity of nicotinamide riboside kinase 1 (NRK1) in CD4+ T cells, which phosphorylates nicotinamide riboside (NR), directing it into the NAD salvage pathway. We identified this increases upon cell stimulation, driven by TCR and CD28 signalling. NRK1 non-redundantly contributes to NAD/H abundance in these cells but suppresses their activation and function. Consistently, NRK1-deficient CD4+ T cells have a hyper-inflammatory phenotype, expressing high levels of effector cytokines, which occurs alongside impaired viability.

Mechanistically, this is linked to NRK1 redistribution to the cytoplasm upon CD4+ T cell activation, where it locally elevates NAD/H levels. This supports glycolysis, but more profoundly impacts cytoplasmic NADP/H generation, thereby determining ROS abundance and nuclear NFAT translocation. During invasive fungal infection, NRK1 activity critically maintains effector CD4+ T cell frequencies within affected tissues, confirming that regulation of immune cell metabolism at the subcellular level determines whole organism immune responses.

Dietary arginine supplementation exacerbates experimental arthritis severity

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Arginine is a basic amino acid whose metabolites such as nitric oxide and polyamines are shown to be important in regulating gastrointestinal, renal, and immune functions. A growing body of evidence is showing that arginine and its byproducts, namely microbially-derived polyamines, are increased in patients with autoimmune arthritis compared to healthy individuals and that they are positively associated with disease severity. The mechanisms underpinning this association remain underexplored. To address this gap, here we investigated the effect of arginine dietary supplementation on immunopathology in experimental arthritis. Using an acute model of antigen-induced arthritis we found increased arginine availability significantly exacerbated arthritis severity.

In remission from arthritis, we found that arginine supplementation led to an increased frequency of pro-inflammatory B cell subsets such as class-switched and germinal centre B cells and a loss of regulatory B cells in the spleen (SP) and gut-associated lymphoid tissues (GALT). In the lymph node draining the inflamed joint (dLN) these changes in peripheral and GALT B cell subset distribution were accompanied by a reduction in regulatory T cells and an expansion of pro-inflammatory neutrophils. In conclusion, we show that dietary arginine supplementation is associated with increased presence of pro-inflammatory cells as well as a loss of regulatory lymphocytes, leading to increased arthritis severity.

Investigation into obesity related defects in MAIT cells - is glutamine the missing link?

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Mucosal associated Invariant T cells or MAIT cells are important innate effectors that produce proinflammatory cytokines such as IFNg and IL17. MAIT cells are not only critical for fighting bacterial and viral infections they are also emerging to be important in defense against cancer. TCR+cytokine induced activation of MAIT cells results in a change in MAIT cell metabolism whereby, MAIT cells show a significant upregulation of glycolysis. This glycolytic reprogramming is essential for MAIT cell function and proliferation. We have also previously shown that mTORC1 and cMyc play an integral role in enabling this metabolic switch. However, little is known about amino acid uptake and requirement to support MAIT cell function and metabolism. Here we show by proteomic analysis that several amino acid transporters including the glutamine transporter SLc1A5 is upregulated in activated MAIT cells. Along with this, the enzymes involved in glutamine lysis such as GLUD1 and GLS are also upregulated. We explore the requirement of glutamine in further detail by utilizing the inhibitor of GLS -CB839 which led to MAIT cell dysfunction in vitro. These data identify an essential role for glutamine and its metabolism in optimal MAIT cell responses upon activation.

Elucidating the link between metabolic profile and NK cell function in the progression of multiple myeloma

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Multiple myeloma (MM) is an incurable plasma cell cancer, preceded by clinically defined precursor conditions: monoclonal gammopathy of uncertain significance (MGUS) and smouldering myeloma (SMM). Both states share many genomic features with MM and thus, progression is thought to be partly influenced by tumour extrinsic factors. It is well established that natural killer (NK) cells are key players in the innate immune response against tumours. Whilst NK cell dysfunction has been described in MM, no clear picture has emerged of the way in which NK cell function is altered in MM progression.

This study aims to elucidate the effect of MM disease progression on patient NK cell function. In comparison to precursor conditions, we observed functional impairment of MM patient NK cells to both cytokine and tumour target stimulation. NK cell function was consistently impaired in bone marrow NK cells than in matched peripheral blood NK cells for all patients. Analysis of NK cell metabolism revealed no differences in mitochondrial mass; but markedly elevated long-chain fatty acid uptake in bone marrow NK cells when compared to matched peripheral blood NK cells. This was particularly evident in bone marrow CD56bright NK cells, where uptake positively correlated with

patient tumour burden. Bone marrow CD56bright NK cells also demonstrated reduced levels of c-Myc, a transcription factor which regulates NK metabolic and functional activity.

Our findings suggest that the diseased bone marrow environment may play a role in altering NK cell metabolic function that contributes to their dysfunctional state.

Poster presentations

P.01 **A transcriptome-wide Mendelian randomization study assessing the causal role of CD4+ T cell gene expression profiles in colorectal cancer development**

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Recent research has identified a potential protective effect of increased circulating lymphocytes counts on colorectal cancer (CRC) development. CD4+T cells, a highly dynamic lymphocyte subtype, undergo remodelling upon activation including gene expression changes. Previous studies investigating their role in cancer risk often use bulk tissue, thus limiting insights to static, non-dynamic relationships. Therefore, the importance of different lymphocyte subtypes and activation states in CRC development remains poorly understood and warrants further investigation.

We performed two genetic epidemiological methods, Mendelian randomization (MR) and genetic colocalization, to evaluate evidence for causal relationships of gene expression on CRC risk in multiple lymphocyte subtypes and stages of activation. Genetic instruments were obtained from single-cell transcriptomic data capturing the transcriptional states of CD4+T cells at rest and at three timepoints during activation. This allowed us to investigate the causal effect of expression of 1,805 genes across lymphocyte activation states on CRC risk (78,473 cases; 107,143 controls), repeating analyses stratified by CRC anatomical subsites and sex.

We identified 6 genes with strong evidence (FDR-P<0.05 in MR analyses and H4>0.8 in genetic colocalization analyses) for a causal role in CRC development. Importantly, we observed differences in causal estimates of gene expression on CRC risk across different CD4+T cell subtypes and activation timepoints, as well as CRC anatomical subsites and sex. Our study demonstrates the importance of capturing the dynamic nature of CD4+T cells in understanding CRC risk. By implementing a comprehensive causal framework, we were able to provide valuable insights into possible candidate genes for CRC prevention.

P.02 **Glucocorticoids inhibit HIF-1α accumulation and metabolic reprogramming in inflammatory macrophages**

Lockwood C [1], Clayton S [1], Booty L [2], O'Neil JD [1], Daley KK [1], Tennant DA [1], Clark A [1]

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Background: Since the 1950s, glucocorticoids have been used to effectively alleviate symptoms of inflammatory diseases. Long-term glucocorticoid use is associated with adverse side effects, many of which have a metabolic basis. Despite the wide-spread use of glucocorticoids, the factors contributing to the beneficial-versus-harmful effects are not fully understood. Our working theory proposes that both beneficial and harmful effects of glucocorticoids result from their physiological role in regulating glucose availability to the brain (which is highly glucose dependent). Glucocorticoids directly target inflammatory pathways to inhibit leukocyte activity. We propose that they also indirectly prevent leukocyte activation by targeting their metabolism.

Methods: Human monocyte-derived macrophages were treated in vitro with LPS and glucocorticoid. Expression of glycolytic regulators was determined by RT-qPCR and Western blotting, whilst metabolism was assessed using the Seahorse bioanalyser.

Results: We show that the synthetic glucocorticoid, dexamethasone, strongly inhibits inflammatory macrophage glycolytic metabolism, glucose uptake and expression of the glucose transporter GLUT1. Additionally, we identified that dexamethasone inhibits LPS-induced activation of hypoxia-inducible factor 1-alpha (HIF-1α), a driver of macrophage glycolytic metabolism.

Conclusions: Through comparing the effects of dexamethasone to pharmacological and genetic manipulation of the HIF-1α pathway, we aim to understand the consequences of dexamethasone-mediated HIF-1α inhibition. Utilising this model system, we hope to understand how dependent the anti-inflammatory effects of glucocorticoids are on HIF-1α and metabolic reprogramming. Understanding this mechanism may not only be therapeutically relevant in chronic inflammatory diseases, but also trauma and cancer, where the effects of synthetic glucocorticoids might involve modulation of HIF-1α function.

P.03 **Dynamics of metabolism in UpCell-differentiated hMDMs**

Olivia Boag, Newcastle University, UK

Macrophages play a crucial role within the immune system, while the dynamics of metabolic patterns in macrophages are not well understood. It is suggested that M1-polarised macrophages have decreased OXPHOS and increased glycolytic demand (Warburg effect). The study aim was to map the dynamics of metabolism and cytokine release in human monocyte derived macrophages (hMDMs).

Human CD14+ monocytes were differentiated into hMDMs in UpCell plates and stimulated up to 20h with either LPS, R848, TNF-α, IFN-γ or cGAMP.

Levels of IL-6, CXCL10 and IL-10 in the media increased over time (2h to 20h), whereas TNF-α concentrations remained stable. Only stimulation with LPS or R848 increased ECAR by 25% at 2h and 4h. Surprisingly, OXPHOS coupling efficiency was not changed by any of the stimuli. Palmitate driven OCR was about 10-15% of total OCR, suggesting slow rate of FAO in hMDMs. GLUT1 expression was significantly increased for all stimuli at 20h suggesting increased glucose uptake. None of the stimuli

induced changes in glycolytic genes expression up to 4h post stimulation. Inflammatory gene expression decreased over time (2h to 20h) for all stimuli.

We show that in hMDMs at the acute stimulation phase OXPHOS does not change, while only LPS and R848 stimulation induce a moderate increase in glycolysis that is not driven by changes in gene expression. Moreover, FAO is generally slow, and likely not the main energy source for hMDMs. Overall, our data suggests that the acute immune response in hMDMs is not driven by the Warburg effect.

P.04 **Development of methods to interrogate subcellular localisation of NRK1-dependent NAD/H synthesis in CD4+ T cells**

Myah Ali [1], Victoria Stavrou [1], Nancy Gudgeon [1], Meghan Teh C [2], Bethany Turley [1], Gareth Lavery [3], Sarah Dimeloe [1]

[1] University of Birmingham, UK; [2] University of Oxford, UK; [3] Nottingham Trent University

CD4+ T cell function is underpinned by dynamic changes in metabolism upon antigen encounter. Substantial increases in cytoplasmic glycolysis provide biosynthetic precursors for clonal expansion and promote cytokine expression. In parallel, elevated mitochondrial glucose and glutamine oxidation generate heightened ATP, metabolic intermediates and reactive oxygen species. Nicotinamide adenine dinucleotide (NAD/H) is a critical redox cofactor for these processes. Its abundance increases in line with increased CD4+ T cell metabolic activity upon stimulation, but synthetic pathways are not fully characterised.

In our laboratory, the role of nicotinamide riboside kinase 1 (NRK1) for NAD/H synthesis in CD4+ T cells has been interrogated, with a focus on the subcellular localisation and activity of this enzyme. To support this, I have established methods to measure NAD/H abundance within subcellular compartments. The first of these employs genetically-encoded fluorescent biosensors, which localise either to the cytoplasm, mitochondria or nuclei and report localised NAD+ abundance. These were previously developed and tested in cell lines, and I have optimised protocols for their use in human primary CD4+ T cells. This has been complemented by a second approach to measure NAD/H abundance within cytoplasmic cellular fractions generated by digitonin permeabilisation of the plasma membrane, which has been applied to human, as well as murine wild-type and NRK1-deficient CD4+ T cells.

Here, I will present an overview of steps taken to establish and validate these methods, and share data generated, which indicate that NRK1 activity particularly promotes cytoplasmic NAD/H abundance in activated CD4+ T cells.

P.05 **Interrogating NMNAT isoform expression and function in human CD4+ T cells**

Bethany Turley, Myah Ali, Victoria Stavrou, Nancy Gudgeon, Taylor Fulton-Ward, Rebecca Mann, Emily Meyrick and Dr Sarah Dimeloe

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CD4+ T cell metabolic reprogramming upon activation supports their immune effector functions. However, dysregulated CD4+ T metabolism is described in autoimmunity, chronic infection and cancer, highlighting potential for therapeutic targeting to restore normal immune function.

Nicotinamide adenine dinucleotide (NAD/H) is a critical metabolic redox cofactor, supporting cytoplasmic glycolysis and mitochondrial substrate oxidation. Its abundance increases in line with increased CD4+ T cell metabolic activity upon stimulation, but synthetic pathways are not fully characterised. Nicotinamide mononucleotide adenylyltransferase (NMNAT) catalyses the final step of NAD synthesis and exists in three isoforms with distinct subcellular localisation. Of these, NMNAT1 and 3 are expressed by CD4+ T cells, but relatively little is known about how they influence activity of these cells.

In this project, we assessed NMNAT1 and 3 expression within resting and activated human CD4+ T cells, observing transcripts for cytoplasmic NMNAT1 to increase significantly upon activation, whilst mitochondrial NMNAT3 transcription decreased. At the protein level, we confirmed increased NMNAT1 abundance in activated CD4+ T cells by western blot, whilst antibodies for NMNAT3 were non-specific. Further flow cytometry analysis identified NMNAT1 associated with CD25 and CD69 expression upon CD4+ T cell stimulation and confirmed both T cell receptor (TCR) and CD28-dependent signals drive upregulation. Of note, NMNAT1 is differentially expressed within CD4+ T cell subsets at rest, being highest in central memory and regulatory cells. Finally, pan-NMNAT inhibition suppressed IFN-γ secretion, indicating activity of these enzymes supports CD4+ T cell function. Future work will interrogate the specific contribution of NMNAT1 by genetic manipulation.

P.06 **Iron is critical for mucosal-associated invariant T cell metabolism and effector functions**

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Mucosal Associated Invariant T (MAIT) cells are a population of innate T cells which play a critical role in host protection against bacterial and viral pathogens. Upon activation, MAIT cells can rapidly respond via both T cell receptor dependent and independent mechanisms, resulting in robust cytokine production. The metabolic and nutritional requirements for optimal MAIT cell effector responses are still emerging. Iron is an important micronutrient, and is essential for cellular fitness, in particular cellular metabolism. Iron is also critical for many pathogenic microbes, including those which activate MAIT cells. However, iron has not been investigated with respect to MAIT cell metabolic or functional responses. In this study we show that MAIT cells require exogenous iron, transported via CD71, for optimal metabolic activity, including their production of ATP. We demonstrate that restricting iron availability by either chelating environmental iron or blocking CD71 on MAIT cells results in impaired cytokine production and proliferation. These data collectively highlight the importance of a CD71-iron axis for MAIT cell metabolism and functionality, an axis which may have implications in conditions where iron availability is limited.

P.07 **The role and regulation of cholesterol metabolism in B cell survival and proliferation**

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High cholesterol levels are a risk factor for developing cardiovascular disease (CVD); which causes approximately 17.9 million deaths per year. Fortunately, the progression of CVD can be prevented by

drugs called statins which inhibit HMG-CoA reductase (Hmgcr), a protein in the cholesterol biosynthesis pathway. Studies suggest that elderly individuals taking statins have lower antibody titers and a higher chance of developing severe respiratory disease following influenza vaccination. This suggests a defect in the ability of these B cells to undergo antibody class-switch recombination (CSR) and promote the adaptive immune response. Research from our lab has shown that short-term lipopolysaccharide (LPS) and IL-4 stimulation upregulates proteins involved in cholesterol metabolism, including Hmgcr. We hypothesised that inhibiting cholesterol metabolism would reduce proliferation, which is required for B cells to undergo CSR. B cells were isolated from wild-type mice and plated in normal or delipidated media to block uptake through the low-density lipoprotein receptor (Ldlr). The cells were treated with inhibitors to block rate-limiting enzymes in the cholesterol biosynthesis pathway – squalene monooxygenase (Sqle) or Hmgcr, and then stimulated using LPS and IL-4. Flow cytometry was used for quantification. Inhibition of Hmgcr and Sqle reduced cell number, size, proliferation, and cholesterol content. However, the changes induced by blocking Hmgcr may also be attributed to blocking protein prenylation. Interestingly, there was a significant increase in cell number, size, and proliferation upon blocking of the Ldlr. Our findings suggest that the activation of a feedback loop involving SREBP2 may be important for regulating changes in intracellular cholesterol levels.

P.08 **Lung tissue control of regulatory T cell phenotype, function and metabolism**

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The phenotype and function of immune cells can be dramatically influenced by their tissue location. Foxp3+ regulatory T cells (Tregs) are a subset of primarily anti-inflammatory CD4+ T cells which play a central role in preventing autoimmunity, promoting resolution of immune and inflammatory responses, and broadly regulating activation of a wide range of immune cells. Tregs show significant heterogeneity within non-lymphoid tissues, displaying context-specific functions such as helping to maintain tissue homeostasis. Immune cell function is dependent on cellular metabolism, which in turn is influenced by the environment in which they reside. Thus far, this has been well characterised in Treg within visceral adipose tissue, which upregulate fatty acid oxidation. However, metabolic adaptations of Treg in other tissues are much less well understood.

Using human and murine lung tissue samples and flow cytometry, we have compared expression of proposed tissue residency markers by pulmonary Tregs and effector CD4+ T cells (Teff). Simultaneously, we have used SCENITH and Met-Flow to evaluate tissue resident and non-resident Tregs and Teff dependence on glycolytic or mitochondrial metabolism.

We have characterised expression of putative tissue residency markers in Tregs and Teff isolated from human and murine lungs and identified differences in immunometabolism within and between defined lung tissue resident and non-resident populations.

Enhanced fundamental understanding of tissue adapted Treg immunometabolism and function may help inform future development of novel therapies for inflammatory disease and help to define how these cells orchestrate immune homeostasis within tissues.

P.09 **Uncovering untapped potential: noncanonical neoepitopes and T-cell metabolism in paediatric acute lymphoblastic leukaemia**

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Tumour-specific antigens, also termed neoepitopes, are ideal targets for cancer precision therapies due to their exclusive presence on cancerous cells and frequent implication in the malignant transformation process, rendering them not easily repressible by the tumour via antigen loss. Nevertheless, presentation of neoantigens is often limited by their capability to efficiently undergo the human leukocyte antigen class I presenting pathway due to possible sequence restrictions. To overcome this, our project harnesses noncanonical neoepitopes, consequently obtaining more potentially antigenic and immunogenic neoepitope sequences. Due to the high prevalence of tumour-specific fusion proteins in paediatric acute lymphoblastic leukaemia, noncanonical neoepitopes could be a promising target for immunotherapy. Using our in vitro-in silico pipeline we have already identified potential neoepitopes, which we are currently validating via distinct ex vivo approaches, including exploring their ability to induce anti-cancer T-cell responses. However, to ultimately harness the most promising targets for immunotherapy, it is crucial to gain a better understanding of the functional profile of patients' T-cells, including differentiation, effector functions and exhaustion levels. The latter are inherently linked to the T-cells' metabolic profile, an area that still lacks comprehensive exploration in children and will be a primary focus of our project. In conclusion, our study aims to improve adoptive cell therapies through identification of novel immunogenic cancer-specific neoantigens and the strengthening of T-cell responses tailored to our patients.

P.10 **Exploring Affinity-Dependent Metabolic Imprinting of Anti-Viral T Cell Responses**

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T cell responses to viral infections are mediated by their recognition of specific viral peptide-MHC complexes by the T cell receptor (TCR). T cell functions are underpinned by changes in metabolism in response to TCR ligation, whereby the metabolic network is reprogrammed to meet the demands of the cell. This project focuses on how the affinity of the TCR determines T cell function, and how TCR affinity influences T cell metabolism to ultimately drive this response.

Specifically, this project explores antigen-specific T cells in the context of Epstein-Barr Virus (EBV). Primary EBV infection affects over 90% of the worldwide population and in some individuals, is associated with the long-term development of cancer and autoimmunity.

To investigate the immune and metabolic phenotypes of EBV antigen-specific T cells, primary cells have been detected ex vivo using peptide-MHC tetramers. Tetramer staining has been combined with metabolic probe staining to assess mitochondrial mass and membrane potential, as well as with SCENITH analysis to explore the cells' dependence on glycolysis and oxidative phosphorylation.

Antigen-specific T cell clones have also been generated in vitro and have been assessed for their variable TCR sequences and functional avidity. A range of unique TCRs and distinct functional

responses towards the same EBV epitope have been identified. These T cell clones will be compared for metabolic differences in response to antigen stimulation by various approaches, including extracellular flux analysis and SCENITH.

P.11 **Lactate regulation of MAIT cell responses**

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Mucosal-Associated Invariant T cells (MAITs) are a subset of innate T cells that have emerged as robust anti-cancer responders. MAITs account for approximately 2-8% of circulating lymphocytes and are potent producers of cytotoxic cytokines and cytolytic molecules. Upon activation, MAITs deploy their killing machinery to destroy cancer cells. Nutrients and metabolites play important roles in immune function. Many

tumours have high glycolytic activity which can result in the exposure of MAITs to increased concentrations of the metabolite lactate. Lactate can be immunosuppressive when coupled with an acidic pH in the tumour microenvironment. However, in this study, we show the lactate anion is well tolerated by MAITs in pH neutral conditions and we report activated MAITs upregulate machinery required to, uptake and metabolise lactate. Activation in the presence of a pH neutral form of lactate supports MAIT cell degranulation and significantly increases MAIT cell production of key effector molecules including IFNγ, and Granzyme B. Moreover, we demonstrate how lactate can be used as a carbon fuel source to drive ATP production and cytokine reponses by MAITs in low glucose conditions. In summary, our studies outline the previously unrecognised role of lactate in shaping the activity of MAIT cell cytotoxicity which may have implications for their use as an ant-**tumour immunotherapeutic agent as well as the understanding of lactate in immune metabolism.**

P.12 **Interrogating control of macrophage metabolic and immune function by TNF-α**

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Anti-TNFα therapies have revolutionised treatment of chronic inflammatory conditions. However, TNFα is also important in initiating and maintaining inflammatory responses to pathogens; this is highlighted by the fact that anti-TNFα treatment increases the risk of reactivating latent mycobacterium tuberculosis. Our group recently identified that TNFα drives metabolic reprogramming of CD4+ T cells upon activation, thereby promoting their immune function. We hypothesised this may also occur in macrophages, which are important for control of latent tuberculosis infection and further, that identifying the mechanistic basis may offer novel opportunities for protection without interruption of effective anti-TNFα therapy. To begin to understand this, I have differentiated human monocyte-derived macrophages and activated them in presence of isotype control or TNF-α blocking antibodies, or alternatively under titration of exogenous recombinant TNF-α. My data indicate that blocking TNFα in macrophages reduces surface expression of costimulatory and antigen-presenting surface molecules (CD40, CD86, MHC class II) and reduces production of IL-10 and IL-6. Using extracellular flux analysis, we also found that macrophages treated with TNFα blockade demonstrate increased levels of mitochondrial oxidative

phosphorylation compared to control cells. Future work will interrogate the other effects of TNFα on macrophage metabolism, for example on expression and activity of NAD+ synthesis pathways and NAD+ consuming enzyme activity.

P.13 **Interrogating the metabolic effects of the antimicrobial peptide cathelicidin on CD4+ T cells**

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Cathelicidin is an antimicrobial peptide secreted by multiple cell types including neutrophils, epithelial cells, mast cells and macrophages. Studies indicate capacity for cathelicidin to alter metabolic activity of diverse cell types. Previous research from our lab has shown that cathelicidin drives Th17 development and protects these cells from apoptosis. As T cell metabolism has previously been linked to T cell differentiation, we hypothesise that the alterations in T cell fate following contact with cathelicidin may relate to changes in metabolism.

In this project, we investigated the metabolic pathways through which cathelicidin regulates T cell metabolism in murine splenic cell cultures.

Single cell suspensions of total splenocytes from C57BL6/JOlaHsD mice were cultured with anti-CD3 stimulation in the presence or absence of Th17 polarising conditions (TGF-β, IL-6, IL-23). The impact of cathelicidin in the presence or absence of metabolic pathway inhibitors was used to identify the effect on Th17 polarisation and cytokine production, and for metabolic capacity by techniques including extracellular flux analysis, mitotracker staining and SCENITH assay. We also assessed signalling through the PI3K/AkT/mTOR signalling pathway, by analysis of phospho-protein abundance.

Our findings confirm previous reports of cathelicidin promoting Th17 differentiation. Preliminary metabolic data indicate cathelicidin alters phospho-mTOR but not upstream phospho-AkT. The data suggests that cathelicidin may exert effects on CD4+T cell differentiation and inflammatory activity via metabolic alteration.

P.14 **Tissue resident CD8+ TRM adapt their metabolic profile to survive and function in the human liver**

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Death rates from chronic liver disease (CLD) are four times higher than they were in 1970. Alcohol, viral hepatitis and obesity are the major drivers of CLD. In the UK, 64 percent of adults are overweight or obese. As such many are at significant risk of developing CLD, for which treatment options are limited. Shifts in the metabolic environment of a tissue influence cell identity, imprint function, and ultimately impact survival and longevity. Accordingly, we are interested in understanding how the local milieu characteristic of those living with CLD effects tissue-resident T-cells (TRM) – highly specialised effectors, that no longer recirculate.

To address this question, we use spectral flow cytometry to understand the metabolic preference of T-cells residing within the liver evaluating what pathways they rely on to survive and function, and

how CLD forces T-cells to adapt. Using our well-defined cohorts we have begun to compare the metabolic profiles of the long-lived TRM compartment (denoted as: CD69 ±CD103) with T-cells infiltrating through, but not resident within the human liver (CD69-CD103-). Firstly, we show that hepatic TRM express a higher density of nutrient transporters (CD98/CD71/CD36) on their surface compared to their infiltrating, non-resident counterparts. This is supported by an enhanced ex vivo uptake of amino acids, transferrin and free fatty acids at rest. Secondly, we have previously shown TRM can be induced in vitro by sequential exposure to IL-15 and TGFb (two prototypical liver cytokines). We now demonstrate that upon differentiation into an "induced-TRM", peripheral CD8+T-cells undergo a metabolic rewiring mimicking the enhanced uptake of transferrin and free fatty acids seen ex vivo. Finally, when assessing the impact of the underlying disease aetiology (for example whether there is a metabolic or viral driver of disease) on the differentiation, proliferation or survival of hepatic T-cells we discovered a significant increase in the retention of CD8+ T-cells with a bona fide resident profile (CD69+CD103+, CD49a+/CXCR6hi) in those individuals living with CLD caused by metabolic disorders. In these same individuals we note a reduction in the capacity of TRM to produce proinflammatory cytokines and chemokines, and a concomitant decrease in their ability to mobilise cytotoxic granules to the surface upon antigen engagement. We are now exploring whether the metabolic profile characteristic of TRM in health and CLD contributes to either their dysfunction or enhanced retention.

Hepatic TRM adapt their metabolic profile to ensure longevity and functionality in this hostile environment. Importantly, by elucidating the metabolic underpinnings of liver-resident T-cells in CLD, we hope to guide new therapeutic strategies to combat liver disease, addressing a significant gap in current treatment regimens.