

Investigating the Environmental Modulators of Mucosal-Associated Invariant T Cell Metabolism and Effector Responses



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Declaration

This thesis has not been submitted whole or in part to this or any university for any other degree, and is the original work of the author except where stated otherwise.

I declare that I have not used Generative Artificial Intelligence or Large Language Models to source, create, structure or phrase the text in this thesis, except and only in the case where expressly permitted in the Maynooth University Department of Biology academic guidelines, or by written guidance from my supervisor.

Signed:

Date: 31st July 2026

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List of Abbreviations

(m)ROS	(Mitochondrial) reactive oxygen species
2-Deoxy-D-Glucose	2DG
5-A-RU	5-amino-6-D-ribitylaminouracil
5-OP-RU	5-(2-oxopropylideneamino)-6-D-ribitylaminouracil
α -GalCer	α -galactosylceramide
AAO	Amino acid oxidation
ACO2	Aconitase
Acetyl-CoA	Acetyl coenzyme A
ADP	Adenosine diphosphate
AGL	Glycogen debranching enzyme
AILD	Autoimmune liver disease
AMPK	AMP-activated protein kinase
APC	Antigen presenting cell
AS	Ankylosing spondylitis
ATF4	Activating transcription factor 4
ATP	Adenosine triphosphate
BCH	2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid
BCL11B	B cell lymphoma/leukaemia 11B
BSA	Bovine serum albumin
BTN	Butyrophilin
CA7S	Cholic acid 7-sulfate
CD71	Transferrin receptor
CLP	Common lymphoid progenitor
CoQ	Coenzyme Q
CPT	Carnitine palmitoyl transferase
CTL	Cytotoxic T lymphocyte
CytC	Cytochrome C
DC	Dendritic cell
DFO	Deferoxamine mesylate salt
DIA	Data-independent acquisition
DN	Double negative
DP	Double positive
DPBS	Dulbecco's phosphate-buffered saline
ECAR	Extracellular acidification rate
eIF	Eukaryotic initiation factor
EOMES	Eomesodermin
ETC	Electron transport chain

FAO	Fatty acid oxidation
FAD(H)	Flavin adenine dinucleotide (reduced form)
FBS	Foetal bovine serum
Fe-S	Iron-sulphur
FeSO ₄	Iron(II) sulfate heptahydrate
FMO	Fluorescence minus one
FSC	Forward scatter area
G6P	Glucose-6-phosphate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLS	Glutaminase
GLUT1	Glucose transporter 1
GYS	Glycogen synthase
HBSS	Hanks' balanced salt solution
HCC	Hepatocellular carcinoma
HK2	Hexokinase 2
HMBPP	(E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate
HPLM	Human Plasma-Like Media
IAV	Influenza A virus
ICB	Immune checkpoint blockade
IFN	Interferon
IFNAR	Interferon- α/β receptor
ILC	Innate lymphoid cell
IL (R)	Interleukin (receptor)
iNKT	Invariant natural killer T
ISG	Interferon-stimulated gene
ISR	Integrated stress response
KDM6B	Lysine demethylase 6B
LDHA	Lactate dehydrogenase A
LNAA	Large neutral amino acid
MACS	Magnetic cell separation
MAIT	Mucosal-associated invariant T
MASLD	Metabolic dysfunction-associated steatotic liver disease
MFI	Mean fluorescence intensity
MG	Methylglyoxal
MHC	Major histocompatibility complex
mTOR(C)	Mammalian target of rapamycin (complex)
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NFAT	Nuclear factor of activated T cells

NK	Natural killer
OCR	Oxygen consumption rate
OxPhos	Oxidative phosphorylation
PAGs	Phosphoantigens
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell death protein 1
PDH	Pyruvate dehydrogenase
PDHK1	Pyruvate dehydrogenase kinase 1
PI3K	Phosphatidylinositol-3-kinase
PKM2	Pyruvate kinase muscle isoform 2
PLZF	Promyelocytic leukaemia zinc finger
PPP	Pentose phosphate pathway
pSS	Primary sjogren's syndrome
PUFA	Polyunsaturated fatty acids
PWO	People with obesity
PYG(B)	Glycogen phosphorylase (brain isoform)
R5P	Ribose-5-phosphate
R	Receptor
S1P	Sphingosine 1-phosphate
SP	Single positive
SRC	Spare respiratory capacity
STAT	Signal transducer and activator of transcription
TCA	Tricarboxylic acid
TCF1	T cell factor 1
Tcm	Central memory T
TCR	T cell receptor
Tem	Effector memory T
TFRC	Transferrin receptor
Tfh	T follicular helper
TGF	Transforming growth factor
Th	T helper
Tm	Memory T
TMB	Tetramethyl benzidine
Trm	Tissue resident memory T
Treg	Regulatory T cell
TSC2	Tuberous sclerosis complex 2

Publications

Included in this thesis:

- **Eimear K. Ryan**, Christy Clutter, Conor De Barra, Benjamin J. Jenkins, Simon O'Shaughnessy, Odhrán K. Ryan, Chloe McKenna, Helen M. Heneghan, Fiona Walsh, David K. Finlay, Linda V. Sinclair, Nicholas Jones, Daniel T. Leung, Donal O'Shea, and Andrew E. Hogan. Iron is Critical for Mucosal-Associated Invariant T Cell Metabolism and Effector Functions. *J Immunol.*, 2024. [doi: 10.4049/jimmunol.2300649](https://doi.org/10.4049/jimmunol.2300649).
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- Ciara L. Flynn, **Eimear K. Ryan**, Cian Davis, Czara Kennedy, Katherine Egan, Aimee Brennan, Philip McEntee, Kirstan Murphy, Niamh Lynam-Lennon, Linda V. Sinclair, Nicholas Jones, Donal O'Shea, Helen M. Heneghan, and Andrew E. Hogan. Defective V δ 2⁺ T cell metabolic and functional responses in obesity are reversed with bariatric surgery. *JCI Insight.*, **in revision**.
- Féaron C. Cassidy, Odhrán K. Ryan, **Eimear K. Ryan**, Ronan Bergin, Amine Toubal, Agnes Lehuens, Helen M. Heneghan, Donal O'Shea, and Andrew E. Hogan. Loss of CD8⁺ MAIT cells underpins reduced MAIT cytotoxicity in obesity. **In preparation**.

Abstract

Mucosal-associated invariant T (MAIT) cells are subset of innate-like unconventional T cells, characterised by the expression of a non-MHC restricted, semi-invariant T cell receptor (TCR); with a significant role in modulating host-protective responses to bacterial and viral pathogens. Upon activation, MAIT cells upregulate a potent effector programme, associated with robust proliferation, and the expression of a milieu of cytokines and cytolytic molecules. Such effector reprogramming is underpinned by proteome remodelling, rapid *de novo* biosynthesis, and increased metabolic flux. Therefore, MAIT cells are reliant on enhanced nutrient uptake to support their capacity to respond appropriately to challenge; and thus, nutrient availability is considered a limiting factor for MAIT cell activation. This is an important consideration to uphold in the context of disease settings such as infection, cancer, and obesity, where environmental nutrient availability can become perturbed. Gaining a complete understanding of the metabolic and nutrient underpinnings of MAIT cell effector responses is thus pertinent to our understanding of how these can responses become dysfunctional in disease.

In this thesis, we aim to elucidate further our current understanding of MAIT cell metabolism, and the nutritional requirements underpinning the effector programme. Specifically, we highlight a novel role for iron uptake in dictating MAIT cell metabolic fitness downstream of TCR triggering, and characterise significant dysfunction under iron-deplete conditions. In addition, we describe for the first time, the metabolic responses of human MAIT cells to innate cytokine stimulation, and characterise a significant divergence from the TCR driven phenotype. Finally, we elucidate the importance of a functioning glutamine-MYC axis in supporting both metabolic rewiring and effector reprogramming in activated human MAIT cells. Overall, this PhD thesis contributes novel insights into MAIT cell metabolism in the context of both TCR-dependent and TCR-independent signalling pathways; and further demonstrates how environmental nutrient availability can shape host immune responses.

CHAPTER 1. General Introduction

1.1. Conventional T Cells

Conventional T lymphocytes comprise a major component of the adaptive immune system, potentiating host immunity through T cell receptor (TCR)-mediated recognition of antigenic peptides presented by Major Histocompatibility Complex (MHC) class I or class II molecules present on antigen presenting cells (APCs), such as dendritic cells (DCs), macrophages, and B cells. Following development in the thymus, T cells express either one of the co-receptors, CD8 or CD4, and depending on this expression profile, the $\alpha\beta$ -TCR is either MHC class I or MHC class II restricted, respective for CD8⁺ or CD4⁺ T cells (Marshall et al., 2018). The TCR expressed by an individual T cell is reactive to one specific MHC-peptide complex, and naïve T cells will continuously circulate the body between the blood, and the secondary lymphoid organs, such as the spleen and the lymph nodes, until they encounter the appropriate peptide-complex expressed on the cell surface of an APC (Piconese et al., 2020). Thus, T cells are important players in mounting antigen-specific immune responses which allow for the elimination of invading pathogens, and the targeted killing of pathogen-infected cells.

1.1.1. Conventional T Cell Development

T cells arise from pluripotent hematopoietic stem cells in the bone marrow, which in turn differentiate into a common lymphoid progenitor (CLP) to subsequently give rise to the development of a heterogenous lymphoid lineage, including Natural Killer (NK) cells, T cells, B cells, and Innate Lymphoid Cells (ILCs) (Kondo et al., 1997, Murphy and Weaver, 2017). Migration of early thymic progenitor cells into the thymus enables T cell development and maturation (Figure 1.1). In phase I, early precursor CD4/CD8 double-negative (DN) thymocytes expressing CD44 undergo extensive proliferation and begin to commit to the T cell lineage upon receiving Notch signals from thymic stromal cells. Notch signalling facilitates expression of a T cell-specific gene programme via transcriptional induction of the genes encoding T cell Factor 1 (TCF1), and GATA3; followed by later induction of the transcription factor, B cell lymphoma/leukaemia 11B (BCL11B). When armed with this T cell gene expression programme, DN2 thymocytes will upregulate expression of the Interleukin (IL)-2 receptor (R) α -chain, CD25, marking irreversible commitment to the T cell fate (Murphy and Weaver, 2017, Hosokawa and Rothenberg, 2018). In phase III, CD44⁺ CD25⁺ DN3 cells undergo

beta-selection, during which cells which will persist must have successfully generated a functional TCR β chain through extensive rearrangement of the associated gene loci, allowing for cell surface expression of a complex containing the newly generated β chain along with a pre-TCR α , and CD3, as a complete pre-TCR. Following a proliferative burst, the cells become further differentiated through downregulation of CD25 and upregulation of the cell surface molecules, CD4 and CD8; and are hereby referred to as double-positive (DP) thymocytes. Finally, rearrangement of the TCR α chain locus allows finally for the generation of a true, productive $\alpha\beta$ -TCR (Murphy and Weaver, 2017, Hosokawa and Rothenberg, 2018).

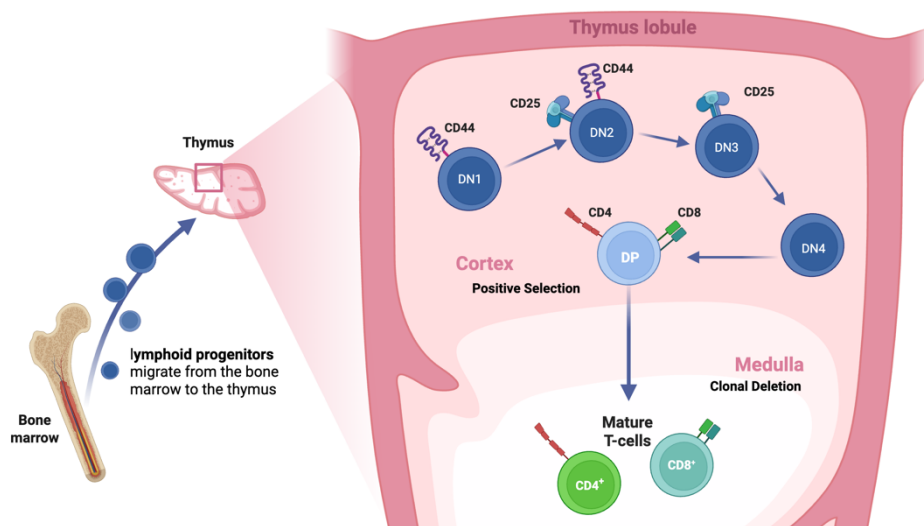


Figure 1.1. Thymic Development of Conventional T Cells. Lymphoid progenitor cells migrate from the bone marrow to the thymus, and undergo a step-wise developmental programme which yields a mature T cell compartment. Mature thymic T cells then egress into the periphery, where they circulate as antigen-naïve conventional T cells. Adapted from “T-Cell Development in Thymus 2”, BioRender.com.

DP thymocytes which express a productive $\alpha\beta$ -TCR are subject to positive selection in the thymic cortex, during cells which engage MHC-self-peptide complexes expressed on thymic epithelial cells with weak affinity die by apoptosis. Those remaining are reactive to self-peptide antigens and in this manner receive a survival signal allowing for their positive selection (Klein et al., 2014, Adu-Berchie et al., 2023). Positively selected thymocytes subsequently migrate to the medulla and are here subject to clonal deletion. DP thymocytes are presented with self-peptides through MHC molecules expressed by medullary thymic

epithelial cells, or by DCs. Cells which express high affinity for self-peptides are here subject to cell death by apoptosis; or otherwise skew towards regulatory T (Treg) cell differentiation. Thus, T cells with reactivity towards antigens which are encountered throughout the body under normal circumstances, are eliminated. Finally, downregulation of either co-receptor during thymic selection results in the generation of single-positive CD4-expressing (MHC-II restricted), or CD8-expressing (MHC-I restricted) T cells (Klein et al., 2014, Adu-Berchie et al., 2023). Further maturation of such naïve T cells in the medulla ensures upregulation of the G-protein-coupled receptor, sphingosine 1-phosphate (S1P)R1, to facilitate recognition of the lipid molecule, S1P. Because S1P is present at high concentrations in the blood, and in the lymph, it thus acts as a chemoattractant in promoting the egress of mature T cells out of the thymus, and into the periphery (Allende et al., 2004, Matloubian et al., 2004, Pappu et al., 2007).

1.1.2. Effector T Cell Phenotypes and Functional Responses

Upon encountering an APC presenting the appropriate MHC-peptide-complex in the secondary lymphoid organs, T cells are primed to proliferate rapidly in a process termed clonal expansion, which ensures the expansive generation of genetically identical effector cells expressing a TCR with exact specificity for the initial activating antigen (Adams et al., 2020). This is critical process which ensures amplification of an antigen-specific immune response targeted at the invading pathogen. Naïve CD4⁺ T cells will differentiate into effector T-helper (Th) cells, which generally lack the ability to exert cytotoxicity, but have a significant role in potentiating immune responses through modulating the activity of other cells, including APCs, and B cells; whilst naïve CD8⁺ T cells differentiate into cytotoxic T lymphocytes (CTLs), which are directly involved in the destruction of infected or malignant-transformed target cells (Marshall et al., 2018). However, cognate antigen-recognition alone is not sufficient to facilitate complete effector T cell activation and differentiation. In addition to TCR binding to the MHC-peptide complex (signal 1), naïve T cells also require interaction between the co-stimulatory molecules present on APCs, such as CD80 or CD86, and the counter receptors present on the T cell surface, for example, CD28, to enhance survival and proliferation (Lenschow et al., 1996); thus, co-stimulation is considered as a necessary ‘signal 2’ in the potentiation of effector T cell responses. Finally, the presence of extracellular cytokines will further influence the type of response elicited downstream of antigen recognition and co-

stimulation (Curtsinger and Mescher, 2010, Sun et al., 2023), and thereby cytokines are considered an important tertiary signal in modulating the activation and differentiation of effector T cells.

There are five major subsets in the CD4⁺ Th cell compartment, namely Th1, Th2, Th17, Treg, and T follicular helper (Tfh) cells. Th cell responses are elicited downstream of antigen recognition and in the presence of a particular extracellular cytokine milieu (Figure 1.2). For example, the Th1 response is elicited in the presence of either IL-12 or interferon (IFN)- γ , which facilitate downstream induction of the Th1-associated master transcription factor T-bet; and subsequent expression of the Th1 effector cytokine, IFN γ (Meitei and Lal, 2023). One role for Th1-derived IFN γ is the potentiation of host-protective responses by polarisation of macrophages towards their pro-inflammatory 'M1' state, enhancing their cytotoxic functionality (Nathan et al., 1983, Pace et al., 1983, Celada et al., 1984). In contrast, the polarisation of T cells towards the Th2 subset is facilitated by the presence of IL-4 and IL-2 at the time of antigen recognition, both of which underpin the induction of the transcription factor GATA3, resulting in a cytokine expression profile characterised by IL-4, IL-5, and IL-13. Accordingly, Th2 cells are associated with host-immune responses against extracellular pathogens, such as helminths, though they are too implicated in the potentiation of immunopathology the context of asthma, and allergy (Walker and McKenzie, 2018, Meitei and Lal, 2023). In the presence of IL-4, transforming growth factor (TGF) β will induce further differentiation of the Th2 subset into the more recently characterised Th9 subset, appropriately named for their high-level expression of the pleiotropic cytokine, IL-9 (Veldhoen et al., 2008, Dardalhon et al., 2008). Th9 cell differentiation and effector responses are underpinned by increased expression of the master transcription factor, PU.1 (Chang et al., 2010, Ramming et al., 2012). A role for the Th9 cell compartment has been described in the context of anti-tumour immunity, whereby Th9-derived IL-9 can promote the activation of tumour-infiltrating DCs, and thus facilitate the potentiation of tumour-specific CD8⁺ CTL responses (Kim et al., 2015). However, similar to the Th2 compartment, Th9 cells have also been implicated in the development of allergic inflammatory disorders (Garn, 2018).

TGF β , in the presence of an inflammatory milieu, will drive the differentiation of Th17 cells; inducing expression of the master transcription factor ROR γ T, which subsequently elicits

high-level expression of IL-17, and other inflammatory cytokines, such as IL-22 (Ivanov et al., 2006, Manel et al., 2008). Th17 cells are protective against extracellular pathogens at mucosal sites, but are also thought to potentiate the pathogenesis of certain chronic inflammatory, and autoimmune diseases (Ohara et al., 2024).

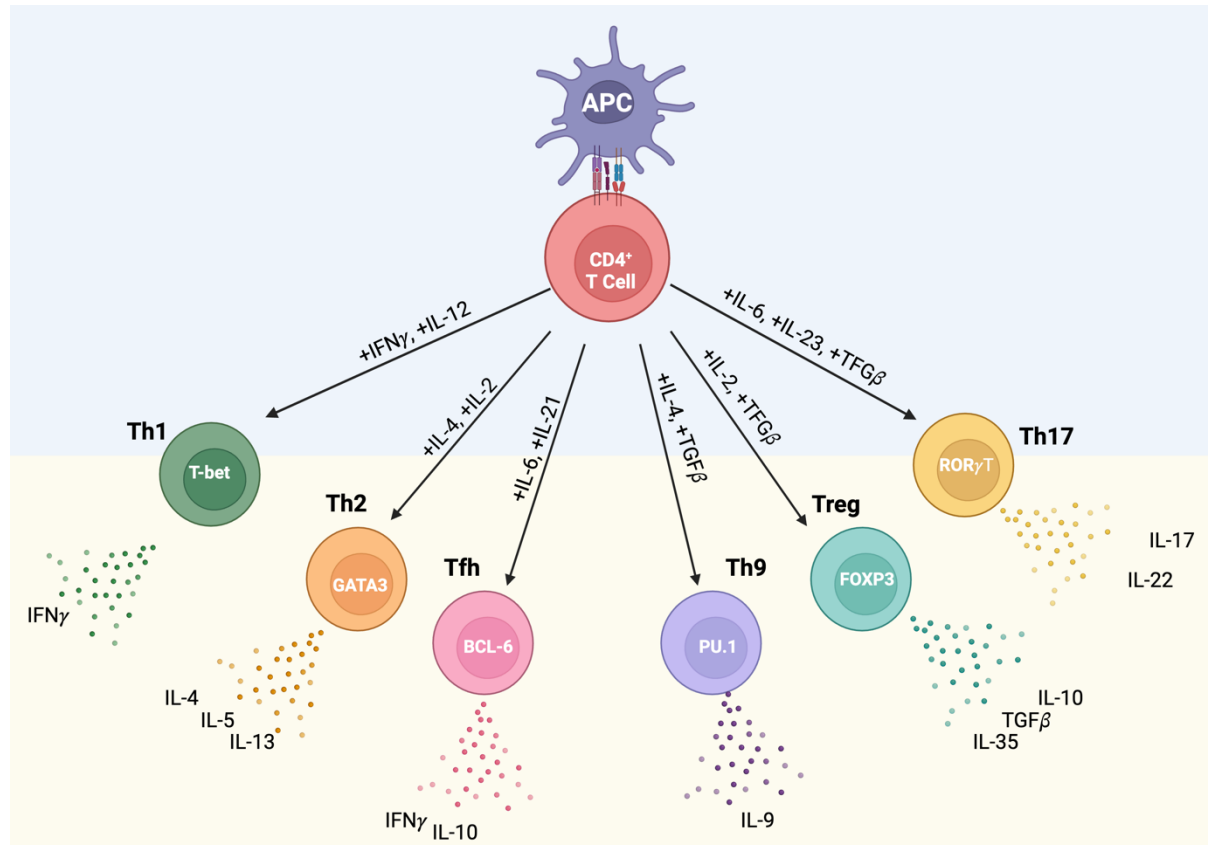


Figure 1.2. Lineage Differentiation of $CD4^+$ Effector T-helper Cells. Naïve $CD4^+$ T cells encounter their cognate antigen in the presence of a polarising cytokine milieu, and differentiate into specific Th cell subsets, including Th1, Th2, Th9, Th17, Tfh, and Treg, cell subsets. The associated functional responses are underpinned by the specific upregulation of a dominating master transcription factor, including T-bet, GATA3, PU.1, ROR γ T, BCL-6, and FOXP3. Adapted from (Meitei and Lal, 2023). Created with Biorender.com.

Peripheral Treg cells are a subset of Th cells characterised by high expression of CD25, and the master transcription factor FoxP3; with an important role in maintaining immune tolerance, and in the negative regulation of inflammatory immune responses (Plitas and Rudensky, 2016). Tregs express an inhibitory cytokine profile characterised by the release of IL-10, TGF β , and IL-35, and their differentiation is also mediated by the presence of TGF β , and IL-2, at the time of TCR engagement and co-stimulation (Chen et al., 2003, Laurence et al., 2007, Meitei and Lal, 2023). Finally, Tfh cells are differentiated in the presence of IL-6 and IL-

21 (Nurieva et al., 2008, Vogelzang et al., 2008), only in the absence of $TGF\beta$ (McCarron and Marie, 2014); and are characterised by high cell surface expression of the checkpoint protein, Programmed cell death protein-1 (PD-1), and the chemokine receptor, CXCR5 (Hardtke et al., 2005, Haynes et al., 2007). Tfh cells secrete a mix of pro- and anti- inflammatory cytokines, including $IFN\gamma$ and IL-10, and are functionally important in the context of B cell-mediated humoral immunity (Meitei and Lal, 2023).

Conversely, effector $CD8^+$ CTLs are directly involved in the clearance of intracellular pathogens, and malignant transformed cells. Upon activation, $CD8^+$ CTLs expand rapidly, and upregulate an inherent cytotoxic effector programme, characterised by secretion of the pore-forming lytic molecule, perforin, alongside cytotoxic granules containing a family of serine proteases, known as granzymes; which collectively induce target cell death via apoptosis (Trapani and Smyth, 2002). Furthermore, $CD8^+$ CTLs are robust producers of type-1 effector cytokines, such as $IFN\gamma$ and $TNF\alpha$ (Wherry et al., 2003), which can potentiate host-immunity in several ways. For example, alongside its role in promoting M1 polarisation, $IFN\gamma$, a potent inducer of JAK-STAT signalling, can elicit the expression of interferon-stimulated genes (ISGs). ISGs control viral, microbial, and parasitic infections by targeting pathways involved in replication, whilst certain ISGs also have proapoptotic functions (Schneider et al., 2014). CTLs will also induce target cell lysis via the Fas/FasL axis. $CD8^+$ effector T cells present with increased cell surface expression of FasL, a ligand for the Fas molecule expressed on the cell surface of transformed cells. Interaction between Fas/FasL mediates initiation of an intracellular caspase cascade in the target cell, thus resulting in apoptotic cell death (Nagata, 1999). Although the most abundant phenotype is indeed this type-1 profile characterised by expression of $IFN\gamma$ and cytolytic molecules, CTL lineage determination and effector responses are also influenced by the cytokine milieu present at the time of antigen-recognition and co-stimulation (Figure 1.3), as with Th cells. Whilst IL-12 is typically involved in the polarisation of the type-1 effector programme, licensing expression of master transcription factors, T-bet and Eomesodermin (EOMES); $CD8^+$ effector T cells can also be polarised towards a type-2 (Tc2) or type-17 (Tc17) response profiles. Specifically, the Tc2 profile is polarised by IL-4, as with Th2 cells, and is similarly underpinned by expression of the transcription factor GATA3; whilst Tc17 polarisation is induced by $TGF\beta$ in the presence of an inflammatory milieu, resulting in the induction of ROR γ T, and robust secretion of IL-17 (Koh et al., 2023).

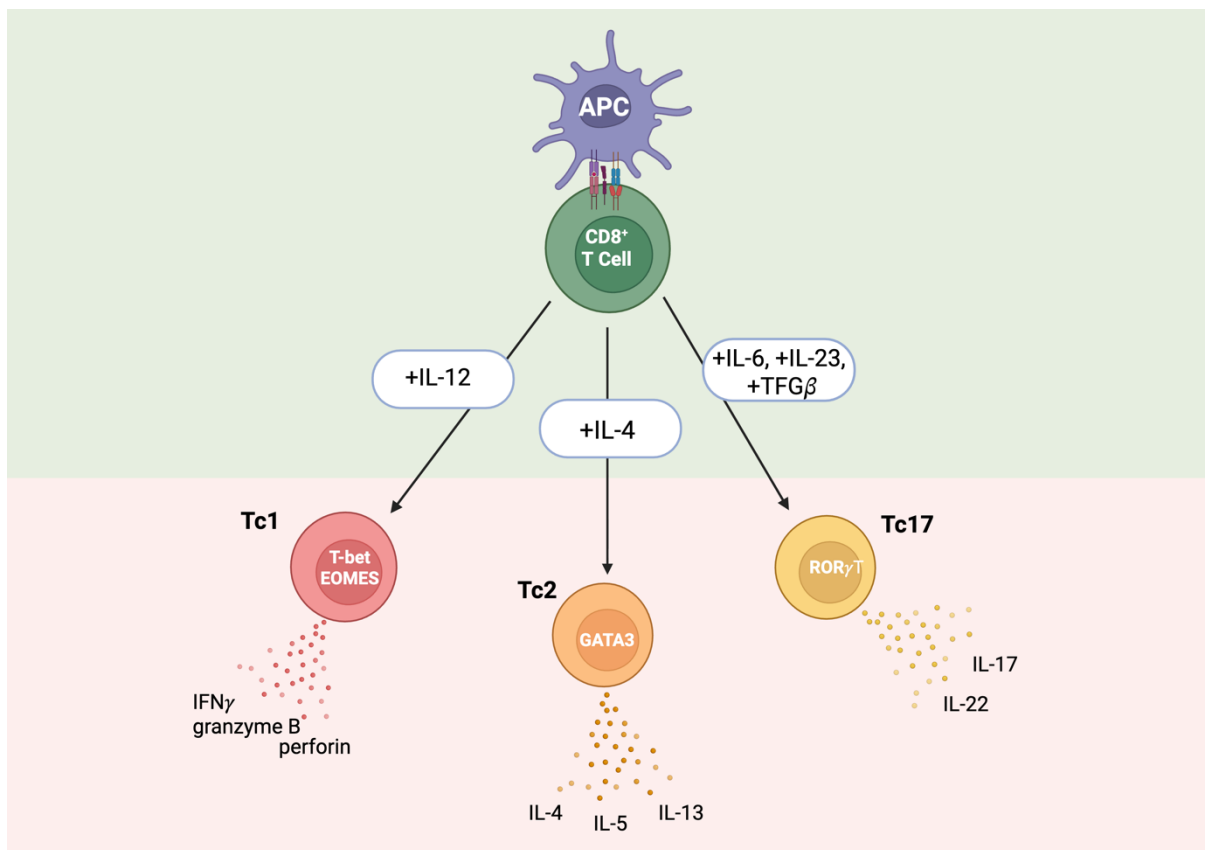


Figure 1.3. CD8⁺ Effector T Cell Differentiation. Upon encountering an appropriate antigen, naïve CD8⁺ T cells will expand and differentiate into cytotoxic effector T lymphocytes (CTLs). CD8⁺ T cells are most abundantly differentiated into cells expressing a type-1 cytotoxic effector programme (Tc1) underpinned by the IL-12-mediated induction of transcription factors T-bet and EOMES; and most notably characterised by a robust IFN γ response. However, CD8⁺ effector T cell responses are dictated by the cytokine milieu present upon antigen-recognition, and thus can take on a type-2 or type-17 effector profile, underpinned respectively by upregulation of GATA3 or ROR γ T, in the presence of appropriate cytokines. Created with Biorender.com

Following contraction of the initial adaptive T cell response, during which approximately 95% of effector T cells are eliminated in a process termed activation-induced cell death, approximately 5% of the effector T cell compartment will remain as memory T (T_m) cells (Ahmed and Gray, 1996); and by the second decade of life, T_m cells comprise up to 35% of the circulating T cell compartment (Cossarizza et al., 1996, Farber et al., 2014). Phenotypically, T_m cells are characterised by the expression of the CD45-RO isoform, and absence of the CD45-RA isoform (CD45RO⁺ CD45RA⁻) (Smith et al., 1986, Sanders et al., 1988). However, there are two broad subsets within the T_m cell lineage, further defined by their localisation, function, and via the expression of surface molecules such as the lymph node homing

chemokine receptor, CCR7. Central memory T (T_{cm}) cells are classified by their constitutive expression of CCR7 (CD45RO⁺ CCR7⁺), akin to that observed on naïve T cells, which facilitates homing to the secondary lymphoid organs (Campbell et al., 2001, Sallusto et al., 2004). Conversely, effector memory T (T_{em}) cells are CD45RO⁺ CCR7⁻ and therefore circulate through non-lymphoid tissues in the periphery, and the blood (Sallusto et al., 2004). T_{em} cells are terminally differentiated, and present with rapid cytolytic activity and robust cytokine expression upon re-stimulation. Conversely, T_{cm} cells present with a robust proliferative capacity, alongside expression of IL-2, which facilitates the generation of newly differentiated effector cells upon antigen re-stimulation (Sallusto et al., 2004). There also exists a subset of long-lived T_m cells that preferentially reside in non-lymphoid tissues, appropriately termed tissue-resident memory T (T_{rm}) cells, which offer constitutive protection against pathogens at barrier sites. T_{rm} cells are poised to respond rapidly upon antigen re-stimulation, and present with a robust proliferative capacity. They are characterised by a unique residency phenotype associated with increased cell surface expression of the lymphocyte activation marker, CD69, and the adhesion molecule, CD103; which promote tissue retention through downregulation of S1PR1, and facilitate epithelial adhesion, respectively (Rodger et al., 2024).

1.2. Unconventional T Cells

Unconventional T cells are a group of T lymphocytes that differ from the conventional subsets in how they are activated, or in the kinetics of their functional responses. Typically, unconventional TCRs are not restricted by the prototypical MHC class I and class II molecules; and instead recognise non-polymorphic antigen-presenting molecules, such as the MHC class I-like molecules, CD1 and MR1. Although classical MHC molecules present peptide antigens, both CD1 and MR1 present non-peptide metabolite antigens, and accordingly, unconventional T cells are predominately characterised by the expression of TCRs that are of limited diversity (Godfrey et al., 2015). Furthermore, unconventional T cells are often capable of mounting rapid innate-like responses to stimulation by innate cytokines, independently of their TCR (Hackstein and Klenerman, 2023). Such robust functional diversity, and a broad cytokine expression pattern, lends to an important role in potentiating the activation of conventional T and B lymphocytes; and so, they are regarded as a bridge between innate and adaptive immune responses (Figure 1.4). In humans, the most well-defined unconventional T

cell subsets in humans include invariant natural killer T (iNKT) cells, $\gamma\delta$ T cells, and mucosal-associated invariant T (MAIT) cells.

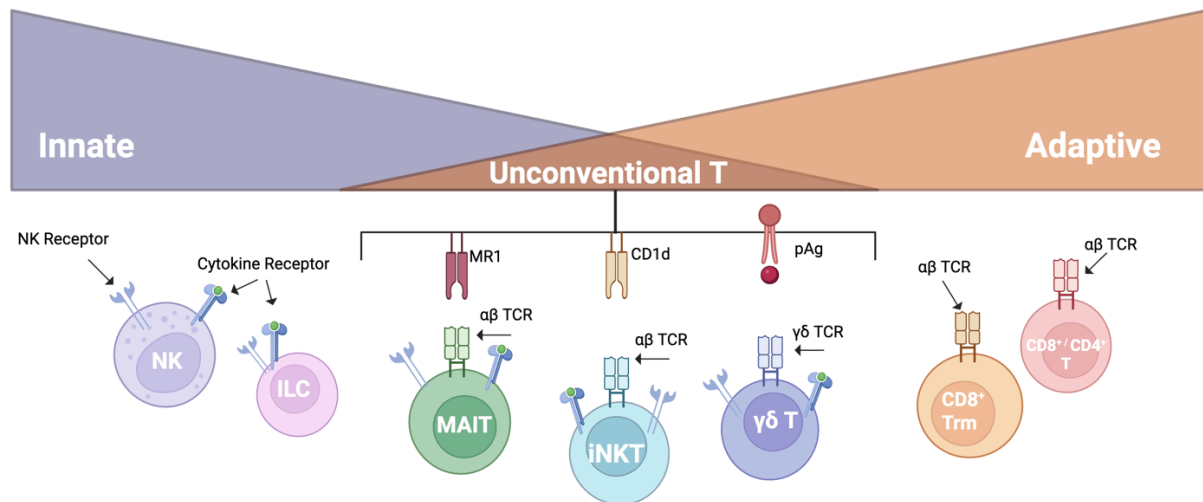


Figure 1.4. Gradient of Innateness. Unconventional T cells, including MAIT cells, iNKT cells, and $\gamma\delta$ T cells, bridge the innate and adaptive immune compartments. These cells are predominately characterised by high surface expression of various cytokine receptors, and a conserved, non-MHC restricted TCR of limited diversity. Adapted from (Riffelmacher and Kronenberg, 2026). Created with Biorender.com

1.2.1. Invariant Natural Killer T Cells

iNKT cells are classified as unconventional T lymphocytes as they present with intermediate expression of a TCR, alongside co-expression of typical NK cell markers, like CD161 and CD56. Their $\alpha\beta$ TCR is composed of a limited repertoire of TCR variable (V) region genes, and in humans, is discernible as a pairing between the invariant V α 24-J α 18 TCR α chain, coupled with a limited V β 11 TCR β chain repertoire (Godfrey et al., 2004). The iNKT cell TCR is restricted by the conserved MHC class I-like molecule, CD1d, which is broadly expressed on many cell types, including dendritic cells, B cells, and epithelial cells (Dougan et al., 2007). CD1d presents non-peptide glycolipid antigens, with the first lipid antigen for iNKT cells identified as the marine sponge-derived glycosphingolipid, α -galactosylceramide (α -GalCer)(Kawano et al., 1997). iNKT cells also recognise a diverse range of bacterial derived glycolipid antigens (Pei et al., 2012); and can mount rapid and robust responses to cytokine stimulation, in particular to IL-12, in the presence of weak TCR signals (Brigl et al., 2011, Wang et al., 2012) . They are potent

effectors upon activation, capable of rapid expansion and the secretion of a diverse range of cytokines, including IFN γ , IL-4, and IL-17 (Crowe et al., 2003, Wilson et al., 2003, Coquet et al., 2008); as well as Fas/FasL-mediated cytotoxicity against CD1d-expressing tumours (Wingender et al., 2010). Accordingly, mouse studies have highlighted iNKT cells as important immunoregulatory cells, with a critical role in defence against infection, cancer, and autoimmunity (Godfrey et al., 2015). Although they comprise a small proportion of the human peripheral blood mononuclear cell (PBMC) compartment, representing approximately 0.1-0.2% of T cells (Gumperz et al., 2002, Montoya et al., 2007), iNKT cells are found to be enriched in the liver, and in human adipose tissue, where they are thought to regulate local inflammation via TCR triggering at the steady state (Lynch et al., 2009, Lynch et al., 2012, Ji et al., 2012, Lynch et al., 2015). In agreement, several studies in mice have too described an enrichment of iNKT cells across peripheral tissues, and it is again hypothesised in this setting that these cells have a key role in maintaining tissue homeostasis and repair (Paiva and Salou, 2025).

1.2.2. $\gamma\delta$ T cells

$\gamma\delta$ T cells are a distinct subset of unconventional T cells which present with a $\gamma\delta$ TCR, and comprise up to 5% of peripheral blood T cells (Parker et al., 1990). In humans, there are three dominant subsets of $\gamma\delta$ T cells, characterised by the expression of specific variable (V) segments in their δ -chains, referred to as V δ 2⁺, V δ 1⁺, and V δ 3⁺ T cells. In adults, V δ 2⁺ T cells, which express a semi-invariant TCR characterised most commonly by V δ 2/V γ 9 chain pairings, are the most abundant subset in the blood; whilst V δ 1⁺ T cells are enriched at peripheral tissues sites, such as in the skin, and the large intestine, and exhibit more variable γ -chain pairings (Godfrey et al., 2015, Provine et al., 2018). A third sub-compartment, V δ 3⁺ T cells, are the least studied of the major human subsets, likely because of their rarity in the peripheral blood; but are also found to be enriched at tissue sites (Rice et al., 2021), and in particular the liver, comprising approximately 20% of the intrahepatic $\gamma\delta$ T cell population (Kenna et al., 2004). Importantly, $\gamma\delta$ T cells can be activated in the absence of antigen processing and presentation on MHC molecules. For example, these cells can directly recognise the intact microbial-derived antigen phycoerythrin, independently of cellular antigen processing and presentation; responding rapidly via the expression of proinflammatory cytokines (Zeng et al., 2012, Vantourout and Hayday, 2013). More

specifically, $V\delta 2^+$ T cells are activated by phosphoantigens (PAgs) derived intracellularly from the host and microbial mevalonate pathways; the most potent microbial-derived antigen being (*E*)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate or HMBPP. Binding of such PAgs to the intracellular domain of the surface protein butyrophilin (BTN) 3A1 stabilises its association with BTN2A1 and induces a conformational change to the extracellular domains, which can be sensed by the $V\delta 2^+$ TCR (Yuan et al., 2023). As such, these cells are implicated in anti-microbial responses, and defence against intracellular pathogens (Morita et al., 2007). $V\delta 1^+$ T cells recognise both MHC-I molecules, and MHC class I-like molecules (Roy et al., 2016, Benveniste et al., 2018, Le Nours et al., 2019); including the stress ligands MICA (Wu et al., 2002) and ULBP (Poggi et al., 2004), suggesting a role for these cells in the elimination of infected or transformed cells. $V\delta 3^+$ cells have also been reported to recognise both CD1d and MR1 molecules in a ligand-independent manner (Mangan et al., 2013, Rice et al., 2021), and a similar functional profile has been proposed for these cells in modulating the clearance of transformed cells.

In contrast with $V\delta 1^+$ T cells, which are more closely related to conventional $\alpha\beta$ T cells in that they exhibit TCR clonal diversity and differentiate from phenotypically naïve to effector cells (Davey et al., 2017); the $CD161^{hi} V\delta 2^+$ T cell compartment are considered more innate-like, expressing a semi-invariant TCR, but also exhibiting robust responses to stimulation with innate cytokine pairings, such as IL-12, IL-18, IL-15, and type I interferons (IFNs) (Provine et al., 2018). This relative ‘innateness’ is likely underpinned by a high-level expression of the innate master transcription factor, promyelocytic leukaemia zinc finger (PLZF); something that is not seen amongst the $V\delta 1^+$ compartment (Provine et al., 2018).

1.3. Mucosal-Associated Invariant T Cells

Although present at low frequencies in mice (Rahimpour et al., 2015), MAIT cells are the most abundant unconventional T cell subset in humans (Godfrey et al., 2015), and are considered important players in the context of human health. MAIT cells are characterised in humans by expression of a semi-invariant TCR, comprising of a conserved α -chain arrangement, $V\alpha 7.2-J\alpha 33/12/20$, paired with a limited repertoire of $V\beta$ chain segments (Porcelli et al., 1993, Tilloy et al., 1999). The MAIT cell TCR is restricted to antigen presentation by the evolutionarily

conserved, non-polymorphic MHC class I-like molecule, MR1 (Treiner et al., 2003); which presents metabolite derivatives of the microbial vitamin B2 (riboflavin) biosynthesis pathway (Kjer-Nielsen et al., 2012, Corbett et al., 2014). Furthermore, MAIT cells are enriched across mucosal and non-mucosal barrier tissues, including in the oral mucosa (Sobkowiak et al., 2019), the lung (Gold et al., 2010), the skin (Constantinides et al., 2019), the female genital mucosa (Gibbs et al., 2017), the gut (Treiner et al., 2003), and the adipose tissue (Carolan et al., 2015). However, these cells are most abundant in the blood and the liver, where they comprise up to 10% of circulating T lymphocytes (Le Bourhis et al., 2010, Gherardin et al., 2018), and up to 45% of the intrahepatic T cell compartment (Dusseaux et al., 2011). Upon activation, MAIT cells are capable of rapid cytotoxic effector responses characterised by the secretion of inflammatory cytokines such as IFN γ , TNF α , and IL-17, and lytic molecules like granzyme B and perforin (Hinks and Zhang, 2020); as well as robust proliferative expansion downstream of TCR triggering (Howson et al., 2018, Parrot et al., 2021, Kedia-Mehta et al., 2023).

1.3.1. MAIT Cell Development

MAIT cells develop in the thymus and, in contrast with conventional T cells, undergo positive selection by DP cortical thymocytes expressing MR1 (Treiner et al., 2003, Martin et al., 2009, Seach et al., 2013); where it is thought that antigens derived from riboflavin-synthesising commensals might play a governing role (Legoux et al., 2019). A process of negative selection has also been proposed, as evident by the depletion of thymic MAIT cell frequencies in germ-free mice upon injection with high doses of the potent MAIT cell TCR agonist, 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) (Legoux et al., 2019). Subsequently, positively selected MAIT cell progenitors are subject to a three-stage thymic maturation programme (Figure 1.5). In stage 1, thymic MAIT cells are majorly DP and are classified as CD161⁺CD27⁻, with a naïve phenotype underpinned by low expression of PLZF, T-bet, and ROR γ T. In stage 2, these cells acquire expression of CD27 and begin to egress from the thymus. However, stage 2 MAIT cells remain phenotypically naïve and unresponsive to stimulation; thus, continue to mature extrathymically. Between stage 2 and stage 3, MAIT cells increase expression of PLZF, which is thought to facilitate their migration into the periphery. Stage 3 MAIT cells dominate in the blood and are classified as CD161⁺CD27^{pos-lo} cells which are predominately CD8 single-positive or DN, presenting with increased

expression of PLZF and T-bet, co-expression of ROR γ T, and functional competence (Koay et al., 2016).

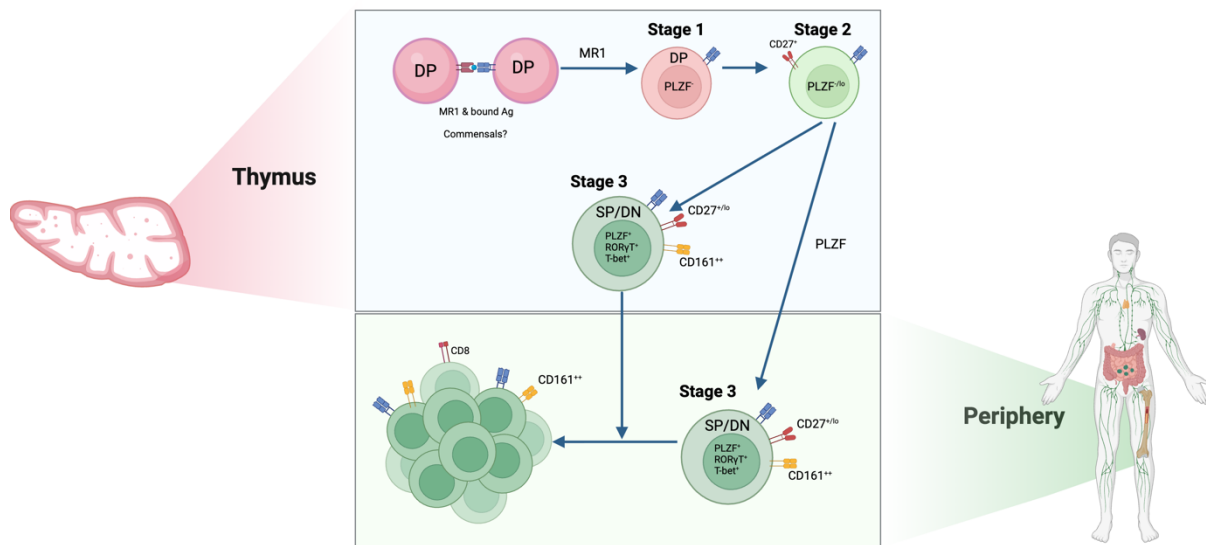


Figure 1.5. MAIT Cell Development. MAIT cells are selected in the thymus by CD4/CD8 double-positive (DP) thymocytes, expressing MR1. Thereafter, these cells are subject to a three-stage developmental programme, egressing from the thymus between stage 2 and stage 3 and continuing to mature in the periphery. Adapted from (Koay et al., 2018). Created with Biorender.com

Circulating MAIT cell frequencies increase in early life (Koay et al., 2016), and continue to increase gradually until approximately 30-40 years of age, when they begin to decline (Novak et al., 2014, Walker et al., 2014). Interestingly however, the prevalence of both the CD39⁺ intestinal and CD56⁺ intrahepatic MAIT cell compartments have been shown to increase with donor age (Kammann et al., 2024).

1.3.2. MAIT Cell Phenotype

Mature human MAIT cells are distinguished by high surface expression of the c-type lectin-like receptor, CD161 (Martin et al., 2009), and the IL-18R (Le Bourhis et al., 2010, Ussher et al., 2014). Circulating MAIT cells are majorly CD8 single-positive (~80%), or DN (~15%), whilst CD4⁺ phenotypes comprise a negligible fraction in healthy individuals (Reantragoon et al., 2013, Gherardin et al., 2018, Dias et al., 2018). As with $\gamma\delta$ T and iNKT cells, the innate-like effector functionality of MAIT cells is underpinned by constitutive expression of the innate master transcription factor PLZF in mature cells (Koay et al., 2016, Mao et al., 2016), alongside co-expression of T-bet, EOMES, Helios, and ROR γ T (Leeansyah et al., 2015); owing towards a

capacity for human MAIT cells to elicit both Tc1 and Tc17-like responses. In this manner, circulating MAIT cells present with a dominant Tc1-like effector profile, whilst mucosal MAIT cells are predominately Tc17-like (Gibbs et al., 2017, Sobkowiak et al., 2019); though such phenotypes are plastic, and dictated by microenvironmental cues. Interestingly, however, the same is not true for murine MAIT cells, which are polarised into distinct subsets in the thymus, and express either T-bet and IFN γ (MAIT1 cells) or ROR γ T and IL-17 (MAIT17 cells) (Koay et al., 2016, Rahimpour et al., 2015, Koay et al., 2019). Circulating human MAIT cells express an effector-memory phenotype, and as such are classified as CD45RO⁺, CD27⁺, CCR7⁻, CD44^{high}, CD62L^{low} (Nel et al., 2021). Further, these cells home to sites of inflammation and infection (Magalhaes et al., 2015, Chen et al., 2017, Salerno-Goncalves et al., 2017, Jouan et al., 2020, Parrot et al., 2020, Sakai et al., 2021); thus express a diverse array of chemokine receptors, including but not limited to CCR9, CCR5, CCR6, CCR2, CXCR6, and CCR4 (Provine and Klenerman, 2020, Wu et al., 2025). However, beyond the context of infection or inflammation, MAIT cells do preferentially home to tissue sites at the steady state, and in mice, have been shown to acquire a tissue-targeting phenotype during thymic development; increasing the expression of chemokine receptors such as CCR8, CCR6, and CXCR6 (Salou et al., 2019). Such residency behaviour is thought to be underpinned by PLZF, which has been shown to modulate the surface expression of adhesion molecules, such as CD62L and lymphocyte function-associated antigen 1 (LFA-1) (Thomas et al., 2011, Mao et al., 2016, Salou et al., 2025).

Tissue resident MAIT cells are characterised by high surface expression of the tissue-egress inhibitory molecule CD69, and the adhesion molecule, CD103 (Nel et al., 2021). However, MAIT cells do exhibit phenotypic heterogeneity across matched tissue sites, such that expression of the adhesion receptor CD103 is increased across intestinal MAIT cell populations, but not those residing in the liver, for example (Kammann et al., 2024). Both circulating and tissue resident MAIT cells express the adhesion molecule CD56 to heterogeneous levels, though CD56 expression is highest on intrahepatic MAIT cells; something that is thought to be attributed to the high presence of IL-7 within the liver microenvironment, and confers an increased effector capacity (Kammann et al., 2024). Thus, although MAIT cells are generally considered a relatively homogenous population, their

phenotype, and concomitantly their functional profile, is shaped by local microenvironments; something that is discussed at breadth in **Paper I** of this thesis.

1.3.3. MAIT Cell Activation

Characteristic of all T lymphocytes, MAIT cells become activated through TCR engagement and require secondary co-stimulation by surface molecules such as CD28 (Turtle et al., 2011). However, in line with their innate status, these cells can also elicit functional responses to stimulation with innate cytokines, such as IL-18 and IL-12, independently of their TCR (Provine and Klenerman, 2020, Sandberg et al., 2023). Nonetheless, a complete response profile is observed in the context of synergistic activation via TCR engagement and co-stimulation with surface molecules and cytokines (Slichter et al., 2016, Leng et al., 2019, Garner et al., 2023). An in-depth analysis of the environmental signals which drive MAIT cell activation and functional responses in the context of both host-defence and immunopathology is presented in **Paper I**.

1.3.3.1. TCR-Dependent: TCR Engagement and Co-Stimulation

The pyrimidine 5-OP-RU is the most potent MAIT cell-activating antigen identified to date, formed when a precursor derived from the microbial riboflavin biosynthesis pathway, 5-amino-6-D-ribitylaminouracil (5-A-RU), is involved in a non-enzymatic condensation reaction with methylglyoxal (Corbett et al., 2014). Similarly, 5-A-RU may condense with glyoxal to generate 5-OE-RU, which also potently activates MAIT cells (Corbett et al., 2014). Various bacterial and fungal microbes are equipped with the riboflavin synthesis pathway (Kjer-Nielsen et al., 2018), with expression of the *RibD* gene necessary for the generation of 5-A-RU (Hartmann et al., 2018), and thus elicit the activation of MAIT cells in an MR1-dependent manner. The importance of the riboflavin pathway in mediating MAIT cell responses to microbial challenge is highlighted by findings which elucidate a correlation between the presence of riboflavin synthesis-related genes and riboflavin production with MR1-mediated responsiveness of MAIT cells to *Streptococcus pneumoniae* (Godfrey et al., 2019). Similarly, microbes such as *Enterococcus faecalis* and *Listeria monocytogenes* lack expression of the riboflavin pathway, and consequently, do not activate MAIT cells (Kjer-Nielsen et al., 2018). Importantly, MR1 is widely expressed by cells of both haematopoietic and non-haematopoietic origin, including DCs, macrophages, B cells, and epithelial cells (Hinks and

Zhang, 2020); meaning there is considerable scope for MAIT cell activation. However, MR1 is not surface expressed in the absence of an appropriate ligand (McWilliam et al., 2016, McWilliam et al., 2020). Recently, host-derived riboflavin catabolites have been shown to bind ER resident MR1 and downregulate its cell surface expression, whilst also competing with MAIT cell agonists such as 5-OP-RU in an antagonistic manner (Abdelaal et al., 2026); with the authors hypothesising that such catabolites might represent an inherent mechanism of protection against the aberrant activation of MAIT cells. Although humans are unable to synthesise riboflavin, the selection of MAIT cells in the thymus is dependent on MR1 and occurs in germ-free mice (Koay et al., 2016), which has suggested the presence of alternative host-derived antigen(s) of MR1. Supporting this, a sulfated bile acid, cholic acid 7-sulfate (CA7S), has recently been found to bind MR1 and elicits a homeostatic signature in human MAIT cells, whilst thymic MAIT cell frequencies are reduced in mice lacking the CA7S synthesis pathway (Ito et al., 2024); highlighting the potential significance for non-riboflavin derived ligands of MR1 in supporting MAIT cell fitness and immune function.

1.3.3.2. TCR-Independent: Cytokine Stimulation

Mature human MAIT cells exhibit constitutively high cell surface expression of the IL-18R (Dusseaux et al., 2011, Ussher et al., 2014) alongside expression of a plethora of other cytokine receptors, including IL-12R (Wallington et al., 2018, Meermeier et al., 2022), IL-15R (Sattler et al., 2015, Tao et al., 2021), IL-7R (Tang et al., 2013), and the interferon- α/β receptor (IFNAR) (Lamichhane et al., 2020). Consequently, MAIT cells are licensed to elicit TCR-independent, partial effector responses to cytokine stimulation, and specifically, to cytokine pairings. Thus, MAIT cells are not limited to their role in immune defence against riboflavin-synthesizing pathogens. Importantly, the activation of human MAIT cells during viral infections is thought to be mediated in a TCR-independent manner through the recognition of innate cytokines derived from APCs, as viruses do not synthesize riboflavin and to date, there are no known viral-derived antigens of MR1 (Van Wilgenburg et al., 2016).

The inflammatory cytokines IL-18 and IL-12 were first highlighted to induce significant IFN γ production from MAIT cells in a TCR-independent, synergistic, manner (Ussher et al., 2014, Jo et al., 2014). In accordance, subsequent studies also highlighted the induction of T-bet (Lamichhane et al., 2019), and the expression of cytotoxic machinery, such as perforin, and

the granzymes B, H, K, A, and M (Lamichhane et al., 2019). Peak activation elicited *in vitro* by IL-12/IL-18 occurs at approximately 24hr, contrasting TCR-driven effector responses which were found to peak at 6hr post-activation (Lamichhane et al., 2019); and thus, highlighting the potential for differential response kinetics. Stimulation of MAIT cells with the type I IFNs, IFN α or IFN β , independent of TCR triggering, also elicits functional responses *in vitro*, namely driving increased expression of IFN γ ; but only when in synergy with either IL-18 or IL-12 (Van Wilgenburg et al., 2016, Provine et al., 2021).

Specifically, the synergistic activity of IL-18 with IL-12, IL-15, and/or IFN α/β has been shown to mediate MAIT cell responses to APCs infected with dengue, hepatitis C, and influenza virus *in vitro* (Van Wilgenburg et al., 2016). This is recapitulated *in vivo* where MAIT cells have been shown to be responsive in the context of several human viral infections, including but not limited to HIV-1, hepatitis, SARS-CoV-2, measles, and dengue; to varying degrees and disease outcomes (Sandberg et al., 2023). Importantly, MAIT cells are protective against lethal influenza A virus (IAV) infection *in vivo*, and in this setting, MAIT cell accumulation in the lung was attenuated by IL-18 deficiency, whilst IL-12, IL-18, IL-15 and IFN α/β also had a role in facilitating MAIT cell activation (Van Wilgenburg et al., 2018).

Beyond the scope of viral infection, MAIT cell responses to certain bacterial pathogens can also occur independently of cognate-antigen recognition. Although the bacterium *Klebsiella pneumoniae* (*K. pneumoniae*) is capable of synthesizing riboflavin (Kjer-Nielsen et al., 2018), it has recently been shown that MAIT cell activation and effector responses to *Klebsiella* are mediated by type I IFNs, independent of the MR1-TCR axis (López-Rodríguez et al., 2023).

1.3.3.3. Transcriptional and Functional Diversity

Human MAIT cells are a single cell population displaying functional plasticity and high adaptability to environmental cues (Garner et al., 2023). Thus, TCR- versus cytokine- activated MAIT cells present with distinct response profiles, underpinned by signal-specific upregulation of differential transcriptional programmes. TCR triggering lends to the specific upregulation of transcription factors involved in Th17 cell differentiation and IL-17 production, including ROR γ T, and Signal Transducer and Activator of Transcription (STAT) 5A (Lamichhane et al., 2019, Garner et al., 2023). Stimulation with cytokine alone drives unique

early upregulation of STAT1, and this is predicted to regulate subsequent activation of *TBX21* (T-bet) (Garner et al., 2023). Both TCR triggering and cytokine stimulation can independently upregulate expression of T-bet, although cytokine stimulation potentiates a more robust IFN γ response (Lamichhane et al., 2019, Garner et al., 2023). However, TCR engagement elicits a more polyfunctional response profile, with increased expression of additional proinflammatory cytokines and chemokines, such as IL-1A, IL-1B, IL-22, GM-CSF, CCL3, and CCL20 (Lamichhane et al., 2019). Transcriptomic analyses have also elucidated a tissue repair signature in human MAIT cells, with TCR-driven expression of genes such as *FURIN*, *VEGF*, and *TGFB1*; a signature that is not induced downstream of cytokine stimulation alone (Hinks et al., 2019, Lamichhane et al., 2019, Leng et al., 2019). It is thus hypothesised that MAIT cells have a role in maintaining tissue integrity and promoting homeostasis at barrier sites, through interactions with commensal-derived ligands of MR1 in the absence of inflammatory co-stimulation, which would otherwise polarize the downstream response towards the inflammatory, anti-microbial profile, observed during infection (Klenerman et al., 2021).

1.4. Metabolism and T cells

T cells are highly adaptable to the surrounding environment. Upon challenge, these cells switch from a state of quiescence to undergo clonal expansion and differentiation into effector subsets; something that involves rapid changes to gene expression, and proteome remodelling to facilitate downstream functional responses (Ron-Harel et al., 2016, Araki et al., 2017, Howden et al., 2019). Cellular metabolism is a central regulator of such processes, and thus nutrient availability comprises a governing signal in the potentiation of effector T cell responses (Raynor and Chi, 2024). Glucose, amino acids, and lipids, as well as the deriving metabolic intermediates and environmental metabolites, each have a role in orchestrating the metabolic programming that underpins T cell homeostasis, and their effector responses (Raynor and Chi, 2024). The importance of such is emphasised in that these cells possess an inherent mechanism to recognise microenvironmental changes to nutrient availability, and to respond accordingly. This is achieved by expression of two key molecules, the mammalian target of rapamycin (mTOR), regarded as a central regulator of immunity (Liu et al., 2015); and AMP-activated protein kinase (AMPK). mTOR is a conserved serine/threonine kinase and the catalytic subunit of two structurally distinct protein complexes, mTORC1 and mTORC2

(Loewith et al., 2002). These complexes act to sense environmental cues such as nutrients, amino acids, and growth factors, and modulate cellular translation, growth, metabolism, and differentiation, accordingly (Weichhart et al., 2015). When intracellular adenosine triphosphate (ATP) levels and nutrient availability become depleted, AMPK, an important negative regulator of mTOR activity (Gwinn et al., 2008, Inoki et al., 2003), is activated to switch off anabolic processes such as protein translation, and to instead potentiate the generation of ATP from catabolic processes, such as fatty acid oxidation (Hardie and Pan, 2002). Thus, subject to the environmental cues present, as well as their specific activation state, cells will heavily favour different metabolic pathways to generate enough ATP to survive, and to promote the most appropriate response profile at that time. The most significant metabolic pathways described to date in the context of T cell homeostasis and effector functioning include glycolysis, the tricarboxylic acid (TCA) cycle, oxidative phosphorylation (OxPhos), the pentose phosphate pathway (PPP), and amino acid metabolism. Furthermore, an importance for the metabolism of the micronutrient iron has more recently been elucidated in the context of T cell metabolism and fitness (Teh et al., 2021, Frost et al., 2021, Kumar et al., 2024, Teh et al., 2025). A general graphical summary of T cell metabolism is presented in Figure 1.6.

1.4.1. Glycolysis

Glycolysis typically describes the uptake of extracellular glucose and its metabolism to pyruvate via sequential enzymatic reactions in the cytosol. It is a rapid, yet relatively inefficient programme in the context of ATP production, generating only two molecules of ATP per one molecule of glucose (O'Neill et al., 2016). However, glycolytic metabolism is used to support cellular biogenesis by providing important intermediates, such as glucose-6-phosphate and 3-phosphoglycerate, which can be further metabolised to support *de novo* synthesis of nucleic acids and amino acids, for example (Dimeloe et al., 2017). Furthermore, glycolysis drives the reduction of nicotinamide adenine dinucleotide (NAD)⁺ to NADH, and NADH can be recycled back to NAD⁺ to sustain the glycolytic flux, when pyruvate, the end product of glycolysis, is reduced into lactate. Termed aerobic glycolysis, this process is favoured by rapidly proliferating cells to support robust levels of biosynthesis (O'Neill et al., 2016). Glycolysis-derived pyruvate can also be oxidised to CO₂ within the TCA cycle to facilitate the subsequent generation of high levels of ATP via OxPhos; however, increasing

mitochondrial ATP production also inhibits the glycolytic flux, which is in part driven by ATP consumption (Lunt and Vander Heiden, 2011). Naive T cells rely on glucose uptake to fuel OxPhos and prevent cellular atrophy (Wofford et al., 2008).

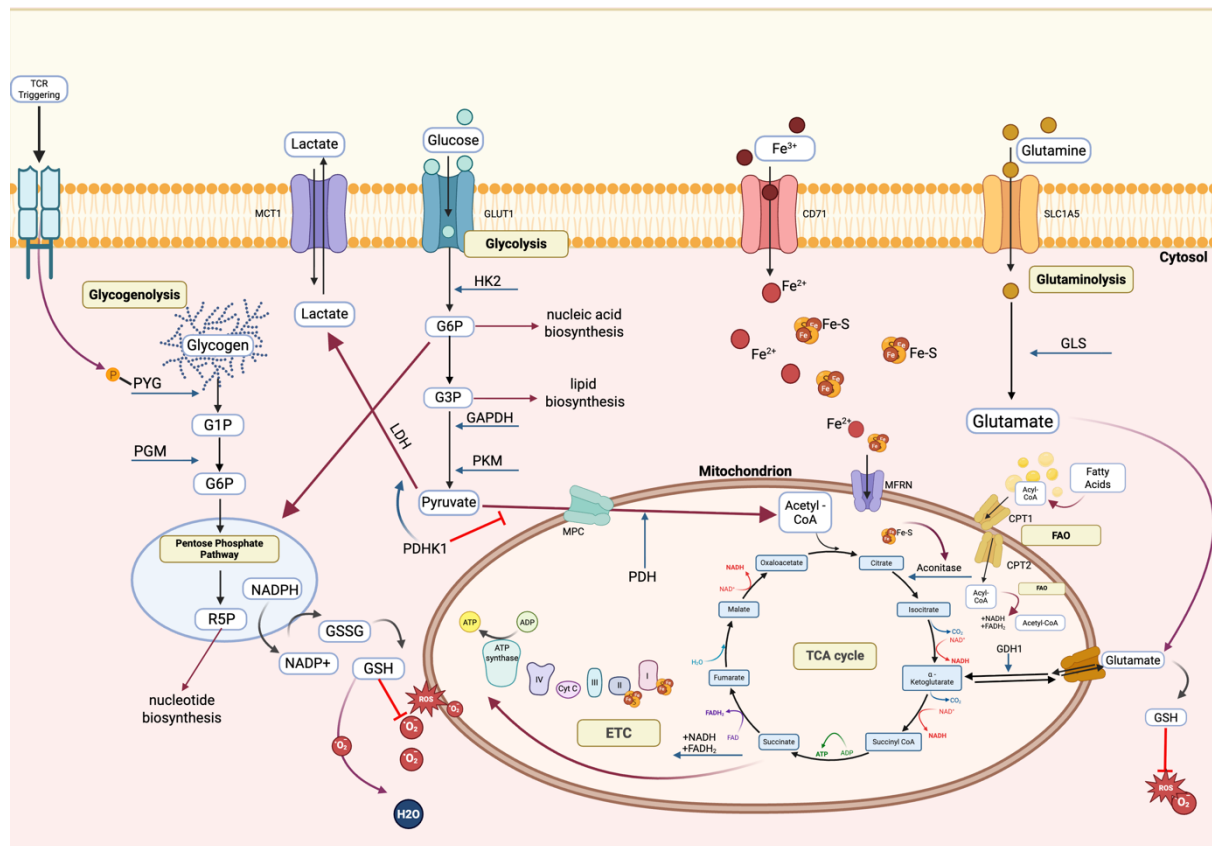


Figure 1.6. T Cell Metabolism – General Overview. T cells rely on several metabolic processes to support cell survival and to potentiate effector response to challenge, including glycolysis, glycogenolysis, TCA cycling, oxidative phosphorylation, and the pentose phosphate pathway. The cellular uptake, breakdown, and generation of nutrient fuels such as glucose, glycogen, iron, amino acids, and fatty acids, sustains the metabolic flux. Created with Biorender.com.

Stimulation of T cells results primarily in the upregulation of aerobic glycolysis through activation of pyruvate dehydrogenase kinase 1 (PDHK1), which inhibits the mitochondrial import of pyruvate, thus diverting pyruvate towards the production of lactate (Menk et al., 2018). Concomitantly, T cell activation coincides with increased cell surface expression of the glucose transporter 1 (Glut1), and glucose uptake; something that requires CD28 signalling to license the phosphorylation of Akt by phosphatidylinositol-3-kinase (PI3K) (Frauwirth et al., 2002, Jacobs et al., 2008). Activation of Akt allows for direct phosphorylation of the inhibitor of mTOR, tuberous sclerosis complex 2 (TSC2) (Inoki et al., 2002, Manning et al., 2002), resulting in the potentiation of mTOR activity. mTOR further enhances glycolytic activity by

modulating the expression of glucose transporters and glycolytic enzymes (Hukelmann et al., 2016, Zeng et al., 2016). The transcription factor Myc is also rapidly induced upon TCR stimulation in conventional T cells (Wang et al., 2011, Preston et al., 2015), modulating the expression of numerous glycolytic enzymes and transporters, including Glut1, hexokinase 2 (HK2), and pyruvate kinase muscle isoform 2 (PKM2) (Wang et al., 2011). Myc also regulates the expression of lactate transporters in conventional T cells, which comprises a critical rate limiting step of the glycolytic flux (Marchingo et al., 2020). Thus, Myc is considered a master regulator of metabolism in conventional T cells and plays a critical role in modulating the switch to glycolysis upon activation.

Human CD4⁺ Tem cells rely on the provision of pyruvate derived from glycolysis towards mitochondrial oxidation for survival (Dimeloe et al., 2016). Beyond cell survival, T cell effector responses are also underpinned by glycolysis. IFN γ production from CD8⁺ Tem cells is dependent on their capacity to rapidly upregulate aerobic glycolysis upon activation (Gubser et al., 2013). Similarly, aerobic glycolysis drives effector responses in naïve CD4⁺ and CD8⁺ T cells upon activation. IFN γ secretion from effector T cells is critically impaired in the absence of glucose, something that has been attributed to GAPDH binding the 3'UTR of IFN γ mRNA when disengaged from glycolysis, thus preventing its translation (Chang et al., 2013). Glycolysis is also important for the differentiation of CD4⁺ effector T cell subsets, and in particular, promotes the generation of both Th1 and Th17 cell subsets (Raynor and Chi, 2024).

1.4.1.1. Glycogenolysis

Recent findings have elucidated a role for glycogenolysis in promoting early recall responses in CD8⁺ Tem cells. Glycogenesis is the metabolic process by which glycogen stores are synthesised from glucose, mediated in part by the enzymatic activity of dephosphorylated glycogen synthase (GYS) (Jiang and Zhang, 2003). Primarily occurring in the skeletal muscle and the liver, this process ensures a carbohydrate reserve that can provide an immediate energy source when metabolic demands are increased; for example, during periods of intense exercise to fuel muscle contraction, or to increase blood glucose concentrations during fasting (Zhang et al., 2021). The degradation of glycogen is referred to as glycogenolysis, whereby endogenous glycogen stores are broken down to yield glucose-6-phosphate (G6P) in the cytosol, through the actions of two key enzymes: glycogen phosphorylase (PYG), and the

glycogen debranching enzyme (AGL) (Adeva-Andany et al., 2016). Interestingly, CD8⁺ Tem cells have recently been shown to store glycogen and initiate glycogenolysis upon activation. Although required as a substrate for HK2 to generate G6P in the first step of glycolysis, glucose uptake does not occur in CD8⁺ Tem cells until 12hr post-activation. Importantly, robust cytokine responses are established 4hr post-activation, whilst the depletion of intracellular glycogen stores is evident at the 1hr timepoint (Zhang et al., 2021). Zhang and colleagues found that the rapid mobilization of glycogen stores is mediated by TCR-dependent phosphorylation of brain isoform of PYG (PYGB) in activated CD8⁺ Tem cells, eliciting a flux of G6P into the glycolytic pathway in the absence of exogenous glucose metabolism to fuel early effector responses (Zhang et al., 2021). Endogenous glycogen therefore comprises a carbon source for rapid glycolysis in activated Tm cells.

1.4.2. The TCA Cycle

The TCA cycle, coupled with OxPhos, constitutes a major source of ATP for most quiescent, or non-proliferating cells. Naïve T cells predominately oxidise substrates derived from glucose, fatty acids, and amino acids, in the mitochondria to fuel ATP production via OxPhos (Wang et al., 2011, van der Windt and Pearce, 2012). The TCA cycle is a central metabolic pathway, linking with numerous others. Notably, pyruvate derived from glycolysis is transported into the mitochondria via the mitochondrial pyruvate carrier, and is converted to acetyl coenzyme A (acetyl-CoA) by pyruvate dehydrogenase (PDH) to initiate the first step of the TCA cycle (Lunt and Vander Heiden, 2011). Similarly, long chain fatty acids are oxidised in the cytosol to generate acyl-CoA, which is transported to the inner mitochondria by the actions of the carnitine palmitoyl transferase (CPT)1 and CPT2. In the mitochondrial matrix, acyl-CoA undergoes β -oxidation, also termed fatty acid oxidation (FAO), to generate NADH and flavin adenine dinucleotide (FAD)H₂, used to support the electron transport chain (ETC), and acetyl-CoA, which is destined for the TCA cycle (Longo et al., 2016, Adeva-Andany et al., 2019). FAO is thought to promote both the development of CD8⁺ Tm cells, and their enhanced spare respiratory capacity (SRC) compared to effector T cells (Pearce et al., 2009, Windt et al., 2012, O'Sullivan et al., 2014). Furthermore, CD4⁺ Treg cells utilise mitochondrial FAO to fuel their differentiation, and suppressive functions *in vitro* (Michalek et al., 2011).

Acetyl-CoA is subject to a condensation reaction with oxaloacetate to form citrate, initiating the commitment stage of the TCA cycle. The oxidation of citrate subsequently lends to the initiation of a series of redox reactions, eventually yielding two major products of the TCA cycle, the electron carriers NADH and FADH₂; both of which transfer electrons to the ETC in the generation of high levels of ATP during OxPhos (O'Neill et al., 2016, Dimeloe et al., 2017). Alongside the production of NADH and FADH₂, the TCA cycle generates critical biosynthetic intermediates, including α -ketoglutarate, oxaloacetate, and citrate, which can be used for anabolic processes such as amino acid and lipid biosynthesis (Dimeloe et al., 2017). The metabolism of glutamine is an important component of normal TCA cycle functioning, as it is used to replenish TCA cycle intermediates like α -ketoglutarate in a process termed anaplerosis. The breakdown of glutamine, or glutaminolysis, begins with the conversion of glutamine to glutamate in the cytosol by the enzyme glutaminase (GLS); which is then transferred to the mitochondria by the mitochondrial glutamate transporter. Here, glutamate is converted to α -ketoglutarate via glutamate dehydrogenase, or through the activity of transaminases (Yang et al., 2017). T cell activation coincides with the rapid uptake of glutamine via cell surface expression of the amino acid transporter, SLC1A5 (Nakaya et al., 2014, Sinclair et al., 2013); and glutamine consumption through oxidative metabolism is concomitantly increased in activated T cells (Wang et al., 2011, Johnson et al., 2018).

Although activated T cells present with a metabolic phenotype dominated by aerobic glycolysis, T cell activation also coincides with the provision of some pyruvate towards the mitochondria to replenish TCA cycle intermediates, which supports cellular activation, proliferation, and the production of IL-2 (Dimeloe et al., 2017). The provision of glutamine towards the TCA cycle also supports the proliferation of effector T cells. Ma and colleagues found that the early proliferation of CD8⁺ effector T cells during *in vivo* infection with *Listeria monocytogenes* is underpinned by the provision of glutamine carbon into the TCA cycle which lends to the production of aspartate, driving *de novo* pyrimidine nucleotide biosynthesis (Ma et al., 2024).

1.4.2.1. Oxidative Phosphorylation

The TCA cycle is tightly coupled to the ETC, yielding high levels of ATP. The ETC consists of five distinct complexes (I, II, III, IV, and V), and two electron carriers (coenzyme Q (CoQ), and

cytochrome C (CytC)) located within the inner mitochondrial membrane. TCA-derived NADH and FADH₂ are oxidised within the ETC to NAD⁺ and FAD⁺, by complex I and complex II, respectively. This series of oxidation reactions is referred to as OxPhos. Free electrons derived from the oxidation of NADH and FADH₂ are transferred via CoQ to complex III, and CytC to complex IV. Once transferred onto complex IV, these electrons are accepted by molecular oxygen, initiating its reduction into water. The redox energy generated through electron transfer facilitates the pumping of protons from complexes I, III, and IV in the mitochondrial matrix, to the intermembrane space, creating an electrochemical proton gradient that results in the generation of ATP, from adenosine diphosphate (ADP) and phosphate, by ATP synthase (complex V) (Dimeloe et al., 2017, Yin and O'Neill, 2021).

Alongside the production of ATP, OxPhos lends to the generation of reactive oxygen species (ROS) at complexes I, II, and III. Mitochondrial ROS (mROS) facilitate T cell activation via induction of the nuclear factor of activated T cells (NFAT) (Sena et al., 2013). Furthermore, the establishment and maintenance of naïve and memory CD8⁺ T cells is dependent on complex III-derived mROS (Steinert et al., 2025). However, high levels of mROS also lend to increasing cellular oxidative stress, ultimately resulting in damage to DNA, proteins, and lipids (Ray et al., 2012). Mitochondrial ETC functioning is also required to support robust T cell proliferation (Sena et al., 2013, Tarasenko et al., 2017, Chen et al., 2022). Specifically, complex III supports antigen-induced proliferation in CD8⁺ T cells (Steinert et al., 2025).

1.4.3. The Pentose Phosphate Pathway

The PPP is a fundamental metabolic pathway occurring in the cytosol which produces the end products ribose-5-phosphate (R5P), an important precursor for nucleotide and amino acid biosynthesis, and nicotinamide adenine dinucleotide phosphate (NADPH). NADPH protects against oxidative stress, thus maintaining the cellular redox balance, and also supports fatty acid and cholesterol biosynthesis (TeSlaa et al., 2023). The PPP is divided into two key compartments, the oxidative compartment, and the non-oxidative compartment. The oxidative PPP describes the initial phase, and involves the conversion of G6P, shunted from early glycolysis, into R5P, yielding two molecules of NADPH. The non-oxidative phase subsequently involves the reversible interconversion of molecules such as ribulose-5-phosphate, and R5P, into molecules such as fructose-6-phosphate, and glyceraldehyde-3-

phosphate; thus, feeding back into glycolysis. The glycolytic intermediates produced in the non-oxidative phase can also be used to generate G6P to support further NADPH production via the oxidative PPP, something termed pentose cycling (TeSlaa et al., 2023). Activated T cells increase glucose flux through the PPP (Wang et al., 2011), and flux through the oxidative branch has been shown to promote memory formation (Zhang et al., 2020) and anti-tumour responses (Chen et al., 2025) in CD8⁺ T cells, via the generation of NADPH. Interestingly, alongside promoting glycolysis, glycogenolysis-derived G6P is also shunted into the PPP in activated CD8⁺ Tm cells to generate NADPH for redox homeostasis (Ma et al., 2018, Zhang et al., 2022); with glycogenolysis, not glucose, facilitating activation of the PPP flux (Zhou et al., 2025).

1.4.4. Amino Acid Metabolism

Alongside the uptake of glutamine to support the TCA cycle flux, T cell activation also coincides with significant proteome remodelling and increases in cellular protein biosynthesis. Thus, activated T lymphocytes must increase their uptake of extracellular amino acids, the building blocks of protein synthesis, in support of this. There are nine essential amino acids, including leucine and methionine, obtained by diet alone and imported into the cell via the expression of amino acid transporters (Sinclair and Cantrell, 2025). Semi-essential amino acids, including glutamine and arginine, are synthesised endogenously; although protein synthesis increases intracellular demand, and as such, enhanced acquisition from the microenvironment is required upon activation (Sinclair and Cantrell, 2025). Of note, an adequate supply of intracellular leucine is critically important for the activity of mTORC1 (Schriever et al., 2013); and this is mediated by upregulated cell surface expression of the large neutral amino acid transporter, SLC7A5, in activated T cells (Sinclair et al., 2013, Hayashi et al., 2013). Accordingly, the loss of SLC7A5 or leucine deprivation impairs metabolic reprogramming, differentiation, proliferation, and cytokine production in activated T cells (Zheng et al., 2009, Sinclair et al., 2013, Hayashi et al., 2013). Similarly, glutamine uptake via SLC1A5 also underpins mTORC1 signalling in activated T cells (Nakaya et al., 2014, Sinclair et al., 2013), and is required to support effector T cell responses, in particular fuelling the production of IFN γ and cellular proliferation (Carr et al., 2010, Nakaya et al., 2014, Johnson et al., 2018, Bopp et al., 2024). Interestingly, Th17 cells exhibit higher rates of glutaminolysis upon activation, and are reliant on this to fuel their differentiation (Johnson et al., 2018). The

metabolism of amino acids therefore comprises a fundamental component of anabolic cellular processing in activated T lymphocytes.

1.4.5. Iron

In the context of T cell metabolism, much emphasis has been placed on elucidating a role for macronutrients such as glucose, amino acids, and lipids, in facilitating T cell homeostasis and host-protective responses. However, robust immune functioning is also underpinned by the availability of micronutrients (Gombart et al., 2020), and recent findings have elucidated a critical role for the micronutrient iron in governing T cell metabolism and fitness.

Cellular iron is generally found in two oxidation states, and accordingly, is referred to as ferric (Fe^{3+}) or ferrous (Fe^{2+}). Switching between these two states ensures that iron can act both as an electron donor, and an electron acceptor; and as such, it is an important co-factor for numerous enzymes involved in redox reactions (Coffey and Ganz, 2017). Thus, iron has an intricate role in several biological processes, including cellular metabolism, notably within the TCA cycle and the ETC (Ben Zichri- David et al., 2025). It is estimated that 6.5% of all human enzymes are iron dependent, and 2% of human genes encode an iron-binding protein. The majority bind iron through heme, or alternatively, bind individual iron ions. Approximately 17% are iron-sulphur (Fe-S) cluster proteins. The mitochondrion, alongside the endoplasmic reticulum, is the most iron-enriched cellular compartment; and in particular contains the most Fe-S proteins (Andreini et al., 2018). Within the ETC, complex I and complex II are highly enriched in Fe-S clusters, and critically depend on binding Fe-S clusters to support their function (Ben Zichri- David et al., 2025). Similarly, functioning of the TCA cycle is dependent on the availability of Fe-S clusters. Notably, the enzyme aconitase (ACO2), which catalyses the isomerization of citrate to isocitrate in the second step of the TCA cycle, is critically dependent on the acquisition of [4Fe-4S] clusters within its substrate binding site to enable its catalytic activity (Ben Zichri- David et al., 2025).

Upon activation, the demand for iron in T cells increases dramatically; with the total iron content of CD8^+ T cells estimated to triple 24hr post-stimulation (Teh et al., 2021). Accordingly, T cells substantially increase cell surface expression of the transferrin receptor, TFRC (CD71) upon activation (Howden et al., 2019, Frost et al., 2021). The binding of

transferrin-bound ferric iron with TFR1 initiates receptor-mediated endocytosis of the complex, and it comprises the major mechanism by which cells acquire iron (Dautry-Varsat, 1986, Frost and Drakesmith, 2025). Teh and colleagues have shown that the majority (60%) of iron atoms in T cells are implicated in OxPhos, and upon activation, the absolute number of iron atoms within the OxPhos pathway is increased (Teh et al., 2021). As such, iron-starvation severely impedes mitochondrial function in T cells, with suppressed TCA cycling owing to reduced activity of ACO2, and succinate dehydrogenase (Teh et al., 2025). Downstream loss of the proton-dependent mitochondrial aspartate carrier, SLC25A12/13, lends to the accumulation of aspartate within the mitochondria and results in significant impairments to proliferation in activated T lymphocytes (Teh et al., 2025). As well as impairing the proliferative capacity of T cells, low serum iron availability (hypoferremia) inhibits T cell effector cytokine production and memory responses to immunization and influenza virus infection *in vivo* (Frost et al., 2021). Thus, the metabolism of iron is of significant importance in the context of T cell fitness, further highlighting nutrient availability as a governing determinant of T cell-mediated immunity.

1.5. MAIT Cell Metabolism

Although T cell metabolism has been extensively described, MAIT cell metabolism remains a novel research focus, and elucidation into the cellular metabolic pathways which govern MAIT cell homeostasis and host-protective functioning remains an emerging field of research. As established, to maintain homeostasis, cells require a consistent supply of exogenous nutrients through modulation of the expression of surface nutrient transporters, which can directly influence downstream metabolic and functional responses (Sinclair and Cantrell, 2025, Raynor and Chi, 2024). **Paper I** of this thesis comprehensively describes our current understanding of MAIT cell metabolism in health and disease, and the importance of nutrient availability as critical 'signal 5' in the context of potentiating MAIT cell fitness. See figure 1.7 for a graphical description of our understanding of MAIT cell metabolism prior to the undertaking of this thesis.

Garnering a complete understanding of the metabolic tendencies of MAIT cells is of importance, as it is likely that in environments where local or systemic metabolism is altered,

such as in the tumour microenvironment, or in obesity, MAIT cell responses may become dysregulated (Kedia-Mehta and Hogan, 2023). For example, hypoferrremia is a common characteristic of obesity, and is induced upon infection (Nemeth et al., 2004, Yanoff et al., 2007), however whether this is linked to the perturbation of MAIT cell responses in such contexts, remains unknown. Elucidation into the iron-requirements of human MAIT cells in the context of TCR-driven effector responses was previously undescribed and comprises the focus of **Paper II** of this thesis. Similarly, the metabolic underpinnings of TCR-independent MAIT cell effector responses have yet to be characterised and thus, this comprises the focus of **Paper III**. Finally, in **Paper IV**, we sought to elucidate the importance of glutaminolysis in supporting MAIT cell fitness and effector responses to TCR challenge, with the role of amino acid metabolism in MAIT cells having remained largely undescribed.

Comprehensive elucidation of the metabolic requirements of MAIT cells could facilitate identification of a metabolic target(s) which could be manipulated to restore their protective functions in certain diseased contexts.

1.5.1. Glycolysis in MAIT Cells

Similar to conventional T cells, the MAIT cell metabolic programme is also underpinned by activity of the nutrient sensor, mTORC, the nutrient-sensitive transcription factor, Myc, and their downstream glycolytic targets (O'Brien et al., 2019, Kedia-Mehta et al., 2023). Upon activation, MAIT cells significantly increase cell surface expression of Glut1, alongside increasing exogenous glucose uptake (Zinser et al., 2018, O'Brien et al., 2019). Expression of both HK2 and lactate dehydrogenase A (LDHA), the enzyme which catalyses the conversion of pyruvate to lactate, are too dramatically increased upon activation (Kedia-Mehta et al., 2023); indicative of a dependency on the exogenous glucose metabolism. Indeed, MAIT cell activation coincides with robust increases in cellular glycolysis, and such metabolic reprogramming is indispensable for the production of IFN γ and Granzyme B (Zinser et al., 2018, O'Brien et al., 2019). Furthermore, MAIT cells undergo robust proliferative expansion downstream of TCR engagement and cytokine co-stimulation, and this proliferative capacity is significantly attenuated upon inhibition of glucose metabolism (Kedia-Mehta et al., 2023). Inhibition of either mTORC1 or Myc induces a loss of glycolysis, and concomitant impairment

to effector functioning in MAIT cells; further highlighting the importance of adequate nutrient sensing for MAIT cell fitness (O'Brien et al., 2019, Kedia-Mehta et al., 2023).

1.5.1.2 Glycogenolysis in MAIT Cells

Recent findings have revealed that MAIT cells are capable of rapid cytotoxic responses *in vitro*. In response to MR1-expressing tumour cell lines pulsed with 5-A-RU-MG, MAIT cells robustly increase cell surface expression of the degranulation marker, CD107A, at the early (2hr) timepoint. Indeed, MAIT cells have the capacity to induce significant target cell death within as little as 2hr; whilst 1.5hr TCR stimulation elicits significantly increased expression of IFN γ (Cassidy et al., 2023). Interestingly, Cassidy *et al.*, revealed that the metabolism of exogenous glucose was dispensable for such rapid cytotoxic responses, as inhibition of HK2 using 2DG did not significantly attenuate expression of CD107A, IFN γ , or MAIT cell cytotoxicity at early timepoints (2-3hr). Similarly, MAIT cells do not increase expression of Glut1 until 6hr post activation (Cassidy et al., 2023). However, MAIT cells do contain the cellular machinery involved in glycogen synthesis (glycogenesis), and glycogenolysis, exhibiting high level expression of GYS1, and PYGB. Indeed, Cassidy and colleagues revealed that endogenous glycogen stores are rapidly mobilised in MAIT cells upon TCR activation; and inhibition of glycogenolysis using the specific PYGB inhibitor, CP91149, resulted in significant impairment to rapid cytotoxic responses. Interestingly, inhibition the glycolytic enzyme GAPDH, which has a critical role in the latter stages of glycolysis, resulted in significantly diminished CD107A expression in early activated MAIT cells; suggesting that these cells are indeed reliant on glycolysis, but not exogenous glucose. Accordingly, CP91149 significantly attenuated metabolic rewiring upon 3hr TCR engagement. Such findings suggest that the metabolism of exogenous glucose is dispensable for rapid TCR-driven metabolic rewiring, with early cytotoxic responses in MAIT cells instead fuelled by the rapid mobilization of endogenous glycogen stores (Cassidy et al., 2023).

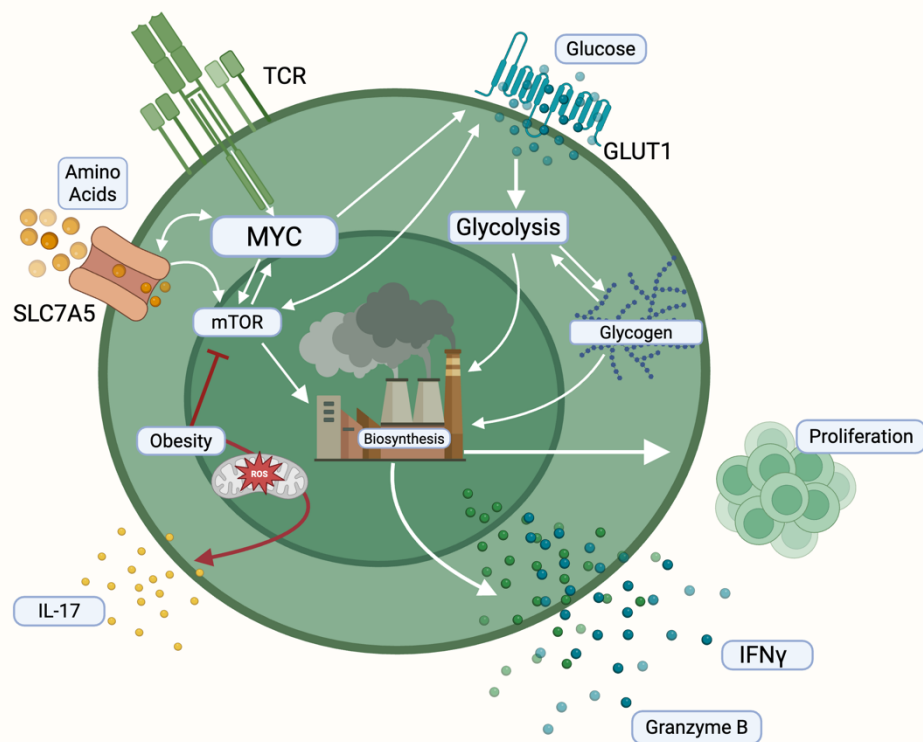


Figure 1.7. MAIT Cell Metabolism – Current Understanding. Human MAIT cells increase cellular uptake of glucose and amino acids, whilst breaking-down endogenous glycogen stores, upon TCR triggering. Increased nutrient availability activates and upregulates expression of both mTOR and Myc, which in turn facilitate a robust metabolic switch towards glycolysis; fuelling de novo biosynthesis and supporting effector functions. In obesity, the nutrient-mTOR-Myc axis is perturbed, thus impeding glycolytic reprogramming. Increased expression of IL-17 has instead been linked with ROS accumulation and dysfunctional mitochondrial respiration. Created with Biorender.com.

1.5.2. TCA Cycling and Oxidative Phosphorylation in MAIT Cells

Early cytotoxic responses in MAIT cells are not affected by inhibition of ATP synthase using Oligomycin (Cassidy et al., 2023), suggesting a independence from mitochondrial respiration in this context; however, whether there is a role for TCA cycling and OxPhos in MAIT cells, resting or upon activation, remains largely undescribed. Obesity is a chronic inflammatory disease associated with impaired insulin signalling, and consequent hyperglycemia (Hildebrandt et al., 2023). However, MAIT cells from people with obesity (PWO) fail to increase glucose uptake upon activation, and do not upregulate glycolysis (O’Brien et al., 2019), perhaps owing to diminished activation of mTORC1 and a loss of Myc (O’Brien et al., 2019, Kedia-Mehta et al., 2023). MAIT cell frequencies are thus significantly reduced in PWO (Magalhaes et al., 2015, Carolan et al., 2015), and exhibit a limited effector capacity *ex vivo*,

with attenuated expression of IFN γ and reduced proliferative expansion (Carolan et al., 2015, O'Brien et al., 2019, Kedia-Mehta et al., 2023). However, IL-17 expression is significantly increased in circulating MAIT cells from PWO (Carolan et al., 2015, Brien et al., 2020, Bergin et al., 2022); suggesting that, unlike IFN γ , the production of IL-17 is not linked to the glycolytic programme. Interestingly, high glucose environments are thought to induce oxidative stress (Papachristoforou et al., 2020), and increasing mROS production from MAIT cells in PWO potentiates the IL-17 response (Brien et al., 2020); suggesting that this population are increasingly engaged in processes pertaining to mitochondrial oxidation. In agreement, Riffelmacher and colleagues have described two subsets of murine, antigen-adapted (aa) pulmonary MAIT cells; characterised as Klr $g1^+$ IFN γ expressing cells underpinned by a glycolytic programme, and CD127 $^+$ IL-17 producers, reliant on mitochondrial oxidation (Riffelmacher et al., 2023). Mitochondrial ETC activity and ATP turnover in CD127 $^+$ aaMAIT cells was linked with the continuous uptake of fatty acids, and subsequent FAO, which fuelled the production of IL-17 (Riffelmacher et al., 2023). Interestingly, the continuous uptake of polyunsaturated fatty acids (PUFAs) by intrahepatic MAIT cells in patients with metabolic dysfunction-associated steatotic liver disease (MASLD) drives oxidative stress and impairments to both glycolysis and OxPhos, lending to diminished functional responses, including reduced expression of IL-17A (Deschler et al., 2025). These data further highlight how the nutrient composition of local microenvironments can shape MAIT cell metabolism, and consequently, downstream effector responses.

1.5.3. Amino Acid Metabolism in MAIT cells

The specific amino acid requirements of MAIT cells in the context of health and disease have yet to be elucidated in full. However, MAIT cells have been shown to upregulate expression of the system L amino acid transporter, SLC7A5, which is a heterodimer of the heavy chain, CD98, and the light chain SLC7A5, downstream of TCR engagement and cytokine co-stimulation (O'Brien et al., 2019, Kedia-Mehta et al., 2023). Inhibition of amino acid transport through system L amino acid transporters using 2-aminobicyclo- (2, 2, 1)-heptane-2-carboxylic acid (BCH) significantly attenuates the activation of mTORC1, and almost completely abrogates the glycolytic switch induced in MAIT cells upon activation (O'Brien et al., 2019). Similarly, the MAIT cell effector capacity is impaired in the presence of BCH, with reduced expression of IFN γ , and diminished proliferative expansion, a likely consequence of the

observed impairments to glycolysis (O'Brien et al., 2019). Expression of Myc is required to facilitate upregulated expression of SLC7A5 in activated MAIT cells, and inhibition of Myc lends to complete attenuation of the associated amino acid flux (Kedia-Mehta et al., 2023). Overall, these data point towards a critical role for the uptake of exogenous amino acids in TCR activated MAIT cells, specifically in the context of potentiating the glycolytic flux which fuels robust downstream effector responses.

Interestingly, the TCA cycle intermediate α -ketoglutarate has been shown to enhance antigen-driven MAIT cell effector responses *in vitro*, attributed in part to its functioning as a co-factor for the chromatin-remodelling enzyme, lysine demethylase 6B (KDM6B) (Howson et al., 2021). However, addition of α -ketoglutarate alongside 5-OP-RU could still partially enhance MAIT cell cytokine responses upon inhibition of KDM6B, suggesting that this process is in part driven by the increased availability of α -ketoglutarate for TCA cycling (Howson et al., 2021). Such findings are indicative of a potential role for glutaminolysis in facilitating MAIT cell effector responses, however, this has yet to be examined directly.

Research Aims

The overarching aim of this thesis is to further elucidate the metabolic underpinnings of MAIT cell fitness, and to garner an enhanced understanding of how microenvironmental cues, such as nutrient availability, can dictate effector responses to challenge.

The specific aims of each study included in this thesis are to:

Paper I: comprehensively review the literature and produce a detailed commentary on the environmental signals which dictate metabolic and functional responses in MAIT cells; further highlighting nutrient availability as a novel governing signal, with a capacity to drive dysregulated responses in certain diseased contexts.

Paper II: examine a role for cellular iron metabolism in supporting MAIT cell fitness, metabolism, and effector responses to TCR challenge.

Paper III: describe the functional kinetics of MAIT cell responses to innate cytokine (IL-18/IFN α) stimulation, and to investigate the metabolic and nutritional demands of this response *in vitro*.

Paper IV: examine the importance of microenvironmental glutamine availability and intracellular uptake in supporting MAIT cell metabolic rewiring downstream of activation.

CHAPTER 2. General Materials and Methods

2.1. Study Cohorts and Ethical Approval

Healthy controls were recruited from St. Vincent's University Hospital. Criteria for recruitment of the healthy control cohorts used in **Paper II, Paper III, and Paper IV** included the ability to provide informed written consent, 18 to 55 years of age, and having a healthy BMI. Exclusion criteria included current or recent infection (<2 weeks), being a current smoker, and the use of immunomodulatory medications. All participants provided full consent prior to taking part in this study. Full ethical approval granted by the Maynooth University Research Ethics Committee and St. Vincent's University Medical Ethics Committee (BSRESC-2024-38575).

2.2. Cell Culture

2.2.1. Cell Culture Media

Across **Paper II, Paper III, and Paper IV**, cell culture media was prepared as follows, and constituted the base medium of all downstream experiments, unless stated otherwise. RPMI 1640 medium +GlutaMAX (Gibco) was supplemented with 10% v/v heat-inactivated Foetal Bovine Serum (FBS) (Gibco), 100 U/ml of Penicillin (Sigma), and 100 μ g/ml Streptomycin (Sigma); hereafter referred to as complete (c)RPMI. In **Paper IV**, glutamine-free RPMI 1640 (Gibco) was used, and supplemented with 2mM L-glutamine (Gibco) where appropriate. In **Paper II**, Human Plasma-Like Media (HPLM) (Gibco) was supplemented with 10% v/v heat-inactivated FBS (Gibco), 100 U/ml of Penicillin (Sigma), and 100 μ g/ml Streptomycin (Sigma). Across all papers, for the expansion of MAIT cell lines, expansion media was prepared using RPMI 1640 +GlutaMAX (Gibco) as the base media, and supplemented with 10% v/v heat-inactivated FBS (Gibco), 100 U/ml of Penicillin, and 100 μ g/ml Streptomycin (Sigma), 20mM HEPES (Gibco), MEM-Non essential amino acid solution (Gibco) diluted to 1X, and 1mM Sodium Pyruvate (Gibco). Hereafter, this media is referred to as MAIT media.

2.2.2. Isolation of Peripheral Blood Mononuclear Cells (PBMC)

In **Paper II, Paper III and Paper IV**, fresh venous blood samples were collected into Lithium Heparin-coated sample tubes (Vacutainer, BD BioSciences) and human PBMC were isolated

using SepMate PBMC isolation tubes (StemCell Technologies). Blood was diluted 1:1 with Dulbecco's Phosphate Buffered Saline (DPBS) (Sigma) and layered over 15ml Lymphoprep (StemCell Technologies). Samples underwent centrifugation at 1200g for 10 minutes, with the breaks off. Plasma and buffy coat layers were poured into 50ml falcons, and spun at 300g for 10 minutes. Plasma was collected, and PBMC were washed with 5ml DPBS. Cells were resuspended to be counted in cRPMI. PBMC were either bio-banked (stored at -70°C, at a concentration of 10×10^6 cells/ml CryoStor cell preservation media (Sigma), or used to expand MAIT cell lines.

2.2.3. MAIT Cell Expansion

In **Paper II**, **Paper III**, and **Paper IV**, freshly-isolated PBMC were plated at 2×10^6 cells/ml in a total volume of 1ml MAIT media, in a 24-well plate. A solution of 5-A-RU-MG was prepared fresh each time. This solution consisted of a working stock of 5-A-RU (gifted) at $100 \mu\text{g/ml}$, and Methylglyoxal (MG)(Sigma) at 10mM/ml , diluted in MAIT media; was used immediately, due to the unstable nature of the reagents. $20 \mu\text{l}$ of the 5-A-RU-MG mix was added to each well, and the cells were subsequently topped with 1ml MAIT media, to bring the final concentration up to 2×10^6 cells/2ml media, and to ensure sufficient mixing. The final in-well concentration of 5-A-RU was $1 \mu\text{g/ml}$, and $100 \mu\text{M/ml}$ MG. The cells were then incubated at 37°C with 5% CO_2 . After 24hr, cells were treated with 6.8ng/ml IL-2 (BioLegend). After 48hr, cells were treated with 34ng/ml IL-2. Cell lines were maintained for a maximum of 14 days, and culture media was replaced with fresh MAIT media containing IL-2 every 3 days, providing a final concentration of 34ng/ml each time. Within this time, if cells became too confluent, the wells were split as follows. $500 \mu\text{l}$ media was removed from the top of the well, so as not to disturb the cells. The cells, now in 1.5ml, were resuspended. $500 \mu\text{l}$ cell suspension was taken and transferred to an empty well. This step was repeated with a second original well, such that the new well now contains 1ml cell suspension, taken from two individual wells. At the end of this procedure, all wells now contain 1ml cell suspension. Cells were then topped with $500 \mu\text{l}$ warm MAIT media, and $500 \mu\text{l}$ IL-2-containing MAIT media, ensuring a final in-well concentration of IL-2 at 34ng/ml . Throughout the culture, media can evaporate. Thus, every effort was taken to constantly ensure that the well volume was maintained at 2ml total volume, allowing sufficient nutrient availability, and the correct concentration of IL-2. If at any point the media became highly acidic (very yellow), 50% of the media was removed, and

replaced with fresh MAIT media. This was done alongside the appropriate IL-2 feed. MAIT cell purity was confirmed using flow cytometry, outlined in subsequent sections, on day 7-8, and again on day 12.

5-AR-U was gifted from Professor Gurdyl Besra, University of Birmingham. It was reconstituted at 10mg/ml in DMSO, aliquoted, and stored at -70°C for up to 12 months. Aliquots of 5-AR-U were never freeze-thawed and were always protected from light. When dissolved in DMSO, 5-AR-U gives off a clear-yellow hue. Degradation of the compound is indicated by a shift towards a pink-purple hue. MAIT cells fail to expand in the absence of either antigen or IL-2, and so all new stock of 5-AR-U was routinely tested against previous stock to control for changes in efficacy.

2.2.4. MAIT Cell Purification

In **Paper II**, **Paper III**, and **Paper IV**, for functional analysis by ELISA, for proteomic analysis, and for RNA sequencing, MAIT cells were isolated from culture via magnetic cell separation (MACS) to ensure high purity (>90%) and to remove debris. MAIT cells were removed from culture and spun at 300g for 10 minutes. Cells were then resuspended in 5mls DPBS and centrifuged at 300g for 10 minutes. Cells were resuspended in cold autoMACS Running Buffer (Miltenyi Biotec) at 100 μ l/1x10⁷cells, and stained with anti-human TCR Va7.2, APC or PE - conjugated antibody (Miltenyi Biotec or BioLegend) at 5 μ l/100 μ l or 10 μ l/100 μ l, respectively. MAIT cells were stained for 10 minutes at 4°C. Cells were washed with 5ml autoMACS buffer, and centrifuged at 300g for 10 minutes. Following centrifugation, cells were resuspended in 80 μ l autoMACS buffer /1x10⁷cells, and treated with anti-APC/PE MACS MicroBeads (Miltenyi Biotec) at 20 μ l/1x10⁷cells. Cells were incubated at 4°C for 15 minutes, and subsequently washed with 3ml autoMACS buffer as previously described. Cells were resuspended in 5ml cold autoMACS buffer. MACS cell isolation was performed using the QuadroMACS Cell Separator (Miltenyi Biotec). LS columns (Miltenyi Biotec) were rinsed with 3ml autoMACS buffer, and the cell suspension was loaded onto the LS column. Following two more column rinses, as previously described, magnetically-labelled MAIT cells were retained in the LS column, whilst flowthrough was discarded. MAIT cells were flushed out of the LS column, and autoMACS buffer was removed following centrifugation at 300g for 10 minutes. Isolated MAIT

cells were rested, and subsequently treated as appropriate for downstream experiments (Figure 2.1).

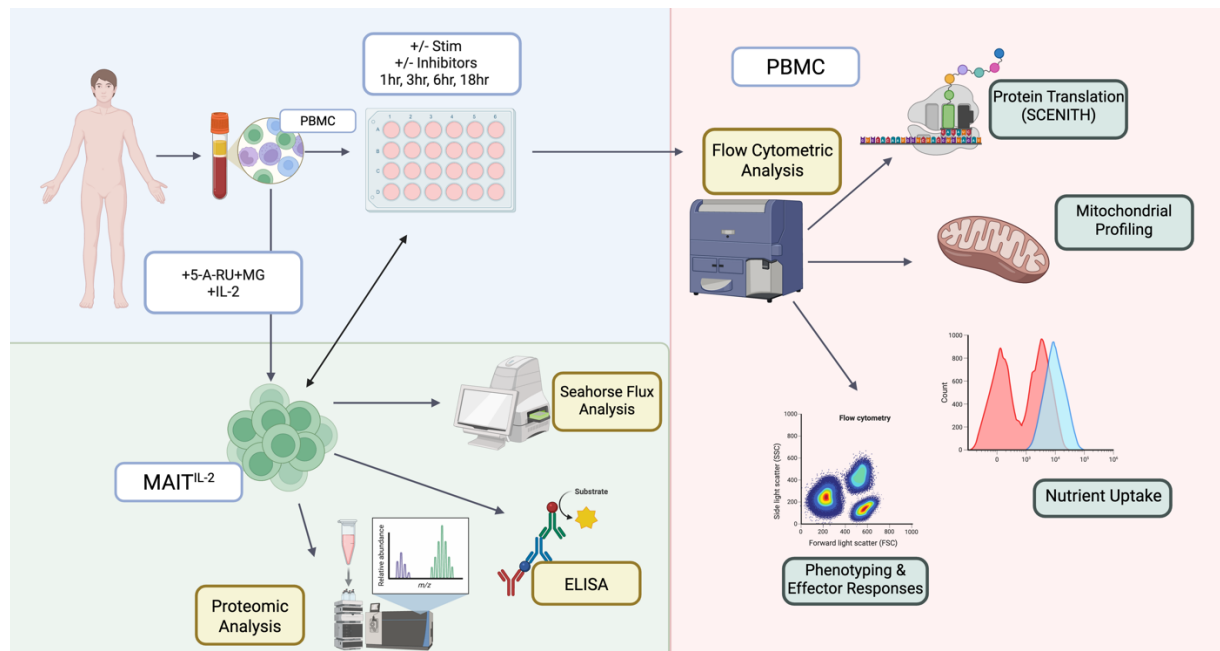


Figure 2.1. Methods Overview. PBMC were isolated from fresh venous blood samples. High-purity MAIT^{IL-2} cell lines were expanded for 12-14 days using 5-A-RU, MG, and IL-2. Experiments performed using MAIT^{IL-2} include Seahorse Extracellular Flux Analysis, ELISA, and Proteomics. MAIT cells were also assessed directly in PBMC using various Flow Cytometry-based protocols, including SCENITH, Mitotracker assays, and Nutrient-Uptake assays. Flow Cytometry was also performed to phenotype MAIT cells in PBMC, and to probe effector responses, including cytokine expression and proliferation. Created with Biorender.com.

2.3. Flow Cytometric Analysis

2.3.1. Preparation of MAIT Cells for Analysis by Flow Cytometry

In **Paper II** for flow cytometric analysis of surface markers and intracellular cytokine expression, and flow-based metabolic assays, PBMC were stimulated with 50ng/ml IL-18 (BioLegend) and anti-CD3/CD28 TCR Dynabeads (Gibco) at a ratio of 2.5:1, cells:beads, for 18hr at 2×10^6 cells /ml in HPLM. Alternatively, cells were stimulated with 50ng/ml IL-18 and 50ng/ml IL-12 (BioLegend) only. To examine the impact of iron-starvation on phenotype and functionality, HPLM was treated with 200 μ M DFO a minimum of 24hr prior to use, to ensure sufficient iron-chelation. PBMC were subsequently activated as outlined above, in the

absence or presence of DFO. In **Paper III** for analysis of intracellular ISG15 expression, intracellular cytokine expression, and Puromycin incorporation, PBMC were plated at 1×10^6 /ml and stimulated with 50ng/ml IL-18, and 250-500ng/ml IFN α 2A (R&D systems) for 6hr or 18hr in cRPMI. Alternatively, cells were activated with anti-CD3/CD28 TCR Dynabeads at a ratio of 2.5:1 cells:beads, 50ng/ml IL-15 (BioLegend), 50ng/ml IL-12, or 50ng/ml IL-33 (BioLegend). To examine the contribution of glycogen and glucose to such responses, PBMC were plated at 1×10^6 /ml in cRPMI, and pre-treated for 1hr in the absence or presence of the HK2 inhibitor 2DG (1mM) (Sigma), or the PYG inhibitor, CP91149 (100 μ M) (Sigma). Cells were subsequently activated as outlined above, for 6hr or 18hr. In **Paper IV**, for analysis of intracellular activating transcription factor 4 (ATF4) expression, intracellular cytokine expression, and Puromycin incorporation, PBMC were plated at 1×10^6 /ml in glutamine-free RPMI 1640 (Gibco), supplemented with 2mM L-glutamine where appropriate, and stimulated for 18hr using Ultra-LEAF purified anti-human CD3 (1 μ g/ml) and Ultra-LEAF purified anti-human CD28 (0.5 μ g/ml) (both BioLegend).

In **Paper III**, for experiments with a 6hr stimulation and to assess intracellular cytokine expression, eBioscience Protein Transport Inhibitor Cocktail (Invitrogen) was added to the culture at a 1X final concentration, 1hr post-stimulation. In **Paper II**, **Paper III**, and **Paper IV**, for experiments with an 18hr stimulation, to assess intracellular cytokine expression, eBioscience Protein Transport Inhibitor Cocktail was added to the culture at a 1X final concentration, 6hr prior to the end of the incubation.

2.3.2. Basic Cell Staining Protocol

In **Paper II**, **Paper III**, and **Paper IV**, following appropriate stimulation as described in section 2.3.1, PBMC were transferred to a 96-well round-bottom plate, and stained for viability using eBioscience Fixable Viability Dye (eFluor506) (Invitrogen), prepared at 1 μ l/1ml cold DPBS, in a total volume of 50 μ l/well. Cells were incubated in the dark at 4°C for 25 minutes. Cells were then washed with 100 μ l DPBS and centrifuged at 400g for 7 minutes. Extracellular markers were stained for in flow buffer (1% FBS in DPBS) in a total volume of 25 μ l/well, with appropriate antibody concentrations prepared as per manufacturer guidelines. Cells were then incubated in the dark at 4°C for 25 minutes. To remove excess antibody, cells were washed with 100 μ l flow buffer, and centrifuged as previously described. Cells were then fixed

using fixation buffer from the True-Nuclear Transcription Factor Buffer Set (BioLegend) made to a 1X concentration, at 100 μ l/well, and incubated at room temperature, in the dark, for 30 minutes. Cells were then permeabilised using permeabilisation buffer from the True-Nuclear Transcription Factor Buffer Set (BioLegend) made to a 1X concentration, at 100 μ l/well. Cells were then centrifuged at 400g for 7 minutes and resuspended once more in 100 μ l permeabilisation buffer, and centrifuged once more as previously described. Intracellular markers were stained for in permeabilisation buffer, in a total volume of 25 μ l/well, with appropriate antibody concentrations prepared as per manufacturer guidelines. Cells were incubated at 4°C, in the dark, for 45 minutes. Cells were then washed with permeabilisation buffer, and resuspended in 200 μ l flow buffer. Cell populations were acquired using an Attune NxT flow cytometer (ThermoFisher Scientific), and analysed using FlowJo version 10.8.2 (TreeStar). Fluorescence minus one (FMO) and unstained controls were prepared for each experiment. Results are expressed either as a percentage of the parent population, using FMO and unstained controls to determine percent positivity; or through the use of the geometric mean to determine the mean fluorescence intensity (MFI) of the relevant marker.

2.3.3. MAIT Cell Transferrin Uptake Assay

In **Paper II**, PBMC (2x10⁶/ml) were stimulated for 18hr with IL-18 and anti-CD3/CD28 TCR Dynabeads, or IL-18 and IL-12, in HPLM, as described in section 2.3.1. The next day, were rested in serum-free HPLM supplemented with 5% Bovine Serum Albumin (BSA) (Sigma) for 2hr. Appropriate cytokines (IL-18 alone (50ng/ml), or IL-18/IL-12 (50ng/ml) were re-added to the culture during this time. Cells were subsequently washed in serum-free HPLM supplemented with 0.5% BSA and incubated with 5 μ g/ml Transferrin-AlexaFluor647 (Invitrogen) for 10 minutes at 37°C. Holo-Transferrin (500 μ g/ml) (Sigma) was used as a competition control. A cold FMO control was kept on ice and treated with 5 μ g/ml Transferrin-AlexaFluor647 (Invitrogen) in ice-cold 0.5% BSA, then incubated at 4°C for 10 minutes. Cells were then washed in ice-cold media supplemented with 0.5% BSA. Cells were stained for viability and extracellular surface markers, fixed, and analysed by flow cytometry, as outlined in section 2.3.2.

2.3.4. MAIT Cell SCENITH Assay

In **Paper II**, PBMC were plated at 2×10^6 /ml in 2ml total volume HPLM, and stimulated with IL-18 and anti-CD3/CD28 TCR Dynabeads for 18hr, in the absence or presence of 200 μ M DFO; as outlined in section 2.3.1. Cells were then seeded into a round-bottomed 96-well plate, whereby each sample was divided across five wells, constituting a control well (untreated with inhibitors), to be treated with 2DG (100 μ M) (Sigma), to be treated with Oligomycin (1 μ M) (Sigma), to be treated with both 2DG and Oligomycin (DGO well), and a well that is not to be treated with inhibitors, or Puromycin (minus Puromycin control). The plate was centrifuged at 400g for 7 minutes, and cells were resuspended in resuspended in 173 μ l HPLM. Following addition of the appropriate inhibitor (2DG or Oligomycin, both, or none), cells were incubated at 37°C for 15 minutes. Subsequently, cells were treated with Puromycin (11 μ M) (Sigma), and incubated at 37°C for a further 25 minutes. Cells were washed with 100 μ l ice-cold DPBS to stop Puromycin incorporation. Cells were then washed with 100 μ l DPBS, and stained for viability, and MAIT cell extracellular markers, as outlined in section 2.3.2. Cells were subsequently fixed and permeabilised as outlined in section 2.3.2. Intracellular staining for Puromycin was achieved as described in section 2.3.2, using the Anti-Puromycin-Alexa Fluor 488 Conjugate Antibody (Sigma), at a titrated concentration of 0.5 μ l/100 μ l permeabilisation buffer, with 25 μ l added to each well. Cells were acquired and analysed by flow cytometry as outlined in section 2.3.2. Calculations used to determine percent glucose-dependence, and fatty-acid oxidation (FAO) and amino-acid oxidation (AAO) capacity were extracted from the pioneering paper by Argüello and colleagues which describes the use of SCENITH to assess the immunometabolic profile of single-cell populations (Argüello et al., 2020).

In **Paper III**, to assess protein translation via puromycin incorporation only, PBMC were plated at 1×10^6 /ml and stimulated with IL-18 and IFN α for 6hr in cRPMI as outlined in section 2.3.1. In **Paper IV**, PBMC were plated at 1×10^6 /ml in RPMI with or without 2mM glutamine as indicated, and activated via anti-CD3/CD28 as outlined in section 2.3.1. Cells were then seeded into a round-bottomed 96-well plate, and centrifuged at 400g for 7 minutes, before being resuspended in 173 μ l of cRPMI media. Cells were treated with Puromycin (11 μ M) and incubated for 25 minutes at 37°C. Following this incubation, 100 μ l of ice-cold DPBS was added

to stop puromycin incorporation. Cells were then washed with 100 μ l DPBS, and stained for viability, MAIT cell extracellular markers, and puromycin, as outlined above.

2.3.5. MAIT Cell Mitochondrial Analysis

In **Paper II**, to assess mitochondrial membrane potential and mitochondrial mass, PBMC were plated at 2×10^6 /ml in HPLM, and activated for 18hr using IL-18 and anti-CD3/CD28 TCR Dynabeads, in the absence or presence of 200 μ M DFO, as outlined in section 2.3.1. The next day, cells were seeded into a 96-well plate, and washed with 100 μ l DPBS. Cells were stained for viability, and MAIT cell extracellular markers, as outlined in section 2.3.2. Cells were washed with DPBS, and stained with 50 μ M MitoTracker Deep Red FM (Invitrogen), and 50 μ M MitoTracker Green FM (Invitrogen), as per manufacturer guidelines. Cells were incubated for 1hr at 37°C. Cells were then washed with 100 μ l DPBS, and resuspended in 200 μ l flow buffer, to be acquired and analysed immediately by flow cytometry, as outlined in section 2.3.2.

2.3.6. MAIT Cell Expansion - Absolute Counts

In **Paper II** for examination into the impact of iron-starvation on MAIT cell proliferative capacity, MAIT cells were expanded as in section 2.2.3, in the absence or presence of Iron(II) sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) (Sigma) at 200 μ M, and/or 200 μ M DFO. Respective treatments were added alongside the IL-2 feed every 3 days. On day 5 of the cell culture, cells were stained for viability and MAIT cell extracellular markers, as outlined in section 2.3.2; and absolute counts were determined using total cell counts, and MAIT cell frequencies, as determined by flow cytometry.

2.3.7. Glutamine Uptake Assay (QUAS-R)

In **Paper IV**, for assessment of glutamine uptake in MAIT cells, PBMC were plated at 1×10^6 /ml in cRPMI and TCR stimulated for 18hr, as outlined in section 2.3.1. Cells were then transferred to FACS tubes, and stained at room temperature for viability and extracellular markers, as outlined in section 2.3.2. Cells were then washed with warm Hanks' Balanced Salt Solution (HBSS)(Gibco), and subsequently resuspended in 225 μ l HBSS and transferred to a 96-well round-bottomed plate, in triplicate at 75 μ l per well. Cells were incubated for 10 minutes at 37°C, and 25 μ l of L-azidohomoalanine (L-AHA) (Jena Bioscience) was added to each well, yielding a final in-well concentration of 100 μ M. Cells were incubated for 8 minutes at 37°C.

One well per sample was also treated with glutamine (10mM) as a competition control, and one well per sample received no AHA, as a background control. Cells were then fixed with 1% paraformaldehyde (PFA) (Santa Cruz) for 30 minutes at room-temperature. Cells were washed twice with DPBS, and permeabilised using a solution of 0.01% Saponin (Sigma) and 1% BSA in DPBS, added at 100µl per well, for 20 minutes at room-temperature in the dark. The click chemistry staining mix was made up as follows during this time. Reagents were added in the following order: 10µl Copper II Sulfate Pentahydrate (Sigma) , 10µl Sodium L-Ascorbate (Sigma), 10µl THPTA (Jena Bioscience), 160µl Aminoguanidine Hydrochloride (Cayman Chemicals), 810µl 0.01% Saponin solution, 2.5µl AZDye 488 Alkyne Fluorophore (Jena Bioscience). Cells were washed and resuspended in 30µl of the click staining mix, and incubated for 1hr at room temperature on a roller, whilst protected from the light. Cells were washed with DPBS and resuspended in 200µl flow buffer, to be analysed by flow cytometry, as outlined in section 2.3.2. Working stock concentrations of the click chemistry reagents are listed below.

Table 2.1. Stock concentrations, appropriate solvent, source, and catalogue number of key click chemistry reagents required to perform QUAS-R glutamine uptake assay, adapted from (Pelgrom et al., 2023).

NAME	STOCK	CATALOGUE NUMBER
4-Azido-L-homoalanine HCl (L-AHA)	400mM, dH2O; diluted to 400µM in HBSS prior to assay	Jena Bioscience, CLK-AA005-10
Aminoguanidine hydrochloride (AG)	1M, dH2O	Cayman Chemicals, 81530
AZDye488 Alkyne Fluorophore	2mM, DMSO	Jena Bioscience, CLK-1277-1
Copper (II) sulfate pentahydrate	100mM, dH2O	Sigma, 209198
Sodium-L-Ascorbate	1M, dH2O	Sigma, A7631
Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA)	100mM, dH2O	Jena Bioscience, CLK-1010-25

2.3.8. MAIT Cell Phenotyping and Functional Assessment

In **Paper II**, **Paper III**, and **Paper IV**, doublets were excluded using the forward scatter height (FSC-H) and forward scatter area (FSC-A) exclusion method (**Figure 2.2, A**). Dead cells were excluded on the basis of positive staining for the eBioscience Fixable Viability Dye (eFluor506) (**Figure 2.2, B**). Subsequently, MAIT cells could be identified in PBMC as being positive for CD3, CD161 and $V\alpha 7.2$ (**Figure 2.2, C-D**).

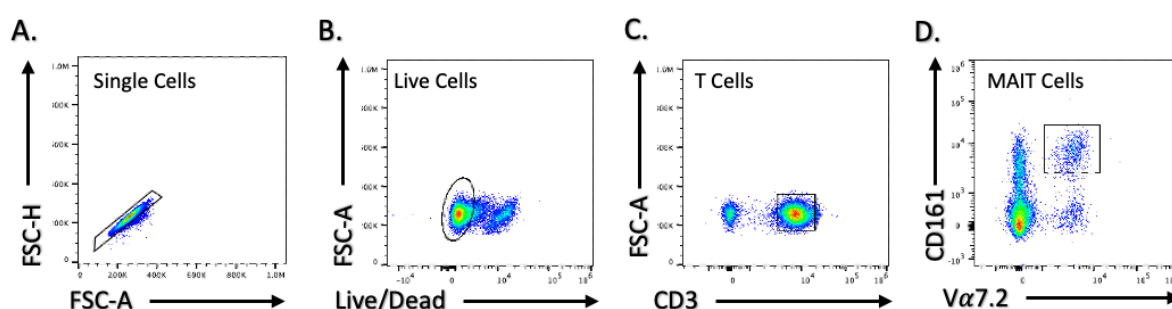


Figure 2.2. Schematic detailing the gating strategy used in *Paper II*, *Paper III*, and *Paper IV* to identify MAIT cells amongst PBMC.

In **Paper II**, **Paper III**, and **Paper IV**, MAIT cells in PBMC were assessed for the expression of numerous extracellular surface markers using specific monoclonal antibodies, listed in Table 2.2. Intracellular cytokine expression, and the expression of metabolic markers, were also assessed using specific monoclonal antibodies listed in Table 2.3.

Table 2.2. Monoclonal antibodies used to assess extracellular surface markers expressed on MAIT cells.

ANTIBODY	SOURCE	CATALOGUE NUMBER
Anti-human CD161 (clone REA631), APC	Miltenyi Biotec	130-113-595
Anti-human CD161 (clone REA631), APC-Vio770	Miltenyi Biotec	130-129-806
Anti-human CD161 (clone REA631), PE-Vio615	Miltenyi Biotec	130-123-483
Anti-human CD3 (clone OKT3), Brilliant Violet 605	BioLegend	317322

Anti-human CD3 (clone REA613), APC	Miltenyi Biotec	130-113-135
Anti-human CD3 (clone REA613), PE-Vio615	Miltenyi Biotec	130-114-520
Anti-human CD3 (clone UCHT1), Brilliant Violet 711	BioLegend	300464
Anti-human CD25 (clone REA570), Pe-Vio615	Miltenyi Biotec	130-123-035
Anti-human CD56 (clone REA196)	Miltenyi Biotec	130-127-383
Anti-human CD69 (clone REA824), APC	Miltenyi Biotec	130-112-614
Anti-human CD71 (clone CY1G4), APC/Cyanine7	BioLegend	334110
Anti-human CD71 (clone REA902), Pe-Vio615	Miltenyi Biotec	130-115-036
Anti-human TCR Va7.2 (clone 3C10), PE	BioLegend	351706
Anti-human TCR Va7.2 (clone REA179), APC	Miltenyi Biotec	130-123-828
Anti-human TCR Va7.2 (clone REA179), PE-Vio770	Miltenyi Biotec	130-123-885
Anti-human TCR Va7.2 (clone REA179), VioBlue	Miltenyi Biotec	130-136-731

Table 2.3. Monoclonal antibodies used to assess intracellular cytokine expression, and the expression of metabolic markers.

ANTIBODY	SOURCE	CATALOGUE NUMBER
Anti-human/mouse Granzyme B (clone QA16A02), PE/Dazzle 594	BioLegend	372216
Anti-human/mouse/rat Granzyme B (clone REA226), FITC	Miltenyi Biotec	130-118-341
Anti-human/mouse/rat Granzyme B (clone REA226), PE	Miltenyi Biotec	130-116-486

Anti-human IFN- γ (clone 4S.B3), Alexa Fluor 488	BioLegend	502515
Anti-human IFN- γ (clone REA600), FITC	Miltenyi Biotec	130-113-497
Anti-human IFN- γ (clone REA600), PE	Miltenyi Biotec	130-113-498
Anti-human ISG15/UCRP (clone 851701), PE	R&D Systems	IC8044P
Anti-puromycin (clone 12D10), Alexa Fluor 488	Sigma	MABE343-AF488
ATF4 Recombinant Rabbit Monoclonal Antibody	Invitrogen	MA5-52598

2.4. Proteomics

2.4.1. Preparation of MAIT Cells for Proteomic Analysis

In **Paper III**, purified MAIT cells were seeded into a 24-well plate at a concentration of 1×10^6 /ml in a total volume of 2ml HPLM, and activated for 18hr using a combination of IL-18 (50ng/ml) and anti-CD3/CD28 TCR Dynabeads (1:1, cells:beads) or a combination of IL-18 (50ng/ml) and IFN α (500ng/ml). Cells were then transferred to 15ml falcons, centrifuged at 400g for 7 minutes, and resuspended in 1ml HBSS. Cells were then transferred to 2ml microcentrifuge tubes and centrifuged at max speed (17000g) for 20 seconds. The supernatant was carefully removed, and cells were resuspended in 1ml HBSS, and centrifuged at 17000g for 20 seconds. The supernatant was aspirated, and cell pellets were immediately flash frozen in liquid nitrogen, and stored at -70°C. Within weeks, cell pellets were shipped to the FingerPrints Proteomics Facility at the University of Dundee and mass spectrometry was carried out.

2.4.2. In Silico Proteomic Analysis

In **Paper II**, a publicly available proteomic dataset was downloaded from PRIDE (accession #PXD041544). Using a previously established protocol from Teh and colleagues (Teh et al.,

2021) a list of human iron-interacting proteins (Andreini et al., 2018) was compared against the complete list of proteins detected in publicly available dataset comparing unstimulated MAIT cells, versus MAIT cells stimulated with a combination of anti-CD3/CD28 TCR Dynabeads, IL-18 and IL-12. Iron atom counts per protein species were estimated by multiplying the protein copy number of each iron-interacting protein by the iron atom counts per protein, which were obtained using the UniProt database cofactor information attributed to each protein. Where iron atom counts were not listed, estimates of iron usage per protein species were assumed to be 1 atom for heme and iron-interacting proteins, and 2 atoms for Fe-S cluster-interacting proteins. The total number of iron atoms required per cell was calculated as the sum of iron atoms required by each protein species.

In **Paper III**, a novel proteomic dataset was generated, using the sample set-up described in 2.4.1, by Data-Independent Acquisition (DIA) mass spectrometry carried out at the FingerPrints Proteomics Facility within the University of Dundee. Heatmaps were generated using the Morpheus data visualisation tool (<https://software.broadinstitute.org/morpheus>) from The Broad Institute. Relevant protein copy numbers were graphed and assessed for statistical significance using GraphPad Prism 10 software for macOS. *P* values and fold changes for data tables were calculated using R Studio (v.2023.12.1+402). Gene Ontology (GO) Enrichment Analysis for Biological Processes was carried out on commonly upregulated ($\log_2\text{fold} > 0.5$, $p \leq 0.05$) proteins between TCR/IL-18 and IL-18/IFN α conditions using the ClusterProfiler package (version 4.10.1) for R Studio. *P* values were adjusted for multiple comparisons using the Benjamini-Hochberg (BH) method to control for the false-discovery rate (FDR). GO values with an adjusted *P* value of < 0.05 were considered significant. A bubble plot mapping the top 10 enriched terms was created using the ClusterProfiler package (version 4.10.1). A list of human mitochondrial proteins expressed the within the generated proteome was extracted by comparing the complete list of proteins detected, with the list of human mitochondrial proteins available from the MitoCarta3.0 inventory, identifiable by the Gene Ontology term GO:0005739. Within this list, proteins involved in the ETC were identified and extracted using the MitoCarta3.0 Mitopathways column reference term 'OXPHOS'. All matches were extracted using R Studio (v.2023.12.1+402), and all tables were exported to Excel. The total summed copy numbers of mitochondrial and ETC proteins were calculated in Excel.

2.5. Seahorse Extracellular Flux Analysis

2.5.1. Hydration of Sensor Cartridge

24hr prior to the beginning of the assay, the Seahorse XF Sensor Cartridge (Agilent) was hydrated. 200 μ l Seahorse XF Calibrant (Agilent) was added to every well, and the cartridge was placed at 37°C without CO₂ overnight. The Seahorse XF Analyser was also switched on, and left to equilibrate overnight.

2.5.2. Preparation of MAIT Cells for Seahorse Assay

In **Paper II**, purified MAIT cells were plated at 1x10⁶/ml in a total volume of 2ml cRPMI, and activated using IL-18 (50ng/ml) and anti-CD3/CD28 TCR Dynabeads (1:1, beads:cells) for 18hr, in the absence or presence of DFO (200 μ M). In **Paper III**, MAIT cells were plated at 1x10⁶/ml in a total volume of 2ml cRPMI, and activated using IL-18 (50ng/ml) and IFN α (500ng/ml) or IL-18 (50ng/ml) and anti-CD3/CD28 TCR Dynabeads (1:1, beads:cells) for 6hr or 18hr. In **Paper III**, MAIT cells were pre-incubated in cRPMI with or without 1mM 2DG and 100 μ M CP91149, or appropriate vehicle controls, for 1hr, and subsequently stimulated with IL-18 (50ng/ml) and IFN α (250ng/ml) for 3hr.

Following all appropriate incubations, MAIT cells were harvested into 15ml falcons and re-counted prior to the beginning of the assay, to ensure equal seeding. Cells were then centrifuged at 400g for 7 minutes and resuspended in 2ml phenol-red -free Seahorse XF RPMI media (Agilent) supplemented with 10mM glucose, 2mM glutamine, and 1mM pyruvate (Agilent), which had been heated to 37°C. Cells were centrifuged at 400g for 7 minutes and resuspended at 2x10⁵/140 μ l Seahorse XF RPMI, supplemented as stated above. Where appropriate, inhibitors (DFO, 2DG, or CP91149), and the appropriate vehicle control, were re-added to the media. Cells were then seeded into a Cell-Tak (Corning) -coated 96-well Seahorse XF cell culture plate (Agilent) at 140 μ l/well, in quintuplicates. Any remaining wells were filled with 140 μ l of warm (37°C) Seahorse XF RPMI. The cell culture plate was then centrifuged at 200g for 3min, with the breaks on, to ensure adhesion of cells in a monolayer at the bottom of the Cell-Tak -coated well. Cells were then incubated at 37°C, without CO₂, for 45 minutes.

During this time, the sensor cartridge was removed from the incubator. The sensors were briefly lifted out of the calibrant to release any bubbles that may have formed during the 24hr incubation period, and placed back again. The sensor cartridge injection ports were then loaded with the appropriate inhibitor solutions. Briefly, four 15ml falcons were filled with 2.5ml warm (37°C) Seahorse XF RPMI, and contained stock concentrations of Oligomycin (Sigma) (8 μ M), FCCP (Sigma) (9 μ M), Rotenone (Sigma) (10 μ M) and Antimycin A (Sigma) (10 μ M), and Monensin (Sigma) (220 μ M). The falcons were kept in a 37°C water bath until use. Using a multichannel pipette, 20 μ l of each inhibitor was then loaded into the appropriate injection port, using the port loading guide provided by Agilent. The injection ports were visually inspected to ensure that the solutions were dispensed correctly. The sensor cartridge was then transferred to the Seahorse XF Analyser to begin the assay calibration.

Following the 45 minute incubation, and upon successful completion of the calibration, the cell culture plate was transferred to the Seahorse XF Analyser, and respiratory parameters (mitochondrial and glycolytic) were measured using oxygen consumption rate (OCR) (pmol/min) and extracellular acidification rate (ECAR) (mpH/min), respectively. During the assay, Oligomycin, FCCP, Rotenone, and Antimycin A were further diluted in-well to a final concentration of 1 μ M. Monensin was further diluted in-well to a final concentration of 20 μ M. Assay results were exported as an Excel file, and metabolic parameters were calculated as per well-established protocols.

2.5.3. Cell-Tak Protocol

To ensure MAIT cells remained adhered to the cell culture microplate during the assay, the microplate was coated with Cell-Tak prior to seeding. A 0.1M solution of sodium bicarbonate (Honeywell Fluka) was prepared in molecular grade water (Sigma). The solution was vortexed until homogenous, and sterile-filtered. The pH was confirmed to be approximately pH 8 using pH-indicator paper (Supelco). Cell-Tak was added to 0.1M sodium bicarbonate solution to a final concentration of 22.4 μ g/ml in 3ml total volume, and 25 μ l of this solution was added to every well of the 96-well cell culture microplate. The plate was left to incubate at room temperature for a minimum of 20 minutes. The Cell-Tak was then washed twice with 200 μ l sterile water, and all liquid was aspirated off with a multichannel pipette, avoiding touching the bottom of the well. The plate was left to dry in the hood with the lid off until use. If the

plate was not used that same day, once dry, it was wrapped in parafilm and kept at 4°C until use the following day.

2.6. Enzyme-Linked Immunosorbent Assay (ELISA)

2.6.1. Sample Set-Up and Reagent Preparation for ELISA

In **Paper II**, purified MAIT cells were plated at 1×10^6 /ml in HPLM, and stimulated using IL-18 (50ng/ml) and anti-CD3/CD28 TCR Dynabeads (1:1 ratio, cells:beads), in the absence or presence of 100 μ M DFO. Alternatively, cells were activated in the absence or presence of an anti-CD71 monoclonal antibody (Invitrogen) at 20 μ g/ml, or the recommended IgG isotype control (Invitrogen). Cells were stimulated for 18hr. Cell supernatants were collected and analysed by ELISA, to determine the concentrations of IFN γ , IL-17, IL-2, and IL-26. Each ELISA kit was used as per manufacturer guidelines. Wash buffer was prepared as a solution of 0.05% (v/v) Tween-20 (Sigma) in PBS, made using dH₂O and DPBS tablets (Oxoid). Reagent diluent was prepared as a solution of 1% (w/v) BSA (Sigma) in DPBS (Sigma). Substrate solution was prepared as a 1:1 mixture of tetramethyl benzidine (TMB) substrate A and TMB substrate B (BioLegend). A 1M solution of sulphuric acid (H₂SO₄) was used as a stop solution, and prepared by adding 5.6ml H₂SO₄ (Sigma) to 94.4ml dH₂O. For detection of IL-26, wash buffer, substrate solution, and stop solution, came with the kit and were appropriately prepared as per manufacturer guidelines. ELISA plates were analysed using a microplate reader (ThermoFisher Scientific) which measures absorbance at 450nm, with the wavelength correction set to 595nm. Assay results were exported to Excel to be analysed.

2.6.2. ELISA Protocol

Briefly, capture antibody was diluted in DPBS to the recommended concentration and added at 25 μ l/well of a 96-well half-area flat-bottomed microplate. The plate was incubated at room temperature overnight, with gentle rocking. The contents of the plate were poured off, and the plate was washed three times with 100 μ l wash buffer. The plate was then blotted, and 75 μ l reagent diluent was added to each well, to block non-specific binding sites. The plate was incubated at room temperature for 1-2hr, with gentle rocking. During this time, cell supernatants were thawed, and standards were prepared in reagent diluent, as per

manufacturer guidelines. Where appropriate, samples were also diluted in reagent diluent. The plate was then washed and blotted as previously described, and samples and standards were added in triplicate, at 25 μ l/well. The plate was then either incubated at room temperature for 2hr with gentle rocking, or incubated at 4°C until use the following day. The contents of the plate were then poured off, and the plate was washed as previously described. Detection antibody was diluted as per manufacturer guidelines in reagent diluent, and added at 25 μ l/well. The plate was then incubated at room temperature with gentle rocking, for 2hr. Subsequently, the contents of the plate were poured off, and the plate was washed as previously described. Streptavidin-horse radish peroxidase was then diluted to the appropriate working concentration, in reagent diluent, as per manufacturer guidelines, and added at 25 μ l/well. The plate was wrapped in tinfoil to protect from the light, and incubated at room temperature for 20 minutes, with gentle rocking. The contents were poured off, the plate was washed and blotted as previously described, and 25 μ l of TMB substrate solution was added to each well. The plate was incubated for a maximum of 20 minutes, at room temperature, and protected from the light. When standards or samples appeared saturated, the reaction was stopped with stop solution, at 12.5 μ l/well. Immediately thereafter, absorbance values were measured.

For detection of human IL-26, a slightly different protocol was required, and this was carried out as per manufacturer guidelines (Cusabio). However, samples were prepared, thawed, and analysed in the same manner as those using the R&D Systems ELISA kits. The kits used within this thesis are outlined in Table 2.4.

Table 2.4. *ELISA kits used in Paper II to assess protein concentrations in MAIT cell supernatants.*

TARGET	KIT & SOURCE	CATALOGUE NUMBER
IFN γ	Human IFN γ DuoSet ELISA, R&D Systems	DY285B
IL-17	Human IL-17 DuoSet ELISA, R&D Systems	DY317
IL-2	Human IL-2 DuoSet ELISA, R&D Systems	DY202
IL-26	Human IL-26 ELISA Kit, Cusabio	CSB-E11716h

2.7. RNA Isolation, cDNA Synthesis, and Real Time -qPCR.

In **Paper III**, to assess mRNA expression levels of MYC, purified MAIT cells were activated with at a concentration of 1×10^6 /ml with a combination of IFN α (500ng/ml) and IL-18 (50ng/ml) or IL-18 (50ng/ml) and anti-CD3/CD28 TCR Dynabeads (1:1, cells:beads) for 6hr. Total RNA was extracted using TRIzol Reagent (Invitrogen) as per manufacturer guidelines. cDNA was generated using the qScript cDNA Synthesis kit (QuantaBio), as per manufacturer guidelines. Real time (RT) -qPCR was performed using PerfeCTa SYBR Green FastMix Reaction Mix (Green Fastmix, ROX) (QuantaBio), and KiCqStart primer sets (Sigma).

2.7.1. RNA Extraction

Briefly, following the appropriate stimulation setup, cells were harvested into 15ml falcons and centrifuged at 400g for 7 minutes. Cell supernatants were discarded, and cell pellets were resuspended in 500 μ l of TRIzol Reagent. Samples were stored at -70°C.

2.7.2. mRNA Isolation

Samples were thawed and allowed to equilibrate to room temperature. 100 μ l of chloroform (Sigma) was added and each sample was vortexed, and rested for 3 minutes. Samples were then centrifuged at 12,000g for 15 minutes. The uppermost liquid interphase was removed and transferred to new tubes, avoiding contamination with the white DNA layer. Isopropanol (ThermoFisher Scientific) was added at 250 μ l/sample, and samples were incubated at room temperature for 10 minutes. Samples were then centrifuged at 12,000g for 10 minutes. Supernatant was removed carefully, so as not to disturb the RNA pellet. Samples were briefly centrifuged once more, and any remaining supernatant was removed. Cell pellets were then resuspended in 1ml of 75% ethanol (Sigma) and samples were centrifuged at 7500g for 5 minutes. Supernatant was again removed, the pellet was resuspended in 20 μ l molecular-grade water, and placed at 60°C on a heating block for 10 minutes.

2.7.3. Quantification of mRNA

The Nanodrop 2000 spectrophotometer (ThermoFisher Scientific) was used to quantify the mRNA concentration of each sample. The RNA nucleic acid setting was used, and absorbance was measured at 260nm and 280nm. Samples were kept on ice, and 1 μ l of molecular-grade

water was put onto the Nanodrop first, as a blank control. Subsequently, 1 μ l of each sample was added to the Nanodrop, to determine the concentration of RNA at Xng/1 μ l. Samples were then diluted in molecular-grade water to achieve a final concentration of 100ng/ μ l.

2.7.4. cDNA Synthesis and RT-qPCR

cDNA was generated using the qScript cDNA Synthesis kit, as per manufacturer guidelines, and each reaction contained 500ng of RNA. Samples were then run on a G-Storm Thermal Cycler (Gene Technologies Ltd). Following cDNA synthesis, each sample was diluted 1 in 5 with molecular-grade water and stored at -20°C. Primers (Table 2.5.) were reconstituted in molecular-grade water to give a working stock concentration of 100 μ M, and stored at -20°C. To analyse mRNA expression by RT-qPCR, samples were thawed, primers were thawed and further diluted to a working stock of 4 μ M in molecular-grade water, and the PerfeCTa SYBR Green FastMix Reaction Mix (Green Fastmix, ROX) was used as per manufacturer guidelines. 8 μ l of the primer-SYBR green mix was added to each well of the 96-well reaction plate (MicroAmp Fast 96-well reaction plate (0.1mL) (Life Technologies), alongside 2 μ l of cDNA. Samples were run in triplicate. HPRT was used as the appropriate housekeeping gene. The plate was sealed, and briefly centrifuged to ensure all reagents were at the bottom of the well. Samples were run on the StepOnePlus RealTime PCR System (Applied Biosystems). Results were exported to Excel and analysed using the $\delta\delta$ CT method to determine relative expression levels.

Table 2.5. Forward and reverse primer sequences used in paper III to assess mRNA expression levels by qPCR.

TARGET	FORWARD SEQUENCE (5'-3')	REVERSE SEQUENCE (5'-3')	SOURCE
Human HPRT	ATAAGCCAGACTTTGTTGG	ATAGGACTCCAGATGTTTCC	Sigma
Human MYC	TGAGGAGGAACAAGAAGAT	ATCCAGACTCTGACCTTTTG	Sigma

2.8. Key Materials

Materials and resources required for the generation of the data presented in this thesis are listed in Table 2.6. Materials which have appeared in previous tables within this chapter are not listed. Similarly, the specific machines, software, or deposited data inventories used, are not listed here.

Table 2.6. List of key materials and resources used in Paper II, Paper III, and Paper IV.

REAGENT	SOURCE	CATALOGUE NUMBER
2-Deoxy-D-Glucose (2DG)	Sigma	D8375
5-amino-6-D-ribitylamouracil (5-A-RU)	Gifted from Professor Gurdyl Besra, University of Birmingham	N/A
Anti-APC MicroBeads	Miltenyi Biotec	130-090-855
Anti-PE MicroBeads	Miltenyi Biotec	130-048-801
Antimycin A	Sigma	A8674
autoMACS® Running Buffer – MACS® Separation Buffer	Miltenyi Biotec	130-091-221
Bovine Serum Albumin (BSA)	Sigma	A7030
Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP)	Sigma	C2920
CD71 (Transferrin Receptor) Monoclonal Antibody (OKT9 (OKT-9)), eBioscience	Invitrogen	14-0719-82
Cell-Tak Cell and Tissue Adhesive	Corning	CLS354240
CP91149	Sigma	PZ0104
CryoStor® cell cryopreservation media	Sigma	C2874
Deferoxamine mesylate salt (DFO)	Sigma	D9533
Dulbecco's Phosphate Buffered Saline (DPBS)	Sigma	D8537
Dynabeads Human T-Activator CD3/CD28 for T Cell Expansion and Activation	Gibco	11131D
eBioscience Fixable Viability Dye eFluor506	Invitrogen	65-0866-14
Fetal Bovine Serum (FBS), Value Heat Inactivated FBS	Gibco	A5256801
Hanks' Balanced Salt Solution (HBSS)	Gibco	14025092
HEPES (1M)	Gibco	15630080
holo-Transferrin human	Sigma	T4132
Human IFN-alpha A (alpha 2a) Protein (carrier-free) for cytokine stimulation	R&D Systems	10984-IF
Human IL-12 (p70) (carrier-free) Recombinant	BioLegend	573004

Human IL-15 (carrier-free) Recombinant	BioLegend	570304
Human IL-18 (carrier-free) Recombinant	BioLegend	592104
Human IL-2 (carrier-free) Recombinant	BioLegend	589106
Human IL-33 (carrier-free) Recombinant	BioLegend	581804
Human Plasma-Like Medium (HPLM)	Gibco	A4899101
LS Columns	Miltenyi Biotec	130-042-401
Luminescent ATP Detection Assay Kit	Abcam	ab113849
Lymphoprep	StemCell Technologies	18061
MACS® Comp Bead Kit, anti-mouse IgK	Miltenyi Biotec	130-097-900
MACS® Comp Bead Kit, anti-REA	Miltenyi Biotec	130-104-693
MEM Non-Essential Amino Acids Solution (100X)	Gibco	11140035
Methylglyoxal	Sigma	M0252
MitoTracker™ Deep Red FM Dye, for flow cytometry	Invitrogen	M46753
MitoTracker™ Green FM Dye, for flow cytometry	Invitrogen	M46750
Molecular-Grade Water	Sigma	W4502
Monensin Sodium Salt	Sigma	M5273
Mouse IgG1 kappa Isotype Control (P3.6.2.8.1), eBioscience	Invitrogen	14-4714-82
Oligomycin A	Sigma	75351
Paraformaldehyde solution 4% in PBS	Santa Cruz	SC-281692
Penicillin-Streptomycin	Sigma	P4333
PerfeCTa SYBR® Green FastMix	QuantaBio	95073
Protein Transport Inhibitor Cocktail	Invitrogen	00-4980-93
Puromycin	Sigma	P7255
qScript cDNA Synthesis Kit	QuantaBio	95047
RPMI 1640 Medium + GlutaMAX	Gibco	61870010
RPMI 1640 Medium No Glutamine	Gibco	21870076
Rotenone	Sigma	R8875
Saponin	Sigma	47036
Seahorse XFe96/XF Pro FluxPak Mini	Agilent	103793-100
Seahorse XF 1.0 M glucose	Agilent	103577-100
Seahorse XF 100 mM pyruvate	Agilent	103578-100
Seahorse XF 200 mM glutamine	Agilent	103579-100
Seahorse XF RPMI medium, pH 7.4	Agilent	103576-100
SepMate™ PBMC Isolation Tubes	StemCell Technologies	85460
Sodium Bicarbonate	Honeywell Fluka	S6014-1KG
Sodium Pyruvate (100 mM)	Gibco	11360070
Sulfuric Acid	Sigma	339741
TMB Substrate Set	BioLegend	421101
Transferrin From Human Serum, Alexa Fluor™ 647 Conjugate	Invitrogen	T23366
TRIzol Reagent	Invitrogen	15596018
True Nuclear Transcription Factor Buffer Set	BioLegend	424401

TWEEN® 20	Sigma	P1379
Ultra-LEAF™ Purified anti-human CD28 Antibody	BioLegend	302934
Ultra-LEAF™ Purified anti-human CD3 Antibody	BioLegend	317326

CHAPTER 3. Paper I

This chapter consists of a first-author literature review, published in *Science Signalling* (2026).

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The Environmental Regulation of Mucosal Associated Invariant T cells – Adding Food for Thought.

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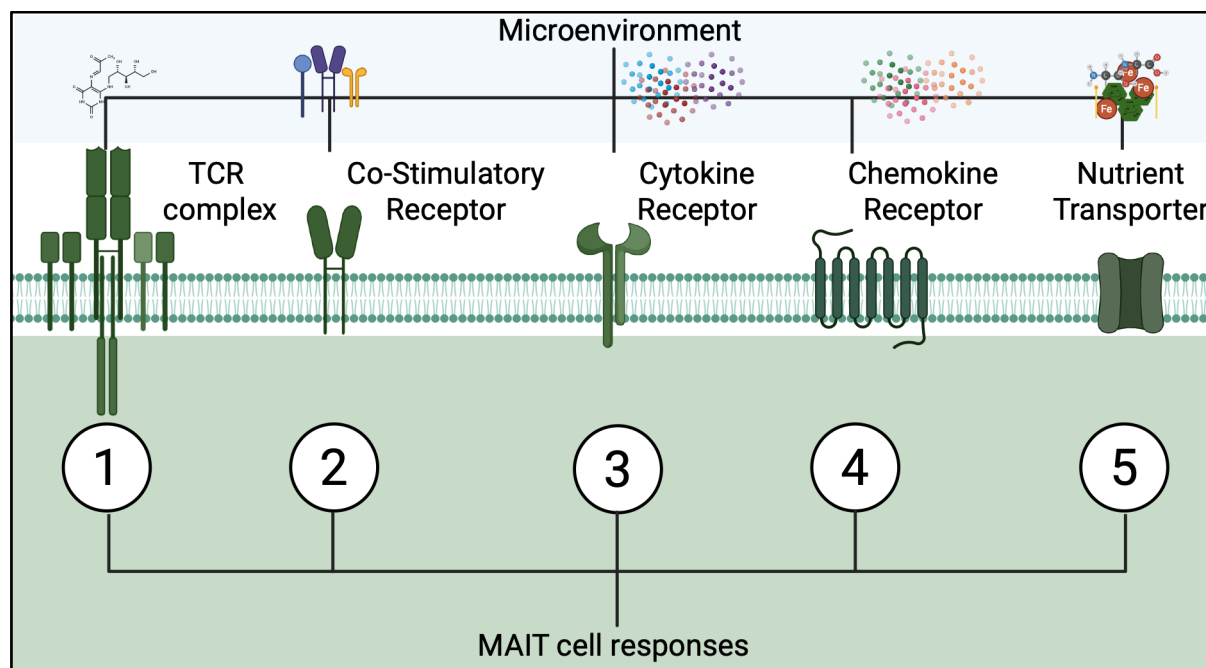
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Graphical Abstract



Abstract

Mucosal-Associated Invariant T (MAIT) cells are a population of innate-like, unconventional T cells characterized by the expression of a semi-invariant, non-MHC restricted T cell receptor (TCR); with a significant role in mediating innate-immune responses to bacterial and viral pathogens. Emerging research continues to describe the plethora of environmental signals which influence the type of response elicited downstream of activation. In this review, we highlight five key factors which dictate MAIT cell metabolic and functional responses, including TCR engagement and co-stimulation, chemokine signalling, cytokine stimulation and nutrient availability. Here, we further define the importance of optimal nutrient availability as 'signal 5' in promoting MAIT cell fitness, and in governing their capacity to respond appropriately to challenge. In understanding the way in which MAIT cells are influenced by their microenvironment, we can continue to identify the potential factors which drive dysregulated responses in hostile circumstances, allowing for restoration of their protective nature in the context of infection, or in the tumour microenvironment.

MAIT Cell Phenotype and Functional Importance

Mucosal-Associated Invariant T (MAIT) cells are a group of unconventional effector T cells that comprise a critical component of the host immune response, capable of eliciting robust host-protective responses against a range of bacterial and viral pathogens¹⁻⁵. MAIT cells are characterized in humans primarily by their expression of a conserved T cell receptor (TCR) alpha chain, *Va7.2 - Ja33*, paired with a limited repertoire of V β -chain segments^{6,7}; and high co-expression of the C-type lectin-like receptor, CD161⁸. MAIT cells are invariant in their restriction by the MHC-class-I -like molecule, MR1, which facilitates the presentation of antigenic derivatives of the microbial riboflavin biosynthesis pathway^{9,10}. MAIT cell frequencies are enriched in the periphery, accounting for 1-10% of circulating T cells in healthy individuals^{11,12}. Circulating human MAIT cells are predominantly CD8 single-positive (SP) (~80%), existing alongside a smaller but significant double-negative (DN) population (~15%)¹²⁻¹⁴. Though CD4 SP and CD4/CD8 double-positive (DP) phenotypes have been confirmed as 'true' MAIT cell subpopulations using MR1 tetramers, circulating CD4+ MAIT cells comprise a minor proportion in healthy individuals^{12,15}. CD8+ MAIT cells are further characterized by constitutively high cell surface expression of the IL-18 receptor (IL-18R)^{16,17}, which facilitates robust effector responses. Of note and to be discussed, MAIT cell activation can be independently elicited by two distinct mechanisms: upon TCR engagement¹⁰ or downstream of stimulation with innate cytokines such as IL-18 and IL-12⁴, though robust functional responses are observed in the context of synergistic signalling through both pathways.

Upon activation, MAIT cells take on an effector phenotype, characterized by the production of a milieu of cytokines including IFN γ , TNF α , and IL-17; as well as lytic molecules, such as perforin and granzyme B¹⁸. Owing to this, activated MAIT cells depict an increased capacity for targeted cell killing, and can elicit cytotoxic responses against infected or malignant-transformed cells¹⁹⁻²¹. Another key attribute of the MAIT cell innate-effector phenotype is their capacity to undergo rapid proliferative expansion downstream of activation, which further underpins their role in innate immune defence²²⁻²⁵. The MAIT cell effector programme

is facilitated by acquired expression of the innate master transcription factor, PLZF, during thymic development²⁶; and the rapid acquisition of a memory phenotype after birth²⁷.

MAIT cells also display a tissue-residency phenotype, and as such they are enriched in the liver, lung, gut, and skin²⁸. This tissue-homing capacity is thought also to be underpinned by PLZF, which has been shown to mediate the expression of homing and adhesion receptors in other cell types^{29,30}. Interestingly, adoptive transfer experiments in mice confirmed that MAIT cells are pre-programmed in the thymus to preferentially home to peripheral tissues³¹. Human MAIT cell frequencies are most abundant in the liver, an important site of immune surveillance and defence against gut-derived pathogens^{11,32,33}. When compared with other hepatic T cells, liver derived MAIT cells present with a distinct tissue residency signature characterized by reduced expression of several chemokine receptors, and the circulatory marker, CD62L; alongside increased expression of the tissue residency marker, CD69³¹. MAIT cells expressing CD56 comprise the dominant population in the liver, armed with a robust effector capacity characterized by a greater responsiveness to stimulation, and an enhanced polyfunctional response profile³⁴, which may have implications both in the context of immune defence, and inflammation-mediated tissue pathology. However, transcriptomic analyses have also revealed that upon activation, MAIT cells express genes involved in tissue angiogenesis and repair, such as *VEGFA* and *AREG*³⁵⁻³⁷. Furthermore, recent studies have elucidated a protective role for MAIT cells in the context of tissue injury, suggesting that MAIT cells have a role in promoting tissue homeostasis and repair³⁸⁻⁴⁰.

Environmental Signals 1 & 2: TCR Triggering and Co-Stimulation

Classically, the first signal for T cell activation is TCR engagement⁴¹ (Figure 1). As highlighted, MAIT cells are defined by their expression of a conserved TCR α -chain which is paired to a limited repertoire of β chains⁹. This semi-invariant TCR is restricted to antigens presented on MR1, and the most potent activating antigen reported to date derives from an intermediate of the microbial vitamin B₂ (riboflavin) pathway, known as 5-amino-6-D-ribitylaminouracil (5-A-RU)^{10,42}. This intermediate generates 5-OP-RU, which is stabilized and presented by MR1. It is worth noting, however, that MR1 in the absence of bound antigen, is not surface

expressed^{43,44}. Therefore, MAIT cells are responsive in environments where their cognate microbial antigen is present, such as in the gut, thymus, skin, liver, and periphery^{39,45,46}. Tumour infiltrating MAIT cells can also display signatures of bacterial activation, highlighting the ubiquitous reach of their antigen⁴⁷. More recently, the first supporting evidence of a host-derived MAIT cell antigen was reported⁴⁸. A sulphated bile acid, cholic acid 7-sulfate (CA7S), was shown to bind MR1 and support MAIT cell development and homeostasis. Deletion of the sulfotransferase 2a family of enzymes responsible for CA7S synthesis resulted in a reduction of thymic MAIT cells in mice; and unlike the microbial derived 5-OP-RU, CA7S did not induce MAIT cell activation but rather drove the expression of a homeostatic gene signature⁴⁸. In addition, host-generated riboflavin catabolites have been identified in human blood with Abdelaal *et al*⁴⁹ elucidating a role for such in regulating MR1 surface expression, and MAIT cell responses. The riboflavin catabolites, 10-formylmethylflavin (FMF), lumichrome, lumiflavin, and alloxazine, were found to bind and stabilize MR1, and in contrast to microbial-derived antigens, prevent the egress of MR1 to the cell surface. These catabolites were also found to be potent competitors of 5-OP-RU, restricting MAIT cell activation in a dose-dependent manner. Overall, the authors hypothesise that host-generated riboflavin catabolites act as regulators of the MR1-MAIT axis to prevent aberrant MAIT cell responses⁴⁹. It should be noted however, that the TCR-MR1 environmental regulation of MAIT cells extends beyond the context of host and microbial derived antigens. Small drug metabolites and drug-like molecules including salicylates and diclofenac are known to bind MR1, and have been shown to impact MAIT cell responses, in both an inhibitory and activatory manner⁵⁰. In addition, several components of cigarette smoke have been shown to bind MR1, resulting in the modulation of MAIT cell responses and subsequently hampering MAIT cells responses to influenza infection⁵¹.

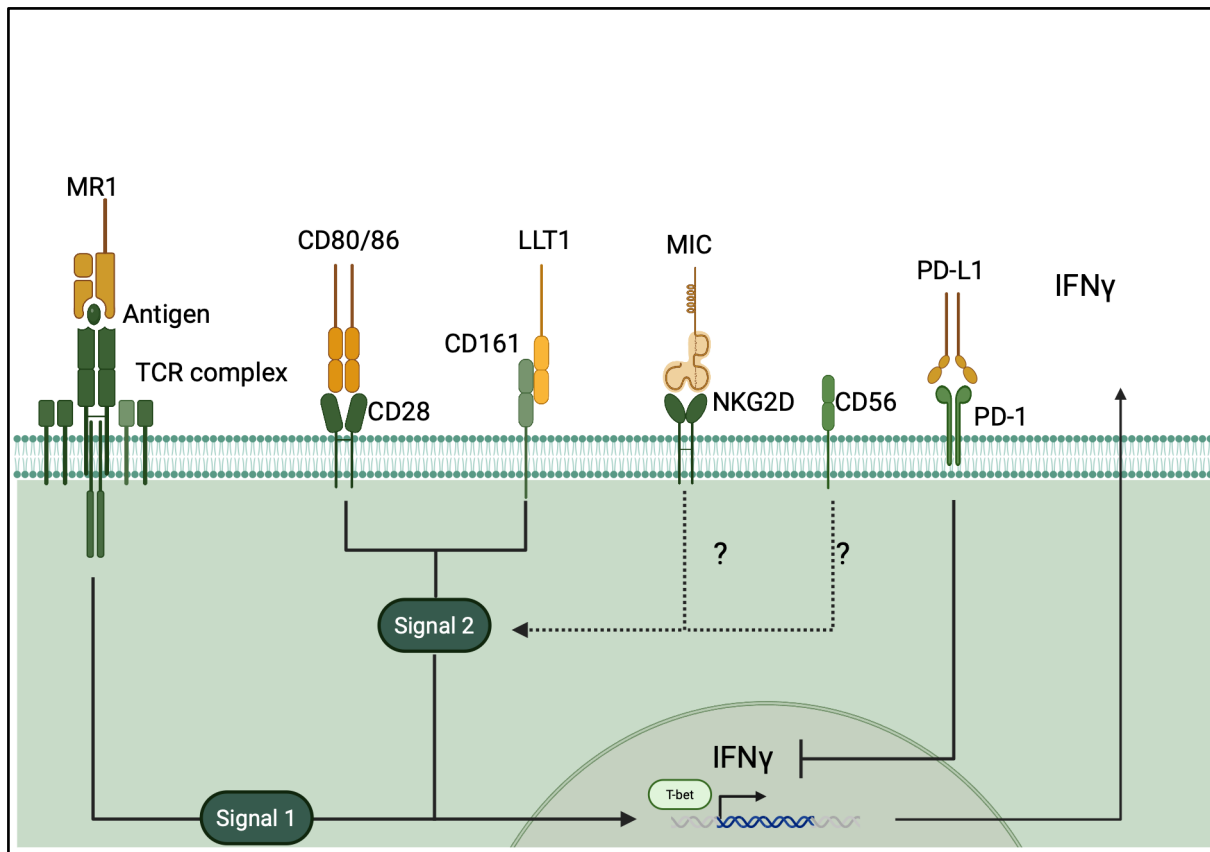


Figure 1. Signal 1 and Signal 2 dictate MAIT cell responses. Overview of T cell receptor (TCR) and co-stimulatory molecule signalling in MAIT cells. MAIT cells express a semi-invariant TCR which recognizes MR1 presentation of the cognate MAIT cell antigen 5-OP-RU leading to intracellular signalling (termed signal 1) and activation of cytokine production by transcription factors such as T-bet. In parallel MAIT cells receive co-stimulation signals through surface expressed molecules such as CD28 and CD161 which can synergize (termed signal 2) with the TCR signal to promote cellular responses. In addition, surface expression of NKG2D and CD56 may modulate MAIT cell responses. Finally, PD-1 is a well-established negative regulator of MAIT cell responses.

Signal 2 for T cell activation comprises of co-stimulatory interactions from membrane bound molecules expressed by cells encountered in the immediate microenvironment (Figure 1). The classical example of such is CD28, which provides co-stimulatory signals after binding specific ligands, such as CD80 or CD86, expressed on surrounding immune cells, like dendritic cells⁵². MAIT cell responses are also supported by CD28 co-stimulation^{53,54}, with Kurioka and colleagues demonstrating that CD3 stimulation alone was not sufficient to drive MAIT cell responses¹⁹. Similarly, Dias and colleagues demonstrated that CD28 co-stimulation boosted human MAIT cell responses to *Escherichia coli* *in vitro*⁵⁵. In contrast, murine MAIT cell responses were not dependent on CD28 signalling in a *Francisella tularensis* infection model⁵⁶. As previously mentioned, MAIT cells are defined by high surface expression of the

co-stimulatory receptor CD161, which interacts with ligands such as LLT1, expressed on surrounding immune cells⁵⁷. Fergusson and colleagues found that CD161 ligation boosted IFN γ and TNF production in MAIT cells, when paired with TCR but not cytokine stimulation⁵⁸. In agreement, loss of CD161 limited the production of IFN γ in CD3/CD28 triggered MAIT cells⁵³.

In line with their innate-like status, MAIT express a range of NK-like receptors including the NKG2 family of molecules^{12,14}, which can act as inhibitory (NKG2A) or activating (NKG2D) receptors⁵⁹. NKG2D recognizes ligands which are increased on cells that are subject to cellular stress, such as on infected, or transformed cells. It is constitutively expressed on conventional human CD8+ T cells, and acts as a co-stimulatory receptor alongside TCR activation to enhance downstream activation and functional responses⁶⁰. More recently, NKG2D has been shown to promote CD8+ T memory cell formation⁶¹⁻⁶³, and antibody-mediated co-stimulation by NKG2D rescued CD4-unhelped CD8+ memory T cell responses from patients with HIV⁶². As such, therapeutics focused on engaging the NKG2D pathway in CD8+ T cells are of clinical interest, and may have implications in the settings of anti-tumour and anti-viral immunity⁶⁰. However, the NKG2D signalling axis has also been implicated in immunopathology. Kim *et al.*, have previously shown that IL-15 derived from hepatitis A-infected cells activates memory CD8+ T cells and licenses TCR-independent cytotoxicity induced by NKG2D and NKp30; which is associated with increased liver injury during acute infection⁶⁴. Similarly, in MAIT cells stimulated with IL-15, NKG2D facilitated TCR-independent cytotoxic responses, which was also linked to hepatocyte injury in the setting of hepatitis A infection⁶⁵. However, whether NKG2D has a co-stimulatory role in potentiating TCR-mediated effector responses in MAIT cells remains to be elucidated, and could be important in the context of developing MAIT cell-focused immunotherapeutics.

Interestingly, MAIT cells also express the adhesion molecule CD56 at heterogenous levels across tissues, with the highest expression observed on hepatic MAIT cells³⁴. Though it is typically considered as a surface marker for the identification of human NK cells, a role for CD56 in mediating functional responses in NK cells has been described. Picard and colleagues have shown that NK cells which are pre-licensed by cytokine stimulation elicit functional responses to antibody-mediated engagement of CD56, as evident by increased degranulation,

and secretion of IFN γ ⁶⁶. CD56-expressing MAIT cells comprise a subpopulation characterised by enhanced effector responses to stimulation⁶⁷⁻⁶⁹, alongside increased expression of the transcription factors PLZF, T-bet, and eomesodermin⁶⁷. MAIT cells which express CD56 have also been shown to bind MR1 tetramer more robustly⁶⁹, however, it is not known whether CD56 has a direct role in the modulation of MAIT cell effector responses through co-stimulatory interactions, or otherwise.

Expression of the checkpoint molecule Programmed Cell Death Protein-1 (PD-1) leads to inhibition of cellular responses upon binding its ligand, PD-L1, and several studies have reported high PD-1 expression on circulating MAIT cells in diseased settings⁷⁰⁻⁷⁷. Ruf and colleagues have identified an axis between PD-L1 expressing tumour-associated macrophages, and MAIT cells in the non-tumour adjacent liver, as a potential driver of dysregulated MAIT cell responses in liver cancer; whereby hepatic MAIT cells present with an impaired tumour-infiltrating capacity, alongside a dysfunctional phenotype associated with high cell surface expression of PD-1 and TIM-3⁷⁴. Administration of an immune checkpoint blockade (ICB) targeting PD-L1 resulted in increased tumour-infiltration by MAIT cells in an *in vivo* mouse model of Hepatocellular Carcinoma (HCC), alongside improved granzyme B production from the intrahepatic MAIT cell fraction *ex vivo*⁷⁴. Similarly, Jarvis and colleagues identified significantly increased PD-1 expression on MAIT cells from patients with pancreatic cancer following *ex vivo* stimulation with the MR1 ligand, 5-A-RU/MG; and blocking PD-1 using the monoclonal antibody pembrolizumab boosted MAIT cell cytotoxicity and IFN γ production *in vitro*⁷¹. Circulating MAIT cell frequencies were associated with responsiveness to PD-1 therapy in patients with metastatic melanoma, and anti-PD-1 therapy increased MAIT cell effector gene expression, and cell surface expression of the chemokine receptor, CXCR4⁷³. Accordingly, the authors hypothesise that anti-PD-1 therapy might facilitate the migration of MAIT cells towards metastases, supported by the fact that MAIT cell numbers were increased in melanoma lesions in those responding to the ICB, versus those who did not respond⁷³. Overall, these data further highlight the PD-1/PD-L1 axis as a plausible driver of MAIT cell dysfunction in cancer, and emphasise the potential in targeting microenvironmental signals to restore MAIT cell functionality in disease settings.

Environmental Signal 3: Cytokines

Cytokines can act in synergy with TCR signalling to potentiate robust downstream effector responses, and in this manner are considered as environmental ‘signal 3’ in modulating MAIT cell functionality. When directly compared with conventional CD8+ memory T cells, MAIT cell effector responses to TCR triggering alone appear weak, even when paired with robust “signal 2” co-stimulation from CD28, for example⁷⁸. Sustained MAIT cell effector responses are seen only when TCR-engagement and co-stimulation are paired with inflammatory signals (signal 3) from cytokines like IL-12, IL-18, and IL-15, for example^{36,78}. Considering that the MAIT cell TCR recognizes antigenic by-products derived from the riboflavin biosynthesis pathway, something that is conserved amongst both commensal and pathogenic bacteria, it is hypothesised that this tertiary requirement of an inflammatory cue provides a level of protection against hyperresponsiveness to commensals at mucosal sites⁷⁹. Thus, cytokines are considered an important microenvironmental ‘signal 3’ in the context of driving MAIT cell functional responses. However, a major divergence of MAIT cells from conventional T cells is seen in their nuanced ability to respond directly to cytokines in a TCR-independent manner¹⁸ (Figure 2). The activation of MAIT cells during human viral infections is thought to occur independently of TCR-triggering through cytokine stimulation alone, as viruses are incapable of synthesizing riboflavin⁴ –the antigenic donor for MAIT cell TCR activation. It has also been shown that MAIT display transcriptional diversity downstream of stimulation with cytokines alone versus when in conjunction with TCR signalling⁶⁸; therefore, we will discuss the role of environmental cytokines as modulators of MAIT cell effector responses within the classical context of ‘signal 3’, and within the context of MAIT cell specific, TCR-independent effector functionality.

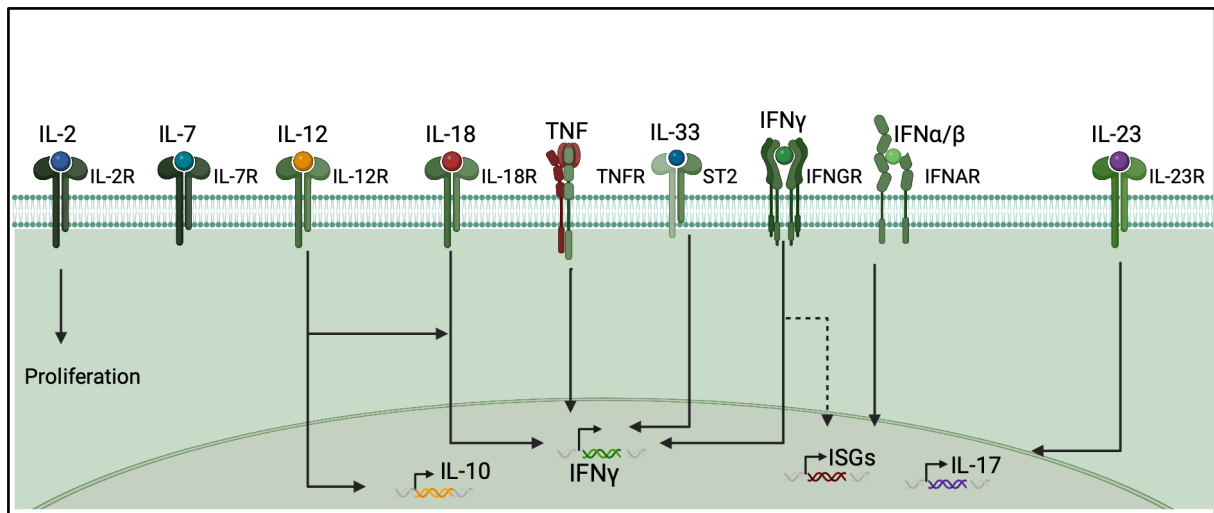


Figure 2. Cytokines (Signal 3) direct MAIT cell functional responses. Overview of cytokine signalling in MAIT cells. MAIT cells express multiple cytokine receptors (both constitutively (as with IL-18R) and after activation (as with the IL-33 receptor ST2)). Upon environmental cytokines binding their cognate, surface expressed receptor, intracellular signals (termed signal 3) promote MAIT cell proliferative responses (through the secretion of IL-2, for example); or lead to transcriptional responses, with increased expression of cytokines or interferon stimulated genes, for example. In most cases, two or more cytokines can synergistically promote MAIT cell responses (as with IL-12 and IL-18). Cytokines can also synergistically modulate TCR-driven MAIT cell responses.

Human MAIT cells express a plethora of cytokine receptors and exhibit the highest resting cell surface expression of the IL-18R out of all T lymphocytes^{17,58}. As such, MAIT cells are pre-licensed to respond to cytokine-stimulation, and in particular, to combinations of cytokines⁸⁰. The first cytokine pairing to be characterized as a potent modulator of MAIT cell functional responses was that of IL-12 and IL-18. Both IL-12 and IL-18 have been highlighted as significant modulators of MAIT cell activation and associated host protective responses during viral infection *in vitro* and *in vivo*⁸¹. Interestingly, IL-18 has been identified as the dominant signal involved in driving MAIT cell host-protective responses to Influenza A virus infection *in vivo*⁵, though it is also speculated to contribute to MAIT cell exhaustion and increased viral pathogenicity in certain disease contexts⁸²⁻⁸⁴. Long-term stimulation of MAIT cells with IL-12/IL-18 has also been shown to induce an exhaustive state *in vitro*, as characterized by significantly increased cell surface expression of CD39, further highlighting their sensitivity to inflammatory cytokine signalling, and their susceptibility to dysfunction in hyperinflammatory settings. *In vitro* stimulation of MAIT cells with a combination of IL-12/IL-18 induces upregulation of Tbet alongside robust IFNγ production, and increased expression of cytolytic molecules including perforin, and granzymes B, H, A, K and M^{17,85,86}. In the context of IL-12/IL-

18 signalling as 'signal 3' in potentiating MAIT cell effector responses, the synergistic activity of IL-12/IL-18 co-stimulation alongside TCR triggering is required to elicit optimal production of IL-17 in MAIT cells *in vitro*⁸⁷. Furthermore, Leng and colleagues confirmed that a combination of IL-12/IL-18 co-stimulation alongside TCR engagement resulted in amplification of the effector profile in human MAIT cells *in vitro*, when compared to that elicited by signal 1 and 2, or signal 3 alone⁷⁹.

Interestingly, autoimmune liver disease (AILD) is associated with chronic inflammation, and high serum levels of proinflammatory cytokines, such as IL-18, IL-12, and IL-1 β . It has been found that repetitive stimulation of MAIT cells with IL-12 and IL-18 *ex vivo* could recapitulate the exhaustive phenotype described in MAIT cells from patients with AILD⁷⁵. IL-17A has been implicated in the development of liver fibrosis in diseased settings, and the frequency of T_H17 cell infiltration in the liver positively correlated with hepatic inflammation and fibrosis grade in autoimmune hepatitis⁸⁸. Böttcher and colleagues noted that repetitive stimulation of MAIT cells with IL-12 elicited secretion of IL-17A, and MAIT cell derived IL-17A was involved in the activation and proliferation of hepatic stellate cells in a coculture system *in vitro*⁷⁵. Overall, these data suggest that the hyperactivation of hepatic MAIT cells through inflammatory cytokine stimulation might potentiate liver fibrosis in diseased settings, thus contributing to the inflammatory burden of the disease.

Though co-stimulation with IL-18/IL-12 drives a dominant inflammatory phenotype *in vitro*, stimulation with IL-12 alone alongside cognate antigen recognition has been shown to induce a regulatory phenotype in human MAIT cells, characterized by upregulation of TIM-3, LAG-3, and PD-1⁸⁹. Furthermore, IL-12 co-stimulation elicits upregulation of c-MAF and a resulting robust increase in the production of IL-10, which is abrogated in the presence of IL-18 signalling⁸⁹; indicative of a role for IL-18 as an inflammatory signal which potentiates the effector phenotype in the context of microbial challenge. In a similar context, administration of IL-12 can polarize MAIT cell responses towards the MAIT1 phenotype in mice infected with *Legionella longbeachae*, an infection which typically skews responses towards the MAIT17 phenotype⁵⁶; further recapitulating the intricate plasticity of MAIT cell responses, and their susceptibility to distinct microenvironmental cues.

Both IL-12 and IL-23 are heterodimeric cytokines which share a common β subunit, IL-12p40, and more recently, MAIT cell responses to IL-23 signalling have begun to be elucidated. Wang and colleagues have previously described diminished MAIT cell accumulation in the lungs of IL-23-knockout mice infected with *Salmonella enterica* serovar *typhimurium* (*S. Typhimurium*), which may suggest a protective role for IL-23 signalling in the context of microbial challenge⁹⁰. Both IL-12 and IL-23 were found to promote the expansion of MAIT1 cells during an *in vivo* model of acute tularemia⁵⁶, whilst IL-23 co-stimulation alongside administration of 5-OP-RU has also been shown to promote the expansion of MAIT17 cells *in vivo*; and skewed human MAIT cell responses towards those associated with the T_H17-like phenotype *in vitro*⁹⁰. Similarly, in patients with primary Sjogren's syndrome (pSS), MAIT cells are depleted in the blood and accumulate in the salivary glands with a diminished T_H1 expression profile⁹¹. *In vitro*, these cells present with increased sensitivity to IL-23 and IL-7 signalling, which induced polarization towards the IL-17 phenotype through overexpression of RORc, or by a STAT3/HIF1- α signalling axis, respectively⁹². Conversely, IL-23 has more recently been shown to license a regulatory phenotype in human MAIT cells *in vitro*, inducing significant production of IL-10 when present as a secondary stimulus alongside TCR engagement; something that the authors hypothesise might be important in the context of commensal antigen recognition, in the absence of a proinflammatory cue from IL-18⁸⁹.

MAIT cells express high levels of the IL-7 receptor³², and like IL-23, co-stimulation with IL-7 has been shown to induce a T_H17-like phenotype in MAIT cells. Intrasinusoidal MAIT cells are licensed to respond robustly to anti-CD3/CD28 through IL-7 co-stimulation, which increases the production of IL-17A and IFN γ ³². Furthermore, stimulation with IL-7 could promote the proliferation of liver-derived MAIT cells *in vitro*⁶⁵. IL-7 is secreted by hepatocytes in response to TLR-signalling⁹³, something which again alludes to the physiological importance of signal 3 in potentiating MAIT cell effector responses to pathogenic challenge. Similarly, in patients with Ankylosing Spondylitis (AS), MAIT cells are enriched in the synovial fluid and present with a robust IL-17 phenotype⁹⁴. *In vitro*, MAIT cells from patients with AS displayed elevated IL-17 production when primed with IL-7 prior to TCR stimulation, which is concomitant with increased levels of IL-7 in the gut and joints of patients with AS⁹⁴. Stimulation with IL-7 alone was sufficient in inducing a cytotoxic capacity in human MAIT cells *in vitro*, licensing

upregulation of granzyme B, and increased expression of perforin, and granzyme A; whilst priming with IL-7 improved MAIT cell sensitivity to antigenic challenge⁹⁵.

More recently, MAIT cell responses to type I IFNs have been elucidated. Previously, it had been shown that MAIT cells are functionally responsive to IFN α / β *in vitro*, only when co-administered with IL-12 or IL-18⁴. Similarly, Pavlovic and colleagues have shown that in response to IFN α signalling alone, MAIT cells increase cell surface expression of the activation marker CD69, but fail to respond functionally, suggesting a preventative mechanism against hyperactivation. Interestingly, IFN α can also synergize with TCR stimulation to drive robust cytokine responses in MAIT cells *in vitro*, and to elicit a cytotoxic capacity as defined by increased expression of perforin, and granzyme B^{4,96}. In mice, MAIT cell responses to *K. pneumoniae*, which include activation, tissue localization, and T_H1-like transcriptional reprogramming, occur independently of MR1, and are instead driven by type I IFNs³. This was further recapitulated *in vitro*, whereby co-culture of human MAIT cells in PBMC with *Klebsiella* induced upregulation of a type I IFN-associated gene signature, as characterized by increased expression of interferon stimulated genes (ISGs), like *MX1* and *IFIT1*. This type I IFN-MAIT cell axis was protective against infection with *K. pneumoniae* in mice³, once more highlighting a physiological importance for MAIT cell responses to type I IFNs. Recently, the Klenerman group reported on MAIT cell responses to SARS-CoV-2 spike peptide challenge in individuals vaccinated with the BNT162b2 vaccine against SARS-CoV-2, which was mediated by memory T cell production of another interferon family member, IFN γ eliciting cytokine responses from MAIT cells including the production of IFN γ ⁹⁷.

Other cytokines which can promote MAIT cell effector responses include IL-15, and IL-2. Both IL-15 and IL-2 can induce the expansion of MAIT1 cells *in vivo*, as characterized by upregulation of Tbet⁹⁸. Similarly, human MAIT cells were shown to proliferate *in vitro* upon stimulation with IL-15 alone, or when administered in synergy with a TCR stimulus^{65,99}, whilst IL-2 can also act as an environmental signal which potentiates antigen responses to facilitate the robust expansion of MAIT cells *in vitro*²⁵. Stimulation with IL-15 alone, but not IL-2, was also sufficient in inducing a robust T_H1-like cytokine expression profile in liver-derived MAIT cells⁶⁵, and acts in synergy with either IL-12 or IL-18 to elicit significant IFN γ production in peripheral blood MAIT cells *in vitro*⁴. Furthermore, IL-15 stimulation of liver-derived MAIT

cells drives robust cytotoxicity *in vitro*, independent of MR1⁶⁵; and IL-15 is thought to act in synergy with IL-12 and IL-18 to promote MAIT cell host-protective responses against Influenza A virus infection *in vivo*⁵.

In addition, a novel role for the tissue-derived alarmin, IL-33, has been described in the context of driving MAIT cell functional responses. IL-33 is a member of the IL-1 family of cytokines¹⁰⁰, and is constitutively expressed in epithelial barrier tissues¹⁰¹. IL-33 acts as an alarmin in that it is released during instances of tissue damage or cellular stress caused by infection, for example. Azzout and colleagues have described an inflammatory response elicited by MAIT cells upon stimulation with IL-33 and IL-12 in combination, as confirmed by increased expression of IFN γ , TNF α , and granzyme B¹⁰². Furthermore, the activation of MAIT cells post liver transplant was found to correlate significantly with serum levels of IL-33¹⁰³. However, MAIT cell activation further correlated with the severity of liver dysfunction following graft reperfusion; indicative of a potentially deleterious role for the MAIT/IL-33 axis during sterile inflammation¹⁰³, further highlighting the complexity of MAIT cell responses to environmental stimuli.

Environmental Signal 4: Chemokines

Although not typically considered as a signal required for T cell activation, chemokines can significantly modulate cellular immune responses within specific microenvironments (Figure 3) by directing the migration of cells to sites of inflammation, in a process referred to as 'homing'. MAIT cells are abundant in the periphery and are enriched across a wide range of human tissues. Moreover, MAIT cells are actively recruited to sites of infection and inflammation^{18,104}. Accordingly, MAIT cells express a wide repertoire of chemokine receptors, including but not limited to CCR2, CCR5, CCR6, and CXCR6^{18,105}, facilitating their homing to sites of inflammation. MAIT cells are most abundant in the liver, and in 2011 Dusseaux and colleagues first reported the chemokine expression profile (CCR5, CCR6, CCR9 and CXCR6) which supports preferential homing to both liver and gut tissues¹¹. MAIT cells also migrate to sites of infection¹⁰⁶⁻¹⁰⁸. In a human challenge with *Salmonella enterica* serovar Typhi, MAIT cell frequencies declined in the periphery and presented with an increased expression of CCR6

and CCR9 in individuals susceptible to infection, suggesting migration to the site of infection¹⁰⁹. In a model of *Francisella tularensis*, MAIT cell accumulated in the infected lung and expressed high levels of CXCR6. Though CXCR6 was found to be dispensable for their recruitment, the associated ligand CXCL16 was sufficient to drive MAIT cell recruitment to the lung, even in the absence of infection¹¹⁰. In patients with mucosal associated cancers, MAIT cells accumulate within the tumour and display significant expression of CCR6 CCR9 and CXCR6^{111,112}, chemokine receptors linked to increased migration to gut tissue, which was further supported by elevated levels of the ligand CCL20 and CXCL16 in these mucosal tumours¹¹². In a recent study Zheng and colleagues comprehensively investigated the chemokine axis responsible for MAIT cell recruitment to the inflamed appendix¹¹³, and reported significant expression of CCR2, CCR4, CCR5, CCR6 and CXCR6 on peripheral blood MAIT cells in healthy controls and in individuals with an inflamed appendix. In addition, they reported expression of CCR1, CCR3 and CXCR1 on appendix infiltrating MAIT cells. Most notably, they demonstrated the accumulation of MAIT cells in patient derived appendix organoids was dependent on CCR1, CCR2 and CCR4¹¹³. In the setting of another inflammatory disease, hidradenitis suppurativa, we reported an accumulation of MAIT cells in the lesions, alongside elevated levels of CCL20 which actively promoted MAIT cell migration¹¹⁴. In patients with pSS, MAIT cells accumulate in the salivary glands¹¹⁵, and presented with increased expression of CCR9/CXCR5, something thought to drive migration towards elevated CCL25/CXCL13 levels¹¹⁶. However, the chemokine-mediated promotion of MAIT cells trafficking is not limited to inflammatory microenvironments. In the setting of pregnancy, MAIT cells are enriched in the intervillous blood of the placenta¹¹⁷. The authors highlighted MIF and CCL25 as recruiting factors, with supporting expression of CXCR4 and *in vitro* migration assays¹¹⁷.

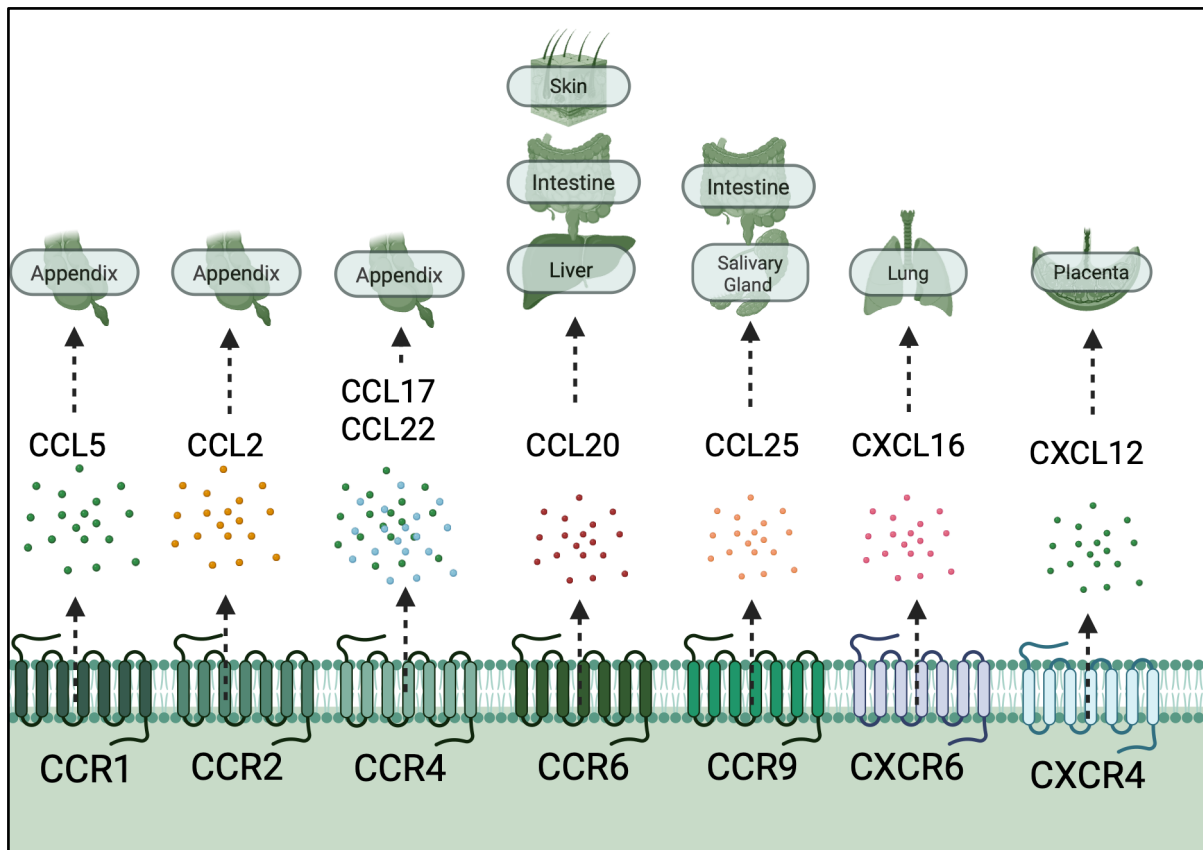


Figure 3. Chemokines as Signal 4 in the regulation of MAIT cell responses. Overview of chemokine responses in MAIT cells. MAIT cells express multiple chemokine receptors (both constitutively and after stimulation, and in a tissue specific manner). Upon environmental chemokine sensing (termed signal 4), MAIT cells expressing the cognate chemokine receptor promote MAIT cell homing to the site of chemokine accumulation.

Although the chemokine/chemokine receptor axis has a long-established role in mediating the homing of MAIT cells towards peripheral tissues, or towards inflammatory sites during infection, it is not yet known whether chemokines, or their receptors, can actively impact MAIT cell activation. For example, downstream of APC-derived chemokine signalling in T cells, the chemokine receptors CCR5 and CXCR4 are recruited into the immune synapse, and here act in a costimulatory manner to enhance T cell activation¹¹⁸. Whether this is relevant in the context of MAIT cell biology remains to be elucidated, and could perhaps be an interesting angle to interrogate in the context of elucidating the environmental regulators of MAIT cell effector responses.

Environmental Signal 5: Nutrients

Nutrients are a more recent addition to the environmental regulation list. Cellular metabolism is now recognized as a critical process that supports and controls immune cell function¹¹⁹. Cellular metabolism supports ATP production, macromolecule synthesis, intracellular signalling and post translational modifications, all of which are essential for immune cell responses. Immune cells can utilize different metabolic pathways depending on their functional needs¹²⁰. Classically, naïve T cells are quiescent and rely on oxidative phosphorylation for the maintenance, while activated T cells that are highly proliferative relying on glycolytic metabolism¹²¹.

Cellular metabolism is fine-tuned by optimal nutrient availability in the microenvironment. To maintain cellular metabolism, cells require an exogenous nutrient supply, which can exert a significant influence on cellular responses¹²². Highlighting the importance of exogenous nutrients, two major regulators of MAIT cell metabolic and functional responses are the nutrient sensor, mTORC1, and the nutrient-sensitive transcription factor, Myc. Myc is well-established as a master regulator of metabolism in conventional T cells, controlling the expression of nutrient transporters, and downstream metabolic pathways^{123,124}. In the absence of either mTORC1 or Myc, MAIT cells fail to engage their metabolic programme, and ultimately their effector functions. Both mTORC1 and Myc are highly sensitive to nutrient availability in the cellular microenvironment.

The cellular metabolic pathways utilized by MAIT cells have been an intense area of research for the past several years ¹²⁵⁻¹²⁷ (Figure 4). After challenge, MAIT cells rapidly respond, growing, proliferating, and producing new effector molecules. These responses are underpinned by the cellular metabolic program which requires a significant supply of nutrients. Glucose was the first nutrient to be highlighted as a critical determinant of MAIT cell functionality. In 2018, the Klenerman group reported that glycolysis was required for granzyme B production by MAIT cells¹²⁵. Shortly after, our group also highlighted the importance of glycolytic metabolism for MAIT cell production of IFN γ ¹²⁶, and demonstrated that this process was fuelled by the uptake of exogenous glucose from the environment.

Limiting glucose availability significantly blunted MAIT cell IFN γ production. Similarly, blocking the first step of glycolysis using 2-Deoxy-D-glucose also limited MAIT cell IFN γ production¹²⁶. Murine MAIT cell subsets display diverging metabolic profiles, though the IFN γ producing MAIT1 subset is dependent on glycolysis and the uptake of glucose, mirroring the human equivalent^{128,129}. In addition, MAIT cells possess glycogen synthase 1 (GYS1), the enzyme responsible for converting glucose into glycogen through glycogenesis. Accordingly, MAIT cells store glycogen, and rely on glycogen-fuelled glycolysis to fuel their rapid functional responses, until the cell can access a sufficient supply of glucose from the environment²¹. Given the importance of glycolysis for MAIT cell effector functioning, it is very likely that environments with a limited supply of glucose, characteristic of some tumour microenvironments (TME), might skew the MAIT cell metabolic profile, and consequently alter MAIT cell functional responses.

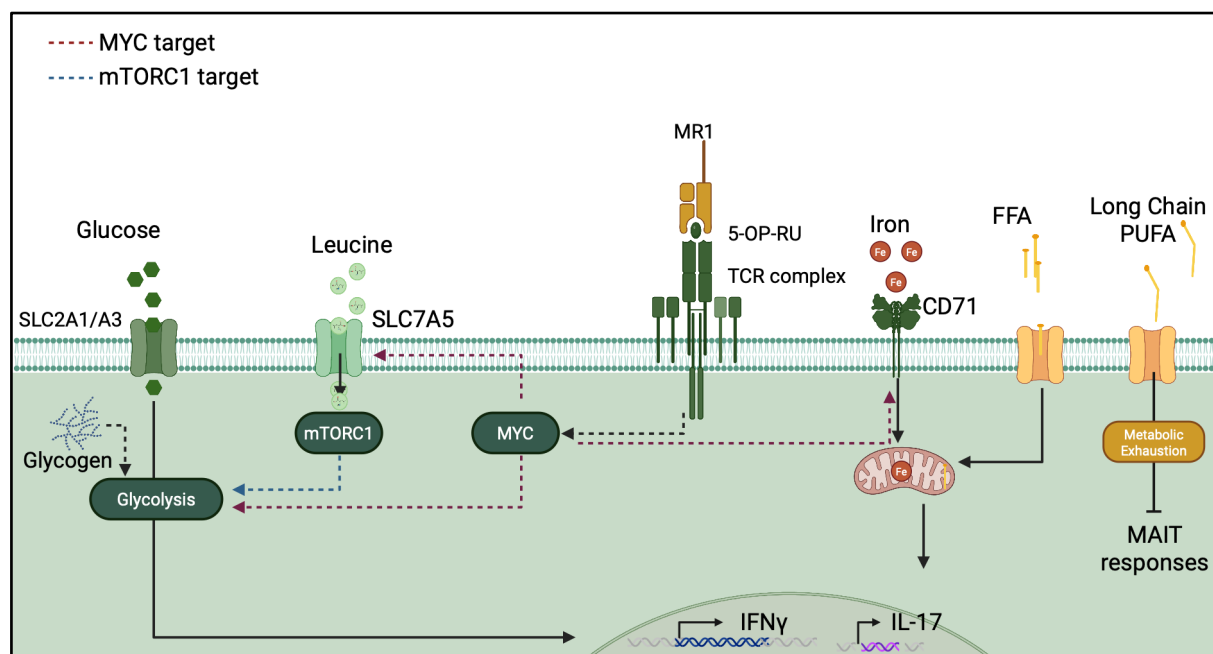


Figure 4. Nutrients as Signal 5 in the regulation of MAIT cell responses. At rest MAIT cells express low levels of nutrient transporters on their surface, but upon TCR stimulation (with the cognate MAIT cell antigen 5-OP-RU, for example) the metabolic regulator, Myc is activated. Activation of the Myc leads to the increased expression of nutrient transporters on the cell surface including glucose transporters such as SLC2A1, the large neutral amino acid transporter SLC7A5, and the iron transporter CD71. Nutrients are sensed by mTOR (termed Signal 5) leading to metabolic reprogramming which supports MAIT cell responses through a glucose – mTOR – glycolysis – IFN γ signalling axis. Conversely, nutrients associated with

disease settings can lead to the suppression of MAIT cells responses through metabolic exhaustion, as is the case for long chain fatty acids, for example.

In humans, IL-17 producing MAIT cells are rare but are often expanded in diseased settings¹³⁰. Obesity is associated with chronic low-grade inflammation which drives impaired insulin signalling, and hyperglycaemia. MAIT cells are dysfunctional in people with obesity, as characterised by diminished glycolytic metabolism, a limited effector capacity, and elevated secretion of IL-17. Interestingly, a high glucose environment increased mitochondrial ROS production by murine T cells, which subsequently resulted in ROS-mediated T_H17 differentiation¹³¹. The authors suggest that high concentrations of glucose might increase mitochondrial ROS production by inducing cellular oxidative stress¹³¹. Similarly, our group has shown that MAIT cells from people with obesity present with elevated levels of mitochondrial ROS, and this too was linked with increased secretion of IL-17¹²⁷. It is also important to note that increased frequencies of IL-17 producing MAIT cells in children with obesity positively correlate with the occurrence of insulin resistance, and *in vitro* experiments confirmed that MAIT cell derived IL-17 could disrupt insulin-mediated glucose uptake¹³². This suggests that although MAIT cells are likely impacted by altered nutrient content in the obese microenvironment, they also contribute to the overall inflammatory burden and may have a role in further potentiating the disturbances to systemic metabolism that are characteristic of the disease.

Iron is an essential micronutrient with a significant role in physiological processes like oxygen transport, energy production and immune regulation¹³³. We have recently highlighted iron as a critical regulator of MAIT cell host protective responses. Upon activation, human peripheral MAIT cells increase cell surface expression of the iron transporter, CD71, which correlates with increased iron uptake¹³⁴. Limiting the availability of iron in the microenvironment, or conversely, blocking iron uptake through use of a monoclonal against CD71, resulted in diminished metabolic capacity, reduced oxygen consumption, and a significant reduction in ATP levels. Iron-starved MAIT cells consequently present with a failure to proliferate and a diminished cytokine expression profile¹³⁴. Once more, these data highlight the importance of optimal nutrient availability as 'signal 5' in the potentiation of MAIT cell host-protective responses. Serum iron levels are controlled by the liver-derived hormone hepcidin, which

downregulates dietary iron absorption from the small intestine, and attenuates the mobilization of recycled iron from macrophages, and from the iron exporter, ferroportin¹³⁵. Inflammatory IL-6 is thought to drive increased production of hepcidin during microbial and viral challenge, as a mechanism of host-protection¹³⁶, though low-grade inflammation is also thought to promote the pathogenesis of chronic hypoferremia in obesity¹³⁷. It is thus plausible that the dysregulation of MAIT cell responses during infection, or in chronic inflammatory conditions like obesity, may in part be driven by disruptions to physiologic iron homeostasis. Alongside iron deficiency, iron overload has also been linked with aberrant immune functioning, and lends to an increased risk of infection and associated mortality¹³³. Iron overload has been shown to impair CD4+ T_H1 differentiation and associated IFN γ production in mice infected with *Candida albicans*¹³⁸, and in a murine model of *S. Typhimurium*¹³⁹. The observed impairments to T_H1 differentiation induced by iron excess in *S. Typhimurium* were linked with iron-mediated upregulation of TIM-3 on the cell surface¹³⁹. Furthermore, in patients with HFE-related hemochromatosis, V δ 2+ T cell frequencies in the blood were significantly reduced, and those which remained presented with increased expression of exhaustion markers, and production of ROS¹⁴⁰. The authors suggest that iron overload lends to constitutive triggering of the V δ 2 TCR, which subsequently results in activation-induced cell death¹⁴⁰. However, it is not yet known whether iron overload has an impact on MAIT cells, or their host-protective responses in infection.

The metabolism of intracellular amino acids is important for TCA cycle processing, and other cellular anabolic processes, such as protein synthesis. Furthermore, the regulation of mTORC1 activity is dictated by availability of amino acids like leucine, glutamine, and arginine^{141,142}. Cellular uptake of large neutral amino acids (LNAAs), like leucine and tryptophan, is controlled in activated T cells by cell surface expression of the System-L amino acid transporter, SLC7A5; and is required for metabolic reprogramming and effector differentiation in response to antigenic challenge¹⁴³. Indeed, our group has previously shown that MAIT cells activated by CD3/CD28 co-stimulation with a combination of IL-12/IL-18 increase cell surface expression of SLC7A5, whilst the associated amino acid flux is a critical determinant of mTORC1 activation, and the MAIT cell proliferative capacity.^{25,126} This pathway is defective in MAIT cells from people with obesity, something which likely contributes to the associated metabolic and functional dysregulation¹²⁶. Furthermore, the glutamate-derived metabolite

α -ketoglutarate is an important TCA cycle intermediate¹¹⁹, and has been shown to enhance MAIT cell effector responses to TCR triggering *in vitro*¹⁴⁴, suggesting an importance for the sensing and uptake of microenvironmental glutamine in the context of potentiating MAIT cell effector responses.

Although IL-17 producing MAIT cells represent a functional state in humans⁶⁸, MAIT17 cells are a distinct subset in mice, and display a divergent metabolic program characterised by an increased dependency on mitochondrial metabolism (OXPHOS)¹²⁹. This metabolic program is thought to be fuelled by exogenous fatty acids, whereby MAIT17 cells present with elevated lipid uptake and intracellular lipid content after challenge¹²⁹. However, it seems that not all lipids are preferential fuels for MAIT cells, especially in humans. Recently, Deschler and colleagues have demonstrated that fatty acids found within the liver microenvironment of patients with MASLD, can accumulate in MAIT cells and induce oxidative stress alongside the formation of intracellular lipid peroxisomes, which ultimately results in metabolic exhaustion, and MAIT cell dysfunction⁷⁶. Interestingly, human MAIT cells exhibit potent anti-tumour cytotoxicity against HCC cell lines *in vitro*, but this is abrogated in the presence of MASLD-associated PUFAs. Similarly, MAIT cells from patients with non-steatotic HCC exhibited increased expression of gene signatures associated with PUFA-induced MAIT cell dysfunction, and this was associated with poor survival. These findings further emphasize how environmental nutrient content can influence the functional plasticity of MAIT cell responses in diseased settings, such as cancer; and highlight the potential for targeting this axis to improve immune function and disease prognosis in such contexts.

Conclusions and Open Questions

MAIT cells are garnering worthy attention for their critical role in host protection, chronic disease and potential therapeutic advantages. Over the past decade approximately 1000 publications focused on MAIT cells have been indexed on PubMed, exponentially increasing our understanding of MAIT cell biology and their role in health and disease. However, when compared to conventional CD8⁺ T cells (with >35,000 publications in the same period) or other unconventional T cell subsets (for example, iNKT cells with >10,000 publications in the

same period) our understanding of MAIT cell biology is still in its infancy. In this review, we summarized the signals which control MAIT cell responses, from antigenic stimulation mediated by their restricted TCR, to the plethora of cytokines which can potentiate their functions. The recent identification of an endogenous antigen⁴⁸, will likely lead to the identification of more. For example, in the setting of cancer, infiltrating MAIT cells have the hallmarks of microbial activation⁴⁷, but higher MR1 expression in some tumours may indicate the presence of cancer specific antigens, which would advance the potential for MAIT cell immunotherapy.

We also add nutrients as an important regulatory factor for MAIT cell responses. A large body of evidence has emerged over the past decade, demonstrating that altered nutrient availability in the tumour microenvironment significantly limits conventional CD8+ T cell anti-cancer responses¹⁴⁵⁻¹⁴⁹. It is well established that tumours are metabolically very active¹⁵⁰, and display a dysregulated uptake of glucose and amino acids from their environment¹⁴⁹⁻¹⁵². The importance of glucose and amino availability for MAIT cell functionality is rapidly emerging^{126,129}, as is the deleterious effect of altered metabolism on MAIT cell responses^{25,76,126}. However, a comprehensive understanding of the complete nutritional requirements which support MAIT cell activation and their capacity to respond functionally, remains to be elucidated. For example, glutamine is the most abundant amino acid and plays a critical role in modulating the effector responses of other innate populations like NK cells, where it has been shown to act as both a signal and a fuel¹⁵³. However, whether MAIT cells need or use glutamine, remains unknown. Today, one of the most exciting aspects of MAIT cell biology is their proposed role in tissue-repair, and homeostasis¹⁵⁴, but again, the nutritional underpinnings of this response are not yet described and will be important if we are to harness the full potential of MAIT cells in inflammatory or hostile environments.

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CHAPTER 4. Paper II

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Candidate Contribution

Figure panels produced via data collection and/or analysis performed by EKR are listed in table I. Manuscript writing was performed by EKR and AEH.

Table I. Paper figures and panels containing the work of the candidate.

Paper II
Figure 1: All Panels
Figure 2: Panels A-B
Figure 3: Panels A-K
Figure 4: Panels A-F, Panels J-K
Figures S1-S3: All Panels

Iron is critical for mucosal-associated invariant T cell metabolism and effector functions

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Abstract

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 human MAIT cells require exogenous iron, transported via CD71 for optimal metabolic activity in MAIT cells, 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 human MAIT cell metabolism and functionality, an axis which may have implications in conditions where iron availability is limited.

Key Points

- Activated MAIT cells increase iron uptake via CD71
- MAIT cell metabolism and functionality is dependent on a CD71-iron axis

Introduction

Mucosal Associated Invariant T (MAIT) cells are a population of non-MHC restricted T cells that are important in the immune defence against bacterial and viral infections^{1, 2, 3, 4, 5}. MAIT cells are rapidly responding T cells that are capable of producing multiple cytokines upon activation such as IFN γ , TNF and IL-17^{1, 6}. MAIT cells are activated when their invariant T cell receptor (TCR) recognises bacterial riboflavin derivatives presented on the MHC like molecule MR1^{5, 6}. In contrast, they can also be activated in a TCR- independent manner, via stimulation with cytokines such as IL-18^{7, 8}. Recently, several studies have highlighted the importance of metabolism for MAIT cell functional responses⁹. Our group and others have demonstrated that MAIT cells are reliant on glycolysis to support their production of IFN γ and Granzyme B^{10, 11, 12}, whereas IL-17 production by MAIT cells has been linked to mitochondrial metabolism^{13, 14}. However, our knowledge of the nutritional requirements of MAIT cells remains limited.

Iron is an essential trace element for all multicellular organisms, and is critical for a range of physiological processes including oxygen transport and energy production¹⁵. Iron is also critical for the majority of microorganisms, and successful iron sequestration is required to establish infection¹⁶. Iron availability is regulated by the liver-derived hormone, hepcidin. Increased hepcidin production occurs in response to infection and inflammation, and results in reduced iron availability, a mechanism of host protection^{17, 18}. However, previous studies have demonstrated that conventional T cell responses are negatively impacted by low iron levels, with reduced proliferation and effector functions^{19, 20}. Whether MAIT cells require iron for their metabolic processes and effector responses is currently unknown.

In the current study, we demonstrate that MAIT cells upregulate their expression of the transferrin receptor, CD71, upon activation, correlating with a significant increase in the uptake of transferrin-bound iron. *In silico* analysis of the MAIT cell proteome reveals that MAIT cells increase their overall iron content upon activation. We demonstrate that MAIT cell metabolism is limited, and the metabolic profile altered, under iron-deplete conditions, and this is associated with a robust reduction in ATP levels. Finally, we demonstrate that extracellular iron-restriction impairs MAIT cell functional responses, leading to a reduced

proliferative capacity and diminished cytokine production. Collectively our data pinpoint MAIT cells as another player in the iron tug-of-war between pathogenic invaders and host immunity.

Materials and Methods

Study Cohorts & Ethical Approval

A total cohort of healthy 30 adult donors were recruited. Inclusion criteria included ability to give informed consent, 18-65 years of age, BMI<28, no current or recent (<2 weeks) infection. Ethical approval was obtained from both St Vincent's University Medical Ethics Committee and Maynooth University Ethics Committee.

Preparation of Peripheral Blood Mononuclear Cells (PBMC) and Flow Cytometric Analysis

PBMC samples were isolated, using SepMate isolation tubes, by density-centrifugation over Lymphoprep (both STEMCELL Technologies), from fresh human-peripheral blood samples. Cell viability was determined using eBioscience Fixable Viability Dye (eFluor506), and MAIT cells were phenotyped using specific extracellular monoclonal antibodies (Miltenyi Biotec and BioLegend), namely CD3, CD161, TCRV α 7.2, and CD71. Where appropriate, cells were fixed and permeabilized according to manufacturer guidelines, using the True-Nuclear Transcription Factor Buffer set (BioLegend). Cell populations were identified using an Attune NXT flow cytometer and analysed using FlowJo version 10.8.2 (TreeStar). Results are expressed either as a percentage of the parent population as indicated and determined using fluorescence minus one (FMO) and unstained controls; or as the mean fluorescence intensity (MFI) of the relevant population.

MAIT Cell Transferrin Uptake Assay

PBMC (2×10^6 /ml) were activated using CD3/CD28 TCR Dynabeads (Gibco) and IL-18 (50ng/ml) or cytokine alone (IL-12/IL-18 both 50ng/ml) for 18 hours as indicated. Cells were rested in serum-free HPLM with 5% BSA for 2 hours. Cells were then washed in serum-free HPLM with 0.5% BSA and incubated with 5 μ g/ml Transferrin-AlexaFluor647 (Invitrogen) for 10 minutes at 37°C. Holo-transferrin (500 μ g/ml, Sigma-Aldrich) was used to competitively control for

transferrin-uptake. Cells were washed in ice-cold HPLM with 0.5% BSA to stop membrane trafficking. Cells were then stained for viability, and MAIT cells or conventional T cells were labelled for extracellular markers, to be analysed by flow cytometry.

In Silico Proteomic Analysis

Publicly available proteomic data set of MAIT cells were downloaded from PRIDE accession number PXD041544 (<https://www.ebi.ac.uk/pride/archive/projects/PXD041544>). A list of human iron interacting proteins was provided by Andreini et al²¹. Using the protocol from Teh and colleagues²², the list of human iron interacting proteins was compared and aligned against the complete list of proteins detected in the human MAIT cell proteomic dataset. Matches were extracted and listed in Table S1. To estimate the iron atom counts per protein species the copy number value of each iron interacting protein was multiplied by the iron atom counts per protein. If available, iron atom counts per protein were obtained using the Uniprot database cofactor information for each protein. Where iron counts were not available, estimates of iron usage per protein species were assumed to be 1 atom for heme and iron interacting proteins and 2 atoms for FE-S cluster interacting proteins. The total number of iron atoms required per cell was calculated as the sum of iron atoms required by each protein species.

MAIT Cell SCENITH Assay

Fresh PBMC (2×10^6 /mL) were activated using CD3/CD28 TCR Dynabeads, and IL-18 (50ng/mL) for 18 hours. Cells were seeded into a 96-well plate, and treated as a control, or with 2-Deoxy-D-Glucose (100mM), Oligomycin (1 μ M), or both. Following incubation at 37°C for 15 minutes, cells were treated with Puromycin (11 μ M) and incubated for a further 25 minutes. Cells were washed with ice-cold PBS to stop puromycin incorporation. Cells were then stained for viability. MAIT cells were stained for extracellular markers and fixed, as outlined above. Staining of puromycin was achieved using anti-puromycin monoclonal antibody (AlexaFluor488, Sigma), in permeabilization buffer (BioLegend).

MAIT Cell Seahorse Assay

Purified MAIT cells (IL-2 expanded) were activated using CD3/CD28 TCR Dynabeads, and IL-18 (50ng/mL) for 18 hours in the absence or presence of DFO (200 μ M, Sigma) and metabolic

analysis was carried out using the Seahorse Extracellular Flux Analyzer XFe96 (Agilent). MAIT cells were resuspended in phenol red-free RPMI media containing 10mM glucose, 2mM glutamine and 1mM pyruvate (Agilent) and plated onto a Cell-Tak (Corning) coated microplate for adhesion. Respiratory parameter (mitochondrial and glycolytic) were measured using OCR (pmol/min) and ECAR (mpH/min), respectively, using injections of oligomycin (1 μ M), FCCP (1 μ M), rotenone and antimycin A (both 1 μ M) and monensin (20 μ M) (All Sigma). Metabolic parameters were calculated as per well-established protocols^{23, 24}.

MAIT Cell Mitochondrial Analysis

Fresh PBMC (2×10^6 /mL) were activated using CD3/CD28 TCR Dynabeads, and IL18 (50ng/mL) for 18 hours, in the absence or presence of DFO (200 μ M, Sigma). Cells were seeded into a 96-well plate, and washed in serum-free buffer. Cells were then stained for viability as outlined above, and MAIT cells were stained for extracellular markers. Cells were then washed, and stained with Mitotracker Deep Red FM (50 μ M, ThermoFisher Scientific) and Mitotracker Green (50 μ M, ThermoFisher Scientific) in PBS, and incubated for 1 hour at 37°C. Cells were subsequently analysed by flow-cytometric analysis.

MAIT Cell ATP Assay

IL-2 expanded MAIT cells (1×10^6 /ml) were stimulated using CD3/CD28 TCR Dynabeads, and IL18 (50ng/mL) for 18 hours. ATP levels were measured using a luminescence ATP assay kit (Abcam). Reagents in the kit were reconstituted as per manufacturer's instructions. A standard curve was also prepared as per the kit's the instructions. MAIT cells were harvested and washed with PBS. 100 μ L of resuspended MAIT cells was added to a black walled, clear bottomed plate. 50 μ L of detergent was added to each well and the plate was placed on an orbital shaker for 5 minutes at 600-700 rpm. 50 μ L of substrate solution was added and the plate was returned to the orbital shaker for 5 minutes at 600-700 rpm. The plate was then covered and placed in the dark for 10 minutes before luminescence was measured on a multimode plate reader (Clariostar).

MAIT Cell Functional Analysis

IL-2 expanded MAIT cells were activated using CD3/CD28 TCR Dynabeads and IL-18 (50ng/ml) for 18 hours in the absence or presence of DFO (100 μ M), or anti-CD71 monoclonal antibody (20 μ g/mL). An appropriate IgG isotype control was used in blocking experiments. After 18 hours, culture supernatants were assessed for IFN γ , IL-17 or IL-26 levels using ELISA.

MAIT Cell *E. coli* stimulation assay

Freshly isolated MAIT cells were incubated with 20 μ g/mL anti-CD71 monoclonal antibody (Invitrogen) for 1 hour. Meanwhile, THP-1 cells (ATCC TIB-202) were pre-pulsed for 1 hour with fixed *E. coli* (DH5 α) at an MOI of 100. At the end of an hour, representative THP-1 cells with and without *E. coli* were counted and titrated to match the number of MAIT cells at a 1:1 ratio, and MAIT cells were cocultured with THP-1 overnight. Four hours before staining for flow cytometry, cells were centrifuged and resuspended in media containing brefeldin A (Invitrogen). Intracellular cytokine (IFN γ and Granzyme B) levels were assessed using flow cytometry (Cytex Aurora).

MAIT Cell Proliferation Analysis

Fresh PBMC (1 x 10⁶ /ml) were stimulated for 24 hours with 1 μ g/mL of 5-ARU and 100 μ M of Methylglyoxal, in the absence or presence of DFO (200 μ M) and FeSO₄•7H₂O (200 μ M, Sigma). After 24 hours, culture media was replaced with fresh culture media containing IL-2 (6.8 ng/mL). After 48 hours, culture media was replaced with fresh culture media containing IL-2 (34 ng/ mL) . On day 5, absolute cell numbers were determined using flow cytometric analysis of MAIT cell frequencies, and total cell counts.

Statistics

Statistical analysis was completed using Graph Pad Prism 6 Software (USA). Data is expressed as mean $\pm$ SEM. Distribution was assessed using Shapiro-Wilk test. We determined differences between two groups using Student T-test (paired or unpaired) or Wilcoxon Signed-Rank test where appropriate. Analysis across 3 or more groups was performed using ANOVA with multiple measures. Correlations were determined using linear regression models and

expressed using Pearson or Spearman's rank correlation coefficient, as appropriate. P values were expressed with significance set at <0.05.

Results

MAIT Cells Increase Expression of the Transferrin Receptor CD71 Upon Activation

First we demonstrate that MAIT cells from PBMC increase transferrin receptor (CD71) surface expression in response to TCR/IL18 stimulation but not cytokine alone. (Fig1 A-D & Figure S1). We then compared CD71 expression on activated MAIT cells and activated conventional T cells and noted higher CD71 expression on conventional T cells (Figure 1E). Next, we investigated whether MAIT cells altered their CD71 expression under restricted iron conditions. In order to do this, we limited iron availability in our culture system using the iron chelator deferoxamine (DFO) which did not impact MAIT cell viability at 18 hours (Figure S2). Resting MAIT cells do not have high levels of CD71 expression however upon iron depletion both the proportion of MAIT cells which express CD71 and the amount of CD71 expressed was increased, and this was also evident in TCR/IL18 activated MAIT cells (Figure 1F-H & Figure S2), suggesting a compensatory mechanism to support iron uptake. Next, we investigated the functionality of CD71, using a transferrin uptake assay, and show increased transferrin uptake (both % of MAIT cells and MFI) by activated MAIT cells, and again noted higher uptake by activated conventional T cells (Figure 1I-M). Finally, we demonstrate a linear relationship between CD71 expression and transferrin uptake in activated MAIT cells (Figure 1N).

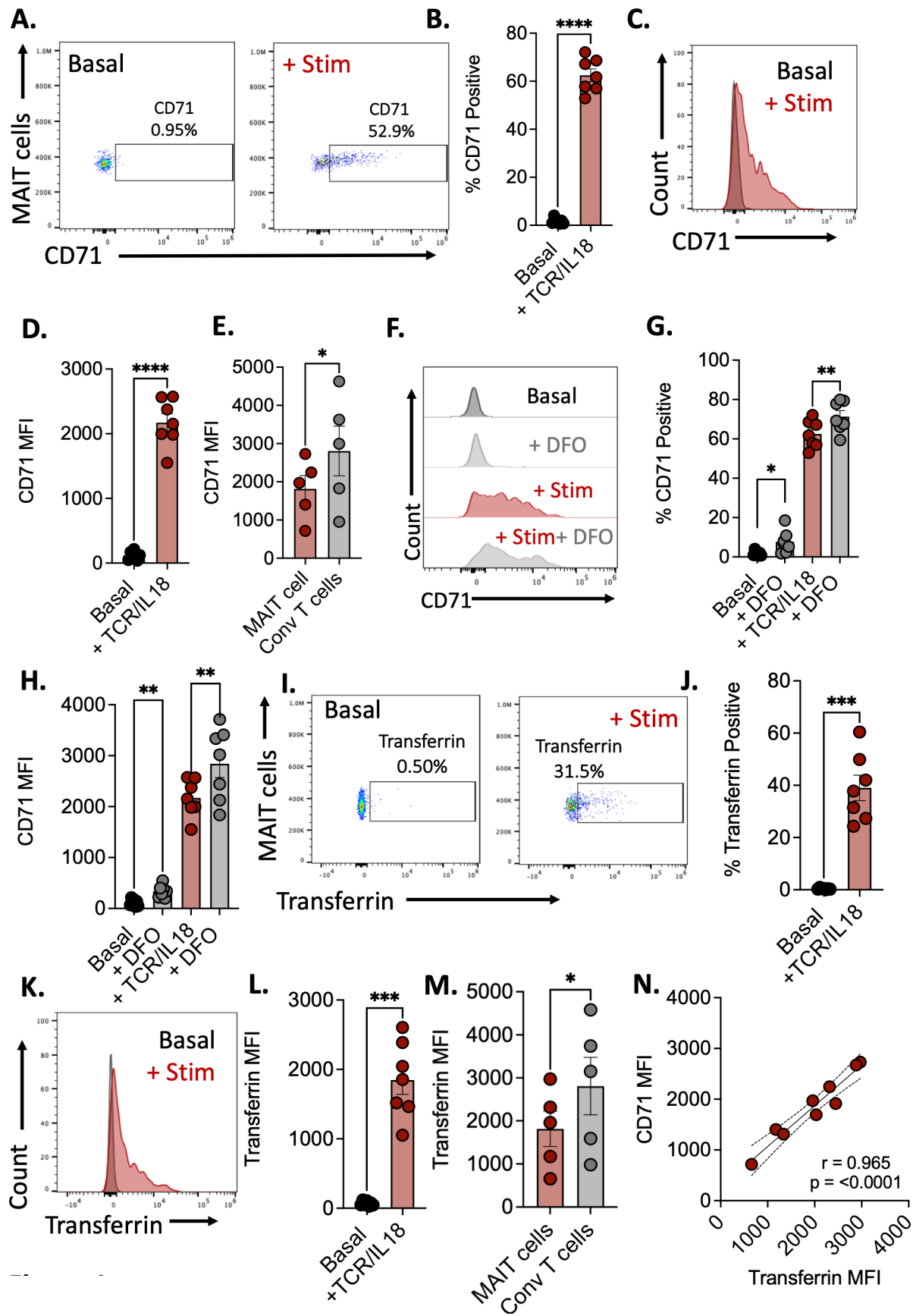


Figure 1. MAIT cells increase CD71 and transferrin uptake upon activation. (A-D) Flow cytometric dot plots, scatter plots, and representative flow cytometric histograms showing CD71 expression in MAIT cells, basal or stimulated for 18 hr with anti-CD3/CD28 TCR beads and IL-18. (E) Scatter plot comparing CD71 expression on MAIT cells and conventional T cells (non-MAIT CD3+ cells) after 18 hours stimulation with anti-CD3/CD28 TCR beads and IL-18. (F-H) Flow cytometric dot plots, scatter plots, and representative flow cytometric histograms showing CD71 expression in MAIT cells, basal or stimulated for 18 hr with anti-CD3/CD28 TCR beads and IL-18, in the absence or presence of the iron chelator, deferoxamine (DFO). (I-L) Flow cytometric dot plots, scatter plots, and representative flow-cytometric histograms showing transferrin content in MAIT cells, basal or stimulated for 18 hr with anti-CD3/CD28 TCR beads and IL-18. (M) Scatter plot comparing transferrin uptake by MAIT cells and conventional T cells (non-MAIT CD3+ cells) after 18 hours stimulation with anti-CD3/CD28 TCR beads and IL-18. (N) Correlation plot showing the relationship between CD71 expression and Transferrin uptake in activated MAIT cells (18 hr with anti-CD3/CD28 TCR beads and IL-18). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

In silico Analysis of the MAIT Cell Proteome Reveals Increased Iron Content Upon Activation

To assess the iron requirements of MAIT cells, we performed *in silico* pathway analysis of a previously published proteomics data-set¹². We found that IL-2 expanded MAIT cells express high basal expression of ferritin, the protein responsible for iron storage, which is reduced upon activation (Figure 2A-B). Next, we determined the number of iron-interacting proteins in MAIT cells, and identified 135 proteins in total, representative of 2.3% of the total proteome (Figure 2C & Table S1). Pathway analysis revealed these proteins were intrinsic to many processes, including a major involvement in cellular metabolism (Figure 2D). Using a recently published algorithm²² which determines cellular iron content, based on number of iron containing proteins multiplied by the number of iron atoms per protein, we determined the predicted iron content from the MAIT cell dataset, and show an approximately 1×10^7 iron atoms in MAIT cells, increasing significantly upon activation (Figure 2E). Finally, we found that the iron content of MAIT cells is associated with Heme and iron-sulphur clusters (Figure 2F-G).

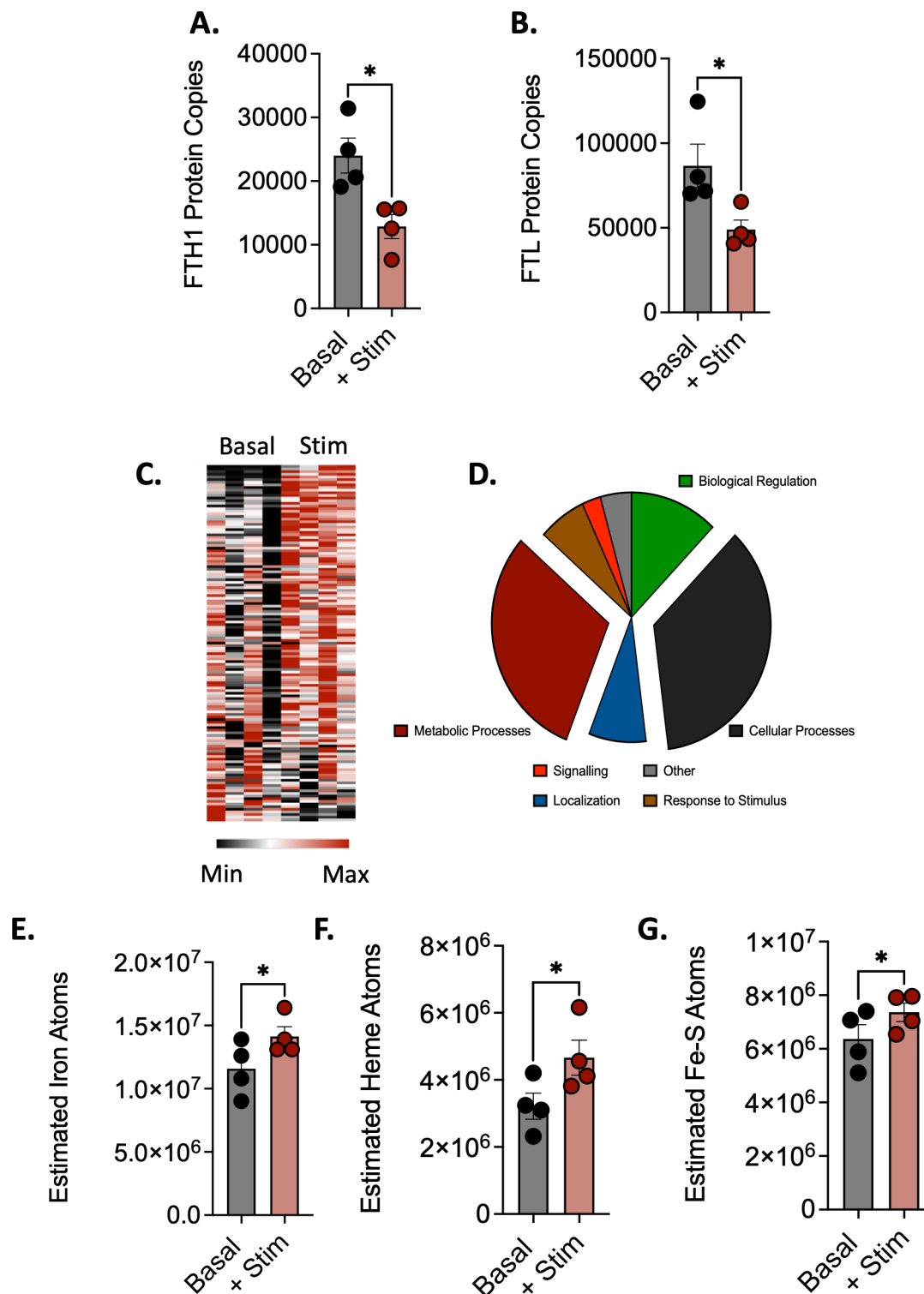


Figure 2. MAIT cells store and utilize iron. (A and B). Scatter plot showing protein copy number of the heavy and light chain subunits of ferritin in IL-2 expanded MAIT cells, basal or stimulated for 18 hr with anti-CD3/CD28 TCR beads and IL-18. (C) Heat map of iron-related protein content in IL-2 expanded MAIT cells, basal or stimulated for 18 hr with anti-CD3/CD28 TCR beads and IL-18. (D) Pie graph showing proportional pathway analysis based on iron-related

*protein content in MAIT cells (analysis performed with Panther). (E-G) Estimated iron, heme and iron-sulphur cluster atoms extrapolated from proteome of IL-2 expanded MAIT cells, basal or stimulated for 18 hr with anti-CD3/CD28 TCR beads and IL-18. * $p < 0.05$.*

Iron is a Critical Co-Factor for MAIT Cell Metabolism

We next investigated if limiting iron availability in our culture system using the iron chelator deferoxamine (DFO) impacted MAIT cell metabolism. Using protein translation as functional readout of metabolism, we demonstrate that activated MAIT cells significantly increase protein translation after stimulation (Figure 3A-B), and protein translation is limited when MAIT cells are activated in the presence of DFO (Figure 3C-D & Figure S2). A recently published method, SCENITH²⁵, demonstrated that the measurement of protein translation, paired with the use of metabolic inhibitors such as 2-deoxyglucose (2DG) and oligomycin, allows for metabolic analysis of cells at a single cell level. Using the SCENITH approach (Figure S2), we demonstrate that iron-restricted MAIT cells increase their dependency on glucose metabolism (Figure 3E), and this was paired with a decreased capacity to undergo oxidative-linked metabolic processes (Figure 3F). With this reduction in oxidative metabolism, we next examined the impact of limiting iron on the mitochondrial phenotype, and observed modest increases in both mitochondrial mass (MitoTracker Green) and membrane potential (MitoTracker Deep Red) (Figure 3G-H). To confirm these observations we next investigated the impact of iron restriction via DFO treatment on MAIT cell metabolism using extracellular flux analysis and noted strong inhibition in the rates of oxidative phosphorylation (Figure 3I-J). We also noted reduced rates of glycolysis (Figure S3). Furthermore, when we tested the mitochondrial capacity of MAIT cells using the FCCP treatment we observed a striking reduction with DFO treatment (Figure 3K). Finally, we examined the impact of low iron availability, using a monoclonal antibody to block CD71, on ATP levels in MAIT cells, and demonstrate that iron restriction limits ATP production. (Figure 3L), a finding supported by our Seahorse analysis (Figure S3).

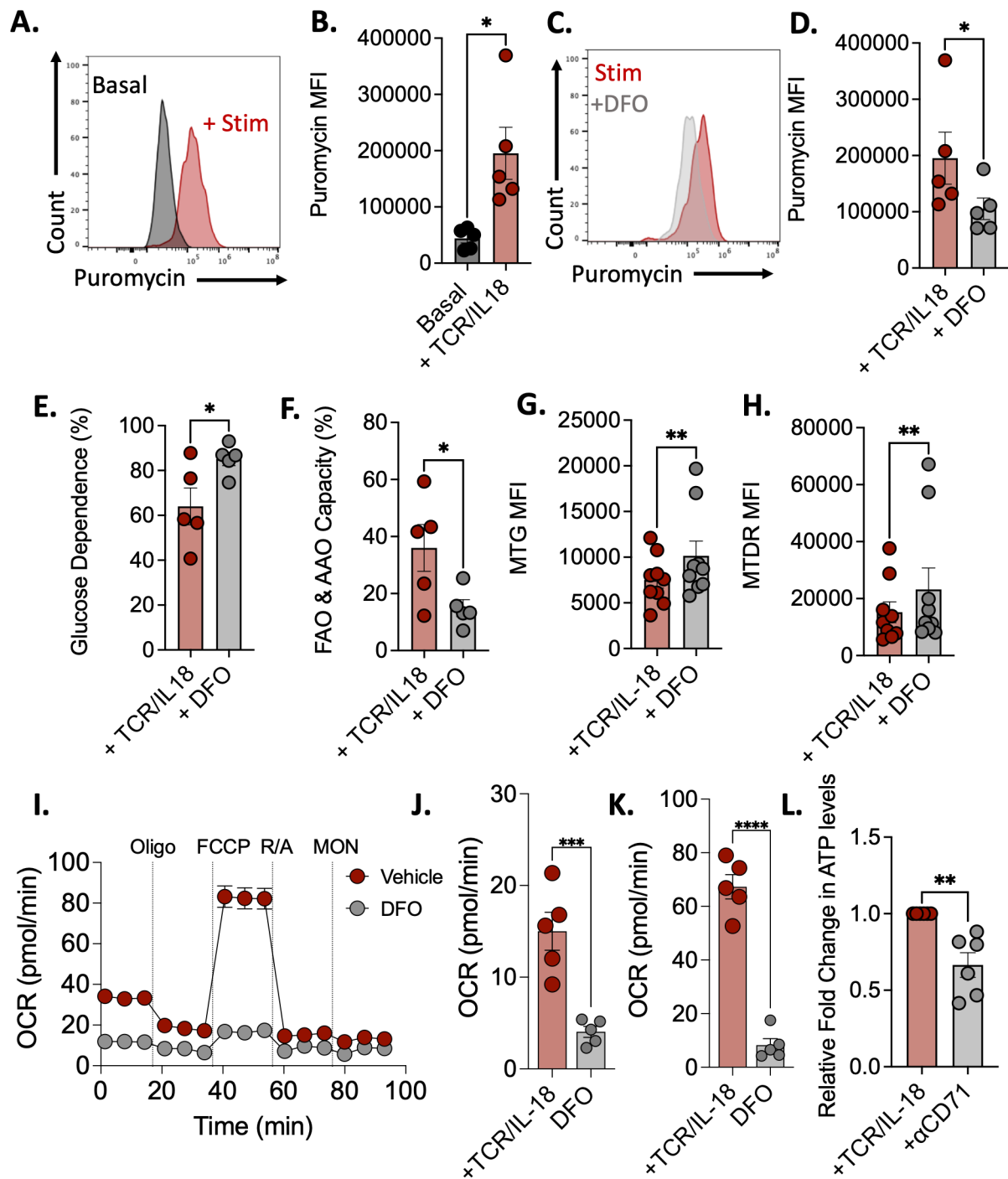


Figure 3. Iron restriction alters MAIT cell metabolism. (A-D) Representative flow cytometric histograms and scatter plots showing puromycin incorporation in MAIT cells, basal or stimulated for 18 hr with anti-CD3/CD28 TCR beads and IL-18, in the absence or presence of the iron chelator, deferoxamine (DFO). (E) Scatter plot showing percentage dependency on glucose metabolism in MAIT cells stimulated for 18 hr with anti-CD3/CD28 TCR beads and IL-18, in the presence or absence of DFO. (F) Scatter plot showing percentage dependency on fatty acid oxidation and amino acid oxidation in MAIT cells stimulated for 18 hr with anti-CD3/CD28 TCR beads and IL-18, in the presence or absence of DFO. (G and H) Scatter plots

showing the mean-fluorescence intensity (MFI) of Mitotracker Green and Mitotracker Deep Red, depicting mitochondrial mass and mitochondrial membrane potential, respectively, in MAIT cells stimulated with anti-CD3/CD28 TCR beads and IL-18 for 18hr, in the presence or absence of DFO. (I-J) Representative Seahorse trace and scatter plot showing oxygen consumption rates (OCR) in MAIT cells stimulated with anti-CD3/CD28 TCR beads and IL-18 for 24hr, in the presence or absence of DFO. (K) Scatter plot showing the mitochondrial capacity (post FCCP treatment) in MAIT cells stimulated with anti-CD3/CD28 TCR beads and IL-18 for 24hr, in the presence or absence of DFO. (L) Scatter plot showing the impact of an anti-CD71 monoclonal with blocking activity on ATP production in MAIT cells stimulated with anti-CD3/CD28 TCR beads and IL-18 for 18hr. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

MAIT Cell Functional Responses Require Exogenous Iron Uptake

We next investigated if MAIT cells require extracellular iron for their functional responses. We assessed the impact of DFO treatment on MAIT cell in PBMC ability to produce IFN γ via flow cytometry and observed a reduction with iron restriction (Figure 4A). Next we investigated the impact of DFO on the cytokine responses of isolated MAIT cells and show reductions in IFN γ , IL-17 and IL-2 levels (Figure 4B-D). To confirm the impact of low iron availability on MAIT cell IFN γ production, we switched our approach to block CD71 and again demonstrate significant reductions in IFN γ levels (Figure 4E). We next assessed the impact of limiting iron availability on MAIT cell production of the antimicrobial cytokine IL-26, and demonstrate significantly reduced levels with CD71 blockade (Figure 4F). We next assessed the impact of iron restriction via CD71 blockade on freshly isolated MAIT cell responses to THP-1 cells infected with *E. Coli* and demonstrated significant reductions in both IFN γ and granzyme B production (Figure 4G-H). With the noted impact of iron restriction on MAIT cell metabolism, we sought to assess the impact of long-term iron restriction on MAIT cell proliferative capacity and viability. We first show that MAIT cells proliferation is inhibited with the addition of the ATP synthase inhibitor oligomycin (Figure 4I), and then demonstrated that MAIT cells fail to proliferate under low iron conditions, and this was paired with a significant reduction in cell viability, which was significantly improved with the addition of FeSO₄, which bypasses CD71 (Figure 4J-K).

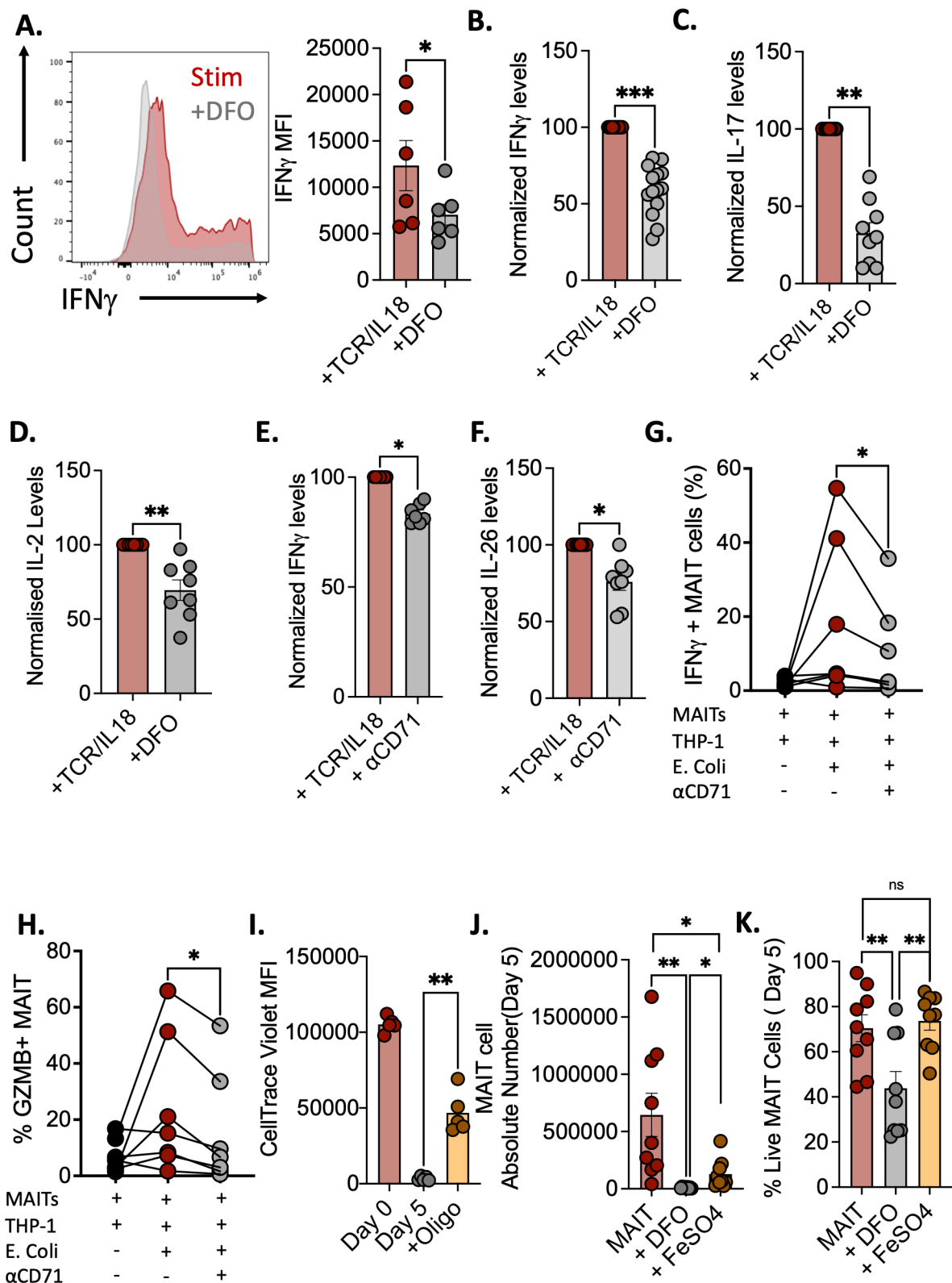


Figure 4. MAIT cells require iron for their functional responses. (A) Representative flow cytometry histogram and scatter plot showing $IFN\gamma$ levels (mean fluorescence intensity (MFI)) in MAIT cells stimulated with anti-CD3/CD28 TCR beads and IL-18 for 18hr, in the absence or presence of the iron chelator, deferoxamine (DFO) measured by flow cytometry. (B-D) Scatter

plots showing IFN γ , IL-17 and IL-2 secreted protein levels (normalised to assay) in IL-2 expanded MAIT cells stimulated with anti-CD3/CD28 TCR beads and IL-18 for 18hr, in the absence or presence DFO, as measured by ELISA. (E and F) Scatter plots showing the impact of an anti-CD71 monoclonal with blocking activity on IFN γ and IL-26 secreted protein levels (normalised to assay) in IL-2 expanded MAIT cells stimulated with anti-CD3/CD28 TCR beads and IL-18 for 18hr, as measured by ELISA. (G-H) Line graph showing IFN γ or granzyme B levels (percentage of parent population) in MAIT cells co-cultured at 1:1 ratio with THP-1 cells alone or pre-pulsed with fixed *E. coli* (DH5 α) for 18hr, in the absence or presence of the anti-CD71 monoclonal with blocking activity, as measured by flow cytometry. (I) Scatter plot showing the impact of the ATP-synthase inhibitor, oligomycin, on MAIT cell proliferative capacity (during a 5 day IL-2 mediated MAIT cell expansion culture), whereby the mean fluorescence intensity (MFI) of the intracellular dye CellTrace Violet decreases with each cycle of cell division. (J and K) Scatter plots showing the impact of long-term iron depletion via DFO, and alternative iron repletion via Iron(II) sulfate heptahydrate ($\text{FeSO}_4 \bullet 7\text{H}_2\text{O}$) on MAIT cell proliferation and viability, during a 5 day IL-2 mediated MAIT cell expansion culture. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Discussion

MAIT cells are a subset of unconventional T cells capable of rapidly responding to stimulation, producing cytokines, lytic molecules and proliferating²⁶. MAIT cells are key mediators of host protection against many bacterial and viral pathogens^{2, 3, 27, 28, 29}. Immune responses are metabolically intense processes, with significant energy demands required to support *de novo* generation of biosynthetic intermediates³⁰. The metabolic processes and nutrient requirements that govern MAIT cell effector responses are rapidly emerging but still incomplete^{10, 11, 12, 13, 31}. Iron is an essential microelement and is critical for almost all living organisms, including humans and the majority of microbes¹⁵. Iron plays a critical role in cellular metabolism and energy production, and is vital for conventional T cell immunity^{19, 20}. The iron requirements of MAIT cells are unknown. In this study, we show that MAIT cell metabolism and functional responses, namely cytokine production and proliferation, are governed by extracellular iron availability. We show that activated MAIT cells increase their expression of CD71, the transferrin receptor, and that iron-restriction significantly diminishes MAIT cell protein translation and ATP production. Consequently, MAIT cells present with a reduced capacity to proliferate, and diminished production of several cytokines central to MAIT cell host protective function.

The majority of bioavailable iron in the circulation is bound to transferrin which is taken into cells via the transferrin receptor TfR1, also known as CD71³². We first examined MAIT cells for the expression of CD71 and found low basal expression but significant increases in expression after stimulation with TCR beads/IL-18 but not IL-12/IL-18 suggesting a TCR dependency, this supports our previous study which highlighted CD71 as a Myc target, which is an important transcription factor upregulated by TCR stimulation in MAIT cells¹². Previous studies have reported similar activation induced increases in CD71 expression on activated T cells and NK cells^{33, 34}. Interestingly, a missense mutation in TFRC, the gene encoding CD71, resulted in immunodeficiency underpinned by defective T cells³⁵. In line with increased CD71 expression, we observed increased uptake of transferrin by activated MAIT cells, similar to that reported in both conventional CD4+ and CD8+ T cells³⁶. MAIT cells are primarily CD8+, and recently it was demonstrated that CD8 receptor expression was critical for optimal TCR driven responses in MAIT cells³⁷. In a recent study by Teh and colleagues, they reported greater iron dynamics in CD8+ T cells compared to CD4+ T cells, which is supported by proteomic data from Howden et al where they demonstrated that CD8+ T cells express more CD71 than CD4+ T cells³³. To investigate the iron dynamics of MAIT cells we performed *in silico* analysis of our publicly available proteomic dataset¹². We found that MAIT cells decrease their levels of ferritin, suggesting mobilization of iron stores³⁸. Using the approach introduced by Teh et al for determining cellular iron content based on proteomic analysis²², we found that MAIT cells contain 135 proteins with iron binding sites and significantly increase their predicted iron content upon activation, similar to that observed in conventional T cell subsets²².

Pathway analysis of the 135 proteins with iron binding sites present in MAIT cells highlighted cellular metabolism as a major process central to these proteins. Similarly, Teh and colleagues found that the majority of intracellular iron in T cells was utilized in oxidative phosphorylation²². Therefore, we next assessed the impact of altered iron availability on MAIT cell metabolism and functional responses. We used two different approaches to limit MAIT cell access to extracellular iron; (1) we utilized the iron chelator deferoxamine (DFO) which removes iron from ferritin³⁹, (2) we utilized a monoclonal antibody specific for CD71 with blocking activity⁴⁰. To assess the impact of low iron availability on MAIT cell metabolism we used two different approaches, Seahorse extracellular flux analysis and a recently published method SCENITH, which monitors rapid changes in protein translation paired with a series of

metabolic inhibitors to profile cellular metabolism at a single cell level²⁵. Using SCENITH, we found that MAIT cells significantly increase protein translation upon activation. This supports our recent publication where we demonstrated significant increases in protein content in activated MAIT cells¹². In the presence of DFO, protein translation was reduced in activated MAIT cells, suggesting that iron supports global MAIT cell metabolism. This was confirmed by our Seahorse data which showed reduced rates of glycolysis and to a greater extent reduced rates of OxPhos. Similarly in activated NK cells, treatment with DFO resulted in reduced cell size, indicative of reduced protein content and metabolism³⁴. Paired with reduced protein translation, limited iron availability also reduced the oxidative capacity of activated MAIT cells, suggesting altered mitochondria, similar to that reported in conventional T cells by Frost and colleagues²⁰. In the same study by Frost *et al*, iron deficiency resulted in reduced ATP production and an accumulation of dysregulated mitochondria in CD8+ T cells²⁰. We observed only modest changes in mitochondrial phenotype, but note the longer timeframe in the study by Frost *et al* compared to our overnight timepoint. We did however find reduced mitochondrial capacity and ATP levels in MAIT cells activated in the presence of limited iron availability.

Iron is critical for the majority of commensal and pathogenic bacteria, and during infection, in response to inflammation, the host restricts the accessibility of iron as a protective measure. Many pathogenic bacteria synthesize iron chelators called siderophores to scavenge host iron, resulting in an iron tug-o-war⁴¹. One of the major roles for MAIT cells is mediating host protection against bacteria^{27, 42, 43, 44}. Interestingly, several bacteria which elicit MAIT cell responses *in vivo*, such as *Salmonella typhimurium*⁴⁵, *Klebsiella pneumoniae*⁴⁶ and *Legionella longbeachae*⁴⁷ utilize siderophores as a virulence factor^{48, 49, 50}. Therefore, we investigated if MAIT cells need iron for their effector functions, the primary of which is cytokine production. Previously, we had found IFN γ the most abundant cytokine produced by MAIT cells, and here we demonstrate that limiting iron availability limits the levels of IFN γ secreted in response to either TCR stimulating beads or THP-1 cells infected with *E. coli*. Similarly, in a model of hepcidin driven hypoferremia, antigen specific CD8 T cells produced less IFN γ than in control mice, highlighting the importance of iron for conventional T cell responses. Interleukin-26 is cytokine with direct antimicrobial activity produced by MAIT cells, and part of MAIT cell responses to infection in the lung²⁸. We found that IL-26 production by MAIT cells was also

reduced under limited iron conditions. Another key effector function required for MAIT cell host defence is the ability to proliferate⁵¹. We found that under iron restriction MAIT cells failed to proliferate, likely linking into the reduced metabolism observed. In CD4 T cells, iron was also required for proliferation, and the authors linked this failure to altered mitochondrial metabolism¹⁹, supporting our data which demonstrated significant reduction in MAIT cell proliferation with ATP synthase inhibition. In summary our study highlights the importance of iron for MAIT cell metabolic and functional responses, and may have implications in conditions where iron availability is limited.

Contributors Statement: EKR performed the experiments, carried out analysis, drafted the manuscript, and approved the final manuscript as submitted. CC, CDB, BJJ, and CMcK performed experiments, carried out analysis and approved the final manuscript as submitted. SOS and DKF performed proteomic analysis, helped with study design, analysis and approved the final manuscript as submitted. OR, HH, and DOS, recruited study participants and helped with study design, analysis and approved the final manuscript as submitted. DOS, FW, DTL, NJ, LVS, and AEH, conceptualized and designed the study, analyzed the data, drafted the manuscript, and approved the final manuscript as submitted.

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Competing Interest: The authors declare no conflict of interest.

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Supplementary Figures and Tables

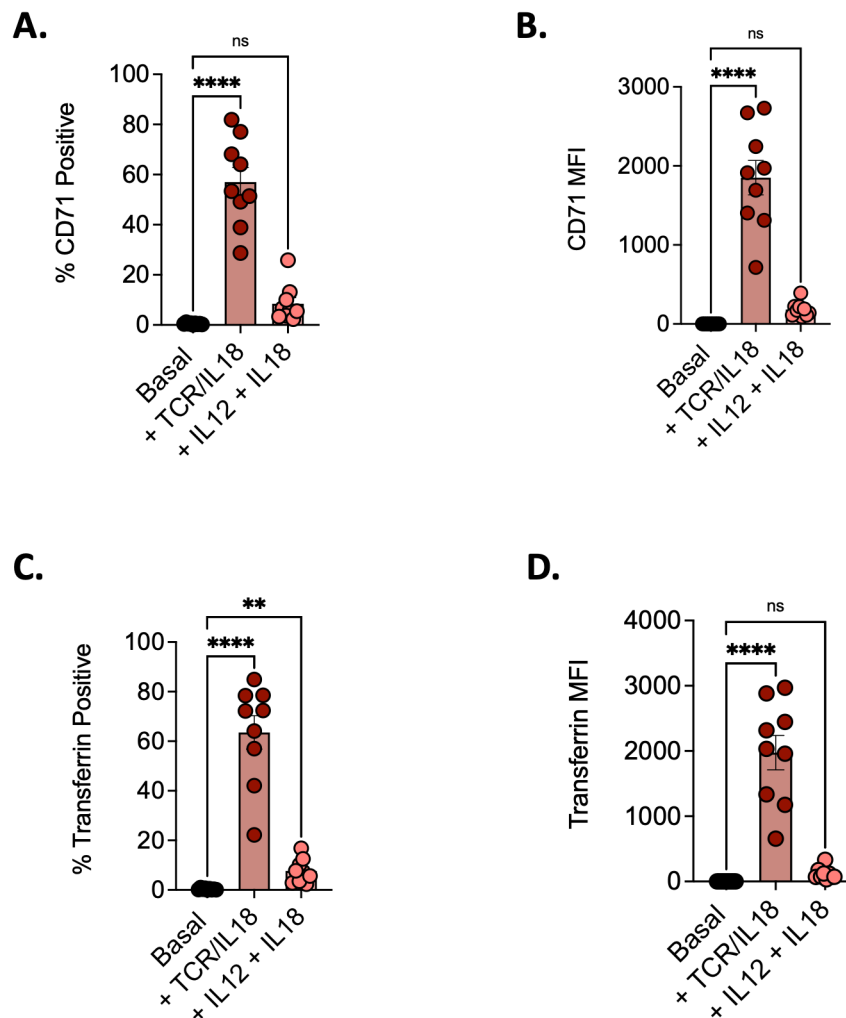


Figure S1. (A-D) Scatter plots showing CD71 expression (both % and MFI) on MAIT cells basally or after 18 hours stimulation with either TCR beads and IL-18 (50ng/ml) or IL-12/IL-18 (both 50ng/ml). ** $p < 0.01$ and **** $p < 0.0001$.

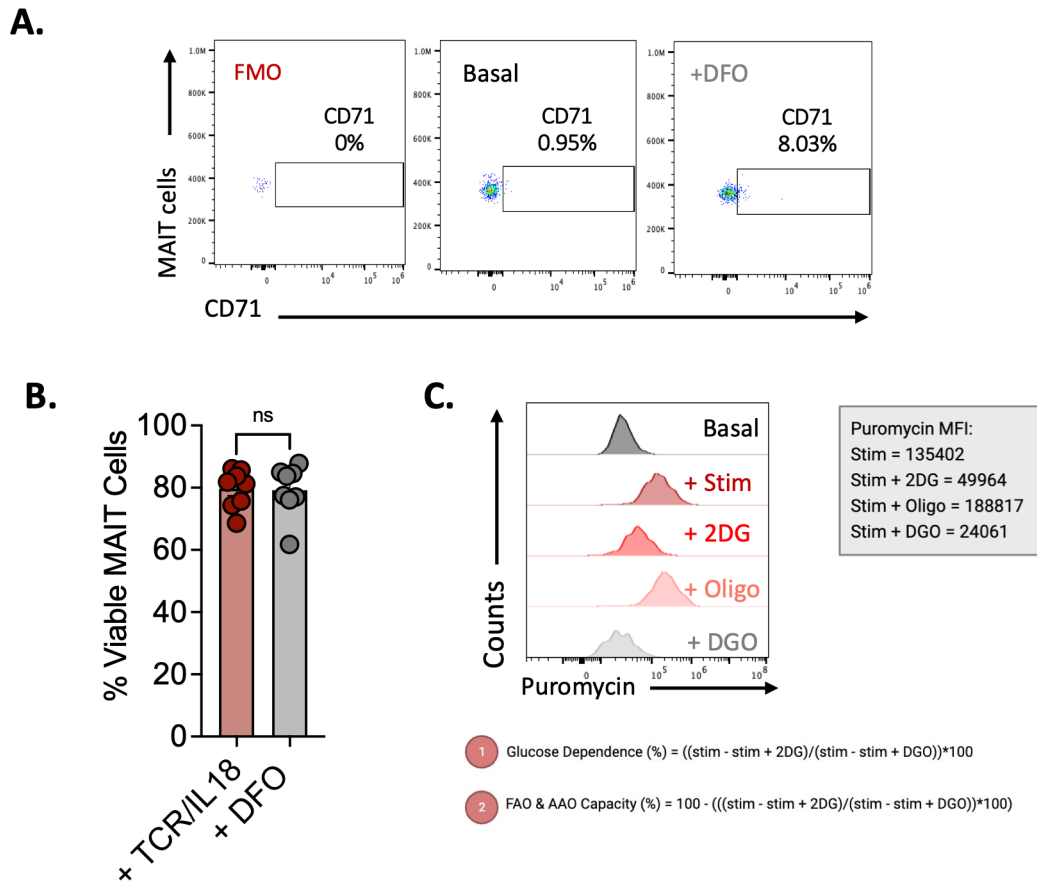


Figure S2. (A) Representative flow cytometry dot plots detailing CD71 expression on MAIT cells basally or after 18 hours of DFO treatment (200 μ M), including fluorescence minus one (FMO) control for CD71. (B) Scatter plot showing the viability of TCR/IL-18 activated MAIT cells in the absence or presence of DFO (200 μ M) for 18 hours. (C) Representative example of SCENITH data including calculations for presented metabolic dependencies. ns = not significant.

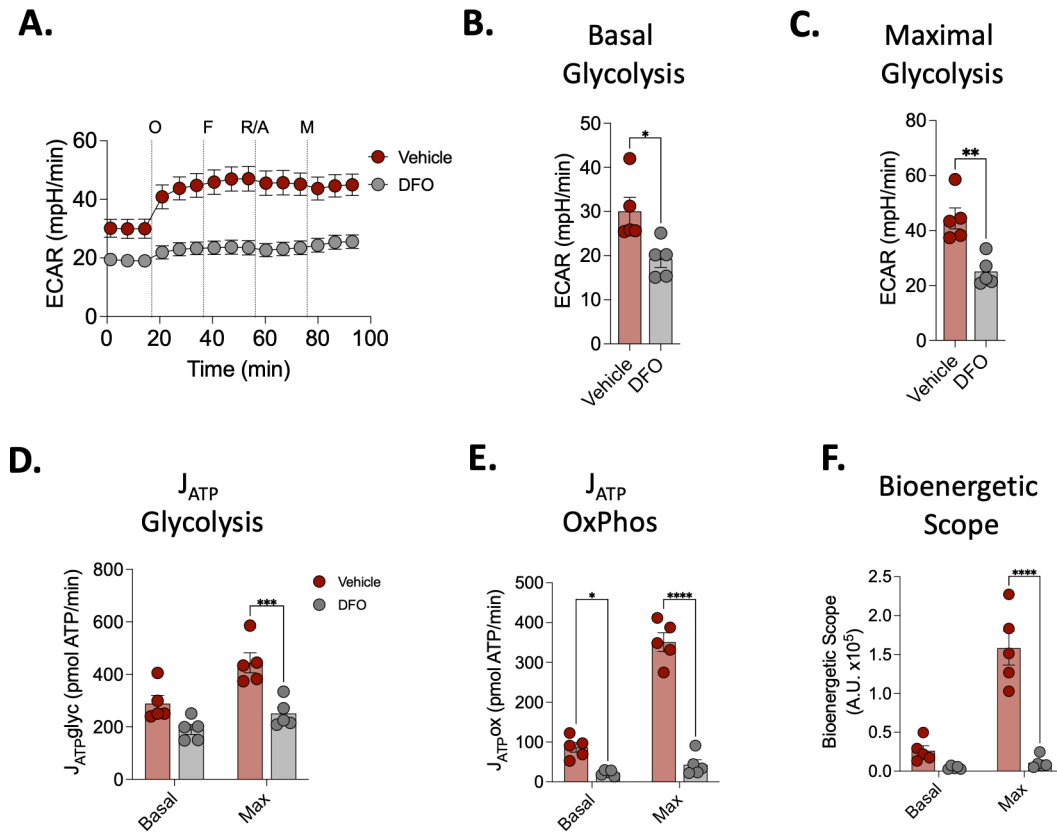


Figure S3. (A) Representative Seahorse trace showing extracellular acidification rate (ECAR) following pre-optimized injections of oligomycin, FCCP, antimycin A/rotenone (all 1 mM) and Monensin (20mM) in MAIT cells activated for 18 hours in the absence or presence of DFO (200 μ M). (B-C) Scatter plots showing basal or maximal glycolytic rates in MAIT cells activated for 18 hours in the absence or presence of DFO (200 μ M). (D-E) Scatter plots showing basal or maximal ATP (J_{ATP}) production linked to glycolysis or oxidative phosphorylation in MAIT cells activated for 18 hours in the absence or presence of DFO (200 μ M). (F) Scatter plot showing basal or maximal bioenergetic scope of MAIT cells activated for 18 hours in the absence or presence of DFO (200 μ M). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

Table S1. List of all iron interacting proteins identifiable in human MAIT Cells

BOLA2	ISCA2	SDHC	FTL
ACO2	PRIM2	CYP51A1	METAP2
UQCRRF51	ISCU	CYB5A	PPP3CA
CISD2	ABAT	PTGES2	METAP1
NDUFS1	NFS1	CYP20A1	RRM2
ABCE1	GLRX5	CYBA	MRE11
SDHB	NFU1	HPX	ADO
NDUFV2	ACO1	NENF	OSGEP
CISD1	DPH2	TBXAS1	P4HA1
NDUFV1	DPYD	RORC	KDM2A
GLRX3	POLA1	SDHD	ALKBH5
NUBP2	NTHL1	JAK3	NIF3L1
NDUFS8	DPH1	HEBP1	FTO
NDUFS2	POLE	MT-CO1	JMJD6
CIAPIN1	ABCB7	PTGIS	RIOX1
NUBP1	FECH	COX15	HPDL
ERCC2	HBA1	HMOX1	MSMO1
NDUFS7	HBB	CYB5R4	RRM2B
POLD1	CYCS	MT-CYB	FTH1
FDX1	ALB	FADS2	RIOX2
ETFDH	CYB5B	INPPL1	PPP3CB
CISD3	COPA	DGCR8	LTF
ELP3	CYC1	HERC2	DOHH
PPAT	PGRMC2	ABCB6	PPP3CC
CDKAL1	HMOX2	AFM	EGLN1
KDM2B	COX5A	CYP4F22	RPE
ASPHD2	CAT	GSTP1	KDM3B
KDM5A	PGRMC1	PPP1CA	PHF8
KDM5B	HCCS	ETHE1	PIR
KDM4A	HEBP2	FXN	OGFOD1
KDM4B	KDM3A	TMPPE	SCD
PHF2	P4HA2	KDM6B	PLOD3
ACP5	SCD5	HIF1AN	ADI1
P3H1	KDM5C	GALT	

CHAPTER 5. Paper III

This chapter consists of a first-author manuscript currently accepted for publication (in press) in *The Journal of Immunology*.

Candidate Contribution

Figure panels produced via data collection and/or analysis performed by EKR are listed in table I. Manuscript was written by EKR and AEH.

Table I. Paper figures and panels containing the work of the candidate.

Paper III
Figure 1: All Panels
Figure 2: All Panels
Figure 3: Panels A-N
Figure 4: All Panels
Figure 5: All Panels
Figures S1-S3: All Panels; Figures S5-S9: All Panels.

Rapid glycolytic metabolism underpins mucosal-associated invariant T cell functional responses to innate cytokines

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Key words: Mucosal-associated invariant T cells, Immunometabolism, Glycogen, Type I Interferon, Anti-Viral.

Abstract

Mucosal Associated Invariant T (MAIT) cells express a semi-invariant T cell receptor (TCR), that recognises bacterial-derived antigens presented on MR1. Upon TCR triggering, MAIT cells respond rapidly, producing a range of effector molecules which facilitate host-protective responses in the context of microbial infections. In contrast, MAIT cell responses to viral infection are instead triggered by the recognition of cytokines, and occur independently of TCR engagement. The molecular and metabolic regulation of MAIT cell TCR responses is rapidly emerging, but there is a paucity of data on cytokine driven responses. Here, using high-resolution, quantitative proteomic analysis, we map the downstream proteome of innate cytokine (IL-18/IFN α) -activated MAIT cells, highlighting robust cytokine-driven remodelling and a signature that is distinct from the TCR-driven response. MAIT cells significantly increase protein biosynthesis in response to innate cytokine stimulation, and rapidly upregulate the production of IFN γ , granzyme B, and ISG15. For the first time, we demonstrate the metabolic kinetics of MAIT cell responses to cytokine stimulation, and highlight a rapid but transient glycolytic burst that is uncoupled from the mitochondria; and contrasts the robust metabolic profile elicited downstream of TCR engagement. Finally, we demonstrate differential contributions from both glycogen and glucose in supporting MAIT cell responses to innate cytokines, and further highlight the importance of nutrient availability as a governing signal for MAIT cell fitness, and effector functioning.

Key Points

- Human MAIT cells rapidly respond to IFN α /IL-18 stimulation
- IFN α /IL-18 stimulation drives a rapid glycolytic burst in human MAIT cells
- MAIT cells responses to IFN α /IL-18 stimulation are fuelled by glucose and glycogen

Introduction

Mucosal-associated Invariant T (MAIT) cells are a population of innate T cells characterised by the expression of a semi-invariant T cell receptor (TCR)¹, which recognises bacterial-derived antigens presented on the MHC-like molecule, MR1². Upon TCR triggering, MAIT cells are capable of rapid and sustained proliferation³, alongside the production of a range of cytokines and lytic molecules^{4, 5}. It is therefore through TCR triggering that MAIT cells develop a host-protective capacity to defend against a range of bacterial infections^{6, 7, 8}.

Cellular immune responses are metabolically expensive processes, where significant energy demands are required to underpin *de novo* generation of biosynthetic intermediates⁹. There are several studies which highlight the importance of metabolism in the regulation of MAIT cell functional responses¹⁰. Previous studies have demonstrated that upon TCR stimulation, MAIT cells are increasingly reliant on glycolysis to support rapid proliferation, and the production of effector molecules such as interferon-gamma (IFN γ) and granzyme B^{3, 11}. Additionally, we have demonstrated that the transcription factor Myc acts as a critical regulator of TCR-driven MAIT cell metabolic switching, through modulation of the expression of nutrient transporters and glycolytic machinery³.

Importantly, MAIT cells have more recently been described as robust innate responders during human viral infections^{12, 13, 14, 15}. Specifically, MAIT cells are capable of eliciting robust responses to viral challenge through the recognition of innate cytokines such as interleukin(IL)-12, IL-18 and type I interferons (such as IFN α)^{12, 16, 17, 18}. The activation of MAIT cells during human viral infections can therefore occur independently of TCR triggering. To date, the metabolic kinetics which dictate MAIT cell responses to innate cytokine stimulation remain to be elucidated.

In this study, using high-resolution quantitative proteomic analysis, we identify significant proteome remodelling in human MAIT cells following co-stimulation with the innate cytokines IL-18 and IFN α . Specifically, the stimulation of human peripheral blood MAIT cells with IL-18/IFN α elicits a rapid burst of *de novo* biosynthesis, and we identify early, yet robust, IFN γ

and granzyme B responses; alongside high level expression of the interferon-stimulated gene (ISG), ISG15. Probing the metabolic profile, we demonstrate that in response to stimulation with IL-18/IFN α , human MAIT cells engage in an early but transient glycolytic burst that is not coupled with mitochondrial remodelling; and contrasts significantly the robust glycolytic phenotype that has been contextualised downstream of TCR signalling and cytokine co-stimulation. Finally, we highlight differential roles for glycogen and glucose in supporting the rapid kinetics of MAIT cell responses to innate cytokines. Overall, our data elucidates the metabolic and functional kinetics of MAIT cell responses to innate cytokine stimulation, and may have implications in the context of human viral infections, during which these responses can become perturbed.

Materials & Methods

Study Cohorts and Ethical Approval

Full ethical approval was obtained from both St Vincent's University Medical Ethics Committee and Maynooth University Ethics Committee. Criteria for recruitment of this healthy control cohort included the ability to give informed consent, being between the ages of 18 to 55, and having a healthy BMI. Exclusion criteria included current or recent infection (<2 weeks), being a current smoker, and the use of immunomodulatory or anti-inflammatory medications. All participants provided full consent.

Preparation of Peripheral Blood Mononuclear Cells (PBMC) and MAIT Cell Expansion

Human PBMC were isolated from fresh blood samples using SepMate™ PBMC isolation tubes. Standard protocol was carried out as per manufacturer guidelines (StemCell Technologies). PBMC were either stored at -70°C or used to expand MAIT cell lines. To expand MAIT cell lines, fresh PBMC were plated at 1×10^6 /ml complete RPMI 1640 (Gibco), and stimulated with 5-Amino-6-(D-ribitylamino)uracil (5-A-RU) (1 μ g/ml; gifted) and methylglyoxal solution (100 μ M/ml; Sigma). After 24hr, culture media was replaced with fresh culture media containing IL-2 (6.8ng/ml; BioLegend). After 48hr, culture media was replaced with fresh culture media containing IL-2 (34ng/ml). Cell cultures were maintained for up to 14 days, and culture media was replaced with IL-2 media (34ng/ml) every 3 days. MAIT cell purity was confirmed using flow cytometry on day 7, and again on day 12, as detailed by Kedia-Mehta

et al., 2023. MAIT cells which have not expanded to high purity (>75%) were not used in downstream experiments.

MAIT Cell Sample Preparation for Proteomic Analysis

IL-2-expanded MAIT cells were further purified using anti-V α 7.2 -APC conjugated antibody (Miltenyi Biotec) and anti-APC microbeads (Miltenyi Biotec) for Magnetic Cell Separation (MACS), to ensure a pure cell population suitable for proteomic analysis. Cells were then stimulated using CD3/CD28 TCR Dynabeads (Gibco) and IL-18 (50ng/ml; BioLegend), or IFN α (500ng/ml; R&D Systems), and IL-18 (50ng/ml) for 18hr. Samples were prepared for proteomic analysis as previously described³. Cell pellets (2 million cells per sample) were lysed at room temperature in 5% SDS, 50 mM TEAB (pH 8.5), and 10 mM TCEP under agitation for 30 min, then boiled for 5 min, and sonicated with a BioRuptor (30-s on, 30-s off $\times$ 15 cycles). Protein concentration was determined using an EZQ protein quantification kit (Invitrogen) according to the manufacturer's protocol. Lysates were alkylated with 20 mM iodoacetamide for 1 hour at room temperature in the dark. The samples were then processed using S-Trap columns (Protifi) according to manufacturer's instructions. An equivalent of 1.5- μ g peptides was injected onto a nanoscale C18 reverse-phase chromatography column coupled to an UltiMate 3000 RSLC nano HPLC system (ThermoFisher Scientific) and an Orbitrap Exploris 480 mass spectrometer (ThermoFisher Scientific) as described in³.

DIA Data Quantification and Proteomic Analysis

Quantification of reporter ions was completed using Spectronaut (Biognosys; Spectronaut 17.6.230428.55965) in library-free (directDIA) mode, searched against the Human Swissprot database (November 2023). Minimum peptide length was set to 7, and maximum peptide length was set to 52, with a maximum of 2 missed cleavages. MS1 and MS2 mass tolerance strategy and XIC IM and RT extraction windows were set to dynamic, all with a correction factor of 1. Trypsin was specified as the digestive enzyme used. The false discovery rate (FDR) at the precursor ion level and protein level was set at 1% (protein and precursor Q value cut-off). The max number of variable modifications was set to 5, with protein N-terminal acetylation and glutamine and asparagine deamidation and methionine oxidation set as variable modifications. Carbamidomethylation of cysteine residues was selected as a fixed modification. For calibration, the MS1 and MS2 mass tolerance strategy was set to the system

default. Machine learning was set across experiments, with a precursor PEP cut-off of 0.1 and a protein Q value cut-off of 0.01. Single-hit proteins were not excluded, with single hits defined by stripped sequence. For quantification, the quantification method was set to QUANT 2.0. Inference correction was set to true, with MS1 min of 2 and MS2 min of 3. The major protein grouping was by protein group ID, and the minor peptide grouping was set to stripped sequence. Major and minor group top N was set to false, with minor and major group quantities set to sum precursor quantity and sum peptide quantity, respectively. Quantity at the MS level was set to MS2, and quantity type was set to area. Proteotypicity filter was set to none, data filtering to Q value, and cross run normalization was switched off. MS2 demultiplexing was automatic, and the run limit for the directDIA library was set to -1, with no profiling strategy or unify peptide peaks strategy. Data filtering and protein copy number quantification were performed in the Perseus software package, version 1.6.6.0. Copy numbers were calculated using the proteomic ruler¹⁹. This method sets the summed peptide intensities of the histones to the number of histones in a typical diploid cell. The ratio between the histone peptide intensity and summed peptide intensities of all other identified proteins is then used to estimate the protein copy number per cell for all the identified proteins. Further filtration of the data was completed to include proteins detected in at least two biological replicates and to exclude proteins identified on the basis of single peptides.

In Silico Proteomic Analysis

P values and fold changes for data tables were calculated using R Studio (v.2023.12.1+402). Heatmaps were generated using the Morpheus data visualization tool from the Broad Institute (<https://software.broadinstitute.org/morpheus>). Relevant protein copy numbers were graphed and assessed for statistical significance using GraphPad Prism 10 software for macOS. Gene Ontology (GO) Enrichment Analysis for Biological Processes was carried out on commonly upregulated ($\log_2\text{fold} > 0.5$, $p \leq 0.05$) proteins between TCR/IL-18 and IL-18/IFN α conditions using the ClusterProfiler package (version 4.10.1) for R Studio. *P* values were adjusted for multiple comparisons using the Benjamini-Hochberg (BH) method to control for the false-discovery rate (FDR). GO values with an adjusted *P* value of < 0.05 were considered significant. A bubble plot mapping the top 10 enriched terms was created using the ClusterProfiler package (version 4.10.1). Mitochondrial proteins were identified using the Gene Ontology term GO:0005739 from the MitoCarta3.0 inventory; and Electron Transport

Chain proteins were identified from within MitoCarta3.0 'Mitopathways' column, using the reference term 'OXPHOS'(<https://personal.broadinstitute.org/scalvo/MitoCarta3.0/human.mitocarta3.0.html>).

MAIT Cell Puromycin Incorporation Assay

MAIT cells were activated with IFN α (500ng/ml; R&D Systems) and IL-18 (50ng/mL; BioLegend) for 6hr. For metabolic analyses, PBMC were pre-treated for 1hr with 2-deoxy-D-glucose (1mM; Sigma), CP91149 (100 μ M; Sigma), and activated as outlined above. Cells were then seeded into a 96-well plate, and treated with puromycin (11 μ M; Sigma) for 25 min at 37°C. Cells were washed with ice-cold phosphate-buffered saline (PBS) (Sigma) to stop puromycin incorporation. Cells were stained for viability, and MAIT cells were phenotyped and assessed for the incorporation of puromycin, as outlined below under "MAIT Cell Flow Cytometric Analysis".

MAIT Cell Seahorse Assay

IL-2 expanded MAIT cells were activated using either CD3/CD28 TCR Dynabeads with IL-18 (50ng/ml), or with IFN α (250-500ng/ml) and IL-18 (50ng/ml) for 3hr, 6hr or 18hr. Metabolic analysis was carried out using the Seahorse Extracellular Flux Analyzer XFe96 (Agilent). MAIT cells were re-counted prior to beginning the Seahorse assay, to ensure resuspension at equal concentrations. Cells were then washed and resuspended in phenol red-free RPMI media, substituted with 10mM glucose, 2mM glutamine, and 1mM pyruvate (all Agilent). Cells were seeded onto a Cell-Tak (Corning)-coated 96-well microplate for adhesion, and incubated in a non CO₂ incubator at 37°C, for 45 minutes. Metabolic parameters (mitochondrial and glycolytic) were measured using OCR (pmol/min), and ECAR (mpH/min), as readouts. Metabolic dependencies were interrogated using the following injections: Oligomycin (1 μ M), FCCP (1 μ M), Rotenone and Antimycin A (both 1 μ M) and Monensin (20 μ M) (all Sigma). Metabolic parameters were calculated as per well-established protocols^{20, 21}. Glycolytic burst is calculated as Maximal Glycolysis minus Basal Glycolysis.

MAIT Cell Flow Cytometric Analysis

PBMC were activated using IFN α (250-500ng/ml; R&D Systems) and IL-18 (50ng/ml; BioLegend) for 6hr or 18hr. For metabolic interrogation studies, PBMC were pre-treated for 1hr with 2-deoxy-D-glucose (1mM; Sigma), or CP91149 (100 μ M; Sigma), prior to stimulation. To interrogate intracellular ISG15 expression with alternative stimuli, MAIT cells in PBMC were also stimulated with anti-CD3/CD28 TCR Dynabeads, IL-15 (50ng/ml; BioLegend), IL-12 (50ng/ml; BioLegend), IL-33 (50ng/ml; BioLegend) or IL-18 (50ng/ml) alone, or in combination as denoted in the appropriate figure legend. Cell viability was determined using eBioscience Fixable Viability Dye (eFluor506; Invitrogen), and gating was carried out on live cells. MAIT cells were phenotyped as being CD3⁺, CD161⁺, and TCRV α 7.2⁺ using extracellular monoclonal antibodies (BioLegend and Miltenyi Biotec). Specific monoclonal antibodies were used to assess phenotyped MAIT cells for the expression of CD69, IFN γ , granzyme B, granulysin, perforin, granzyme A, and granzyme K (all Miltenyi Biotec or BioLegend), Puromycin (Sigma), and ISG15 (R&D Systems). Where appropriate, cells were fixed and permeabilised according to manufacturer guidelines, using the True-Nuclear Transcription Factor Buffer set (BioLegend). For assessing the expression of IFN γ and granzyme B at a 6hr timepoint, Protein Transport Inhibitor Cocktail (Invitrogen) was added 1hr post-stimulation. For assessing the expression of IFN γ and granzyme B at an 18hr timepoint, cells were stimulated overnight, and Protein Transport Inhibitor Cocktail (Invitrogen) was added for the final 6hrs. Cell populations were acquired using an Attune NxT flow cytometer, and analysed using FlowJo version 10.8.2 (TreeStar). Results are expressed as either a percentage of the parent population, as determined using fluorescence minus one (FMO) and unstained controls; or through use of geometric mean to determine the mean fluorescence intensity (MFI) of the relevant marker.

In Silico RNA Sequencing Analysis

Raw gene counts for human peripheral blood MAIT cells isolated from individuals one day prior to and one day following vaccination with ChAdOx1 MenB.1 (n = 14 donors) were downloaded from Gene Expression Omnibus, accession number GSE158835. Gene counts were normalised and differential expression analysis between pre- and post-vaccination timepoints was assessed. Similarly, single-cell sequencing raw data from a human COVID-19 challenge study²² was downloaded and ISG15 expression assessed in both peripheral, and nasal, MAIT cells from individuals with sustained SARS-CoV-2 infection.

RNA Isolation, cDNA Synthesis, and Real-Time PCR

IL-2 (BioLegend) expanded MAIT cells were activated with 500ng/ml IFN α (R&D Systems) and 50ng/ml IL-18 (BioLegend) for 6hr. Total RNA was extracted using TRIzol Reagent (Invitrogen) as per manufacturer guidelines. cDNA was generated using the qScript cDNA Synthesis kit (QuantaBio). Real time RT-qPCR was performed using PerfeCTa SYBR Green FastMix Reaction Mix (Green Fastmix, ROX™) (QuantaBio), and KiCqStart primer sets (Sigma).

Statistics

Statistical analysis was completed using Graph Pad Prism 10 software for macOS. Data is expressed as the mean $\pm$ SEM. Distribution was assessed using Shapiro-Wilk test. Outliers were determined using ROUT analysis. We determined differences between two groups using Student t-test (paired or unpaired) or one sample t and Wilcoxon test where appropriate. Analysis across 3 or more groups was performed using one-way ANOVA, or Friedman test, where appropriate. P values were expressed with significance set at <0.05.

Results

Innate Cytokine Stimulation Drives Significant Proteomic Remodelling in Human MAIT Cells

To investigate the molecular and metabolic responses of MAIT cells to innate cytokine stimulation, we performed high-resolution quantitative proteomic analysis of expanded human MAIT cell lines in the absence (basal) or presence of IL-18/IFN α for 18hr, detecting 7634 proteins (Figure 1A-B). We observed a significant remodelling of the MAIT cell proteome in response to IL-18/IFN α stimulation with 479 proteins increasing in abundance (Figure 1A-B). The data demonstrate a strong anti-viral signature, with significant increases in proteins such as signal transducer and activator of transcription 1 (STAT1), STAT2, and IRF9 (Figure 1C-E). Furthermore, we observed substantial increases in the expression of numerous ISGs, including but not limited to ISG15, MX1, and USP18 (Figure 1F-G); indicative of interferon-stimulated gene factor 3 (ISGF3) complex formation. As expected, we did not identify upregulation of STAT1, STAT2, or IRF9 upon stimulation with TCR/IL-18 (Figure 1C-E), and as such, observed distinct proteome remodelling (Figure S1). Even more so than IL-18/IFN α , stimulation with TCR/IL-18 also drives robust remodelling of the MAIT cell proteome, with

839 proteins increasing in abundance, compared to unstimulated MAIT cells (Figure S1). Of these proteins, approximately 206 are commonly upregulated downstream of both TCR triggering, and IL-18/IFN α signalling, with pathway analysis revealing a shared enrichment of processes pertaining to ribosomal biogenesis and cellular biosynthesis (Figure S1).

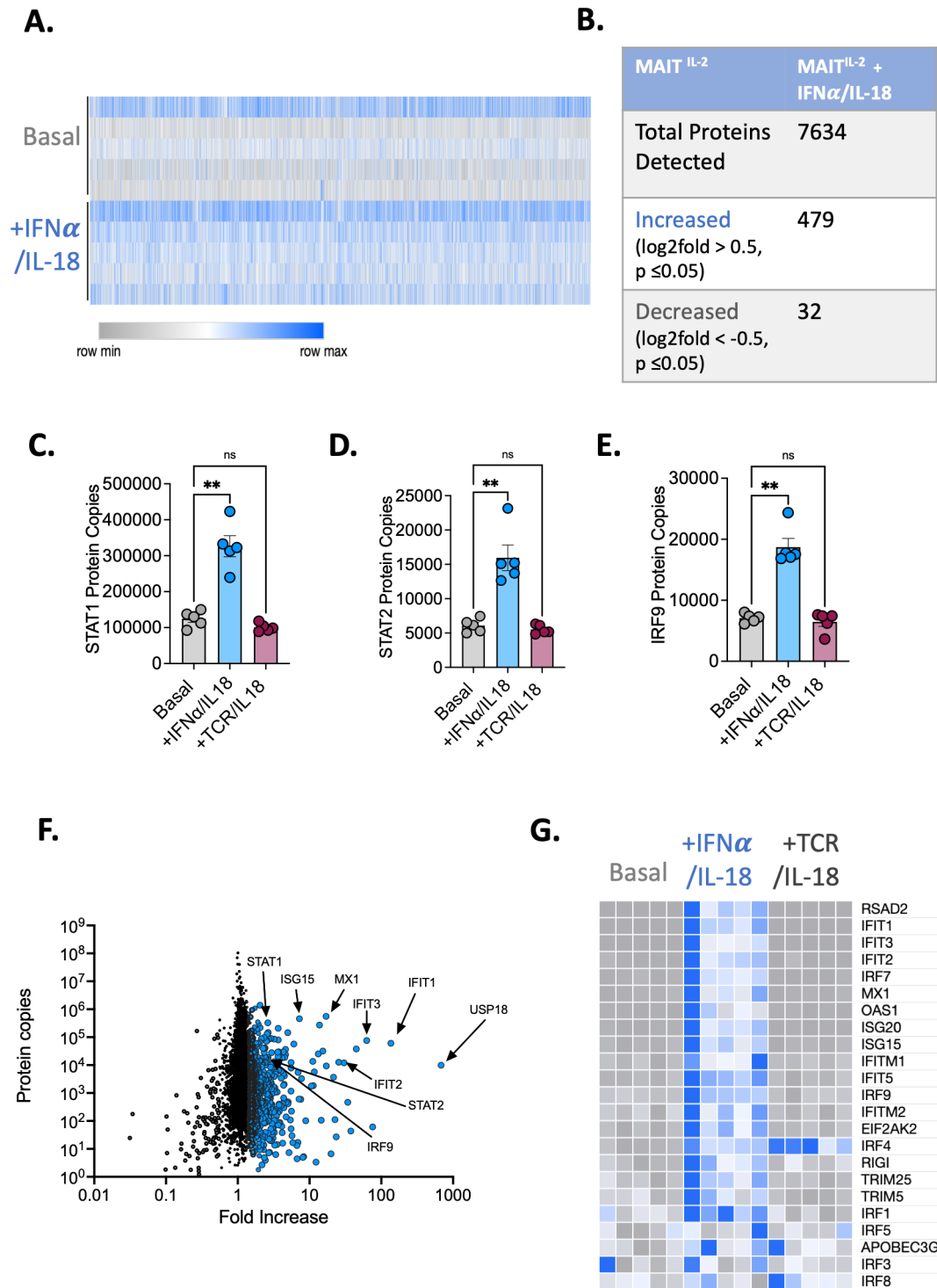


Figure 1. Stimulation with IL-18/IFN α drives significant proteomic remodelling in human MAIT Cells. (A) Heatmap of the proteome of IL-2 expanded MAIT (MAIT^{IL-2}) cells or MAIT^{IL-2} cells stimulated with IL-18/IFN α for 18hr (n=5 per group). Relative protein abundance is graded from grey (low) to blue (high) per row. (B) Table outlining the total number of proteins detected in MAIT^{IL-2} cells, and their change upon stimulation with IL-18/IFN α for 18hr; as determined by quantitative proteomics. (C-E) Scatterplots comparing protein copy expression of the components of the ISGF3 protein complex (STAT1, STAT2, and IRF9) in MAIT^{IL-2} cells, and

*MAIT^{IL-2} cells stimulated with IL-18/IFN α , or MAIT^{IL-2} cells stimulated with anti-CD3/CD28 TCR beads/IL-18, for 18hr; as determined by quantitative proteomics. (F) Scatterplots depicting protein copy number against fold increase of the proteome of MAIT^{IL-2} cells or MAIT^{IL-2} stimulated with IL-18/IFN α for 18hr. Blue circles represent MAIT^{IL-2} stimulated proteins that have increased 1.5 fold or more over MAIT^{IL-2} cells. (J) Heatmap depicting the relative expression of interferon-stimulated genes (ISGs) in MAIT^{IL-2} cells, and MAIT^{IL-2} cells stimulated with IL-18/IFN α , or MAIT^{IL-2} cells stimulated with anti-CD3/CD28 TCR beads/IL-18, for 18hr; as determined by quantitative proteomics. Relative protein abundance is graded from grey (low) to blue (high) per row. * = $p \leq 0.05$, ** = $p \leq 0.01$, ns = not significant, $p > 0.05$, as determined using paired t test, or one-way ANOVA, where appropriate.*

Innate Cytokine Stimulation Increases MAIT Cell Biosynthesis

Pathway analysis of our proteomic dataset revealed an enrichment of the translation initiation signature in IL-18/IFN α stimulated MAIT cells (Figure 2A). To investigate this further, we examined the expression of the 5' cap translational machinery (Figure 2B), and found substantial increases in the expression of associated proteins, eIF4G1, eIF4A1, and PABPC1, downstream of IL-18/IFN α stimulation (Figure 2C-F). Additionally we note downregulated expression of two known repressors of this pathway, 4E-BP1/2 and PDCD4 (Figure 2G-H). In agreement with the findings from our enrichment analysis, we compared the ratio of 4E-BP1/2 to eIF4E, and PDCD4 to eIF4A, in basal and IL-18/IFN α stimulated MAIT cells (Figure 2I-J), and found substantially lowered ratios following stimulation. An excess of free eIF4E and eIF4A protein copies in innate cytokine activated MAIT cells is indicative of an increased capacity to perform of cap-dependent translation. In agreement, we identified a significant increase in MAIT cell size following just 6hr stimulation with IL-18/IFN α , and noted concomitant with a significant increase in total cellular protein content (Figure 2K-L). Finally, to directly probe the rates of *de novo* protein synthesis occurring in human peripheral-blood MAIT cells in response to stimulation with IL-18/IFN α , we measured the incorporation of puromycin into newly synthesized proteins using flow cytometry, and highlighted significantly increased rates of protein biosynthesis 6hr post-stimulation (Figure 2M-N). Taken together, these data demonstrate that MAIT cells respond rapidly to stimulation with innate cytokines, presenting with marked proteome remodelling and robust *de novo* biosynthesis.

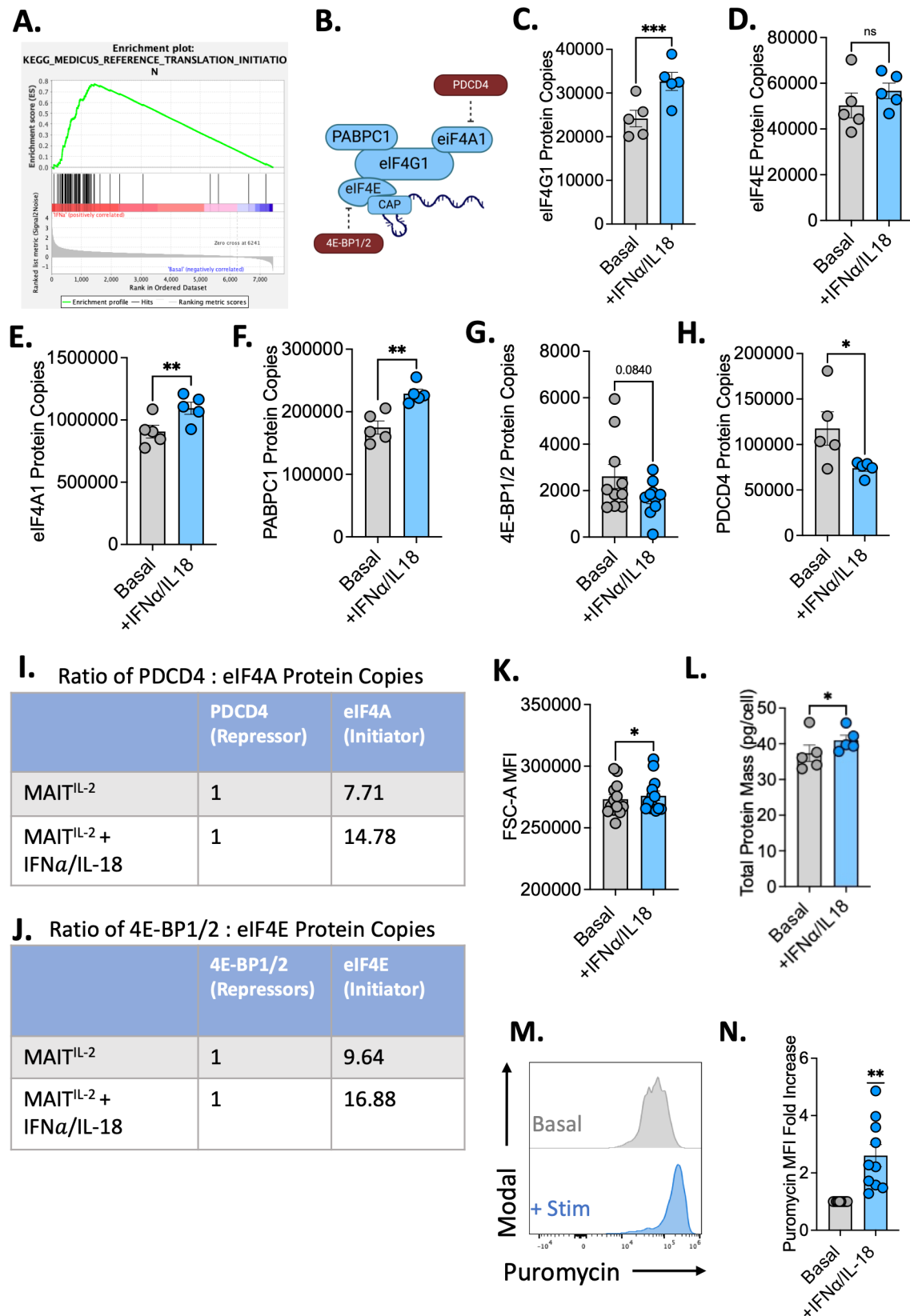


Figure 2. Stimulation with IL-18/IFN α drives significant cellular biosynthesis in human MAIT cells. (A) GSEA plot showing increased proteomic expression of translation initiation-related proteins in MAIT^{IL-2} cells stimulated with IL-18/IFN α for 18hr. Data extrapolated using quantitative proteomic analysis. (B) Schematic of the eukaryotic initiation factor 4F (eIF4F)

*cap-binding complex. (C-F) Scatterplots depicting protein copy numbers of the positive regulatory components of the eIF4F translation initiation complex, in MAIT^{IL-2} cells or MAIT^{IL-2} cells stimulated with IL-18/IFN α for 18hr; as determined by quantitative proteomics. (G-H) Scatterplots depicting protein copy numbers of the negative regulatory components of the eIF4F translation initiation complex, in MAIT^{IL-2} cells or MAIT^{IL-2} cells stimulated with IL-18/IFN α for 18hr; as determined by quantitative proteomics. (I-J) Tables showing the ratio of PDCD4 to eIF4A or 4E-BP1/2 to eIF4E in MAIT^{IL-2} cells or MAIT^{IL-2} cells stimulated with IL-18/IFN α for 18hr; as determined by quantitative proteomics. (K) Scatterplots showing forward scatter (FSC) mean fluorescence intensity (MFI) of MAIT cells, basal or stimulated with IL-18/IFN α for 6hr. (L) Scatterplots showing protein mass in MAIT^{IL-2} cells or MAIT^{IL-2} cells stimulated with IL-18/IFN α for 18hr. (M-N) Representative flow cytometric histograms and scatterplots showing puromycin incorporation in MAIT cells, basal or stimulated with IL-18/IFN α for 6hr. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, ns = not significant, $p > 0.05$, as determined using paired t test, or one sample t and Wilcoxon test, where appropriate.*

Innate Cytokine Stimulation Mediates Rapid Effector Responses in Human MAIT Cells

Having identified significant proteome remodelling at 18hr, alongside robust protein biosynthesis at 6hr, we sought to further investigate the functional kinetics of MAIT cell responses to innate cytokine stimulation via flow cytometric analysis, looking directly at human peripheral blood MAIT cells in PBMC (see Figure S2 for relevant gating strategy). Stimulation with IL-18/IFN α elicited significant early (6hr) production of the key effector cytokine, IFN γ (Figure 3B), and although we identified significant IFN γ production at 18hr (Figure 3A and C), we noted no difference in the magnitude of the response at the 6hr and 18hr timepoints (Figure 3D). Such findings suggest that IFN γ is rapidly expressed in MAIT cells upon stimulation with innate cytokines, but this is not a sustained response. As expected, IL-18/IFN α elicited a rapid increase in the production of granzyme B, apparent at 6hr (Figure 3F); and there was a significant increase in the magnitude of the response at 18hr compared to 6hr (Figure 3A and G-H), suggesting that the granzyme B response is established early, and comprises a sustained arm of the MAIT cell effector response to innate cytokine stimulation. We next probed the kinetics of the ISG response elicited downstream of stimulation with IL-18/IFN α using flow cytometry, and confirmed the rapid induction of key anti-viral protein ISG15 in human peripheral blood MAIT cells, which was evident as early as 6hr post-stimulation (Figure 3I-J). A substantial increase in the magnitude of the response was observed at the 18hr timepoint (Figure 3I-J), highlighting slower response kinetics when compared with IFN γ . As expected, ISG15 is robustly induced by IFN α , and it is further upregulated by the synergistic activity of IL-18 (Figure S3). Interestingly, IL-18 does not

synergize with TCR triggering to enhance the expression of ISG15, though we identify some upregulation upon activation with TCR beads alone, and upon stimulation with IL-15, but not IL-12 or IL-33 (Figure S3). Such findings further highlight a distinct role for the synergistic activity of IL-18 in potentiating MAIT cell responses to IFN α , and characterise robust ISG15 expression as a component of MAIT cell innate effector responses to IL-18/IFN α . Highlighting the divergence from conventional T cells, and the rapid innate responsiveness of MAIT cells, we found significantly increased expression of ISG15 in CD8⁺ peripheral blood MAIT cells when compared with non-MAIT CD8⁺ conventional T cells, 18hr post-stimulation with IL-18/IFN α ; and this response followed a 'gradient of innateness', whereby NK cells expressed higher levels of ISG15 than MAIT cells (Figure 3K-M).

To further corroborate our findings and demonstrate this response profile of increased IFN γ , granzyme B, and ISG15 expression in MAIT cells in the context of a more physiologically relevant challenge, we performed *in silico* analysis of two publicly available bulk and single-cell RNA sequencing datasets. Reanalysis of a 2021 study by Provine and colleagues¹⁶ highlighted robust increases in MAIT cell specific granzyme B and ISG15, but not IFN γ , expression post-ChAdOx1 (chimpanzee adenovirus Ox1) vector challenge (Figure 3N-Q); whilst reanalysis of single-cell sequencing data from a human COVID-19 challenge study²², revealed significant increases in ISG15 expression by both peripheral and nasal MAIT cells from individuals with sustained SARS-CoV-2 infection (Figure S4).

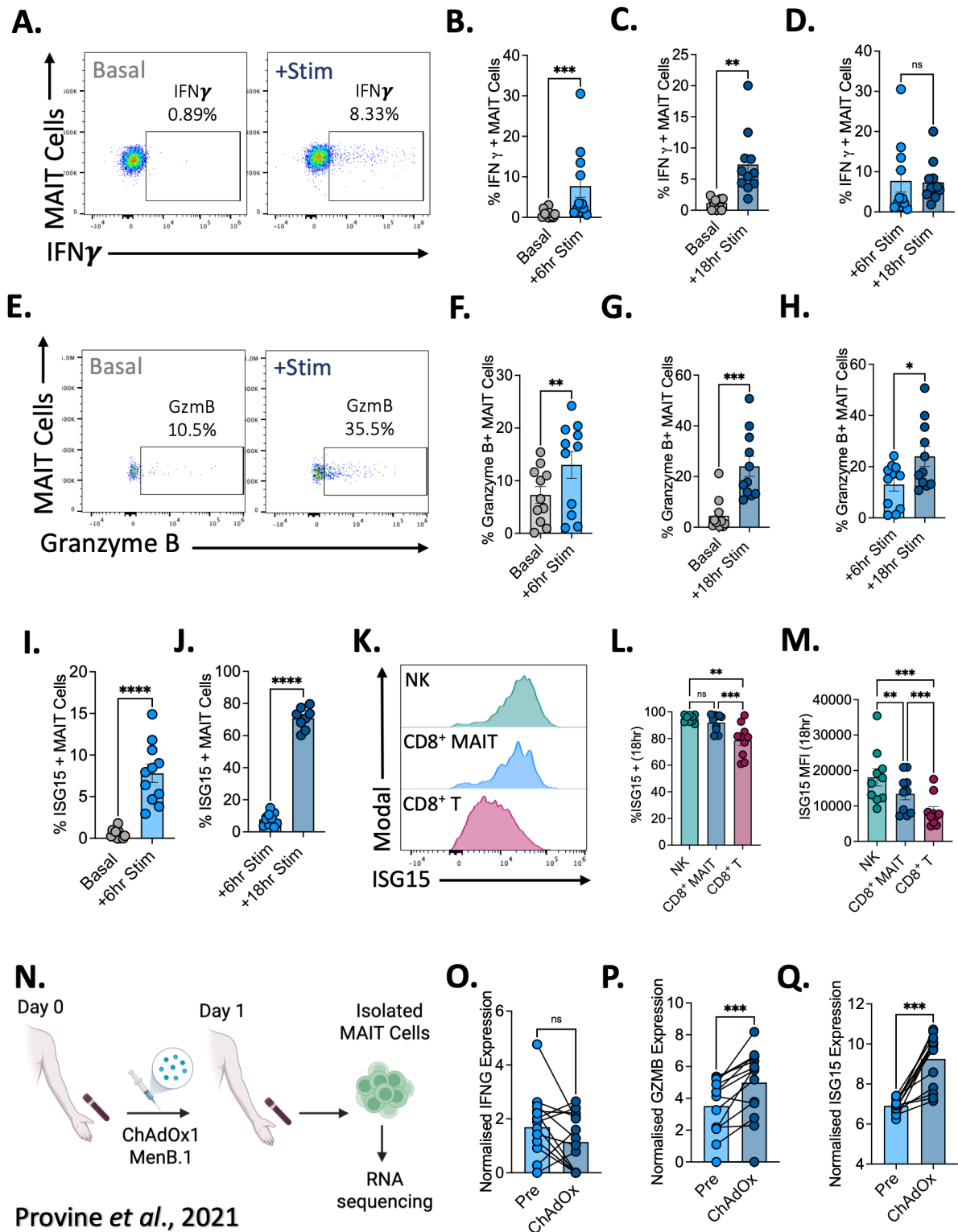


Figure 3. Stimulation with IL-18/IFN α mediates rapid effector responses in human MAIT cells. (A) Representative flow cytometric dotplots depicting percent IFN γ expression in MAIT cells basal or stimulated with IL-18/IFN α for 18hr. (B-D) Scatterplots depicting percent IFN γ expression in MAIT cells, basal or stimulated with IL-18/IFN α for 6hr or 18hr. (E) Representative flow cytometric dotplots detailing percent expression of granzyme B in MAIT

cells, basal or stimulated with IL-18/IFN α for 18hr. (F-H) Scatterplots depicting percent expression of granzyme B in MAIT cells basal or stimulated with IL-18/IFN α for 6hr or 18hr. (I-J) Scatterplots depicting percent ISG15 expression in MAIT cells, basal or stimulated with IL-18/IFN α for 6hr or 18hr. (K-M) Representative flow cytometric histograms and scatterplots detailing ISG15 expression, percent expression or mean fluorescence intensity (MFI), in NK cells, CD8⁺ MAIT cells, and CD8⁺ non-MAIT conventional T cells in PBMC, basal or following 18hr stimulation with IL-18/IFN α . (N) Schematic illustrating a brief description of the gene expression dataset generated by Provine and colleagues¹⁶. (O-Q) *In silico* analysis of this gene expression dataset, deposited in the Gene Expression Omnibus under GSE158835, detailing *IFNG*, *GZMB*, and *ISG15*, expression in *ex vivo* MAIT cells isolated from the peripheral blood mononuclear cells of human volunteers pre- and post (24hr)- vaccination with the adenoviral vaccine vector, ChAdOx1. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$, ns = not significant, $p > 0.05$, as determined using paired or unpaired t test, or one-way ANOVA, where appropriate.

Given that stimulation of MAIT cells with IL-18/IFN α appears to drive a Th1-like/cytotoxic effector phenotype, we sought also to evaluate whether the stimulation of human MAIT cells with IL-18/IFN α might elicit expression of other cytotoxic/cytolytic molecules, beyond granzyme B. Unsurprisingly, in both expanded MAIT cell lines, and directly amongst MAIT cells in PBMC, IL-18/IFN α signalling resulted in significantly increased expression of the lytic molecule perforin at the 18hr timepoint (Figure S5). Interestingly, analysis of our proteomic dataset also revealed increased expression of granzyme H in expanded human MAIT cell lines downstream of stimulation with IL-18/IFN α (Figure S5). However, we did not identify increased expression of granzyme A, granzyme K, granzyme M, or granulysin, in expanded human MAIT cell lines, or amongst MAIT cells in PBMC, downstream of IL-18/IFN α signalling; but do note high-level basal expression of these effector molecules in human MAIT cells (Figure S5). All together, these data highlight the rapid functional kinetics of MAIT cell effector responses to IL-18/IFN α , characterised in particular by the robust upregulation of granzyme B, and ISG15.

Innate Cytokine Stimulation Drives an Early Glycolytic Burst in Human MAIT Cells

Our group and others have previously defined the metabolic responses elicited in MAIT cells downstream of TCR triggering^{3, 11}; however to date, the metabolic response of MAIT cells to innate cytokine stimulation remains unknown. Thus, to investigate the metabolic kinetics of MAIT cell responses to IL-18/IFN α , we carried out Seahorse Extracellular Flux Analysis on

expanded human MAIT cell lines across 6hr and 18hr timepoints. Here, we show significantly increased rates of glycolysis (as determined by baseline extracellular acidification rates, ECAR) upon stimulation with innate cytokines (IL-18/IFN α), or TCR Dynabeads and IL-18 (TCR/IL-18) at 6 hours (Figure 4A-B). Importantly, we did not observe increased oxygen consumption rates in either condition, at the 6 hour timepoint (Figure 4C-D). We next investigated whether this same finding could be recapitulated at the later timepoint of 18hr. In line with our previous findings^{3, 23}, TCR/IL-18 stimulated MAIT cells engaged in sustained, and robust levels of glycolysis 18hr post-stimulation; but also presented with enhanced mitochondrial OxPhos, as determined by analysis of basal oxygen consumption rates. However, in contrast to our findings at 6hr, there was no difference in the metabolic profiles between unstimulated or IL-18/IFN α -stimulated MAIT cells at the 18hr timepoint, suggestive of a more transient metabolic response (Figure 4E-H). Previous work from our group identified Myc as a major regulator of the glycolytic switch induced in TCR activated MAIT cells³. Accordingly, we investigated whether this was also true for innate cytokine stimulated MAIT cells. Interestingly, we found that MAIT cells do not significantly enhance Myc mRNA expression upon stimulation with IL-18/IFN α . Importantly, we did show robust TCR-driven increases, thus corroborating our previous findings (Figure S6); and suggesting that Myc is not a significant transcriptional regulator of the transient glycolytic burst elicited in MAIT cells upon the recognition of innate cytokines in the absence of TCR engagement. We further corroborated our findings by evaluating expression of Myc target genes within the IL-18/IFN α MAIT cell proteome, and similarly, did not observe robust increases in Myc target gene expression (Figure S6).

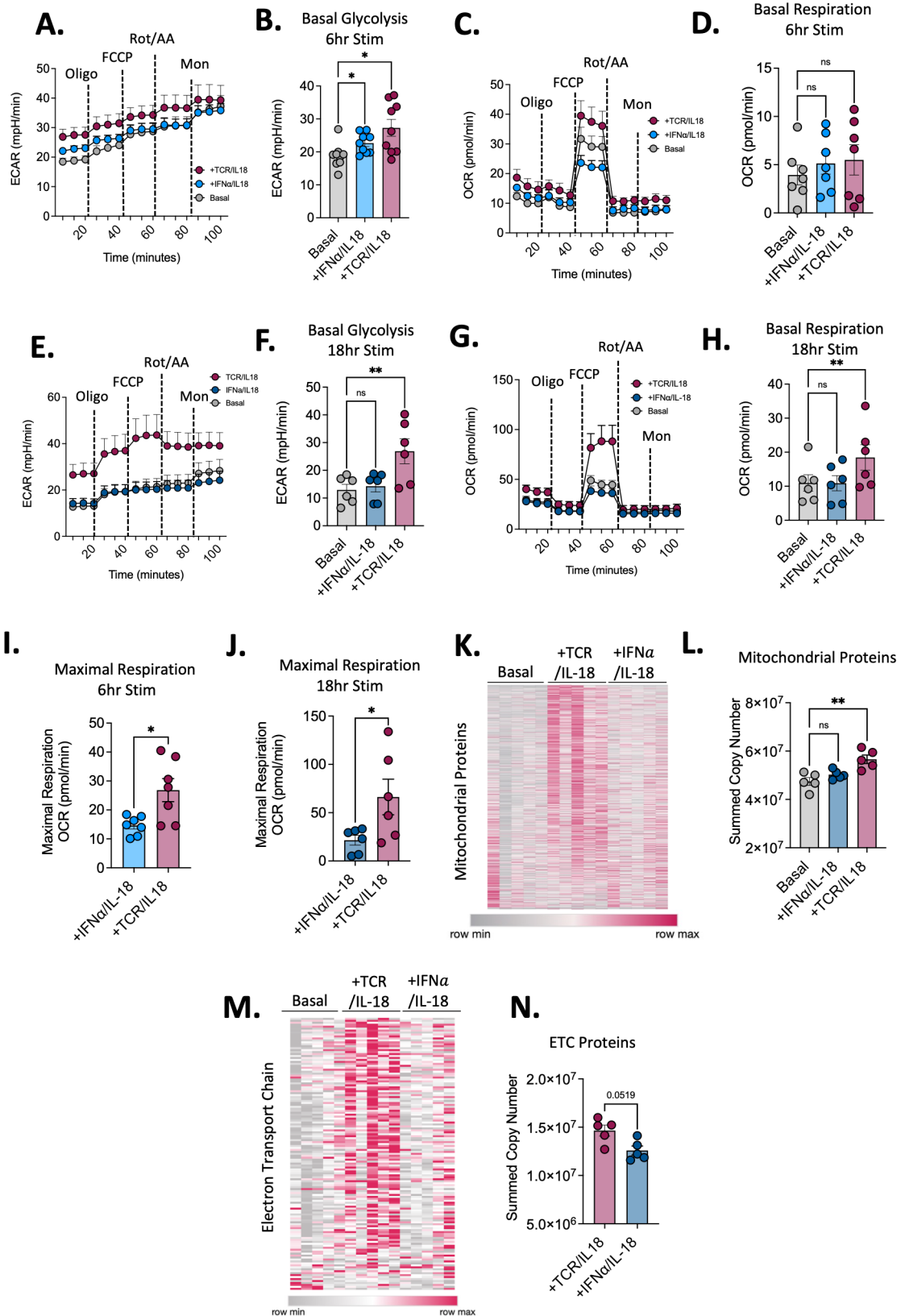


Figure 4. Innate cytokine stimulation drives an early glycolytic burst in human MAIT cells. (A-B) Representative Seahorse trace and scatterplots ($n=9$) depicting basal extracellular acidification rates (ECAR) in MAIT^{L-2} cells, and MAIT^{L-2} cells stimulated with IL-18/IFN α or anti-

CD3/CD28 TCR beads/IL-18 for 6hr. (C-D) Representative Seahorse trace and scatterplots (n=7) depicting basal oxygen consumption rates (OCR) in MAIT^{IL-2} cells, and MAIT^{IL-2} cells stimulated with IL-18/IFN α or anti-CD3/CD28 TCR beads/IL-18, for 6hr. (E-F) Representative Seahorse trace and scatterplots (n=6) depicting basal extracellular acidification rates (ECAR) in MAIT^{IL-2} cells, and MAIT^{IL-2} cells stimulated with IL-18/IFN α , or anti-CD3/CD28 TCR beads/IL-18, for 18hr. (G-H) Representative Seahorse trace and scatterplots (n=6) depicting basal oxygen consumption rates (OCR) in MAIT^{IL-2} cells, and MAIT^{IL-2} cells stimulated with IL-18/IFN α or anti-CD3/CD28 TCR beads/IL-18, for 18hr. (I-J) Scatterplots showing maximal respiration in MAIT^{IL-2} cells, and MAIT^{IL-2} cells stimulated with or IL-18/IFN α , or anti-CD3/CD28 TCR beads/IL-18, for 6hr or 18hr. (K) Heatmap of mitochondrial proteins (Gene Ontology GO:0005739; MitoCarta3.0) expressed in MAIT^{IL-2} cells basal or stimulated with anti-CD3/CD28 TCR beads/IL-18, or IL-18/IFN α for 18hr. (L) Summed protein copy number of mitochondrial proteins expressed in MAIT^{IL-2} cells basal or stimulated with anti-CD3/CD28 TCR beads/IL-18, or IL-18/IFN α for 18hr. (M) Heatmap of electron transport chain proteins (ETC; MitoCarta3.0 'OXPHOS') expressed in MAIT^{IL-2} cells basal or stimulated with anti-CD3/CD28 TCR beads/IL-18, or IL-18/IFN α for 18hr. (N) Summed protein copy number of electron transport chain proteins in MAIT^{IL-2} cells basal or stimulated with anti-CD3/CD28 TCR beads/IL-18, or IL-18/IFN α for 18hr. * = $p \leq 0.05$, ** = $p \leq 0.01$, ns = not significant, $p > 0.05$, as determined using one-way ANOVA, Friedman test, or paired t test, where appropriate.

At both 6hr and 18hr timepoints, we observed a significantly diminished maximal respiratory capacity in IL-18/IFN α stimulated MAIT cells in comparison to that driven downstream of TCR/IL-18 signalling (Figure 4I-J). Furthermore, just 6hr stimulation with IL-18/IFN α significantly impaired the spare respiratory capacity (SRC) of MAIT cells, when compared to those unstimulated, or treated with TCR/IL-18 (Figure S7), suggesting an independence from mitochondrial respiration in IL-18/IFN α -treated MAIT cells. To investigate this further, we performed further analysis of the MAIT cell proteome, probing mitochondrial protein expression. Indeed, stimulation of MAIT cells with IL-18/IFN α did not result in an upregulation of mitochondrial protein content, contrasting significantly the remodelling elicited downstream of TCR/IL-18 signalling (Figure 4K-L). Furthermore, we confirmed reduced expression of proteins involved in the electron transport chain (ETC), and across the individual complexes, in IL-18/IFN α activated MAIT cells, when compared with TCR/IL-18-stimulated MAIT cells (Figure 4 M-N, Figure S7); confirming a reduced capacity for OxPhos in MAIT cells stimulated with innate cytokines.

Collectively, these data demonstrate, for the first time, that MAIT cells respond metabolically to IL-18/IFN α stimulation via an early glycolytic burst which is transient in nature, and uncoupled from mitochondrial respiration.

Glucose and Glycogen are Critical Determinants of MAIT Cell Responses to Innate Cytokine Stimulation

Having described an early glycolytic burst upon stimulation with IL-18/IFN α , we next sought to identify key nutrients which might govern the metabolic and functional response kinetics of innate cytokine activated MAIT cells. Given the importance of glucose for MAIT cell fitness downstream of TCR engagement, we first examined the impact of the hexokinase II (HK2) inhibitor, 2-deoxy-D-glucose (2DG), on the metabolic profile of IL-18/IFN α activated human MAIT cell lines, to gauge the contribution of glucose to early metabolic activity (3hr). We observed a significantly diminished glycolytic capacity in the presence of 2DG, with impairments to basal glycolysis, maximal glycolysis, and estimated ATP production deriving from glycolysis (Figure 5A-D); suggesting that glucose critically underpins the early glycolytic response characteristic of innate cytokine -activated MAIT cells. Interestingly, 2DG did not significantly impact the magnitude of the glycolytic burst i.e., the capacity for MAIT cells to jump from basal glycolysis to maximal glycolysis upon injection of the ionophore, Monensin (Figure 5E). MAIT cells activated with IL-18/IFN α in the presence of 2DG were increasingly engaged in OxPhos, and significantly increased their rates of ATP production linked to OxPhos (Figure S8). Interestingly, 2DG had no significant impact on maximal respiration, or SRC in IL-18/IFN α treated MAIT cells (Figure S8). Furthermore, we investigated the impact of HK2 inhibition on the levels of protein translation directly amongst MAIT cells in PBMC following stimulation with IL-18/IFN α , and interestingly, did not observe a significant reduction in protein biosynthesis in the presence of 2DG (Figure 5F-G). These data suggest that although glucose is fuelling the early glycolytic profile, it may not be a critical determinant of the capacity for rapid biosynthesis following the stimulation of MAIT cells with IL-18/IFN α . To investigate this further, we examined expression of IFN γ , granzyme B, and ISG15 in peripheral blood MAIT cells following stimulation with IL-18/IFN α in the presence of 2DG, via flow cytometry. Interestingly, 2DG had no impact on the expression of IFN γ from these cells (Figure 5H), but significantly attenuated the production of granzyme B, and ISG15 (Figure 5I-J). As previously highlighted in Figure 3, the IFN γ response of IL-18/IFN α treated MAIT cells is rapid, but not increased in magnitude beyond the 6hr timepoint. Conversely, expression of both granzyme B and ISG15 is significantly increased and more established by 18hr. Thus taken together, these data suggest that glucose underpins the early metabolic profile, alongside

robust functional responses; however, early functional responses with more rapid response kinetics are potentially fuelled by an alternative nutrient source.

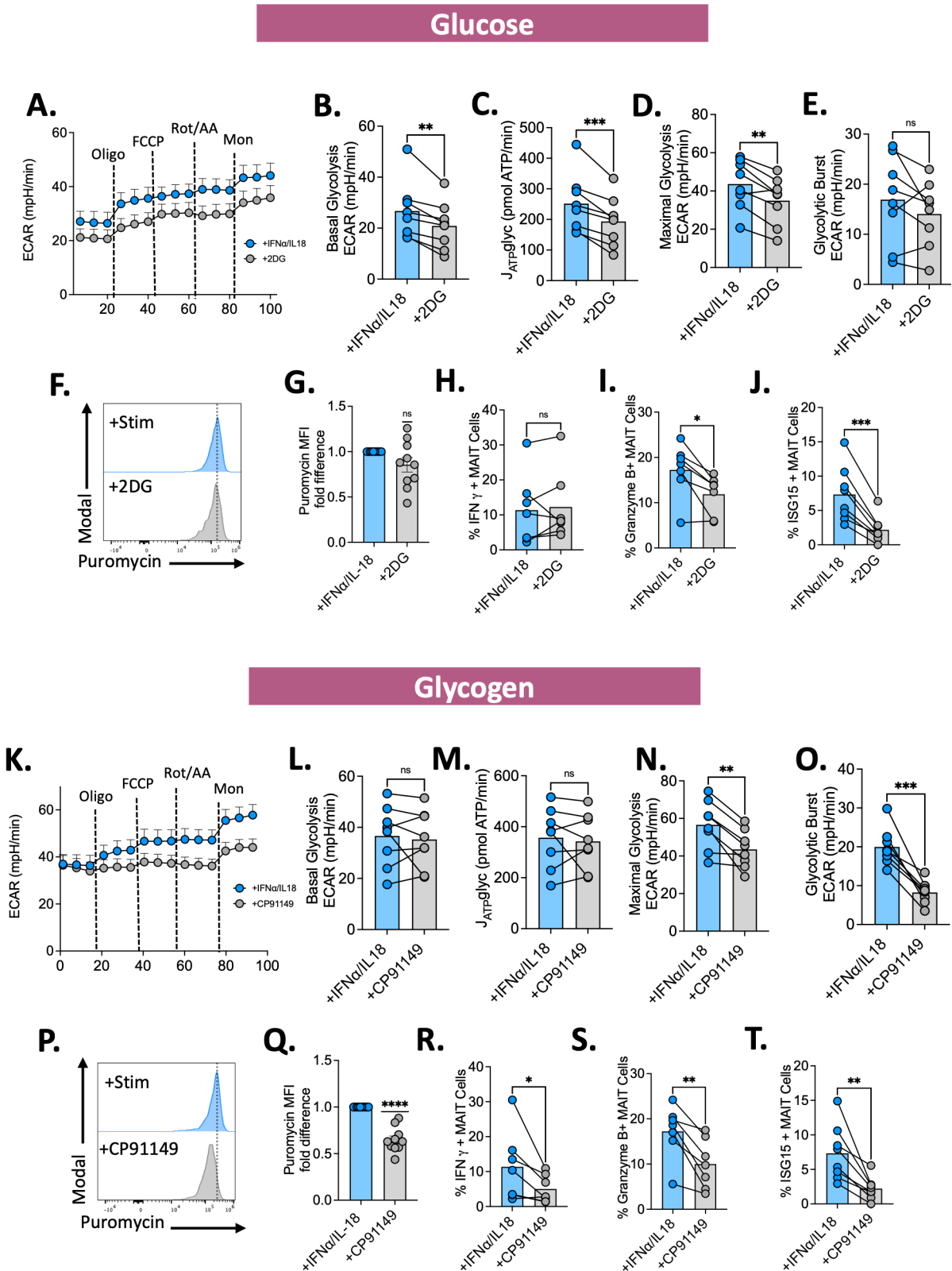


Figure 5. Glucose and glycogen are critical determinants of human MAIT cell metabolic and functional responses to stimulation with IL-18/IFN α . (A-B) Representative Seahorse trace and

scatterplots (n=8) depicting basal extracellular acidification rates (ECAR) in MAIT^{IL-2} cells stimulated with IL-18/IFN α for 3hr, in the absence or presence of the Hexokinase II inhibitor 2-Deoxy-D-glucose (2DG). (C) Scatterplot detailing estimated ATP production linked to glycolysis in MAIT^{IL-2} cells stimulated with IL-18/IFN α for 3hr, in the absence or presence of 2DG. (D) Scatterplot showing maximal ECAR, elicited upon injection of Monensin, in MAIT^{IL-2} cells stimulated with IL-18/IFN α for 3hr, in the absence or presence of 2DG. (E) Scatterplot detailing the glycolytic burst (maximal ECAR minus basal ECAR) in MAIT^{IL-2} cells stimulated with IL-18/IFN α for 3hr, in the absence or presence of 2DG. (F) Representative flow cytometric histograms and scatterplots detailing puromycin incorporation, as a surrogate marker for protein translation, in human peripheral blood MAIT cells stimulated with IL-18/IFN α for 6hr, in the absence or presence of 2DG. (H-J) Scatterplots depicting percent IFN γ , granzyme B, and ISG15, expression in peripheral blood MAIT cells, basal or stimulated with IL-18/IFN α for 6hr in the absence or presence of 2DG. (K-L) Representative Seahorse trace and scatterplots (n=8) depicting basal extracellular acidification rates (ECAR) in MAIT^{IL-2} cells stimulated with IL-18/IFN α for 3hr, in the absence or presence of the glycogen phosphorylase inhibitor, CP91149. (M) Scatterplot detailing estimated ATP production linked to glycolysis in MAIT^{IL-2} cells stimulated with IL-18/IFN α for 3hr, in the absence or presence of CP91149. (N) Scatterplot showing maximal ECAR, elicited upon injection of Monensin, in MAIT^{IL-2} cells stimulated with IL-18/IFN α for 3hr, in the absence or presence of CP91149. (O) Scatterplot detailing the glycolytic burst (maximal ECAR minus basal ECAR) in MAIT^{IL-2} cells stimulated with IL-18/IFN α for 3hr, in the absence or presence of CP91149. (P-Q) Representative flow cytometric histograms and scatterplots detailing puromycin incorporation, as a surrogate marker for protein translation, in peripheral blood MAIT cells stimulated with IL-18/IFN α for 6hr, in the absence or presence of CP91149. (R-T) Scatterplots depicting percent expression of IFN γ , granzyme B, and ISG15, in peripheral blood MAIT cells basal or stimulated with IL-18/IFN α for 6hr in the absence or presence of CP91149. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$, ns = not significant, $p > 0.05$, as determined using paired t test, or one sample t and Wilcoxon test, where appropriate.

In humans, endogenous glycogen stores are metabolised into glucose-6-phosphate through the activity of two key enzymes, glycogen phosphorylase (PYG) and glycogen debranching enzyme (AGL). Glycogen-derived glucose-6-phosphate can directly feed into the glycolytic pathway, bypassing HK2 activity. We have previously shown that rapid (2hr) MAIT cell TCR-specific anti-tumour responses are underpinned by the breakdown of glycogen. To probe whether glycogen might fuel rapid, TCR-independent responses in MAIT cells, we examined the expression of key enzymes involved in the glycogenolysis pathway within our proteomic dataset, and again confirmed robust expression of this pathway in MAIT^{IL-2} cells. In particular, we noted high expression of the brain isoform of PYG (PYGB), and a trending increase in both AGL and PYGB levels upon stimulation with IL-18/IFN α (Figure S9).

We first sought to assess the impact of inhibiting glycogenolysis on the early glycolytic activity of IL-18/IFN α activated MAIT cells, using the specific PYG inhibitor, CP91149. Interestingly, we demonstrated that CP91149 did not impact the levels of basal glycolysis, or estimated glycolysis-linked ATP production (Figure 5K-M), in MAIT cell lines following 3hr stimulation with IL-18/IFN α ; further emphasising that it is glucose which fuels the early metabolic activity of these cells. However, the inhibition of glycogenolysis did result in a diminished glycolytic capacity following stimulation with IL-18/IFN α , as observed via impairment to maximal ECAR, and a diminished glycolytic burst (Figure 5N-O). Similarly, treatment of MAIT cell lines with CP91149 did not impact basal OCR, or estimated OxPhos-linked ATP production following IL-18/IFN α stimulation; however, inhibition of glycogenolysis significantly attenuated both maximal respiration, and the SRC, in IL-18/IFN α -stimulated MAIT cell lines (Figure S8). Interestingly, inhibition of glycogenolysis using CP91149 also resulted in significant impairment to protein biosynthesis in peripheral blood MAIT cells following stimulation with IL-18/IFN α (Figure 5P-Q); and resulted in an attenuated early (6hr) cytokine expression profile, with a loss of IFN γ , granzyme B, and ISG15 (Figure 5R-T). We thus propose that glycogen is a major nutrient facilitator of the early biosynthesis elicited in MAIT cells downstream of stimulation with innate cytokines.

Overall these data highlight robust metabolic and functional responses in human MAIT cells in response to TCR-independent stimulation with innate cytokines. Treatment of human MAIT cells with IL-18/IFN α elicits rapid but transient response kinetics distinct from those driven downstream of TCR engagement, and we propose that such responses are differentially underpinned by the metabolism of glucose, and the breakdown of endogenous glycogen stores.

Discussion

MAIT cells are a subset of unconventional effector T cells capable of rapid and robust functional responses⁵. The sensing of innate cytokines by MAIT cells, independent of their TCR, confers a capacity to respond in the context of human viral infections¹². Recently, MAIT cells have been shown to respond to type I interferons with robust functional responses^{8, 16}.

However, such robust effector functioning would drive significant energy demands, and increase the cellular need for bio-intermediates in order to fuel *de novo* protein biosynthesis. Although the metabolic processes and nutritional requirements that govern MAIT cell effector responses to TCR stimulation are rapidly emerging^{3, 11, 24, 25}, the metabolic regulation of MAIT cell responses to cytokine stimulation has not yet been reported.

In this study, we demonstrate for the first time using high-resolution quantitative proteomics, significant remodelling of the MAIT cell proteome downstream of activation with IL-18/IFN α ; mapping both the molecular and metabolic responses. We demonstrate that MAIT cells respond rapidly to stimulation with IL-18/IFN α , which coincides with a transient glycolytic burst, and early functional responses characterised here by the production of IFN γ , and robust expression of granzyme B, and ISG15. Moreover, we show that IL-18/IFN α driven responses in human MAIT cells are underpinned by glucose, which fuels early metabolic activity and supports sustained functional responses; as well as the breakdown of endogenous glycogen stores, which we have shown to fuel rapid biosynthesis, and effector responses.

Stimulation of human MAIT cells with IL-18/IFN α elicits robust increases in the protein copy expression of STAT1, STAT2, and IRF9. The tyrosine phosphorylation of STAT1 and STAT2 elicits heterodimer formation and their association with IRF9, which then collectively forms the ISGF3 complex. Translocation of a ISGF3 into the nucleus, allows for binding of interferon-stimulated response elements (ISREs) in the ISG promoters, thus inducing their transcription^{26, 27, 28}. Corroborating this, we find ISG protein expression to be some of the most upregulated within our proteomic dataset downstream of IL-18/IFN α signalling, thus conferring an anti-viral state within the cell itself, through the inhibition of viral entry, translation, and replication^{29, 30, 31, 32}. We also demonstrate robust expression of the ubiquitin-specific protease (USP), USP18, a negative regulator of type I IFN-signalling³³; suggesting that by 18hrs, IFN α -activated MAIT cells might display desensitisation to type I signalling.

Unsurprisingly, pathway analysis of the proteome of IL-18/IFN α -stimulated MAIT cells revealed a strong translation-initiation signature, which further emphasises their robust

innate responsiveness. Initiation of cap-dependent translation is facilitated by formation of the eukaryotic initiation factor 4F (eIF4F) complex; an essential cap-binding complex which recruits translation initiation factors and ribosomal subunits to the 5'UTR of mRNA^{34, 35}. Formation of the eIF4F complex is underpinned by binding of eIF4G to eIF4E. Importantly, we find eIF4G protein copies to be significantly increased in MAIT cells downstream of stimulation with IL-18/IFN α , alongside increased expression of PABP and EIF4A. Translation efficiency is enhanced through interaction of EIF4G with the poly-A-tail binding protein, PABP³⁶, and the EIF4A helicase which is required to unwind secondary structures in the 5' UTR³⁷. Collectively, this EIF4F cap-binding complex is a rate-limiting factor of translation initiation, and is negatively regulated by the activities of the tumour-suppressor protein PDCD4³⁸, and the 4E-binding proteins (4E-BPs)^{39, 40}. Importantly, we confirm much lower protein copy ratios of 4E-BP1/2 to eIF4E, and PDCD4 to eIF4A, in basal versus IL-18/ IFN α -activated MAIT cells; indeed suggesting an increased capacity for *de novo* biosynthesis. A recent study by Pavlovic and colleagues noted rapid increases in transcriptional activity in MAIT cells downstream of IFN α -stimulation⁴¹. Indeed, we find that MAIT cells increase in size following 6hr stimulation with IL-18/IFN α ; whilst using puromycin incorporation as a surrogate marker for protein translation⁴² confirmed increased translational activity at this same timepoint.

We confirm that MAIT cells elicit rapid effector in response to the synergistic activity of IL-18/IFN α , displaying robust expression of IFN γ and granzyme B at 6hr post-stimulation. Although IFN γ is rapidly expressed in MAIT cells in response to innate cytokine stimulation, the response is modest and does not follow the same trajectory as granzyme B; which is robustly expressed in IL-18/IFN α stimulated MAIT cells, and is further increased at 18hr, following the initial biosynthetic burst. Interestingly, we show that MAIT cells exhibit high level expression of the interferon-stimulated gene ISG15 following specific stimulation with IFN α , and this is further supported by the synergistic activity of IL-18. Further, CD8⁺ MAIT cells had significantly increased expression of ISG15 when compared to CD8⁺ conventional T cells, highlighting once more their innate-like divergence. Critically, ISG15 is a ubiquitin-like protein which exerts intracellular anti-viral activity through a process termed ISGylation. Post-translational modifications induced by ISG15 are achieved via direct conjugation of ISG15 molecules to lysine residues of newly synthesised proteins⁴³. It is via this ubiquitin-like

function that ISG15 has been proposed to modulate the activity and/or expression of both host and viral proteins. For example, ISGylation of the influenza A virus (IAV) virulence factor, NS1, attenuates viral replication⁴⁴. Thus, the robust expression of ISG15 elicited downstream of IL-18/IFN α signalling in human MAIT cells might comprise a critical signature of their proposed anti-viral functioning. Corroborating this, *in silico* analysis of two publicly available datasets confirmed robust ISG15 expression in MAIT cells in the context of challenge with the ChAdOx1-vaccine vector¹⁶, and during human SARS-CoV-2 infection²². Furthermore, ISG15 can be secreted and has been proposed to act in a cytokine-like manner, where it can potentiate effector responses from both NK cells and T cells *in vivo*⁴⁵; thus MAIT cell derived ISG15 could potentially drive enhanced effector responses from immune cells in the surrounding microenvironment.

Significant energy demands derive from increasing activity of the protein synthesis machinery^{46, 47}. Our group and others have shown that TCR triggering initiates a metabolic switch in MAIT cells, characterised by robust increases in glycolysis^{3, 11, 23, 48}. However, to date, MAIT cell metabolism has not been characterised in the context of TCR-independent signalling. Here, we demonstrate that MAIT cells rapidly engage in glycolysis upon stimulation with IL-18/IFN α , but do not concurrently upregulate mitochondrial respiration; suggesting an uncoupling from the mitochondria, and the provision of pyruvate towards the production of lactate. In an attempt to further characterise the kinetics of this response, we again interrogated the metabolic profile of IL-18/ IFN α -activated MAIT cells, but at the later timepoint of 18hr. Interestingly, we demonstrate that at 18hr, MAIT cells stimulated with IL-18/IFN α are no longer engaging in increased metabolic activity; a signature that markedly contrasts the metabolic response elicited downstream of TCR/IL-18 triggering²³, which coincides with robust increases in glycolysis and OxPhos, at this same timepoint. We also highlight restricted maximal and impaired spare respiratory capacities in IL-18/IFN α - stimulated MAIT cells compared to both TCR stimulated, and unstimulated cells; suggesting that stimulation of MAIT cells with IL-18/IFN α restricts mitochondrial metabolism. Concomitant analysis of our proteomic dataset allowed for surrogate mapping of the mitochondrial capacities of both TCR/IL-18 and IL-18/IFN α – activated MAIT cells, which revealed robust remodelling and upregulation of the global mitochondrial proteome in TCR-activated, but not innate cytokine activated MAIT cells. Furthermore, there are a number of

studies^{49, 50, 51} describing type I IFN-associated impairments to mitochondrial fitness. Taken together, such findings support our hypothesis that IL-18/IFN α drives a transient glycolytic burst in MAIT cells which is uncoupled from the mitochondria, and elicits modest effector responses when compared with those attributed to antigen recognition and co-stimulation.

Downstream of TCR triggering, MAIT cells are critically reliant on the metabolism of glucose to fuel their effector functioning^{11, 24, 52}. Similarly, we show that the early glycolytic burst elicited downstream of innate cytokine stimulation is underpinned by the metabolism of exogenous glucose, and this further supports robust functional responses, specifically the high level expression of granzyme B, and ISG15. Interestingly however, we show that glucose is dispensable for the rapid biosynthesis triggered upon stimulation with innate cytokines, and inhibition of glycolysis using 2DG does not attenuate rapid IFN γ production. Glycogenolysis has previously been shown to fuel rapid (2hr) glycolysis in human MAIT cells following TCR triggering, which elicits early anti-tumour cytotoxicity in the absence of exogenous glucose uptake²⁵. Similarly, early recall responses in CD8⁺ memory T cells are fuelled by glycogen⁵³ and thus, we investigated a role for glycogen in facilitating the rapid responses of MAIT cells to innate cytokine stimulation. Importantly, we confirm that it is glucose, not glycogen, which underpins the initial metabolic burst in response to IL-18/IFN α . However, we highlight glycogen as a critical fuel which facilitates the rapid biosynthesis occurring in human MAIT cells following stimulation IL-18/IFN α . Specifically, we show that robust expression of IFN γ , granzyme B, and ISG15 is facilitated by the rapid mobilisation of endogenous glycogen stores.

Although glycogen has previously been shown to fuel glycolysis in the context of early TCR-driven responses⁵³, we propose here that the glycogenolysis pathway is preferentially used to provide precursors which support rapid and robust biosynthesis in the context of MAIT cell responses to innate cytokines, but does not feed into glycolysis. Recently, Zhou and colleagues have highlighted that glucose-6-phosphate derived from glycogenolysis is the primary substrate involved in fuelling the pentose phosphate pathway (PPP) flux in CD8⁺ memory T cells, something that the author's propose is preferentially upregulated to support the rapid generation of NADPH⁵⁴. It remains unknown whether glycogenolysis is preferentially coupled with the PPP in MAIT cells, but it is interesting to speculate that this

might comprise a key component of the rapid biosynthesis observed in innate cytokine activated MAIT cells, which is at least partially independent from the early glycolytic flux.

Collectively, our data details for the first time the metabolic kinetics of the MAIT cell responses to innate cytokines, highlighting substantial differences when compared to the TCR-driven metabolic profile. Furthermore, we highlight a role for the metabolism of both glucose and glycogen in facilitating the rapid metabolic and functional response kinetics to IL-18/IFN α . Understanding the metabolic profile, and nutrient underpinnings of MAIT cell responses to innate cytokine stimulation are of importance, and may provide further insight into the mechanisms by which MAIT cell anti-viral responses can become perturbed.

Contributors Statement: EKR performed the experiments, carried out the analysis, drafted the manuscript, and approved the final manuscript as submitted. BJJ, RB, RSC, NKM, CF, and SR performed experiments, carried out analysis, and approved the final manuscript as submitted. CD, HH and DOS recruited study participants and helped with study design, analysis, and approved the final manuscript as submitted. LVS performed the proteomic analysis and approved the final manuscript as submitted. LG, AA, IP and PK performed *in silico* analysis of bulk and single cell RNA-sequencing datasets and approved the final manuscript as submitted. DOS, NJ, LVS & AEH conceptualized and designed the study, analysed the data, drafted the manuscript, and approved the final manuscript as submitted.

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Data Availability: All data needed to evaluate the conclusions in the paper are present in the paper or the Materials. All data are available from the corresponding authors in line with ethical approval. The mass spectrometry proteomic data *will* be deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier XXX-XXX-XXX.

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Supplementary Figures and Tables

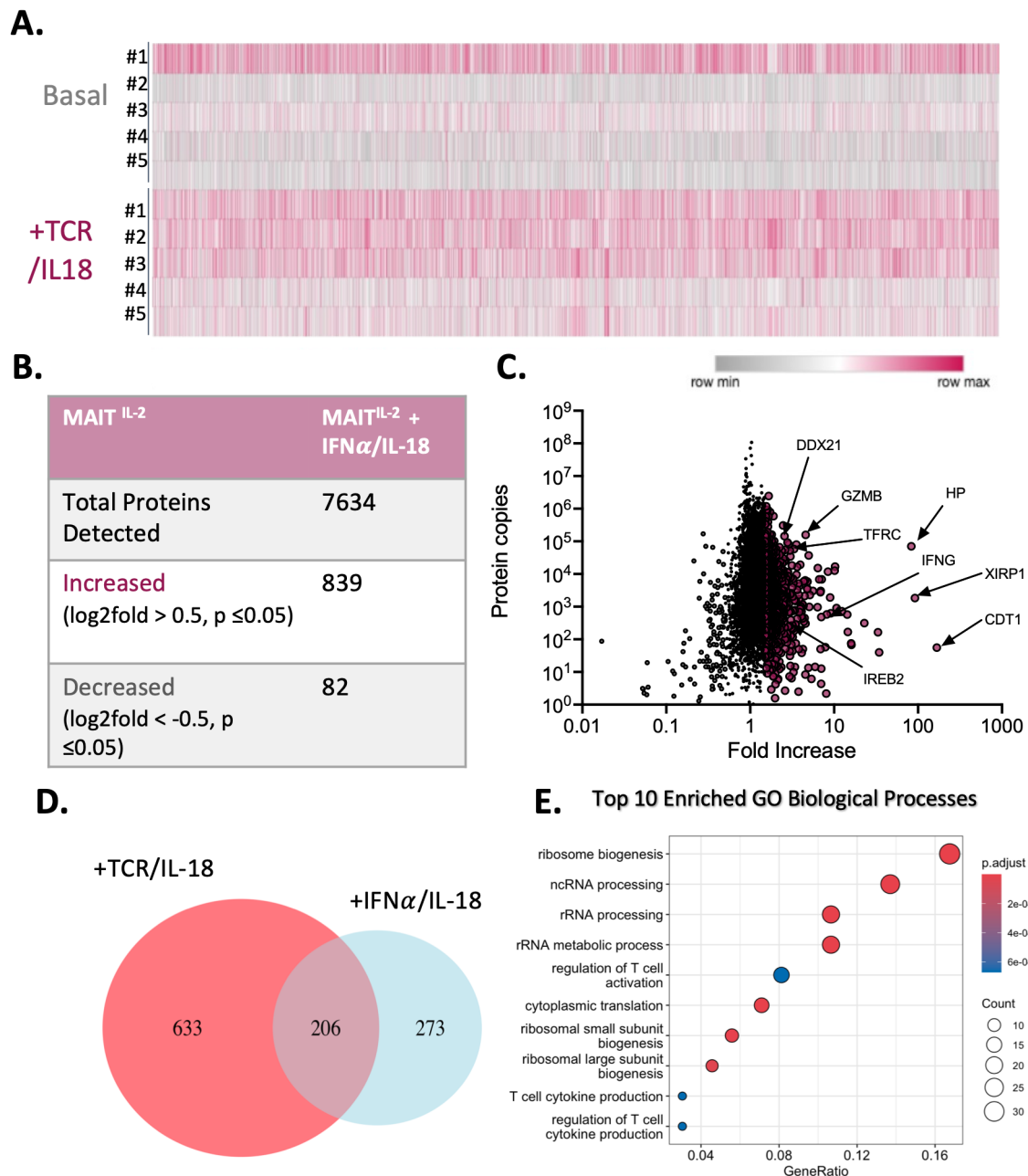


Figure S1. (A) Heatmap of proteome of IL-2 expanded MAIT (MAIT^{IL-2}) cells or MAIT^{IL-2} cells stimulated with anti-CD3/CD28 TCR beads/IL-18 for 18hr (n=5 per group denoted as donor #1, #2, #3, #4 and #5). Relative protein abundance is graded from grey (low) to purple (high) per row. (B) Table outlining total number of proteins detected in MAIT^{IL-2} cells, and their change upon stimulation with anti-CD3/CD28 TCR beads/IL-18 for 18hr, as determined by quantitative proteomics. (C) Scatterplots depicting protein copy number against fold increase of the proteome of MAIT^{IL-2} cells or MAIT^{IL-2} stimulated with anti-CD3/CD28 TCR beads/IL-18 for 18hr.

Purple circles represent MAIT^{L-2} stimulated proteins that have increased 1.5-fold or more over MAIT^{L-2} cells. (D) Venn-diagram depicting the number of proteins that are increased ($\log_2\text{fold} > 0.5$, $p \leq 0.5$) in MAIT^{L-2} cells upon stimulation with anti-CD3/CD28 TCR beads/IL-18 (red) or IL-18/IFN α (blue) for 18hr. (E) Bubble plot of Gene Ontology Biological Processes enrichment analysis highlighting the top 10 biological processes enriched amongst proteins that are commonly upregulated (panel D) in MAIT^{L-2} cells following stimulation with either IL-18/IFN α or anti-CD3/CD28 TCR beads/IL-18 for 18hr.

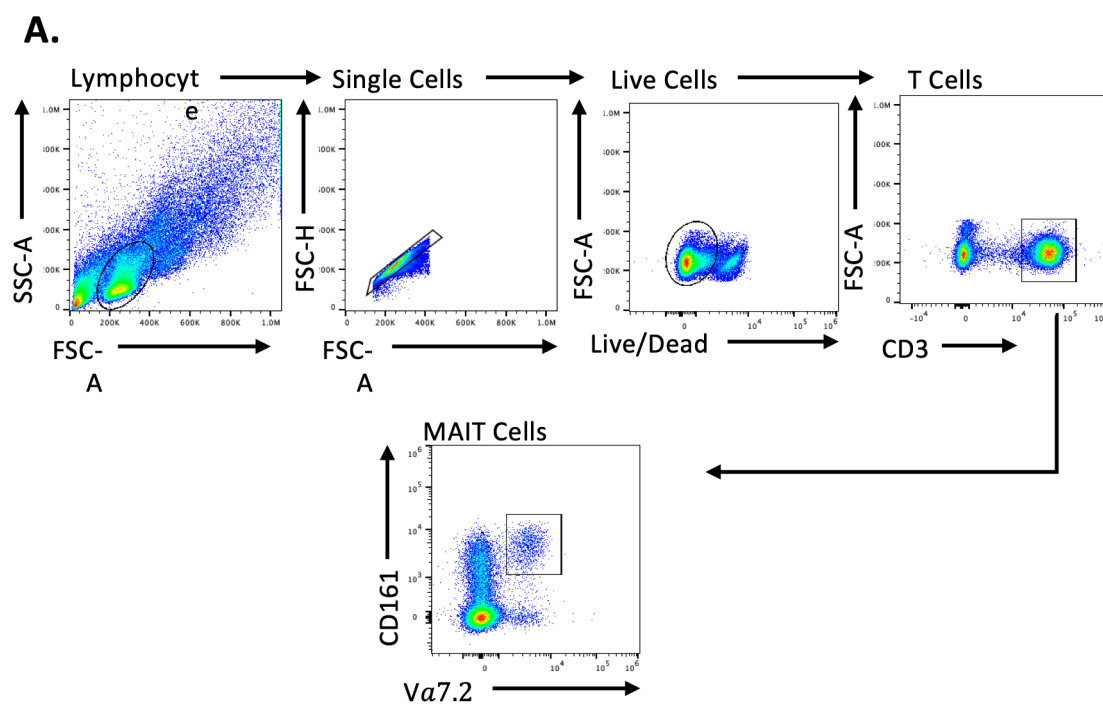


Figure S2. (A) Flow cytometric dot plots detailing the gating strategy used to identify MAIT cells.

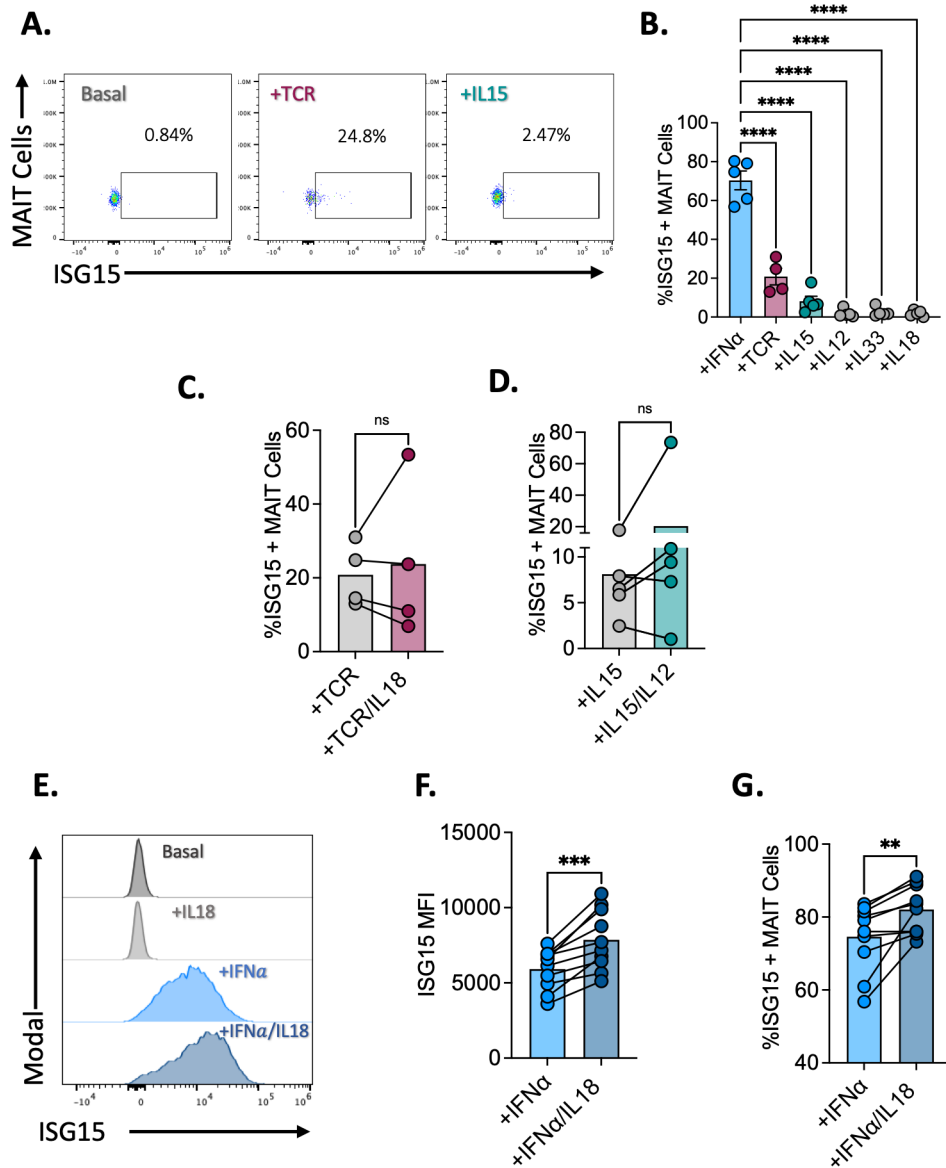


Figure S3. (A-B) Representative dotplots and scatterplots showing percent expression of ISG15 in MAIT cells, basal or treated with anti-CD3/CD28 TCR beads, IFN α , IL-15, IL-12, IL-33, or IL-18 for 18hr. (C-D) Scatterplots showing percent expression of ISG15 in MAIT cells upon stimulation with anti-CD3/CD28 TCR beads, alone or with IL-18, or treated with IL-15 alone, or with IL-12, for 18hr. (E-G) Representative flow cytometric histograms and scatterplots showing ISG15 mean fluorescence intensity (MFI) or percent expression of ISG15 in MAIT cells upon stimulation with IFN α alone, or with IL-18, for 18hr. ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** $p \leq 0.0001$, ns = not significant, $p > 0.05$, as measured by one-way ANOVA, paired t test or Wilcoxon signed-rank test, where appropriate.

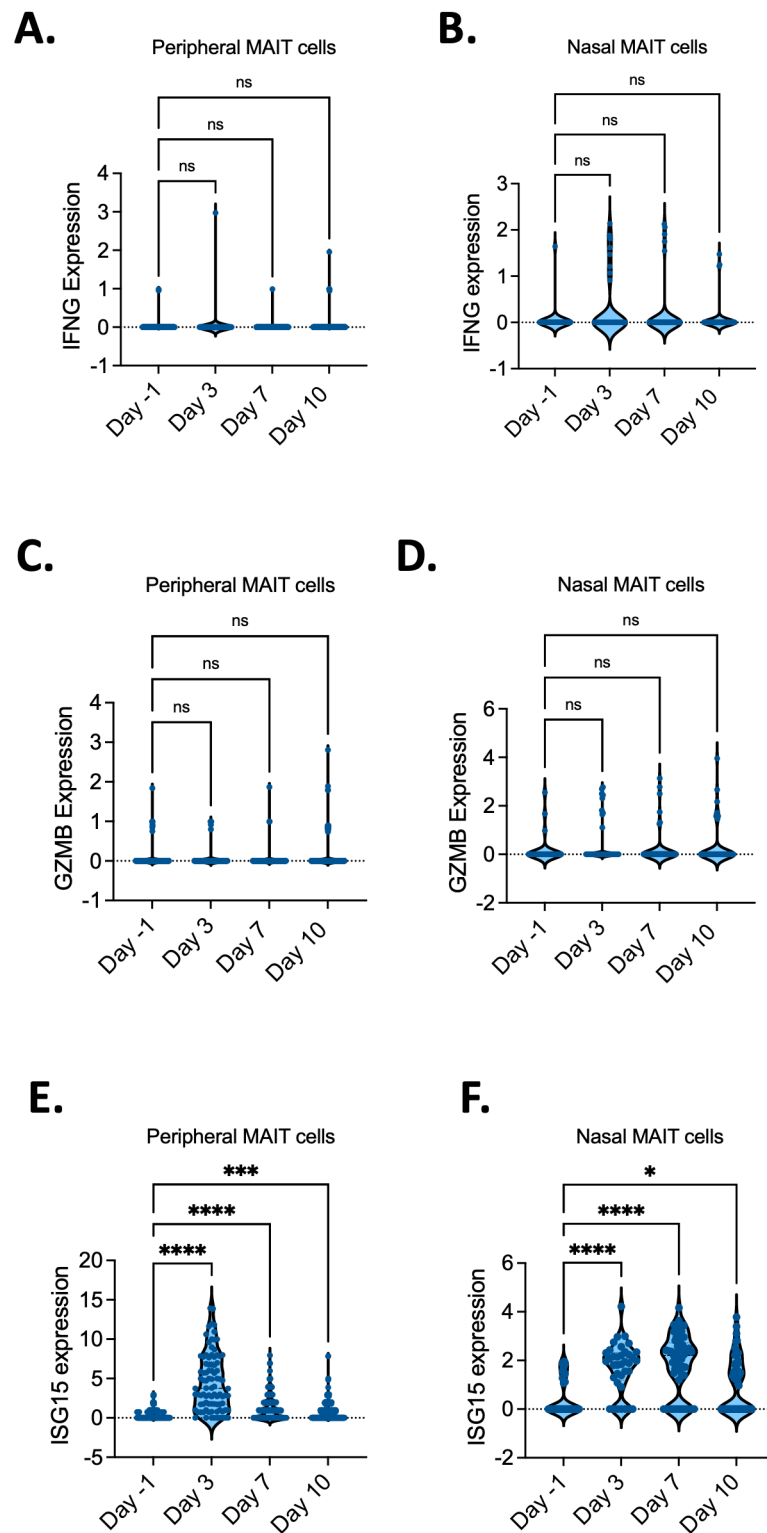


Figure S4. (A-F) *In silico* analysis of a previously published dataset (<https://www.covid19cellatlas.org>) showing IFNG, GZMB, and ISG15 gene expression in peripheral and nasal MAIT cells from seronegative individuals challenged with pre-Alpha

*SARS-CoV-2. * = $p \leq 0.05$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$, ns = not significant, $p > 0.05$, as determined using one-way ANOVA.*

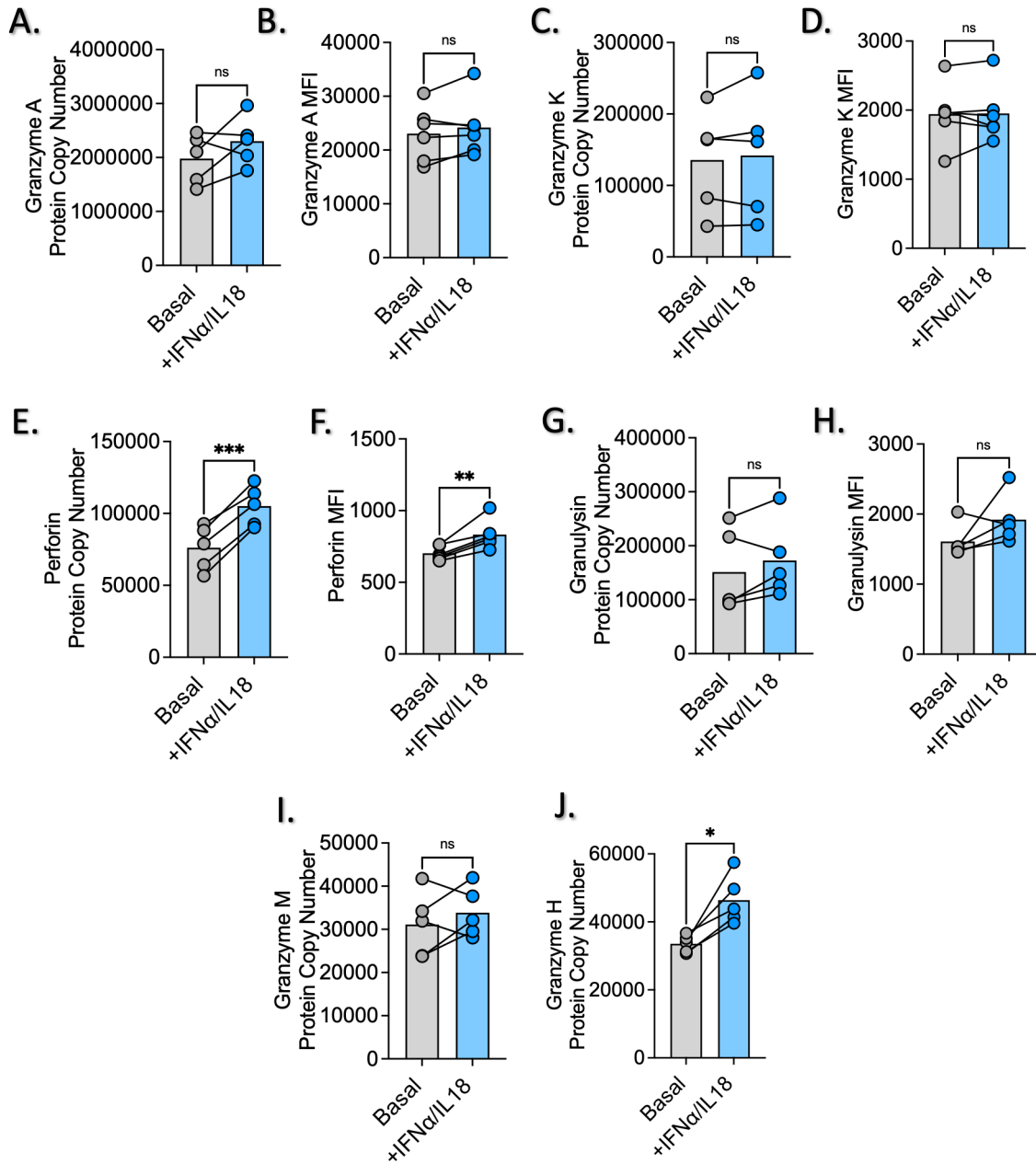


Figure S5. (A-H) Scatterplots showing granzyme A, granzyme K, perforin, or granulysin expression in MAIT^{IL-2} cells or MAIT cells in PBMC, basal or stimulated with IL-18/IFN α for 18hr. (I-J) Scatterplots showing granzyme M and granzyme H expression in MAIT^{IL-2} cells basal or stimulated with IL-18/IFN α for 18hr. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, ns = not significant, $p > 0.05$, as determined using paired t test.

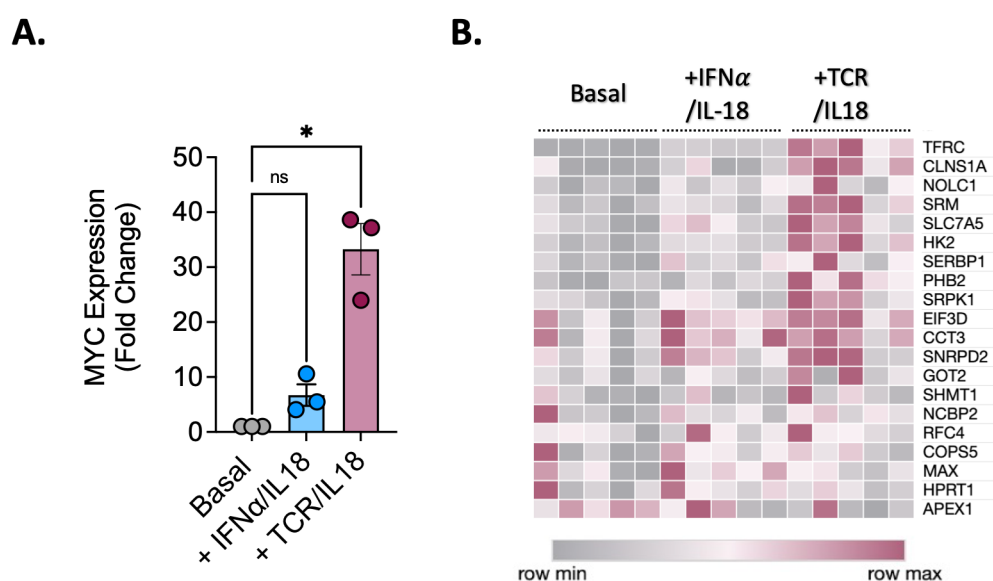


Figure S6. (A) Scatterplots comparing relative expression of MYC mRNA in MAIT^{IL-2} cells, basal or stimulated with IL-18/IFN α or anti-CD3/CD28 TCR beads/IL-18 for 6 hours. (B) Heatmap showing MYC target gene expression, as determined by quantitative proteomics analysis, in MAIT^{IL-2} cells, basal or stimulated with IL-18/IFN α or anti-CD3/CD28 TCR beads/IL-18 for 18 hours. * = $p \leq 0.05$, ns = not significant, $p > 0.05$, as identified by one-way ANOVA.

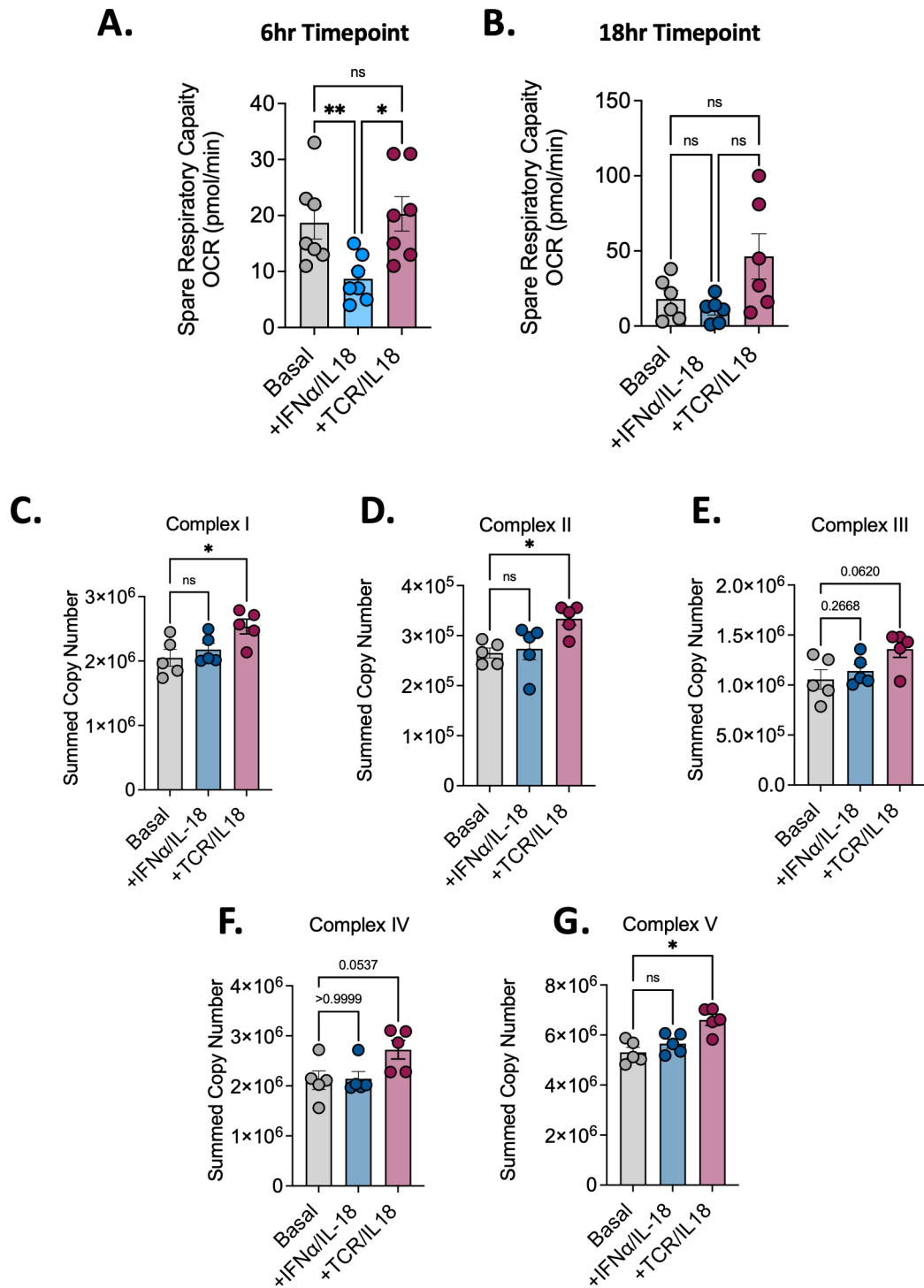


Figure S7. (A-B) Scatterplots showing spare respiratory capacity (SRC) in MAIT^{L-2} cells basal or stimulated with IL-18/IFN α or anti-CD3/CD28 TCR beads/IL-18 for 6hr or 18hr. (C-G) Scatterplots showing summed copy number of proteins associated with Complex I-V of the electron transport chain (ETC) expressed in MAIT^{L-2} cells, basal or stimulated with IL-18/IFN α or anti-CD3/CD28 TCR beads/IL-18 for 18hr. * = $p \leq 0.05$, ** = $p \leq 0.01$, ns = not significant, $p > 0.05$, as determined by one- way ANOVA..

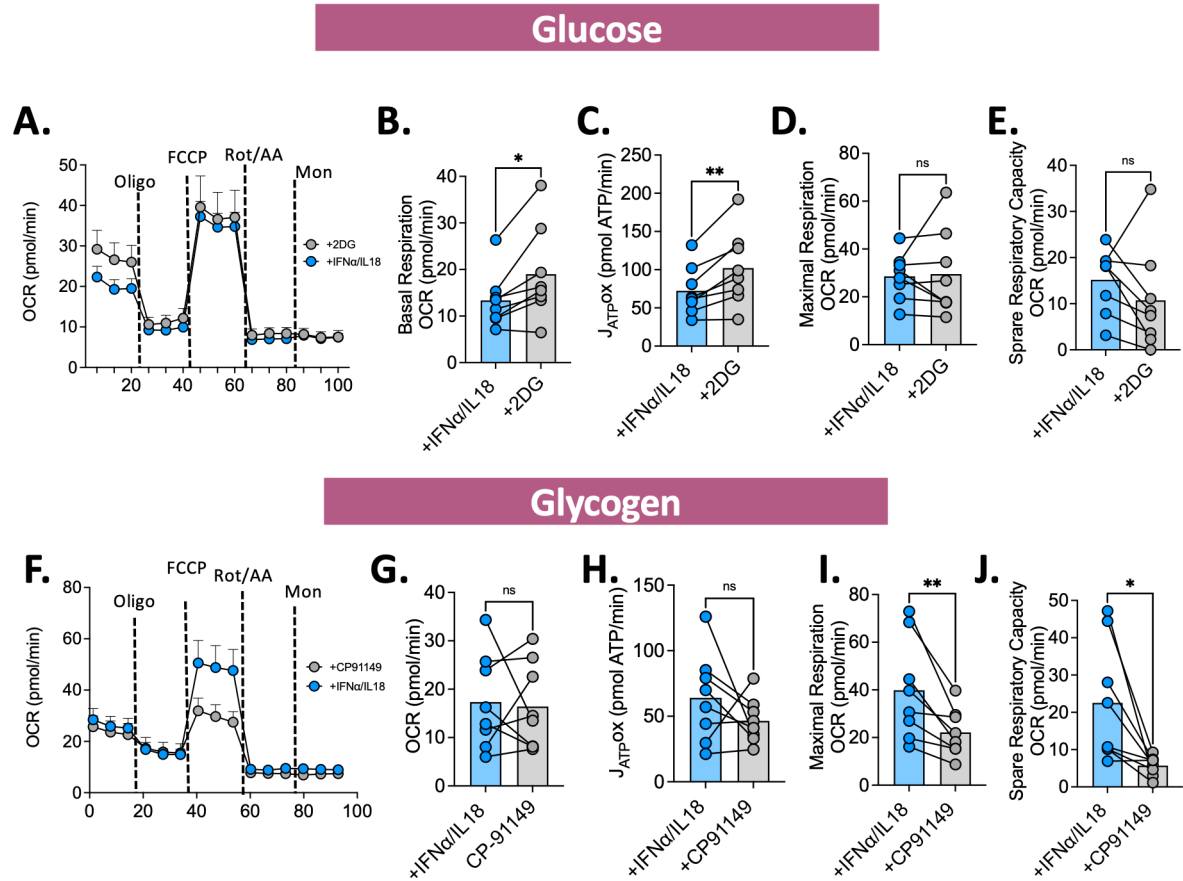


Figure S8. (A-B) Seahorse Oxygen Consumption Rate (OCR) trace and scatterplots ($n=8$) depicting basal OCR in MAIT^{L-2} cells stimulated with IL-18/IFN α for 3hr, in the absence or presence of 2DG. (C) Scatterplot depicting estimated ATP production linked to oxidative phosphorylation in MAIT^{L-2} cells stimulated with IL-18/IFN α for 3hr, in the absence or presence of 2DG. (D-E) Scatterplots depicting maximal respiration and spare respiratory capacity in MAIT^{L-2} cells stimulated with IL-18/IFN α for 3hr, in the absence or presence of 2DG. (F-G) Seahorse Oxygen Consumption Rate (OCR) trace and scatterplots ($n=8$) depicting basal OCR in MAIT^{L-2} cells stimulated with IL-18/IFN α for 3hr, in the absence or presence of CP91149. (H) Scatterplot depicting estimated ATP production linked to oxidative phosphorylation in MAIT^{L-2} cells stimulated with IL-18/IFN α for 3hr, in the absence or presence of CP91149. (I-J) Scatterplot showing maximal respiration and spare respiratory capacity in MAIT^{L-2} cells stimulated with IL-18/IFN α for 3hr, in the absence or presence of CP91149. * = $p \leq 0.05$, ** = $p \leq 0.01$, ns = not significant, $p > 0.05$, as determined using paired t test.

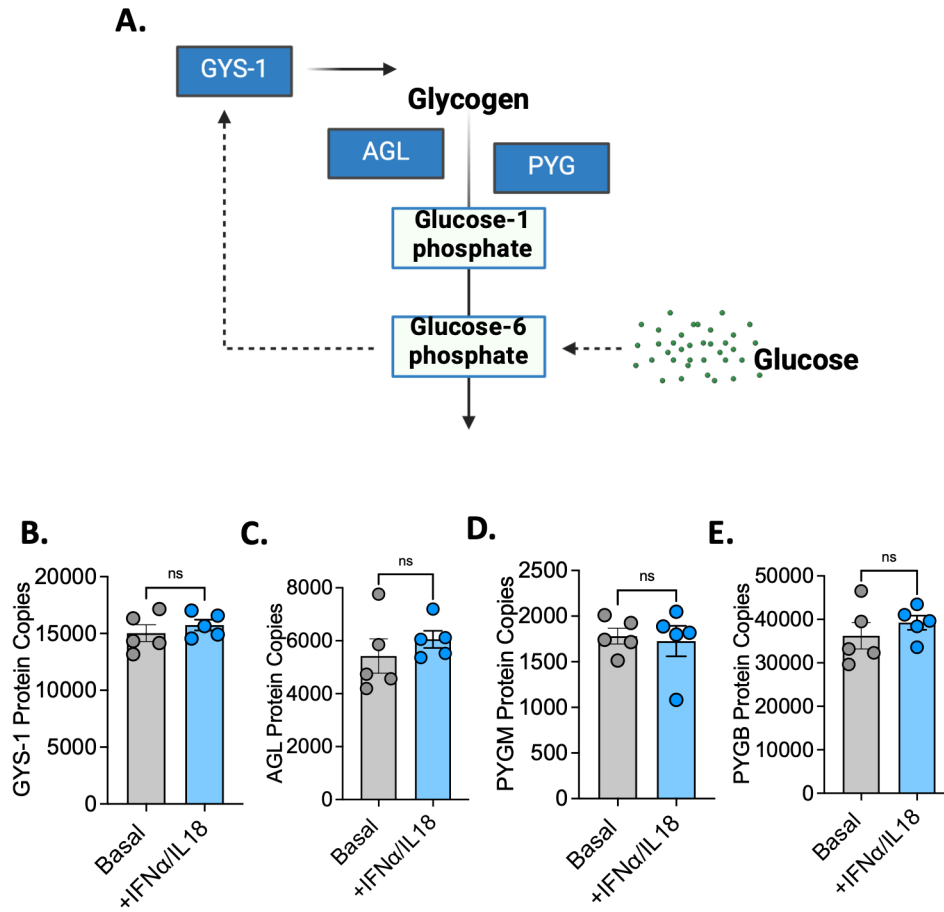


Figure S9. (A) Schematic detailing the major enzymes of the glycogenolysis pathway (B-E) Scatterplots detailing the number of protein copies of enzymes involved in the glycogenolysis pathway in MAIT^{IL-2} cells, basal or stimulated with IL-18/IFN α for 18 hours ($n=5$ individual donors). ns = not significant, $p > 0.05$ as measured by paired t test.

CHAPTER 6. Paper IV

This chapter consists of a second-author manuscript, currently in preparation.

Candidate Contribution

Figure panels produced via data collection and/or analysis performed by EKR are listed in Table I. This manuscript was written by EKR and AEH.

Table I. *Paper figures and panels containing the work of the candidate.*

Paper IV
Figure 1: Panels M-N
Figure 2: Panel A, Panels D-N
Figure 3: Panels I-J.
Figure 4: Panels A-B, Panels F-I.

Glutamine uptake is required for Myc signalling and metabolic fitness in human mucosal-associated invariant T cells.

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Running title: Glutamine is critical for MAIT cell metabolic responses

Key words: Mucosal-associated invariant T cells, Glutamine, Immunometabolism, Myc, ATF4

Abstract

Mucosal Associated Invariant T (MAIT) cells are an abundant subset of innate-like unconventional T cells that elicit host-protective immune responses against bacterial and viral pathogens. In addition, MAIT cells are emerging as potent anti-cancer effectors. These cells are responsive to both T cell receptor (TCR) and TCR-independent stimuli, and upon activation, upregulate a robust effector programme. Such effector responses are underpinned by proteome remodelling and *de novo* biosynthesis; and thus coincide with significant metabolic rewiring, and microenvironmental nutrient acquisition. Our understanding of the metabolic and nutritional requirements which underpin MAIT cell effector functioning remains incomplete, but comprises a rapidly emerging area of research. Glutamine is the most abundant amino acid, with a multifaceted role in supporting cellular *de novo* biosynthesis pathways, redox homeostasis, and mitochondrial fitness. Enhanced glutamine metabolism can be harnessed by rapidly proliferating tumour cells, and glutamine can become limited within the tumour microenvironment. In this study, we demonstrate that human MAIT cells enhance glutamine uptake via SLC1A5 upon activation, which facilitates upregulated expression of metabolic regulator, Myc. Using an *in vitro* model of glutamine-deprivation, we show that MAIT cells fail to upregulate Myc, or engage in metabolic rewiring, in the absence of glutamine. Instead, we highlight upregulated expression of the stress-responsive transcription factor, ATF4. Consequently, we demonstrate repressed protein translation, and significant defects to both proliferation, and expression of IFN γ , under glutamine-deprivation. Overall, our data suggest that human MAIT cells are highly sensitive to perturbations in environmental glutamine availability, and may have significant implications in disease settings, where glutamine availability can become limited.

Key points:

- Activated MAIT cells increase glutamine uptake via SLC1A5
- MAIT cell metabolism and effector responses are dependent on a glutamine-Myc axis

Introduction

Mucosal-associated invariant T (MAIT) cells are a group of unconventional T cells characterised by the expression of a semi-invariant T cell receptor (TCR) which recognises conserved metabolite antigens presented on the major histocompatibility complex (MHC)-I-like molecule, MR1¹⁻⁴. MAIT cells are capable of launching host-protective effector responses against several bacterial pathogens in a TCR-dependent manner^{5,6}; and elicit rapid responses to stimulation with innate cytokines, independent of their TCR, conferring their capacity to respond during human viral infections⁷⁻⁹. More recently, MAIT cell responses have begun to be characterised in the setting of anti-tumour immunity¹⁰, and thus, these cells are considered important players in the context of human health.

MAIT cell effector responses downstream of TCR triggering are supported by significant proteome remodelling and *de novo* biosynthesis¹¹, and these anabolic processes thus substantially enhance the demand for environmental nutrient acquisition. Accordingly, MAIT cells increase the uptake of large neutral amino acids (LNAAs) downstream of TCR triggering^{11,12}, likely because the intracellular supply of amino acids is rate limiting for protein translation¹³. Several studies have now elucidated the deleterious impact of altered nutrient availability / cellular metabolism on human MAIT cell fitness and effector responses^{11,12,14-17}, and thus it is of interest to further characterise the metabolism of amino acids in human MAIT cells, and the impact of deprivation on their effector capacity.

Glutamine is the most abundant amino acid in the body, contributing significantly to cellular *de novo* biosynthesis pathways, facilitating mitochondrial redox homeostasis, and acting as an important mitochondrial respiratory fuel required to sustain the tricarboxylic acid (TCA) cycle flux¹⁸. Importantly, the demand for intracellular glutamine is increased significantly in activated T lymphocytes, such that they consume more glutamine than they can synthesise; and thus, are reliant on glutamine uptake from the surrounding microenvironment¹⁹. Similarly, some rapidly proliferating tumour cells are “glutamine addicted”²⁰, presenting with increased expression of glutamine transporters due to constitutive oncogene activity of Myc²¹. Thus, glutamine deprivation within the tumour microenvironment (TME) likely

restricts immune functioning, and could represent a plausible target for therapeutic intervention^{22,23}.

In this study we demonstrate that human MAIT cells increase cellular glutamine uptake downstream of TCR triggering, and enhance their expression of the machinery involved in cellular glutamine metabolism. Using a model of glutamine deprivation, we demonstrate attenuated Myc expression in activated MAIT cells in the absence of glutamine, as well a state of nutrient-stress characterised by marked upregulation of the integrated stress response (ISR) pathway. Consequently, the capacity for MAIT cells to engage in a high level metabolic switch in response to TCR challenge is impaired under glutamine deplete environments; suggesting that these cells do not have the capacity, or the plasticity, to compensate metabolically for the loss of glutamine. MAIT cells also fail to launch appropriate effector responses in the absence of glutamine, demonstrating reduced *de novo* biosynthesis, impaired proliferation, and limited expression of the key effector cytokine, interferon (IFN)- γ . Taken together, these data suggest that glutamine signalling is required to sustain the expression of Myc in human MAIT cells downstream of TCR signalling, supporting metabolic fitness and upregulation of the effector programme. Therefore, MAIT cells might be particularly sensitive to dysfunction in environments where glutamine availability can become limited, such as within the TME.

Materials & methods

Study Cohorts and Ethical Approval

Criteria for recruitment of our cohort included the ability to give informed written consent, aged between 18-60 years, and BMI<28. Exclusion criteria included current or recent infection (<2 weeks), the use of immunomodulatory or anti-inflammatory medications, and being a current smoker. All participants provided full consent, and full ethical approval was granted from St. Vincent's University Medical Ethics Committee, and Maynooth University Ethics Committee.

Preparation of Peripheral Blood Mononuclear Cells (PBMC) and MAIT Cell Expansion

Human PBMC were isolated from fresh venous blood samples by density centrifugation using SepMate™ PBMC isolation tubes, as per manufacturer guidelines (StemCell Technologies). PBMC were either stored at -70°C or used to expand MAIT cell lines as previously described. Expanded MAIT cell lines are referred to as MAIT^{IL-2} cells throughout this manuscript, to differentiate from analyses performed directly *ex vivo* on MAIT cells in PBMC.

RNA Sequencing & Analysis

MAIT^{IL-2} cells were further purified using anti-V α 7.2 -APC conjugated antibody (Miltenyi Biotec) and anti-APC microbeads (Miltenyi Biotec) for Magnetic Cell Separation (MACS). Cells were then stimulated with Ultra-LEAF Purified anti-human CD3 (1 μ g/ml) (BioLegend) and Ultra-LEAF Purified anti-human CD28 (0.5 μ g/ml) (BioLegend) for 18hr in glutamine-free RPMI 1640 (Gibco) supplemented with 10% Dialysed FBS (Gibco); in the absence or presence of a 2mM L-Glutamine supplement (Gibco). Cells were harvested, and mRNA was extracted using the RNeasy kit (Qiagen) as per manufacturer guidelines. mRNA was quantified using a Nanophotometer N60 (Implen). Samples were processed by Novogene to isolate mRNA using poly-T oligo-attached magnetic beads. After fragmentation, the first strand cDNA was synthesized using random hexamer primers, followed by the second strand cDNA synthesis using dUTP for strand specific library. The library was checked using Qubit and real-time PCR for quantification and bioanalyzer for size distribution detection. Quantified libraries were pooled and sequenced using NovaSeq X Plus Series (PE150). Quality control: Clean reads were obtained by removing reads that contained adapter, reads containing poly-N, and low quality reads from the raw data. Q20, Q30 and GC content of the clean data was also determined. All downstream analysis was performed on clean data reads. Mapping to the reference genome: Reference genome and gene model annotation files were downloaded from the genome website. HISAT2 (2.2.1) was used to build the index of the reference genome and HISAT2 was used to align paired-end clean reads to the reference genome. Quantification of gene expression: FeatureCounts (2.0.6) was used to count the reads numbers mapped to each gene. Differential expression analysis between two groups was performed using the DESeq2 R package (1.42.0). *P* values were adjusted using the Benjamini-Hochberg (BH) method to account for the false-discovery rate. The threshold for significant differential expression was an adjusted *P* value of <0.05. Further analysis on differentially expressed genes used the

ClusterProfiler R Studio Package (4.10.1) and Gene Set Enrichment Analysis (GSEA) software, to perform Gene Ontology (GO) enrichment analysis and KEGG enrichment analysis (<http://www.genome.jp/kegg/>). Heatmaps were generated based on differentially expressed genes, using the Morpheus data visualization tool from the Broad Institute (<https://software.broadinstitute.org/morpheus>).

In Silico RNA sequencing and Proteomic Analysis of Publicly Available Datasets

In silico analysis of glutamine metabolism machinery was investigated using a previously published RNA sequencing dataset on MAIT cells²⁴. *In silico* analysis was also performed on a published MAIT cell proteomic dataset downloaded from PRIDE accession number PXD041544 (<https://www.ebi.ac.uk/pride/archive/projects/PXD041544>).

QUAS-R Glutamine Uptake Assay

For single-cell analysis of glutamine uptake via SLC1A5 we used the previously published QUAS-R click-chemistry based approach. Briefly, MAIT cells in PBMC were stimulated with CD3 (1 μ g/ml) and CD28 (0.5 μ g/ml) (BioLegend) for 18 hours, and subsequently stained for viability and surface markers. After which, cells were treated with L-azidohomoalanine (L-AHA) (Jena Bioscience) to a final concentration of 100 μ M. For each sample, a glutamine control (10mM) and a minus AHA control were used; to decipher SLC1A5-mediated uptake, and to account for background noise. Following incubation, cells were fixed with 1% paraformaldehyde (Santa Cruz), permeabilised with a 0.01% Saponin solution (Sigma), and resuspended in the click staining mix (Copper II Sulfate Pentahydrate (1mM) (Sigma), Sodium L-Ascorbate (10mM) (Sigma), THPTA (1mM) (Jena Bioscience), Aminoguanidine Hydrochloride (10mM) (Cayman Chemicals), AZDye 488 Alkyne Fluorophore (5 μ M) (Jena Bioscience)). Cells were incubated for 1hr at room temperature, and analysed by flow cytometry.

ATF4 Expression

To assess the expression of activating transcription factor 4 (ATF4) in human peripheral-blood MAIT cells, PBMC were stimulated for 18hr using anti-human CD3 (1 μ g/ml) and anti-human CD28 (0.5 μ g/ml) (BioLegend), in glutamine-free RPMI 1640 (Gibco) supplemented with 10% Dialysed FBC (Gibco) in the absence or presence of 2mM L-Glutamine (Gibco). Cells were then

washed with PBS, and stained for extracellular surface markers (CD3, CD161, and V α 7.2). Cells were fixed and permeabilised using the True-Nuclear Transcription Factor Buffer Set, as per manufacturer guidelines (BioLegend). Cells were then stained with ATF4 recombinant rabbit monoclonal antibody as per manufacturer guidelines (Invitrogen) for 1hr at room temperature, prior to staining with a PE-conjugated goat-anti rabbit IgG secondary (Jackson ImmunoResearch) for a further 45 minutes. Cells were then analysed by flow cytometry.

Surface Markers and Cytokine Expression

To phenotype MAIT cells for activation and to assess effector functioning, human peripheral-blood MAIT cells (in PBMC) were stimulated for 18hr using anti-CD3/CD28 as previously described. Cells were stimulated in glutamine-free RPMI 1640 (Gibco) supplemented with 10% Dialysed FBS (Gibco) in the absence or presence of 2mM L-Glutamine (Gibco). Protein Transport Inhibitor Cocktail (Invitrogen) was added to a 1X concentration for the final 6hr of the culture; before cells stained for viability using eBioscience fixable viability dye (eFluor506, BioLegend). Cells were then stained for extracellular surface markers (CD3, CD161, V α 7.2, CD25 and CD69), washed, and then fixed, and permeabilised using the True-Nuclear Transcription Factor Buffer Set, as per manufacturer guidelines (BioLegend). Subsequently, cells were stained for expression of IFN γ (Miltenyi), and analysed by flow cytometry.

Puromycin Incorporation Assay

Cellular rates of protein translation were directly assessed in human peripheral-blood MAIT cells, using the incorporation of Puromycin as a surrogate marker for translational activity. PBMC were stimulated as before in glutamine-free RPMI 1640 (Gibco) supplemented with 10% Dialysed FBC (Gibco) in the absence or presence of 2mM L-Glutamine (Gibco). Cells were then treated with Puromycin (11 μ M) (Sigma) for 25 minutes. Cells were washed with ice-cold PBS, stained for viability and extracellular markers (CD3, CD161 and V α 7.2 TCR), and fixed and permeabilised, as previously described. Puromycin uptake was assessed using an Alexa Fluor 488 -conjugated anti-Puromycin monoclonal antibody (Sigma) via flow cytometry.

Flow Cytometric Analysis

Cell populations were acquired using an Attune NxT flow cytometer, and analysed using FlowJo version 10.8.2. (TreeStar). Results are expressed as either a percentage of the parent

population, as determined using fluorescence minus one (FMO) and unstained controls; or through the geometric mean to determine the mean fluorescence intensity (MFI) of a relevant marker, within the appropriate population.

Western Immunoblot Analysis

MAIT^{IL-2} cells were further purified using anti-V α 7.2 -APC conjugated antibody (Miltenyi) and anti-APC microbeads (Miltenyi) for Magnetic Cell Separation (MACS). Cells were then stimulated for 18hr using Ultra-LEAF Purified anti-human CD3 (1 μ g/ml) / anti-human CD28 (0.5 μ g/ml) (BioLegend) in glutamine-free RPMI 1640 (Gibco), supplemented with 10% Dialysed FBS (Gibco); in the absence or presence of 2mM L-Glutamine (Gibco). Cells were harvested in Radioimmunoprecipitation Assay Buffer (RIPA buffer) (Sigma) supplemented with phosphatase and protease inhibitors as per manufacturer guidelines (Abcam). Protein concentration was quantified using Bradford reagent (Sigma), resuspended in reducing 4x Laemmli sample buffer (Bio-Rad) and heated to 98°C for 10 min. Samples were then resolved on 10% polyacrylamide gels and semi-dry transferred to nitrocellulose membranes. Membranes were blocked for 30 minutes at room temperature in 5% non-fat milk in Tris-Buffered Saline (TBS). Subsequently, membranes were incubated with either mouse anti-human β -Actin (Sigma) or rabbit anti-human c-Myc (Cell Signalling Technologies). All primary antibodies were incubated overnight at 4°C. Membranes were washed three times in TBS-T for 15 minutes. Secondary antibodies were incubated for 1hr at room temperature, either goat anti-rabbit or goat anti-mouse (Abcam). Membranes were again washed three times in TBS-T for 15 minutes, and incubated for 5 minutes with chemiluminescence substrate (ThermoFisher Scientific). Membranes were developed using the ChemiDoc Imaging System from BioRad.

Analysis of Protein Expression

ImageLab software (BioRad) was used to quantify the relative protein expression of c-Myc. Normalisation was carried out as follows: the adjusted per/well volume values were divided by the corresponding well values in the control blot (β -actin). Fold change was then calculated by dividing the normalised value by the control value run for c-Myc. Statistical analysis was performed in GraphPad Prism.

MAIT Cell Seahorse Assay

MAIT^{IL-2} cells were activated for 18hr with anti-CD3/CD28 antibodies in the presence or absence of 2mM L-glutamine, as previously described. Metabolic analysis was carried out using the Seahorse Extracellular Flux Analyzer XFe96 (Agilent). Briefly, MAIT cells re-counted prior to beginning the Seahorse assay, to ensure resuspension at equal concentrations. Cells were then washed and resuspended in phenol red-free RPMI media, supplemented with 10mM glucose and 1mM pyruvate; with or without 2mM glutamine (media and supplements all Agilent). Cells were seeded onto a Cell-Tak (Corning)-coated 96-well microplate for adhesion, and incubated in a non CO₂ incubator at 37°C, for 45 minutes. Metabolic parameters (mitochondrial and glycolytic) were measured using OCR (pmol/min), and ECAR (mpH/min), as readouts. Metabolic dependencies were interrogated using the following injections: Oligomycin (2μM), FCCP (1μM), Rotenone and Antimycin A (both 1μM) and 2DG (30mM). Metabolic parameters were calculated as per well-established protocols.

CellTrace CFSE Assay & Absolute Counts

Freshly-isolated PBMC were stained with 1μM CellTrace CFSE (Invitrogen) in pre-warmed PBS (Sigma), at a concentration of 1x10⁶ cells/ml. Cells were stained for 10 minutes at room temperature. Cells were then washed with PBS and prepared for expansion as outlined under “Preparation of Peripheral Blood Mononuclear Cells (PBMC) and MAIT Cell Expansion”, in the absence or presence of 2mM L-Glutamine. The culture was maintained for 7 days, and cells were then harvested, stained for extracellular markers, and analysed by flow cytometry as outlined under “Flow Cytometric Analysis”. To determine absolute MAIT cell counts, cells were counted manually, and absolute counts were quantified using the percentage of MAIT cells (CD3⁺, CD161^{Hi}, Vα7.2⁺) in the culture, as determined by flow cytometric analysis.

ELISA

MAIT^{IL-2} cells were further purified using anti-Vα7.2 -APC conjugated antibody (Miltenyi) and anti-APC microbeads (Miltenyi) for Magnetic Cell Separation (MACS). Cells were then activated for 18hr with anti-human CD3 (1μg/ml) and anti-human CD28 (0.5μg/ml) (BioLegend) in the absence or presence of 2mM glutamine, as previously described. After 18hr, culture supernatants were collected, and assessed for expression of IFNγ using the Human IFNγ ELISA Kit from R&D Systems, as per manufacturer guidelines.

Statistical Analysis

General statistical analysis was completed using Graph Pad Prism 10 software for macOS. Data is expressed as the mean $\pm$ SEM. Distribution was assessed using Shapiro-Wilk test. Outliers were determined using ROUT analysis. We determined differences between two groups using Student t-test (paired). P values were expressed with significance set at ≤ 0.05 .

Results

MAIT cells increase cellular glutamine uptake and expression of machinery involved in glutamine metabolism following activation

To assess whether the demand for glutamine is increased in MAIT cells following activation, we carried out *in silico* analysis of a publicly available MAIT cell RNA sequencing dataset²⁴ and probed the expression of machinery involved in cellular glutamine metabolism pre- and post-challenge. These analyses highlighted increased gene copy expression of the major cellular glutamine importer, SLC1A5, following the stimulation of peripheral blood mononuclear cells (PBMC) with either 5-A-RU, or *E.coli* (Figure 1B-C), and downstream of innate cytokine (IL-18/IL-12) stimulation (Figure 1D). We also noted substantial increases in the gene expression of enzymes involved in cellular glutamine metabolism, and this was most pronounced in the context of TCR triggering (Figure 1B-C); suggesting that the cellular uptake and metabolism of glutamine is required to support MAIT cell fitness downstream of TCR challenge, in particular. We confirmed these findings on the protein level performing re-analysis of a publicly available MAIT cell proteomic dataset generated by Kedia-Mehta *et al.*,¹¹ (Figure 1E) which demonstrated robust increases in the expression of SLC1A5 and the enzymes involved in cellular glutamine metabolism upon activation (Figure 1F-L). Finally, we implemented the use of the recently developed click chemistry-based flow cytometry assay, QUAS-R²⁵, to directly assess the capacity for human MAIT cells to increase cellular glutamine uptake via SLC1A5 upon TCR triggering. In support of our *in silico* analyses, we found that human peripheral blood MAIT cells increase cellular glutamine uptake via SLC1A5 in response to TCR stimulation (Figure 1M-N). Taken together, these data suggest enhanced glutamine metabolism in human MAIT cells downstream of TCR signalling.

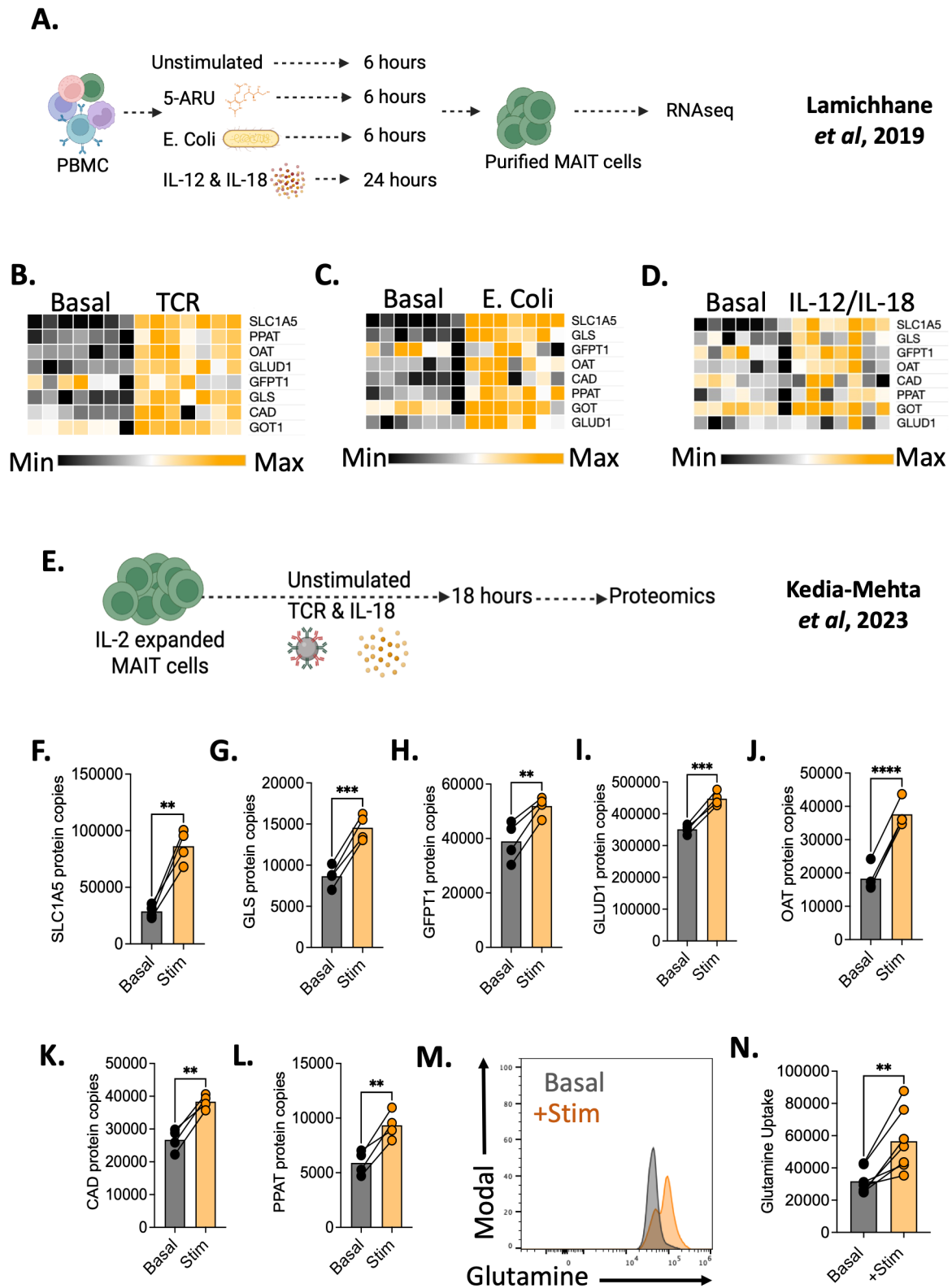


Figure 1. MAIT cells upregulate glutamine uptake and expression of machinery involved in cellular glutamine metabolism downstream of activation. (A) Schematic depicting a brief overview of the experimental setup used in the generation of three publicly available RNA sequencing datasets deposited in the NCBI Sequence Read Archive (SRA) deposited under the

accession number PRJNA559574, and produced by Lamichhane et al., 2019²⁴. (B-D) Heatmap depicting the relative expression of genes involved in cellular glutamine metabolism in isolated human MAIT cells following stimulation of PBMC with 5 μ M 5-A-RU, or 10BpC *E.coli* for 6hr, and IL-18/IL-12 (both 50ng/ml) for 18hr; as extracted from RNA sequencing datasets produced by Lamichhane et al., 2019. Relative abundance is graded from black (low) to orange (high) per row. (E) Schematic detailing the experimental setup used to generate the MAIT cell proteome produced by Kedia-Mehta et al., 2023¹¹, which was deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD041544. (F-L) Scatterplots copy number expression of proteins involved in glutamine metabolism, in MAIT^{L-2} cells, basal or stimulated with anti-CD3/CD28 beads and IL-18 for 18hr; as extracted from the proteome produced by Kedia-Mehta et al., 2023. (M-N) Representative flow cytometric histogram and dotplots showing glutamine uptake in peripheral blood MAIT cells, basal or following stimulation of PBMC with anti-CD3/CD28 antibodies for 18hr. ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$, as determined using paired t test.

Glutamine deprivation attenuates expression of Myc in TCR activated MAIT cells and triggers activation of the integrated stress response (ISR)

To further probe the role of glutamine in human MAIT cells, we investigated the transcriptional profile of MAIT cells in a model of glutamine deprivation. We performed pathway analysis on the differentially expressed genes (DEGs) that were upregulated in the absence of glutamine, and noted an enrichment of pathways pertaining to cellular nutrient levels, starvation, glutamine transport, and cellular stress (Figure 2A). We have previously identified the nutrient-dependent transcription factor Myc as a critical regulator of metabolism in human MAIT cells¹¹. In conventional T cells, an increased supply of glutamine downstream of TCR triggering is required to sustain the expression of Myc^{26,27}, and therefore, we investigated the Myc signalling axis in our glutamine deprived MAIT cells and noted attenuated expression of *MYC*, whilst gene set enrichment analysis (GSEA) highlighted concomitant downregulation of Myc target gene expression (Figure 2B-D). Interestingly, we have previously identified Myc as a critical regulator of amino acid metabolism in human MAIT cells; suggesting a feed-forward axis by which Myc also modulates increased cell surface expression of the LNAA transporter, SLC7A5, and thus facilitates increased amino acid uptake downstream of activation¹¹. Strikingly, however, we identified global increases in the gene expression of amino acid transporters, including the Myc targets, *SLC7A5*, *SLC3A2* (CD98), *SLC1A5*; and another cellular glutamine transporter, SLC38A2 (Figure 2E-F). In Figure 2A, we highlighted an enrichment of genes involved in ISR signalling, and a hallmark of the ISR pathway is the selective translation and activation of the basic leucine zipper transcription

factor, Activating Transcription Factor 4 (ATF4). ATF4 can modulate cellular responses to nutrient starvation by preferentially upregulating the expression of amino acid transporters, including SLC3A2, SLC7A5, and SLC38A2²⁸. Indeed, we highlighted a significant increase in *ATF4* and summed ATF4 target gene expression in glutamine-starved MAIT^{IL-2} cells within our RNA sequencing dataset (Figure 2G-H). Importantly, we confirmed this finding using flow cytometric analysis in glutamine deprived MAIT cells (Figure 2I-J). An overarching consequence of the ISR is the global repression of translation initiation^{29,30}. To this end, we identified increased expression of *PDCD4*, a repressor of 5'cap dependent translation initiation, and concomitant downregulation of the translation initiation factors (Figure 2K-L). Finally, we demonstrated significantly reduced rates of protein translation in glutamine MAIT cells stimulated using a puromycin incorporation assay (Figure 2M-N). Together these data demonstrate a loss of Myc signalling under glutamine-deplete conditions with activation of the compensatory ATF4 signalling pathway.

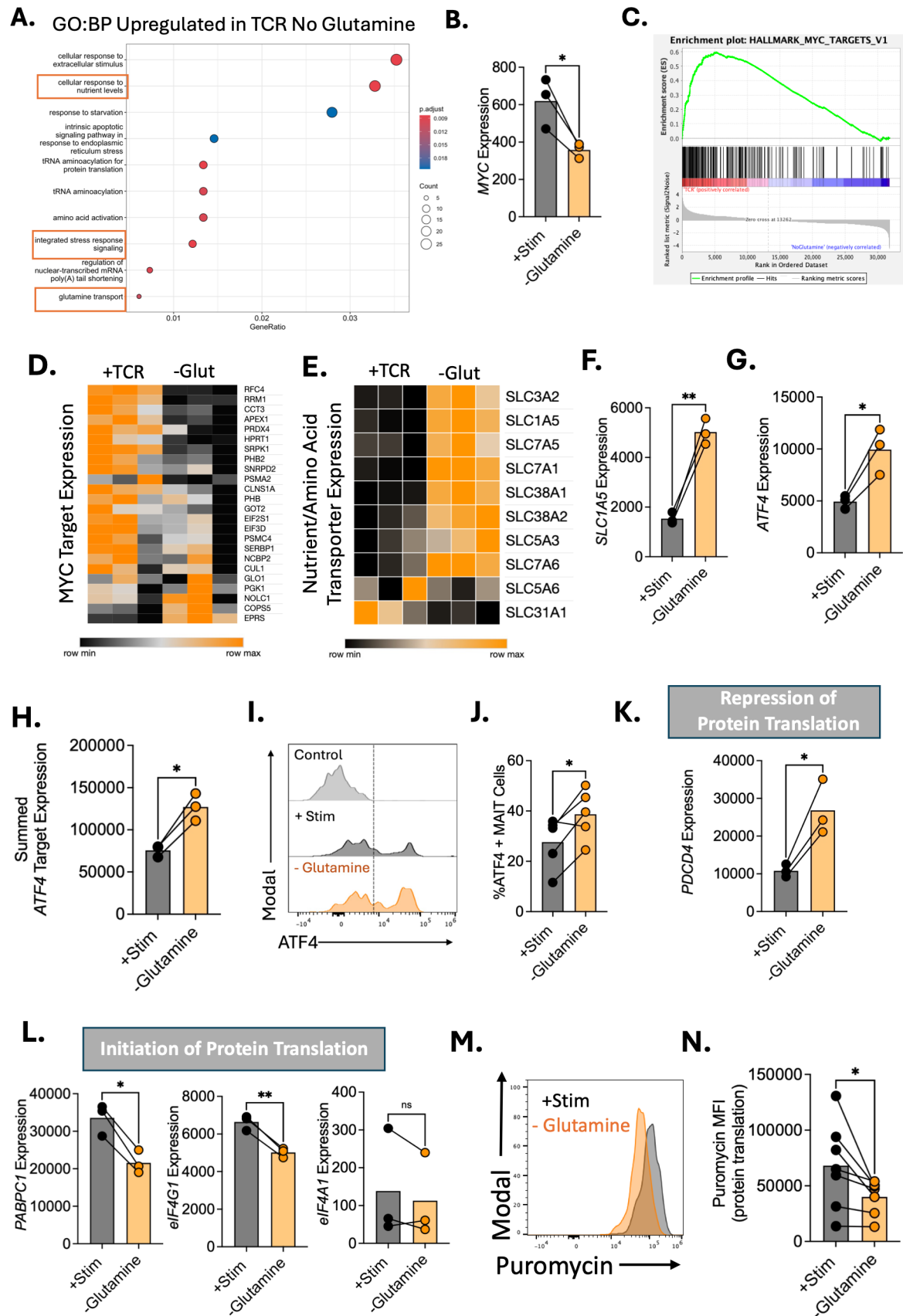


Figure 2. Glutamine deprivation attenuates MYC signalling in activated MAIT cells and triggers upregulation of the cellular integrated stress response (ISR) pathway. (A) Dotplot

showing the top 10 Gene Ontology (GO) Biological Processes (BP) terms enriched in MAIT^{IL-2} cells stimulated with anti-CD3/CD28 antibodies for 18hr, in the absence of 2mM glutamine. Based on differentially-expressed genes that were upregulated in the absence of glutamine. (B) Scatterplots showing normalised MYC gene count expression in MAIT^{IL-2} cells stimulated with anti-CD3/CD28 antibodies for 18hr, in the presence or absence of 2mM glutamine. (C) GSEA plot showing expression of Myc targets MAIT^{IL-2} cells stimulated with anti-CD3/CD28 antibodies for 18hr, in the absence or presence of 2mM glutamine. (D) Heatmap depicting the relative expression of Myc target genes in MAIT^{IL-2} cells stimulated with anti-CD3/CD28 antibodies for 18hr, in the presence or absence of 2mM glutamine. Relative abundance is graded from black (low) to orange (high). (E) Heatmap depicting the relative gene expression of various nutrient/amino acid transporters in MAIT^{IL-2} cells stimulated with anti-CD3/CD28 antibodies for 18hr, in the presence or absence of 2mM glutamine. (F-G) Scatterplots detailing SLC1A5 and ATF4 normalised gene count expression in MAIT^{IL-2} cells stimulated with anti-CD3/CD28 antibodies for 18hr, in the presence or absence of 2mM glutamine. (H-I) Flow cytometric histograms and scatterplots showing ATF4 expression in peripheral blood MAIT cells stimulated with anti-CD3/CD28 antibodies for 18hr, in the presence or absence of 2mM glutamine. (J) Scatterplots showing summed gene count expression of ATF4 targets in MAIT^{IL-2} cells stimulated with anti-CD3/CD28 antibodies for 18hr, in the presence or absence of 2mM glutamine. (K) Scatterplots depicting normalised gene count expression of PDCD4 in MAIT^{IL-2} cells stimulated with anti-CD3/CD28 antibodies for 18hr, in the presence or absence of 2mM glutamine. (L) Scatterplots showing normalised gene count expression of protein translation initiation machinery in MAIT^{IL-2} cells stimulated with anti-CD3/CD28 antibodies for 18hr, in the presence or absence of 2mM glutamine. (M-N) Flow cytometric histograms and scatterplots showing puromycin incorporation in peripheral blood MAIT cells upon stimulation of PBMC with anti-CD3/CD28 antibodies for 18hr, in the presence or absence of 2mM glutamine. * = $p \leq 0.05$, ** = $p \leq 0.01$, ns = not significant, $p > 0.05$, as determined using paired t test.

Glutamine deprivation limits the metabolic flux in human MAIT cells downstream of TCR challenge

To further investigate the loss of MYC gene expression observed via RNA sequencing analysis, we performed western blotting analysis and observed a loss of Myc protein expression in glutamine deprived MAIT cells (Figure A-B). Given the established importance of Myc in facilitating robust metabolic rewiring in activated human MAIT cells, we next performed Seahorse Extracellular Flux Analysis and demonstrate significant reductions in global metabolic activity in glutamine deprived MAIT cells (Figure 3C-H). Specifically, MAIT cells failed to upregulate both glycolysis and oxidative phosphorylation (OxPhos) (Figure 3C-D and Figure 3F-G) under glutamine-deplete conditions; and presented with significant impairments to both the maximal glycolytic capacity (Figure 3E), and spare respiratory capacity (SRC) (Figure 3H). Sinclair *et al.*, have recently reported that the autophagic flux is increased in activated CD8⁺ T cells in absence of even a singular amino acid³¹. In accordance, we noted

substantially increased gene expression of the machinery involved in autophagy in MAIT^{IL-2} cells activated for 18hr in the absence of L-glutamine (Figure 3I); and most notably, of *MAP1LC3B* (Figure 3J), which encodes a central protein in autophagy³². Taken together, these data demonstrate that glutamine deprivation impairs Myc signalling in TCR activated MAIT cells, which corresponds with defective cellular metabolism.

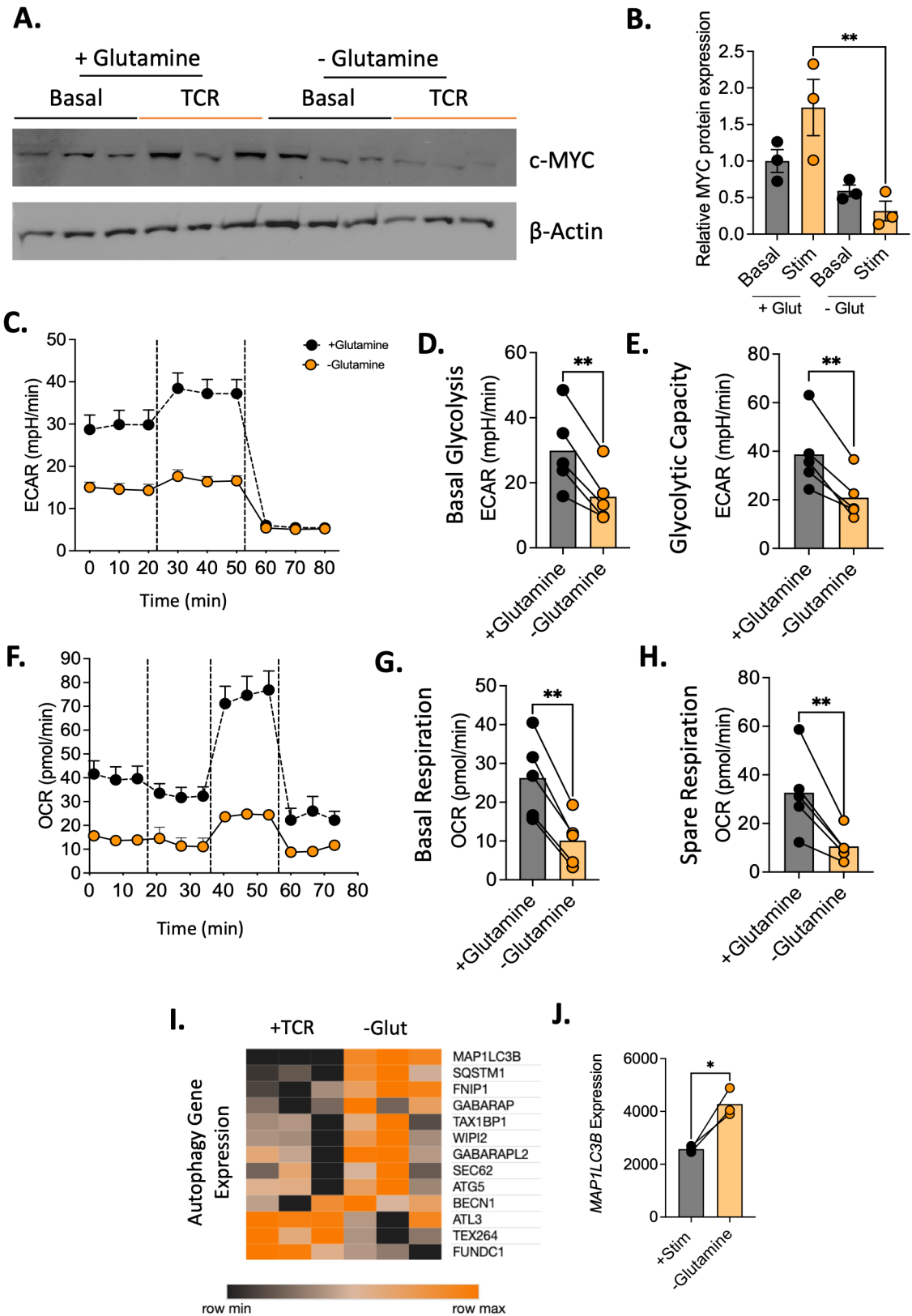


Figure 3. Activated MAIT cells fail to upregulate *Myc* and are metabolically exhausted in the absence of glutamine. (A) Western blot of *Myc* expression in MAIT^{L-2} cells (three individual

donors) basal, or stimulated with anti-CD3/CD28 antibodies, in the presence or absence of 2mM glutamine and (B) quantified relative expression. (C-E) Representative Seahorse trace and scatterplots detailing basal glycolytic rates, as determined by baseline extracellular acidification rate (ECAR) in MAIT^{L-2} cells stimulated for 18hr, in the presence or absence of 2mM glutamine. Scatterplots detailing glycolytic capacity (ECAR post-oligomycin) in MAIT^{L-2} cells stimulated for 18hr, in the presence or absence of 2mM glutamine. (F-G) Representative Seahorse trace and scatterplots showing basal respiration (as determined by baseline oxygen consumption rates (OCR) minus non-mitochondrial (post-rotenone/antimycin A) OCR) in MAIT^{L-2} cells stimulated for 18hr, in the presence or absence of 2mM glutamine. (H) Scatterplots detailing spare respiratory capacity (as determined by post-FCCP OCR minus baseline OCR) in MAIT^{L-2} cells stimulated for 18hr, in the presence or absence of 2mM glutamine. (I) Heatmap showing relative expression of autophagy-related genes in MAIT^{L-2} cells stimulated for 18hr, in the presence or absence of 2mM glutamine, and (J) normalised gene count expression of MAP1LC3B. * = $p \leq 0.05$, ** = $p \leq 0.01$, as determined using paired *t* test.

MAIT cells require glutamine to launch appropriate effector responses downstream of TCR challenge

MAIT cells are reliant on a robust metabolic switch towards glycolysis to fuel their effector responses downstream of TCR signalling^{11,12}; whilst OxPhos is also required to fuel clonal expansion in antigen-activated MAIT cells¹⁶. Considering our findings depicting a loss of normal metabolic functioning in human MAIT cells in the absence of glutamine, we sought to probe the associated effector profile. MAIT cell growth 72hr post anti-CD3/CD28 triggering was markedly attenuated in the absence of glutamine, as determined by reduced cell size (Figure 4A). These data are indicative of a significant impairment to *de novo* biosynthesis, which lend towards a diminished proliferative capacity, which was noted in the absence of glutamine over a 5-day proliferation assay (Figure 4C-E). Further, glutamine deprivation impaired activation in peripheral blood MAIT cells in response to TCR triggering, as measured by cell surface expression of CD25 and CD69, 18hr post stimulation (Figure 4F-G). Finally, expression of key effector cytokine, IFN γ , is significantly attenuated amongst peripheral blood MAIT cells in PBMC, and in MAIT^{L-2} cells, 18hr post stimulation in the absence of L-glutamine (Figure 4H-J). Thus, MAIT cell effector responses to TCR challenge are profoundly limited under glutamine-deplete conditions.

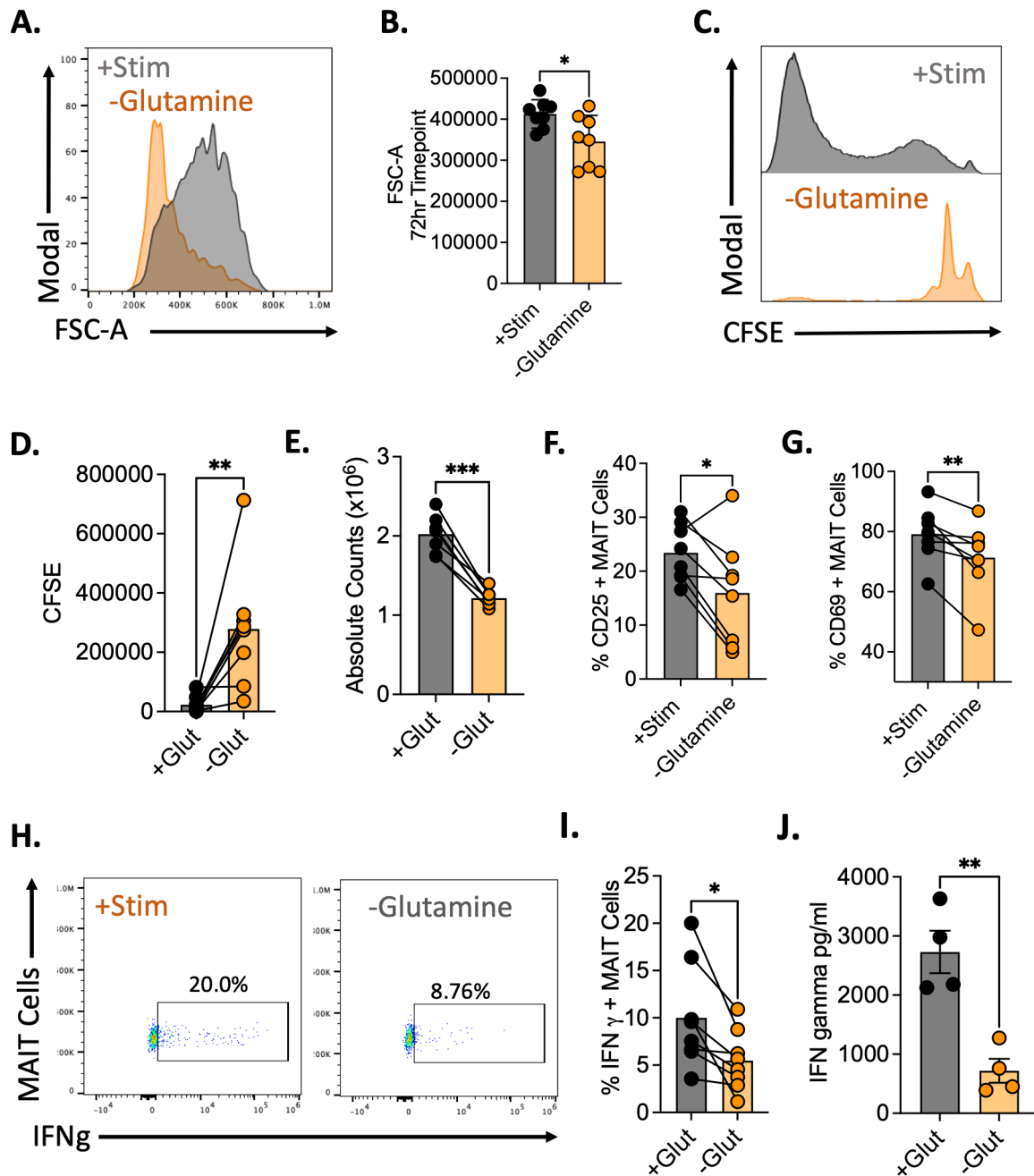


Figure 4. MAIT cell effector responses to TCR challenge are impaired in the absence of glutamine. (A-B) Representative flow cytometric histograms and scatterplots detailing MAIT cell size, as determined by the mean fluorescence intensity (MFI) of the forward scatter area (FSC-A) of peripheral blood MAIT cells upon stimulation of PBMC for 18hr using anti-CD3/CD28 antibodies, in the presence or absence of 2mM glutamine. (C-E) Representative flow cytometric histograms and scatterplots detailing MAIT cell proliferation, as determined by the mean fluorescence intensity of CellTrace CFSE, in MAIT^{IL-2} cells expanded for 7 days in the presence or absence of 2mM glutamine. (F-G) Scatterplots detailing the percent expression of activation markers CD25 and CD69 in peripheral blood MAIT cells following 18hr stimulation of PBMC with anti-CD3/CD28 antibodies, in the presence or absence of 2mM glutamine. (H-I)

*Representative flow cytometric dotplots and scatterplots detailing percent expression of IFN γ in peripheral blood MAIT cells following stimulation of PBMC with anti-CD3/CD28 antibodies in the presence or absence of 2mM glutamine, for 18hr; and (J) IFN γ expression in MAIT^{L-2} cells as measured by ELISA. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, as determined by paired t test.*

Discussion

Glutamine is the most abundant amino acid in the body and is required for several metabolic processes including *de novo* biosynthesis, facilitating mitochondrial redox homeostasis, and as a tricarboxylic acid (TCA) cycle fuel¹⁸. However the role of glutamine in supporting human MAIT cell responses is unknown. In this study, we have identified a critical role for glutamine uptake in mediating human MAIT cell fitness downstream of TCR activation. Specifically, we highlight that glutamine signalling is indispensable for the expression of Myc in activated human MAIT cells, and thus facilitates metabolic reprogramming and effector responses to challenge.

Conventional T cells are strongly dependent on the metabolic regulator Myc to modulate the expression of key transporters and enzymes involved in glutamine metabolism following activation²⁷. We have previously identified Myc as key metabolic regulator in human MAIT cells, but the role of glutamine metabolism in MAIT cells has not yet been described. Here, we highlight increased cellular SLC1A5-mediated glutamine uptake in human MAIT cells following TCR triggering, whilst pathway analysis of a previously published RNA sequencing dataset²⁴ revealed concomitant increases in the machinery involved in cellular glutamine metabolism. We next sought to ascertain a role for glutamine in activated human MAIT cells, and using an *in vitro* model of glutamine deprivation, we demonstrated significant attenuation of the activity of Myc at the transcriptional level in glutamine-deplete conditions. In conventional T cells, a feed-forward axis exists through which an influx of cellular amino acids stabilises the expression of Myc, which in turn drives an increased expression of amino acid transporters, such as SLC7A5, and SLC1A5²⁷. Indeed, we have previously highlighted SLC7A5 as a downstream target of Myc in human MAIT cells, suggesting that glutamine deprivation would attenuate the expression of amino acid transporters. However, we identified substantially increased gene expression of a number of amino acid transporters,

including SLC7A5 and SLC1A5, in human MAIT cells that had been activated in the absence of glutamine; suggesting that there is a compensatory mechanism at play.

Pathway analysis of the genes upregulated in glutamine-deprived MAIT cells highlighted an enrichment of the cellular integrated stress response (ISR) pathway. Cells respond to nutrient-stress and metabolic perturbations through upregulation of the evolutionarily conserved ISR, comprising of a four major stress-sensing kinases that converge on the selective phosphorylation of the eukaryotic translation initiation factor (eIF)-2 α ^{29,30}. Although phosphorylation of eIF2 α results in the global repression of protein translation, a secondary consequence is the selective translation of ATF4, a stress-responsive transcription factor which can elicit the upregulation pro-survival pathways³³. Indeed, direct analysis of our RNA sequencing dataset revealed significant increases in *ATF4* and ATF4 target gene expression in MAIT cells activated in the absence of glutamine; and we corroborated this via flow cytometry, confirming increased ATF4 expression in TCR stimulated peripheral blood MAIT cells in the absence of glutamine. Further, we noted reduced gene expression of translation initiation factors, and directly confirmed reduced rates of cellular protein translation in glutamine-deprived MAIT cells; thus, substantiating our claim that the ISR is globally upregulated in glutamine-deprived MAIT cells downstream of TCR challenge. Importantly, ATF4 has been shown to drive the transcription of genes involving amino acid transport, including *SLC3A2* and *SLC7A5*²⁸. Thus, we hypothesise that the upregulation of ATF4 might attempt to compensate for the loss of Myc signalling in glutamine-deprived MAIT cells in driving the increased gene expression of amino acid transporters.

We have previously reported the importance of the Myc signalling axis in facilitating the high level glycolytic switch observed downstream of activation in human MAIT cells¹¹. Considering the loss of Myc activity in glutamine deprived MAIT cells, and parallel upregulation of ATF, we sought to probe the metabolic phenotype elicited in the absence of glutamine. Indeed, glutamine-deprived MAIT cells failed to upregulate either glycolysis or OxPhos in response to TCR stimulation; and presented with severe metabolic defects, as characterised by reductions in both maximal glycolytic and spare respiratory capacities. These data suggest that although ATF4 elicits increased gene expression of amino acid transporters in the absence of glutamine/Myc signalling, MAIT cells still fail to compensate metabolically. Sinclair *et al.*, have

recently reported that the activation of CD8⁺ T cells correlates with repression of the cellular autophagic flux to protect against the autophagic-degradation of key effector molecules such as perforin, and granzymes³¹. However, this autophagic flux is shaped by amino acid availability, such that when the availability of amino acids is limited, cellular autophagy is rapidly upregulated to ensure the appropriate allocation of resources towards energy-conserving pathways, and to promote T cell survival³¹. Thus, autophagy limits anabolic metabolism in T cells, remodelling mitochondrial protein expression to maintain integrity rather than increasing the respiratory flux; and repressing the glycolytic flux via degradation of glucose transporters³¹. In accordance, we noted that MAIT cells substantially increased the gene expression of machinery involved in autophagy when activated in the absence of glutamine, suggesting perhaps another mechanism by which the metabolic flux is repressed in MAIT cells in glutamine-deprived environments. Taken together, these data confirm that glutamine deprivation impairs Myc signalling in TCR activated MAIT cells, which likely corresponds with an impaired amino acid flux, that appears not to be fully compensated for by the ISR pathway.

Finally, we sought to elucidate the functional defects that would likely occur considering such perturbation of the metabolic phenotype in the absence of glutamine; and indeed, MAIT cells fail to elicit appropriate effector responses to TCR challenge under glutamine-deplete conditions. Specifically, MAIT cells failed to undergo proliferative expansion, and presented with reduced surface expression of activation markers, CD25 and CD69 downstream of activation. Furthermore, IFN γ expression was significantly attenuated in the absence of glutamine. These data align with our previous observations detailing impaired proliferation and IFN γ expression in human MAIT cells following perturbation of the Myc signalling axis.

Overall, we describe the importance of the glutamine-MYC signalling axis in modulating robust metabolic rewiring and effector responses in human MAIT cells. Although we did not probe the direct role of glutaminolysis, or alpha-ketoglutarate for example, in fuelling MAIT cell metabolism downstream of activation, we do highlight the importance of enhanced glutamine acquisition in response to TCR challenge. Therefore, we propose that this may represent an axis susceptible to perturbation in disease environments, such as within the TME, or in the context of obesity³⁴, where glutamine availability can be limiting.

Contributors Statement: NKM, EKR, CLF, SR, and RB performed the experiments, carried out the analysis, and approved the final manuscript as submitted. NKM and EKR also drafted the manuscript. CK, HH and DOS recruited study participants and helped with study design, analysis, and approved the final manuscript as submitted. GB provided reagents. NKM, LVS, NJ, DOS & AEH conceptualized and designed the study, analysed the data, drafted the manuscript, and approved the final manuscript as submitted.

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Data Availability: All data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials. All data are available from the corresponding authors in line with ethical approval.

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CHAPTER 7. General Discussion

In this thesis, I have explored how microenvironmental signals can shape MAIT cell metabolism and effector responses. **Paper I** provides a comprehensive review of the environmental cues that are currently understood to modulate MAIT cell fitness. In this review, we discuss the multifaceted roles of TCR engagement, surface-molecule mediated co-stimulation, chemokine signalling, and cytokine stimulation, in facilitating MAIT cell effector functioning. Importantly, we highlight nutrient availability and metabolism as a novel ‘signal 5’ which governs MAIT cell homeostasis, and their capacity to respond appropriately to challenge. In **Paper II**, we examine the iron requirements of TCR-challenged MAIT cells, and explore the impact of iron-restriction on MAIT cell fitness, something that might aid in our understanding of how MAIT cell responses might become dysregulated in certain diseased settings, particularly where iron homeostasis is altered. In **Paper III**, using high-resolution proteomic analysis, we conduct a comparative examination of TCR versus TCR-independent MAIT cell functional responses. Furthermore, we explore the metabolic underpinnings of MAIT cell responses to IL-18/IFN α , which could enhance our understanding of the regulation of MAIT cell responses to viral challenge. Finally, in **Paper IV**, we elucidate a critical role for glutamine uptake in facilitating metabolic rewiring in human MAIT cells, highlighting the importance of glutamine signalling in sustaining the expression of Myc upon activation.

As established, MAIT cells can become activated through two mechanisms: via TCR engagement and co-stimulation mediated by surface molecules and/or innate cytokines (Turtle et al., 2011, Le Bourhis et al., 2013, Slichter et al., 2016, Leng et al., 2019), or through the recognition of a milieu of cytokines, such as IL-18, IL-12, and IFN α , independently of the MR1-TCR axis (Van Wilgenburg et al., 2016). Critically, the type of response elicited downstream is majorly dictated by the activating stimuli (Garner et al., 2023), as explored further in **Paper I**, **Paper II**, **Paper III**, and **Paper IV**.

In **Paper III**, we sought to gain an in-depth, comparative view of MAIT cell responses to innate cytokine (IL-18/IFN α), versus anti-CD3/CD28 TCR triggering and IL-18 co-stimulation (TCR/IL-18), using high-resolution quantitative proteomic analysis. Indeed the activation of MAIT cells downstream of TCR/IL-18 elicits a more global remodelling of the MAIT cell proteome, with over 830 proteins increased in abundance. However, in agreement with findings from Garner and colleagues who identified that stimulation of MAIT cells with innate cytokines induces a

distinct gene regulatory network, with specific upregulation of STAT1, for example (Garner et al., 2023); we identified that stimulation of MAIT cells with IL-18/IFN α elicits distinct remodelling of the proteome, with a global upregulation of approximately 479 proteins, and the cytokine-specific upregulation of approximately 270 proteins. Specifically, we showed that IL-18/IFN α elicit upregulation of STAT1, STAT2, and IRF9 transcription factors, and a proteome dominated by the expression of ISGs, such as ISG15, ISG20, MX1, and USP18; suggesting ISGF3 complex formation. Importantly, this phenotype was not observed downstream of TCR/IL-18 signalling, and was specific to the synergistic activity of IL-18/IFN α . Although we found the expression of IFN γ to be modest in innate cytokine treated MAIT cells compared to that elicited downstream of TCR triggering and co-stimulation (**Paper II, Paper III, and Paper IV**), we emphasised that MAIT cells respond robustly to stimulation with IL-18/IFN α , and crucially, the response kinetics are rapid in nature, with an early and robust induction of *de novo* biosynthesis.

MAIT cell activation corresponds with the upregulation of a host-protective response profile, and there are numerous studies, in both mice and humans, which suggest a non-redundant role for MAIT cells in mediating host immune defence against bacterial and viral pathogens (Godfrey et al., 2019, Sandberg et al., 2023). For instance, MAIT cells have been shown to be protective *in vivo* against pathogens such as *K. pneumoniae*, *Streptococcus pneumoniae*, *Francisella tularensis*, and *Legionella longbeachae* (Georgel et al., 2011, Meierovics et al., 2013, Kurioka et al., 2018, Wang et al., 2018, Zhao et al., 2021); and higher MAIT cell numbers correlate with increased protection against lethal IAV infection *in vivo* (Loh et al., 2016, Van Wilgenburg et al., 2018). Critically, the host-protective capacity of MAIT cells likely resides in their ability to rapidly produce an array of effector cytokines and cytolytic molecules. MAIT cells mediate protection against fatal pulmonary *Legionella* infection in an IFN γ -dependent manner (Wang et al., 2018); whilst MR1-deficient mice present with a diminished cytokine milieu in the lung upon infection with the live vaccine strain of *F. tularensis*, something that corresponds with increased bacterial burden and perturbed disease clearance (Meierovics et al., 2013). Furthermore, the ‘secretome’ of γ chain cytokine -stimulated MAIT cells has recently been shown to facilitate the clearance of carbapenemase-producing *E.coli* *in vitro*, restoring the antimicrobial efficacy of imipenem (Yiting et al., 2025). MAIT cell protection against lethal IAV infection *in vivo* is mediated, at least in part, by their secretion of IFN γ (Van

Wilgenburg et al., 2018). MAIT cells also exhibit a robust capacity to proliferate *in vitro*, and infection in humans with *Salmonella enterica* serovar Paratyphi A drives the clonal expansion of antigen-specific MAIT cells with enhanced effector responses (Howson et al., 2018); thus, proliferation also comprises an important arm of their effector functioning. More recently, MAIT cells have been shown to secrete IL-26 *in vitro* upon TCR triggering or cytokine stimulation (Lamichhane et al., 2019, Meermeier et al., 2022), and although not commonly probed as a functional readout, we corroborated these findings in **Paper II**, noting the secretion of IL-26 from expanded human peripheral-blood MAIT cells *in vitro* following stimulation via TCR/IL-18. Critically, IL-26 exerts direct bactericidal effects against extracellular pathogens, including *K. pneumoniae*, via its pore-forming properties (Meller et al., 2015); highlighting another mechanism by which MAIT cells exert their host-protective functioning. Emphasising their capacity to protect against viral pathogens, in **Paper III**, we identified high level expression of ISG15 in MAIT cells upon stimulation with IL-18/IFN α , and corroborated our findings *in vivo*, whereby *in silico* analysis of two publicly available RNA sequencing datasets confirmed increased expression of ISG15 in human MAIT cells upon challenge with the adenoviral vaccine vector, ChadOx1, and in both nasal and peripheral blood MAIT cells isolated from individuals infected with SARS-CoV-2. Typically, ISG15 acts as a ubiquitin-like modifier in that it is covalently conjugated onto newly synthesised target proteins, both host and viral, in a process termed ISGylation, something that ultimately impedes viral replication (Durfee et al., 2010, Perng and Lenschow, 2018). However, ISG15 also exists in an unconjugated form which can be secreted from the cell, and may act in a cytokine-like manner to induce effector responses from surrounding NK cells and T cells (Recht et al., 1991, D'Cunha et al., 1996, Swaim et al., 2017, Iglesias-Guimaraes et al., 2020). Given that the robust expression of ISG15 comprises a significant component of MAIT cell responses to IL-18/IFN α , it is plausible that MAIT cells could potentiate host immunity in part via the secretion of ISG15, and this perhaps represents an axis which should be directly assessed in future studies. Specifically, whether ISG15 is secreted from MAIT cells in response to stimulation with IL-18/IFN α was not assessed in this study, and could be an interesting angle to interrogate in the context of human viral infections.

Although these data highlight the robust host-protective properties of MAIT cells, and emphasise their role in host immune defence, MAIT cell responses can become dysregulated

and/or impaired in chronic disease settings, and may instead contribute to host pathology. Of note, and as outlined in **Paper I**, the role of metabolism in governing MAIT cell fitness is significant, but represents an axis vulnerable to environmental disturbances. Alterations to the nutrient microenvironment *in vivo*, or impaired access to nutrients, can perturb MAIT cell metabolic and functional responses (Kedia-Mehta and Hogan, 2023); and this has been linked with disease pathogenesis in the setting of obesity, and MASLD. MAIT cells from PWO present with impaired glucose handling, and consequently fail to respond appropriately to challenge, with a diminished proliferative capacity and a loss of IFN γ (O'Brien et al., 2019, Kedia-Mehta et al., 2023). Further, MAIT cells from PWO display exacerbated mROS production, indicative of mitochondrial dysfunction and thought to drive the increased expression of IL-17 (Brien et al., 2020). Critically, in children with obesity, IL-17 expressing MAIT cells are directly correlated with the occurrence of insulin resistance, and MAIT cell derived IL-17 could disrupt insulin mediated glucose uptake *in vitro*; highlighting a potential feedback loop by which altered systemic metabolism drives MAIT cell dysfunction, and this in turn potentiates further metabolic defects (Bergin et al., 2022). Further corroborating this, mice lacking MR1 are protected against metabolic dysfunction in the context of diet-induced obesity (Toubal et al., 2020). Similarly, the uptake of microenvironmental PUFAs by hepatic MAIT cells in patients with MASLD lends to metabolic exhaustion, characterised by a diminished capacity to perform glycolysis or mitochondrial respiration, and ultimately resulting in attenuated functional responses and cell death by ferroptosis (Deschler et al., 2025). Importantly, high expression of this MAIT-PUFA gene signature is associated with poor survival in patients with Hepatocellular Carcinoma (HCC) (Deschler et al., 2025).

Such findings highlight the importance of describing a comprehensive view of MAIT cell metabolism in health and disease, with the aim of elucidating pathways which might contribute towards MAIT cell dysfunction; and thus could represent a plausible target for therapeutic intervention. Although MAIT cell metabolism is a rapidly emerging area of research, our understanding of the metabolic processes which underpin MAIT cell fitness remains incomplete, and thus comprised the focus of **Paper II**, **Paper III**, and **Paper IV**.

Something that had not been elucidated prior to the undertaking of this thesis was the role of OxPhos in supporting homeostasis and fitness in human MAIT cells. MAIT cells engage in a

robust glycolytic programme downstream of activation, which fuels their effector phenotype; and it has previously been shown that mitochondrial respiration does not support early cytotoxic responses in human MAIT cells in response to TCR challenge (Cassidy et al., 2023). However, in **Paper II** and **Paper III**, we highlighted that MAIT cells concomitantly upregulate their rates of OxPhos alongside glycolysis following TCR/IL-18 stimulation. Specifically, in **Paper III**, we highlighted robust remodelling of the mitochondrial proteome in MAIT cells following activation via TCR/IL-18, and these cells were increasingly engaged in mitochondrial respiration at 18hr post-activation. Furthermore, in **Paper II**, we demonstrated that inhibition of ATP synthase using Oligomycin significantly attenuated the proliferative capacity of these cells *in vitro*, highlighting a role for mitochondrial respiration in supporting MAIT cell fitness.

It has recently been shown that the majority of iron atoms found in conventional CD8⁺ T cells are implicated in OxPhos (Teh et al., 2021), and thus, in **Paper II**, we hypothesised that mitochondrial respiration in human MAIT cells could comprise an axis that is particularly sensitive to iron deficiency *in vivo*. Iron deficiency is the most common micronutrient deficiency worldwide, with the incidence rate currently estimated at 24.3% (Gardner et al., 2023). Although iron is predominately used *in vivo* to sustain erythropoiesis, iron deficiency is associated with an increased risk of infection, and infection-associated mortality (Mohus et al., 2018, Mottelson et al., 2024, Frost and Drakesmith, 2025). Further emphasising the significance of the iron-immune axis, a missense mutation in the *TFRC* gene, which disrupts internalisation of the TFRC-transferrin complex, results in only mild anaemia, but causes a rare combined immunodeficiency disorder in children, and consequent recurrent infections (Jabara et al., 2016). Elucidation into how iron influences immune function has been a relatively recent undertaking, and whether iron supports MAIT cell fitness was previously unknown.

In **Paper II**, given that stimulation with TCR/IL-18 elicited profound remodelling of the MAIT cell proteome, and drove increased mitochondrial respiratory activity (**Paper III**), we sought to identify whether the demand for intracellular iron content was concomitantly increased. Indeed, we showed substantially upregulated surface expression of the cellular iron transporter CD71 (TFRC), and a correlating increase in cellular iron uptake downstream of activation. Interestingly, the expression of CD71 was further enhanced, in both unstimulated

and stimulated MAIT cells, when iron-availability was restricted, suggesting a constitutive importance of the CD71-MAIT axis. Furthermore, analysis of a publicly available proteomic dataset (Kedia-Mehta et al., 2023) confirmed that MAIT cells store iron in the form of ferritin; a protein composed of 24 subunits of a heterogenous mix of ferritin heavy (FTH) and ferritin light (FTL) chains, which can store up to 4,300 atoms of iron (Galy et al., 2024). Following activation, protein copy numbers of FTH and FTL were significantly reduced, suggesting that TCR-engagement triggers the mobilisation of intracellular iron stores. Similarly, proteomic-analysis revealed a concomitant increase in total cellular iron content following TCR/IL-18-mediated activation. Taken together, these data confirmed our hypothesis that TCR-activated MAIT cells readily metabolise and require iron to support their effector responses.

Critically, pathway analysis revealed a substantial contribution of iron-containing proteins to processes pertaining to metabolism, and thus we hypothesised that robust increases in cellular metabolism in TCR-activated MAIT cells are underpinned, in part, by the acquisition of iron. Protein translation is an energetically expensive process, and approximately 50% of cellular energy produced by metabolic processes is consumed by the protein synthesis machinery; thus protein translation is used as a surrogate to assess cellular metabolic activity (Argüello et al., 2020). Restricting exogenous iron availability significantly attenuated global translation rates in TCR-activated MAIT cells, and as a whole, protein synthesis was further uncoupled from processes pertaining to mitochondrial oxidation; indicative of a role for iron in facilitating mitochondrial fitness and responses to TCR triggering in human MAIT cells. Indeed, we found that MAIT cells activated in iron-deplete conditions presented with an attenuated capacity to engage in OxPhos downstream of TCR triggering, and exhibited a dramatic loss in both their spare-respiratory and maximal-respiratory capacities. Accordingly, directly blocking transferrin uptake via CD71 resulted in a significant loss in ATP production downstream of activation. Interestingly, although glycolysis is considered as an iron-independent pathway given that the core glycolytic enzymes do not use iron as a cofactor (Teh et al., 2024), we noted a concomitant loss of glycolysis and glycolytic capacity in MAIT cells activated in iron-deplete conditions. It has recently been shown that low iron availability induces a loss of signalling through both mTORC1, and Myc in activated CD8⁺ T cells (Teh et al., 2025), and considering the importance of mTORC1 and Myc in facilitating the glycolytic burst in activated MAIT cells (O'Brien et al., 2019, Kedia-Mehta et al., 2023), it is plausible that

the diminished glycolytic profile observed in TCR-activated iron-starved MAIT cells is attributed to a consequential loss of these two critical metabolic factors.

Interestingly, several bacterial pathogens, including those which elicit MAIT cell responses, rely on the use of siderophores as virulence factors, through which they sequester host microenvironmental iron (Crouch et al., 2008, Bachman et al., 2009, Cianciotto, 2015, Chen et al., 2017, Wang et al., 2018, López-Rodríguez et al., 2023). Conversely, inflammatory cytokines such as IL-6 and IL-1 β , drive increased expression of the iron-regulatory hormone, hepcidin, and consequently induce hypoferremia (Nemeth et al., 2004, Kanamori et al., 2017). Hepcidin drives the internalisation of the iron exporter, ferroportin, which is expressed on duodenal enterocytes and erythrophagocytic macrophages, and thus inhibits dietary iron uptake, and iron recycling (Frost and Drakesmith, 2025). In this manner, infections are often associated with inflammatory hypoferremia, and of note, increased hepcidin levels were highlighted as a predictive marker of disease severity and mortality in COVID-19 (Nai et al., 2021). Whether this inflammation-iron axis might impact MAIT cell fitness during severe infection *in vivo* remains unknown, and thus this might comprise an interesting consideration to uphold in prospective studies. In **Paper II**, we found that limiting access to exogenous iron during activation severely attenuated MAIT cell host-protective responses *in vitro*. Specifically, iron-depletion perturbed the cytokine expression profile, resulting in the loss of IFN γ , IL-17, IL-2, IL-26 and granzyme B; and completely abrogated the proliferative response to antigen challenge, something that was likely underpinned by the concomitant loss in mitochondrial fitness. Interestingly, mitochondrial health, ATP production and proliferation in CD8⁺ T cells from mice with a missense mutation in the *TFRC* gene could be improved via administration of FeSO₄, an alternate iron source with bypasses CD71 (Frost et al., 2021). We similarly showed a partial rescue in both proliferation and cellular viability upon administration of FeSO₄ to iron-starved MAIT cells during a 5 day culture *in vitro*, suggesting some improvement to mitochondrial function, although this was not assessed directly.

Overall, **Paper II** describes for the first time the influence of the CD71-MAIT axis on MAIT cell metabolic fitness and effector functioning (Figure 7.1), and is perhaps something that should be considered when probing MAIT cell dysfunction in diseased settings; given the global prevalence of iron deficiency, and chronic inflammatory diseases such as obesity.

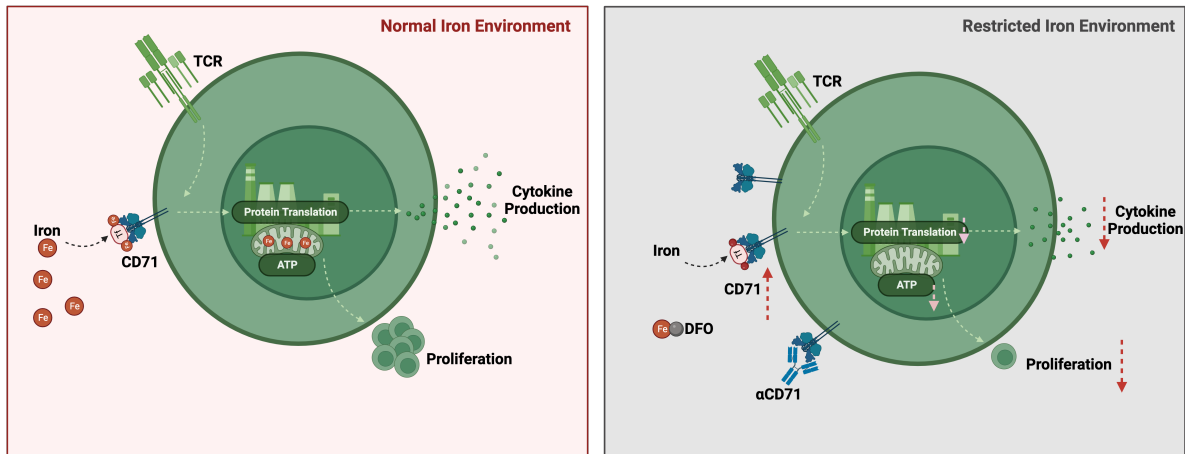


Figure 7.1. Graphical summary of Paper II. TCR triggering drives upregulated cell surface expression of the iron transporter, CD71, and increases the demand for cellular iron uptake. Iron supports cellular metabolism and de novo protein biosynthesis in human MAIT cells, thus facilitating their capacity to respond appropriately to challenge. In restricted-iron environments, the CD71-iron-MAIT axis is perturbed, resulting in impaired metabolic rewiring and diminished effector responses. Created with Biorender.com.

Previous studies have highlighted that upon activation, MAIT cells upregulate a sustained glucose-fuelled glycolytic programme, which underpins their expression of IFN γ and granzyme B in response to TCR challenge, as well as their capacity to proliferate (Zinser et al., 2018, O'Brien et al., 2019, Kedia-Mehta et al., 2023). Importantly, the capacity for MAIT cells to upregulate this programme is underpinned by expression of nutrient sensor, mTORC1, and the nutrient-sensitive transcription factor, Myc; such that inhibition of either mTORC1 or Myc impedes MAIT cell glycolytic reprogramming, and ultimately attenuates the TCR-driven effector profile (O'Brien et al., 2019, Kedia-Mehta et al., 2023). Our group have previously highlighted diminished expression of Myc in peripheral-blood MAIT cells isolated from PWO, and linked this with a defective glycolytic programme which partially underpins MAIT cell dysfunction (Kedia-Mehta et al., 2023). Interestingly, CD71 has also been identified as a downstream target of Myc (O'Donnell et al., 2006), thus it is plausible that this axis might also underpin some of the dysfunction observed in obesity; even in the absence of inflammatory hypoferraemia.

The cellular uptake of amino acids via surface expression of the LNAA transporter, SLC7A5, sustains the expression of Myc downstream of activation in conventional T cells (Marchingo et al., 2020). Similarly, upregulation of Myc elicits increased expression of amino acid

transporters in T cells, sustaining the amino acid flux; and thus there exists a critical feed-forward axis (Marchingo et al., 2020). In agreement, SLC7A5 was previously identified as a downstream target of Myc in activated MAIT cells (Kedia-Mehta et al., 2023), and MAIT cells do increase the influx of amino acids following activation in a Myc-dependent manner (O'Brien et al., 2019, Kedia-Mehta et al., 2023); however, whether this feed-forward axis exists in MAIT cells has not been described.

In **Paper IV**, to describe the role of amino acid metabolism in human MAIT cells, we sought to elucidate a role for glutamine, the most abundant amino acid, in supporting MAIT cell fitness during activation (Figure 7.2.). Indeed, we identified a substantial increase in the expression of machinery involved in cellular glutamine metabolism downstream of activation; whilst MAIT cells significantly increased glutamine uptake via SLC1A5 in response to anti-CD3/CD28-mediated TCR triggering. Thus, these data are indicative of a role for glutamine in supporting MAIT cell responses to challenge. Importantly, we demonstrated that glutamine deprivation significantly attenuated Myc protein expression downstream of activation. However, we identified a parallel increase in the gene expression of a number of amino acid transporters, including known Myc targets SLC7A5, and SLC1A5 (Wang et al., 2011, Marchingo et al., 2020). We therefore hypothesised that there was an alternative mechanism at play, which might compensate for the loss of Myc signalling under cellular nutrient-stress. Corroborating this, pathway analysis of our RNA sequencing dataset highlighted an enrichment of the integrated stress response (ISR) pathway amongst glutamine-deprived MAIT cells downstream of TCR engagement. Upregulation of the cellular ISR is driven by the sensing of environmental stressors, including nutrient deprivation (Costa-Mattioli and Walter, 2020). The most significant downstream consequence of the ISR pathway is phosphorylation of the translation initiation factor, eIF2 α , which results in a global repression of translation initiation, but specifically induces the translation of the stress-responsive transcription factor, ATF4 (Harding et al., 2000, Wek, 2018). Interestingly, ATF4 can drive the expression of cellular amino acid transporters, including SLC7A5 (Harding et al., 2003). Thus, we hypothesised that glutamine-deprived MAIT cells experience nutrient-stress, which triggers activation of the ISR to potentially allow for an increased amino acid flux via ATF4, in the absence of Myc. Indeed, we validated our sequencing data and confirmed significantly increased protein expression of ATF4 in glutamine-deprived MAIT cells, alongside repressed protein translation rates;

indicative of activation of the ISR. These data suggest that MAIT cells are sensitive to microenvironmental glutamine availability, and just 18hr glutamine deprivation drives increased cellular stress during activation.

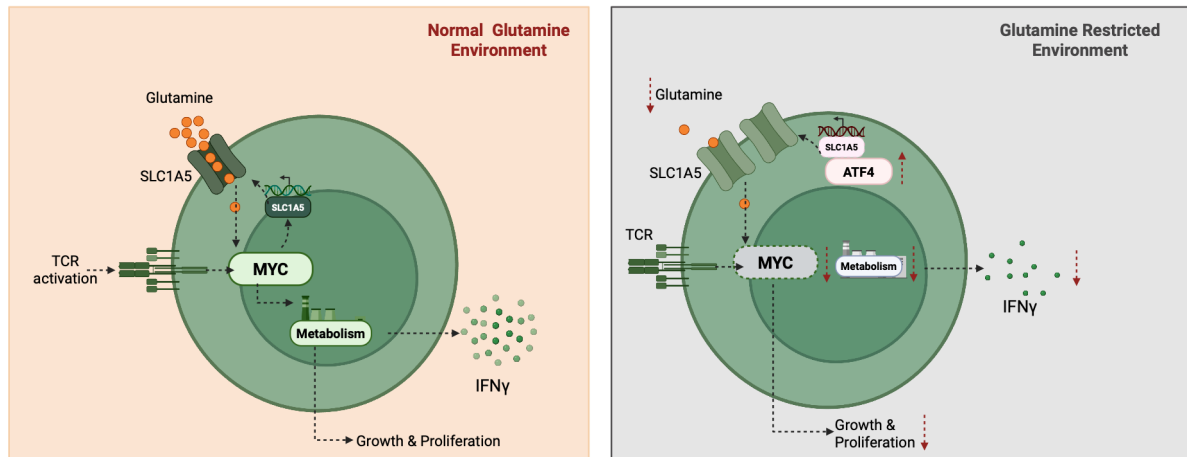


Figure 7.2. Graphical summary of Paper IV. MAIT cells enhance glutamine uptake upon activation, which regulates the expression of MYC, and in turn potentiates robust metabolic reprogramming and effector responses. In glutamine-deplete environments, this axis is perturbed, and the expression of MYC is attenuated. Instead, increased expression of ATF4 facilitates the transcription of pro-survival genes, including cellular nutrient transporters. Global protein translation and metabolic rewiring are repressed, resulting in dysfunctional effector responses. Created with Biorender.com.

Because ATF4 can modulate increased expression of a number of different nutrient transporters, we sought to identify whether MAIT cells could compensate metabolically for this loss of a singular amino acid. On the contrary, glutamine-deprived MAIT cells exhibited pronounced metabolic exhaustion, failing to upregulate either glycolysis or OxPhos in response to TCR triggering. In parallel, we confirmed that MAIT cell effector responses were significantly attenuated in the absence of glutamine, as highlighted by a failure to proliferate, and the reduced expression of IFN γ . As previously described, activation of the Myc signalling axis is required to facilitate the glycolytic switch that occurs downstream of activation in MAIT cells, and thus the repressed metabolic phenotype observed in the absence of glutamine is likely attributed to the preceding loss of Myc. However, ATF4 can also regulate cellular autophagy (B'Chir et al., 2013), and inducing autophagy in activated CD8⁺ T cells has recently been found to repress metabolic rewiring (Sinclair et al., 2025). In **Paper IV**, we did observe robust increases in the gene expression of the machinery involved in cellular autophagy in MAIT cells activated in the absence of glutamine; however, we did not validate this on the

protein level. Nonetheless, it is interesting to speculate that ATF4 might also upregulate the autophagic flux in MAIT cells activated under glutamine-restricted environments; and thus, might represent a mechanism by which glutamine-deprivation further represses metabolic rewiring in human MAIT cells, in conjunction with attenuation of Myc.

Overall, in **Paper IV**, we identify glutamine as a critical signalling molecule required to facilitate the expression of Myc downstream of TCR triggering in human MAIT cells. MAIT cells fail to compensate, and do not exhibit any capacity for metabolic plasticity, in glutamine-depleted environments; and consequently, fail to launch appropriate effector responses to TCR challenge. Critically, these data imply that MAIT cells are particularly sensitive to environmental glutamine depletion, and thus might have implications in the context of anti-tumour immunity. Some malignant transformed cells exhibit a level of “glutamine addiction”, relying on glutamine as a primary substrate for TCA cycling to sustain their high level proliferative capacity (Wise and Thompson, 2010); thus, it is thought that glutamine levels may become limiting in the tumour microenvironment (Zhou et al., 2017) Further, several tumour-infiltrating populations, including conventional T cells and NK cells, also require glutamine to sustain immune homeostasis; and thus, there is an environmental “Tug O’ War” between both cancer and immune cells for access to free glutamine (Pallett et al., 2021). Furthermore, alterations to glutamine availability are not exclusive to cancer, and have been reported in the setting of obesity, and COVID-19; two diseases that have been linked with dysfunctional MAIT cell responses (Petrus et al., 2020, Parrot et al., 2020, Jouan et al., 2020).

Although **Paper IV** further emphasises an importance for the Myc signalling axis in modulating robust metabolic responses in MAIT cells, in **Paper III**, we demonstrated that MAIT cells do not significantly increase their expression of Myc following stimulation with IL-18/IFN α . This was further corroborated through proteomic analysis, which revealed little alteration to Myc target gene expression following stimulation with IL-18/IFN α . Indeed, it has previously been shown that stimulation of human MAIT cells with either IL-18 or IL-12 alone does not elicit increased expression of MYC (Kedia-Mehta et al., 2023); although this could have also been because MAIT cells are more responsive to cytokine pairings. However, in **Paper II**, we demonstrated that the dual stimulus of IL-18/IL-12 did not drive increased surface expression of CD71, a downstream target of Myc, in human MAIT cells; and accordingly, these cells

exhibited a reduced capacity to take in transferrin, as compared with TCR/IL-18 activated MAIT cells. Taken together, these data suggested that MAIT cell responses to innate cytokine stimulation exhibit some level of independence from the Myc signalling axis; and we thus hypothesised that innate cytokines might drive a diverging metabolic profile from that elicited downstream of TCR triggering.

As evident in **Paper I**, prior to the undertaking of this thesis, MAIT cell metabolism had been described only in the context of TCR signalling; thus in **Paper III**, we sought to elucidate the metabolic kinetics and the nutritional underpinnings of MAIT cell responses to innate cytokines, using the dual stimulus of IL-18/IFN α . Indeed, in **Paper III**, we highlighted differential kinetics in the metabolic response profiles of TCR- versus innate-cytokine-activated MAIT cells, and this was most apparent at the 18hr timepoint (Figure 7.3). In line with previous findings, we confirmed robust glycolytic activity in MAIT cells downstream of TCR/IL-18 stimulation at the 18hr timepoint, and in agreement with our findings from **Paper II**, we highlighted a concomitant increase in OxPhos. However, MAIT cells stimulated with IL-18/IFN α exhibited metabolic quiescence at this same timepoint, thus mirroring the metabolic profile of a resting MAIT (Zinser et al., 2018). Conversely, we found that IL-18/IFN α drove a rapid, but modest, glycolytic burst which was evident as early as 3hr post-stimulation, but this was not sustained. Perhaps owing to this transient metabolic phenotype, we found the glycolytic burst elicited downstream of IL-18/IFN α to be uncoupled from the mitochondria, noting no concomitant increase in mitochondrial respiration at any of the observed timepoints. We further corroborated this finding within our proteomic dataset, where we found no remodelling of the mitochondrial proteome downstream of stimulation with IL-18/IFN α ; something that markedly contrasted the robust upregulation elicited through stimulation with TCR/IL-18. Conversely, just 6hr stimulation with IL-18/IFN α induced a loss in SRC, as compared with unstimulated cells, suggesting that this innate cytokine pairing might actually hinder MAIT cell mitochondrial fitness. Given that in **Paper II**, we highlighted a governing role for mitochondrial respiration in underpinning MAIT cell proliferation in response to TCR challenge, these data could have implications in the context of chronic viral infection; and in particular, in the context of secondary microbial challenge. Supporting this hypothesis, early exposure to type I IFNs during acute viral infection in mice has been shown to attenuate the proliferative capacity of bystander CD8⁺ T cells in response to a subsequent

cognate antigen challenge (Marshall et al., 2011); and several papers have highlighted cellular defects in mitochondrial fitness upon chronic exposure to type I IFNs (Gkirtzimanaki et al., 2018, Lei et al., 2021, Buang et al., 2021).

Interestingly, when probing the nutrient underpinnings of MAIT cell responses to IL-18/IFN α , we found that whilst glucose fuelled the early glycolytic burst, and supported robust functional responses in the context of ISG15 and granzyme B; the inhibition of exogenous glucose metabolism using 2DG had no impact on early (6hr) protein biosynthesis, and specifically, exogenous glucose was dispensable for the production of IFN γ . It has previously been shown that glycogen fuels early (2hr) MAIT cell cytotoxicity, and partially fuels the robust glycolytic burst elicited in TCR stimulated MAIT cells (Cassidy et al., 2023). Indeed, in **Paper III**, we found that the inhibition of glycogenolysis significantly attenuated rapid protein biosynthesis and effector responses in response to IL-18/IFN α , and unlike exogenous glucose, was indispensable for the expression of IFN γ . However, contrasting our previous findings in the context of TCR triggering, we found that in the setting of IL-18/IFN α , early glycolytic activity was uncoupled from glycogenolysis. Taken together, these findings suggest that MAIT cell responses to IL-18/IFN α are underpinned by the preferential nutrient partitioning of glucose and glycogen. We found that early glycolytic metabolism supports prolonged and robust functional responses, as glucose was indispensable for the high-level expression of ISG15 and granzyme B, which is increased in magnitude over the course of 18hr; but glycogenolysis can independently fuel the rapid biosynthetic burst, without feeding into the glycolytic programme.

Zhou and colleagues have recently found that the glycogenolytic flux is preferentially coupled with the PPP in activated CD8⁺ Tm cells, to ensure the efficient generation of NADPH which is used to maintain cellular redox homeostasis and facilitate Tm cell persistence in response to challenge (Zhou et al., 2025). In **Paper III**, we established that the glycogenolytic flux is not coupled to glycolysis in innate cytokine activated MAIT cells, contrasting that previously observed in TCR stimulated MAIT cells. Given the impact of IL-18/IFN α on the MAIT cell SRC, it would be interesting to evaluate in future studies whether this cytokine stimulus might drive preferential upregulation of the glycogen-PPP coupled flux in activated MAIT cells in an

attempt to counter potential IFN α -induced defects in mitochondrial fitness, and to support cellular biosynthesis.

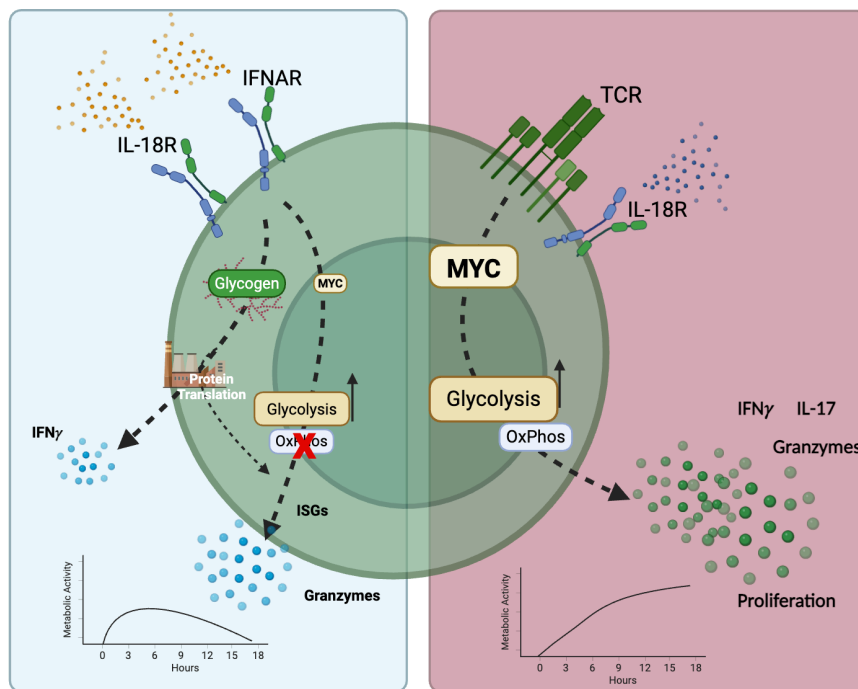


Figure 7.3. Graphical summary of Paper III. Stimulation of human MAIT cells with IL-18/IFN α independent of TCR triggering does not license upregulation of the metabolic regulator Myc, and as such elicits only a modest glycolytic burst, uncoupled from the mitochondria. Such metabolic rewiring is not sustained, and by 18hr, these cells are metabolically quiescent. Downstream effector responses are rapid, but modest, limited predominately to the expression of IFN γ , granzyme B, and ISGs. Such innate-cytokine driven responses are fuelled by the partitioning of glycogen and glucose. In contrast, TCR triggering and cytokine co-stimulation elicits strong upregulation of Myc, which in turn facilitates robust and sustained metabolic rewiring, alongside polyfunctional effector responses. Created with Biorender.com.

These data describe, for the first time, the metabolic and nutritional underpinnings of MAIT cell responses to innate cytokine stimulation, and thus may enhance our understanding of the regulation of MAIT cell host-protective responses during the early stages of viral infection; although these findings should be assessed further *in vivo*. For example, *in vivo*, it is likely that MAIT cells would encounter these cytokines sequentially rather than in tandem, such that the expression of IFN α is thought to occur via faster response kinetics than those eliciting expression of IL-18; and likely involves activation of a milieu of different cell types (Lee et al., 2017, Provine et al., 2021, Sandberg et al., 2023). Thus, the metabolic and functional response profiles described here might not be fully recapitulated within the human system. Further, it

would be of interest to probe whether MAIT cell responses to innate cytokines are dysregulated in conditions where glucose availability is perturbed. For example, given that glucose handling is altered in MAIT cells from PWO (O'Brien et al., 2019), it would also be of importance to examine whether there remains a capacity to create endogenous glycogen stores, and if not, whether this may also contribute towards MAIT cell dysfunction and the increased susceptibility of PWO to morbidity and mortality associated with infection (Nyberg et al., 2026). Finally, probing this metabolic axis in MAIT cells during severe viral infections, such as in COVID-19, could shed light on the mechanisms which underpin dysfunction *in vivo*, and thus may elucidate a plausible target for intervention.

Concluding Remarks

MAIT cells are innate-like effector T cells comprising a significant arm of the unconventional T cell compartment in humans; demonstrating high abundance in the blood and the liver, but also populating barrier tissue sites, such as the skin, gut, and lung. Importantly, these cells express a semi-invariant TCR which recognises metabolite derivatives of the microbial riboflavin biosynthesis pathway, presented on MR1; and in this manner, are capable of launching robust host-protective responses against a range of bacterial pathogens. Furthermore, MAIT cells present with high surface expression of numerous innate cytokine receptors, and it is thought that via cytokine stimulation alone, independent of TCR signalling, these cells are responsive in the context of human viral infections. It has more recently been revealed that MAIT cells exert robust tumour-specific cytotoxicity; whilst a role for these cells in promoting tissue homeostasis and repair has also been proposed. However, MAIT cell responses can become dysregulated, and have been shown to contribute to the pathogenic burden of disease in certain settings. Thus, MAIT cells are critical players in the context of both health, and pathology.

Importantly, effector T cell responses are underpinned by significant proteome remodelling and *de novo* biosynthesis; and thus, are metabolically expensive processes, which drive an increased demand for bio-intermediates, and nutrients. Therefore, nutrient availability is considered a governing environmental and cellular signal which can dictate the downstream metabolic profile, and the capacity to respond appropriately to challenge. Although cellular metabolism has been extensively characterised in conventional T cell subsets, the field of MAIT cell metabolism remains in its infancy. Garnering a comprehensive understanding of the nutritional requirements and metabolic underpinnings of MAIT cell effector responses is important if we are to harness their full potential in the context of host protection; and could shed further light on how such responses can become dysregulated in disease settings. Accordingly, the aim of this PhD thesis was to further characterise the nutritional underpinnings of MAIT cell effector responses to challenge; and to compare, for the first time, the metabolic profiles elicited downstream of TCR versus innate cytokine (TCR independent) signalling pathways.

In **Paper I**, we carried out a comprehensive review of the literature and synthesised the environmental signals that can influence MAIT cell functional responses. In addition, we provided a novel commentary that further highlights nutrient availability as a critical signal dictating MAIT cell fitness; suggesting that alterations to the nutrient-MAIT axis have the potential to drive dysregulated effector responses in diseased contexts.

In **Paper II**, we described a novel role for cellular iron metabolism in fuelling MAIT cell effector responses to TCR challenge. We highlighted that MAIT cells increase cellular iron uptake via CD71 downstream of TCR triggering and cytokine co-stimulation, and metabolise iron to support mitochondrial health and global cellular metabolism. Limiting the availability of exogenous iron severely attenuated metabolic fitness, and consequently impaired downstream effector responses; including global cytokine expression, and the capacity to proliferate.

In **Paper III**, we further characterised the TCR-independent responses of human MAIT cells to IL-18/IFN α challenge, generating a quantitative proteomic dataset which allows for comparative functional analyses between TCR/IL-18 and IL-18/IFN α triggered MAIT cells. We described, for the first time, the metabolic profile underpinning MAIT cell responses to stimulation with IL-18/IFN α ; and highlighted significant differences in both the kinetics of the response, and the metabolic pathways upregulated, when compared to TCR/IL-18 challenged MAIT cells. Finally, we uncovered a novel role for the partitioning of glycogen and glucose in fuelling rapid functional responses to innate cytokines.

In **Paper IV**, we probed the glutamine-Myc signalling axis in human MAIT cells, and characterised a critical role for this axis in supporting MAIT cell fitness. We found that glutamine-deprivation attenuates the expression of Myc, which consequently impedes global metabolic rewiring and effector responses in the context of TCR challenge.

Taken together, this PhD thesis contributes novel insights into the metabolic regulation of human MAIT cells in the context of both TCR-dependent and TCR-independent signalling pathways; and highlights the importance of environmental nutrient availability for MAIT cell effector functioning. These data may have significant implications when probing the

dysregulation of MAIT cell responses in disease settings, such as in infection, cancer, and obesity.

Limitations & Future Perspectives

Although the studies presented within this thesis provide novel insights into the metabolic regulation of human MAIT cells, there are several limitations that I wish to acknowledge.

A major limitation faced with examining immune cell metabolism *in vitro* using protocols such as Seahorse Flux Analysis is that they require high cell numbers of a single cell population; and thus is it difficult to probe the metabolic phenotypes of rare cell populations directly *ex vivo*. Therefore, we used expanded MAIT cell lines to generate some of the metabolism data presented in this thesis, and it must be acknowledged that these cells may differ from true naïve cell populations in their resting metabolic and functional capacities. Ideally, isolated MAIT cell populations would be profiled directly *ex vivo*, however this also poses issues. In addition to low yields, FACS sorting can impact cellular metabolism due to the lack of available metabolites and nutrients present in sorting fluid. Nonetheless, we did validate our findings on *ex vivo* MAIT cells in PBMC, using single-cell metabolic assays for flow cytometry.

Another limitation of this thesis is the use of peripheral blood MAIT cells in an *in vitro* setting. *In vitro* culture systems will never truly recapitulate the *in vivo* niche in terms of nutrient availability, metabolite content, and cell-cell crosstalk influenced by spatial proximity, for example. On this point, MAIT cells preferentially populate tissue sites, are recruited towards sites of inflammation and infection, and have recently been shown to exhibit heterogeneity across tissue sites. For example, intrahepatic MAIT cells are thought to be trained by IL-7 signalling which impacts downstream effector responses, and therefore this would likely shape the metabolic phenotype. It is in this manner important to consider that MAIT cell metabolism is likely not fixed to the TCR versus TCR-independent programmes presented here. Although challenging, future work should focus on delineating the metabolic tendencies of tissue-specific human MAIT cell compartments, as this could provide further insight into their functional capacities, and their potential to be harnessed therapeutically.

In **Paper II**, we described the critical importance of exogenous iron availability in dictating the capacity for MAIT cells to respond appropriately to challenge. In this paper, we speculated

that these data could have significant considerations when probing the dysregulation of MAIT cell responses *in vivo*, for example during infection; however, this was beyond the scope of our study and is indeed a limitation. It would thus be of interest for future work to incorporate information on patient serum transferrin, iron, or hepcidin levels to determine whether this indeed correlates with increased morbidity or mortality associated with disease; and thus, whether this presents an axis that could be targeted therapeutically to restore MAIT cell dysfunction *in vivo*.

In **Paper III**, we highlighted a role for glycogen in fuelling rapid biosynthesis in MAIT cells in response to IL-18/IFN α signalling; but suggest that glucose is the preferential fuel used to support early metabolic activity, and some downstream effector responses. Thus, we propose a model of nutrient partitioning. To further corroborate this, our study however could have benefited from the use of stable-isotope glucose tracing to track the allocation of glucose during the response cascade. Furthermore, although we used stimulation with innate cytokines to gauge an understanding of the metabolic underpinnings of MAIT cell responses during human viral infections, this is a surrogate, and again, may not fully recapitulate MAIT cell biology *in vivo*. Thus, future studies might aim to further corroborate our findings using single-cell based *ex vivo* analyses from patient samples; such as SCENITH and other flow-cytometry based metabolic assays.

In **Paper IV**, we described a critical role for glutamine in sustaining the expression of Myc, and facilitating metabolic rewiring and effector responses, in human MAIT cells. Although we thus demonstrated that glutamine signalling has a pivotal role in potentiating MAIT cell responses to challenge, we did not probe whether glutamine also fuels metabolism, for example TCA cycling, in human MAIT cells. Critically, it has been demonstrated that NK cells require glutamine for the expression of Myc, and downstream metabolic rewiring and cytotoxic responses; but this is independent of glutaminolysis (Loftus et al., 2018). Future directives should therefore aim to ascertain whether MAIT cell effector responses exhibit similar independence from glutamine “fuelling”, as there is increasing interest in targeting glutamine metabolism in cancer using inhibitors of glutaminolysis to attenuate tumour progression. In theory, this should also increase microenvironmental glutamine availability, which would benefit intra-tumoral immune cells that rely on glutamine signalling for expression of Myc.

CHAPTER 8. General Bibliography

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CHAPTER 9. Appendix

9.1. Paper I PDF

IMMUNOLOGY

Environmental regulation of mucosal-associated invariant T cells: Adding food for thought

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Mucosal-associated invariant T (MAIT) cells are a population of innate-like, unconventional T cells characterized by the expression of a semi-invariant, non-MHC-restricted T cell receptor (TCR), which play an important role in mediating innate immune responses to bacterial and viral pathogens. Emerging research continues to describe the many environmental signals that influence the types of responses elicited downstream of MAIT cell activation. In this Review, we highlight five key factors that determine the metabolic and functional responses of MAIT cells, including TCR engagement, costimulation, chemokine signaling, cytokine stimulation, and nutrient availability. We further define the importance of optimal nutrient availability as “signal 5” in promoting MAIT cell fitness and in governing their capacity to respond appropriately to challenge. In understanding the ways in which MAIT cells are influenced by their microenvironment, we can continue to identify the potential factors that drive dysregulated responses in hostile circumstances, enabling restoration of their protective nature in the context of infection or in the tumor microenvironment.

MAIT CELL PHENOTYPE AND FUNCTIONAL IMPORTANCE

Mucosal-associated invariant T (MAIT) cells are a group of unconventional effector T cells that constitute a critical component of the immune response, and they elicit robust, host-protective responses against a range of bacterial and viral pathogens (1–5). MAIT cells are characterized in humans primarily by their expression of a conserved T cell receptor (TCR) α chain, V α 7.2-J α 33, paired with a limited repertoire of V β -chain segments (6, 7), and high coexpression of the C-type lectin-like receptor, CD161 (8). MAIT cells are invariant in their restriction by the major histocompatibility complex (MHC) class I-like molecule MR1, which facilitates the presentation of antigenic derivatives of the microbial riboflavin biosynthesis pathway (9, 10). MAIT cell frequencies are enriched in the periphery, accounting for 1 to 10% of circulating T cells in healthy individuals (11, 12). Circulating human MAIT cells are predominantly CD8 single-positive (SP) (~80%), existing alongside a smaller but substantial double-negative (DN) population (~15%) (12–14). Although CD4 SP and CD4/CD8 double-positive (DP) phenotypes have been confirmed with MR1 tetramers as being true MAIT cell subpopulations, circulating CD4⁺ MAIT cells constitute a minor proportion in healthy individuals (12, 15). CD8⁺ MAIT cells are further characterized by constitutively high cell surface abundance of the interleukin-18 (IL-18) receptor (IL-18R) (16, 17), which facilitates robust effector responses. Note that MAIT cell activation can be independently elicited by two distinct mechanisms: upon TCR engagement (10) or downstream of stimulation with innate cytokines, such as IL-18 and IL-12 (4), although robust functional responses are observed in the context of synergistic signaling through both pathways.

Upon activation, MAIT cells take on an effector phenotype, which is characterized by the production of a range of cytokines, including interferon- γ (IFN- γ), tumor necrosis factor- α (TNF α), and IL-17, as well as lytic molecules, such as perforin and granzyme B (18). Because of this, activated MAIT cells demonstrate an increased capacity for targeted cell killing and can elicit cytotoxic responses against infected

or malignant transformed cells (19–21). Another key attribute of the MAIT cell innate-effector phenotype is the capacity to undergo rapid proliferative expansion downstream of activation, which further underpins the role of MAIT cells in innate immune defense (22–25). The MAIT cell effector program is facilitated by acquired expression of the innate master transcription factor promyelocytic leukemia zinc finger (PLZF) during thymic development (26) and the rapid acquisition of a memory phenotype after birth (27).

MAIT cells also display a tissue residency phenotype, and as such, they are enriched in the liver, lung, gut, and skin (28). This tissue-homing capacity is also thought to be underpinned by PLZF, which mediates the expression of homing and adhesion receptors in other cell types (29, 30). Adoptive transfer experiments in mice confirmed that MAIT cells are preprogrammed in the thymus to preferentially home to peripheral tissues (31). Human MAIT cell frequencies are most abundant in the liver, an important site of immune surveillance and defense against gut-derived pathogens (11, 32, 33). When compared with other hepatic T cells, liver-derived MAIT cells exhibit a distinct tissue residency signature that is characterized by the reduced abundance of several chemokine receptors and the circulatory marker CD62L, together with the increased expression of the tissue residency marker CD69 (31). MAIT cells expressing CD56 constitute the dominant population in the liver, armed with a robust effector capacity characterized by a greater responsiveness to stimulation and an enhanced polyfunctional response profile (34), which may have implications both in the context of immune defense and inflammation-mediated tissue pathology. However, transcriptomic analyses revealed that upon activation, MAIT cells express genes involved in tissue angiogenesis and repair, such as *VEGFA* and *AREG* (35–37). Furthermore, studies have elucidated a protective role for MAIT cells in the context of tissue injury, suggesting that MAIT cells have a role in promoting tissue homeostasis and repair (38–40).

ENVIRONMENTAL SIGNALS 1 AND 2: TCR ENGAGEMENT AND COSTIMULATION

Classically, the first signal for T cell activation is TCR engagement (41) (Fig. 1). MAIT cells are defined by their expression of a conserved

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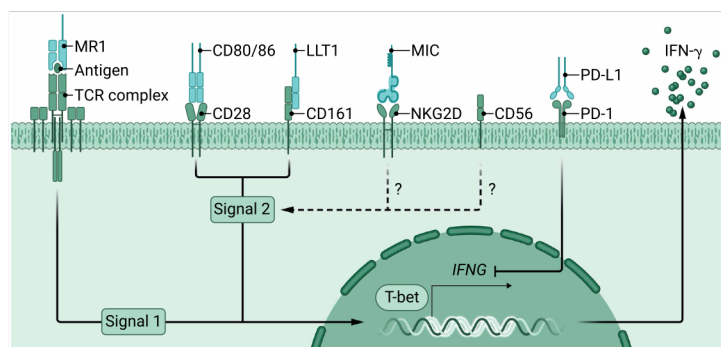


Fig. 1. Signals 1 and 2 directing MAIT cell responses. Overview of TCR and costimulatory molecule signaling in MAIT cells. MAIT cells express a semi-invariant TCR that recognizes the cognate MAIT cell antigen 5-OP-RU presented by MR1, leading to intracellular signaling (termed signal 1) and the activation of transcription factors, such as T-bet, that lead to cytokine production. In parallel, MAIT cells receive costimulation signals through cell surface-expressed molecules, such as CD28 and CD161, which can synergize (termed signal 2) with the TCR-dependent signal to promote cellular responses. In addition, the surface expression of NKG2D and CD56 may modulate MAIT cell responses. Last, the coreceptor PD-1 is a well-established inhibitor of MAIT cell responses.

TCR α chain that is paired to a limited repertoire of β chains (9). This semi-invariant TCR is restricted to antigens presented on MR1, and the most potent activating antigen reported to date derives from an intermediate of the microbial vitamin B₂ (riboflavin) pathway, known as 5-amino-6-D-ribitylaminouracil (5-A-RU) (10, 42). This intermediate generates 5-OP-RU, which is stabilized and presented by MR1. Note, however, that MR1 in the absence of bound antigen is not expressed at the cell surface (43, 44). Therefore, MAIT cells are responsive in environments in which their cognate microbial antigen is present, such as in the gut, thymus, skin, liver, and periphery (39, 45, 46). Tumor-infiltrating MAIT cells can also display signatures of bacterial activation, highlighting the ubiquitous reach of their antigen (47). Supporting evidence of a host-derived MAIT cell antigen has been reported (48). A sulfated bile acid, cholic acid 7-sulfate (CA7S), binds to MR1 and supports MAIT cell development and homeostasis. The deletion of the sulfotransferase 2a family of enzymes responsible for CA7S synthesis results in a reduction in the number of thymic MAIT cells in mice, and unlike the microbial 5-OP-RU, CA7S does not induce MAIT cell activation but rather stimulates the expression of a homeostatic gene signature (48). In addition, host-generated riboflavin catabolites have been identified in human blood, and Abdelaal *et al.* (49) elucidated a role for such catabolites in regulating MR1 surface expression and MAIT cell responses. The riboflavin catabolites 10-formylmethylflavin, lumichrome, lumiflavin, and alloxazine bind to and stabilize MR1, and in contrast with microbial-derived antigens, they prevent the transport of 5-OP-RU, restricting MAIT cell activation in a dose-dependent manner. On the basis of these findings, the authors hypothesized that host-generated riboflavin catabolites act as regulators of the MR1-MAIT cell axis to prevent aberrant MAIT cell responses (49). However, note that the TCR-MR1-mediated environmental regulation of MAIT cells extends beyond the context of host- and microbial-derived antigens. Small-drug metabolites and drug-like molecules, including salicylates and diclofenac, bind to MR1 and affect MAIT cell responses in both an inhibitory and stimulatory manner (50). In addition, several components

of cigarette smoke bind to MR1, resulting in the modulation of MAIT cell responses and subsequently hampering MAIT cell responses to influenza infection (51).

Signal 2 for T cell activation consists of costimulatory interactions from membrane-bound molecules expressed by cells encountered in the immediate microenvironment (Fig. 1). The classical example of such is CD28, which provides costimulatory signals after binding to specific ligands, such as CD80 or CD86, expressed on surrounding immune cells, such as dendritic cells (52). MAIT cell responses are also supported by CD28 costimulation (53, 54), with Kurioka *et al.* (19) demonstrating that CD3 stimulation alone is not sufficient to drive MAIT cell responses. Similarly, Dias *et al.* (55) demonstrated that CD28 costimulation enhances human MAIT cell responses to *Escherichia coli* in vitro. In contrast, murine MAIT cell responses are not dependent on CD28 signaling in a *Francisella tularensis* infection model (56).

As mentioned earlier, MAIT cells are defined by the high surface abundance of the costimulatory receptor CD161, which interacts with ligands such as LLT1, expressed on surrounding immune cells (57). Fergusson *et al.* (58) found that CD161 ligation enhances IFN- γ and TNF production by MAIT cells when paired with TCR stimulation but not cytokine stimulation. Consistent with this, loss of CD161 limits the production of IFN- γ by CD3/CD28-stimulated MAIT cells (53).

Consistent with their innate-like status, MAIT cells express a range of natural killer (NK)-like receptors, including the NKG2 family of molecules (12, 14), which can act as inhibitory (NKG2A) or activating (NKG2D) receptors (59). NKG2D recognizes ligands that are increased in abundance on cells subjected to cellular stress, such as infection or malignant transformation. NKG2D is constitutively expressed on conventional human CD8⁺ T cells and acts as a costimulatory receptor together with the activated TCR to enhance downstream activation and functional responses (60). NKG2D promotes the formation of CD8⁺ memory T cells (61–63), and antibody-mediated costimulation by NKG2D rescues CD8⁺ memory T cell responses, in the absence of CD4⁺ T cell help, in patients with HIV (62). As such, therapeutics that engage the NKG2D pathway in CD8⁺ T cells are of clinical interest and may have implications in the settings of antitumor and antiviral immunity (60). However, the NKG2D signaling axis is also implicated in immunopathology. Kim *et al.* (64) showed that IL-15 derived from hepatitis A-infected cells activates memory CD8⁺ T cells and licenses TCR-independent cytotoxicity induced by NKG2D and Nkp30, which is associated with increased liver injury during acute infection. Similarly, in MAIT cells stimulated with IL-15, NKG2D facilitates TCR-independent cytotoxic responses, which are linked to hepatocyte injury in the setting of hepatitis A infection (65). However, whether NKG2D has a costimulatory role in potentiating TCR-mediated effector responses in MAIT cells remains to be elucidated and could be important in the context of developing MAIT cell-specific immunotherapeutics.

MAIT cells also express the adhesion molecule CD56 at varying amounts across tissues, with the greatest abundance observed on

hepatic MAIT cells (34). Although CD56 is typically considered to be a surface marker for the identification of human NK cells, it plays a role in mediating their functional responses. Picard *et al.* (66) showed that NK cells that are prelicensed by cytokine stimulation elicit functional responses to antibody-mediated engagement of CD56, as evidenced by increased degranulation and secretion of IFN- γ . CD56-expressing MAIT cells constitute a subpopulation characterized by a more robust innate-like phenotype, with enhanced effector responses to stimulation (34, 67, 68) together with increased expression of the transcription factors PLZF, T-bet, andomesoderm (67). MAIT cells that express CD56 bind MR1 tetramer more robustly (34); however, it is not known whether CD56 has a direct role in the modulation of MAIT cell effector responses through costimulatory interactions or otherwise.

Expression of the checkpoint molecule programmed cell death protein-1 (PD-1) leads to inhibition of cellular responses upon binding to its ligand, PD-L1, and several studies have reported high PD-1 abundance on circulating MAIT cells in diseased settings (69–76). Ruf *et al.* (73) identified an axis between PD-L1-expressing tumor-associated macrophages and MAIT cells in the non-tumor-adjacent liver as a potential driver of dysregulated MAIT cell responses in liver cancer, whereby hepatic MAIT cells exhibit an impaired tumor-infiltrating capacity together with a dysfunctional phenotype associated with high cell surface amounts of PD-1 and T cell immunoglobulin and mucin-domain containing-3 (TIM-3). The administration of an immune checkpoint blockade (ICB) targeting PD-L1 results in increased tumor infiltration by MAIT cells in a mouse model of hepatocellular carcinoma (HCC), together with improved granzyme B production from the intrahepatic MAIT cell fraction *ex vivo* (73). Similarly, Jarvis *et al.* (70) identified substantially increased PD-1 expression on MAIT cells from patients with pancreatic cancer after *ex vivo* stimulation with the MR1 ligand 5-A-RU/MG and that blocking PD-1 with the monoclonal antibody pembrolizumab enhances MAIT cell cytotoxicity and IFN- γ production *in vitro*. Circulating MAIT cell frequencies are associated with responsiveness to PD-1 therapy in patients with metastatic melanoma, and anti-PD-1 therapy increases MAIT cell effector gene expression and the cell surface expression of the chemokine receptor CXCR4 (72). Accordingly, the authors hypothesized that anti-PD-1 therapy might facilitate the migration of MAIT cells toward metastases, which is supported by the fact that MAIT cell numbers are increased in melanoma lesions in those patients responding to the ICB compared with those who did not respond (72). Together, these data further highlight the PD-1/PD-L1 axis as a plausible driver of MAIT cell dysfunction in cancer and emphasize the potential for targeting microenvironmental signals to restore MAIT cell functionality in disease settings.

ENVIRONMENTAL SIGNAL 3: CYTOKINES

Cytokines can act in synergy with TCR signaling to potentiate robust downstream

effector responses and, in this manner, are considered an environmental “signal 3” in modulating MAIT cell functionality. When directly compared with the responses of conventional CD8⁺ memory T cells, MAIT cell effector responses to TCR stimulation alone appear weak, even when paired with robust signal 2 costimulation from CD28, for example (77). Sustained MAIT cell effector responses are seen only when TCR engagement and costimulation are paired with inflammatory signals (signal 3) from cytokines, such as IL-12, IL-18, and IL-15, for example (36, 77). Considering that the MAIT cell TCR recognizes antigenic by-products derived from the riboflavin biosynthesis pathway, which is conserved among both commensal and pathogenic bacteria, it is hypothesized that this tertiary requirement of an inflammatory cue provides a level of protection against hyper-responsiveness to commensals at mucosal sites (36). Thus, cytokines are considered an important microenvironmental signal 3 in the context of driving MAIT cell functional responses. However, a major divergence of MAIT cells from conventional T cells is their nuanced ability to respond directly to cytokines in a TCR-independent manner (18) (Fig. 2). The activation of MAIT cells during viral infections in humans is thought to occur independently of TCR stimulation through cytokine stimulation alone because viruses are incapable of synthesizing riboflavin (4), the antigenic donor for MAIT cell TCR activation. MAIT cells display transcriptional diversity downstream of stimulation with cytokines alone compared with when they were stimulated with cytokines in conjunction with TCR signaling (68); thus, we discuss the role of environmental cytokines as modulators of MAIT cell effector responses in the classical context of signal 3 and in the context of MAIT cell-specific, TCR-independent effector functionality.

Human MAIT cells express many cytokine receptors and exhibit the highest resting cell surface expression of the IL-18R among all T lymphocytes (17, 58). As such, MAIT cells are prelicensed to respond to cytokine stimulation and, in particular, to combinations of cytokines (78). The first cytokine pairing to be characterized as a potent modulator of MAIT cell functional responses was that of IL-12 and IL-18. Both IL-12 and IL-18 are important modulators of MAIT cell

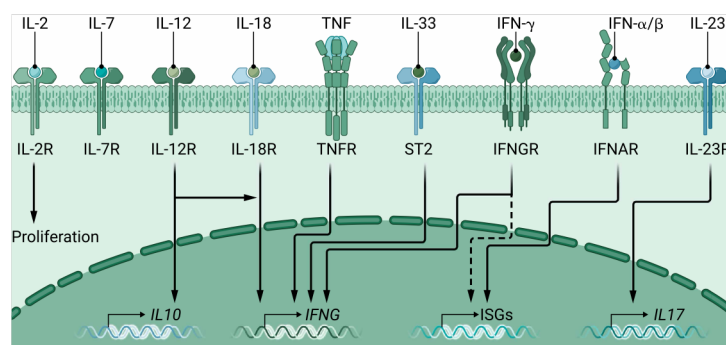


Fig. 2. Cytokines act as signal 3 to direct MAIT cell responses. Overview of cytokine signaling in MAIT cells. MAIT cells express multiple cytokine receptors both constitutively (for example, IL-18R) and after activation (for example, the IL-33 receptor ST2). When cytokines in the microenvironment bind to their cognate cell surface receptors, intracellular signals (termed signal 3) promote MAIT cell proliferative responses (for example, through the secretion of IL-2) or induce transcriptional responses, leading to the increased expression of cytokines or ISGs. In most cases, two or more cytokines (for example, IL-12 and IL-18) can synergistically promote MAIT cell responses. Cytokines can also synergistically modulate TCR-dependent MAIT cell responses.

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activation and associated host protective responses during viral infection in vitro and in vivo (79). IL-18 is the dominant signal involved in driving MAIT cell host-protective responses to influenza A virus infection in vivo (5), although it is also speculated to contribute to MAIT cell exhaustion and increased viral pathogenicity in certain disease contexts (80–82). Long-term stimulation of MAIT cells with IL-12 and IL-18 induces an exhaustive state in vitro, as characterized by the substantially increased cell surface abundance of CD39, further highlighting the sensitivity of MAIT cells to inflammatory cytokine signaling and their susceptibility to dysfunction in hyperinflammatory settings. The stimulation of MAIT cells with a combination of IL-12 and IL-18 induces the increased expression of T-bet, together with substantial IFN- γ production, as well as increased expression of cytolytic molecules, including perforin and granzymes B, H, A, K, and M (17, 37, 58). In the context of considering IL-12 and IL-18 signaling as signal 3 in potentiating MAIT cell effector responses, the synergistic activities of both IL-12 and IL-18 together with TCR stimulation are required to elicit the optimal production of IL-17 by MAIT cells in vitro (83). Furthermore, Leng *et al.* (36) confirmed that a combination of IL-12 and IL-18 together with TCR engagement results in amplification of the effector profile in human MAIT cells in vitro when compared with that elicited by signals 1 and 2, anti-CD3 and anti-CD28, or signal 3, cytokine stimulation, alone.

Autoimmune liver disease (AILD) is associated with chronic inflammation and high serum concentrations of proinflammatory cytokines, such as IL-18, IL-12, and IL-1 β . Repeated stimulation of MAIT cells with IL-12 and IL-18 ex vivo recapitulates the exhaustive phenotype of MAIT cells derived from patients with AILD (74). IL-17A is implicated in the development of liver fibrosis in diseased settings, and the frequency of T helper 17 (T_H17) cells that infiltrate the liver positively correlates with hepatic inflammation and fibrosis grade in autoimmune hepatitis (84). Böttcher *et al.* (74) noted that repeated stimulation of MAIT cells with IL-12 elicits the secretion of IL-17A, and MAIT cell–derived IL-17A supports the activation and proliferation of hepatic stellate cells in a coculture system in vitro. Together, these data suggest that the enhanced activation of hepatic MAIT cells through stimulation by inflammatory cytokines might potentiate liver fibrosis in diseased settings, thus contributing to the inflammatory burden of the disease.

Although costimulation of MAIT cells with IL-18 and IL-12 drives a dominant inflammatory phenotype in vitro, stimulation with IL-12 alone together with cognate antigen recognition induces a regulatory phenotype in human MAIT cells, which is characterized by the increased abundance of TIM-3, lymphocyte-activation gene 3 (LAG-3), and PD-1 (85). Furthermore, costimulation with IL-12 induces the increased expression of c-MAF and a resulting robust increase in the production of IL-10, which is abrogated in the presence of IL-18 signaling (85), indicative of a role for IL-18 as an inflammatory signal that potentiates the effector phenotype in the context of microbial challenge. In a similar context, the administration of IL-12 polarizes MAIT cell responses toward the T-bet⁺ ROR γ t[–] (retinoic acid–related orphan receptor γ t–negative) “MAIT1” phenotype in mice infected with *Legionella longbeachae*, an infection that typically skews responses toward the ROR γ t⁺ “MAIT17” phenotype (56), further recapitulating the intricate plasticity of MAIT cell responses and their susceptibility to distinct microenvironmental cues.

Both IL-12 and IL-23 are heterodimeric cytokines that share a common β subunit, IL-12p40, and MAIT cell responses to IL-23 are becoming clearer. Wang *et al.* (86) described reduced MAIT cell accumulation

in the lungs of IL-23–knockout mice infected with *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*), which suggests a protective role for IL-23 in the context of microbial challenge. Both IL-12 and IL-23 promote the expansion of MAIT1 cells in an in vivo model of acute tularemia (56), whereas IL-23 costimulation together with administration of 5-OP-RU promotes the expansion of MAIT17 cells in vivo and skews human MAIT cell responses toward those associated with the T_H17-like phenotype in vitro (86). Similarly, in patients with primary Sjögren’s syndrome (pSS), MAIT cells are depleted in the blood, and those that remain exhibit a reduced capacity to produce the T_H1 cell–associated cytokines IFN- γ and TNF α (87). In vitro, these cells exhibit increased sensitivity to IL-23 and IL-7, which also stimulate polarization toward a T_H17 cell–like phenotype through overexpression of ROR γ c or by a signal transducers and activators of transcription 3–hypoxia-inducible factor 1- α signaling axis, respectively (88). Conversely, IL-23 licenses a regulatory phenotype in human MAIT cells in vitro, inducing substantial production of IL-10 when it is present as a secondary stimulus with TCR engagement, a finding that the authors hypothesized might be important in the context of commensal antigen recognition in the absence of a proinflammatory cue from IL-18 (85).

MAIT cells express high amounts of the IL-7R, and similarly to IL-23, costimulation with IL-7 induces a T_H17 cell–like phenotype in MAIT cells. Intrasinusoidal MAIT cells are licensed to respond robustly to CD3 and CD28 stimulation through IL-7 costimulation, which increases the production of IL-17A and IFN- γ (32). Furthermore, stimulation with IL-7 promotes the proliferation of liver-derived MAIT cells in vitro (65). IL-7 is secreted by hepatocytes in response to Toll-like receptor (TLR) signaling (89), a finding suggestive of the physiological importance of signal 3 in potentiating MAIT cell effector responses to pathogenic challenge. Similarly, in patients with ankylosing spondylitis (AS), MAIT cells are enriched in the synovial fluid and exhibit an IL-17–dominant phenotype (90). In vitro, MAIT cells from patients with AS produce increased amounts of IL-17 when primed with IL-7 before TCR stimulation, which is concomitant with increased amounts of IL-7 in the guts and joints of patients with AS (90). Stimulation with IL-7 alone is sufficient to induce cytotoxic capacity in human MAIT cells in vitro, licensing the increased production of granzyme B, perforin, and granzyme A, and priming with IL-7 improves MAIT cell sensitivity to antigenic challenge (91).

MAIT cell responses to type I IFNs have been elucidated. Previously, it was shown that MAIT cells are functionally responsive to IFN- α/β in vitro only when the IFNs are co-administered with IL-12 or IL-18 (4). Similarly, Pavlovic *et al.* (92) showed that in response to IFN- α signaling alone, MAIT cells increase the cell surface abundance of the activation marker CD69 but fail to respond functionally, suggesting a preventative mechanism against hyperactivation. Furthermore, IFN- α synergizes with TCR stimulation to drive robust cytokine production by MAIT cells in vitro and to elicit cytotoxic capacity as defined by the increased expression of perforin and granzyme B (4, 92). In mice, MAIT cell responses to *Klebsiella pneumoniae*—which include activation, tissue localization, and T_H1 cell–like transcriptional reprogramming—occur independently of MR1 and are instead stimulated by type I IFNs (3). This finding was further recapitulated in vitro, whereby the coculture of human MAIT cells from peripheral blood mononuclear cells with *K. pneumoniae* induced a type I IFN–associated gene signature, as characterized by increased expression of IFN-stimulated genes (ISGs), such as *MX1* and *IFIT1*. This type I IFN–MAIT cell axis is protective against infection with

K. pneumoniae in mice (3), once more highlighting the physiological importance of MAIT cell responses to type I IFNs. Amini *et al.* (93) reported on MAIT cell responses to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike peptide challenge in individuals vaccinated with the BNT162b2 vaccine against SARS-CoV-2, which is mediated by memory T cell production of another interferon family member, IFN- γ , eliciting cytokine responses from MAIT cells including the production of IFN- γ .

Other cytokines that can promote MAIT cell effector responses include IL-15 and IL-2. Both IL-15 and IL-2 induce the expansion of MAIT1 cells *in vivo*, as characterized by the increased expression of Tbet (94). Similarly, human MAIT cells proliferate *in vitro* upon stimulation with IL-15 alone or when it is administered together with a TCR stimulus (65, 95), whereas IL-2 can also act as an environmental signal that potentiates antigen responses to facilitate the robust expansion of MAIT cells *in vitro* (25). IL-15 alone, but not IL-2, is also sufficient to induce a robust T_H1 cell-like cytokine expression profile in liver-derived MAIT cells (65), and it acts in synergy with either IL-12 or IL-18 to elicit substantial IFN- γ production by peripheral blood MAIT cells *in vitro* (4). Furthermore, the stimulation of liver-derived MAIT cells with IL-15 drives robust cytotoxicity *in vitro*, independently of MR1 (65), and IL-15 is thought to act in synergy with IL-12 and IL-18 to promote MAIT cell host-protective responses against influenza A virus infection *in vivo* (5).

In addition, a role for the tissue-derived alarmin IL-33 has been described in the context of driving MAIT cell functional responses. IL-33 is a member of the IL-1 family of cytokines (96) and is constitutively expressed in epithelial barrier tissues (97). IL-33 acts as an alarmin in that it is released during instances of tissue damage or cellular stress caused by infection, for example. Azzout *et al.* (98) described an inflammatory response elicited by MAIT cells upon stimulation with a combination of IL-33 and IL-12, as confirmed by the increased production of IFN- γ , TNF α , and granzyme B. Furthermore, the activation of MAIT cells after liver transplant significantly correlates with serum amounts of IL-33 (99). However, MAIT cell activation further correlates with the severity of liver dysfunction after graft reperfusion, which indicates a potentially deleterious role for the MAIT cell-IL-33 axis during sterile inflammation (99), further highlighting the complexity of MAIT cell responses to environmental stimuli.

ENVIRONMENTAL SIGNAL 4: CHEMOKINES

Although not typically considered as signals required for T cell activation, chemokines can substantially modulate cellular immune responses in specific microenvironments by directing the migration of cells to sites of inflammation in a process referred to as “homing” (Fig. 3). MAIT cells are abundant in the periphery and are enriched across a wide range of human tissues. Moreover, MAIT cells are actively recruited to sites of infection and inflammation (18, 100). Accordingly, MAIT cells express a wide repertoire of chemokine receptors, including, but not limited to, CCR2, CCR5, CCR6, and CXCR6 (18, 101), facilitating their homing to sites of inflammation. MAIT cells are most abundant in the liver, and Dusseaux *et al.* (11) first reported a chemokine expression profile (CCR5, CCR6, CCR9, and CXCR6) that supports preferential homing to both liver and gut tissues. MAIT cells also migrate to sites of infection (102–104). In a human challenge with *S. enterica* serovar Typhi, MAIT cell frequencies decline in the periphery and exhibit an increased expression of CCR6 and

CCR9 in individuals susceptible to infection, suggesting migration to the site of infection (105). In a model of *F. tularensis*, MAIT cells accumulate in the infected lung and express high amounts of CXCR6. Although CXCR6 is dispensable for MAIT cell recruitment, its cognate ligand CXCL16 is sufficient to drive recruitment to the lung, even in the absence of infection (106).

In patients with mucosal-associated cancers, MAIT cells accumulate in the tumor and express substantial amounts of CCR6, CCR9, and CXCR6 (107, 108), chemokine receptors linked to increased migration to gut tissue, which was further supported by the increased amounts of the ligands CCL20 and CXCL16 in these mucosal tumors (108). Zheng *et al.* (109) investigated the chemokine axes responsible for MAIT cell recruitment to the inflamed appendix and reported substantial expression of CCR2, CCR4, CCR5, CCR6, and CXCR6 on peripheral blood MAIT cells in healthy controls and in individuals with an inflamed appendix. In addition, the authors reported the expression of CCR1, CCR3, and CXCR1 on appendix-infiltrating MAIT cells. Note that they demonstrated that the accumulation of MAIT cells in patient-derived appendix organoids depends on CCR1, CCR2, and CCR4 (109). In the setting of another inflammatory disease, hidradenitis suppurativa, we reported an accumulation of MAIT cells in the lesions, together with increased amounts of CCL20, which promotes MAIT cell migration (110). In patients with pSS, MAIT cells accumulate in the salivary glands (111) and exhibit increased amounts of CCR9 and CXCR5, which are thought to drive migration toward increased amounts of the ligands CCL25 and CXCL13 (112). However, the chemokine-mediated promotion of MAIT cell trafficking is not limited to inflammatory microenvironments. In the setting of pregnancy, MAIT cells are enriched in the intervillous blood of the placenta (113). The authors of that study highlighted MIF and CCL25 as recruiting factors, with supporting expression of CXCR4 and *in vitro* migration assays (113).

Although the chemokine–chemokine receptor axis has a long-established role in mediating the homing of MAIT cells toward peripheral tissues or toward inflammatory sites during infection, it is not yet known whether chemokines, or their receptors, affect MAIT cell activation. For example, downstream of antigen-presenting cell (APC)–derived chemokine signaling in T cells, the chemokine receptors CCR5 and CXCR4 translocate to the immunological synapse, where they function in a costimulatory manner to enhance T cell activation (114). Whether this is relevant in the context of MAIT cell biology remains to be determined and could perhaps be an interesting angle to interrogate in the context of elucidating the environmental regulators of MAIT cell effector responses.

ENVIRONMENTAL SIGNAL 5: NUTRIENTS

Nutrients are a relatively new addition to the environmental regulation list. Cellular metabolism is now recognized as a critical process that supports and controls immune cell function (115). Cellular metabolism supports adenosine 5'-triphosphate (ATP) production, macromolecule synthesis, intracellular signaling, and posttranslational modifications, all of which are essential for immune cell responses. Immune cells can use different metabolic pathways depending on their functional needs (116). Classically, naïve T cells are quiescent and rely on oxidative phosphorylation (OXPHOS) for their maintenance, whereas activated T cells that are highly proliferative rely on glycolytic metabolism (117).

Cellular metabolism is fine-tuned by optimal nutrient availability in the microenvironment. To maintain cellular metabolism, cells

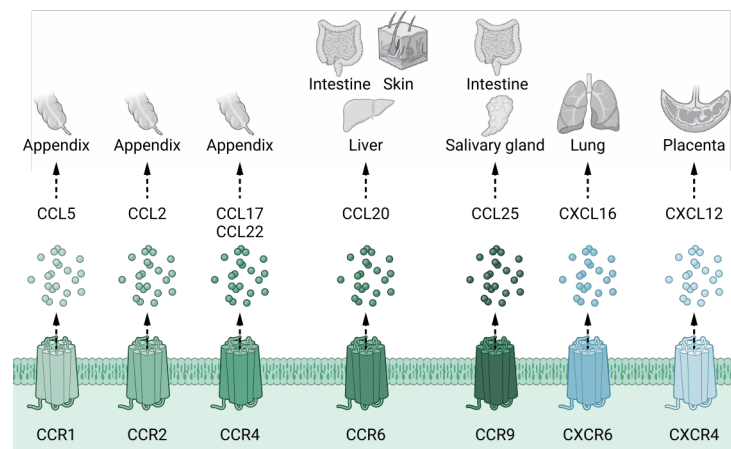


Fig. 3. Chemokines provide signal 4, directing MAIT cell responses. Overview of chemokine responses in MAIT cells. MAIT cells express multiple chemokine receptors both constitutively and after stimulation and in a tissue-specific manner. Upon sensing chemokines (termed signal 4) in their environment, MAIT cells expressing the cognate chemokine receptors promote MAIT cell homing toward the site of chemokine accumulation.

require an exogenous nutrient supply, which can exert a substantial influence on cellular responses (118). Highlighting the importance of exogenous nutrients, two major regulators of MAIT cell metabolic and functional responses are the nutrient sensor mTORC1 and the nutrient-sensitive transcription factor MYC. MYC is well established as a master regulator of metabolism in conventional T cells, controlling the expression of genes encoding nutrient transporters and regulators of metabolic pathways (119, 120). In the absence of either mTORC1 or MYC, MAIT cells fail to engage their metabolic program and ultimately their effector functions. Both mTORC1 and MYC are highly sensitive to nutrient availability in the cellular microenvironment.

The cellular metabolic pathways used by MAIT cells have been an intense area of research for the past several years (Fig. 4) (121–123). After challenge, MAIT cells rapidly respond, growing, proliferating, and producing new effector molecules. These responses are underpinned by the cellular metabolic program, which requires a substantial supply of nutrients. Glucose was the first nutrient to be highlighted as a critical determinant of MAIT cell functionality. In 2018, Zinser *et al.* (121) reported that glycolysis is required for granzyme B production by MAIT cells. Shortly thereafter, we highlighted the importance of glycolytic metabolism for MAIT cell production of IFN- γ (122) and demonstrated that this process is fueled by the uptake of exogenous glucose from the environment. Limiting glucose availability substantially blunts MAIT cell production of IFN- γ . Similarly, blocking the first step of glycolysis with 2-deoxy-D-glucose also limits MAIT cell IFN- γ production (122). Murine MAIT cell subsets display diverging metabolic profiles, although the IFN- γ -producing MAIT1 cell subset is dependent on glycolysis and the uptake of glucose, similarly to their human counterparts (124, 125). In addition, MAIT cells use glycogen synthase 1 (GYS1), the enzyme that converts glucose into glycogen through glycogenesis. Accordingly, MAIT cells store glycogen and rely on glycogen-fueled glycolysis to fuel their rapid functional responses until the cell can access a sufficient supply of glucose from the

environment (21). Given the importance of glycolysis for MAIT cell effector function, it is very likely that environments with a limited supply of glucose, characteristic of some tumor microenvironments (TMEs), might skew the MAIT cell metabolic profile and consequently alter MAIT cell functional responses.

In humans, IL-17-producing MAIT cells are rare but are often expanded in diseased settings (126). Obesity is associated with chronic, low-grade inflammation, which drives impaired insulin signaling and hyperglycemia. MAIT cells are dysfunctional in people with obesity, as characterized by diminished glycolytic metabolism, a limited effector capacity, and increased secretion of IL-17. A high-glucose environment increases mitochondrial reactive oxygen species (ROS) production by murine T cells, subsequently resulting in ROS-mediated T_H17 cell differentiation (127). The authors of that study suggested that high concentrations of glucose might increase mitochondrial ROS production by inducing cellular oxidative stress (127). Similarly, we showed that MAIT cells from people with obesity have increased amounts of mitochondrial ROS, which is linked with increased secretion of IL-17 (123). Note that the increased frequencies of IL-17-producing MAIT cells in children with obesity positively correlate with the occurrence of insulin resistance, and in vitro experiments confirmed that MAIT cell-derived IL-17 disrupts insulin-mediated glucose uptake (128). This suggests that although MAIT cells are likely affected by altered nutrient content in the obese microenvironment, they also contribute to the overall inflammatory burden and may have a role in further potentiating the disturbances to systemic metabolism that are characteristic of the disease.

Iron is an essential micronutrient with an important role in physiological processes, such as oxygen transport, energy production, and immune regulation (129). We previously highlighted iron as a critical regulator of MAIT cell host-protective responses. Upon activation, human peripheral MAIT cells increase their cell surface expression of the iron transporter CD71, which correlates with increased iron uptake (130). Limiting the availability of iron in the microenvironment or conversely blocking iron uptake with a monoclonal antibody against CD71 reduces metabolic capacity and cellular ATP production. Iron-starved MAIT cells consequently fail to proliferate and they exhibit a reduced cytokine expression profile (130). Once more, these data highlight the importance of optimal nutrient availability as “signal 5” in the potentiation of MAIT cell host-protective responses. Serum iron concentrations are controlled by the liver-derived hormone hepcidin, which reduces dietary iron absorption from the small intestine and attenuates the mobilization of recycled iron from macrophages and from the iron exporter ferroportin (131). The inflammatory cytokine IL-6 is thought to drive increased production of hepcidin during microbial and viral challenge as a mechanism of host protection (132), although low-grade inflammation is also thought to promote the pathogenesis of chronic hypoferrremia in obesity (133). It is thus plausible that the dysregulation of MAIT

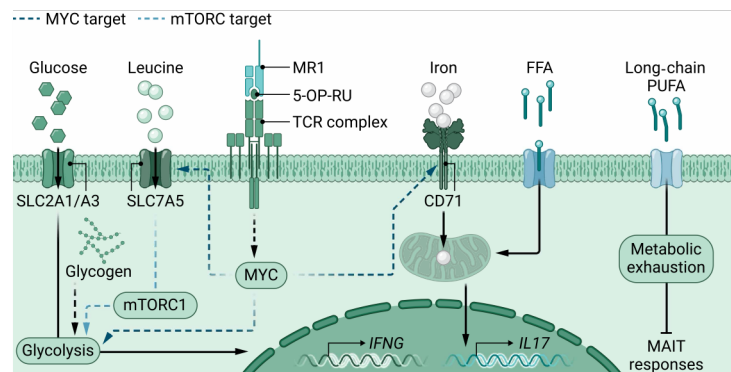


Fig. 4. Nutrients as signal 5 for MAIT cells. At rest, MAIT cells express low amounts of nutrient transporters on their surface; however, upon TCR stimulation with the cognate MAIT cell antigen 5-OP-RU, the metabolic regulator MYC is activated. Activation of MYC leads to the increased expression of nutrient transporters on the cell surface, including glucose transporters such as SLC2A1, the LNA transporter SLC7A5, and the iron transporter CD71. Nutrients are sensed by mTOR (termed signal 5), leading to metabolic reprogramming, which supports MAIT cell responses through a glucose-mTOR-glycolysis-IFN- γ signaling axis. Conversely, nutrients associated with disease settings can suppress MAIT cell responses through metabolic exhaustion, as is the case for long-chain fatty acids, for example.

cell responses during infection or in chronic inflammatory conditions such as obesity may in part be driven by disruptions to physiologic iron homeostasis.

In addition to iron deficiency, iron overload has also been linked with aberrant immune functioning and leads to an increased risk of infection and associated mortality (129). Iron overload impairs the differentiation of T_H1 cells and associated IFN- γ production in mice infected with *Candida albicans* (134) and in a murine model of *S. Typhimurium* (135) infection. The observed impairments to T_H1 cell differentiation induced by iron excess in *S. Typhimurium* infection are linked with the iron-mediated increase in expression of TIM-3 on the cell surface (135). Furthermore, in patients with HFE-related hemochromatosis, $V\delta 2^+$ T cell frequencies in the blood are significantly reduced, and those that remain exhibit increased expression of exhaustion markers and production of ROS (136). The authors suggest that iron overload leads to constitutive stimulation of the $V\delta 2$ TCR, which subsequently results in activation-induced cell death (136). However, it is not yet known whether iron overload has an effect on MAIT cells or their host-protective responses in infection.

The metabolism of intracellular amino acids is important for tricarboxylic acid (TCA) cycle processing and for other cellular anabolic processes, such as protein synthesis. Furthermore, the regulation of mTORC1 activity is determined by the availability of amino acids, such as leucine, glutamine, and arginine (137, 138). Cellular uptake of large neutral amino acids (LNAs), such as leucine and tryptophan, is controlled in activated T cells by the cell surface expression of the system L amino acid transporter SLC7A5 and is required for metabolic reprogramming and effector cell differentiation in response to antigenic challenge (139). We previously showed that MAIT cells activated by CD3/CD28 costimulation with a combination of IL-12 and IL-18 increase their cell surface expression of SLC7A5, whereas the associated amino acid flux is a critical determinant of mTORC1 activation and MAIT cell proliferative capacity. (25, 122) This pathway is defective in MAIT cells isolated from people with obesity, which likely

contributes to the associated metabolic and functional dysregulation (122). Furthermore, the glutamate-derived metabolite α -ketoglutarate is an important TCA cycle intermediate (115) and enhances MAIT cell effector responses to TCR stimulation in vitro (140), suggesting that the sensing and uptake of microenvironmental glutamine is important in the context of potentiating MAIT cell effector responses.

Although IL-17-producing MAIT cells represent a functional state in humans (68), MAIT17 cells are a distinct subset in mice and display a divergent metabolic program that is characterized by an increased dependency on mitochondrial metabolism (OXPHOS) (125). This metabolic program is thought to be fueled by exogenous fatty acids, whereby MAIT17 cells exhibit increased lipid uptake and intracellular lipid content after challenge (125). However, it seems that not all lipids are preferred fuels for MAIT cells, especially in humans. Deschler *et al.* (75) demon-

strated that fatty acids found in the liver microenvironment of patients with metabolic dysfunction-associated steatotic liver disease (MASLD) accumulate in MAIT cells and induce both oxidative stress and the formation of intracellular lipid peroxisomes, which ultimately results in metabolic exhaustion and MAIT cell dysfunction. Human MAIT cells exhibit potent antitumor cytotoxicity against HCC cell lines in vitro, but this is abrogated in the presence of MASLD-associated polyunsaturated fatty acids (PUFAs). Similarly, MAIT cells from patients with nonsteatotic HCC exhibit increased expression of gene signatures associated with PUFA-induced MAIT cell dysfunction, which is associated with poor survival. These findings further emphasize how environmental nutrient content can influence the functional plasticity of MAIT cell responses in diseased settings, such as cancer, and highlight the potential for targeting this axis to improve immune function and disease prognosis in such contexts.

CONCLUSIONS AND OPEN QUESTIONS

MAIT cells are garnering worthy attention for their critical roles in host protection and chronic disease and because of their potential therapeutic advantages. Over the past decade, about 1000 publications focused on MAIT cells have been indexed in PubMed, vastly increasing our understanding of the biology of MAIT cells and their role in health and disease. However, when compared with conventional $CD8^+$ T cells (for which there were >35,000 publications in the same period) or other unconventional T cell subsets (for example, invariant NKT cells with >10,000 publications) our understanding of MAIT cell biology is still in its infancy. In this Review, we summarized the signals that control MAIT cell responses, from antigenic stimulation mediated by their restricted TCR to the plethora of cytokines that can potentiate their functions. The identification of an endogenous antigen for MAIT cells (48) will likely lead to the identification of further examples. For example, in the setting of cancer, infiltrating MAIT cells have the hallmarks of microbial activation

(47), but increased MR1 expression in some tumors may indicate the presence of cancer-specific antigens, which could advance the potential for MAIT cell immunotherapy.

We also added nutrients as important regulatory factors for MAIT cell responses. A large body of evidence has emerged over the past decade to demonstrate that altered nutrient availability in the TME substantially limits the antitumor responses of conventional CD8⁺ T cells (141–145). It is well established that tumors are metabolically very active (146) and display dysregulated uptake of glucose and amino acids from their environment (145–148). The importance of glucose and amino acid availability for MAIT cell functionality is rapidly emerging (122, 125), as is the deleterious effect of altered metabolism on MAIT cell responses (25, 75, 122). However, a comprehensive understanding of the complete nutritional requirements that support MAIT cell activation and their capacity to respond functionally remains to be elucidated. For example, glutamine is the most abundant amino acid and plays a critical role in modulating the effector responses of other innate immune cell populations, such as NK cells, for which it acts as both a signal and a fuel (149). However, whether MAIT cells need or use glutamine remains unknown. Today, one of the most exciting aspects of MAIT cell biology is these cells' proposed role in tissue repair and homeostasis (150); however, the nutritional underpinnings of these functions are not yet described, which will be important to achieve if we are to harness the full potential of MAIT cells in inflammatory or hostile environments.

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Environmental regulation of mucosal-associated invariant T cells: Adding food for thought

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9.2. Paper II PDF

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 that play a critical role in host protection against bacterial and viral pathogens. Upon activation, MAIT cells can rapidly respond via both TCR-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 that activate MAIT cells. However, iron has not been investigated with respect to MAIT cell metabolic or functional responses. In this study, we show that human MAIT cells require exogenous iron, transported via CD71 for optimal metabolic activity in MAIT cells, 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 human MAIT cell metabolism and functionality, an axis that may have implications in conditions where iron availability is limited. *The Journal of Immunology*, 2024, 212: 1706–1713.

Mucosal-Associated Invariant T (MAIT) cells are a population of non-MHC-restricted T cells that are important in the immune defense against bacterial and viral infections (1–5). MAIT cells are rapidly responding T cells that are capable of producing multiple cytokines upon activation, such as IFN- γ , TNF, and IL-17 (1, 6). MAIT cells are activated when their invariant TCR recognizes bacterial riboflavin derivatives presented on the MHC-like molecule MR1 (5, 6). In contrast, they can also be activated in a TCR-independent manner, via stimulation with cytokines such as IL-18 (7, 8). Recently, several studies have highlighted the importance of metabolism for MAIT cell functional responses (9). Our group and others have demonstrated that MAIT cells are reliant on glycolysis to support their production of IFN- γ and granzyme B (10–12), whereas IL-17 production by MAIT cells has been linked to mitochondrial metabolism (13, 14). However, our knowledge of the nutritional requirements of MAIT cells remains limited.

Iron is an essential trace element for all multicellular organisms and is critical for a range of physiological processes, including

oxygen transport and energy production (15). Iron is also critical for the majority of microorganisms, and successful iron sequestration is required to establish infection (16). Iron availability is regulated by the liver-derived hormone hepcidin. Increased hepcidin production occurs in response to infection and inflammation, and results in reduced iron availability, a mechanism of host protection (17, 18). However, previous studies have demonstrated that conventional T cell responses are negatively impacted by low iron levels, with reduced proliferation and effector functions (19, 20). Whether MAIT cells require iron for their metabolic processes and effector responses is currently unknown.

In this study, we demonstrate that MAIT cells upregulate their expression of the transferrin receptor, CD71, upon activation, correlating with a significant increase in the uptake of transferrin-bound iron. In silico analysis of the MAIT cell proteome reveals that MAIT cells increase their overall iron content upon activation. We demonstrate that MAIT cell metabolism is limited, and the metabolic profile altered, under iron-deplete conditions, and this is associated with a robust reduction in ATP levels. Finally, we

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Abbreviations used in this article: DFO, deferoxamine; FCCP, carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone; HPLM, human plasma like media; MAIT, Mucosal-Associated Invariant T; MFI, mean fluorescence intensity.

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demonstrate that extracellular iron restriction impairs MAIT cell functional responses, leading to a reduced proliferative capacity and diminished cytokine production. Collectively, our data pinpoint MAIT cells as another player in the iron tug-of-war between pathogenic invaders and host immunity.

Materials and Methods

Study cohorts and ethical approval

A total cohort of 30 healthy adult donors was recruited. Inclusion criteria included ability to give informed consent, 18–65 y of age, body mass index <28, and no current or recent (<2 wk) infection. Ethical approval was obtained from both St Vincent's University Medical Ethics Committee and Mayo University Ethics Committee.

Preparation of PBMCs and flow cytometric analysis

PBMC samples were isolated, using SepMate isolation tubes, by density centrifugation over Lymphoprep (both STEMCELL Technologies), from fresh human peripheral blood samples. Cell viability was determined using eBioscience Fixable Viability Dye (eFluor506), and MAIT cells were phenotyped using specific extracellular mAbs (Miltenyi Biotec and BioLegend), namely, CD3, CD161, TCR α 7.2, and CD71. Where appropriate, cells were fixed and permeabilized according to manufacturer guidelines, using the True-Nuclear Transcription Factor Buffer set (BioLegend). Cell populations were identified using an Attune NXT flow cytometer and analyzed using FlowJo version 10.8.2 (TreeStar). Results are expressed either as a percentage of the parent population as indicated and determined using flow minus one and unstained controls, or as the mean fluorescence intensity (MFI) of the relevant population.

MAIT cell transferrin uptake assay

PBMCs (2×10^6 /ml) were activated using CD3/CD28 TCR Dynabeads (Life Technologies) and IL-18 (50 ng/ml) or cytokine alone (IL-12/IL-18, both 50 ng/ml) for 18 h as indicated. Cells were rested in serum-free human plasma like media (HPLM) with 5% BSA for 2 h. Cells were then washed in serum-free HPLM with 0.5% BSA and incubated with 5 μ g/ml Transferrin-AlexaFluor647 (Invitrogen) for 10 min at 37°C. Holo-transferrin (500 μ g/ml; Sigma-Aldrich) was used to competitively control for transferrin uptake. Cells were washed in ice-cold HPLM with 0.5% BSA to stop membrane trafficking. Cells were then stained for viability, and MAIT cells or conventional T cells were labeled for extracellular markers, to be analyzed by flow cytometry.

In silico proteomic analysis

A publicly available proteomic dataset of MAIT cells was downloaded from PRIDE accession number PXD041544 (<https://www.ebi.ac.uk/pride/archive/projects/PXD041544>). A list of human iron-interacting proteins was provided by Andreini et al. (21). Using the protocol from Teh et al. (22), the list of human iron-interacting proteins was compared and aligned against the complete list of proteins detected in the human MAIT cell proteomic dataset. Matches were extracted and listed in Supplemental Table I. To estimate the iron atom counts per protein species, we multiplied the copy number value of each iron-interacting protein by the iron atom counts per protein. If available, iron atom counts per protein were obtained using the UniProt database cofactor information for each protein. Where iron counts were not available, estimates of iron usage per protein species were assumed to be 1 atom for heme and iron-interacting proteins and 2 atoms for FE-S cluster-interacting proteins. The total number of iron atoms required per cell was calculated as the sum of iron atoms required by each protein species.

MAIT cell SCENITH assay

Fresh PBMCs (2×10^6 /ml) were activated using CD3/CD28 TCR Dynabeads and IL-18 (50 ng/ml) for 18 h. Cells were seeded into a 96-well plate, and treated as a control, or with 2-deoxy-D-glucose (100 mM), oligomycin (1 μ M), or both. After incubation at 37°C for 15 min, cells were treated with puromycin (11 μ M) and incubated for a further 25 min. Cells were washed with ice-cold PBS to stop puromycin incorporation. Cells were then stained for viability. MAIT cells were stained for extracellular markers and fixed, as outlined earlier. Staining of puromycin was achieved using anti-puromycin mAb (AlexaFluor488; Sigma) in permeabilization buffer (BioLegend).

MAIT cell Seahorse assay

Purified MAIT cells (IL-2 expanded) were activated using CD3/CD28 TCR Dynabeads and IL-18 (50 ng/ml) for 18 h in the absence or presence of deferrioxamine (DFO; 200 μ M; Sigma), and metabolic analysis was carried

out using the Seahorse Extracellular Flux Analyzer XFe96 (Agilent). MAIT cells were resuspended in phenol red-free RPMI media containing 10 mM glucose, 2 mM glutamine, and 1 mM pyruvate (Agilent) and plated onto a Cell-Tak (Corning)-coated microplate for adhesion. Respiratory parameters (mitochondrial and glycolytic) were measured using oxygen consumption rate (pmol/min) and extracellular acidification rate (mpH/min), respectively, using injections of oligomycin (1 μ M), carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) (1 μ M), rotenone and antimycin A (both 1 μ M), and monensin (20 μ M) (all Sigma). Metabolic parameters were calculated as per well-established protocols (23, 24).

MAIT cell mitochondrial analysis

Fresh PBMCs (2×10^6 /ml) were activated using CD3/CD28 TCR Dynabeads and IL-18 (50 ng/ml) for 18 h in the absence or presence of DFO (200 μ M; Sigma). Cells were seeded into a 96-well plate and washed in serum-free buffer. Cells were then stained for viability as outlined earlier, and MAIT cells were stained for extracellular markers. Cells were then washed and stained with MitoTracker Deep Red FM (50 μ M; ThermoFisher Scientific) and MitoTracker Green (50 μ M; ThermoFisher Scientific) in PBS and incubated for 1 h at 37°C. Cells were subsequently analyzed by flow cytometric analysis.

MAIT cell ATP assay

IL-2 expanded MAIT cells (1×10^6 /ml) were stimulated using CD3/CD28 TCR Dynabeads and IL-18 (50 ng/ml) for 18 h. ATP levels were measured using a luminescence ATP assay kit (Abcam). Reagents in the kit were reconstituted as per manufacturer's instructions. A standard curve was also prepared as per the kit's instructions. MAIT cells were harvested and washed with PBS. A total of 100 μ l of resuspended MAIT cells was added to a black-walled, clear-bottomed plate. A total of 50 μ l of detergent was added to each well, and the plate was placed on an orbital shaker for 5 min at 600–700 rpm. A total of 50 μ l of substrate solution was added, and the plate was returned to the orbital shaker for 5 min at 600–700 rpm. The plate was then covered and placed in the dark for 10 min before luminescence was measured on a multimode plate reader (CLARIOstar).

MAIT cell functional analysis

IL-2 expanded MAIT cells were activated using CD3/CD28 TCR Dynabeads and IL-18 (50 ng/ml) for 18 h in the absence or presence of DFO (100 μ M) or anti-CD71 mAb (20 μ g/ml). An appropriate IgG isotype control was used in blocking experiments. After 18 h, culture supernatants were assessed for IFN- γ , IL-17, or IL-26 levels using ELISA.

MAIT cell Escherichia coli stimulation assay

Freshly isolated MAIT cells were incubated with 20 μ g/ml anti-CD71 mAb (Invitrogen) for 1 h. Meanwhile, THP-1 cells (ATCC TIB-202) were pre-pulsed for 1 h with fixed *E. coli* (DH5 α) at a multiplicity of infection of 100. At the end of an hour, representative THP-1 cells with and without *E. coli* were counted and titrated to match the number of MAIT cells at a 1:1 ratio, and MAIT cells were cocultured with THP-1 overnight. Four hours before staining for flow cytometry, cells were centrifuged and resuspended in media containing brefeldin A (Invitrogen). Intracellular cytokine (IFN- γ and granzyme B) levels were assessed using flow cytometry (Cytex Aurora).

MAIT cell proliferation analysis

Fresh PBMCs (1×10^6 /ml) were stimulated for 24 h with 5 μ g/ml 5-Amino-6-(D-ribitylamino)uracil and 100 μ M methylglyoxal, in the absence or presence of DFO (200 μ M) and FeSO $_4 \cdot 7$ H $_2$ O (200 μ M; Sigma). After 24 h, culture media were replaced with fresh culture media containing IL-2 (6.8 ng/ml). After 48 h, culture media were replaced with fresh culture media containing IL-2 (34 ng/ml). On day 5, absolute cell numbers were determined using flow cytometric analysis of MAIT cell frequencies and total cell counts.

Statistics

Statistical analysis was completed using GraphPad Prism 6 Software. Data are expressed as SEM. Distribution was assessed using Shapiro–Wilk test. We determined differences between two groups using Student *t* test (paired or unpaired) or Wilcoxon signed-rank test where appropriate. Analysis across three or more groups was performed using ANOVA with multiple measures. Correlations were determined using linear regression models and expressed using Pearson or Spearman rank correlation coefficient, as appropriate. The *p* values were expressed with significance set at <0.05.

Results

MAIT cells increase their expression of the transferrin transporter CD71 upon activation

First, we demonstrate that MAIT cells from PBMCs increase transferrin receptor (CD71) surface expression in response to TCR/IL-18 stimulation, but not cytokine alone (Fig. 1A–D, Supplemental Fig. 1). We then compared CD71 expression on activated MAIT cells and activated conventional T cells and noted higher CD71 expression on conventional T cells (Fig. 1E). Next, we investigated whether MAIT cells altered their CD71 expression under restricted iron conditions. To do this, we limited iron availability in our culture system using the iron chelator DFO, which did not impact MAIT cell viability at 18 h (Supplemental Fig. 2). Resting MAIT cells do not have high levels of CD71 expression; however, upon iron depletion, both the proportion of MAIT cells that express CD71 and the amount of CD71 expressed were increased, and this was also evident in TCR/IL-18 activated MAIT cells (Fig. 1F–H, Supplemental Fig. 2), suggesting a compensatory mechanism to support iron uptake. Next, we investigated the functionality of CD71, using a transferrin uptake assay; showed increased transferrin uptake (both % of MAIT cells and MFI) by activated MAIT cells; and again noted higher uptake by activated conventional T cells (Fig. 1I–M). Finally, we demonstrated a linear relationship between CD71 expression and transferrin uptake in activated MAIT cells (Fig. 1N).

In silico analysis of the MAIT cell proteome reveals increased iron content upon activation

To assess the iron requirements of MAIT cells, we performed in silico pathway analysis of a previously published proteomics dataset (12). We found that IL-2 expanded MAIT cells express high basal expression of ferritin, the protein responsible for iron storage, which is reduced upon activation (Fig. 2A, 2B). Next, we determined the number of iron-interacting proteins in MAIT cells and identified 135 proteins in total, representative of 2.3% of the total proteome (Fig. 2C, Supplemental Table I). Pathway analysis revealed these proteins were intrinsic to many processes, including a major involvement in cellular metabolism (Fig. 2D). Using a recently published algorithm (22) that determines cellular iron content, based on number of iron-containing proteins multiplied by the number of iron atoms per protein, we determined the predicted iron content from the MAIT cell dataset and showed $\sim 1 \times 10^7$ iron atoms in MAIT cells, increasing significantly upon activation (Fig. 2E). Finally, we found that the iron content of MAIT cells is associated with heme and iron-sulfur clusters (Fig. 2F, 2G).

Iron is a critical cofactor for MAIT cell metabolism

We next investigated whether limiting iron availability in our culture system using the iron chelator DFO impacted MAIT cell metabolism. Using protein translation as functional readout of metabolism, we demonstrate that activated MAIT cells significantly increase protein translation after stimulation (Fig. 3A, 3B), and protein translation is limited when MAIT cells are activated in the presence of DFO (Fig. 3C, 3D, Supplemental Fig. 2). A recently published method, SCENITH (25), demonstrated that the measurement of protein translation, paired with the use of metabolic inhibitors such as 2-deoxyglucose (2DG) and oligomycin, allows for metabolic analysis of cells at a single-cell level. Using the SCENITH approach (Supplemental Fig. 2), we demonstrate that iron-restricted MAIT cells increase their dependency on glucose metabolism (Fig. 3E), and this was paired with a decreased capacity to undergo oxidative-linked metabolic processes (Fig. 3F). With this reduction in oxidative metabolism, we next examined the impact of limiting iron on the mitochondrial phenotype, and we observed modest increases in

both mitochondrial mass (MitoTracker Green) and membrane potential (MitoTracker Deep Red) (Fig. 3G, 3H). To confirm these observations, we next investigated the impact of iron restriction via DFO treatment on MAIT cell metabolism using extracellular flux analysis, and we noted strong inhibition in the rates of oxidative phosphorylation (Fig. 3I, 3J). We also noted reduced rates of glycolysis (Supplemental Fig. 3). Furthermore, when we tested the mitochondrial capacity of MAIT cells using the FCCP treatment, we observed a striking reduction with DFO treatment (Fig. 3K). Finally, we examined the impact of low iron availability, using an mAb to block CD71, on ATP levels in MAIT cells, and we demonstrated that iron restriction limits ATP production (Fig. 3L), a finding supported by our Seahorse analysis (Supplemental Fig. 3).

MAIT cell functional responses require extracellular iron

We next investigated whether MAIT cells require extracellular iron for their functional responses. We assessed the impact of DFO treatment on MAIT cell in PBMC ability to produce IFN- γ via flow cytometry, and we observed a reduction with iron restriction (Fig. 4A). Next, we investigated the impact of DFO on the cytokine responses of isolated MAIT cells and show reductions in IFN- γ , IL-17, and IL-2 levels (Fig. 4B–D). To confirm the impact of low iron availability on MAIT cell IFN- γ production, we switched our approach to block CD71 and again demonstrate significant reductions in IFN- γ levels (Fig. 4E). We next assessed the impact of limiting iron availability on MAIT cell production of the antimicrobial cytokine IL-26, and we demonstrated significantly reduced levels with CD71 blockade (Fig. 4F). We next assessed the impact of iron restriction via CD71 blockade on freshly isolated MAIT cell responses to THP-1 cells infected with *E. coli*, and we demonstrated significant reductions in both IFN- γ and granzyme B production (Fig. 4G, 4H). With the noted impact of iron restriction on MAIT cell metabolism, we sought to assess the impact of long-term iron restriction on MAIT cell proliferative capacity and viability. We first showed that MAIT cell proliferation is inhibited with the addition of the ATP synthase inhibitor oligomycin (Fig. 4I) and then demonstrated that MAIT cells fail to proliferate under low iron conditions, and this was paired with a significant reduction in cell viability, which was significantly improved with the addition of FeSO₄, which bypasses CD71 (Fig. 4J, 4K).

Discussion

MAIT cells are a subset of unconventional T cells capable of rapidly responding to stimulation, producing cytokines and lytic molecules, and proliferating (26). MAIT cells are key mediators of host protection against many bacterial and viral pathogens (2, 3, 27–29). Immune responses are metabolically intense processes, with significant energy demands required to support de novo generation of biosynthetic intermediates (30). The metabolic processes and nutrient requirements that govern MAIT cell effector responses are rapidly emerging but still incomplete (10–13, 31). Iron is an essential microelement and is critical for almost all living organisms, including humans and the majority of microbes (15). Iron plays a critical role in cellular metabolism and energy production, and it is vital for conventional T cell immunity (19, 20). The iron requirements of MAIT cells are unknown. In this study, we show that MAIT cell metabolism and functional responses, namely, cytokine production and proliferation, are governed by extracellular iron availability. We show that activated MAIT cells increase their expression of CD71, the transferrin receptor, and that iron restriction significantly diminishes MAIT cell protein translation and ATP production. Consequently, MAIT cells present with a reduced capacity to proliferate

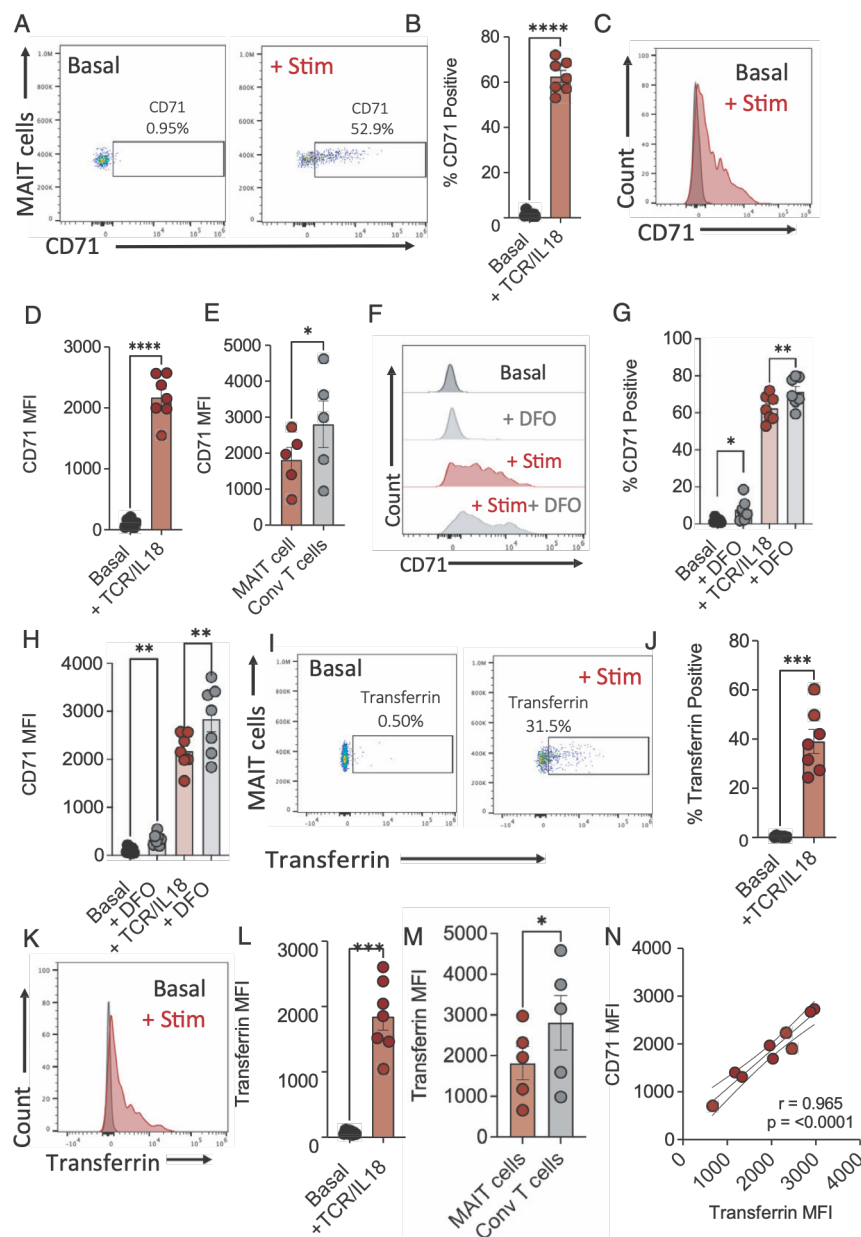


FIGURE 1. MAIT cells increase CD71 and transferrin uptake upon activation. (A–D) Flow cytometric dot plots, scatterplots, and representative flow cytometric histograms showing CD71 expression in MAIT cells, basal or stimulated for 18 h with anti-CD3/CD28 TCR beads and IL-18. (E) Scatterplot comparing CD71 expression on MAIT cells and conventional T cells (non-MAIT CD3⁺ cells) after 18-h stimulation with anti-CD3/CD28 TCR beads and IL-18. (F–H) Flow cytometric dot plots, scatterplots, and representative flow cytometric histograms showing CD71 expression in MAIT cells, basal or stimulated for 18 h with anti-CD3/CD28 TCR beads and IL-18, in the absence or presence of the iron chelator, DFO. (I–L) Flow cytometric dot plots, scatterplots, and representative flow cytometric histograms showing transferrin content in MAIT cells, basal or stimulated for 18 h with anti-CD3/CD28 TCR beads and IL-18. (M) Scatterplot comparing transferrin uptake by MAIT cells and conventional T cells (non-MAIT CD3⁺ cells) after 18-h stimulation with anti-CD3/CD28 TCR beads and IL-18. (N) Correlation plot showing the relationship between CD71 expression and transferrin uptake in activated MAIT cells (18 h with anti-CD3/CD28 TCR beads and IL-18). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

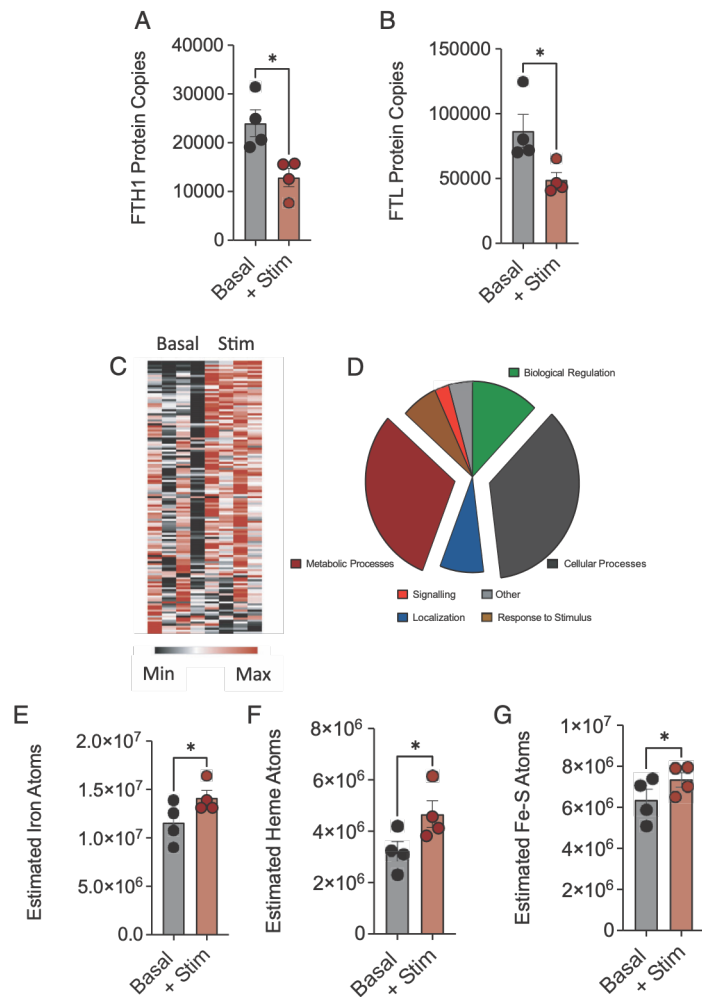


FIGURE 2. MAIT cells store and use iron. **(A and B)** Scatterplot showing protein copy number of the H and L chain subunits of ferritin in IL-2 expanded MAIT cells, basal or stimulated for 18 h with anti-CD3/CD28 TCR beads and IL-18. **(C)** Heatmap of iron-related protein content in IL-2 expanded MAIT cells, basal or stimulated for 18 h with anti-CD3/CD28 TCR beads and IL-18. **(D)** Pie graph showing proportional pathway analysis based on iron-related protein content in MAIT cells (analysis performed with Panther). **(E–G)** Estimated iron, heme, and iron-sulfur cluster atoms extrapolated from proteome of IL-2 expanded MAIT cells, basal or stimulated for 18 h with anti-CD3/CD28 TCR beads and IL-18. **p* < 0.05.

and diminished production of several cytokines central to MAIT cell host protective function.

The majority of bioavailable iron in the circulation is bound to transferrin, which is taken into cells via the transferrin receptor TfR1, also known as CD71 (32). We first examined MAIT cells for the expression of CD71 and found low basal expression but significant increases in expression after stimulation with TCR beads/IL-18, but not IL-12/IL-18, suggesting a TCR dependency; this supports our previous study that highlighted CD71 as an MYC target, which is an important transcription factor upregulated by TCR stimulation in MAIT cells (12). Previous studies have reported similar activation-induced increases in CD71 expression on activated T cells and NK cells (33, 34). Interestingly, a missense mutation in *TFRC*, the gene encoding CD71, resulted in immunodeficiency underpinned by defective T cells (35). In line with increased CD71 expression, we observed increased uptake of transferrin by activated MAIT cells, similar to that reported in both conventional CD4⁺ and CD8⁺ T cells (36). MAIT cells are primarily CD8⁺, and recently it was

demonstrated that CD8 receptor expression was critical for optimal TCR-driven responses in MAIT cells (37). In a recent study by Teh and colleagues (22), they reported greater iron dynamics in CD8⁺ T cells compared with CD4⁺ T cells, which is supported by proteomic data from Howden et al. (33), where they demonstrated that CD8⁺ T cells express more CD71 than CD4⁺ T cells. To investigate the iron dynamics of MAIT cells, we performed in silico analysis of our publicly available proteomic dataset (12). We found that MAIT cells decrease their levels of ferritin, suggesting mobilization of iron stores (38). Using the approach introduced by Teh et al. (22) for determining cellular iron content based on proteomic analysis, we found that MAIT cells contain 135 proteins with iron binding sites and significantly increase their predicted iron content upon activation, similar to that observed in conventional T cell subsets (22).

Pathway analysis of the 135 proteins with iron binding sites present in MAIT cells highlighted cellular metabolism as a major process central to these proteins. Similarly, Teh et al. (22) found that the majority of intracellular iron in T cells was used in oxidative

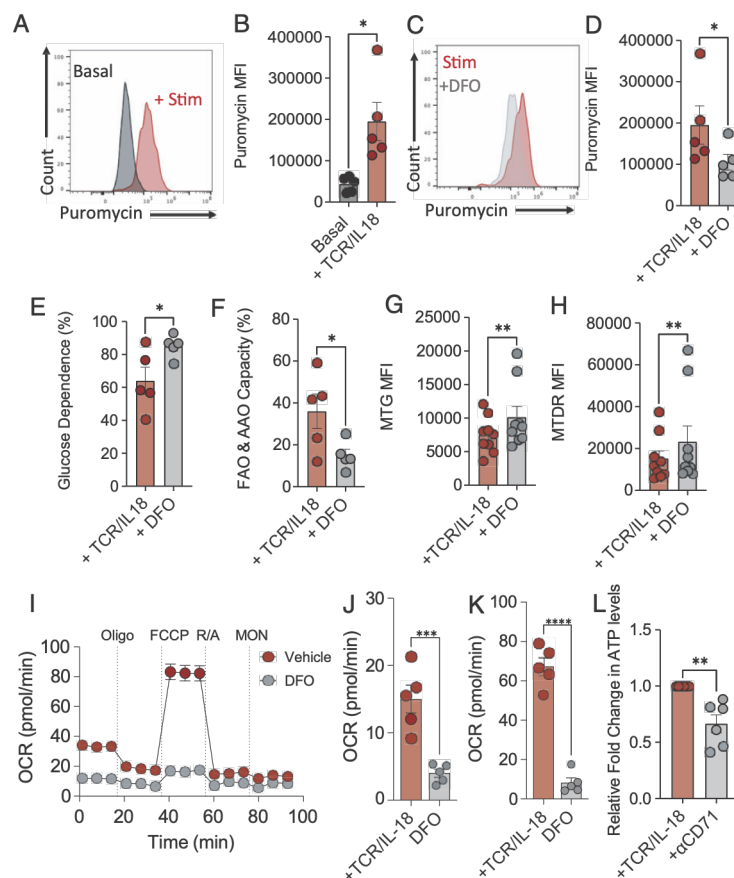
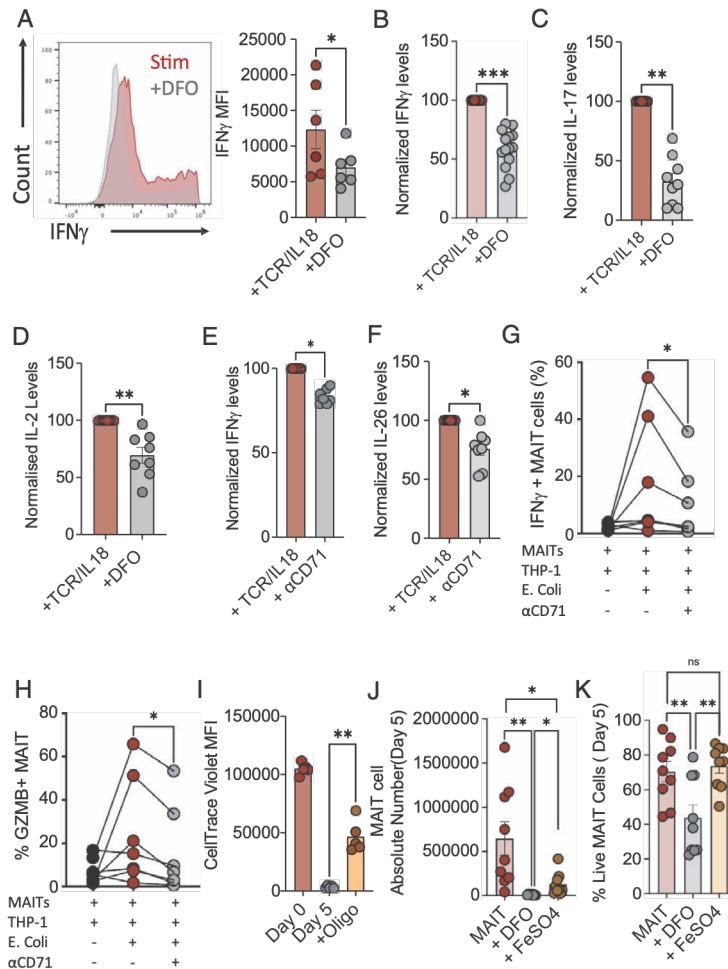


FIGURE 3. Iron restriction alters MAIT cell metabolism. (**A–D**) Representative flow cytometric histograms and scatterplots showing puromycin incorporation in MAIT cells, basal or stimulated for 18 h with anti-CD3/CD28 TCR beads and IL-18, in the absence or presence of the iron chelator, DFO. (**E**) Scatterplot showing percentage dependency on glucose metabolism in MAIT cells stimulated for 18 h with anti-CD3/CD28 TCR beads and IL-18, in the presence or absence of DFO. (**F**) Scatterplot showing percentage dependency on fatty acid oxidation and amino acid oxidation in MAIT cells stimulated for 18 h with anti-CD3/CD28 TCR beads and IL-18, in the presence or absence of DFO. (**G** and **H**) Scatterplots showing the MFI of MitoTracker Green and MitoTracker Deep Red, depicting mitochondrial mass and mitochondrial membrane potential, respectively, in MAIT cells stimulated with anti-CD3/CD28 TCR beads and IL-18 for 18 h, in the presence or absence of DFO. (**I** and **J**) Representative Seahorse trace and scatterplot showing oxygen consumption rates (OCRs) in MAIT cells stimulated with anti-CD3/CD28 TCR beads and IL-18 for 24 h, in the presence or absence of DFO. (**K**) Scatterplot showing the mitochondrial capacity (after FCCP treatment) in MAIT cells stimulated with anti-CD3/CD28 TCR beads and IL-18 for 24 h, in the presence or absence of DFO. (**L**) Scatterplot showing the impact of an anti-CD71 mAb with blocking activity on ATP production in MAIT cells stimulated with anti-CD3/CD28 TCR beads and IL-18 for 18 h. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

phosphorylation. Therefore, we next assessed the impact of altered iron availability on MAIT cell metabolism and functional responses. We used two different approaches to limit MAIT cell access to extracellular iron: (1) we used the iron chelator DFO, which removes iron from ferritin (39); and (2) we used an mAb specific for CD71 with blocking activity (40). To assess the impact of low iron availability on MAIT cell metabolism, we used two different approaches: Seahorse extracellular flux analysis and a recently published method, SCENITH, which monitors rapid changes in protein translation paired with a series of metabolic inhibitors to profile cellular metabolism at a single-cell level (25). Using SCENITH, we found that MAIT cells significantly increase protein translation upon activation. This supports our recent publication where we

demonstrated significant increases in protein content in activated MAIT cells (12). In the presence of DFO, protein translation was reduced in activated MAIT cells, suggesting that iron supports global MAIT cell metabolism. This was confirmed by our Seahorse data, which showed reduced rates of glycolysis and to a greater extent reduced rates of Oxidative Phosphorylation. Similarly, in activated NK cells, treatment with DFO resulted in reduced cell size, indicative of reduced protein content and metabolism (34). Paired with reduced protein translation, limited iron availability also reduced the oxidative capacity of activated MAIT cells, suggesting altered mitochondria, similar to that reported in conventional T cells by Frost et al. (20). In the same study by Frost et al. (20), iron deficiency resulted in reduced ATP production and an

FIGURE 4. MAIT cells require iron for their functional responses. **(A)** Representative flow cytometry histogram and scatterplot showing IFN- γ levels (MFI) in MAIT cells stimulated with anti-CD3/CD28 TCR beads and IL-18 for 18 h, in the absence or presence of the iron chelator, DFO, measured by flow cytometry. **(B–D)** Scatterplots showing IFN- γ , IL-17, and IL-2 secreted protein levels (normalized to assay) in IL-2 expanded MAIT cells stimulated with anti-CD3/CD28 TCR beads and IL-18 for 18 h, in the absence or presence of DFO, as measured by ELISA. **(E and F)** Scatterplots showing the impact of an anti-CD71 mAb with blocking activity on IFN- γ and IL-26 secreted protein levels (normalized to assay) in IL-2 expanded MAIT cells stimulated with anti-CD3/CD28 TCR beads and IL-18 for 18 h, as measured by ELISA. **(G and H)** Line graph showing IFN- γ or granzyme B levels (percentage of parent population) in MAIT cells cocultured at 1:1 ratio with THP-1 cells alone or prepulsed with fixed *E. coli* (DH5 α) for 18 h, in the absence or presence of the anti-CD71 mAb with blocking activity, as measured by flow cytometry. **(I)** Scatterplot showing the impact of the ATP synthase inhibitor, oligomycin, on MAIT cell proliferative capacity (during a 5-d IL-2-mediated MAIT cell expansion culture), whereby the MFI of the intracellular dye CellTrace Violet decreases with each cycle of cell division. **(J and K)** Scatterplots showing the impact of long-term iron depletion via DFO and alternative iron repletion via Iron(II) sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) on MAIT cell proliferation and viability during a 5-d IL-2-mediated MAIT cell expansion culture. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



accumulation of dysregulated mitochondria in CD8⁺ T cells. We observed only modest changes in mitochondrial phenotype, but note the longer time frame in the study by Frost et al. (20) compared with our overnight time point. We did, however, find reduced mitochondrial capacity and ATP levels in MAIT cells activated in the presence of limited iron availability.

Iron is critical for the majority of commensal and pathogenic bacteria, and during infection, in response to inflammation, the host restricts the accessibility of iron as a protective measure. Many pathogenic bacteria synthesize iron chelators called siderophores to scavenge host iron, resulting in an iron tug-of-war (41). One of the major roles for MAIT cells is mediating host protection against bacteria (27, 42–44). Interestingly, several bacteria that elicit MAIT cell responses in vivo, such as *Salmonella typhimurium* (45), *Klebsiella pneumoniae* (46), and *Legionella longbeachae* (47), use siderophores as a virulence factor (48–50). Therefore, we investigated whether MAIT cells need iron for their effector functions, the primary of which is cytokine production. Previously, we had found IFN- γ to be the most abundant cytokine produced by MAIT cells, and in this

study we demonstrate that limiting iron availability limits the levels of IFN- γ secreted in response to either TCR-stimulating beads or THP-1 cells infected with *E. coli*. Similarly, in a model of hepcidin-driven hypoferrremia, Ag-specific CD8 T cells produced less IFN- γ than in control mice, highlighting the importance of iron for conventional T cell responses. IL-26 is a cytokine with direct antimicrobial activity produced by MAIT cells and part of MAIT cell responses to infection in the lung (28). We found that IL-26 production by MAIT cells was also reduced under limited iron conditions. Another key effector function required for MAIT cell host defense is the ability to proliferate (51). We found that under iron restriction, MAIT cells failed to proliferate, likely linking into the reduced metabolism observed. In CD4 T cells, iron was also required for proliferation, and the authors linked this failure to altered mitochondrial metabolism (19), supporting our data, which demonstrated significant reduction in MAIT cell proliferation with ATP synthase inhibition. In summary, our study highlights the importance of iron for MAIT cell metabolic and functional responses and may have implications in conditions where iron availability is limited.

Disclosures

The authors have no financial conflicts of interest.

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