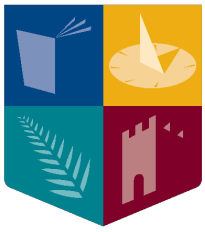
Characterisation of Mucosal Associated Invariant T cells function and metabolism in health and obesity



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B.Sc. (Hons)

Thesis submitted to National University of Ireland Maynooth in fulfilment of the requirements for the degree of Doctor of Philosophy

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Table of contents

List of figures

List of tables

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Publications arising from this thesis

Obesity reduces mTORC1 activity in mucosal-associated invariant T cells, driving defective metabolic and functional responses

Aisling O'Brien*, Roisin M. Loftus*, **Marta M. Pisarska***, Laura M. Tobin, Ronan Bergin, Nicole A.W. Wood, Cathriona Foley, Arimin Mat, Frances C. Tinley, Ciaran Bannan, Gary Sommerville, Natacha Veerapen, Gurdyal S. Bersa, Linda V Sinclair, Paul N. Moynagh, Lydia Lynch, David K. Finlay, Donal O'Shea and Andrew E. Hogan. The Journal of Immunology. 2019: 202(12):3404-3411.
*Joint first authorship

Interleukin-17 producing mucosal-associated invariant T cells- emerging players in chronic inflammatory diseases?

Marta M. Pisarska, Margaret R. Dunne, Donal O'Shea and Andrew E. Hogan. European Journal of Immunology. 2020; 50(8):1098-1108. Review.

Manuscripts in preparation

Glycolysis supports proliferation and expansion of mucosal-associated invariant T cells

Marta M. Pisarska, Nicole A.W. Wood, Nidhi Kedia-Mehta and Andrew E. Hogan.

Manuscript in preparation

List of Abbreviations

2DG	2-Deoxy-D-Glucose
5-ARU	5-Amino-6-D-Ribityaminouracil
5-OE-RU	5-(20oxoethyludeneamino)-6-D-Ribitylaminouracil
5-OP-RU	5-(2-Oxopropulideneamino)-6-D-Ribitylaminouracil
6-FP	6-Formyl Pterin
Acetyl Co-A	Acetyl Coenzyme-A
AMPK	AMP-activated protein kinase
AP-1	Activator Protein-1
APC	Antigen Presenting Cell
ASCT2	Alanine, Serine, Cysteine Transporter 2
AT	Adipose Tissue
ATM	Adipose Tissue Macrophages
ATP	Adenosine Triphosphate
BCH	2-amino-2-norbornanecarboxylic acid
Blimp-1	B-Lymphocyte-Induced Maturation Protein -1
BMI	Body Max Index
BSA	Bovine Serum Albumin
C/EBPδ	CAAT/Enhancer-Binding Protein Gamma
CCL	CC Chemokine Ligand
CCR	CC Chemokine Receptor
CD	Cluster of Differentiation
CFSE	Carboxyfluorescein Succinimidyl Ester
CTV	Cell Trace Violet
CVD	Cardiovascular Disease
DEPTOR	DEP Domain Containing mTOR Interacting Protein

DMSO	Dimethyl Sulfoxide
EAE	Experimental Autoimmune Encephalomyelitis
ECAR	Extracellular Acidification Rate
ELISA	Enzyme-linked immunosorbent assay
Eomes	Eomesodermin
ETC	Electron Transport Chain
FCS	Foetal Calf Serum
FFA	Free Fatty Acids
FMO	Fluorescence minus one
FSC	Forward Scatter
FTO	Fat Mass and Obesity Associated (gene)
FVD	Fixable Viability Dye
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GLS	Glutaminase
GLUT	Glucose Transporter
HBSS	Hank's Balanced Salt Solution
HCV	Hepatitis C Virus
HFD	High Fat Diet
HIF-1α	Hypoxia Inducible Factor-1α
HIV	Human Immunodeficiency Virus
HKII	Hexokinase II
HLA-DR	Human Leukocyte Antigen- DR
IBD	Inflammatory Bowel Disease
IFNγ/α/β	Interferon γα/β
IGRP	Islet-Specific Glucose-6-Phosphate Catalytic Subunit-Related Protein

IL	Interleukin
ILC2	Innate Lymphoid Cell 2
iNKT	Invariant natural killer T cell
JNK	C-Jun N-Terminal Kinase
LAT	L-Type Amino Acid Transporter
LDHA	Lactate dehydrogenase A
MAIT	Mucosal Associated Invariant T cell
MCP-1	Monocyte Chemoattractant Protein-1
MG	Methylglyoxal
MHC	Major Histocompatibility Complex
mLST8	Mammalian Lethal with Sec13 Protein 8
MR1	MHC class I related protein 1
mROS	Mitochondrial Reactive Oxygen Species
MS	Multiple Sclerosis
mTOR	Mammalian Target of Rapamycin
mTORC1	Mammalian Target of Rapamycin Complex 1
mTORC2	Mammalian Target of Rapamycin Complex 2
NFAT	Nuclear Factor of Activated T Cells
NFκB	Nuclear Factor-Kappa Light Chain Enhanced of Activated B Cells
NK	Natural Killer (cells)
NOD	Non-Obese Diabetic
OxPhos	Oxidative Phosphorylation
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PD-1	Programmed Cell Death Protein-1
PEP	Phosphoenolpyruvate

PFA	Paraformaldehyde
PKM2	Pyruvate kinase 2
PLZF	Promyelocytic Leukaemia Zinc Finger
PPP	Pentose Phosphate Pathway
PRSA40	Proline-Rich AKT Substrate 40kda
RA	Rheumatoid Arthritis
Rheb	Ras Homolog Enriched in Brain
RORγT	RAR-Related Orphan Receptor Gamma γT
RT-qPCR	Real Time Quantitative Polymerase Chain Reaction
SERCA	Sarcoendoplasmic Reticulum Calcium ATPase
SGLT	Sodium-Glucose Linked Transporters
siRNA	Small Interfering RNA
SLC	Solute Carrier
SLE	Systemic Lupus Erythematosus
SNAT	Sodium Coupled Neutral Amino Acid Transporter
T-bet	T-Box Protein Expressed in T Cells
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TCR	T Cell Receptor
TNF-α	Tumour Necrosis Factor Alpha
TSC2	Tuberous Sclerosis Complex 2
WAT	White Adipose Tissue
WHO	World Health Organisation
WT	Wild Type
α-KG	α-Ketoglutarate

Abstract

Obesity has developed into a worldwide pandemic, affecting individuals regardless of their socio-economic status, gender or age. It is associated with development of multiple co-morbidities including type 2 diabetes mellitus, which have been shown to be underpinned by inflammation. Indeed, excessive adiposity has been shown to be associated with a large dysregulation of immune cell function, including altered phenotype of mucosal-associated invariant T (MAIT) cells. MAIT cells from people with obesity were shown to have enhanced IL-17 production, accompanied by impaired IFNγ expression. As of now, very little is known about the control of MAIT cell function, therefore more research is required to understand their biology in order to identify the altered process, which leads to their dysfunction in obesity. Immunometabolism of MAIT cells became the focus of this thesis as previous data indicated it as a key biological process that controls immune cell function. Here we confirmed previously published data, showing that MAIT cells are potent producers of IFNγ and IL-17 cytokines and that MAIT cells can proliferate and expand upon stimulation. In addition, we reported that MAIT cells enhance glycolysis upon activation, which in turn is controlled by the influx of amino acids into the cells via LAT1 amino acid transporters. Glycolytic metabolism was required for successful IFNγ production as well as proliferation and expansion of MAIT cells. People with obesity have impaired IFNγ expression and MAIT cell expansion that was accompanied by impaired glycolytic metabolism. Collectively our data indicates that MAIT cells effector functions are in part controlled by the intrinsic metabolic pathways including the glycolytic metabolism. Defect in glycolysis or glycolysis-associated pathways as observed in obesity, leads to their dysfunction and altered immune cells responses.

Chapter One

General Introduction

1.1 Obesity

1.1.1. Obesity Overview

Obesity is a chronic progressive disease, which has evolved into a global pandemic (Bray et al., 2017; WHO, 2020a). Between 1975 to 2016 the number of individuals living with obesity has almost tripled. As of 2016 it is estimated that 650 million adults are living with obesity worldwide (WHO, 2020a). Initially, obesity affected only high-income countries, but it has quickly spread to the middle-income and low-income countries with the number of cases of obesity having increased in the vast majority of countries worldwide between 1975 and 2014 (Collaboration(NCD-RisC), 2016).

Obesity is described as an excessive accumulation of adipose tissue (AT) which is disproportionate to the height of an individual (CDC, 2020). Diagnosis of the disease in adults is most commonly based on the calculation of Body Mass Index (BMI), due to the lack of more precise and freely available diagnostic tools (Rothman, 2008; Shah and Braverman, 2012; WHO, 2020a). BMI is calculated by dividing the weight of the individual in kilograms by their height in meters squared. This equation allows for a crude estimation of an individual's nutritional status, proportion of AT and classification into one of the following categories as set out by World Health Organisation (WHO): underweight BMI of <18.5 kg/m^2, normal weight as 18.5-24.9 kg/m^2, pre-obesity/overweight as 25-29.9 kg/m^2 and obesity as a BMI >30 kg/m^2 (WHO, 2020a). This equation is inherently flawed, as it doesn't take into consideration other factors, which would aid the assessment of the severity of obesity and its impact on the individual's health, such as localisation of the AT deposits, muscle density or sex (Bray et al., 2018; Rothman, 2008). BMI measurement can be supported by the measurement of the waist circumference, which correlates with the amount of visceral AT and increased risk of development of cardiovascular disease (CVD) risk, type 2 diabetes mellitus (T2DM) and overall mortality (Cerhan et al., 2014; Jeon et al., 2019; Mamtani et al., 2013; Nazare et al., 2015; Siren et al., 2012). Initially, the primary cause of obesity was identified as an energy imbalance, where calorie intake exceeded the calorie expenditure (WHO, 2020a). Since then, multiple studies have highlighted the complexity and heterogenicity of this disease, depicting how multiple factors affect disease development (González-Muniesa et al., 2017; Swinburn et al., 2011). Susceptibility of an individual to weight gain is largely

influenced by their environment, behaviour, socio-economic status and biological factors. Calorie intake is greatly affected by food availability and accessibility, as well as the marketing strategies employed by the food companies, which promote snacking and consumption of highly processed, calorie dense foods (González-Muniesa et al., 2017; Swinburn et al., 2011). On the other hand, calorie expenditure has been reduced as a result of improvement of the public transport systems and sedentary lifestyle, resulting in a much-decreased amount of time which is dedicated to physical activity (González-Muniesa et al., 2017; Swinburn et al., 2011). Weight gain is also affected by biological factors such as sleep deprivation, location of AT or genes (Goodarzi, 2018; Swinburn et al., 2011; Vishvanath and Gupta, 2019). In recent years, mutations in fat mass and obesity associated (FTO) gene and genes coding for proteins involved in the leptin-melanocortin pathway such as leptin, leptin receptor and melanocortin 4 receptor, have been identified in people with obesity (Pigeyre et al., 2016). The contribution of the genetic variation to the development of obesity yet remains to be fully elucidated.

The rate of obesity in Ireland is growing at an alarming rate. Pineda *et al.* have estimated that without appropriate interventions, by 2025 Ireland will become the country with the highest rate of obesity in Europe (Pineda et al., 2018). This is in line with an earlier study by Keaver *et al*., which estimated that by 2030 as many as 89% of Irish men and 85% of Irish women will be living with overweight or obesity (Keaver et al., 2013). Healthy Ireland Survey revealed that 37% of Irish adults are overweight and a further 23% are living with obesity (Department of Health and MRBI IPSOS, 2015). It also revealed bad behavioural habits in regard to calorie intake by adults. Out of those surveyed, 65% reported to consume snack food or sugar drinks daily, whereas little under half of the participants met the minimum level of recommended physical activity of 150 minutes per week (Department of Health and MRBI IPSOS, 2015). Although obesity has significant implications on a person's health, the economic consequences cannot be overlooked. It has been estimated that the direct and indirect costs of obesity in Ireland in 2009 accounted for €1.13 billion (Perry et al., 2012). Keaver *et al.* projected that by 2030 this bill will be greatly exceeded, as the direct healthcare cost of obesity alone in Ireland will reach staggering €5.4 billion (Keaver et al., 2013). Although obesity was initially considered an adult disease, a substantial

increase of cases in the paediatric population has been observed worldwide (WHO, 2020a). As reported by the Childhood Obesity Surveillance Initiative, at least 1 in 5 Irish primary school children were living with overweight or obesity (Bel-Serrat et al., 2017). The Children's Sport participation and Physical Activity study showed only 17% of primary and 10% of post-primary school children met the national guidelines for physical activity (Woods et al., 2018). If paired with bad eating habits, this may contribute to the increasing rates of obesity in children. It is now well established that childhood obesity strongly tracks into adulthood (Evensen et al., 2016; Gordon-Larsen et al., 2004; Wang et al., 2008; Ward et al., 2017). Study by Simmonds *et al*. estimated that 55% of children with obesity will become obese adolescents, and 80% of obese adolescents will become obese adults, highlighting the need for early interventions (Simmonds et al., 2016). As obesity is affecting children at a very young age, obesity-associated co-morbidities such as T2DM, begin to manifest much earlier than previously observed, often still in childhood or adolescence (Mayer-Davis et al., 2017). Unfortunately, treatment options of obesity are still limited and include lifestyle interventions, pharmacological treatments and bariatric surgery (Bray et al., 2018). None of these methods are fully successful, as studies have shown that following each of these treatment regimes, weight can be regained (Felsenreich et al., 2016; Franz et al., 2007). Undisputedly, prevention of obesity starting in early childhood years and development of safe and non-invasive treatments are desperately needed.

1.1.2. Obesity associated co-morbidities

Obesity is associated with an extensive list of co-morbidities, ranging from T2DM, CVD, development of multiple types of cancers and mental health problems (Figure 1.1) (Avgerinos et al., 2019; Daousi et al., 2006; Mannan et al., 2016; Pantalone et al., 2017).

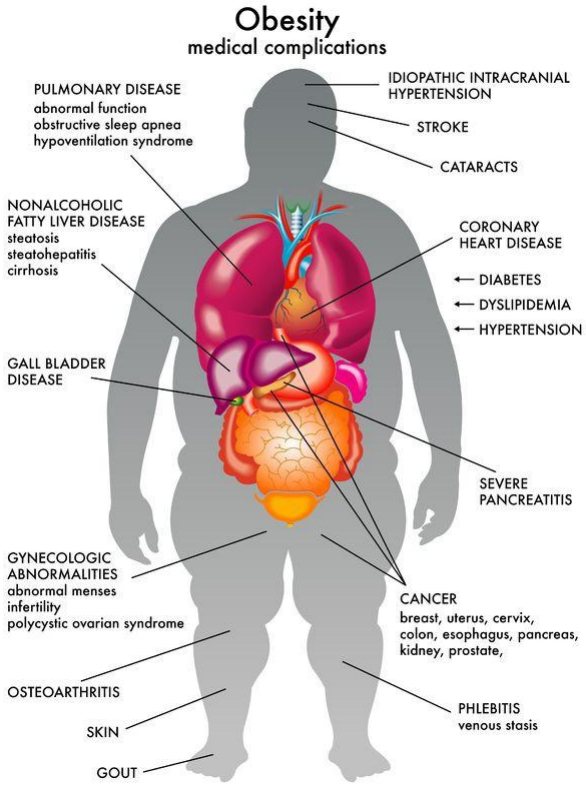


Figure 1.1. Obesity associated co-morbidities. Obesity contributes to development of multiple diseases such as T2DM, CVD and multiple types of cancers (Doheny, 2015).

Individuals who are living with overweight or obesity are at a major risk of developing T2DM, in fact up to 90% of cases are weight related (Daousi et al., 2006; Iglay et al., 2016; Public Health England, 2014). T2DM is characterised by a defect in insulin secretion and/or impaired insulin sensitivity of tissues, which ultimately leads to chronic hyperglycaemia (DeFronzo et al., 2015). Although pathogenesis of T2DM in obesity is still not fully elucidated, it is known to be underpinned by low-grade inflammation. White adipose tissue (WAT) has been identified as the main source of pro-inflammatory mediators in obesity. It was shown to release high amounts of molecules such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, leptin and monocyte chemoattractant protein-1 (MCP-1). This leads to the recruitment of inflammatory immune cells, which contribute to the establishment of the pro-inflammatory environment and insulin resistance in the AT (Longo et al., 2019; Mantzoros et al., 2011; Zatterale et al., 2020; Zorena et al., 2020).

TNF-α is one of the best studied cytokines in the establishment of T2DM and was shown to be implicated in the onset of insulin resistance by inducing a defect in

insulin signalling (Aguirre et al., 2000; Hotamisligil et al., 1995, 1993). Despite the promising data, clinical trials which focused on the neutralisation of TNF-α in individuals with T2DM, no improvement in insulin sensitivity after treatment was reported (Bernstein et al., 2006; Dominguez et al., 2005; Ofei et al., 1996). Although other inflammatory cytokines have been also implicated in the development of T2DM and insulin resistance (McArdle et al., 2013), the role of immune cells and immune derived factors in this disease is yet to be fully understood.

Obesity is also a significant risk factor associated with the development of CVD (Khan et al., 2018). Excessive weight was shown to alter not only the function of the cardiovascular system, but also the structure of the heart. Individuals with obesity displayed a larger left ventricular mass and worse systolic function, both of which are likely to contribute to the development of hypertension (Bello et al., 2016; Kotchen, 2010; Wu et al., 2019). Failure to regulate blood pressure leads to further complications, such as coronary heart disease, heart failure, stroke and renal disease (Landsberg et al., 2013). Dyslipidaemia, often diagnosed alongside obesity, is strongly linked to atherosclerosis (Duncan et al., 2019; Klop et al., 2013). Excessive exposure to high levels of low-density lipoprotein cholesterol is the main risk factor associated with development of atherosclerosis in humans, as it has been shown to initiate atherogenesis. Over time the plaques deposited on the arterial wall, increase in size, accumulating more lipids. This eventually leads to a substantial restriction of the blood flow and increases a risk of plaque rupture, which can cause acute thrombosis and myocardial infarction. Development of atherosclerosis is accompanied by an inflammatory response in immune cells (Libby et al., 2019). This highlights the common denominator between the pathogenesis of CVD and T2DM in the context of obesity – altered immune response and pro-inflammatory environment.

It is acknowledged that obesity is positively correlated with the development of certain cancers such as colorectal, ovarian, gallbladder, kidney, post-menopausal breast and liver (Bhaskaran et al., 2014; Lauby-Secretan et al., 2016). Recently, it was reported that excessive body weight is attributed to 3.9% of all cancers worldwide (Sung et al., 2018). The contribution of excess adiposity to cancer development is complex, as multiple types of cancer and multiple

factors are involved (Bhaskaran et al., 2014; Sung et al., 2018). Chronic inflammation, which persists in obesity, favours tumour development, through the disruption of tissue homeostasis and dysregulation of immune cell function (Y. S. Lee et al., 2018; Quail and Dannenberg, 2019). Although inappropriate activation of the immune system is closely related to obesity, excessive adiposity is also associated with a loss of immune cell function. People with obesity have a defect in natural killer cell mediated killing of the tumour cells *in vitro* (Michelet et al., 2018; Tobin et al., 2017). In addition, chronic stimulation of T cells in obese AT induces expression of exhaustion markers such as programmed cell death protein-1 (PD-1) and leads to dampened anti-tumour responses. However, using PD-1 checkpoint inhibitors, anti-tumour activity in subjects with obesity can be restored (Canter et al., 2018; Z. Wang et al., 2019). Once again, this illustrates how the immune system dysregulation caused by obesity underpins the development of another co-morbidity.

Although obesity has a detrimental effect on the body, the impact of obesity on mental health is often overlooked. It has been reported that more than half of adults with obesity admitted to experience stigmatisation by their co-workers, whereas children with obesity have over 60% higher chance of being bullied (WHO, 2017). Mistreatment of individuals due to their weight can lead to the development of mental health diseases. A study by van Vuuren *et al*. found that adolescents who are overweight displayed more suicidal thought tendencies than their lean counterparts (Van Vuuren et al., 2019). A meta-analysis which looked at the relationship between weight stigma and mental health, reported a medium or large correlation between weight-related stigma and symptoms of depression, anxiety and psychological distress (Emmer et al., 2020; Luppino et al., 2010). Discrimination and prejudice of the individuals with excessive weight is undisputable, although in 2016 almost 4 in 10 adults were living with overweight and 1 in 8 were living with obesity, indicating a high prevalence of this condition in society (WHO, 2020a). Hence, appropriate actions must be taken to end the stigma of obesity.

1.2 Immune system in health and obesity

1.2.1 Immune system and inflammation

The aim of the immune system is to provide the host with protection from invading microorganisms and injury. Upon pathogen entry or tissue damage, an inflammatory response is initiated, led by the innate immune system which include cells like monocytes, neutrophils and dendritic cells (Figure 1.2) (Delves et al., 2011). This results in the production of pro-inflammatory cytokines and chemokines and recruitment of other leukocytes in order to contain the infection (Coico and Sunshine, 2015; Delves et al., 2011). The innate immune response is supported by the more specialised adaptive arm of the immune system, which assists in the eradication of the infection and aims to provide long-term protection from the pathogen via memory (Figure 1.2) (Coico and Sunshine, 2015; Delves et al., 2011). The adaptive immune system is composed of T lymphocytes, which are classified as cytotoxic $CD8^+$ cells and cytokine producing helper $CD4^+$ cells, and B lymphocytes, which support the immune response by producing antigen specific antibodies.

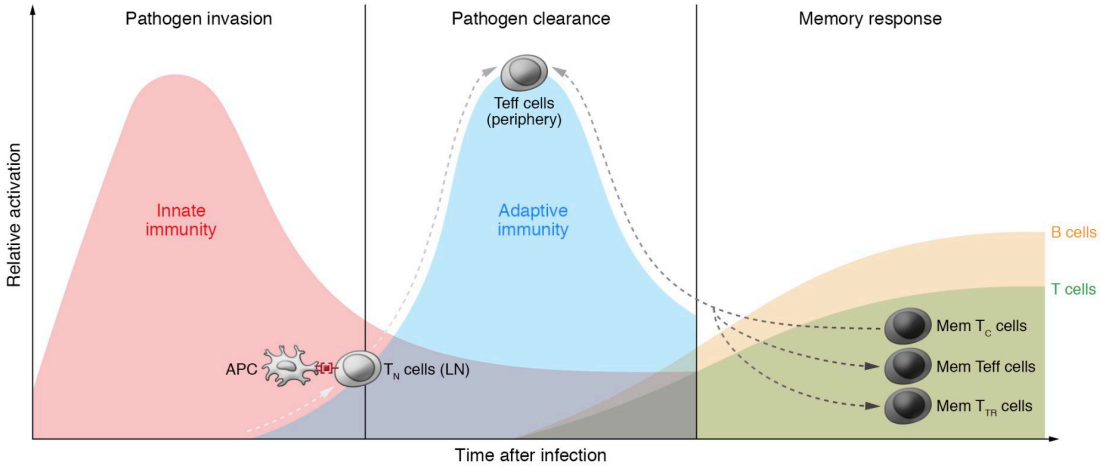


Figure 1.2 Response of the immune system to infection with pathogen. Upon infection, the innate immune system is activated upon recognition of pathogen associated molecular patterns and damage associated molecular patterns by antigen presenting cells (APC). This is followed by the engagement of the adaptive immune system in order to successfully restore homeostasis and provide long-term protection to the host thorough formation of immunological memory(Khader et al., 2019) .

Activation of the adaptive arm of the immune system leads to clonal expansion of the cell population and the formation of effector cells, providing a more specific memory response upon re-challenge with the pathogen and therefore a better protection to the host (Coico and Sunshine, 2015; Delves et al., 2011).

As research in the area of immunology is quickly advancing, it becomes clear that the initial classification of cells into the innate and adaptive immune system requires reconsideration. In recent years more subtypes of immune cells have been discovered and termed as unconventional T cells, which include cells such as mucosal associated invariant T (MAIT) cells, γδ cells and invariant natural killer T (iNKT) cells (Figure 1.3) (Godfrey et al., 2015). These T cells have been classified as "unconventional" as they are not restricted by major histocompatibility complex (MHC) molecules like classical T cells. However, they still in part rely on the antigen presentation and recognition by their T cell receptor (TCR), but in addition these cells can be activated through cytokine stimuli alone (Godfrey et al., 2015). Furthermore, they don't recognise protein antigens. MAIT cells recognise vitamin B metabolites in the context of MHC class I related protein 1 (MR1) complex, γδ cells recognise lipid antigens and phosphoantigens presented by CD1c & CD1d molecules, whereas iNKT cells respond to lipid antigens in the context of CD1d (Godfrey et al., 2015). This provides the immune system with another rapid response to pathogens, bridging the innate and adaptive systems.

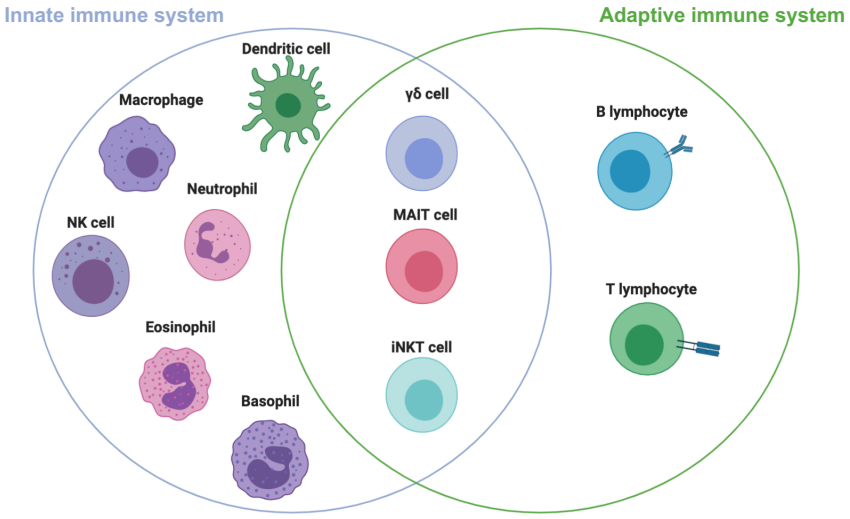


Figure 1.3 Cells of the immune system. Immune cells can be divided into innate and adaptive immune systems, however some cells such as MAIT cells, don't fit into either of these categories possess feature of both systems. (Adapted from Bonam *et al.*, 2017).

Inflammation is deemed to be beneficial to the host in many circumstances. Acute inflammation is a short-lived response, which is followed by resolution to achieve tissue homeostasis (Coico and Sunshine, 2015). Acute inflammation can become chronic if the agent, which stimulates the immune response cannot be removed (Chen et al., 2018). Sustained low-grade inflammation underpins the

development of multiple inflammatory diseases such as T2DM, rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (Guo et al., 2018; Moulton et al., 2017; Wu and Ballantyne, 2020). However, the cause of many of these diseases remains to be elucidated. Identifying the primary triggers of an inappropriate immune response would aid the development of successful treatments. Currently, our understanding of inflammatory diseases is insufficient, and we are limited to the treatment of symptoms. Current therapies aim to dampen down the immune response by using immunomodulatory drugs such as anti-TNF-α and anti-IL-17A, however they don't eradicate the triggers of such diseases (Bissonnette et al., 2018; Kimball et al., 2016; Thaçi et al., 2015; Wang et al., 2016). Advancing research to understand the pathophysiology of the immune response during inflammation and identifying the antigens, which stimulate the cells, will aid in developing successful treatments.

1.2.2 Adipose tissue associated inflammation and immune cell dysregulation in obesity

Obesity is associated with a systemic, low-grade chronic inflammation (Hotamisligil, 2017; Y. S. Lee et al., 2018). It has been well established that this inappropriate activation of the immune system strongly contributes to the development of insulin resistance and T2DM (Zatterale et al., 2020). The origin and cause of this inflammation is still to be fully elucidated and researchers are currently investigating multiple sites including liver, pancreas, muscles. However, studies have strongly focused on the AT as the primary site of inflammation (Y. S. Lee et al., 2018).

For decades, AT has been considered merely as an energy storing tissue. As research progressed, it has been demonstrated that AT is capable of producing and releasing a variety of factors such as adipokines and hormones and is enriched in immune cells, which ultimately lead to the re-classification of AT as an endocrine organ (Ouchi et al., 2011; Rodríguez et al., 2015). Nonetheless, AT plays a very important role in energy homeostasis in a lean individual. It provides long-term storage of excess nutrients, which are released during demand for energy (Ouchi et al., 2011). From the point of view of evolution, this is a very good process of saving nutrients for periods of starvation, where they can be easily accessed and used for survival. Today in the westernised world, this process is

not as crucial, due to the wide availability of food, which often requires very little effort to obtain. Instead, this very process is now leading to over-storing of energy, contributing to the development of diseases. AT adapts to the excess of nutrients by undergoing structural remodelling and expansion. Hypertrophy and hyperplasia of the adipocytes occurs. This leads to increased oxygen demand and therefore the induction of angiogenesis which was shown to be supported by the resident adipose tissue macrophages (ATM) (Figure 1.4) (Cho et al., 2007; Sun et al., 2011; White and Ravussin, 2019). Expansion of the AT is associated with an increase in immune cell infiltration, release of pro-inflammatory factors from the adipocytes and leukocytes leading to inflammation (Figure 1.4). Chronic over-nutrition causes those changes to be sustained for a long-time causing hypoxia, fibrosis and sustained inflammation, leading to AT dysfunction (Zatterale et al., 2020).

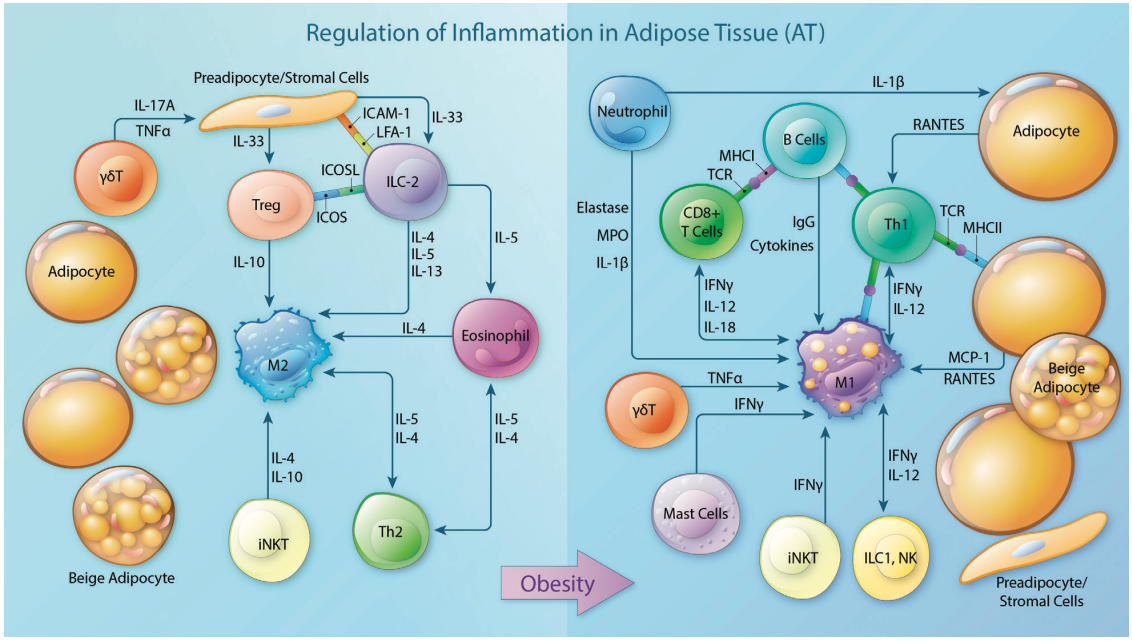


Figure 1.4. Changes in adipose tissue in obesity. Obesity leads to hypertrophy and hyperplasia of adipocytes and recruitment of immune cells, resulting in chronic inflammation (Wu and Ballantyne, 2020).

In obesity, adipocytes alter their secretory profile and produce higher levels of leptin but downregulate the production of adiponectin (Considine et al., 1996; Hanley et al., 2007; Ryan et al., 2003). In addition to the role of these hormones in maintaining energy homeostasis in humans, both impact the function of immune cells. Leptin was shown to have pro-inflammatory properties. It can stimulate macrophages to produce pro-inflammatory cytokines such as IL-6 and TNF-α (Acedo et al., 2013), promote chemotaxis of neutrophils (Montecucco et

al., 2006), and enhance the differentiation of T cells into Th1 and Th17 subsets (Gerriets et al., 2016; Reis et al., 2015; Rodríguez et al., 2007). On the other hand, adiponectin can upregulate the secretion of IL-10 from macrophages, suppress M1 type in favour of M2 polarisation and decrease production of interferon γ (IFNγ) and IL-17A by $CD4^+$ T cells (Ohashi et al., 2010; Surendar et al., 2019; Wolf et al., 2004). Taking into consideration the opposing effects of leptin and adiponectin, it can be hypothesised that the imbalance of these hormones observed in obesity, contributes to the inflammatory state. In addition, production of AT tissue derived TNF-α was found to be increased in obesity (Cao et al., 2008; Hotamisligil et al., 1993). As TNF-α was shown to have a negative impact on insulin sensitivity, this further suggests that changes in the AT and the pro-inflammatory environment induced by obesity is contributing to development of T2DM (Aguirre et al., 2000; Hotamisligil et al., 1995, 1993).

Alterations in the function of AT is not the only change observed in obesity. Infiltration of immune cells plays one of the key roles in initiating and maintaining low-grade inflammation. Both arms of the immune system are implicated, however macrophages have received a lot of attention as they are enriched in the AT of people and mice with obesity (Harman-Boehm et al., 2007; Weisberg et al., 2003). In mice, macrophages were shown to make up 40-50% of immune cells in obese AT vs 5-10% observed in the AT of lean mice (Weisberg et al., 2003). Macrophages can be broadly classified as pro-inflammatory M1 or anti-inflammatory M2. In obesity, AT is largely enriched in the M1 subtype of macrophages which produce pro-inflammatory cytokines such as IL-6, TNF-α, IL-1β (Figure 1.4) (Lumeng et al., 2007). Their recruitment is likely driven by secretion of chemokines by AT such as of MCP-1, and death of adipocytes, which must be removed to maintain tissue homeostasis and resolve inflammation. (Guilherme et al., 2008; Vishvanath and Gupta, 2019). The association between inflammatory macrophages and insulin resistance in obesity is well established (Catrysse and van Loo, 2018). They are primary producers of TNF-α, and it has been shown that the depletion of these cells normalizes insulin sensitivity (Patsouris et al., 2008). As more evidence is mounting, it is now becoming clearer that macrophages in the AT cannot be strictly identified as M1 or M2 (Kratz et al., 2014), but rather their phenotype is more flexible and lies on a spectrum, which changes our approach in investigating the role of macrophages in the AT. The

adaptive arm of the immune system is also implicated in creating this pro-inflammatory environment. CD3 expressing lymphocytes are the second most abundant cells in obese AT after the macrophages. Of those lymphocytes, an increase in both CD8 and CD4 expressing T cells is observed (Y. S. Lee et al., 2018). Although the sequence in which the cells arrive in the AT is still to be elucidated, some evidence suggests that the accumulation of $CD8^+$ T cells precedes the increase in ATM (Nishimura et al., 2009). T cells contribute to the AT inflammation by releasing Th1 and Th17 type cytokines such as IFNγ and IL-17, which can induce recruitment of M1 macrophages to the tissue (Fabbrini et al., 2013; Winer et al., 2009). These changes were found to be accompanied by a decrease in T reg cells, which are known for their anti-inflammatory functions, suggesting further loss of control over inflammation (Winer et al., 2009). Other immune cells have been also identified to be altered in obese AT. MAIT cells were shown to accumulate in human AT in obesity and display a type 17 phenotype characterised by predominant production of IL-17 cytokines rather type 1 phenotype which in turn is identified by IFNγ production. This suggests that MAIT cells are involved in maintaining the inflammatory environment in AT (Carolan et al., 2015; Magalhaes et al., 2015). Similarly, IL-17 producing γδ T cells were also found to be enriched in AT of obese mice and were shown to regulate T reg populations in this tissue (Kohlgruber et al., 2018). On the other hand, populations of innate lymphoid cells 2 (ILC2) and iNKT cells in obese AT is diminished (Brestoff et al., 2015; Lynch et al., 2012). As ILC2 and iNKT cells support M2 profile of macrophages and contribute to the anti-inflammatory profile of AT, it suggests that a reduction in their frequencies may favour pro-inflammatory environment in obese AT (Lynch et al., 2015; Molofsky et al., 2013).

The primary trigger of inflammation in obese AT is yet to be discovered, however some hypotheses have been put forward. Increased circulation of free fatty acids (FFA) in obesity is likely caused by the eruption of the adipocytes during AT expansion (Arner and Rydén, 2015; Ni et al., 2015). FFA have been shown to initiate the pro-inflammatory immune response by binding to toll like receptor 4 on adipocytes and macrophages and activate nuclear factor-kappa light chain enhanced of activated B cells (NFκB) and c-Jun N-terminal kinase (JNK) signalling (Shi et al., 2006). In addition, FFA were shown to contribute to hypoxia, by uncoupling of mitochondrial oxidative metabolism in adipocytes. This leads to

deprivation of oxygen in the cell, enhancement in hypoxia inducible factor-1α (HIF-1α) expression and activation of the pro-inflammatory pathways within the adipocyte (Lee et al., 2014). Increased gut permeability has been also reported in obesity, which has been suggested to be one of the multiple triggers of systemic chronic inflammation in this disease (Toubal et al., 2020). In fact, an increase in lipopolysaccharide circulation in individuals with obesity has been reported and correlated with development of T2DM (De La Serre et al., 2010; Trøseid et al., 2013). The onset of obesity is multifactorial therefore, the trigger of its associated inflammation is hypothesised to be affected by multiple events. Identification of the triggers of the disease as well as the sequence of immune cell infiltration into the AT and factors involved in initiation of the immune response will aid in further examination of the causes of development of diabetes and other inflammation associated diseases in obesity, hopefully leading to a successful treatment.

1.3 Mucosal Associated Invariant T cells

1.3.1 Mucosal Associated Invariant T cells overview

MAIT cells are a type of unconventional adaptive T cells, which display innate properties and they don't strictly belong to the innate or adaptive category. Initially they have been identified to be enriched at mucosal tissues, hence their name, however it is now well established they are abundant at multiple sites including liver, AT and lungs (Dusseaux et al., 2011; Lezmi et al., 2019; Tobin et al., 2017). They can be also readily identified in the peripheral blood, where they compromise 1-10% of T cells (Le Bourhis et al., 2010).

MAIT cells are defined by the semi-invariant, highly conserved, αβ TCR, which is accompanied by high expression of CD161 molecules. MAIT cell TCR is composed of TRAV1-2 (Vα7.2) and TRAJ33/12/20 (Jα33/12/20) alpha chains paired with a limited number of beta chains (Lepore et al., 2014; Porcelli et al., 1993; Tilloy et al., 1999). In contrast to classical T cells, MAIT cells were shown to be restricted by MR1, rather than MHC-I or MHC-II antigen presenting complexes (Treiner et al., 2003). The antigen presented by MR1 to MAIT cells has been unidentified for many years. The first clue of the potential origin of the antigens was uncovered by Gold *et al.* and Le Bourhis *et al*. who showed that MAIT cells respond to a wide array of gram- positive and gram-negative bacteria

as well as some strains of yeast (Gold et al., 2010; Le Bourhis et al., 2010). It wasn't until 2012, when the landmark study Kjer-Nielsen *et al.* identified the antigen of MAIT cells as metabolites of vitamin B_2 and B_9, which are produced by multiple strains of microorganisms, utilising the riboflavin synthesis pathway (Kjer-Nielsen et al., 2012). This antigen is very unique to microorganisms, as humans don't synthesise vitamin B, providing the host with an advantage in detecting these pathogens. The most potent antigen which activates MAIT cells is synthesised from a 5-amino-6-D-ribityaminouracil (5-ARU), an intermediate produced during riboflavin synthesis and methylglyoxal (MG), a metabolite produced during glycolysis, which together form 5-(2-oxopropulideneamino)-6-D-ribitylaminouracil (5-OP-RU) (Corbett et al., 2014).

MAIT cells development begins in the thymus, where they undergo thymic selection. In contrast to T cells, which are selected by binding to thymic epithelial cells, MAIT cells bind to double positive ($CD4^+/CD8^+$) cortical thymocytes, which express MR1 molecule (Seach et al., 2013). The process of thymic selection of MAIT cells still remains largely unexplored. It is yet to be established whether MR1 is loaded with any antigen, and if so whether it is low or high affinity and where it originates from. Future studies should also investigate whether thymic negative selection applies to MAIT cells and how and if overreactive MAIT cells are removed. Following the successful positive selection, MAIT cells undergo a three-step maturation process which begins in the thymus. A study by Koay *et al*. has significantly contributed to our current understanding of MAIT cell maturation in human and mice. They classified the different stages of development based on surface markers. Stage 1 of MAIT cell were defined as $CD27^-$ $CD161^-$, stage 2 as $CD27^+$ $CD161^-$ and stage 3 by $CD27^{lo/-}$, $CD161^+$ and co-expression of IL-18R (Koay et al., 2016).

The mouse study showed that progression through the three stages is depended on the MR1 expression, as addition of anti-MR1 antibody successfully inhibited this process. The progress from stage 2 to stage 3 was described to be of particular importance in functional development of MAIT cells. Promyelocytic leukaemia zinc finger (PLZF), a transcription factor shared by the cells of the innate immune system, was found to be absolutely necessary for MAIT cell maturation from stage 2 to stage 3 and development of their functional competence. PLZF-null mice completely lacked stage 3 MAIT cell population and

residual stage 1 and stage 2 cells failed to produce cytokine upon stimulation in contrast to stage 3 MAIT cells from wild type (WT) mouse (Koay et al., 2016). Expression of PLZF, T-box protein expressed in T cells (T-bet) and RAR-related orphan receptor gamma γT (RORγT) transcription factors increased upon MAIT cell maturation to stage 3. In human MAIT cells, T-bet and RORγT were co-expressed in contrast to murine MAIT cell development, where these two transcription factors were mutually exclusive (Koay et al., 2016). To assess the functional capacity of stage 3 MAIT cells, Koay *et al.* stimulated samples obtained from matched blood and thymus and measured their cytokine production. More than 80% of stage 3 MAIT cells from peripheral blood produced IFNγ and TNF-α, in comparisons to <25% of stage 3 MAIT cells derived from the thymus. The superiority of peripheral blood stage 3 MAIT cells in cytokine expression, indicates that MAIT cells leaving the thymus are somewhat functionally competent, with further maturation of the cells occur extrathymically (Koay et al., 2016). A more recent study by Koay *et al*. focused on the transcriptional profile of MAIT cells during the three stages of development and maturation, using single-cell RNA sequencing in mice and humans (Koay et al., 2019). This study confirmed previous data that murine MAIT cells at stage three cells show a more mature prolife and express more *Zbtb16* (PLZF), *Rorc* (RORγT), and *IL18r* (IL-18 receptor) but less *Ccr7* than stage 1 cells. In addition, upregulation of other genes was identified at stage 3 such as *GzmB* (Granzyme B), *IL7R* (CD127) and chemokine receptors *Ccr6*, *Ccr2, Cxcr6* which bind C-C Motif Chemokine Ligand (CCL) 20, CCL2 and CCL16 respectively. The increase in the chemokine receptors was confirmed on the protein level by flow cytometry (Koay et al., 2019). Upon closer investigation of the stage 3 MAIT cells, this study was able to differentiate between type 1 and type 17 MAIT cells and found a significant difference in the expression of genes between those two subtypes of cells.

Mature MAIT cells can be identified in the peripheral blood and display a memory phenotype identified by the lack of the expression of CC-chemokine receptor (CCR)7, CD62L and CD45RA (Dusseaux et al., 2011; Gold et al., 2013). They are largely $CD8^+$, with a small proportion being double negative of $CD4^+$ (Gherardin et al., 2018b; Kurioka et al., 2017; Walker et al., 2012). As discussed in more detail in chapter four, MAIT cells can be activated via engagement of their TCR with MR1 bound to an antigen or via cytokine stimulation alone (Le Bourhis

et al., 2010; Leng et al., 2019; Van Wilgenburg et al., 2016). MAIT cells express a wide range of cytokine receptors including IL-2R, IL-12R, IL-15R or IL-18R, suggesting their activation can be induced by a various cocktails of cytokines in the absence of TCR antigens (Sattler et al., 2015; Wallington et al., 2018; Wilgenburg et al., 2018). Indeed, MAIT cells were shown to respond with enhanced IFNγ production when stimulated with a combination of cytokines namely IL-12 and IL-18 or IL12 and IL-15, whereas stimulation with single cytokines did not induce cytokine production or led to their very weak expression (Ussher et al., 2014; Van Wilgenburg et al., 2016). This shows plasticity of MAIT cells in responding to a potential threat, as cytokine stimulation alone, allows the cells to become activated in the absence of TCR antigens. This response may be very beneficial during viral infections or infections with bacteria which do not produce riboflavin; however, the non-specific MAIT cell activation may be harmful and contribute to the development of autoimmune and inflammatory diseases. Activation of MAIT cells induces rapid secretion of pro-inflammatory cytokines such as IFNγ, TNF-α and IL-17 and chemokines CCL3 and CCL4 which support recruitment of other immune cells to the site of inflammation and their activation (Hinks et al., 2019; Lamichhane et al., 2019). In addition, MAIT cells were shown to produce other effector molecules such as granzyme B and perforin, indicating their role in direct killing of cells infected with a pathogen (Dusseaux et al., 2011; Lamichhane et al., 2019; Wilgenburg et al., 2018). Indeed, MAIT cells were shown to kill bacterially infected cells in *in vitro* studies (Kurioka et al., 2015; Le Bourhis et al., 2010), however their role seems to extend beyond infections. MAIT cells exposed to MR1 expressing cancer cells lines were shown to induce killing of the cancer cells, indicating their role in cancer surveillance (Gherardin et al., 2018a; Won et al., 2016).

Although MAIT cells have been discovered relatively recently, their pleotropic functions and highly conserved TCR indicate a key role in the immune protection of the host. More research into MAIT cell biology and function is required to fully understand the role they play in bridging the innate and adaptive immune system.

1.3.2 MAIT cells in infection

MAIT cells mount an immune response to bacterial and yeast riboflavin biosynthesis metabolites in the context of MR1 molecules expressed by antigen

presenting cells. Study by Le Bourhis *et al*. in 2010 was one of the first to highlight the important contribution of MAIT cells to the immune response against microbial infections (Le Bourhis et al., 2010). This study showed MAIT cells respond to APC infected with *E. coli* in an MR-1 dependent manner (Le Bourhis et al., 2010). The response of MAIT cells to multiple bacteria strains has since been investigated in much more detail. Meierovics *et al.* reported MAIT cell activation and cytokine production during infection with *Francisella Tularensis*, which was dependent on MR1 and IL-12 signals (Meierovics et al., 2013). MAIT cells were also found to be implicated during infections with bacteria from the mycobacterium family. Initially, MAIT cells were shown to be reduced in the peripheral blood of patients with active infection with *Mycobacterium tuberculosis* and exhibited defective IFNγ production in comparison to the healthy controls (Jiang et al., 2016; Kwon et al., 2015). In contrast, Suliman *et al*. and Paquin-Proulx *et al.* reported no significant difference in the frequencies of MAIT cells or their IFNγ production between individuals with active infection versus healthy controls (Paquin-Proulx et al., 2018; Suliman et al., 2020). This indicates that the role of MAIT cells in tuberculosis infection may differ between the stages of infection, and more research is required to clearly establish their involvement in the response against this pathogen. Additionally, MAIT cells are capable of responding to multiple other bacteria via TCR-MR1 engagement such as *Klebsiella pneumoniae* and *Staphylococcus aureus* as well as yeast strains including *Candida albicans* (Le Bourhis et al., 2010). Although the majority of these studies identified MAIT cells as a major subtype of T cells mediating beneficial immune response, the opposite has been observed with *Helicobacter pylori* in mice. During *H. pylori* infection in WT mice, MAIT cells accumulated in the mucosa of the stomach and contributed to gastritis, whereas changes in gastric pathology were ameliorated in MR1 deficient mice, suggesting a pathogenic role of MAIT cells in this infection (D'Souza et al., 2018).

While the involvement of MAIT cells in mounting an effective immune response against bacterial infection wasn't surprising, as their primary antigen was identified to be a bacterial metabolite, MAIT cells were also shown to respond during viral infections. Early reduction in MAIT cell frequency has been shown in the peripheral blood of individuals infected with human immunodeficiency virus (HIV) and Hepatitis C virus (HCV) and this decrease was reported to be

irreversible in adults despite successful antiviral treatment during the chronic stage of the disease (Barathan et al., 2016; Cosgrove et al., 2013; Eberhard et al., 2014; Spaan et al., 2016). A study of HIV by Lal *et al.* showed that MAIT cells were highly activated at the peak of viremia, which was followed by an expansion of the population and enhancement of its effector functions. Decreases in the MAIT cell frequencies and impairment of function were observed at about one-year post infection, suggesting that early treatment of HIV patients, could help to rescue MAIT cell population and preserve their effector functions for a longer time (Lal et al., 2020). On the other hand, MAIT cells from peripheral blood of individuals with HCV infection displayed an activated phenotype measured by the CD69 expression and a significant increase in the expression of markers such as human leukocyte antigen- DR (HLA-DR) and PD-1, suggesting their exhausted phenotype (Barathan et al., 2016; Hengst et al., 2016). MAIT cells are also involved during influenza virus infection. Studies by Van Wilgenburg *et al.* and Loh *et al.* have demonstrated a decrease in the frequency of circulating MAIT cells and upregulation of IFNγ and granzyme B during the infection (Loh et al., 2016; Van Wilgenburg et al., 2016). The direct role of MAIT cells in response to influenza was demonstrated in MR1 deficient mice, which upon infection with H1N1 showed a higher mortality rate than controls. Adoptive transfer of MAIT cells into the MR1 deficient mice, extended the lifespan of infected mice, suggesting a non-redundant role of MAIT cells in influenza infections (Wilgenburg et al., 2018). All these studies demonstrate that MAIT cells are implicated in multiple bacterial and viral infections, making them an interesting target for vaccine development. However, more studies are required to establish their exact role, as discussed, they have a beneficial role in multiple infection, but like in the case of *H. pylori* they might cause excessive inflammation and contribute to development of pathologies.

1.3.3 MAIT cells in inflammatory and autoimmune diseases

The role of MAIT cells in autoimmune and inflammatory diseases is still under investigation, however an alteration in peripheral blood MAIT cell frequencies and function has been observed in a range of diseases including, RA or inflammatory bowel disease (IBD) (Figure 1.5) (Hayashi et al., 2016; Tominaga et al., 2017).

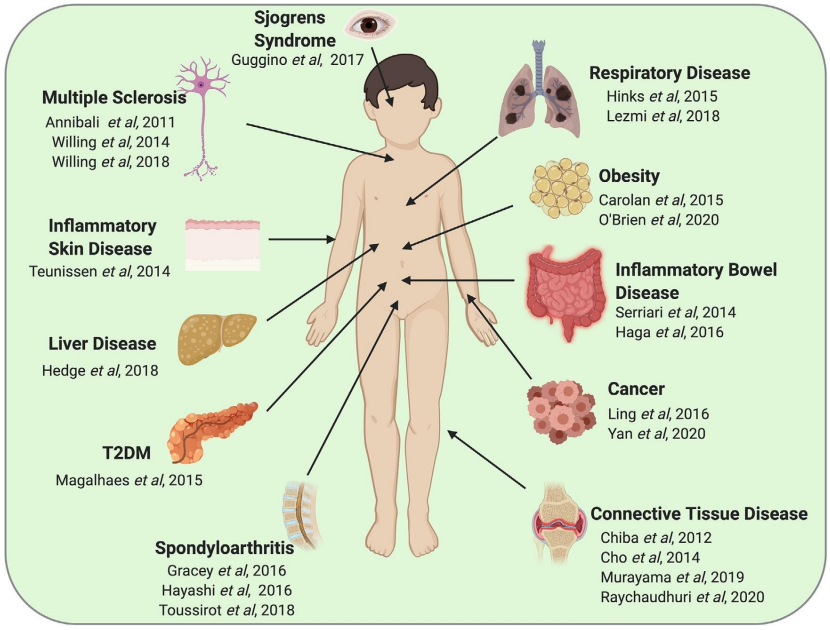


Figure 1.5 MAIT cells in inflammatory and autoimmune diseases. MAIT cell phenotype and function is altered in multiple inflammatory and autoimmune diseases, where they largely present with an IL-17 phenotype (Pisarska et al., 2020).

Despite multiple studies, the role of MAIT cells in multiple sclerosis (MS) remains controversial. One of the primary T cells implicated in MS are Th17 cells, however, MAIT cells were shown to contribute to IL-17 production in MS (Abrahamsson et al., 2013; Willing et al., 2018). In addition, they were also shown to overexpress migratory molecules, suggesting their ability to migrate across the blood brain barrier (Salou et al., 2016). Indeed, MAIT cells were found to accumulate in central nervous system lesions supporting the hypothesis that they may be implicated in the pathology of the disease (Illés et al., 2004; Salou et al., 2016; Willing et al., 2014). It is not clear whether the frequency of MAIT cells ih MS is altered due to conflicting reports. Willing *et al.* and Miyazaki *et al.* have shown a decrease in the frequencies of MAIT cells in the peripheral blood of MS subjects (Miyazaki et al., 2011; Willing et al., 2014), whereas other studies have shown an increase or no difference in MAIT frequencies in the peripheral blood of MS individuals (Ammitzbøll et al., 2020; Annibali et al., 2011; Salou et al., 2016; Sugimoto et al., 2016). In search for the cause of RA, the role of MAIT cells has been also evaluated. In comparison to the healthy controls, MAIT cell frequencies in the peripheral blood of RA patients vary between studies, with some reporting a decrease or no change (Cho et al., 2014; Gracey et al., 2016; Koppejan et al., 2019). However, an increase in MAIT cell frequencies has been identified in the

synovial fluid versus blood of individuals with RA, suggesting their migration to the inflamed tissue (Cho et al., 2014; Gracey et al., 2016). Indeed, TNF-α and IL-1β found at high concentrations in the synovial fluid of RA patients were shown to trigger MAIT cell migration into the tissue through the induction of migration-associated molecules on the endothelium (Kim et al., 2017). The negative effect of MAIT cells on the disease development was confirmed in a mouse model of arthritis, in which MR1 deficient mice developed less severe disease. Repopulation of MR1 deficient mice with MAIT cells caused an exacerbation of the disease (Chiba et al., 2012).

Similarly to RA, MAIT cell frequency is reduced in the peripheral blood of SLE patients (Chiba et al., 2017; Cho et al., 2014), which display impaired IFNγ production (Cho et al., 2014). A direct relationship between MAIT cells and development of SLE has been depicted in a mouse study of SLE, which reported that MR1 knockout mice had lower levels of autoantibodies than controls, whereas co-incubation of MAIT cells & B cells from a murine lupus mouse model, showed an enhancement in the production of antibodies which are a hallmark of the disease (Murayama et al., 2019).

MAIT cells are known to be involved in multiple inflammatory diseases such as IBD. The pathophysiology of the disease is not fully understood; however, immune system dysfunction strongly underpins the development of this disorder (Neurath, 2019). IBD individuals with ulcerative colitis or Crohn's Disease displayed a very similar phenotype of MAIT cells observed in RA. Patients with IBD had reduced frequency of peripheral blood MAIT cells which largely displayed IL-17 phenotype, with only Hiejima *et al.* reporting no difference in IL-17A production (Haga et al., 2016; Hiejima et al., 2015; Serriari et al., 2014; Tominaga et al., 2017). In addition, IBD patients presented with enhanced accumulation of MAIT cells in the inflamed mucosa (Haga et al., 2016; Serriari et al., 2014; Tominaga et al., 2017). Together with MS, RA and IBD reports, this further suggests that MAIT cells may be actively recruited from the blood to the site of inflammation. Studies of immune cells in the skin revealed that MAIT cells form a small population of the resident immune cells in the normal healthy skin (Li et al., 2017; Teunissen et al., 2014). However, investigation of the skin of individuals with psoriasis or dermatitis herpetiformis reported the presence of IL-

17 producing MAIT cells in the inflamed skin areas (Teunissen et al., 2014), suggesting that during inflammation, MAIT cells are likely to contribute to the pro-inflammatory environment.

MAIT cell frequency in the peripheral blood, lungs tissue and bronchioalveolar lavage of adults with asthma was shown to be reduced in comparison to the healthy controls and correlated with the disease severity and administration of inhaled corticosteroids (Hinks et al., 2015). It is unknown whether the reduction of MAIT cell frequency was directly related to the disease pathology or inhaled corticosteroids treatment, however, this reduction in MAIT cell frequency may contribute to an increased susceptibility of infection in this cohort. A study of children with asthma by Lezmi *et al*. identified a higher frequency of IL-17 producing MAIT cells, in children who had one or more severe exacerbations during a twelve-month period versus children who did not. In addition, the frequency of IL-17 producing MAIT cells positively correlated with the number of exacerbations of the disease (Lezmi et al., 2018). More recently, Lezmi *et al*. obtained similar results in a study of children with severe asthma, showing an enhancement in the frequency of IL-17A producing MAIT cells in their bronchoalveolar lavage samples, which positively correlated with the number of exacerbation (Lezmi et al., 2019). Recently it was identified that chronic stimulation of MAIT cells can result in IL-13 production (Kelly et al., 2019). This suggests Th2 cells in asthma may not be the only source of the allergy associated cytokines which contribute to the severity of disease.

Majority of the diseases discussed here can be characterised by a reduction in the frequencies of MAIT cells in the peripheral blood and their enhanced IL-17 phenotype, suggesting their migration to the site of inflammation and aggravation of the pro-inflammatory response. More research is required to identify the trigger which alters the phenotype of MAIT cells and to find targets to ameliorate their pro-inflammatory response.

1.3.4 MAIT cells in diabetes and obesity

Type 1 and type 2 diabetes mellitus are considered chronic inflammatory diseases, which involve an array of immune cells, primarily B and T lymphocytes (DeFronzo et al., 2015; Katsarou et al., 2017). Like in the vast number of

autoimmune diseases, the primary factors which contribute to the onset of the disease are yet to be identified. The microbiome was shown to play an important role in development of type 1 diabetes mellitus (T1DM) (Alkanani et al., 2015; Wen et al., 2008). As MAIT cells are associated with gut mucosal sites and are in close interactions with the microbiota, this suggests their possible involvement in the pathogenesis of T1DM.

Analysis of juvenile T1DM revealed no difference in the proportion of MAIT cells in peripheral blood between diabetic or control subjects, however T1DM individuals had a significantly larger subset of $CD27^-$ MAIT cells in comparison to the controls. In addition, $CD27^-$ MAIT cells from healthy donors produced more IL-17A than $CD27^+$ MAIT cells (Harms et al., 2015). The same group also reported a reduction in MAIT cell frequency in seroconverted patients (without T1DM yet) in comparison to the control group (Harms et al., 2018). Rouxel *et al.* reported a similar finding whereby MAIT cell frequencies were reduced in the peripheral blood of children with recent-onset T1DM versus healthy children. MAIT cells from children who recently developed T1DM expressed exhaustion markers of inflammation such as PD-1 and they produced less IFNγ, but more IL-17 and granzyme B upon challenge *in vitro* in comparison to controls (Rouxel et al., 2017). In addition, experiments performed with purified donor MAIT cells and β-cell lines demonstrated the ability of MAIT cells to kill them (Rouxel et al., 2017). In a mouse model of T1DM, non-obese diabetic (NOD) mice had an increased migration of MAIT cells into the pancreas during development of T1DM, accompanied by an increase in the production of IFNγ and Granzyme B. Interestingly, NOD mice showed changes in the gut during the progression of diabetes. $MR1^{-/-}$ NOD mice displayed a deterioration of gut integrity, favouring translocation of bacteria and possibly ultimately promoting T1DM development (Rouxel et al., 2017). On the other hand, Kuric *et al.* noted no difference in MAIT cell frequency in areas of insulitis or its proximity in patients with early onset of T1DM as compared to pancreas of healthy donors. They reported low expression of Vα7.2 MAIT cell TCR mRNA levels in the pancreas of both healthy and diseased donors (Kuric et al., 2018). These results contradict the previous findings, which suggested a strong role of MAIT cell in the development of T1DM. More research is required to assess the level of involvement of MAIT cells in the development of T1DM.

MAIT cells may also be involved in the development of T2DM, however this area is not very well researched. A reduction in MAIT cell frequencies have been reported in individuals with T2DM (Magalhaes et al., 2015; Zhang et al., 2019). Resting peripheral blood MAIT cells from individuals with T2DM exhibited a more activated phenotype as assessed per CD25 expression and upon stimulation expressed higher levels of IL-2, granzyme B and IL-17. In addition, MAIT cells in the AT of individuals with T2DM displayed enhanced IL-17 production, suggesting their contribution to AT inflammation (Magalhaes et al., 2015). More recent study, by Zhang *et al*. assessed the expression of OX40 by MAIT cells in T2DM. OX40 is a co-stimulatory molecule expressed by T cells, whose engagement leads to their activation and differentiation. This study reported significantly higher frequency of $OX40^{+}$ MAIT cells from individuals with T2DM, which was negatively correlated with MAIT cell frequencies. In addition, engagement of OX40 leads to the induction of apoptosis by MAIT cells, suggesting that over-expression of OX40 may be leading to the diminished frequencies observed in subjects with T2DM (Zhang et al., 2019). The knowledge of MAIT cell activation and function in T2DM remains very limited, therefore more studies are required to identify their role in the development of the disease.

Development of T2DM is closely associated with obesity, and in fact MAIT cells from people with obesity displayed very similar phenotype. A significant reduction in MAIT cell frequencies has been recorded in peripheral blood of people with obesity or obesity and T2DM (Carolan et al., 2015; Magalhaes et al., 2015). As described in detail in the introduction to chapter 4, these MAIT cells displayed an activated phenotype, with an enhanced IL-17 production and a defect in expression of IFNγ in comparison to the lean controls (Carolan et al., 2015; Magalhaes et al., 2015). Although it has been established that MAIT cells are highly dysfunctional in obesity, currently it remains unknown what causes such profound alterations in their frequencies and function. As MAIT cells have been discovered relatively recently, more basic research is required to establish what mechanisms control their activation. This will establish which of the pathways are dysfunctional in MAIT cells from individuals with obesity and hence identify targets which would aim to eradicate those flaws.

1.4 Immunometabolism

For a long time, the function and metabolism of immune cells have been investigated separately, overlooking the strong connection between these two areas. Immunologists have gained more insight into how metabolism of an organism can influence immune cells during the early studies of nutrient deprivation, obesity and obesity related diseases such as T2DM. Although it may seem obvious now, the disruption to nutrient availability and metabolism of such nutrients by immune cells is critical to their function, it hasn't been that evident for many years. One of the first clues that indicated the importance of metabolism in immune cell functions was reported as early as 1950s (Oren et al., 1963; Pachman, 1967; Sbarra AJ and Karnovsky M. L., 1959) however, research in the area didn't escalate until about 10 years ago. This is when immunometabolism emerged as a new exciting area of immunology.

Cell metabolism is very complex and involves multiple pathways, which are closely linked. A defect in one of those pathways may have further downstream consequences on whole cell metabolism and function. The aim of these pathways is to provide energy and biosynthetic intermediates which are required for the cell survival, activation and proliferation (Dimeloe et al., 2017; Munford and Dimeloe, 2019; O'Neill et al., 2016). It has been described that the metabolism of T cells varies greatly depending on their activation state. In naïve T cells in the quiescent state or during memory formation, their energy production is largely supported by the Kreb's cycle and mitochondrial oxidative phosphorylation (OxPhos), which are known to generate large amounts energy through adenosine triphosphate (ATP) production (Munford and Dimeloe, 2019; O'Neill et al., 2016).

Upon stimulation, T cells undergo metabolic reprogramming and rapid upregulation of glycolysis. This is known as the Warburg effect, which was first discovered by Otto Warburg in 1920s in cancer cells (Warburg, 1925). Although OxPhos is upregulated in activated T cells, glycolysis becomes the dominant pathway (Palmer et al., 2015; Sena et al., 2013). Glucose is one of the primary fuels required for cell function. It is obtained from the extracellular environment of immune cells and its uptake is facilitated by glucose transporters such as GLUT1 (Jacobs et al., 2008; Macintyre et al., 2014). Glycolysis breaks down a molecule

of glucose into pyruvate in a series of enzymatically controlled steps. It is considered to be an inefficient source of energy production as it only yields two net ATP energy molecules per one molecule of glucose, however it provides multiple intermediates, which can feed into other metabolic pathways. In this way, glycolysis contributes to the synthesis of nucleotides by feeding into the pentose phosphate pathway (PPP) as well as the synthesis of fatty acids by feeding into the Krebs's cycle or synthesis of amino acids by fuelling the serine synthesis pathway (Munford and Dimeloe, 2019; O'Neill et al., 2016). This shows that the upregulation of glycolysis upon activation is more beneficial to the cell, as it provides it with enough energy, but also yields biosynthetic precursors required for its proliferation and exertion of its functions. Upregulation of glycolysis can also happen more swiftly than the upregulation of OxPhos, providing the cell with the advantage of faster response to stimuli (O'Neill et al., 2016). The importance of glucose and glycolysis in T cell activation was demonstrated by multiple studies. Mice deficient in the GLUT1 receptor on their T cells demonstrated reduced proliferation capacity and impaired glycolysis upon stimulation (Macintyre et al., 2014). Incubation of T cells in media in which glucose was substituted for galactose, resulted in a defect in their IFNγ production (Chang et al., 2013). Stimulation of human T cells demonstrated an increase in glycolysis during the period of 24-72 hours as measured by extracellular acidification rate (ECAR), whereas incubation of T cells with 2-deoxy-d-glucose (2DG), inhibitor of hexokinase 2, the first enzyme of glycolysis, significantly reduced IFNγ production (Renner et al., 2015).

Glycolysis is dependent on mammalian target of rapamycin complex 1 (mTORC1) and complex 2 (mTORC2) activation, although the action of mTORC2 is less understood (Saxton and Sabatini, 2017). Mammalian target of rapamycin (mTOR) plays a central role in T cell metabolism and has multiple functions. It is upregulated upon T cell activation and acts as a nutrient sensor in the cells. It integrates the signals of nutrient status and antigen signalling to orchestrate the activation of appropriate pathways. It detects levels of amino acids such as leucine, promotes lipid synthesis, as well as RNA transcription (Salmond, 2018; Saxton and Sabatini, 2017). Multiple studies have demonstrated the role of mTOR activation in glycolytic metabolism. Inhibition of mTOR activation with rapamycin was shown to impair their expression of glucose transporters GLUT1

and GLUT3 by cytotoxic T lymphocytes and impair glucose uptake (Hukelmann et al., 2016). mTORC1 signalling implicated in aerobic glycolysis is still not very clear, however it is thought to be controlled by the regulation of Myc and HIF1α transcription factors (Finlay et al., 2012; Wang et al., 2011). As mentioned earlier, mTOR is sensitive to amino acid levels. In the past few years, studies have focused on elucidating the role of amino acids in T cell metabolism. It was found that upon activation, cells not only increase the flux of glucose, but the uptake of amino acids is higher (Carr et al., 2010; Nakaya et al., 2014). Stimulation of the cells leads to an increase in the expression of amino acid transporters (Carr et al., 2010; Sinclair et al., 2013). The most well studied amino acid transporter in lymphocytes to date is the L-type amino acid transporter 1 (LAT1). LAT1 is a heterodimer of CD98 and solute carrier (SLC)7A5. It is an antiporter which transports extracellular leucine into the cell in exchange of intracellular glutamine (Hsu and Dzhagalov, 2019). It is known to be upregulated on T cells upon stimulation via TCR/CD28 and is controlled by the expression of Myc (Hayashi et al., 2013; Sinclair et al., 2013). It can transport several amino acids, however it plays a major role in transporting leucine, as leucine is required for mTORC1 activity (Saxton et al., 2016; Sinclair et al., 2013). The role of LAT1 was strongly highlighted in a study by Cantrell lab, which showed the deletion of SLC7A5 in mouse T cells leads to the impairment of cell proliferation and differentiation into CD4 and CD8 cells (Sinclair et al., 2013). Similar results were obtained by Hayashi *et al.* where LAT1 expression on human T cells was disrupted by small interfering RNA (siRNA). They have reported a reduction in the uptake of amino acids and impaired cytokine production by T cells (Hayashi et al., 2013). There is mounting evidence highlighting a role for glutamine in T cell activation and proliferation (Carr et al., 2010; Johnson et al., 2018; Sener et al., 2016). Glutamine is involved in multiple metabolic aspects of the cell. It is transported into the cell via multiple transporters belonging to the solute carrier family including SLC1A5, SLC3A2, SLC7A5 and SLC38A1, however many more glutamine transporters exist (Carr et al., 2010; Nakaya et al., 2014; Wang et al., 2011). Upon entry into the cell, glutamine can be converted to α-ketoglutarate and fed into the Kreb's cycle or it can fuel acetyl Coenzyme A (Acetyl Co-A) production. It can also be used in the generation of glutathione, a natural anti-oxidative or enter the serine biosynthesis pathway (Altman et al., 2016). Glutamine was shown to be utilised by cells upon activation and proliferation

(Carr et al., 2010; Wang et al., 2011). Deprivation of this amino acid during stimulation of naïve CD4 T cells resulted in T reg differentiation, despite Th1 cytokine signal. It was shown that α-ketoglutarate, a metabolite derived from glutamine metabolism, acts as a CD4 T cell differentiation controller, as addition of α-ketoglutarate analogue to the cultured lead to Th1 differentiation (Klysz et al., 2015). This was further confirmed by Johnson *et al.* who found that the inhibition of glutaminase, an enzyme which converts glutamine to glutamate, promoted T cell differentiation into Th1 type, while impairing Th17 differentiation, indicating Th17 cells are dependent on glutaminase and glutamine influx during cell activation (Johnson et al., 2018). These studies highlight the importance of amino acids, not only as building blocks required for cell function, but as a signalling molecule which together with the external stimulation signals largely impact the cell function.

Identifying this close relationship between immune cell function and metabolism opened a new avenue in targeting immune cells in inflammatory diseases. As mentioned before, inhibition of glycolysis with 2DG has profound effects on T cell function and leads to the impairment of its activation. The effects of the inhibitor have been further explored and studied by Yin *et al*. in a mouse model of SLE. Combined treatment of the SLE mice with 2DG, the glycolysis inhibitor and metformin which was shown to impair OxPhos by inhibiting the complex I of electron transport chain, resulted in the downregulation of metabolism in T effector cells, with no effect in the control mice (Owen et al., 2000; Wheaton et al., 2014; Yin et al., 2015). However, since then, the mode of action of metformin has been under review (Wang et al., 2019). Yin *et. al* also found a decrease in the production of anti-nuclear antibodies and anti-double stranded DNA IgG, leading to remittance of the SLE phenotype (Yin et al., 2015). Liu *et al*. investigated the effect of 2DG in a mouse model of Guillain-Barre syndrome, an inflammatory demyelinating disease of the peripheral nervous system. They found that the administration of 2DG prevented progression of the disease and inhibited its initiation (Liu et al., 2018). Treatment with 2DG was also applied to the NOD mouse model of an autoinflammatory diabetes and resulted in an increase in β cell granularity and a reduction in T cells reactive to diabetes antigen, the islet-specific glucose-6-phosphate catalytic subunit-related protein (IGRP) (Garyu et al., 2016). As GLUT1 plays a very important role in immune cell

function, inhibition of GLUT1 mediated transport of glucose is another potential target in treatment of inflammatory diseases. Inhibition of GLUT1 with WZB117 inhibitor in early-stage memory T cells derived from patients with T1DM inhibited the expansion of this cell subset (Vignali et al., 2018). The investigations of using glycolysis inhibitors to treat autoimmune diseases are in very early days. mTOR inhibitors such as Sirolimus are currently approved for use in the clinics to prevent organ transplant rejection (Nguyen et al., 2019). A recent clinical trial of Sirolimus in active SLE over 12 months found an expansion in Treg and CD8 memory T cell populations in the treated patients and improved disease activity (Lai et al., 2018). Administration of rapamycin in a murine model of EAE has also shown promising results. The treatment increased the percentage of Treg cells in the rapamycin treated group versus untreated. The percentage of Th17 cells, which are considered to be highly pathogenic in MS and EAE, were found to be decreased in the rapamycin treated group as well as infiltration of IL-17 expressing cells in the spinal cord of the EAE mice was reduced in comparison to the controls (Li et al., 2019). These studies show a potential in controlling inappropriate immune cell activation, as is observed in diabetes and obesity. Although these results are very promising, these approaches must be considered very carefully, not only immune cells use glycolysis and mTOR to exert their functions. The side effects of these drugs could be very widespread, causing increased susceptibility to infections and cancer development, due to the dampening of the immune system. The effect of these inhibitors must also be considered on cells in other tissues and organs, as widespread inhibition of such vital life pathways like glycolysis, may impair the function of whole organs.

Metabolism has been shown to be an integral part of the immune cell function; however, upon undertaking this thesis no data has been published on the metabolism of MAIT cells. In this thesis we aimed to identify the metabolic requirements of these cells by investigating the role of glycolysis and glycolysis related pathways in MAIT cell activation. We further examined how these pathways are involved in supporting cytokine production, proliferation and expansion of these cells. It has been established in the literature that people with obesity present with a severe impairment of MAIT cell function in comparison to the control population (Carolan et al., 2015; Magalhaes et al., 2015). Therefore, this study also aimed to investigate the metabolism of MAIT cells from people

with obesity and compare it to control individuals in order to determine whether there are any differences, which may be negatively affecting MAIT cell function. Identification of any alterations could open new avenues of targeted treatments to restore MAIT cell function in individuals with obesity.

Chapter Two

Materials and Methods

2.1. Ethics

Ethical approval for this study and collection of samples from people with obesity and healthy controls was granted by Medical Research Ethics Committee from St. Vincent's University Hospital Dublin. Local ethics were also obtained from Maynooth University Research Ethics committee.

2.2. Study Design

This was a cross-sectional investigative comparative study. Control blood samples were obtained from St. Vincent's University Hospital Dublin and Irish Blood Transfusion Service. People with obesity were recruited from the Weight Management Clinic in Loughlinstown (part of the St. Vincent's Healthcare group). All recruited control individuals (apart from blood samples obtained from IBTS) and people with obesity were recruited into the study after giving informed and written consent. The variation in the number of samples obtained from the control individuals and people in obesity occurs due to the variability in sample availability and sample quality for each of the groups. As control samples were more accessible and of better quality, than samples from people with obesity, more individuals have been included in the control groups for each experiment.

2.3. Isolation of human peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated using gradient density centrifugation from venous blood samples collected from control lean individuals or people with obesity into lithium heparin coated tubes (vacutainer, BD Bioscience); or from donated buffy coats from the Irish Blood Transfusion Service. The blood was diluted 1:1 with sterile phosphate buffered saline (PBS, Sigma Aldrich) for venous blood samples and 1:5 for buffy coats and carefully layered on top of 15mls of Lymphoprep™ medium (StemCell Technologies) in a 50mls tube (Sarstedt) (Figure 2.1). The blood preparation was centrifugated at 1800 rpm for 20 minutes, with low acceleration and break turned off as shown in Figure 2.1. After centrifugation, the buffy layer was removed into a new sterile tube and washed twice in 20mls of PBS at 1800 rpm for 8 minutes with high acceleration and deacceleration. Depending on the pellet size, cells were resuspended in a volume of complete RPMI (cRPMI) 1640 1X (GIBCO, ThermoFisher Scientific) containing 2%v/v HEPES buffer solution 1M (GIBCO), 10% v/v heat inactivated foetal calf serum (FCS) (GIBCO), 1X antibiotic-antimycotic (100untis/mL of penicillin, 100μg/mL of streptomycin, 250ng/mL Gibco Amphotericin B) (GIBCO) 1mM Sodium Pyruvate and non-essential amino acids diluted to 1X(GIBCO)) and the cells were counted as per section 2.4 and Figure 2.2.

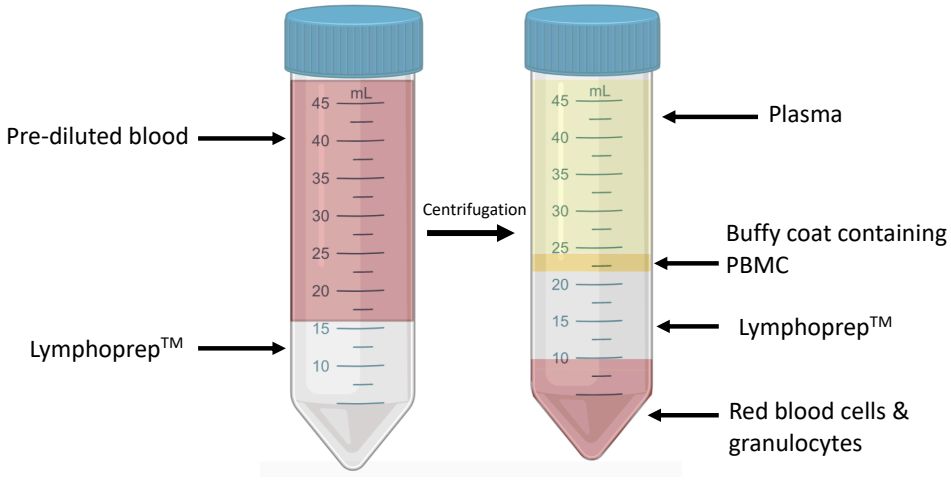


Figure 2.1 Isolation of peripheral blood mononuclear cells. A diagram showing layering of the blood and compartmentalisation of the blood cells after centrifugation. Image created with BioRender.

2.4. Cell enumeration

The cells were stained using trypan blue solution (GE Healthcare) using 1:10 or 1:20 dilution and 10µl of the stained cell suspension was applied to a haemocytometer counting chamber. Using a dye exclusion test, cells which didn't take up the dye (white in colour) were considered to be viable and only those cells were counted in the middle square of the haemocytometer as shown in figure 2.2. To calculate the number of the cells in suspension per 1mL the following formula was used:

$$Cells/mL = Cell\ count\ per\ middle\ square \times\ dilution\ factor\ \times 10^4$$

The cells were resuspended at 2×10^6 cells/mL in cRPMI for further analysis.

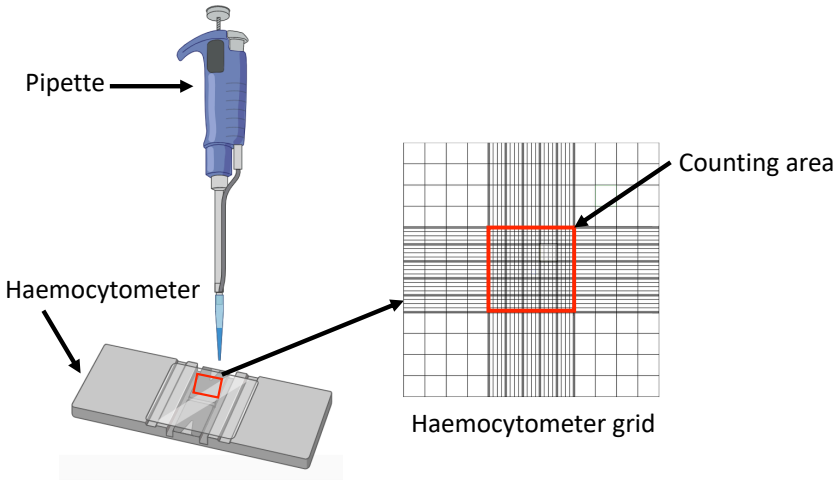


Figure 2.2 PBMC counting. Trypan blue stained PBMC were applied to the haemocytometer and counted as per diagram. Image created with BioRender.

2.5. MAIT cell proliferation and expansion

2.5.1. Cell Trace Violet Staining

MAIT cell proliferation was measured with CellTrace™ Violet (CTV) cell proliferation kit for use on flow cytometer (ThermoFisher Scientific). The required number of freshly isolated PBMC was pelleted by centrifugation at 1800rpm for 8 minutes and the supernatant was completely removed. CTV solution was prepared immediately prior to use, by adding 20µL of dimethyl sulfoxide (DMSO) to the supplied vial with the stain, to obtain 5mM stock. Next, the dye was diluted to 5µM working concentration by adding appropriate amount of the stock solution

into pre-warmed PBS. The cells were gently resuspended and stained at 10^6 cells per mL of the PBS-dye solution. Cells were incubated for 20 min at room temperature, protected from light with circular agitation. Next, five times the original staining volume of cRPMI was added to the tubes and incubated for 5 minutes at room temperature, which allowed for the removal of any unbound dye. The cells were pelleted at 1800rpm for 8 minutes and resuspended in warm cRPMI culture media at $2x10^6$ cells per ml and re-counted. Cells were incubated for at least 10 minutes to allow acetate hydrolysis of the dye, before proceeding with the experimental set up.

2.5.2. TCR only induced MAIT cell expansion & proliferation over 5 days

PBMC were stained with CTV as per section 2.5.1 for assessment of MAIT cell proliferation, or PBMC remained unstained for measurement of MAIT cells expansion. A sample of $0.25\text{-}0.5x10^6$ cells was set aside to stain for flow cytometry to measure MAIT cell frequency or CTV uptake prior to proliferation or expansion. Next, PBMC were plated at $1x10^6$/mL in a 48 well flat bottom plate, in a total volume of 1mL of cRPMI. Cells were stimulated with 1μg/mL of 5-ARU (synthesised by and received from Professor Gurdynal Besra) and 100μM of MG (Sigma) for 18 hours. On day 1 (18 hours after stimulation), 50μL of the media was removed from all the wells and replaced with 50μL of fresh complete media. Replacement of 50μL of the media was carried out, to ensure consistency between these experiments and experiments of MAIT cell expansion and proliferation, which were supplemented with IL-2 on day 1 in 50μL volume (please see section 2.5.4). On day 4, 500μL of media was removed and replaced with 500μL of fresh complete media. On day five, each well was resuspended by pipetting and 100μL of cells were removed and stained for flow cytometry as per section 2.10.5 MAIT cell frequencies were determined as the percentage of $CD3^+$ cells whereas CTV was on MAIT the cell population using geometric mean.

2.5.3. TCR and IL-2 induced MAIT cell expansion & proliferation over 5 days

CTV stained (proliferation) or unstained (expansion) PBMC were plated at $1x10^6$/mL in a 48 well flat bottom plate, in a total volume of 1mL of cRPMI. Cells were stimulated with 1μg/mL of 5-ARU and 100μM of MG for 18 hours in the presence or absence of the following inhibitors: 2DG (10mM) (Sigma), rapamycin

(20nM) (Sigma), oligomycin (2μM) (Sigma), antimycin A (4μM) (Sigma) or CB-839 (10μM) (Sigma). To assess the effect of 2-amino-2-norbornanecarboxylic acid (BCH) (50mM) (Sigma) on MAIT cell proliferation and expansion, the same experimental procedure was followed, except the complete media was diluted with Hanks balanced salt solution (HBSS)(Gibco) in 1:2 ratio. On day 1 (18 hours after stimulation), 750μL of the media was removed from all the wells and replaced with 750μL of fresh complete media supplemented with low dose of IL-2 (6.75ng/mL). On day 4, 500μL of media was removed and replaced with 500μL of fresh complete media supplemented with high dose of IL-2 (33.33ng/mL). On day 5, 18 hours post high dose IL-2, 100μL was removed from each well and stained for flow cytometry as per section 2.10.6 (Figure 2.3). MAIT cell frequencies and CTV uptake and dilution were assessed prior to expansion and on day 5 as described in section 2.5.2. To assess the effect of nutrient restriction on MAIT cell proliferation and expansion, the cells were stained (proliferation)/unstained (expansion) and the same protocol was followed as described above, with the exception that for the duration of the experiment cells were kept in their respective nutrient restricted media. i.e., 10mM, 5mM and 1mM glucose or 10mM galactose.

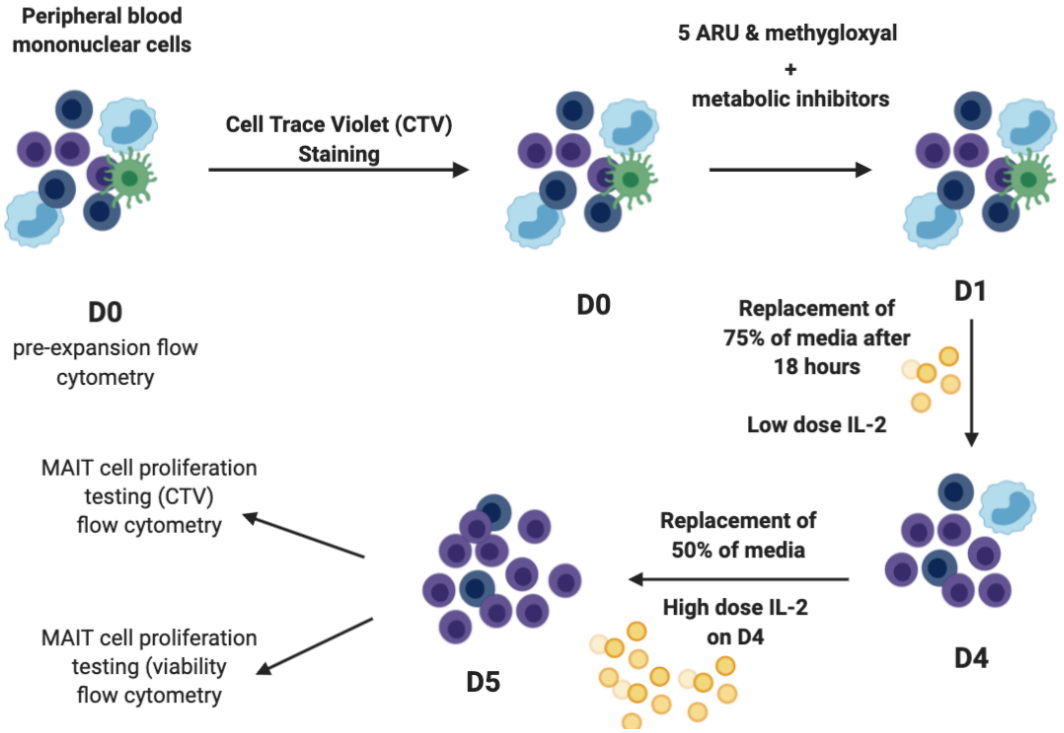


Figure 2.3 Plan of MAIT cell proliferation experiment. Schematic showing the experimental set up to investigate the effect of metabolic inhibitors on MAIT cell proliferation. Image created with BioRender.

2.5.4. TCR & IL-2 induced MAIT cell expansion over 7 days

PBMC were plated at $1x10^6$/mL in a 48 well flat bottom plate, in a total volume of 1mL of cRPMI. A sample of $0.25\text{-}0.5x10^6$ cells was stained for flow cytometry prior to expansion of MAIT cells. Cells were stimulated with 1μg/mL of 5-ARU and 100μM of MG for 18 hours. On day 1 (18 hours after stimulation), 50μL of the media was removed from all the wells and replaced with 50μL of fresh cRPMI supplemented with low dose IL-2 media (6.75ng/mL). On day 4, 500μL of media was removed and replaced with 500μL of fresh cRPMI supplemented with high dose IL-2 media (33.3ng/mL). On day 7, each well was resuspended by pipetting and a sample of cells was removed for counting. Next 100μL of cells were removed and stained for flow cytometry as per section 2.10.6. Based on cell counts and MAIT cell percentage in samples pre- and post-expansion, absolute numbers of MAIT cells in the culture were calculated. Fold expansion was calculated by dividing the number of MAIT cells present in the culture on day 7 by the number of MAIT cells prior to stimulation.

2.5.5. TCR & IL-2 induced MAIT cell expansion – 28 days

PBMC were plated at $2x10^6$ cells in a 24 flat well plate in a total volume of 2mL of cRPMI. Cells were stimulated with 1μg/mL of 5-ARU and 100μM of MG for 18 hours. Following the stimulation, on day 1, low dose of IL-2 at 6.75ng/mL was added into each well, followed by a high dose of IL-2 at 33.3ng/mL on day 4,7,11,14,18 and 21. During the high dosage feeding, 1mL of supernatant was removed and replaced with fresh media containing the high dose of IL-2. Expansion of MAIT cells was checked prior to expansion and on day 4,7,11,14,18,21 and 28 by flow cytometry as per table 2.10.5.

2.5.6. MAIT cell expansion for Seahorse, ELISA and real time RT-qPCR

PBMC were plated at $2x10^6$ cells in a 24 flat well plate in a total volume of 2mL cRPMI. Cells were stimulated with 1μg/mL of 5-ARU and 100μM of MG for 18 hours. Following the stimulation, on day 1 low dose of IL-2 at 6.75ng/mL was added into each well, followed by a high dose of IL-2 at 33.3ng/mL on day 4,7,11 and 14. Expanded MAIT cells were harvested between day 13 and 18 and the culture was purified using magnetic isolation as per section 2.6.1 and 2.6.2

2.6. Preparation of expanded MAIT cells for experiments

2.6.1. MAIT cell magnetic labelling

Expanded MAIT cells were removed from the culture between day 13 and 18 for purification. The cells were counted as previously described in section 2.2 and the desired number of cells were removed for isolation. The cells were centrifuged at 300g for 10 min (as all centrifugation steps in this protocol) to remove all media and then spun in Auto MACS running buffer (BSA/EDTA/0.9%Azide/PBS) (Miltenyi Biotec) using 2mL of the buffer per 10^7 of cells. The supernatant was completely removed, and the cells were resuspended in 100μL of MACS buffer per 10^7 of cells. 10μL of APC anti-human TCR Vα7.2 conjugated antibody (BioLegend) was added per 10^7 of cells, mixed well and incubated for 10 min in the fridge. The cells were washed with MACS buffer as above and after removing the supernatant, the cells were resuspended in 80μL of MACS buffer per 10^7 of cells and 20μL of Anti-APC Microbeads (Miltenyi Biotec) were added per 10^7 of cells. Cells were incubated for further 15min in the fridge, washed as above and resuspended in 500μL of MACS buffer.

2.6.2. MAIT magnetic separation

To isolate the labelled MAIT cells, LS columns (Miltenyi Biotec) and a magnetic separator (Miltenyi Biotec) were used. The LS columns were carefully placed into the magnet holders and prepared by rinsing with 3mL of MACS buffer. Next 500μL of the labelled cell suspension was applied to the column, which was then rinsed three times with 3mL of MACS buffer. The fraction of unlabelled cells was collected and discarded. To recover the labelled cells of interest, the column was removed from the separator and placed over a suitable tube. 5mL of MACS buffer as added into the column reservoir and immediately flushed out with the plunger supplied with the column. The cells were spun out of the MACS buffer via centrifugation at 300g for 10mins, resuspended in full media and counted. The purity of the isolated cells was determined by extracellular staining as per section 2.4.3 and run on the Attune NxT flow cytometer. Purity for purifications exceeded 95%.

2.7. MAIT cell molecular analysis

2.7.1. mRNA expression experimental set up

MAIT cells were expanded, harvested and purified as per sections 2.5.5, 2.6.1 and 2.6.2. Isolated MAIT cells were plated at $3x10^6$ per well in a 24 flat bottom plates in a total volume of 1mL of cRPMI and rested overnight. Next, cells were stimulated for 4 hours with TCR microbeads (Dynabeads™ Human T activator CD3/CD28, prepared as per manufacturer's instructions) (ThermoFisher Scientific) in 1:1 ratio of beads to the number of cells and with IL-12/IL-18 at 50ng/mL each (BioLegend) (Figure 2.4) in a total volume of 1.5mL. At the end of the stimulation period, the cells were removed into 2ml sterile tubes (Sarstedt) and centrifuged at 400g for 10 minutes. The supernatant was removed, the pellets were resuspended in 500μL of RNA Later™ Stabilisation solution (Invitrogen) and stored at 4°C overnight, before freezing at -20°C for long term storage.

For assessment of the effect of BCH inhibition on MAIT cell activation on the molecular level, expanded and purified MAIT cells rested overnight in a flask. The following day, cells were recounted, resuspended in cRPMI and plated at $3x10^6$ per well in a 24 flat bottom plates. BCH (50mM) or HBSS was added to appropriate wells for 1 hour prior to MAIT cell activation, ensuring that the media is diluted 1:1 with HBSS for the duration of the experiment. After 1 hour, MAIT cells were stimulated with TCR Dynabeads and cytokines (IL12/18 at 50ng/ml) in a total volume of 1.5mL for further 4 hours and harvested as described above.

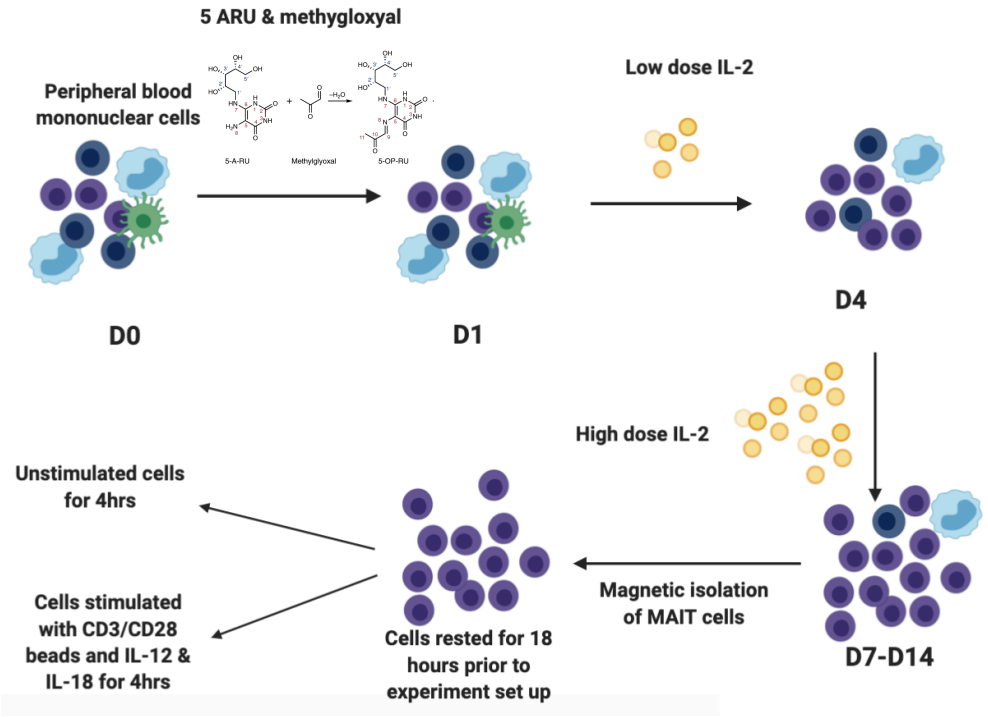


Figure 2.4 Diagram showing experimental plan for analysis of MAIT cell mRNA expression. Experimental set up used to investigate the expression of mRNA by MAIT cells upon stimulation. Image created with BioRender.

2.7.2. mRNA isolation

Isolation of mRNA from MAIT cells was performed using E.Z.N.A Total RNA kit I (Omegabio-tek). Manufacturer's protocol was followed. Briefly, HiBind RNA Mini Column were inserted into the provided 2mL collection tube and labelled. MAIT cells in RNA Later™ were pelleted at 10,000g for 5 minutes and the supernatant was removed. Next 350μL of TRK Lysis buffer was added to the pellet and the cells were homogenised by pipetting and vortexing, followed by the addition of 350μL of 70% ethanol (Sigma) (Figure 2.5). Suspension was vortexed thoroughly and the total volume of 700μL of the sample was transferred to the HiBind RNA Mini Column, which was centrifuged at 10,000g for 1 minute. The filtrate was discarded, and the membrane was washed with 500μL of RNA Wash Buffer I, followed by centrifugation at 10,000g for 30 seconds. Next, 500μL of RNA Wash Buffer II was added to the columns. The tubes were centrifuged for 10,000g for 1 minute and the filtrate was discarded. Wash with RNA Wash Buffer II was repeated once more, as above. Then the columns were spun on maximum speed for 4 minutes to dry the HiBind RNA Mini Column membrane. Next the columns were transferred into labelled 1.5mL RNAse free tubes and 40μL of DEPC water

was applied straight to the middle of the membrane. The tubes were centrifuged at maximum speed for 2 minutes to elute mRNA (Figure 2.5).

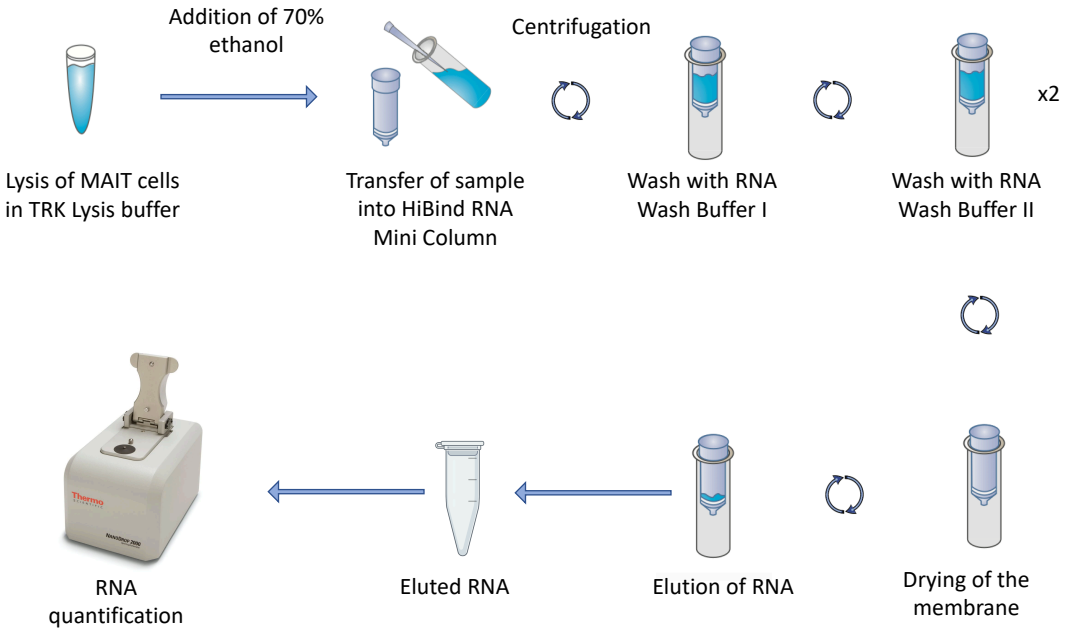


Figure 2.5 Isolation and quantification of RNA. Diagram depicting steps involved in RNA isolation using E.Z.N.A Total RNA kit I and RNA quantification with Nanodrop2000. Adapted from E.Z.N.A. Total RNA kit I manual.

2.7.3. mRNA quantification

After mRNA isolation, mRNA was quantified using Nanodrop™ 2000 spectrophotometer (ThermoFisher Scientific). Nanodrop software settings were set up to measure RNA nucleic acids and the instrument was blanked using 1µL of DEPC water. All samples were measured and concentration of RNA (µg/µL) and 260:230 ratios were noted. RNA was diluted to the desired concentration, uniform for each batch of the samples between 35ng-100ng/µL, depending on the initial concentration of RNA in the samples.

2.7.4. cDNA synthesis

Synthesis of cDNA was performed using qScript cDNA Synthesis kit (QuantaBio). Each reaction contained 500ng of RNA. Total volume of each reaction was equal to 20µL, composed of up to 15µL of RNA (if RNA was very concentrated this volume was made up to 15µL with molecular grade nuclease free water), 4µL of concentrated master mix (tittered primer blend, qPCR optimised dNTP blend, flexible magnesium titration) and 1µL of qScript reverse transcriptase. The cDNA

reaction was prepared in 0.2mL Corning Thermowell GOLD PCR tubes (Sigma) and ran on G- Storm Thermal Cycler (Gene Technologies Ltd). First the samples were heated up to 22^{o}C for 5minutes, next the temperature was increased to 42^{o}C for 30 minutes and 85^{o}C for further 5 minutes, before the temperature was dropped to 0^{o}C. After cDNA synthesis, samples were diluted 1:5 with molecular grade water (Sigma) and kept at -20^{o}C.

2.7.5. Realtime RT-qPCR

Real time RT-qPCR was performed using PerfeCTa SYBR Green FastMix Reaction Mix (Green Fastmix, ROX^{TM}) (QuantaBio) and primers as per table 2.1 below (Sigma). Primers were resuspended in molecular grade water (Sigma) as per manufacturer's instructions to obtain a stock of 100μM, and then further diluted with molecular grade water to 4μM working solution. A master mix of the SYBR Green and primers was prepared, which contained 5μL of SYBR Green Enzyme Mix, 1.25μL of forward primer, 1.25μL of reverse primer, 0.5μL of molecular grade water per sample. 8μL of the master mix was added into appropriate wells in the 96 well reaction plate (MicroAmp Fast 96-well reaction plate (0.1mL) (Life Technologies) and 2μL of the cDNA template was added. After addition of the master mix and cDNA template, the plate was covered with an adhesive coverslip (MicroAmp Optical Adhesive film) (ThermoFisher Scientific) and spun for 5 seconds at 1800rpm. The plate was loaded into a PCR instrument (Applied Biosystems) and appropriate program was used as follows. The temperature was increased to 95^{o}C for 30 seconds, this was followed by the cycle of 95^{o}C for 10 seconds, 57^{o}C for 15 seconds and 70^{o}C for 10 seconds. This cycle was repeated 45 times for all primers and tests. Samples were run in duplicate for each treatment and target. A melt curve was also performed from 60^{o}C to 95^{o}C at 2^{o}C steps to assess primer integrity. Controls genes used included IPO8 (coding for nuclear import protein, which transports miRNA), TBP (coding for TATA-box binding protein; transcription factor) and RPL13A (coding for a ribosomal component), based on a study by Ledderose *et al*. and recommendations from Doreen Cantrell's laboratory, Dundee (Ledderose et al., 2011). Results were analysed using ΔΔCT method. Average of the three control genes was used to normalise the expression of the gene of interest to the housekeeping genes. Results of the control subjects and people with obesity were further normalised to their own basal expression.

Table 2.1. Sequences of forward and reverse primers

Name	Forward sequence 5'-3'	Reverse sequence 5'-3'
TBP	GCCAAGAGTGAAGAACAG	GAAGTCCAAGAACTTAGCTG
IPO8	CCACTTGACCTTGACAATAG	CAATCTTCTTCTTTGCCTCTG
RPL13A	GTCTGAAGCCTACAAGAAAG	TGTCAATTTTCTTCTCCACG
SLC2A1	AGTTCTACAACCAGACATGG	CAGGTTCATCATCAGCATTG
SLC2A3	TCACTATTTAGGAGCCTACG	CTATGAAATGCTCATCCTTGG
SLC2A4	CTATGAAATGCTCATCCTTGG	TCAGAATGCCGATAACAATG
SLC3A2	CACTGATTATGACTGTGGAC	CCAGAGCATCCTATTTTAGAG
SLC7A5	ACATTGTGCTGGCATTATAC	CTGTAGGGGTTGATCATTTC
SLC7A8	GTTCAAGACATCTTCACAGC	CCTTTGCATATCTGTACAATCC
SLC43A1	CATGTGTCTGTGTATGTGTG	TTTTCAGTCTCTCACTTCCC
SLC43A2	CTCAACTGCTTCTTTAACTGG	CAGCTGAACTTGATCTTCAC
SLC1A5	GTAAAGATCATCACCATCCTG	CCAGGATCAAGGAGATATGG
SLC38A1	CTTGTTGGTGATCTTCATACC	AGCCCAAATTCTTTGAGTTC
SLC38A2	TGTTCTTTCTGATTGTGGTC	TGCAAGAGTCATTTTCAGTC
HK2	GAAAGCAACTFTTTGAGAAG	CAATGTCTGAGATGTCTTTGG
PKM	ATGGTTGATATGGTGTTTGCG	ATGTTGATATGGTGTTTCGC
IFNY	CATTCAGATGTAGCGGATA	ATTCATGTCTTCCTTGATGG
IL17A	CATTGGTGTCACTGCTAC	TCGGTTGTAGTAATCTGAGG
IL17F	TGGAATTACACTGTCACTTG	GAACGGAATTCATGGAGAGT
IL22	CTGTGAGCTCTTTCCTTATG	AACGTCTGTGTTGTTATCAG
IL32	AATGCAAAATGCAGAATCAG	GTAGAGGAGTGAGCTCTG
TBX21	CTTTCCAAGAAACCCAGTTC	GTCAACAGATGTGTACATGG
RORC	TTTTGAAGGCAAATACGGTG	AGTGGGAGAAGTCAAAGATG

2.8. ELISA

2.8.1. ELISA Experimental set up

MAIT cells were expanded and purified as per section 2.5.5, 2.6.1 and 2.6.2. Next MAIT cells were counted and plated at $1x10^6$ per well, in 48 flat bottom well plate (Sarstedt) in a total volume of 1mL. To assess the metabolic requirements for MAIT cell function, cells were stimulated with TCR Dynabeads 1:1 bead to cell ratio (Dynabeads™ Human T activator CD3/CD28) (ThermoFisher Scientific) and with IL-12/IL-18 at 50ng/mL each (BioLegend) in the presence or absence of 2DG (10mM), Rapamycin (20nM) and CB-839 (10μM) in cRPMI for 24 hours. To investigate the requirement of amino acid transport via LAT1, cells were stimulated with TCR Dynabeads (Dynabeads™ Human T activator CD3/CD28)

(ThermoFisher Scientific) and with IL-12/IL-18 at 50ng/mL each (BioLegend) in the presence or absence of BCH (50mM) for 24 hours. For this experimental set up the 1mL volume of cells consisted of complete RPMI diluted 1:2 with HBSS. Following the 24-hour stimulation, the plate was spun at 300g for 10 minutes and 900μL of the supernatant was collected and frozen.

2.8.2. ELISA testing

Prior to the testing all the reagents were resuspended as per manufacturer's instructions for IFNγ, IL-17, IL-17F, IL-22, IL-32 ELISA kits (DuoSet ELISA development systems; R&D). Capture antibody was diluted to the required working concentration in PBS. NUNC-Immuno 96 well plate was immediately coated with 50μL of the diluted antibody per well (ThermScientific). The plate was sealed and incubated at room temperature overnight. As per figure 2.6, the following day the plate was washed with a wash buffer (PBS & 0.05% Tween20). Each well was filled with approximately 300μL of the wash solution. This process was repeated three times and was followed by gentle tapping of the plate against a clean towel to blot any residual fluid out of the wells. Every wash step in this protocol was performed as described here. Next the plate was blocked using 150μL of reagent diluent (1% bovine serum albumin (BSA) in PBS; filtered) per well and incubated at room temperature for an hour. Next the standards and samples were prepared. Standard curve samples were prepared by adding appropriate amount of the reconstituted standard into a total volume of 1mL of reagent diluent to achieve the recommended concertation of top standard for each separate analyte measured. Next, a six-point serial dilution of the top standard was performed in reagent diluent and for the last standard point reagent diluent was used. If required, samples were diluted in reagent diluent in a 96 well plate. Following the blocking step, the plate was washed and blotted as descried above and 50μL of standards or samples was added to the appropriate wells. The plate was incubated in the fridge at 4^{o}C overnight. Next day, the plate was washed, and the detection antibody was diluted to the recommended concentration. 50μL of the solution was added into all wells and the plate was incubated for two hours at room temperature and then washed. HRP-Avidin was diluted to the recommended concentration and 50μL of the solution was added into the wells. The plate was incubated at room temperature, protected from light for 20 minutes, which was followed by the wash step. Next TMB substrate

solution was prepared by adding equal volumes of Substrate A and Substrate B (BioLegend) and 50µL of the solution was added to the wells. The plate was incubated at room temperature protected from light up to 20 minutes and the reaction was stopped using 50µL of stop solution (1M Sulphuric acid) (Sigma-Aldrich). The plate was lightly tapped to ensure mixing of the reagents. Optical density was determined using a microplate reader (ThermoFisher) and measurements were taken at 450nm and 540nm. Raw data was analysed in Prism 9 Software.

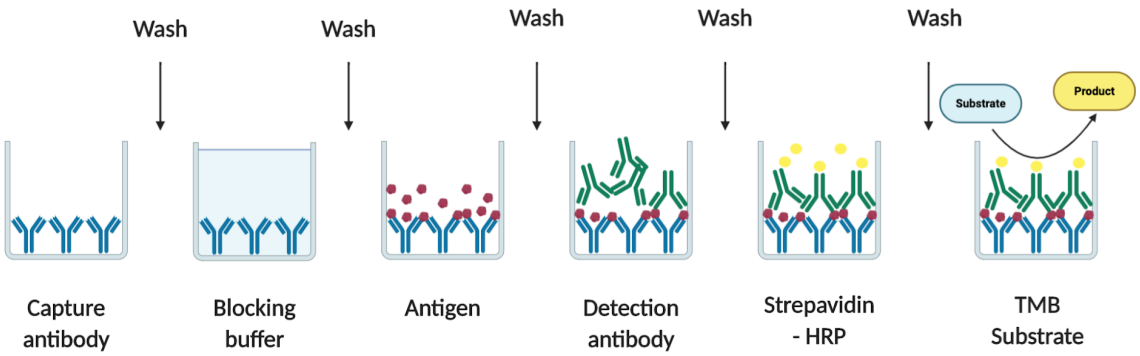


Figure 2.6 ELISA steps. Diagram depicting steps involved in ELISA testing using R&D Duoset ELISA kit. Image created with BioRender.

2.9. Seahorse

2.9.1. Seahorse experimental set up

MAIT cells were expanded and purified as per section 2.5.5, 2.6.1 and 2.6.2. Once successfully expanded, the cells were removed from the culture and plated in a 24 well plate at $2x10^6$ per well in a total volume of 1mL. To investigate the glycolysis levels in control individuals and people with obesity, cells were plated in complete RPMI and stimulated with IL-12/IL-18 each at 50ng/mL and TCR microbeads at 25ng/mL (T cell Activation/Expansion kit, prepared as per manufacturer's instructions) (Miltenyi Biotec) for 18 hours. To investigate the impact of inhibition of amino acid influx, the cells were plated in complete media which was diluted 1:2 with HBSS. To the inhibitor well, BCH (50mM) was added and the cells were stimulated as above.

2.9.2. Seahorse protocol

Seahorse XFe96 sensor cartridge (Agilent) was hydrated with 200µL per well with Seahorse XF Calibrant Solution (Agilent) and left at 37°C in a non-CO_2 incubator for 18 hours prior to use. Seahorse XF phenol-free DMEM media, pH 7.4 (Agilent) was prepared by adding glucose and glutamine to final concentration of 10mM

and 2mM respectively. This media has limited buffering capacity, therefore is sensitive to any changes in the pH, which is used to calculate ECAR. Media was kept at 37°C. Next Cell-Tak (Agilent) for 96 well plate was prepared at a final concentration of 3.5μg/cm^2. 2979μL of sodium bicarbonate (Sigma) at 0.1M pH8, 16.7μL of cell-tak at 1.85mg/ml (Agilent), and 4.65μL of 1M sodium hydroxide (Sigma-Aldrich) were mixed and 25μL of the solution was added to each well of the XF96 cell culture microplates plate (Agilent). The plate was incubated for 20 minutes at room temp. Next Oligomycin (Sigma), FCCP (Sigma), Rotenone (Sigma), 2DG (Sigma) inhibitors were prepared as per table 2.2 and kept on ice. Then, cells were harvested, counted and the exact number of cells was removed from each sample treatment. The cells were pelleted and washed twice in 500μL of pre-warmed seahorse DMEM media at pH 7.4 (Agilent) at 1800rpm for 8 minutes and resuspended at 2x10^5 cells per 180μL of pre-warmed seahorse media. The cells-tak was washed with sterile deionised water (Baxter), by adding 170-200μL of water into each well and aspirating water with p200 multichannel pipette. Ensure no droplets of water remain in the wells before proceeding. The samples were plated in quadruplicates at 2x10^5 cells per 180μL per well. 180μL of seahorse media was also added to all unused wells. The plate was spun at 300g without break for 3 minutes and the wells were check under the microscope to ensure even monolayer of the cells in the wells. The plate was left in a non-CO_2 incubator, while the inhibitors were loaded into the injection ports of the sensor cartridge in the following order - oligomycin, FCCP, Rotenone, 2DG as per table 2.2, using template guide. The plate was calibrated on the Seahorse XF instrument (Agilent), the location of the sample wells was defined, and protocol was selected. The protocol used to run was defined as adding 4 injections of the inhibitors into the cells in the order of oligomycin, FCCP, Rotenone and 2DG. Once the calibration was completed, the plate with the cells was loaded onto the instrument. The results were exported into prism file and analysed in prism.

Table 2.2. Seahorse inhibitors injection. A table showing injection ports sequence and final concentrations of the inhibitors in the seahorse wells.

Ports	Compounds	Working stock	Starting well volume	Injecting Volume	Final concertation
Port A	Oligomycin	20μM	180	20	2μM
Port B	FCCP	10μM	200	22	1μM
Port C	Rotenone	1.21μM	222	20	0.1μM
Port D	2DG	393mM	240	20	30mM

2.10. Flow Cytometry

2.10.1. MAIT cell glycolytic metabolism

Isolated PBMC plated at 0.5-1x10^6 (depending on the experiment) in a 96 round bottom well plate (Sarstedt) in a total of 250μL of complete RPMI per well for analysis of MAIT cell metabolism associated proteins. To assess expression of GLUT1, CD98, HKII, LDHA and PKM2 and cells were incubated in the media alone (control) or with IL-12/IL-18 each at 50ng/mL and TCR microbeads at 25ng/mL (T cell Activation/Expansion kit, prepared as per manufacturer's instructions) (Miltenyi Biotec) or TCR Dynabeads 1:1 bead to cell ratio (Dynabeads^{TM} Human T activator CD3/CD28) for 18 hours at 37^{o}C. After 18 hours, the cells were harvested and stained for flow cytometric analysis as per table 2.3 and section 2.10.5 or 2.10.6 using extracellular or extracellular and intracellular staining protocols.

2.10.2. MAIT cell amino acid requirements for activation

To assess MAIT cell cytokine production, PBMC were plated at 0.5-1x10^6 in a 96 round bottom well plate (Sarstedt) in a total volume of 250μL. To investigate IFNγ production, PBMC were incubated in cRPMI with 12/IL-18 each at 50ng/mL and TCR Dynabeads 1:1 bead to cell ratio (Dynabeads^{TM} Human T activator CD3/CD28) for 18 hours at 37^{o}C in the presence of 1x of protein transport inhibitor cocktail (Brefeldin A and Monensin) (Invitrogen). To investigate the role of amino acid influx on MAIT cell metabolism and function, PBMC were plated as above in full media diluted 1:2 with HBSS for investigating the role of amino acid influx on MAIT cell metabolism and function. Cells were incubated in cRPMI:HBSS alone (control) or with IL-12/IL-18 (each 50ng/mL) and TCR microbeads at 25ng/mL (T cell Activation/Expansion kit, prepared as per manufacturer's instructions) (MiltenyiBiotec) in the presence or absence of BCH (50mM) for 18 hours at 37^{o}C in the presence of the 1x of protein transport inhibitor

cocktail (Brefeldin A and Monensin) (Invitrogen). After 18 hours, the cells were harvested for extracellular and intracellular flow cytometric staining.

2.10.3. Expression of CD25

To investigate the effect of MAIT cell activation on the high affinity IL-2 receptor (CD25) expression PBMC were plated at $0.5x10^6$ in a 96 round bottom well plate (Sarstedt) in a total of 250μL of complete RPMI per well. Cells were incubated in the media alone (control) or were stimulated with TCR Dynabeads 1:1 bead to cell ratio (Dynabeads^{TM} Human T activator CD3/CD28) or in combination with IL-12/IL-18 (each 50ng/mL) and TCR Dynabeads in the presence or absence of 2DG (10mM) for 18 hours at 37^{o}C. After stimulation, the cells were harvested and stained for flow cytometric analysis as per table 2.3 and section 2.10.5 using extracellular staining protocols.

2.10.4. Antibodies and panels used in this thesis

Staining used in this thesis is detailed as per table 2.3. Whereas details of the clones of flow cytometric antibodies are detailed in table 2.4.

Table 2.3. Flow cytometry panels for studying of MAIT cells

	BL1	YL1	YL4	RL1	RL3	VL1	VL2	VL4
Excitation laser (nm)	480	516	516	638	638	405	405	405
Emission filter (nm)	530/30	585/16	780/60	670/14	780/60	440/50	512/25	530/30
MAIT Purification				Va7.2				
MAIT Frequencies			Va7.2	CD161*	CD161*		CD3	
CTV		CD3	Va7.2	CD161	CD8	CTV		
Viability		CD3	Va7.2	CD161	CD8		Viability dye	
LDHA, GLUT1,HKII	LDHA	GLUT1	Va7.2	HKII	CD161		CD3	
PKM2		PKM2	Va7.2	CD161			CD3	
CD98	CD98		Va7.2	CD161			CD3	
pS6		pS6		MAIT Tetramer			CD3	
IFNγ	IFNγ		Va7.2	CD161			CD3	
Kynurenine			CD3	MAIT Tetramer		Kynurenine		
CD25		CD3	Va7.2	CD161				CD25

* CD161 APCCy7 used for only for IL-2 expansion experiments

Table 2.4. Clones of antibodies used in this thesis

Antibody	Fluorochrome	Clone	Manufacturer
Vα7.2	PE-Vio770	REA179	Miltenyi Biotec
Vα7.2	PE/Cyanine7	3C10	BioLegend
Vα7.2	APC	3C10	BioLegend
CD161	APC	REA631	Miltenyi Biotec
CD161	APC/Cyanine7	NKR-P1A	BioLegend
CD8	APC-Vio770	REA734	Miltenyi Biotec
CD3	PE	REA613	Miltenyi Biotec
CD3	VioGreen	REA613	Miltenyi Biotec
CD3	PE-Vio770	REA613	Miltenyi Biotec
IFNγ	FITC	45-15	Miltenyi Biotec
CD98	FITC Bright	REA387	Miltenyi Biotec
GLUT1	PE	EPR3915	Abcam
HKII	AlexaFluor647	EPR20839	Abcam
LDHA	AlexaFluor488	EP1563Y	Abcam
PKM2	PE	EPR10138(B)	Abcam
pS6	PE	REA454	Miltenyi Biotec
CD25	BV711	M-A251	BioLegend

2.10.5. Extracellular and intracellular staining in tubes

Cells were harvested from the culture (between 0.5-1x10^6), transferred into labelled 5mls FACS tubes (BD Falcon) and washed in 1ml of stain buffer (PBS with 1% (v/v) FCS) at 1800 rpm for 8 minutes. The supernatant was removed, and the cell pellets were stained with the fluorochrome conjugated surface antibodies (as per table 2.3) as per recommendations. Cells were vortexed and incubated in a total volume of 100μL of stain buffer per test, at 4^{o}C for 20-30 minutes in darkness. Next, cells were washed as described above and fixed in a 300μL of a 1X fix working solution (True-Nuclear Transcription Factor Buffer set) (BioLegend) vortexed and incubated for 30 minutes at 4^{o}C. Then, 1mL of 1X Perm Buffer was added to the tubes (True-Nuclear Transcription Factor Buffer set) (BioLegend) and centrifuged at 1800 rpm for 10 minutes. If no further staining was required, the cells were resuspended in 300μL of staining buffer and ran on flow cytometer. If cells required intracellular staining, intracellular antibodies conjugated to the fluorochromes were added to the washed cells (as per table 2.3), as per previous lab optimisation protocol, vortexed and incubated in a total volume of 100μL of 1X Perm Buffer (True-Nuclear Transcription Factor Buffer

set) (BioLegend) per test, at 4°C for 30 minutes in darkness. Next, 1mL of stain buffer was added and the tubes were centrifugated at 1800rpm for 8 minutes. The pellet was resuspended in 300μL of stain buffer for flow cytometry analysis. Unstained and fluorescence minus one (FMO) controls were stained and ran with every experiment. The instruments used for analysis included AttuneNxT (Thermofisher). Data was analysed with FlowJo software.

2.10.6. Extracellular and intracellular staining in plates

Cells were removed from the culture plate (between 0.5-1x10^6 cells) and placed in a new 96 round bottom well plate for staining. The cells were centrifuged for 5min at 1500rpm to remove media. Next 250μL of staining buffer was added to the wells and the cells were spun as detailed above. The fluorochrome conjugated surface antibodies were added to each well as per recommendations, in a total volume of 50-100μL staining volume, mixed well. The plate was incubated at 4°C for 20-30 minutes in darkness and was washed by adding 150-200μL of staining buffer to the wells by spinning at 1500rpm for 5 minutes. The supernatant was discarded. The cells were fixed in 150μL of 1X fix working solution (True-Nuclear Transcription Factor Buffer set) (BioLegend) for 30 minutes, at 4°C in darkness, and then 100μL of 1X Perm Buffer was added to the wells (True-Nuclear Transcription Factor Buffer set) (BioLegend) and centrifuged at 1500 rpm for 5 minutes and the supernatant was discarded. If no intracellular staining was required, cells were resuspended in 200μL of staining buffer, transferred to tubes, topped up to 300μL total volume and ran on flow cytometer. If staining for intracellular targets, after discarding the supernatant intracellular antibodies conjugated to the fluorochromes were added to the cells in a master mix of 100μL per well, which was made up in 1X Perm Buffer (True-Nuclear Transcription Factor Buffer set) (BioLegend). The plate was incubated at 4°C for 30 minutes in darkness. Next 150μL of staining buffer was added into each well and the cells were spun at 1500rpm for 5minutes. The supernatant was removed, and the cell were resuspended in 200μL of staining buffer, next they were transferred to tubes, topped up to 300μL of staining buffer and ran on included AttuneNxT (ThermoFisher Scientific) with unstained and FMO controls. Data was analysed with FlowJo software.

2.10.7. Gating of MAIT cells

PBMC isolated as per section 2.3 were stained for extracellular MAIT cell surface markers as per section 2.10.5 or 2.10.6 and table 2.3. First lymphocytes were identified and gated using forward and side scatter. Next doublets were excluded using FSC-area and FSC-height parameters (Figure 2.7). This allowed for further characterisation and identification of MAIT cells among the lymphocyte population using the specific fluorochrome bound antibodies and/or MAIT cell tetramer. MAIT cells were first identified by the positive expression of CD3, a surface T cell marker. From the CD3 positive population, MAIT cells were identified by their high expression of CD161 molecule and the co-expression of Vα7.2 TCR on their cell surface (Figure 2.7) or by binding to MAIT cell tetramer (Figure 2.8).

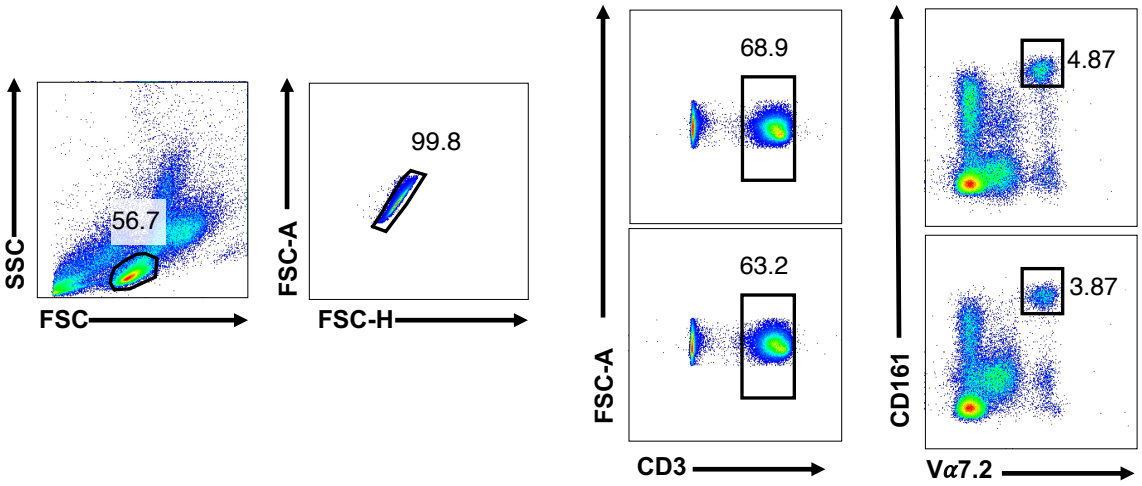


Figure 2.7 MAIT cell identification using flow cytometric antibodies. Identification of MAIT cells using extracellular antibodies specific to CD3, CD161 and Vα7.2 conjugated to appropriate fluorochromes.

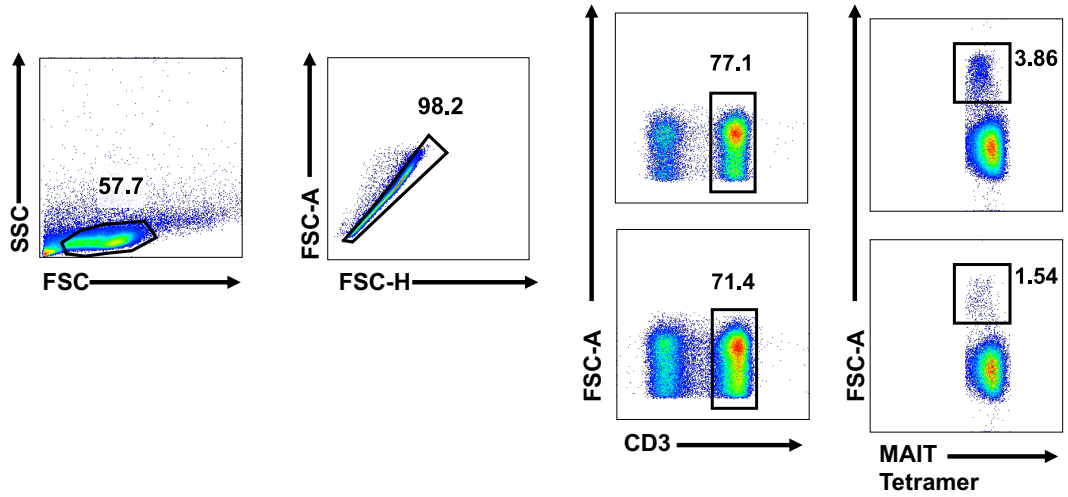


Figure 2.8 MAIT cell identification using flow cytometric antibodies and MAIT cell tetramer.

2.10.8. Viability staining

Cells were stained with a fixable viability dye eFluor^{TM}506 (FVD) (eBioscience, Invitrogen) prior to extracellular or intracellular staining. Cells (between 0.5-1x10^6) were moved to a new 96 well round bottom plate for staining, spun at 1500rpm for 5 minutes. The supernatant was removed, the cells were washed twice with 250μL of protein free and azide free PBS by centrifugation at 1500 rpm for 5 minutes and the supernatant was decanted. FVD dye was diluted 1:1000 in PBS and the cells were quickly resuspended in 100μL of the working solution. The plate was incubated in the refrigerator, protected from light for 30 minutes. Next the cells were washed twice with 150μL of flow staining buffer, centrifuging at 1800rpm for 5 minutes. After this step, other antibody-based flow cytometry staining was performed as per section 2.10.6.

2.10.9. Kynurenine Assay

Kynurenine (Sigma) stock of 800μM was made up fresh in HBSS. 2 aliquots of the stock were prepared where 1 was kept at 37^{o}C whereas the other was kept at 4^{o}C. Also, 2 aliquots of HBSS were prepared and kept at 37^{o}C or 4^{o}C prior to the beginning of the assay. Cryopreserved cells or expanded MAIT cells were counted as per section 2.4. First, 1x10^6 cells per condition were stained with extracellular flow cytometry antibodies (without the fixation and permeabilisation step) as per section 2.10.5. After staining, cells were resuspended in 200μL per treatment of pre-warmed HBSS and kept in the water bath at 37^{o}C or in 200μL of

cold HBSS and kept on ice. For the first control, cells and the reagents were kept at 37°C. To the 200μL of cells, 200μL of HBSS was added and kept at 37°C. For the second control, cell and reagents were kept at 4°C. To the 200μL of cells, 100μL of HBSS and 100μL of kynurenine was added and kept at 4°C. For kynurenine uptake, cells and reagents were kept at 37°C. 100μL of Kynurenine and 100μL of HBSS were added to the 200μL of the cells and kept at 37°C. To investigate the effect of BCH on kynurenine uptake, cells and reagents were kept at 37°C. 100μL of HBSS, 100μL of Kynurenine and 100μL of BCH at 25mM was added to the 200μL of the cells. The addition of the warm reagents was performed sequentially in the water bath, whereas the addition of the cold reagents was performed on ice at the same time. The cells and reagents were incubated for 4 minutes and the reaction was stopped by adding 125μL of 4% paraformaldehyde (PFA) (Sigma) and incubated for further 30 minutes at room temperature protected from light. Next the cells were washed twice in PBS buffer with 0.5% of BSA and resuspended in 300μL of the buffer for flow cytometry. Kynurenine was read of the violet laser at 405nm with 440/50nm filter.

Chapter Three

Results

Functional Characterisation of MAIT cells

3.1 Introduction

3.1.1. MAIT cells

MAIT cells are a non-classical subset of T lymphocytes in humans. To date, MAIT cells have been identified at multiple sites, including the liver, where they comprised up to 45% of total T cells (Dusseaux et al., 2011), and peripheral blood where they represent up to 10% of circulating T cells (Gherardin et al., 2018b; Le Bourhis et al., 2010). In humans, MAIT cells were also found in lungs, female reproductive tract, skin or adipose tissue (Carolan et al., 2015; Gibbs et al., 2017; Hinks et al., 2016). Peripheral blood MAIT cells are typically $CD8^+$ or double negative, with a very small population expressing CD4 (Gherardin et al., 2018b; Kurioka et al., 2017; Walker et al., 2012). They also display an effector-memory phenotype, as identified by the lack of CCR7, CD62L, CD45RA (Dusseaux et al., 2011; Gold et al., 2013).

MAIT cells are defined by the expression of a highly conserved, semi-invariant $\alpha\beta$ T cell receptor, which is accompanied by high expression of CD161 (C-type lectin-like receptor) molecules (Dusseaux et al., 2011; Lepore et al., 2014; Porcelli et al., 1993; Tilloy et al., 1999). In humans, the alpha chain is composed of TRAV1-2 (Vα7.2) and TRAJ33/12/20 (Jα33/12/20) (Vα19- Jα33 in mice) and is paired with a limited number of beta chains (Lepore et al., 2014; Porcelli et al., 1993; Tilloy et al., 1999). The confined TCR repertoire indicates a narrow antigen range, suggesting that MAIT cell response can be very rapid. The alpha chain rearrangement was first identified by Porcelli *et al.*in 1993, during analysis of TCR expression by human peripheral blood $CD4^-$ $CD8^-$ cells (Porcelli et al., 1993). Later Tilloy *et al*. described a homologous Vα7.2-Jα33 in the murine T cell repertoire, suggesting that this TCR receptor is highly conserved among species (Tilloy et al., 1999). The MAIT cell TCR recognises MR1, another highly conserved molecule across species. MR1 is associated with B_2-microglobulin and is expressed by antigen presenting cells or epithelial cells (Gold et al., 2010; Treiner et al., 2003; Yamaguchi and Hashimoto, 2002). The type of ligand presented by the MR1 molecule remained unknown for a long time, however some studies have suggested they are expressed during microbial infections (Gold et al., 2010; Le Bourhis et al., 2010). Landmark study by Kjer-Nielsen *et al.* in 2012 finally revealed that MR1 presents non-peptide antigens to MAIT cells, which were identified as vitamin B metabolites produced by various strains of

bacteria (Kjer-Nielsen et al., 2012). During that study, the primary ligand which bound to MR1 molecule was named as 6-formyl pterin (6-FP), a derivative of folic acid. Binding of 6-FP to MR1 lead to increase in the expression of the antigen presenting complex on the cell surface, but it didn't induce MAIT cell activation (Kjer-Nielsen et al., 2012). Upon closer investigation of the riboflavin biosynthesis, it's precursor 5-A-RU was pinned as the key intermediate, which induced MAIT cell activation. Free amine of 5-A-RU binds non-enzymatically to methylglyoxal, metabolite of glycolysis, to form a more potent 5 OP-RU antigen. 5 A-RU can also bind with glyoxal to form less potent 5-(20oxoethyludeneamino)-6-D-ribitylaminouracil (5-OE-RU) (Corbett et al., 2014). Riboflavin is synthesised by certain bacteria and yeast such as *Staphylococcus* or *Candida* species, but not by humans, making it a unique antigen capable of inducing a response to variety of microbial infections (Le Bourhis et al., 2010). Up to date, a limited number of other MAIT cell antigens have been identified. Some drugs were found to bind to the MR1 molecule such as derivatives of salicylic acid or metabolites of an anti-inflammatory drug diclofenac but these have limited effects on MAIT cell activation (Keller et al., 2017). Activation of MAIT cells via their engagement with MR1 has been shown to be indispensable during infections with riboflavin synthesising microorganisms (Figure 3.1). This has been elegantly shown by Le Bourhis *et al.* during infection with *E. coli*, where addition of anti-MR1 antibodies, prevented MAIT cell activation (Le Bourhis et al., 2010).

MAIT cells are activated independently of their TCR via cytokine stimulation (Figure 3.1). Indeed, MAIT cells were shown to express a variety of cytokine receptors including IL-2R, IL-7R, IL-12R, IL-15R, IL-18R and IL-23R, suggesting they can respond to a range of stimuli (Figure 3.2) (Dusseaux et al., 2011; Gracey et al., 2016; Sattler et al., 2015; Wallington et al., 2018; H. Wang et al., 2019; Wilgenburg et al., 2018). Study by Ussher *et al.* and van Wilgenburg *et al.* reported that MAIT cells stimulated by IL-12 or IL-18 alone do not modulate expression of IFNγ, however combination of these cytokines enhanced production IFNγ (Ussher et al., 2014; Van Wilgenburg et al., 2016). Van Wilgenburg *et al.* has explored the effect of a larger number of cytokines on IFNγ production by MAIT cells. They found that incubation of the cells with a combination of IL-12 and IL-15 led to similar IFNγ production as the IL-12/IL-18 stimulation, whereas combination of IFN-α or -β with IL-12, IL-15 or IL-18 led to

variable results (Van Wilgenburg et al., 2016). MAIT cells were also shown to be activated independently of TCR engagement in a mouse study, which investigated the role of MAIT cells in influenza infections (Wilgenburg et al., 2018). MR1 deficient mice who received adoptive transfer of MAIT cells have shown no significant difference in their activation in comparison to the wild type mice, whereas deletion of receptors for IL-12, IL-18, IL-15 and IFNα cytokines significantly impacted MAIT cell activation (Wilgenburg et al., 2018). TCR independent activation of MAIT cells is very beneficial in response against pathogens, which don't produce antigens recognised by these cells. These include viruses or bacteria and yeast which don't use riboflavin synthesis pathway. Due to this mechanism, MAIT cell can provide a much greater protection to the host. Although MAIT cells can be activated in a TCR dependent or independent manner, it is very likely that *in vivo* they combine both signals where available, to reach their full effector capacity. Studies *in vitro* have shown that stimulation of MAIT cells using TCR and cytokine stimulation can enhance their activation. For example, Gracey *et al.* have shown that MAIT cells primed with IL-7 prior to TCR stimulation significantly enhanced their cytokine and granzyme B production (Gracey et al., 2016).

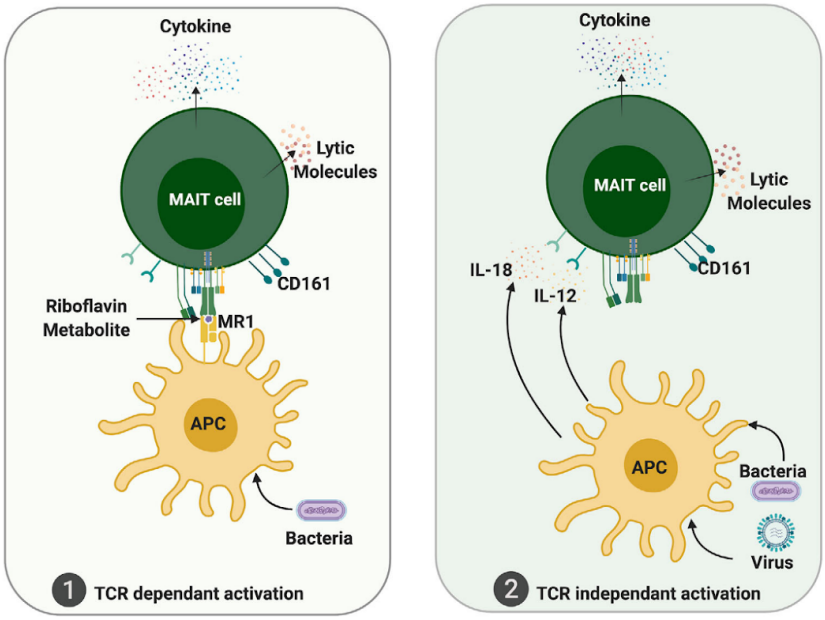


Figure 3.1. MAIT cell activation. MAIT cells can be activated via TCR-dependent or independent manner. This results in MAIT cell proliferation and production of effector molecules such as cytokines and cytotoxic mediators (Pisarska et al., 2020).

PLZF is crucial transcription factor required for the innate function and cytokine production of MAIT cells, however it is co-expressed with multiple other transcription factors including RORγT governing type17 response; T-bet, Eomesodermin (Eomes) and B-lymphocyte-induced maturation protein -1 (Blimp-1) associated with Type 1 immunity and CAAT/enhancer-binding protein gamma (C/EBPδ) required for the expression of appropriate tissue homing molecules (Koay et al., 2016; C. H. Lee et al., 2018; Leeansyah et al., 2015) (Figure 3.2). In contrast to T cells, MAIT cell RORγT and T-bet were shown to be co-expressed (Kurioka et al., 2015; Leeansyah et al., 2015). Expression of T-bet implicated that MAIT cells might engage in type 1 immune response by producing IFNγ. Upon stimulation MAIT cells indeed significantly upregulated their IFNγ production (Dusseaux et al., 2011; Van Wilgenburg et al., 2016). Similarly, to CD8 T cells, activation of MAIT cells led to higher expression of cytotoxic proteins, which in T cells were controlled by T-bet, Eomes and Blimp-1 transcription factors. Upon stimulation MAIT cells produced granzyme B, granzyme A, granzyme K and perforin (Dusseaux et al., 2011; Kurioka et al., 2015; Van Wilgenburg et al., 2016), whereas granulysin was expressed but the levels were not modified upon activation (Le Bourhis et al., 2013; Sobkowiak et al., 2019). This indicates direct killing capabilities of MAIT cells. Indeed, MAIT cells primed with *E. coli* were shown to kill antigen presenting cells infected with this microorganism in an MR1 dependent manner (Kurioka et al., 2015). MAIT cells also expressed RORγT and IL-17, markers typical for type 17 phenotype (Dusseaux et al., 2011; Hinks et al., 2019; Lamichhane et al., 2019). IL-17 is a major pro-inflammatory cytokine, implicated in multiple immune mediated diseases. It was originally identified as a cytokine produced by the cells of the adaptive system, namely Th17 cells, however as new data emerged, it became clear that MAIT cells also contribute to the production of IL-17 in many inflammatory diseases (Pisarska et al., 2020). Similarly, to conventional T cells, MAIT cells express IL-23R, which is known to facilitate IL-23 signalling and differentiation of cells towards type 17 profile. It is not yet fully understood whether IL-23 may contribute to the transition of MAIT cells to type 17 cells, however IL-23 acts directly on the cells and enhances their proliferation (Raychaudhuri et al., 2020; H. Wang et al., 2019). Other less described cytokines expressed by MAIT are IL-17F and IL-22, of which both are members of the IL-17 family. Lu *et al.* who investigated the role of MAIT cells in pneumonia in children, have reported higher expression of IL-17F producing

MAIT cells in the samples of bronchial lavage versus blood in children with infection (Lu et al., 2020). On the other hand, Gibbs *et al*. identified IL-17 and IL-22 producing MAIT cells in mucosa of the female genital tract and suggested this phenotype may be protective against microbial infections (Gibbs et al., 2017).

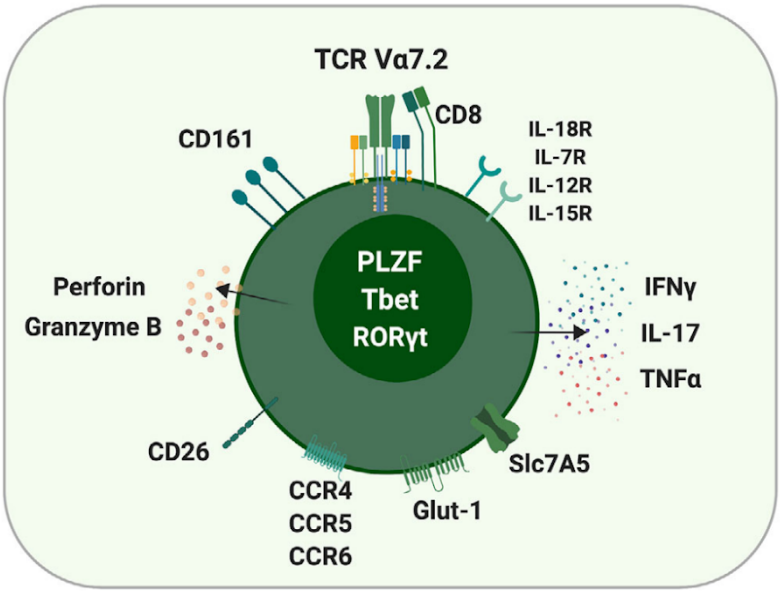


Figure 3.2. Phenotype of MAIT cells. MAIT cells express Vα7.2 TCR receptor which is accompanied by a range of cytokine and chemokine receptors. In addition, they express a combination of transcription factors and cytokines which are typical for responses of Th1, Th17 and innate cells (Pisarska et al., 2020).

Activation of MAIT cells has been shown to induce their expansion and proliferation, although the data is still very limited. Studies which have investigated MAIT cell responses in bacterial infections, reported that MAIT cells are capable of expansion upon activation (Howson et al., 2018; Wong et al., 2019). Howson *et al.* has shown that during infection of human subjects with *S. enterica*, MAIT cells displayed clonal expansion which was followed by a contraction phase (Howson et al., 2018). Similar findings were reported by Wong *et al.* who investigated the impact of *M. Tuberculosis* on MAIT cell function and has shown an oligoclonal expansion of MAIT cells in response to the infection (Wong et al., 2019). Other studies measured MAIT cell proliferation using Ki67 marker in order to compare their activation and have shown that MAIT cells from individuals with IBD or liver cirrhosis displayed more proliferative in comparison to healthy controls (Hegde et al., 2018; Serriari et al., 2014). Recently, Gutierrez-Arcelus *et al*. have shown that MAIT cells can proliferate in response to CD3/CD28 bead stimulation over 5 days of culture as depicted by carboxyfluorescein succinimidyl ester (CFSE) assay (Gutierrez-Arcelus et al.,

2019). Up to date, the control of MAIT cell expansion and proliferation has not been explored in detail.

3.1.2. MAIT cells in obesity

The impact of obesity on MAIT cell phenotype and function still remains largely unexplored. As new studies emerged, it became more evident that obesity is associated with alterations in MAIT cell distribution and a switch to more pro-inflammatory phenotype. Magalhaes *et al*. and Carolan *et al.* who assessed MAIT cells in the peripheral blood of people with obesity, revealed a significant decrease in their frequencies in comparison to the control individuals, whereas study by Li *et al*. reported no difference in the percentage of MAIT cells between the two groups (Carolan et al., 2015; Li et al., 2020; Magalhaes et al., 2015). The inconsistency in the frequencies reported by these studies may be due to the difference in the BMI in the cohorts of people with obesity. Cohorts used by Magalhaes *et al*. and Carolan *et al.* had much higher BMI than the subjects used in the study by Li *et al*. In fact, frequencies of MAIT cells were shown to be negatively correlated with BMI, where in some people with obesity MAIT cells have not been detected (Magalhaes et al., 2015), however this requires further investigation as Carolan *et al.* reported no association between those two parameters (Carolan et al., 2015). People with obesity who underwent bariatric surgery, were shown to increase the population of their MAIT cells in the peripheral blood, suggesting that this defect is reversible upon weight loss (Magalhaes et al., 2015).

In addition, MAIT cells from people with obesity were shown to be largely dysfunctional and displayed dysregulation in the proportion of the produced cytokines. Carolan *et al.* reported a decrease in the IFNγ expression by peripheral blood MAIT cells from people with obesity, whereas other studies have shown no apparent defect in the production of this cytokine (Carolan et al., 2015; Magalhaes et al., 2015; Toubal et al., 2020). In contrast, peripheral blood and AT MAIT cells from people with obesity displayed strongly enhanced IL-17 production in comparison to the control individuals (Carolan et al., 2015; Magalhaes et al., 2015; O'Brien et al., 2020). Similar results were obtained in a mouse study by Toubal *et al.* where high-fat diet (HFD) mice had a higher proportion of IL-17 producing MAIT cells in the ependymal fat in comparison to

mice fed on normal diet (Toubal et al., 2020). Collectively this suggests that MAIT cells from people with obesity may have a defect in the production of IFNγ, however the enhancement in IL-17 may be contributing to the inflammation observed in obesity. Indeed Toubal *et al.* has demonstrated a close relationship between MAIT cells and macrophages, which are present in the AT (Toubal et al., 2020). Coculture of MAIT cells with M1 macrophages, significantly enhanced MAIT cell IL-17 production, whereas activated MAIT cells promoted polarisation of macrophages into M1 phenotype (Toubal et al., 2020). This mechanism may possibly be contributing to the sustained AT inflammation observed in obesity. Although gut dysbiosis has been reported in people with obesity, very little is known about possible causes and mechanisms which are involved in the development of this condition (Boulangé et al., 2016; Muscogiuri et al., 2019). Recently, Toubal *et al.* has shown that MAIT cells contribute to the alteration in the gut microbiota. In HFD mice, MAIT cells were shown to increase gut inflammation and leakiness, whereas transfer of the microbiota from HFD mice induced dysfunction of the mucosa, leakiness of the gut and enhanced inflammation of the gut in the recipient mice (Toubal et al., 2020).

As immunometabolism became a focal point of many immunological studies, a recent study focused on the relationship between MAIT cell metabolism and their dysfunction in obesity. O'Brien *et al.* have shown that MAIT cells from people with obesity had elevated levels of mitochondrial reactive oxygen species (mROS) and mitochondrial membrane potential in comparison to the lean controls. Reducing mROS using MitoTEMPO and MitoQ antioxidants, was shown to reduce IL-17 production by MAIT cells from people with obesity, suggesting that metabolic alterations in MAIT cells may be contributing to their dysfunction (O'Brien et al., 2020).

These studies show that obesity significantly affects MAIT cell frequencies and function in the peripheral blood as well as other tissues including AT. The alterations in their phenotype are shown to support systemic inflammation in obesity, which may further lead to development of conditions such as T2DM. More studies are required to elucidate the causes of the altered MAIT cell function and to identify potential targets for treatment to ameliorate their pro-inflammatory responses in obesity and regain homeostasis.

3.2. Specific aims of this chapter

The specific aim of this chapter was the characterisation of MAIT cells in cohorts of adults with a healthy or obese BMI, and therefore to:

1. Enumerate MAIT cell frequencies in peripheral blood
2. Determine the proliferative capacity of MAIT cells
3. Characterise MAIT cell cytokine profiles

3.3. Results

3.3.1. MAIT cell identification

MAIT cells have been previously reported to represent 1-10% of peripheral blood T cells in healthy individuals (Carolan et al., 2015; Gherardin et al., 2018b; Le Bourhis et al., 2010). To confirm their frequencies in the peripheral blood of our cohorts of adults with a healthy or obese BMI we utilized multi-colour flow cytometry.

The gating strategy used to identify MAIT cells is shown in materials and methods figure 2.7. Briefly, PBMC isolated from control individuals were stained using antibodies against surface antigens conjugated to appropriate fluorochromes namely CD3 (VioGreen), CD161 (APC) and Vα7.2 (PeCy7). To identify MAIT cells, we first selected lymphocytes based on their morphology (forward and side scatter), next doublets were excluded using forward scatter (FSC)-area and FSC-height parameters. Finally, MAIT cells were first identified by the positive expression of CD3, high expression of CD161 molecule and the co-expression of Vα7.2 TCR on their cell surface (Figure 3.3 A&B). MAIT cell frequencies in the cohort of control individuals ranged from 0.53% to 13.7% and displayed a mean of 3.2% (Figure 3.3 A&B).

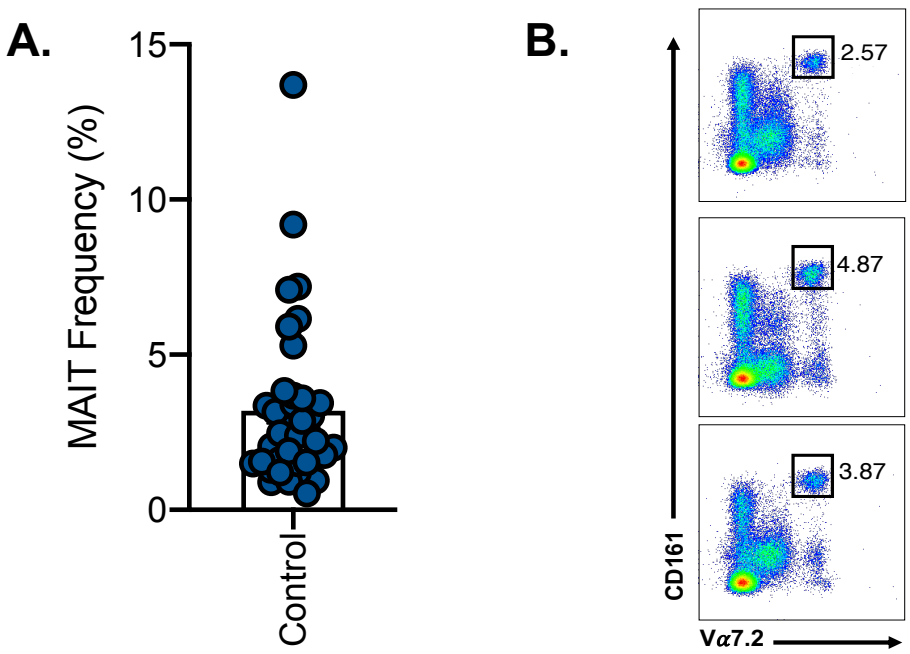


Figure 3.3 MAIT cell identification using flow cytometry. (A) Scatter plot of MAIT cell frequencies in the peripheral blood of control individuals (n=39). (B) Representative dot plots showing MAIT cells from 3 control individuals as percentage of $CD3^+$ cells. MAIT cells identified as $V\alpha7.2^+$ and $CD161^{hi}$.

3.3.2. People with obesity display lower MAIT cell frequencies in the peripheral blood

Previous work from our lab and others has shown obesity related alterations in the frequencies and functions of a range of immune cells, including MAIT cells (Carolan et al., 2015; Magalhaes et al., 2015). After establishing the range of MAIT cell frequencies in the control individuals (Figure 3.3), next we investigated the frequencies of these immune cells in people with obesity.

People with obesity had significantly lower frequencies of MAIT cells in the peripheral blood, in comparison to the control individuals (Figure 3.4). MAIT cell frequencies in people with obesity ranged from 0.11% to 4.9% and had a mean of 1.2%. This data is in line with previously published studies, which have shown a significant decrease in circulating MAIT cells in people with obesity (Carolan et al., 2015; Magalhaes et al., 2015).

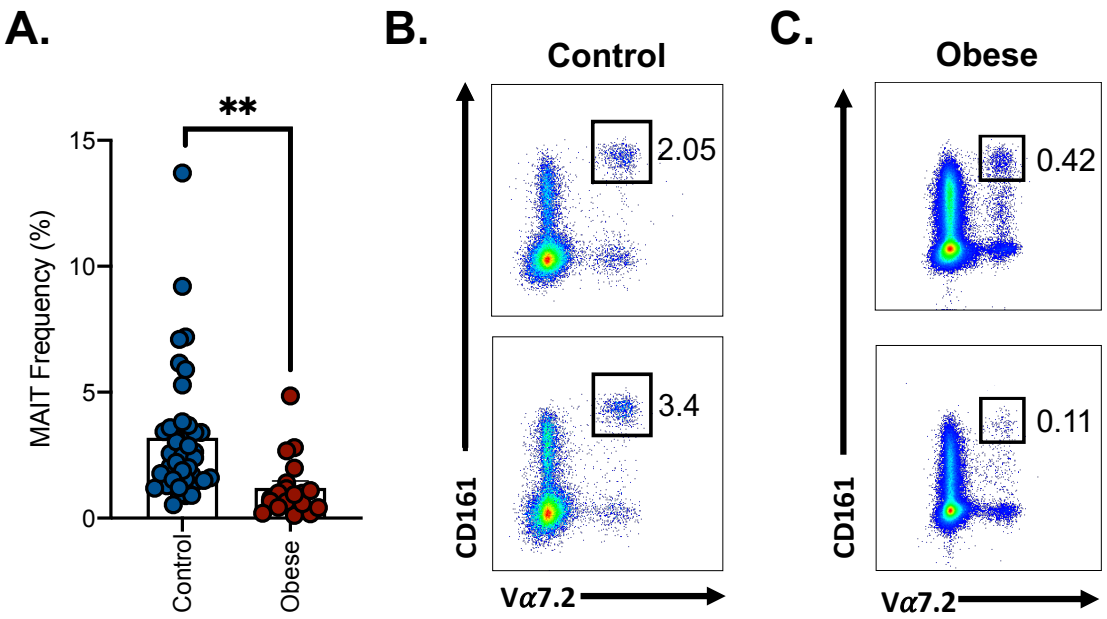


Figure 3.4 MAIT cell frequencies in control subjects and individuals with obesity. (A) Scatter plot of MAIT cell frequencies in peripheral blood of control individuals (n=39) and individuals with obesity (n=19). Representative dot plots showing MAIT cell frequencies of (B) two control and (C) two obese individuals as percentage of $CD3^+$ cells. MAIT cells identified as $V\alpha7.2^+$ and $CD161^{hi}$. Statistical analysis performed using unpaired student's t-test, ** $p \leq 0.01$.

3.3.3. TCR stimulation induces MAIT cell proliferation

Proliferation of lymphocytes plays a crucial role in response to microbial infections. MAIT cell proliferation remains largely unexplored and to date very little evidence is recorded in the literature. Recently, Gutierrez-Arcelus *et al*. investigated the function and proliferation of multiple innate immune cells including MAIT cells and suggested that innate immune cells are capable of proliferation in response to TCR stimulation, albeit to a much lower extend than cells of the adaptive immune response (Gutierrez-Arcelus et al., 2019). The proliferation capacity of MAIT cells was also demonstrated in a study by Howson *et al.*. This group utilised an *in vivo* approach of controlled infection in humans with *S. Paratyphi* A. They found that at the peak of infection, MAIT cells from the peripheral blood of the participants were in a state of proliferation, which they measured by Ki67 (Howson et al., 2018). Although the proliferation capacity of MAIT cells has been identified in response to a general stimulation, we decided to explore MAIT cell proliferation in response to the MAIT cell specific antigen, 5-ARU in combination with MG. 5-ARU has been identified as an intermediate in the riboflavin metabolism pathway, and was shown to combine with MG, a by-product of glycolysis to form a highly 5-OP-RU (Corbett et al., 2014).

MAIT cell expansion was assessed by investigating the proportion of MAIT cells expressed as percentage of all T cells present in the culture five days after activation. Stimulation of PBMC with 5-ARU-MG resulted in very limited, non-significant expansion of MAIT cells by day five (Figure 3.5 A&B). To investigate this further, MAIT cell proliferation was also assessed using CTV proliferation assay. CTV is a fluorescent dye, which allows the traceability of cell proliferation, through dye dilution and analysis using flow cytometry. CTV stained PBMC were activated with 5-ARU-MG and incubated for 5 days. Although the expansion of MAIT cells was shown to be limited (Figure 3.5 A&B), CTV proliferation assay revealed that MAIT cells were dividing (Figure 3.5 C&D). This data demonstrates that in response to a pathogen derived antigen MAIT cells display limited expansion, but enhanced proliferation However, the modest increase in the proportion of MAIT cells in the culture suggests limited capability to expand in this experimental setting, possibly due to the lack of other signals which could be originating from other immune and non-immune cells during active bacterial infection.

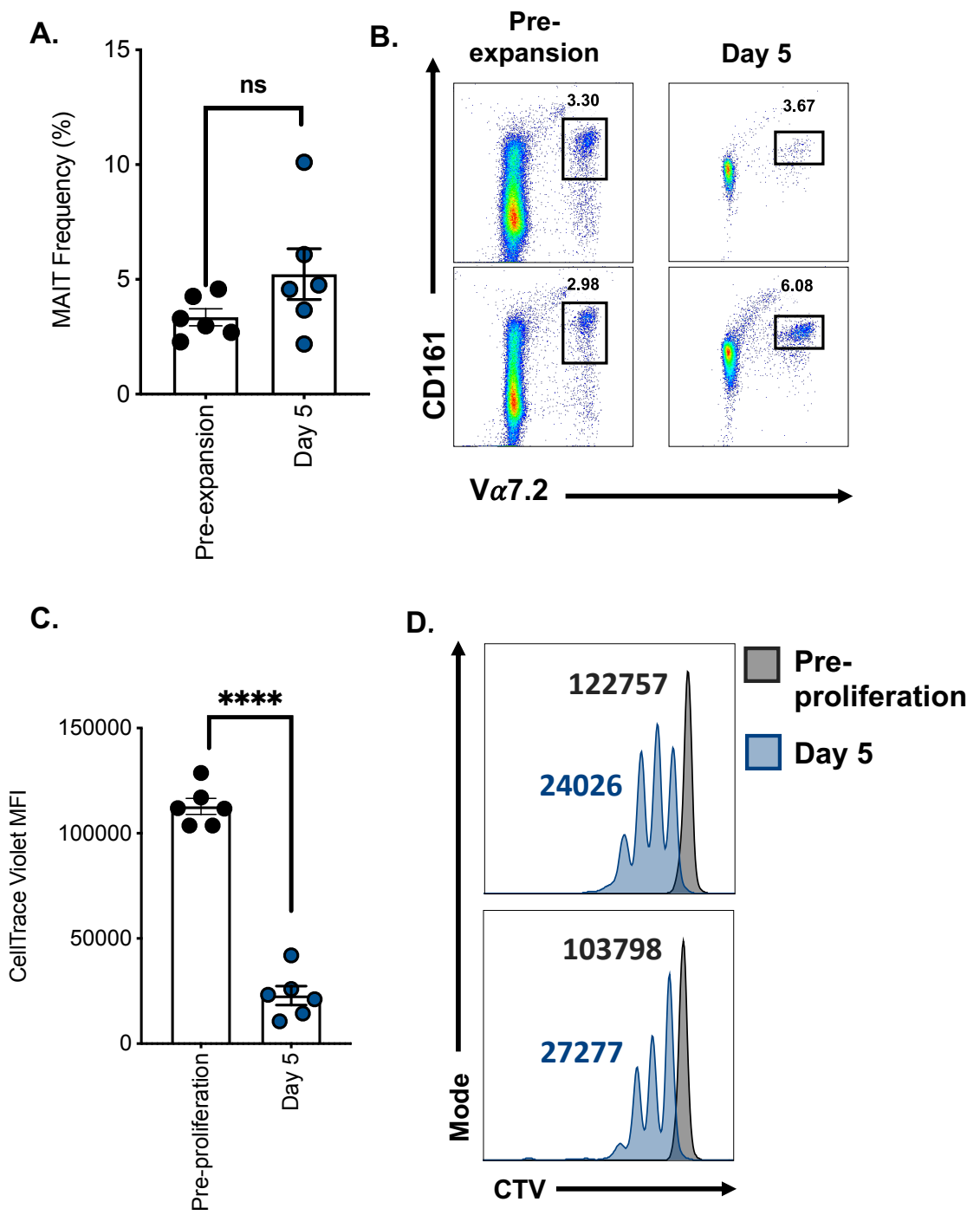


Figure 3.5 MAIT cells proliferate in response to 5-ARU-MG. (A) Scatter plot of MAIT cell proliferation from control subjects represented as a percentage of $CD3^+$ population. (B) Data depicts MAIT cells prior to proliferation (pre-proliferation) and on day 5 of proliferation. PBMCs were unstimulated (pre-proliferation) or stimulated (day 5) with 5-ARU (1µg/mL) and MG (100µL) (C) Scatter plot and (D) representative histograms of MAIT cell proliferation as measured by the uptake and dilution of CTV (5µM). Data depicts CTV uptake prior to expansion (pre-proliferation) and on day 5 of culture. Statistical analysis performed using student's paired T-test, ns- not significant, ****p≤0.0001.

3.3.4. TCR triggering upregulates expression of IL-2 receptor on MAIT cell surface

Stimulation of MAIT cells has been shown to enhance the expression of multiple cytokine and chemokine receptors such as IL-2R, IL-12R, IL-18R, CCR2, CCR4 or CCR6 (Chen et al., 2019; C. H. Lee et al., 2018; Sattler et al., 2015; Van Wilgenburg et al., 2016). Binding of the specific ligands to their receptors can shape the immune response to achieve the optimal protection of the host. IL-2 is known to be an immunomodulatory cytokine that can regulate multiple processes in immune cells such as controlling the fate of $CD4^+$ T cell differentiation, modulation of $CD8^+$ T cell function as well as T cell proliferation (Ross and Cantrell, 2018). In the context of MAIT cell proliferation, next we investigated whether stimulation of MAIT cells through TCR alone or in combination with IL-12/IL-18 cytokines for 18 hours can induce high affinity IL-2 receptor (IL-2R/CD25) expression on MAIT cell surface. TCR triggering alone was sufficient to significantly enhance IL-2R expression on MAIT cell surface (Figure 3.6 A&B). Addition of IL-12 and IL-18 in combination with TCR stimulation further enhanced IL-2R expression in comparison to TCR stimulation alone (Figure 3.7 A&B). This data demonstrates a TCR-mediated increase in the expression of IL-2R, which might facilitate the engagement of MAIT cells in proliferation.

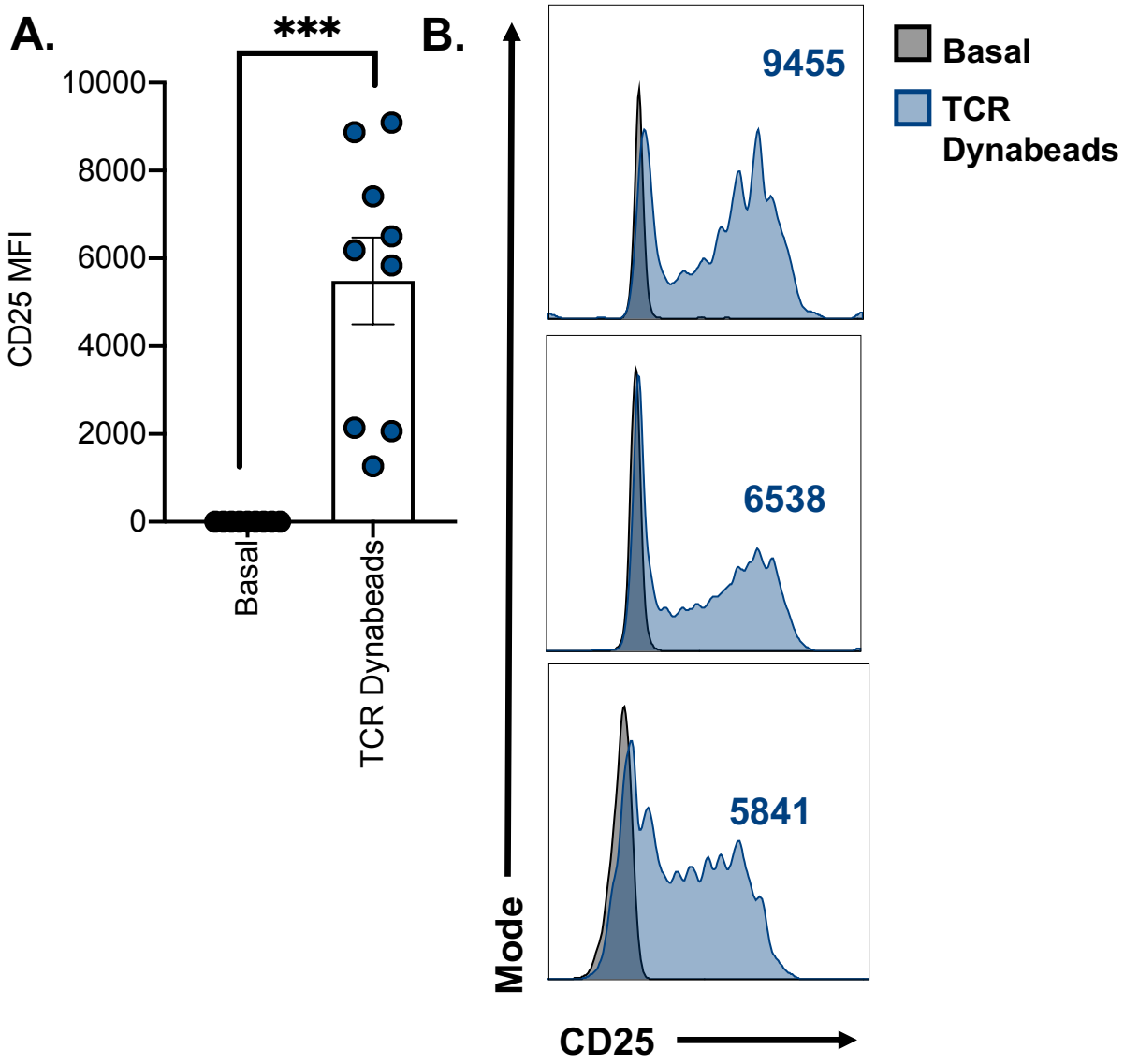


Figure 3.6 TCR stimulation of MAIT cells increases IL-2 receptor (CD25) expression. (A) Scatter plot and (B) representative histograms of CD25 (IL-2R) expression by MAIT cells from control individuals, basally or upon stimulation with TCR Dynabeads (1:1 bead to cell ratio) for 18 hours. Statistical analysis performed using paired student's t-test, ***p≤0.001.

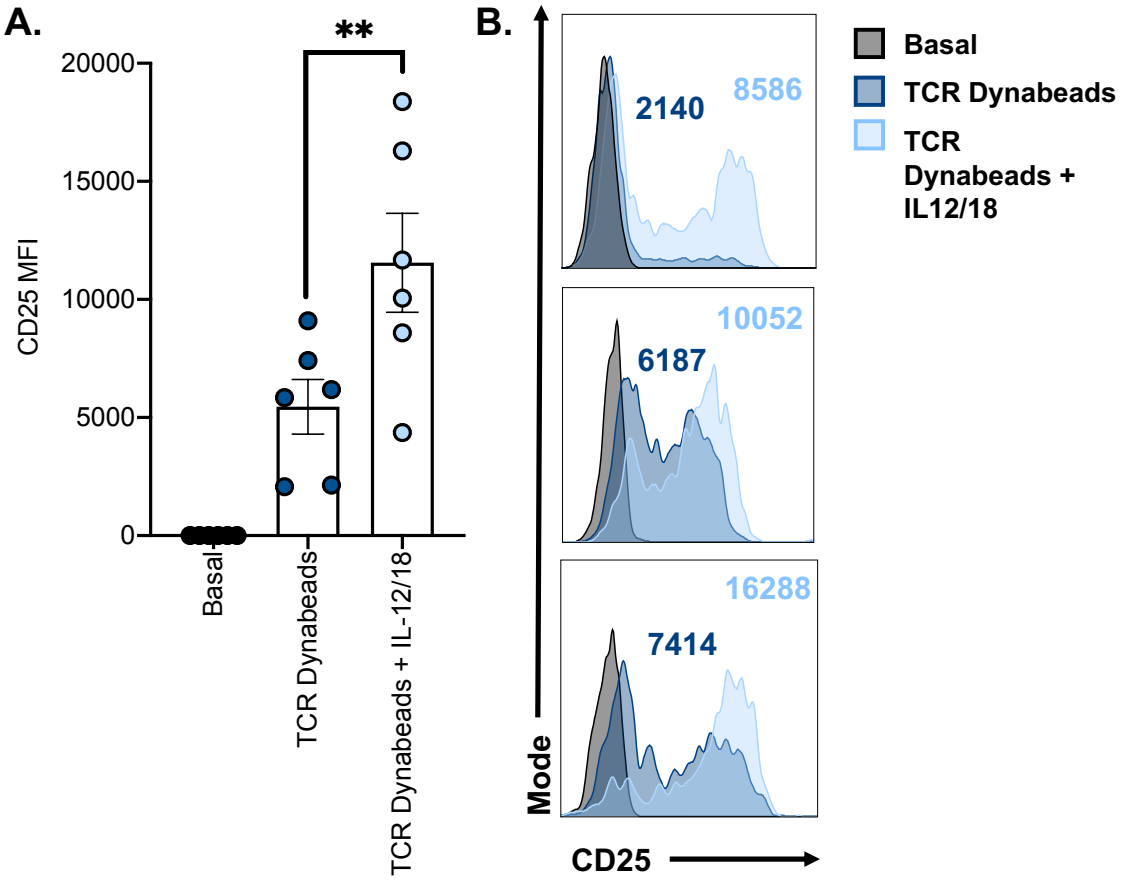
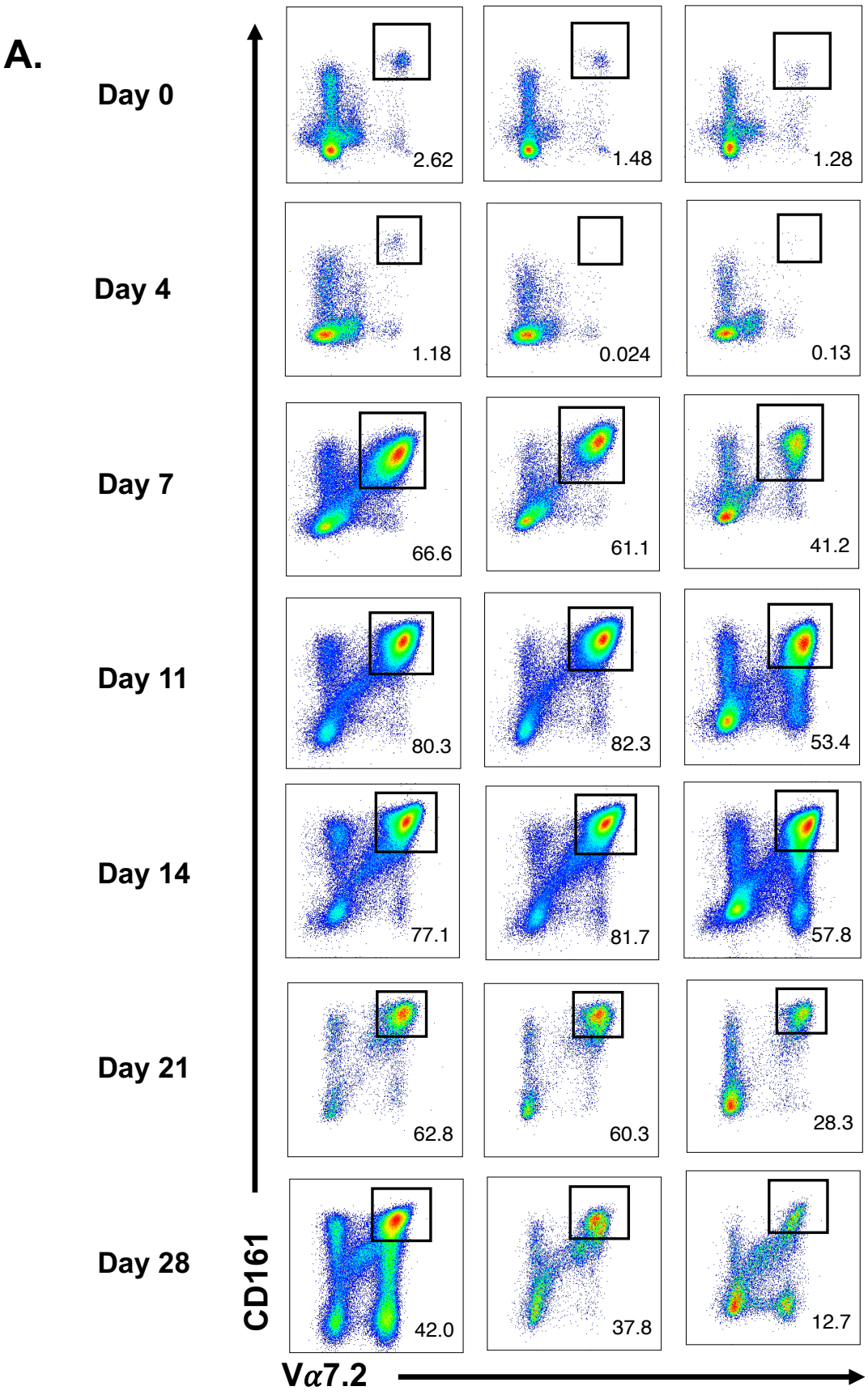


Figure 3.7 TCR triggering in combination with IL-12 and IL-18 cytokine stimulation enhances expression of IL-2 receptor (CD25) by MAIT cells. (A) Scatter plot and (B) representative histograms of CD25 expression by MAIT cells from control individuals, basally, upon stimulation with TCR Dynabeads (1:1 bead to cell ratio) with or without IL-12/IL-18 (50ng/mL each) for 18 hours. . Statistical analysis performed using paired student's t-test, **p≤0.01.

3.3.5. IL-2 supplementation enhances MAIT cell proliferation and expansion

Stimulation of MAIT cells via their TCR was shown to induce MAIT cell proliferation with a limited expansion (Figure 3.5) and a significant upregulation of IL-2R (Figure 3.6). To further investigate the proliferative capabilities of MAIT cells, we investigated the effect of exogenous IL-2 supplementation in combination with 5-ARU-MG activation on MAIT cell proliferation and expansion. Activation of PBMC with 5-ARU-MG and supplementation with exogenous IL-2 over a period of 28 days resulted in the significant expansion of MAIT cells from day 7 onwards (Figure 3.8 A&B). Expansion of MAIT cells peaked around day 11 after initial stimulation, which was followed by a period of contraction, where despite IL-2 supplementation, the percentage of MAIT cells in the culture decreased (Figure 3.8 A&B). To confirm this further, MAIT cell proliferation was also assessed using CTV, where stained PBMC were stimulated with 5-ARU-MG and IL-2 for five days. It was confirmed that MAIT cells were capable of significant proliferation over a five-day period in the presence of exogenous IL-2, where between five and six generations could be identified with CTV (Figure 3.9). This suggests that additional factors such as IL-2, which may be produced during infection *in vivo*, are required to support and enhance MAIT cells proliferation.


A.
Day 0
Day 4
Day 7
Day 11
Day 14
Day 21
Day 28
2.62
1.48
1.28
1.18
0.024
0.13
66.6
61.1
41.2
80.3
82.3
53.4
77.1
81.7
57.8
62.8
60.3
28.3
42.0
37.8
12.7
CD161
Vα7.2

B.

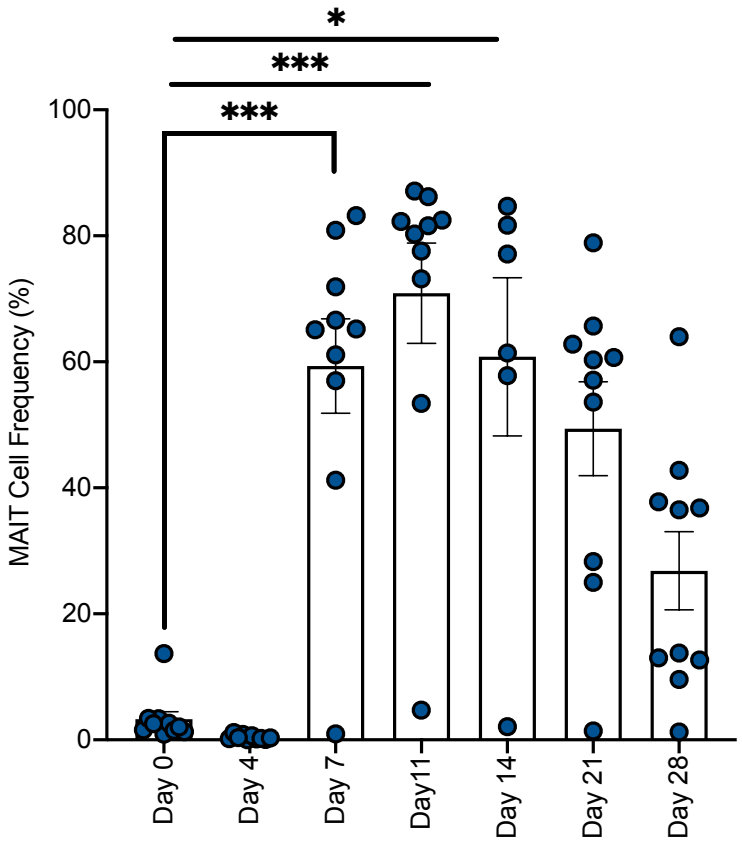


Figure 3.8 MAIT cells expand in response to 5-ARU-MG and IL-2 over 28 days. (A) Representative dot plots of MAIT cell proliferation from 3 control subjects observed over 28 days of culture in response to 5-ARU-MG and IL-2. PBMCs were stimulated with 5-ARU (1µg/mL) and MG (100µL) on day 0 and fed with a low dose of IL-2 (6.75ng/mL) on day 1 followed by high doses of IL-2 (33.3ng/mL) on days 4, 7, 11, 14,18 and 21. (B) Scatter plot of MAIT cell expansion from control subjects (n=10; n=6 on day 14) over 28 days of culture. Statistical analysis performed using one-way ANOVA with Tukey's correction, *p≤0.05, ***p≤0.001.

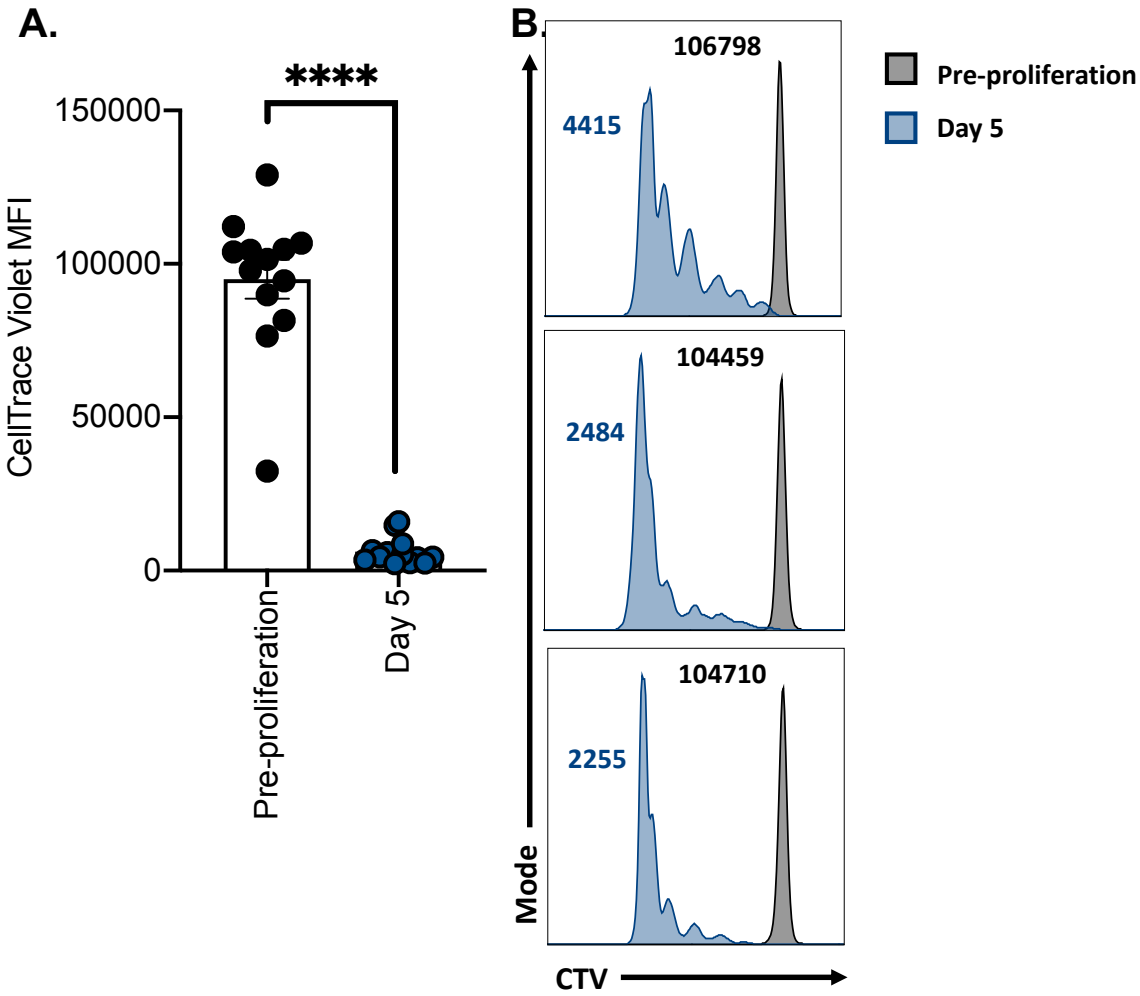
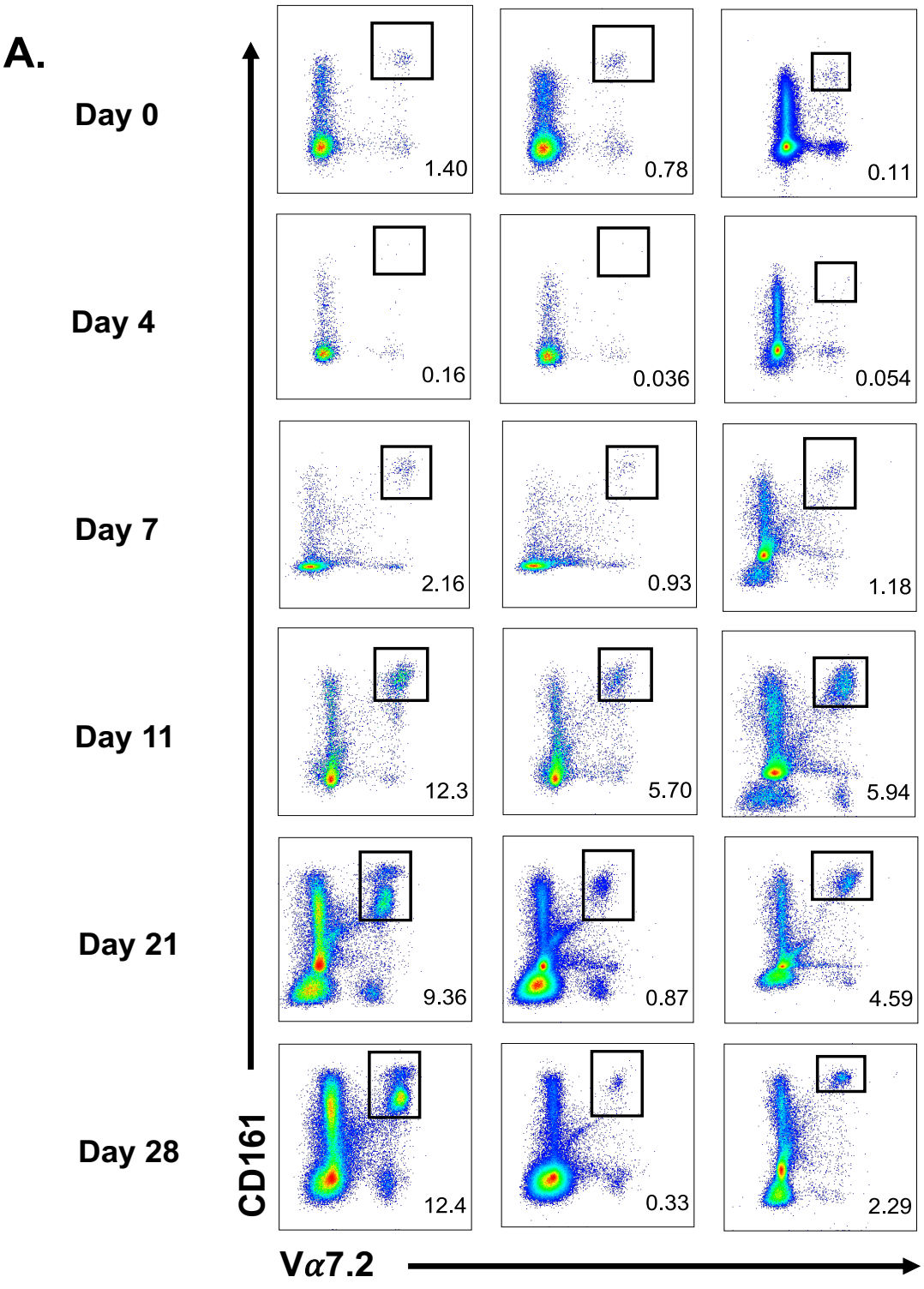


Figure 3.9 MAIT cells proliferate in response to 5-ARU-MG and IL-2. (A) Scatter plot of MAIT cell proliferation as measured by uptake and dilution of Cell Trace Violet (CTV) at 5μM, on day 0 and day 5 of culture. PBMCs were unstimulated (pre-proliferation) or stimulated (day 5) with 5-ARU (1μg/mL) and MG (100μL) on day 0 and received a low dose of IL-2 (6.75ng/μL) on day 1 followed by a high dose of IL-2 (33.3ng/μL) on day 4 (n=11). (B) Representative histograms from 3 control subjects of MAIT cell proliferation over 5 days of culture. Statistical analysis performed using paired student's t-test, ****p<0.0001.

3.3.6. MAIT cells from people with obesity display limited expansion capacity in response to 5-ARU-MG and IL-2

It is now well established that people with obesity have reduced frequencies of peripheral blood MAIT cells (Figure 3.4) (Carolan et al., 2015; Magalhaes et al., 2015). In addition to reduction in the frequencies, MAIT cells from people with obesity were shown to have impaired function as assessed by their cytokine production. However, up to now no one has investigated whether the proliferative capacity of MAIT cells in these individuals is affected (Carolan et al., 2015; Magalhaes et al., 2015).

To assess expansion of MAIT cells from individuals with obesity and control subjects over 28 days, PBMC were treated as described in section 3.3.5. MAIT cell expansion from individuals with obesity was significantly lower in comparison to control individuals (Figure 3.10 A&B). To confirm this further, we stimulated PBMC from people with obesity and control individuals with 5-ARU-MG and IL-2 over 7 days and determined their fold expansion. MAIT cells from people with obesity displayed significantly lower fold increase at days 7 after cell activation, further indicating a defect in MAIT cell expansion in people with obesity (Figure 3.10 C). To investigate this in more detail, MAIT cell proliferation was measured using CTV assay as described in section 3.3.5 and has shown no significant difference in proliferation rates of MAIT cells between people with obesity and control individuals (Figure 3.11 A&B). This data suggests that the expansion capacity of MAIT cells from people with obesity is impaired, but their proliferation by day five is yet unaffected.


A.
Day 0
Day 4
Day 7
Day 11
Day 21
Day 28
1.40
0.78
0.11
0.16
0.036
0.054
2.16
0.93
1.18
12.3
5.70
5.94
9.36
0.87
4.59
12.4
0.33
2.29
CD161
Vα7.2

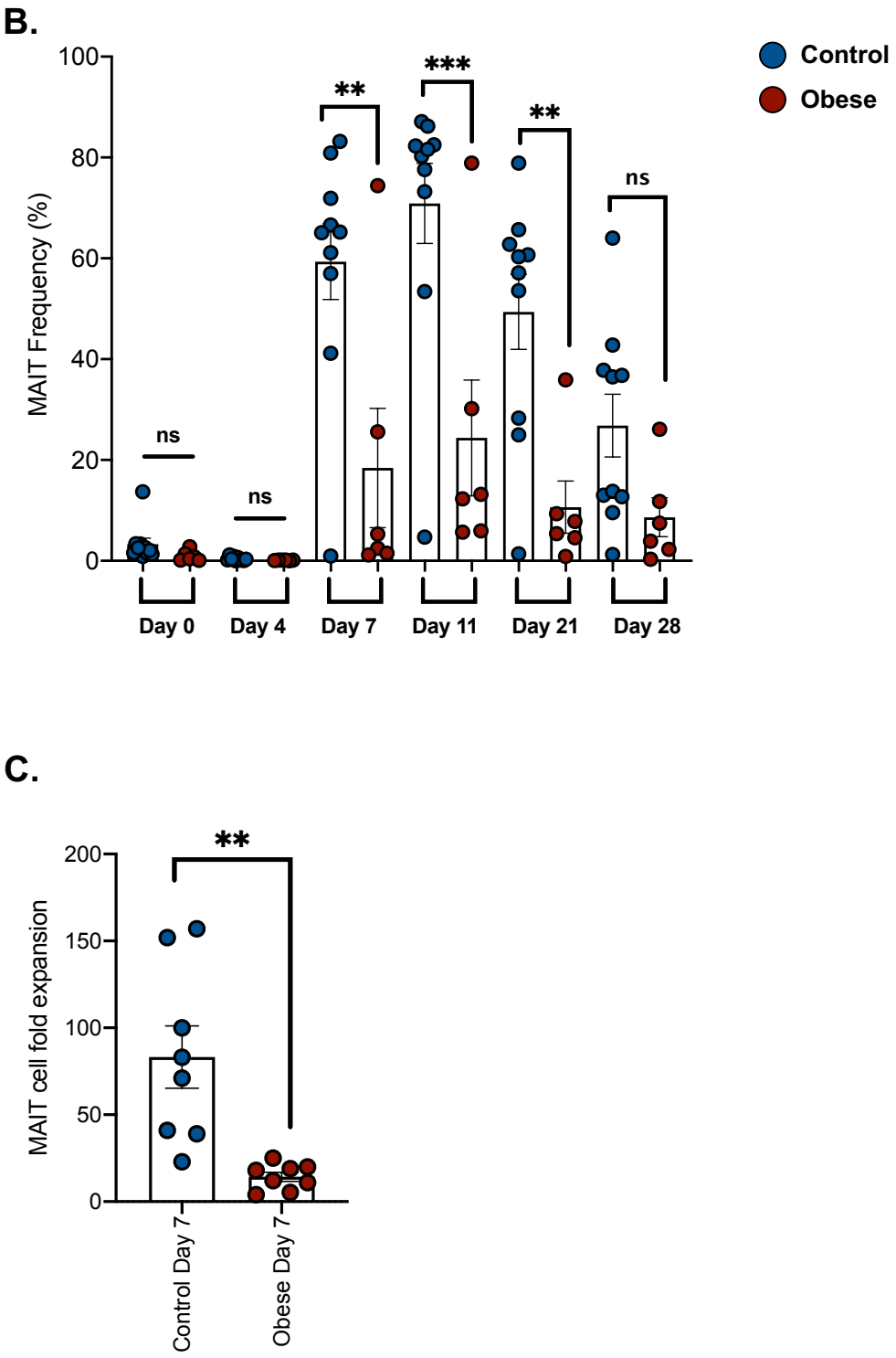


Figure 3.10 MAIT cells from people with obesity show impaired expansion in response to 5-ARU-MG and IL-2. (A) Representative dot plots of MAIT cell expansion from 3 individuals with obesity, observed over 28 days of culture in response to 5-ARU-MG and IL-2. PBMCs were stimulated with 5-ARU (1µg/mL) and MG (100µL) on day 0 and received a low dose of IL-2 (6.75ng/µL) on day 1 followed by high doses of IL-2 (33.3ng/µL) on days 4, 7, 11, 14, 18 and 21 (B) Scatter plots of control (n=10) and people with obesity (n=5) expansion over 28 days of culture. (C) Scatter plot of MAIT cell fold expansion of control subjects (n=8) and people with obesity (n=8) over 7 days of culture. Statistical analysis performed using one-way ANOVA with Tukey's correction or unpaired student's t-test, **p≤0.01, ***p≤0.001, ****p≤0.0001.

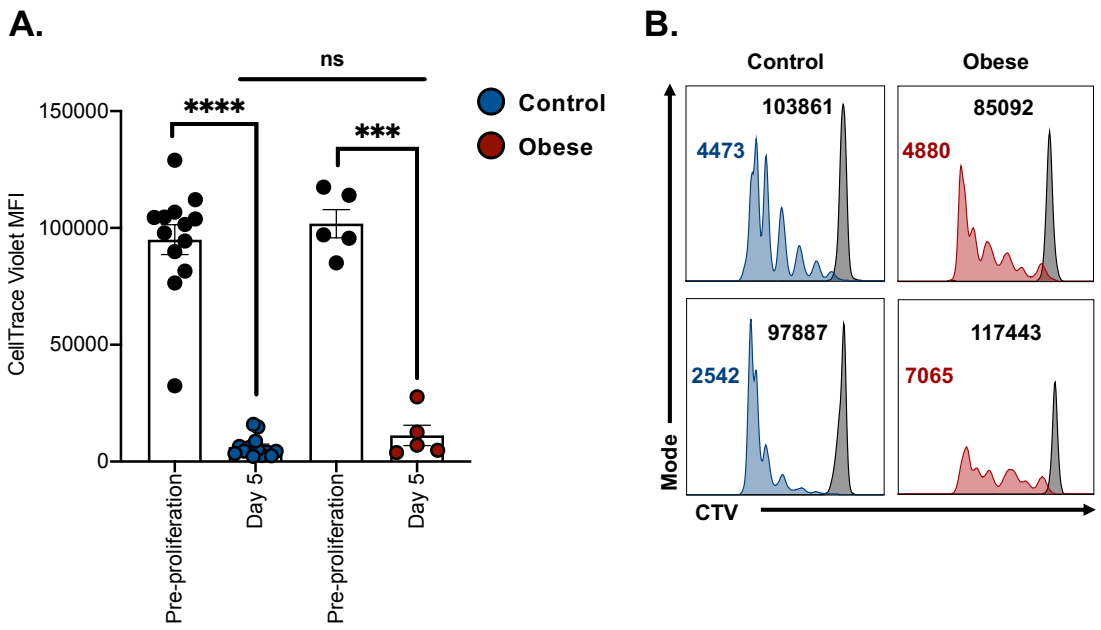


Figure 3.11 MAIT cells from people with obesity show similar proliferation profile in response to 5-ARU-MG and IL-2. (A) Scatter plot of MAIT cell proliferation as measured by uptake and dilution of Cell Trace Violet (CTV) at 5μM, on day 0 and day 5 of culture from control individuals (n=11) and people with obesity (n=5). PBMCs were unstimulated (pre-proliferation) or stimulated (day 5) with 5-ARU (1μg/mL) and MG (100μL) on day 0 and received a low dose of IL-2 (6.75ng/μL) on day 1 followed by a high dose of IL-2 (33.3ng/μL) on day 4. (B) Representative histograms from 2 control and 2 subjects with obesity of MAIT cell proliferation over 5 days of culture. Statistical analysis performed using one-way ANOVA with Tukey's correction ***p≤0.001, ****p≤0.0001.

3.3.7. MAIT cells produce IFNγ

Previous studies have reported MAIT cells are potent producers of multiple cytokines including IFNγ and can express a wide repertoire of transcription factors (Koay et al., 2019; Leeansyah et al., 2015). Expression of IFNγ has been shown to be induced with TCR dependent and independent stimulation, suggesting its production by MAIT cells can be induced in a variety of infections (Hinks et al., 2019; Ussher et al., 2014; Van Wilgenburg et al., 2016). Transcription control of MAIT cell cytokine expression has been only investigated in the past five years. Koay *et al*. reported co-expression of T-bet and RORγT at stage 3 of MAIT cell maturation in humans (Koay et al., 2016). Lamichhane *et al.* investigated the transcription profile of activated $CD8^+$ MAIT cells activated via TCR or cytokines stimulation and reported an upregulation of T-bet and RORγT in MAIT cells on both a transcriptional and protein level (Lamichhane et al., 2019).

Here, we sought to confirm these findings in our cohort by assessing the cytokine and transcription factor profile of peripheral blood MAIT cells. Stimulation of PBMC using TCR Dynabeads, IL-12 and IL-18 for 18 hours, significantly increased the percentage of IFNγ producing MAIT cells (Figure 3.12 A&B). To determine whether the enhancement in IFNγ production was due to the direct effect of TCR and cytokine activation on MAIT cells, rather than indirect effect exerted by other cells in the PBMC culture, we stimulated expanded MAIT cells using TCR Dynabeads and cytokines for 4 hours for mRNA analysis or 24hours to measure protein expression. Upon activation MAIT cells significantly upregulated protein and mRNA expression of IFNγ (Figure 3.12 C&D). This was accompanied by significant enhancement in the expression of T-bet (*TBX21*) mRNA in our cohort of control individuals following stimulation (Figure 3.12 E). Overall, these results indicate that TCR and cytokine co-stimulation of MAIT cells increase their expression of T-bet which is likely to facilitate the upregulation of IFNγ synthesis (Figure 3.12 A-D).

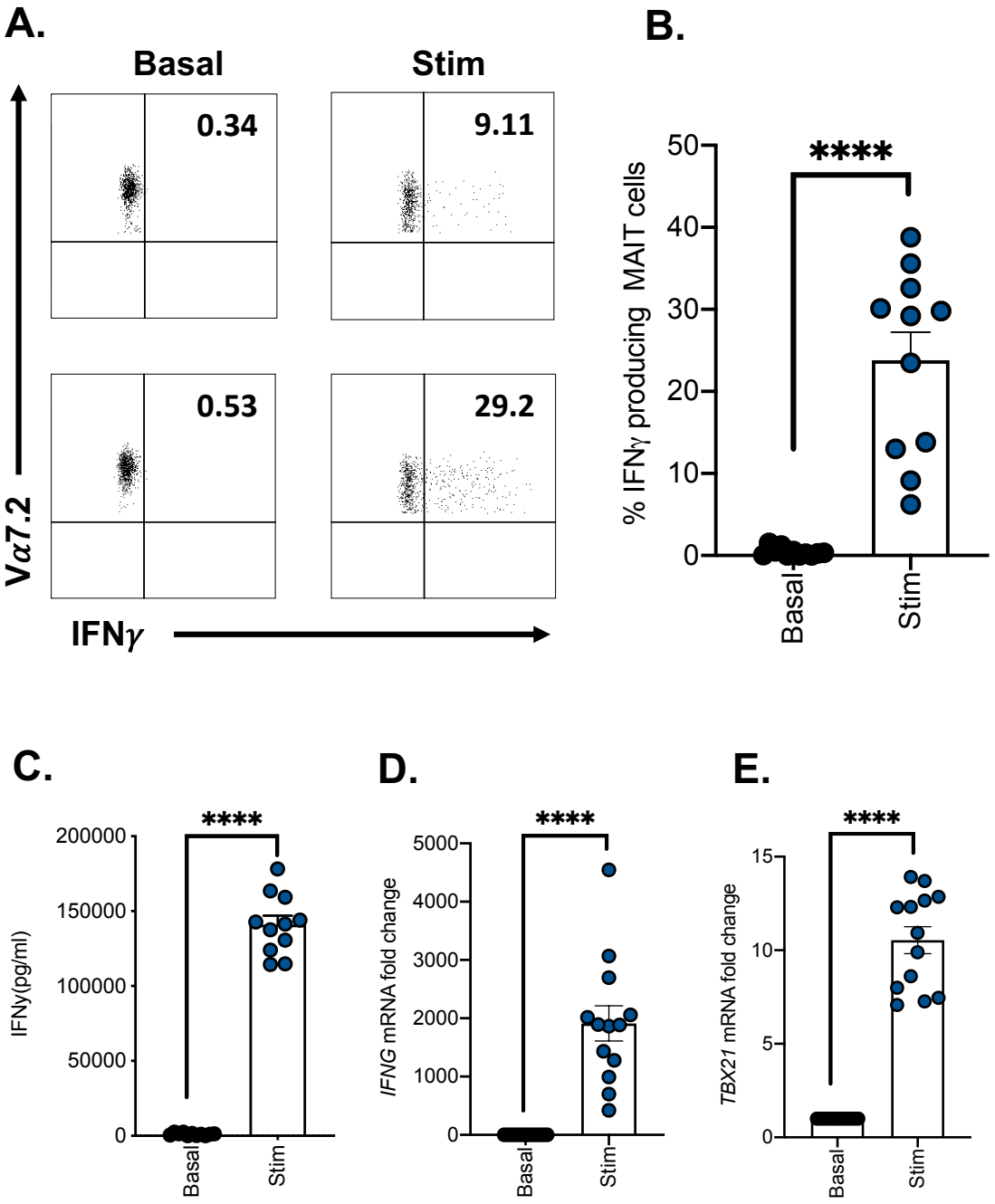


Figure 3.12 MAIT cell produce IFNγ upon stimulation. (A) Representative dot plots and (B) scatter plot showing MAIT cell IFNγ production by control individuals basally or upon stimulation with TCR microbeads (Milteneyi 25ng/mL), IL-12 and IL-18 (50ng/mL each) for 18 hours (n=11). (C) Scatter plot showing IFNγ production (pg/mL) by purified MAIT cells basally and upon stimulation with TCR Dynabeads (Dynabeads; 1:1 ratio), IL-12 and IL-18 (50ng/mL each) for 24 hours (n=11). (D) and (E) Scatter plots showing normalized expression of *IFNG* and *TBX21* mRNA by expanded and purified MAIT cells in response to TCR microbeads (Dynabeads; 1:1 ratio) IL-12 and IL-18 (50ng/mL each) for 4 hours (n=13). Statistical analysis performed using paired student's t-test, ****p≤0.0001.

3.3.8. MAIT cells produce IL-17A

As described in chapter one, MAIT cells are involved in a range of bacterial and viral infections (Lal et al., 2020; Le Bourhis et al., 2010; Spaan et al., 2016). However, in addition to their role in host protection, MAIT cells have also been implicated in a range of chronic inflammatory diseases, such as obesity or MS (Carolan et al., 2015; Willing et al., 2018). In particular, type 17 MAIT cells have been highlighted as a potentially pathogenic subset. Th17 cells were originally established as the primary producers of IL-17, however as research in this area progressed, MAIT cells were identified to be a significant contributor to the pool of this cytokine during inflammation.

In order to further explore MAIT cell function, next we investigated the ability of MAIT cells to produce IL-17 in our cohort of control individuals. Expanded MAIT cells, activated as per section 3.3.7 significantly increased IL-17A protein production (Figure 3.13 A) and mRNA expression (Figure 3.13 B). This is in line with previously published data, which presented MAIT cells as potent producers of IL-17A (Pisarska et al., 2020).

As previously discussed in section 3.3.6, Koay *et al.* and Lamichhane *et al.* reported co-expression of T-bet and RORγT transcription factors by MAIT cells. Having confirmed that *TBX21* (T-bet) mRNA is upregulated upon stimulation of MAIT cells from our cohort of control individuals (Figure 3.12 E), next we sought to investigate whether expression of mRNA coding for RORγT follows a similar pattern to *TBX21*. Indeed, stimulation of expanded MAIT cells as per section 3.3.7 significantly increased *RORC* mRNA expression (Figure 3.13 C). This data shows that TCR and cytokine stimulation is sufficient to increase mRNA expression for RORγT and enhance expression of IL-17A by MAIT cells.

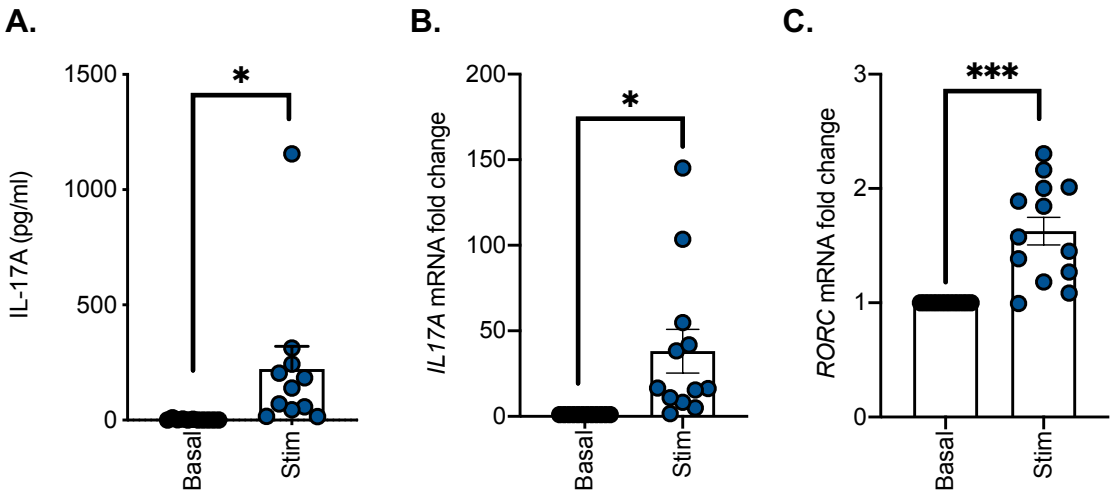


Figure 3.13 MAIT cells produce IL-17A upon stimulation. (A) Scatter plot showing IL-17A production (pg/mL) by purified MAIT cells from control individuals basally and upon stimulation with TCR Dynabeads (Dynabeads; 1:1 ratio), IL-12 and IL-18 (50ng/mL each) for 24 hours (n=11). (B) Scatter plots showing normalized expression of *IL17A* and (C) *RORC* mRNA by expanded and purified MAIT cells in response to TCR microbeads (Dynabeads; 1:1 ratio) IL-12 and IL-18 (50ng/mL each) for 4 hours (n=13). Statistical analysis performed using paired student's t-test, *p≤0.05, ***p≤0.001.

3.3.9. MAIT cell produce less IFNγ and more IL-17 cytokines in obesity

Dysregulation of MAIT cell cytokine production has been reported in obesity. MAIT cells were shown to have impaired production of IFNγ and enhanced IL-17 expression in comparison to their lean counterparts (Carolan et al., 2015; Magalhaes et al., 2015). Here we sought to confirm these findings in our cohort of people with obesity.

Expanded MAIT cells from people with obesity or control individuals were stimulated with TCR Dynabeads, IL-12 and IL-18 cytokines for 4 hours for mRNA analysis or 24 hours for the measurement of protein expression. MAIT cells from people with obesity displayed lower IFNγ production upon activation in comparison to the controls (Figure 3.14 A). This was also reflected at the transcriptional level where stimulated MAIT cells from people with obesity had lower levels of *IFNG* mRNA in comparison to the control individuals (Figure 3.14 B). As we have shown that transcription of *TBX21* mRNA is significantly upregulated upon MAIT cell activation (Figure 3.12 E), next we investigated whether transcription of *TBX21* is impacted in people with obesity. We showed that the expression of mRNA coding for T-bet upon activation was lower in people in obesity in comparison to the control individuals (Figure 3.14 C). Investigation of IL-17 expression by MAIT cells from individuals with obesity, demonstrated a significant increase in IL-17 protein expression upon activation in comparison to the control individuals (Figure 3.15 A), however no significant difference was observed in the expression of mRNA for IL-17 or RORγT (Figure 3.15 B&C). These results further confirm dysregulation in cytokine production by MAIT cells in obesity and provide additional information regarding the transcriptional phenotypic differences between the two cohorts.

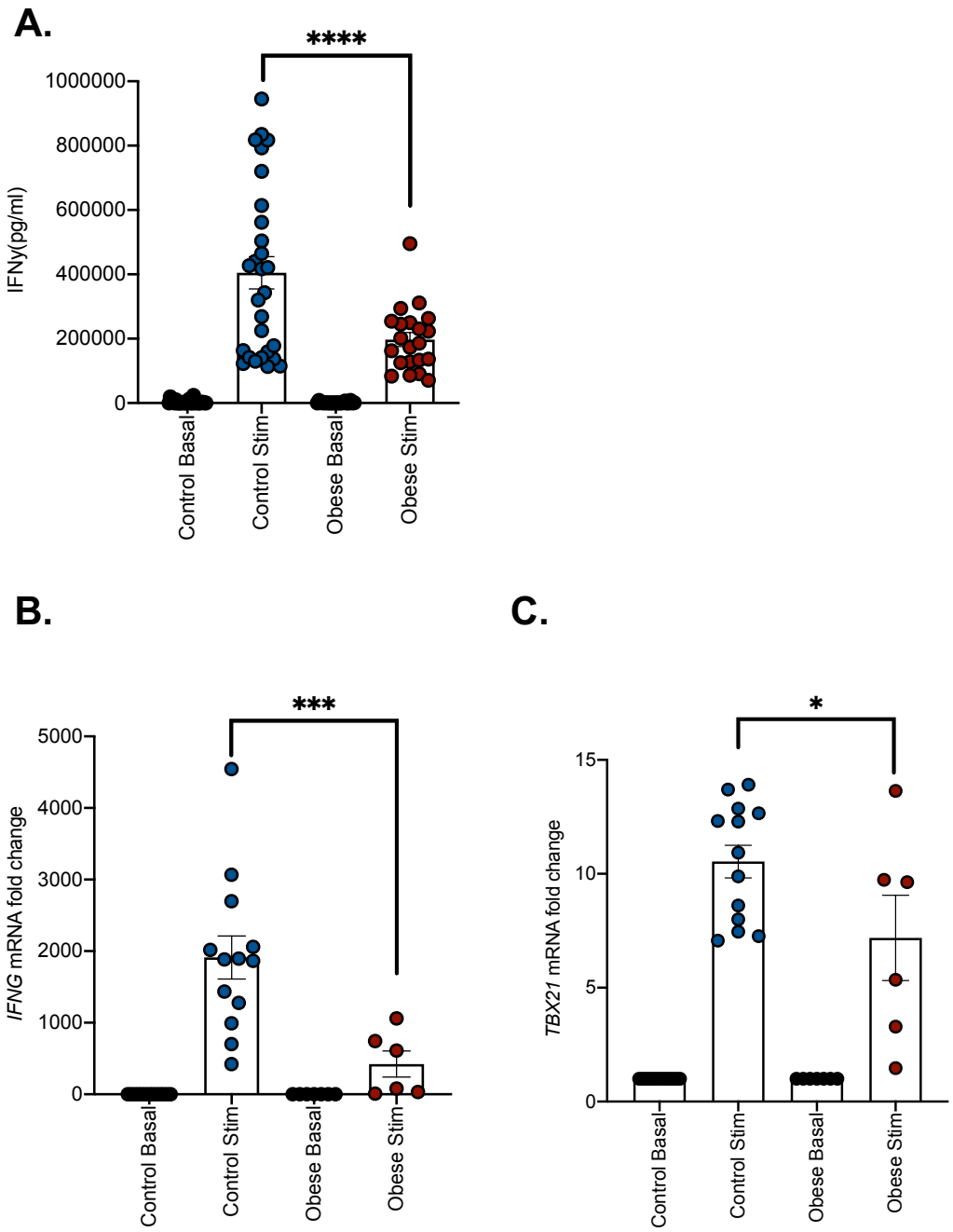


Figure 3.14 MAIT cell production of IFNγ upon stimulation is reduced in obesity. (A) Scatter plots of IFNγ protein production by control individuals and individuals with obesity in response to TCR microbeads (Dynabeads; 1:1 ratio) IL-12 and IL-18 (50ng/mL each) for 24 hours (control n=28; obese n=21). (B) Scatter plots of *IFNG* (IFNγ) and (C) *TBX21* (T-bet) mRNA transcription by MAIT cells from healthy and people with obesity basal or upon stimulation with TCR Dynabeads (1:1 bead to cell ratio), IL-12 and IL-18 (50ng/mL each) for 4 hours (control n=13; obese n=6). Statistical analysis performed using one-way ANOVA with Tukey's correction, *p≤0.05, ***p≤0.001, ****p≤0.0001.

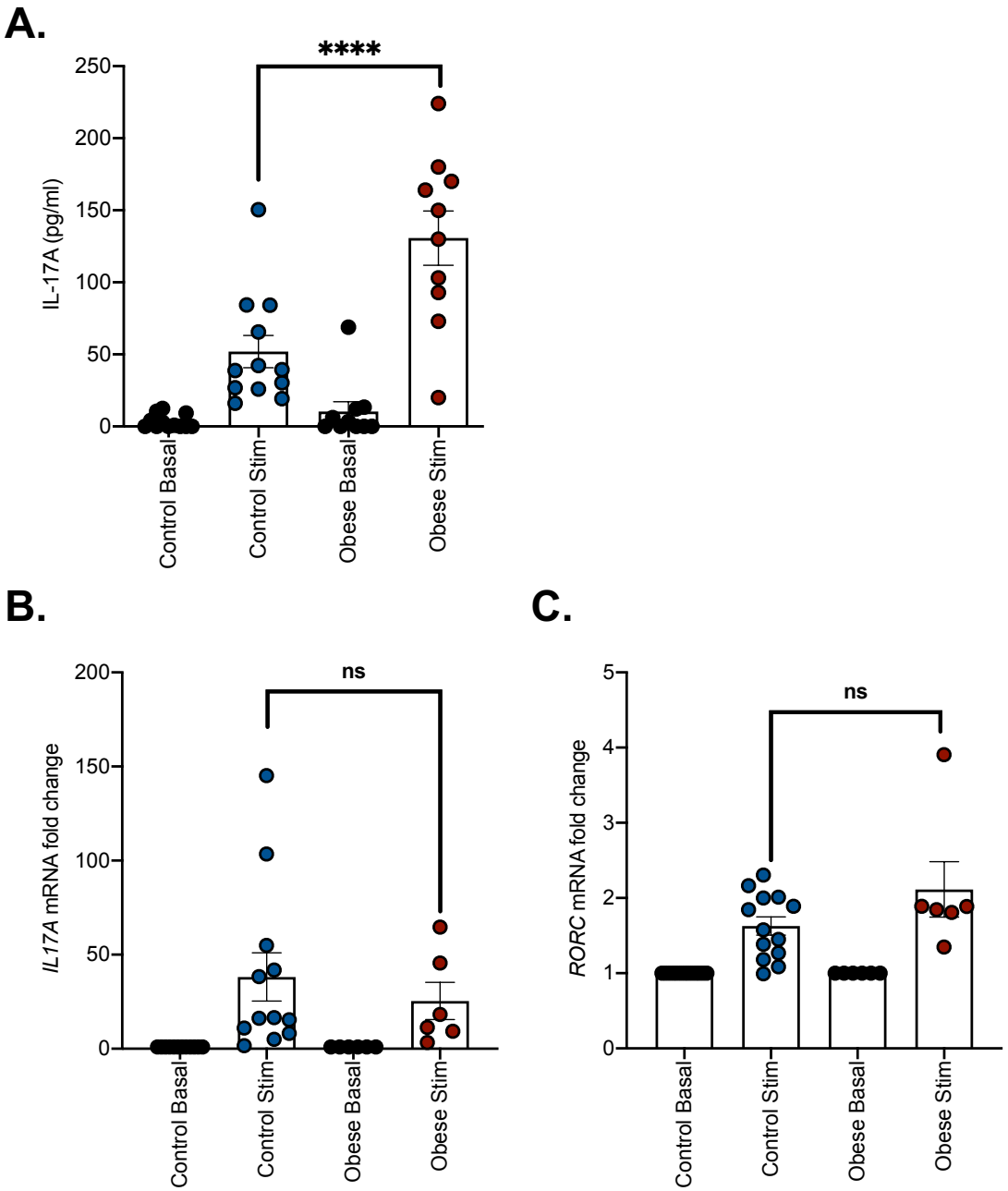


Figure 3.15 MAIT cells from individuals with obesity, display enhanced IL-17 protein production, but no difference in the transcription of *IL17A* & *RORC* upon stimulation in comparison to the controls. (A) Scatter plots showing IL-17A protein production by control individuals (n=12) and individuals with obesity (n=10) in response to TCR microbeads (Dynabeads; 1:1 ratio) IL-12 and IL-18 (50ng/mL each) for 24 hours. (B) Scatter plots showing normalized expression of *IL17A* (controls n=12; obese n=6) and (C) *RORC* (controls n=13; obese n=6) mRNA by expanded and purified MAIT cells from controls and individuals with obesity in response to TCR microbeads (Dynabeads; 1:1 ratio) IL-12 and IL-18 (50ng/mL each) for 4 hours. Statistical analysis performed using one-way ANOVA with Tukey's correction, ****p≤0.0001.

3.3.10. MAIT cells produce an expanded repertoire of cytokines

At present, the majority of the studies that investigated the role of MAIT cells in the immune system, have largely focused on the production of the well-studied cytokines, IFNγ, IL-17A and TNF-α (Provine and Klenerman, 2020). Interestingly, it has been reported that MAIT cells are capable of producing other cytokines such as IL-22 and IL-17F (Gibbs et al., 2017; Lu et al., 2020; Toussirot et al., 2018). To further characterise MAIT cells in the control subjects, expression of IL-17F, IL-22 and IL-32 was measured. Expanded MAIT cells were activated as described in section 3.3.9. MAIT cells displayed an increase in their expression of protein and mRNA for IL-17F and IL-22 cytokines (Figure 3.16 A&B; D&E). A more modest increase in IL-32 was noted (Figure 3.16 C), while the transcription of *IL32* mRNA was reduced upon activation (Figure 3.16 F). Overall, these results indicate that although MAIT cells are potent producers of IFNγ, they are also capable of producing other cytokines such as IL-17F, IL-22 and IL-32. As these cytokines play a role in inflammation, the contribution of MAIT cells to the levels produced during an immune response should be further investigated.

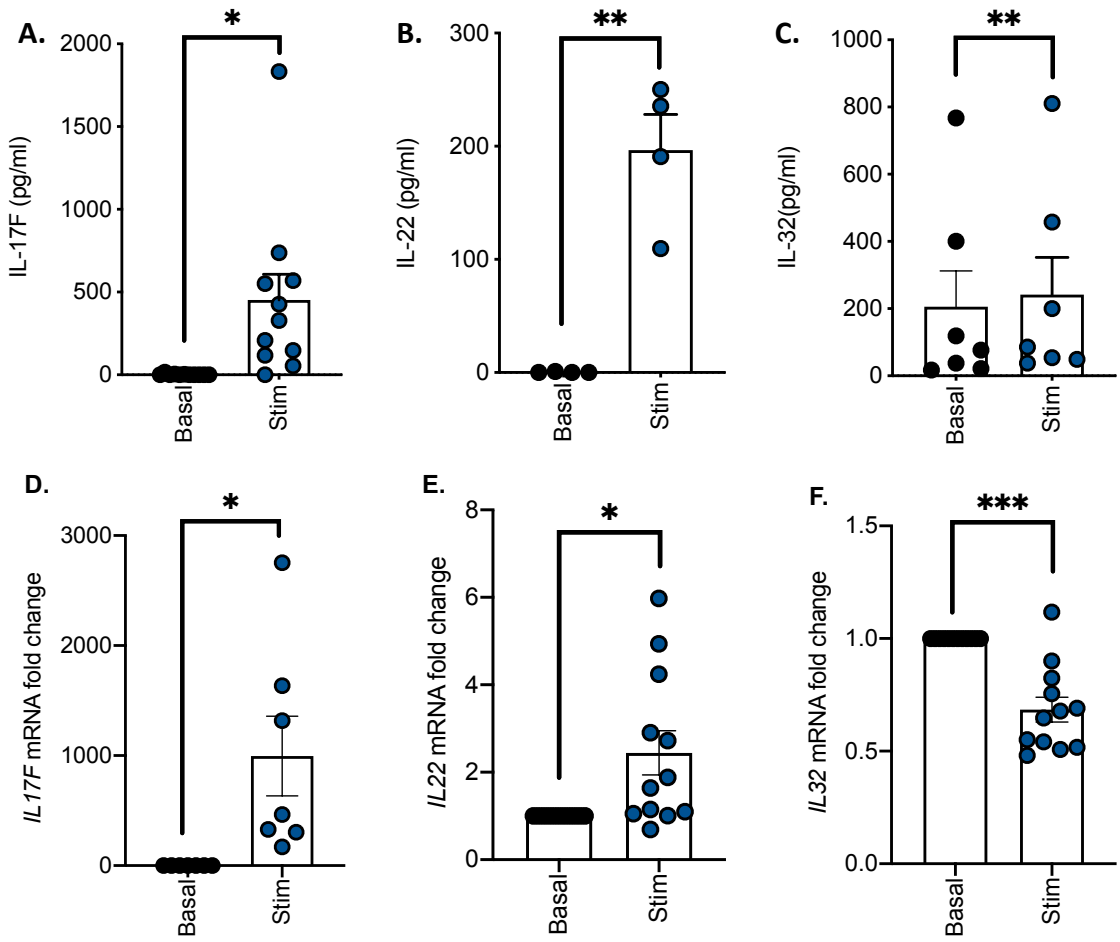


Figure 3.16 Stimulation of MAIT cells induces the production of IL-17F, IL-22 and IL-32 cytokines. (A-C) Scatter plots showing concentration of IL-17F, IL-22 & IL-32 in the cell supernatants, produced by the expanded and purified MAIT cell upon stimulation with TCR microbeads (Dynabeads; 1:1 ratio) IL-12 and IL-18 (50ng/mL each) for 24 hours (IL-17F n=11; IL-22 n=4; IL-32 n=7). (D-F) Scatter plots showing normalized expression of *IL17F*, *IL22*, *IL33* by expanded and purified MAIT cells in the control population in response to TCR microbeads (Dynabeads; 1:1 ratio) IL-12 and IL-18 (50ng/mL each) for 4 hours (IL-17F n=7; IL-22 n=12; IL-32 n=12) Statistical analysis performed using paired student's t-test, *p≤0.05, **p≤0.01, ***p≤0.001.

3.3.11. Individuals with obesity display no significant difference in the expression of other immune cytokines

Investigations around MAIT cells phenotypes in people with obesity has focused on IFNγ and IL-17, the most studied cytokines produced by these cells. Here, we decided to examine whether alteration of MAIT cell function extends beyond the imbalances observed with IFNγ and IL-17.

Expanded MAIT were activated as per section 3.3.9. MAIT cells from people with obesity produced IL-17F and IL-32 protein upon stimulation, but no significant difference was observed in the expression between people with obesity and control individuals (Figure 3.17 A&B). mRNA expression coding for IL-17F mirrored its protein synthesis and showed no significant alteration in transcription between the two cohorts (Figure 3.17 C). On the other hand, stimulation of MAIT cells from people with obesity revealed a defect in the downregulation of *IL32* mRNA expression in comparison to the control subjects. In fact, MAIT cells from people with obesity produced higher levels of IL-32 protein upon stimulation in comparison to the control individuals, however the difference in the expression was not significant (Figure 3.17 B&D). Investigation of IL22 transcription upon stimulation of MAIT cells from people with obesity revealed no significant difference in the expression between the two cohorts (Figure 3.17 E), however this data must be confirmed on the protein level. Collectively, this data shows non-significant alterations in expression of these three cytokines by MAIT cells from people with obesity. Increasing the number of experiments on MAIT cells from people with obesity and control individuals may provide more definite answers in the future.

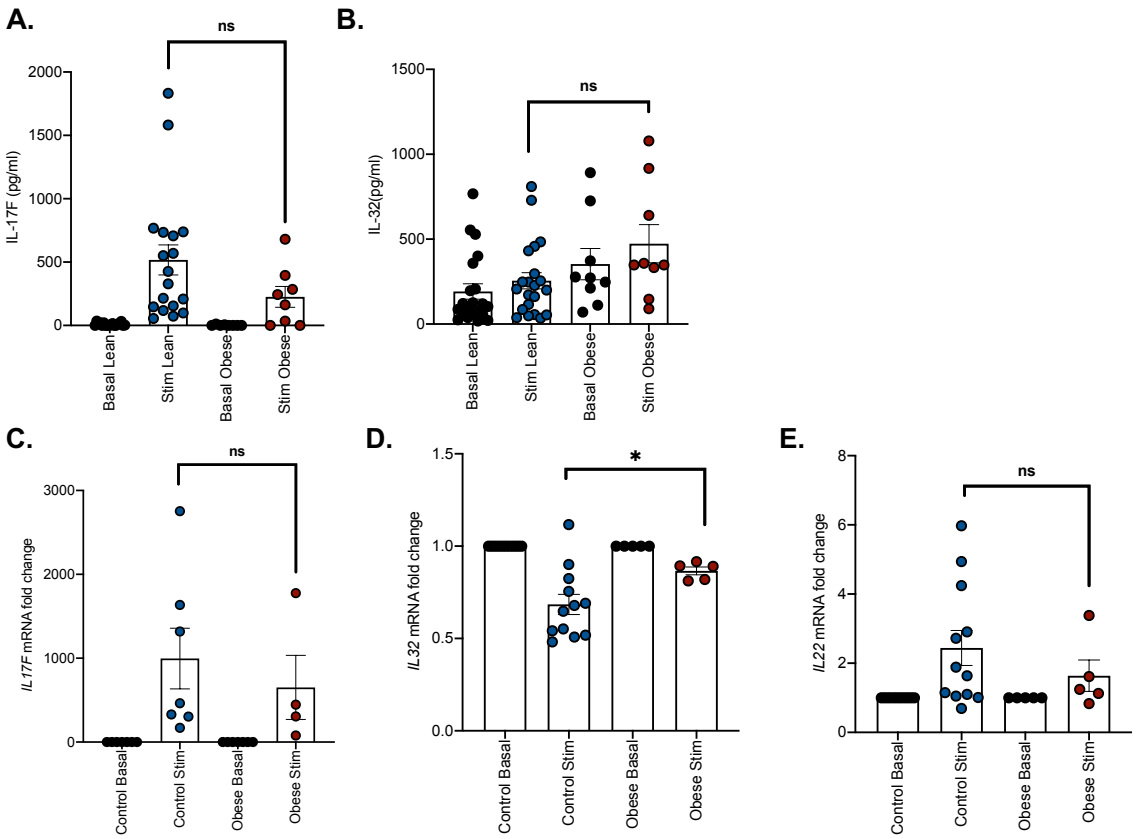


Figure 3.17 Stimulation of MAIT cells reveals no difference in *IL17F* and *IL22* mRNA transcription by the cells from healthy and obese individuals but shows impairment in *IL32* transcription. (A-B) Scatter plots showing concentration of IL-17F and IL-32 in the cell supernatants, produced by the expanded and purified MAIT cell upon stimulation with TCR microbeads (Dynabeads; 1:1 ratio) IL-12 and IL-18 (50ng/mL each) for 24 hours (IL-17F control =18, obese n=8; IL-32 control =21, obese n=9). (C-E) Scatter plots showing normalized expression of *IL17F*, *IL22*, *IL32* by expanded and purified MAIT cells in the control population in response to TCR microbeads (Dynabeads; 1:1 ratio) IL-12 and IL-18 (50ng/mL each) for 4 hours (*IL17F* control =7, obese n=4; *IL32* control =12, obese n=5; *IL22* control =12, obese n=5). Statistical analysis performed using paired student's t-test, *p≤0.05, **p≤0.01, ***p≤0.001.

3.4 Discussion

MAIT cells comprise 1-10% of circulating T cells in healthy individuals (Gherardin et al., 2018b; Le Bourhis et al., 2010). In line with these publications, we report that MAIT cell frequencies in the peripheral blood of our cohort of control individuals ranged from 0.5% to 13.7%. To further characterise MAIT cells, next we described their proliferative and expansion capacity in response to TCR activation. Stimulation of MAIT cells with their cognate antigen resulted in limited proliferation. This data aligns with previous studies which demonstrated MAIT cell proliferation *in vitro* in response to *E. coli* or 5-OP-RU (Dias et al., 2016; Kurioka et al., 2017; Rahimpour et al., 2015). In agreement with published data, TCR stimulation enhanced IL-2 receptor expression, suggesting it may play a role in MAIT cell function and proliferation (Van Wilgenburg et al., 2016; Vorkas et al., 2018). It has been well established that IL-2 supports T cell proliferation, therefore we hypothesized that the increase in IL-2R combined with antigen stimulation and exogenous IL-2 supplementation may enhance proliferation of MAIT cells. Indeed, here we showed that the combination of TCR and IL-2 signals led to significantly greater expansion and proliferation of MAIT cells *in vitro*. This suggested that appropriate combination of signals *in vivo* can potentially drive expansion of MAIT cells in response to a pathogen.

Next, we examined the repertoire of cytokines and transcription factors expressed by MAIT cells upon activation. IFNγ a cytokine which is highly implicated in supporting anti-microbial responses, has been shown to be upregulated upon TCR and/or cytokine stimulation (Dusseaux et al., 2011; Van Wilgenburg et al., 2016; Wang et al., 2018). In line with these findings, we demonstrated that the activation of MAIT cells using TCR, IL-12 and IL-18 led to the transcription of mRNA coding for IFNγ and T-bet, followed by an increase in IFNγ protein. This is consistent with the study published by Lamichhane *et al*. who reported an increase in the expression of mRNA coding for IFNγ and T-bet upon stimulation of MAIT cells (Lamichhane et al., 2019). Another cytokine produced by MAIT cells which has been of particular interest is IL-17, due to its potent pro-inflammatory properties (Pisarska et al., 2020). IL-17^{+} MAIT cells have been confirmed at the site of inflammation in multiple diseases including MS and RA (Kim et al., 2017; Willing et al., 2018). In agreement with published data, stimulation of MAIT cells upregulated IL-17A production and enhanced

transcription of mRNA coding for RORγT (Lamichhane et al., 2019). Collectively, this data showed that the activation of MAIT cells induced a very rapid alteration in the transcription events. MAIT cells respond by increasing the expression of transcription factors, most likely to facilitate the increased demand for synthesis of these cytokines in response to the activation signals. As the research progressed, the range of cytokines produced by MAIT cells expanded. Recently, other cytokines of the IL-17 cytokine family, IL-17F and IL-22 have gained more attention (Gibbs et al., 2017; Lamichhane et al., 2019; Toussirot et al., 2018). In line with Lamichhane *et al.*, we showed an enhancement in the synthesis of IL-17F and IL-22 upon MAIT cell stimulation (Lamichhane et al., 2019). In addition, these cells expressed IL-32, a cytokine which has not been previously described to be produced by MAIT cells. These results illustrate that MAIT cells can produce a much wider repertoire of pro-inflammatory cytokines, although further studies are required to explore what other effector molecules are expressed by these cells.

After the characterisation of MAIT cell functional responses in the control subjects, next we sought to describe their activity in people with obesity. In line with other studies, people with obesity presented with lower MAIT cell frequencies in the peripheral blood in comparison to the control subjects (Carolan et al., 2015; Magalhaes et al., 2015; Toubal et al., 2020) and displayed severely impaired expansion upon cell activation. Surprisingly, no difference was observed in the rate of proliferation assessed using CTV. This may be due to the limitations associated with the CTV assay. This method allowed for tracking MAIT cell proliferation only up to five days, whereas the defects in MAIT cells expansion from people in obesity has been observed seven days after activation and was sustained for up to 14 days. To date proliferation of MAIT cells in obesity was only briefly assessed in adipose tissue using Ki67 expression. MAIT cells from individuals with obesity were shown to have higher proliferative capacity in the AT in comparison to the peripheral blood, however this study didn't compare Ki67 expression between people with obesity and lean controls (Magalhaes et al., 2015). Toubal *et al.* has shown that MAIT cells from the adipose tissue of the control and HFD mice displayed no difference in proliferation as measured by expression of Ki67, however proliferation capacity from the peripheral blood was not assessed (Toubal et al., 2020). We propose that the impairment in MAIT cell

expansion in people with obesity may be contributing to the low frequencies of MAIT cells observed in their peripheral blood. However, other factors such as activation induced cell death or MAIT cell migration to the site of inflammation should be studied in more detail to provide a more comprehensive explanation.

As obesity had a profound impact on MAIT cell frequencies and proliferation, next we assessed their cytokine production upon cell activation. In comparison to the control individuals, MAIT cells from people with obesity displayed lower expression of IFNγ which was accompanied by decreased transcription of T-bet transcription factor. This is in line with Carolan *et al.* who demonstrated an impairment in the frequencies of IFNγ producing MAIT cells in obesity (Carolan et al., 2015). Magalhaes *et al.* displayed contradicting results, as in their hands people with obesity had a higher proportion of IFNγ producing MAIT cells in comparison to the lean controls (Magalhaes et al., 2015). This discrepancy may be due to the fact Magalhaes used a pan activator of cells (PMA/Ionomycin), whereas our study used a more specific approach in activating MAIT cells. This data indicates that the deficiency in IFNγ expression by MAIT cells from people with obesity occurs at the transcription level. This cytokine plays a central role in fighting microbial infections, therefore loss of IFNγ may contribute to the greater susceptibility to infections associated with obesity. In contrast to IFNγ, MAIT cells from people with obesity produced more IL-17 cytokine than the control individuals but displayed no difference in the transcription of mRNA for IL-17A or RORγT. This suggested that the defect, which leads to enhanced IL-17 production occurs at the post-transcriptional level. Similar observations were published by Carolan *et al.* and Magalhaes *et al*., who also reported an enhancement in IL-17 production by MAIT cells in obesity. There is growing evidence indicating that overexpression of IL-17 is harmful and contributes to the chronic inflammation observed in obesity and therefore may contribute to the development of T2DM (Chehimi et al., 2017; Liu and Nikolajczyk, 2019). Further studies have revealed that MAIT cells from people with obesity display lower (non-significantly) expression of IL-17F and IL-22 in comparison to the controls. In addition, MAIT cells from people with obesity failed to suppress transcription of *IL32* mRNA upon stimulation, which resulted in an enhanced albeit non-significant expression of IL-32 cytokine in comparison to the controls. This data suggested that IL-17F and IL-22 may not play an important role in maintaining

the pro-inflammatory environment in obesity, however the role of IL-32 should be further investigated.

Collectively this study has provided more insight into MAIT cell proliferation and cytokine production. We show that MAIT cells can proliferate and express a wide range of cytokines and transcription factors, whereas MAIT cells from people with obesity display a marked deficiency in MAIT frequencies and expansion and significant alterations in the cytokine expression (Figure 3.18). This may lead to the lower frequencies, susceptibility to infections and inflammation observed in obesity.

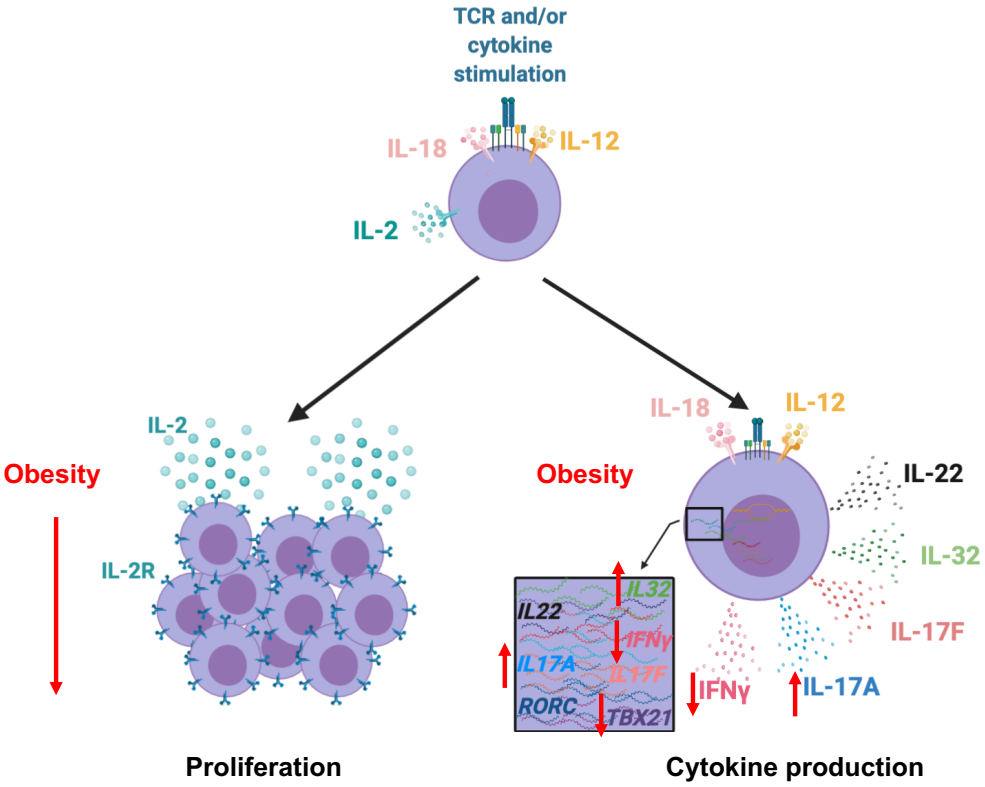


Figure 3.18 Summary of MAIT cell function in control and people with obesity. Stimulation MAIT cells enhances expression of IL-2 receptor, induces their proliferation, transcription of T-bet and RORγT transcription factors and production of IFNγ, IL-17A, IL-17F, IL-22 and IL-32 cytokines. MAIT cells from people with obesity (red) display diminished proliferation and alterations in the expression of multiple cytokines and transcription factors.

Chapter Four

Results

MAIT cell metabolism

4.1. Introduction

4.1.1. Glycolysis in immunometabolism

As the field of immunometabolism advances, it becomes clear that glycolysis is essential to support immune cell survival and function. Glucose is one of the few fundamental nutrients required for T cell function, therefore it is no surprise glycolysis has become a focal point of multiple studies of immunometabolism. In order for glucose to enter the cell, it requires glucose transporters. Glucose transporters can be put into two main categories of sodium-glucose linked transporters (SGLT) and facilitated diffusion glucose transporters (GLUT) (Navale and Paranjape, 2016). The category of glucose transporters which are expressed by immune cells and are largely studied are GLUTs. This family of transporters consists of 12 members and to date, T cells were found to express GLUT1, 3, 4, 6 and 8 (Kavanagh Williamson et al., 2018; Macintyre et al., 2014), of which GLUT1 is the major and most thoroughly studied glucose transporter in T cells (Palmer et al., 2016). Surface expression of this transporter is markedly increased upon their TCR & CD28 activation (Jacobs et al., 2008; Ricciardi et al., 2018; Wieman et al., 2007). Expression of the transporter is controlled at multiple stages including transcription and trafficking to the cell membrane, however shuffling of GLUT1 to the cell surface was shown to be the key event responsible for the rapid upregulation of its expression (Jacobs et al., 2008; Macintyre et al., 2014).

Upon uptake of glucose, the carbohydrate enters glycolysis, which takes place in the cytosol of the cell and is central to cell metabolism. This process breaks down glucose into pyruvate, which further feeds into the Kreb's cycle to contribute to the energy production for cellular function via oxidative phosphorylation (Almeida et al., 2016). Glucose and glycolysis are crucial in T cell function. One of the first scientists who identified that glycolysis is linked to T cell activity was Robson MacDonald in 1977. Using glycolysis inhibitor 2DG, MacDonald reported inhibition of the cytotoxic activity of T cells (Robson MacDonald, 1977). More recent studies have shown that indeed glycolysis plays a central role in T cells during activation. CD8 effectors cells, Th1 and Th17 cells can substantially upregulate their glycolysis upon stimulation (Gubser et al., 2013; Michalek et al., 2011), whereas the substitution of glucose with galactose in the culture media impairs IFNγ production by CD4 T cells (Chang et al., 2013). Inhibition of

glycolysis with 2DG promotes the development of Treg rather than Th17 cells, highlighting the role of glycolysis in determining the fate of naïve T cells (Shi et al., 2011). As more studies emerge, it is becoming clear that for some aspects of T cell activation, especially IFNγ production, the role of glycolysis extends beyond energy production.

A study by Chang *et al.* identified a key role for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which is one of the multiple enzymes involved in glycolysis, in T cell functional responses. In the resting state of T cells, GAPDH binds to IFNγ mRNA and prevents its translation. Upon cell activation and triggering of glycolysis, GAPDH dissociates from the IFNγ mRNA strand and reverts back to fulfil its function in the glycolysis pathway (Chang et al., 2013). IFNγ is also controlled metabolically at the transcription level in T cells. Acetylation of histones affects the access of transcription enzymes to the IFNγ gene. Acetyl Co-A is produced during oxidation of pyruvate, the end product of glycolysis and can be used to acetylate proteins, including histones (Peng et al., 2016). An intermediate of glycolysis, phosphoenolpyruvate (PEP), was also found to impact IFNγ production. PEP inhibits the activity of endoplasmic reticulum calcium transporter sarcoendoplasmic reticulum calcium ATPase (SERCA), which shuffles calcium from the cytoplasm to endoplasmic reticulum. This leads to the activation of the calcium dependent nuclear factor of activated T cells (NFAT) signalling and transcription of IFNγ (Ho et al., 2015). Although glycolysis plays a role in multiple ways to impact IFNγ production, metabolic intermediates produced as a result of this process, may also be supporting lymphocytes in their metabolism and function. Pyruvate, the end product of glycolysis, can be used in synthesis of amino acids and fatty acids (Figure 4.1) (O'Neill et al., 2016), however it has been also shown to play a role in CD4 effector memory cell survival by stabilising mitochondrial membrane potential to prevent apoptosis. Dimeloe *et al.* have shown that independely of oxygen availability, disruption of glycolysis results in an increased expression of apoptotic proteins. This was associated with decreased mitochondrial membrane potential, which was shown to be rescued by addition of pyruvate(Dimeloe et al., 2016). However, pyruvate isn't the only product of glycolysis, which supports the cell metabolism. Metabolic intermediates produced during this process, such as glucose-6-phosphate can enter PPP to produce ribose, which is required for

nucleotides synthesis (Figure 4.1) (O'Neill et al., 2016). This suggests that the involvement of glycolysis in the control of immune cells function is more complex than first anticipated. Current findings uncover a very exciting area for further research, emphasising the extensive influence of metabolic processes on immune responses.

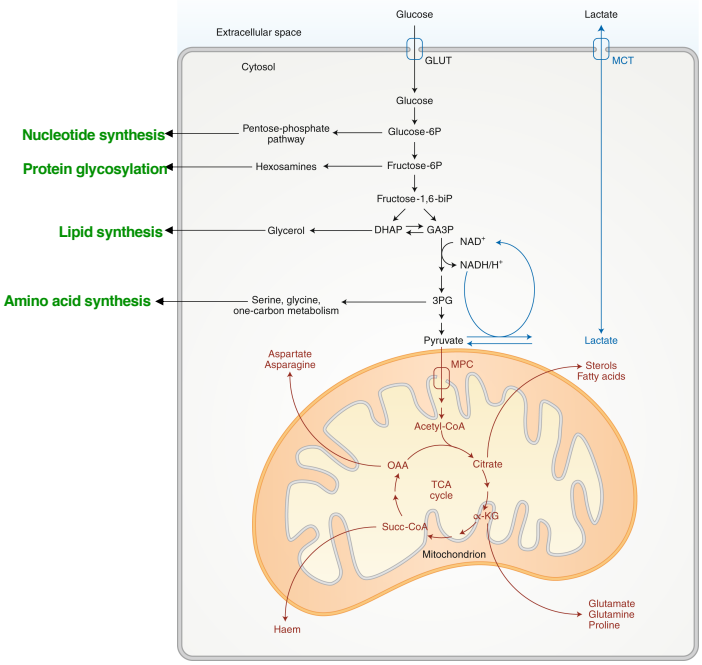


Figure 4.1 Glycolysis. Glucose is metabolised to pyruvate during glycolysis, which can enter Kreb's cycle. Glycolysis also leads to synthesis of multiple intermediates, which can feed into other metabolic pathways and contribute to the production of macromolecules (in green). Adapted from DeBerardinis and Chandel, 2020.

4.1.2. mTORC1

mTOR is an evolutionarily conserved serine/ threonine kinase, which regulates the activation and function of multiple proteins (Saxton and Sabatini, 2017). mTOR integrates an array of signals including growth factor signals, oxygen level, cell energy, nutrient availability and antigen receptor signalling. Based on the received signals, it controls cell metabolism, growth, differentiation and apoptosis (Salmond, 2018; Saxton and Sabatini, 2017). mTOR is the key catalytic component of two complexes it can be associated with, namely mTORC1 and mTORC2 (Figure 4.2) (Salmond, 2018). Although these complexes share three subunits- mTOR, mLST8 (mammalian lethal with sec13 protein 8) and DPETOR (DEP domain containing mTOR interacting protein). mTORC1 further associates with Raptor (regulatory protein associated with mTOR) and PRSA40 (proline-rich AKT substrate 40kDa), whereas mTORC2 is defined by the presence of Rictor (rapamycin insensitive companion of mTOR), mSIN1, or Protor-1/2 (Saxton and

Sabatini, 2017). Out of these two complexes, mTORC1 is shown to be of particular importance in immune cell function and control of glycolysis (Delgoffe et al., 2011, 2009; Finlay et al., 2012; Sinclair et al., 2013)

mTORC1 plays a substantial role in regulating cell growth, proliferation and activation. Activation of T cells via TCR or cytokine stimulation increases mTORC1 activity indicating a role for mTORC1 in T cell function (Rao et al., 2010; Ray et al., 2015). Expression of mTORC1 is shown to influence the fate of T cells, as CD4 T cells which lack mTORC1 fail to develop into Th1, Th2 or Th17, but rather favour Treg differentiation (Delgoffe et al., 2011, 2009). On the other hand, enhanced activity of mTORC1 in CD8 T cells leads to highly activated effector cells, which fail to transition into memory cells (Pollizzi et al., 2015). This indicates that tight control of mTORC1 is essential for maintaining T cell homeostasis.

As multiple factors can activate mTORC1, the regulation of this kinase is very complex. Among various points of control, changes in the cellular environment such as nutrient, energy and oxygen availability, can affect the activity of the kinase via the action of AMP-activated protein kinase (AMPK) (Saxton and Sabatini, 2017). Glucose restriction was shown to activate AMPK, leading to the phosphorylation of raptor or tuberous sclerosis complex 2 (TSC2) (regulator of mTORC1) and attenuation of the mTORC1 complex (Rolf et al., 2013; Saxton and Sabatini, 2017). Although mTORC1 is sensitive to glucose concentration, it was also shown to be absolutely required for control of glycolytic metabolism. mTORC1 is required to maintain high levels of glycolytic activity to support T cell function (Finlay et al., 2012). Treatment of T cells with rapamycin, an inhibitor of mTORC1, impairs glucose uptake induced by TCR signalling. It also prevents the upregulation of glucose transporters and glycolytic enzymes (Finlay et al., 2012; Hukelmann et al., 2016). Similar findings were obtained by Donnelly *et al.* in a study of natural killer (NK) cells, which demonstrated that stimulation of mTORC1 is responsible for the control of enhanced glycolysis and production of IFNγ and Granzyme B (Donnelly et al., 2014).

Amino acids were also shown to affect mTORC1 signalling. Although amino acids are required for protein synthesis and their enhanced import during cell activation is important to facilitate the increased nutrient demands of the cells, it is the

concentration of leucine, which directly affects the activity of mTORC1(Figure 4.2) (Saxton and Sabatini, 2017). The presence of amino acids activates Rag, a GTPase, which in turn mediates the translocation of mTORC1 to the lysosomes. This facilitates the lysosome bound Ras homolog enriched in brain (Rheb) to activate mTORC1 (Figure 4.2) (Saxton and Sabatini, 2017). Activity of Rag is controlled by the interaction between Sestrin2, a protein which controls cell metabolism and GATOR, which is a Rag activating protein (Figure 4.2) (Kim et al., 2015). Briefly, Kim *et al.* reported that Sestrin2 binds to GATOR2 resulting in its inhibition. GATOR2 is a negative regulator of GATOR1, therefore this interaction results in the liberation of GATOR1 from GATOR2 complex and its activation. The release of GATOR1 inhibits the activity of the Rag protein resulting in suppression of mTORC1 activity (Kim et al., 2015). Sestrin2 itself is a leucine sensor. Leucine binds to Sestrin2, leading to the freeing of GATOR2, which binds to GATOR1, allowing Rag mediated activation of mTORC1 (Chantranupong et al., 2014; Saxton et al., 2016; Wolfson et al., 2016). This method of mTORC1 control was shown to be relevant in T cells. Sinclair *et al.* described that deletion of amino acid transporter LAT1, which transports leucine, leading to the downregulation of mTORC1 activity in murine T cells (Sinclair et al., 2013).

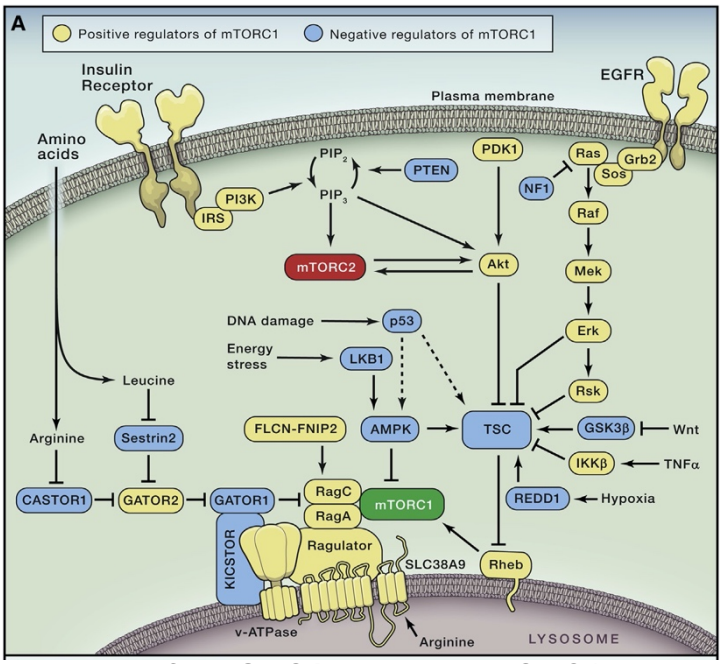


Figure 4.2. Regulation of mTORC1 activity. mTORC1 is tightly controlled by cellular and environmental signals such as growth factors, nutrient availability, DNA damage or energy availability. All combined signals incorporated by mTORC1 decide on the faith of the cell (Saxton and Sabatini, 2017).

4.1.3. Amino acid metabolism

Although glucose and glycolysis are central in T cell homeostasis and function, the role of amino acid metabolism cannot be underestimated. Amino acids are essential building blocks of proteins, but their role in the cell function was reported to be far more complex**.** It has been established that interruption to amino acid transport or metabolism impairs immune cell function, however the mechanisms behind this altered immune response have begun to emerge in recent years (Ma et al., 2017; Rodriguez et al., 2007; Sinclair et al., 2013). The role of multiple amino acids is currently under investigation in the field. However, leucine and glutamine have been the focus of this study due to their effects on T cell activation (Nakaya et al., 2014; Sinclair et al., 2013; Wolfson et al., 2016).

Amino acids are transported into the cells by a wide range of membrane bound transporters. Leucine, one of the essential amino acids, was found to be transported into the cells by LAT1 (Hsu and Dzhagalov, 2019). LAT1 belongs to a family of four L-type amino acid transporters, which are sodium independent and transport neutral amino acids. In addition to leucine, they facilitate the influx of isoleucine, valine, phenylalanine and methionine (Hsu and Dzhagalov, 2019). This family of transporters includes LAT1 (SLC7A5), LAT2 (SLC7A8), LAT3 *(*SLC43A1) and LAT4 (SLC43A2), with LAT 1 is the most studied member of this family. LAT1 forms a heterodimer with CD98 (SLC3A2) and was shown to be an antiporter, which imports leucine in exchange for intracellular glutamine (Hsu and Dzhagalov, 2019). SLC7A5 was found to be upregulated in human and murine T cells upon stimulation (Hayashi et al., 2013; Sinclair et al., 2013). Study by Sinclair *et al.* elegantly showed that T cells require LAT1 for differentiation into the effector cells. Deletion of SLC7A5 in mice resulted in a reduction in T cell differentiation into Th1, Th17 and CTL cells, however, Treg differentiation remained unaffected (Sinclair et al., 2013). As leucine was shown to be transported via LAT1 and the activation of mTOR, the defect in T cell proliferation and differentiation in the absence of SLC7A5 expression was linked to the lack of this amino acid and failure to induce the appropriate metabolism response to support the cell function (Sinclair et al., 2013). Further investigation into the association between amino acid transport via LAT1 and T cell function revealed that SLC7A5 deficient T cells failed to reprogram their metabolism and resembled c-Myc null cells. It was shown that an influx of amino acids via LAT1 L-system

transporter was required to sustain the expression of c-Myc transcription factor in T cells, which is critical for cell metabolic reprogramming, proliferation and survival (Gnanaprakasam and Wang, 2017; Sinclair et al., 2013). This was also confirmed by another study, which investigated the role of amino acids for NK cell function and activation. They also highlighted a role for glutamine, rather than leucine in supporting the c-Myc expression (Loftus et al., 2018). Furthermore, Hayashi *et al.* confirmed the upregulation of LAT1 expression by human primary T cells upon stimulation. In line with the findings by Sinclair *et al*., Hayashi showed that the inhibition of LAT1 with a specific inhibitor, JPH203, or transfection with LAT1 specific siRNA, resulted in a decrease of leucine uptake and cytokine production by activated T cells. This study showed that TCR stimulation of T cells upregulates LAT1 in a NFκB and activator protein-1 (AP-1) dependent manner (Hayashi et al., 2013), whereas Sinclair *at al*. suggested SLC7A5 expression is controlled by ERK/MAPK and NFAT pathway (Sinclair et al., 2013). Another study by Sinclair *et al*., which studied the transport of kynurenine, a tryptophan metabolite, via SLC7A5 in T cells, depicted a very comprehensive method to investigate uptake of this metabolite and indirectly assess the function of the amino acid transporter. This paper formed the basis for the investigation of this amino acid metabolism and uptake by MAIT cell for this thesis (Sinclair et al., 2018). Expression patterns and function of other amino acids transporters including LAT2-4 in the cells of the immune system remains largely unexplored. Similarly, to LAT1, LAT2-4 transport large neutral amino acids (Babu et al., 2003; Bodoy et al., 2005; Yan et al., 2020). In addition, LAT2 was shown to also support transport of small neutral amino acids such as serine or alanine (Yan et al., 2020). Although the specific amino acids transported by LAT3 remain to be elucidated, it has been shown that LAT4 transports essential and branched chained amino acids such as phenylalanine, methionine and leucine or valine (Bodoy et al., 2005). Further research is required to assess the expression, function and importance of amino acid transporters other than LAT1 for the cells of the immune system. In addition, more detailed studies are vital to assess, whether all neutral amino acids are equally transported by all four transporters.

Among other amino acids, glutamine has also attracted a lot of attention from researchers in the context of immunometabolism. Glutamine is synthesised de novo by the cell or absorbed from the diet and imported into the cells through a

range of glutamine transporters, such as sodium coupled neutral amino acid transporters (SNAT)1-4 (SLC38A1-SLC38A4), and the most heavily studied glutamine importer Alanine, Serine, Cysteine Transporter 2 (ASCT2/SLC1A5) (Hsu and Dzhagalov, 2019). Upon entry, glutamine can be used for multiple purposes such as generation of energy thorough feeding into the Kreb's cycle. Mitochondrial glutaminase enzyme converts glutamine to glutamate, which can be further metabolised to α-ketoglutarate and enter the Kreb's cycle to produce ATP and contribute to the fatty acid synthesis via reductive carboxylation (Altman et al., 2016). In addition, glutamine can be also directed for synthesis of nucleotides, glutathione or O-GlcNAcylation of proteins (Altman et al., 2016).

Uptake of glutamine as well as expression of the glutamine transporters and enzymes involved in glutaminolysis are upregulated upon T cell stimulation, which in turn are dependent on the c-Myc pathway (Carr et al., 2010; Nakaya et al., 2014; Wang et al., 2011). The importance of glutamine uptake was highlighted by Nakaya *et al*., who showed that the deletion of ASCT2 in mice led to impaired differentiation of Th1 and Th17 cells, and failure to produce their signature cytokines (Nakaya et al., 2014). ASCT2 was also required for successful mTOR signalling, suggesting that the influx of glutamine via this transporter is important for the intracellular pool of glutamine, to be exchanged for leucine by transport via LAT1. Although glutamine is required for T cell proliferation, surprisingly deficiency in ASCT2 does not negatively affect proliferation (Carr et al., 2010; Nakaya et al., 2014), suggesting other glutamine transporters must be implicated. Indeed, Carr *et al.* and Raposo *et al.* have reported that T cell stimulation leads to the upregulation of other amino acid transporters, namely SNAT1 (SLC38A1) and SNAT2 (SLC38A2) (Carr et al., 2010; Raposo et al., 2015), whereas inhibition of SNAT glutamine transporters, reduces T cell proliferation and production of IFNγ and IL-2 (Raposo et al., 2015).

Research in the area of amino acid metabolism and immune response was shown to be very exciting, allowing for better understanding of the relationship between cell metabolism and immune cell function. Study of amino acids revealed their complex roles in the activity of the cell. It is now known, they are not merely substrates used for protein synthesis, but can also act as signalling molecules to support the immune cell function. Although a progress has been

made in understanding the role of amino acids in T cells, up to date there were no studies published, which would investigate their importance in MAIT cell function and activation.

4.2. Specific aims of this chapter

The specific aim of this chapter was the characterisation of MAIT cell metabolism in both healthy individuals and people with obesity.

Therefore, to:

1. Investigate nutrient transporter expression by MAIT cells in health and obesity
2. Elucidate metabolic pathways used by MAIT cells
3. Determine metabolic regulators of MAIT cell metabolism
4. Compare MAIT cell metabolism rates between healthy individuals and people with obesity

4.3. Results

4.3.1. MAIT cells express glucose transporters GLUT1, GLUT3 and GLUT4

As outlined, research has provided us with substantial evidence, which shows T cells require glucose and glycolysis to successfully perform their effector functions. It has been shown that stimulation of T cells leads to substantial upregulation of glycolysis, suggesting that glucose is one of the key nutrients, which supports T cell activation (Gubser et al., 2013; Michalek et al., 2011; Wang et al., 2011). In order for glucose to be metabolised, it must be transported into the cell. Among the 14 GLUT glucose transporters, which have been identified to be expressed by humans, T cells have been shown to express GLUT1, 3, 4, 6 and 8 (Kavanagh Williamson et al., 2018; Macintyre et al., 2014). At present, the majority of studies investigating a role of GLUT1 and GLUT3 in T cell function have reported an upregulation of their expression upon T cell stimulation (Hukelmann et al., 2016; Macintyre et al., 2014; Palmer et al., 2015). To the best of our knowledge, the expression of glucose transporters by MAIT cells has not yet been described.

We first determined the expression of glucose transporters GLUT 1, 3 and 4 on expanded MAIT cells using real time RT-qPCR. Expanded MAIT cells were found to express mRNA for these three GLUT transporters (Figure 4.3 A, D&E). Stimulation of expanded MAIT cells with TCR Dynabeads, IL-12 and IL-18 cytokines for 4 hours showed no significant difference in the *SLC2A1* mRNA level coding for GLUT1 in comparison to the basal expression (Figure 4.3 A) However, as GLUT1 is the best studied glucose transporter in T cells and its expression is known to be controlled at the point of trafficking of the complex from the cytosol to the cell surface, we decided to measure the expression of GLUT1 protein on the surface of MAIT cells by flow cytometry (Wieman et al., 2007). GLUT1 protein was expressed on the cell membrane of MAIT cells, and its expression was significantly decreased upon stimulation of PBMC via TCR, and cytokines after 18 hours (Figure 4.3 B&C). Analysis of mRNA expression of the other two glucose transporters by expanded MAIT cells stimulated via TCR and cytokines for 4 hours revealed significant increase in the transcription of *SLC2A3* (GLUT3) and *SLC2A4* (GLUT4) (Figure 4.3 D&E). This data indicates that MAIT cells express mRNA for the four major transporters tested and display a limited alteration in their expression upon stimulation at the chosen timepoints.

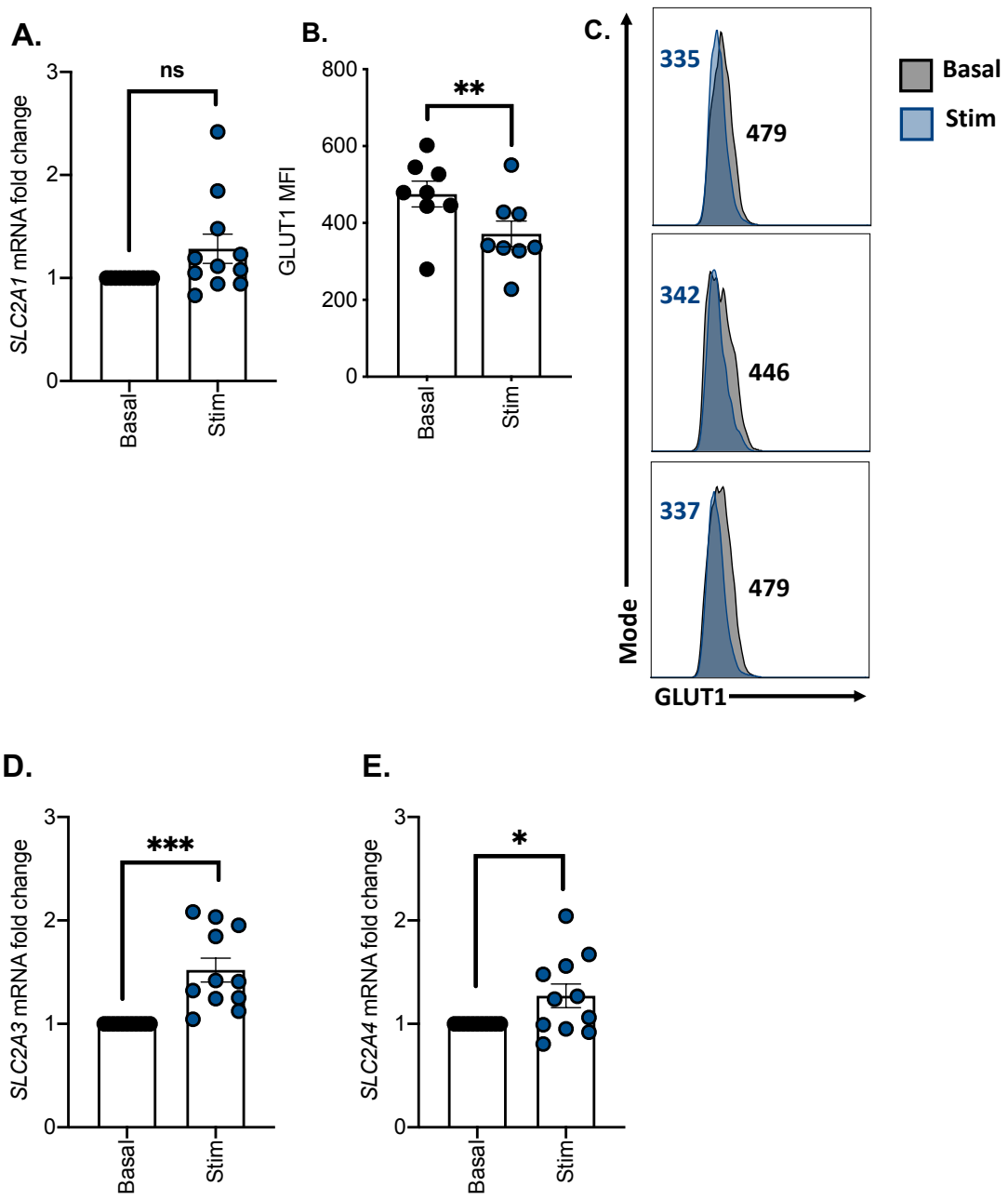


Figure 4.3 MAIT cells express mRNA and protein for glucose transporter GLUT1 and mRNA for GLUT3&4. (A) Scatter plot of normalized MAIT cell mRNA expression of glucose transporter GLUT1(*SLC2A1*) by purified MAIT cells basally and upon stimulation with TCR Dynabeads (cell to bead ratio 1:1), IL-12 and IL-18 (at 50ng/mL each) for 4 hours in the control population sample(n=11). (B) Scatter plot of surface MFI GLUT1 expression by MAIT cells from a PBMC culture; unstimulated or stimulated with TCR Dynabeads (cell to bead ratio 1:1) IL-12 and IL-18 (at 50ng/mL each) for 18 hours (n=8). (C) Representative histograms of two control samples showing GLUT1 expression on MAIT cells basally or upon stimulation with TCR Dynabeads cell to bead ratio 1:1), IL-12 and IL-18 (at 50ng/mL each) for 18 hours. (D-E) Scatter plot of normalized MAIT cell mRNA expression of glucose transporters GLUT3 and GLUT4 (*SLC2A3-4*) by purified unstimulated MAIT cells and cells stimulated with Dynabeads (cell to bead ratio 1:1), IL-12 and IL-18 (at 50ng/mL each) for 4 hours in the control population sample (n=11). Statistical analysis performed using paired student's t-test, ns-not significant, * p≤0.05, ** p≤0.01, *** p≤0.001.

4.3.2. MAIT cells from individuals with obesity display no difference in glucose transporters mRNA expression

MAIT cells from the control individuals expressed GLUT1, 3 and 4 glucose transporters and displayed modulation of their expression upon activation (Figure 4.3), therefore next investigated the impact of obesity on the expression of these glucose transporters by MAIT cells. Similarly, to control subjects, MAIT cells from people with obesity expressed mRNA for all three glucose transporters (Figure 4.4 A-C). Expanded MAIT cells from people with obesity, stimulated as per section 4.3.1 displayed no significant differences in the expression of mRNA coding for GLUT1, 3 and 4, when compared to the cohort of control individuals (Figure 4.4 A-C). This investigation showed that during early stages of MAIT activation, people with obesity display no defect in transcription of mRNA for glucose transporters GLUT1, 3 and 4.

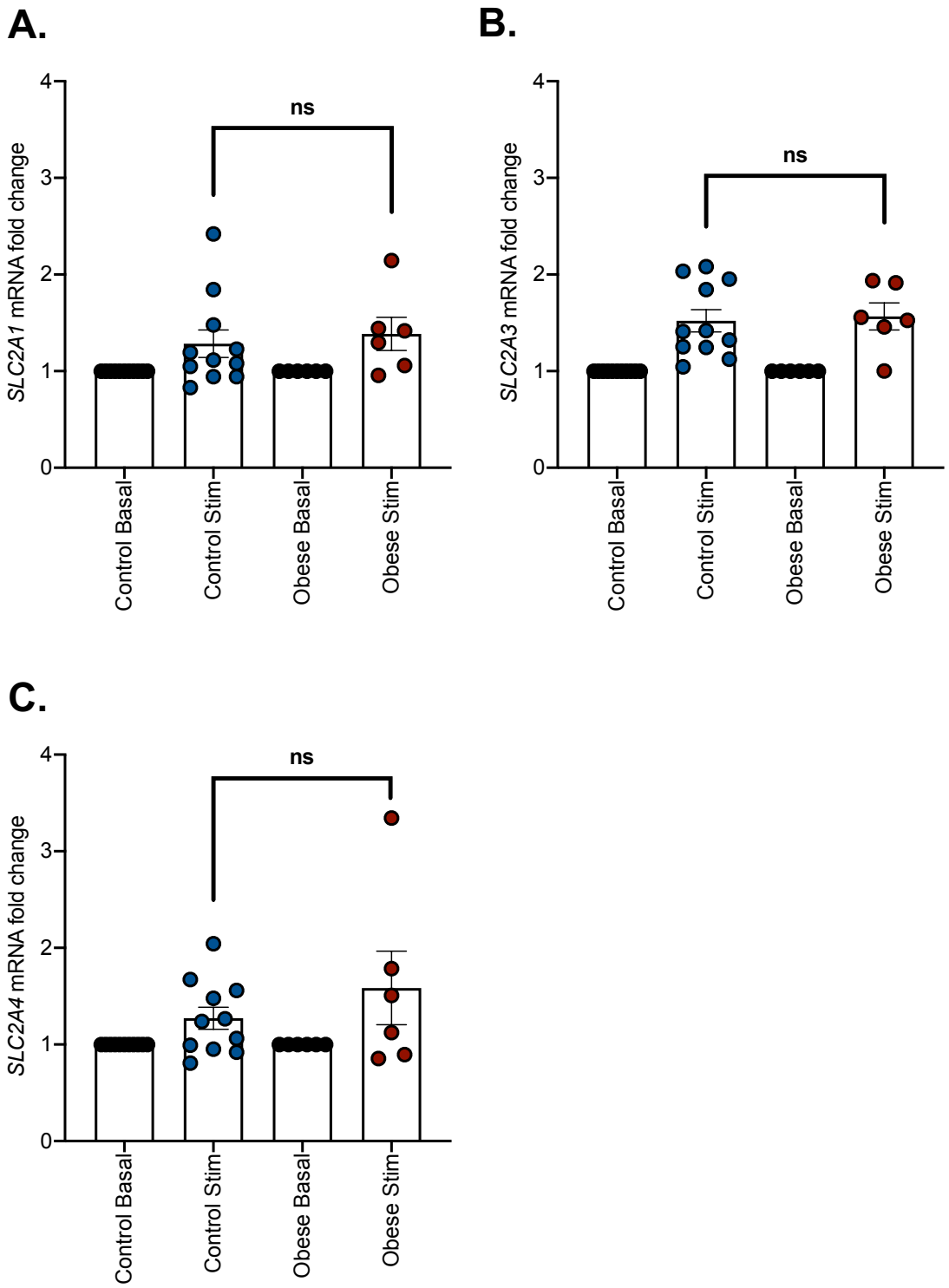


Figure 4.4 MAIT cells from people with obesity showed no difference in mRNA expression for GLUT glucose transporters. (A-C) Scatter plot of normalized MAIT cell mRNA expression of glucose transporters *SLC2A1, SLC2A3 and SLC2A4* (GLUT1,3 and 4) by purified unstimulated MAIT cells and cells stimulated with Dynabeads (cell to bead ratio 1:1), IL-12 and IL-18 (at 50ng/mL each) for 4 hours derived from the controls and people with obesity (control n=11; obese n=6. Statistical analysis performed using one-way ANOVA with Tukey's correction, ns-not significant.

4.3.3. MAIT cells express glutamine transporters ASCT2, SNAT1 and SNAT2

Having established the expression of the major glucose transporter, we next investigated if MAIT cells express glutamine transporters. Glutamine is a non-essential amino acid, implicated in multiple cell processes including the production of ATP by feeding into Kreb's cycle, synthesis of nucleic acids by a provision of biosynthetic precursors and in protein O-GlcNAcylation (Altman et al., 2016; Hsu and Dzhagalov, 2019). Although glutamine can be synthesised de novo, it may also be obtained from the diet, requiring transport via glutamine transporters (Hsu and Dzhagalov, 2019). Glutamine can be transported in and out of the cells through a range of amino acid transports. ASCT2 and LAT1 have been reported as the main glutamine transporter in T lymphocytes (Hsu and Dzhagalov, 2019; Nakaya et al., 2014), however the family of SNAT, especially SNAT1 and SNAT2 were also shown be involved in glutamine transport in T cells (Hsu and Dzhagalov, 2019).

Expression of the glutamine transporters was measured by real time RT-qPCR and expanded MAIT cells expressed mRNA coding for ASCT2, SNAT1 and SNAT2 transporters (Figure 4.5 A-C). Upon stimulation with TCR Dynabeads, IL-12 and IL-18 for 4 hours, MAIT cells greatly increased their expression of *SLC1A5* (ASCT2) neutral amino transporter (Figure 4.5 A). More modest increases in the expression of *SLC38A1* (SNAT1) and *SLC38A2* (SNAT2) were observed (Figure 4.5 B&C). MAIT cells were shown to display a similar pattern in the expression of glutamine transporters to the reported expression of these transporters by T cells. In addition, upregulation of transcription of the glutamine transporter *SLC1A5* (ASCT2) this early into activation of MAIT cells suggests that the transport of glutamine into MAIT cells may play an important role in their activation and function.

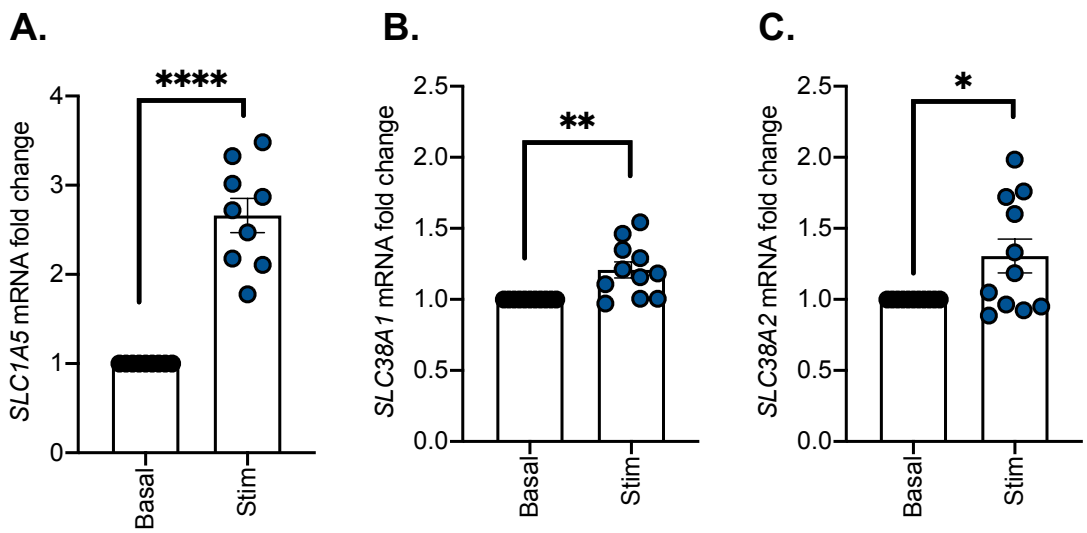


Figure 4.5 MAIT cells express mRNA for glutamine transporters ASCT2 and SNAT1&2. (A-C) Scatter plots of normalized MAIT cell mRNA expression of glutamine transporters (A) *SLC1A5* (ASCT2) (n=9) and (B-C) *SLC38A1-2* (SNAT1-2) (n=11) by purified unstimulated MAIT cells and cells stimulated with TCR Dynabeads (cell to bead ratio 1:1), IL-12 and IL-18 (at 50ng/mL each) for 4 hours in the control population sample. Statistical analysis performed using paired student's t-test, ns-not significant, *p≤0.05, **p≤0.01, **** p≤0.0001.

4.3.4. MAIT cells from individuals with obesity display no difference in expression of mRNA for glutamine transporters

We next decided to extend our investigations in obesity by investigating the mRNA expression pattern of ASCT2, SNAT1 and SNAT2 glutamine transporters on MAIT cells from people with obesity. Expanded MAIT cells from individuals with obesity were stimulated as per section 4.3.3. MAIT cells expressed *SLC1A5 (*ASCT2*), SLC38A1(*SNAT1) and *SLC38A2 (*SNAT2) mRNA at levels comparable to the control individuals (Figure 4.6 A-C). This suggests that similarly to the expression of mRNA for glucose transporters (Figure 4.4), MAIT cells from people with obesity, display no defect in the transcription of mRNA for glutamine transporters at 4 hours post activation with TCR Dynabeads, IL-12 and IL-18.

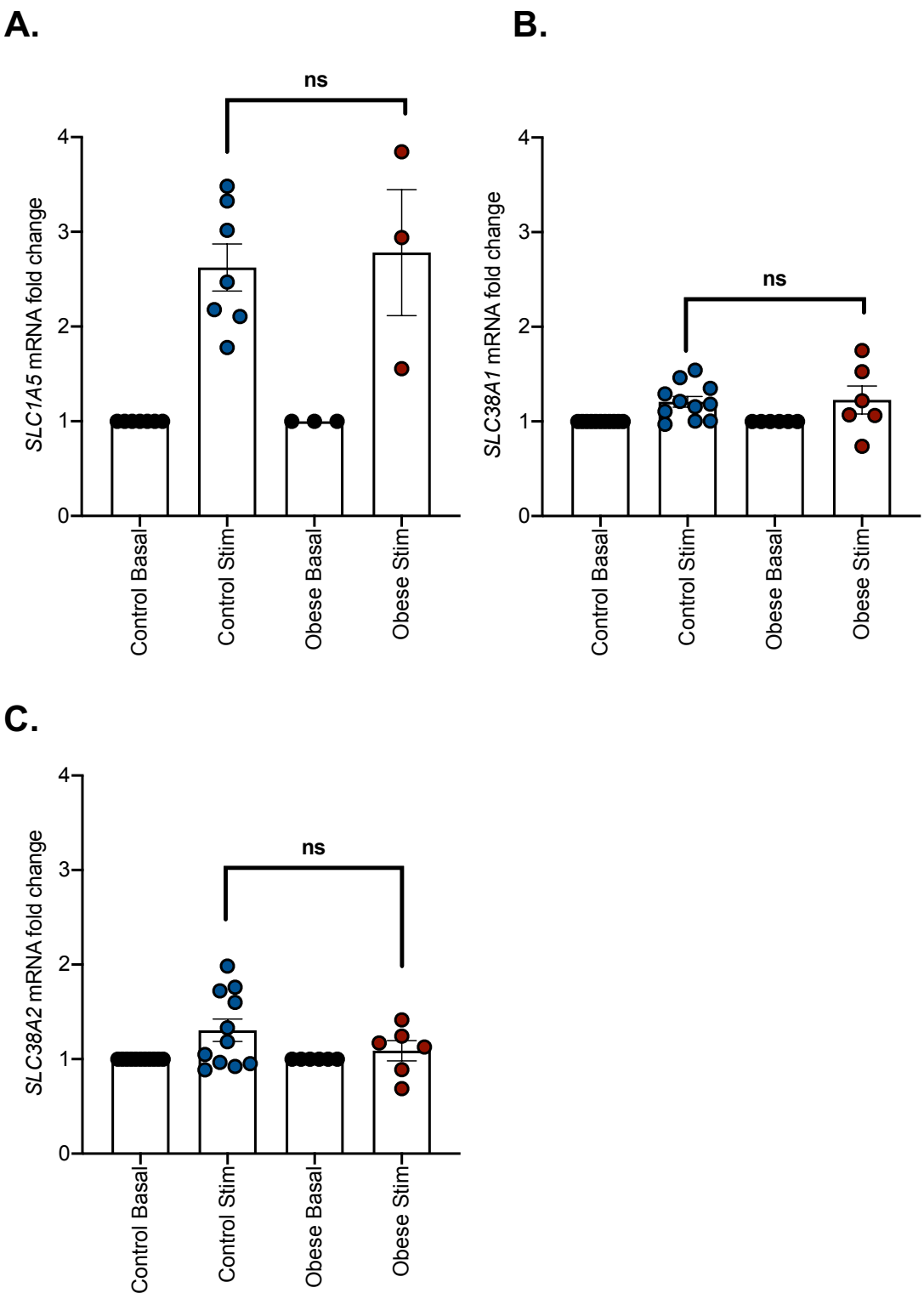


Figure 4.6 MAIT cells from donors with obesity showed no difference in mRNA expression for SNAT glutamine transporters. (A-C) Scatter plot of normalized MAIT cell mRNA expression of glutamine transporters (A) *SLC1A5* (ASCT2), (B) *SLC38A1* (SNAT1) and (C) *SLC38A2* (SNAT2*)* by purified unstimulated MAIT cells and cells stimulated with Dynabeads (cell to bead ratio 1:1), IL-12 and IL-18 (at 50ng/mL each) for 4 hours by the controls and individuals with obesity (*SLC1A5* control n=7; obese n=3; *SLC38A1 &2* control n=11; obese n=6). Statistical analysis performed using one-way ANOVA with Tukey's correction, ns-not significant.

4.3.5. MAIT cells express amino acid transporters LAT1- LAT4

We next decided to investigate another family of amino acid transporters; L-type amino acid transporters. Out of this family, LAT1 is the best studied amino acid transporter. LAT1 is expressed by T cells and becomes upregulated upon stimulation (Sinclair et al., 2013). LAT1 is defined as an anti-porter, as it imports neutral essential amino acids into the cells in the exchange for glutamine. This particular amino acid transporter was shown to play an important role in mTORC1 activation, as it facilitates the influx of leucine, which in turn facilitates activation of mTORC1, a master regulator of the cells (Sancak et al., 2008; Wolfson et al., 2016). Functional LAT1 transporter is a heterodimer composed of CD98 heavy chain which was shown to be associated with LAT1. Stimulation of expanded MAIT cells with TCR Dynabeads, IL-12 and IL-18 for 4 hours resulted in a small but significant increase in *SLC3A2* (CD98) mRNA expression (Figure 4.7 A). Much larger increase in the transcription level in comparison to the resting state of the cell was observed with *SLC7A5* (LAT1), suggesting that this transporter may also play an important role in MAIT cell function (Figure 4.7 B).

We next assessed whether the increases in mRNA expression of LAT1 components are reflected at the protein level using flow cytometry and a CD98 specific antibody. CD98 protein was expressed on MAIT cells and was upregulated upon stimulation of PBMC with TCR Dynabeads, IL-12 and IL-18 cytokines for 18 hours (Figure 4.7 C&D). This data shows that the increase in the transcription of LAT1 (*SLC7A5*) & CD98 (*SLC3A2*) is reflected on the protein level, further reinforcing that this amino acid transporter may be central to MAIT cell function. Investigation of the expression of other LAT amino acid transporters showed a varied response. There was no significant difference in the expression of *SLC7A*8 (LAT2) mRNA upon stimulation, whereas a significant increase in the transcription of *SLC43A1* (LAT3) was observed (Figure 4.7 E&F). *SLC43A2* (LAT4) expression was significantly downregulated upon stimulation in comparison to the transcription in the resting cells (Figure 4.7 G). These results are in line with previously published data, which demonstrated upregulation of LAT1 expression upon stimulation by T cells. This suggests that amino acids transported via LAT1 are of particular importance for not only for T cell function but also may play a role in supporting function of MAIT cells.

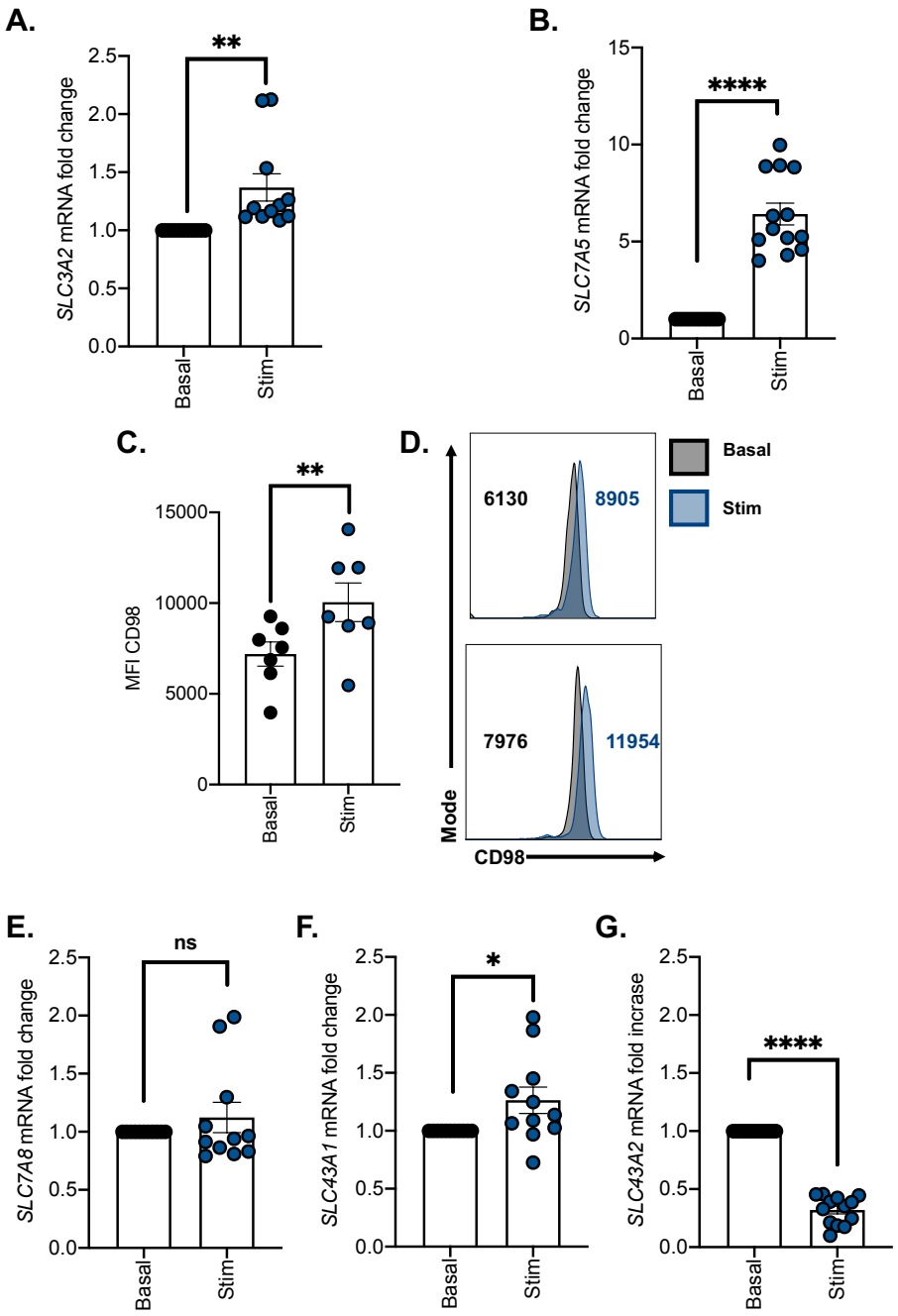


Figure 4.7. MAIT cells express mRNA and protein for CD98 subunit of LAT1 amino acid transporter and mRNA for LAT2-4. (A) Scatter plot of normalized MAIT cell mRNA expression of *SLC3A2* (CD98) and (B) *SLC7A5* (LAT1) by purified unstimulated MAIT cells and cells stimulated with Dynabeads (cell to bead ratio 1:1), IL-12 and IL-18 (at 50ng/mL each) for 4 hours in the control population sample (n=11). (C) Scatter plot of surface MFI CD98 expression by MAIT cells from a PBMC culture and (D) representative histogram of two control samples showing MFI CD98 surface expression; unstimulated or stimulated with TCR microbeads at 25ng/mL (Miltenyi), IL-12 and IL-18 (at 50ng/mL each) for 18 hours in the control population sample (n=7). (E-G) Scatter plot of normalized MAIT cell mRNA expression of amino acid transporters *SLC7A5, SLC43A1, SLC43A2* (LAT2-4) by purified unstimulated MAIT cells and cells stimulated with Dynabeads (cell to bead ratio 1:1), IL-12 and IL-18 (at 50ng/mL each) for 4 hours in the control population sample (n=11). Statistical analysis performed using paired student's t-test, ns-not significant, *p≤0.05, ** p≤0.01, **** p≤0.0001.

4.3.6. MAIT cells in obesity express lower level of LAT1, but show no significant difference in transcription of other amino acid transporters

We again extended our investigations into obesity. Stimulation of expanded MAIT cells from people with obesity as per section 4.3.5 led to modest upregulation of *SLC3A2* (CD98) mRNA transcription and there was no difference noted between the two cohorts (Figure 4.8 A). However, the expression of *SLC7A5* mRNA was diminished in people with obesity (Figure 4.8 B). We also noted decreased expression of CD98 protein on the cell surface of MAIT cells from people with obesity in comparison to the control individuals (Figure 4.8 C&D). Next, we investigated whether MAIT cells from people with obesity have altered transcription of the other three LAT transporters. Stimulation of the expanded MAIT cells as per section 4.3.5 showed no difference in the expression of mRNA for LAT2 (*SLC7A*8) or LAT3 (*SLC43A1*) between two cohorts (Figure 4.9 A&B). Expression of SLC43A2 (LAT4) mRNA showed a similar trend of downregulation of transcription upon cell activation, however MAIT cells from people with obesity displayed an impaired expression of the LAT4 transcription in comparison to the cohort of control individuals (Figure 4.9 C). Collectively, this data shows that MAIT cells from people with obesity show a defect in the expression of LAT1 amino acid transporter, which may have downstream impact on the transport of amino acids into the cells and thus may lead to their impaired function.

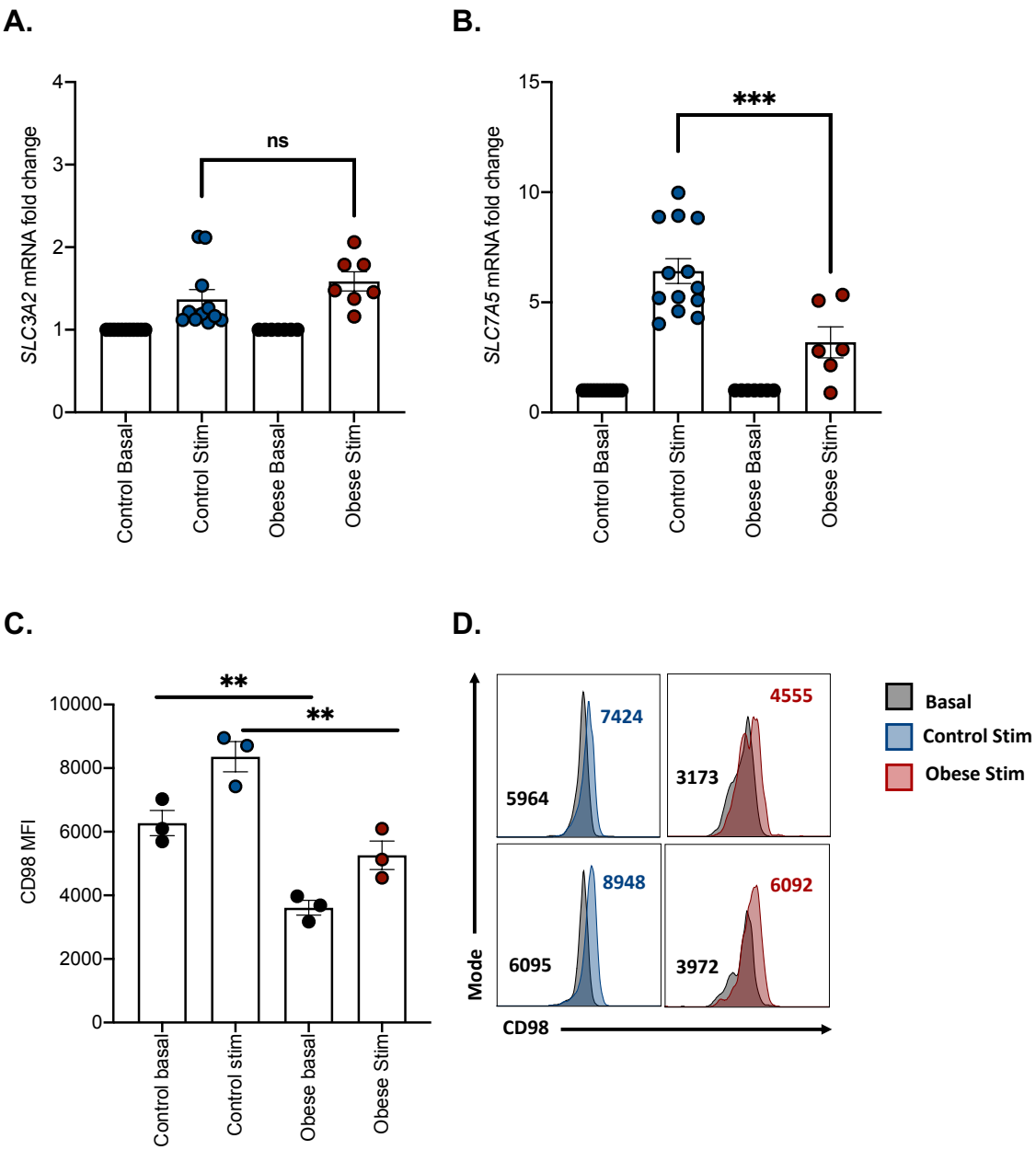


Figure 4.8 MAIT cells from obese donors show lower expression of LAT1 transporter. (A)Scatter plot of normalized MAIT cell mRNA expression of *SLC3A2* (CD98) and (B) *SLC7A5* (LAT1) by purified unstimulated MAIT cells and cells stimulated with Dynabeads (cell to bead ratio 1:1), IL-12 and IL-18 (at 50ng/mL each) for 4 hours by control and obese individuals (lean n=11; obese n=6). (C) Scatter plot of surface MFI CD98 expression by MAIT cells from a PBMC culture (lean n=3; obese n=3) and (D) representative histogram of two control samples showing CD98 surface expression; unstimulated or stimulated with TCR microbeads at 25ng/mL (Milteneyi), IL-12 and IL-18 (at 50ng/mL each) for 18 hours by the control and obese samples (n=3). Statistical analysis performed using one-way ANOVA with Tukey's correction, ns-not significant, ** p≤0.01, *** p≤0.001.

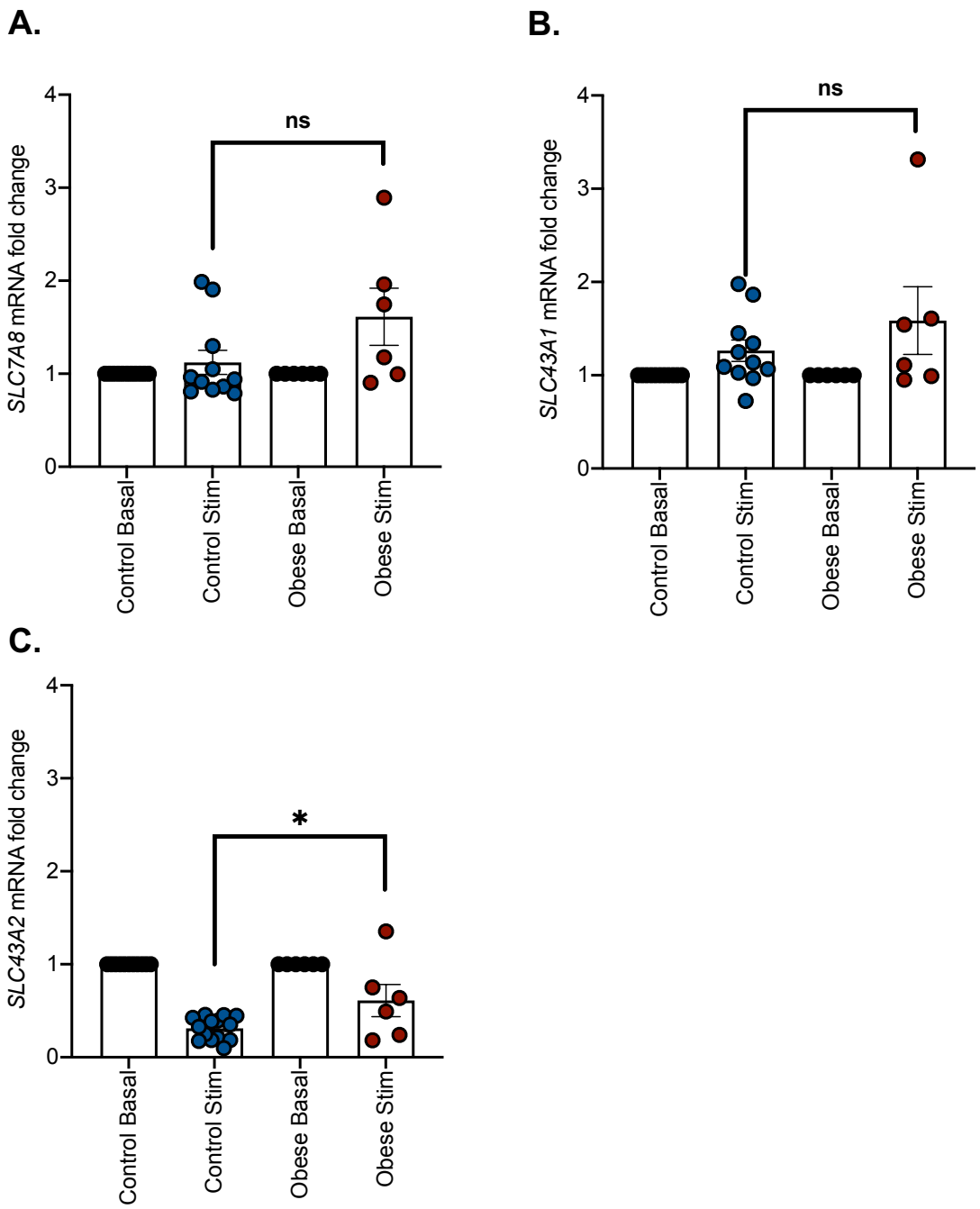


Figure 4.9 MAIT cells from obese donors express similar levels of LAT2-4 mRNA level to the control samples. (A-C) Scatter plot of normalized MAIT cell mRNA expression of glucose transporters *SLC7A5, SLC43A1, SLC43A2* (LAT2-4) by purified unstimulated MAIT cells and cells stimulated with Dynabeads (cell to bead ratio 1:1), IL-12 and IL-18 (at 50ng/mL each) for 4 hours from controls and individuals with obesity (lean n=11; obese n=6). Statistical analysis performed using one-way ANOVA with Tukey's correction, ns-not significant, *p≤0.05.

4.3.7. Stimulation of MAIT cells increases their size

Having determined the expression of the major nutrient transporters on MAIT cells and the impact of activation, we next aimed to define the metabolic profile of MAIT cells. A study by Howden *et al.,* which investigated the proteome of $CD4^+$ and $CD8^+$ T cells found that naïve $CD8^+$ had a higher protein content than naïve $CD4^+$ cells. This was reflected in the FSC measurement, where $CD8^+$ cells were larger than $CD4^+$ cells (Howden et al., 2019). The authors suggested the size of the cells was proportional to the protein content of that cell. Upon cell activation the protein content increases, of which a substantial proportion of the proteins are related to metabolism (Howden et al., 2019). Therefore, this data suggests that the size of a cell can be used to estimate the metabolic activity. The size of immune cells can be easily evaluated using the flow cytometry by measuring the FSC. Based on this observation from Howden *et al.*, we assessed whether the size (therefore metabolic activity) of MAIT cells was altered upon stimulation with 5-ARU and MG for five days and observed an increase in MAIT cell size (Figure 4.10 A&B). This indicates that MAIT cells upregulate the synthesis of a large amount of proteins upon stimulation, suggesting high engagement in metabolic activity.

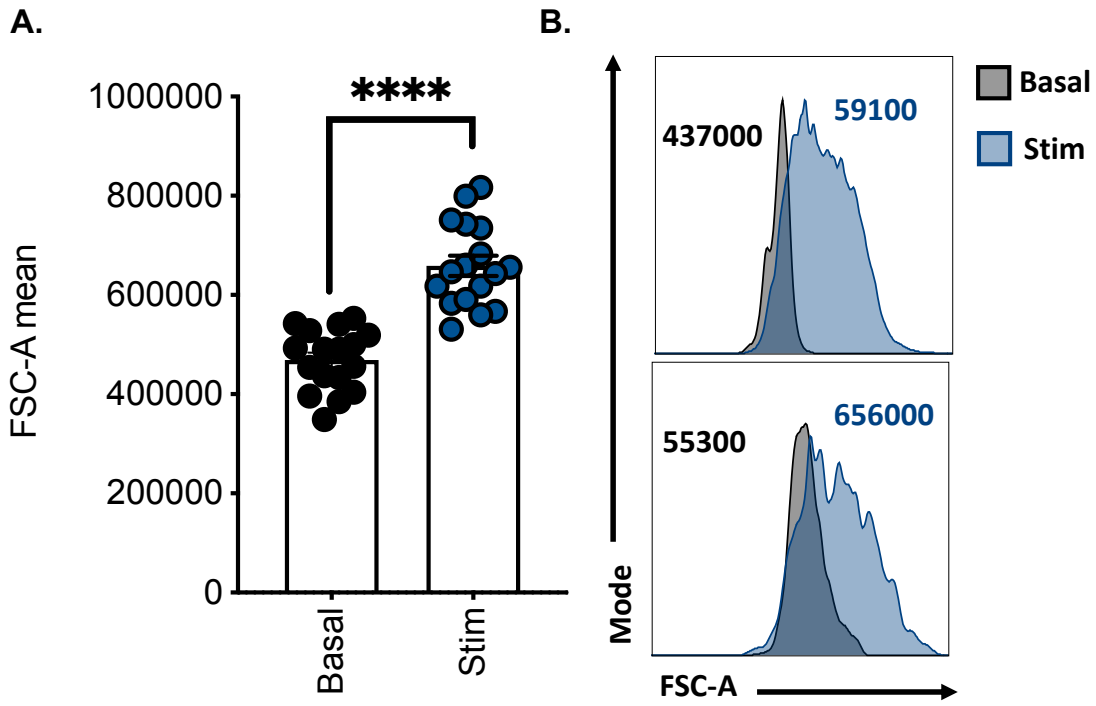


Figure 4.10 MAIT size increases upon extended stimulation. (A) Scatter plot and mean MAIT cell size (FSC-A) basally and after 5 days of stimulation. PBMC were unstimulated (basal) or stimulated (stim) with 5-ARU (1µg/mL) and MG (100µL) on day 0 and received a low dose of IL-2 (6.75ng/µL) on day 1 followed by a high dose of IL-2 (33.3ng/µL) on day 4 (n=17). (B) Representative histograms from 2 control subjects of MAIT cell size after 5 days of culture. Statistical analysis performed using paired student's t-test, ****p≤0.0001.

4.3.8. MAIT cells upregulate glycolysis upon stimulation

As discussed in section 4.1, the role of glucose and glycolysis in T cell function is very well established (Cham et al., 2008; Chang et al., 2013; Jacobs et al., 2008; Shi et al., 2011). Having demonstrated the expression of glucose transporters by MAIT cells, we next investigated the level of glycolysis in resting and stimulated MAIT cells using extracellular flux analysis (Seahorse). Extracellular flux analysis allows for the measurement of glycolysis rate by measuring the ECAR. Upon transport of glucose into the cell, the molecule is metabolised through glycolysis to pyruvate and lactate, releasing protons during this process. As the protons are exported from the cell, this leads to acidification of the cell culture media. The concentration of these protons is measured during seahorse assay and is directly proportional to the rate of glycolysis. Expanded MAIT cells were unstimulated or stimulated with TCR microbeads, IL-12 and IL-18 for 18 hours prior to the seahorse analysis. Once placed on the seahorse instrument, data on the rates on MAIT cell glycolysis was collected by measuring ECAR over an hour. During the real-time measurement oligomycin, and ATP synthase inhibitor, was injected 15 minutes into the incubation to inhibit oxphos and forced the cells to fully switch to use glycolytic metabolism to support their function. At 35 minutes 2DG was injected to inhibit glycolysis and show that the ECAR measured in the experiments was due to the glycolysis and no other cell processes. Here, we demonstrated increased rates of ECAR by MAIT cells upon stimulation (Figure 4.11 A&B). This data shows that MAIT cells engage in glycolytic metabolism upon activation. In addition to ECAR rates, glycolytic capacity was measured by adding oligomycin, which inhibits ATP synthase and thus OxPhos. This forces the cell to use glycolysis to its maximal capacity in order to produce ATP to sustain cell function. There was no significant difference in the glycolytic capacity between basal and stimulated MAIT cells, however a trend towards higher glycolytic capacity upon stimulation was observed (Figure 4.11 C).

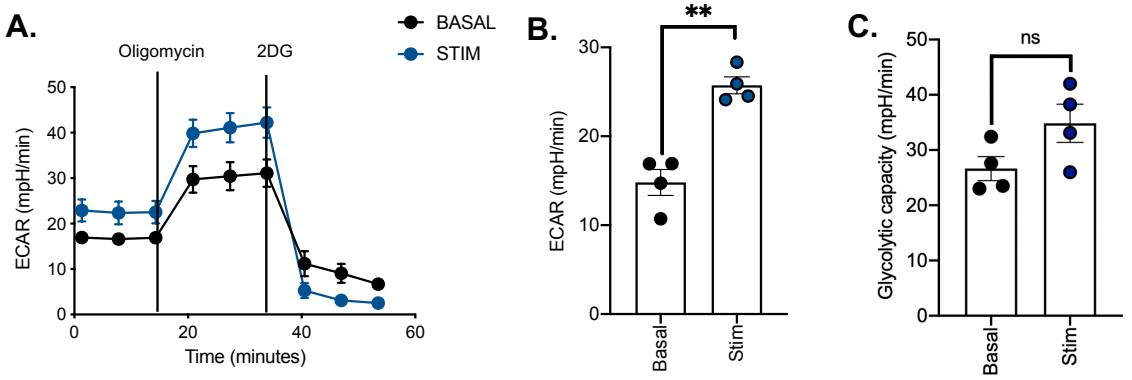


Figure 4.11 MAIT cells upregulate glycolysis upon stimulation. (A) Representative Seahorse trace and (B) scatter plot of ECAR rate as wells as (C) scatter plot of glycolytic capacity of MAIT cells basally or upon stimulation with TCR microbeads (Miltenyi 25ng/mL) and IL-12/IL-18 cytokines (at 50ng/mL each) for 18 hours (n=4). Statistical analysis performed using paired student's t-test, ns-not significant, **p≤0.01.

4.3.9. Stimulation of MAIT cells modulates expression of enzymes of glycolysis

With our observation that MAIT cells increase their glycolytic rate upon stimulation (Figure 4.11), we next aimed to confirm this finding by investigating the expression of key glycolytic enzymes, such as Hexokinase 2 (HK2), Pyruvate kinase (PKM) and Lactate dehydrogenase A (LDHA). Upon stimulation of expanded MAIT cells with TCR Dynabeads, IL-12 and IL-18 for 4 hours we observed increased mRNA expression of *HK2* and *PKM* indicating enhanced glycolytic metabolism (Figure 4.12 A&B). To assess whether the increase in transcription of these enzymes is reflected on the protein level, we used flow cytometry and noted increased MAIT cell HK2 expression upon stimulation of PBMC with TCR Dynabeads, IL-12 and IL-18 for 18 hours (Figure 4.12 C&F). In contradiction to the molecular results, PKM2 expression at 18 hours post stimulation was shown to be unchanged (Figure 4.12 D&G). In addition, we investigated the protein level of LDHA in activated MAIT cells and found significantly enhanced expression of this enzyme (Figure 4.12 E&H). In summary this data suggests that MAIT cells upon stimulation rapidly engage a glycolytic metabolic programme.

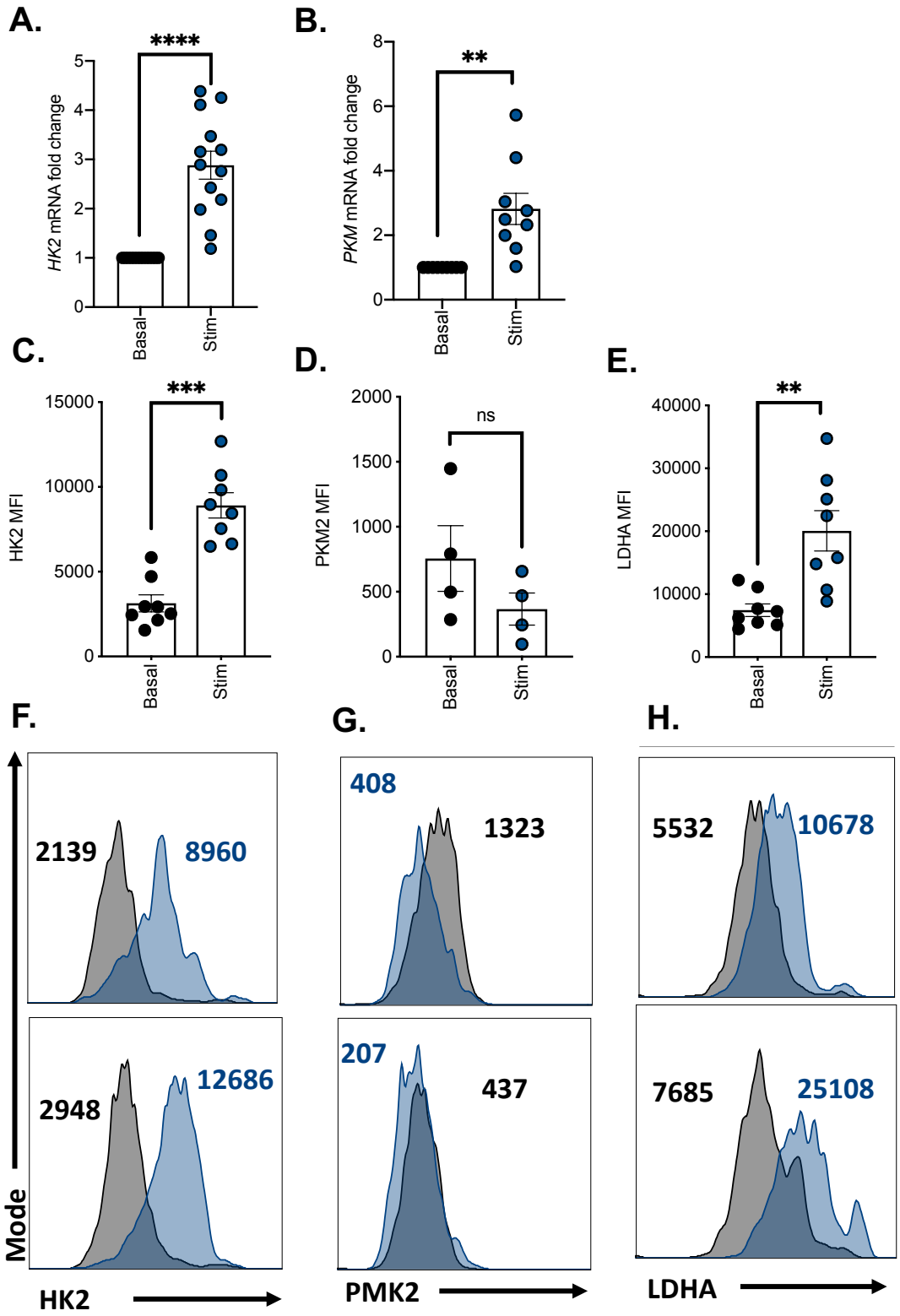


Figure 4.12 MAIT cells upregulate expression HKII and LDHA, but not PKM2 protein expression upon stimulation (A-B) Scatter plot of *HK2* (n=13) and *PKM* (n=9) mRNA produced by purified MAITs basally upon stimulation with TCR Dynabeads (1:1 cell to bead ratio) and IL-12/IL-18 (50ng/mL each) cytokines for 4 hours. (C-E) Scatter plot and (F-H) representative histograms of HK2 (n=8), PKM2 (n=4) and LDHA (n=8) enzymes respectively, produced by MAIT cells from a PBMC culture basally and upon stimulation with TCR Dynabeads and IL-12/IL-18 cytokines for 18 hours (n=8). Statistical analysis performed using student's t-test, ns-not significant, **p≤0.01, ***p≤001, ****p≤0001.

4.3.10. MAIT cells from people with obesity display impaired glycolysis

Having demonstrated that glycolytic metabolism is central to MAIT cell activation (Figure 4.11 & 4.12), we next investigated the impact of obesity on MAIT cell metabolism. Stimulation of expanded MAIT cells from people with obesity with TCR Dynabeads, IL-12 and IL-18 for 18 hours did not increase their rates of glycolysis (Figure 4.13 A&B). MAIT cells form people with obesity also failed to increase their glycolytic capacity upon stimulation. In addition, glycolytic capacity of stimulated MAIT cells from people with obesity was significantly lower in comparison to the control group (Figure 4.13 C). To explore this failure to upregulate glycolysis in more detail, expression of mRNA for glycolytic enzymes was measured. In comparison to the control cohort, we noted no differences in the expression of either *HK2* or *PKM* upon stimulation for 4 hours with TCR Dynabeads and cytokines (Figure 4.14 A&B). Despite the fact that MAIT cells from people with obesity displayed no impairment in the expression of mRNA for glucose transporters in comparison to the control individuals (Figure 4.4), this data indicates a clear defect in the rates of glycolysis. Collectively this data indicates that obesity not only impacts MAIT cells proliferation and cytokine production (chapter three), but also glycolytic metabolism.

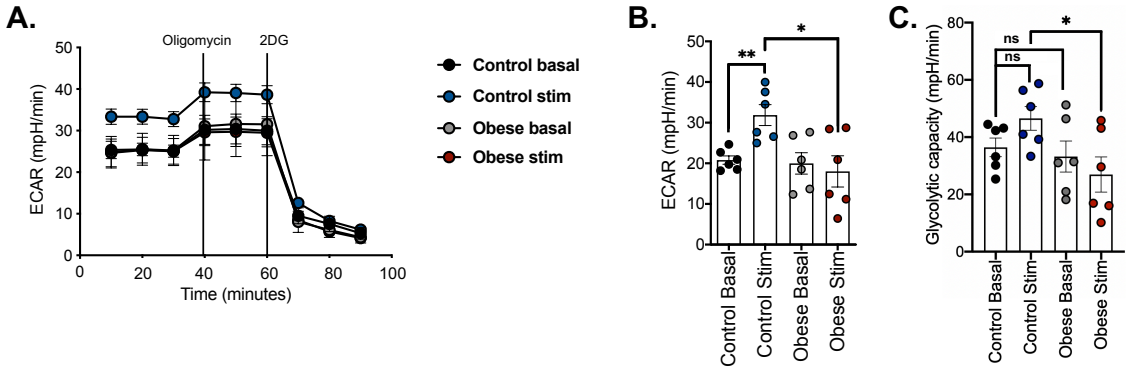


Figure 4.13 MAIT cells from people with obesity don't upregulate glycolysis upon stimulation. (A) Representative Seahorse trace and (B) a scatter plot of MAIT cells ECAR rate as wells as (C) a scatter plot of glycolytic capacity of MAIT cells basally or upon stimulation with TCR microbeads (Miltenyi 25ng/mL) and IL-12/IL-18 cytokines (at 50ng/mL each) for 18 hours (control n=6; obese n=6). Statistical analysis performed using one-way ANOVA with Tukey's correction, ns-not significant, *p≤0.05, **p≤0.01.

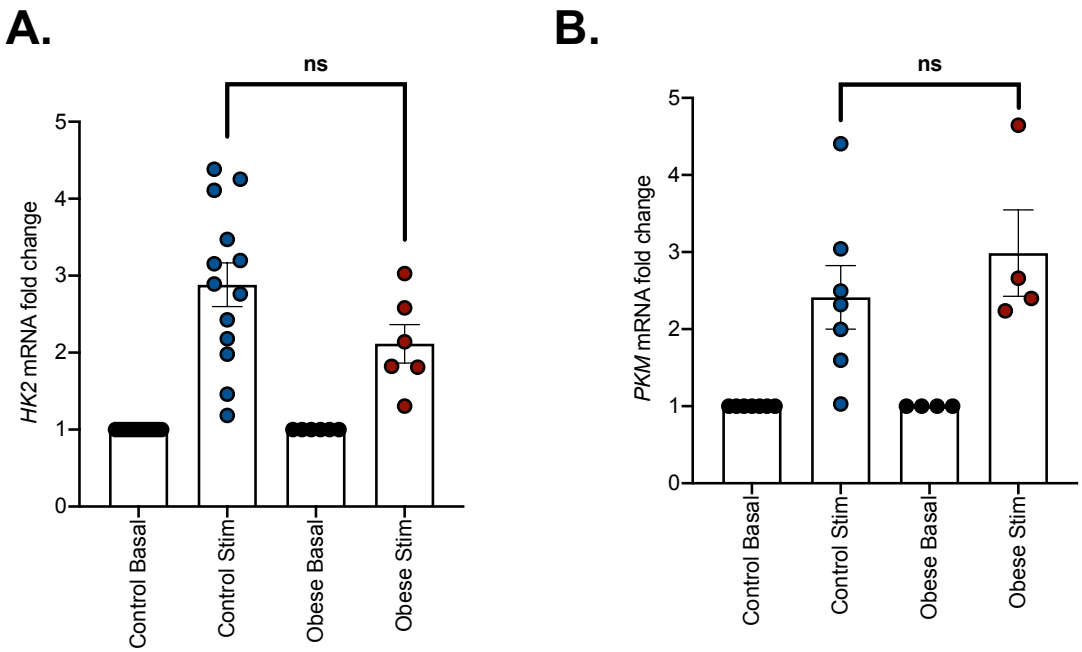


Figure 4.14 MAIT cells from people with obesity display a non-significant alteration mRNA expression of HKII and PKM2 enzymes. Scatter plot of (A) *HK2* (control n=13, obese n=6) and (B) *PKM* (control n=9, obese n=4) mRNA expression by purified MAITs from control and obese cohort, basally and upon stimulation with TCR Dynabeads (1:1 cell to bead ratio) and IL-12/IL-18 (50ng/mL each) cytokines for 4 hours. Statistical analysis performed using one-way ANOVA with Tukey's correction, ns- not significant.

4.3.11. MAIT cells actively use LAT1 amino acid transporters

We next investigated a role for LAT1 in MAIT cell metabolism, as it was the most abundantly expressed transporter on activated MAIT cells (Figure 4.7 A-D). Although LAT1 is known to transport multiple amino acids including leucine, kynurenine a metabolite of t-tryptophan is reported to be a substrate of LAT1 (Sinclair et al., 2018). Kynurenine was shown to be auto fluorescent, and this feature was used by Sinclair *et al.* to develop a kynurenine uptake assay, which allows for the measurement of kynurenine uptake via LAT-1 by immune cells using flow cytometry (Sinclair et al., 2018). In this study we used the kynurenine assay developed by Sinclair *et al.* to assess the activity of the LAT1 amino acid transporter in MAIT cells and the efficiency BCH to inhibit transport via LAT1 (Sinclair et al., 2018). We observed significant kynurenine uptake over 4-minute incubation by basal MAIT cells from PBMC samples (Figure 4.15 A&B) and expanded MAIT cells (Figure 4.15 C&D), which was significantly reduced upon incubation with LAT1 specific BCH inhibitor (Figure 4.15 A&B; Figure 4.15 C&D). This data indicates that LAT1 expressed by MAIT cells is active and capable of importing LAT1 specific substrates, whereas BCH was shown to specifically inhibit LAT1 mediated transport.

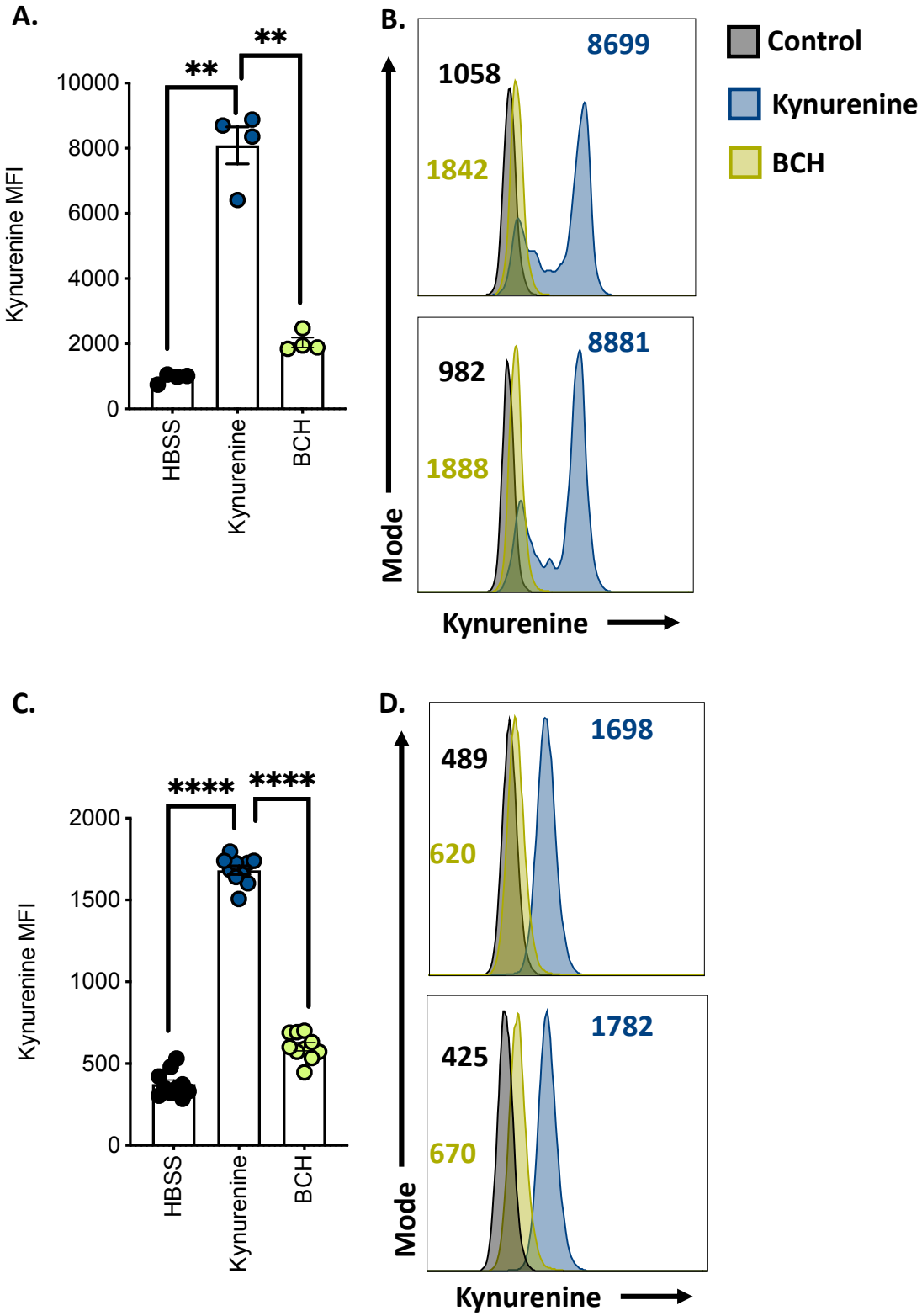


Figure 4.15 MAIT cells import kynurenine via LAT1 amino acid transporter. (A) Scatter plot and (B) representative histograms of basal uptake of kynurenine (200μM) in the presence or absence of BCH (20mM) by MAIT cells from PBMC of healthy individuals during 4 minutes of incubation (n=4) (C) Scatter bar and (D) representative histograms of basal uptake of kynurenine (200μM) by expanded MAIT cells from healthy individuals in the presence or absence of BCH (20mM) during 4 minutes (n=10). Statistical analysis performed using one-way ANOVA with Tukey's correction, **p≤0.01, ****p≤0.001.

4.3.12. MAIT cells from people with obesity display defective LAT1 mediated transport

Previously we had demonstrated a decrease in the expression of LAT1 transporter by MAIT cells from people with obesity (Figure 4.8). Therefore, we next investigated Kynurenine uptake by MAIT cells from people with obesity and demonstrated impaired kynurenine uptake in comparison to healthy controls (Figure 4.16 A&B). Collectively this data suggests that lower expression of LAT1, leads to this reduced uptake of kynurenine and potential large neutral amino acids.

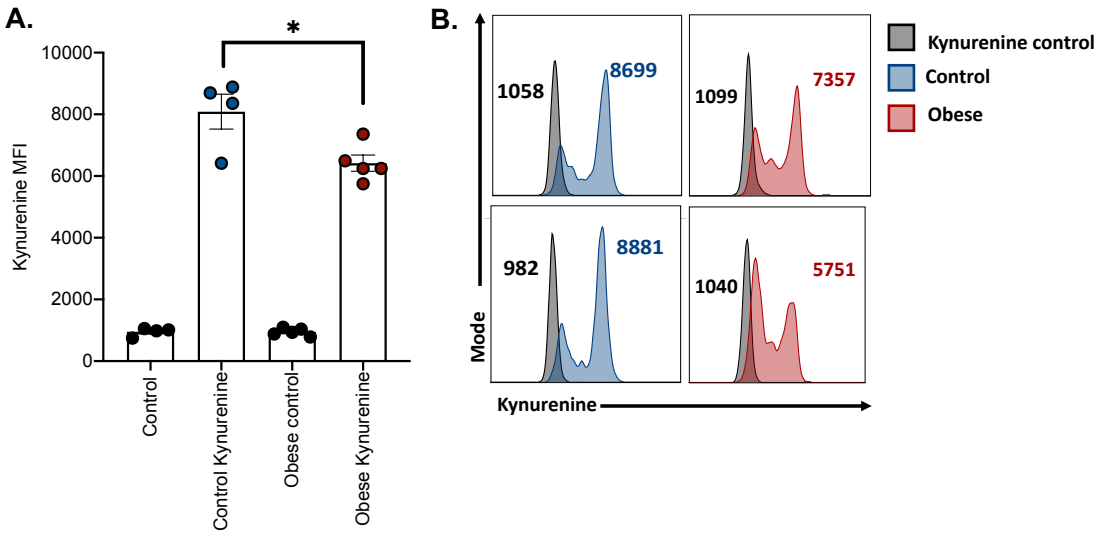


Figure 4.16 Obese MAIT cells have impaired uptake of kynurenine via LAT1 amino acid transporter. (A) Scatter plot and (B) representative histograms of basal uptake of kynurenine by MAIT cells from PBMCs culture of control and obese individuals (n=4). Statistical analysis performed using one-way ANOVA with Tukey's correction, *p≤0.05.

4.3.13. MAIT cells require amino acid influx via LAT1 to activate mTORC1 and glycolysis

Published studies have shown that LAT1 is responsible for the influx of leucine into T cells, which was shown to activate mTOR, a master regulator of cellular metabolism. We first investigated if MAIT cells utilize mTOR by assessing mTORC1 activity as measured by the phosphorylation of S6, a downstream target of mTORC1. Upon stimulation of MAIT cells with TCR microbeads, IL-12 and IL-18 for 18 hours, phosphorylation of S6 was significantly increased (Figure 4.17 A&B). We next assessed the impact of LAT1 inhibition on mTORC1 activity and noted a non-significant reduction in the phosphorylation of S6 protein (Figure 4.17 C&D). As mTOR is known to orchestrate the metabolism of T cells, including the control of glycolysis, next we decided to investigate whether limiting amino acid influx will alter the rate of glycolysis. To address this question, we used the ECAR assay as described in section 4.3.8. Expanded MAIT cells were unstimulated or stimulated with TCR microbeads, IL-12 and IL-18 for 18 hours in the presence or absence of BCH before the cells were put on the Seahorse instrument for analysis. Limiting the amino acid influx using BCH resulted in the significant reduction in glycolysis as well as glycolytic capacity (Figure 4.18 A,B,C). This data suggests that amino acids imported via LAT1, most likely leucine, are required for activation of mTORC1, which in turn is required for upregulation of glycolysis.

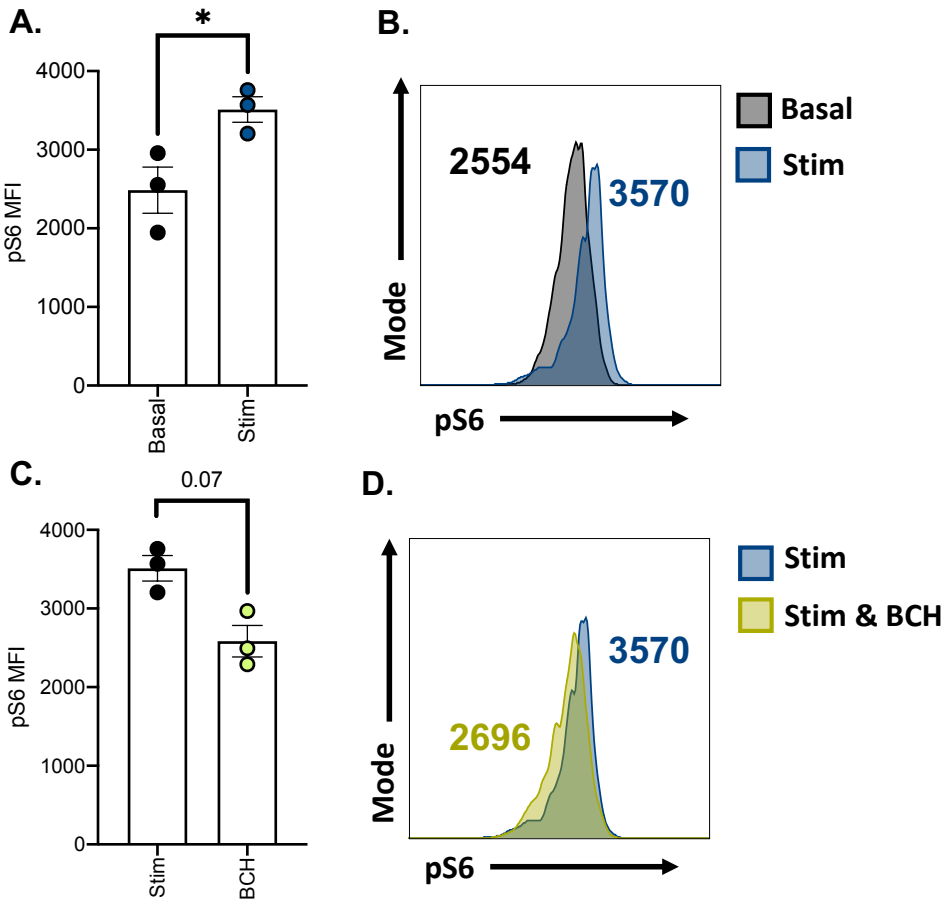


Figure 4.17 Amino acid transport via LAT1 is important for mTOR activation. (A) Scatter plot and (B) representative histogram of pS6 expression by MAIT cells from PBMCs culture of control individuals. Cells unstimulated or stimulated with TCR microbeads at 25ng/mL (Milteneyi), IL-12 and IL-18 (at 50ng/mL each) for 18 hours (n=3). (C) Scatter plot and (D) representative histogram of pS6 expression by MAIT cells from PBMCs culture of control individuals. Cells stimulated with TCR microbeads at 25ng/mL (Milteneyi), IL-12 and IL-18 (at 50ng/mL each) for 18 hours with or without BCH inhibitor (50mM) (n=3). Statistical analysis performed using student's t-test, *p≤0.05.

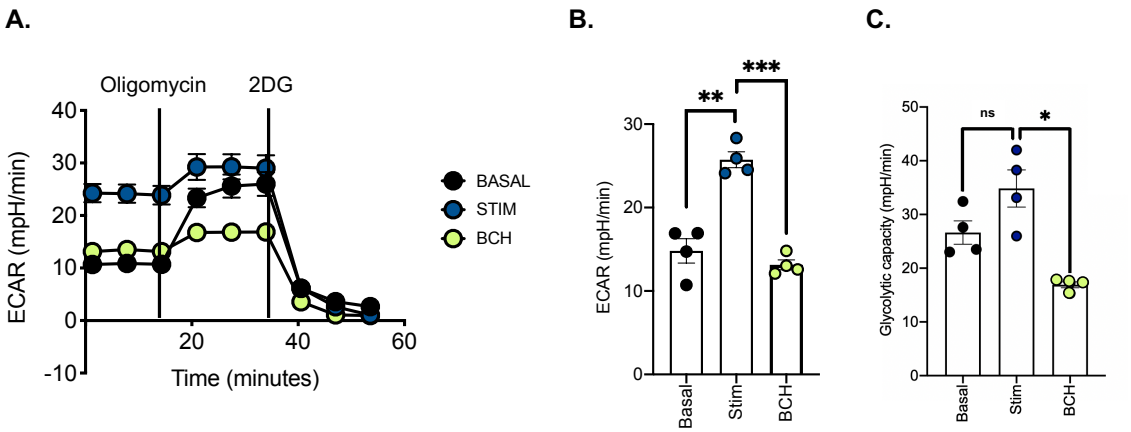


Figure 4.18 Amino acid transport via LAT1 is necessary for upregulation of glycolysis. (A) Representative Seahorse trace and (B) a scatter plot of MAIT cells ECAR rate as wells as (C) a scatter plot of glycolytic capacity of MAIT cells basally or upon stimulation with TCR microbeads (Miltenyi 25ng/mL) and IL-12/IL-18 cytokines (at 50ng/mL each) in the presence or absence of BCH (50mM) for 18 hours. Cells cultured in media diluted 1:2 with HBSS (n=4). Statistical analysis performed using one-way ANOVA with Tukey's correction, ns-not significant, **p≤0.01, ***p≤0.001.

4.4. Discussion

Over the past decade, research in the area of immunometabolism has massively increased. Elaborate studies have illustrated that T cell function heavily relies on the precisely orchestrated cell metabolism. This aspect of immune cell activity has been largely taken for granted and overlooked for many years, however it is now well established that altered cell metabolism can have detrimental effects on immune cell function. Therefore, in this study we aimed to elucidate for the first time, which metabolic pathways control MAIT cell activation.

We found that MAIT cells express mRNA for glucose transporters GLUT1, 3 and 4, the expression of which was modestly upregulated at an early point during their activation. The literature suggests that glucose transporters expression is controlled during transcription and protein synthesis, as stimulation of T cells increased the mRNA and protein levels (Cretenet et al., 2016; Frauwirth et al., 2002; Kavanagh Williamson et al., 2018). Our data indicates that the stimulation of MAIT cells also leads to the initiation of early metabolic reprogramming, to support the influx of glucose by increasing transcription of some of the GLUT transporters. Parallel work from our lab has shown that the activation of MAIT cells increases uptake of glucose after overnight stimulation (O'Brien et al. 2019; Figure 1D). Together these results illustrate an important role for glucose in supporting MAIT cell activation.

Studies in T cells have established that glycolysis is the dominant pathway used by effector T cells during activation (Chang et al., 2013; Gubser et al., 2013; Michalek et al., 2011; Procaccini et al., 2016). Our study shows that MAIT cells, a potent effector cell subset, also upregulate the rate of glycolysis upon stimulation, however there is no significant difference in the glycolytic capacity between basal and stimulated cells. This was elucidated by measuring the extracellular acidification rate, which assessed the glycolytic rate in MAIT cells in real time. Enhancement of glycolysis was accompanied by significant upregulation of hexokinase 2, the first enzyme of the glycolytic pathway. On the other hand, short-time stimulation of MAIT cells significantly increased the expression of transcripts for pyruvate kinase, the last enzyme of the glycolysis pathway. However, after overnight stimulation, the level of PKM2 enzyme was non-significantly lower in comparison to the resting cells. This suggested that the

transcription of this enzyme is more important at earlier timepoints in MAIT cell activation. Expression of LDHA, an enzyme involved in the conversion of pyruvate into lactic acid was higher after overnight stimulation, suggesting that MAIT cells were still converting pyruvate into lactate. Collectively this data is in line with results published on effector T cell metabolism. It indicates that MAIT cells engage in a similar metabolic programme upon activation, during which they significantly increase glycolysis, likely to support the demand for ATP production and provision of intermediates required for the synthesis of proteins such as cytokines or lipids and nucleic acids required for cell division.

Recent research has highlighted the importance of leucine in controlling mTORC1 activation, which is responsible for control of glycolysis in T cells (Saxton et al., 2016; Sinclair et al., 2013; Wolfson et al., 2016), therefore next we decided to investigate how the import of this amino acid affects the glycolytic metabolism of MAIT cells. Leucine is imported into the cells via large amino acid transporters, of which LAT1 mediates is majority of the transport (Maimaiti et al., 2020; Scalise et al., 2018; Sinclair et al., 2013), therefore we measured this transporter expression by MAIT cells. Transcription of mRNA for both components of LAT1 heterodimer was significantly increased upon MAIT cell stimulation, which was reflected on the protein level. This is in line with the data reported by Sinclair *et al*. who demonstrated enhancement of LAT1 expression by conventional $CD8^+$ T cells upon stimulation (Sinclair et al., 2013). Transcription of other members of the LAT family of transporters varied upon stimulation. The observed diversity in the expression pattern may occur as a result of the induction of different amino acid transporters at different timepoints. Although LAT1-3 presented a trend towards upregulation of transcription of those transporters, expression of LAT4 has been downregulated. LAT4 has been shown to transport a range of amino acids including essential and branched chained amino acids, suggesting that transport of these amino acids via LAT4 may not play a critical role in activated MAIT cells (Bodoy et al., 2005). As upregulation of LAT1 has been the most prominent, next we evaluated it's transport capacity using a kynurenine assay developed by the Cantrell lab (Sinclair et al., 2018). Kynurenine, a metabolite of tryptophan, is a substrate of LAT1. The fluorescent properties of kynurenine allowed for a rapid measurement of its uptake by the immune cells, and hence allowed for indirect assessment of the efficiency of

transport of amino acids via this amino acid transporter (Sinclair et al., 2018). In line with the data published by Sinclair *et al.*, our study found that LAT1 expressed by MAIT cells is functional and imports kynurenine into these cells. To assess the effect of amino acid transport via LAT1 on MAIT cell glycolytic metabolism, we limited the influx of amino acids using BCH, a LAT1 specific inhibitor. Despite the activation signals, restraining of the amino acid transport resulted in a significant reduction in the rate of glycolysis, which resembled the rate of unstimulated MAIT cells, as well as significant reduction of glycolytic capacity was observed. This data indicated that LAT1 mediated amino acid transport is indispensable for modulation of the glycolysis rate.

mTORC1 is the master regulator of metabolism, which controls multiple processes including glycolysis (Salmond, 2018). Direct inhibition of mTORC1 activity with rapamycin during T cell stimulation or deletion of mTORC1 component Rheb, has been shown to have a detrimental effect on glycolysis, leading to impairment in its upregulation (Hukelmann et al., 2016; Pollizzi et al., 2015). mTORC1 is sensitive to the influx of leucine, which is largely mediated by LAT1 amino acid transporter (Saxton et al., 2016; Sinclair et al., 2013; Wolfson et al., 2016). Sinclair *et al.* has elegantly shown that T cells absolutely require LAT1 for metabolic reprogramming, and control of mTORC1 activity (Sinclair et al., 2013). Here we hypothesised that blocking LAT1 during MAIT cell stimulation, may result in impairment in activation of mTORC1 complex. Indeed, activation of mTORC1 as measured by phosphorylation of S6 protein, was reduced upon MAIT cell activation in the presence of LAT1 inhibitor. This was confirmed in expanded MAIT cells (O'Brien et al. 2019 Figure 4I), where the inhibition of LAT1 mediated transport, resulted in significant reduction in mTORC1 activity. This is in line with study published by Loftus *et al*. on NK cells where the blocking of amino acid transport via LAT1, led to the reduction in pS6 protein expression and c-Myc activation (Loftus et al., 2018). Parallel work from our lab has also revealed that the inhibition of mTORC1 with rapamycin in MAIT cells, significantly impairs their glycolytic metabolism as shown through the measurement of extracellular acidification rate (O'Brien et al. 2019 Figure 3E). Similar reports were published by Hukelmann *et al*. and Donnely *et al*. in T cells and NK cells respectively, indicating mTORC1 is crucial for controlling of glycolysis in lymphocytes (Donnelly et al., 2014; Hukelmann et al., 2016).

Collectively this data shows that MAIT cells undergo metabolic reprogramming upon stimulation. Activation of MAIT cells increases the expression of LAT1 amino acid receptor on the cell surface, which facilitates the uptake of amino acids including leucine, to support the activated state of the cell. Detection of leucine by mTORC1 increases its activity and leads to upregulation of the glycolysis rate (Figure 4.19).

People with obesity display a significant defect in MAIT cell proliferation and cytokine production, however the underpinning mechanism responsible for those defects has not yet been described. Having established some of the metabolic pathways required for successful MAIT cell activation, next we investigated those in people with obesity. MAIT cells from people with obesity displayed a defect at each point of this metabolic pathway. In comparison to the control individuals, MAIT cells from people with obesity displayed diminished expression of LAT1. This most likely leads to the reduced uptake of kynurenine and hence other LAT1 substrates such as leucine. This results in lower accumulation of leucine in the cell, which is sensed by mTORC1. Lower levels of the amino acid concentration may mistakenly provide a signal to the cell indicating there are not enough nutrients available for the cell to engage in activation. The failure of leucine to reach the appropriate threshold required to induce mTORC1 activity leads to a failure to upregulate glycolysis (Figure 4.19). More research is required to elucidate why the expression of LAT1 amino acid transporter is lower in people with obesity. A defect in LAT1 expression may be due to only partial engagement of the cells in a signalling pathway which is activated upon cell stimulation to upregulate the expression of mRNA for LAT1 and failure to export fully formed LAT1 transporters onto the surface of the cell.

Alterations in the function and metabolism of immune cells from people with obesity are not limited to MAIT cells. Children with obesity were shown to have a defect in their cytotoxic activity and presented with an activated metabolic phenotype in comparison to their lean counterparts (Tobin et al., 2017). This study found that NK cells from children with obesity have enhanced basal glycolysis in comparison to lean children and failed to upregulate it any further upon stimulation. This was accompanied by higher activation of mTORC1 basally and failure to significantly upregulate its activity after 18 hours stimulation. These

results indicate that resting NK cells from children with obesity are highly glycolytic, but fail to enhance their metabolism upon activation, possibly leading to the alteration in their function (Tobin et al., 2017). The failure of NK cells to increase their glycolytic metabolism upon activation, in part resembles the findings from our study of MAIT cells, which are not capable of enhancing their glycolytic metabolism to the levels observed in the control subjects. Study by Michelet *et al.* suggested this alteration in NK cell metabolism in people with obesity is due to their intracellular accumulation of lipids, which were shown to significantly decrease their mTORC1 activity, glycolysis and impaired their cytotoxicity (Michelet et al., 2018). In the future, lipid handling by MAIT cells and its impact on their metabolism should also be investigated. These studies further indicate that obesity has a significant impact on immune cells function and metabolism and highlights that immune cell metabolism is closely related to their function.

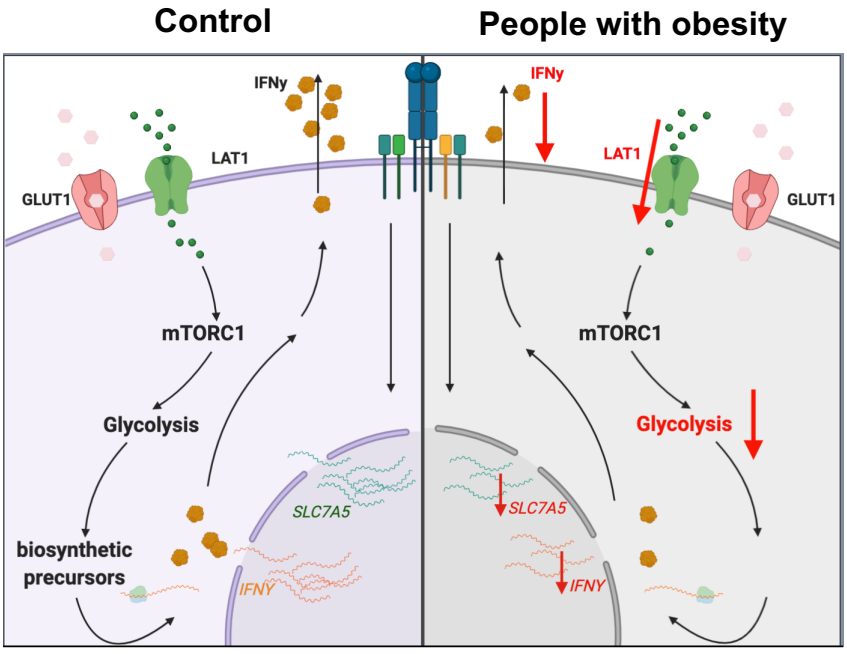


Figure 4.19 Summary of findings of chapter four. Upon stimulation of MAIT cells from control individuals (left), *IFNγ* and *SLC7A5* mRNA is transcribed. Expression LAT1 transporter is upregulated, which allows for amino acid influx. Transport of amino acids such as leucine, activates mTORC1, which enhances glycolysis producing multiple metabolic precursors. This supports increased IFNγ production. MAIT cells from people with obesity (right) upon stimulation display lower levels of *IFNY* and *SLC7A5* transcription. In addition, LAT1 expression and transport is also impaired, leading to defects in mTORC1 activation. This in turn results in failure to enhance glycolysis, leading to lower level of metabolic precursors and hence lower IFNγ production.

Chapter Five

Results

Metabolic requirements for MAIT cell function

5.1 Introduction

5.1.1 Immunometabolism and cell function

Recognition of cellular metabolism as one of the crucial processes that impacts the activity of immune cells has led to substantial advancements in our understanding of mechanisms which control immune cell function. Understanding the complex web of interlinked metabolic pathways poses a great challenge in identifying their direct or indirect association with a particular cellular response. It is now known that the metabolism of immune cells doesn't stay constant during its lifespan. Metabolism itself is very flexible and is capable of adjusting to the energetic and biosynthetic demands of the cell at any given time (Almeida et al., 2016). Extensive research has identified glycolysis and OxPhos as two key pathways implicated in supporting the immune function of T cells. The metabolic state of quiescent or effector T cells were shown to be quite distinct. During quiescent state, cells primarily engage in Kreb's cycle and OxPhos, to produce high amounts of ATP, whereas stimulation results in rapid metabolic reprogramming, leading to the strong enhancement of glycolysis, which becomes the dominant metabolic pathway (O'Neill et al., 2016). This provides the cell with energy in the form of ATP and a range of metabolic intermediates, which directly or indirectly contribute to T cell effector function as described in chapter four. As a result, recent research of T cell metabolism has been largely focused on the consequences brought on by the disturbance of glycolysis and glycolysis-related pathways during cell activation. Indeed, glycolysis was described to support the production of cytokines by T cells and impact their proliferation. Glucose restriction or inhibition of glycolysis reduced the capability of T cells to produce IFNγ (Jacobs et al., 2008; Renner et al., 2015). Similar effects were brought on by inhibition of mTORC1 or amino acid influx, which indirectly led to the restriction of glycolytic metabolism (Pollizzi et al., 2015; Sinclair et al., 2013; Yang et al., 2013). The relationship between the glycolytic metabolism and T cell cytokine production has been explored in detail in chapter four, therefore here we will focus on the association between T cell metabolism and their proliferation. The involvement of metabolism in the production of T cell effector molecules is beginning to unravel, however details of how metabolism affects T cell proliferation is still relatively poorly explored. T cell proliferation is dependent on a range of metabolic processes including glycolysis and OxPhos. As early as 1994, glucose was reported to be essential for T cell proliferation. (Greiner et al.,

1994). This finding has been revisited since by multiple researchers. Jacobs *et al.* showed that glucose is required for T cell proliferation, as a reduction of glucose below 0.5mM in the culture media, significantly affected their proliferation capacity (Jacobs et al., 2008). Chang *et al.* also demonstrated that activated T cells rely on glycolysis as their incubation in media without sugars, strongly inhibited their proliferation. Substitution of glucose with galactose, which forced the cells to switch from glycolysis to OxPhos to support their function, slowed down T cell proliferation, but didn't inhibit it (Chang et al., 2013), as it has been observed with direct treatment of T cells with 2DG (Cham et al., 2008). This is in line with an earlier study by Delgoffe *et al.* which investigated the impact of Rheb deficiency (mTOCR1) on T cell function in mice. This study showed that mTORC1 deficiency causes retardation of T cell proliferation, but not a complete inhibition (Delgoffe et al., 2011).

As cells have been shown to switch between glycolysis and OxPhos depending on their energetic and biosynthetic requirements, the role of OxPhos has been investigated in T cell proliferation. Blocking of OxPhos using complex I, III or ATP synthase inhibitors does not lead to complete obstruction of proliferation. In fact, Chang *et al.* this study has also presented that inhibition of OxPhos, lead to enhancement of glycolysis, likely to compensate for the loss of ATP and biosynthetic precursors induced by the inhibitors (Chang et al., 2013). Study by Sena *et al.* has shown that CD4 T cells lacking complex III have retained their ability to proliferate during lymphopenia but failed to respond to TCR induced proliferation. Authors of this publication suggested this defect is likely due to the lack of mROS required for T cell activation, and not due to the inability to generate biosynthetic precursors or ATP (Sena et al., 2013). This data suggests that T cells rely on both glycolysis and OxPhos to proliferate, however more research is required to elucidate the exact contribution of each of these pathways in supporting T cell proliferation.

Amino acids play a role in T cell proliferation. Deletion of SLC7A5, a component of LAT1 amino acid transporter in a mouse model severely impacted the proliferation of T cells *in vitro* (Sinclair et al., 2013). Based on the literature and results obtained in chapter 4, it can be hypothesised that deletion of SLC7A5 leads to lower influx of leucine, leading to lower activation of mTORC1 and lower

rates of glycolysis. Again, this highlights the importance of glycolytic metabolism in T cell proliferation. The role of glutamine in T cell proliferation was also investigated. ASCT2 glutamine transporter was shown to be dispensable to T cell proliferation, as deletion of ASCT2, did not affect T cell proliferation (Nakaya et al., 2014). Although ASCT2 was not deemed indispensable, subsequent study by Sener *et al.* showed glutamine is required for proliferation, as media with no glutamine abrogated this process. In addition, inhibition of glutaminase, an enzyme which catalyses the metabolism of glutamine to glutamate, also led to significant impairment of T cell proliferation (Sener et al., 2016). Collectively these studies indicate that glutamine metabolism, similarly to glycolysis and OxPhos supports T cell proliferation.

MAIT cells, as a novel subset of T cells, are still very poorly understood in comparison to the conventional T cells. The past decade has led to a greater appreciation of this subset and their role in disease and health, however the mechanisms controlling MAIT cell functions remains largely unexplored. Using the knowledge gained during the investigation of MAIT cell metabolism in chapter four and the evidence presented in the literature discussed above on the associations between immune cell function and cell metabolism, here we decided to investigate whether MAIT cell metabolism also plays a role in supporting their effector functions.

5.2 Specific aims of this chapter

The specific aim of this chapter was to identify the metabolic requirements for MAIT cell functional responses.

Therefore to:

1. Investigate how glucose availability and glycolysis affects is related to MAIT cell proliferation, expansion and IFNγ production
2. Elucidate how amino acid influx affects MAIT cell proliferation, expansion and IFNγ production
3. Determine whether OxPhos is implicated in supporting MAIT cell proliferation and expansion

5.3 Results

5.3.1 MAIT cells use glucose to support proliferation

Having identified that MAIT cells engage in glycolytic metabolism upon activation, we next investigated the metabolic requirements for MAIT cell proliferation in the control individuals. We first cultured MAIT cells in culture media with varying levels of glucose (10mM, 5mM and 1mM) for 5 days and assessed MAIT cell proliferation via cell trace violet, CTV. MAIT cells proliferated readily at the standard concentration of 10mM glucose (Figure 5.1 A&B). Reduction of glucose to 5mM did not affect MAIT cell proliferation (Figure 5.1 C&D), however a reduction of glucose concentration in the media to 1mM slowed proliferation (Figure 5.1 C&D). As MAIT cell proliferation was dependent on IL-2 (Figure 3.5, 3.6, 3.8), next we investigated whether glucose restriction (1mM) impacts the levels of CD25. In glucose restricted conditions (1mM glucose), reduced IL-2R expression was noted in comparison to 10mM glucose (Figure 5.2 A&B). To confirm the requirement of glucose for MAIT cell proliferation and expansion, we substituted glucose in our culture media with galactose, which limits the rate of glycolysis in the cell. We demonstrate that in galactose culture media, MAIT cell expansion was greatly reduced in comparison to glucose culture media during the five-day incubation period (Figure 5.3 A&B). These results show that MAIT cells require an influx of appropriate amounts of glucose to facilitate their proliferation and expression of IL-2R, which allows them to respond to proliferative signals in the culture.

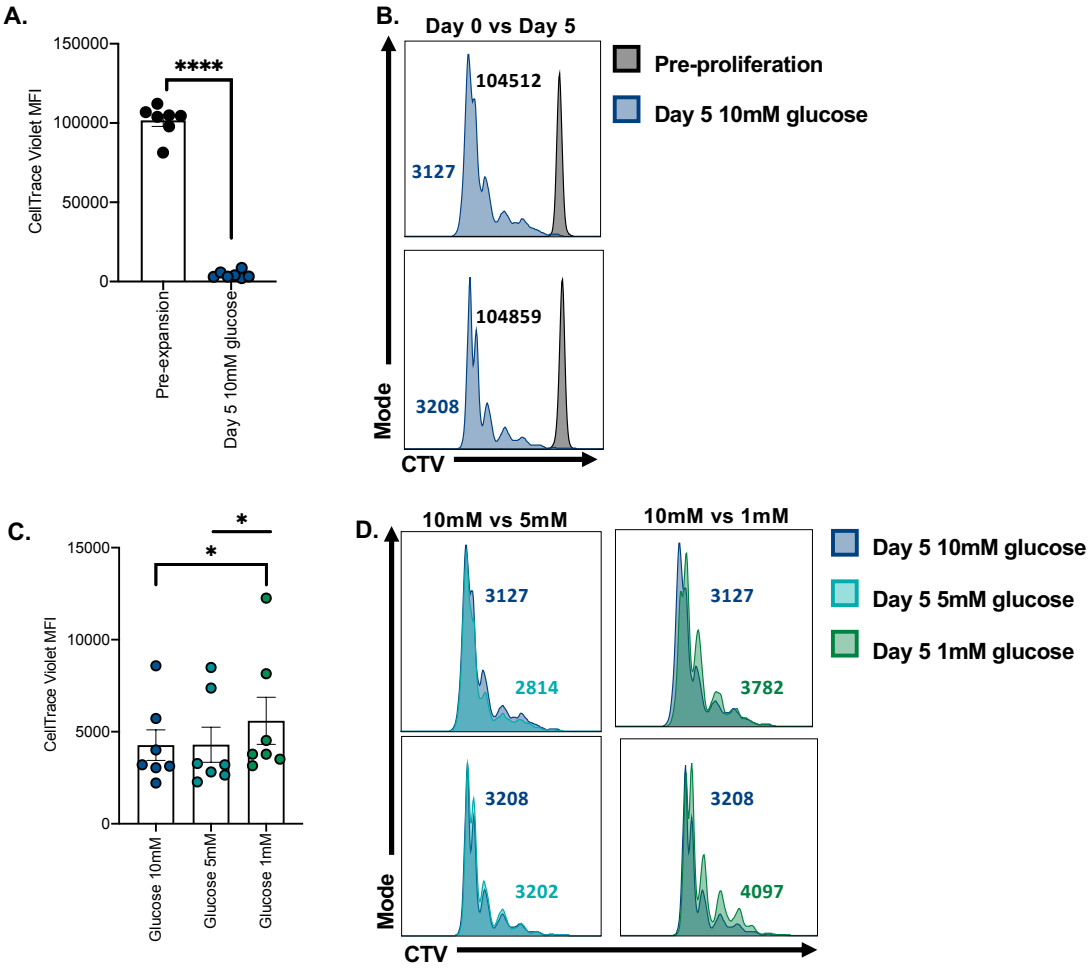


Figure 5.1 MAIT cell proliferation is restricted by glucose availability. (A) Scatter plot and (B) representative histograms of two control samples of MAIT cell proliferation as measured by uptake and dilution of Cell Trace Violet (CTV) at 5μM on day 0 and day 5 of culture. PBMCs were incubated in 10mM glucose full media and were unstimulated (Day 0) or stimulated (Day 5) with 5-ARU (1μg/mL) and MG (100μL) on day 0 and received a low dose of IL-2 (6.75ng/mL) on day 1 followed by a high dose of IL-2 (33.3ng/mL) on day 4 (n=7). (C) Scatter plot of MAIT cell proliferation at 10mM, 5mM and 1mM glucose on day 5 of culture (n=7). (D) Representative comparison histograms of MAIT cell proliferation on day 5 with glucose at 10mM and 5 mM or 10mM and 1mM. Statistical analysis performed using paired student's t-test or one-way ANOVA with Tukey's correction, * p≤0.05, ****p<0.0001.

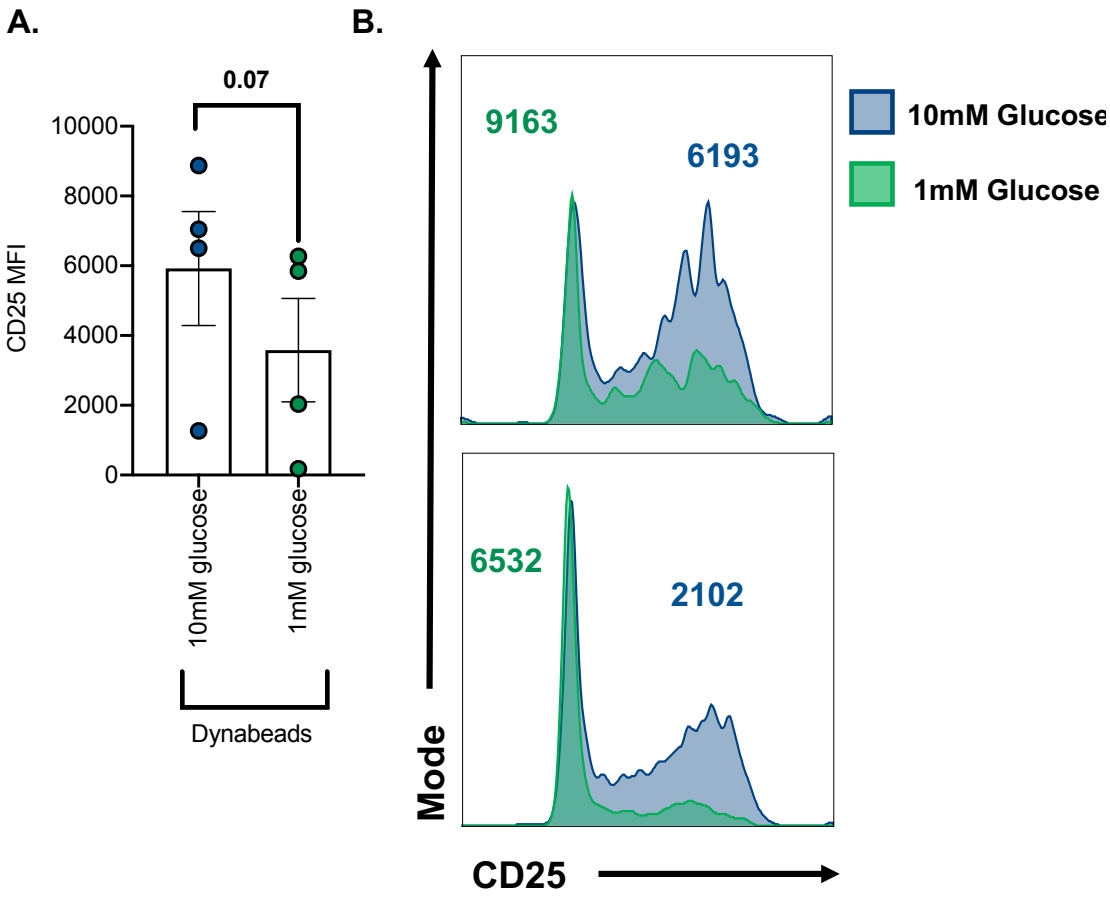


Figure 5.2 Restriction of glucose negatively impacts CD25 surface expression by MAIT cells. (A) Scatter plot of CD25 (IL-2R) expression by MAIT cells. Cells were stimulated with TCR Dynabeads (1:1 cell to bead ratio) in the presence of 10mM or 1mM concentration of glucose. (B) Representative histograms of 2 control samples showing CD25 expression upon stimulation with TCR Dynabeads in the presence of 10mM or 1mM concentration of glucose (n=4). Statistical analysis performed using paired student's t-test, ns- non-significant.

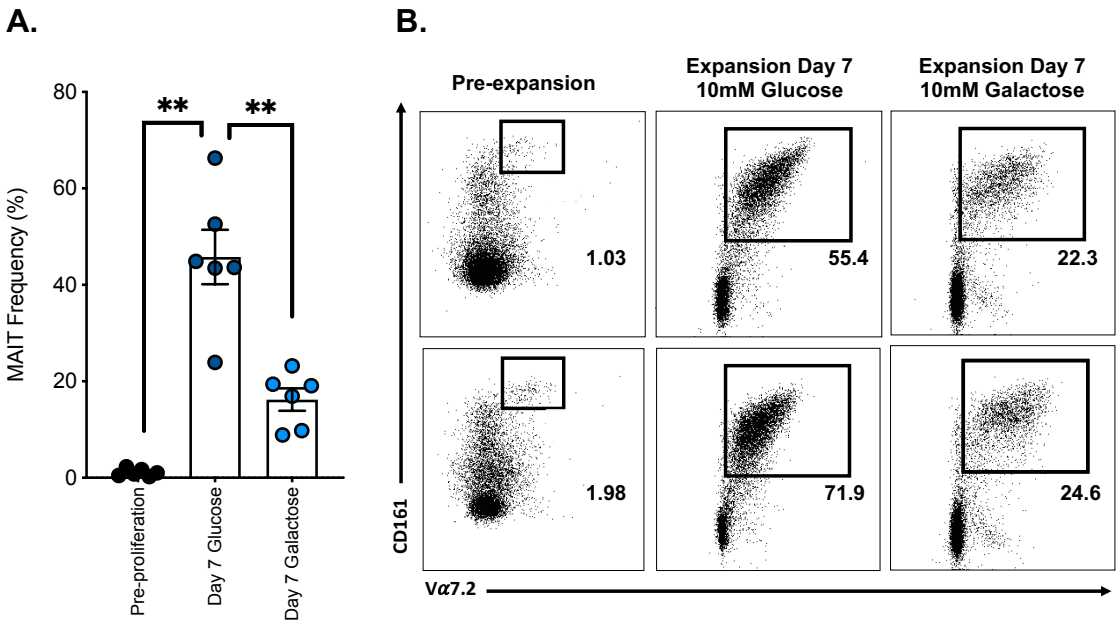


Figure 5.3 MAIT cells expansion is restricted by substitution of glucose with galactose. (A) Scatter plot and (B) representative dot plots of two control samples of MAIT cell expansion as measured by the percentage of CD3 cells on day 0 and day 7 of culture. PBMCs were incubated in 10mM glucose or 10mM glutamine media and were unstimulated (Day 0) or stimulated with 5-ARU (1µg/mL) and MG (100µL) on day 0 and received a low dose of IL-2 (6.75ng/mL) on day 1 followed by a high dose of IL-2 (33.3ng/mL) on day 4 (n=6). Statistical analysis performed using paired student's t-test or one-way ANOVA with Tukey's correction, * p≤0.05, ****p<0.0001.

5.3.2 MAIT cells require glycolysis for proliferation

As shown in section 5.3.1 glucose supports proliferation of MAIT cells and expression of IL-2R. Therefore, to further explore metabolic requirements for MAIT cell proliferation, we decided to investigate the significance of glycolysis in this process using a 2-deoxy-d-glucose inhibitor. 2DG is a glucose analogue, which enters glycolysis, just as glucose does, however 2DG inhibits hexokinase 2, the first enzyme of glycolysis and suppresses the entire pathway. PBMC were stained with CTV for analysis of MAIT cell proliferation or remained unstained for the assessment of MAIT cell expansion. Next cells were activated with 5-ARU-MG, in the presence or absence of 2DG for the first 18 hours of culture. Then cells were stimulated with IL-2 and cultured for a total of 5 days and analysed by flow cytometry. Here we demonstrated that glycolysis inhibition significantly reduced MAIT cell proliferation and expansion (Figure 5.4 A-D). As expression of IL-2R was shown to be sensitive to glucose concentration, next we investigated whether inhibition of glycolysis with 2DG also impacts the level of IL-2R present on MAIT cell surface. Similar to restriction of glucose (Figure 5.2 A&B), stimulation of MAIT cells as per section 5.3.1 in the presence or absence of the glycolysis inhibitor for 18 hours significantly reduced IL-2R expression upon stimulation (Figure 5.5 A&B). This data suggests that glycolysis is central to MAIT cell proliferation, as inhibition of this metabolic process halts expansion of MAITs cells, which in part may be due to the reduction in the expression of IL-2R.

5.3.3 MAIT cells require an influx of amino acids via LAT1 for proliferation

Activated MAIT cells were shown to be highly dependent on the transport of amino acids via LAT1 transporter in order to enhance their rate of glycolysis (Figure 4.18). As glucose and glycolysis were shown to play a central role in MAIT cell proliferation (Figure 5.1, 5.3 & 5.4), we next investigated the requirement of amino acid transport via LAT1 for MAIT cells proliferation. PBMC were prepared and stimulated as described in section 5.3.2 in the presence or absence of BCH. Inhibition of amino acid transport by blocking the activity of LAT1 transporter using BCH, significantly reduced MAIT cell proliferation (Figure 5.6 A&B) and expansion (Figure 5.6 C&D). Collectively with data obtained in chapter four, these results highlight the importance of amino acid influx for MAIT cell function.

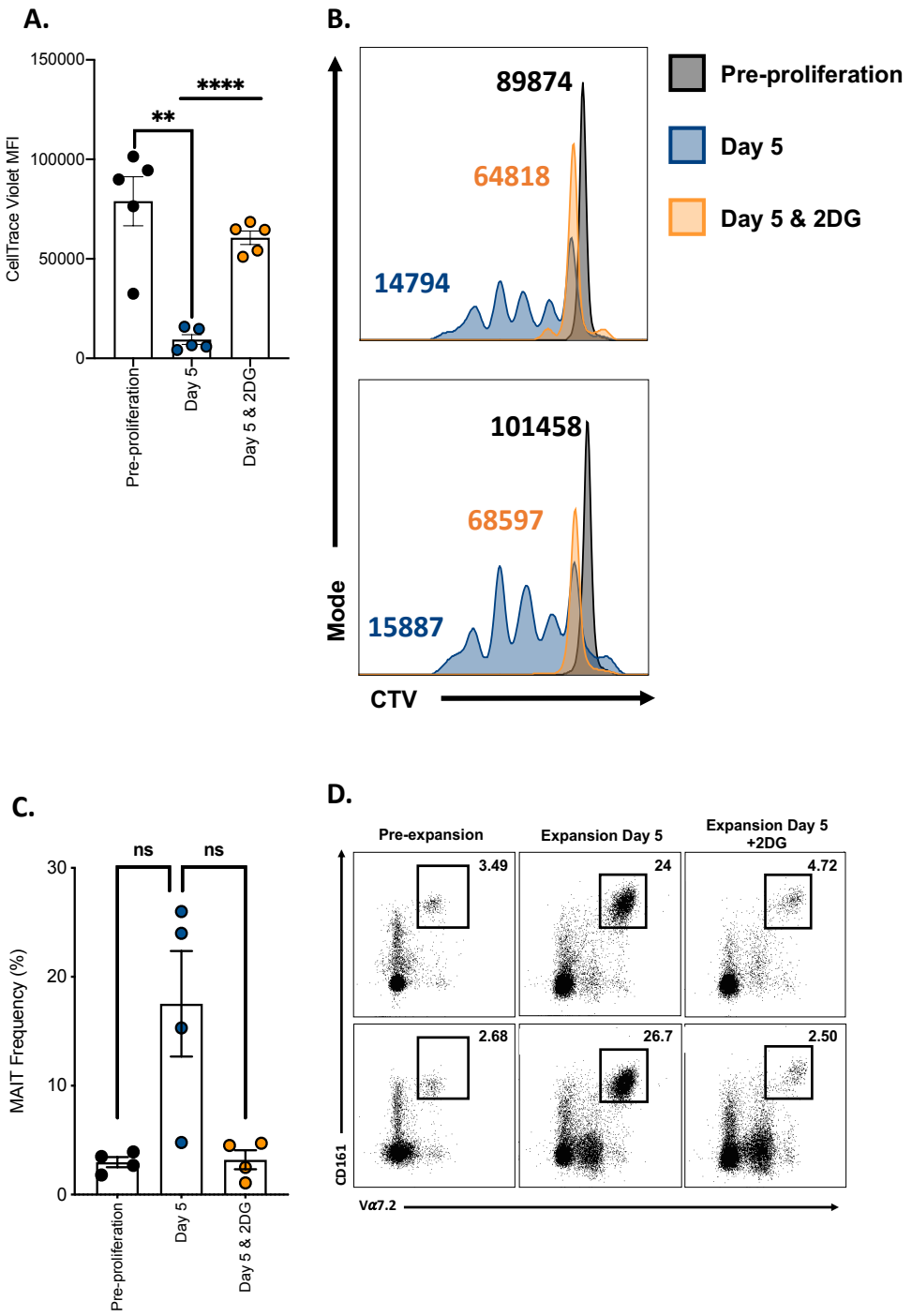


Figure 5.4 MAIT cells require glycolysis to proliferate in response to 5-ARU-MG and IL-2. (A) Scatter plot and (B) representative histograms of 2 control subjects showing MAIT cell proliferation over 5 days of culture with or without the addition of 2DG (10mM) as measured by uptake and dilution of Cell Trace Violet (CTV) at 5μM, on day 0 and day 5 of culture. (C) Scatter plot and (D) representative dot plots of MAIT cell expansion over 5 days of culture with or without the addition of 2DG (10mM) as measured by the percentage of CD3 population. For both experiments, PBMCs were unstimulated (Day 0) or stimulated (Day 5) with 5-ARU (1μg/mL) and MG (100μL) on day 0 and received a low dose of IL-2 (6.75ng/μL) on day 1 followed by a high dose of IL-2 (33.3ng/μL) on day 4 in the presence or absence of 2DG (10mM) (n=4/5). Statistical analysis performed using one-way ANOVA with Tukey's correction, ** p≤0.01, **** p≤0.0001.

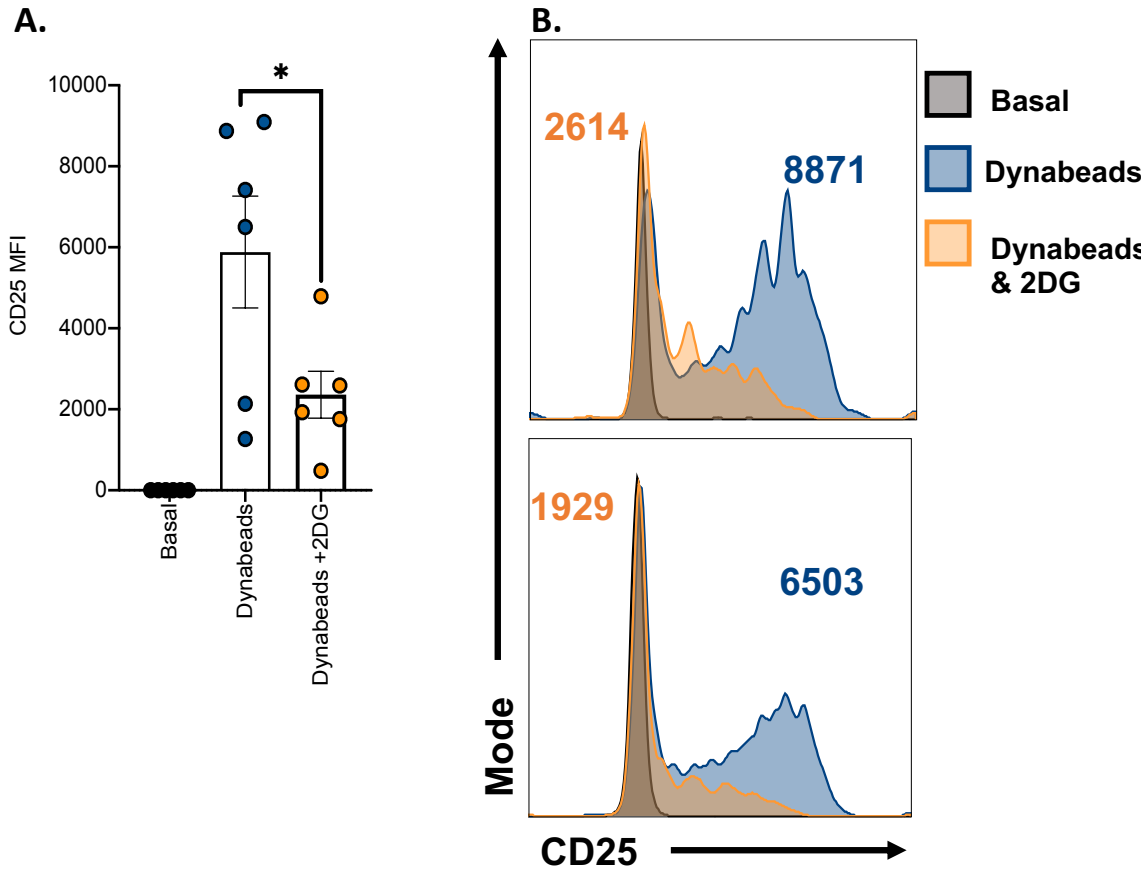


Figure 5.5 MAIT cells require glycolysis to express IL-2 receptor on the cell surface. (A) Scatter plot of CD25 (IL-2R) expression by MAIT cells. Cells were untreated or stimulated with TCR Dynabeads (1:1 cell to bead ratio) in the presence or absence of 2DG (10mM) for 18 hours and analysed by flow cytometry. (B) Representative histograms of 2 samples showing CD25 expression upon stimulation with TCR Dynabeads with or without the addition of 2DG (10mM) (n=6). Statistical analysis performed using one- ANOVA with Tukey's correction, *p≤0.05.

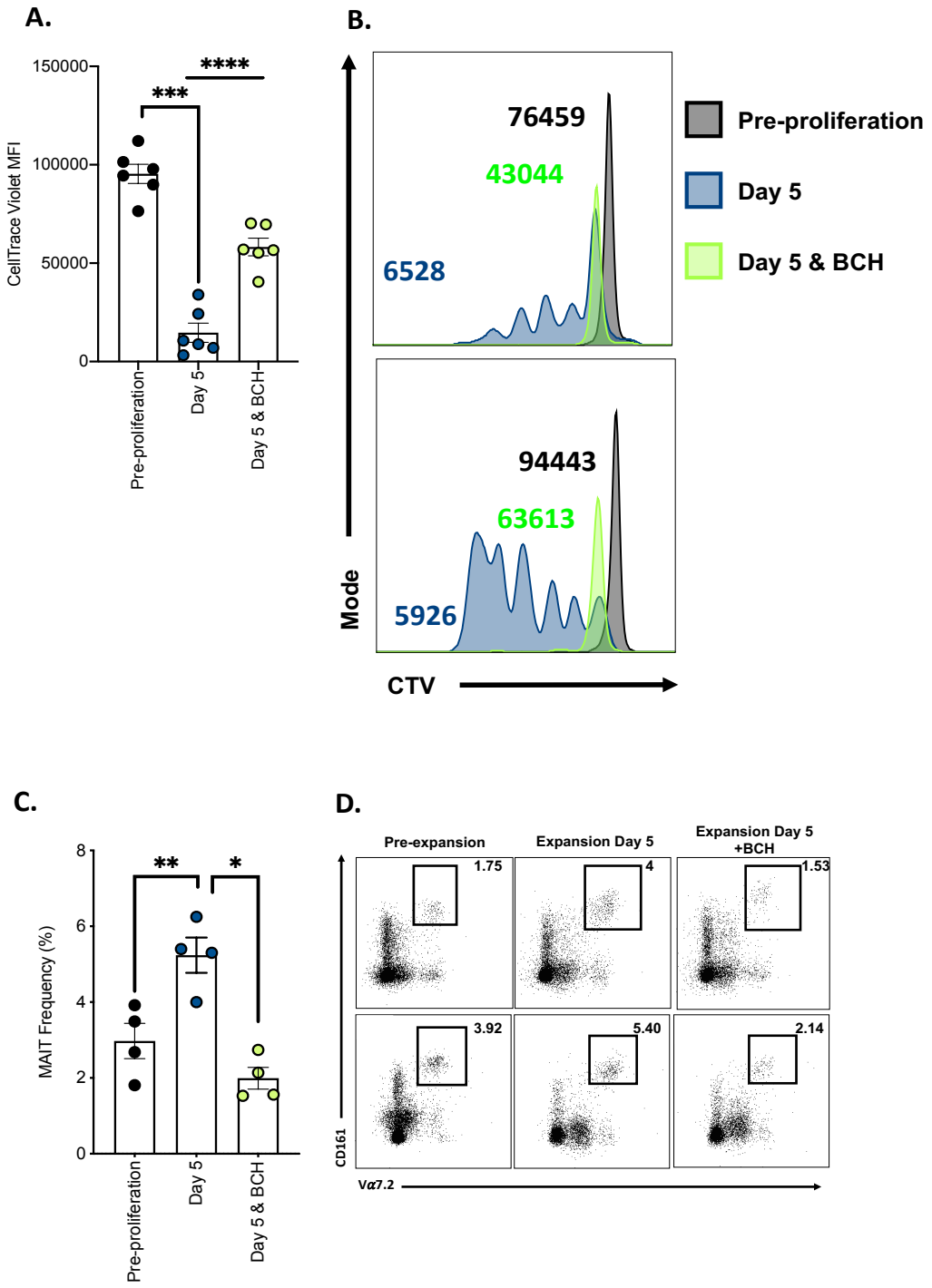


Figure 5.6 MAIT cells require amino acid influx to proliferate in response to 5-ARU-MG and IL-2. (A) Scatter plot and (B) representative histograms of 2 control subjects showing MAIT cell proliferation over 5 days of culture with or without the addition of BCH (50mM) as measured by uptake and dilution of Cell Trace Violet (CTV) at 5μM, on day 0 and day 5 of culture. (C) Scatter plot and (D) representative dot plots of MAIT cell expansion over 5 days of culture with or without the addition of BCH (50mM) as measured by the percentage of CD3 population. For both experiments, PBMCs were unstimulated (Day 0) or stimulated (Day 5) with 5-ARU (1μg/mL) and MG (100μL) on day 0 and received a low dose of IL-2 (6.75ng/μL) on day 1 followed by a high dose of IL-2 (33.3ng/μL) on day 4 in the presence or absence of BCH (50mM) (n=6/4). Statistical analysis performed using one-way ANOVA with Tukey's correction, ** p≤0.01, **** p≤0.0001.

5.3.4 MAIT cells require mTORC1 for proliferation

Influx of amino acids was shown to be important for the activation of mTORC1 in MAIT cells (Figure 4.17). mTORC1 is known to orchestrate metabolism of T cells. Indeed, previous studies in T cells have demonstrated that the activation of mTORC1 in part relies on the uptake of leucine, which is transported into the cells via LAT1. Activation of mTORC1, was in turn shown to be required for upregulation of glycolysis during activation. As both glycolysis and amino acids were shown to be required for MAIT cell proliferation, next we investigated the role of mTORC1 on MAIT cell proliferation. PBMC were stained with CTV for analysis of MAIT cell proliferation or remained unstained for the assessment of MAIT cell expansion. Then cells were stimulated with 5-ARU-MG, in the presence or absence of Rapamycin for the first 18 hours of culture. Then cells were supplemented with IL-2, cultured for a total of 5 days and analysed by flow cytometry. Rapamycin, a specific mTORC1 inhibitor, significantly limited MAIT cell proliferation (Figure 5.7 A&B) and expansion (Figure 5.7 C&D). This data indicates that activation of mTORC1 is required for progression of MAIT cell proliferation.

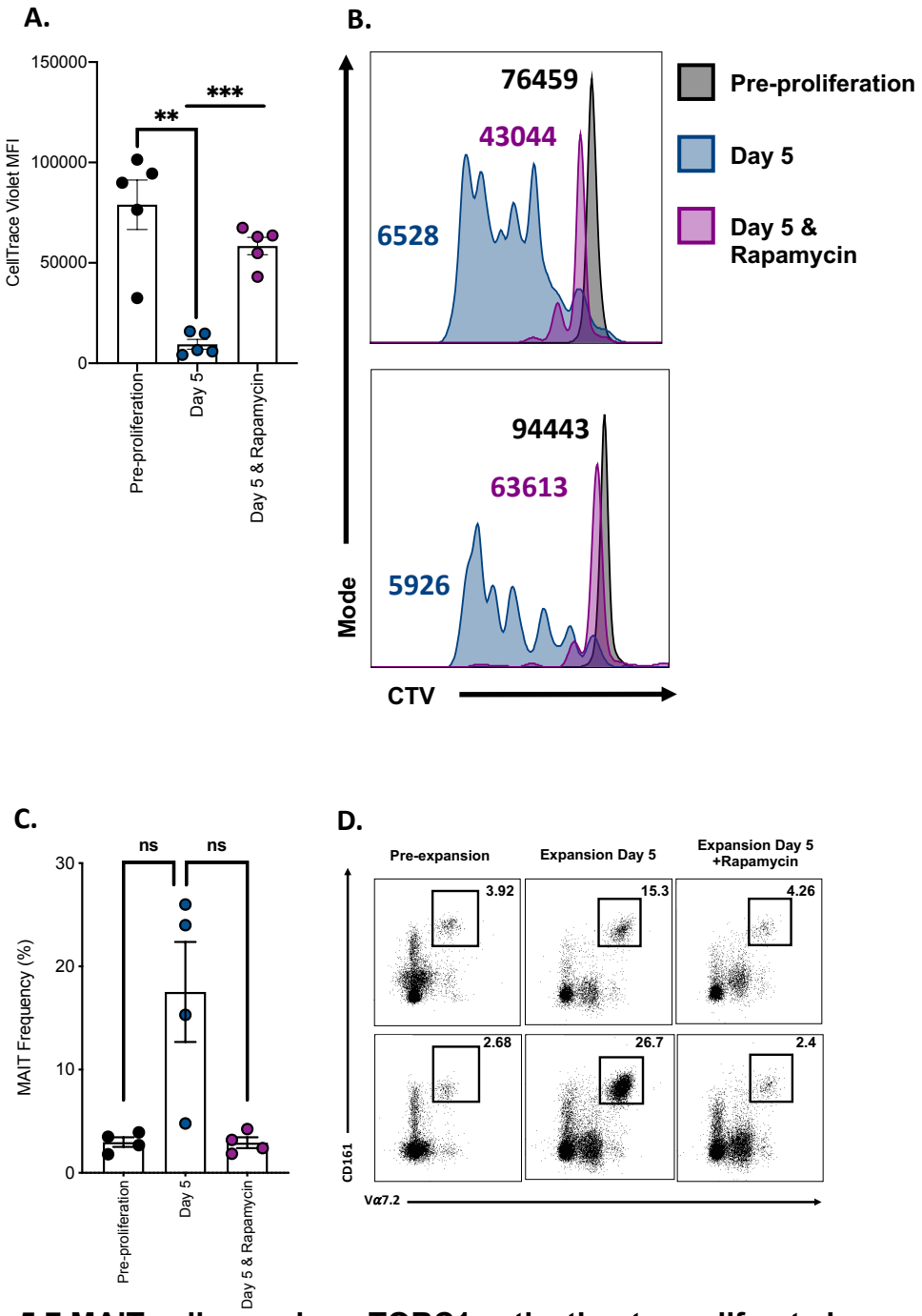


Figure 5.7 MAIT cells require mTORC1 activation to proliferate in response to 5-ARU-MG and IL-2. (A) Scatter plot and (B) representative histograms of 2 control subjects showing MAIT cell proliferation over 5 days of culture with or without the addition of Rapamycin (20nM) as measured by uptake and dilution of Cell Trace Violet (CTV) at 5μM, on day 0 and day 5 of culture. (C) Scatter plot and (D) representative dot plots of MAIT cell expansion over 5 days of culture with or without the addition of Rapamycin (20nM) as measured by the percentage of CD3 population. For both experiments, PBMCs were unstimulated (Day 0) or stimulated (Day 5) with 5-ARU (1μg/mL) and MG (100μL) on day 0 and received a low dose of IL-2 (6.75ng/μL) on day 1 followed by a high dose of IL-2 (33.3ng/μL) on day 4 in the presence or absence of Rapamycin (20nM) (n=6/4). Statistical analysis performed using one-way ANOVA with Tukey's correction, ** p≤0.01, **** p≤0.0001.

5.3.5 MAIT cells require glutamine metabolism and OxPhos for proliferation

Having identified the dependency of MAIT cell proliferation on a LAT1-mTORC1-Glycolysis axis, we extended our investigations to other metabolic pathways. OxPhos has been reported to play a role in supporting conventional T cell proliferation (Bailis et al., 2019; Sena et al., 2013). Therefore, we decided to investigate the role of OxPhos in MAIT cell proliferation.

OxPhos is the most effective way of producing large amounts of energy in the form of ATP by the cell, and proliferation is a very energy and nutrient demanding process. In this series of experiments, we decided to explore whether the inhibition of particular components of the OxPhos machinery to limit ATP production impacts the rate of MAIT cell proliferation. Two inhibitors of the electron transport chain were used - oligomycin, an ATP synthase inhibitor or antimycin A, an inhibitor of complex III of electron transport chain (ETC). PBMC were prepared and stimulated as described in section 5.3.4 in the presence or absence of oligomycin or antimycin A for the first 18 hours of the experiment and then cells were analysed using flow cytometry. Inhibition of ATP synthase with oligomycin significantly reduced MAIT cell proliferation and expansion by day 5 of culture (Figure 5.8 A-D). Halt in the proliferation occurred early in proliferation, similar to what was observed following treatment with 2DG (Figure 5.4) and BCH (Figure 5.6), as signified by one to two discrete peaks shown on the representative histograms (Figure 5.8 B). Treatment of cells with antimycin A, the inhibitor of complex III of the ETC, slowed MAIT cell proliferation and expansion, however to a lesser extent (Figure 5.9 A-D). As depicted by the representative histograms in Figure 5.9 B, inhibition of complex III of ETC still allowed for MAIT cell expansion into multiple generations, shown by the multiple individual peaks on the graph.

This data suggests that the generation of ATP via the ETC is necessary for effective MAIT cell proliferation. Inhibition of the complex III of ETC has a negative impact on MAIT cell expansion, however it may still allow for ATP production. As previously mention in section 4.3.3, glutamine can feed into the Kreb's cycle and hence support OxPhos. In order for glutamine to enter the Kreb's cycle, it must undergo glutaminolysis, an enzymatic process during which glutamine is broken

down into α-ketoglutarate (α-KG), an intermediate of the Kreb's cycle (O'Neill et al., 2016). It has been shown that glutaminase (GLS), the first enzyme involved in this process, can be inhibited with a small molecule inhibitor CB-839 in leukaemia cells, resulting in their reduced proliferation and function. To assess the role of glutamine in supporting MAIT cell proliferation, we used a glutaminase inhibitor CB-839. PBMC were prepared and stimulated as described above in the presence or absence of CB-839 Treatment with CB-839 slowed MAIT cell proliferation (Figure 5.10 A&B), resulting in significantly reduced MAIT cell expansion (Figure 5.10 C&D). Overall, these results indicate a role for OxPhos in supporting MAIT cell proliferation, most likely through provision of ATP required to support the process of proliferation.

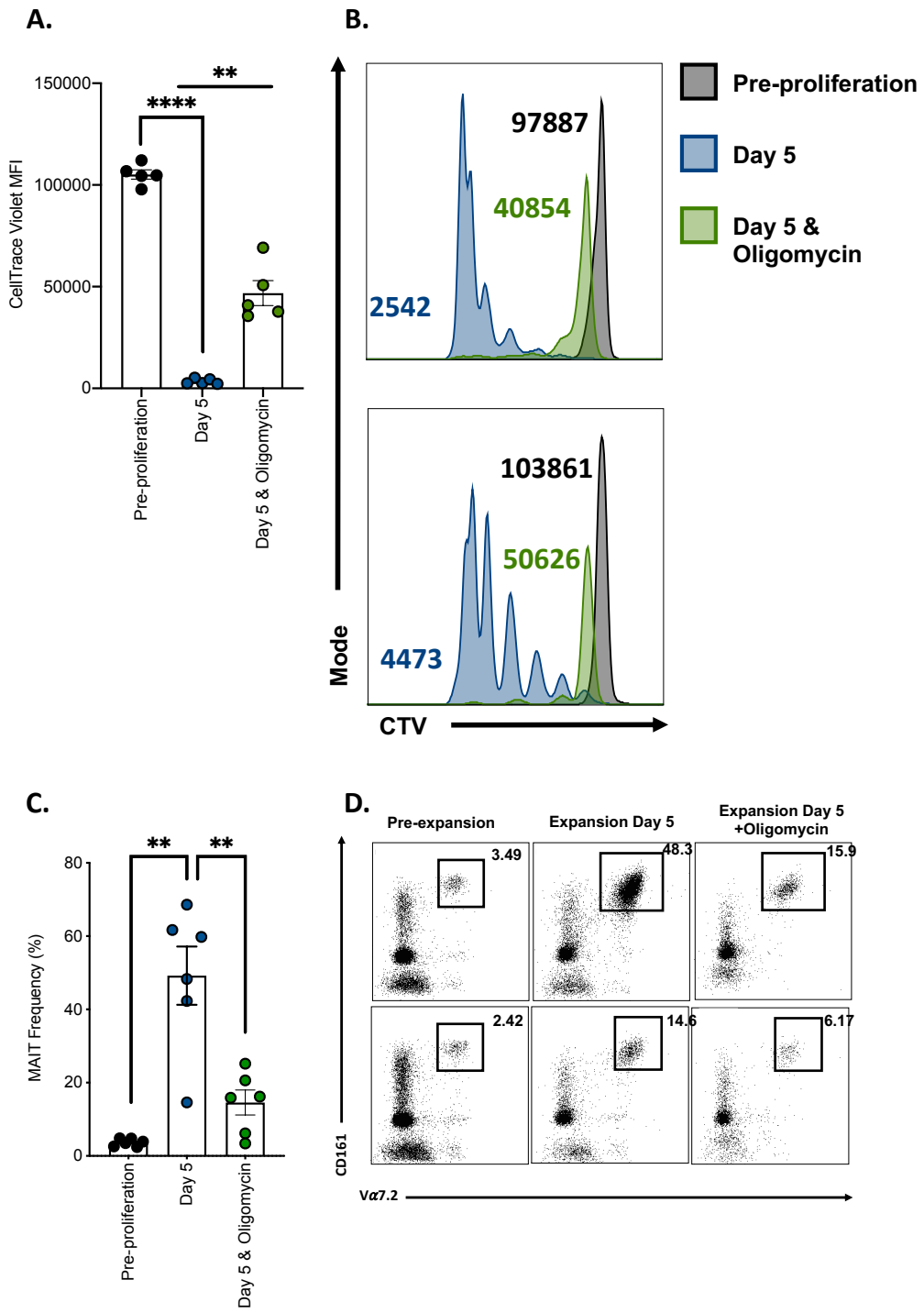


Figure 5.8 MAIT cells require active ATP synthase to proliferate in response to 5-ARU-MG and IL-2. (A) Scatter plot and (B) representative histograms of 2 control subjects showing MAIT cell proliferation over 5 days of culture with or without the addition of oligomycin (2µM) as measured by uptake and dilution of Cell Trace Violet (CTV) at 5µM, on day 0 and day 5 of culture. (C) Scatter plot and (D) representative dot plots of MAIT cell expansion over 5 days of culture with or without the addition of oligomycin (2µM) as measured by the percentage of CD3 population. For both experiments, PBMCs were unstimulated (Day 0) or stimulated (Day 5) with 5-ARU (1µg/mL) and MG (100µL) on day 0 and received a low dose of IL-2 (6.75ng/µL) on day 1 followed by a high dose of IL-2 (33.3ng/µL) on day 4 in the presence or absence of oligomycin (2µM) (n=6). Statistical analysis performed using one-way ANOVA with Tukey's correction, ** p≤0.01, **** p≤0.0001.

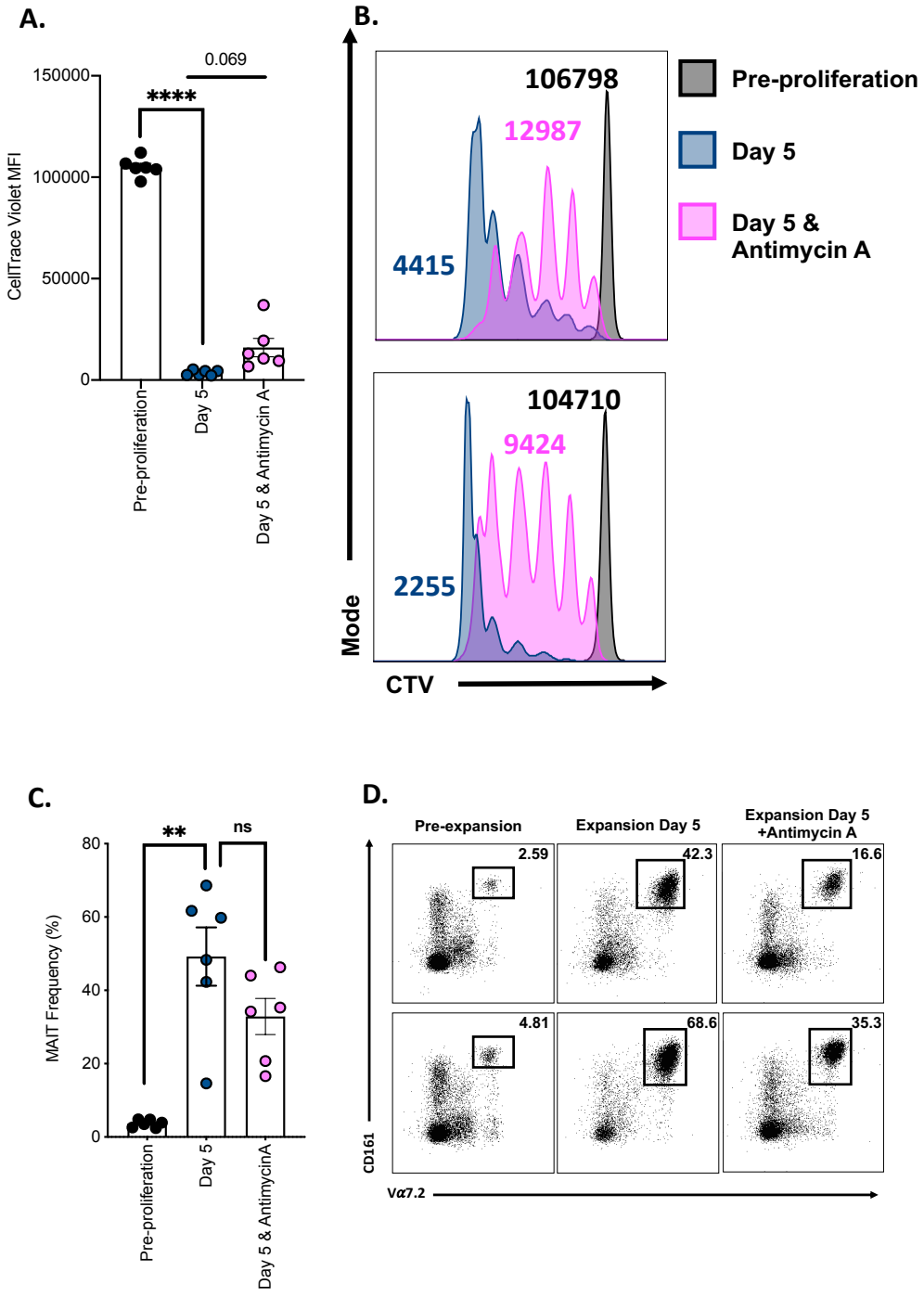


Figure 5.9 MAIT cells require OxPhos complex III to proliferate in response to 5-ARU-MG and IL-2. (A) Scatter plot and (B) representative histograms of 2 control subjects showing MAIT cell proliferation over 5 days of culture with or without the addition of Antimycin A (4μM) as measured by uptake and dilution of Cell Trace Violet (CTV) at 5μM, on day 0 and day 5 of culture. (C) Scatter plot and (D) representative dot plots of MAIT cell expansion over 5 days of culture with or without the addition of Antimycin A (4μM) as measured by the percentage of CD3 population. For both experiments, PBMCs were unstimulated (Day 0) or stimulated (Day 5) with 5-ARU (1μg/mL) and MG (100μL) on day 0 and received a low dose of IL-2 (6.75ng/μL) on day 1 followed by a high dose of IL-2 (33.3ng/μL) on day 4 in the presence or absence of Antimycin A (4μM) (n=6). Statistical analysis performed using one-way ANOVA with Tukey's correction, ** p≤0.01, **** p≤0.0001.

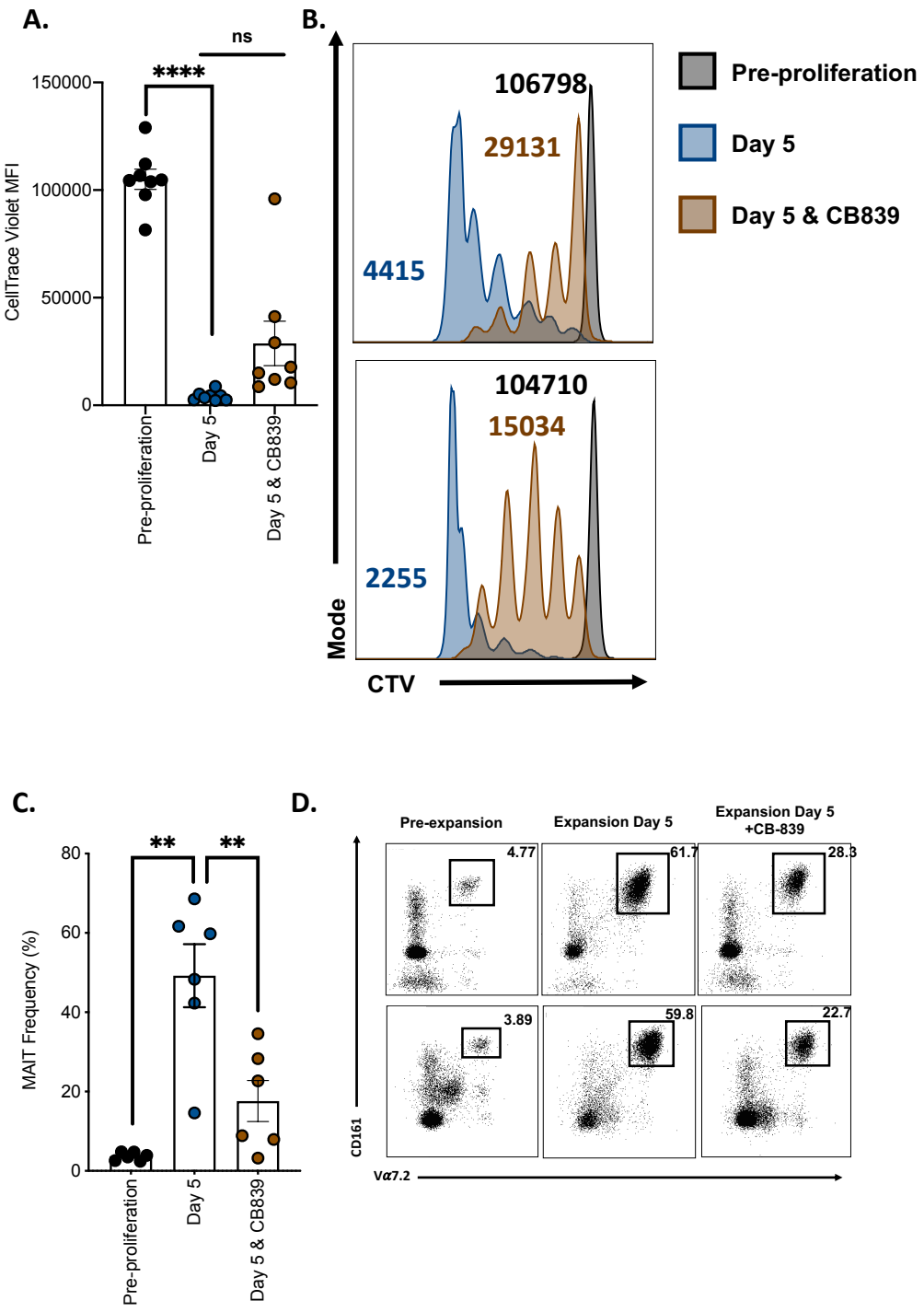


Figure 5.10 MAIT cells require glutamine metabolism to proliferate in response to 5-ARU-MG and IL-2. (A) Scatter plot and (B) representative histograms of 2 control subjects showing MAIT cell proliferation over 5 days of culture with or without the addition of CB-839 (10μM) as measured by uptake and dilution of Cell Trace Violet (CTV) at 5μM, on day 0 and day 5 of culture. (C) Scatter plot and (D) representative dot plots of MAIT cell expansion over 5 days of culture with or without the addition of CB-839 (10μM) as measured by the percentage of CD3 population. For both experiments, PBMCs were unstimulated (Day 0) or stimulated (Day 5) with 5-ARU (1μg/mL) and MG (100μL) on day 0 and received a low dose of IL-2 (6.75ng/μL) on day 1 followed by a high dose of IL-2 (33.3ng/μL) on day 4 in the presence or absence of CB839 (10μM) (n=8/6). Statistical analysis performed using one-way ANOVA with Tukey's correction, ** p≤0.01, **** p≤0.0001.

5.3.6 Glycolysis is required for IFNγ production

We next investigated the metabolic requirements for MAIT cell cytokine production, their primary effector function. Previous studies in T cells have shown that glycolysis is crucial for cytokine production. Renner *et al.* have shown that production of IFNγ is intrinsically dependent on glycolytic metabolism (Renner et al., 2015). To investigate expanded MAIT cells were stimulated with TCR Dynabeads, IL-12 and IL-18 in the presence or absence of 2DG for 24 hours and the supernatants were tested using ELISA. Here we demonstrate a strong reduction in IFNγ production by MAIT cells (Figure 5.11 A). This data shows that MAIT cells upregulate glycolysis upon cell activation, which is required for their production of IFNγ.

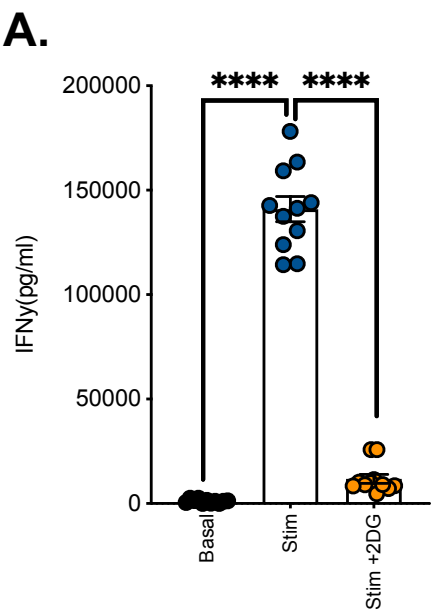


Figure 5.11 Blocking glycolysis with 2DG reduces IFNγ production by MAIT cells. (A) Scatter plot of IFNγ production by in vitro expanded and purified MAIT cells upon stimulation with TCR Dynabeads (1:1 bead to cell ration) and IL-12/IL-18 (50ng/mL each) cytokines for 24 hours in the presence or absence of 2DG (10mM) for 24 hours (n=13). Statistical analysis performed using student's t-test, ****p<0.0001.

5.3.7 Amino acid transport is required for IFNγ production

In chapter 4, we identified a LAT1-mTOR-glycolysis axis in activated MAIT cells. We next investigated if LAT1 activity was required for MAIT cell cytokine production. Previous studies have demonstrated that the inhibition of LAT1 function in T cells leads to a decrease in their cytokine production (Hayashi et al., 2013), however, this has not been investigated in MAIT cells. Using PBMC from healthy controls we show that upon activation with TCR microbeads, IL-12 and IL-18 for 18 hours MAIT cells increased their IFNγ production (Figure 5.12 A&B), and with inhibition of the amino acid flux via LAT1, we observed a reduction in IFNγ expression by MAIT cells (Figure 5.12 C&D). To confirm that the LAT1 inhibitor BCH was acting directly on MAIT cells, MAIT cells were expanded. Upon stimulation with TCR Dynabeads, IL-12 and IL-18 for 24 hours, MAIT cells produced high levels of IFNγ (Figure 5.12 E), however IFNγ levels were significantly reduced in the presence of the LAT1 inhibitor BCH (Figure 5.12 E). We next investigated whether the inhibition of amino acid influx had any impact on early transcription of mRNA coding for IFNγ and T-bet mRNA. Expanded MAIT cells were pre-treated with BCH for 1 hour and then stimulated with TCR Dynabeads, IL-12 and IL-18 for further 4 hours. Here we demonstrate no impact on the transcription of mRNA for IFNγ or T-bet (Figure 5.12 F&G). The mean fold increase of *IFNG* transcription was higher than transcription of *IFNG* in the absence of the inhibitor, however this difference was not significant (Figure 5.12 F). To investigate whether the inhibition of amino acids influx has any effect on the transcription of key glycolytic enzymes HKII and PKM, expression of mRNA for both of these enzymes was measured on expanded MAIT cells treated as described for Figure 5.12 F&G. Incubation of cells in the presence of BCH upon cell stimulation, revealed no difference in the transcription level of those two enzymes of glycolysis (Figure 5.13 A&B). In summary, this data shows that the transport amino acids via LAT1 is essential for IFNγ production, but no difference in transcription was observed at 4 hours. Inhibition of amino acid flux had also no effect on the expression of key glycolytic enzymes at this timepoint.

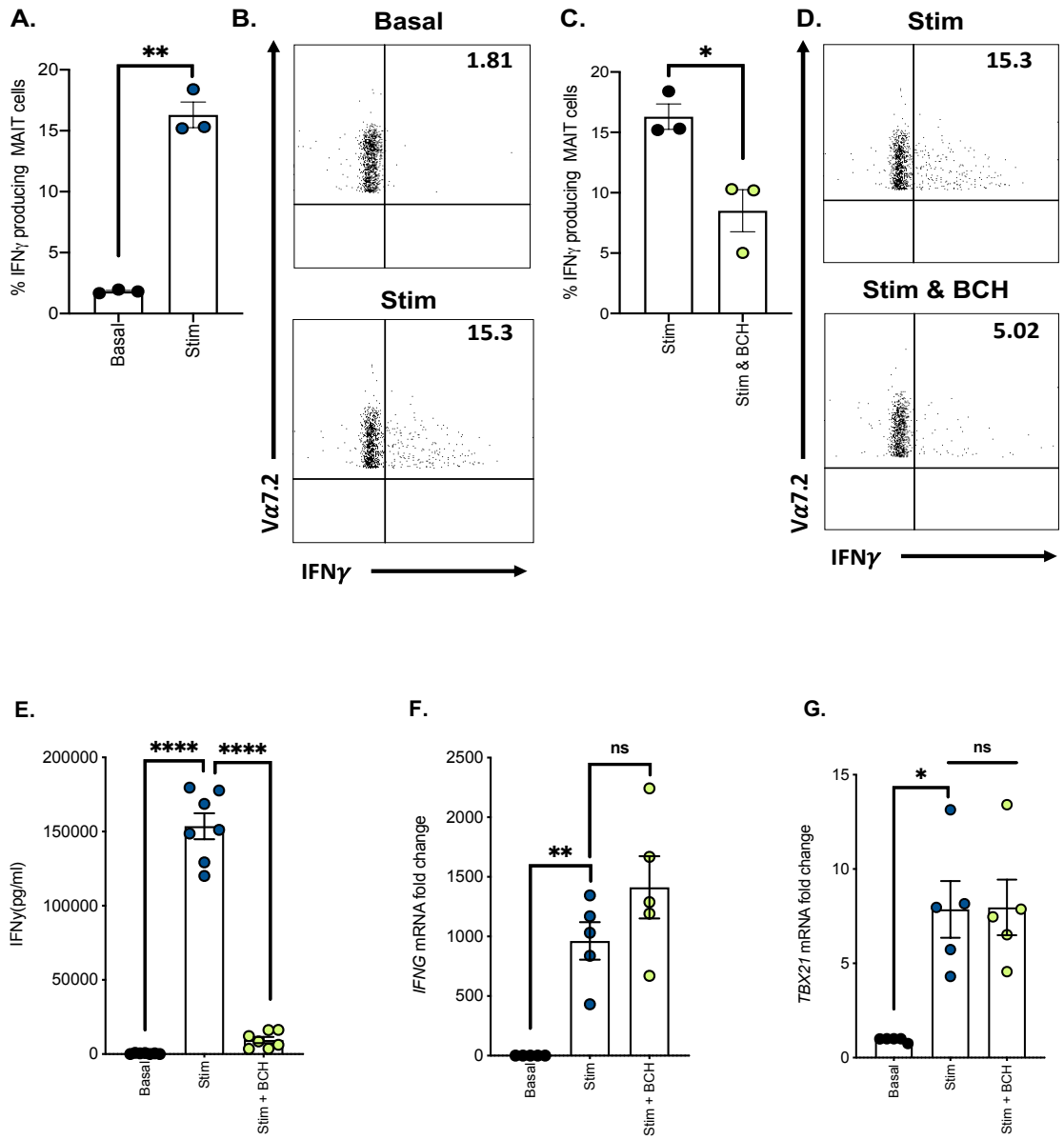


Figure 5.12 Blocking amino acid transport by BCH reduces IFNγ production by MAIT cells. (A) Scatter plot and (B) representative dot plots of IFNγ production from PBMCs unstimulated or stimulated with TCR microbeads at 25ng/mL (Milteneyi), IL-12 and IL-18 (at 50ng/mL each) for 18 in the control individuals (n=3). (C) Scatter plot and (D) representative dot plots of IFNγ production from PBMCs stimulated with TCR microbeads at 25ng/mL (Milteneyi), IL-12 and IL-18 (at 50ng/mL each) with or without addition of BCH (50mM) for 18 hours in the control population sample (n=3). (E) Scatter plot of IFNγ production by in vitro expanded and purified MAIT cells upon stimulation with TCR Dynabeads (1:1 bead to cell ration) and IL-12/IL-18 (50ng/mL each) cytokines for 24 hours in the presence or absence of BCH (50mM) for 24 hours (n=7). (F) Scatter plot of *IFNG* (n=4) and (G) *TBX21* (n=5) mRNA transcription by in vitro expanded and purified MAIT cells upon stimulation with TCR Dynabeads (1:1 bead to cell ration) and IL-12/IL-18 (50ng/mL each) cytokines for 4 hours in the presence or absence of BCH (50mM) for 1 hour prior to stimulation. Statistical analysis performed using student's t-test, or one-way ANOVA with Tukey's correction, ns- non-significant, *p<0.05, **p<0.01, ****p<0.0001

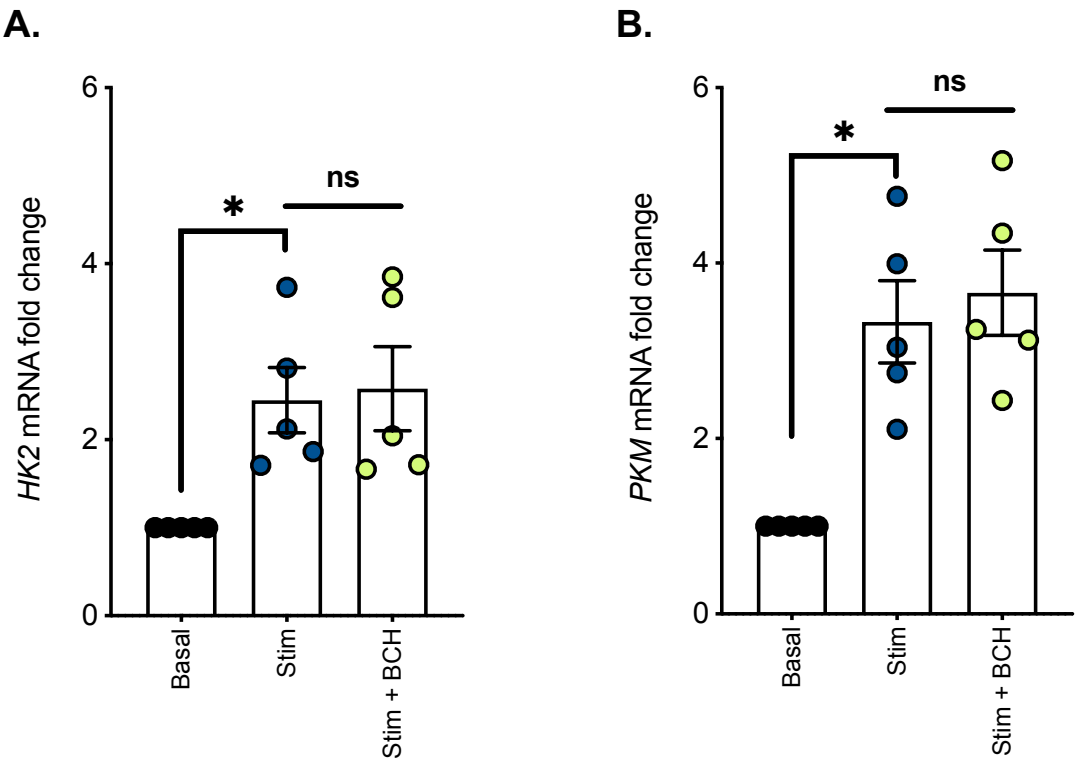


Figure 5.13 Blocking amino acid transport using BCH doesn't affect the transcription of *HK2* or *PKM* by MAIT cells at an early stage during activation. (A) Scatter plot of *HK2* mRNA and (B) *PKM* transcription by *in vitro* expanded and purified MAIT cells upon stimulation with TCR Dynabeads (1:1 bead to cell ration) and IL-12/IL-18 (50ng/mL each) cytokines for 4 hours in the presence or absence of BCH for 1 hour prior to stimulation(50mM) (n=5). Statistical analysis performed using one-way ANOVA with Tukey's correction, ns-non-significant, *p<0.05.

5.3.8 mTORC1 is required for IFNγ production

We next investigated inhibition of mTORC1 has any effect on IFNγ production using the specific inhibitor rapamycin Expanded MAIT cells were stimulated as per section 5.3.6 in the presence in Rapamycin inhibitor for 24 hours and IFNγ production was measured by ELISA. A significant decrease in the production of IFNγ cytokine by MAIT cells in the presence of mTORC1 inhibitor was observed (Figure 5.14 A). Together this data shows that activity of mTORC1 is required for upregulation of glycolysis, which in turn is required for successful cytokine production.

5.3.9 Glutamine metabolism supports IFNγ production

Inhibition of glutamine metabolism limited MAIT cell proliferation (Figure 5.10); however, no studies have been performed to investigate whether the inhibition of glutamine metabolism affects MAIT cell cytokine production. We decided to investigate the effect of GLS inhibition on MAIT cell IFNγ production. Expanded MAIT cells were stimulated as per section 5.3.6 in the presence in CB-839 inhibitor for 24 hours and IFNγ was measured by ELISA. Inhibition of GLS with CB-839 resulted in a modest reduction in IFNγ production (Figure 5.15 A). Although the effect of CB-839 was modest in comparison to inhibition of glycolysis or mTORC1 with 2DG or Rapamycin respectively (Figure 5.11, 5.14 & 5.15), our data suggests a role for glutamine in supporting the MAIT cell function. This is further supported by the observed upregulation of ASCT2 amino acid transporter mRNA expression during the early stages of MAIT cell activation, what may support uptake of glutamine into the cells

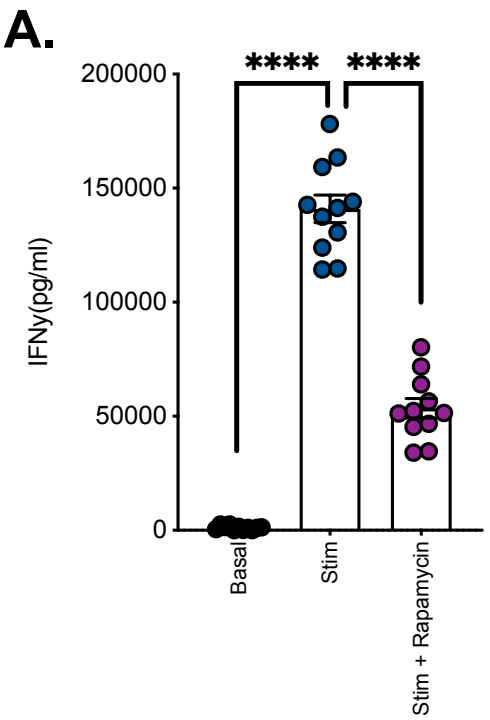


Figure 5.14 Inhibition of mTORC1 with rapamycin reduces IFNγ production by MAIT cells. (A) Scatter plot of IFNγ production by in vitro expanded and purified MAIT cells upon stimulation with TCR Dynabeads (1:1 bead to cell ration) and IL-12/IL-18 (50ng/mL each) cytokines for 24 hours in the presence or absence of Rapamycin (20nM) for 24 hours (n=13). Statistical analysis performed using one-way ANOVA with Tukey's correction, ****p<0.0001.

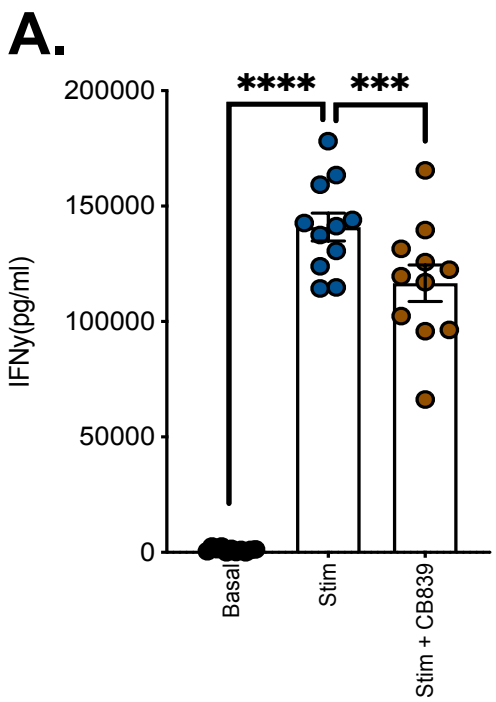


Figure 5.15 Inhibition of glutaminase with CB-839 reduces IFNγ production by MAIT cells. (A) Scatter plot of IFNγ production by in vitro expanded and purified MAIT cells upon stimulation with TCR Dynabeads (1:1 bead to cell ration) and IL-12/IL-18 (50ng/mL each) cytokines for 24 hours in the presence or absence of CB839 (10μM) for 24 hours (n=13). Statistical analysis performed using one-way ANOVA with Tukey's correction, ****p<0.0001.

5.4 Discussion

This study shows that MAIT cells engage in multiple interconnected metabolic pathways upon activation, suggesting that early metabolic reprogramming is required to facilitate their effector functions. Multiple studies in T cells and NK cells have shown that cell metabolism is in fact required for optimal proliferation and cytokine production (Bailis et al., 2019; Chang et al., 2013; Keating et al., 2016; Loftus et al., 2018; Renner et al., 2015). Therefore, we investigated the metabolic requirements for MAIT cell functions within this chapter.

Although the rate of metabolism can be controlled at multiple stages, nutrient and substrate availability is one of the key limiting steps for any enzymatic reaction. Here we show that by restricting glucose availability to 1mM, this leads to impaired MAIT cell proliferation and expansion. This is in line with Jacobs *et al*. who demonstrated that glucose restriction negatively affects T cell proliferation (Jacobs et al., 2008). To further explore the role of glucose on MAIT cell function, we substituted glucose for galactose. Supplying galactose as the only source of carbohydrates, it urges the cell to favour OxPhos over glycolysis (Chang et al., 2013; Weinberg et al., 2010). MAIT cells cultured in galactose containing media displayed delayed expansion in comparison to the cells cultured in media with glucose. Chang *et al*. have recorded very similar findings in T cells, where the incubation of T cells in the presence of galactose slowed down their proliferation.

To further investigate the role of glucose and its metabolism on MAIT cell function, we inhibited glycolysis using 2DG. The presence of 2DG in culture led to a very strong inhibition of MAIT cell proliferation and IFNγ production in comparison to the cells cultured without the inhibitor. In line with these results, Procaccini *et al.* have reported a significant reduction in proliferation of T cells when activated in the presence of 2DG (Procaccini et al., 2016), whereas others have reported that glycolysis and glucose is required for their IFNγ production (Dimeloe et al., 2016; Renner et al., 2015; Yin et al., 2015). In addition, restriction of glucose or inhibition of glycolysis resulted in the reduction in IL-2 receptor expression by MAIT cells upon stimulation. As we showed (in chapter four) exogenous supplementation of IL-2 strongly enhances MAIT cell proliferation, we therefore hypothesised that the level of glycolysis affects the expression of IL-2 receptor and hence contribute to the impaired proliferation of MAIT cells.

Collectively, our study provides extensive data, which supports the hypothesis that glycolysis is central to MAIT cell function as inhibition of glycolysis, through the withdrawal or restriction of glucose, induces a significant defect in their proliferation and IFNγ production. Here we demonstrate that an abundance of glucose and glycolysis are important at the early stages of MAIT cell activation, although more studies are required to assess what metabolic events occur at later timepoints. The role of augmented glycolysis in MAIT cell activation remains to be fully elucidated, however studies conducted on T cells, have shown that the metabolic intermediates derived from glycolysis are the primary reason for the metabolic switch. For example, the first intermediate of glycolysis, glucose-6-phosphate, can enter the pentose phosphate pathway to synthesise new nucleic acids, essential for cell proliferation. Other intermediates of glycolysis can contribute to amino acids synthesis, which may play a role in the augmented production of cytokines during cell activation (O'Neill et al., 2016). Intermediates of glycolysis were shown to be directly related to IFNγ production in T cells (Ho et al., 2015; Peng et al., 2016). In addition, studies of cell cycle and cell metabolism reported that cell cycle progression is sensitive to nutrient availability, where restriction of glucose or inhibition of glycolysis prevented progression of the cell cycle (Kalucka et al., 2015). This suggests that inadequate rate of glycolysis or extracellular glucose concentration, may provide a signal to the cell indicating that there are insufficient nutrients for cell activation to take place, hence preventing cell proliferation and cytokine production. This is of particular importance in the hypoglycaemic settings *in vivo*, such as tumour microenvironment. Restricted access of MAIT cells to glucose may hinder their potential anti-tumour responses (Godfrey et al., 2018; Vacchini et al., 2020).

As this study has shown that amino acid influx is critical for the upregulation of glycolysis upon MAIT cell activation, next we investigated the role of LAT1 mediated amino acid transport on MAIT cell function. Inhibition of LAT1 using BCH resulted in a restriction of proliferation and expansion of MAIT cells. This is in line with the results described in chapter four, which depicted the inhibition of amino acid influx via LAT1, leads to suppressed mTORC1 activation, that occurs most likely due to hindered transport of leucine. Low activity of mTORC1 prevents enhancement of glycolysis, which we show is crucial for MAIT cell function. Limitation of leucine import most likely contributes to the defect in MAIT cell

proliferation. Interruption in the import of methionine via LAT1, an amino acid, which is required for methylation of DNA, process required for T cell proliferation and differentiation may also fortify this effect (Sinclair et al., 2019). In our study, LAT1 amino acid transport was also required for IFNγ protein production by MAIT cells. Blocking of amino acid influx upon MAIT cell stimulation didn't affect early transcription of mRNA coding for IFNγ and T-bet or the transcription of glycolytic enzymes, HK2 or PKM. This suggests that despite suboptimal uptake of amino acids, MAIT cells become activated and initiate transcription of mRNA for the proteins required to support their effector functions. This suggests that impairment in upregulation of glycolysis and IFNγ synthesis in the presence of BCH occurs due to the alterations which occur at translational level. Low intracellular concentration of leucine and other amino acids prevents MAIT cells from enhancing glycolysis via mTORC1, leading to the impairment in IFNγ production. Low cytokine production may be in part due to low levels of amino acids required to fulfil translation of the mRNA of the effector proteins. This is in line with the literature, as the studies showed that T cells also require amino acids to support cytokine production (Hayashi et al., 2013; Ron-Harel et al., 2019; Sinclair et al., 2019, 2013).

Leucine has been described to be required to activate mTORC1, which in turn is responsible for the regulation of glycolysis. As amino acid transport was shown to be required for MAIT cell function, next we investigated how the inhibition of mTORC1 with rapamycin would affect MAIT cell proliferation and IFNγ production. Similar to the inhibition of glycolysis and amino acid influx, preventing mTORC1 activation, decreased the proliferative capacity and production of IFNγ by MAIT cells. This is in line with studies which have demonstrated a reduction in production of IFNγ and proliferation of T cells when incubated in the presence of mTORC1 inhibitor (Chaoul et al., 2015; Herrero-Sánchez et al., 2016; Kurebayashi et al., 2012), which has been further confirmed in *in vivo* studies of Rheb or Raptor deficient mice (Pollizzi et al., 2015; Yang et al., 2013). Collectively, this data shows that MAIT cells enhance their glycolytic metabolism during activation to support their effector functions. Any defect in this pathway, that controls the glycolytic rate in the cell, leads to severe impairment of MAIT cell proliferation and IFNγ production.

It is now widely accepted that T cells use OxPhos as their primary source of energy in their quiescent state but switch to glycolysis upon activation (O'Neill et al., 2016). Data published on T cell metabolism indicates that engagement of the cells in OxPhos during proliferation is important (Bailis et al., 2019; Sena et al., 2013). After establishing that MAIT cells rely on glycolytic metabolism for their cytokine production, proliferation and expansion, next we focused on the role of OxPhos in MAIT cell function. Study performed by O'Brien *et al.* in our laboratory and described in her thesis, has shown that OxPhos was not required for production of IFNγ by MAIT cells upon stimulation. In contrast to these results, here we have shown that although OxPhos may not be critical to MAIT cell cytokine production, it plays a role in their proliferation and expansion. Blocking of ATP synthase using oligomycin, resulted in the inhibition of MAIT cell proliferation and expansion, whereas the inhibition of complex III of ETC, resulted in a retardation of proliferation and expansion, rather than inhibition. The differences observed between the inhibition of two different components in ETC was most likely due to the role they fulfil in this reaction. Inhibition of ATP synthase has a more severe impact as it is required for production of all ATP generated during the respiration. Inhibition of complex III most likely only slows down the reaction, but still produces ATP. As a result, ETC provides the cell with a limited amount of energy, leading to suboptimal proliferation. This is in line with the data published by Bailis *et al.* who reported significant defect in the proliferation of T cells after inhibiting complex III of ETC (Bailis et al., 2019). In addition, we investigated the importance of glutamine in supporting MAIT function, by inhibiting GLS with CB-839. Inhibition of glutamine metabolism slowed down MAIT cell expansion, proliferation and IFNγ production, but had a modest effect in comparison to direct or indirect inhibition of glycolysis. This is in line with a study by Carr *et al*. which showed that glutamine is essential for proliferation and cytokine production (Carr et al., 2010). Johnson *et al.* have also shown that a lack of glutamine in media strongly halts T cell proliferation, whereas inhibition of GLS with CB-839 modestly slows it down (Johnson et al., 2018).

Collectively this data provides extensive evidence that MAIT cells undergo an early metabolic reprogramming upon activation to support cytokine production and proliferation. Glycolytic metabolism was shown to be indispensable for MAIT cell function. Inhibiting any of the steps required for the modulation of glycolytic

metabolism or direct inhibition of glycolysis resulted in profound defects in cell proliferation and IFNγ production (Figure 5.16). In this study, we showed that MAIT cells from people with obesity have dysregulated glycolytic metabolism and fail to adjust glycolysis upon stimulation. We suggest that this deficiency underpins the defect in proliferation and IFNγ production by MAIT cells from people with obesity. In addition, OxPhos was also shown to support MAIT cell proliferation, therefore this also should be assessed in MAIT cells in people with obesity. Defects in both metabolic pathways may be responsible for the observed impairment in MAIT cell proliferation from people with obesity and thus lead to the reduced frequencies observed in the peripheral blood and adipose tissue (Carolan et al., 2015; Magalhaes et al., 2015). Although this study provides a plausible explanation for the reduction of MAIT cell frequencies observed in people with obesity, to elucidate this further activation induced cell death of MAIT cells in people with obesity should also be assessed to fully explain lower MAIT cells frequencies.

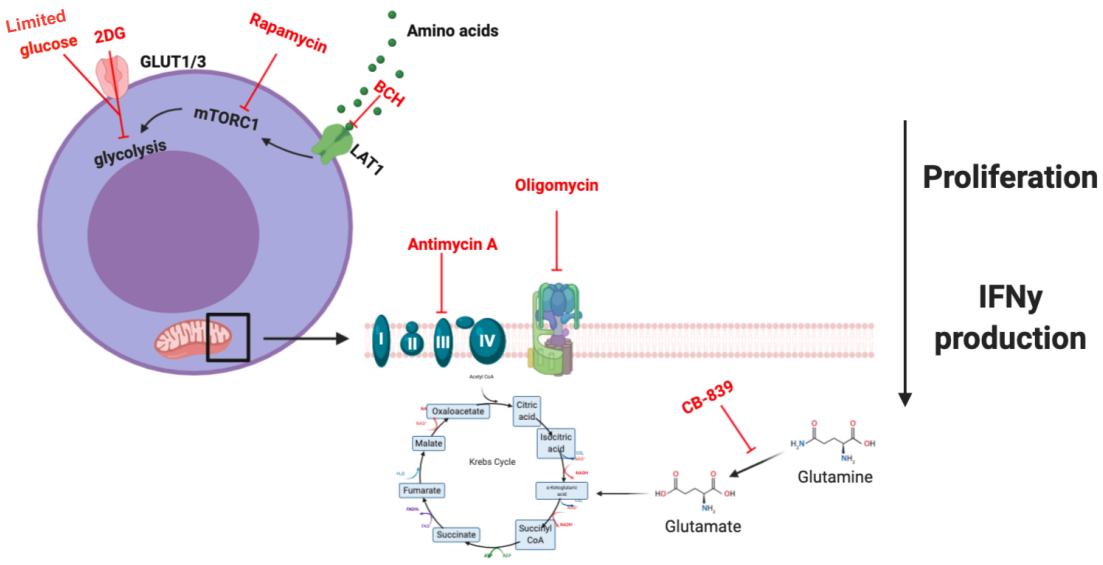


Figure 5.16 Summary of results of chapter five. MAIT cells from control individuals require glycolytic metabolism for proliferation and IFNγ production. Inhibition of any step in the pathway which controls the rate of glycolysis was shown to have a profound impact on MAIT cell function. OxPhos was shown to also support MAIT cell proliferation, however to a lower extent

Chapter Six

General Discussion

6. Discussion

Nearly thirty years after the discovery of MAIT cells, they still remain largely unexplored in comparison to other cells of the innate and adaptive immune system. Their high frequencies in multiple human tissues and pleotropic functions suggest they play an important role in the protection of their host and maintenance of tissue homeostasis.

Advancements in the research of MAIT cell biology and function were possible due to the development of monoclonal antibodies against Vα7.2 TCR and identification of MAIT cell antigens. This led to the development of tetramers and flow cytometric antibodies, which allowed for more detailed studies (Corbett et al., 2014; Martin et al., 2009; Rahimpour et al., 2015; Reantragoon et al., 2013). MAIT cell phenotype and function have been described in healthy and diseased individuals, and it has been shown they are susceptible to the alterations that may occur in their environment during the development of illnesses (Provine and Klenerman, 2020). In healthy individuals, MAIT cells respond to TCR dependent or independent stimulation, through the upregulation of a large number of effector proteins including cytokines, chemokines and cytotoxic molecules (Provine and Klenerman, 2020). Production of these mediators has been found to support the resolution of microbial infection (Chua et al., 2012; Meierovics and Cowley, 2016). Expression of chemokines induces recruitment of other immune cells to the site of inflammation, which are likely to become stimulated by cytokines such as IFNγ, TNF-α and IL-17, produced by activated MAIT cells (Meierovics et al., 2013; Meierovics and Cowley, 2016; Provine and Klenerman, 2020). These cytokines play an important role in eradiation of the infection. IFNγ acts on macrophages and enhancing phagocytic ability, whereas TNF-α and IL-17 induce the production of more pro-inflammatory cytokines and chemokines, other immune cells as well as epithelial and mesenchymal cells and hence exacerbate inflammation (Gaffen et al., 2014; Wu et al., 2014).

As MAIT cells were identified as potent producers of cytokines, the expression of them has been used as markers of their function in the state of disease. Dysregulation of MAIT cell function has been identified in multiple inflammatory and autoimmune diseases (Cho et al., 2014; Gracey et al., 2016; Willing et al., 2018). The first alteration observed in many diseases in which the phenotype of

MAIT cells is changed, is a decrease in their frequencies in the peripheral blood of individuals with diseases such as RA, IBD or SLE (Chiba et al., 2017; Hayashi et al., 2016; Serriari et al., 2014). Furthermore, blood MAIT cells largely display a pro-inflammatory profile with enhanced IL-17 production and a variable IFNγ expression (Pisarska et al., 2020). In RA and IBD increased frequency of type 17 MAIT cells has been reported at the sites of inflammation in the synovial fluid, gut mucosa or sclerotic lesions respectively, suggesting their migration and possible contribution to the inflammatory process (Gracey et al., 2016; Tominaga et al., 2017).

Investigations of MAIT cell function in obesity demonstrated severe alterations (Carolan et al., 2015; Magalhaes et al., 2015; Toubal et al., 2020). Activation of peripheral blood MAIT cells from people with obesity *in vitro* revealed a significant impairment in their IFNγ production as compared to the controls, whereas IL-17 levels were strongly enhanced (Carolan et al., 2015; Magalhaes et al., 2015). In addition, AT MAIT cells from people with obesity also displayed enhanced IL-17 but diminished IFNγ production (Carolan et al., 2015; Magalhaes et al., 2015). A very similar phenotype of peripheral blood MAIT cells was observed in colorectal cancer patients, who displayed a reduction in peripheral blood MAIT cell frequencies with impaired IFNγ but enhanced IL-17 production (Ling et al., 2016). IFNγ production by MAIT cells has been reported to be diminished in the adenocarcinoma and hepatocellular carcinoma lesions (Duan et al., 2019; Melo et al., 2019; Sundström et al., 2015). This suggests that cancer and obesity induce similar changes in MAIT cell function.

In this study, we confirmed lower frequencies of MAIT cells in the peripheral blood in people in obesity in comparison to the control individuals, which also displayed altered functional phenotype. In line with previously published data, MAIT cells from people with obesity presented with impaired IFNγ production but enhanced IL-17 synthesis (Carolan et al., 2015; Magalhaes et al., 2015). Here, for the first time, we report that in addition to the alterations in the cytokine production, peripheral blood MAIT cells from people with obesity display impaired MAIT cell expansion upon activation. This suggests that the failure of MAIT cells to expand may be in part responsible for the lower frequencies observed in the peripheral blood of people with obesity. What's more, this data suggests that obesity has

even bigger consequences on immune cell function. In fact, MAIT cells are not the only population affected by excessive weight gain. Michelet *et al.* and Tobin *et al.* reported impaired cytokine production and killing capabilities of NK cells from people with obesity (Michelet et al., 2018; Tobin et al., 2017).

Although changes in MAIT cell biology have been described in multiple disease settings, no clear mechanism controlling their function has yet been established. Recent progression in the field of immunometabolism identified an extra layer of control points of immune cell activation. Immune cell function is closely related to cell metabolism, where the disruption to the glycolytic metabolism or OxPhos pathways results in the disruption of their activation or effector functions (O'Neill et al., 2016). Resting T cells have been shown to primarily use Kreb's cycle and OxPhos to support energy production and survival (O'Neill et al., 2016). However, stimulation of T cells was shown to induce early metabolic reprogramming and metabolic switch to the use of glycolytic metabolism, which has been hypothesised to be more beneficial to the cell upon activation, due to the generation of metabolic intermediates during glycolysis (O'Neill et al., 2016). These intermediates can be used to support other metabolic pathways in order to synthesise amino acids, fatty acids or other molecules required to facilitate the activated state of the cell (O'Neill et al., 2016). Inhibition of glycolysis or glycolysis related pathways or restriction of glucose was shown to significantly reduce IFNγ production of stimulated T cells as well impair their differentiation (Chang et al., 2013; Hayashi et al., 2013; Renner et al., 2015; Sinclair et al., 2013). Here we have hypothesised that MAIT cells from people with obesity have impaired metabolism, which leads to altered MAIT cell function. Due to the lack of data on MAIT cell metabolism, we first aimed to characterise the metabolic requirements for MAIT activation in control individuals.

For the first time, we have shown that MAIT cells express a range of glucose and amino acid transporters, which synthesis level is modulated upon cell stimulation. This suggested that MAIT cells upregulate the expression of nutrient transporters to enhance the transport of glucose and amino acids to facilitate their activated state. Indeed, previous experiments performed by our lab have shown that the stimulation of MAIT increases the influx of nutrients, as measured by 2NBDG uptake (O'Brien, Loftus & Pisarska *et al.*, 2019; Figure 1 C&D). Potential

enhanced transport of glucose further suggested its role in supporting MAIT cell activation. Indeed, measurement of the rates of glycolysis, by assessing ECAR rates revealed significant upregulation in the glycolytic metabolism upon MAIT cell stimulation. This was further supported by the measurement of the expression of glycolytic enzymes, which were also enhanced upon activation. To test the hypothesis that metabolism of MAIT cells supports their function, we investigated the impact of disrupting glycolysis in MAIT cells during their activation. Restriction of glucose in the culture media or inhibition of glycolysis with 2DG significantly impaired MAIT cell proliferation and expansion. In addition, incubation of cells with 2DG significantly inhibited IFNγ production. Collectively, this strongly indicates that MAIT cells heavily rely on glycolytic metabolism upon their activation to exert their effector function. These results are in line with the research performed on T cells, which illustrated that upon stimulation, T cells undergo metabolic reprogramming, switching from OxPhos to glycolytic metabolism to support their function (O'Neill et al., 2016). Stimulation of T cells was shown to upregulate their glycolysis rates, whereas the limitation of glucose in the culture media or direct disruption of the glycolysis led to significant impairment in T cell cytokine production and proliferation (Chang et al., 2013; Gubser et al., 2013; Renner et al., 2015). It still remains to be elucidated why glycolysis is strongly favoured over OxPhos during the activation of lymphocytes however some hypotheses have been put forward. Although glycolysis is much less energy efficient than OxPhos, it provides a wide range of intermediates which can be used to *de novo* synthesis of amino acids, nucleic acids and fatty acids (O'Neill et al., 2016). These molecules in turn are thought to play a role in the production effector molecules such as cytokines or in providing building blocks required for cell proliferation and division. On the other hand, components of glycolysis have been identified to have a very direct role in supporting IFNγ synthesis. For example, in resting T cells, GAPDH enzyme was shown to bind to IFNγ mRNA, preventing it from transcription. Activation of T cells was shown to recruit GAPDH to facilitate enhanced glycolysis, freeing IFNγ mRNA for translation (Chang et al., 2013). More research is required to establish the direct relationship between the glycolysis and immune cell activation and function.

To explore MAIT cell metabolism further, we decided to investigate the role of amino acids in supporting MAIT cell function, as expression of LAT1 and ASCT2

transporters appeared to be largely enhanced early into their activation. A study of T cells by Sinclair *et al.* highlighted the importance of leucine transport in their glycolytic metabolism, therefore, we next focused on the LAT1 mediated transport of amino acids. Here, for the first time we have shown that LAT1 mediated transport of amino acids during MAIT cell activation is required for the upregulation of their glycolytic rate. Previous studies in T cells have established that leucine is required for the activation of mTORC1, which in turn is required for the upregulation of glycolysis (Finlay et al., 2012; Saxton et al., 2016). Therefore, we sought to investigate the impact of limiting amino acid influx on mTORC1 activation. Indeed, blocking of LAT1 diminished the activation of mTORC1. Collectively, this indicates that the stimulation of MAIT cells leads to the upregulation of LAT1, which facilitates the enhanced transport of amino acids, including leucine into the cell. Leucine supports the activation of mTORC1, which in turn enhances the rate of glycolysis. Parallel work from our lab has shown that mTORC1 activation is required for the upregulation of glycolysis upon MAIT cell stimulation, as inhibition of mTORC1 activity with rapamycin resulted in a failure of MAIT cells to modulate glycolysis rate upon stimulation (O'Brien, Loftus, Pisarska et al, 2019; Figure 3E&F). Inhibition of mTORC1 activity or amino acid influx had a strong negative impact on MAIT cell proliferation, expansion and IFNγ production, which mimics the effect obtained with the direct inhibition of glycolysis. This further indicates that both of these factors control the glycolytic metabolism in MAIT cells.

Although studies of T cell activation strongly focused on glycolytic metabolism, OxPhos has been shown to support T cell proliferation (Chang et al., 2013). To assess whether it also plays a role in the proliferation of MAIT cells, we used inhibitors of ETC and found that ATP synthase plays a particularly important role for MAIT cell proliferation, suggesting ATP derived during OxPhos is involved in supporting MAIT cell proliferation. On the other hand, inhibition of complex III of ETC, allowed for MAIT cell proliferation albeit at a slower rate. Similarly, preventing metabolism of glutamine, which plays a role in replenishing intermediates of Kreb's cycle, and hence supports OxPhos, led to impairment of MAIT cell proliferation, expansion and IFNγ production, but to a much lower extent than the inhibition of glycolytic metabolism. Collectively, this data indicates that OxPhos supports MAIT cell expansion and proliferation, however, plays a

less significant role than glycolysis in supporting MAIT cell function upon activation.

In this thesis, we have shown that glycolytic metabolism is central to MAIT cell activation and is absolutely required for successful MAIT cell proliferation, expansion and IFNγ production. As MAIT cells from people with obesity presented with severe impairment of both of these functions, we decided to compare their cell metabolism to the metabolism of the control individuals to identify any potential flaws, which may lead to the impairment in their function. Indeed, we found that MAIT cells from people with obesity fail to upregulate glycolysis upon stimulation. In addition, parallel work from our lab revealed that the activation of MAIT cells from people with obesity increases mTORC1 activation, but to a lower extent than in the control individuals (O'Brien, Loftus, Pisarska et al, 2019; Figure 3J). This suggests that inability to control the activity of mTORC1 leads to failure to enhance the rate of glycolysis. To elucidate this further, we measured the expression of LAT1, and the transport capabilities of this amino acid transporter required for the influx of leucine to activate mTORC1. We found that the expression of LAT1 by MAIT cells is lower in people with obesity, which likely leads to the observed lower transport capabilities. Collectively, data derived from the control individuals and people with obesity indicates that a defect in glycolytic metabolism displayed by MAIT cells from people with obesity underpins their impaired function. In this study we show that MAIT cells from people with obesity have impaired expression of LAT1 transporter, which likely leads to lower influx of amino acids including leucine. Lower availability of leucine probably leads to impaired mTORC1 activation, which further prevents glycolysis from being upregulated. Low rate of glycolysis fails to support stimulated MAIT cells, limiting the production of biosynthetic precursors which are likely to be required to enhance cytokine production and proliferation.

Here, we propose a mechanism responsible for impaired MAIT cell function in people in obesity, which may be translated into multiple disease settings, where MAIT cells present with a defect in activation, cytokine production or proliferation. Identification of the mechanism(s) controlling some of the effector functions exerted by MAIT cells will allow for the identification of targets, which could be

used to ameliorate immunometabolism in MAIT cells and hence restore their function. This data provokes multiple questions regarding MAIT immunometabolism and their function in diseases. Future research should focus on elucidating what induces the alterations in MAIT cell glycolytic metabolism in people with obesity. Here we hypothesise that inadequate influx of leucine due to the reduction in LAT1 expression, leads to a cascade of events which eventually results in impaired MAIT cell function. To further confirm this, measurement of leucine uptake by MAIT cells from individuals with and without obesity should be performed. In addition, further research should also investigate why the expression of LAT1 is impaired in MAIT cells with people with obesity. This defect was shown to be present at the transcriptional level, therefore investigations should focus on identifying which signalling pathway is responsible for inducing LAT1 transcription and whether it is also impaired in obesity. Sinclair *et al.* has also shown that direct TCR activation of T cells increases expression of LAT1, but the mechanism behind this upregulation remains to be elucidated (Sinclair et al., 2013).

Independently of this research, there could be also other causes for impaired glycolytic metabolism in MAIT cells, which should be considered. In obesity, the microenvironment of the cells is often dramatically altered (Fuster et al., 2016; Quail and Dannenberg, 2019). As obesity is closely related to the development of T2DM, in multiple cases, immune cells may be exposed to hyperglycaemia if the individual is unaware, they have diabetes, or their diabetes is badly controlled. Study by Hu *et al*. showed that the incubation of peripheral blood mononuclear cells in media with high glucose concentrations impairs their cytokine production, suggesting excessive nutrient concentration may impact immune cell function (Hu et al., 2018). It has been shown that individuals with obesity display elevated FFA concentration (Arner and Rydén, 2015; Madak-Erdogan et al., 2019; Ni et al., 2015). An excellent paper by Michelet *et al.,* revealed that obesity induced intracellular lipid accumulation in NK cells. Increased uptake of lipids led to serious impairment of their immunometabolism characterised by lower activation of mTORC1, defect in enhancement of glycolysis upon cell activation and production of cytotoxic granules and IFNγ production. This resembled a very similar phenotype to the one of MAIT cells described in this study. Therefore, the

impact of other nutrients on MAIT cell metabolism and function should also be investigated in the future.

As obesity is associated with an elevation in multiple pro-inflammatory factors, long-term exposure to these may impact MAIT cell function and metabolism. Leptin concentration in the peripheral blood has been shown to be elevated in people in obesity (Adeyemi and Abdulle, 2000; Considine et al., 1996; Farr et al., 2015). The consequences of high leptin are yet to be fully understood; however, leptin was shown to have pro-inflammatory properties on the cells of the immune system and modulate cell function (Wrann et al., 2012). A study by Wrann *et al*. has shown that short term incubation of NK in the presence of leptin increased their IFNγ secretion and cytotoxic capabilities, whereas long-term exposure of NK cells to leptin impaired their function (Wrann et al., 2012). This suggests that long term exposure to leptin could be altering immune cell metabolism, by inducing a defect in glycolysis and hence lead to lower IFNγ production.

Although our study provides a very valuable insight into MAIT cell function and metabolism it has limitations, which must be taken into consideration when interpreting these results. This research was performed using solely only human samples. Although primary cells are a wonderful resource, as they allow us to investigate the biology of MAIT cells directly from the cohort of interest, they introduce a large amount of variation, which can be observed in some of the data presented in this thesis. Although MAIT cells are readily detected in the peripheral blood, they are very enriched in tissues, hence the profile of these cells may alter based on their location in the body. This study uses only peripheral blood samples; therefore, these results can only be interpreted in the context of blood. Although our obesity cohort is very well characterized clinically, our control population is less well defined. Although the fitness of each blood donor is assessed before blood donation, it cannot be assumed that these donors don't have any underlying conditions or excessive weight, which could skew the data. Another point of note is the degree of obesity within our cohort, the mean BMI is 47 which is firmly in the morbid/severely obese category. Therefore, our conclusions around the impact of obesity may not reflect BMIs in the 30-40 range.

All of the experiments were performed *in vitro*, which poses another limitation to this study. All *in vitro* studies aim to resemble the *in vivo* environment as much as possible, for example, the incubation of cells at 37^{o}C or by providing them with an abundance of nutrients normally present in their environment. After conducting this study, I believe we should reconsider the culture media we use, especially for immunometabolism studies. Standard culture media contains 10mM (10mmol/L) glucose. This concentration of glucose in human blood would be considered as a hyperglycaemia, as normal fasting blood glucose is considered to be below 5.6 mmol/L (American Diabetes Association, 2020; WHO, 2020b). This means that all *in vitro* assays which investigate immune cell activation or in fact immunometabolism are performed in hyperglycaemic conditions, which we consider to be harmful *in vivo*. Immune restriction assays performed in this thesis, which used 10mM, 5mM and 1mM glucose concentrations, have shown that MAIT cell proliferation was not impacted at 5mM, which would resemble normal blood glucose. This suggests that 5mM concentration of glucose could be sufficient to support the activation of immune cells *in vitro*, providing the cells with conditions, which would be closer to their normal environment. It is important to consider than *in vivo*, under normal conditions, blood glucose is tightly controlled by the action of pancreas and insulin, keeping it at a constant concentration of around 5mM to maintain homeostasis. Setting up of the experiments in the media containing 5mM may lead to the deprivation of glucose during the experiment depending on the activity of the cells and glucose consumption rates and hence "hypoglycaemic" environment. More basic research should be performed to assess the most optimal conditions of culture, to obtain the most accurate results possible.

This study used primary human MAIT cells for all investigations. Murine models, especially genetically modified mice would help to conclusively validate our results. Unfortunately, mice display lower frequencies of MAIT cells than humans, suggesting they may not play as an essential role in their immunity as they do in humans (Chen et al., 2017; Kawachi et al., 2006; Rahimpour et al., 2015). Therefore, data obtained from mouse studies may not fully reflect the biology of MAIT cells in humans. In addition, mice display a homogenous population of subjects, which is a major difference between studies conducted on human samples, as each human is genetically different. This genetic variation is an

important factor, especially during development of drugs or treatments as well as the heterogenicity of the human population, may largely affect their responses. What's more, mouse studies are conducted in largely controlled environments, whereas humans cannot be controlled in such a way. The differences in the lifestyles of individuals, albeit introduces variations into the data, better reflects the studied cohorts. Mouse studies provide a very valuable tool when investigating biology of MAIT cells in the whole organism, however I believe studying MAIT cell metabolism and function in the primary cells provides an advantage over mouse models in this setting.

This study has advanced the knowledge of MAIT cell biology and function. Here we show that MAIT cell metabolism is an integral part of their activation. Stimulation of MAIT cells enhances their glycolytic metabolism, which is sensitive to amino acid influx via LAT1 amino acid transporters. Interruption of this metabolic pathway at any point, results in diminished IFNγ production as well as impaired expansion and proliferation of these cells. In this study we show that MAIT cells from people with obesity present with impaired glycolytic metabolism and effector functions, therefore we propose that a defect in glycolysis is responsible for the altered function of MAIT cells from obese individuals. This study describes a novel metabolic axis which controls MAIT cell function, which could be targeted in the future to restore MAIT cell function in people in obesity and other diseases which present with a similar MAIT cell phenotype.

Chapter Seven

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Appendix

Obesity Reduces mTORC1 Activity in Mucosal-Associated Invariant T Cells, Driving Defective Metabolic and Functional Responses

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Obesity underpins the development of numerous chronic diseases, such as type II diabetes mellitus. It is well established that obesity negatively alters immune cell frequencies and functions. Mucosal-associated invariant T (MAIT) cells are a population of innate T cells, which we have previously reported are dysregulated in obesity, with altered circulating and adipose tissue frequencies and a reduction in their IFN-γ production, which is a critical effector function of MAIT cells in host defense. Hence, there is increased urgency to characterize the key molecular mechanisms that drive MAIT cell effector functions and to identify those which are impaired in the obesity setting. In this study, we found that MAIT cells significantly upregulate their rates of glycolysis upon activation in an mTORC1-dependent manner, and this is essential for MAIT cell IFN-γ production. Furthermore, we show that mTORC1 activation is dependent on amino acid transport via SLC7A5. In obese patients, using RNA sequencing, Seahorse analysis, and a series of in vitro experiments, we demonstrate that MAIT cells isolated from obese adults display defective glycolytic metabolism, mTORC1 signaling, and SLC7A5 aa transport. Collectively, our data detail the intrinsic metabolic pathways controlling MAIT cell cytokine production and highlight mTORC1 as an important metabolic regulator that is impaired in obesity, leading to altered MAIT cell responses. *The Journal of Immunology*, **2019, 202: 3404–3411.**

Obesity is a global epidemic, impacting over 600 million adults and 150 million children (1). Obesity is linked to numerous chronic diseases, including type II diabetes, cardiovascular disease, and certain cancers (2). Underpinning the obesity-driven development of chronic disease is systemic inflammation and immune cell dysregulation (3, 4). We have previously reported the negative impact of obesity on several immune cell populations, including invariant NK T cells, NK cells, dendritic cells, and mucosal-associated invariant T (MAIT) cells (5–8).

MAIT cells are a population of non-MHC–restricted T cells that are important in the immune defense against bacterial and viral infections. MAIT cells are early-responding T cells that are capable of rapidly producing multiple cytokines upon activation, such as IFN-γ, TNF-α, and IL-17 (9). MAIT cells are activated when their invariant TCR recognize bacterial derivatives presented on the MHC-like molecule MR1 (9, 10). MAIT cells can also be activated in a TCR-independent manner via cytokine stimulation (11). Dysregulated MAIT cell cytokine profiles have been reported in several diseases, including obesity, arthritis, and viral

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A.O., R.M.L., M.M.P., L.M.T., N.A.W.W., F.C.T., R.B., and C.B. performed the experiments and carried out analysis and approved the final manuscript as submitted. A.M. recruited patients and controls. N.V. and G.S.B. provided MAIT cell reagents and aided in the design of MAIT cell activation experiments. G.S. and C.F. performed and analyzed the RNA sequencing. A.E.H., L.V.S., P.N.M., D.K.F., R.M.L., L.L., and D.O. conceptualized and designed the study, analyzed the data, drafted the manuscript, and approved the final manuscript as submitted.

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Abbreviations used in this article: BCH, 2-aminobicyclo- (2, 2, 1)-heptane-2-carboxylic acid; 2DG, 2-deoxyglucose; ECAR, extracellular acidification rate; FMO, flow minus-1; MAIT, mucosal-associated invariant T; OxPhos, oxidative phosphorylation; ps6, ribosomal S6 protein; RNA-Seq, RNA sequencing.

infection (8, 12–15). The pathways controlling MAIT cell cytokine responses are still under investigation.

It is now well established that cellular metabolism plays a critical role in determining immune cell responses (16). Extrinsic and intrinsic signals result in immune cell metabolic reprogramming, which provides both the energy and biological intermediates required for a robust immune response. Although different lymphocyte subsets can adopt distinct metabolic signatures, in general, effector lymphocytes are characterized by elevated glucose metabolism with high rates of glycolysis and oxidative phosphorylation (OxPhos) (16).

Currently, there is a paucity of data regarding the metabolic profile of MAIT cells or the importance of cellular metabolism in driving MAIT cell effector functions. In this study, we show that MAIT cells use glycolytic metabolism upon activation. Importantly, we show the requirement of this elevated rate of MAIT cell glycolysis for cytokine production. Using RNA sequencing (RNA-Seq) and Seahorse analysis, we show impaired glycolytic metabolism in MAIT cells from obese individuals. We show that MAIT cell glycolysis is dependent on mTORC1 activation, and in obesity mTORC1 activation is reduced. Finally, we show that mTORC1 activation in MAIT cells is dependent on amino acid transport via SLC7A5. Collectively, our data show for the first time, to our knowledge, the intrinsic metabolic pathways controlling MAIT cell cytokine production. Furthermore, we implicate a novel amino acid–dependent regulatory mechanism that is concurrent with impaired MAIT cell functions in obesity.

Materials and Methods

Study cohorts

A cohort of 30 obese adults (mean body mass index 45.1) was enrolled into this study, along with an age- and sex-matched cohort of healthy lean controls (mean body mass index 24.7) (Supplemental Table I). Exclusion criteria included patients with comorbid inflammatory conditions outside of type II diabetes. Patients who smoke or are on anti-inflammatory medications, GLP-1 therapies, or sodium-glucose cotransporter 2 inhibitors were also excluded.

Preparation of PBMCs and flow cytometric analysis

PBMC samples were isolated by density centrifugation from fresh venous blood samples. MAIT cell staining (1×10^6 PBMC) was performed using specific surface mAbs (all Miltenyi Biotec), namely, CD3, CD161, CD8, and TCRVα7.2 (Supplemental Fig. 3). Cell populations were acquired using a BD FACSCanto II and analyzed using FlowJo software (Tree Star). Results are expressed as a percentage of the parent population, as indicated, and determined using flow minus-1 (FMO) and unstained controls.

MAIT cell RNA-Seq and analysis

Highly pure MAIT cells were isolated from peripheral blood samples using FACS (MoFlo XDP Sorter). RNA was extracted from cells using the RNeasy Mini Isolation Kit (Qiagen) and analyzed for quality control and quantification purposes using the Agilent Bioanalyzer and RNA Pico assay (Agilent Technologies). RNA-Seq libraries were prepared using the SMART-Seq v4 Ultra Low Input RNA Kit (Clontech Laboratories), and next-generation sequencing was carried out using Illumina NextSeq 500 Single-End 75 bp (SE75) chemistry. Single-end RNA-Seq reads were aligned to the human hg38 reference genome using Tophat2 (17) and Bowtie (18) and the Ensembl gene annotation downloaded from the BioMart Web site. The Qlucore Omics Explorer 3.2 software package was used for visualization of RNA-Seq data. Qlucore Omics software converted imported bam files into read counts and then normalized these across the samples using the trimmed mean of M-values method (19). Principal component analysis was performed using the default setting: mean = 0, variance = 1 normalization based on 503 genes that passed filtering by variance set at the level of 0.25. Gene set enrichment analysis ranked genes based on the correlation between their expression, and the normalized enrichment score reflected the degree to which Hallmark gene sets from the Molecular Signatures Database were overrepresented from the ranked gene list (20). Differential gene expression analysis was implemented in the Qlucore, which performed a two-group comparison between lean and obese MAIT cells. Genes downregulated in obesity within the glycolysis and MTORC1 gene sets with a p value <0.05 and $\log_2$ fold change >1.5 were represented in the heat maps generated in Qlucore. https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE126169.

MAIT cell cytokine production analysis

MAIT cell cytokine production was determined by intracellular flow cytometry. Briefly, PBMC (1×10^6) were cultured in the presence of a protein transport inhibitor in media alone (control) or stimulated with either TCR microbeads (Miltenyi Biotec), IL-12, IL-18, IL-12/18 (all BioLegend), or a combination of TCR microbeads and IL-12/IL-18 for 18 h at 37°C. Additionally, metabolic inhibitors were added as indicated: rapamycin (20 nM), 2-deoxyglucose (2DG) (10 μM), oligomycin (2 μM), or 2-aminobicyclo- (2, 2, 1)-heptane-2-carboxylic acid (BCH) (50 mM). For acute glucose restriction experiments, PBMC were stimulated in media containing less than 1 mM glucose for 18 h, after which cells were investigated for intracellular cytokine production by flow cytometry, as previously described.

MAIT cell cytokine production by ELISpot

MAIT cell cytokine production was also determined by ELISpot. Briefly, 96-well ELISpot plate(s) were precoated with mAbs to IFN-γ overnight at 4°C. Plates were washed in PBS to remove coating Ab and incubated at 37°C for 1 h in complete RPMI. Previously isolated MAIT cells (2×10^5) were cultured on the precoated plates either basally or stimulated with IL-12/IL-18 and TCR microbeads at 37°C for 18 h. ELISpot plates were then washed with PBS/Tween for a minimum of six washes. Following this, 50 μl of biotinylated mouse anti-human IFN-γ was added and incubated at room temperature for 3 h. ELISpot plates were washed in wash buffer, and antibiotin-labeled Ab was added to the wells and incubated at room temperature for 2 h. Finally, prefiltered BCIP/NBT solution (50 μl) was added to each well and incubated at room temperature for 5 min or until distinct dark spots appeared in the positive-control wells. Plates were rinsed with tap water and allowed to air dry overnight. Cytokine expression was analyzed using a semiautomated ELISpot plate reader.

MAIT cell glucose consumption analysis

Fresh PBMC (1×10^6 ml) were activated as previously described for 18 h; cells were then washed and transferred to glucose-free media in the presence of the fluorescent glucose analogue 2NBDG (Life Technologies) for 2 h. Additionally, glut 1 expression was examined on basal and activated MAIT cells using an mAb specific for Glut-1 (R&D Systems). FMO and unstimulated PBMC were used as negative controls.

MAIT cell Seahorse analysis

For real-time analysis of the extracellular acidification rate (ECAR) and oxygen consumption rate of purified MAIT cells cultured under various conditions, a Seahorse XF-96 Analyzer (Seahorse Bioscience) was used. In brief, 200,000 purified, expanded MAIT cells were adhered to a CellTaq (BD Pharmingen)–coated 96-well XF Cell Culture Microplate (Seahorse Bioscience). Sequential measurements of ECAR and oxygen consumption rate following addition of the inhibitors (Sigma-Aldrich) oligomycin (2 μM), FCCP (0.5 μM), rotenone (100 nM) plus antimycin A (4 μM), and 2DG, (30 mM) allowed for the calculation of basal glycolysis, glycolytic capacity, basal OxPhos, and maximal mitochondrial capacity. Each cell culture condition was evaluated in quadruplicate, and 14 measurements were made per sample.

MAIT cell mTOR analysis

PBMC (1×10^6 ml) were activated as previously described for 18 h. mTOR activity in MAIT cells and $CD8^+$ T cells was examined via the phosphorylation of ribosomal S6 protein (pS6) (Cell Signaling Technology) by flow cytometry. Rapamycin treatment was used as a control for pS6 specificity and to determine the requirement of mTORC1 for IFN-γ production. FMO and unstimulated PBMC cells were used as negative controls.

MAIT cell amino acid uptake and BCH experiments

PBMC or MAIT cells were used to investigate kynurenine uptake as described in (21). Briefly, after surface Ab staining of samples, cells were resuspended in 200 ml of warmed HBSS (1×10^6 cells) and incubated in a water bath at 37°C. Kynurenine (200 mM in HBSS), BCH (20 mM in HBSS), and HBSS were warmed to 37°C and added as appropriate. Uptake was stopped after 4 min by PFA (final concentration 1%) for 30 min at room temperature in the dark. After fixation, cells were washed twice in

PBS/0.5% BSA and resuspend in PBS/0.5% BSA prior to acquisition on flow cytometer. The 405-nm laser and 450/50 BP filter were used for kynurenine fluorescence detection. For SLC7A5 inhibition experiments, the concentration of amino acids in RPMI 1640 was diluted two-fold using HBSS (Invitrogen) in the presence or absence of BCH (50 mM; Sigma-Aldrich).

Statistics

Statistical analysis was completed using GraphPad Prism 6 Software. Data are expressed as SEM. We determined differences between two groups using Student *t* test and Mann–Whitney *U* test where appropriate. Analysis across three or more groups was performed using ANOVA. 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.

Study approval

Ethical approval was granted by the Medical Research Ethics Committees at St. Vincent's University Hospital. All patients gave written informed consent prior to partaking in the study.

Results

Increased rates of glycolysis are essential for driving MAIT cell IFN-γ production

MAIT cells are potent producers of cytokine (IFN-γ and TNF-α among the most abundant). We show that in a stimulation-dependent manner, a large proportion of MAIT cells produce IFN-γ and TNF-α but not IL-17, with the most robust response seen after stimulation with anti-CD3/CD28–coated beads (TCR beads) and the cytokines IL-12/IL-18 (Fig. 1A, Supplemental Figs. 1A, 3). The metabolic pathways used by MAIT cells during their cytokine production are currently undefined. We show that MAIT cells express the major glucose transporter Glut-1 and upregulate the uptake of the fluorescent 2DG analogue 2NBDG upon activation in a stimulus-dependent manner (Fig. 1B–D). These data suggested that MAIT cells upregulate glucose metabolism. Therefore, we next performed detailed metabolic analysis using the Seahorse extracellular flux analyzer. Interestingly, we found MAIT cells specifically upregulate their rates of glycolysis (Fig. 1E, 1F), but not OxPhos (Supplemental Fig. 2B), upon activation. In addition, the glycolytic capacity, ECAR following oligomycin injection, of MAIT cells also increased significantly upon stimulation, which suggests that these cells are increasing the expression of the glycolytic machinery (Fig. 1E). Therefore, overall there was a pronounced shift toward glycolytic metabolism in activated MAIT cells. To assess if cellular glycolysis was required for cytokine production by MAIT cells, we used low doses of the specific glycolytic inhibitor 2DG. The data showed that MAIT cell IFN-γ production was significantly reduced when the rate of glycolysis was limited with 2DG (Fig. 1G, 1H). Interestingly, 2DG treatment had no impact on TNF-α production by MAIT cells (Fig. 1I). In contrast, inhibition of mitochondrial respiration with the ATP synthase inhibitor oligomycin did not reduce IFN-γ production by MAIT cells (Supplemental Fig. 2D, 2E) but did reduce TNF-α (Supplemental Fig. 2F). To further test the requirement for glucose metabolism in driving MAIT cell cytokine responses, we cultured MAIT cells under acute glucose restriction and showed a strong inhibition of IFN-γ, further

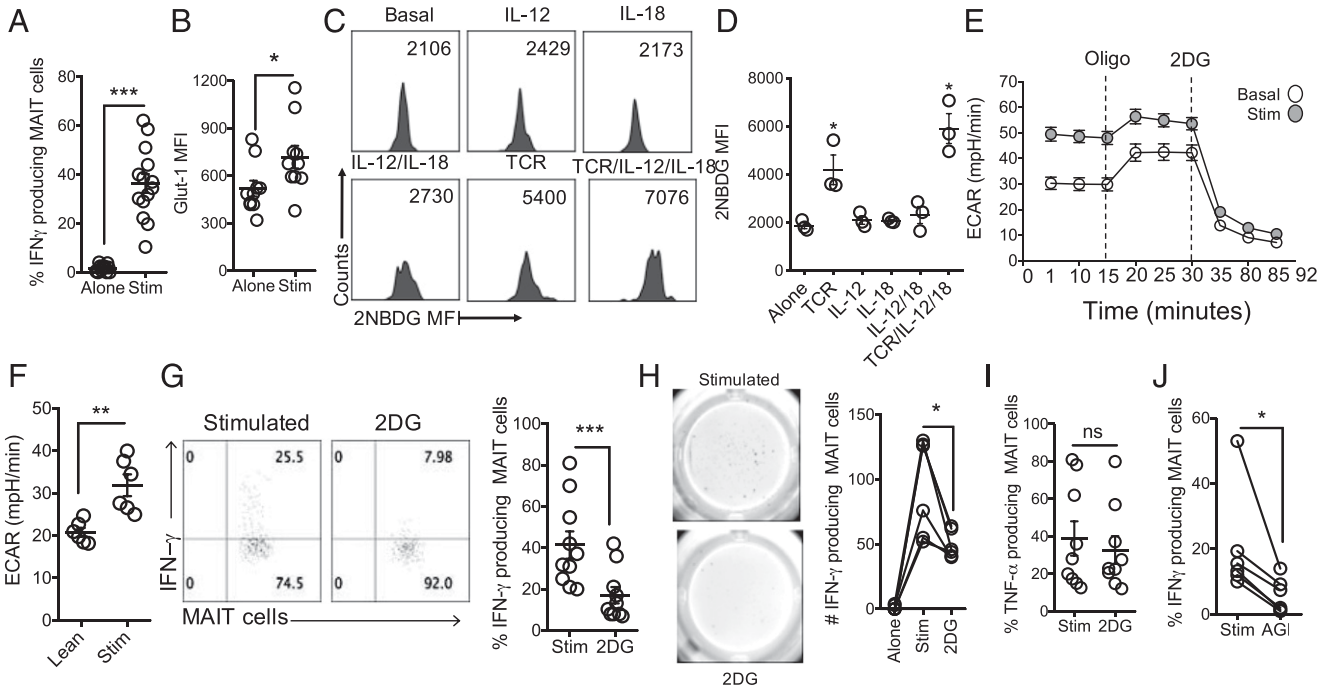

FIGURE 1. MAIT cells use glycolytic metabolism during IFN-γ production. (**A**) Scatter plot showing MAIT cell production of IFN-γ in a cohort of lean adults (unstimulated versus stimulated with TCR microbead/IL-12/IL-18). (**B**) Scatter plot showing Glut-1 expression (MFI) on either basal or stimulated MAIT cells from lean adults ($n = 10$). (**C** and **D**) Representative histograms and scatter plot showing 2NBDG uptake by MAIT cells stimulated with either TCR microbeads, IL-12, IL-18, both IL-12 and IL-18, or TCR microbeads and IL-12/IL-18 ($n = 3$). (**E** and **F**) Scatter plot and representative Seahorse traces displaying MAIT cells ECAR rates in either basal (open circles) or stimulated (gray circles) MAIT cells from lean adults. Data representative of six independent experiments. (**G**) Representative flow cytometry dot plots and scatter plot displaying the effect of 2DG treatment on IFN-γ production by stimulated MAIT cells ($n = 10$). (**H**) Representative ELISpot wells and scatter plot displaying the effect of 2DG treatment (bottom panel) on IFN-γ production by stimulated MAIT cells ($n = 5$). (**I**) Scatter plot displaying the effect of 2DG treatment on TNF-α production by stimulated MAIT cells ($n = 10$). (**J**) Scatter plot showing impact of acute glucose restriction (AGR) on IFN-γ production by stimulated MAIT cells from lean adults ($n = 5$). Data representative of five independent experiments. Statistical comparisons using Student *t* test or ANOVA. Data representative of a minimum of 10 independent experiments unless otherwise stated. Error bars represent SEM. $*p < 0.05$, $**p \leq 0.01$, $***p < 0.001$. MFI, mean fluorescence intensity.

supporting our findings that MAIT cell IFN-γ production is dependent on glycolytic metabolism (Fig. 1J).

Defective IFN-γ production by MAIT cells in obesity parallels defective cellular metabolism

We have previously reported defective MAIT cells in obese individuals, including impaired IFN-γ production, which we confirm in an expanded cohort of obese adults (Fig. 2A). Interestingly, in our obese cohort, we did not observe defective $CD8^+$ T cell IFN-γ production (Fig. 2B). To investigate the mechanism(s) driving defective IFN-γ production by MAIT cells in obesity, we performed RNA-Seq of MAIT cells isolated from five lean and four obese individuals. Normal enrichment score analysis highlighted a reduction in the glycolytic metabolism pathway (Fig. 2C). To investigate the significance of the reduced glycolytic metabolism in obesity, Glut-1 expression and 2NBDG uptake assays were performed. The data showed that MAIT cells from obese adults show no significant difference in Glut-1 expression (Fig. 2D). However, there was a significant decrease in the uptake of 2NBDG in stimulated MAIT cells from obese individuals when compared with healthy adults (Fig. 2E). Detailed metabolic analysis was used to confirm the alterations in MAIT cell glycolytic metabolism between healthy and obese MAIT cells. In line with the reduced glycolytic gene expression and glucose uptake, activated MAIT cells from obese adults do not increase their rate of glycolysis (Fig. 2F, 2G). Indeed, MAIT cells from obese adults show reduced glycolytic capacity when compared with lean adults (Fig. 2H). Furthermore, acute glucose restriction had no additional impact/reduction on IFN-γ production in MAIT cells from obese individuals in comparison with the significant effect observed in MAIT cells from lean individuals (Fig. 2I). These data confirm that MAIT cells from obese adults are not performing increased rates of glycolysis during their activation.

mTORC1 activity is essential for driving MAIT cell glycolysis and is reduced in obesity

The signaling complex mTORC1 acts as both a nutrient sensor and a central metabolic regulator of effector immune cells. Studies have shown that mTORC1 is intrinsically linked with glycolytic metabolism in effector lymphocytes (22, 23). Strikingly, our RNA-Seq data revealed defective mTORC1 signaling. (Fig. 3A). To investigate a role for mTORC1 in driving MAIT cell metabolism, we measured mTORC1 activity in MAIT cells upon stimulation and demonstrate a robust increase as measured via the phosphorylation of pS6 (Fig. 3B, 3C). Importantly, these elevated levels of pS6 were prevented by the mTORC1 inhibitor rapamycin, demonstrating the specificity of this assay for mTORC1 signaling (Fig. 3D). We next investigated if mTORC1 signaling is required for increased rates of glycolysis in activated MAIT cells. Indeed, MAIT cells stimulated in the presence of rapamycin had significantly lower levels of glycolysis (Fig. 3E, 3F). As expected, rapamycin treatment had no significant impact on the ECAR of MAIT cells

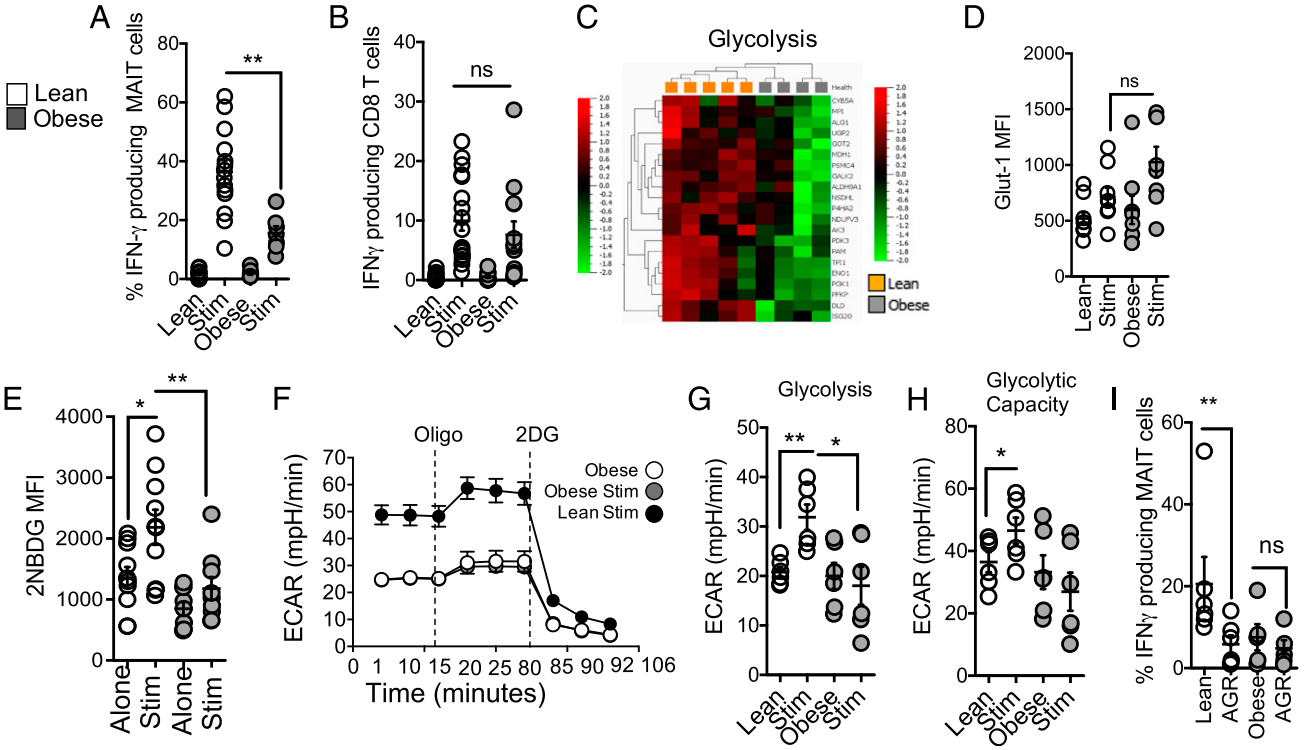

FIGURE 2. MAIT cells from obese adults display defective glycolytic metabolism. (**A**) Scatter plot displaying MAIT cell production of IFN-γ in a cohort of lean (white circles) and obese (gray circles) adult (unstimulated versus stimulated with TCR microbead/IL-12/IL-18) ($n = 15$). (**B**) Scatter plot displaying CD8 T cell production of IFN-γ in a cohort of lean (white circles) and obese (gray circles) adult (unstimulated versus stimulated with TCR microbead/IL-12/IL-18) ($n = 15$). (**C**) Heat map depicting differentially downregulated genes (p value < 0.05) within the glycolysis gene set in MAIT cells isolated from lean and obese donors ($n = 5$ and 4, respectively). (**D**) Scatter plot showing Glut-1 MFI on either basal or stimulated MAIT cells from lean (white circles) or obese (gray circles) donors ($n = 10$). (**E**) Scatter plot showing 2NBDG MFI on either basal or stimulated MAIT cells from lean (white circles) or obese (gray circles) donors. (**F**) Representative Seahorse trace displaying MAIT cell ECAR rates in either basal (open circles) or stimulated (gray circles) MAIT cells from an obese adult with representative stimulated lean donor (black circles). (**G** and **H**) Scatter plot displaying glycolysis levels (ECAR) or glycolytic capacity (ECAR post–oligomycin treatment) from lean (white circles) or obese (gray circles) donors ($n = 6$). (**I**) Scatter plot showing impact of acute glucose restriction (AGR) on IFN-γ production by stimulated MAIT cells from lean (white circles) or obese (gray circles) adults. Data representative of a minimum of five independent experiments unless otherwise stated. Statistical comparisons using Student t test or ANOVA. Error bars represent SEM. $*p < 0.05$, $**p \leq 0.01$. MFI, mean fluorescence intensity.

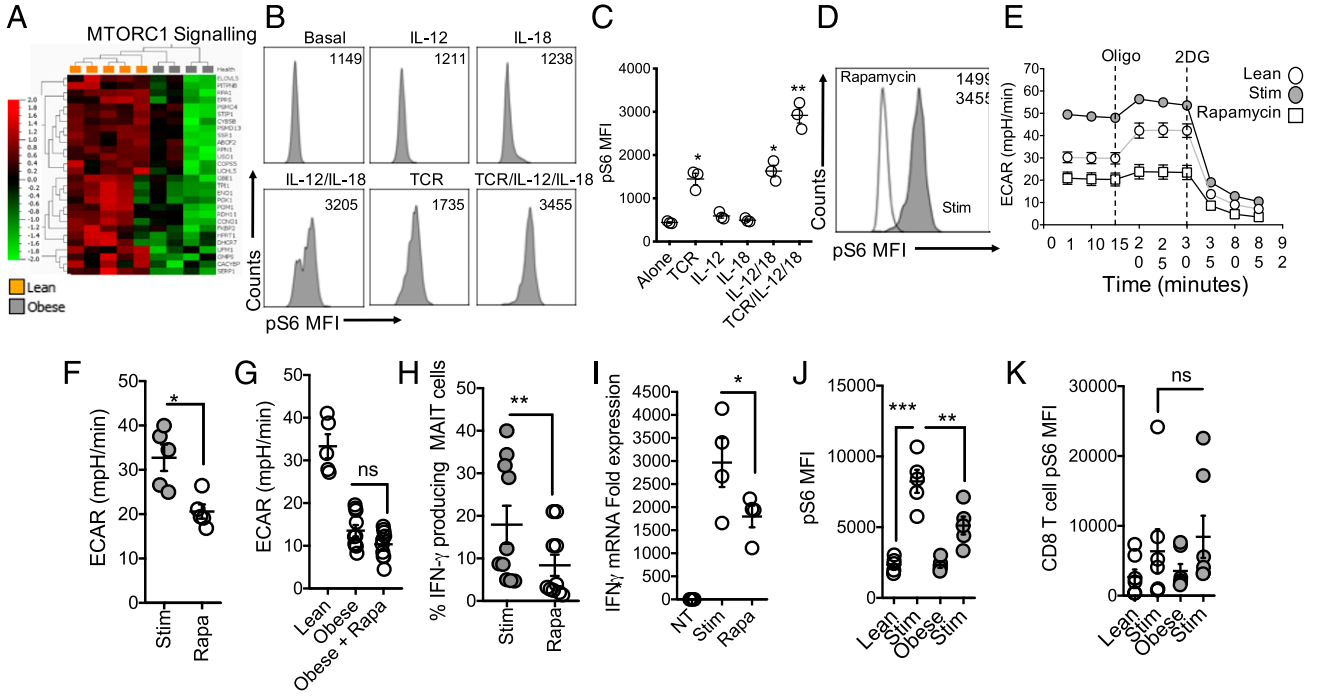

FIGURE 3. MAIT cell glycolytic metabolism is dependent on mTORC1 activation, which displays defects in obesity. (**A**) Heat map depicting differentially downregulated genes (p value < 0.05) within the mTORC1 gene set in MAIT cells isolated from lean and obese donors ($n = 5$ and 4, respectively). (**B** and **C**) Representative histogram and scatter plot showing the activation of mTORC1 (phosphorylation of S6) in MAIT cells stimulated with either TCR microbeads, IL-12, IL-18, both IL-12 and IL-18, or TCR microbeads, IL-12 and IL-18 ($n = 3$). (**D**) Representative histogram showing the inhibition of S6 phosphorylation by rapamycin in stimulated MAIT cells. (**E**) Representative Seahorse traces displaying MAIT cell ECAR rates in either basal (open circles), stimulated (gray circles), or rapamycin-treated (open squares) in MAIT cells from lean donors. (**F**) Scatter plot displaying ECAR levels in stimulated (gray circles) or rapamycin-treated (white circles) MAIT cells from lean donors. (**G**) Scatter plot detailing effect of rapamycin on ECAR rates in stimulated obese MAIT cells. (**H**) Scatter plot detailing effect of rapamycin on IFN-γ production by stimulated MAIT cells from lean donors. (**I**) Scatter plot detailing effect of rapamycin on IFN-γ mRNA in MAIT cells from lean donors. (**J**) Scatter plot showing pS6 MFI in either basal or stimulated MAIT cells from lean (white circles) or obese (gray circles) donors. (**K**) Scatter plot showing pS6 MFI in either basal or stimulated CD8 T cells from lean (white circles) or obese (gray circles) donors. Data representative of a minimum of five independent experiments unless otherwise stated. Statistical comparisons using Student t test or ANOVA. Error bars represent SEM. $*p < 0.05$, $**p \leq 0.01$, $***p < 0.001$.

from obese donors (Fig. 3G). We show that rapamycin treatment of activated MAIT cells from lean individuals inhibits IFN-γ, and this regulation occurs at an mRNA level (Fig. 3H, 3I). Together, these data demonstrate that mTORC1 is required for metabolic and functional MAIT cell responses. Indeed, the data show that activation-induced mTORC1 activity was significantly reduced in MAIT cells from obese adults (Fig. 3J). Similar to $CD8^+$ T cell IFN-γ production, we observed no difference in $CD8^+$ T cell mTORC1 activity between lean and obese donors (Fig. 3K). Collectively, these data highlight an important role for mTORC1 signaling in MAIT cell metabolism and highlight obesity-associated defects in mTORC1 as a possible driver of defective MAIT cell function.

mTORC1 activity is dependent on amino acid flux via SLC7A5, which is diminished in obesity

The data thus far highlight a crucial role for mTORC1 signaling in controlling MAIT cell metabolism and function. We next investigated the upstream regulators of mTOR activity in MAIT cells in the hopes that it may shed light on the cause behind the defective mTORC1 signaling observed in obese MAIT cells. Previous studies have highlighted the importance of amino acid transport through the system L amino acid transporter, SLC7A5, in controlling mTOR activity in primary T cells (24). To assess whether amino acid transport controls mTORC1 activity in MAIT cells, we first investigated amino acid transport in MAIT cells using a recently described flow cytometric–based assay (21). We show that MAIT cells readily uptake exogenous amino acids (as determined by active transport of the fluorescent system L transporter substrate kynurenine) (Fig. 4A, 4B). Furthermore, we show that treatment with the system L amino acid transport inhibitor BCH reduced kynurenine uptake by MAIT cells (Fig. 4C, 4D). We next investigated if MAIT cells from obese donors had altered amino acid uptake. Interestingly, we observed a significant decrease in uptake through system L transport compared with MAIT cells from lean donors (Fig. 4E, 4F). To investigate this reduction, we measured the expression of the predominant system L amino acid transporter expressed in T cells, SLC7A5, and show reduced mRNA expression in obese donors (Fig. 4G). Finally, using BCH treatment, we investigated the impact of this diminished amino acid uptake on mTORC1 activity and glycolytic metabolism in MAIT cells from healthy donors. We observed reduced mTORC1 activation and strong abrogation of glycolytic metabolism with BCH (Fig. 4H–K). As expected, we observed reduced IFN-γ production by MAIT cells treated with BCH (Fig. 4L). These data implicate for the first time, to our knowledge, a crucial role for amino acid availability in regulating MAIT cell metabolism and function in obesity.

Discussion

In this study, we show definitively that MAIT cells increase their rates of glucose uptake and glycolysis upon activation. We also show that this increased rate of MAIT cell glycolysis is essential for driving IFN-γ production. Furthermore, we identify mTORC1 as a key metabolic regulator that controls the rate of MAIT cell

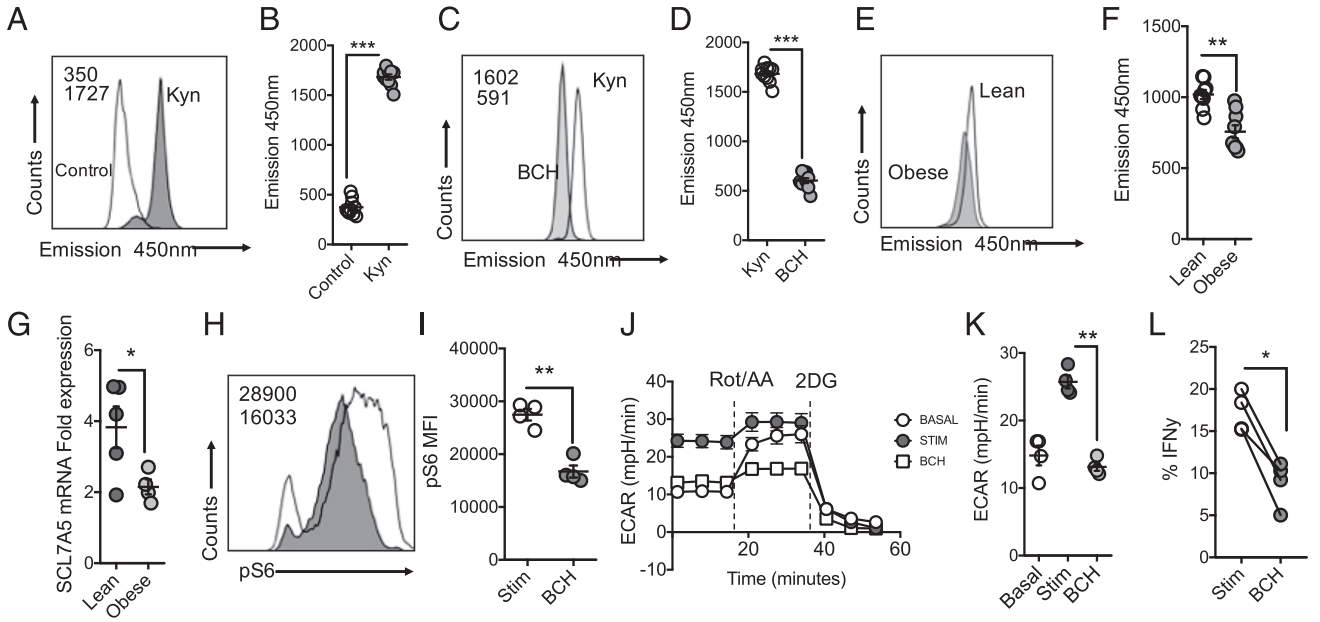

FIGURE 4. mTORC1 activation in MAIT cells is dependent on amino acid uptake via SLC7A5, which is defective in obesity. (**A** and **B**) Representative histogram and scatter plot showing Kynurenine (Kyn) uptake by MAIT cells compared with HBSS control (no Kyn) ($n = 10$). (**C** and **D**) Representative histogram and scatter plot showing the effect of BCH (50 mM) on Kyn uptake by MAIT cells ($n = 10$). (**E** and **F**) Representative histogram and scatter plot showing Kyn uptake by MAIT cells from a lean (open histogram) or obese donor (gray histogram) ($n = 10$). (**G**) Scatter plot showing SLC7A5 mRNA expression in MAIT cells from lean or obese donors ($n = 5$). (**H** and **I**) Representative histogram and scatter plot showing the effect of BCH (50 mM) on the activation of mTORC1 (pS6) in TCR-/IL-12–/IL-18–activated MAIT cells ($n = 4$). (**J** and **K**) Representative Seahorse trace and scatter plot showing the effect of BCH (50 mM) on the ECAR of TCR-/IL-12–/IL-18–activated MAIT cells ($n = 4$). (**L**) Scatter plot showing the effect of BCH (50 mM) on IFN-γ production by TCR-/IL-12–/IL-18–activated MAIT cells ($n = 4$). Data representative of a minimum of five independent experiments unless otherwise stated. Statistical comparisons using Student *t* test or ANOVA. Error bars represent SEM. $*p < 0.05$, $**p \leq 0.01$, $***p < 0.001$.

metabolism and thereby effector molecule production. We show that mTORC1 activation is under the control of amino acid uptake by the cell and expression of the system L amino acid transporter, SLC7A5. We previously reported that MAIT cells isolated from obese individuals are functionally defective. To understand the mechanisms at play, we used RNA-Seq to identify alterations in the gene expression of MAIT cells isolated from obese individuals. These analyses highlighted alterations in both glycolytic and mTORC1 pathways in MAIT cells isolated from obese individuals. Indeed, we confirm our sequencing data by showing reduced rates of glycolysis and mTORC1 activity in MAIT cells from obese adults, which parallel the loss of IFN-γ production reported in obesity (8). Finally, we provide evidence that defects in amino acid transport contribute to the described defective MAIT cell metabolism in obese patients.

MAIT cells are potent effector T cells, capable of rapidly producing multiple cytokines (9). During viral infections, activation of the MAIT cell is independent of the TCR but dependent on IL-18. Data from Slichter and colleagues (11) show that MAIT cells respond weakly to TCR stimulation in the absence of secondary stimulation by cytokine. Our data support this finding, showing that MAIT cells respond most strongly when stimulated via the TCR and cytokine receptors, specifically IL-18 and IL-12.

The pathways regulating MAIT cell cytokine production are poorly understood; however, a recent study by Zinser and colleagues (25) provided evidence that MAIT cell production of granzyme B was dependent on glycolytic metabolism. In other effector lymphocytes, it is well established that the cytokine profiles are regulated by intrinsic cellular metabolism (26–28). Activated T cells have been shown to have a large glucose requirement, especially Th 1 and 17 subsets along with $CD8^+$ cells (27). We show that MAIT cells express the glucose transporter Glut-1 and upon cytokine stimulation increase their uptake of the fluorescent 2DG analogue 2NBDG. Like the cytokine responses, MAIT cells increased 2NBDG uptake with TCR stimulation; however, the greatest increases in glucose uptake were observed with TCR–cytokine costimulation. This is in keeping with the requirement for greater amounts of energy and biological intermediates following TCR/cytokine stimulation.

Studies in effector populations such as $CD8^+$ T cells and NK cells have demonstrated that the cytokine production by these cells is dependent on glycolytic metabolism (20, 27). We investigated the metabolic pathways used by MAIT cells using the Seahorse extracellular flux analyzer and show that MAIT cells are glycolytic cells, increasing their rates of glycolysis upon activation. Interestingly, in contrast to NK cells, MAIT cells do not display parallel increases in OxPhos (28). To link MAIT cell glycolytic metabolism to cytokine production, we used the glycolytic pathway inhibitor 2DG and show that at low doses it strongly inhibits MAIT cell production of IFN-γ. We also show that acute restriction of glucose during MAIT cell activation significantly impacted their activation, confirming their glycolytic requirements. Interestingly, 2DG treatment did not impact TNF-α production by MAIT cells, whereas treatment with oligomycin reduced TNF-α production but not IFN-γ. Previous studies in macrophages have described the differential regulation of TNF-α, showing that 2DG blocks IL-1β but not TNF-α. This suggests differential metabolic regulation of TNF-α production in MAIT cells.

We and others have previously reported that MAIT cells are defective in obese adults, although the mechanism(s) underpinning their dysregulation are unknown (8, 29). To elucidate the mechanisms driving the alterations in cytokine production, we performed RNA-Seq of MAIT cells isolated from lean and obese adults. We observed alterations in the expression over 400 genes between lean and obese donors, and principal component analysis

separated MAIT cells isolated from lean donors from MAIT cells isolated from obese donors. Pathway analysis revealed changes in several cellular metabolism pathways, including the downregulation of genes associated with glycolytic metabolism in MAIT cells isolated from obese individuals. To confirm our sequencing data, we performed a series of experiments, which show for the first time, to our knowledge, that glycolytic metabolism is defective in MAIT cells isolated from obese adults. This parallels our recent study showing defective glycolytic metabolism in NK cells from obese adults (30). In contrast to NK cells and the MAIT data from obese adults presented in this study, we previously reported that in a cohort of obese children there was altered NK cell metabolism with elevated rates of glycolysis, which may suggest that chronic activation of glycolytic machinery in childhood obesity results in a failure later in life (31).

The mTOR complex is highlighted both as an important signaling complex and regulator of metabolism in immune populations such as $CD8^+$ T cells and NK cells (20, 26, 32). Our data identify mTORC1 as a key regulator of MAIT cell metabolism. In this study, we show that MAIT cells increase mTORC1 activity upon activation, which is essential for the elevated levels of MAIT cell metabolism upon activation. Finally, our sequencing data highlighted reductions in mTORC1 signaling in MAIT cells isolated from obese individuals. We show diminished mTORC1 activation in MAIT cells but not classical $CD8^+$ T cells isolated from obese individuals as compared with lean controls. Previous studies show that inhibition of mTORC1 with rapamycin results in reduced effector immune cell functions (20, 33). Indeed, inhibition of mTORC1 activation with rapamycin inhibited IFN-γ production, which is in line with reduced rate of glycolysis observed in rapamycin-treated MAIT cells and the requirement of glycolysis for MAIT cell IFN-γ production. These findings contribute to our understanding as to why MAIT cell functional responses are impaired in obesity.

To investigate why mTOR activity is diminished in MAIT cells from obese donors, we investigated the upstream regulators of mTOR. Previous studies by our collaborators have extensively shown that AKT is dispensable for T cell metabolism and NK cell metabolism but highlight amino acid transport as an important regulator of mTORC1 activity (24, 26, 34, 35). To this end, we show that MAIT cell activation of mTORC1 and subsequent upregulation of glycolytic metabolism and IFN-γ production is dependent on amino acid transport into the cell. We also show that MAIT cells from obese donors have reduced expression of the system L amino acid transporter SLC7A5 and thus reduced amino acid uptake. Numerous studies have highlighted the dysregulation of obesity on amino acid metabolism, in particular branched chained amino acids such as leucine, which is the candidate amino acid implicated in the activation of mTOR in T cells (36–41).

Collectively, our data show that MAIT cells use glycolytic metabolism during their production of IFN-γ and that this process is regulated by mTORC1, which in turn is dependent on amino acid flux into the cell. In obesity, RNA-Seq of MAIT cells revealed downregulation of genes involved in both glycolytic metabolism and mTORC1 signaling. We confirmed these alterations using a series of in vitro experiments. These findings provide a mechanism for the reported alterations in MAIT cell IFN-γ production in obesity and may help us to understand the alterations of MAIT cells in other human diseases such as hepatitis C virus, malignant myeloma, and colorectal cancer (42–44). Finally, understanding the metabolic pathways controlling MAIT cell activation may highlight novel targets for controlling their functions in disease (45).

Disclosures

The authors have no financial conflicts of interest.

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HIGHLIGHTS

REVIEW

Interleukin-17 producing mucosal associated invariant T cells - emerging players in chronic inflammatory diseases?

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Mucosal associated invariant T (MAIT) cells are a population of evolutionarily conserved T cells, which express an invariant T cell receptor (TCR) and represent a significant subset of innate-like T cells in humans, yet their role in immunity is still emerging. Unlike conventional αβ T cells, MAIT cells are not restricted by MHC molecules, but instead uniquely recognize microbially derived vitamin metabolites presented by the MHC-I like molecule MR1. MAIT cells are enriched in mucosal sites and tissues including liver and adipose tissue where they are thought to play an important role in immunosurveillance and immunity against microbial infection. In addition to their putative role in antimicrobial immunity, recent research on MAIT cells, in particular IL-17 producing MAIT cells, has demonstrated their involvement in numerous chronic inflammatory conditions. In this review, we give an overview of the work to date on the function and subsets of MAIT cells. We also examine the role of IL-17 producing MAIT cells in chronic inflammatory diseases ranging from autoimmune conditions, metabolic diseases to cancer. Furthermore, we discuss the most recent findings from the clinic that might help deepen our understanding about the biology of MAIT cells.

Keywords: chronic inflammation · IL-17 · mucosal-associated invariant T cells

Mucosal-associated invariant T cells

Mucosal associated invariant T (MAIT) cells comprise a unique population of evolutionarily conserved T cells, first described in humans by Porcelli and colleagues in the early 1990s, what was further confirmed by another landmark study by Tilloy et al. in 1999 [1, 2]. These cells are characterized by expression of the invariant TCR-α chain, Vα7.2 (TRAV1-2-TRAJ33, TRAJ20, or TRAJ12) in human, or Vα19 (TRAV1-TRAJ33) in mice. This TCR chain is typically associated with a limited array of TCR-β chains, e.g., Vβ2 or Vβ13 in human, or Vβ6 and Vβ8 in mice [3]. This constrained gene usage of the TCR suggests the ability of these cells to target conserved antigens. MAIT cell antigen recognition is not restricted by MHC, instead the MHC class

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I-like molecule (MR1) is recognized by the MAIT TCR [3–5]. The MR1 complex typically presents metabolites derived from the biosynthesis of riboflavin (vitamin B2), a process mediated by many bacterial and yeast species, but not viruses or human cells [6]. This observation is consistent with the finding that MAIT cells are activated by riboflavin synthesizing bacteria, such as *Salmonella* and other *Enterobactericaeae* species [7]. In vitro experiments have identified a number of these riboflavin metabolites as MAIT cell antigens, including stimulatory antigens 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU), 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU), 6,7-dimethyl-8-D-ribityllumazine (RL-6,7-diMe), and 7-hydroxy-6-methyl-8-D-ribityllumazine (RL-6-Me-7-OH) [8]. The potency of these antigens varies, with a non-stimulatory MR1 ligand 6-formyl-pterin (6-FP), a natural breakdown by-product of folic acid, also described [6]. The most potent of these stimulating antigens are 5-OP-RU and 5-OE-RU, which are both generated from the riboflavin precursor 5-amino-6-D-ribitylaminouracil (5-A-RU) through non-enzymatic condensation with small ubiquitous metabolites methylglyoxal and glyoxal, respectively [8, 9]. Identification of these potent antigens has allowed for development of highly specific antigen-loaded MR1 tetramers, for improved identification of both human and mouse MAIT cells [10–13].

In humans, MAIT cells are detectable in cord blood and frequencies increase into adulthood before decreasing after the sixth decade of life [14, 15]. MAIT cells are widely distributed throughout the human body, comprising a substantial proportion of T cells in blood (1–10%), intestinal tissues [16, 17], lungs [18], skin [19], adipose tissue [20], and in particular the liver, where MAIT cells can account for up to 50% of all T cells [21]. In a study of human fetal tissues, MAIT cells were found to be rare in fetal thymus, spleen, and mesenteric lymph nodes but enriched in small intestine, liver, and lung [22]. MAIT cell development proceeds in a step-wise process, where thymic selection precedes peripheral expansion and is dependent on commensal microbes and MR1 expressing B cells [23, 24]. Subsequent studies showed that MAIT cell selecting thymocytes express MR1 and both CD4 and CD8, and that MR1 deficient mice lack MAIT cells [25]. Elaborate studies using MR1 loaded tetramers in mice showed that the MAIT cell compartment is composed of three prominent populations, defined by expression of CD24 and CD44 [26]. Of these populations, the $CD24^{-}/CD44^{+}$ subset was the only functionally active population and could be further subdivided into two distinct subsets of $Tbet^{+}$ and $ROR\gamma t^{+}$ MAIT cells. In humans, the stages of thymic maturation of MAIT cells were differentiated by CD27 and CD161 expression. Stage 1 was defined by lack of expression of CD161 and CD27, at stage 2 cells remained $CD161^{-}$ but also gained expression of CD27, whereas at the most mature stage of thymic development MAIT cells were defined as $CD161^{+}$ and $CD27^{pos\text{-}lo}$. In contrast to the stage 3 of murine MAIT cell maturation, human MAIT cells were observed to co-express $Tbet^{+}$ and $ROR\gamma t^{+}$. Upon in vitro stimulation, only a small subset of stage 3 thymic MAIT cells responded by cytokine production in comparison to the stage 3 MAIT cells found in blood, suggesting that further maturation of these cells takes place outside of the thymus [24].

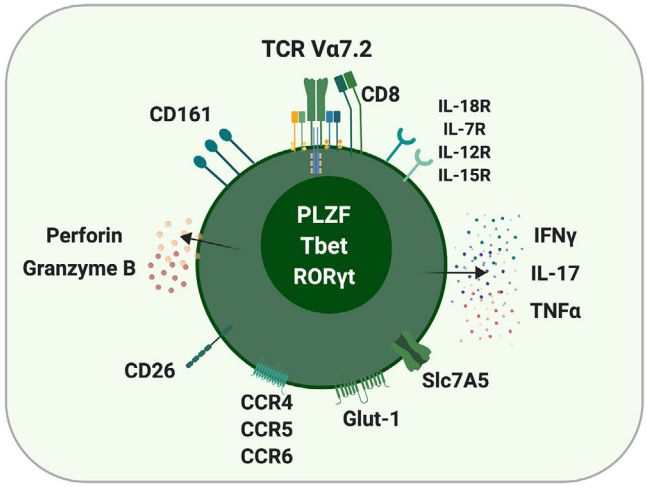

Figure 1. MAIT cell phenotype. In addition to their expression of the invariant TCRVα7.2, MAIT cells can be defined by their expression of several surface molecules including CD161 and CD26. MAIT cells also express several cytokine and chemokine receptors on their surface, including IL-18R and CCR6. Also expressed on the surface of MAIT cells are the nutrient transporters GLUT-1 and SLC7A5, two key transporters in MAIT cell metabolism. MAIT cells can be further characterized by their expression of transcription factors such as PLZF, Tbet, and RORγt, which control the production of cytokines such as IFN-γ and IL-17 during activation. Finally, MAIT cells can also express lytic molecules including perforin and granzyme B.

MAIT cell phenotype and functions

Like other so-called unconventional T cells, such as γδ T cells and invariant NK T (iNKT) cells, MAIT cells possess attributes of both innate and adaptive immunity, e.g., rapid effector function upon activation by conserved ligands and recognized by a classical, albeit semi-invariant, TCR. Human MAIT cells are classically defined by expression of an invariant Vα7.2 TCR chain and high levels of the C-type lectin CD161 [3, 7]. MAIT cells can express CD8, either CD8αα or CD8αβ isoforms, CD4 or can be double negative for both CD4 and CD8, with CD8 expressing MAIT cells representing the majority [27, 28]. The $CD4^{-}/CD8^{-}$ MAIT cell population has recently been described as a functionally distinct MAIT cell subset, producing higher levels of IL-17 and lower levels of IFN-γ [28]. Consistent with their presence in numerous tissue types, MAIT cells express several tissue homing molecules, including CCR5, CCR6, and CXCR6 [29]. MAIT cells also express multiple cytokine receptors, including IL-7R, IL-12R, and IL-18R [7, 30–32]. Several studies have outlined a requirement for cytokine priming or stimulation for full MAIT cell activation, in particular by cytokines IL-7, IL-12, and IL-18 [30, 33] (Fig. 1).

Similar to other unconventional T cell subsets, MAIT cells can be activated independently of their TCR, specifically by cytokines alone [34–36]. This means that MAIT cell activation is not solely reliant on TCR ligation alone, which results in a relatively weak

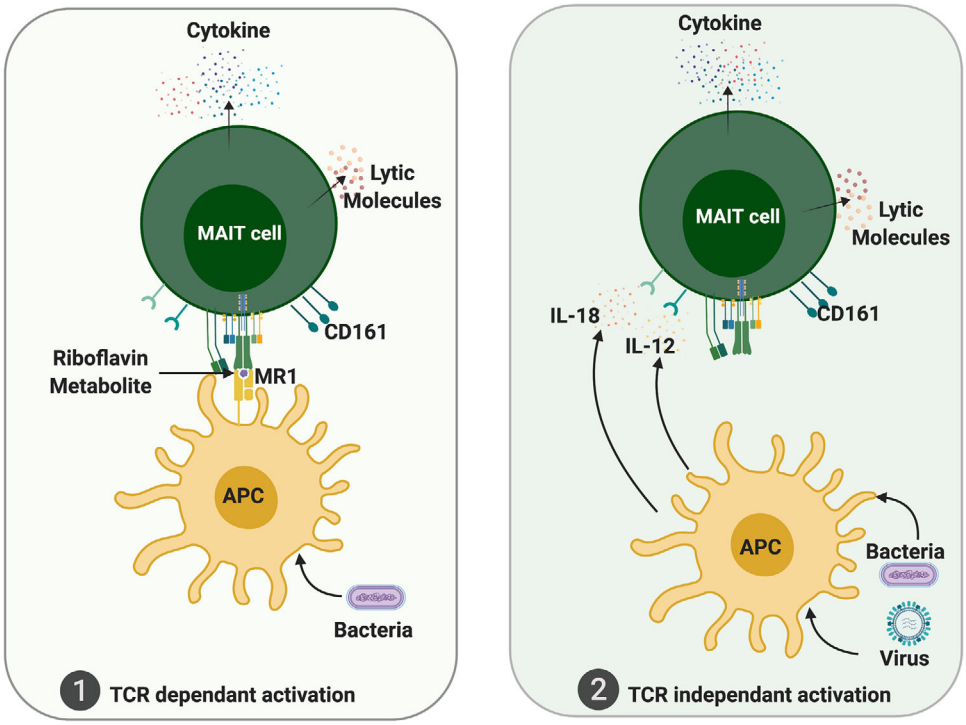

Figure 2. MAIT cell activation. MAIT cells can be activated in a MR1 dependent manner (1) where bacterial ligands are presented by antigen presenting cells (APC) on MR1 and recognized by the invariant Vα7.2 TCR resulting in the production of cytokine and lytic molecules. MAIT cells can also be activated in a MR1 independent manner (2) by inflammatory cytokine such as IL-12 and IL-18 that are produced by innate cells in the presence of viral or bacterial infection, again resulting in the production of effector molecules.

response. Therefore, these cells can respond to a wider set of stimuli than MR1 presented antigens alone, and in a synergistic manner where multiple signals are present [34, 36, 37] (Fig. 2).

Upon activation, MAIT cells can produce multiple cytokines and can be generally divided into MAIT cells that primarily produce Th1 type cytokines including IFN-γ and TNF-α (MAIT1 cells), and MAIT cells that predominantly produce Th17 type cytokines such as IL-17A (MAIT17 cells) [20, 29, 38, 39]. In the human protein atlas, it was noted that MAIT cells expressed among the highest levels of the protein coding gene RORC, which is the transcription factor responsible for IL-17 production [40]. Several other transcription factors have been detailed in MAIT cells including PLZF and Tbet [41]. In addition to rapid cytokine production, MAIT cells possess a full complement of cytolytic effector molecules such as granzymes (granzyme A and B) and perforin, which allow them to lyse bacteria-infected cells [42]. Upon activation, MAIT cells rapidly display this cytotoxic phenotype, which is heightened through TCR interactions and cytokines. The cytotoxic ability of these cells is tightly regulated however, and is ultimately dependent on MR1 ligation, which allows control of bacterial infection while reducing immune-mediated pathology [42]. Numerous recent studies have highlighted the importance of intrinsic metabolic pathways in controlling immune cell functions [43]. We have recently reported on the metabolic pathways controlling MAIT cell cytokine production [38]. Similar to $CD8^+$ T cells and NK cells, MAIT cells increase their rate of glycolysis upon activation, which provides the biological intermediates needed for effector molecule production [38, 44]. MAIT cells express the major glucose transporter GLUT-1 and neutral amino acid transporter SLC7A5 upon stimulation increase their nutrient uptake [38]. Inhibition of glycolysis in MAIT cells reduced the capacity to produce IFN-γ and granzyme B, highlighting the importance of metabolism in MAIT cell effector function [38, 44]. Mammalian target of rapamycin (mTOR) is a well-characterized regulator of glycolysis in effector cells such as NK cells [45]. Upon activation, MAIT cells increase signaling through mTOR and inhibition with rapamycin results in reduced rates of glycolysis and cytokine production, further highlighting the importance of these intrinsic metabolic pathways in MAIT cells [38], (Fig. 3).

MAIT cells in host defense

The ability of MAIT cells to recognize bacterial metabolites via the highly conserved MR1 molecule suggests that these cells play a critical role in immune defense against microbial infections. MR1 KO mice show higher bacterial burden following injection with either *Escherichia coli* or *Mycobacterium abscessus*, thus implicating MAIT cells in immunity against these pathogens [7]. Furthermore, MR1 deficient mice exposed to low dose aerosol infection with *Mycobacterium bovis* demonstrated a higher bacterial burden compared to MR1 sufficient mice. This effect evens out by day 30 post-infection, however, suggesting that MAIT cells drive early containment of mycobacterial infections in the lung [35].

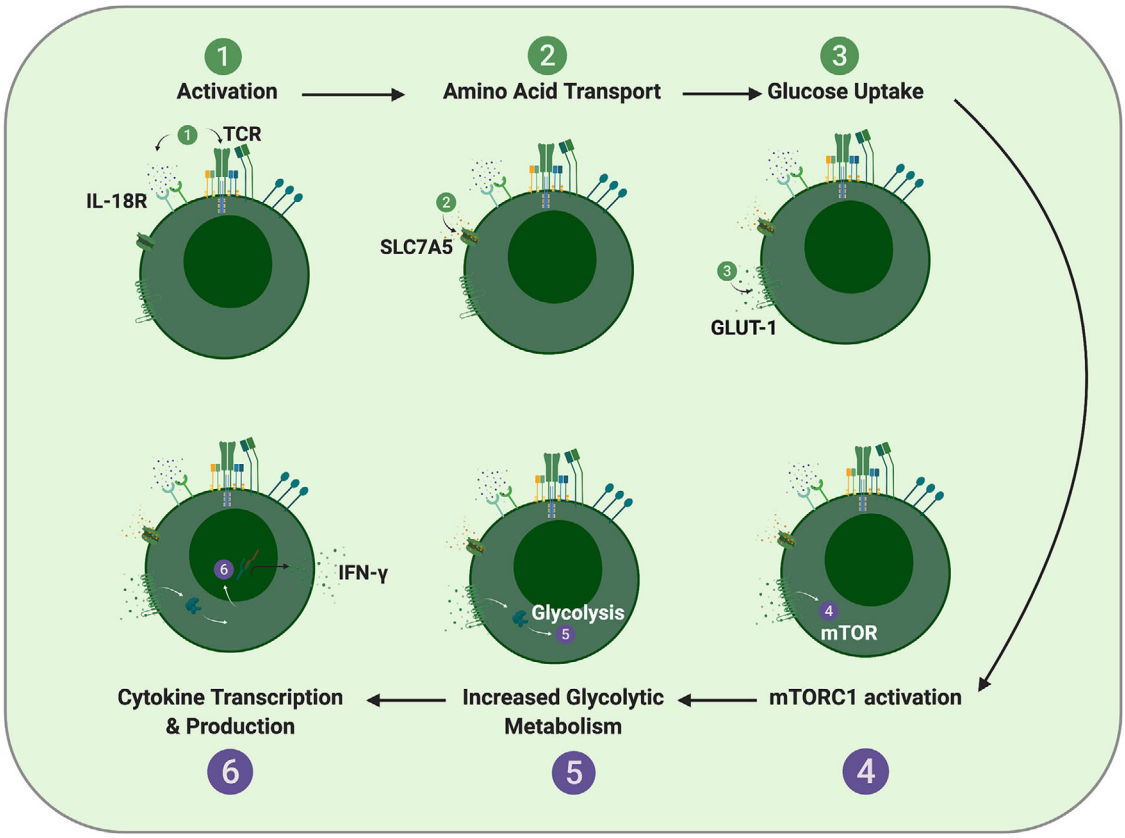

Figure 3. MAIT cell metabolism. Upon activation, MAIT cells increase their consumption of amino acids and glucose (1), which are transported into MAIT cells via SLC7A5 and GLUT-1, respectively (2-3). Upon sensing nutrients mTORC1 activation (4) leads to increased rates of glycolytic metabolism (5) that provides the intermediates for the production of molecules such as IFN-γ and granzyme B (6).

MAIT cells are reduced in the blood but elevated in the lungs of humans with *Mycobacterium tuberculosis* (Mtb), and respond to Mtb-infected MR1 expressing lung epithelial cells [18]. In children with active Mtb infection, MAIT cell frequencies are decreased in the peripheral blood but unlike in Mtb infected adults, they do not accumulate in the lung [46]. MAIT cells have also been shown to respond in a TCR-dependent manner to Gram-negative bacteria such as *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Salmonella enterica*, as well as Gram-positive bacteria including *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Clostridioides difficile,* but do not respond to *Streptococcus pyogenes* or *Enterococcus faecalis* [7, 47–51]. A decrease in circulating MAIT cells has been reported in a number of bacterial infection settings, with lower MAIT cell presence correlating with severity of infection. This was observed in cystic fibrosis patients during *Pseudomonas aeruginosa*, active Mtb infection, in the mite-borne disease scrub typhus, and in critically ill patients admitted to intensive care units with sepsis, who are also more susceptible to nosocomial infections [48, 52–55].

In addition to their broad antibacterial properties, a role for MAIT cells in viral infection has also emerged, detailing how MAIT cells become activated in a TCR-independent, IL-18-dependent manner in response to human viral infections including influenza, hepatitis C (HCV), and dengue virus [36, 56, 57]. Indeed, MAIT cell activation and IL-18 levels correlated with disease severity in acute dengue infection [56]. It was observed that MAIT cell activation was further enhanced in the presence of other proinflammatory cytokines such as IL-12 and type I IFNs [56]. This cytokine-driven mechanism of MAIT cell activation has also been demonstrated in an in vitro model of Zika virus infection, and resulted predominantly in IFN-γ production [57]. A similar IFN-γ response was observed in HCV infection, which resulted in a reduction of in vitro HCV replication [36]. MAIT cells were also shown to be important in the immune response against severe H1N1 influenza infection in mice, with MAIT cell deficient $MR1^{-/-}$ mice showing higher mortality and greater weight loss than control groups [58].

MAIT cells in chronic inflammatory disease

Acute inflammation is a critical process in the protection and repair process, tightly regulated to resolve after injury or infection. However, numerous studies have provided empirical evidence that many diseases are caused by a systemic and non-resolving inflammation termed chronic inflammation [59]. These diseases are increasing in prevalence and dramatically impacting morbidity and mortality. Chronic inflammatory diseases include rheumatoid

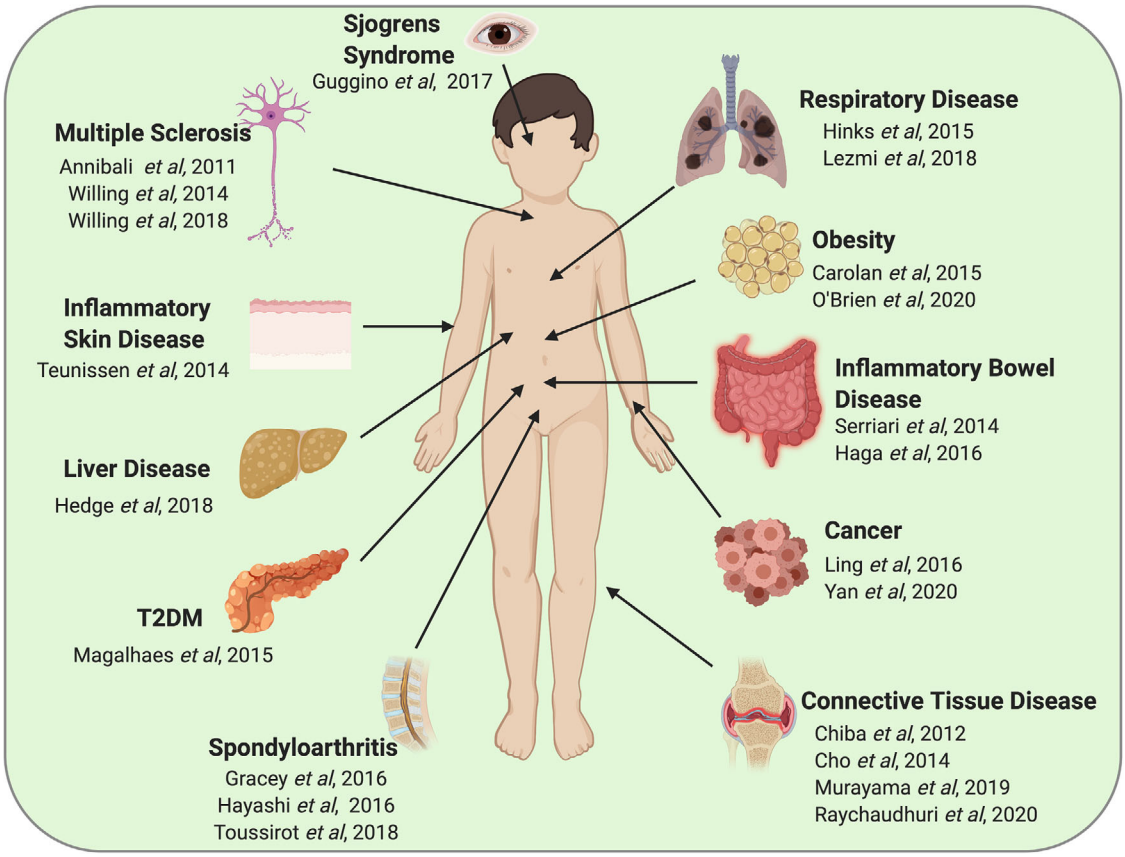

Figure 4. MAIT17 cells in chronic inflammatory disease. Schematic outlining the body of works describing increased MAIT17 cells in chronic inflammatory conditions.

arthritis, non-alcoholic fatty liver, and type II diabetes mellitus. The role of MAIT17 cells in the development and pathogenesis of these conditions is summarized in Figure 4 and discussed herein.

Respiratory disease

Asthma is a respiratory disease characterized by chronic airway inflammation with increased mucus production in the bronchioles, and is associated with activation of an allergen-specific T helper type 2 (T_H2) immune response [60]. Recently, IL-17 has emerged as a pathogenic player in asthma, in particular in severe asthma and immediate responses to bronchodilators [61]. The role of MAIT cells in asthma is unclear with reported reduced frequencies in blood, sputum, and lung biopsies of patients with asthma [62]. In this study, the authors conclude that a loss of MAIT cells in severe asthma might increase susceptibility to bacterial infection, which might impact asthma pathology, suggesting a protective role. However, in a study by Lezmi and colleagues, it was noted that MAIT17 cells were increased in the BALF of asthma patients, and were associated with exacerbations, suggesting a potential pathogenic role [63]. The exact role of MAIT cells in respiratory disease remains to be elucidated, however their relative abundance in humans and association with disease severity, suggests MAIT17 cells may be a major player in asthma pathology either directly or indirectly.

Connective tissue disease

MAIT cells have been implicated in several rheumatoid diseases, including rheumatoid arthritis, systemic lupus erythematosus, and spondyloarthritis [64–68]. Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune condition which affects the joints. Several immune cell populations have been implicated in the pathogenesis of disease including T cells, B cells, and macrophages [69]. Studies in RA patients revealed significant decreases in peripheral MAIT cells observed in comparison to healthy controls, and these decreases are correlated with disease activity scores [64]. MAIT cells have been shown to migrate into the synovial fluid in RA, mediated by IL-1β and TNF-α driven expression of E-selectin at that site [70]. Chiba and colleagues demonstrated an improvement in disease severity in a murine model of collagen induced arthritis in MAIT cell deficient mice, which was paired with a reduction in IL-17 production [71]. Systemic lupus erythematosus (SLE) is an inflammatory autoimmune condition, which impacts several organs including skin, joints and the CNS [72]. Similar to RA, alterations in MAIT cell frequencies were associated with

disease severity in patients [64]. In a murine model of SLE, IL-17 producing MAIT cells were expanded and their deletion resulted in improved disease severity [73]. Spondyloarthritis is a group of chronic inflammatory conditions, which can affect the joints of the spine and pelvis. Several inflammatory cytokines have been implicated in the pathogenesis of the disease including TNF-α and IL-17 [74]. Three separate studies have investigated MAIT cells in ankylosing spondylitis (AS), with each observing reduced MAIT cell frequencies in the circulation [65, 66, 75]. In one of the studies, Gracey and colleagues reported an increase in MAIT cell numbers in the synovial fluid of patients with AS [65]. All three studies also reported that IL-17 production by MAIT cells was higher, suggesting that MAIT cells play a role in pathogenesis [76]. Sjögren syndrome (SjS) is a systemic autoimmune inflammatory disease that primarily affects the exocrine glands resulting in the severe dryness of mucosal surfaces, principally in the mouth and eyes [77]. MAIT cells were found to infiltrate the salivary glands of patients with SjS, displaying an altered phenotype and IFN-γ production, but no difference in IL-17 was reported [78]. In a subsequent report, Guggino and colleagues showed increased MAIT17 cells in salivary gland tissue of patients with SjS, and provided evidence for activation of the IL-7/IL-23 pathways in polarization of MAIT17 cells in SjS [79]. Based on the studies above, it appears that MAIT cells play an important role in the pathogenesis of connective tissue disease and may represent a novel therapeutic target.

Neurological disease

Multiple sclerosis (MS) is a chronic immune-mediated condition affecting the CNS in which T cells play a central pathogenic role in destruction of the myelin sheath [80]. In humans, CD8 T cells comprise the most abundant cell type in the CNS of MS patients in contrast to animal models [71] and MAIT cells account for approximately 5% of these CNS-infiltrating cells [81]. MAIT17 cells are increased in both the CNS and periphery of MS patients, which indicates that MAIT cells may be driving the inflammation associated with this autoimmune disorder [82]. A recent study by Willing et al. has suggested a role for IL-7 in augmentation of IL-17 production by MAIT cells in MS, where increased surface IL-7R expression on MAIT cells correlated with their IL-17 production [82]. Further evidence for MAIT17 cells in the pathogenesis of MS comes from the identification of an expanded population in MS patients of $CCR6^+$, $CD8^+$, $CD161^{hi}$, $CD3^+$, $IL\text{-}17^+$ cells, which bear all the hallmarks of MAIT cells [83]. The exact role for MAIT cells in the pathogenesis of MS remains to be elucidated but their increased production of IL-17 may highlight them as a potential therapeutic target.

Inflammatory skin disease

Recent studies have begun to elucidate a role for MAIT cells in psoriasis, one of the most common immune-mediated chronic inflammatory skin disorders [84]. Evidence suggests that IL-17A and T_H17-related cytokines are critical in the pathogenesis of this systemic disease [85]. A study by Teunissen et al. shows the presence of $CD8^+$ MAIT cells in the dermis and epidermis of psoriatic plaques as well as healthy skin, with MAIT cells accounting for a proportion of $IL\text{-}17A^+$ $CD8^+$ T cells in plaques [19]. IL-17 producing MAIT cells were predominantly found in the psoriatic lesions and were almost absent in healthy skin. These results identify an additional source of IL-17 in psoriatic skin that may be contributing to the disease [19]. Psoriatic arthritis (PsA) is an inflammatory condition, which affects the joints of up to 25% of psoriasis patients. In the majority of cases, psoriasis precedes the onset of PsA [86]. MAIT cells are found to be enriched in the synovial fluid compared to blood of patients with PsA, and upon stimulation produced more IL-17 than in other rheumatoid conditions [68]. The contribution of MAIT cells to other IL-17-driven inflammatory skin diseases such as atopic dermatitis and hidradenitis suppurativa are unknown to date and require further investigation.

Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the gastrointestinal tract encompassing two separate diseases: Crohn's disease (CD) and ulcerative colitis (UC) [87]. Both conditions are driven by over production of inflammatory mediators including TNF-α, IL-23, and IL-17 [88]. Serriari and colleagues investigated the frequencies of MAIT cells in cohorts of patients with either UC or CD and reported reduced frequencies in the circulation compared to healthy controls, which was paired with an increase of MAIT cells in the inflamed tissue [89]. In vitro, MAIT cells from both UC and CD produced more IL-17 [89]. These findings were supported by a subsequent study by Haga and colleagues, who reported reduced circulating MAIT cell frequencies and increased IL-17 production in UC patients [90]. Furthermore, increased MAIT cell levels in the inflamed mucosa correlated with disease activity, suggesting a possible pathogenic role for MAIT cells.

Metabolic disease

Obesity is emerging as the number one cause of preventable death in the Western world, underpinning the development of many chronic diseases, such as type 2 diabetes mellitus (T2DM), cardiovascular disease, and many cancers [91]. Obesity has also been identified as a confounding factor in other chronic diseases including psoriasis, arthritis, and cancer [92]. We and others have previously reported reduced MAIT cell frequencies in the periphery of adults living with obesity [20]. MAIT cells isolated from the periphery of these patients displayed a potent IL-17 phenotype, i.e., significantly higher IL-17 production and reduced IFN-γ production following stimulation compared to healthy controls [20, 93]. This predominant IL-17 phenotype may be contributing to the chronic inflammation seen in obesity and insulin resistance

[94]. MAIT cells were found at higher frequencies in adipose tissue compared to blood, in both lean and obese individuals. MAIT cells in healthy adipose tissue produce IL-10, however this production is reduced in MAIT cells from the adipose tissue of obese patients. [20] MAIT cells may therefore play a regulatory role in healthy individuals and initiate a more inflammatory response through their pro-IL-17 phenotype in obese individuals. Following bariatric surgery (1-year post-surgery) MAIT cell levels were increased in blood compared to presurgical levels, however the levels detected were still lower than healthy controls [93]. The data associated with childhood obesity is slightly different. MAIT cell frequencies in the blood of obese children appear to be significantly higher when compared to their lean counterparts. However, as obese children progress into adulthood, their MAIT cell frequency declines [20]. This is not observed in the lean cohort, suggesting that MAIT cell numbers ultimately decline with the progression of obesity. This altered frequency is associated with both increased fasting insulin and insulin resistance, factors driving the development of type 2 diabetes [20]. Similar to adult obesity, increased MAIT cell IL-17 production was also seen in the pediatric obese cohort [20]. Recently, we have demonstrated that alterations in mitochondria ROS are linked to IL-17 production in MAIT cells from patients with obesity. Using several mitochondrial antioxidants, we were able to limit the production of IL-17 by MAIT cells from patients with obesity in vitro [95]. Non-alcoholic fatty liver disease (NAFLD) is characterized by the excess accumulation of fat in hepatocytes, which can drive fibrosis, progressing to cirrhosis [96]. In cohorts of patients with cirrhotic NAFLD, MAIT cell frequencies were reduced in both the periphery and the liver, but displayed elevated MAIT17 cells compared to controls [97]. Using an in vitro system, it was demonstrated that MAIT cells could drive a pro-inflammatory human hepatic myofibroblast phenotype in a IL-17/TNF-a dependent manner, suggesting MAIT cells are a pro-fibrogenic population [97]. The contribution of MAIT cells to the progression of metabolic disease remains to be elucidated but due to their abundance in humans, they may represent a novel therapeutic target.

Cancer

MAIT cells have been detected within many solid tumor types, including breast, lung, liver, thyroid, colorectal, kidney, brain, stomach, and esophagus [98–102] and in multiple myeloma [11]. These emerging studies have demonstrated that MAIT cells are capable of tumor cell lysis in vitro, however, the role of MAIT cells within cancer, and in particular MAIT17 cells, remains unclear. Ling and colleagues report elevated IL-17 production by MAIT cells in colorectal tumors, when compared to a healthy control group [99]. The authors also found increased IL-17 production by MAIT cells co-cultured in vitro with colorectal cancer cell lines, but also observed an MR1 dependent inhibition of the tumor cell cycle in this setting, suggesting a more complex role for MAIT17 cells in cancer [99]. In contrast, a recent study by Yan and colleagues provided evidence for the promotion of tumor metastasis by MAIT cells, in particular MAIT17 cells [103]. Using two murine models, the authors showed increased tumor expression of MR1, which promoted MAIT cell activation and production of IL-17, which subsequently inhibited NK cell antitumor activity [103]. More work is required in order to define the role of MAIT cells in cancer, including in infection driven cancers, and to properly elucidate their immunotherapeutic potential.

Conclusion

MAIT cells represent a significant proportion of the human $CD8^+$ T cell compartment, yet their place in immunity is only beginning to be understood. In recent years, the volume of studies into MAIT cell biology has exponentially increased, largely due to greater availability of antibodies and tetramers. Many of these studies have examined the role of MAIT cells in disease settings, giving new insights into the role played by MAIT cells in disease. The most striking observation common to many different disease states, is the significant alteration in MAIT cell frequency and cytokine profile. In particular, we have discussed the prevalence of MAIT17 cells in chronic inflammatory diseases. Currently, we do not fully understand the environmental signals and molecular pathways driving this expansion of MAIT17 cells. Several studies have implicated IL-7 as a licencing factor for MAIT17 cells [65, 79, 82], however, this was not specific to IL-17 as other studies detailed IL-7 licencing of IFN-γ and Granzyme B [30, 104]. In addition to IL-7 priming of MAIT17 cells, some studies detailed additional cytokines such as IL-12 or IL-23 [79]. Further work will be required to establish the exact cytokine signals driving MAIT cells and if these are common or disease specific. As discussed, our group has highlighted the importance of immunometabolism in controlling MAIT cell cytokine responses, we highlighted altered mitochondria as a potential driver of IL-17 in MAIT cells from patients with obesity [38, 95]. Altered mitochondria haven been linked to IL-17 production in conventional T cells [105–107], however additional work will again be required to determine if this is a major mechanism driving IL-17 production in MAIT cells, and also to elucidate the environmental signals driving mitochondrial dysregulation. It is clear that despite the recent surge in MAIT cell studies, including numerous studies on MAIT17 cells, improved insight into the pathways controlling MAIT17 cells is required.

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Abbreviations: **CD**: Crohn's disease · **IBD**: inflammatory bowel disease · **iNKT**: invariant NK T cells · **MAIT**: mucosal associated invariant T cells · **Mtb**: *Mycobacterium tuberculosis* · **MS**: multiple sclerosis · **mTOR**: mammalian target of rapamycin · **PsA**: psoriatic arthritis · **RA**: rheumatoid arthritis · **SjS**: Sjögren syndrome · **T2DM**: type 2 diabetes mellitus · **TCR**: T cell receptor · **UC**: ulcerative colitis

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