MUCOSAL-ASSOCIATED INVARIANT T (MAIT) CELL HETEROGENEITY IN PERIPHERAL BLOOD AND BRONCHOALVEOLAR COMPARTMENT: IMPLICATIONS FOR TB AND HIV IMMUNITY

By

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PREFACE

I, **Sharon Khuzwayo** performed the work described in this thesis, most of which was performed at the Africa Health Research Institute (AHRI) and the HIV Pathogenesis Programme (HPP), Nelson R. Mandela School of Medicine, University of KwaZulu-Natal (UKZN), Durban, South Africa, from May 2016 until March 2020. I performed certain parts of the work detailed in this thesis at the VA Portland Health Care Centre, Oregon Health and Science University (OHSU), Portland (OR), United States of America from January to April 2017. The work was done under the supervision of Dr Emily Wong and Prof. Thumbi Ndung'u. This work has not been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others, it is duly acknowledged in the text.

Sharon Khuzwayo (Student):  **Date** **18-12-2020**

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PLAGIARISM DECLARATION

I, **Sharon Khuzwayo** declare that:

i. The research reported in this dissertation, except where otherwise indicated, is my original work.

ii. This dissertation has not been submitted for any degree or examination at any other university.

iii. This dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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PUBLICATIONS

"TRAV1-2+ CD8+ T-cells including oligoconal expansions of MAIT cells are enriched in the airways in human tuberculosis."

Emily B. Wong, Marielle C. Gold, Erin W. Meermeier, Bongiwe Z. Xulu , **Sharon Khuzwayo**, Zuri A. Sullivan, Eisa Mahyari, Zoe Rogers, Hénrik Kløverpris, Prabhat K. Sharma, Aneta H. Worley, Umesh Lalloo, Prinita Baijnath, Anish Ambaram, Leon Naidoo, Moosa Suleman, Rajhmun Madansein, James E. McLaren, Kristin Ladell, Kelly L. Miners, David A. Price, Samuel M. Behar, Morten Nielsen, Victoria O. Kasprowicz, Alasdair Leslie, William R. Bishai, Thumbi Ndung'u and David M. Lewinsohn.

Commun Biol. 2019 Jun 5;2:203. DOI: 10.1038/s42003-019-0442-2.

"MR1-restricted MAIT cells from the human lung mucosal surface have distinct phenotypic, functional, and transcriptomic features that are preserved in HIV infection."

Sharon Khuzwayo, Maphe Mthembu, Erin W. Meermeier, Sanjay M. Prakadan, Samuel W. Kazer, Thierry Bassett, Kennedy Nyamande, Dilshaad Fakey Khan, Priya Maharaj, Mohammed Mitha, Moosa Suleman, Zoey Mhlane, Dirhona Ramjit, Farina Karim, Alex K. Shalek, David M. Lewinsohn, Thumbi Ndung'u, Emily B. Wong.

BioRxiv 2020.11.19.389858. DOI: 10.1101/2020.11.19.389858

"Human lung-resident mucosal-associated invariant T cells are abundant, antimicrobial, and cytokine responsive"

Erin W. Meermeier, Christina L. Zheng, Jessica G. Tran, Shogo Soma, Aneta H. Worley, David I. Weiss, Robert L. Modlin, Gwendolyn Swarbrick, Elham Karamooz, **Sharon Khuzwayo**, Emily B. Wong, Marielle C. Gold, David M. Lewinsohn.

Nat Commun 2020. (Under review)

PRESENTATIONS

Oral presentations

"Phenotypic heterogeneity of mucosal-associated invariant T (MAIT) cell clones from bronchoalveolar lavage fluid"

Sharon Khuzwayo, Erin Meermeier, David Lewinsohn, Thumbi Ndung'u, Emily Wong.

6th South African Immunology Society (SAIS) Conference 3-6 September 2017, Gordons Bay (South Africa)

"Cloning and characterisation of lung and peripheral blood MAIT cells"

Sharon Khuzwayo, Erin Meermeier, David Lewinsohn, Thumbi Ndung'u, Emily Wong.

Sub-Saharan African Network for TB/HIV Research Excellence (SANTHE) Research Day 6-7 June 2018, Hillcrest (South Africa)

"Lung mucosal MAIT cells are phenotypically, functionally and transcriptionally distinct from peripheral blood MAIT cells"

Sharon Khuzwayo, Maphe Mthembu, Erin Meermeier, Thierry Bassett, Sanjay Prakadan, Alex Shalek, David Lewinsohn, Thumbi Ndung'u, Emily Wong.

SANTHE Research Day 18-19 June 2020 (Virtual)

Poster presentations

"Phenotypic heterogeneity of mucosal-associated invariant T (MAIT) cell clones from bronchoalveolar lavage fluid"

Sharon Khuzwayo, Erin Meermeier, David Lewinsohn, Thumbi Ndung'u, Emily Wong.

7th Infectious Diseases in Africa (IDA) Symposium 7-9 September 2017, Gordons Bay (South Africa)

"Characterisation of Mucosal-Associated Invariant T (MAIT) Cell Clones from Bronchoalveolar Lavage (BAL) Fluid"

Sharon Khuzwayo, Erin Meermeier, David Lewinsohn, Thumbi Ndung'u, Emily Wong.

3rd SANTHE Annual Consortium Meeting 26-27 September 2018, Kigali (Rwanda)

"Lung mucosal MAIT cells are phenotypically, functionally and transcriptionally distinct from peripheral blood MAIT cells"

Sharon Khuzwayo, Erin Meermeier, Maphe Mthembu, Thierry Bassett, David Lewinsohn, Thumbi Ndung'u, Emily Wong.

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And above all else, to God my creator, without whom I would be lost.

ABBREVIATIONS

5-OP-RU	5-(2-oxopropylideneamino)-6-D-ribitylaminouracil
6FP	6-formyl pterin
APC	Antigen presenting cell
ART	Antiretroviral therapy
BAL	Bronchoalveolar lavage
BALT	Bronchus-associated lymphoid tissue
BCG	Bacille Calmette-Guérin
BCR	B cell receptor
CD	Cluster of differentiation
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DURT	Donor-unrestricted T (cell)
ELISpot	Enzyme-linked immuno-spot assay
FACS	Fluorescence activated cell sorting
FMO	Fluorescence minus one
HIV	Human immunodeficiency virus
ICS	Intracellular cytokine staining
IFN	Interferon
Ig	Immunoglobulin
IGRA	Interferon gamma release assay
IL	Interleukin
INOS	Inducible nitric oxide synthase
KO	Knock out
LCL	Lymphoblastoid cell line
LDA	Limiting dilution assay
LTBI	Latent TB infection
MAIT	Mucosal associated invariant T (cell)
MALT	Mucosa-associated lymphoid tissue
MHC	Major histocompatibility complex
Mo-DC	Monocyte-derived dendritic cell
MR1	MHC class I-related protein 1

Mtb	*Mycobacterium tuberculosis*
NALT	Nasopharynx-associated lymphoid tissue
NK	Natural killer (cell)
NLR	Nucleotide-binding oligomerisation domain (NOD)-like receptors
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PD-1	Programmed cell death protein 1
PHA	Phytohaemagglutinin
PLWH	People living with HIV
PMA	Phorbol myristate acetate
SFU	Spot forming unit
SIV	Simian immunodeficiency virus
TB	Tuberculosis
TCR	T cell receptor
TGF-β	Transforming growth factor β
TIM-3	T cell immunoglobulin and mucin domain-containing protein 3
TH	T helper (cell)
TFH	Follicular helper T (cell)
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAV	T cell receptor alpha variable
TRAJ	T cell receptor alpha joining
Treg	Regulatory T (cell)
TST	Tuberculin skin test
WT	Wild type

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Tuberculosis (TB) is the leading cause of infectious mortality globally and the leading cause of death in people living with human immunodeficiency virus (HIV). Understanding the mechanisms leading to impaired anti-TB immunity at mucosal sites and how this is altered during HIV infection, may assist in the development of more effective TB vaccines or immune-based therapies. Mucosal-associated invariant T (MAIT) cells are depleted and dysfunctional in the peripheral blood during HIV infection, but little is known about HIV's impact on their quantity and quality at the lung's mucosal surface, the site of TB infection. We aimed to characterise phenotypic, functional and transcriptomic features of MAIT cells in the peripheral blood and lung mucosa of people with latent *Mycobacterium tuberculosis* (*Mtb*) infection and HIV co-infection.

Matched peripheral blood and bronchoalveolar lavage fluid were collected from consenting participants with confirmed latent TB infection, either with or without HIV. Characterisation of MHC class I-related protein 1 (MR1) 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) tetramer-positive T cells from the lung was performed by cloning, immunophenotyping, functional assays and T cell receptor sequencing. The phenotype, function and transcriptome of MAIT cells from both compartments were characterised by surface marker staining, intracellular cytokine staining, as well as single-cell and bulk RNA-sequencing of MR1 5-OP-RU tetramer-positive T cells from HIV-negative and HIV-positive individuals.

Peripheral blood MAIT cells were characterised by the CD161++CD26++ phenotype and produced more interferon (IFN)-γ ($P = 0.016$) than bronchoalveolar MAIT cells in HIV-negative individuals. Bronchoalveolar MR1 5-OP-RU tetramer-positive cells, included subpopulations with the atypical CD161-CD26++ and CD161-CD26- phenotypes. T cell cloning demonstrated that cells from the typical and atypical CD161/CD26 phenotypic subpopulations all had MR1-restricted function and MAIT cell consistent T cell receptors. In HIV infection, the frequency of both peripheral blood and bronchoalveolar MAIT cells was reduced ($P = 0.035$ and $P = 0.047$ respectively), with peripheral blood MAIT cells producing less interleukin (IL)-17 ($P = 0.025$) and expressing higher levels of the inhibitory co-stimulatory molecule T cell immunoglobulin and mucin domain-containing protein (TIM)-3. Interestingly, the phenotype and function of bronchoalveolar MAIT cells remained relatively unchanged by HIV. Single-cell transcriptional analysis confirmed MAIT cell heterogeneity in the bronchoalveolar compartment which contained two distinct transcriptional subsets, one associated with typical MAIT cell features and effector functions and the other associated with alternative MAIT cell functions including tissue-repair.

We report previously undocumented phenotypic and transcriptional heterogeneity in bronchoalveolar MAIT cells, which were also less pro-inflammatory than those in peripheral blood. HIV infection led to depletion of MAIT cells in both compartments, but phenotypic and functional alterations were more pronounced in the peripheral blood compartment. The preservation of function and heterogeneity in bronchoalveolar MAIT cells may represent a potential avenue for therapeutic targeting to restore normal MAIT cell function in people living with HIV. This data suggests that understanding immune responses requires compartment-specific analyses, which may lead to the development of more effective vaccines and immunotherapies targeted at inducing immune responses at the site of infection.

TABLE OF CONTENTS

CHAPTER 1

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1. Global burden of HIV and TB

Mycobacterium tuberculosis (*Mtb*) and the type-1 human immunodeficiency virus (HIV-1) are the leading causes of morbidity and mortality globally. *Mtb* infection leads to tuberculosis (TB) disease and was responsible for approximately 10 million infections and 1.5 million deaths in 2018 (WHO, 2019). Untreated HIV infection leads to the acquired immunodeficiency syndrome (AIDS), and approximately 1.7 million people became newly infected with HIV and 770 000 died of AIDS-related diseases in 2018 (UNAIDS, 2019). Of the lives TB claimed in 2018, approximately 251 000 of these were in HIV-positive people accounting for one third of deaths and making TB the greatest cause of death in people living with HIV (PLWH). In southern Africa, the rate of HIV co-infection among people with TB is higher than 50% in some areas (**Figure 1.1**), highlighting the significant impact of these two epidemics in this region (Karim *et al.*, 2009). Although the advent of antiretroviral therapy (ART) has significantly improved life expectancy and resulted in global declines in HIV-related mortality (Bor *et al.*, 2013; Trickey *et al.*, 2017), infection with HIV still profoundly alters individuals' immune responses to pulmonary infections such that HIV infection remains the greatest risk factor for acquiring TB even in those on ART (Harries *et al.*, 2010). Thus, even in the age of ART, improved vaccines and therapeutics are thus urgently needed to restore optimal immunity against respiratory pathogens in people living with HIV.

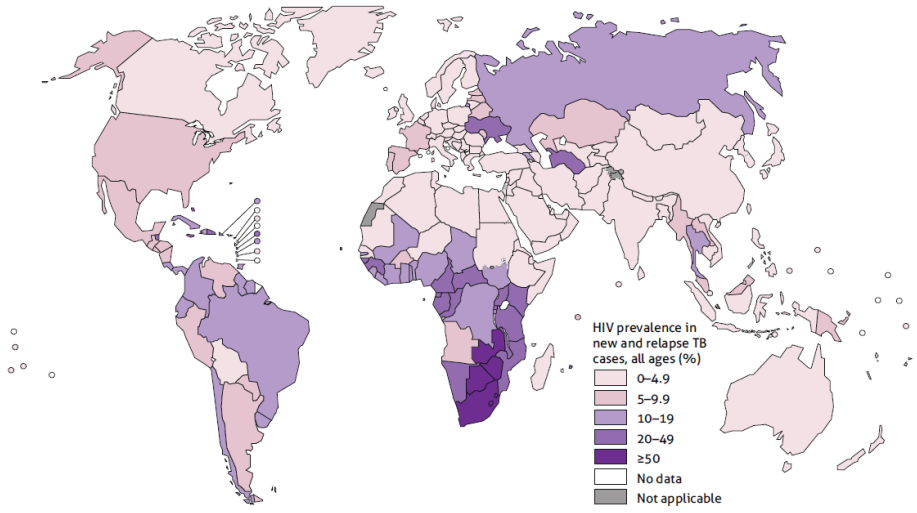


Figure 1.1: The global prevalence of HIV infection in new and relapse TB cases in 2018 indicating that prevalence exceeds 50% in southern Africa (from WHO (2019)).

1.2. Overview of the immune system

The immune system serves to protect the host from invading pathogens which is achieved by the discrimination of self from non-self. Broadly, the immune system is divided into two functional components, the innate and the adaptive arms. The innate arm of the immune system is the first line of defence and acts rapidly and non-specifically. The adaptive arm is slower in response and generates immune memory that is antigen-specific (Bellanti *et al.*, 2012; Actor, 2019). The cellular components of the innate immune system comprise phagocytes including macrophages and neutrophils which ingest foreign material and infected cells; antigen presenting cells (APCs) such as dendritic cells (DCs) which present foreign material to T cells thereby activating the adaptive immune system; and cytotoxic cells such as natural killer (NK) cells that can kill virally infected cells (Bellanti *et al.*, 2012; Actor, 2019). The innate arm detects pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) on invading microbes or infected cells via non-specific pattern recognition systems including toll-like receptors (TLRs) and Nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs) (Chaplin, 2010; Turvey and Broide, 2010).

The cellular components of the adaptive immune system are the B lymphocytes, which mediate humoral immunity, and the T lymphocytes, which mediate cellular immunity. These cells detect specific peptide antigens using a diverse repertoire of B cell receptors (BCRs), also known as surface immunoglobulins (Igs), or T cell receptors (TCRs) (Bellanti *et al.*, 2012; Actor, 2019). T cells are divided into two major categories: cytotoxic (CD8+) T cells, which recognise antigen presented on major histocompatibility complex (MHC) class I molecules and mediate the lysis of infected cells, and T helper (CD4+) cells which recognise antigen presented on MHC class II (**Figure 1.2.1**). T helper (Th) cells are further divided mainly into Th1, Th2, Th17, regulatory T (Treg) cells and follicular helper T (Tfh) cells and have varying functions depending on Th cell lineage (Bellanti *et al.*, 2012; Actor, 2019). Th1 cells primarily produce interferon (IFN)-γ and interleukin (IL)-2 under the control of T-box transcription factor 21 (T-bet) and assist cytotoxic lymphocytes. Th2 cells provide B cell help via the production of the cytokines IL-4 and IL-5, for example, under the control of GATA binding protein 3 (GATA-3). Th17 cells, express the transcription factor Retinoic Acid-Related orphan receptor gamma t (RORγt), mostly produce IL-17 and IL-22 and are involved in immune responses at the skin and mucosa. Tregs produce IL-10 and TGF-β as directed by Forkhead box P3 (FOXP3) and are important for dampening immune responses (Bellanti *et al.*, 2012; Mak *et al.*, 2014a; Actor, 2019). Tfh cells are found in secondary lymphoid tissues where they assist in B cell activation and affinity maturation. The transcription factor B-cell lymphoma 6 (Bcl6) is important for Tfh and germinal centre development (Crotty, 2014; Mak *et al.*, 2014a; Actor, 2019).

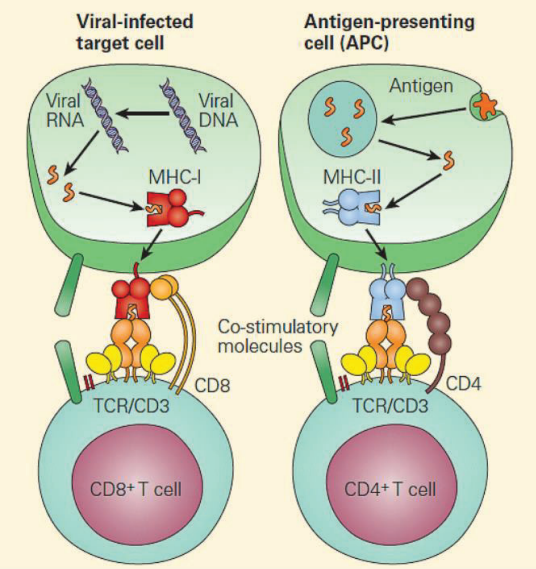


Figure 1.2.1: T cell recognition via major histocompatibility (MHC) molecules. CD8+ T cell recognition of endogenous antigen presented on MHC class I (left) and CD4+ T cell recognition of exogenous antigen presented on MHC class II (right). In both CD4+ and CD8+ T cells, effector functions are activated by synaptic interactions between T cell receptors and MHC molecules and the strength of downstream signalling pathways is modulated by co-stimulatory and co-inhibitory molecules (from Bellanti *et al.* (2012)).

Peripheral immune responses are facilitated by interactions between APCs and lymphocytes in peripheral lymph nodes. Interactions that prime mucosal responses on the other hand, occur within the tissue at organised structures known as the mucosa-associated lymphoid tissues (MALTs) (Mak *et al.*, 2014b). The respiratory mucosa, is composed of the nasopharyngeal- and bronchi-associated lymphoid tissues (NALT and BALT, seen in **Figure 1.2.2**) (Mak *et al.*, 2014b). Most responses at the respiratory mucosa are orchestrated locally by cells such as alveolar macrophages, DCs and γδ T cells (Bellanti *et al.*, 2012; Mak *et al.*, 2014b). These cells ensure that moderate inflammatory responses are maintained to minimise exaggerated responses to harmless environmental microbes, reduce potential damage to the delicate epithelial layers of the mucosa and ensure that responses are rapidly resolved to ensure a return to normal tissue structure and function (Chaplin, 2010; Branchett and Lloyd, 2019). The primary cytokines at the respiratory mucosa include IL-10, IL-35 and TGF-β, which are produced by macrophages and bronchial epithelial cells, and assist in the dampening of inflammatory responses at this site (Bellanti *et al.*, 2012; Murphy and Weaver, 2016; Branchett and Lloyd, 2019). These cytokines also stimulate the production of IgA by local plasma cells which can neutralise and prevent adherence of microbes to respiratory epithelia (Mak *et al.*, 2014b; Murphy and Weaver, 2016). In cases where these innate immune responses are breached DCs present antigen to

naïve T cells in the draining lymph nodes, an additional site where mucosal immune responses can be primed, activating the adaptive arm of the immune response (Mak *et al.*, 2014b; Murphy and Weaver, 2016).

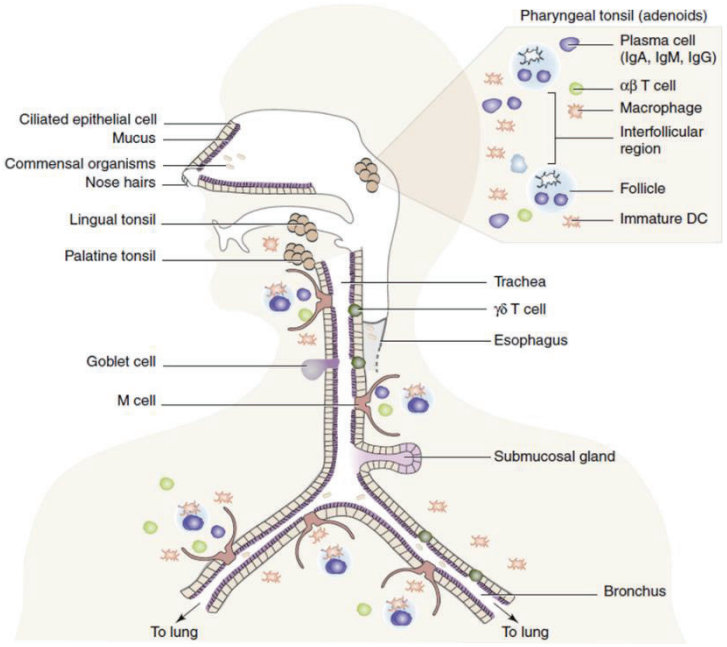


Figure 1.2.2: The nasopharyngeal- and bronchi-associated lymphoid tissue (NALT and BALT) components of the respiratory mucosa. Immune responses at this site are predominantly coordinated by cells including macrophages and γδ T cells. Local plasma cells secrete immunoglobulins, for example IgA, which promote tolerance, while dendritic cells present antigen to naïve T cells to initiate adaptive immune responses if mucosal tissues become compromised (from Mak *et al.* (2014b)).

1.3. HIV's impact on the immune system

In addition to directly infecting and depleting CD4+ T cells throughout the body, untreated HIV infection increases susceptibility to opportunistic infections by causing disruptions to mucosal barriers (Brenchley and Douek, 2008). Another hallmark of HIV infection is chronic immune activation which is characterised by a number of factors including shorter T cell survival rates (Hellerstein *et al.*, 1999), hyperactivation of B cells (Lane *et al.*, 1983) and increased T cell expression of activation markers such as HLA-DR and CD38 (Kestens *et al.*, 1992). HIV infection results in the significant depletion of CD4+ T cells systemically and in mucosal tissues, which has been well studied in the gastrointestinal tract (GIT). Depletion of CD4+ T cells in the GIT is associated with altered barrier integrity, as a result of the influx of inflammatory cells, and leads to the translocation of microbial

products from the gut and contributes to systemic activation (Mehandru *et al.*, 2004; Brenchley *et al.*, 2006). HIV also increases serum levels of pro-inflammatory cytokines, most notably type I interferons, which leads to the recruitment of more target cells to be infected by HIV and drives persistent immune activation (Sokoya *et al.*, 2017). In addition, HIV infection results in the significant recruitment of cells including B cells, CD8+ T cells and γδ T cells into the lung, dysregulating the composition of immune cell subsets at the lung mucosa, which may potentially increase susceptibility to respiratory infections in PLWH (Mwale *et al.*, 2018). The effect of HIV on the function of these cell subsets is detailed below in section 1.6. Infection with HIV thus results in dysregulated cytokine production, systemic depletion of CD4+ T cells, immune subset alterations at the lung mucosa and promotes T cell exhaustion via persistent activation.

1.4. The spectrum of TB infection

The consequences of encounter with *Mtb* have been recognised to be heterogenous and to result in a spectrum of infection and disease states (Barry *et al.*, 2009; O'Garra *et al.*, 2013; Pai *et al.*, 2016). Exposure to *Mtb* may (1) be insufficient to establish infection, may (2) be cleared by the innate immune system or may (3) be cleared at the site of infection and thus not lead to any systemic alterations (**Figure 1.4**). In all three of these cases, the assessment of host responses to *Mtb* antigens by tuberculin skin test (TST) and IFN-γ release assay (IGRA) would yield negative results (Barry *et al.*, 2009; O'Garra *et al.*, 2013; Pai *et al.*, 2016). The TST measures skin induration following intradermal injection with purified protein derivative (PPD) from *Mtb*. PPD also contains antigens from non-tuberculous mycobacteria including *Mycobacterium bovis* Bacille Calmette-Guérin (BCG). The IGRA measures IFN-γ released by T cells following exposure to *Mtb* antigens found within the region of difference 1 (RD1) locus. The IGRA is thus more specific as the RD1 locus is found in virulent *Mtb* but not in BCG and many other environmental mycobacteria (Barry *et al.*, 2009; O'Garra *et al.*, 2013; Pai *et al.*, 2016). Exposure to *Mtb* may alternatively (4) lead to latent TB infection. This is a state in which the bacterial encounter has led to the formation of measurable T cell mediated responses in individuals, resulting in the conversion of both TST and IGRA to positive results. Latently TB infected people represent a heterogenous population where the individual may have cleared infection (green in **Figure 1.4**), may be harbouring the bacteria in a quiescent state (yellow) or have subclinical disease which may or may not lead to the development of symptoms but is characterised by intermittent culture positivity (orange) (Barry *et al.*, 2009; O'Garra *et al.*, 2013; Pai *et al.*, 2016). Lastly, a subset of individuals (5) will develop active TB disease, either through reactivation of latent infection or as a direct result of the initial encounter with *Mtb*. These individuals are usually sputum culture positive and have mild to severe symptoms (Barry *et al.*, 2009; O'Garra *et al.*, 2013; Pai *et al.*, 2016).

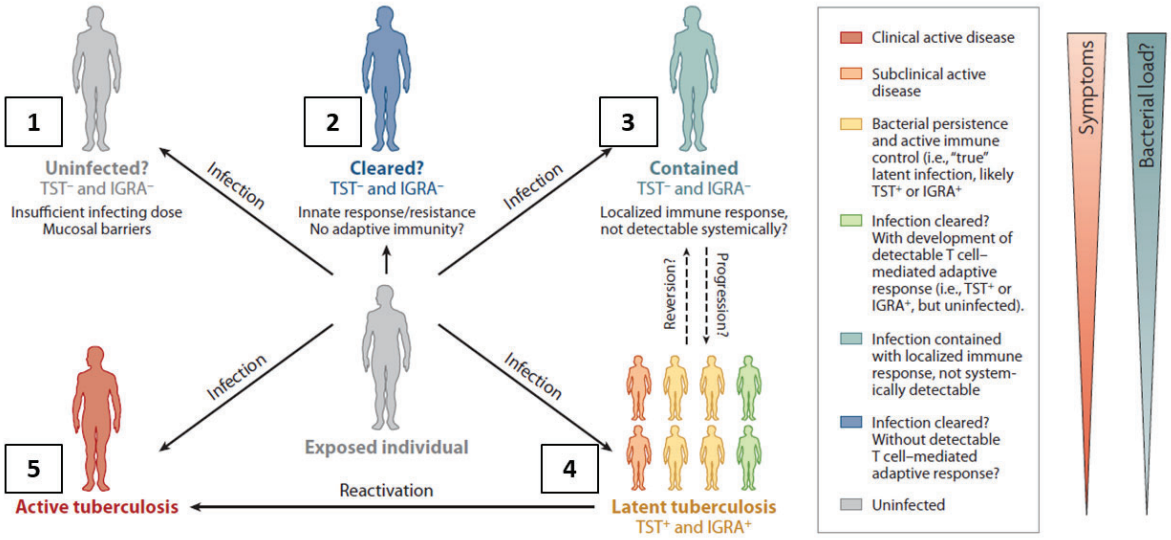


Figure 1.4: Consequences of exposure to *Mycobacterium tuberculosis* (*Mtb*). Individuals encountering *Mtb* may (1) remain uninfected, (2) clear infection via innate mechanisms or (3) eliminate the infection via local immune responses that are not measurable by tuberculin skin test (TST) and IFN-γ release assay (IGRA). In all three scenarios individuals would be TST- and IGRA-negative. Individuals may also (4) develop latent TB infection characterised by conversion of TST and IGRA or (5) develop active TB via reactivation of latent infection or from initial encounter with *Mtb* (from Barry *et al.* (2009) and O'Garra *et al.* (2013)).

1.5. Immune response to TB infection

The natural human immune response to *Mtb* is thus complex and the mechanisms which lead to bacterial control or loss of control are not fully understood. It is however, generally agreed that Th1 responses from CD4+ T cells (primarily via IFN-γ production) are important for the successful containment of *Mtb* (Nunes-Alves *et al.*, 2014; Singhania *et al.*, 2018). Mice lacking CD4+ T cells are highly susceptible to TB infection (Orme *et al.*, 1993; Caruso *et al.*, 1999; Mogues *et al.*, 2001) and in humans, depletion of CD4+ T is associated with TB disease in HIV infected individuals (Geldmacher *et al.*, 2010). Studies using mice have also shown the importance of IFN-γ in mediating beneficial responses to *Mtb* (Cooper *et al.*, 1993; Flynn *et al.*, 1993) and in humans, individuals with genetic defects to IFN-γ signalling are more susceptible to mycobacterial infections (Ottenhoff *et al.*, 1998; Casanova and Abel, 2002). Thus, an important component of the human protective immune response to *Mtb* is thought to be IFN-γ produced by CD4+ T cells and its activation of macrophages which then eliminate *Mtb* via fusion with the phagolysosome or by nitric oxide production (**Figure 1.5**) (Nunes-Alves *et al.*, 2014).

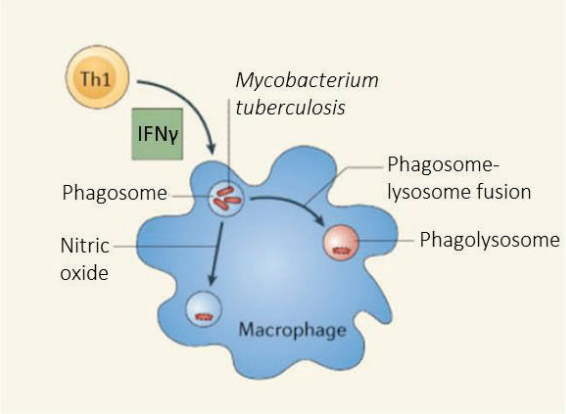


Figure 1.5: Overview of the immune response required for the containment of *Mycobacterium tuberculosis* (*Mtb*). CD4+ T cells produce IFN-γ which activates macrophages to kill *Mtb* via nitric oxide production or phagolysosome fusion (from Nunes-Alves *et al.* (2014)).

Although IFN-γ production by CD4+ T cells has been shown to be important in protective immune responses to *Mtb*, vaccine strategies which have successfully produced such responses have failed to confer protection (Tameris *et al.*, 2013). This has highlighted that a lot still must be learned about aspects of the human immune response that confer protection in naturally occurring TB immunity (Nunes-Alves *et al.*, 2014). Recent developments in human TB vaccine trials with positive outcomes have now opened the possibility of identifying correlates of protective immunity and this remains an area undergoing active investigation (Nemes *et al.*, 2018; Van Der Meeren *et al.*, 2018).

1.6. HIV-induced alterations on TB immunity

HIV increases the risk of acquiring respiratory pathogens, including *Mtb*, through multiple immunological mechanisms. Disruption of barrier integrity, chronic pulmonary inflammation, disruption of the microbiome and depletion of specific cell subsets have been implicated (Jambo *et al.*, 2014a; Brune *et al.*, 2016; Cribbs *et al.*, 2016). HIV increases susceptibility to TB by selectively depleting and impairing the function of *Mtb*-specific CD4+ T cells during early HIV infection (Geldmacher *et al.*, 2008; Kalsdorf *et al.*, 2009; Geldmacher *et al.*, 2010; Jambo *et al.*, 2011). *Mtb*-specific CD4+ T cells are important not only for prevention of the establishment of *Mtb* infection, but also for containment during latent TB infection (Cooper, 2009; Nunes-Alves *et al.*, 2014). The rapid decline in CD4+ T cells during the early phases of HIV infection is accompanied by increasing risk of TB disease, which reaches as much as 20-fold greater than HIV uninfected controls, during chronic untreated infection (**Figure 1.6**) (Bell and Noursadeghi, 2018). The lifetime risk of TB disease remains as high as 5-fold above control levels even in individuals who are on otherwise successful

ART. Improved TB case finding is thus needed, especially in PLWH who have a high burden of undiagnosed TB (Hoog *et al.*, 2011).

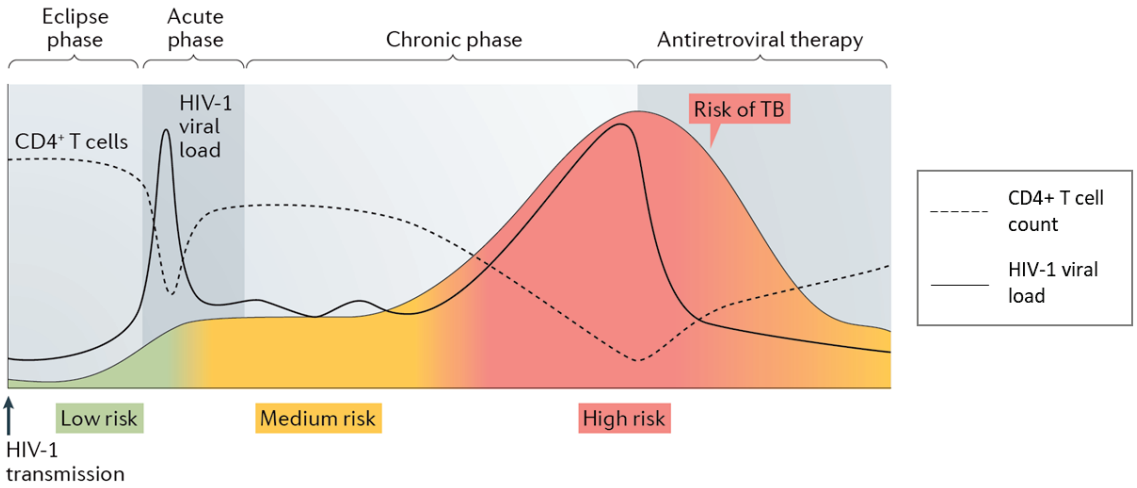


Figure 1.6: Increased risk of active TB acquisition in HIV infection. CD4+ T cell decline during acute HIV infection leads to the initial increase in TB risk. TB risk is at its highest during chronic phase of untreated HIV, and remains as much as 5-fold higher than baseline during antiretroviral therapy (from Bell and Noursadeghi (2018)).

HIV also disrupts anti-TB immunity via interactions with macrophages, which are essential for the clearance of *Mtb*. HIV has been shown to preferentially infect small alveolar macrophages and reduce their capacity to phagocytose bacteria, which may predispose individuals to respiratory infections including TB (Jambo *et al.*, 2014b). Macrophage phagocytosis and autophagosome maturation can both be inhibited by the HIV accessory protein Nef. This inhibition allows for greater mycobacterial replication and prevents degradation of HIV virions by autophagy pathways (Kyei *et al.*, 2009; Mazzolini *et al.*, 2010). HIV infected macrophages also produce lower amounts of IL-10 which is needed to prevent exaggerated immune responses to *Mtb*. This failure in immune regulation leads to increased HIV replication by recruiting additional lymphocytes to the site of infection during this pro-inflammatory response (Tomlinson *et al.*, 2014). Overall, HIV has been shown to have multiple mechanisms by which it abrogates naturally occurring TB immunity. Further understanding of these effects, with a focus on expanding our understanding of the HIV-induced alterations to immune system components that can be targeted for restored function, is critical to the long-term goal of improving TB immunity and reducing TB morbidity and mortality in PLWH.

1.7. Unconventional T cells

Unconventional T cells, a diverse group of T lymphocytes also known as donor-unrestricted T cells (DURTs), are united by their lack of classical MHC-restriction (La Manna *et al.*, 2020). The T cell receptors of unconventional T cells interact with alternative antigen-presenting molecules which have some homology to MHC-class molecules but have significantly less variation across the human population. The DURTs include natural killer T (NKT) cells, germline-encoded mycolyl-reactive (GEM) T cells, and other classes of CD1-restricted T cells which recognise lipid and glycolipid antigens presented on molecules in the CD1 family which include, CD1a, CD1b, CD1c and CD1d (Godfrey *et al.*, 2015). Mucosal-associated invariant T cells, which will be discussed in the next section, interact with small molecule antigens presented on MHC class 1-related protein 1 (MR1) (Godfrey *et al.*, 2015; Van Rhijn and Moody, 2015; La Manna *et al.*, 2020). Interest in understanding the biology of these cells, which have been said to bridge the innate and adaptive arms of the immune system, has increased due to their involvement in many infectious diseases including TB and HIV (La Manna *et al.*, 2020). Additionally, these cells have been noted to be enriched in the skin and at mucosal surfaces and to respond more rapidly than conventional T cells to stimuli. These properties and their donor-unrestricted nature stimulated interest in them as potential candidates for novel universal vaccines and therapies (Godfrey *et al.*, 2015; Van Rhijn and Moody, 2015).

1.8. Mucosal-associated invariant T cells

MAIT cells are a class of evolutionarily conserved, innate-like unconventional T lymphocytes. MAIT cells were first discovered as a subpopulation of CD4-CD8- T cells which possessed an invariant T cell receptor rearrangement Vα7.2-Jα14 (TRAV1-2/TRAJ33) (Porcelli *et al.*, 1993). Tilloy *et al.* (1999) next described these cells in mice, cattle, and humans, finding that in mice and cattle they were characterised by invariant Vα19-Jα33 α chain rearrangement, homologous to the Vα7.2-Jα33 rearrangement in humans. These authors also confirmed the preferential usage of Vβ2 (TRBV20) and Vβ13 (TRBV6) gene families by these cells in humans. This novel T cell subset was later found to be enriched in the gut lamina propria of mice and humans and thus received the name 'mucosal-associated invariant T cells' (Treiner *et al.*, 2003). Subsequent studies found that MAIT cells were enriched at many other mucosal sites including the lungs and the liver (further discussed in section 1.10). MAIT cells were also found to be restricted by the non-polymorphic MR1 molecule (**Figure 1.8**), the most conserved of the MHC-related molecules in mammals (Treiner *et al.*, 2003). These findings suggested a conserved function of MAIT cells, likely relating to the maintenance of immune responses at mucosal sites.

Unlike conventional T cells which respond to peptide antigen, MAIT cells respond to intermediates of vitamin B biosynthesis pathways (**Figure 1.8**), which are absent in mammals and present in certain microbes. Kjer-Nielsen *et al.* (2012) were the first to identify MAIT cell ligands through mass-spectrometric analysis of MR1-bound antigens, with the first ligand discovered being folic acid (vitamin B9) derivative, 6-formylpterin (6-FP). Binding of 6-FP to MR1 did not result in MAIT cell activation, while the ribityllumazine-based ligands, riboflavin (vitamin B2) derivatives, identified in this study were capable of activating MAIT cells. Pyrimidine-based ligands, 5-OP-RU (5-(2-oxopropylideneamino)-6-D-ribitylaminouracil) and 5-OE-RU (5-(2-oxoethylideneamino)-6-D-ribitylaminouracil), derived from 5-A-RU (5-amino-6-D-ribitylaminouracil) can also activate MAIT cells (Corbett *et al.*, 2014). The most studied of these has been 5-OP-RU from which MAIT identifying tetramers were created (Corbett *et al.*, 2014).

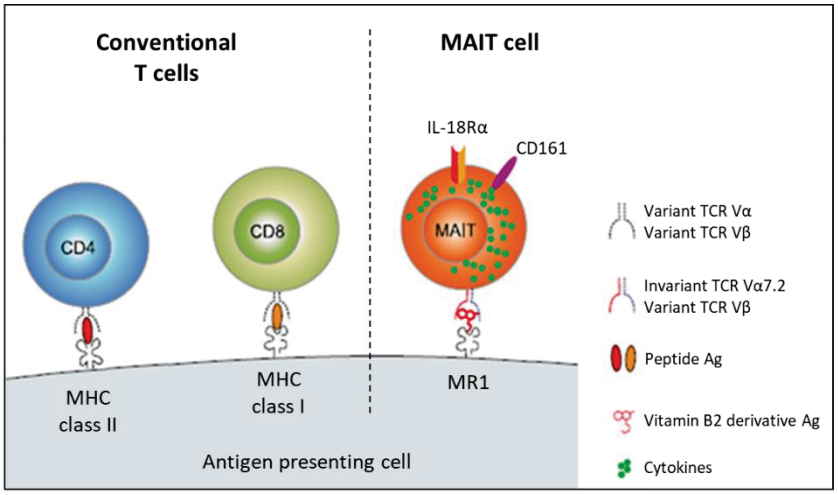


Figure 1.8: Mucosal-associated invariant T (MAIT) cell restricting molecule, stimulating antigen and T cell receptor (TCR) in comparison to those of conventional T cells. MR1-restricted MAIT cells respond to vitamin B derived antigen via their semi-invariant TCR (from Y.-W. Park and Kee (2015)).

The ability of MAIT cells to recognise non-polymorphic antigens in a donor-unrestricted manner opens possibilities for their use in novel, universal vaccine strategies that would not be limited by the rich diversity of MHC class I and II molecules within the human population that complicates vaccine design and which targets conventional CD4+ or CD8+ T cells (Van Rhijn and Moody, 2015). Elucidation of MAIT cell activating ligands enabled the development of MR1 tetramers to identify and characterise MAIT cells from samples derived from peripheral blood and tissue sites (Reantragoon *et al.*, 2013). Using these MR1 tetramers, which stain both human and mouse MAIT cells, the heterogeneity of MAIT cell TCRs was confirmed with TRAJ12 and TRAJ20 segments also

being identified in addition to the canonical TRAJ33 segment (Gold *et al.*, 2010; Reantragoon *et al.*, 2013). Further work is required to expand knowledge about the repertoire of MAIT activating ligands which appears to include additional metabolites which may be specific to certain pathogens. Characterisation of these and utilisation of alternative ligand-specific MR1 tetramers may reveal greater diversity in MAIT cell function than is currently recognised based on use of the MR1 5-OP-RU tetramer alone (Harriff *et al.*, 2018).

1.9. Methods of defining MAIT cells

1.9.1. Surface marker and tetramer definitions

MAIT cells in healthy peripheral blood are characterised by the high expression of the C-type lectin CD161 and high expression of the dipeptidyl peptidase CD26 (Martin *et al.*, 2009; Le Bourhis *et al.*, 2010; P.K. Sharma *et al.*, 2015). Prior to the advent of MR1 tetramers, surface expression of these surrogate markers was often used in combination with TRAV1-2 and conventional T cell lineage markers CD3, CD4 and CD8 to identify MAIT cells. Although MAIT cells were first identified from the proportion of CD4-CD8- T lymphocytes, assessment of CD4/CD8 co-receptor usage has shown that MAIT cells are predominantly CD8α+ (CD8αβ+ and CD8αα+) in the blood of most individuals (Walker *et al.*, 2012; Gherardin *et al.*, 2018). A minority of MAIT cells are also CD4-CD8- cells and a significantly smaller proportion are CD4+CD8- cells (Gherardin *et al.*, 2018). The typical profile of MAIT cells in peripheral blood is thus: CD3+, mostly CD4-, CD161++, CD26++ and TRAV1-2+ (discussed below in section 1.9.2).

Whether the above markers can accurately define MAIT cells in different tissues and at various disease states is unclear (Godfrey *et al.*, 2019). Additionally, it is also challenging to determine whether MAIT cells may vary in their expression of these surface markers. Leeansyah *et al.* (2013) and Eberhard *et al.* (2014) observed a decrease in CD161 expression on MAIT cells in HIV infected individuals, raising questions about whether cells with downregulated CD161 expression were in fact MAIT cells. Following the advent of MR1 tetramers, it is now possible to identify MAIT cells with greater certainty (Reantragoon *et al.*, 2013; Gherardin *et al.*, 2018). The currently available tetramers used for MAIT cell identification come loaded with MAIT activating ligands derived from 5-A-RU such as 5-OP-RU and 5-OE-RU, or with non-activating ligand 6-FP which is used as a control tetramer (Reantragoon *et al.*, 2013; Corbett *et al.*, 2014). A longitudinal analysis of peripheral blood samples from HIV-positive individuals confirmed an increase in CD161-negative T cells but found that these did not bind the MR1 5-OP-RU tetramer (Fernandez *et al.*, 2015). The discordance in these findings suggests that caution should be exercised when interpreting results generated before the

advent of tetramers. Recent findings have demonstrated the previously unappreciated diversity of MAIT cell ligands (Keller *et al.*, 2017; Harriff *et al.*, 2018) and revealed the existence of MR1 5-OP-RU tetramer-dim and -negative cells which possess other phenotypic and functional MAIT cell features (Wong *et al.*, 2019; Pomaznoy *et al.*, 2020). Therefore, although this thesis focuses on MR1 5-OP-RU tetramer-positive MAIT cells, it must be noted that these may comprise only a subset of all human MAIT cells and may also include a small subset of MR1 tetramer-binding γδ T cells (Le Nours *et al.*, 2019). The use of the T cell receptor definition (discussed below in Section 1.9.2) can thus be helpful in excluding these cells from analyses of MR1 5-OP-RU tetramer-positive MAIT cells.

1.9.2. T cell receptor usage in MAIT cell definitions

Because MAIT cells utilise a semi-invariant TCR-α chain, they may also be detected using monoclonal antibodies against the TRAV1-2 gene segment (Martin *et al.*, 2009; Reantragoon *et al.*, 2013). Other MAIT cell surrogate surface markers are also included (for example, CD161 or CD26) to increase the reliability of this method. Using MR1 tetramers to determine the accuracy by which surface markers are able to identify peripheral blood MAIT cells, Gherardin *et al.* (2018) found that surface markers are able to identify most, but not all, MAIT cells and that the best markers for identifying MAIT cells was a combination of CD161 and TRAV1-2.

MAIT cells may also be identified using paired TCRα and TCRβ sequencing. The TRAV1-2 variable gene segment is often paired with a limited number of joining segments, namely TRAJ33, TRAJ12 and TRAJ20, and MAIT cells β-chain usage is also limited, with the TRBV20 and TRBV6 genes families being the most frequently utilised (Porcelli *et al.*, 1993; Tilloy *et al.*, 1999; Reantragoon *et al.*, 2013; Lepore *et al.*, 2014). The CDR3α region of the MAIT cells is of conserved length and typically has a tyrosine at the 95th position (Tyr95α) (Tilloy *et al.*, 1999; Reantragoon *et al.*, 2012; Gold *et al.*, 2014). The features of the MAIT CDR3α can therefore be used to identify MAIT cells using a web-based tool "MAIT Match", which compares the input CDR3α sequence to reference sequences on the server and assigns a similarity score. The closer the score to 1.0 the greater the similarity of the sequence to known MAIT cell CDR3α sequences (Wong *et al.*, 2019).

1.10. MAIT cell development and effector functions

Akin to conventional T cells, MAIT cells develop in the thymus (Tilloy *et al.*, 1999; Martin *et al.*, 2009). There, MAIT cells are selected by MR1-expressing CD4+CD8+ thymocytes, in contrast to conventional T cells, which are trained on MHC Class I and II-expressing thymocytes (Seach *et al.*, 2013). Thymic MAIT cells display a naïve phenotype which is also seen in the cord blood and occur

at low frequencies (Martin *et al.*, 2009; Murphy and Weaver, 2016). The frequency of MAIT cells in the periphery rapidly expands soon after birth, a process dependent on interactions with commensal flora and the metabolite ligands that they generate, and is accompanied by the acquisition of a MAIT cell memory phenotype (Martin *et al.*, 2009). Koay *et al.* (2016), using MR1 tetramers, identified a three-stage progression in the development of MAIT cells (**Figure 1.10.1**). The first stage, which occurs in the thymus, is characterised by low expression of CD27 and CD161. CD27 expression is acquired during the second stage. Stage 2 MAIT cells migrate into the periphery where they comprise a minority of circulating MAIT cells. A high level of CD161 expression is then acquired during the third and final stage (Koay *et al.*, 2016). Mature, stage 3 MAIT cells comprise the majority of circulating MAIT cells and express the transcription factor PLZF (promyelocytic leukaemia zinc finger) and interleukin 18 receptor (IL-18R) (Koay *et al.*, 2016). In mice, mature MAIT cells leaving the thymus predominantly express T-bet or RORγt, with these programmes directing MAIT cell subsets to specific peripheral sites (Koay *et al.*, 2016). For example, T-bet+ MAIT cells are directed to the liver and spleen, while RORγt+ MAIT cells are directed to the lungs (Legoux *et al.*, 2019). Interestingly, this polarisation appears to be absent in humans with MAIT cells capable of expressing multiple transcription factors simultaneously (Leeansyah *et al.*, 2015; Koay *et al.*, 2016). The mechanisms governing the targeting of MAIT cells to peripheral tissues and their maintenance at these sites thus remain unclear in humans. It has been speculated that microbial ligands derived from commensal bacteria at mucosal sites drive these processes as is the case in mice (Godfrey *et al.*, 2019; Lantz and Legoux, 2019), but the determinants of species-specific differences remain unresolved.

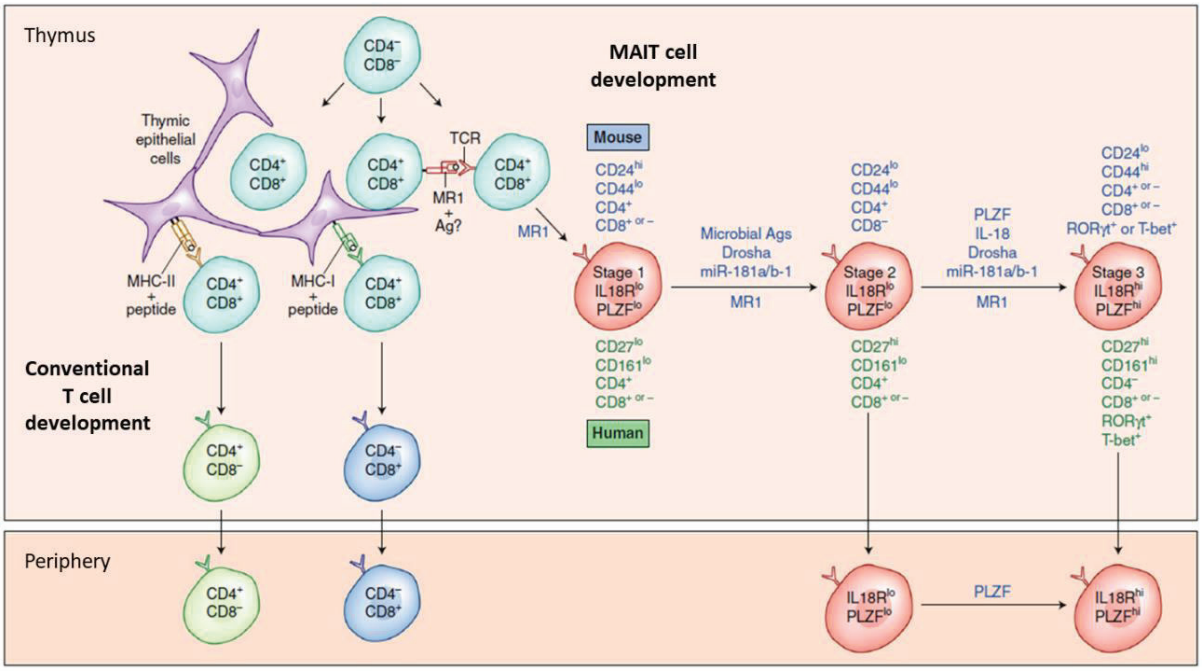


Figure 1.10.1: Outline of MAIT cell development contrasted to conventional T cell development. MAIT cells develop in the thymus through three stages of maturity as outlined by Koay *et al.* (2016). Stage 1 and 2 MAIT cells can be found in the thymus with a proportion of stage 2 MAIT cells being found in the peripheral circulation. Most peripheral blood MAIT cells are stage 3, functionally mature MAIT cells (from Godfrey *et al.* (2019)).

A recent study by Swarbrick *et al.* (2020) characterising MAIT cells in neonates has highlighted the importance of the expression of typical MAIT cell markers CD26 and TRAV1-2 in addition to CD161. The expression of these markers was increased in infants and correlated with the acquisition of effector functions in mature MAIT cells. MAIT effector functions occur as a result of activation. In MAIT cells activation may occur via direct activation of the MAIT cell TCR by MR1-presented antigen in a TCR-independent manner by cytokines binding to their cognate cytokine receptors (**Figure 1.10.2**). MAIT cells utilise MR1-dependent activation for the sensing of riboflavin synthesising bacteria and MR1-independent activation to sense secondary inflammation triggered by viruses and bacteria which do not synthesise riboflavin. Cytokines capable of activating MAIT cells include IL-18, IL-12, IL-15, and type I interferons (further discussed in Section 1.11.3) (Ussher *et al.*, 2014; Sattler *et al.*, 2015; van Wilgenburg *et al.*, 2016).

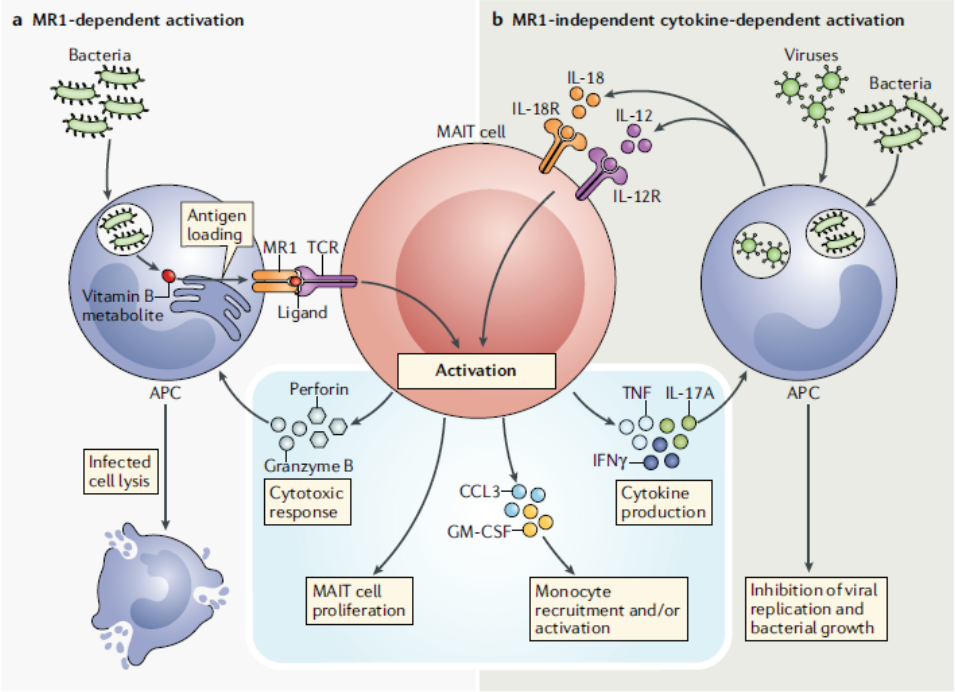


Figure 1.10.2: Modes of MAIT cell activation. Engagement of vitamin B derived antigen with the MAIT cell T cell receptor leads to MR1-dependent activation of MAIT cells (left), while the binding of cytokines to their cognate receptors results in cytokine-dependent activation (right). MR1-dependent activation allows for responses to vitamin B synthesising bacteria, while cytokine dependent activation enables perception of viruses and bacteria which do not synthesise bacteria (from Toubal *et al.* (2019)).

Activated MAIT cells produce a range of chemokines and cytokines which enable them to perform their functions (**Figure 1.10.3**). MAIT cell activation leads to effector functions which fall into three distinct categories (Provine and Klenerman, 2020). The first, which is associated with MR1- and cytokine-dependent co-activation, is direct cytotoxic T cell effector functions that lead to the lysing of infected cells or the killing of free-living bacteria (Gold *et al.*, 2010; Le Bourhis *et al.*, 2010; Boulouis *et al.*, 2020). The second recognised category, which can be mediated by cytokine-dependent activation alone, includes early production of cytokines that leads to the activation and differentiation of conventional lymphocytes as part of the adaptive immune response (i.e. signal amplification) (Meierovics and Cowley, 2016; Salio *et al.*, 2017). The third and most recently discovered category of MAIT cell function is wound healing and tissue repair and is associated with MR1-dependent activation (Constantinides *et al.*, 2019; Hinks *et al.*, 2019; Leng *et al.*, 2019).

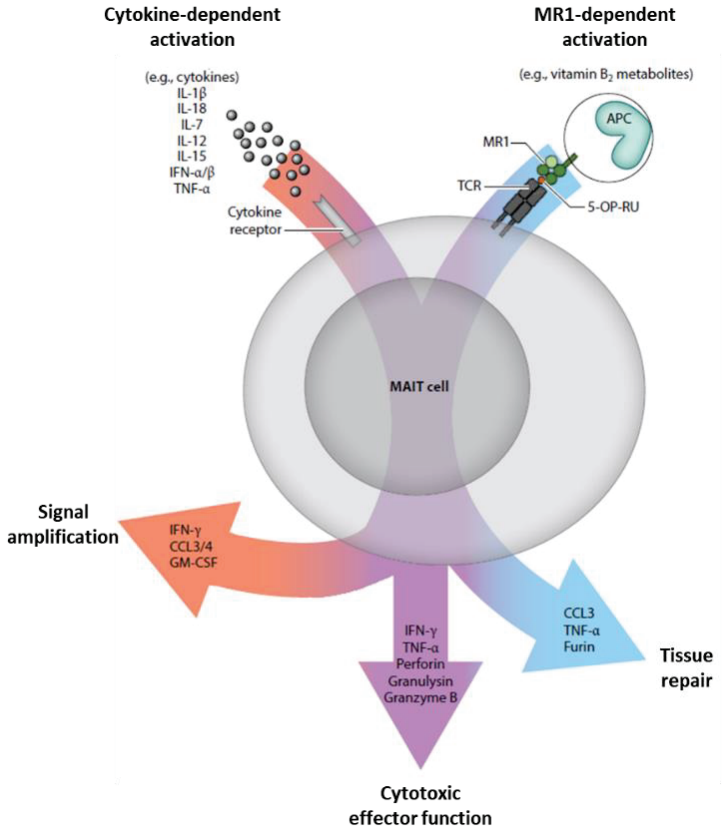


Figure 1.10.3: Activation of MAIT cells to perform effector functions in three main categories. Cytokine-dependent activation leads to signal amplifying functions of MAIT cells (orange); MR1- and cytokine-dependent activation results in cytotoxic effector functions (purple) and MR1-dependent activation is associated with tissue repair functions (blue) (from Provine and Klenerman (2020)).

The first function of MAIT cells to be recognised was production of cytokines and cytotoxic molecules. Le Bourhis *et al.* (2010) and Gold *et al.* (2010) were among the first to show that MAIT cells, when activated by bacterially infected cells, produce pro-inflammatory cytokines including IFN-γ and TNF-α (**Figure 1.10.3**). MAIT cells at mucosal sites have been shown to produce IL-17 and IL-22 (Dusseaux *et al.*, 2011; Gibbs *et al.*, 2017; Sobkowiak *et al.*, 2019). Additional cytokines produced by MAIT cells upon activation include MIP-1β and IL-2, while only low amounts of Th2 cytokines (e.g., IL-4, IL-5 and IL-10) have been reported in a limited number of studies (Le Bourhis *et al.*, 2010; Lepore *et al.*, 2014). MAIT cells produce granzymes, granulysin and perforin to mediate cytotoxicity in response to bacterial infection (Lepore *et al.*, 2014; Kurioka *et al.*, 2015). Through production of these products MAIT cells can lyse bacterially infected cells and also kill free-living *Escherichia coli* (*E. coli*) (Le Bourhis *et al.*, 2013; Boulouis *et al.*, 2020).

MAIT cells were next noted to promote the differentiation of monocytes into monocyte-derived dendritic cells (Mo-DCs) via the production of GM-CSF (Meierovics and Cowley, 2016). In a MR1 deficient mouse model, Meierovics and Cowley (2016) demonstrated that GM-CSF production by MAIT cells was required for the recruitment of CD4+ T cells into the lungs during *Francisella tularensis* (*F. tularensis*) live vaccine strain (LVS) infection. MAIT cells are also able to promote the maturation of primary and monocyte-derived DCs via a secondary mechanism which is CD40L- and MR1-dependent (Salio *et al.*, 2017). This mechanism results in functional, IL-12 producing DCs, and also leads to the transactivation of NK cells in a manner dependent on MR1 and IL-18. As part of their signal amplification functions, MAIT cells have also been shown to be capable of providing help to B cells. This was first demonstrated *ex vivo* where the addition of supernatants from activated MAIT cells to autologous B cells led to increases in the frequencies of antibody-secreting plasmablasts (Bennett *et al.*, 2017). This function was MR1-dependent and likely as a result of plasmablast differentiation from memory B cells (Bennett *et al.*, 2017). More recently, SIV vaccination and subsequent SIV infection in rhesus macaques resulted in SIV-specific tissue-resident memory B cells and later on SIV-specific antibody production that correlated with MAIT cell frequencies in the peripheral blood and bronchoalveolar compartments (Rahman *et al.*, 2020). These findings thus add to the view of MAIT cells as being important in orchestrating early response to infection to bridge the innate and adaptive arms of the immune system.

Most recently, assessment of the MAIT cell transcriptome in mice and humans has identified a tissue repair signature resembling that of commensal-specific H2-M3-restricted cells in mice (Linehan *et al.*, 2018). This tissue repair signature is dependent on TCR-mediated signalling and results in the expression of *VEGFA* and *TGFB1,* for example, which are involved in angiogenesis and extracellular matrix production, as well as *FURIN* and *MMP10* which are involved in chemotaxis and tissue remodelling respectively (Constantinides *et al.*, 2019; Hinks *et al.*, 2019; Leng *et al.*, 2019). Functionally, *E.coli* stimulated MAIT cells were able to repair a scratch in a colorectal cell line monolayer (Leng *et al.*, 2019), thus suggesting the importance of MAIT cells, not only as mediators of host defence but as players in the maintenance of tissue homeostasis.

1.11. MAIT cell responses to pathogens

1.11.1. Bacterial infection

MAIT cells have a varied response to a wide range of bacterial pathogens. Early studies recognized that MAIT cells became activated in the presence of cells infected with bacteria such as *E. coli* and *F.*

tularensis, but not others like *Enterococcus faecalis* (*E. faecalis*) and *Listeria monocytogenes* (*L. monocytogenes*) (Gold *et al.*, 2010; Le Bourhis *et al.*, 2010; Meierovics *et al.*, 2013). The mechanism for this discrimination between microbes was initially a mystery. After crystallization of MR1 by Kjer-Nielsen and colleagues in 2016, it became evident that MAIT cells sense bacteria via MR1-dependent presentation of microbially derived riboflavin intermediates which are not present in all bacteria. MAIT cells activated by *E. coli*-infected monocytes produced IFN-γ in a manner proportional to the *E. coli* multiplicity of infection (MOI) suggesting a dose-dependent relationship between microbially-derived small molecular ligand and effector function. Data from the MR1-deficient mouse model suggests that MAIT cells play a role in controlling infections with MAIT cell activating microorganisms. MR1-deficient mice have higher bacterial burdens and are less capable of controlling *E. coli* infection when compared to TCR-transgenic mice with more numerous MAIT cells (Le Bourhis *et al.*, 2010). In murine *F. tularensis* infection, MAIT cell frequency increased in the lungs of wild-type (WT) mice. These MAIT cells were also able to produce higher amounts of IFN-γ, TNF, IL-17A and inducible nitric oxide synthase (iNOS) in comparison to those from MR1-deficient mice. In MR1-deficient mice, cytokine and iNOS production were decreased and MAIT cell frequencies in the lung were lower, correlating with higher colony forming units (CFUs) of *F. tularensis* and delayed clearance (Meierovics *et al.*, 2013). These findings suggest that MAIT cells may be important in the early control of bacterial infections, however in MR1-deficient animal models it is difficult to ascertain whether the observed effects are due to MAIT cell deficiency or other effects of MR1.

1.11.2. Mycobacterial infection

Mycobacterial metabolic pathways include production of the riboflavin intermediates that activate MAIT cells. MAIT cells can be activated by *Mycobacterium abscessus* (*M. abscessus*)-infected APCs to produce IFN-γ (Le Bourhis *et al.*, 2010). In the mouse model, MR1-deficient mice were found to have higher *M. abscessus* burdens after experimental infection, suggesting that MAIT cells might be important in the control of mycobacterial infection (Le Bourhis *et al.*, 2010). In the murine model of *M. bovis* BCG infection, MAIT cells respond to infection by producing IFN-γ in a manner dependent on IL-12p40. WT mice in this study controlled infection more efficiently than MR1-deficient mice during the early stages of infection (Chua *et al.*, 2012).

In humans, Gold *et al.* (2010) found that a significant proportion of *Mtb*-specific CD8+ T cells in people with latent TB were MAIT cells. This finding has recently been confirmed and expanded in a transcriptomic analysis of *Mtb*-specific CD8+ T cells by Pomaznoy *et al.* (2020), which was also observed in latently TB infected individuals. Interestingly, Pomaznoy *et al.* (2020) found both MR1 5-OP-RU tetramer-positive and MR1 5-OP-RU tetramer-negative *Mtb*-reactive T cells. These groups of

Mtb-reactive T cells had distinct transcriptional profiles (further discussed in Chapter 4.1.2). During latent TB infection MAIT cells are reduced in the peripheral blood, as compared to healthy controls and are further depleted during active TB (Gold *et al.*, 2010). This reversible depletion in the periphery is in contrast to the lung where MAIT cells are enriched in TB infected adults, suggestive of redistribution to the site of infection (Gold *et al.*, 2010; Le Bourhis *et al.*, 2010; P.K. Sharma *et al.*, 2015; Wong *et al.*, 2019). During active TB, peripheral blood MAIT cells are less functional, producing lower amounts of TNF than their bronchoalveolar counterparts (Wong *et al.*, 2019). In the non-human primate model, intravenous BCG administration was shown to induce protective immunity to subsequent *Mtb* infection. In this model, bronchoalveolar MAIT cells (along with conventional CD4+ and CD8+ T cells) were found to expand and activate a Type II IFN gene cassette in in early infection, suggesting a role in the establishment of protective immunity (Darrah *et al.*, 2020). Interestingly, other recent studies in the rhesus macaque and murine models have shown that MAIT cells do not accumulate in the airways after experimental infection with *Mtb*, and when accumulation is artificially induced, they do not contribute to protection (Bucsan *et al.*, 2019; Vorkas *et al.*, 2020; Yu *et al.*, 2020). Taken together, these findings suggest that MAIT cells are capable of responding to *in vivo* mycobacterial infection, but that their role in response to infection is complex, involves interaction with both the innate and adaptive arms of the immune response, and requires additional work to be fully understood.

1.11.3. Viral infections

MAIT cell activation in response to viral infection is independent of TCR-mediated recognition of antigen and relies on cytokine-mediated stimulation (Ussher *et al.*, 2014). MAIT cells are able to respond to a range of viral infections including dengue, influenza and hepatitis C viruses (van Wilgenburg *et al.*, 2016). MAIT cells increased production of IFN-γ and granzyme B in response to coculture with virally infected APCs. TNF-α was only marginally increased in these experiments. Activation of MAIT cells is predominantly dependent on IL-18 acting synergistically with IL-12 and/or IL-15 (Sattler *et al.*, 2015; van Wilgenburg *et al.*, 2016). Type I interferons (IFN-α and IFN-β) are also capable of stimulating cytokine production in MAIT cells, but only in the presence of IL-18 or IL-12 (van Wilgenburg *et al.*, 2016). Individuals infected with dengue, influenza or hepatitis C virus have lower frequencies of circulating MAIT cells in comparison to healthy controls (Barathan *et al.*, 2016; Loh *et al.*, 2016; van Wilgenburg *et al.*, 2016). Cytolytic activity in MAIT cells was increased, as measured by granzyme B production, in all virally infected patients with CD38 expression, indicative of activation, also being elevated in dengue- and hepatitis C-infected patients (Barathan *et al.*, 2016; van Wilgenburg *et al.*, 2016). Levels of both granzyme B and CD38 increased over time until convalescence where they then decreased to healthy control levels (van Wilgenburg *et*

al., 2016). Overall, these studies indicate that MAIT cells respond to viral infection, though the specific role that they play in these infections requires further investigation.

1.12. MAIT cell phenotypes and functions in the mucosa

Originally named for their enrichment in mucosal tissues of the gastrointestinal tract, MAIT cells are found at notably high frequencies in many mucosal sites throughout the human body, making up as much as 60% of CD3+CD4- T cells found in the jejunum (Reantragoon *et al.*, 2013). MAIT cells highly express CCR6, CXCR6 and CCR5, as well as intermediate levels of CCR9 confirming homing to tissue sites, particularly the liver and gut (**Figure 1.12**) (Dusseaux *et al.*, 2011). A common feature of MAIT cells at mucosal sites is the high expression of T cell activation and tissue-retention markers such as HLA-DR, CD69, PD-1 and α4β7 (Gibbs *et al.*, 2017; Sobkowiak *et al.*, 2019). CD4-CD8- and CD4+ MAIT cells were also found to be the more dominant subsets in the oral mucosa in contrast to the peripheral blood where CD8+ MAIT cells were more common (Sobkowiak *et al.*, 2019). MAIT cell frequency varies depending on mucosal site. For example, X. Tang *et al.* (2013) confirmed the enrichment of MAIT cells in the liver, Booth *et al.* (2015) observed a lower frequency of MAIT cells in the lamina propria of the stomach, and Sobkowiak *et al.* (2019) found there to be no differences in the frequencies of MAIT cells in the oral mucosa compared to those in the peripheral blood. Mucosal MAIT cells are also capable of cytokine production upon bacterial activation, with a preference towards IL-17 and IL-22 production (X. Tang *et al.*, 2013; Gibbs *et al.*, 2017; Sobkowiak *et al.*, 2019). Mucosal MAIT cells also produced IFN-γ, TNF-α, granzyme B and perforin but at lower levels than those from the peripheral blood (Gibbs *et al.*, 2017; Sobkowiak *et al.*, 2019). T cell receptor usage also differs between compartments with the canonical TRAJ33 gene being most frequently used by peripheral blood MAITs, while the TRAJ12 was more dominant at various tissue sites including the gut, liver and kidney (Lepore *et al.*, 2014). No bias towards TRAJ12 usage was found in oral mucosal MAIT cells, however greater TRAJ diversity was observed in comparison to the peripheral blood (Sobkowiak *et al.*, 2019). These findings suggest a site-specific role of MAIT cells in antimicrobial defence at mucosal sites, as well as in the maintenance of barrier integrity.

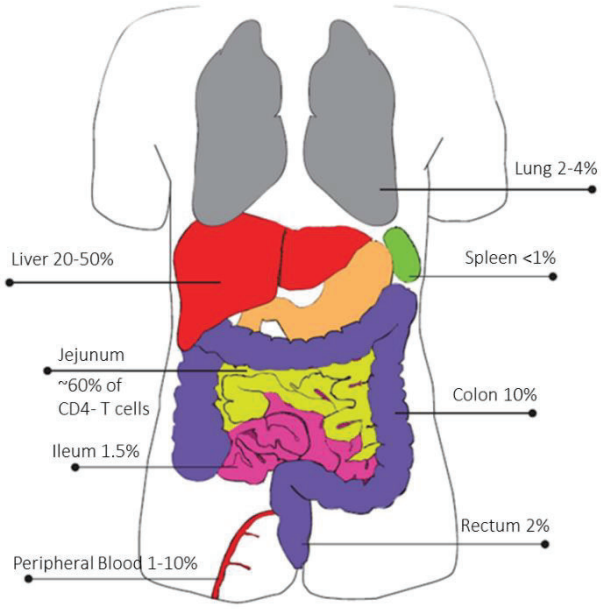


Figure 1.12: MAIT cell distribution at various anatomical sites. MAIT cells are enriched at mucosal sites making up a significant proportion of T cells especially at sites such as the liver and gut lamina propria in healthy individuals (taken from Kurioka *et al.* (2016)).

1.13. MAIT cell phenotypes and functions in the respiratory mucosa

Respiratory MAIT cells have been shown to exhibit a tissue-resident, memory phenotype characterised by the expression of CD103, CD69 and CD45RO (Mvaya *et al.*, 2019; Boulouis *et al.*, 2020; Lu *et al.*, 2020). CD103-expressing MAIT cells were found at higher frequencies in the airways of healthy adults when compared to the peripheral blood (Mvaya *et al.*, 2019). In children however, no significant differences were seen in the frequency of CD103+CD69+ MAIT cells when comparing the bronchoalveolar compartment to the blood (Lu *et al.*, 2020). Contrasting observations were made when comparing the functional responses of MAIT cells across the respiratory mucosa. More polyfunctional (IFN-γ+, TNF+ and IL-17A+) MAIT cells were found in the airway versus peripheral blood of adults, while nasopharyngeal MAIT cells were lower producers of granulysin, granzymes A and B, and perforin (Mvaya *et al.*, 2019; Boulouis *et al.*, 2020). The latter finding is in line with observations made by Lu *et al.* (2020), who showed that MAIT cells in the respiratory mucosa of healthy children produced significantly less IFN-γ, IL-17F, IL-21, IL-22 and IL-23 than those in matched peripheral blood. Notably, during adult TB infection and childhood pneumonia infection, MAIT cells are enriched at the lung mucosa and are more pro-inflammatory than those in the peripheral blood (Wong *et al.*, 2019; Lu *et al.*, 2020). Taken together these findings suggest that respiratory mucosal MAIT cells may display a certain phenotype during homeostatic conditions but

have the potential to mediate functional responses during bacterial infections. The literature on respiratory MAITs is less extensive than that on peripheral and gut-resident MAIT cells and many questions remain unanswered about MAIT cells in this compartment in human health and disease.

1.14. The impact of HIV on MAIT cells

Peripheral blood MAIT cells are depleted early in the course of HIV infection (Cosgrove *et al.*, 2013; Leeansyah *et al.*, 2013; Wong *et al.*, 2013) with the remaining MAIT cells displaying higher levels of markers of activation and exhaustion including CD38, HLA-DR and TIM-3. Leeansyah *et al.* (2013) reported that MAIT cells from people living with HIV are less functional and produce lower amounts of TNF, IL-17 and IFN-γ in response to *E. coli* infection (Leeansyah *et al.*, 2013). However, a subsequent study, using MR1 tetramer-defined MAIT cells, reported no significant difference in the amounts of IFN-γ and TNF-α produced by MAIT cells from HIV-positive individuals compared to those from healthy controls (Fernandez *et al.*, 2015). MAIT cells in the latter study were gated on from total lymphocytes, and the individuals assessed earlier during infection, which may both contribute to the differences in findings. Cosgrove *et al.* (2013), Leeansyah *et al.* (2013) and Wong *et al.* (2013) all reported that MAIT cells were not restored following otherwise effective ART. In a more recent study by X. Tang *et al.* (2020), partial restoration of MAIT cell function was observed via a slight increase in IFN-γ production following ART, while Sortino *et al.* (2018) found IL-7 treatment to be sufficient in restoring the frequency of peripheral blood MAIT cells. Nonetheless, significant depletion of MAIT cells, which as discussed above appear to play diverse roles in antimicrobial immunity, has been observed during early HIV infection and it has been hypothesised that this may increase the susceptibility of individuals to secondary infections including TB.

There have been very few studies investigating the fate of MAIT cells at tissue sites during HIV infection. Cosgrove *et al.* (2013), Eberhard *et al.* (2014) reported declines in the frequencies of MAIT cells at the gut mucosa, the lymph nodes and the airway lumen respectively, while Leeansyah *et al.* (2013) reported MAIT cell preservation in the rectal mucosa. Notably, Greathead *et al.* (2014) found the frequency of colon-resident MAIT cells to be restored following ART. Even fewer studies have described the consequences of HIV infection on bronchoalveolar MAIT cells. Bronchoalveolar MAIT cells were found to be decreased in simian immunodeficiency virus (SIV) infected rhesus macaques (Vinton *et al.*, 2016), and in HIV infected humans with increasing viral loads (Mvaya *et al.*, 2019). In this study by Mvaya *et al.* (2019), ART was found to restore MAIT cell frequencies in the lung, with HIV infection not having had profound impacts on the cytokine production of these cells. This study was limited by sample size, an expected difficulty in studies utilizing human bronchoscopy, and hence

further work is required in confirming and extending these findings to fully characterise MAIT cell quantity and quality at the lung mucosa, the site of TB infection.

1.15. Study research question, hypothesis, and aims

It is clear that HIV infection leads to the depletion and dysfunction of peripheral blood MAIT cells. The impact of HIV on the frequency and function of bronchoalveolar MAIT cells, which appear to be active in mediating local responses to *Mtb*, is less known. We sought to characterise the phenotype, function and transcriptome of bronchoalveolar MAIT cells from healthy individuals with latent *Mtb* infection and to determine the impact of HIV infection on the frequency, phenotype and function of these cells.

1.15.1. Hypothesis

We hypothesised that bronchoalveolar MAIT cells are phenotypically, functionally, and transcriptionally distinct from those in the peripheral blood and that HIV infection would abrogate compartment-specific features.

1.15.2. Aim 1 and objective

First, we aimed to characterise MAIT cells from the bronchoalveolar compartment and to compare their frequency, phenotype, and function to their peripheral blood counterparts in healthy humans with latent TB infection.

MR1 tetramer was used to identify MAIT cells from bronchoalveolar lavage (BAL) fluid and peripheral blood of HIV-negative people with latent TB infection. MR1 tetramer-positive MAIT cells were characterised using surface phenotyping, cytokine and cytolytic enzyme production to understand the compartment-driven differences in MAIT cell phenotype and function. T cell clones were cultivated from the bronchoalveolar compartment to confirm MAIT cell identity of subpopulations displaying atypical expression of classical MAIT cell markers. The identity of these T cell clones as MAIT cells was assessed by performing surface staining, functional assays to assess MR1-dependent activity, and by T cell receptor sequencing.

1.15.3. Aim 2 and objective

Second, we aimed to characterise HIV-driven differences in MAIT cells from the BAL fluid and peripheral blood of HIV-negative and HIV-positive participants with latent TB infection.

We characterised MAIT cell phenotype and function in both the bronchoalveolar and peripheral blood compartments and compared the characteristics of MAIT cells from HIV-negative and HIV-positive participants. The goal of this aim was to define the impact of HIV infection, specifically on bronchoalveolar MAIT cells, with the eventual goal of trying to understand how HIV might alter anti-TB immune responses involving MAIT cells.

1.15.4. Aim 3 and objective

Third, we aimed to use bulk population and single-cell transcriptomics to understand the functional differences between bronchoalveolar and peripheral MAIT cells.

MR1 tetramer-positive MAIT cells were sorted into single-cells and mini-populations from which mRNA was extracted, reversed transcribed and subjected to RNA-sequencing. After alignment to the human genome, the expression of genes was analysed using supervised and unsupervised methods to determine the transcriptional features and heterogeneity of bronchoalveolar and peripheral MAIT cells.

1.16. Study design

Paired peripheral blood and BAL fluid samples were collected from consenting adult participants living in KwaZulu-Natal, South Africa. Participants were enrolled through two bronchoscopy cohorts (details provided in Chapter 3). All participants were latently TB infected and either HIV-negative or HIV-positive. This study design allowed for direct comparisons to be made between compartments (i.e. peripheral vs bronchoalveolar compartment) as well as within compartment between groups based on HIV infection status (i.e. HIV-negative vs HIV-positive).

1.17. Thesis outline

This thesis is organised as follows:

Chapter 1 reviews the literature pertaining to the major points discussed in this thesis. These include the global burden of HIV and TB and how these two diseases impact the human host and its immune system. MAIT cells are then introduced, including methods for defining them, their development, their anatomical distribution, their effector functions and what is known about their responses to pathogens.

Chapter 2 presents the methods and findings pertaining to the first aim of the study, which was to characterise the compartment-driven differences in MAIT cells from the peripheral blood and

bronchoalveolar compartment of healthy individuals using flow cytometry and T cell cloning to define phenotype and function.

Chapters 3 presents the methods and findings pertaining to the second aim of the study, which was to characterise HIV-driven differences on peripheral blood and bronchoalveolar MAIT cells by comparing the frequency, phenotype, and function of cells from both compartments of HIV-negative and HIV-positive individuals.

Chapter 4 presents the methods and findings pertaining to the third aim, which characterised the transcriptomic features of MAIT cells from the peripheral blood and bronchoalveolar compartments using both bulk population and single-cell RNA sequencing.

Chapter 5 discusses the key findings of this thesis, synthesising the data presented in Chapters 2 - 4, drawing overall conclusions and placing the findings in the context of previously published literature. This chapter also discusses study limitations and future directions that are suggested by this and related recent work.

CHAPTER 2

CHAPTER 2: THE PHENOTYPE AND FUNCTION OF MAIT CELLS IN THE PERIPHERAL BLOOD AND AT THE LUNG MUCOSAL SURFACE

2.1. Introduction

There is mounting evidence suggesting that MAIT cells potentially play a role in the defence against respiratory pathogens including *Mtb*. Very little is known, however, about MAIT cell phenotype and function in the bronchoalveolar compartment of healthy humans – the site of *Mtb* infection. To address this gap, we sought to compare the frequency, surface phenotype and function of MAIT cells from paired bronchoalveolar lavage fluid and peripheral blood of HIV-negative people with evidence of latent TB infection.

2.1.1. Identification of MAIT cell phenotypes in the lung mucosal and peripheral blood compartments

The detailed study of individual immune cell subsets has traditionally been reliant on the ability to identify a specific combination of surface markers that can be used in flow cytometry to distinguish the subset. Prior to the invention of MR1 tetramers, MAIT cells were identified based on their use of the TRAV1-2 α-chain of the T cell receptor (TCR), their high expression of CD161 and expression of the cytokine receptor IL-18Rα (Martin *et al.*, 2009; Le Bourhis *et al.*, 2010; Reantragoon *et al.*, 2013). High expression of CD26 has also been consistently found on circulating MAIT cells and some studies have used high levels of co-expression of CD161 and CD26 as a way to identify MAIT cells (P.K. Sharma *et al.*, 2015; Suliman *et al.*, 2019). There are several limitations in using combinations of surface markers to define cellular subsets. One limitation is that the expression levels of these markers can vary during activation and other perturbations of cellular function; CD161 expression has been noted to vary on MAIT cells during activation and in states of infection (Gold *et al.*, 2010; Leeansyah *et al.*, 2013; Eberhard *et al.*, 2014). Another limitation is that by defining cells based on their expression of certain proteins, it becomes impossible to assess levels of expression of that protein on that immune subset. Finally, there is the risk that these approaches will fail to identify atypical subsets that feature previously undescribed surface phenotypes (Gherardin *et al.*, 2016; Meermeier *et al.*, 2016; Pomaznoy *et al.*, 2020). The advent of the MR1 5-OP-RU tetramer makes it possible to assess the surface and functional phenotypes of MAIT cells that bind the tetramer without bias based on previous phenotypic definitions. Using this method does however exclude the possibility of identifying MAIT cells that recognise alternative small molecule ligands which may have biological significance (Harriff *et al.*, 2018). The use of the MR1 5-OP-RU tetramer does for now remain the most standard method for identifying MAIT cells (Gherardin *et al.*, 2018) and thus using this approach, we aimed to determine the surface and functional phenotypes of MR1 5-OP-RU

tetramer-positive MAIT cells (hereafter referred to as “MR1 tetramer” and “MAIT cells” respectively) from the lung mucosal and peripheral blood compartments of healthy, HIV-negative humans with evidence of latent TB infection.

2.1.2. The use of T cell cloning and functional assays in assessing MAIT cell phenotype and function

In addition to surface and tetramer staining of MAIT cells (discussed in Chapter 1, section 1.9), another technique that can be employed to improve the understanding of MAIT cell biology is T cell cloning. This method not only increases the MAIT cell numbers to enable functional experimentation on greater numbers of cells, but also allows for the characterisation of clonally expanded MAIT cell subpopulations with varied specificities (Cansler *et al.*, 2020). A key component of generating MAIT cell clones is confirmation of the clone’s cell identity by assessing MR1-restricted function. Previously, this has been done with the use of anti-MR1 blocking antibodies to inhibit the function of MR1-restricted cells, and more recently using MR1-knocked out cell lines generated using CRISPR/Cas9 technology (Gold *et al.*, 2014; Laugel *et al.*, 2016).

Using this technique Gold *et al.* (2010) and Lepore *et al.* (2014) identified a proportion of MAIT cells which utilise alternative TRAJ gene segments (TRAJ12 and TRAJ20) to the previously reported TRAJ33 segment (Porcelli *et al.*, 1993; Tilloy *et al.*, 1999). Lepore *et al.* (2014) also showed a skewing towards TRAJ12 usage in tissue-resident MAIT cells, suggestive of varied antigen exposure at different anatomical sites and confirmed MAIT cell bias towards using TRBV6 and TRBV20 genes. In a follow up study, Lepore *et al.* (2017) identified MR1-restricted T cells which did not recognize microbial ligands but rather self-antigens presented on cancerous cells. These studies, although limited to the investigation of MAIT cell clones generated only from the peripheral blood, were able to expand the knowledge of MAIT cell TCR usage and effector functions. Thus, the focus of this chapter is on the characterisation of bronchoalveolar MAIT cells, using surface and functional phenotyping as well as T cell cloning.

2.2. Methods

2.2.1. Study participants

Matched peripheral blood and bronchoalveolar lavage (BAL) fluid samples were collected from HIV-negative participants undergoing either research (n = 23) or clinically indicated (n = 8) bronchoscopies at Inkosi Albert Luthuli Central Hospital (IALCH) in Durban, South Africa.

Participants had all provided written consent for study procedures and ethical approval of the study protocol had been received from the University of KwaZulu-Natal (UKZN) Biomedical Research Ethics Committee (BREC) (protocol numbers BF503/15 and BE037/12) and the Partners Institutional Review Board. The screening of HIV-negative participants occurred concurrently with that of HIV-positive participants (further discussed in Chapter 3) and thus all individuals were subject to the same study procedures which did not include the use of an HIV rapid test. Healthy participants in the research bronchoscopy cohort were HIV-negative (negative HIV ELISA and undetectable HIV viral load) with latent TB infection (LTBI) as determined by a positive QuantiFERON-TB Gold Plus (QFT-Plus) assay (Qiagen) (background subtracted *Mtb*-peptide pool stimulated IFN-γ > 0.35 IU/mL). Participants from the clinically indicated bronchoscopy cohort had their HIV status defined by HIV ELISA and viral load. The characteristics of the study participants from both cohorts are shown in **Table 2.2.1**. Participants with a history of TB as well as those with radiographic signs consistent with active TB, or those with symptoms of respiratory infection (cough, fever, shortness of breath) were excluded from the research bronchoscopy cohort. In the clinical bronchoscopy cohort, participants with respiratory infections were excluded based on positive bacterial, fungal or mycobacterial BAL fluid cultures. These individuals were in a good state of health overall, ensuring that they were comparable to those in the research bronchoscopy cohort and allowing for a greater number of participants to be assessed in subsequent analyses. As these cohorts were established in a TB endemic region with the majority of adult individuals already having encountered *Mtb*, QFT-negative individuals were excluded. This was to ensure that the HIV-positive and -negative arms were comparable and to prevent the inclusion of individuals with false QFT-negative results which are frequent in HIV-positive individuals with low CD4 counts (Ndzi *et al.*, 2016).

Table 2.2.1: Demographic and clinical characteristics of healthy, HIV-negative participants from the research bronchoscopy and clinically indicated (clinical) bronchoscopy cohorts.

	Research bronchoscopy cohort	**Clinical bronchoscopy cohort**
N	23	8
Age, years (IQR*)	32 (25 - 36)	45 (29.25 - 54.75)
Sex, number of females (%)	16 (69.6%)	2 (25%)

2.2.2. Preparation of peripheral and bronchoalveolar lymphocytes

BAL fluid was combined 1:1 with complete RPMI (cRPMI) media (10% fetal bovine serum, 200 mM L-glutamine, 10 000 U/mL penicillin-streptomycin and 250 µg/mL amphotericin) or left undiluted and transported back to the laboratory on ice. Samples were processed under BSL-3 conditions, within 3 hours of collection. BAL fluid was first filtered through a 40 µm strainer (BD Pharmingen) and the strained fluid then centrifuged. The pelleted lymphocytes were resuspended in cRPMI media for immediate characterisation or cryopreserved for later use. Concurrently, peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using the Histopaque® (Sigma-Aldrich) gradient centrifugation method. PBMC isolation was performed according to the manufacturer's instructions, where plasma was first removed from centrifuged whole blood which was then diluted (1:1 with sterile PBS), overlaid on the Histopaque® (Sigma-Aldrich), centrifuged with no brakes and the cell layer washed before being resuspended in cRPMI for immediate use or cryopreserved. Differential cell counts were performed on sample aliquots from both the BAL fluid and peripheral blood compartments and the cellular composition of the BAL fluid also microscopically assessed after cytocentrifugation to ensure there was no contamination by cells of the upper respiratory tract (Muema *et al.*, 2020).

2.2.3. Flow cytometry panel for characterising MAIT cells

Surface staining was performed on matched PBMCs and BAL lymphocytes. Samples were first stained with either 1:500 of MR1 5-OP-RU tetramer (PE) or MR1 6-FP tetramer (PE) (NIH Tetramer Core Facility), for 40 minutes in the dark at room temperature (RT). This was followed by staining with a fixable viability dye (Live/Dead Aqua, Invitrogen) and one of two surface staining antibody cocktails for 20 minutes at 4°C (listed in **Table 2.2.3**).

Table 2.2.3: List of antibody cocktails used to characterise MAIT cells from each of the study cohorts.

Clinical bronchoscopy cohort				
Antibody target	**Fluorophore**	**Clone**	**Manufacturer**	**Dilution ratio**
Live/Dead	Aqua	NA	Invitrogen	1:800
CD3	PE-CF594	UCHT1	BD Biosciences	1:100
CD4	BV711	OKT4	BioLegend	1:50
CD8	APC-H7	SK1	BD Biosciences	1:200
CD161	PE-Cy7	HP-3G10	BioLegend	1:50
CD26	FITC	BA5b	BioLegend	1:50
HLA-DR	BV605	L243	BioLegend	1:100
PD-1	BV421	EH12.1	BD Biosciences	1:50
CD69	BUV395	FN50	BD Biosciences	1:50
Research bronchoscopy cohort				
Antibody target	**Fluorophore**	**Clone**	**Manufacturer**	**Dilution ratio**
Live/Dead	Aqua	NA	Invitrogen	1:800
CD3	BV650	OKT3	BioLegend	1:50
CD4	BV711	OKT4	BioLegend	1:50
CD8	PE-Texas Red	3B5	Thermo Fisher	3:100
CD161	PE-Cy7	HP-3G10	BioLegend	1:100
CD26	FITC	BA5b	BioLegend	1:50
PD-1	BV421	EH12.1	BD Biosciences	1:50
TIM-3	BV785	F38-2E2	BioLegend	1:50

In addition to the MR1 5-OP-RU tetramer, CD3, CD4, CD8, CD161 and CD26 were included in the panel in order to define the T cell compartment and assess the surface phenotype of MAIT cells. We also included HLA-DR, PD-1 and TIM-3 to define the activation and inhibitory status of MAIT cells in both compartments. Samples were then acquired on the FACSAria™ III (BD Biosciences) and the data analysed using FlowJo™ (v10.4).

MAIT cells were gated on from live, single lymphocytes that were CD3+ and CD4-, and bound to the MR1 5-OP-RU tetramer (**Figure 2.2.3.1**). The MR1 5-OP-RU gate was defined using the MR1 6-FP tetramer, and unstained or fluorescence minus one controls (FMOs) used to define gates for HLA-DR, PD-1 and TIM-3 staining (**Figure 2.2.3.2**).

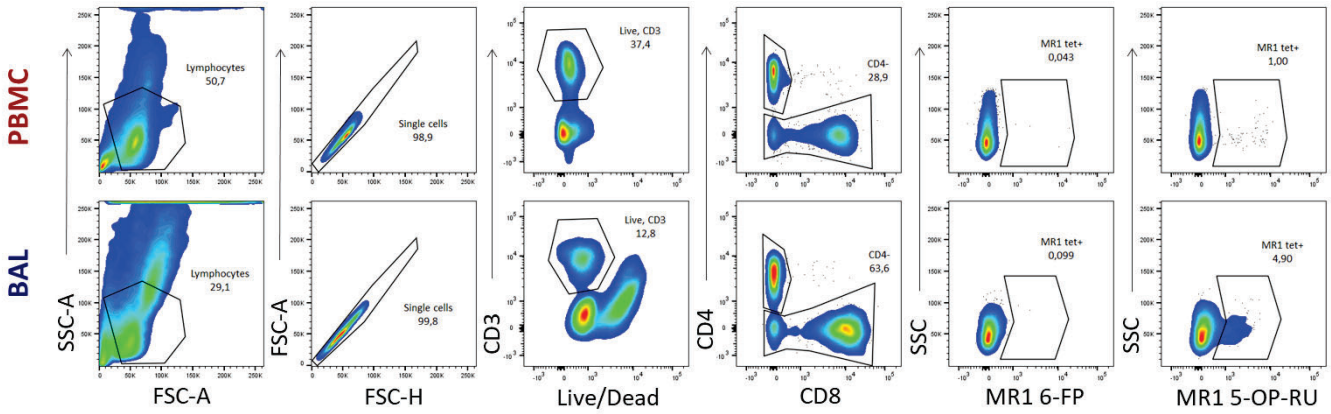


Figure 2.2.3.1: Gating strategy used for the phenotypic characterisation of peripheral blood and bronchoalveolar MAIT cells.

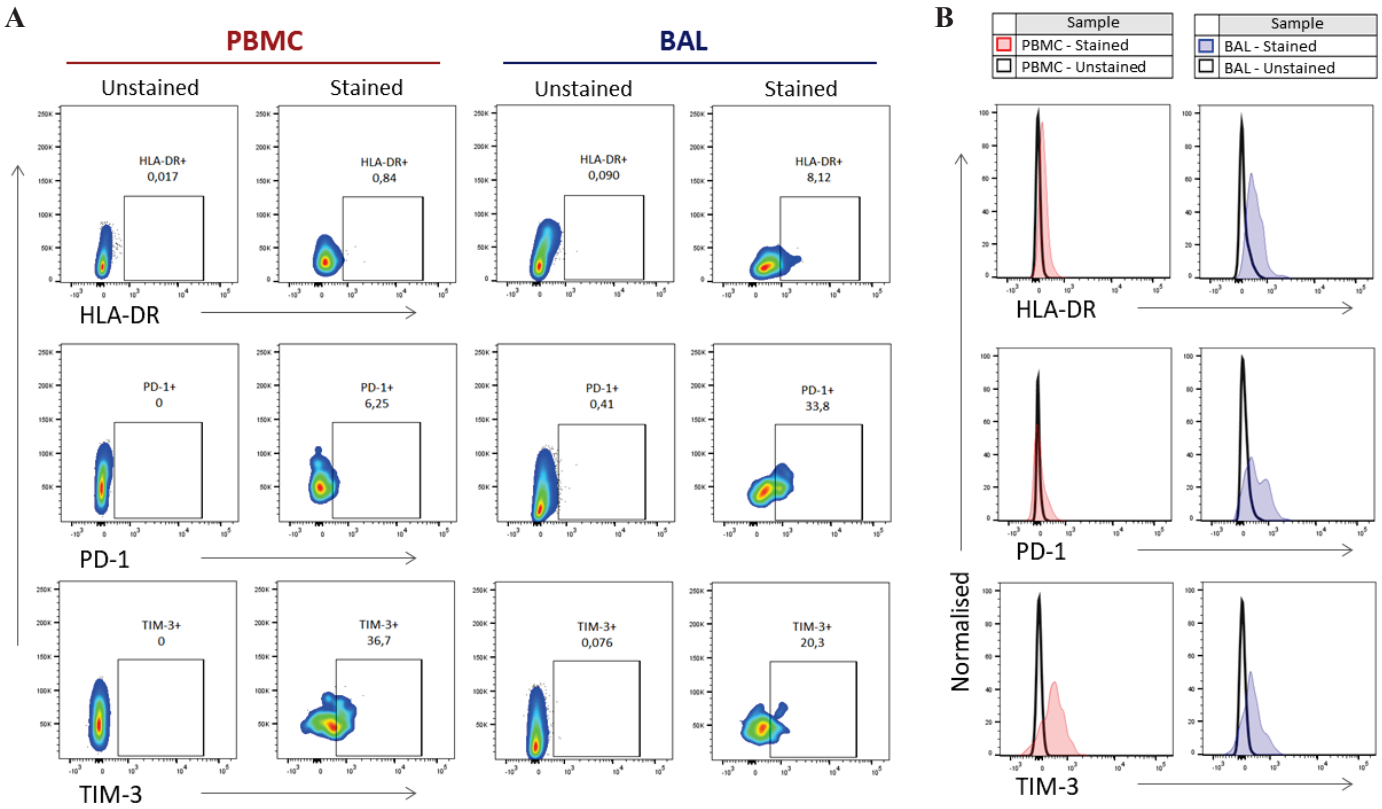


Figure 2.2.3.2: (**A**) Staining controls used to define gates for HLA-DR, PD-1 and TIM-3 positive MAIT cells in the peripheral blood (PBMC) and bronchoalveolar (BAL) compartments, and (**B**) characteristic staining intensity of each marker in peripheral blood and bronchoalveolar MAIT cells.

2.2.4. Determination of MAIT cell functional capacity

The functional capacity of MAIT cells was determined using intracellular cytokine staining (ICS). Using this method, we stained for IFN-γ, IL-17 and granzyme B to measure Th1 and Th17 cytokine responses as wells as cytolytic granule production, respectively. Freshly isolated paired BAL lymphocytes and PBMCs were simultaneously stimulated *ex vivo* using PMA (25 ng/mL) and ionomycin (500 ng/mL) together with 2µl/mL of BD GolgiStop™. Cell were stimulated for 6 hours at 37°C in 96-well microplates, when sufficient cell numbers were available. Because we had insufficient cell numbers to perform multiple stimulations, we were unable to perform MR1-specific stimulations. We instead used PMA/ionomycin stimulations as these have been shown to induce IL-17 production, which is of particular interest in tissue resident MAIT cells (Dusseaux *et al.*, 2011). After 6 hours, plates were washed in PBS before being stained with either MR1 6-FP (PE) or MR1 5-OP-RU (PE) tetramer (NIH Tetramer Core Facility). Tetramer staining was performed in the dark at RT for 40 minutes, followed by surface staining at 4°C for 20 minutes. The surface staining antibodies used are listed at the top of **Table 2.2.4**. Cells were then fixed (Fix & Perm Medium A, Invitrogen) in the dark at RT for 15 minutes and permeabilised (Fix & Perm Medium B, Invitrogen) for 20 minutes at 4°C. Intracellular antibodies were added during permeabilisation and are listed at the bottom of **Table 2.2.4**. Cells were PBS washed, acquired using the LSRFortessa™ (BD Biosciences) and the data then analysed using FlowJo (v10.4). IFN-γ and IL-17 data were background subtracted (presented as "inducible") and because PMA/ionomycin stimulation does not result in significant production of granzyme B by MAIT cells (Kurioka *et al.*, 2015; Dias *et al.*, 2018), unstimulated granzyme B data are presented ("constitutive").

Table 2.2.4: Surface and intracellular markers used to perform functional characterisation of MAIT cells.

	Antibody target	Fluorophore	Clone	Manufacturer	Dilution ratio
Surface antibodies	Live/Dead	Aqua	NA	Invitrogen	1:800
	CD4	BV711	OKT4	BioLegend	1:25
	CD8	APC-H7	SK1	BD Biosciences	1:100
	CD161	PE-Cy7	HP-3G10	BioLegend	1:50
	CD26	FITC	BA5b	BioLegend	1:50
Intracellular antibodies	CD3	AlexaFluor700	UCHT1	BioLegend	1:50
	IFN-γ	PE-Dazzle594	4S.B3	BioLegend	1:50
	IL-17	BV421	BL168	BioLegend	1:50
	Granzyme B	AlexaFluor647	GB11	BioLegend	1:50

MAIT cells were gated on from live, single, CD3+CD4- lymphocytes, as shown in **Figure 2.2.3**, using the MR1 6-FP tetramer to define the MR1 5-OP-RU gate. Gate placement for each cytokine and cytolytic molecule was determined using an unstimulated control each for the peripheral blood and BAL fluid samples as indicated in **Figure 2.2.4**.

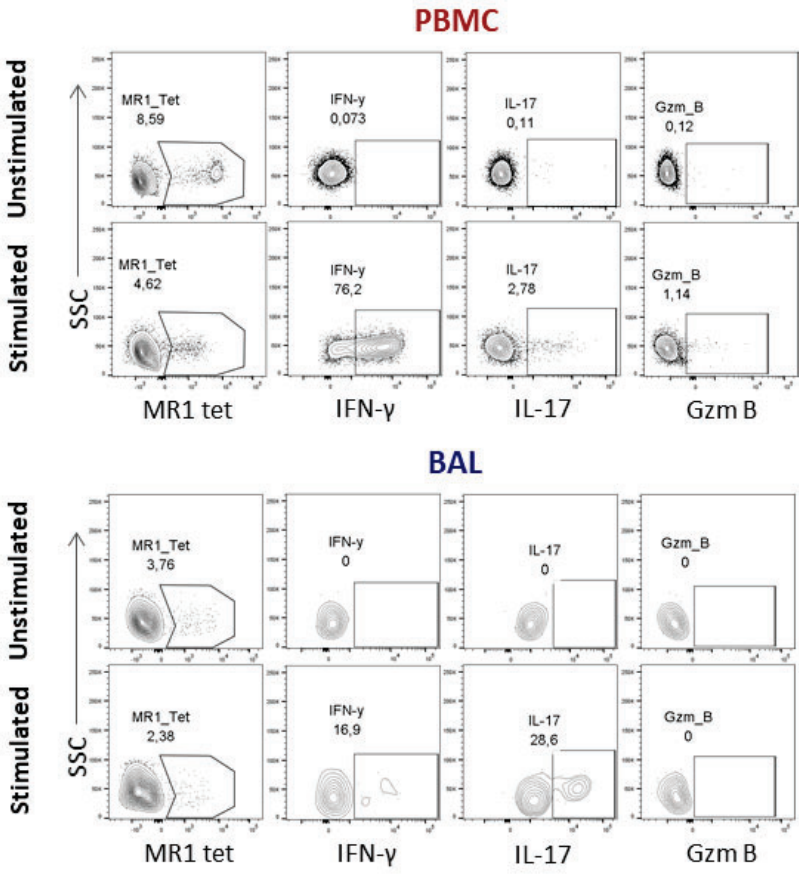


Figure 2.2.4: Gating strategy used to define cytokine producing CD3+CD4- MR1 5-OP-RU tetramer-positive cells from the peripheral blood (PBMC) and bronchoalveolar compartment (BAL) observed in the unstimulated (top row) and stimulated (bottom row) conditions.

2.2.5. Bronchoalveolar MAIT cell enrichment and sorting for T cell cloning

In order to evaluate the characteristics of T cells cloned from subsets expressing different combinations of CD161 and CD26, T cell cloning was performed on bronchoalveolar cells of one participant who displayed distinctive heterogeneity in the expression of these markers. Briefly, cryopreserved BAL-derived mononuclear cells were thawed and resuspended in cRPMI media containing 10% fetal bovine serum (FBS), 200 mM L-glutamine, 10 000 U/mL penicillin-streptomycin, 250 µg/mL amphotericin and 1M HEPES buffer, with 100 µL of DNase (3 mg/mL).

Cells were centrifuged, resuspended in cRPMI containing 10% human serum (HuS) and counted using the haemocytometer method. After centrifugation, cells were resuspended in PBS containing 2% FBS and 1mM EDTA at 5 x 10^7 cells/mL, EasySep™ Human T cell Enrichment Kit (Stemcell Technologies) enrichment cocktail added, and samples incubated for 10 minutes at RT. Thoroughly vortexed magnetic particles were then added followed by a 5 minute incubation at RT. Samples were topped up with buffer (PBS with 2% FBS and 1mM EDTA), pipette-mixed and incubated for 5 minutes at RT in the EasySep™ magnetic block. Samples were then transferred to a fresh tube and staining performed for subsequent sorting. T cell enriched BAL fluid samples were then stained with 1:500 MR1 5-OP-RU tetramer (PE, NIH tetramer Core Facility) for 40 minutes in the dark at RT. This was followed by staining with a cocktail of surface antibodies and live/dead viability dye (listed in **Table 2.2.5**) and incubation for a further 20 minutes at 4°C. CD3 was excluded from the master-mix to minimize the T cell stimulation that may occur through the staining and sorting processes.

Table 2.2.5: List of antibodies used to sort bronchoalveolar MR1 5-OP-RU tetramer-positive MAIT cells to perform limiting dilution T cell cloning.

Antibody target	Fluorophore	Clone	Manufacturer	Dilution ratio
Live/Dead	Aqua	NA	Life Technologies	1:250
CD4	FITC	OKT4	BioLegend	1:25
γδTCR	FITC	5A6.E9	Invitrogen	1:25
CD26	PE-Cy7	BA5b	BioLegend	1:25
CD161	AlexaFluor647	HP-3G10	BioLegend	1:50

MAIT cells were sorted using an Influx flow cytometer (BD Biosciences). FSC and SSC were used to define the lymphocyte population, trigger pulse width and FSC to identify single cells and the live/dead viability stain to exclude dead cells (**Figure 2.2.5**). CD4 and γδTCR were used to define the dump channel and only CD4- γδTCR- and MR1 tetramer-positive cells were carried forward. Subpopulations expressing varying levels of CD161 and CD26 were then identified and sorted from these MR1 tetramer-positive cells.

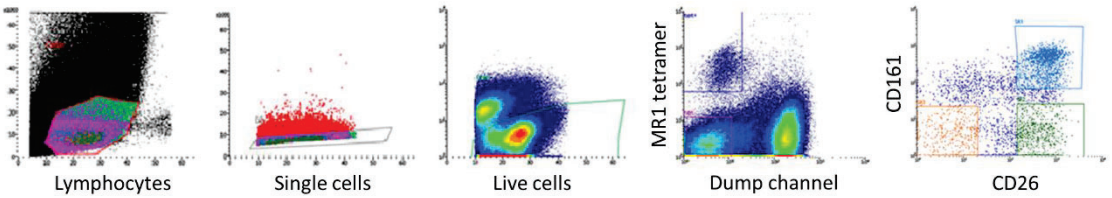


Figure 2.2.5: Sorting strategy used to obtain bronchoalveolar MR1 5-OP-RU tetramer-positive MAIT cells of different phenotypic subpopulations for liming dilution T cell cloning.

2.2.6. Cloning by limiting dilution assay

MR1 5-OP-RU tetramer-positive cells from each of three phenotypic subsets (CD161+CD26+, CD161-CD26+ and CD161-CD26-) were washed twice in antibiotic containing cRPMI with %10 human serum (HuS) and rested overnight in media containing low-dose recombinant human (rh)IL-2 (0.5 ng/mL). Limiting dilution cloning was performed the next morning as described by Cansler *et al.* (2020), with the aim of seeding cells at one cell per well in 96-well plates with RPMI media containing 10% HuS, 2% L-glutamine and 0.1% gentamycin. PBMCs and cells from a lymphoblastoid cell line (LCLs) were irradiated using 3000 cGy and 6000 cGy respectively (X-Rad 320, Precision X-ray), and added to wells as feeder cells. PBMCs were added at 1.5 x 10^5 cells/well and LCLs at 3 x 10^4 cells/well. The feeder-containing media that cells were seeded into, also included rhIL-2 (5 ng/mL), rhIL-7 (0.5 ng/mL), rhIL-12 (0.5 ng/mL), rhIL-15 (0.5 ng/mL) (BioLegend) and soluble anti-CD3 (0.03 µg/mL) (eBiosciences). In additional plates, cells were seeded at densities of 3, 10 and 30 cells/well as growth controls. Plates were then wrapped in foil to prevent excessive media evaporation and then incubated for 2 - 3 weeks at 37°C in 5% CO_2. Plates were assessed at weekly intervals for growth and the best-looking cells in terms of roundness and fullness of growth (termed “buttons”), were taken forward for identity confirmation and further characterisation (**Figure 2.2.6**).

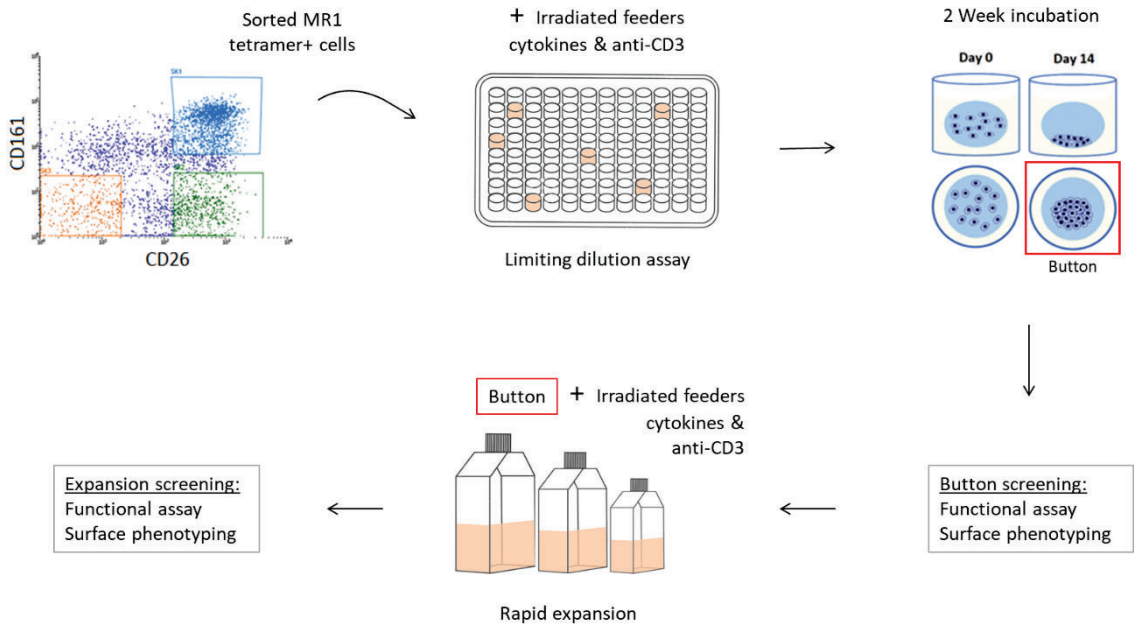


Figure 2.2.6: Workflow used for T cell cloning beginning with sorting and limiting dilution assay followed by phenotypic and functional screening. Clones confirmed to be MAIT cell clones are rapid expanded and an additional round of screening performed on expanded clones.

With the goal of identifying plates in which single cells had grown into clonal populations, plates were taken forward only when less than 30% of the wells had evidence of growth as this would indicate clones of single-cell origin according to Poisson distribution. Cell "buttons" growing from the bottom of each well were divided into four. One quarter was used for surface phenotyping, another quarter for functional confirmation of MR1-restricted activity, another quarter for expansion and the last quarter stored in liquid nitrogen for future experiments.

2.2.7. Characterisation of clonal growth

2.2.7.1. Phenotypic assessment of clonal growth

Resulting buttons were subjected to surface 1:500 MR1 5-OP-RU tetramer (PE, NIH Tetramer Core Facility) staining for 40 minutes in the dark at RT and followed with a master-mix of monoclonal antibodies and live/dead viability dye (listed in **Table 2.2.7.1**) for an additional 20 minutes at 4°C in the dark.

Table 2.2.7.1: Antibodies used to perform phenotypic confirmation of MAIT cell identity from liming dilution buttons and later, expanded clones.

Antibody target	Fluorophore	Clone	Manufacturer	Dilution ratio
Live/Dead	Aqua	NA	Life Technologies	1:200
CD3	PerCP/Cy5.5	UCHT1	BioLegend	1:50
CD4	BV785	OKT4	BioLegend	1:50
CD8	FITC	RPA-T8	BD Biosciences	1:100
CD26	PE-Cy7	BA5b	BioLegend	5:200

After staining, cells were fixed overnight in 1% PFA and acquired using the LSR II (BD Biosciences) the following morning. CD3+ cells were gated on from live, single lymphocytes and then all other gates (CD4 vs CD8, MR1 tetramer and CD26) gated on from the CD3 gate (**Figure 2.2.7.1**).

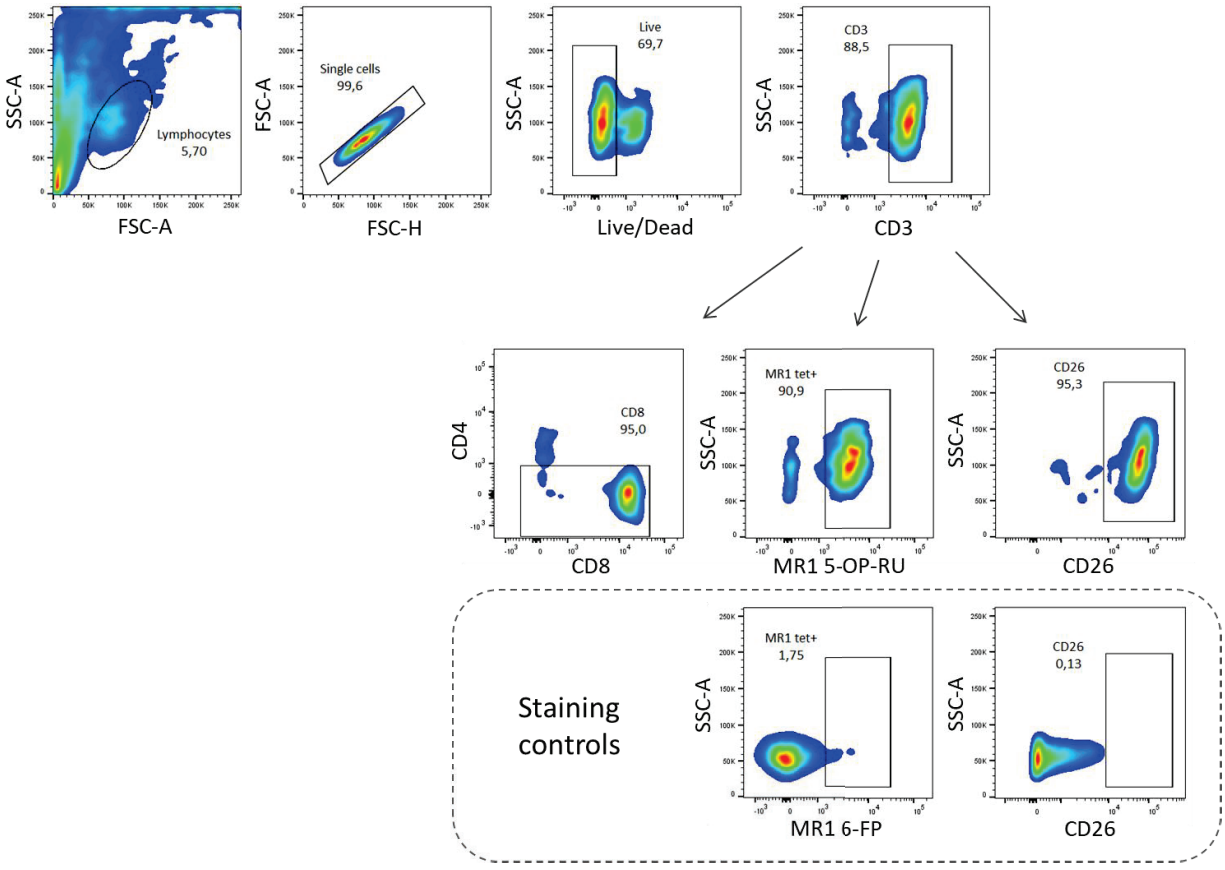


Figure 2.2.7.1: Gating strategy used for the phenotypic confirmation of MAIT cell identity from cloned MAIT cell buttons. CD4/CD8, MR1 5-OP-RU tetramer and CD26 expression were determined from total CD3+ T cells, using healthy donor cells to define the MR1 tetramer (MR1 6-FP) and CD26 gates.

2.2.7.2. Functional assessment of clonal growth

Functional assessment of clonal growth was performed by IFN-γ ELISpot assay. In this assay, T cells are exposed to infected wild type (WT) or MR1-knocked out (KO) antigen presenting cells (APCs) and IFN-γ production is measured as a read-out of those clonal populations which respond to mycobacterially-infected APCs in a MR1-dependent manner. (**Figure 2.2.7.2**).

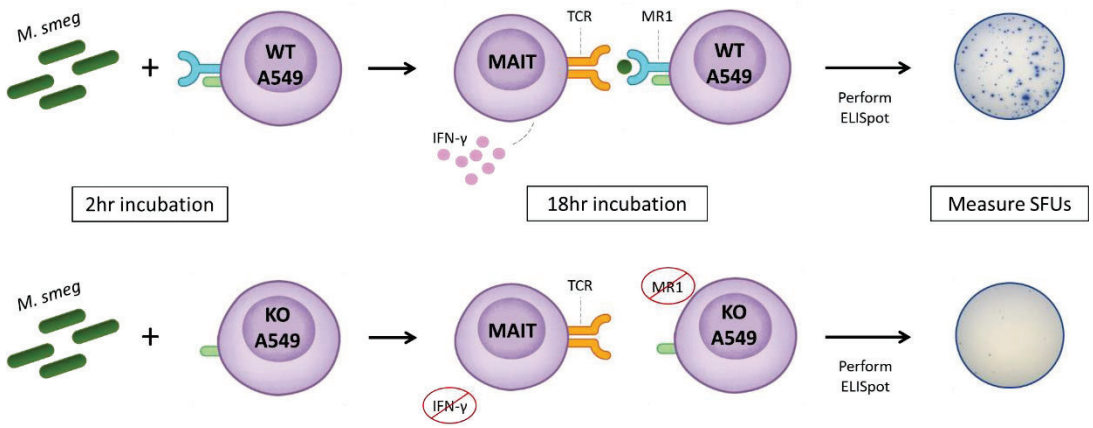


Figure 2.2.7.2: Principle of the MR1-restriction functional assay. IFN-γ produced by MAIT cells upon encounter with *Mycobacterium smegmatis* (*M.smeg*) infected wild type (WT) A549s is measured in spot forming units (SFUs). MAIT cells are unable to produce IFN-γ when incubated with MR1-knocked out (KO) A549s and no SFUs seen.

ELISpot plates were prepared by first coating with 10 µg/mL anti-human IFN-γ coating antibody (Mabtech, clone 1-D1K) and incubating at 4°C overnight. The following morning, plates were washed with 1x PBS, blocked for 1 hour at RT using antibiotic-free cRPMI containing 10% HuS and then WT or MR1-KO A549s added (Laugel *et al.*, 2016). *Mycobacterium smegmatis* (*M.smeg*) was added using a multiplicity of infection (MOI) of 1:3, the infection allowed to proceed for 2 – 3 hours and the potential MAIT cell clones then added. Lasty, a PHA control (10 µg/mL) was added and plates incubated at 37°C overnight. Plates were washed with 1x PBS-T the next morning, before being coated with 3 µg/mL streptavidin-ALP secondary antibody (Mabtech, clone 7-B6-1) and then incubated at RT for 2 hours. Additional 1x PBS-T washes were performed, detection substrate added, and spots allowed to develop. Rinses with deionized (DI) water were performed, and the plates dried for 45 minutes before spots were quantified using an ELISpot reader (Autoimmun Diagnostika GmbH) with accompanying software.

2.2.8. MAIT clone expansion and identity confirmation

Clonal growth confirmed to contain MAIT cells by surface and functional phenotyping were then rapidly expanded in T12.5 culture flasks. Irradiated LCLs (2.5 x 10^6 cells/flask) and PBMCs (12.5 x 10^6 cells/flask) were added to cell clones with 0.03 µg/mL of anti-CD3 in media containing 10% HuS. The next day media was supplemented with 2 ng/mL rhIL-2 and the cells then washed on the 5^{th} day of expansion to remove the anti-CD3. Cultures were then supplemented every second/third day with rhIL-2 and the expansion continued another 5 – 7 days. A small volume was removed from the flask for identity confirmation of the expanded cells. Confirmation was performed as described above using surface phenotyping (section 2.2.7.1) and a functional assay (section 2.2.7.2). Clones confirmed to have remained MAIT cells during expansion were then transferred to larger T25 flasks for continued expansion, in which case fresh media, cytokines and feeder cells were added, and the cells cryopreserved thereafter for later experimentation.

2.2.9. DNA isolation and T cell receptor sequencing

In order to determine the T cell receptor sequencing of the T cell clones, genomic DNA was extracted from T cell clones using the Qiagen DNeasy Mini Kit (Spin-Column Protocol) according to the manufacturer's instructions. This was done by thawing 1 x 10^6 cell/mL clone aliquots, centrifuging and resuspending cells in proteinase K-containing PBS. Buffer AL was added, and the tubes mixed by vortexing before being incubated at 56°C for 10 minutes. Ethanol (>99%) was added, the tubes vortexed and the samples transferred to spin-columns. Buffer AW1 was added after samples had been centrifuged and the flow-through discarded. Samples were centrifuged and the flow through discarded to allow for the addition of buffer AW2. Buffer AW2 was again removed by centrifugation and buffer AE added. Tubes were incubated for 1 minute at RT and then centrifuged. The resulting DNA was then quantified using the ND-1000 (NanoDrop®, Thermo Scientific). The ImmunoSEQ assay (Adaptive Biotechnologies) was used to perform paired TCRα and TCRβ sequencing (Lepore *et al.*, 2014). MAIT cell identity was confirmed by assessing the CDR3α sequences using a web-based tool called MAIT Match (http://www.cbs.dtu.dk/services/MAIT_Match). Using this tool, clone sequences were compared to reference MAIT cell sequences in the database and a similarity score between 0 and 1 calculated. Clones with similarity scores close to 1.0 were more likely to be MAIT cells, with MAIT Match scores of 0.95 and above considered to be MAIT cell consistent TCR sequences (Wong *et al.*, 2019).

2.2.10. Statistical analysis

All statistical analyses were performed on Prism 8.0.0 (GraphPad Software) using Mann-Whitney *U* tests to assess differences between compartments (peripheral blood vs BAL fluid) and data are represented as medians and interquartile ranges. $P < 0.05$ indicated by *, $P < 0.01$ by **, $P < 0.001$ by *** and $P < 0.0001$ by ****.

2.3. Results

2.3.1 Phenotypic heterogeneity of bronchoalveolar MAIT cells from healthy individuals

We first determined whether bronchoalveolar MAIT cells (defined as CD3+CD4- MR1 5-OP-RU tetramer-positive cells, **Figure 2.2.3**) were tissue-resident T cells by comparing their expression of CD69 to that of peripheral blood MAIT cells in a subset of individuals from the clinical bronchoscopy cohort. A significantly higher proportion of CD69 expressing MAIT cells were found in the bronchoalveolar compartment when compared to those in the peripheral blood [median of 93.65% and interquartile range (IQR) of 74.98 – 97.50% in the bronchoalveolar compartment and median of 14.30% and IQR of 4.26 – 17.35% in peripheral blood ($P = 0.0002$)] (**Figure 2.3.1.1, A**). We next compared the frequency of MAIT cells in the peripheral blood to those in the bronchoalveolar compartment and found that in healthy, HIV-negative participants without any active respiratory infection, MAIT cells frequencies in peripheral blood were similar to those in the bronchoalveolar compartment [median of 1.74% and IQR of 0.74 – 3.53% in peripheral blood and median of 1.15% IQR of 0.44 – 2.22% in the bronchoalveolar compartment ($P = 0.3027$)] (**Figure 2.3.1.1, B**).

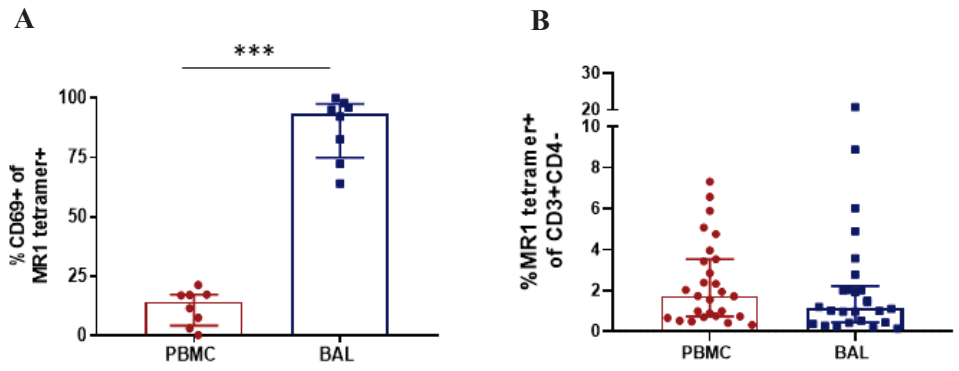


Figure 2.3.1.1: (A) Frequency of CD69+ MR1 tetramer-positive CD3+CD4- MAIT cells in the peripheral blood (PBMC) and bronchoalveolar (BAL) compartments (n = 9). **(B)** Frequency of MR1 tetramer-positive CD3+CD4- MAIT cells in the PBMC (n = 27) and BAL compartments (n = 26).

Paired surface staining of MAIT cells from the peripheral blood and bronchoalveolar compartment of healthy controls using surface markers that are typically highly expressed in MAIT cells, CD161 and CD26, showed surprising heterogeneity of expression of these markers on MAIT cells in the bronchoalveolar compartment (**Figure 2.3.1.2, A**). Assessment of all the healthy HIV-negative participants from the research bronchoscopy cohort demonstrated that the vast majority of peripheral blood MAIT cells had the expected CD161++CD26++ phenotype (94.2%, 87.9 – 97.9%) and that this phenotype was significantly less frequent (64.95%, 39.0 – 77.88%) among MAIT cells derived from the bronchoalveolar fluid ($P = 0.0002$) (**Figure 2.3.1.2, B**).

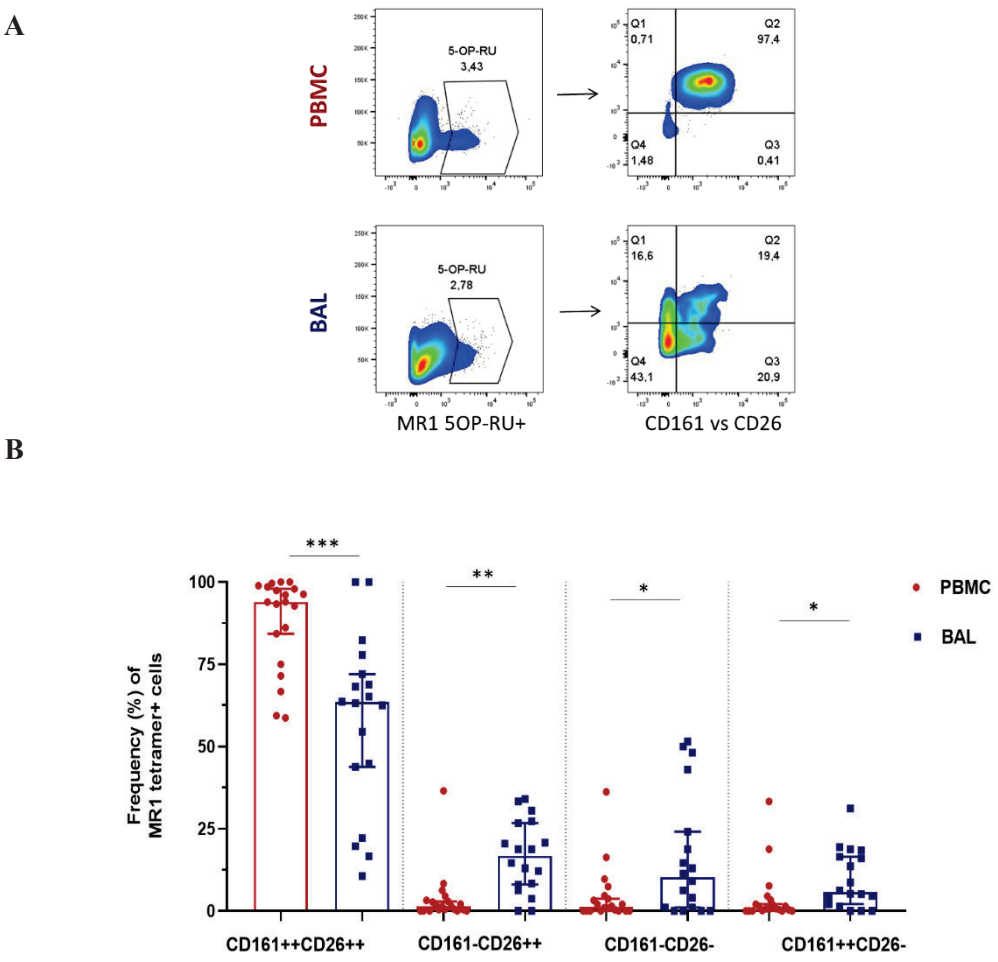


Figure 2.3.1.2: CD161 and CD26 surface staining of peripheral blood and bronchoalveolar MAIT cells. (**A**) Representative plots from each compartment and (**B**) frequency (%) of each subpopulation in the study cohort (n = 20 in PBMC, n = 18 in BAL).

Although populations of CD161-negative and CD26-negative MAIT cells were very rare in the peripheral blood, these cell populations were more frequent in the BAL fluid. MR1 tetramer-positive cells in the bronchoalveolar compartment contained detectable frequencies of cells falling into these categories with medians of 13.5% for CD161-CD26++ (IQR: 7.41 – 27.23%), 10.25% for CD161-CD26- (IQR: 1.86 – 18.58%) and 7.66% for CD161++CD26- (IQR: 1.94 – 15.7%) cells. These phenotypic subpopulations were significantly more frequent in the bronchoalveolar compartment compared to matched peripheral blood samples, with $P = 0.0034$, 0.0084 and 0.0013 for each subpopulation, respectively.

2.3.2. Confirmation of MAIT cell identity of phenotypically atypical MR1 tetramer-positive bronchoalveolar T cells

There have been reports of CD161-low/negative MAIT cells (Leeansyah *et al.*, 2013; Eberhard *et al.*, 2014), but because MAIT cells with a CD161-negative and CD26-negative phenotype have not been previously described, we sought to confirm the identify of these phenotypically heterogenous MR1 tetramer-positive cells by cultivating T cell clones from these phenotypic subpopulations to allow assessment of their functional characteristics and T cell receptor usage. A participant with prominent CD161 and CD26 heterogeneity in the bronchoalveolar compartment was selected and cells were cloned from the CD161++CD26++, CD161-CD26++ and CD161-CD26- subpopulations of bronchoalveolar T cells (**Figure 2.3.2.1**). The frequency of CD161+CD26- cells was generally low and so this population was excluded from further analysis.

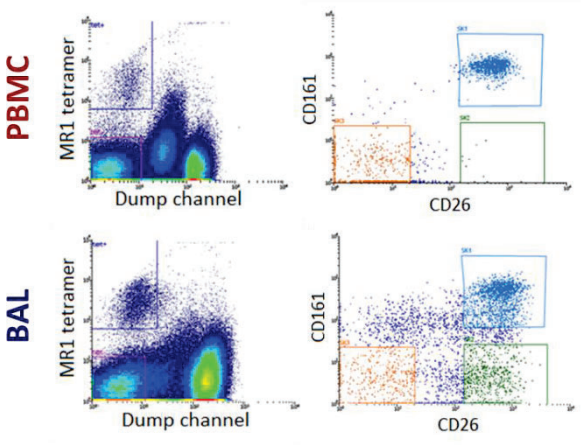


Figure 2.3.2: CD161 and CD26 surface staining of MR1 5-OP-RU tetramer-positive MAIT cells from PID1004 comparing the staining patterns of peripheral blood (PBMC) and bronchoalveolar (BAL) MAIT cells.

2.3.2.1. Phenotypic assessment of T cell clone identity

Limiting dilution cloning yielded 27 buttons and of these 23 were viable. After the first round of expansion, 15 buttons were found to either not have clonally expanded or to not have MR1-restricted function. Eight clones were thus taken forward for a second round of expansion and confirmatory analyses. The phenotypic characteristics of the remaining eight bronchoalveolar T cell clones are shown below (**Figure 2.3.2.1**). Although the intensity of MR1 5-OP-RU staining varied between the clones, clones from all three phenotypic subpopulations were MR1 5-OP-RU tetramer-positive and CD26++, consistent with MAIT cell identify. Interestingly, following this round of expansion, CD26 expression was observed even in clones which had been sorted from the CD161-CD26- population. CD161 expression has been shown to be downregulated during MAIT cell cultivation (Gold *et al*., 2010) and as such was not included in the clone phenotyping panel. Consistent with most human MAIT cells, the clones were predominantly CD8+ although one bronchoalveolar T cell clone (E1) was CD4-CD8-. No CD4+ staining was observed among the clones. These findings suggested that all eight of these clones were MAIT cells.

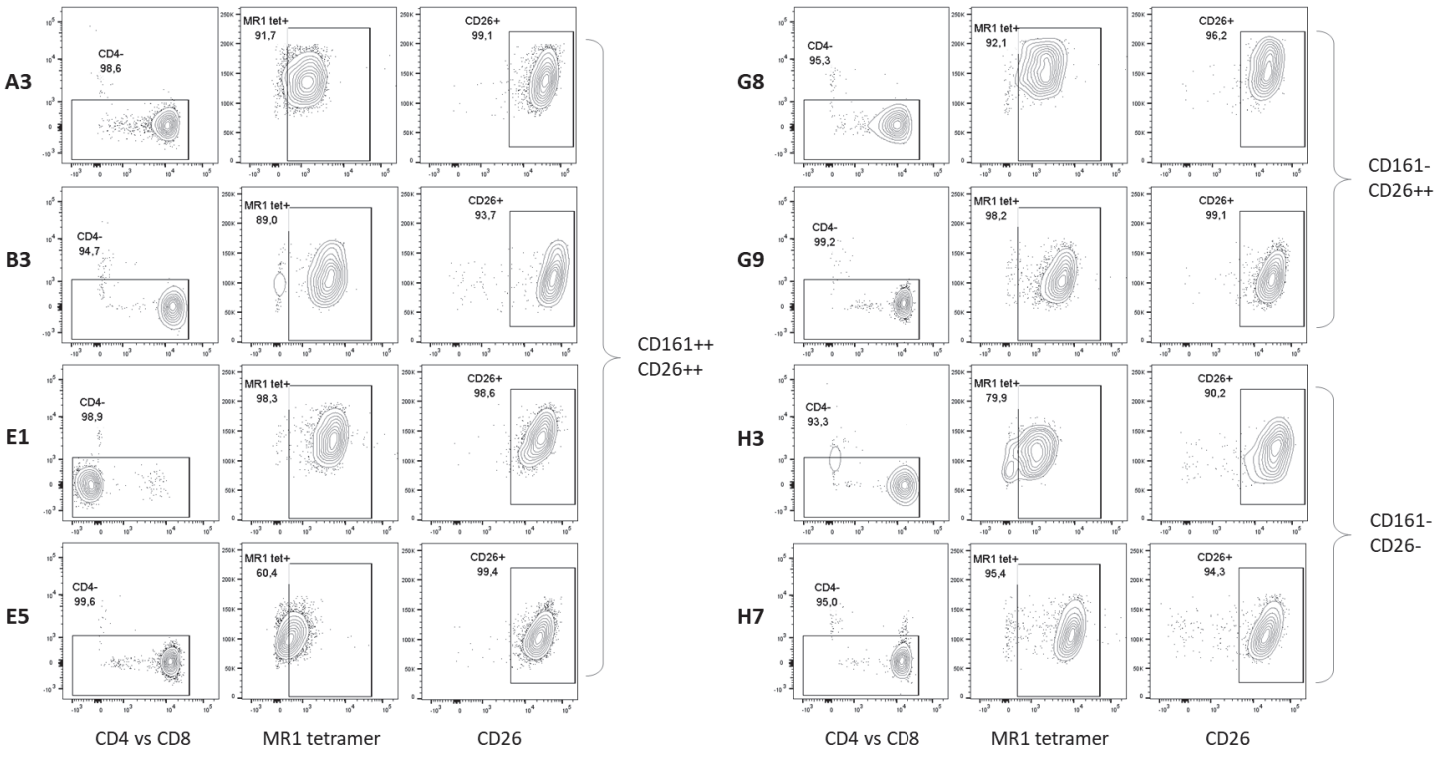


Figure 2.3.2.1: The phenotypic characterisation of bronchoalveolar T cell clones generated from different phenotypic subpopulations showing the post-expansion staining with CD4, CD8, MR1 5-OP-RU tetramer and CD26.

2.3.2.2. Functional determination of T cell clone identity

These eight bronchoalveolar T cell clones were next subjected to a second round of functional analyses to determine their responsiveness to mycobacterial-infected antigen presenting cells and whether this response was MR1-dependent. All of the bronchoalveolar T cell clones demonstrated production of IFN-γ in response to *M. smeg* infected WT A549s (**Figure 2.3.2.2**). Additionally, all of the bronchoalveolar T cell clones showed abrogation of IFN-γ production when co-cultured with *M. smeg* infected MR1-KO A549s, suggesting a reliance on MR1-mediated antigen presentation for IFN-γ production consistent with MAIT cell identity.

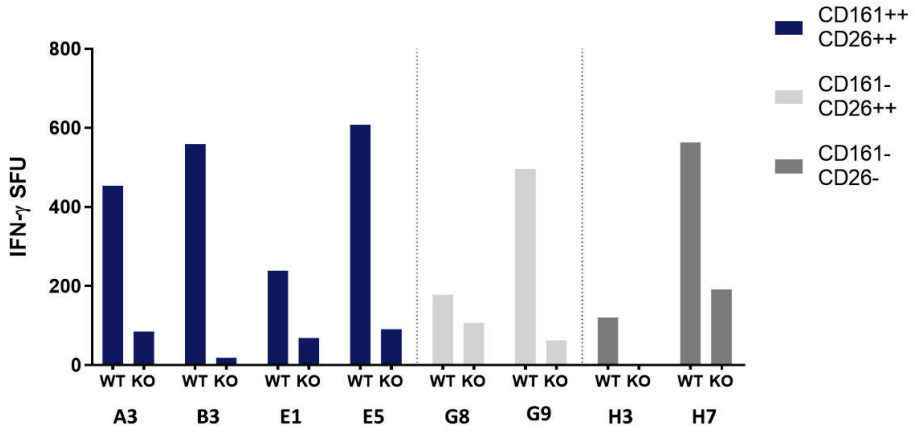


Figure 2.3.2.2: IFN-γ ELISpot assay assessing the MR1-restricted function of bronchoalveolar T cell clones derived from three phenotypic subpopulations. Clones were co-cultured with *Mycobacterium smegmatis* (*M. smeg*) infected wild type A549s (WT) as well as *M. smeg* infected MR1-knocked out A549s (KO) and the IFN-γ produced measured in spot forming units (SFU).

2.3.2.3. T cell receptor assessment of T cell clones

Next the eight bronchoalveolar T cell clones were subjected to TCRα and TCRβ sequencing which, revealed that all bronchoalveolar T cell clones utilised MAIT-consistent T cell receptors. T cell clones from all three phenotypic subpopulations of origin used the TRAV1-2 gene segment paired with the TRAJ33 joining region (**Table 2.3.2.3**). CDR3α lengths were conserved with very few amino acid differences across clones and all eight TCRα sequences were assigned a similarity score of 1.0 using MAIT match consistent with MAIT cell identity (Wong *et al.*, 2019).

Table 2.3.2.3: T cell receptor sequences of bronchoalveolar T cell clones derived from different phenotypic subpopulations.

Population of origin	Clone	TCRα Sequence			MAIT match score	TCRβ Sequence		
		TRAV	CDR3α	TRAJ		TRBV	CDR3β	TRBJ
CD161++ CD26++	A3	1-2	CAVKDSNYQLIW	33	1.00	6-1	CASSPQGAGGQEQYV	2-7
	B3	1-2	CAVTDSNYQLIW	33	1.00	6-5	CASSYEGGGQPQHF	1-5
	E1	1-2	CAALDSNYQLIW	33	1.00	6-4	CASSDGEGQPQHF	1-5
	E5	1-2	CAAMDSNYQLIW	33	1.00	30-1	CAWSHSDRDLNEQYF	2-7
CD161- CD26++	G8	1-2	CAVRDSNYQLIW	33	1.00	6-1	CASSEGETYGYTF	1-2
	G9	1-2	CAVMDSNYQLIW	33	1.00	15-1	CATSGDQEPAEAFF	1-1
CD161- CD26-	H3	1-2	CAAMDSNYQLIW	33	1.00	3	CASSQASGGEETQYF	2-5
	H7	1-2	CAAMDSNYQLIW	33	1.00	20	CSAKRGGASNEQFF	2-1

CDR3β lengths were more diverse with the most frequently utilised TRBV segments belonging to the TRBV6 gene family. This was observed in four out of the eight clones, with three of these coming from the clones which had been generated from the CD161++CD26++ population. The remaining clones utilised TRBV30-1, TRBV15-1, TRBV3 and TRBV20 respectively.

2.3.3. Bronchoalveolar MAIT cells display functional inhibition compared to peripheral counterparts

Mitogenic stimulation of bronchoalveolar MR1 5-OP-RU tetramer-positive cells from healthy HIV-negative controls with PMA/ionomycin resulted in significantly lower IFN-γ production than that seen in matched peripheral blood MAIT cells ($P = 0.0156$) (**Figure 2.3.3.1, A**). A similarly low percentage of peripheral blood and bronchoalveolar MAIT cells produced IL-17 upon mitogenic stimulation (**Figure 2.3.3.1, B**) and the percentage of cells constitutively producing granzyme B was also low in both compartments (**Figure 2.3.3.1, C**).

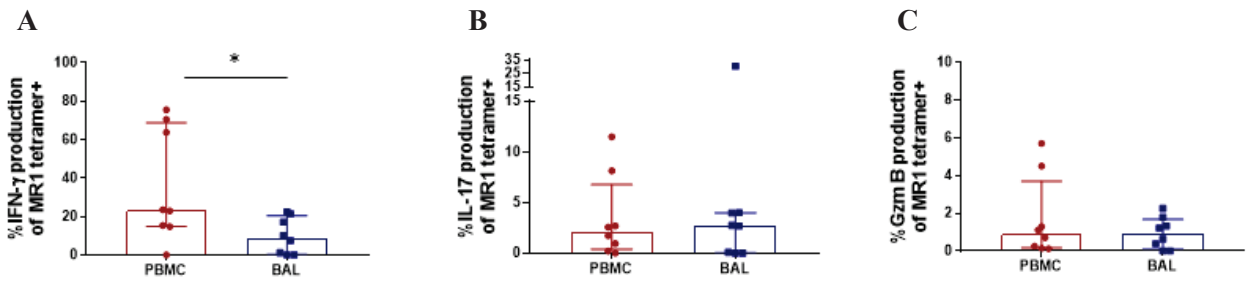


Figure 2.3.3.1.: *Ex vivo* staining of peripheral blood and bronchoalveolar MR1 5-OP-RU tetramer-positive cells for the production of (**A**) inducible IFN-γ, (**B**) inducible IL-17 and (**C**) constitutive granzyme B following 6 hour stimulation with PMA/ionomycin (n = 8).

MAIT cells have been reported to be producers of a range of cytolytic molecules, including granzyme B, when stimulated. The low granzyme B production we observed was thus surprising. We therefore performed a similar analysis using MR1 tetramer-negative CD8+ T cells from the same participants and anatomical compartments and found these to produce significantly more granzyme B in both compartments ($P < 0.0001$ in both cases) suggesting that the antibody and staining protocol were correctly optimised and that the low levels of granzyme B staining in MR1 tetramer-positive cells in both compartments was, in fact, due to low levels of the protein being produced constitutively in MAIT cells (**Figure 2.3.3.2, C**). Comparison to MR1 tetramer-negative CD8+ T cells demonstrated that there was greater inducible IFN-γ production by conventional bronchoalveolar CD8+ T cells compared to MAIT cells ($P = 0.0014$) (**Figure 2.3.3.2, A**), and higher inducible IL-17 production in peripheral blood MAIT cells compared to matched MR1 tetramer-negative CD8+ T cells ($P = 0.0409$) (**Figure 2.3.3.2, B**).

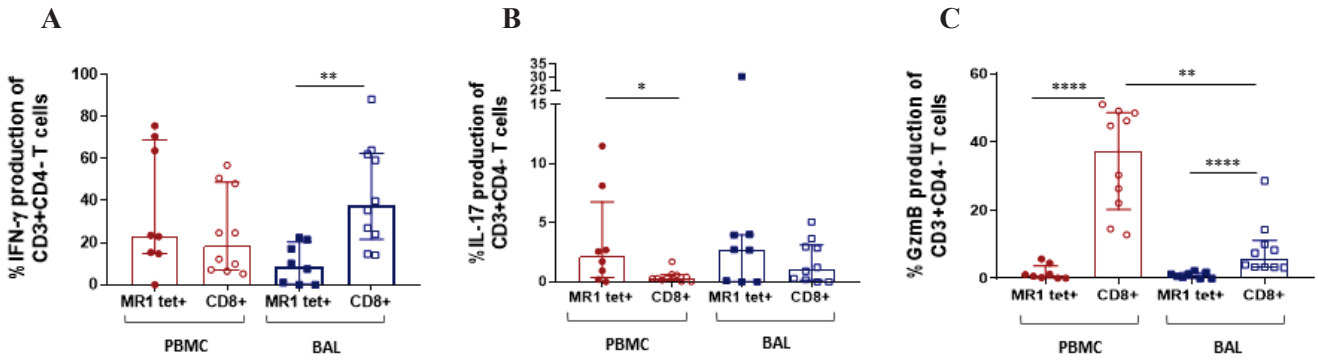


Figure 2.3.3.2: Proportion of (**A**) Inducible IFN-γ, (**B**) inducible IL-17, (**C**) constitutive granzyme B producing MR1 tetramer-positive MAIT cells (n = 8) in the peripheral blood (PBMC) and bronchoalveolar lavage (BAL) fluid in contrast to matched conventional CD8+ T cells (n = 10) from healthy participants.

We next assessed the surface expression of HLA-DR (a marker of T cell activation), PD-1 and TIM-3 (inhibitory co-receptors) on MAIT cells from both compartments to determine whether activation or inhibition might contribute to the reduced pro-inflammatory function we observed in bronchoalveolar MAIT cells. We found that the frequency of MAIT cells expressing all three of these markers was significantly higher in the bronchoalveolar compartment ($P = 0.0104$, $P < 0.0001$, and $P = 0.0417$ respectively) (**Figure 2.3.3.3, A - C**). Analysis of the median fluorescent intensities (MFIs) of HLA-DR, PD-1 and TIM-3 also showed these markers to be elevated in bronchoalveolar MAIT cells ($P = 0.0207$, $P < 0.0001$ and $P = 0.0903$ respectively), although this was significant only for HLA-DR and

PD-1 (**Figure 2.3.3.3, D - F**). These data suggest that higher levels of expression of HLA-DR, PD-1, and TIM-3 in bronchoalveolar MAIT cells may serve to inhibit IFN-γ production and prevent excessive inflammation at this mucosal site.

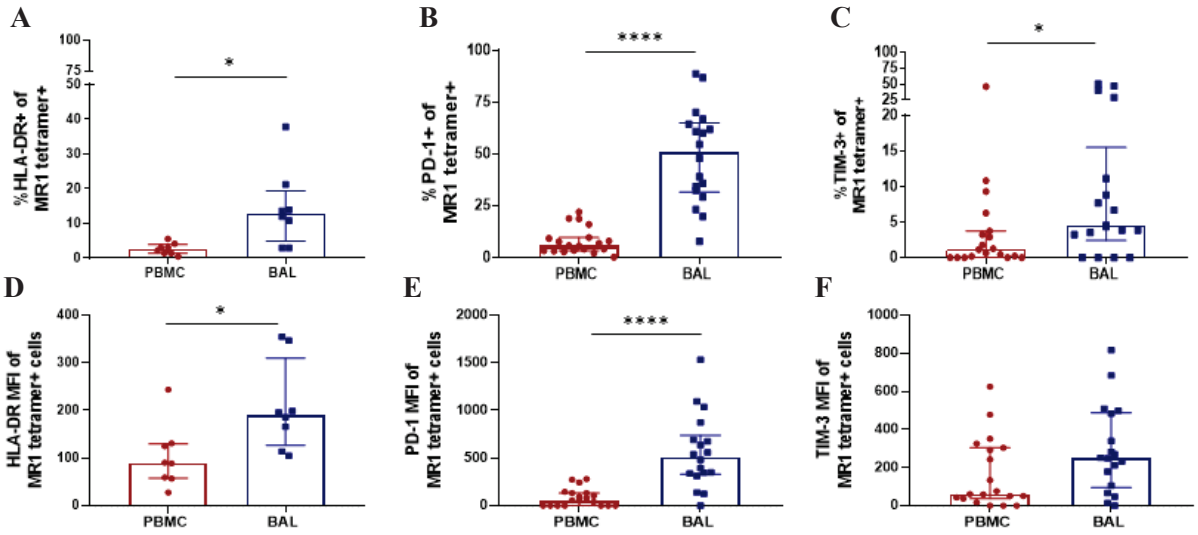


Figure 2.3.3.3: Frequency of (**A**) HLA-DR (n = 8), (**B**) PD-1 (n = 18) and (**C**) TIM-3 (n = 18) expressing peripheral blood (PBMC) and bronchoalveolar (BAL) MR1 tetramer-positive MAIT cells, and median fluorescent intensity (MFI) of (**D**) HLA-DR, (**E**) PD-1 and (**F**) TIM-3 in peripheral blood and bronchoalveolar MAIT cells.

2.4. Discussion

Previous work describing human MAIT cell phenotype and function has largely focused on peripheral blood responses despite the evidence that MAIT cells may play a role in the response to infection at mucosal surfaces. We aimed to address this gap in knowledge by examining MAIT cell phenotype and function in the peripheral blood and lung mucosa of healthy humans. Using MR1 5-OP-RU tetramers to define MAIT cells independently of any particular protein marker, we found that bronchoalveolar MAIT cells in healthy individuals feature previously undescribed phenotypic heterogeneity in the surface expression of MAIT cell markers CD161 and CD26. Limiting dilution cloning experiments demonstrated that these phenotypically atypical cells were, in fact, MR1-restricted MAIT cells that utilised canonical MAIT cell TCRα TRAV1-2/TRAJ33. Functional assay of MR1 5-OP-RU tetramer-positive MAIT cells demonstrated that bronchoalveolar MAIT cells are less pro-inflammatory than their peripheral blood counterparts. Interestingly this decreased pro-inflammatory character was accompanied by higher levels of expression of inhibitory co-receptors in

the bronchoalveolar compartment, suggesting that bronchoalveolar MAIT cells may be functionally inhibited.

CD161 and CD26 expression are associated with effector function. In NK cells, cytotoxic function and the capacity to respond to antigen in a TCR-independent manner relying on IL-12 and IL-18 stimulation, is associated with CD161 expression (Kurioka *et al.*, 2018). Conversely, recent data has shown a link between CD161 expression and functional inhibition in tumour-infiltrating T cells (Mathewson *et al.*, 2021). Cytotoxic function in these cells was enhanced following blockade of the CD161-CLEC2D (the ligand for CD161) pathway (Mathewson *et al.*, 2021). In our setting, CD161 may be associated with MAIT cell function as greater pro-inflammatory potential was observed in peripheral blood MAIT cells which also had significantly greater expression of CD161 than those in the bronchoalveolar compartment. Further work is however required to determine the functional significance of CD161 expression in MAIT cells. Lowered CD161 expression has been observed following successive rounds of MAIT cell cultivation as a result of the stimulatory nature of the culturing environment (Gold *et al.*, 2010; Freeman *et al.*, 2017). Based on these experimentally induced fluctuations in CD161 expression we excluded CD161 from panels used for the phenotypic confirmation of MAIT cell clones and also opted to perform all phenotypic and functional characterisation of MAIT cells from both bronchoscopy cohorts directly *ex vivo*.

Notably, we observed a shift in CD26 expression with the result that all clones, regardless of population of origin, expressed high levels of CD26 after *ex vivo* expansion. This suggests that MAIT cell surface phenotype is not inherent but may be shaped by stimulatory and/or inhibitory signals in the local environment or in *ex vivo* culture conditions (Gold *et al.*, 2010; Leeansyah *et al.*, 2013; Freeman *et al.*, 2017). NK cell cytotoxicity has also been associated with CD26 expression. The ability of NK cells to lyse tumours was significantly decreased in CD26/DPP4-defecient rats (Klemann *et al.*, 2016). CD26 expression is also an indicator of T-helper cell subset polarisation and is highly expressed on Th17 cells, while being lowly expressed or absent on regulatory T (Treg) cells (Bengsch *et al.*, 2012; Salgado *et al.*, 2012). In $V\delta2^+$ T cells CD26 expression has recently been shown to be associated with cytokine responsiveness, where cells were able to more rapidly produce IFN-γ in response to IL-12/IL-18 stimulation (Wragg *et al.*, 2020). Additionally, we observed a trend of lower MR1 5-OP-RU tetramer staining intensity in the bronchoalveolar compartment, in contrast to peripheral blood, suggestive of MAIT cells with varied antigen specificities in this compartment (Harriff *et al.*, 2018). Compartmental differences in MR1 tetramer staining intensity may also result from activation, as is seen following *in vitro* stimulation, and TCR downregulation in bronchoalveolar MAIT cells (Wong *et al.*, 2019). Bronchoalveolar MAIT cells may thus have alternative functions,

not relating to cytotoxicity (such as immunoregulatory functions) or may have altered phenotypes as a consequence of antigen exposure at the lung mucosal surface.

We cultured eight T cell clones from the bronchoalveolar compartment and characterised these clones to confirm their identify as MAIT cells. All expanded clones utilised TRAV1-2/TRAJ33, consistent with MAIT cell identity, and had MAIT Match scores of 1.0. We did not observe any TRAJ12 usage, which was reported to be more frequently used by tissue-resident MAIT cells as compared to those in the peripheral blood (Lepore *et al.*, 2014). The eight clones we cultivated had variable TCR-β usage, with a bias towards the TRBV6 family (observed in 4 out of 8 clones) as has been previously shown (Tilloy *et al.*, 1999; Reantragoon *et al.*, 2013; Lepore *et al.*, 2014). One clone utilised TRBV20, another dominant TRBV gene family, with the remaining three clones using lesser characterised TRBV30-1, TRBV15-1, and TRBV3 gene segments. Recently, the role of the TCRβ in mediating interactions with the MR1-antigen complex has become more widely appreciated, with López-Sagaseta *et al.* (2013) showing the importance of the CDR3β in altering binding affinity to the MR1-ligand complex. Gold *et al.* (2014) and Reantragoon *et al.* (2013) showed that the heterogeneity of the MAIT cell TCRβ repertoire was greater than previously reported and that certain microbial responses were characterised by specific TCRβ chains. These findings point towards the potential for heterogeneity in antigen recognition in bronchoalveolar MAIT cell subpopulations.

In healthy individuals, bronchoalveolar MAIT cells were less pro-inflammatory, producing lower levels of inducible IFN-γ, a finding consistent with those of Boulouis *et al.* (2020) and Lu *et al.* (2020) from the respiratory mucosa. Lower IFN-γ and TNF-α production was also reported by Sobkowiak *et al.* (2019) in oral mucosal MAIT cells, which in their study was paired with increases in the expression of HLA-DR and PD-1. As we also observed an increase in the expression of activation and inhibitory markers in the bronchoalveolar compartment compared to the peripheral blood, these findings together thus suggest that in a state of health, mucosal MAIT cells may be less inflammatory than those in the circulation. Higher expression of both activation and inhibitory markers as observed in the bronchoalveolar compartment, may represent an adaptation of lung resident MAIT cells enabling them to remain poised for the maintenance of mucosal immunity while also minimising exaggerated responses to commensal microbes (Sobkowiak *et al.*, 2019).

In summary, bronchoalveolar MAIT cells in healthy individuals displayed phenotypic heterogeneity in their surface expression of CD161 and CD26 and were less pro-inflammatory when compared to those in the peripheral blood. We used T cell cloning to confirm that bronchoalveolar MR1 tetramer-

positive cells with atypical phenotypes *ex vivo* had MAIT cell identity as demonstrated by T cell clone surface phenotyping, MR1-dependent functional response and TCR sequencing. These MAIT cell clones showed limited diversity in CD4/CD8 co-receptor usage but utilised three TRBV genes which are yet to be characterised. Further work is required to determine whether differences in TCR usage contribute to greater ligand discrimination or represent functional heterogeneity and specialisation amongst bronchoalveolar MAIT cell subpopulations.

CHAPTER 3

CHAPTER 3: STUDIES OF PAIRED PERIPHERAL AND BRONCHOALVEOLAR MAIT CELLS REVEAL HIV-DRIVEN ALTERATIONS IN BOTH COMPARTMENTS

3.1. Introduction

3.1.1. Peripheral blood MAIT cell alterations in HIV

MAIT cells are capable of mounting responses to viral infections in a T cell receptor (TCR)-independent, cytokine-mediated manner (Ussher *et al.*, 2014; van Wilgenburg *et al.*, 2016). MAIT cells in the peripheral blood have been shown to be numerically depleted during HIV, have increased expression of activation markers and inhibitory co-stimulatory molecules such as HLA-DR and TIM-3, and produce lower levels of cytokines including TNF and IFN-γ following bacterial stimulation (Cosgrove *et al.*, 2013; Leeansyah *et al.*, 2013). These alteration in frequency and function were also accompanied by phenotypic alterations. A decline in the frequency of CD161-expressing MAIT cells was observed along with an expansion of CD161-negative MAIT cells in HIV infected individuals (Leeansyah *et al.*, 2013; Eberhard *et al.*, 2014). Interestingly, a subsequent study by Fernandez *et al.* (2015) showed that MR1 tetramer-defined MAIT cells were not functionally impaired (assessed by measurement of IFN-γ and TNF-α) in HIV infected individuals. There thus appears to be some discordance in findings where different methods of defining MAIT cells are used. In the present chapter, the frequency of MAIT cells was determined in HIV infected individuals using the MR1 tetramer definition of MAIT cells, the phenotype determined by surface staining with CD161 and CD26, the functional capacity determined by cytokine and cytolytic molecule assessment paired with staining for markers of activation and inhibition.

3.1.2. Mucosal MAIT cell alterations in HIV

Few studies have assessed the impact of HIV on bronchoalveolar MAIT cells. Both Vinton *et al.* (2016) and Mvaya *et al.* (2019) demonstrated decreases in MAIT cell frequencies in the bronchoalveolar compartment in SIV and HIV infection respectively, that was associated with increases in viral loads in the case of HIV infection. In contrast to findings at the peripheral blood, bronchoalveolar MAIT cell frequency was increased following 1 year of ART, with cytokine production being generally unchanged in HIV-positive versus HIV-negative participants (Mvaya *et al.*, 2019). The functional profile of MAIT cells was not determined in SIV-infected rhesus macaques. Understanding MAIT cell function is important as these cells may be important in the early events that lead to the establishment of effective immune response, which is of added importance in immune compromised individuals. Due to the limited evidence regarding the fate of human MAIT cells during HIV infection, we thus aimed to determine the quantity and quality of bronchoalveolar MAIT cells in

HIV-infected individuals by comparing overall frequencies and the surface and functional phenotypes of bronchoalveolar MAIT cells to those in the peripheral blood.

3.2. Methods

3.2.1. Study participants

Paired peripheral blood and bronchoalveolar lavage (BAL) fluid samples were collected from both HIV-negative and HIV-positive participants undergoing either research (n = 37) or clinically indicated (n = 17) bronchoscopies at Inkosi Albert Luthuli Central Hospital (IALCH) in Durban, South Africa. Participants had all provided written consent for study procedures and ethical approval of the study protocol had been received as described in Chapter 2. The participants in the research bronchoscopy cohort were divided into two groups: 1) "Healthy controls" were HIV-negative (negative HIV ELISA and undetectable HIV viral load) participants with latent TB infection (LTBI) as determined by a positive QuantiFERON-TB Gold Plus (QFT-Plus) assay (Qiagen) (background subtracted IFN-γ > 0.35 IU/mL), and 2) "HIV-positive" participants were those recently diagnosed with HIV (i.e. positive HIV ELISA and detectable HIV viral load), who were otherwise healthy with good functional status, ART-naïve, and who had LTBI by the QFT-Plus assay. Participants with a history of TB or with radiographic signs consistent with active TB or those with symptoms of respiratory infection (cough, fever, shortness of breath) were excluded from this cohort. In the clinically indicated bronchoscopy cohort, participants had their HIV status defined by HIV ELISA and viral load. The characteristics of HIV-negative and HIV-positive study participants from both cohorts are shown in **Table 3.2**. Participants with respiratory infections were excluded from the clinical bronchoscopy cohort based on culture positive confirmation of bacterial, fungal or mycobacterial infection in BAL fluid samples.

Table 3.2: Demographic and clinical characteristics of HIV-negative and HIV-positive participants from the research bronchoscopy and clinically indicated (clinical) bronchoscopy cohorts.

	Research bronchoscopy cohort			Clinical bronchoscopy cohort		
	HIV-	HIV+	*P* -value	HIV-	HIV+	*P* -value
N (%)	23 (62.2%)	14 (37.8%)	0.3333	8 (47%)	9 (53%)	0.6667
Age, years (IQR*)	32 (25 - 36)	30 (21.75 - 35.25)	0.4998	45 (29.25 - 54.75)	34 (32 - 51)	0.833
Sex, number of females (%)	16 (69.6%)	9 (64.3%)	0.3333	2 (25%)	4 (44.4%)	0.3333
Median viral load, copies/mL (IQR*)	N/A	25 229 (9768 - 60 331)	N/A	N/A	304 (<20 - 480 000)	N/A
CD4 count, cells/µL (IQR*)	1005 (833.3 - 1308)	445 (193.8 - 615.3	<0.0001	ND	276 (96 - 589)	N/A

*Interquartile range.

ND - not done

3.2.2. Preparation of peripheral and bronchoalveolar lymphocytes

As detailed in Chapter 2, BAL fluid was combined 1:1 with complete RPMI (cRPMI) media or left undiluted for transportation back to the laboratory which was done on ice. Samples were processed under BSL-3 conditions, within 3 hours of collection. BAL lymphocytes were isolated and enumerated as previously described. Peripheral blood mononuclear cells (PBMCs) were concurrently isolated from whole blood using the Histopaque® (Sigma-Aldrich) gradient centrifugation method according to the manufacturer's instructions. Both BAL lymphocytes and PBMCs were resuspended in cRPMI media for immediate characterisation or cryopreserved for later use.

3.2.3. Flow cytometry panel for characterising MAIT cells

Surface staining was performed on matched PBMCs and BAL lymphocytes as previously described in Section 2.2.3. Briefly, samples were first tetramer stained, and then stained with a fixable viability dye. This was followed by staining with one of two surface antibody cocktails as listed in **Table 2.2.3**.

MAIT cells were gated on as previously described (Section 2.2.3) and the gates for the MR1 5-OP-RU tetramer, HLA-DR, PD-1 and TIM-3 staining defined using the relevant controls (**Figure 2.2.3.1** and **Figure 2.2.3.2**).

3.2.4. Determination of MAIT cell functional capacity

Intracellular cytokine staining (ICS) was used to determine the functional capacity of both peripheral blood and bronchoalveolar MAIT cells as fully detailed in Section 2.2.4. MAIT cells were tetramer stained following PMA/ionomycin stimulation, and then surface and intracellular staining performed as per antibody mastermixes listed in **Table 2.2.4**. MAIT cells were gated on as shown in **Figure 2.2.3** and the gates for IFN-γ, IL-17 and granzyme B staining defined as shown in **Figure 2.2.4**.

3.2.5. Statistical analysis

All statistical analyses were performed on Prism 8.0.0 (GraphPad Software) using Mann-Whitney *U* tests to assess differences between disease state (i.e. HIV-negative vs HIV-positive samples). Data are represented as medians and interquartile ranges. $P < 0.05$ indicated by *, $P < 0.01$ by **, $P < 0.001$ by *** and $P < 0.0001$ by ****.

3.3. Results

3.3.1. HIV causes depletion of peripheral blood and bronchoalveolar MAIT cells and abrogates compartmental functional differences

We performed MR1 tetramer staining on HIV-positive and HIV-negative participants to assess the impact of HIV infection on MAIT cell phenotype and function. We confirmed MAIT cell depletion in the peripheral blood of HIV-positive participants ($P = 0.0349$) (**Figure 3.3.1.1, A**) and found that MAIT cells in the bronchoalveolar compartment were also depleted in these individuals ($P = 0.0471$) (**Figure 3.3.2.1, B**). The median MR1 tetramer-positive cell frequencies declined from 1.74% (0.74 – 3.53%) to 0.88% (0.16 – 2.49%) of CD3+CD4- T cells in the peripheral blood and from 1.15% (0.44 - 2.22%) to 0.38% (0.21 – 1.58%) in the bronchoalveolar compartment.

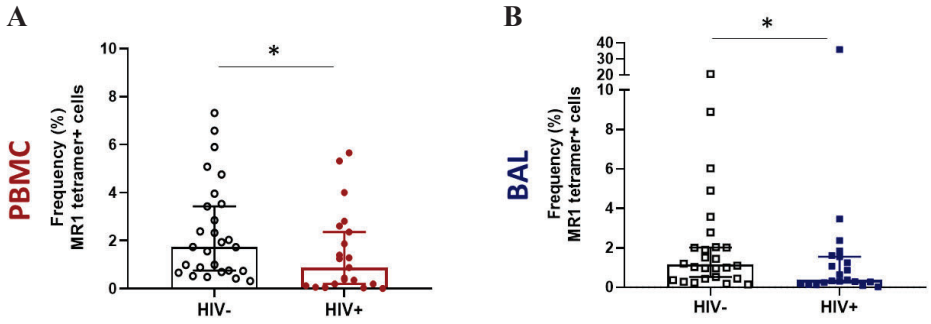


Figure 3.3.1.1: Depletion of MR1 5-OP-RU tetramer-positive MAIT cells in the (**A**) peripheral blood ($P = 0.0349$) and (**B**) bronchoalveolar compartments ($P = 0.0471$) in HIV infection.

We observed that MAIT cells in the peripheral blood remained dominated by the CD161++CD26++ phenotype when assessing the expression of CD161 and CD26 on MAIT cells from HIV-positive participants. HIV infection caused a small change in the phenotype of peripheral blood MAIT cells as evidence by a decrease in the CD161++CD26++ subpopulation ($P = 0.0202$) from 94.2% (87.9 – 97.9%) to 84.3% (74.83 – 94.43%), paired with an increase in the CD161-CD26- subpopulation ($P = 0.0104$) from 1.35% (0 – 3.73%) to 5.38% (2.48 – 12.95%) (**Figure 3.3.1.2, A**). The phenotypic heterogeneity of MAIT cells in the bronchoalveolar compartment was maintained during HIV infection. The proportion of bronchoalveolar MAIT cells with the typical CD161++CD26++ phenotype was significantly reduced from a median of 64.95% (39.0 – 77.88%) to 37.63% (15.68 – 52.7%) ($P = 0.0176$) and the frequencies of the atypical phenotypes marginally increased (**Figure 3.3.1.2, B**).

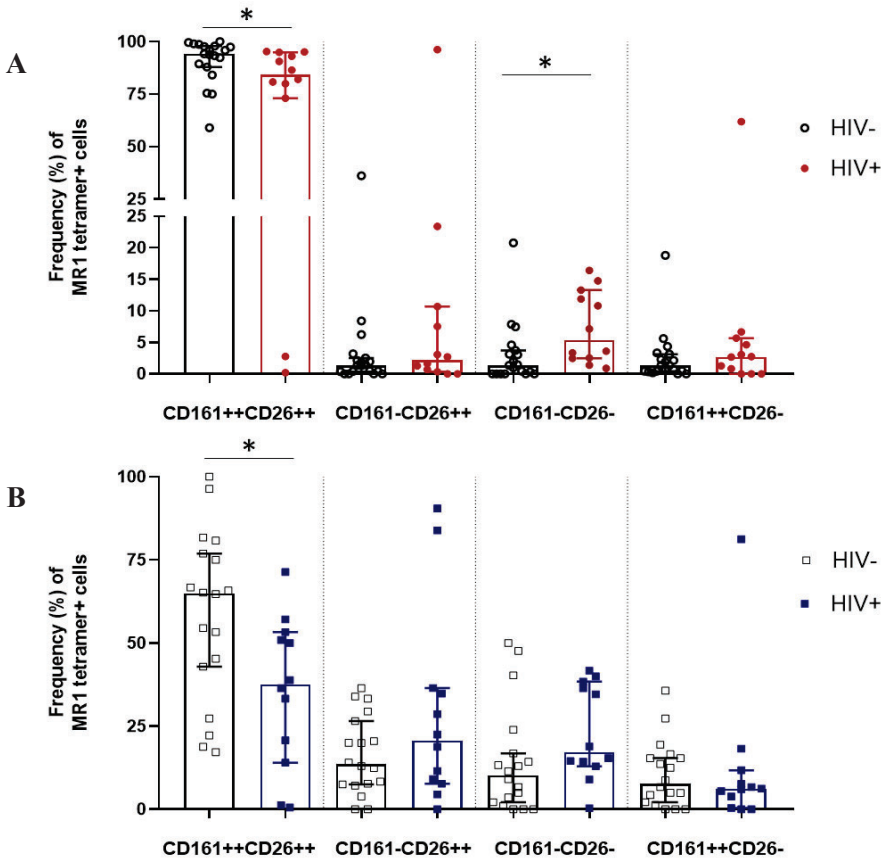


Figure 3.3.1.2: HIV-induced alterations in the phenotypic profiles of MAIT cells from the (**A**) peripheral blood (red) and (**B**) bronchoalveolar compartment (blue). CD161 and CD26 surface staining was performed on MR1 tetramer-positive MAIT cells from the peripheral blood and bronchoalveolar compartments and the staining patterns compared in HIV-negative and HIV-positive participants.

3.3.2. HIV abrogates compartmental functional differences of MAIT cells

Assessment of the functional capacity of *ex vivo* stimulated MAIT cells showed that IL-17 producing MAIT cells were significantly reduced ($P = 0.0247$) in the peripheral blood in HIV (**Figure 3.3.2.1, B**). We observed a non-significant trend towards reduced IFN-γ production in (**Figure 3.3.2.1, A**) this compartment. There were no significant differences in the production of inducible IFN-γ or IL-17 in HIV-positive participants in the bronchoalveolar compartment. HIV did not cause any alterations in the production of constitutive granzyme B by peripheral blood or bronchoalveolar MAIT cells (**Figure 3.3.2.1, C**).

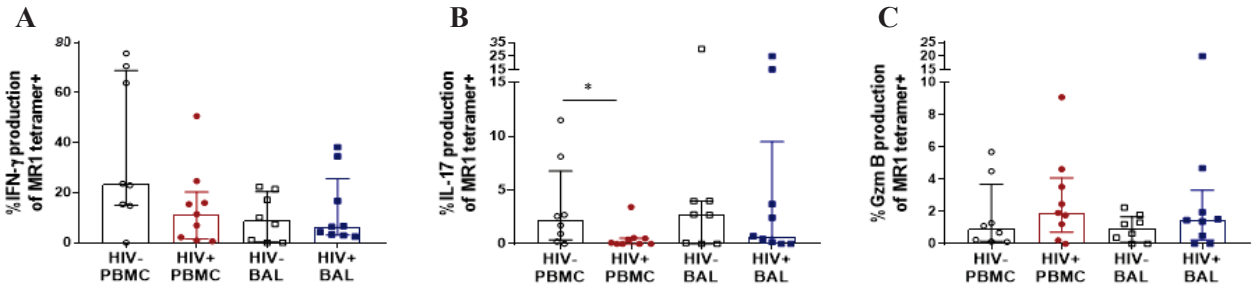


Figure 3.3.2.1: Intracellular cytokine staining for (**A**) inducible IFN-γ, (**B**) inducible IL-17 and (**C**) constitutive granzyme B production in HIV-negative (n = 8) and HIV-positive (n = 9) peripheral blood and bronchoalveolar MAIT cells following 6 hour stimulation with PMA/ionomycin showing reduced functional capacity in peripheral blood MAIT cells during HIV infection.

There were no HIV-mediated differences in expression of the activation marker HLA-DR in either peripheral blood or bronchoalveolar MAIT cells (**Figure 3.3.2.2, A**). There was a trend towards increased PD-1 expression in the peripheral blood of HIV-positive participants ($P = 0.0531$), while its expression remained unchanged in the bronchoalveolar compartment (**Figure 3.3.2.2, B**). TIM-3 expression also showed a trend towards being more highly expressed in both compartments of HIV infected participants (**Figure 3.3.2.2, C**) but was significantly increased only in the peripheral blood ($P = 0.0171$). Analysis of HLA-DR, PD-1 and TIM-3 MFI on the other hand showed no significant HIV-induced alterations in either the peripheral blood or bronchoalveolar compartments (**Figure 3.3.2.2, D - E**).

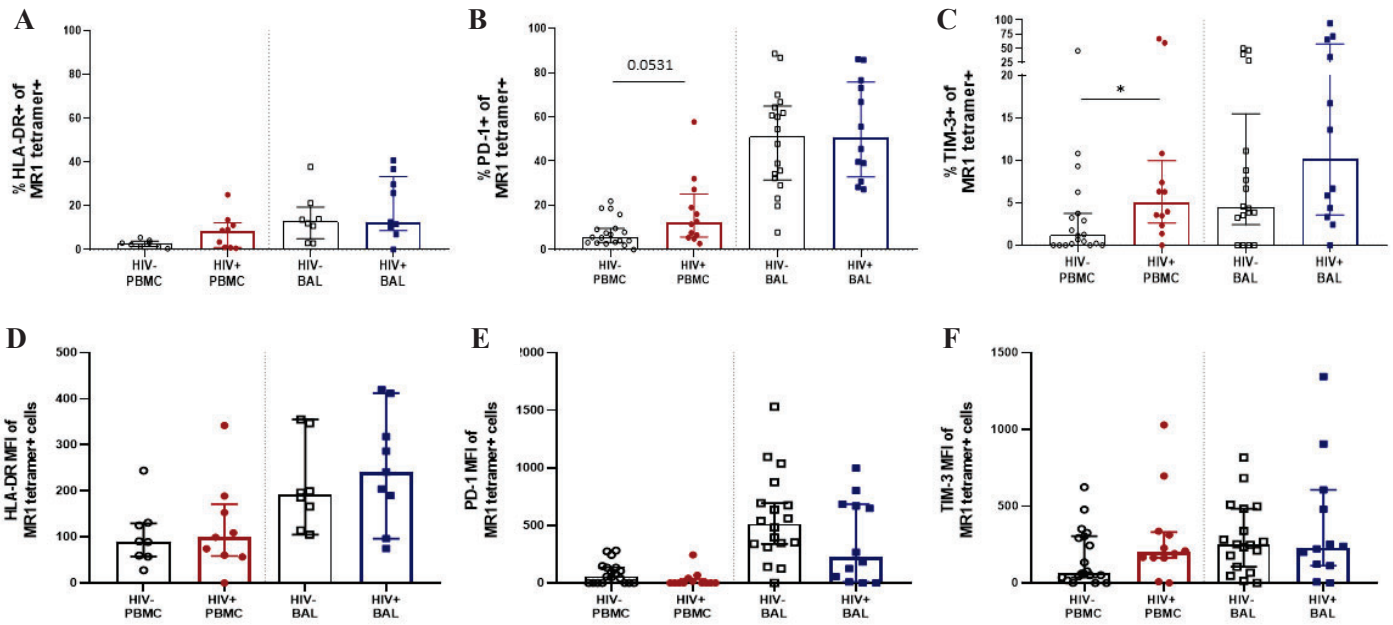


Figure 3.3.2.2: The frequency of (**A**) HLA-DR, (**B**) PD-1 and (**C**) TIM-3 expressing MR1 tetramer-positive MAIT cells, and median fluorescent intensity (MFI) of (**D**) HLA-DR, (**E**) PD-1 and (**F**) TIM-3 staining in the peripheral blood (PBMC) and bronchoalveolar lavage (BAL) fluid of HIV-negative and HIV-positive individuals.

Thus, HIV infection reduced the functional capacity and induced the expression of inhibitory receptors of peripheral blood MAIT cells. In the bronchoalveolar compartment, however, where functional capacity is lower and inhibitory markers are higher in healthy, HIV-negative individuals, we observed little additional impact of HIV-infection on MAIT cell function.

3.4. Discussion

Our study of paired bronchoalveolar and peripheral blood MR1 5-OP-RU tetramer-positive MAIT cells in a cohort of otherwise healthy HIV-negative and HIV-positive participants from Durban, South Africa, demonstrated that in people living with untreated HIV, MAIT cells are depleted in both the peripheral blood and bronchoalveolar compartments. This numerical depletion in HIV-positive participants was accompanied by decreases in the frequency of CD161++CD26++ MAIT cells in both compartments, and an increase in the alternative phenotypes in the bronchoalveolar compartment and the CD161-CD26- sub-population in the peripheral blood. The functional capacity of peripheral blood MAIT cells was altered in people living with untreated HIV, as seen by a reduction in relative

proportions of IL-17 producing MAIT cells, while that of bronchoalveolar MAIT cells remained unchanged. The decreased functional capacity observed in peripheral blood MAIT cells was accompanied by a rise in the expression of inhibitory co-stimulatory markers. In the bronchoalveolar compartment inhibitory marker expression remained at similarly high levels as that observed in HIV-negative individuals.

In HIV-positive individuals we noted a decline in peripheral blood MAIT cells in HIV infection, as has been previously reported (Cosgrove *et al.*, 2013; Leeansyah *et al.*, 2013; Wong *et al.*, 2013). We also observed a decline in MAIT cell frequency in the bronchoalveolar compartment in line with findings by Mvaya *et al.* (2019) who had used a phenotypic definition (CD8+CD161++TRAV1-2+) to measure MAIT cells in humans and Vinton *et al.* (2016) who had used the MR1 tetramer to identify MAIT cells in rhesus macaques. There was a reduction in the frequency of CD161++CD26++ MAIT cells in both the bronchoalveolar and peripheral blood compartments, which in the peripheral blood was associated with an increase in the frequency of CD161-CD26- MAIT cells. An increase in CD161-negative MAIT cells following the reduction in CD161-expressing MAIT cells has been reported by Leeansyah *et al.* (2013) and Eberhard *et al.* (2014). These authors attributed this finding to persistent MAIT cell activation in HIV infected individuals, which may also be the case in our setting as we observed peripheral blood MAIT cells to be less functional and more inhibited, as demonstrated by the reduction in IL-17 producing cells and higher TIM-3 surface expression, in line with these reports (Cosgrove *et al.*, 2013; Leeansyah *et al.*, 2013; Eberhard *et al.*, 2014). Because the expression of both CD161 and CD26 is associated with cytokine responsiveness and cytotoxic effector function, the loss of CD161++CD26++ MAIT cells may be an indicator of increased susceptibility to respiratory infection in HIV infected individuals (Kurioka *et al.*, 2018; Wragg *et al.*, 2020). Interestingly, in the bronchoalveolar compartment, the phenotypic heterogeneity and functional capacity of MAIT cells was not significantly altered by HIV, in line with findings by Mvaya *et al.* (2019), and the high expression of activation and inhibitory markers observed in HIV-negative individuals was maintained.

In conclusion, although both compartments demonstrated MAIT cell depletions associated with untreated HIV, the phenotypic heterogeneity and function of bronchoalveolar MAIT cells remained unaltered by HIV infection, in contrast to HIV's more marked impact on the function and expression of inhibitory co-stimulatory markers in peripheral MAIT cells. We found that MAIT cell heterogeneity at the lung's mucosal surface is preserved even in the face of untreated HIV infection. It is possible that therapeutics directed at the restoration of MAIT cell numbers in the lung could be considered as part of a strategy for improving lung mucosal immunity in HIV infected individuals.

Notably, others have shown that MAIT cell frequency at the lung mucosa can be restored following successful ART, in contrast to findings in the peripheral blood (Mvaya *et al.*, 2019). Further work is required to determine if early ART initiation may promote the preservation of MAIT cells at the lung's mucosal surface and thus contribute to the maintenance of host defence at this site.

CHAPTER 4

CHAPTER 4: COMPARTMENTAL AND HIV-INDUCED TRANSCRIPTOMIC FEATURES OF PERIPHERAL AND BRONCHOALVEOLAR MAIT CELLS

4.1. Introduction

4.1.1. Transcriptional approaches

Whole genome transcriptomic analysis enables quantitative analysis of the genes that a cell or population of cells is actively transcribing in preparation for translation into proteins. From this information, cellular functions can be inferred. Methods such as microarrays and quantitative PCR (qPCR) can also be used to characterise the transcriptional state of cells (Wang *et al.*, 2009; Papalexi and Satija, 2018). A limitation of these methods is the need to know the target RNA sequences which is not required using modern next-generation sequencing (NGS) technologies. NGS, which is a high-throughput method that can be used on lower input samples enables differential gene expression analysis across the entire genome (F. Tang *et al.*, 2009; Soneson and Delorenzi, 2013). RNA-sequencing can be divided into two categories: bulk and single-cell RNA sequencing methods. In bulk RNA-sequencing the transcriptomes of populations of cells are determined. This enables comparison of cellular populations in health versus disease or between anatomical compartments. This method, however, averages the expression of transcripts from many cells and can miss subtle differences between cell types within a population (Treutlein *et al.*, 2014; Buettner *et al.*, 2015; Grün *et al.*, 2015). Single-cell transcriptomics is a rapidly expanding field and a powerful way to identify heterogeneity within cellular populations. Although single-cell transcriptomics can be prone to greater transcriptional noise generated by stochastic fluctuations in gene expression, it can detect lowly expressed genes (Chang *et al.*, 2008; Dillies *et al.*, 2013). In addition, plate-based protocols such as SMART-Seq allow for the assessment of full-length transcripts and the detection of splice variants, in contrast to pooled approaches, for example CEL-Seq, which sequence only the 3' end (Papalexi and Satija, 2018). Thus, the assessment of subtle variations in the transcriptomes of related cells with low starting material is a good application for modern single-cell RNA-sequencing technologies.

Methods of analysis can be divided based on level of organisation, for example transcriptomic analysis can be performed at the exon-level (assessment of differential exon usage), the transcript-level (assessment of isoform usage), the gene-level (determination of differential gene expression) and the sample-level (clustering of samples based on similarity) (Katz *et al.*, 2010; Soneson and Delorenzi, 2013; Kanitz *et al.*, 2015). This chapter will focus on the gene- and sample-level methods of transcriptomic analysis. Differential expression of genes can be determined between two sample types or two conditions following normalisation, to account for differences in transcript length, sequencing depth and GC-content (Evans *et al.*, 2018). Gene expression can be measured in transcripts per million mapped reads (TPM), reads per kilobase per million mapped reads (RPKM), or

fragments per kilobase per million mapped reads (FPKM) all of which account for differences in gene lengths and sequencing depths (Dillies *et al.*, 2013). In addition to normalisation of reads, adjustments for multiple testing are required. The most popular methods include the Bonferroni correction, which controls for the family-wise error rate (FWER), and the Benjamini-Hochberg correction which controls for false discovery rates (FDR) (Korthauer *et al.*, 2019). The newer FDR methods are more commonly used as they are able to control for type I errors while still maintaining power enough to detect true positives unlike the FWER-base methods (Korthauer *et al.*, 2019). Similarity measures can be used to determine correspondence between two transcriptomes and be used for sample clustering analyses. Correlation matrixes, most commonly constructed using the Pearson or Spearman rank correlations, perform pairwise transcriptome assessment from normalised gene expression data (Soneson and Delorenzi, 2013). Similarities between samples can then be visualised using heatmaps, alternatively dimension reduction methods, such as the principal component analysis (PCA), t-stochastic neighbour embedding (t-SNE) or uniform manifold approximation and projection (UMAP) can be used prior to similarity assessment. All three methods aim to convert the high-dimensional structure of the data into a low-dimension representation where transcriptionally similar datapoints lie closer together. (Van Der Maaten and Hinton, 2008). Computation of UMAP outputs is fast, reproducible and can detect subtle differences between populations, that may otherwise be missed by t-SNE, making it well-suited to perform dimensionality reduction on single-cell data (Becht *et al.*, 2019).

4.1.2. Transcriptomic features of MAIT cells

Although MAIT cells can be enriched in tissue sites, the characterisation of typical MAIT cell genes has mostly been based on assessment of the peripheral blood transcriptome. In addition to expressing T cell lineage surface marker genes such as *CD3Z* and *CD8A*, MAIT cells also express *KLRB1* (CD161) and *DPP4* (CD26) amongst others (M. Sharma *et al.*, 2020). Effector function has been well characterised in peripheral blood MAIT cells at the protein level as described in Chapter 1, section 1.10. At the transcriptomic level MAIT cells in the peripheral blood have been observed to express *TNF*, *GNLY* (granulysin), *GZMA*, *GZMB* and *GZMK* (granzymes A, B and K), as well as *PRF1* (perforin), in line with protein-level findings (D. Park *et al.*, 2019; Lu *et al.*, 2020; M. Sharma *et al.*, 2020). Some characteristic cytokine receptors expressed by MAIT cells include *IFNGR1* (IFN-γ receptor 1, CD119), *IL18R1* (IL-18 receptor 1), *IL7R* (IL-7 receptor, CD127) (Dusseaux *et al.*, 2011; M. Sharma *et al.*, 2020). MAIT cells express high levels of tissue homing transcripts *CCR5*, *CXCR6*, *CCR6* and moderate levels of *CXCR4* and *CCR9* (Dusseaux *et al.*, 2011; D. Park *et al.*, 2019). Classical effector transcription factors such as *TBX21* (T box transcription factor 21, T-bet) and *EOMES* (Eomesodermin) are expressed by MAIT cells, as well as transcription factors required for

innate T cell activation and proliferation, such *ZBTB16* (promyelocytic leukaemia zinc finger protein, PLZF), *RORC* (retinoic acid-related orphan receptor γt, RORγt) and *IKZF2* (Helios) (Leeansyah *et al.*, 2015; Gibbs *et al.*, 2017; D. Park *et al.*, 2019). Typical peripheral blood MAIT cells express signalling molecules such as *SLAMF1* (signalling lymphocytic activation molecule family member 1) and *TRAF1* (TNF receptor associated factor 1), and the multidrug resistance transporter *ABCB1* (Dusseaux *et al.*, 2011; Pomaznoy *et al.*, 2020; M. Sharma *et al.*, 2020). More recently the expression of genes such as *VEGFA* (vascular endothelial growth factor A) involved in angiogenesis, and *MMP25* (matrix metalloproteinase-25) involved in tissue remodelling, have been observed in MAIT cells as part of a tissue repair signature (Constantinides *et al.*, 2019; Hinks *et al.*, 2019; Leng *et al.*, 2019). Genes including *RUNX3*, *ITGA2* and *CCL22* have also been reported to be expressed by a novel subset of atypical MR1 tetramer-negative T cells which had a phenotype consistent with that of MAIT cells (TRAV1-2+CD161+CD8+). These gene, which are involved in innate immunity, tissue residency and homing, had an *Mtb*-specific signature and were upregulated in MAIT cells from latently TB infected individuals in contrast to TB negative controls (Pomaznoy *et al.*, 2020). Peripheral blood MAIT cells thus express T cell lineage markers, effector molecules to perform cytolytic function, a variety of cytokine receptors which enable TCR-independent signalling, a range of chemokine receptors for homing to mucosal sites, and in some cases, transcripts related to tissue repair or other atypical functions.

MAIT cells at different mucosal sites have been shown to display an activated transcriptional profile characterised by the expression of *CD38*, *PDCD1* (PD-1) and *CD40LG* (Slichter *et al.*, 2016; Lu *et al.*, 2020). MAIT cells at these sites also express an array of integrins including *ITGAE* (CD103), and *ITGA1* (CD49a), as well as homing markers such as *CCR6* and *CXCR6*, but not *CCR7* (X. Tang *et al.*, 2013; Lu *et al.*, 2020). Mucosal MAIT cells also express cytotoxicity-related effector genes like *IFNG, IL17A* and *TNF* but were unable to produce these cytokines following TCR-stimulation alone (X. Tang *et al.*, 2013; Slichter *et al.*, 2016). Interestingly, a study characterising respiratory mucosal MAIT cells in children with community acquired pneumonia (CAP) found that TCR-stimulation was sufficient to stimulate IL-17A production, and that Th17 differentiation transcription factors such as *BATF* and *STAT3* were upregulated in bronchoalveolar MAIT cells as compared to peripheral blood (Lu *et al.*, 2020). This observation suggests the tight regulation of cytotoxic function in the mucosal MAIT cells of healthy individuals in order to prevent excessive inflammation, and in disease mucosal MAIT cells may be primed toward Th17-mediated immune responses at these sites. Because the number of studies describing transcriptomic features of mucosal MAIT cells, specifically those at the lung mucosa, is limited we employed both bulk and single-cell transcriptomic approaches to determine the transcriptomic differences between bronchoalveolar and peripheral blood MAIT cells and also further characterise the heterogeneity of bronchoalveolar MAIT cells.

4.2. Methods

4.2.1. Cell sorting

Cells were prepared for sorting by surface staining paired bronchoalveolar lymphocytes and peripheral blood mononuclear cells (PBMCs) using 1:500 of MR1 5-OP-RU (PE) (NIH Tetramer Core Facility) for 40 minutes in the dark at room temperature (RT). Cells were then stained for 20 minutes at 4°C using a master-mix containing 1:800 of Live/Dead viability dye (Aqua, Invitrogen) and the following monoclonal antibodies: 1:100 of anti-CD3 PE-CF594 (UCHT1, BD Biosciences), 1;50 of anti-CD4 BV711 (OKT4, BioLegend), 1:200 of anti-CD8 APC-H7 (SK1, BD Biosciences), and 1:50 each of anti-CD26 FITC (BA5b, BioLegend) and anti-CD161 PE-Cy7 (HP-3G10, BioLegend). Cells were washed, resuspended in FACS buffer (5% FBS in 1x PBS) and either bulk sorted into populations of 100 cells per well (in quadruplicate wherever possible) or single-cell sorted. During a single sorting session and using fresh cells, MAIT cells for each individual were sorted into a 96-well microplate with the first two rows being reserved for mini-populations (one row each for peripheral blood MAIT cells and bronchoalveolar MAIT cells) and the last six rows containing single-cells (three rows per compartment) (**Figure 4.2.1**). Cells were sorted using the FACSAria™ III (BD Biosciences) in purity mode, using a sorting strategy that had been optimized and confirmed to result in > 85% purity. MR1 tetramer-positive MAIT cells, which were gated on from CD3+CD4- live lymphocytes, were sorted into 96-well microplates containing 10 µL of 1% 2-mercaptoethanol RLT buffer (Qiagen). Plates were snap frozen and cells stored at -80°C until further use.

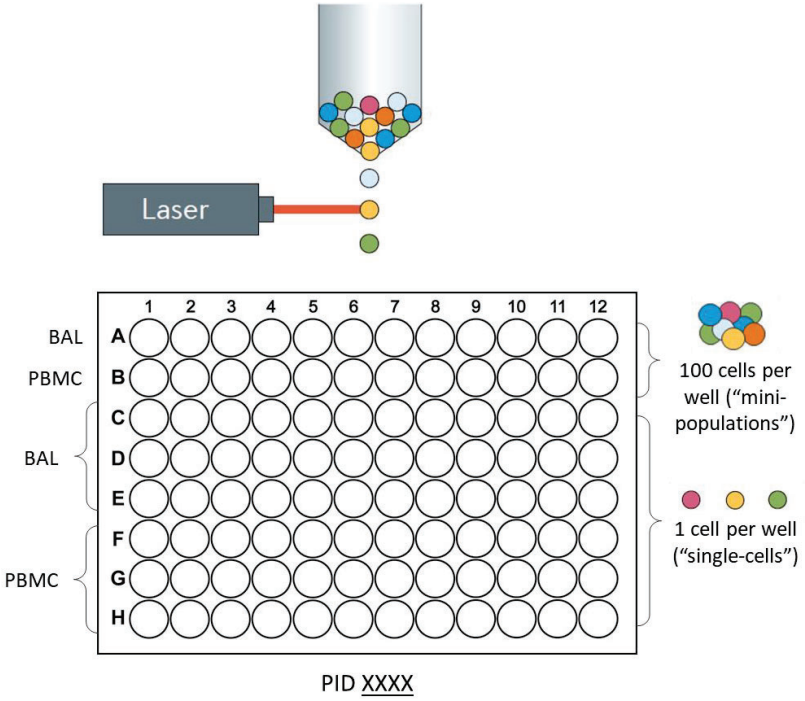


Figure 4.2.1: Fluorescence activated cell sorting (FACS) of MR1 tetramer-positive MAIT cells for each participant identifier (PID). MAIT cells were sorted into 100-cell mini-populations (collected in quadruplicate) for bulk RNA-sequencing (rows A and B of 96-well plate) and single cells for single-cell RNA sequencing (rows C to H of 96-well plate) for each PID (adapted from Papalexi and Satija (2018)).

4.2.2. Single-cell and bulk RNA-sequencing

RNA capture and library preparation were performed on 100-cell mini-populations and single-cell sorted MR1 tetramer-positive MAIT cells from six HIV-negative and six HIV-positive individuals using the SMART-Seq2 approach (Trombetta *et al.*, 2014). Sorted cell populations or single cells were thawed and SPRI magnetic beads (Agencourt RNAClean XP, Beckman Coulter) used to isolate full-length RNA by adding 2.2 times the volume of the beads (at RT) to each sample well, pipette-mixing and incubating for 10 minutes. Microplates were incubated on a 96-well plate magnet before the supernatant was remove and the beads then washed three times with freshly made 80% ethanol. Beads were allowed to dry before the addition of 4.5 µL of SMART CDS Primer II A mastermix per well. Sealed microplates were incubated at RT from 1 minute, centrifuged at 800g for 1 minute and then incubated for 3 minutes at 72°C. Plates were placed on ice, 5.5 µL of SMARTer II A Oligo mastermix added per well, samples pipette-mixed and plates then centrifuged (800g for 1 minute). Samples were placed in a thermal cycler (please refer to Trombetta *et al.* (2014) for cycling conditions) and cDNA then synthesized from these RNA sequences. Plates were stored at 4°C and

whole transcriptome amplification (WTA) was then performed by RT-PCR amplification the following morning. Plates were centrifuged (800g for 1 minute), 40 µL/well of IS PCR Primer mastermix added, samples pipette-mixed and centrifuged before WTA in a thermal cycler (conditions outlined in Trombetta *et al.* (2014)). Plates were centrifuged, 0.8 times the volume of RT DNA SPRI beads (Agencourt AMPure XP, Beckman Coulter) added, and samples pipette-mixed. Plates were incubated for 5minutes on the benchtop and then for a further 5 minutes on a 96-well plate magnet. Samples were washed three times with 80% ethanol, beads then dried and resuspended in 20 µL of TE buffer, and eluent transferred to a clean microplate. The quality of the WTA product was determined by assessing fragment size using the BioAnalyzer (Agilent Technologies), using 1 µL of PCR product. The concentration of the WTA product was then quantified, using the Qubit® fluorometer and accompanying assay kit (Thermo Fisher), and then samples diluted to 0.1 – 0.2 ng/µL in TE buffer. Nextera XT sequencing libraries were constructed by amplification of DNA once 10 µL each of i7 and i5 index primers had been added. Libraries were pooled into a single 1.5 mL tube and then purified using 0.9 µL of DNA SPRI beads (Agencourt AMPure XP, Beckman Coulter). The quality and quantity of the sequencing libraries were then assessed using 1 µL of PCR product for the BioAnalyzer and another 1 µL of product for the Qubit® fluorometer before the sequencing was performed on the NextSeq500 (Illumina).

4.2.3. Data analysis

Single-cell and bulk RNA sequence data was demultiplexed with bcl2fastq v2.17, aligned to the human genome using TopHat and the counts were estimated using RSEM (Li and Dewey, 2011). Differential gene expression analysis and data visualisation of the single-cell data was performed using Seurat R package (3.0.1) (Butler *et al.*, 2018), while DESeq2 R package (1.16.1) (Love *et al.*, 2014) and Prism 8.0.0 were used for the bulk RNA-sequencing analysis. Genes were considered differentially expressed when fold change > 1 or < -1 and FDR *q*-value < 0.05 (Benjamini-Hochberg correction). Pathway analysis was performed using pathfindR R package (1.5.1) using the gene ontology (GO) database. Pathways were considered enriched if they contained 10-300 genes (Ulgen *et al.*, 2019). Analyses were then performed on single-cell data by assessing the expression of typical MAIT cell genes (D. Park *et al.*, 2019; Salou *et al.*, 2019; M. Sharma *et al.*, 2020). Two lists of genes expressed in atypical MR1 tetramer-negative TRAV1-2+CD161+CD8+ T cells (Pomaznoy *et al.*, 2020) and tissue repair (Constantinides *et al.*, 2019; Hinks *et al.*, 2019; Leng *et al.*, 2019) was compiled from the available literature. As described below, these lists were used to analyse both the single-cell and bulk RNA-sequencing datasets.

4.3. Results

4.3.1. Compartmentally distinct transcriptomic profiles of MAIT cells in health

From the six HIV-negative individuals that were selected for RNA-sequencing analysis, data from three individuals failed to meet quality control requirements and were excluded from further analyses on the basis of a low percentage of reads mapping to the genome and/or genes with low counts. Bulk RNA-sequencing revealed there to be a total of 474 genes differentially expressed between the transcriptomes of bronchoalveolar and peripheral blood MAIT cells in HIV-negative individuals, 171 were upregulated in bronchoalveolar MAIT cells and 303 were upregulated in peripheral blood. Genes upregulated in the peripheral blood included *TGFA*, *PIK3R1* and *CX3CR1*, as well as *TGFBR3*, *PXN* and *SMAD3* (**Figure 4.3.1.1**). These genes are significantly enriched in the positive regulation of protein kinase B signalling (pathway cluster 3, **Figure 4.3.1.2**) and in TGFβ receptor signalling (cluster 7), respectively. These pathways are involved in cell growth, survival and cellular differentiation. Conversely, genes that were upregulated in the bronchoalveolar compartment included *HSPA1A*, *HSPA1B*, *HSPA6*, and *ALDH3B1* (**Figure 4.3.1.1**) which are involved in thermal and oxidative stress mediation (cluster 4) (**Figure 4.3.1.2**), as well as genes including *TLR4* (toll-like receptor 4) and *IL1B* which are involved in the regulation of IL-8 (neutrophil chemoattractant, CXCL8) production (cluster 4). These data suggest the maintenance of homeostasis (cellular differentiation and growth) in the peripheral blood, compared to mediation of stress responses and innate sensing in the lung environment. Insufficient genes were detected to enable confident analysis of within compartment transcriptomic profiles of MAIT cells across HIV status.

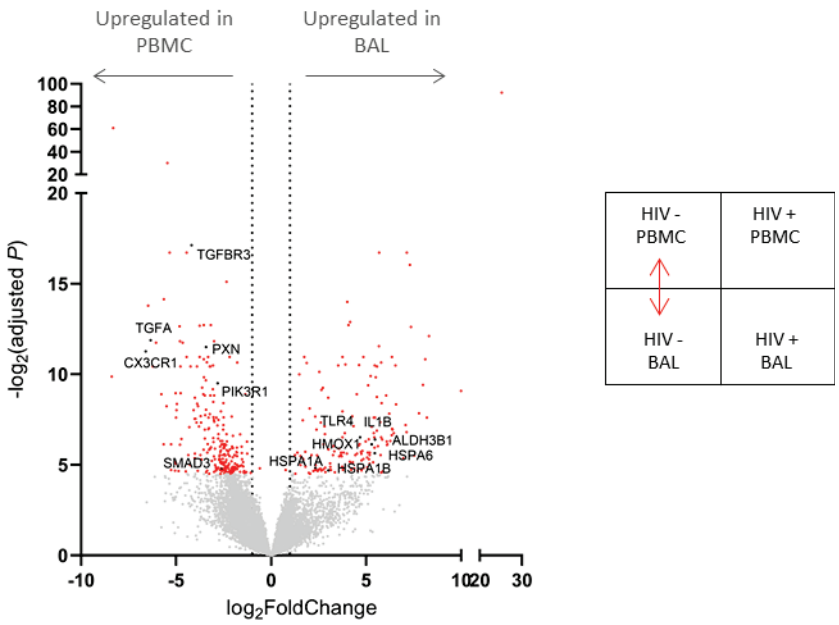


Figure 4.3.1.1: Bulk RNA-sequence analysis of MR1 5OP-RU tetramer-positive MAIT cells from the peripheral blood (PBMC) and bronchoalveolar lavage (BAL) fluid of HIV-negative participants. Volcano plot showing significantly expressed genes (highlighted in orange) above cut-off of adjusted $P = 0.05$ (left) and grid indicating the comparison (right).

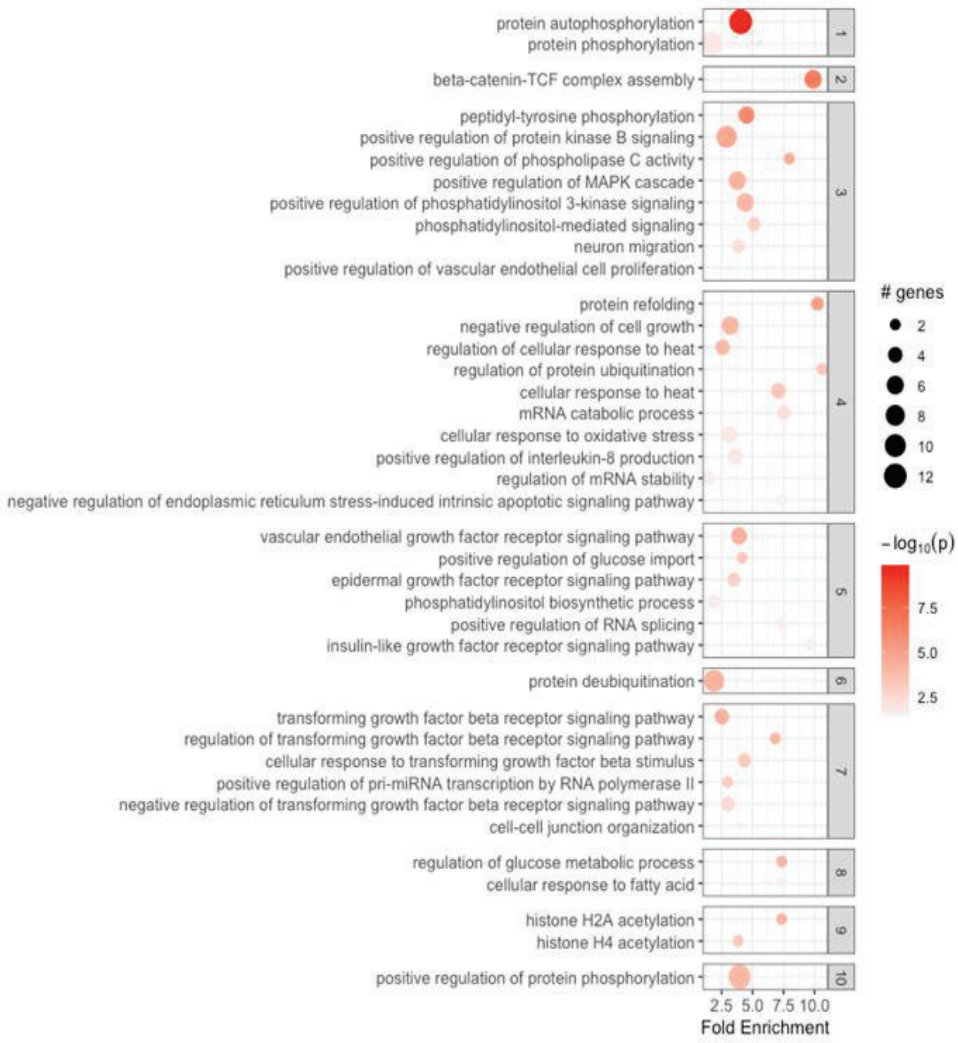


Figure 4.3.1.2: The top 10 significantly enriched pathway clusters (grouped by relatedness) among differentially expressed genes from the comparison of peripheral blood and bronchoalveolar MR1 5OP-RU tetramer-positive MAIT cells of healthy, HIV-negative people (n = 3).

4.3.2. The transcriptomic profiles of peripheral blood and bronchoalveolar MAIT cells in people with HIV.

Because we did not detect sufficient numbers of differentially expressed genes across HIV-infection groups within each individual compartment to perform pathway analysis, we next compared the bulk transcriptomic profiles across compartment (i.e. peripheral blood versus bronchoalveolar MAIT cells) in HIV infected individuals. This analysis revealed there to be a total of 2813 differentially expressed genes between the two compartments. The majority of these genes were upregulated in the peripheral blood (2588) with only 225 being upregulated in bronchoalveolar MAIT cells. Peripheral blood

MAIT cells in HIV infected individuals increased their expression of genes including *NFKBIA*, *MAP3K8* and *RIPK2*, as well as *TNF*, *TNFRSF14* and *CD27* (**Figure 4.3.2.1**). These genes are enriched in IL-1 mediated signalling and TNF-mediated signalling respectively (cluster 5) (**Figure 4.3.2.2**). Genes which were noted to also be upregulated in the peripheral blood were *RPS23* and *EIF2S1*, involved in stress granule assembly, and *IL10RA* and *IFNAR2* which have been implicated in anti-inflammatory signalling. In the bronchoalveolar compartment, *HLA-DRB1*, *HLA-DRB5* and *HLA-DRA* were upregulated in the HIV-positive participants (**Figure 4.3.2.2**). These genes are involved in antigen processing and presentation (cluster 5). Genes which were also upregulated in bronchoalveolar MAIT cells include *IL1B*, *CXCL2* and *CD80*, which are involved in cytokine-mediated signalling pathway (**Supplementary Table 4.5.1**). Overall, in HIV-positive participants, peripheral blood MAIT cells appear to be balancing cytokine-mediated pro-inflammatory response with anti-inflammatory responses, while bronchoalveolar MAIT cells are maintaining antigen sensing in the local environment.

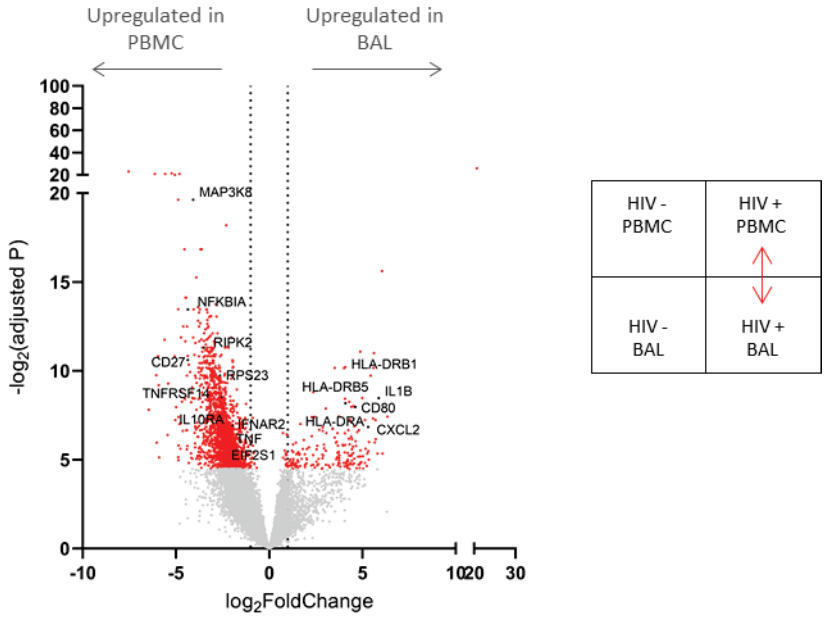


Figure 4.3.2.1: Population transcriptomic analysis of MR1 tetramer-positive MAIT cells from the peripheral blood and bronchoalveolar compartments of HIV-positive individuals. Volcano plot showing significantly expressed genes (highlighted in orange) above cut-off of adjusted $P = 0.05$ (left) and grid indicating the comparison (right).

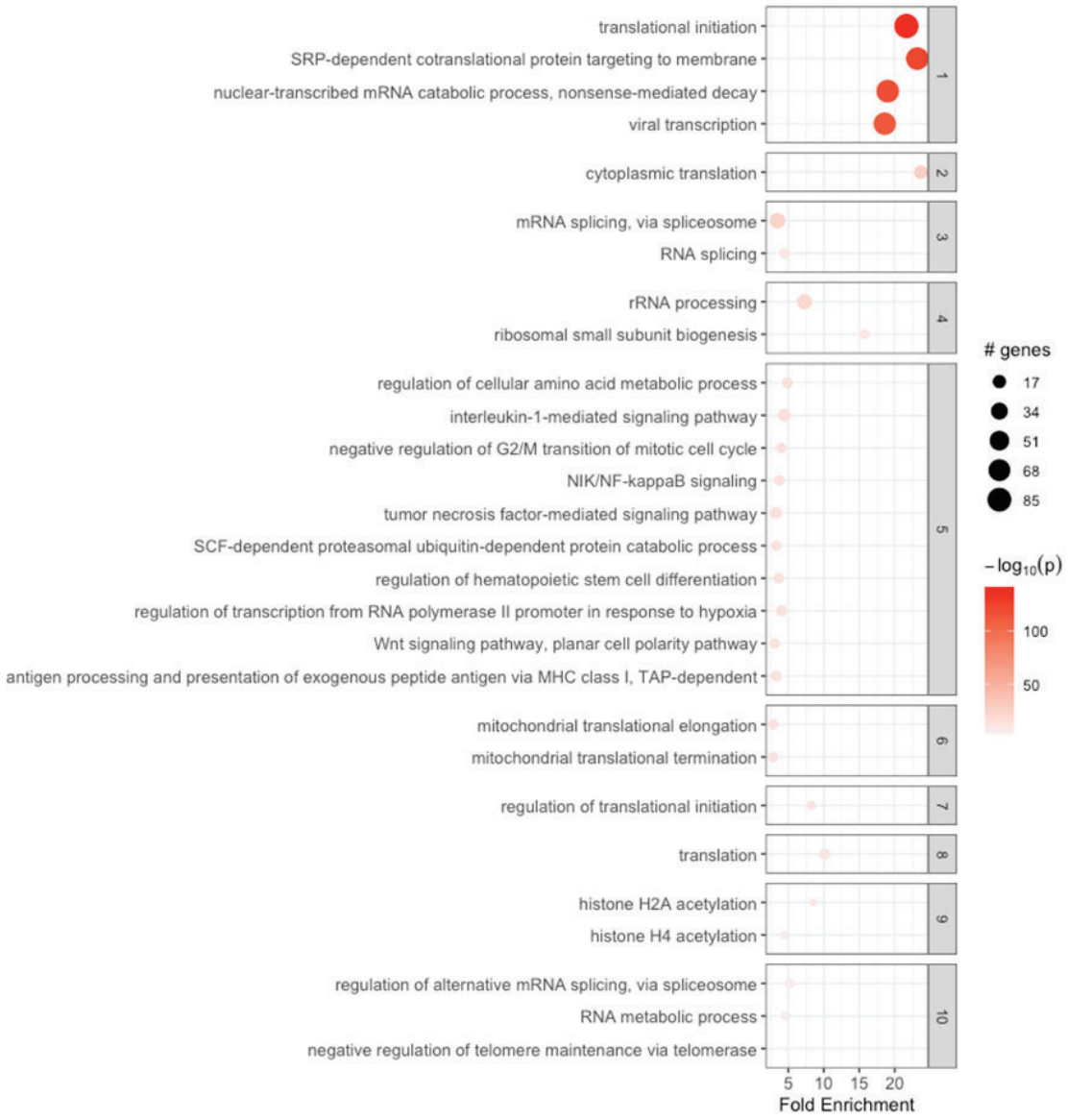
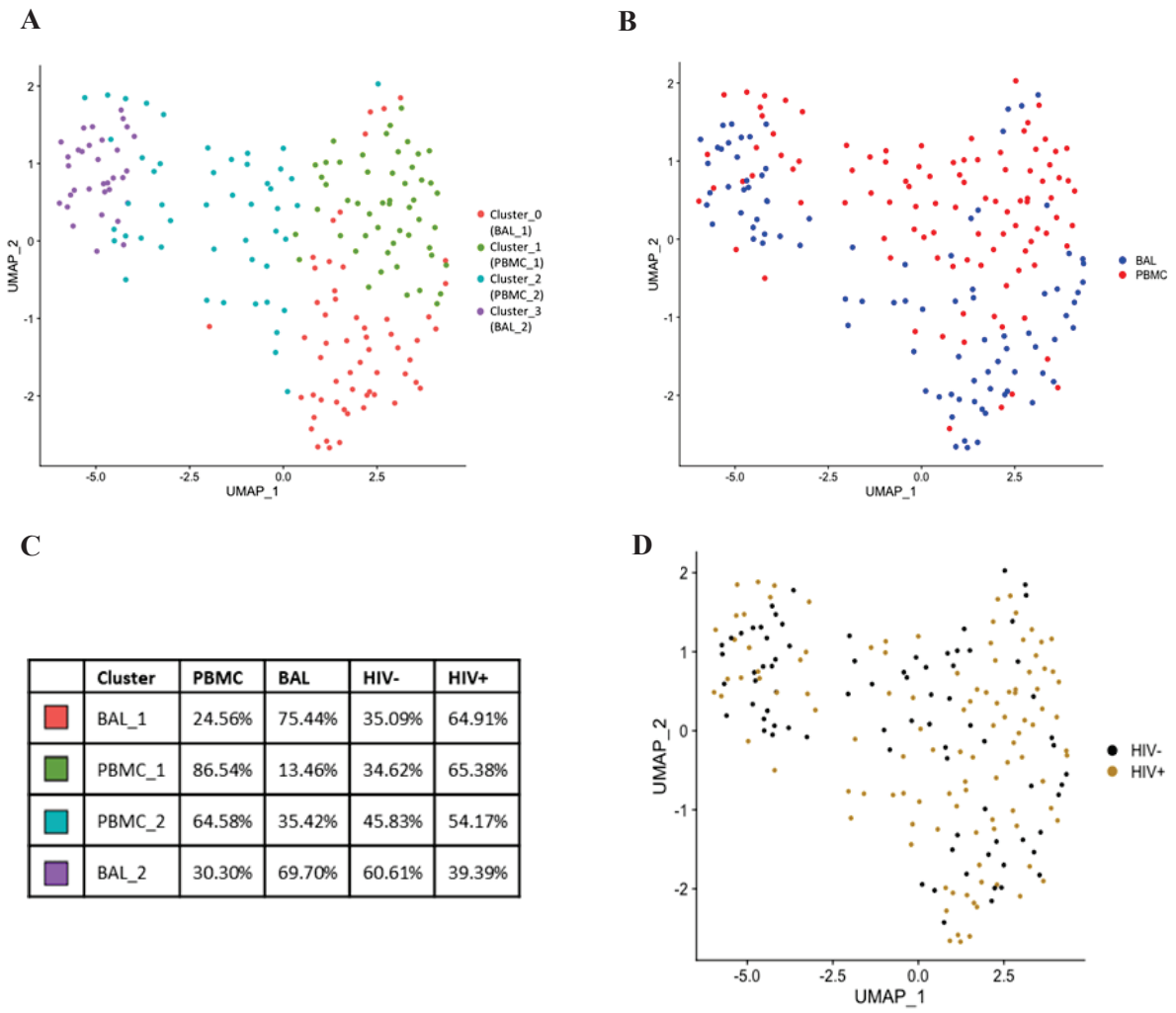


Figure 4.3.2.2: Bubble graph showing the top 10 significantly enriched pathway clusters (grouped by relatedness) comparing MAIT cells from the peripheral blood and bronchoalveolar lavage fluid of HIV-positive individuals (n = 6).

4.3.3. Unsupervised single- cell RNA-sequencing reveals transcriptionally distinct MAIT cell subpopulations

Unsupervised analysis of 190 MR1 tetramer-positive cells, pooled from HIV-negative (n = 3) and HIV-positive (n = 6) samples from both compartments, revealed that MAIT cells separate into four distinct transcriptional clusters based on unique transcriptional profiles (**Figure 4.3.3.1.A**). Clustering was highly driven by compartment (**Figure 4.3.3.1.B**) with Cluster_0 (coral) and Cluster_3 (purple)

composed predominantly of bronchoalveolar MAIT cells (75.44% and 69.70% respectively) and Cluster_1 (lime green) and Cluster_2 (turquoise) composed predominantly of peripheral blood MAIT cells (86.54% and 64.58% respectively) (**Figure 4.3.3.1.C**). The clusters were thus renamed the "BAL_1" (coral) and "BAL_2" (purple), and "PBMC_1" (lime green) and "PBMC_2" (turquoise) subsets.



	Cluster	PBMC	BAL	HIV-	HIV+
	BAL_1	24.56%	75.44%	35.09%	64.91%
	PBMC_1	86.54%	13.46%	34.62%	65.38%
	PBMC_2	64.58%	35.42%	45.83%	54.17%
	BAL_2	30.30%	69.70%	60.61%	39.39%

Figure 4.3.3.1: (**A**) Unsupervised clustering of peripheral blood and bronchoalveolar MR1 tetramer-positive MAIT cells from HIV infected and uninfected participants. (**B**) UMAP plot showing compartment of origin for each cell. (**C**) Summary of characteristics of MR1 tetramer-positive MAIT cell transcriptomic subsets. (**D**) UMAP plot showing assignment of HIV status to MAIT cell transcriptomic subsets.

HIV status did not appear to drive the clustering patterns observed, with all four transcriptional subsets comprised of cells from HIV-negative and HIV-positive participants (**Figure 4.3.3.1.D**). MAIT cell subsets were represented by cells from the nine different individuals with the distribution of subsets varying between individuals (**Figure 4.3.3.2**).

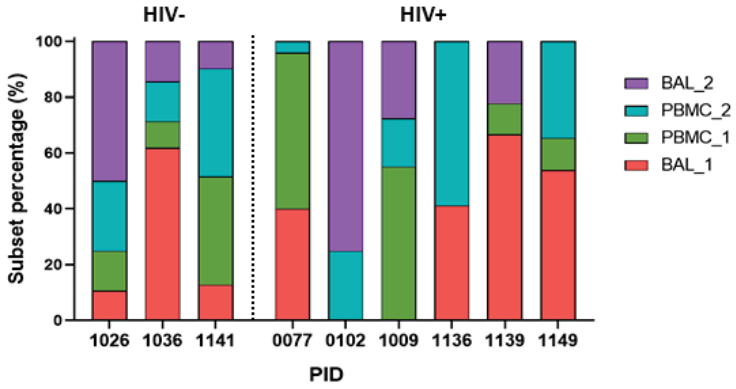


Figure 4.3.3.2: Distribution of MAIT cell transcriptomic subsets (%) across participants (PID = participant identifier).

The expression of CD161 (*KLRB1*) and CD26 (*DPP4*) was next assessed due to earlier observations of heterogenous expression of these surface markers in the bronchoalveolar compartment (Chapter 2). This analysis revealed an interesting pattern in the expression of *KLRB1* and *DPP4* by the MAIT cell subsets generated by unsupervised transcriptomic clustering. Cells from the BAL_1 and PBMC_1 subsets both co-expressed *KLRB1* and *DPP4* (**Figure 4.3.3.3**). PBMC_2 on the other hand, contained cells expressing only *KLRB1*, while the BAL_2 subset contained cells that expressed neither *KLRB1* nor *DPP4*.

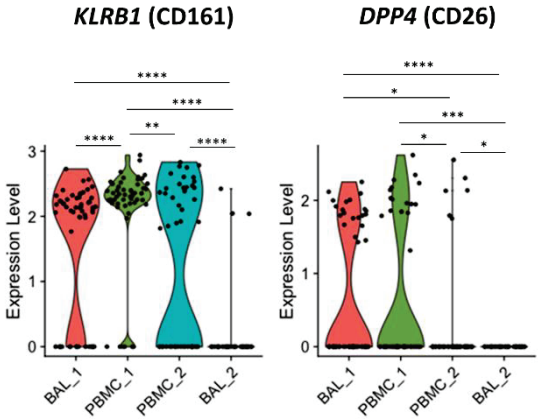


Figure 4.3.3.3: Violin plots showing the expression of typical MAIT cell transcripts *KLRB1* (CD161) and *DPP4* (CD26) by each transcriptomic subset.

4.3.4. Unique transcriptomic subset revealed in the bronchoalveolar compartment

We next analysed the expression of genes known to be characteristic of the MAIT cell transcriptome (i.e. "typical" MAIT genes) in the 4 clusters. This revealed expression of these genes in the BAL_1 and PBMC_1 clusters/subsets. The PBMC_2 subset also expressed many typical MAIT cell genes, while very few of these were expressed by the BAL_2 subset (**Figure 4.3.4.1**).

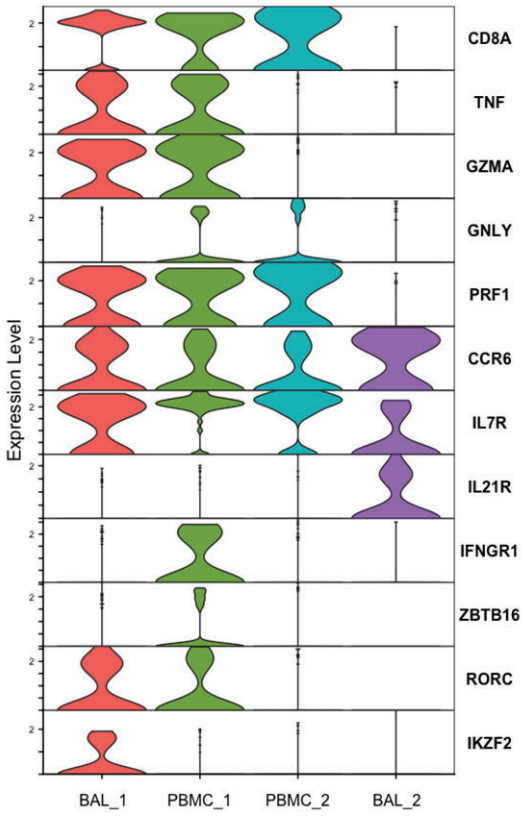


Figure 4.3.4.1: Violin plot showing the expression of typical MAIT cell genes by MR1 5-OP-RU tetramer-positive MAIT cells from each transcriptomic subset.

Following our observation that typical MAIT cell genes were rarely expressed in the BAL_2 subset, we next determined the expression of known macrophage genes in this cluster to rule out the possibility that this cluster represented alveolar macrophage (AM) contamination, as AMs are a highly prevalent and sticky population in BAL fluid. There was very little expression of macrophage genes such as *CD163*, *FCN1* and *VSIG4* across all four transcriptional subsets (**Figure 4.3.4.2**) and we thus concluded that the BAL_2 subset did not consist of AM contaminants.

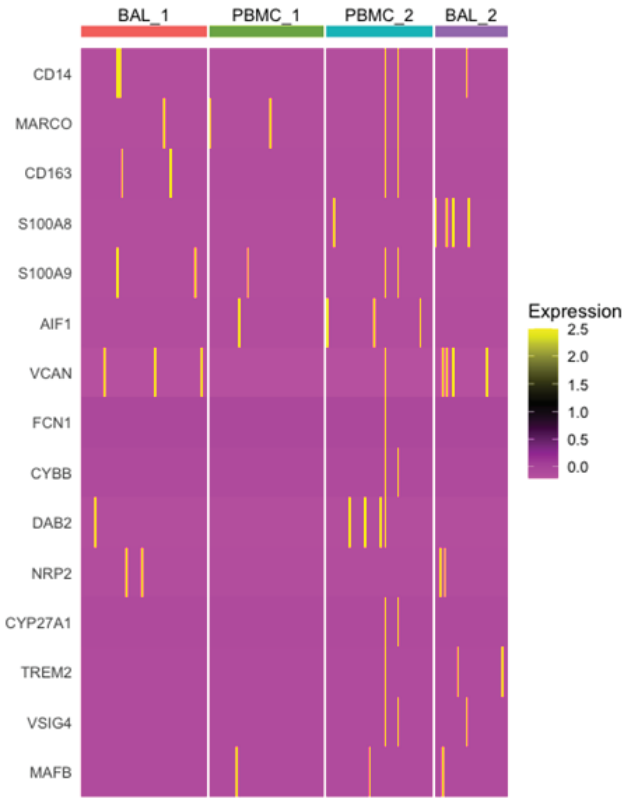


Figure 4.3.4.2: Heatmap showing the expression of signature macrophage genes across the four transcriptional MR1 tetramer-positive MAIT cell subsets.

To try to figure out the transcriptional characteristics of the BAL_2 cluster, we next considered a list of genes found to be elevated in a population of recently described atypical MR1 tetramer-negative TRAV1-2+CD161+CD8+ T cells (Pomaznoy *et al.*, 2020) (full list of differentially expressed genes listed in **Supplementary Table 4.5.2**). Analysis of the genes from this list that were represented in the single-cell dataset showed that these genes, termed "atypical" MAIT genes were more frequently expressed in BAL_2 than in the other MAIT cell transcriptional subsets (**Figure 4.3.4.3**). This suggests resemblance of the BAL_2 subset to these novel MAIT cells with atypical gene expression. Notably, the PBMC_2 subset also expressed some but not all of the typical MAIT cell genes and a few of the atypical MAIT cell genes, suggesting intermediate character. The peripheral blood is thus composed of two closely related MAIT cell subsets with transcriptomic similarities, while in the bronchoalveolar compartment there are two transcriptionally distinct subsets, suggesting greater transcriptomic heterogeneity in the lung mucosal compartment.

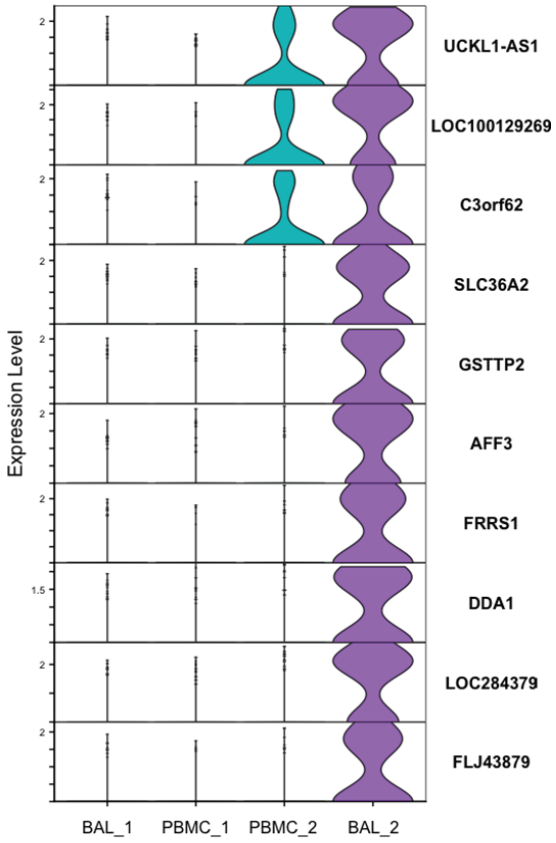


Figure 4.3.4.3: Violin plot of the expression of atypical MR1 tetramer-negative TRAV1-2+CD161+CD8+ T cell genes by MR1 tetramer-positive MAIT cells from each transcriptomic subset.

Following the observation that MAIT cells in the BAL_2 subset completely lacked the expression of typical MAIT effector genes such as *TNF*, *GZMA* (granzyme A), and *PRF1* (perforin) (**Figure 4.3.4.1**), we next sought to establish the functional profile of this subset. To determine whether these atypical MAIT cells expressed a tissue repair gene signature we assembled a list of genes obtained from recent literature describing MAIT cells with this function (Constantinides *et al.*, 2019; Hinks *et al.*, 2019; Leng *et al.*, 2019) (genes listed in **Supplementary Table 4.5.3**). This analysis demonstrated that the majority of the tissue repair genes expressed in the single-cell data set were upregulated by MAIT cells from the BAL_2 subset (**Figure 4.3.4.4**). These findings suggest that the atypical MAIT cell subset in the bronchoalveolar compartment may possess tissue repair functions.

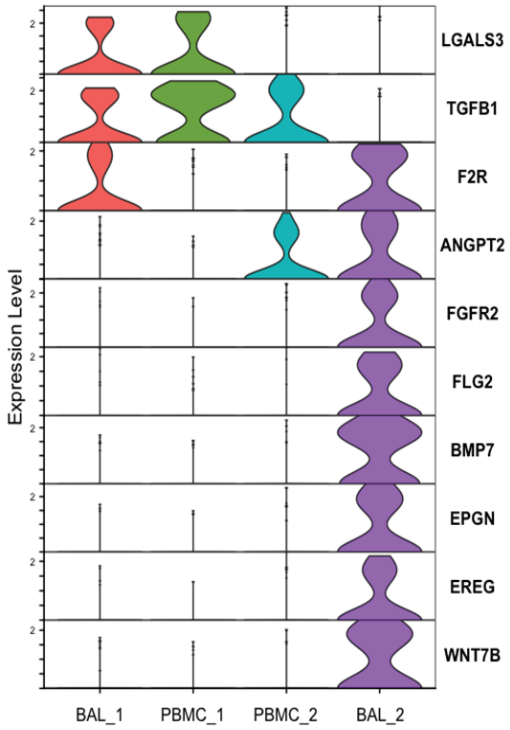


Figure 4.3.4.4: Violin plot showing the expression of MAIT tissue repair genes by MR1 5-OP-RU tetramer-positive MAIT cells from different transcriptomic subsets.

We next assessed the transcriptomic expression of HLA-DR, PD-1 and TIM-3 as our protein-level data had shown that bronchoalveolar MAIT cells express more of these markers of activation and inhibition. This analysis demonstrated that the BAL_2 subset displayed higher expression of *HAVCR2* (TIM-3) which may contribute to the low expression of effector gene transcripts seen in this subset (**Figure 4.3.4.5)**. No differences were noted in the expression of *HLA-DRB1* (HLA-DR). *PDCD1* (PD-1), was low across all transcriptomic subsets, potentially due to limitations of single-cell sequencing. Overall, these findings suggest that, according to their transcriptional profiles, three of our unsupervised subsets (BAL_1, PBMC_1 and PBMC_2) resemble typical MAIT cells, while one subset (BAL_2) had a completely distinct transcriptional character that had similarities with two recently reported subpopulations of MAIT cells with alternative phenotypes.

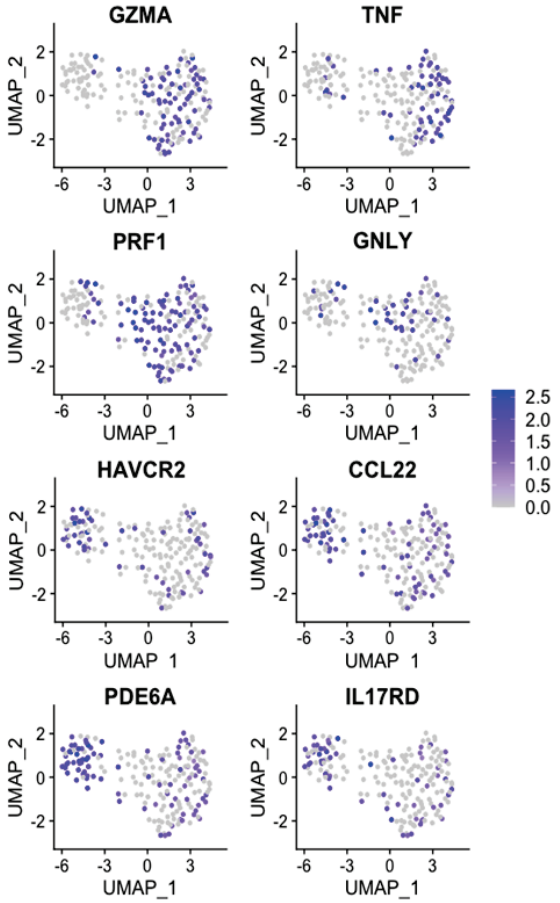


Figure 4.3.4.5: UMAP plots showing genes of interest expressed by MAIT cells from the BAL_2 subset, including MAIT effector genes, genes expressed by atypical MR1 tetramer-negative TRAV1-2+CD161+CD8+ T cells and tissue repair genes.

4.3.5. Confirmation of findings from the single-cell analyses in bulk RNA-sequence data

Because the single cell data did not support a full analysis of the 217 genes differentially expressed by MR1 tetramer-negative TRAV1-2+CD161+CD8+ T cells in people with LTBI (**Supplementary Table 4.5.2**), we next returned to the bulk RNA-sequencing data. Here, we hypothesised that the two BAL MAIT cell clusters would have been combined, and that this would mean that the transcriptional signatures of atypical MAITs should be enriched in the BAL derived MAIT cells compared to the peripheral counterparts. We found that 82% of the genes that were downregulated by these atypical MR1 tetramer-negative T cells were downregulated in the bulk BAL MAIT cells, while 62% of the genes upregulated by atypical TRAV1-2+CD161+CD8+ T cells were also upregulated in the BAL MAIT cells. **Figure 4.3.5.1** shows the top 10 down- and upregulated atypical TRAV1-

2+CD161+CD8+ T genes by bulk-sorted MAIT cells. These results suggest an enrichment of atypical MAIT cells in the bronchoalveolar compartment.

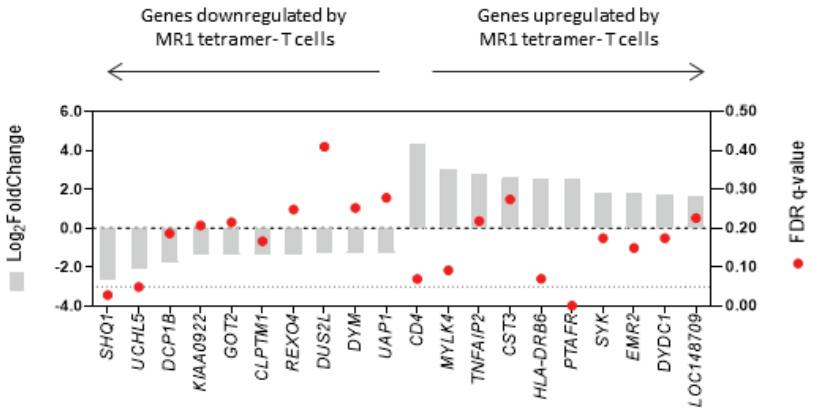


Figure 4.3.5.1: Population RNA-sequence analysis showing the enrichment of atypical MR1 tetramer-negative TRAV1-2+CD161+CD8+ T gene signature in MR1 tetramer-positive bronchoalveolar MAIT cells when compared to peripheral blood MAIT cells in HIV-negative individuals. The top 10 downregulated and top 10 upregulated atypical genes shown.

Analysis of a combined list of MAIT tissue repair genes obtained from literature showed that a tissue repair gene signature was similarly upregulated in bronchoalveolar MAIT cells when comparing to peripheral blood MAIT cells (**Figure 4.3.5.2**), with 67% of these tissue repair genes being upregulated in bronchoalveolar MAIT cells (**Supplementary Table 4.5.3**).

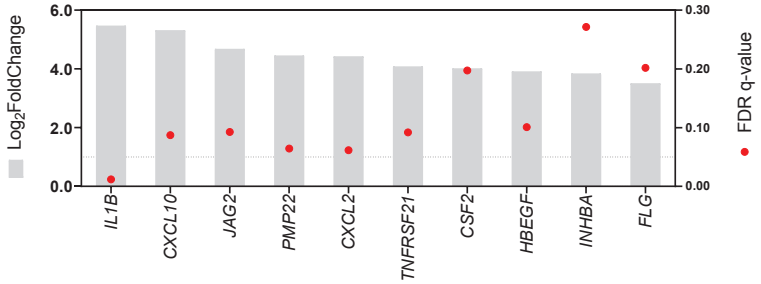


Figure 4.3.5.2: Bulk RNA-sequence analysis showing the upregulation of MAIT tissue repair genes in bronchoalveolar MAIT cells versus peripheral blood MAIT cells in HIV-negative individuals.

4.4. Discussion

Peripheral blood MAIT cells have been shown to transcriptionally express genes such as *IL18R1*, *KLRB1* and *ZBTB16* which are associated with cytokine responsiveness, innateness, as well as possession of an effector memory phenotype (Martin *et al.*, 2009; Koay *et al.*, 2016; Kurioka *et al.*, 2018). Less is known about the transcriptional profile of MAIT cells at the lung mucosa. We performed bulk RNA-sequencing on bronchoalveolar MAIT cells to compare their transcriptomes to those of matched peripheral blood MAIT cells in order to address this gap, and also assessed alterations in transcriptomic features in HIV infection. In healthy individuals, peripheral blood MAIT cells upregulated genes such as *PIK3R1* and *TGFBR3* which are involved in the maintenance of growth and survival, while the genes upregulated in the bronchoalveolar compartment (*HSPA1A* and *HSPA1B*) are those involved in stress response and chemoattraction. Protein kinase B (PKB) signalling was a significantly enriched pathway in healthy peripheral blood MAIT cells. Activation of the PKB signalling pathway is important for maintaining effector function in T cells. This pathway increases differentiation and proliferation while suppressing the expansion of T regulatory cells (Gerriets *et al.*, 2016). Most surveillance at the lung mucosa is dependent on the innate arm of the immune system to rapidly detect and discern between harmful and harmless microbes (Hartl *et al.*, 2018). Bronchoalveolar MAIT cells, in contrast to peripheral blood MAIT cells, upregulated genes involved in the recruitment of neutrophils, which serve a key antimicrobial function at the lung (Kruger *et al.*, 2015). These findings suggest that bronchoalveolar MAIT cells may participate in responses to commensals in health and highlight the divergence in adaptation of MAIT cells to their compartment of origin.

Cytokine mediated signalling pathways (IL-1 and TNF) were enriched for in peripheral blood MAIT cells in HIV infection, a deviation from the pathways involved in homeostatic maintenance that were observed in HIV-negative samples. Notably, *IFNAR2* and *IL10RA* expression was also upregulated in the bulk transcriptome of peripheral blood MAIT cells from HIV-positive individuals. The expression of these receptors has been associated with decreased MAIT cell function, as measured by IFN-γ and granzyme B production, with blockade of IFNAR2 and IL10R in *in vitro* HIV infection restoring MAIT cell function (X. Tang *et al.*, 2020). Stress granule (SG) formation was upregulated in peripheral blood MAIT cells. SGs consist of untranslated mRNA which form during cellular stress and are involved in IFN signalling and may thus be involved in antiviral responses (Onomoto *et al.*, 2012). In contrast, bronchoalveolar MAIT cells from HIV-positive individuals maintained their surveillance of the local environment as had been the case in HIV-negative individuals. This was indicated by the enrichment of pathways associated with antigen processing and presentation. The most notable upregulated genes in these pathways were HLA-DR gene transcripts which may contribute to the activated profile observed in mucosal MAIT cells (Sobkowiak *et al.*, 2019).

We used single-cell RNA-sequencing to interrogate MAIT cell heterogeneity at the lung. Unsupervised clustering analysis revealed the existence of four MAIT cell subsets, two from each compartment, with the two peripheral blood subsets showing somewhat similar transcriptional profiles while the bronchoalveolar subsets displaying completely distinct profiles. One bronchoalveolar subset displayed typical MAIT cell features similar to those found in the major peripheral blood subset and those that have been previously described in peripheral blood MAIT cells (Dusseaux *et al.*, 2011; D. Park *et al.*, 2019; M. Sharma *et al.*, 2020). The second bronchoalveolar MAIT cell subset displayed none of the typical MAIT cell transcripts and instead shared transcriptional characteristics of recently described atypical MR1 tetramer-negative TRAV1-2+CD161+CD8+ T cells (Pomaznoy *et al.*, 2020), as well as tissue repair MAIT cells (Constantinides *et al.*, 2019; Hinks *et al.*, 2019; Leng *et al.*, 2019). The BAL_2 subset displayed no expression of MAIT cell markers *KLRB1* (CD161) and *DDP4* (CD26). In line with data linking cytotoxic effector function to the expression of *KLRB1* and *DPP4* (Salgado *et al.*, 2012; Kurioka *et al.*, 2018), the expression of typical MAIT effector genes such as *GZMA*, *TNF*, *GNLY* and *PRF1* was absent from the BAL_2 subset. MAIT cells from the BAL_2 subset resembled the recently described MR1 tetramer-negative TRAV1-2+CD161+CD8+ T cells which were enriched in latently TB infected individuals (Pomaznoy *et al.*, 2020). The BAL_2 subset not only expressed atypical genes such as *PDE6A* and *IL17RD*, but also expressed the tissue repair gene *CCL22* (Constantinides *et al.*, 2019; Hinks *et al.*, 2019; Leng *et al.*, 2019), which when expressed with *IL17RD* may also play an immunoregulatory role. The expression of CCL22 promotes interactions between dendritic cells (DCs) and Tregs which then leads to the dampening of immune responses (Rapp *et al.*, 2019), while IL17RD reduces pro-inflammatory signalling via TLRs as well as the expression of pro-inflammatory genes like *IL-6* (Mellett *et al.*, 2012; Mellett *et al.*, 2015). Some typical MAIT cell transcripts were expressed by cells in the BAL_2 subset, such as *IL21R*, which on T cells is associated with maintained function during chronic infection (Fröhlich *et al.*, 2009), with IL-21R$^{-/-}$ mice having increased susceptibility to mycobacterial infection (Booty *et al.*, 2016). Exposure of bronchoalveolar MAIT cells to greater microbial diversity in the respiratory tract, may lead to the expansion of a subset of MAIT cells with atypical features and greater ligand discrimination, similar to the MR1 tetramer-negative TRAV1-2+CD161+CD8+ T cells observed in the peripheral blood of people with latent TB (Pomaznoy *et al.*, 2020). Alternatively, MAIT cell heterogeneity may occur as a result of exposure to non-microbial environmental cues at the lung mucosa. Atypical and MAIT tissue repair transcripts were also enriched in bulk-sorted bronchoalveolar MAIT cell populations, potentially as a result of the averaging of the transcriptional features of the two BAL-resident MAIT cell subpopulations. In contrast to typical pro-inflammatory MAIT cell functions the BAL_2 subset,

which was maintained in HIV infected individuals, may play an alternative function contributing to the prevention of excessive tissue damage in the bronchoalveolar compartment.

Bulk RNA-sequencing revealed that the transcriptomes of MAIT cells from healthy individuals are compartmentally different and that differences are maintained in HIV infection. Bronchoalveolar MAIT cells displayed an activated transcriptional profile during HIV, which suggests that MAIT cells at the lung are poised towards orchestration of responses to a range of potentially harmful antigens. Single-cell RNA-sequencing revealed the existence of a distinct subset of MAIT cells in the bronchoalveolar compartment that expresses low levels of most typical MAIT cell effector genes. This subset was characterised by expression of genes that have been associated with atypical MR1 tetramer-negative TRAV1-2+CD161+CD8+ T cell, as well as genes associated with alternative tissue repair and inhibitory functions and was present in HIV infected individuals. Thus, bronchoalveolar MAIT cells display transcriptomic heterogeneity that is not observed in the peripheral blood, and that is maintained during HIV infection. Further work is required to determine if these atypical bronchoalveolar MAIT cells possess an immunoregulatory function and if they contribute to the maintenance of homeostasis in the lung environment. Overall, these results suggest the relative preservation of MAIT cell transcriptional heterogeneity at the lung's mucosal surface, even in the face of untreated HIV infection.

4.5. Supplementary material

Supplementary Table 4.5.1: Enriched pathways when comparing the transcriptomes of bronchoalveolar to peripheral blood MAIT cells from HIV-positive individuals

	ID	Term Description	Fold Enrichment	Lowest *P* value	Highest *P* value
1	GO:0006413	translational initiation	21.66	3.63E-142	3.70E-102
2	GO:0006614	SRP-dependent co-translational protein targeting to membrane	23.19	1.04E-123	7.54E-84
3	GO:0000184	nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	19.01	1.42E-119	3.36E-80

4	GO:0019083	viral transcription	18.59	6.69E-113	1.21E-76
5	GO:0002181	cytoplasmic translation	23.69	1.25E-26	3.50E-22
6	GO:0000398	mRNA splicing, via spliceosome	3.49	1.53E-24	1.10E-10
7	GO:0006364	rRNA processing	7.27	3.01E-20	4.24E-12
8	GO:0006521	regulation of cellular amino acid metabolic process	4.88	7.12E-15	7.12E-15
9	GO:0070498	interleukin-1-mediated signalling pathway	4.46	1.48E-14	1.48E-14
10	GO:0010972	negative regulation of G2/M transition of mitotic cell cycle	4.02	1.48E-14	1.48E-14
11	GO:0038061	NIK/NF-kappaB signalling	3.75	2.92E-14	2.92E-14
12	GO:0033209	tumour necrosis factor-mediated signalling pathway	3.27	8.50E-14	8.50E-14
13	GO:0031146	SCF-dependent proteasomal ubiquitin-dependent protein catabolic process	3.35	8.56E-14	8.56E-14
14	GO:1902036	regulation of hematopoietic stem cell differentiation	3.66	1.14E-13	1.14E-13
15	GO:0061418	regulation of transcription from RNA polymerase II promoter in response to hypoxia	4.06	1.14E-13	1.14E-13
16	GO:0060071	Wnt signalling pathway, planar cell polarity pathway	3.11	1.72E-13	1.72E-13
17	GO:0002479	antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent	3.32	2.89E-13	2.89E-13
18	GO:1901990	regulation of mitotic cell cycle phase transition	3.15	4.72E-13	4.72E-13

19	GO:0031145	anaphase-promoting complex-dependent catabolic process	4.15	5.31E-13	5.31E-13
20	GO:0016579	protein de-ubiquitination	2.43	6.80E-13	2.07E-12
21	GO:0070125	mitochondrial translational elongation	2.89	3.33E-12	1.96E-05
22	GO:0070126	mitochondrial translational termination	2.86	3.71E-12	2.08E-05
23	GO:0043488	regulation of mRNA stability	3.59	4.29E-12	4.29E-12
24	GO:0002223	stimulatory C-type lectin receptor signalling pathway	2.61	7.38E-12	7.38E-12
25	GO:0090263	positive regulation of canonical Wnt signalling pathway	2.40	1.57E-11	1.57E-11
26	GO:0038095	Fc-epsilon receptor signalling pathway	2.53	1.78E-11	1.09E-10
27	GO:0043161	proteasome-mediated ubiquitin-dependent protein catabolic process	2.72	2.71E-11	2.71E-11
28	GO:0006446	regulation of translational initiation	8.29	3.00E-11	0.00819551
29	GO:0055085	transmembrane transport	2.47	6.42E-11	6.42E-11
30	GO:0050852	T cell receptor signalling pathway	3.83	7.87E-11	7.87E-11
31	GO:0042274	ribosomal small subunit biogenesis	15.79	1.16E-10	1.20E-07
32	GO:0090090	negative regulation of canonical Wnt signalling pathway	2.30	1.24E-10	1.24E-10
33	GO:0006412	translation	10.13	1.25E-10	6.12E-05
34	GO:0000165	MAPK cascade	1.95	1.37E-10	1.37E-10
35	GO:0008380	RNA splicing	4.46	5.55E-10	0.00022469

36	GO:0000209	protein polyubiquitination	2.42	7.96E-10	1.87E-09
37	GO:0043968	histone H2A acetylation	8.50	2.18E-09	3.60E-06
38	GO:0043967	histone H4 acetylation	4.42	8.92E-08	6.30E-05
39	GO:0000381	regulation of alternative mRNA splicing, via spliceosome	5.23	7.26E-07	0.00591534
40	GO:0045727	positive regulation of translation	5.53	1.19E-06	0.00034899
41	GO:0042273	ribosomal large subunit biogenesis	15.79	1.72E-06	0.02551783
42	GO:0006626	protein targeting to mitochondrion	4.42	1.04E-05	1.04E-05
43	GO:0016070	RNA metabolic process	4.61	1.15E-05	0.00054646
44	GO:0006405	RNA export from nucleus	4.04	1.18E-05	0.00087265
45	GO:0006977	DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest	4.09	1.82E-05	0.02222064
46	GO:0031295	T cell co-stimulation	5.02	2.28E-05	0.01661571
47	GO:0000715	nucleotide-excision repair, DNA damage recognition	6.28	2.30E-05	2.30E-05
48	GO:0043123	positive regulation of I-kappaB kinase/NF-kappaB signalling	2.87	3.03E-05	3.03E-05
49	GO:0006283	transcription-coupled nucleotide-excision repair	3.03	4.73E-05	4.73E-05
50	GO:0031124	mRNA 3'-end processing	3.01	5.39E-05	5.39E-05
51	GO:0007249	I-kappaB kinase/NF-kappaB signalling	3.21	6.16E-05	6.16E-05
52	GO:0000338	protein deneddylation	8.29	9.17E-05	0.00034996

53	GO:0006357	regulation of transcription by RNA polymerase II	1.93	0.00010914	0.00093596
54	GO:0032088	negative regulation of NF-kappaB transcription factor activity	2.27	0.00012051	0.00012051
55	GO:0000723	telomere maintenance	2.67	0.00015572	0.00017949
56	GO:0006122	mitochondrial electron transport, ubiquinol to cytochrome c	5.02	0.00017955	0.00017955
57	GO:0075522	IRES-dependent viral translational initiation	11.05	0.00020749	0.00020749
58	GO:0006396	RNA processing	5.23	0.00021247	0.00028374
59	GO:0034644	cellular response to UV	4.61	0.00023612	0.00024703
60	GO:0048025	negative regulation of mRNA splicing, via spliceosome	8.50	0.00056735	0.01838962
61	GO:0060261	positive regulation of transcription initiation from RNA polymerase II promoter	3.45	0.00060765	0.00060765
62	GO:0016477	cell migration	1.71	0.00063321	0.00063321
63	GO:2000059	negative regulation of ubiquitin-dependent protein catabolic process	7.90	0.00072157	0.00072157
64	GO:0070102	interleukin-6-mediated signalling pathway	4.61	0.00074518	0.00074518
65	GO:0032727	positive regulation of interferon-alpha production	3.07	0.00074518	0.00074518
66	GO:0042752	regulation of circadian rhythm	2.86	0.00077246	0.00077246
67	GO:0006303	double-strand break repair via nonhomologous end joining	2.83	0.00078359	0.00078359

68	GO:0043280	positive regulation of cysteine-type endopeptidase activity involved in apoptotic process	4.04	0.00085054	0.04242608
69	GO:0002218	activation of innate immune response	2.76	0.00086715	0.00086715
70	GO:0071850	mitotic cell cycle arrest	6.91	0.00089936	0.00110564
71	GO:0006406	mRNA export from nucleus	2.56	0.00094992	0.00349836
72	GO:0006606	protein import into nucleus	4.81	0.00134511	0.00225024
73	GO:0032211	negative regulation of telomere maintenance via telomerase	4.88	0.00134511	0.00586051
74	GO:0006367	transcription initiation from RNA polymerase II promoter	0.89	0.00138659	0.01803859
75	GO:0002230	positive regulation of defence response to virus by host	2.51	0.00140335	0.00140335
76	GO:0016236	macroautophagy	2.28	0.0016021	0.0016021
77	GO:0048024	regulation of mRNA splicing, via spliceosome	4.61	0.00160783	0.00160783
78	GO:0050821	protein stabilization	1.96	0.00166902	0.02185718
79	GO:0019221	cytokine-mediated signalling pathway	2.57	0.00229924	0.00229924
80	GO:0090383	phagosome acidification	1.97	0.00248201	0.00248201
81	GO:0006986	response to unfolded protein	6.91	0.00291886	0.01242528
82	GO:0007050	cell cycle arrest	2.94	0.0029905	0.0029905
83	GO:0032743	positive regulation of interleukin-2 production	8.29	0.00462598	0.00689065
84	GO:0035722	interleukin-12-mediated signalling pathway	4.12	0.00486233	0.02486741

85	GO:0043433	negative regulation of DNA-binding transcription factor activity	3.25	0.00584908	0.00584908
86	GO:0071902	positive regulation of protein serine/threonine kinase activity	2.51	0.0058633	0.0058633
87	GO:0016567	protein ubiquitination	2.36	0.00612536	0.04702936
88	GO:0050434	positive regulation of viral transcription	4.09	0.0065601	0.0065601
89	GO:0036499	PERK-mediated unfolded protein response	10.05	0.00658952	0.00692261
90	GO:0006470	protein dephosphorylation	1.89	0.00682176	0.00682176
91	GO:0051092	positive regulation of NF-kappaB transcription factor activity	2.39	0.00708816	0.00708816
92	GO:1904837	beta-catenin-TCF complex assembly	2.86	0.00716661	0.01470473
93	GO:1902895	positive regulation of pri-miRNA transcription by RNA polymerase II	1.73	0.00730515	0.00730515
94	GO:0006409	tRNA export from nucleus	1.78	0.00766288	0.00766288
95	GO:0070423	nucleotide-binding oligomerization domain containing signalling pathway	3.77	0.01018062	0.01213094
96	GO:0070317	negative regulation of G0 to G1 transition	1.45	0.01062211	0.01062211
97	GO:0000079	regulation of cyclin-dependent protein serine/threonine kinase activity	5.12	0.01179279	0.01448229
98	GO:0045071	negative regulation of viral genome replication	4.36	0.01183304	0.01487152

99	GO:0006974	cellular response to DNA damage stimulus	1.94	0.01222742	0.01222742
100	GO:0070979	protein K11-linked ubiquitination	3.07	0.01309194	0.01309194
101	GO:0016032	viral process	1.45	0.01431352	0.01431352
102	GO:0045785	positive regulation of cell adhesion	1.42	0.01549585	0.01549585
103	GO:0017148	negative regulation of translation	4.40	0.01661571	0.02932885
104	GO:0031397	negative regulation of protein ubiquitination	2.76	0.01922683	0.01922683
105	GO:0010212	response to ionizing radiation	3.07	0.0192304	0.0192304
106	GO:0006397	mRNA processing	1.35	0.02073	0.02073
107	GO:0032922	circadian regulation of gene expression	2.42	0.02185074	0.02185074
108	GO:0006979	response to oxidative stress	2.13	0.02349459	0.02349459
109	GO:0035666	TRIF-dependent toll-like receptor signalling pathway	3.81	0.02397036	0.02854822
110	GO:0032981	mitochondrial respiratory chain complex I assembly	0.91	0.02682311	0.02682311
111	GO:0075733	intracellular transport of virus	2.88	0.029149	0.029149
112	GO:0006338	chromatin remodelling	2.09	0.02923053	0.02923053
113	GO:0006366	transcription by RNA polymerase II	2.16	0.03230014	0.03230014
114	GO:0042981	regulation of apoptotic process	3.54	0.03381756	0.03381756
115	GO:0019058	viral life cycle	3.68	0.03444562	0.03444562
116	GO:0048010	vascular endothelial growth factor receptor signalling pathway	1.36	0.03806579	0.04469115
117	GO:0030433	ubiquitin-dependent ERAD pathway	2.40	0.03990967	0.03990967

118	GO:0072711	cellular response to hydroxyurea	3.45	0.04208717	0.04208717
119	GO:0043517	positive regulation of DNA damage response, signal transduction by p53 class mediator	9.21	0.04356896	0.04356896
120	GO:0035456	response to interferon-beta	12.28	0.04356896	0.04356896
121	GO:0070262	peptidyl-serine dephosphorylation	5.53	0.04839773	0.04839773
122	GO:0007005	mitochondrion organization	2.42	0.04901329	0.04901329

Supplementary Table 4.5.2: Genes differentially expressed by atypical MR1 tetramer-negative TRAV1-2+CD161+CD8+ T cells from latently TB infected individuals and similarly differentially expressed by bronchoalveolar MAIT cells as compared to peripheral blood MAIT cells.

Downregulated genes			**Upregulated genes**		
Gene	**Log_2Fold Change**	**Adjusted *P***	**Gene**	**Log_2Fold Change**	**Adjusted *P***
SHQ1	-2.6701	0.0284	*CD4*	4.2899	0.0694
UCHL5	-2.1101	0.0491	*MYLK4*	3.0350	0.0921
DCP1B	-1.7564	0.1866	*TNFAIP2*	2.7483	0.2181
KIAA0922	-1.3915	0.2070	*CST3*	2.5643	0.2736
GOT2	-1.3571	0.2150	*HLA-DRB6*	2.5482	0.0698
CLPTM1	-1.3420	0.1664	*PTAFR*	2.5395	0.0009
REXO4	-1.3369	0.2481	*SYK*	1.7857	0.1743
DUS2L	-1.2888	0.4092	*EMR2*	1.7575	0.1492
DYM	-1.2671	0.2520	*DYDC1*	1.6938	0.1745
UAP1	-1.2588	0.2778	*LOC148709*	1.6510	0.2260

ADK	-1.2569	0.2680	*UCKL1-AS1*	1.2333	0.1327
SF3B4	-1.2379	0.2404	*FFAR2*	1.2208	0.1693
G6PD	-1.1753	0.2812	*TMEM170B*	1.1443	0.1714
SUCLG2	-1.1519	0.3105	*LOC284379*	1.1325	0.1008
TBC1D14	-1.0872	0.3873	*POU5F1*	1.1270	0.1433
EHMT1	-1.0569	0.2885	*PARD6G*	1.1220	0.0832
C17orf62	-1.0178	0.3102	*C4orf26*	1.1031	0.1397
DDB1	-1.0110	0.3546	*INMT*	1.0740	0.1059
PSMC4	-0.9982	0.3227	*NLRP12*	1.0387	0.1513
MDH2	-0.9210	0.3924	*ITGA2*	1.0100	0.2177
FNTA	-0.8901	0.4044	*SLC16A12*	0.9879	0.3541
NFYB	-0.8863	0.3046	*LOC100506385*	0.9696	0.2255
P4HTM	-0.8772	0.4338	*CNNM1*	0.9499	0.2671
SAE1	-0.8015	0.4571	*BHMT2*	0.9432	0.1588
RHBDD2	-0.7844	0.4511	*MBOAT2*	0.9405	0.2610
PSMC2	-0.7532	0.4045	*NDST3*	0.8965	0.2561
DENND2D	-0.6513	0.5297	*C9orf66*	0.8714	0.2814
KCNA3	-0.6052	0.5338	*IL17RD*	0.8516	0.2422
CASP8	-0.5869	0.2509	*GSTTP2*	0.8512	0.3037
AP3S2	-0.5843	0.4798	*FBLIM1*	0.8419	0.1897
KDELR2	-0.5626	0.5786	*LOC100292680*	0.8375	0.5657
YTHDF2	-0.5545	0.5784	*FOXP4*	0.8366	0.2492
ARCN1	-0.4939	0.4761	*ADCY1*	0.8257	0.3401
ATF2	-0.4807	0.6711	*PART1*	0.8200	0.2501
HADHB	-0.4558	0.6642	*EMX2OS*	0.8016	0.2076

SSR1	-0.3165	0.4241	*TMEM17*	0.7826	0.3050
CCR1	-0.3157	0.8277	*IAPP*	0.7728	0.2636
ACTR3	-0.1953	0.8609	*C21orf62*	0.7703	0.1990
ACAD11	-0.1938	0.8798	*LOC100129269*	0.7659	0.2206
CNDP2	-0.1604	0.8786	*S1PR3*	0.7407	0.4457
PTPN22	-0.1474	0.8947	*C14orf105*	0.7352	0.3690
-	-	-	*RAB3B*	0.7261	0.2027
-	-	-	*PTK6*	0.7249	0.2904
-	-	-	*MSRB3*	0.7112	0.2961
-	-	-	*C1orf140*	0.7106	0.3481
-	-	-	*LOC100128338*	0.7095	0.2399
-	-	-	*FRRS1*	0.7081	0.3074
-	-	-	*ST6GAL2*	0.7039	0.3916
-	-	-	*FCAR*	0.7026	0.2764
-	-	-	*PSME4*	0.6888	0.4503
-	-	-	*AGMO*	0.6763	0.4114
-	-	-	*OLFML2A*	0.6762	0.2584
-	-	-	*CEACAM8*	0.6610	0.4789
-	-	-	*KREMEN1*	0.6568	0.2485
-	-	-	*LOC100287792*	0.6423	0.2394
-	-	-	*SYNPO2*	0.6343	0.3566
-	-	-	*LAMC2*	0.6269	0.5295
-	-	-	*TLCD2*	0.5985	0.2482
-	-	-	*CABP4*	0.5924	0.2043
-	-	-	*IRGQ*	0.5852	0.1588

-	-	-	*TTL*	0.5747	0.6595
-	-	-	*VSTM4*	0.5738	0.3236
-	-	-	*TRIM58*	0.5723	0.2671
-	-	-	*PDE6A*	0.5612	0.3774
-	-	-	*LOC100128682*	0.5426	0.2764
-	-	-	*LOC729603*	0.5388	0.3410
-	-	-	*CACNG8*	0.5231	0.2724
-	-	-	*EMP2*	0.5182	0.3263
-	-	-	*CEACAM22P*	0.4735	0.3803
-	-	-	*MYLK3*	0.4354	0.3615
-	-	-	*FLJ43879*	0.4261	0.4326
-	-	-	*LOC284950*	0.4236	0.4711
-	-	-	*CHST6*	0.3997	0.4104
-	-	-	*SLC36A2*	0.3795	0.5738
-	-	-	*POM121L10P*	0.3724	0.4466
-	-	-	*LOC100128288*	0.3585	0.5745
-	-	-	*KLB*	0.3401	0.5633
-	-	-	*NWD1*	0.3397	0.5703
-	-	-	*SLC15A2*	0.2983	0.6820
-	-	-	*FKBP9*	0.2884	0.6537
-	-	-	*ARGFX*	0.2784	0.4804
-	-	-	*CPA4*	0.2259	0.7484
-	-	-	*NAPSB*	0.2116	0.9262
-	-	-	*TSIX*	0.2041	0.7849
-	-	-	*ARSD*	0.1710	0.8481

-	-	-	*C3orf62*	0.1082	0.8512
-	-	-	*LOC400548*	0.0885	0.9055
-	-	-	*FHDC1*	0.0711	0.9386
-	-	-	*LOC286437*	0.0688	0.9345
-	-	-	*AFF3*	0.0464	0.9434
-	-	-	*S100PBP*	0.0087	0.9931

Supplementary Table 4.5.3: Enrichment of MAIT tissue repair genes in bronchoalveolar MAIT cells from HIV-negative individuals as compared to peripheral blood MAIT cells.

Gene	**Log_2Fold Change**	**Adjusted *P***
IL1B	5.4659	0.0119
CXCL10	5.3120	0.0871
JAG2	4.6732	0.0926
PMP22	4.4515	0.0644
CXCL2	4.4232	0.0616
TNFRSF21	4.0764	0.0918
CSF2	4.0119	0.1972
HBEGF	3.9078	0.1008
INHBA	3.8365	0.2714
FLG	3.5022	0.2018
APOE	3.3867	0.2262
WNT10A	3.1996	0.2962
ADM	2.7396	0.5015
ZBTB7C	2.3640	0.4231

ENG	2.0479	0.1762
LGALS3	2.0072	0.0746
ADAMTS2	1.7944	0.2769
SYK	1.7857	0.1743
CXCL12	1.5037	0.6055
IGF1	1.2513	0.1127
CSF1R	1.2232	0.6028
PDGFA	1.0010	0.6487
THBS1	0.9028	0.5151
FGFR2	0.8919	0.2309
CSF1	0.7786	0.5034
CCL3	0.7190	0.6362
BMP7	0.6525	0.3417
DISP1	0.5697	0.7446
EREG	0.5116	0.5041
FLG2	0.4911	0.6169
LEP	0.4810	0.7144
EPGN	0.4482	0.5569
WNT7B	0.3903	0.6004
APP	0.3797	0.8234
ANGPT2	0.2230	0.7109
CRISPLD2	0.1924	0.8829
HIF1A	0.1474	0.9027
IFT172	0.0849	0.9665
VEGFB	0.0816	0.9475

CHAPTER 5

CHAPTER 5: DISCUSSION AND CONCLUSIONS

5.1. Synthetic discussion of novel findings

5.1.1. Overview of the context

MAIT cells are a relatively recently described group of innate-like T cells that recognise bacterially derived riboflavin metabolites presented by the highly conserved MR1 protein (Tilloy *et al.*, 1999; Treiner *et al.*, 2003; Gold *et al.*, 2010; Le Bourhis *et al.*, 2010; Kjer-Nielsen *et al.*, 2012). MAIT cells recognise and respond to *Mtb*, although the significance of this *in vivo* and whether this interaction is mediated via the recognition of intermediates generated by the riboflavin biosynthesis pathway remains unclear (Harriff *et al.*, 2018). In mouse models, MAIT cells in the lungs have been shown to play a role in the establishment of a coordinated response to respiratory pathogens, while in the lungs of TB infected humans, MAIT cells have been found to be enriched and to display pro-inflammatory function (Meierovics and Cowley, 2016; Wong *et al.*, 2019). It has been demonstrated that MAIT cells make up a large proportion of *Mtb*-reactive CD8+ T cells in the peripheral blood of humans, suggesting their potential importance in anti-TB immunity (Gold *et al.*, 2010; Pomaznoy *et al.*, 2020). Nonetheless, the specific role of MAIT cells in protection against human respiratory infections, including TB, remains unclear (Bucsan *et al.*, 2019; Vorkas *et al.*, 2020; Yu *et al.*, 2020). MAIT cells are also able to respond to viral infection through TCR-independent recognition which is mediated predominantly by IL-18 and IL-12 stimulation (Ussher *et al.*, 2014; Loh *et al.*, 2016; van Wilgenburg *et al.*, 2016). HIV infection is known to alter respiratory immunity to infections including TB, which is the leading cause of death for people living with HIV worldwide (WHO, 2019). MAIT cells have been shown to be depleted and functionally exhausted in the peripheral blood during HIV infection (Cosgrove *et al.*, 2013; Leeansyah *et al.*, 2013). The impact of HIV on MAIT cells at the lung's mucosal surface, the site of TB infection, remains poorly understood (Mvaya *et al.*, 2019). The aim of this study was thus to understand the unique features of MAIT cells in the bronchoalveolar compartment of healthy humans with latent *Mtb* infection and the impact of HIV infection on MAIT cells in the lung mucosal and peripheral compartments.

The initial hypothesis of the study was that bronchoalveolar MAIT cells are phenotypically, functionally, and transcriptionally distinct from those in the peripheral blood and that HIV infection would abrogate compartment-specific features. To address this, we compared the surface phenotype, functional capacity, and transcriptome of bronchoalveolar MAIT cells to those in the peripheral blood. We also compared MAIT cells from HIV-negative individuals to those from HIV-positive individuals to better understand the impact of HIV infection on MAIT cells at the respiratory mucosal surface. We began by assessing the expression of CD161 and CD26 on MR1 tetramer-positive MAIT cells

from both compartments and paired this with T cell cloning of bronchoalveolar MAIT cells to confirm that the unexpected heterogeneity of these markers in the lung was actually due to the heterogenous expression of these markers on MAIT cells. The cloning experiments confirmed that MR1 tetramer-positive cells expressing low levels of these two typical MAIT cell markers did indeed have MR1 mediated function and TCRα chains that confirmed their identity as MAIT cells. We thus concluded that bronchoalveolar MAIT cells showed previously undescribed heterogeneity of CD161 and CD26. The function of MAIT cells was determined using an intracellular cytokine staining panel that was designed to elucidate three known functions of MAIT cells: Th1 cytokine production (IFN-γ) and Th17 cytokine production (IL-17), and production of cytolytic effector molecules (granzyme B). We also measured MAIT cell activation and inhibition by measuring surface expression of HLA-DR, PD-1, and TIM-3. Having characterised the differences between bronchoalveolar and peripheral blood MAIT cells using these approaches, we next assessed the impact of untreated HIV-infection on these parameters. Finally, the MAIT cell transcriptome was assessed using bulk RNA-sequencing to determine overall differences between compartments and using single-cell RNA-sequencing to assess the heterogeneity of lung-resident MAIT cells. The main findings of these analyses are discussed below.

5.1.2. Bronchoalveolar MAIT cells are phenotypically heterogeneous

Assessment of MR1 tetramer-positive MAIT cells in healthy individuals revealed there to be no differences in the frequencies of MAIT cells between the peripheral blood and bronchoalveolar compartment. Our previous work had shown significant enrichment of MAIT cells in the bronchoalveolar space, but this was during active infection with a MAIT ligand producing pathogen (Wong *et al.*, 2019). We thus conclude that during a state of normal health the frequency of MAIT cells in the peripheral blood and bronchoalveolar space is roughly similar and that compartment-specific expansions may occur during active response to infection. Staining of MR1 tetramer-positive cells from both compartments with MAIT cell markers CD161 and CD26, typically expressed at high levels on peripheral blood MAIT cells, revealed interesting patterns of surface staining between the two compartments. In healthy peripheral blood the majority of MAIT cells had the typical CD161++CD26++ phenotype (Martin *et al.*, 2009; Dusseaux *et al.*, 2011; P.K. Sharma *et al.*, 2015), while those in the bronchoalveolar compartment displayed unexpected and previously undescribed heterogeneity in the expression of these markers. MR1 tetramer-positive bronchoalveolar MAIT cells included CD161-CD26++, CD161-CD26- and CD161++CD26- phenotypic subpopulations. Previous studies had reported CD161-low or CD161-negative MAIT cells in the context of HIV infection (Leeansyah *et al.*, 2013; Eberhard *et al.*, 2014), but there has been controversy about whether these CD161-negative or -low cells were truly MAIT cells. We therefore undertook T cell cloning of

bronchoalveolar MR1 tetramer-positive MAIT cells with atypical phenotype to determine the identity of these cells. Cells cloned from the CD161++CD26++, CD161-CD26++ and CD161-CD26- subpopulations all displayed MR1-restricted IFN-γ production in response to *M.smeg* infection of non-HLA-matched antigen presenting cells consistent with the functional definition of MAIT cells. Additionally, all eight clones utilized TRAV1-2/TRAJ33 TCRα chains, all consistent with MAIT cell identity (Gold *et al.*, 2010; Wong *et al.*, 2019). We thus conclude that bronchoalveolar MAIT cells from healthy individuals feature previously unreported phenotypic heterogeneity in their expression of CD161 and CD26.

5.1.3. Single-cell transcriptomics reveals distinct bronchoalveolar MAIT cell subsets

To better understand the phenotypic heterogeneity of bronchoalveolar MAIT cells single-cell RNA-sequencing was performed. This analysis included 190 MR1 tetramer-positive cells derived from paired bronchoalveolar and peripheral compartments of nine individuals. The unsupervised clustering of these cells revealed four distinct transcriptional clusters based on unique gene expression profiles. Compartment of origin contributed significantly to clustering patterns with the four transcriptional clusters consisting of two subsets from each compartment, BAL_1 and BAL_2 from the bronchoalveolar compartment and PBMC_1 and PBMC_2 from the peripheral blood. These transcriptomic subsets displayed interesting patterns of differential expression of the two typical MAIT cell genes, *KLRB1* (CD161) and *DPP4* (CD26), with two transcriptomic subsets (BAL_1 and PBMC_1) characterised by transcriptional co-expression of both of these genes. The PBMC_2 subset, on the other hand, expressed *KLRB1* alone and the BAL_2 subset expressed neither. Assessment of other typical MAIT cell genes, including those associated with Th1/cytolytic effector functions, typical cytokine receptors and typical transcription factors, further elucidated interesting patterns of expression between the four transcriptional subsets (Fergusson *et al.*, 2014; D. Park *et al.*, 2019; Lu *et al.*, 2020; Pomaznoy *et al.*, 2020; M. Sharma *et al.*, 2020). In addition to co-expressing *KLRBI* (CD161) and *DPP4* (CD26), the BAL_1 and PBMC_1 subsets generally expressed most typical MAIT cell genes. The PBMC_2 subset expressed some typical genes and appeared to be a transitional subset. However, the BAL_2 subset expressed very few of the typical MAIT cell genes and none of the genes associated with typical MAIT cell effector functions. After ruling out the possibility of alveolar macrophage contamination of the MR1 tetramer-positive cells, we hypothesised that the BAL_2 subset could be composed of atypical MAIT cells in line with our earlier observation of phenotypically atypical MR1 tetramer-positive cells in the bronchoalveolar compartment. Assessment of the expression of genes found in a recently identified population of atypical MR1 tetramer-negative TRAV1-2+CD161+CD8+ T cells (Pomaznoy *et al.*, 2020) demonstrated that these atypical genes were rarely expressed by any of the typical (BAL_1 and PBMC_1) or transitional cells

(PBMC_2) and were expressed by nearly all the cells in the distinct BAL_2 subset. The BAL_2 subset also expressed genes associated with the recently described MAIT cell tissue repair function (Constantinides *et al.*, 2019; Hinks *et al.*, 2019; Leng *et al.*, 2019). Overall, three unsupervised subsets (BAL_1, PBMC_1 and PBMC_2) shared the expression of many genes that are typical of MAIT cells, while the fourth transcriptomic subset (BAL_2) shared similarities with two groups of recently published alternative MAIT cell phenotypes. Enrichment of atypical and tissue repair gene signatures were confirmed in bronchoalveolar MAIT cells using bulk RNA-sequencing. Consistent with the greater phenotypic heterogeneity observed in bronchoalveolar MAIT cells, these data point towards greater transcriptomic heterogeneity in the bronchoalveolar compartment which consists of two transcriptionally distinct subsets (BAL_1 and BAL_2), in contrast to the peripheral blood which is composed of two closely related subsets (PBMC_1 and PBMC_2).

5.1.4. Bronchoalveolar MAIT cells are functionally inhibited in comparison to peripheral blood MAIT cells

We performed intracellular cytokine staining on non-specifically stimulated matched peripheral blood and bronchoalveolar MAIT cells from healthy, HIV-negative individuals to compare the functional capacity of MAIT cells from these two compartments. Mitogenic stimulation of MAIT cells resulted in a significantly lower proportion of IFN-γ producing bronchoalveolar MAIT cells compared to those in matched peripheral blood. The proportion of IL-17 producing MAIT cells was relatively low in both peripheral blood and bronchoalveolar compartments upon stimulation (though higher than that of conventional CD8+ T cells), and constitutive granzyme B producing MAIT cells were also low in both compartments. Activation and inhibitory marker expression was assessed to determine whether the reduced pro-inflammatory function of bronchoalveolar MAIT cells could be associated with functional repression. HLA-DR, PD-1 and TIM-3 expression were all significantly higher in bronchoalveolar MAIT cells compared to those in peripheral blood. Bulk RNA-sequencing revealed the enrichment of pathways which are associated with the maintenance of effector function in T cells and the suppression of regulatory T cell expansion in the peripheral blood compartment (Gerriets *et al.*, 2016), supportive of the pro-inflammatory profile observed in MAIT cells from this compartment at the protein level. Single-cell RNA-sequencing, on the other hand, showed that bronchoalveolar MAIT cells in the BAL_2 subset failed to express any genes associated with Th1 cytokine or cytolytic function but did express genes including *IL17RD* and *CCL22* which are associated with immunoregulatory function. Notably, TIM-3 was highly expressed on the BAL_2 subset. These findings suggest that overall reduced pro-inflammatory function in bronchoalveolar MAIT cells could be a consequence of higher expression of co-inhibitory markers and immunoregulatory genes in the BAL_2 subset. These findings are in line with the observations of enhanced regulatory functions in

respiratory mucosal lymphocytes which serve to minimise excessive inflammation and prevent damage to mucosal layers (Hartl *et al.*, 2018; Branchett and Lloyd, 2019). The maintenance of barrier integrity may be further promoted by MAIT cells of the BAL_2 subset which potentially poses tissue repair functions.

5.1.5. Bronchoalveolar MAIT cells are depleted during HIV infection but phenotype, function and transcriptome are preserved

We next sought to evaluate the impact of HIV infection on MAIT cell frequency, phenotype, and function in the peripheral blood and bronchoalveolar compartment. MR1 tetramer staining confirmed MAIT cell depletion in the peripheral blood and revealed depletion in the bronchoalveolar compartment. HIV infection led to alterations in the surface expression of CD161 and CD26 in MAIT cells from both compartments. The peripheral blood remained dominated by the CD161++CD26++ phenotype, but a decrease in this phenotype was noted along with an increase in the CD161-CD26- subpopulation. The CD161++CD26++ phenotype was also reduced among bronchoalveolar MAIT cells, however, the frequencies of the atypical phenotypes increased in this compartment although non-significantly. These phenotypic alterations were paired with reduced functional capacity in the peripheral blood, demonstrated by a trend towards the reduction of inducible IFN-γ production and a significant decrease in inducible IL-17 production. Notably, the functional capacity of bronchoalveolar MAIT cells remained unaltered in HIV infection. A trend towards increased PD-1 expression was observed in peripheral blood as well as a significant increase in TIM-3 expression in this compartment in people with HIV. HIV infection thus led to reduced functional capacity and increased inhibitory receptor expression in peripheral blood MAIT cells. Single-cell RNA-sequencing demonstrated that the transcriptionally distinct bronchoalveolar MAIT cell subsets were also present in HIV infected individuals suggesting that transcriptomic heterogeneity at the lung's mucosal surface is maintained even in HIV. Interestingly, the BAL_2 subset exclusively expressed *IL21R* which is required by T cells to maintain function during chronic infection and has been shown to be important against mycobacterial infection in mice (Fröhlich *et al.*, 2009; Booty *et al.*, 2016). IL-21R expression may therefore be important for the maintenance of T cell functions geared towards tissue repair and the maintenance of barrier integrity at the lung mucosa, especially in HIV-infected individuals who are more at risk of acquiring respiratory infections. Our results support previous findings showing the profound effect of HIV infection on the frequency, phenotype, and function of peripheral blood MAIT cells. We found that HIV infection had little additional impact on the function of bronchoalveolar MAIT cells which maintained both phenotypic and transcriptomic heterogeneity in untreated HIV.

5.2 Conclusion

5.2.1 Conclusion and explanatory model

In summary, we found that MR1 tetramer-positive MAIT cells in the bronchoalveolar compartment display previously unreported phenotypic and transcriptional heterogeneity. These findings are summarised in **Figure 5.2**. Surface marker staining with CD161 and CD26 demonstrated greater heterogeneity in the expression of these markers in bronchoalveolar MAIT cells in contrast to those in the peripheral blood. Single-cell transcriptomic analysis suggests the existence of two transcriptionally distinct MAIT cell subsets at the lung mucosa, one of which expresses low levels of most typical MAIT cell effector genes and is characterised by expression of alternative tissue repair and inhibitory genes. We confirmed previous findings that HIV infection reduces the frequency of peripheral blood MAIT cells, reduces the functional capacity and induces expression of inhibitory receptors. The frequency of bronchoalveolar MAIT cells was also reduced in HIV, but in contrast to the effect of HIV on peripheral blood MAIT cells, the functional capacity and inhibitory marker expression on bronchoalveolar MAIT cells remained little changed by HIV. Single cell RNA-sequencing showed that people with HIV had both the typical and atypical MAIT cell subpopulations in their bronchoalveolar compartment. In summary, bronchoalveolar MAIT cells are phenotypically, functionally, and transcriptionally distinct from those in the peripheral blood. HIV infection significantly alters the frequency and function of peripheral blood MAIT cells, while the phenotypic and transcriptional heterogeneity of bronchoalveolar MAIT cells remained preserved even in the face of untreated HIV infection. These findings may have potential implications for immunotherapies in people with HIV infection. Depletion of key immune subsets has been postulated as a mechanism by which HIV increases susceptibility to TB infection (Geldmacher *et al.*, 2008; Kalsdorf *et al.*, 2009; Geldmacher *et al.*, 2010; Jambo *et al.*, 2011). It is possible that restoring perturbed immune subsets might have a therapeutic effect in people with HIV infection. Therapies aimed at expanding MAIT cells specifically in the bronchoalveolar compartment, where they are phenotypically and functionally preserved, may represent a strategy for restoring optimal lung mucosal immunity. It may be important in this regard that we found that HIV numerically depleted but did not abrogate the novel phenotypic or transcriptional heterogeneity of the lung mucosal MAIT cells found in HIV-negative people. This may suggest that it is possible to fully restore the full complement of MAIT cells, including the novel tissue-repair subset described, in people with HIV infection. Further work is needed to confirm the features and function of this subset and to determine whether the residual MAIT cells found in people living with HIV may have the ability to replicate and/or be repopulated. Additionally, because the exact role of MAIT cells in human immunity remains incompletely understood (Bucsan *et al.*, 2019; Vorkas *et al.*, 2020; Yu *et al.*, 2020), future work will be required to define the exact role that MAIT cells play in TB and HIV pathogenesis

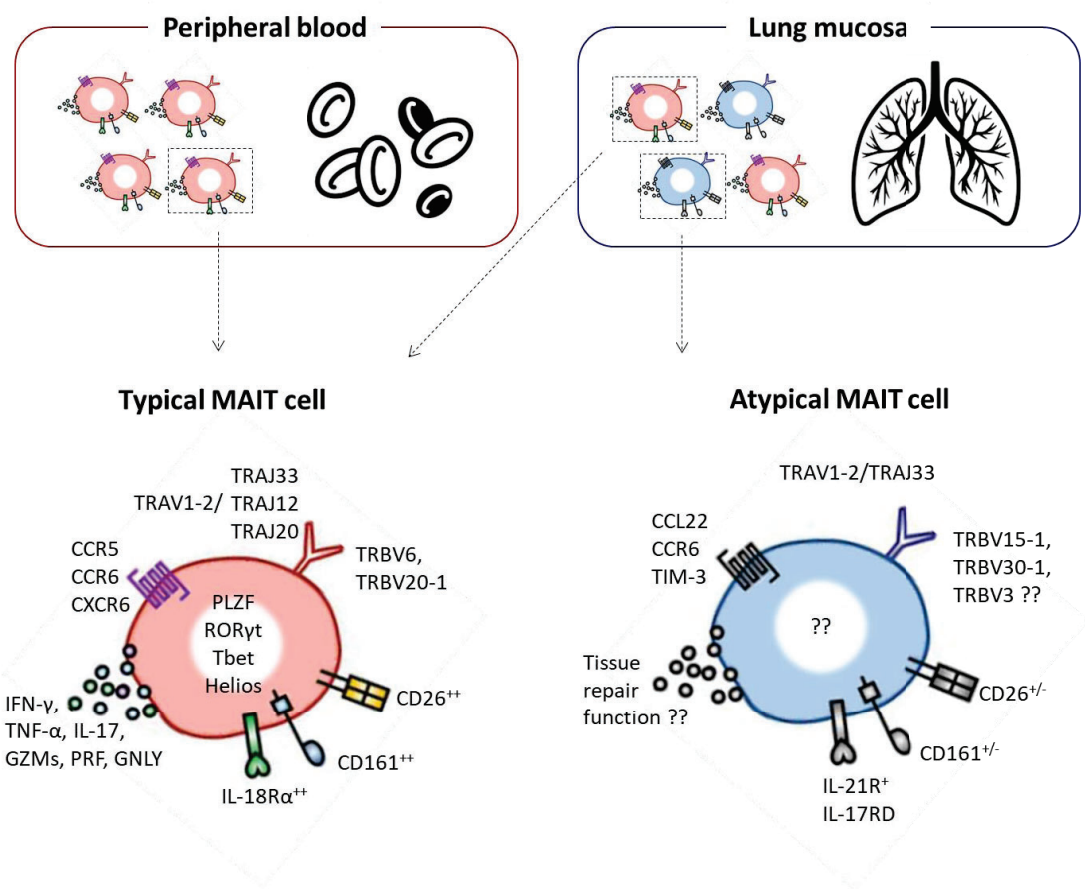


Figure 5.2: Model showing the phenotypic and functional characteristics of MAIT cells in the peripheral blood and lung mucosa. The peripheral blood compartment is dominated by MAIT cells with typical MAIT cell characteristics, while those at the lung mucosa are more heterogenous and include the phenotypically and transcriptionally atypical MAIT cells described here (adapted from Godfrey *et al.* (2019)).

5.2.2 Limitations

This study had a number of limitations, chief among which was a small sample size. To overcome this limitation, we combined individuals from the research and clinical bronchoscopy cohorts which may have impacted the overall findings. Further studies will be required to describe MAIT cell features in healthy individuals versus in those with clinical conditions. The small sample size also prevented the assessment of the impact of gender on MAIT cell frequency and function, and is a further limitation of the study. In addition to sample size, the number of cells available to perform functional assays was limited. The number of MAIT cells obtained specifically from the bronchoalveolar compartment was low and functional assays were therefore performed on a subset of the cohort using mitogenic, rather

than MAIT cell ligand specific, stimulation. Future work and the strengths of the conclusion would be improved by functional experiments on larger numbers of participants. The inability to generate MAIT cell clones from matched peripheral blood was also a limitation as this would have enabled direct comparisons of MAIT cell clones from the two compartments. The ability to fully determine the transcriptional characteristics of the novel BAL_2 subset was limited by the poor representation of a number of key genes from our single-cell dataset, including T cell receptor genes. It would be very interesting in future to determine accurate surface markers for the BAL_2 subsets and to specifically interrogate the cytokine production of cells from this group to determine if the aggregate lower IFN-γ production from the BAL MAIT cells was predominantly driven by the BAL_2 subset. Another limitation was that MAIT cells in this study were also exclusively defined by the MR1 5-OP-RU tetramer. There is growing evidence that the MR1 5-OP-RU tetramer may neglect to identify MAIT cells that respond to alternate small molecule antigens (Harriff *et al.*, 2018). This may be particularly relevant in *Mtb*, where MR1 tetramer-negative atypical TRAV1-2+CD161+CD8+ T cells were recently identified to represent a substantial proportion of *Mtb*-reactive CD8+ T cells (Pomaznoy *et al.*, 2020). Lastly, all the HIV-positive participants included in this study were antiretroviral therapy naive, leaving important questions about the quantity and quality of MAIT cells in people on effective ART unanswered.

5.2.3. Future directions

Recent work has uncovered the previously unappreciated heterogeneity of MAIT cells including those with varied CD8 expression i.e. CD8+ and CD4-CD8- (Brozova *et al.*, 2016; Dias *et al.*, 2018), those with low or no MR1 tetramer binding affinity (Wong *et al.*, 2019; Pomaznoy *et al.*, 2020), as well as those which are TRAV1-2-negative (Gherardin *et al.*, 2016; Meermeier *et al.*, 2016; Koay *et al.*, 2019). CD8+ expressing MAIT cells were found to express higher levels of activating receptors (CD16 and NKG2D), be more pro-inflammatory (IFN-γ) and have greater cytotoxic potential (granzyme B and CD107a) in comparison to CD4-CD8- MAIT cells (Brozova *et al.*, 2016; Dias *et al.*, 2018). MAIT cells which were tetramer low/negative had greater pro-inflammatory capacity (TNF-α) than their peripheral blood counterparts (Wong *et al.*, 2019) and had greater TCR-β diversity during mycobacterial infection (Pomaznoy *et al.*, 2020). MAIT cells utilising alternative TRAV gene segments had unique MR1-restricted antigen specificity recognising antigens other than 5-OP-RU and 6-FP (Gherardin *et al.*, 2016; Meermeier *et al.*, 2016; Koay *et al.*, 2019). The differences in functional capacity and diversity in ligand discrimination between MAIT cells from different subpopulations highlighted by these studies warrants further investigation to determine the role of the phenotypically and transcriptionally atypical bronchoalveolar MAIT cells described here in states of health and in the response to infection. A limitation of the study was that we did not perform *in vitro* assessment of the

functional characteristics of the BAL_2 MAIT cell subset and such experiments would be a productive next step. Whether these BAL_2 MAIT cells represent a unique subpopulation with varied antigen specificity, superior tissue repair capabilities, or an increased ability to maintain functional responses to chronic pulmonary infections remains to be determined. The study was limited by the number of cells available to perform functional assays as discussed above. Because long-term cultivation may alter phenotypic and functional characteristics of MAIT cells, pooling bronchoalveolar MAIT cells from multiple donors may represent a viable alternative which would enable full functional characterisation of rare subpopulations in future. Previous studies have shown the deleterious effects of excessive inflammation in driving TB disease in immune-compromised individuals (Barber *et al.*, 2019) and thus the potential immunoregulatory function of atypical bronchoalveolar MAIT cells may be useful in minimising these effects in HIV infected people. Notably, Mvaya *et al.* (2019) showed that, in contrast to MAIT cells in the peripheral blood (Cosgrove *et al.*, 2013; Leeansyah *et al.*, 2013; Wong *et al.*, 2013), MAIT cells in the bronchoalveolar compartment were restored following 1 year of antiretroviral therapy. Whether this restoration leads to restored numbers, specifically of atypical bronchoalveolar MAIT cells which may be important in maintaining immunoregulatory and tissue repair functions in HIV infected individuals who are at increased risk of respiratory infections such as TB, remains to be determined.

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