Activation of MAIT cells by antigen presenting cells: A comprehensive analysis and assessment of the impact of HIV and TB disease on these interactions.

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Declaration

I, Avuyonke Balfour, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Abstract

Thesis title: Activation of MAIT cells by antigen presenting cells: A comprehensive analysis and assessment of the impact of HIV and TB disease on these interactions.

MAIT cells are non-classical, innate-like T lymphocyte subsets, which recognize microbial vitamin B metabolites and rapidly respond by producing pro-inflammatory cytokines such as IFN-γ, TNF-α and cytotoxic molecules, which may result in the killing of bacteria-infected cells. Unlike conventional T cells, MAIT cells can be activated by either antigen presentation on MR1 or directly by cytokines. MAIT cells play a protective role against bacterial infections in mice but so far, no human studies have confirmed a direct role of MAIT cells in the control of bacterial infections or prevention of disease progression. Circulating MAIT cell numbers decrease in patients with active TB, but findings regarding functional changes have been conflicting. The aims of this study were to assess the cellular interactions between antigen presenting cells and MAIT cells, and determine the effect of TB, HIV and HIV-associated TB on MAIT cell numbers, activation, inhibitory and functional profile.

We recruited 26 healthy controls (HC) without HIV or TB disease, 30 people with HIV only, 30 with active TB only, and 26 with HIV-associated TB. All TB patient samples were obtained before treatment. Blood was collected from all participants and peripheral blood mononuclear cells (PBMC) isolated from the blood and cryopreserved. Later, PBMC were thawed, rested and stimulated with BCG expressing GFP (BCG-GFP) and heat-killed (HK) *M.tb.* Media only, LPS and PHA were used as stimulation controls. The numbers, functional profile, inhibitory and activation status of MAIT cells were determined by flow cytometry. Soluble cytokines were measured by ELISA and multiplex Luminex assays.

For HIV-infected participants with no TB and patients with HIV-associated TB, the median CD4 counts were 501 cells/µL and 228 cells/µL, and HIV viral loads were 1673 copies/mL and 66509 copies/mL, respectively. 63% and 69% of HIV infected patients were on ART in the HIV alone and HIV/TB groups, respectively. We observed lower frequencies of circulating MAIT cells in individuals with active TB only or HIV only compared to HC. HIV/TB resulted in lower but nonsignificant MAIT cell frequencies and numbers compared to HC. In response to stimulation with whole mycobacteria, MAIT cells were more highly activated (expressing high HLA-DR) in people with TB and HIV-associated TB compared to HC. Furthermore, MAIT cells from people with active TB only had significantly upregulated PD-1 expression compared to HC. MAIT cells from individuals with active TB and HIV-associated TB had a lower capacity to degranulate (express CD107a) and produce IFN-γ compared to HC. HIV-infection alone did not affect these functions. The levels of soluble IFN-α2 were reduced in the groups with HIV only and active TB only while IFN-γ was reduced in all patient groups. Blocking experiments revealed that MAIT cell activation in response to BCG was primarily through MR1 antigen presentation pathway. The level of monocyte and dendritic cell infection (expression of GFP) was similar in all groups. We observed a positive correlation between monocyte infection and the frequencies of MAIT cells producing IFN-γ in people with active TB only. We also observed a positive correlation between the amount of soluble IL-12 and the proportion of MAIT cells producing IFN-γ and CD107a in HC and in people with HIV infection, respectively.

Our data show that HIV, TB and or HIV/TB result in a decrease in circulating MAIT cells, impaired functional profile, and a significant alteration in activation status and inhibitory potential. The MR1 antigen presentation was the predominant pathway for MAIT cell activation. There was also a relationship observed between MAIT cell activation and magnitude of innate response to mycobacterial antigens. These results provide further evidence of the potential role of MAIT cells in infectious disease pathogenesis and demonstrate how HIV and TB alter their function.

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“***If I have seen further, it is by standing on the shoulders of giants***.”

-Sir. Issac Newton

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List of abbreviations

5-AR-U	5-amino-6-Dribitylaminouracil
5-MOP-RU	methyl-2-oxopropylideneamino)-6-D-ribitylaminouracil
5-OE-RU	5-(2-oxoethylideneamino)-6-D-ribitylaminouracil
5-OP-RU	5-(2-oxopropylideneamino)-6-D-ribitylaminouracil
6-FP	6-formylpterin
AF	Alexa Fluor
APC	Allophycocyanin
APCs	Antigen presenting cells
ART	Antiretroviral treatment
BAL	Bronchoalveolar Lavage
BCG	Bacillus Calmette–Guérin
BV	Brilliant Violet
CCL2	C-C Motif Chemokine Ligand 2
CD	Cluster of differentiation
CFP-10	Culture filtrate protein
CTLA	Cytotoxic T-lymphocyte-associated protein 4
CXCL-10	C-X-C chemokine receptor-10
CXR	Chest X-ray
DCs	Dendritic cells
ECD	Phycoerythrin-Texas Red
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ESAT-6	Early secretory antigenic target
FBS	Fetal Bovine Serum
FMO	Fluorescence minus one
GALT	Gut-associated lymphoid tissue
GCP	Good clinical practice
GFP	Green fluorescent protein
Hb	Hemoglobin

HCS	Human cytokine standard
HIV	Human Immunodeficiency Virus
HK-M.tb	Heat-killed M.tb
HRP	Horseradish peroxidase
HTLV-1	Human T-Cell Lymphotropic Virus
IFN-γ	Interferon gamma
IGRA	Interferon gamma release assay
IL	Interleukin
IQR	Interquartile range
LAG	Lymphocyte-activation gene 3
LAL	Limulus amebocyte lysate
LAM	Lipoarabinomannan
LDL	Lower than detectable limit
LPS	Lipopolysaccharide
LTBI	Latent Tuberculosis Infection
M.tb	*Mycobacterium tuberculosis*
MAIT	Mucosal associated invariant T cell
mDCs	Myeloid dendritic cells
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
MR1	MHC-related protein 1
NHLS	National Health Laboratory Service
NK	Natural Killer
NO2	Nitrogen Dioxide
OOR	Out of range
PAMPs	Pathogen-associated molecular pattern molecules
PBMCs	Peripheral blood mononuclear cell
PD-1	Programmed cell death protein 1
pDCs	Plasmacytoid dendritic cell
PE	Phycoerythrin

PE-Cy7	Phycoerythrin-Cyanine
PerCP-Cy5.5	Peridinin-chlorophyll proteins cyanin 5.5
PHA	Phytohaemagglutinin
PPD	Purified protein derivative
PRR	Pathogen recognition receptors
TAP	Transporter associated with antigen processing
TB	Tuberculosis
TCR	T cell receptor
Th	T-helper
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
TST	Tuberculin skin test
WHO	World Health Organization

List of figures

List of tables

Table of Contents

Chapter 1: Introduction and literature review

1.1 Introduction

The 2018 Global TB report from the World Health Organization (WHO), reported that in 2017 alone, there were an estimated 10.0 million new incident cases of active tuberculosis (TB). Of these, 5.8 million, 3.2 million and 1 million cases were in men, women and children, respectively. An estimated 60% of these new cases were from developing countries such as India, China, Pakistan, Indonesia, Nigeria and South Africa. The WHO also reported that of all these new cases, 9% of cases were in Human Immunodeficiency Virus (HIV) positive individuals, of which 72% of them were from the African continent (WHO Global TB Report, 2018). Figure 1.1 below is a diagrammatic representation of the estimated global TB incidence case per 100 000 people in 2017, in different countries.

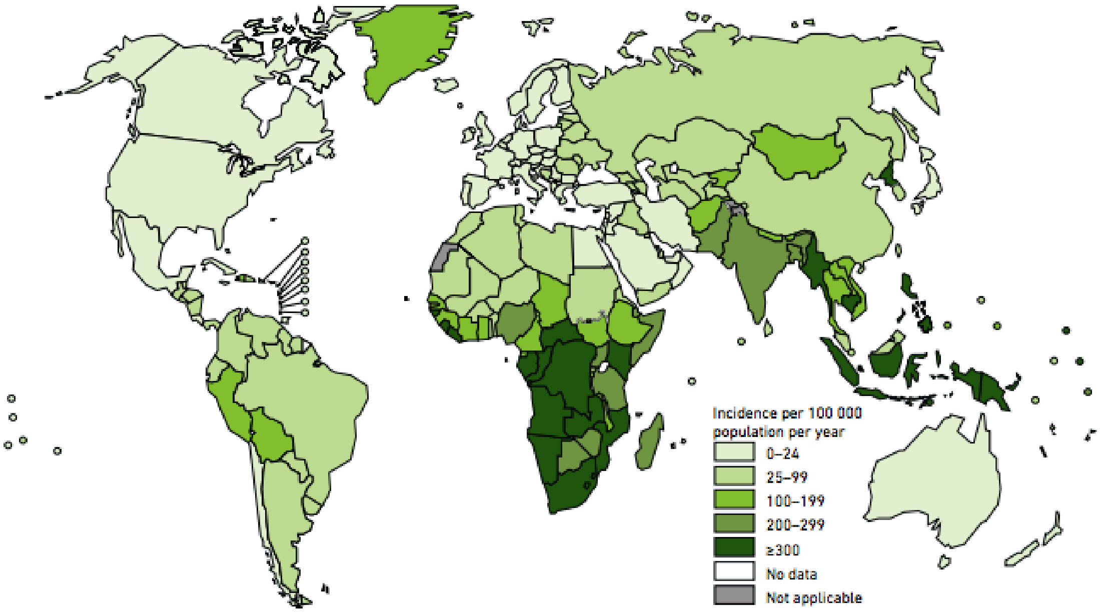


Figure 1.1: Representative diagram showing the global estimates of TB incidence cases per 100 000 people annually as reported by the WHO for the year 2017. Intensity of green colour is representative of the higher incidence countries. As shown in the diagram, South Africa is one of the countries with the highest incidence cases of above 300 per 100 000 people annually. (Image adapted from WHO Global TB Report, 2018).

The WHO also reported that in 2017, TB disease resulted in 1.3 million deaths globally with an estimated 300 000 of these deaths occurring in individuals with the HIV. Although TB remains a global concern, there has been significant progress towards reducing the global disease burden. There has been a 29% reduction in the number of deaths due to TB from 2000 (1.8 million deaths) to 2017 (1.3 million deaths). Among individuals living with HIV, deaths due to TB have almost been halved in this same period, with a 44% reduction from 534 000 to 300 000 deaths (Figure 1.2). The HIV-negative TB mortality rates, which is defined as the number of TB deaths among HIV negative individuals per 100 000 people per annum, were reportedly decreased by 42% from 2000 to 2017 with an average annual reduction of 3%.

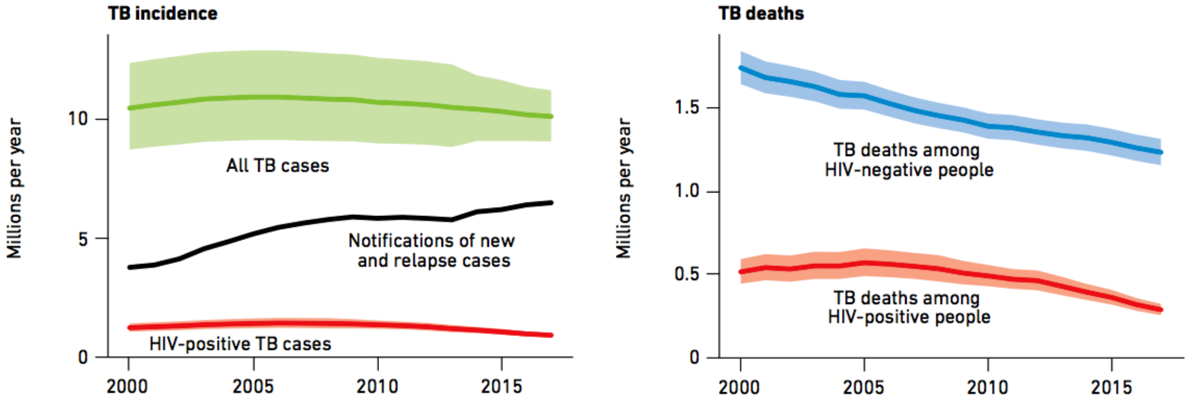


Figure 1.2: Graphical representation of the global TB incidence and the numbers of TB deaths among both HIV-negative and HIV positive individuals between 2000 and 2017. The green line is indicative of the global TB case numbers and the uncertainty intervals are represented as the lightly shaded areas. Black line shows the number of cases notified by TB programmes while the red line shows the number of TB cases among individuals living with HIV. B) Shows the progress which has been made in combating the deaths due to the global TB epidemic. TB deaths among HIV-negative individuals are represented in the blue line and TB deaths among individuals living with HIV are represented by the red line (Image adapted from WHO Global TB Report, 2018).

1.2 The pathogenesis of TB disease

Tuberculosis is a disease which is caused by transmission of *Mycobacterium tuberculosis* (*M.tb*), an airborne pathogen which is transmitted through the inhalation of aerosol droplets (expelled by individuals infected with *M.tb*) into the respiratory tract (Pai *et al.*, 2016). These then travel to the lower respiratory tract and as part of the first line of defense, the bacilli are recognized and ingested by alveolar macrophages in a process of receptor-mediated phagocytosis. During this process, specific molecules on *M.tb*, referred to as pathogen associated molecular patterns (PAMPs) are recognized by pathogen recognition receptors

(PRR) on host cells leading to the ingestion/phagocytosis of the *M.tb* bacilli (Van Crevel *et al.*, 2011).

Failure of the cells to contain and eliminate the bacteria and thus the infection results in the progression of infection to the lung parenchyma. This leads to a cascade of signals which ultimately result in the recruitment of other immune cells to the site of infection in an effort to control the infection. Infected antigen presenting cells (APCs) such as dendritic cells (DCs) and monocytes traffic into pulmonary lymph nodes. The APCs produce cytokines such as TNF-α, IL-1, IL-6 and IL-12 to prime the Th_1 cytokine secreting cells. They also produce chemokines such as CCL2 and CXCL10 which recruit cells such as B cells, T cells, neutrophils and Natural killer cells (NK) to the site of infection leading to the formation of granuloma structures (Slight and Khader, 2013; Korb *et al.*, 2016)**.** The interactions between APCs and T cells and exposure to *M.tb* antigens leads to sensitization of the adaptive immune system (Steinman *et al.*, 1988; Boom *et al.*, 2003).

Inside these granulomas, with cells from both the innate immune system (macrophages, monocytes, DCs, neutrophils and NK cells) and the adaptive immune system (B and T cells) working in concert, the host immune system may be able to successfully control the infection. This is achieved by keeping the bacteria in an inactive state by modulating/inhibiting bacterial replication thus limiting growth and preventing the spread of these bacilli (Flynn and Chan, 2005; Korb *et al.*, 2016)**.** This may result in the infected individual not displaying any symptoms of disease.

In a minority of individuals, the immune system fails to control the infection and as a result, they develop active TB in which they present with clinical symptoms of TB disease and these include; night sweats, coughing, weight loss and chest pains (Meintjes and Wilkinson, 2010). Figure 1.3 below is a representation of the cellular events which occur upon transmission of *M.tb*-containing bacilli.

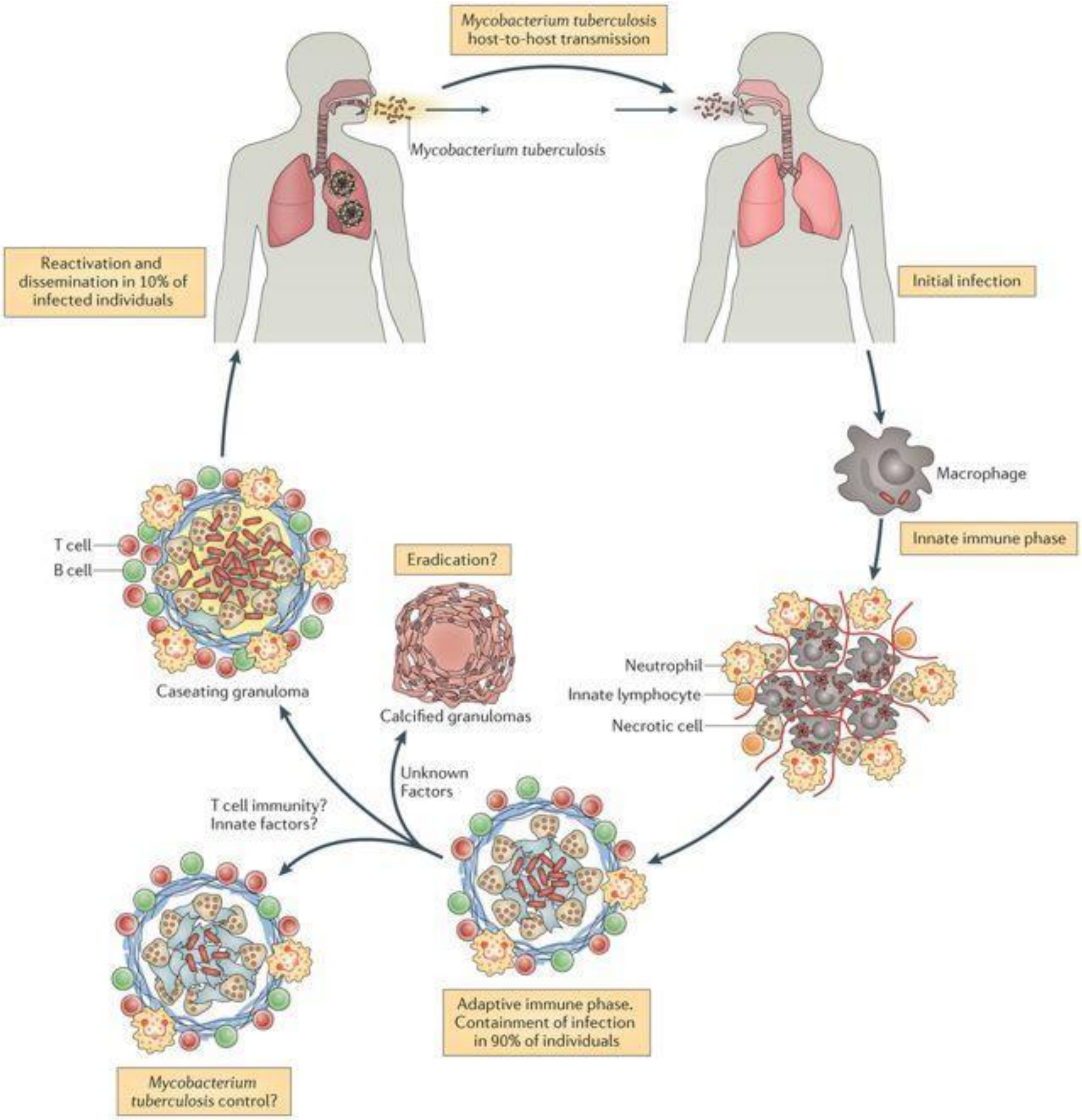


Figure 1.3: Diagrammatic representation of the pathogenesis of TB. *M.tb* is transmitted through inhalation of aerosol droplets. Within the lungs, innate immune cells such alveolar macrophages phagocytose the bacilli in an effort to restrict the growth and replication of the bacilli and this leads to the recruitment of cells such as neutrophils, B and T cells and other cells and ultimately formation of granuloma structures which keep the bacilli in quiescent phase and the individual may not progress to active disease. Imbalances within these structures may occur in a subset of the population resulting in the dissemination of the previously contained bacilli and the development of active TB disease (Image adapted from Nunes-Alves *et al.*, 2014).

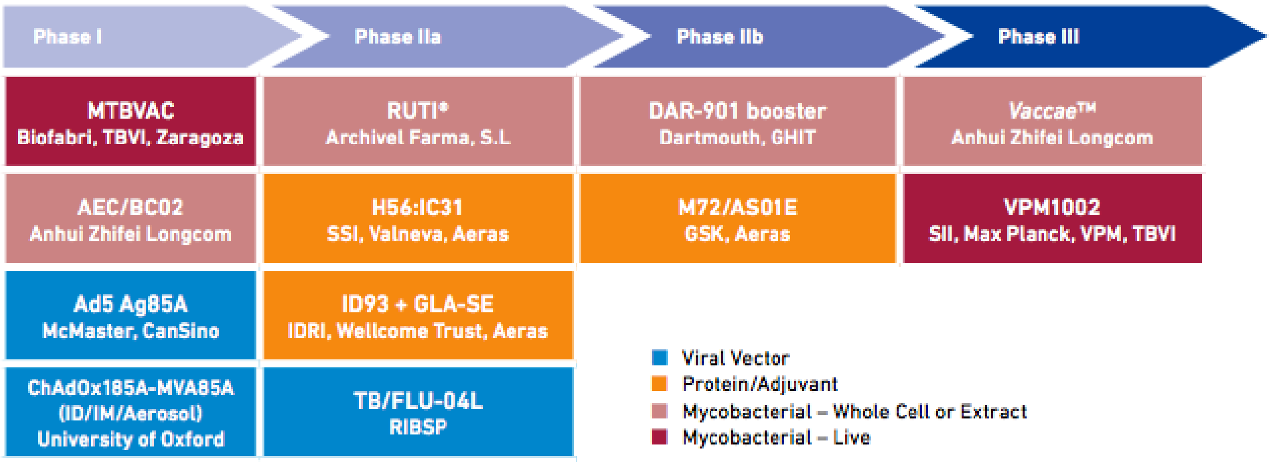
1.3 Prevention and treatment of TB disease

1.3.1 Vaccination

To date, the only licensed vaccine to prevent the development of disseminated or extrapulmonary TB such as TB meningitis and miliary TB, is the Bacille Calmette-Guerin (BCG) vaccine (Pai *et al.*, 2016; Khoshnood *et al.*, 2018)**.** The vaccine is administered to newborns and has been shown to be effective in children, but its efficacy is thought to decline over time and it is thought to offer protection for up to 10 years (Abubakar *et al.*, 2013; Pai *et al.*, 2016).

In adults, the BCG vaccine has been shown to have variable efficacy of between 0-80% (Pai *et al.*, 2016). This variable efficacy in adults and the high incidence of TB disease has prompted the search for new and more effective vaccines which confer protection against TB disease. According to the 2018 WHO Global TB Report, there are currently 12 vaccine candidates in clinical trials (Figure 1.4) and these can be classified into either viral vector, whole cell/mycobacterial lysates or adjuvanted sub-unit vaccines.

A) Viral vector vaccines include Ad5 Ag85A, ChAdOx185A-MVA85A and TB/FLU-04L and these are made up viral vectors expressing *M.tb* protein antigens such as AG85A and ESAT-6.
B) Whole cell/mycobacterial lysate vaccines include M.vaccae, DAR-901 and RUTI and these comprise either live attenuated *M.tb* which have had the virulence genes deleted, or whole cell lysates of non-pathogenic mycobacteria (M.vaccae) or lysates of *M.tb* fragments.
C) Adjuvanted sub-unit vaccines including AEC/BC02, H56:IC31, ID93+GLA-SE and M72/AS01E are vaccines expressing *M.tb* antigens which are conjugated to an adjuvant which functions to boost the immune responses.



Phase I	Phase IIa	Phase IIb	Phase III
MTBVAC Biofabri, TBVI, Zaragoza	RUTI® Archivel Farma, S.L	DAR-901 booster Dartmouth, GHIT	Vaccae™ Anhui Zhifei Longcom
AEC/BC02 Anhui Zhifei Longcom	H56:IC31 SSI, Valneva, Aeras	M72/AS01E GSK, Aeras	VPM1002 SII, Max Planck, VPM, TBVI
Ad5 Ag85A McMaster, CanSino	ID93 + GLA-SE IDRI, Wellcome Trust, Aeras		
ChAdOx185A-MVA85A (ID/IM/Aerosol) University of Oxford	TB/FLU-04L RIBSP		

Figure 1.4: The Global TB vaccine pipeline showing the current TB vaccines which are in phase I-III clinical trials as reported by the WHO. Viral vector vaccines are shown in blue, adjuvanted vaccines in orange, whole cell (mycobacterial) or cell extract derived vaccines are in pink and live mycobacteria derived vaccines are maroon (Image adapted from WHO Global TB Report, 2018).

1.3.2 Diagnosis of TB disease

For simplicity of diagnosis in clinical settings, TB is categorized as either latent or active (active TB) (Esmail *et al.*, 2014; Pai *et al.*, 2016). However, due to the dynamic interactions between host responses and the pathogen (which are thought to result in different clinical outcomes), TB disease is better appreciated as a spectrum (Robertson *et al.*, 2012; Pai *et al.*, 2016).

This spectrum ranges from 1) persons who have had exposure and cleared infection through the involvement of the innate and adaptive immune systems, 2) persons who have not cleared infection resulting in the maintenance of bacteria in a quiescent state, and both these groups (1 and 2) are said to have latent TB infection, 3) persons that are sputum culture positive but do not present with any clinical symptoms and categorized as having subclinical TB, and lastly 4) persons with symptoms of TB disease and diagnosed with active TB (Pai *et al.*, 2016).

Currently, MTB infection/exposure is diagnosed using 2 methods, the Tuberculin skin test (TST) and Interferon gamma release assay (IGRA). These tests are both based on measuring the cell-mediated immune responses to mycobacterial antigens as a result of previous exposure/sensitization (Pai *et al.*, 2014).

Active TB can be screened for by using Chest X-rays (CXR) and diagnosed using sputum smear microscopy, sputum culture, and molecular tests such as GeneXpert MTB/RIF Ultra (Pai *et al.*, 2016). CXR are used as a screening tool to examine the pulmonary lesions which may result due to TB disease. CXR are used for screening rather than diagnosis because they are not specific for TB disease and this is especially difficult in individuals living with HIV because these individuals may present with normal CXR (Palmieri *et al.*, 2002; Meintjes and Wilkinson, 2010).

Sputum smear microscopy and sputum culture are used for the microbiological confirmation of TB, GeneXpert MTB/RIF or GeneXpert MTB/RIF Ultra are also used as molecular tests and have been shown to have a higher accuracy in individuals living with HIV compared to sputum smear microscopy and have thus been endorsed by the WHO as a first-line test for TB (Churchyard *et al.*, 2015). The use of lipoarabinomannan (LAM) for urine LAM assays has also been recommended by the WHO for the detection and diagnosis of active TB, especially disseminated TB in HIV positive individuals with very low CD4 counts (Lawn *et al.*, 2012; Lawn and Gupta-Wright, 2015).

Molecular and culture-based methods also extend in their capacity, to the detection of drug resistant TB. For example, the GeneXpert MTB/ RIF is used as a detection tool for the diagnosis of active TB and for the detection of drug resistant TB (rifampicin resistant TB) (Pai *et al.*, 2016). In addition to GeneXpert MTB/RIF, liquid culture methods and genotyping tools such as GenoType MTBDRplus and NIPRO are also used as detection tools for isoniazid and rifampicin-resistant TB and MTBDRsl for the detection of fluoroquinolone resistant TB and other second line injectable drugs (Pai *et al*., 2016).

1.3.3 Treatment of TB disease

Currently, the treatment for active drug-sensitive TB disease is a 6-month course (at minimum) of Rifampicin, Isoniazid, Pyrazinamide and Ethambutol in which all 4 drugs are taken for the first 2 months of treatment and only Isoniazid and Rifampicin are taken during the last 4 months of treatment.

1.4 Cells involved in host responses to *M.tb* infection

The innate immune system is a specialised component of the immune system for host defences against microbial pathogens (Brubaker *et al.*, 2015; Gasteiger *et al.*, 2017). The cells of the innate immune system use PRR to sense the intra and extracellular environment for the presence of invading microbes (Bonilla and Oettgen, 2010; Brubaker *et al.*, 2015). The second component of the immune system is the adaptive immune system and it acts as a second line of defence specialised for pathogen-specific responses and immunological memory (Bonilla and Oettgen, 2010).

This section of the thesis will describe some of the different cell subsets involved in protective responses to pathogens and more specifically the different roles that these cells play in against *M.tb* infection. These cells include monocytes, dendritic cells(DCs), B cells, T cells (CD4, CD8, natural killer T (NKT) and mucosal-associated invariant (MAIT) cells).

1.4.1 Antigen presenting cells

1.4.1.1 Monocytes

Monocytes are bone marrow-derived antigen presenting cells that play a crucial function in the innate immune response to pathogens such as *M.tb* (Castaño *et al.,* 2011)**.** They are present in blood and have the capacity to differentiate into macrophages and dendritic cells (Boyette *et al.*, 2017).

Monocytes are generally characterized by the surface expression of CD14, a Toll-like receptor (TLR) 4 co-receptor which mediates lipopolysaccharide (LPS) signaling (Ziegler-Heitbrock, 2006). They can be further divided into 3 phenotypically and functionally diverse subsets; classical monocytes, intermediate monocytes and non-classical monocytes by the co-expression of the CD14 and CD16, an Fc gamma receptor (Ziegler-Heitbrock, 2006). Classical monocytes make up between 80 and 95% of monocytes in circulation and have a high expression of CD14 and low expression of CD16 ($CD14^{++}CD16^{-}$), the intermediate monocytes population makes up between 2 and 8% of monocytes in circulating and expresses moderate levels of CD14 and CD16 ($CD14^{+}$ $CD16^{+}$) and non-classical monocytes comprise between 2-

11% of the total circulating monocyte population and are characterized by the low expression of CD14 and the high expression of CD16 ($CD14^{-}CD16^{++}$) (Thomas *et al.*, 2017; Sampath *et al.*, 2018).

In microbial infection such as TB, differences in the monocyte populations were observed between healthy controls and those with TB disease, the frequencies of non-classical and intermediate monocytes have been reported to increase in individuals with TB while classical monocytes were depleted (Castaño *et al.*, 2011). Upon activation, monocytes have the capacity to produce cytokines such as IL-10, IL-12, IL-1β and TNF-α (Ziegler-Heitbrock, 2006)**.**

1.4.1.2 Dendritic cells

Dendritic cells are regarded as one of the key types of antigen presenting cells (Sinha *et al.*, 2007; Madan-Lala *et al.*, 2014) which function by surveying sites of pathogen entry and are usually the first cells to rapidly respond to infection (Shey *et al.*, 2015). These cells are derived from bone marrow progenitor cells and can be found in blood, lymphoid tissues and epithelial tissues where they function as immune sentinels.

Upon recognition of respective pathogen/antigen, DCs become activated and migrate from the peripheral tissues to the draining lymph nodes while simultaneously undergoing maturational changes from immature DCs to mature DCs. During this process, Major histocompatibility complex (MHC) antigen presentation is increased with the concomitant upregulation of co-stimulatory molecules such as CD40, CD80 and CD86 and type 1 helper (Th_1)-inducing cytokines such as IL-12 (Madan-Lala *et al.*, 2014). These activated DCs then stimulate and activate cells of the adaptive immune system such as B and T cells by presenting peptide-derived antigens on respective MHC molecules (Madan-Lala *et al.*, 2014)**.**

DCs are potent APCs and play a crucial role in the priming of T cell Th_1 effector functions via the production of IL-12. They are found at the sites of infection with *M.tb* and have the capacity to recognize, process and present antigens to T cells (Mihret, 2012). Infection of monocyte-derived DCs with *M.tb*-activated DCs led to an increased expression of MHC-I & II, CD40, CD80 and CD58 (Mihret *et al.*, 2011; Henderson*et al.,* 2019). This also increased the expression of cytokines including IL-12, TNF-α, IL-1 and IL-6 which are linked to cell maturation and DC function (Mihret *et al*, 2011). The importance of DCs has also been demonstrated in mouse models in which mice lacking DCs failed to control bacterial growth in their lungs as a result of defective CD4 T cell response (due to impaired capacity to prime CD4 T cells) (Tian *et al.*, 2005).

Dendritic cells can be classified into 2 broad categories; classical DCs (myeloid DCs) and non-classical DCs (plasmacytoid DCs and Langherhans cells).

Classical dendritic cells

Classical dendritic cells/myeloid dendritic cells (mDCs) are located in tissues such as spleen, lymph nodes, bone marrow as well as in non-lymphoid tissues (Mildner and Jung, 2014). They are characterized by high expression of CD11c and low expression of CD123 (Collin *et al.*, 2013; Shey *et al.*, 2015), furthermore, they can be divided into CD1c$^+$ (CD11c$^+$ CD1c$^+$) and CD141$^+$ (CD11c$^+$ and CD141$^+$).

A) CD1c$^+$ DCs have PRR for antigen recognition and are more able to stimulate and activate CD4 T cells (reviewed by Shey *et al.*, 2015). Upon activation, these cells have the capacity to produce TNF-α, IL-18, IL-10, IL-12 and IL-23 (Collin *et al.,* 2013).

B) CD141$^+$ DCs, also have PRR for pathogen recognition and are able to effectively cross-present MHC-1 to CD8 T cells (Collin *et al.*, 2013; Shey *et al.*, 2015) and upon stimulation, produce TNF-α, CXCL10 and are considered inferior producers of IL-12p70 compared to CD1$^+$ mDCs.

Non-classical dendritic cells

Plasmacytoid dendritic cells (pDCs)

These DCs are characterized by the low expression of myeloid markers, CD11c and CD11b, and have a higher expression of CD123, CD303, CD304 (Collin and Bigley, 2018) and are primarily found in blood (Mildner and Jung, 2014). These cells are able to respond to viral infections by detecting viral nucleic acids and produce type 1 interferon (Collin *et al.*, 2013; Shey *et al.*, 2015).

Langerhans cells

Langerhans cells are a class of dendritic cells that are typically located on the epidermal layers of the skin and have the capacity for self-renewal (Collin *et al.,* 2013; Mildner and Jung, 2014; Deckers *et al.*, 2018) and are identified by the high expression of CD1a and Langherin, a c-type lectin receptor (Collin *et al.*, 2013; Shey *et al.*, 2015). They differentiate into migratory DCs and are thought to function to maintain epidermal tissue homeostasis by promoting the tolerance of commensal bacteria (Collin *et al*., 2013).

1.4.1.3 B cells

B cells are recognized for their role in humoral immunity where they differentiate into memory and plasma cells which produce antigen-specific antibodies in response to activation by activated CD4 or other APCs (Rodríguez-Pinto, 2005)**.** They also function as antigen presenting cells with the ability to internalize antigens via the B cell receptor (BCR). They process these antigens and present them to CD4 T cells using MHC II molecules (Hoffman *et al.,* 2016).

In mouse models, B cells have been associated with priming of CD4 T cells, alongside dendritic cells (Constant *et al.*, 1995)**.** Additionally, B cells are also furnished with B7 and CD40 molecules which are costimulatory molecules which bind to CD28 and CD40 ligands on T cells (Constant *et al.*, 1995; Hoffman *et al*., 2016) and express TLR which recognise PAMPs on pathogens. The importance of B cells as APCs has been demonstrated by Crawford *et al.*, (2006) in which they observed that mice with B cells deficient in MHC-II molecules had significantly reduced T cell clonal expansion and differentiation into effector T cells.

Furthermore, the role of B cells is more prominent in the context of mucosal-associated invariant T (MAIT) cells as these cells were initially described to play a critical role in the selection and or expansion of MAIT cells (Treiner *et al.*, 2003). This will be discussed in a later section of the literature review.

1.4.2 T cells (Conventional and non-conventional)

Although host immune responses lead to the *M.tb* bacilli being phagocytosed, it is well known that the outcome of the initial infection of macrophages and DCs is highly dependent on 1) host immune defenses such as bactericidal activity of the host phagocytes (innate cells) and the T cell responses, and 2) on the pathogen defenses and virulence factors resulting in immune evasion (Sasindran and Torrelles, 2011).

M.tb has developed the capacity and mechanisms for immune evasion which allow the pathogen to proliferate in the lungs and survive in host cells (Sasindran and Torrelles, 2011). The persistent infection and destruction of host phagocytes by *M.tb* leads to further cell-mediated immune stimulation in which blood monocytes and other innate immune cells are activated to kill and eliminate the pathogen. This cell mediated immunity involves activation of T lymphocytes which have the capacity to elicit more specific response through the detection of the pathogen specific peptide antigens presented on MHC molecules (van Crevel *et al.*, 2002).

The activated T cells have the capacity to activate the killing of intracellular pathogens such as *M.tb* by macrophages through the production of nitric oxide, or T cells may kill infected cells through cytotoxic molecules such as perforin and granzymes B (van Crevel *et al.,* 2002; Kurioka *et al.*, 2015). These effector T cells and their effector molecules play a pivotal role in preventing the uncontrolled growth of *M.tb,* even though the relative contribution of each of the T cell subsets is yet to be determined (Napier *et al.*, 2015; Saeidi *et al.*, 2016).

1.4.2.1 CD4 T cells

CD4 T cells comprise a functionally diverse class of T cells which are traditionally classified into T helper 1 cells (Th_1), and T helper 2 (Th_2) subsets, although there are other subsets such as Th_{17}, Th_{22}, regulatory T cells (T_{regs}) and follicular helper T cells (T_{fh}) (Luckheeram *et al.*, 2012). The Th_1 and Th_2 cell lineages differ due to their cytokine profiles with Th_1 cells associated with responses to intracellular pathogens such as *M.tb* and produce cytokines such as IFN-γ and IL-2. IFN-γ is crucial for the activation of antigen presenting cells such as macrophages and this activation leads to the activation of IFN-γ-responsive genes within the macrophages which activate them to kill the intracellular pathogen (Flynn, 1993). Another Th_1 cytokine is IL-2 and it is important for the regulation of cellular immunity and IL-2 produced by CD4 T cells has been shown to promote the proliferation of cytotoxic T cells and the development of memory CD8 T cells (Dai *et al.*, 2000; Kim *et al.*, 2006).

Th_2 cells are associated with responses to extracellular pathogens and produce cytokines such as IL-4, IL-5, IL-9 which have a role in allergic responses and inflammation and IL-10 which is involved in the regulation of the immune response by inhibiting Th_1 cell responses (Couper *et al.*, 2008).

The importance of CD4 T cells in *M.tb* infection is universally accepted and they are known to play a role against *M.tb* infection (Boom *et al.*, 2003). *M.*tb antigens are presented by MHC-II to CD4 T cells which are an important source of IFN-γ, IL-2 and TNF-α (Flynn and Chan, 2001). These cells activate macrophages and other APCs through cytokines and through CD40-CD40L interactions which ultimately leads to the activation of other immune cells (Mihret, 2012).

The importance of CD4 T cells in the immune response against *M.tb* infection is evident in people with HIV infection (Mihret, 2012), which is characterized by the progressive depletion of CD4 T cells (Mihret, 2012; Okoye and Picker, 2013; Walker *et al.*, 2019). In persons with HIV infection, there is a rapid progression of TB disease and this is partly attributed to the depletion and dysfunction of CD4 T cells (Walker *et al.*, 2019). Mice lacking CD4 T cells have also been shown to lack the ability to control of *M.tb* infection (Muller *et al.*, 1987; Caruso *et al.*, 2019).

1.4.2.2 CD8 T cells

CD8 T cells are another subset of T cells that have a crucial function in the immune response against intracellular pathogens such as viruses and bacterial pathogens such as *M.tb* (Berg and Forman, 2006; Mittrücker *et al.*, 2014)**.** They recognize peptide molecules in complex with MHC-I molecules on antigen presenting cells and these interactions activate CD8 T cells into effector CD8 T cells (Gold *et al.,* 2015)**.**

CD8 T cells are predominantly described as cytotoxic T cells due to their ability to produce cytotoxic molecules such as perforin which acts to perforate cells generating pores for delivery of other proteins into target cells (Lin and Flynn, 2015; Voskoboinik *et al.*, 2015).They also have the capacity to produce cytotoxic granules which contain molecules such as granzymes which enter the target cell through the help of perforin and induce cell death by activating apoptotic pathways (Voskoboinik *et al.,* 2015). Another molecule within the granules is granulysin and it has the ability to directly kill microbes such as *M.tb* in the extracellular and intracellular environments by disrupting their cellular membranes (Stenger *et al.*, 1998).

Similarly, to CD4 T cells, CD8 T cells also have the ability to produce cytokines such as IFN-γ, TNF-α, IL-10 and IL-2 (Silva *et al.*, 2014; Lin and Flynn, 2015).

CD8 T cells have been shown to produce less IFN-γ than CD4 T cells in response to *M.tb* stimulation, however, CD8 T cells have been shown to play a crucial role in protective immunity during the later stages of *M.tb* infection (Flynn *et al.*, 1992; Chen *et al.*, 2009). Once stimulated by *M.tb*, CD8 T cells produce IFN-γ and cytotoxic molecules molecules which allow CD8 T cells to aid in the control of intracellular bacterial growth within macrophages by activating or lysing infected macrophages (Canaday *et al.*, 2001).

1.4.2.3 Natural killer (NK) T cells

CD4 and CD8 T cells are considered classical or conventional T cells because they recognize peptide antigens that are presented by classical MHC class II and I molecules, respectively (Godfrey *et al.*, 2015; Joosten *et al.*, 2016). In addition to these conventional T cells, there are other T cell subsets which are known as donor unrestricted T (DURT) cells as they recognize different classes of antigens presented to them by non-classical MHC molecules (Godfrey *et al.*, 2015).

Among the DURT are NK T cells, which are an evolutionary conserved subset of T cells that express a TCR α/β, Vα24-Jα18 paired with Vβ11 (Godfrey *et al.*, 2010; Kee *et al.*, 2012; Kim *et al.,* 2019). NKT cells recognize glycolipids such as α-galactosylceramide (α-GalCer) instead of the classical peptide antigens, these glycolipids are presented by an MHC-like molecule, CD1d (Sada-ovalle *et al.*, 2008; Godfrey *et al.*,2010).

NKT cells have the capacity to rapidly produce cytokines such as IFN-γ, IL-4, IL-10, IL-17, TNF-α and IL-2 (Wu *et al.*, 2015). This capacity coupled with the CD1d restriction allows NKT cells to interface between the innate and adaptive immune system, thus they have both a regulatory and effector function (Venkataswamy *et al.*, 2010; Kee *et al.*, 2012).

Results from mouse models suggest that NKT cells may play a role in protection against *M.tb* infection and have shown that α-GalCer incorporation into treatment or into BCG has the ability to improve clinical outcomes of mice (Sada-ovalle *et al.*, 2008) and the immune responses of the mice infected with *M.tb*, respectively (Venkataswamy *et al.*, 2010). This is achieved through NKT-mediated T cell priming (Chackerian *et al.*, 2002; Kee *et al.*, 2012).

Some other non-conventional T cells include 1) the gamma delta (γδ) T cells which are capable of recognizing a variety of antigens, even without presentation by MHC molecules (Chien *et al.*, 2014; Lawand *et al.*, 2017), 2) MHC-Ib restricted T cells which recognize antigens presented by MHC-Ib molecules (HLA-E, HLA-F and HLA-G) (Godfrey *et al.*, 2015; Joosten *et al.*, 2016), and 3) MAIT cells which recognize vitamin B metabolites that are presented by MR1 molecules.

The focus of this study however, will only be on MAIT cells.

1.4.2.4 MAIT cells as a relevant T cell subset in immunity

MAIT cells are a subset of T cells that play a role in TB immunity. These are non-classical and innate-like T lymphocytes that are characterized by the expression of the invariant T cell receptor (TCR), Vα7.2 (TRAV1-2) (Sundström *et al.*, 2015; Jiang *et al.*, 2016). These cells are also characterized by their high expression of IL-12 and IL-18 receptors (CD212 and CD218a, respectively) as well as CD161, a C-type lectin receptor (Howson *et al.*, 2015; Loh *et al.*, 2016). MAIT cells have also been characterized by their co-expression of CD161 and CD26 (Sharma *et al.*, 2015) and can also be identified through the use of MR1-loaded tetramers (Corbett *et al.*, 2014).

MAIT cells recognize and are highly reactive to vitamin B metabolites, mostly those derived from the riboflavin (vitamin B2) pathways found in yeast and bacteria. These metabolites are displayed and presented by the non-classical MHC class-1 related protein, MR1 on APCs (Israeli, 2012; Kjer-Nielsen *et al.*, 2012).

A large proportion of MAIT cells are CD8$^+$ T cells (some expressing CD8αα and others CD8αβ) with a smaller number of MAIT cells displaying the CD4$^+$ and CD4/CD8 double negative (DN) T cell phenotypes (Leeansyah *et al.*, 2013). MAIT cells make up 1-10% of the overall population of circulating T cells and are abundant (up to 50% of T cells) in tissues such as the liver, intestines and the lungs (Martin *et al.*, 2009; Meierovics *et al.,* 2013; Saeidi *et al.*, 2016).

1.5 Development of T cells

1.5.1 Development of conventional T cells

In order to be fully functional, T cells have to undergo distinct stages of development under which they are continually selected, and defective cells are eliminated. These ensure that they are immune tolerant and non-reactive to self-antigens (Baldwin *et al.*, 1999; Takaba and Tagayaki, 2017)These processes differ between conventional and non-conventional T cells.

The development of T cells into differentiated and functional T cells begins in the thymus in where the cells undergo rearrangement of their TCR followed by positive and negative selection by thymic epithelial cells whereby naïve CD4$^+$CD8$^+$ double positive (DP) T cells are exposed to self-peptide-MHC complexes (Klein *et al.*, 2014; Shah and Zúñiga-pflücker, 2019). During this phase of development, the immature DP T cells are selected based on their affinity to the peptide-MHC complexes and those with high affinities and weak affinities to these complexes are eliminated while those with moderate affinities acquire MHC-restriction.

The T cells progress to the next phase which is negative selection whereby the T cells encounter APCs such as DCs, and all T cells that recognize self-antigens presented by the APC (autoreactive T cells) during this stage of negative selection are eliminated (Takaba and Takayanagi, 2017). The cells that survive selection at this stage migrate to the periphery and circulate between blood, lymph and secondary lymphoid organs in a naïve state (Cose *et al.*, 2006; Hunter *et al.*, 2016), this is followed by priming and clonal expansion of the cells upon recognition of antigens presented by APC (typically DCs) on appropriate peptide-HLA complex (Klein *et al.*, 2014).

This process of antigen presentation to the T cells leads to the differentiation from naïve into effector/memory phenotypes in which the cells either acquire an effector function and respond to the stimulation or differentiate into memory cells.

1.5.2 Development and thymic maturation of MAIT cells

The development of MAIT cells is similar to that of conventional T cells (section 1.5.1 above) in that MAIT cells also develop in the thymus with a naïve phenotype. However, selection of MAIT cells differs from that of conventional T cells because MAIT cells undergo selection by CD4^{+}CD8^{+} DP thymocytes which express MR1 (Martin *et al.*, 2009; Seach *et al.*, 2013; Koay *et al.*, 2016), while conventional T cells are selected by thymic epithelial cells (Le Bourhis *et al.*, 2011; Seach *et al.*, 2013). MAIT cells are thought to undergo a 3-stage pathway whereby they mature within the thymus (Koay *et al.*, 2016).

In stage 1 after the selection by DP thymocytes, human MAIT cells emerge as CD27^{-}CD161^{-}IL-18R^{-} (while mouse MAIT cells emerge in stage 1 as CD24^{+}CD44^{-}CD218^{-}) immature cells. In stage 2 the immature cells interact with MR1 and Drosha, a microRNA molecule which regulates the development of MAIT cells (Koay *et al.*, 2016), and differentiate into CD27^{+} CD161^{-}IL-18R^{-} (or CD24^{-}CD44^{-}CD218^{-} in mice). At stage 3, MAIT cells start expressing PLZF, CD161 and IL-18R (CD218^{+}) as they undergo peripheral expansion from the thymus (Koay *et al.*, 2016). The final step after the intrathymic development of MAIT cells involves the interaction of MAIT cells with B cells and commensal flora which induce the expansion of these cells in the periphery into a differentiated effector/memory phenotype (Martin *et al.*, 2009) (Figure 1.5).

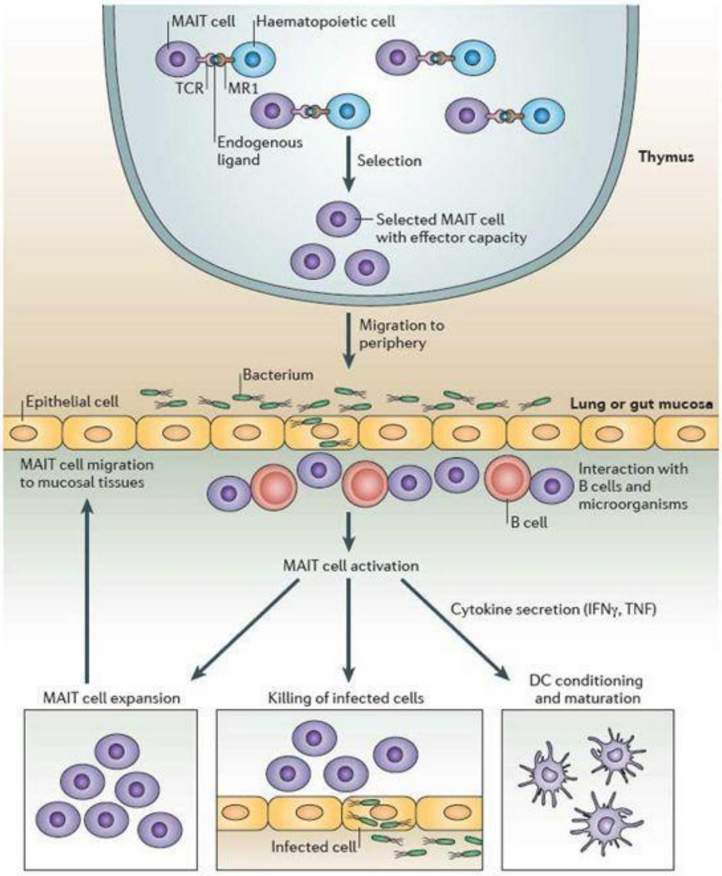


Figure 1.5: Developmental pathway for MAIT cells from the thymus to the periphery. Diagram showing the process of MAIT cell maturation and development where they initially undergo selection by MR1 expressing DP thymocytes and undergo the 3-stage developmental pathway. Following this, MAIT cells exit the thymus as effector cells through interactions with B cells and commensal flora, expand to circulation and mucosal tissues where they carry out their effector function and contribute to the control of bacterial infection (Image adapted from Gold and Lewisohn, 2013).

It is thought that since MAIT cells are present in cord blood (Martin *et al*., 2009) and in the thymuses of germ-free mice (although in small frequencies and absent from circulation) (Koay *et al*., 2016), the presence of bacteria or commensal flora is not required for thymic selection of MAIT cells suggesting the presence of an unidentified ligand within the thymus which allows for intra-thymic selection. While the ligand has not been defined, it has been proposed that there may be an endogenous self-antigen produced within the thymus which allows for selection in absence of commensal flora (Huang *et al.*, 2008; Koay *et al.*, 2016).

1.5.3 Antigen presentation

1.5.3.1 Classical antigen presentation

The ability of the immune system to constantly monitor and survey the cellular environment (intracellular and extracellular) for the presence of foreign agents such as pathogens is a crucial mechanism to fight infection (McWilliam and Villadangos, 2017). The innate immune system uses PRR to recognize PAMPS on pathogens in the extracellular environments (Medzhitov and Janeway, 1997; Shey *et al.*, 2018). Because some pathogens are intracellular and have evolved to occupy different cellular compartments, the immune system employs different pathways to survey the different cellular compartments for the presence of foreign antigen (Huang *et al.*, 2008).

Different molecules are involved in these processes and as a result, the recognition of antigen by antigen presenting molecules leads to distinct cellular pathways which activate different types of T cells depending on the antigen and its location within the cell (Huang *et al.*, 2008; McWilliam and Villadangos, 2017). Classical antigen presentation pathways involve MHC class I or class II molecules while non-classical pathways involve MHC-like molecules such as CD1, and MR1 in the case of MAIT cells (Gold*et al.,* 2015). For classical T cell activation, 3 signals are thought to be required; 1) the recognition and binding of the MHC-peptide complex to the T cell receptor, 2) binding of co-stimulatory molecules such as CD86 and CD40 on APCs which bind to CD28 and CD40L on T cells, and 3) cytokine production by APCs, required for T cell polarization (Gold *et al*., 2015). This activation ultimately leads to the differentiation and development of T cells with different effector functions (Sasindran and Torrelles, 2011; Gold *et al*., 2015).

1.5.3.2 Antigen presentation to MAIT cells

MR1 antigen presentation and MR1 dependent MAIT cell activation

The mechanisms for MAIT cell activation differ from the classical T cell activation in that MAIT cells can be activated either through an MR1-dependent manner in which MR1 from APCs presents the ligand and are recognized by MAIT cell TCR (Vα7.2) (Le Bourhis *et al.*, 2011; Howson *et al.*, 2015), or by an MR1-independent manner in which MAIT cells are directly activated by cytokines (such as IL-12, IL-18 and IFN-α) produced by infected/activated APCs as shown in Figure 1.6A. (Saeidi *et al.*, 2016; Wong *et al*., 2017).

Early studies on the intracellular mechanisms which are used for MR1 presentation discovered that although MR1 and MHC-I molecules share a high sequence homology and structural similarity, MR1 uses a distinct pathway from MHC-I antigen presentation (Huang *et al.*, 2008; McWilliam *et al.*, 2016). This was demonstrated by Huang *et al*., (2008) who conducted transfection experiments on TAP (a transporter protein responsible for delivering peptides from cytosol to endoplasmic reticulum (ER) for MHC-I binding) from deficient cell lines in order to assess the effect of TAP on the overall expression of MR1 on cell surface. They found that the absence of TAP had no impact on MR1 surface expression and MAIT cell activation (Huang *et al*., 2008). Furthermore, the absence of MHC-I associated ER chaperons such as tapesin and calreticulin (CRT) which mediate the MHC-antigen complex formation also had no effect on MR1 surface expression and MAIT cell activation (Huang *et al*., 2008; Martin *et al*., 2009).

The expression of MR1 was also determined to be independent of the proteasome pathway as proteasome inhibitors did not affect the pathway and only brefeldin A, which inhibits protein transport from ER to golgi apparatus, was able to inhibit MR1 expression and MAIT cell activation (Huang *et al*., 2008). These initial findings suggested that MR1 antigen presentation pathway was different from that of MHC-I in that it was independent of TAP, ER chaperones and proteasome. However, the presentation of MR1 on cell surface did involve transport from ER to golgi complex.

MR1 is ubiquitously expressed in all cell types but the cell surface expression is dependent on ligand availability (Huang *et al*., 2009; McWilliam *et al*., 2016). In the absence of its ligand, MR1 is localized in the ER and only trafficked from the ER to the cell surface after ligand recognition and binding and through an endocytic pathway similar to that of MHC class II antigen presentation (Huang *et al*., 2008; McWilliam *et al*., 2016).

MR1-independent activation

Due to the activation of MAIT cells by pathogens both enriched and lacking the riboflavin synthesis pathway and in other inflammatory disease, Ussher *et al.,* (2014) postulated that MAIT cells could be activated through an alternative pathway, a cytokine pathway involving IL-12/IL-18 as had been observed for NK cells (Gorski *et al.*, 2006), this was hypothesized because MAIT cells express high levels of the receptors for IL-12, IL-18 and IFN-α (Spaan *et al.*, 2016; Van Wilgenburg *et al.*, 2016).

The role of IL-12 and IL-18 on MAIT cell activation was therefore investigated by initially stimulating PBMCs with IL-12 or IL-18 or both. CD161$^+$ T cells were activated and were responsive to cytokine stimulation and produced increased amounts of IFN-γ, but this was observed when both cytokines were used together as opposed to when they were used individually (Ussher *et al.*, 2014). This activation was MR1-independent and blocking with MR1 did not affect IFN-γ production by MAIT cells in the cytokine stimulated cells even though it had an effect on *E.coli* stimulated cells (*E.coli* is capable of producing metabolites which are recognised by MR1 in order to activate MAIT cells (Kjer-Nielsen *et al*., 2012)). These responses were also TCR independent as they could only be inhibited by a p38-MAPK inhibitor but not by cyclosporine which is an inhibitor of TCR signaling (Ussher *et al*., 2014).

To further test these mechanisms for MAIT cell activation, *E.coli* and *Enteroccocus faecalis* (*E.feacalis*), which does not have riboflavin pathway were used for stimulation. From these experiments, blocking of *E.coli* stimulated THP-1 cells with either MR1 or IL-12/IL-18 blocking antibodies reduced the IFN-γ production from MAIT cells while in *E.feacalis*, MR1 blocking had no effect on MAIT cell IFN-γ production but IL-12/IL-18 blocking reduced IFN-γ production (Ussher *et al*., 2014).

Further evidence of this cytokine dependent pathway has also been demonstrated by Van Wilgeburg *et al*., (2016) in which they investigated the role of MAIT cells in viral infections and found that MAIT cells were highly activated (upregulated CD69 and CD38) and upregulated IFN-γ production upon incubation with influenza virus-incubated APCs in an MR1 independent manner as was expected since viruses do not produce riboflavin metabolites necessary for MR1 dependent activation.

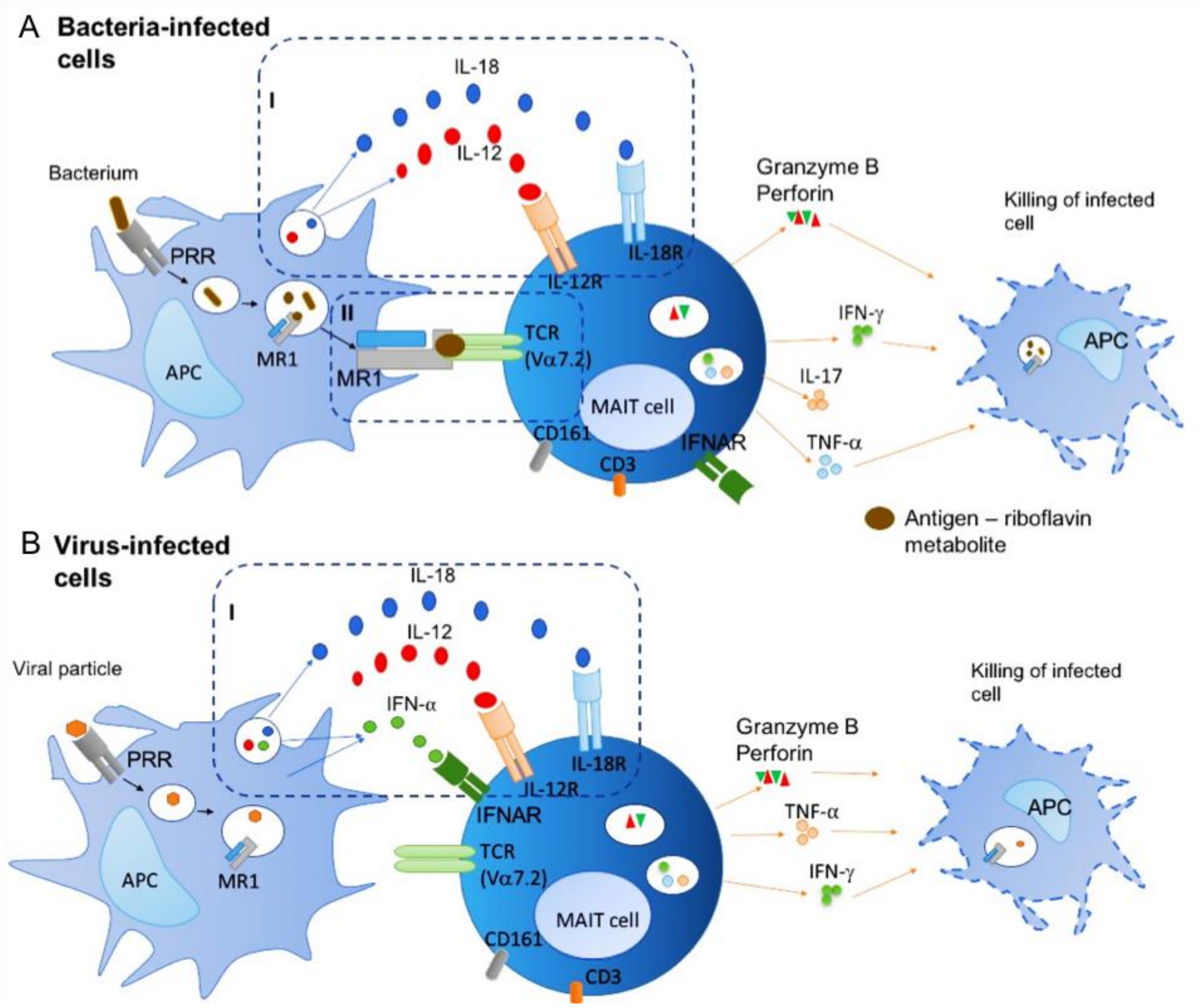


Figure 1.6: Mechanisms involved in the activation of MAIT cells in the presence of riboflavin pathway-containing bacteria. A) In the presence of riboflavin pathway containing bacteria. I) MR1-Independent pathway in which antigen presenting cells produce pro-inflammatory cytokines such as IL-12 and IL-18 which in turn activate MAIT cells to produce cytokines II) MR1 dependent pathway in which vitamin B metabolites released from phagocytosed intracellular pathogen are recognized by MR1 molecules inside antigen presenting cells and are presented on the cell surface for recognition by MAIT cell TCR (Vα7.2) which activates MAIT cells to produce cytokines such as IFN-γ, TNF-α, IL-17 and cytotoxic molecules such as granzyme B and perforin which lyse and kill the infected cells. B) In the presence of pathogens lacking the riboflavin synthesis pathway such as HIV, infected antigen presenting cells produce cytokines such as IL-12, IL-18 and IFN-α which in turn activate MAIT cells in an MR1-independent mechanism resulting in the production of effector cytokines such as IFN-γ, TNF-α and the production of cytotoxic molecules which kill the virus infected cells. (Image adapted from Shey *et al*., 2018).

1.5.4 MR1 ligands: discovery and binding to MR1 and TCR

Prior to 2012, the nature of the MR1 ligand was unknown and was speculated to be similar to that of the MHC or CD1d molecules of peptide and lipid nature, respectively (Huang *et al*., 2008; Kjer-Nielsen *et al*., 2012). Huang *et al*., (2008) speculated that the ligand could be low molecular weight molecules because these were precipitated with MR1 and had the capacity to activate MAIT cells in an MR1 dependent manner. In a paper by Kjer-Nielsen and colleagues (2012), the MR1 ligands were identified as vitamin B metabolites. This discovery was made after a series of experiments which led to the identification of a metabolite of folic acid which was able to facilitate the proper refolding of denatured MR1 to form an MR1-antigen complex.

Through mass spectroscopy (MS) and chromatography, the compound was identified as 6-formyl pterin (6-FP) and was demonstrated to be able to induce the surface expression of MR1 on C1R cells (Kjer-Nielsen *et al.*, 2012). The crystal structure of the MR1-antigen complex revealed that the antigen binding cleft of MR1 was different from that of MHC-I which binds peptide due to a predominantly polar binding cleft and to that of CD1d which binds lipid antigens which has a hydrophobic cleft (Kjer-Nielsen *et al*., 2012). MR1 was different in that its cleft, although solvent-exposed like HLA-A2 (MHC-I), had a mixture of hydrophobic and charged residues. Furthermore, the cleft was smaller than CD1d thus making it unsuitable for larger ligands (Huang *et al*., 2008; Kjer-Nielsen *et al*., 2012).

The binding of the ligand to the MR1 antigen binding cleft was determined to be through;
1) hydrophobic interactions between the ligand and tyrosine residues in the cleft, **2)** van der Waals forces between ionic groups on ligand and the amino acid groups on the MR1 cleft, and **3)** covalent bond between the formyl group on ligand and lysine residues (Lys^{43}) on MR1 forming a Schiff base which is critical to the MR1-ligand complex (Kjer-Nielsen *et al.*, 2012; Eckle *et al.*, 2015; Keller *et al.*, 2017). The amino acid residues associated with Van der Waals interactions and Schiff base formation are highly conserved across MR1 from all species suggesting the conserved nature of antigen recognition by MR1.

Even though 6-FP was found to be effective in binding MR1 and inducing the cell surface expression of MR1, the complex was unable to activate MAIT cells which suggested that the pterin analogues served as building blocks for MAIT activating ligand (Kjer-Nielsen *et al.*, 2012; Corbett *et al.*, 2014). The analysis of supernatants from *Salmonella typhimurium* (*S.typhimurium*) enabled the identification of other vitamin B metabolites that were closely related to 6-FP but had an extra ribityl group and had the ability to activate Jukart MAIT cells. This result suggested that the ribityl group had a role in MAIT TCR recognition of the MR1-antigen complex.

5-amino-6-d-ribitylaminouracil (5-A-RU) was identified as a precursor to the MR1 ligands and the condensation of this compound leads to the formation of unstable pyramidine compounds, 5-(1-methyl2-oxopropylideneamino)-6-D-ribitylaminouracil (5-MOP-RU), 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU) and 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) before forming ribitylumazines identified by Kjer-Nielsen *et al*., (2012). These pyramidines were bound and stabilized by MR1 binding through covalent bonding, Schiff base formation with Lys^{43} and these complexes were recognized by MAIT cell TCR through hydrogen bonding with Tyr^{95} (Corbett *et al*., 2014). Tetramers synthesized using these compounds were successful in identifying human MAIT cells (Reantragoon *et al.*, 2013; Gherardin *et al.*, 2018; Harriff *et al.*, 2018).

In addition to these ligands, synthetic ligands were generated from 6-FP through modification of the group. These 6-FP variants had the capacity to bind and increase the surface expression of MR1 but had no effect on MAIT cell activation and through competition experiments, one of the variants had a higher affinity for MR1 binding cleft, could stabilize MR1 at cell surface and greatly inhibit MAIT cell activation (Soudais *et al.*, 2015).

Through these studies, MR1 ligands are now divided into 2 classes; MAIT activating and MAIT non-activating ligands (McWilliam and Villadangos, 2017). MAIT activating ligands are derived from Vitamin B2 and include pyramidine compounds: 5-OE-RU and 5-OP-RU which are formed through condensation of 5-A-RU with small molecules such as methylglyoxal compounds (Corbett *et al.*, 2014). These ligands are able to form a covalent Schiff base with MR1 Lys^{43}, are presented efficiently at the cell surface in complexes which can be recognized by MAIT cell TCR and thus activate MAIT cells (Corbett *et al.*, 2014; Keller *et al.*, 2017; McWilliam and Villadangos, 2018). MAIT non-activating ligands include 6-FP and its variants such as acetyl-6-FP which are Vitamin B9 derived (folic acid) (McWilliam and Villadangos, 2018). These ligands are able to facilitate binding to MR1 and can be expressed at the cell surface but are not recognized by MAIT cell TCR and thus, do not activate MAIT cells (Kjer-Nielsen *et al.*, 2012; Soudais *et al.*, 2015). They can however inhibit recognition of MR1-antigen complex by MAIT cell TCR through competition for MR1 binding cleft.

This understanding of MAIT cell ligands and their interactions with MR1 and MAIT cell TCR has led to the recognition of drugs and drug-like molecules such as the salicylates and diclofenac which have the ability to regulate MAIT cell functions in mice (Keller *et al.,* 2017).

1.6 The antimicrobial function of MAIT cells

The understanding of MR1-MAIT cell axis and the conserved nature of the MR1 molecule, its antigens and their associations with MAIT cell TCRs suggests that MAIT cells may play a role in responses to microbial infections (Le Bourhis *et al.*, 2010).

The function of these cells, however, had not been characterized until Gold *et al.*, (2010) generated CD8 T cell clones from people with active TB, LTBI and healthy uninfected people. They found that non-classically restricted T cells were of higher numbers in healthy people and these cells could not be restricted by CD1d molecules or HLA-A, HLA-G and HLA-Ia molecules but were only restricted by MR1. Furthermore, the cells could not be activated by TLR-mediated DC stimulation. Phenotypic analysis of these cells revealed the expression of the MAIT TCR, Vα7.2-Jα33 in humans (Vα19-Jα33 in mice) (Gold e*t al.*, 2010). Interestingly, these cells were responsive to DCs stimulated with *M.tb* cell wall fractions compared to cell filtrate protein and these responses could be abrogated by MR1 blocking and proteinase treatment.

The specificity of the MAIT cells was assessed by infecting DCs with a variety of bacteria. The cells were responsive to DCs infected with *Mycobacteria smegmatis, E.coli, S.typhimurium, Staphylococcus aureus* but not to DCs infected with virus or those infected with *Listeria* *monocytogenes,* which after the identification of MAIT cell ligands, is known to lack the riboflavin pathway therefore is unable to produce vitamin B metabolites. Furthermore, MAIT cells were also observed to be able to recognize *M.tb* infected A549 epithelial cells in an MR1 dependent manner (Gold *et al*., 2010). This MR1 dependent MAIT cell responses to bacteria-infected APCs was also observed for mouse MAIT cells, for example; iVα19 transgenic mice were activated in *E.coli* stimulated DCs from MR1 wild-type mice compared to MR1$^{-/-}$ mice (Le Bourhis *et al*., 2010).

Le Bouris *et al.* (2010), demonstrated the anti-microbial role of human and mouse MAIT cells and found that human MAIT cells were highly activated by monocytes in the presence of *E.coli*, and their numbers were reduced in blood of people with bacteria-associated diseases such as TB (Gold *et al.*, 2010; Le Bourhis *et al.*, 2010) and MAIT cells were enriched in the lung lesions from TB infected people and ascitic fluids, suggesting that these cells migrate to sites of infection where they perform their antimicrobial role (Le Bourhis *et al*., 2010; Meierovics *et al*., 2013).

In addition to the broad range of bacteria that Gold and colleagues (2010) reported that MAIT cells were responsive to, Le Bourhis *et al*.,(2010) made similar findings and found a broad range of responses to gram-negative and gram-positive bacteria such as *Klebsiella pneumoniae* and *Lactobacillus acidophilus*. Furthermore, fungi such as *Saccharomyces cerevisiae, Candida glarabat*a and *Candida albicans* activated MAIT cells in an MR1 dependent manner whereas DCs infected with viruses such as Herpes simplex virus, Newcastle disease virus, encephalomyocarditis did not activate MAIT cells even though they activated DCs (Le Bourhis *et al.*, 2010).

MAIT cells can be activated in an MR1-independent manner through the production of cytokines such as IL-12 and IL-18 by APCs, however, the production of cytokines (and MAIT activating cytokines) was not assessed by the researchers and so it is unclear why MAIT cells were not activated by virus-infected DCs. When co-cultured with BCG infected macrophages, MAIT cells effectively reduced intracellular BCG growth within the macrophages and enhanced the production of nitric oxide (NO_2) by the macrophages and were a potent source of IFN-γ and may thus play a role in the control of bacterial infection (Chua *et al.*, 2012).

1.7 Antimicrobial role of MAIT cells *in vivo*

In vivo evidence of the role of MAIT cells was demonstrated by intraperitoneal infection of wild type (WT) and MR1 knockout iVα19 transgenic mice. Mice lacking MR1 had higher bacterial burden in their spleen after 3 days of infection with *E.coli* and *Mycobacterium abscessus* compared to WT transgenic mice (Le Bourhis *et al*., 2010). Similar results were obtained after infection of mice with BCG in which MR1 knockout mice had higher bacterial burden in their lungs compared to wild type mice at 10 days post infection (Chua *et al*., 2012).

In a mouse model of *F.tularensis* live vaccine strain (LVS) pulmonary infection, MAIT cells expanded during the course of infection and transcripts for Vα19-Jα33 were highly enriched at 14 days after infection and this peak in expansion of MAIT cells was associated with reduction of bacterial numbers suggesting that MAIT cells expanded greatly in the late stages of LVS infection and were retained, even after clearance of the bacteria (Meierovics *et al.,* 2013). Finally, when Vα19-Jα33 transcripts were compared from different tissues in the infected mice, MAIT cells were found to preferentially accumulate in the lungs, the site of LVS infection which supports the observations that the cells migrate to the site of infection in order to control bacterial growth (Le Bourhis *et al.*, 2010; Meierovics *et al.,* 2013; Gold *et al*., 2015).

1.8 MAIT cells in disease

1.8.1 MAIT cells in Tuberculosis

As discussed in section 1.6 and 1.7 above, MAIT cells are thought to be important in providing protective immunity against fungal and bacterial infection, including TB in humans (Ussher *et al.*, 2014; Reantragoon *et al.*, 2016). While MAIT cells are important in the control of TB, TB also has an effect on MAIT cell frequencies and function.

1.8.1.2 Effect on MAIT cell frequencies

There appears to be an association between MAIT cells and TB disease, numerous studies have reported a decline in circulating MAIT cell frequencies in peripheral blood of patients with active TB while the frequencies of MAIT cells accumulated in the lungs of the same patients (Jiang *et al.*, 2016). Numerous studies have demonstrated that frequencies of MAIT cells were reduced in peripheral blood of individuals with active TB compared to healthy controls (Wong *et al.*, 2013; Jiang *et al.*, 2014; Kwon *et al.*, 2015)**,** while those with LTBI were observed to have similar frequencies to healthy controls (Wong *et al.*, 2013; Jiang *et al.*, 2014). When Jiang and colleagues, (2014) assessed the memory phenotypes of these MAIT cells, they found that individuals with active TB had higher frequencies of effector memory ($CD45RO^{+}CCR7^{-}$) MAIT cells in blood compared to healthy controls while and central memory ($CD45RO^{+}CCR7^{+}$) MAIT cells were similar.

1.8.1.3 Effect on MAIT cell function

Interestingly, when they analysed MAIT cell functions, Jiang *et al.*, (2014) found that MAIT cells from individuals with active TB had enhanced function compared to healthy controls with a greater production of IFN-γ and TNF-α in response to BCG stimulation. In the same study, however, when the researchers stimulated MAIT cells with *E.coli*, they observed different results to BCG stimulated MAIT cells, the cells were functionally defective with lower IFN-γ and TNF-α production in individuals with active TB compared to healthy controls (Jiang *et al.*, 2014)**.** Kwon *et al.*, (2015) found that the residual MAIT cells were functionally impaired, producing lower IFN-γ relative to healthy controls and MAIT cells from individuals with non-tuberculous mycobacteria (NTM).

In another study, MAIT cell function (IFN-γ production) was retained in individuals with LTBI compared to healthy controls and in individuals with active TB, MAIT cells exhibited an impaired function in response to *M.tb* antigen stimulation (Jiang *et al.*, 2016)**.** Furthermore, both these studies observed higher PD-1 expression in MAIT cells of individuals with active TB compared to healthy controls (Jiang *et al.*, 2014; Kwon *et al.*, 2015) and to individuals with NTM (Kwon *et al.,* 2015). Jiang *et al.,* (2014) also showed that blockade of the signaling pathway by anti-PD-1 antibodies restored the functional capacity of the MAIT cells (Jiang *et al.*, 2014). These results therefore suggested that the reduction in IFN-γ production in MAIT cells may be due to inhibition by the PD-1 signaling pathway (Jiang *et al.*, 2014 and Kwon *et al.*, 2015). This has also been suggested by Schmaler *et al.*, (2018) who observed that MAIT cells in the gut had significantly higher PD-1, CTLA-4 and LAG-3 expression compared to MAIT cells in the gut, and the researchers proposed that the expression of these inhibitory markers by the MAIT cells is a regulatory mechanism from continuous antigen exposure.

1.8.2 MAIT cells in HIV infection

1.8.2.1 Effect on MAIT cell frequencies

The functions and immune activity of MAIT cells is not only limited to responses to bacterial and fungal pathogens, but it also extends to viral pathogens such as HIV. Unlike yeast and bacteria that contain the antigens required to activate MAIT cells in a MR1-dependent manner through MR1 antigen presentation, viruses lack these metabolites and are only able to activate MAIT cells in the MR1-independent manner by eliciting the production of cytokines from activated APCs (Ussher *et al.*, 2014; Van Wilgenburg *et al.*, 2016). The cytokines produced include IL-12, IL-18, IFN-α (which bind to MAIT cell IL-12R, IL-18R and IFNAR receptors, respectively).

The effect of HIV on MAIT cell frequencies and function have been investigated by several studies where they observed that circulating MAIT cells are reduced in people with HIV-1 infection and this depletion was not related to viral load or CD4 counts and could not be restored after anti-retroviral treatment (ART) (Leeansyah *et al.*, 2013; Wong *et al.*, 2013; Saeidi *et al.*, 2015). Furthermore, Saedi *et al.*, (2015), did not observe any significant differences in MAIT cell frequencies between treatment (ART) naïve and ART positive individuals. In addition to studying MAIT cells in peripheral blood, Eberhard *et al.*, (2014) investigated MAIT cells in the lymph nodes of HIV+ individuals and found that the frequencies were also reduced in these sites.

In a study conducted on children between 3-18 years, Khaitan *et al.*, (2016) observed that the frequencies of MAIT cells were also reduced in HIV positive children compared to healthy controls, irrespective of the ART status. Interestingly, they observed that contrary to observations from studies conducted in adults, the frequencies of MAIT cells in children gradually recovered after 10-12 months of ART.

It has been suggested that the reduction in MAIT cell frequencies could be either due to activation-induced cell death as a result of continuous antigen exposure and caspase actions as MAIT cells were found to have a higher expression of activated caspases (Eberhard *et al.*, 2014). They also reported that in individuals with HIV, there was an increase in CD161$^-$ Vα7.2$^+$ population relative to healthy controls which could be due to downregulation of CD161 as a result of this activation. The researchers proposed that this reduction in circulating MAIT cells in the context of HIV infection could be due to the recruitment to inflamed mucosal surfaces since HIV infection is known to compromise mucosal barriers such as the gastrointestinal tract (Leeansyah *et al.*, 2013; Eberhard *et al.*, 2014)**.** Furthermore, the decrease could be due to an up-regulation of gut-homing markers such as CCR9 (Eberhard *et al*., 2014).

Leeansyah *et al*., (2013), Ebehard *et al*., (2014) and Saeidi *et al*., (2015), all suggested that this loss of MAIT cells in HIV infected individuals from circulation may compromise the mucosal barriers thus causing microbial translocation which ultimately results in chronic activation of the immune system, this is supported by their findings of high frequencies of highly activated MAIT cells in highly viremic individuals (Eberhard *et al*., 2014)**.**

1.8.2.2 Effect on MAIT cell functions.

Residual MAIT cells were found to be highly activated (upregulated CD69 expression) and functionally impaired (lower IFN-γ, TNF-α and IL-17 production) in individuals with HIV and this function could be partially restored following long-term ART (Leeansyah *et al*., 2013)**.** The expression of PD-1 was also found to be elevated in ART naïve individuals compared to controls and to individuals on ART treatment (Saeidi *et al*., 2015)**.**

Individuals with HIV infection only (irrespective of ART status) and ART naïve individuals with HIV-associated TB were observed to have lower CCR6 expression compared to healthy controls while CCR5 expression was observed to be similar to healthy controls (all except for individuals receiving ART who had significantly lower CCR5 expression (Saeidi *et al*., 2015)). In the context of HIV infection, CCR6 is important for the recruitment of T cells into secondary lymphoid tissues, and the presence of HIV has been reported to cause an increase in CCR6 ligand which can lead to the apoptosis of CCR6$^+$ T cells.

1.9 Study rationale

While several studies have described the reduction in MAIT cell numbers and frequencies in different infectious diseases and how their function changes in response to bacterial stimulation, their exact roles in cellular immunity against TB are yet to be well defined. Several studies have shown a decline in MAIT cells in peripheral blood of patients with active TB and other infectious diseases. However, there are conflicting reports of whether the function of MAIT decreases or increases even though numbers decrease (Fernandez *et al.*, 2015; Jiang *et al.*, 2016).

In the presence of the pathogen during infection, APCs become activated and respond by phagocytosing the pathogen, antigen presentation and cytokine production occurs, which ultimately activate T cells including MAIT cells. Although studies have investigated the changes in MAIT cells in the context of infectious diseases such as TB*,* little is known about how the magnitude of innate cell infection and activation determines the magnitude of activation of MAIT cells.

For our study, we aimed to characterize both innate and MAIT cell responses (activation and function) and to investigate the relationship between the magnitude of innate immune responses (as a result of mycobacterial infection) and the resulting magnitude of MAIT cell responses. We aimed to investigate the function of MAIT cells in an autologous system in which the infection of APCs and the activation of MAIT cells occur within the same sample (as it would happen in vivo) as opposed to the few studies which have investigated MAIT cell activation in heterologous systems in which APCs are infected and activated separately and are then co-cultured with MAIT cells, and MAIT activation is evaluated.

It has been reported that shorter periods of stimulation only result in the MR1-dependent activation while longer periods of stimulation from 18-20 hours also involve the MR1-independent MAIT cell activation (Ussher *et al.*, 2014). This suggests that MR1 activation is required for immediate MAIT cell activation and both the MR1 and the cytokine-driven activation are required for later activation (Shey *et al.*, 2018). The relative contributions of these 2 pathways towards overall MAIT cell activation are not well defined and so we aimed to evaluate these mechanisms by blocking either the cytokine dependent pathway (MR1-independent activation) or the MR1 antigen presentation pathway (MR1-dependent activation) following BCG or *M.tb* stimulation in order to assess this relative contribution towards the overall activation of MAIT cells.

Finally, we wanted to understand how HIV infection, TB disease and the HIV-associated TB affect MAIT cell frequencies, activation status and the interactions of MAIT cells with innate cells in peripheral blood.

1.10 Aims of study

The focus of the study was to assess the cellular interactions between APCs and MAIT cells and how these interactions change with HIV, TB and HIV-associated TB.

Aim 1: Assessment of the interactions and the magnitude of responses between innate immune cells and magnitude of MAIT cell activation in response to infection with *M.tb* or BCG.

Aim 2: Evaluation of the mechanisms through which innate immune cells stimulated *in vitro* with BCG/*M.tb* mycobacteria are able to elicit MAIT cell activation.

Aim 3: Evaluation of the impact of TB, HIV or HIV-associated TB on the functions and interactions between innate immune cells and MAIT cells.

Chapter 2: Optimisation, Materials and Methods

2.1. Participant recruitment and enrolment

This was a cross sectional study which recruited 112 participants into 4 different groups, 1) healthy controls (HIV-TB-; n=26), 2) individuals with HIV only (HIV+TB-; n=30), 3) active TB only (HIV-TB+; n=30), and 4) HIV-associated TB (HIV+TB+; n=26). A summary of the study groups is shown in Table 2.1 below.

Participants with active TB were identified using symptoms screening, chest X-rays (CXR), sputum culture, sputum smear and GeneXpert tests. The participants were recruited before commencement of TB treatment or after taking a maximum of 3 doses of TB treatment. For participants classified into **HIV-TB-** and **HIV+TB-** by CXR and symptom screening, sputum samples were collected to ensure that they were culture negative. Quantiferon TB Gold assay, which is an IGRA was also used on participants from these 2 groups to assess asymptomatic *M.tb* infection. HIV+ study participants were recruited irrespective of their anti-retroviral treatment (ART) status, which was documented.

Table 2.1: Participant groups evaluated in the study

	Healthy controls (HIV-TB-)	**HIV only (HIV+TB-)**	**Active TB only (HIV-TB+)**	**HIV-associated TB (HIV+TB+)**
Sample size	26	30	30	26
HIV infection	No	Yes	No	Yes
Active TB	No	No	Yes	Yes

Ethical approval for this study was obtained from the Faculty of Health Sciences Human Research Ethics Committee of the University of Cape Town (HREC Ref: 011/2017). Clinical research workers obtained written informed consent from all study participants according to Good Clinical Practice (GCP) guidelines. All study participants were recruited at Site B clinical research facility in Khayelitsha, Cape Town. Participants over the age of 18 were recruited into the study. We excluded participants that declined HIV testing, were pregnant, received more than 3 doses of TB treatment (for TB patient groups), had symptomatic anemia (Hemoglobin levels [Hb]< 8 g/dl together with symptoms of anemia) or had asthma or chronic obstructive pulmonary disease.

2.1.1. HIV testing

HIV testing was performed on all participants with no documented HIV results, especially the HIV-uninfected groups. Study clinicians evaluated the hospital folders of all of our participants for evidence of laboratory diagnosis of HIV such as HIV viral load testing, and participants with evidence of laboratory HIV diagnosis were not re-tested prior to enrolment into the study. Participants without any evidence of HIV diagnosis were tested for HIV using standard clinical HIV testing after having received appropriate counselling. Samples were also collected for the assessment of CD4 counts and HIV viral load testing for HIV-infected groups.

2.1.2. Tuberculosis diagnosis

All study participants underwent a chest X-ray (CXR). Study clinicians screened study participants for symptoms of active TB and reviewed their hospital records for their clinical history. Participants with active TB were enrolled on the basis of evidence of a laboratory test result (TB culture and GeneXpert) confirming the presence of active TB. Participants with a negative HIV test result and with no symptoms of active TB (as determined from symptom screening) were enrolled into HIV**-TB- group**. Participants with a positive HIV test result and no symptoms of active TB disease and CXR not suggestive of active TB were enrolled into the **HIV+TB- group**. Because HIV infection may mask TB symptoms (Wood *et al.*, 2007), sputum samples were also collected from all HIV-infected individuals with no symptoms of TB and sent to the National Health Laboratory Services (NHLS) for TB culture and GeneXpert to exclude TB disease as mentioned on section 2.1 above. Participants with a negative HIV test result and a microbiologically confirmed diagnosis of TB were recruited into the **HIV-TB+ group**. Finally, participants with a positive HIV test result and a microbiologically confirmed diagnosis of TB were enrolled into **HIV+TB+ group**.

2.1.3. Sample collection

Up to 50 ml of blood was collected from all study participants into Sodium heparin (NaHep) blood collection tubes (BD Biosciences®) and transported to the University of Cape Town CIDRI-Africa laboratory within 2 hours of collection for sample processing. A maximum of 50 mL of blood was collected from study participants with Hb levels above 10 g/dl and the volume of blood collected was reduced to maximum of 30 mL in participants with Hb levels between 8 g/dl and 10 g/dl, and as mentioned above in section 2.1 above, participants with Hb levels below 8 g/dl were excluded from the study.

Additional blood samples were collected from all HIV positive participants into appropriate Vacutainer blood collection tubes for CD4 count and Viral load testing by the NHLS. Sputum samples were collected from both TB- groups (HIV-TB- and HIV+TB- group) and were also transported to NHLS for GeneXpert (GXP) and TB culture for diagnosis of TB. In the TB+ groups (HIV-TB+ and HIV+TB+ groups), participants were enrolled into the groups on the basis of sputum tests or other clinical specimens that were sent by the clinic and that were GeneXpert, culture or smear positive.

2.2. QuantiFERON – TB Gold Plus (QFT-Plus) assay (QIAGEN)

The QuantiFERON – TB Gold Plus (QFT-Plus) assay is a method for the determination of *M.tb* infection/exposure by assessing the cell-mediated responses to mycobacterial antigens (CFP-10, ESAT-6). This assay is based on the detection of IFN-γ production in response to antigenic stimulation. The antigens are thought to only be able to stimulate T cells from individuals that have been exposed or infected with *M.tb* and are unable to stimulate T cells from uninfected individuals or BCG-vacccinated individuals without Mtb infection and is therefore thought to be very reliable for the diagnosis of LTBI or at least infection by *M.tb* complex since the presence of other mycobacterial infections such as *Mycobacterium kansasii, Mycobacterium szulgai* and *Mycobacterium marinum* may also elicit responses because these organism have these peptides.

In order to identify individuals with and LTBI (in the 2 groups without active TB), the QFT-Plus assay was used to measure the amounts of IFN-γ produced in response to antigen stimulation.

To do this, 1 ml of blood was transferred from NaHep blood collection tubes into each of the 4 QFT-Plus blood collection tubes; Nil (negative control), TB1 & TB2 (antigen tubes) and MIT (positive control), for each of the participants. The tubes were put on a rotator for 5-10 minutes for proper mixing of blood and antigens and incubated in a humidified incubator at 37°C with 5% CO_2 for 18-20 hours. Following incubation, the tubes were centrifuged at 2000 g (RCF) for 15 minutes and the plasma transferred into Sarstedt tubes and stored at -80°C.

The assay was conducted using QuantiFERON-TB Gold (QFT) 2 plate ELISA kit and was carried out according to the manufacturer's instructions. Briefly, kit reagents (except for conjugate 100X concentrate) and stored thawed plasma samples were all allowed to sit at room temperature for at least an hour prior to running assay. The Human IFN-γ standard was prepared by resuspending in 2.35 ml deionised water to make a concentration of 8 IU/ml and this was gently mixed to ensure adequate solubilisation. 4 tubes were prepared for working standards and labelled with respective concentrations (4, 1, 0.25, 0 IU/ml) and 150 µl of green diluent was added into these 4 tubes.

To make the working standards, 150 µl of the reconstituted standard was transferred into the first standard (4 IU/ml). Serial dilutions were then conducted by transferring 50 µl from 4 IU/ml into the 2nd standard and from this to the 3rd standard as shown in Figure 2.1 below. The 0 IU/ml standard contained green diluent only and no IFN-γ. 1X wash buffer was prepared by adding 25 ml of 20X wash buffer into 475 ml deionised water (1:20 dilution) and this solution was mixed. To prepare working conjugate, 100 µl of conjugate 100X concentrate was mixed with 7 ml of green diluent.

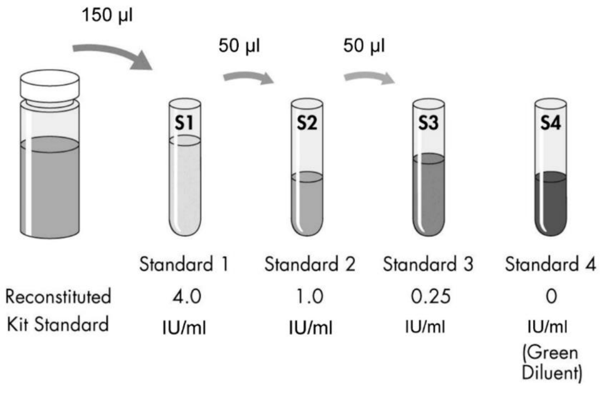


Figure 2.1: Serial dilutions for the preparation of IFN-γ working standards for QFT-Plus ELISA assay (Image adapted from product manufacturer; quantiferon.com).

A volume of 50 µl of working conjugate was added into wells of the ELISA plates with multichannel pipettes. This was followed by addition of 50 µl of plasma samples and duplicate standards into respective wells. The plates were covered with adhesive plate sealers and foil and then shaken on a plate shaker at 500 rpm for 1 minute at room temperature to allow adequate mixing of samples and conjugate in wells. Plates were then incubated for 2 hours at room temperature.

After incubation, plates were washed 6 times with wash buffer using the BioTek ELx50/8 automated plate washer. Plates were blotted with paper towel to remove residual wash buffer and after blotting, 100 µl of enzyme substrate solution was transferred into each well and again, plates were shaken for 1 minute and incubated for 30 minutes at room temperature. Following incubation, 50 µl of stop solution was added and mixed into the plates by gently pipetting into the wells with multichannel pipette and the Optical density (OD) was measured on an iMark Microplate reader (BioRad) at 450 nm. OD values/readings were transferred into QFT-PLUS Analysis software to calculate the IFN-γ concentrations.

The result of the quantiferon assay was considered positive if the background subtracted concentration of IFN-γ in the antigen tubes (TB1-Nil or TB2-Nil) for each participant was at least 0.35 IU/ml and the background subtracted concentration was at least 25% of the background concentration (Nil), and if the background concentration was below 8.0 IU/ml. The result was considered negative if the background subtracted concentrations of IFN-γ in the antigen tubes were either below 0.35 IU/ml or 25% of Nil tube, and the concentrations of the background subtracted Mitogen (Mit-Nil) tubes were at least 0.5 IU/ml.

2.3. Isolation of peripheral blood mononuclear cells (PBMC) from blood

PBMCs from participant blood samples were isolated by density gradient centrifugation using the Ficoll-Paque method. Briefly, blood was aseptically diluted with an equal volume of 1X PBS (SIGMA ALDRICH®) (1:1), gently mixed and 30 mL of diluted blood was layered on 15 ml Ficoll-Paque (GE Healthcare) in a 50 ml Falcon tube (NEST) to a final volume of 45 ml. Tubes were then centrifuged at 1000 xg for 20 minutes at room temperature in a Heraeus Megafuge

40/40R (Thermo Fisher Scientific) with deceleration set to zero and maximum acceleration. Some plasma was removed and discarded while the PBMC layer was gently collected into appropriately labelled 50 ml Falcon tubes and washed twice with RPMI-1640 (SIGMA ALDRICH®), and centrifuged at 400 xg for 10 minutes at room temperature. The cell pellet was resuspended in 2 ml RPMI 1640 and an equal volume of freezing medium (20% DMSO (SIGMA ALDRICH) in HYCLONE FBS (GE LIFE SCIENCES)) was added while gently shaking Falcon tubes. 1 ml of resuspended cells were immediately transferred into appropriately labelled cryogenic vials (CORNING) on ice, transferred into chilled CoolCell freezing containers and transferred to -80°C freezer overnight. Finally, the vials were transferred and cryopreserved in liquid nitrogen (LN) for long term storage the following day.

2.4. Flow cytometry: sample preparation and acquisition

2.4.1. Thawing of PBMCs

Cyropreserved vials (2 per participant) were removed from liquid nitrogen storage and thawed in a 37°C water bath and immediately added into appropriately labelled Falcon tubes containing 8 ml of R10 media (10% FBS, 1% Penicillin/ Streptomycin, 1% HEPES buffer and 50 mM 2-β-mercaptoethanol). Tubes were centrifuged at 400 xg for 10 minutes at room temperature and pellets were resuspended in 10 ml R10 media and incubated for 6 hours in a humidified incubator at 37° C with 5% CO_2. Following resting, samples were centrifuged at 500 xg for 5 minutes and the pellets resuspended in 1 ml modified R10 media (Penicillin/Streptomycin-deficient R10 media). Cell counts and viability were determined with trypan blue staining using a TC20 automated cell counter (BioRad®). Concentrations of cells/ml were determined, and cells were either diluted or concentrated to give a final concentration of 10 million cells/ml.

2.4.2 Stimulation of PBMCs

From final cell suspensions above, 100 µl of cells containing 1 million cells for each participant and for each stimulation condition were transferred into 96-well round-bottom plates and cells were stimulated with the following antigens: lipopolysaccharide (LPS) (100 ng/ml final concentration), Bacillus Calmette–Guérin (which is the attenuated Mycobacterium bovis) expressing green fluorescent protein (BCG-GFP) (BCG-GFP; 1x10^6 CFU/million cells [multiplicity of infection (MOI)=1] and MOI=5), (Heat-killed-Mtb) HK-Mtb (5x10^5 CFU/million cells, MOI=0.5), and phytohaemagglutinin (PHA) (4µg/mL). Modified R10 medium was used for the unstimulated condition (without any antigen) to serve as a background control. The total culture volume with cells and antigens was 200 µl.

Plates were then incubated for 18 hours in a humidified incubator at 37° C, with 5% CO_2. Following incubation, 100 µl of supernatant was collected and transferred to Sarstedt tubes and stored at -80° C and later used for soluble cytokine measurements. Cells were then re-stimulated with 100 µl re-stimulation cocktail containing respective antigens, anti-CD107a antibody, golgi blockers (golgi stop [Monensin] and golgi plug [Brefeldin A]) (BD Biosciences) and incubated for an additional 6 hours following which the plates were covered in aluminium foil and stored at 4° C in preparation for sample staining.

2.4.3. Phenotypic and functional characterisation of cells by flow cytometry

Flow cytometry is a multi-parameter, single cell assay that is used to measure and characterize different properties of cells such as cell size and internal complexity, it can also be used to analyse the expression of cell surface markers and the expression of intracellular molecules such as cytokines. Cellular properties such as size and internal complexity can be determined due to the ability of cells to scatter light while expression of molecules is quantified by the use of fluorochrome-conjugated antibodies which are specific to the cell molecule of interest.

Cells are then stained with these different antibodies and the stained samples are acquired on a flow cytometer during which, as samples pass flow through, they are activated by different laser beams which results in the excitation of the fluorochromes to higher energy levels. As these return to their ground state, they emit light which is detected by photodetectors and converted into a digital signal that can be analysed by flow cytometry software (BD FACSDiva software).

2.4.4 Staining for flow cytometry

Staining for flow cytometry was conducted in 5 ml FACS tubes (CORNING). Stimulated cells were transferred to FACS tubes, washed with 2 ml 1X PBS and centrifuged at 500 xg for 5 minutes. Supernatants were decanted, the cell pellets were stained with 50 µl of viability dye (diluted 1:100; Invitrogen) and incubated in the dark for 10 minutes at room temperature. Samples were then washed with 2 ml of FACS buffer (1X PBS containing 2% FBS) and centrifuged. Following viability staining, cells were stained for extracellular markers (Table 2.2) with 50 µl of extracellular antibody cocktail and incubated in the dark for 30 minutes at room temperature.

Following incubation, cells were washed with 2 ml of FACS buffer and centrifuged. Cells were then permeabilised with 100 µl Cytofix/Cytoperm (BD Biosciences®) and incubated in the dark for 10 minutes at room temperature and subsequently washed with 2 ml of 1X Permwash Buffer (BD Biosciences®) diluted in distilled water and centrifuged. Intracellular staining was conducted for the detection of cytokines and surface markers which may have been internalised during stimulation (Table 2.3) by adding 50 µl of intracellular antibody cocktail followed by incubation in the dark for 1 hour at room temperature. Finally, the cells were washed with 2 ml of 1X Permwash buffer, centrifuged and resuspended in 200 µl of 1% formaldehyde (reconstituted in 1X PBS from 16 % paraformaldehyde (Thermo Fisher®)), covered in aluminium foil and stored at 4° C until acquisition (within 48hours from staining completion).

Table 2.2: Extracellular markers used for staining cells

Marker	**Fluorochrome**	**Clone**	**Manufacturer**	**Amount per tube (µl)#**
CD4	BV686	SK3	BD Biosciences	2
Vα7.2	AF-647	3C10	Biolegend	0.5
CD40	PerCP-Cy5-5	5C3	Biolegend	0.5
CD161	BV605	HP-3G10	Biolegend	5

Table 2.3: Markers used for intracellular cytokine staining

Marker	**Fluorochrome**	**Clone**	**Manufacturer**	**Amount per tube (µl)**[#]
CD3	ECD	UCHT1	Beckman Coulter	1
CD8	APC-H7	SK1	BD Biosciences	2
CD14	PE-Cy7	M5E2	BD Biosciences	4
CD11c	BV650	B-ly6	BD Biosciences	1
HLA-DR	AF-700	L243	Biolegend	1
IL-2	PE	C11.5	Biolegend	0.5
IFN-γ	V450	B27	BD Biosciences	1

[#] These antibody volumes were determined from titration experiments described in section 2.9

2.4.5 Preparation of compensation tubes

Compensation tubes were prepared by labelling FACS tubes with respective fluochromes in the flow cytometry panel. Two drops of positive beads (Anti-mouse, Ig k from BD Biosciences®) were added into tubes followed by 2 µl of each antibody used in the panel and an unstained control with negative beads. Tubes were incubated for at least 15 minutes before washing by centrifugation at 500 xg for 5 minutes in 1ml Permwash Buffer. These were then resuspended in 200 µl 1% formaldehyde and stored at 4° C in aluminium foil until acquisition with the samples.

2.4.5 Flow cytometry acquisition

Acquisition of samples was performed on the BD LSR Fortessa flow cytometer (BD Biosciences®) using the FACS Diva software. Compensation was conducted for each of the fluorochromes in the panel by running compensation tubes and the unstained control (5000 events collected). Following acquisition of the compensation tubes and the successful calculation of compensation by software, samples were then acquired. A total of 500 000 events were acquired per sample.

2.5. Blocking experiments to assess the relative contribution of pathways towards MAIT cell activation

MAIT cells are distinct from conventional T cells in that their activation is independent of MHC-I or MHC-II molecules but rather they can be activated through MR-1 dependent or MR-1 independent (cytokine dependent) mechanisms (Ussher *et al.*, 2014). To assess the relative contributions of these, we conducted blocking experiments using anti-MR-1 (5 µg/ml) blocking antibody to block the MR-1 dependent MAIT cell activation, anti-IL-12 (5 µg/ml), anti-IL-18 (3 µg/ml) blocking antibodies to block cytokine-dependent MAIT cell activation.

PBMCs from 10 healthy controls (HIV-TB-) were thawed, rested and counted as per section 2.4.1 above. Before stimulation (as described in section 2.4.2) with BCG-GFP or media only, the cells were incubated for an hour at 37°C, in 5% CO_2, with 3 separate blocking antibody cocktails: either anti-MR1 only, combination of anti-IL-12 and anti-IL-18, or anti-MR1 with anti-IL-12 and anti-IL-18 together. Following incubation, cells were stimulated with BCG (MOI=1) according to section 2.4.2 and stained with a modified panel shown in Table 2.4. As controls, cells were left unstimulated (media only) or stimulated with BCG (MOI=1) without addition of blocking antibodies.

Table 2.4: Flow cytometry panel used for blocking experiments

Marker	**Fluorochrome**	**Clone**	**Manufacturer**	**Amount per tube (µl)**
CD107a	BV711	H4A3	Biolegend	0.5
Live/Dead Dye	BV510	Aqua fluorescent reactive dye	Invitrogen	0.5
Vα7.2	AF-647	3C10	Biolegend	0.5
CD161	BV605	HP-3G10	Biolegend	5
CD3	ECD	UCHT1	Beckman Coulter	1
CD4	BV686	SK3	BD Biosciences	2
CD8	APC-H7	SK1	BD Biosciences	2
IFN-γ	V450	B27	BD Biosciences	1

2.6. Identification of MAIT cells using MR1 tetramers loaded with 5-OP-RU

The identification of MR1 ligands has allowed for the alternative identification of MAIT cells through ligand-loaded MR1 tetramers. We aimed to use these tetramers to identify MAIT cells and compare with results obtained using monoclonal antibodies (CD161 and Vα7.2). MR1 tetramer loaded with 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU, a potent MAIT cell activating ligand) conjugated to Allophycocyanin (APC) fluorochrome, was used to identify and determine MAIT cell frequencies between the different groups. For this purpose, we selected 10 participants each from HIV-TB-, HIV+TB- and HIV-TB+ groups and 5 participants from the HIV+TB+ group.

PBMCs were thawed and rested for 2 hours, followed by staining for viability and washing. Following washing, 50 µl of 5-OP-RU-loaded MR1 tetramer or 6-FP loaded MR1 tetramer control (diluted 1:100) was added and incubated at 37°C for 30 minutes. Cells were then washed and stained for extracellular markers (Table 2.5). Following extracellular staining, cells were fixed with 1% formaldehyde and acquired. Samples were then acquired using the BD LSR Fortessa flow cytometer.

Table 2.5: Panel to identify MAIT cells using MR1 tetramer. PD1 was also included to compare expression of this marker between the groups of participants

Marker	Fluorochrome	Clone	Manufacturer	Amount per tube (µl)
Live/Dead Dye	BV510	Aqua fluorescent reactive dye	Invitrogen	0.5
MR1-5-OP-RU Or 6-FP control	APC	37006 hMR1	NIH	0.5
CD161	BV605	HP-3G10	Biolegend	5
CD3	ECD	UCHT1	Beckman Coulter	1
CD4	BV686	SK3	BD Biosciences	2
CD8	APC-H7	Sk1	BD Biosciences	2

PD1	BV711	EH12.2H7	Biolegend	1
CD14	PE-Cy7	M5E2	BD Biosciences	5

2.7. Soluble cytokine measurements

2.7.1. 6-Plex Luminex panel for cytokine measurements

The quantification of soluble cytokines produced during PBMC stimulation was performed using a 6-plex Luminex Milliplex map kit (EMD; Merck) for measuring the following cytokines: IFN-α2, IFN-γ, IL-12p40, IL-12p70, IL-10 and TNF-α.

2.7.1.1. Principle of Luminex assay

Luminex technology is a method to measure the concentrations of multiple analytes of interest within the same sample. This is done using colour-coded polystyrene or magnetic beads with pre-coated analyte-specific capture antibodies. Each individual bead is distinctly colour-coded with specific concentrations of 2 individual dyes and thus can be individually identified. Samples containing the analytes are mixed with the analyte-specific beads in order to capture the analytes onto the surface of the beads. These are then incubated. Biotinylated antibodies are then added to bind the beads containing analyte, followed by Streptavidin-PE conjugated reporter molecules that bind to the biotynlyated detection antibodies. The beads are then read on a Bioplex machine in which beads are identified and analyte concentration is determined from the intensity of the reporter molecule.

2.7.1.2 Reagent preparation

Culture supernatants collected after 18 hours of stimulation (section 2.4.2) were thawed and used (without dilution and run as single wells) for the assay. The kit and all content were equilibrated at room temperature for at least an hour prior to conducting the assay. To prepare the antibody bead mixture, we initially sonicated all analyte beads for 30 seconds after which the beads were vortexed for at least 1 minute, 60 µl of each of the antibody beads was added into the mixing bottle and made up to 3 ml with 2.64 ml of bead diluent. The suspension was vortexed again before use.

We then prepared quality control samples 1 and 2 separately by dissolving these with 250 µl deionized water, the vials were inverted several times and vortexed to adequately resuspend the samples. Human cytokine standard (HCS) was prepared by resuspending the standard in 250µl deionized water to make to a final volume of 10 000 pg/ml. The vial was also inverted several times and vortexed, following which it was allowed to stand for at least 10 minutes prior to preparation of working standards. A total of 6 working standards were prepared by labelling Eppendorf tubes with corresponding concentrations (2000 pg/ml, 400 pg/ml, 80 pg/ml, 16 pg/ml, 3.2 pg/ml and 0 pg/ml) and added 200 µl of assay buffer into each tube. Next, 50 µl of reconstituted HCS was added into the tube with 2000 pg/ml tube and serial dilutions were prepared by transferring 50 µl from this tube to the 400 pg/ml tube, and from this tube to the next tube until the 3.2 pg/ml tube. Assay buffer only (with no HCS) was used for the background (0 pg/ml) standard.

The assay was performed according to the manufacturer's instructions. Briefly, 200 µl of wash buffer was added into each well of the 96 well plate, the plate was then sealed and placed in a plate-shaker at 850 rpm for 10 minutes at room temperature to wet the surface of the wells. Wash buffer was then discarded, and the plate was blotted with paper towel to remove residual wash buffer. 25 µl of quality control samples and working standards were added into respective wells on the plate followed by an equal volume (25 µl) of R10 media into these wells. 25 µl of assay buffer followed by 25 µl of undiluted supernatants were added into all the sample wells. After this step, the mixed beads were then vortexed and 25 µl of beads were transferred into each well with intermittent shaking of beads between additions. The plates were then covered with plate sealers and foil and incubated on a plate shaker at 850 rpm for 2 hours at room temperature.

Following incubation, plates were washed twice using the Bioplex wash station (Biorad®) with 1X washing buffer and after washing the plates, 25 µl of detection antibodies were added into each well followed by another incubation of the plates for 1 hour on a plate shaker. This was then followed by the addition of 25 µl of Streptavidin-PE into the wells and incubation of plates for 30 minutes. Finally, the plates were then washed twice with 1X wash buffer and 150 µl sheath fluid was added into plates followed by data acquisition on a Bioplex 200 system (Biorad®) using Bioplex manager software.

2.7.2 IL-18 ELISA asssay

Soluble IL-18 was also measured in the supernatants. The IL-18 was measured using the Human IL-18/IL-1FA Quantikine ELISA Assay (R&D Systems®).

2.7.2.1. Principle of ELISA assay

The Enzyme-Linked Immunosorbent Assay (ELISA) is a method for determining the concentration of an analyte of interest through the use of antibodies. For our ELISA experiments, we used a sandwich ELISA immunoassay which is a type of ELISA assay which measures the concentration of an analyte between 2 layers of antibodies. In this assay, the sample containing analyte is added into a 96-well plate that has been pre-coated with analyte-specific capture antibodies which function to immobilize the specific analyte in the sample to the surface of the 96-well plate. Biotinylated detection antibodies specific to the single analyte of interest are then added to bind to analyte, thus forming a sandwich of capture antibodies, analyte and detection antibodies. Enzyme linked secondary antibodies that bind to these detection antibodies are then added into the plates followed by a solution of enzyme substrate. The substrate is converted by the enzyme, leading to development of a coloured solution which is proportional to the concentration of the analyte in the well. An acid solution is then added to the plate to stop this enzymatic reaction, the colour development stops and the resultant colour is measured to determine the concentrations of cytokines in the wells.

2.7.2.2. ELISA assay

This protocol was carried out as per the manufacturer's instructions. Briefly, all reagents and samples were allowed to equilibrate to room temperature for an hour prior to use. Wash buffer was prepared by diluting 20 ml of wash buffer concentrate with 480 ml of deionised water. Next, a 50 ml solution of Calibrator Diluent RD5P was prepared by adding 10 ml of

Calibrator Diluent into 40 ml deionised water, making a 1:5 dilution. Human IL-18 standards were prepared by firstly resuspending the 10 000 pg/ml standard with 450 µl of the diluted Calibrator Diluent RD5P (1:5) and vortexed for at least 1 minute and allowed to stand for at least 15 minutes before use in the assay. To prepare working standards, 200 µl of calibrator diluent was added into 7 correspondingly labelled tubes (1000, 500, 250, 125, 62.5, 31.3, 15.6 pg/ml). 200 µl RD5P only was used as the 0 pg/ml standard. 50 µl of the 10 000 pg/ml standard was transferred into the 1000 pg/ml standard, serial dilutions were then prepared by transferring 200 µl from 1000 pg/ml to the 500 pg/ml tube and this process was repeated until the last tube labelled 15.6 pg/ml.

Into the 96-well plates, 50 µl of assay diluent RD1-43 was then transferred into each well of the plate using a multi-channel pipette. This was followed by addition of 50 µl of standards and samples into respective wells. The plates were covered with adhesive plate sealers and incubated at room temperature for 2 hours on a horizontal plate shaker at 850 rpm. The plates were then washed 4 times on a BioTek ELx50/8 automated plate washer with 400 µl of wash buffer. Plates were then blotted using paper towels to remove residual assay diluent and unbound sample. Following washing, 200 µl of Human Total IL-18 conjugate was added into each of the wells of the plates and plates were again covered with adhesive plate sealers and incubated at room temperature for 1 hour on a plate shaker. Plates were again washed, following which, 200 µl of Streptavidin-HRP was added into each well and the plates incubated at room temperature for 30 minutes on a plate shaker. Plates were washed again 200 µl of substrate solution was added, and plates were sealed with adhesive plate sealers and foil and incubated at room temperature for 20 minutes. Finally, 50 µl of stop solution was added into each well and plates were read at 450 nm using the iMark Microplate reader (Biorad).

2.8. Data analysis

For our experiments, we allocated a similar number of study participants from all 4 different groups in each Flow cytometry run and in each Luminex and ELISA plate. This was done in order to minise technical biases in the flow cytometry experiments and the soluble cytokine measurements.

After running each Luminex and ELISA plate, the standard curve for the particular plate was immediately generated using Bioplex Manager Software version 6.1 and Microplate Manager Software version 6.3 for Luminex and ELISA plates, respectively. These generated final concentrations for each individual plate, and these concentrations were then exported in excel format and analysed for final results.

2.8.1. Flow cytometry data

Flow cytometry data were analysed using FlowJo version 9.9.2 (FlowJo LLC). Frequencies and or median fluorescent intensity (MFI) were used for all analyses. Background values were subtracted from stimulated values for all frequencies of cells expressing IFN-γ, IL-12p40 or CD107a.

2.8.2. Soluble cytokine measurements

A total of 5 plates were run for soluble cytokine measurements with both Luminex and ELISA. Cytokine concentrations below the lower limit of detection (OOR<) were assigned the average of the lowest extrapolated value measured for each analyte between the 5 plates. A similar analysis was done for cytokine concentrations above the upper limit of detection (OOR>) where an average of the highest extrapolated values measured for each analyte in the 5 plates was used. For ELISAs (because the output is different from luminex), the OOR values were either assigned half the lower value of the standard or the highest value of the standard.

2.8.3 Power considerations

Based on previous studies, a minimum sample size of 25 participants per group (in the 4 patient groups) was calculated and would be adequately powered (80%) to show significant differences at $p<0.05$ with an 8% difference in MAIT cell IFN-γ responses (ie. change from 25% to 17% with standard deviation of 10%).

2.8.4 Statistical analyses

Statistical analyses were conducted using Graphpad Prism version 7. The non-parametric paired Wilcoxon test was used for the statistical comparisons of paired datasets while the non-parametric Mann-Whitney test was used for comparison of unpaired datasets. The non-parametric Spearman rank correlation was used for the assessment of correlation. Data analysed was untransformed. Significant p values were regarded as values less than or equal to 0.05 ($p \leq 0.05$).

2.9. Optimisation of flow cytometry experiments

Before finalising the details of the experiments and antibody volumes described above, several optimisation steps were followed including titrations of antibodies and MR1 tetramers, fluorescent-minus-one (FMO) experiments, optimal voltages for the panel (application setting), testing reagents for endotoxin contamination, determining the optimal concentration of BCG-GFP to use for stimulation and determining the kinetics of IL-12 production for optimal MAIT cell activation.

It is important to mention that for the optimisation of the flow cytometry panel, certain experimental parameters differed from the final experimental setup. These include:

1) Duration of stimulation; during optimisation, PBMCs were stimulated for 6 (with golgi inhibitors) or 8 (4 hours without golgi inhibitors and 4 hours with golgi inhibitors) hours in sections 2.9.1- 2.9.4. This was then changed to 18 hours without golgi inihbitors with a further 6 hours with golgi-inhibitors, after the assessment of soluble IL-12 kinetics and the 18-hour stimulation was used in the final experimental setup as mentioned in section 2.4.2 above.
2) Concentrations of LPS and PHA used during optimisation were 50 ng/ml and 2 μg/ml, these concentrations were increased to 100 ng/ml and 4 μg/ml for the final experimental setup so as to increase antigen responses.
3) TNF-α (TNF-α-PE-Cy7, clone: MAb11) was only used for the optimisation experiments for the assessment of cellular responses and it was excluded from the final flow cytometry panel. Although TNF-α would have been a arker for T cell and APC responses (and indirect marker for APC activation),we opted to use IFN-γ and CD107a for T ell responses and CD40 as a direct marker for APC activation.

2.9.1. Antibody titrations

Antibody titrations were performed for all new antibodies that were being added into the flow cytometry panel. The purpose of these titrations was to obtain the optimal concentrations (saturation titres) of antibodies to be used to stain samples. The optimal titre is considered as the titre which yields the best separation between positive and negative cell populations with minimal non-specific antibody binding (Hulspas, 2010). The flow cytometry panel used in the study was adapted from another T cell panel currently being used in our research group. Several MAIT and innate cell specific antibodies were added, and antibody titrations were done for these antibodies including CD161-BV605, Vα7.2-AF647, IL-12-PE, CD11c-BV650 and CD40-PerCP-Cy-5-5 antibodies.

For CD161, the volume was 5 µl while for Vα7.2, 0.5 µl and 1µl had similar indexes (Fig. A1, appendix) but 0.5 µl was selected because the volume yielded optimal separation (staining index) at minimal antibody concentrations.

Upon completion of antibody titrations, optimal voltages for each panel were determined (as described in the appendix) and the application setting for the panel was developed and used throughout the study with daily quality control of the Fortessa used for acquisition.

2.9.2. The effect of cell culture media on background cellular responses

Different Fetal Bovine Serum (FBS) and cell culture media may introduce variability into results due to endotoxin levels. The presence of endotoxin may lead to background activation of innate cells and thus, erroneous results. We therefore wanted to test different FBS lots available in our lab from different manufacturers in order to choose the FBS lot with minimal endotoxin level inducing minimal background responses for use in our experiments.

To do this, two R10 media were prepared; 1 with FBS from Biochrome (Catalague number: S 0615) and the other with FBS from Hyclone (Catalogue number: SV30160). PBMCs were thawed from 5 donors and split equally between the 2 media. Cells were then stimulated in the respective media in 96-well round bottom plates with LPS (50 ng/ml) and PHA (2µg/ml), media without antigen was used as a background control (unstimulated condition). Plates were incubated for 6 hours at 37°C at 5% CO_2. Samples were then stained and acquired using the BD LSR Fortessa and analysed using FlowJo.

From these results (Fig. A2, appendix), we decided to use Hyclone FBS because it resulted in lower background responses. This was important because we wanted to minimise non-specific APC activation as much as possible because monocytes activated by media alone would activate MAIT cells and this would lead to incorrect observations and conclusions. Secondly, with Hyclone FBS, there were higher antigen-specific responses on APCs in response to LPS stimulation. This was important because it meant that this FBS (Hyclone) was, for our purposes, superior because there were lower interferences, and antigen specific responses were much more evident compared to Biochrome FBS which may have contained components which potentially inhibited stimulation of APCs.

Blood collection tubes were tested for endotoxin contamination using the Limulus amebocyte lysate (LAL) assay. In order to conduct the assay, 2 blood collection tubes were randomly selected from each batch of tubes and 1 ml of endotoxin free water (Lonza) and these were placed on a rotator for 10 minutes at room temperature, this is done to adequately mix water into tubes. The tubes were then incubated at 37°C for 2 hours following which, the LAL assay was conducted according to the manufacturer's instructions (Hycult® Biotech). This is routinely done in our laboratory for all blood collection tubes and if tubes from any pack had endotoxin levels above 0.12 EU/ml all tubes in that pack are discarded.

2.9.3. BCG titration

In order study the relationships between APC infection and MAIT cell responses, we used BCG expressing the green fluorescent protein (GFP) (BCG-GFP) so as to assess the levels of infection in APCs, their responses to stimulation and their ability to activate MAIT cells. We used 3 donors to determine the optimal concentrations/MOI of BCG to use by preparing a series of 6 2-fold serial dilutions of BCG in R10 media.

For these experiments, both the MOI=1 and MOI=5 were selected to be used in the full panel as MOI=1 yielded the greatest IFN-γ and TNF-α expression by MAIT cells and MOI=5 resulted in highest levels of monocyte infection and function (Fig. A3 and A4).

2.9.4. Assessment of kinetics of IL-12

In addition to MR-1 dependent activation, MAIT cells can be activated through an IL-12 and IL-18 dependent mechanism. In order to measure the cytokine mediated MAIT cell activation, we investigated the kinetics of IL-12 production to determine the time point at which IL-12 was optimally produced by innate cells which would represent the optimal period it takes to get sufficient MAIT cell activation through the pathway. PBMCs from 5 donors were stimulated with LPS, BCG (MOI=1 & MOI=5) and PHA and split into 5 plates (for 5 time points: 12, 14, 16, 18, 20 hrs). At each time point (representing hours of stimulation), supernatants were collected into Sarstedt tubes and stored at -80°C for IL-12 quantification by ELISA.

From these experiments, we then chose the 18-hour time-point as the optimal duration of stimulation because the concentrations of soluble IL-12 and the frequencies of CD107a, IFN-γ and HLA-DR increased over time, with the higher concentrations of IL-12 being between 18 and 20 hours and the frequencies of MAIT cells being higher after 18 hours of stimulation (Fig. A5).

2.9.5 Fluorescence minus one (FMO) controls

The use of FMO controls is essential in the development of a polychromatic flow cytometry panel. This is because an FMO control reveals the level of fluorescence spillover into a specific channel as a result of the spectral overlap between different fluorochromes (Maecker & Trotter, 2006). During an FMO experiment, the FMO control is prepared through staining a sample with all the antibodies in the panel except the one being assessed. This gives an indication of the contribution of other fluorochromes into the signal of the unstained FMO control.

For our FMO experiments, we stimulated PBMCs for 18 hours with LPS and PHA, removed supernatants and added re-stimulation cocktail and incubated for an additional 6 hours. From these samples, we prepared FMO controls for CD161, Vα7.2, CD8, CD4, CD11c, CD107a, CD40, IFN-γ and IL-12.

In summary, we found that there was minimal spectral overlap in each of our FMO controls, with the overlap ranging between 0-0.24 in every FMO control except for the IL-12 FMO (Table 2.6). In IL-12 FMO (PE FMO), the frequencies of IL-12 were similar to frequencies of IL-12 in other FMOs which was unusual, however, this could have been due to the fact that the frequencies of APCs expressing IL-12 were generally low, the frequencies of IL-12 were also much lower in the CD14 FMO, possibly due to the absence of a huge proportion of APCs as identified with CD14. Because the frequencies of IL-12 in the IL-12 FMO were similar to frequencies in other channels, IL-12 was excluded from further analyses. We also found that in each FMO control channel, the cell/cytokine of the particular FMO was greatly lower and there was minimal variation in the frequencies of all of the other cells/cytokines in the FMO. Because of this minimal overlap, we concluded that our flow cytometry panel was reliable for the assessment of cells and cytokines. No FMO controls were prepared for HLA-DR, CD3 and GFP and the frequencies of these markers were similar in all the different channels with minor fluctuations for GFP and HLA-DR (Table A1).

2.9.6. Blocking antibody titrations

The optimal concentration of MAIT cell activation blocking antibodies were determined through the titration of blocking antibodies using PBMCs from 2 donors and these were obtained from the WPBTS. Two-fold serial dilutions were used for all 3 blocking antibodies in order to determine the optimal concentrations for blocking. Blocking antibodies were added into plates containing PBMCs, the plates were incubated for 1 hour, stimulation with BCG (MOI=1) was carried out as described in section 2.4.2.

From these experiments, we chose to use concentrations of 5 µg/ml for anti-MR1 and anti-IL-12 and 2 µg/ml for anti-IL-18. These are indicated by the red boxes in Fig. A7.

2.9.7. Tetramer titration

For the titration of the MR-1 loaded tetramer, we initially sought to determine the optimal concentration of tetramer to use and the optimal temperature at which to stain cells. To prepare the tetramer titers, we prepared a series of 8 2-fold serial dilutions for MR-1 tetramers**.** For this, we used PBMCs from 2 donors from WPBTS buffy coats.

The summary plots show that the staining index of the MR1 tetramer was highest at higher concentrations of tetramer and significantly decreased from 100X dilution to 4000X. The red square in the chart represents the concentrations at which the staining index were highest for all the donors and at the 2 temperatures. The 80-fold dilution, highlighted by the red square, was selected for tetramer staining experiments (Fig. A8). The chart also shows the 2 different temperatures at which the cells were stained with tetramers. It was observed that staining indexes were higher at 37 °C for both donors and as such, cells were stained with tetramers at 37 °C during staining experiments.

2.9.8. Contribution towards project

The flow cytometry panel that we used for the study was adapted from a previous panel that had been used for a different study. We added extra antibodies in order to conduct our study. I optimised this flow cytometry panel by conducting the antibody titration experiments, FBS testing experiments, determination of IL-12 kinetics, the culturing and determination of the optimal MOI of BCG to use for our experiments, conducting the FMO experiments and determing the application settings which were used for our flow cytometry acquisition.

My role in the project also included the receipt and processing of all participant blood samples, the isolation of PBMC samples with the assistance of my supervisor (Dr. Muki Shey) and the stimulation of blood samples for QFT-TB Gold Plus ELISA assay. I was responsible for stimulating, staining, acquiring PBMC samples and the analysis of the samples. I conducted the Luminex assays, IL-18 ELISA assay and the QFT-ELISA assays. Following completion of primary flow cytometry panel, I conducted the titration experiments for the blocking antibodies which were used in the study. My supervisor, Dr. Muki Shey, conducted the optimisation experiments for MR1 tetramer in which he carried out titration of the MR1 tetramer to determine the optimal concentrations and incubation conditions to use in staining experiments, in addition to this, he also conducted the tetramer experiments on participant PBMCs, these experiments were aimed at validating the frequencies of MAIT cells which were determined using Vα7.2 antibody.

I conducted the flow cytometry analyses for these experiments and 1) determined the optimal concentrations and temperature to use for the staining experiments on participant PBMC samples and 2) conducted group comparisons using the MR1 tetramer stained MAIT cells. With the guidance and assistance of my supervisors (Dr. Muki Shey and Prof. Graeme Meintjes) I have been responsible for the analysis, interpretation and the write up of all the results from flow cytometry experiments, soluble cytokine assays, blocking experiments and the MR1 tetramer staining experiments. In addition to these contributions, I designed the images which were used for mechanisms of MAIT cell activation in Fig. 1.6 in Chapter 1.

Chapter 3: Results

3.1 Clinical description of study participants

We recruited a total of 112 participants for the study, 26 healthy controls (ie. HIV negative and without active TB), 30 individuals with HIV only, 30 with active TB only (ie. HIV negative) and 26 with both HIV and active TB (HIV-associated TB). The median age for healthy controls was 33 years of age, individuals with HIV only had a median age of 37, in individuals with active TB only the median age was 33 and, finally, in individuals with HIV-associated TB, the median age was 38. 92 % of healthy controls had a positive QFT results (classified as a QFT result above 0.35 IU/ml and above 25% of the QFT result for the unstimulated (Nil) control) while 79% individuals with HIV only had a positive QFT result.

In the groups with HIV only and with HIV-associated TB, the median CD4 counts were 501 and 228 cells/µL, and their median HIV viral loads were 1673 and 66509 copies/ml, respectively. In the group of individuals with HIV only, 11 individuals had HIV viral loads below the detectable limits (LDL) and only 7 had HIV viral loads below detectable limits in individuals with HIV-associated TB. Furthermore, 63% of individuals with HIV only were on anti-retroviral therapy (ART) and in the group with HIV-associated, 69% were on ART. A summary of the clinical characteristics of the participant groups can be found in Table 3.1 below**.**

Table 3.1: Demographic and clinical characteristics of study participants

	Healthy controls (n=26)	**HIV only (n=30)**	**Active TB only (n=30)**	**HIV with active TB (n=26)**
Females	17 (65.4%)	23 (76.7%)	5 (16.7%)	14 (48.3%)
Age	33 (25-40)	37 (29-43)	33.5 (23-44.25)	38 (30.25-42.75)
QFT+	23 (92%)	19 (79.2%)	ND	ND
QFT –	2 (8%)	5 (20.8%)	ND	ND
CD4 count (cells/µL)	ND	501 (368-647)	ND	228 (99-323)
Viral load (copies/ml)	ND	1673 (67-30382) LDL=11 (36.7%)	ND	66509 (2313-146981) LDL=7 (26.9%)

ND = Not done

LDL = Lower than detectable limit

Median (interquartile range) or number (percentage) are shown

3.2 Association of TB, HIV and HIV-associated TB with cellular frequencies

The first step was to examine the frequencies and or numbers of the various immune cell populations and determine if TB, HIV or HIV associated TB had any effect on these frequencies To do this, we sought to initially determine the frequencies of MAIT cells, non-MAIT T cells (CD4 and CD8 T cells) and APCs (monocytes and dendritic cells) in healthy controls and then investigated how these were affected by HIV, TB disease and HIV-associated TB. The gating strategy for analysis of the flow cytometry results is shown in Figure 3.1 below.

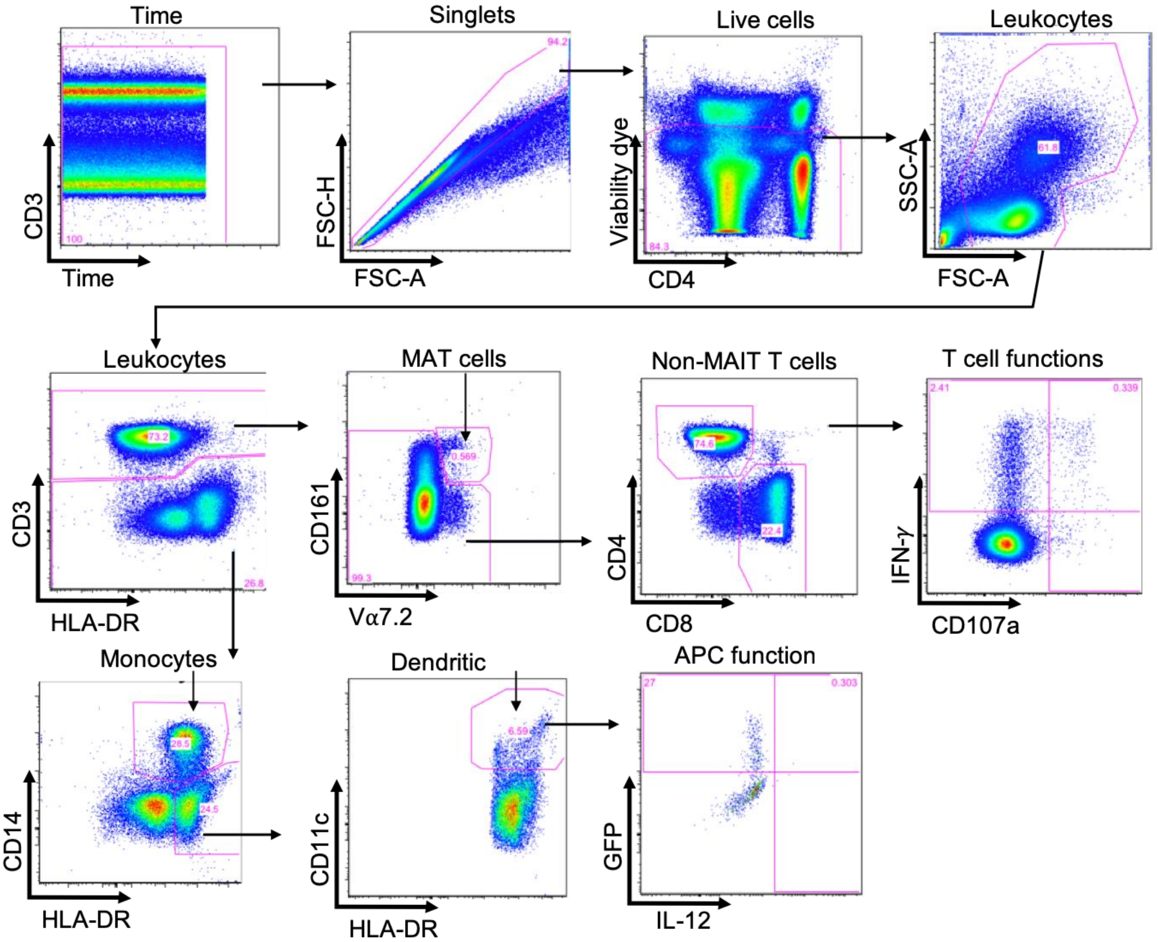


Figure 3.1: Flow plots depicting the gating strategy that was used for the analysis of the flow cytometry panel. Initial gating was done on the time gate to ensure uniform acquisition of samples over time. From this gate, we gated for singlet cells only using FSC-A vs FSC-H gate. We then identified live cells in viability dye vs CD4 gating with dead cells excluded as they stained positive for the viability dye. Leukocytes were identified by gating for FSC-A vs SSC-A. From the leukocytes, we identified T cells by gating for CD3 vs HLA-DR, T cells were identified as all CD3+ cells. From the total T cells, we

identified MAIT cells by gating for CD161 vs Vα7.2and MAIT cells were identified as CD161+ Vα7.2+ cells. From the non-MAIT T cells, we gated on CD4 vs CD8 to identify CD4 and CD8 T cells, respectively. From the CD3- HLA-DR+ cells, we identified monocytes by gating for CD14 vs HLA-DR and monocytes identified as CD14+ HLA-DR+ cells. Dendritic cells were identified from the CD14- HLA-DR+ compartment as CD11c+. We further assessed T cell functions and activation by the expression of CD107a, IFN-γ and HLA-DR and assessed APC infection by assessing the frequencies of GFP+ APCs.

3.2.1. MAIT cell frequencies and numbers

MAIT cells were identified by the co-expression of CD161 and Vα7.2. and were gated from total CD3+ T cells as shown in the representative flow cytometry plots in Figure 3.2A.

When the frequencies of MAIT cells were compared between healthy controls and the different groups, Mann-Whitney U test showed that the frequencies of MAIT cells were significantly reduced in individuals with HIV only (p=0.0440), compared to healthy controls. An even greater reduction in MAIT cell frequencies was observed in individuals with active TB only (p=0.0232) compared to health controls (Figure 3.2B). Interestingly, MAIT cell frequencies in individuals with both HIV and active TB were similar to healthy controls.

Examination of the cell counts revealed trends in lower numbers of MAIT cells in all the groups compared to healthy controls, although the results were only significant in group with active TB only (p=0.0184) (Figure 3.2).

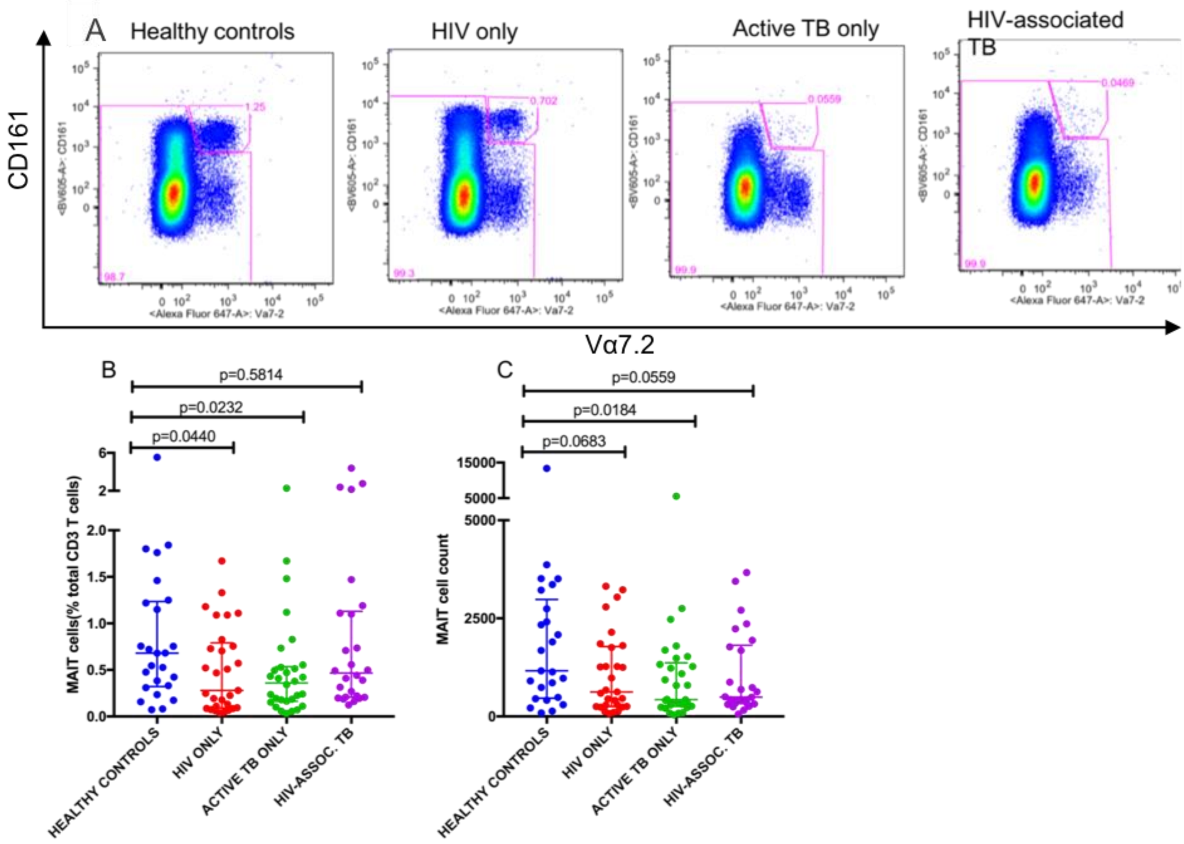


Figure 3.2: Frequencies of MAIT cells and the association of HIV, active TB and HIV associated TB with the frequencies of peripheral blood MAIT cells. A) Representative flow cytometry plots showing the identification of MAIT cells. B) Frequencies of MAIT cells in healthy controls, individuals with HIV only, active TB only and HIV-associated TB. C) MAIT cell counts in healthy controls, individuals with HIV only, active TB only and HIV-associated TB. Data presented as scatter plots with lines representing the median and IQR. HIV-Assoc. TB = HIV Associated Tuberculosis. Blue circles represent healthy controls, red=HIV only, green-active TB, purple=HIV-associated TB.

3.2.2 CD4 T cell frequencies and numbers

In comparison to healthy controls, Mann-Whitney U test revealed that the frequencies of CD4+ T cells were significantly reduced in individuals with HIV only ($p<0.0001$) and individuals with HIV-associated TB ($p<0.0001$) (Figure 3.3A). This same pattern was observed in the numbers of CD4+ T cells in which the numbers were reduced in individuals with HIV only ($p=0.0004$) and individuals with HIV-associated TB ($p<0.0001$) (Figure 3.3B).

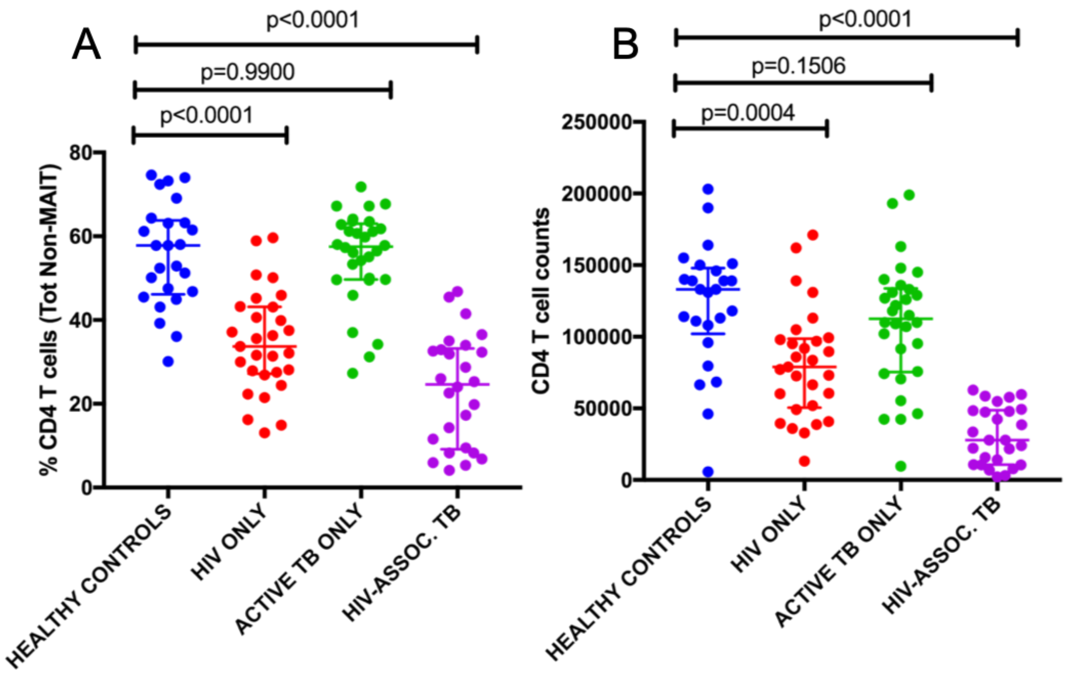


Figure 3.3: Frequencies of CD4 T cells and the association of HIV, active TB and HIV associated TB with the frequencies of CD4 T cells. A) Frequencies of CD4 T cells in healthy controls, individuals with HIV only, active TB only and HIV-associated TB. B) CD4 T cell counts in Healthy controls, individuals with HIV only, active TB only and in both HIV-associated TB. Data presented as scatter plots with lines representing the median and IQR.

3.2.3. CD8 T cell frequencies and numbers

Using the Mann-Whitney U test, we observed that the frequencies of CD8 T cells were significantly higher in individuals with HIV only (p<0.0001) and in individuals with HIV-associated TB (P<0.0001) compared to healthy controls (Figure 3.4A) and the numbers of CD8+ T cells were higher only in individuals with HIV only (p<0.0001) compared to healthy controls (Figure 3.4B).

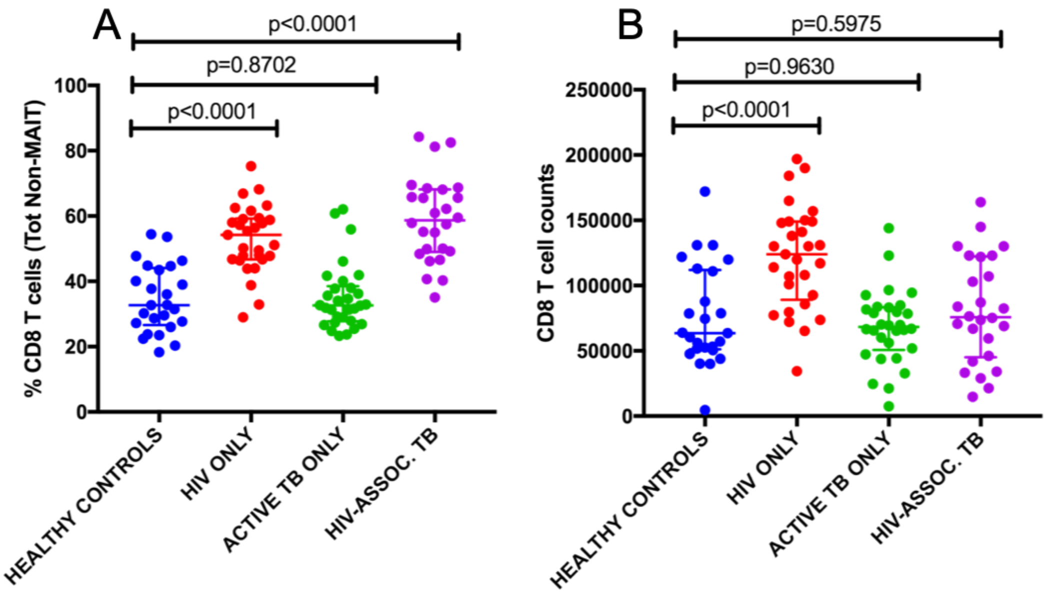


Figure 3.4: Frequencies of CD8 T cells and the association of HIV, active TB and HIV associated TB with the frequencies of CD8 T cells. A) Frequencies of CD8 T cells in healthy controls, individuals with HIV only, active TB only and HIV-associated TB. B) CD8 T cell counts in Healthy controls, individuals with HIV only, active TB only and in HIV-associated TB. Data presented as scatter plots with lines representing the median and IQR.

3.2.4 Monocyte frequencies and numbers

The Mann-Whitney U test revealed that the frequencies of CD14+ monocytes were higher in the individuals with active TB only (p=0.0001) compared to healthy controls while the individuals with HIV only and individuals with HIV-associated TB had similar frequencies to healthy controls (Figure 3.5A). The numbers of monocytes were also significantly higher in individuals with active TB only (p=0.0004) compared to healthy controls (Figure 3.5B).

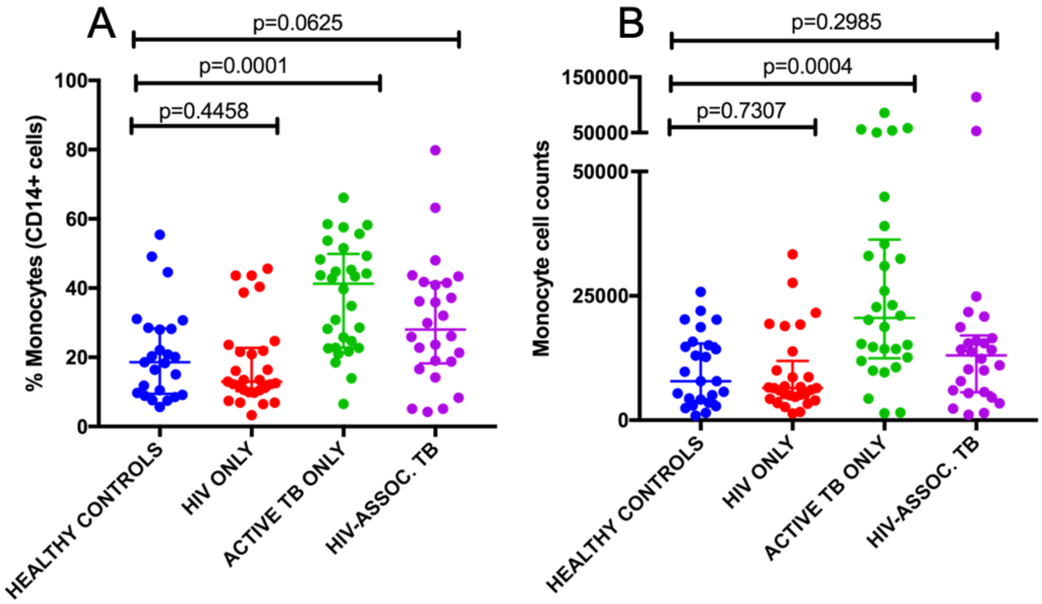


Figure 3.5: Frequencies of monocytes and the association of HIV, active TB and HIV associated TB with the frequencies of monocytes. A) Frequencies of monocytes in healthy controls, individuals with HIV only, active TB only and HIV-associated TB. B) Monocyte cell counts in healthy controls, individuals with HIV only, active TB only and in both HIV-associated TB. Data presented as scatter plots with lines representing the median and IQR.

3.2.5 Dendritic cell frequencies and numbers

In DCs, no significant differences were observed between the frequencies or numbers of CD11c+ DCs between the groups and healthy controls (Figure 3.6).

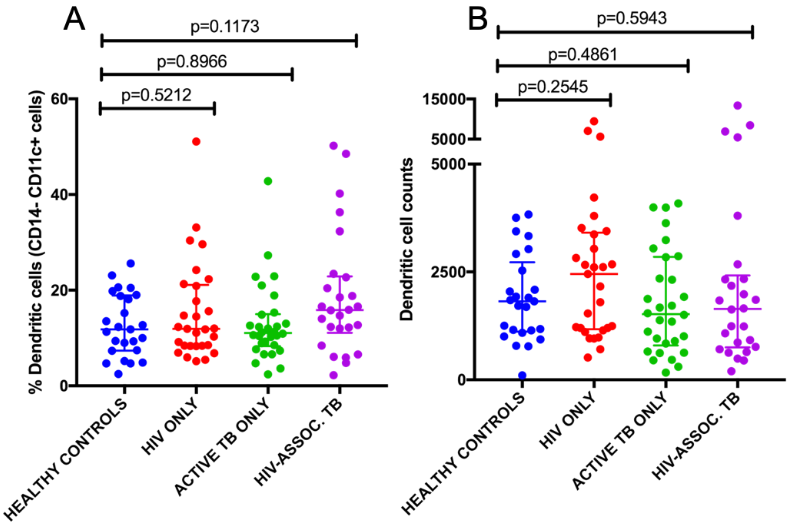


Figure 3.6: Frequencies of dendritic cells and the association of HIV, active TB and HIV associated TB with the frequencies of dendritic cells. A) Frequencies of dendritic cells in healthy controls, individuals with HIV only, active TB only and HIV-associated TB. B) Cell counts for dendritic cells in healthy controls, individuals with HIV only, active TB only and in both HIV-associated TB. Data presented as scatter plots with lines representing the median and IQR.

3.2.6 Association between Latent TB Infection (LTBI) and MAIT cell frequencies

LTBI was assessed in individuals without active TB using the QFT-Plus assay and the frequencies of MAIT cells were compared between participants with LTBI and participants without LTBI, the assessment was done in the healthy controls and the individuals with HIV only. There were similar frequencies of MAIT cells between individuals with LTBI and those without LTBI, in each group, however only 2 individuals were QFT- in healthy controls (Figure A9). Because most of the study participants were latently infected with only 2 participants that were QFT-, it is diffifucult to ascertain the effect of LTBI on MAIT cell frequencies based on these data.

3.2.7 Effect of CD4 Counts and HIV Viral load

In individuals with HIV infection, measurements of CD4 counts have been important for decision making because CD4 count measurements are considered good predictors of death, risk of opportunistic infections and disease progression (Ford *et al.*, 2017; Scorgie *et al.*, 2019; Siberry *et al*., 2014). Measurements of CD4 counts are also considered a great method to assess the immune status of infected persons (Ford *et al*., 2017), this is because HIV leads to the destruction of CD4 T cells and this is coupled with the deterioration/dysfunction of the immune system (Immune responses and possible causes of CD4 T cell depletion (Mishra *et al*., 2009).

In the 2 groups of individuals with HIV, namely; individuals with HIV only and individuals with HIV-associated TB, we assessed the effects of CD4 count and HIV viral loads on the frequencies and numbers of MAIT cells. For CD4 counts, we divided participants into 3 groups; those with CD4 counts below 200 cells/µl, those with CD4 counts between 200-350 cells/µl and finally, those with CD4 counts above 350 cells/µl. These were chosen from the previous WHO recommendation and were divided into the different categories because it is known that decreasing CD4 counts are associated with weakening immune system and progression to AIDS (Post *et al*., 2012). To assess the effects of HIV viral load on the frequencies and numbers of MAIT cells, we divided HIV viral loads from participants from the 2 HIV groups (HIV only and HIV-associated TB) into participants with HIV viral loads below 1000 copies/ml and participants with HIV viral loads above 1000 copies/ml.

In individuals with HIV only, only one individual had a CD4 count below 200 cells/µl, and the frequencies and numbers of MAIT cells were similar between the 3 CD4 count categories. There were also no differences in the frequencies of MAIT cells between participants with HIV viral loads below 1000 copies/ml and above 1000 copies/ml. In individuals with HIV-associated TB, the frequencies of MAIT cells were significantly higher in individuals with CD4 counts below 200 cells/µl compared to individuals with CD4 counts above 200 cells/µl (p=0.0206) (Figure 3.7A). There were no significant differences in the frequencies of MAIT cells in between individuals with CD4 counts below 200 cells/µl and those with CD4 counts above 350 cells/µl. In this same group, the frequencies and numbers of MAIT cells were significantly elevated in individuals with HIV viral loads above 1000 copies/ml (MAIT cell frequencies [p=0.0214], MAIT cell numbers [p=0.0183]) (Figure 3.7B). No significant differences were observed in the frequencies of MAIT cells between individuals with HIV only and those with HIV-associated TB in the different CD4 count categories and VL categories.

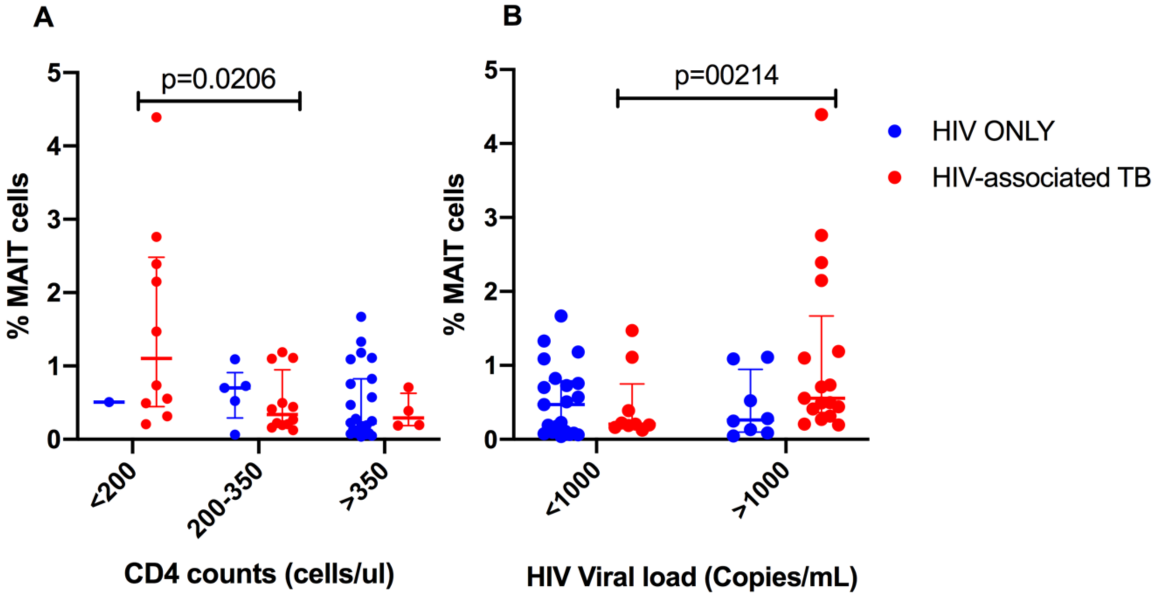


Figure 3.7: The association of CD4 counts and HIV viral loads with the frequencies and numbers of MAIT cells in people with HIV infection (HIV only and HIV-associated TB). A) Shows the frequencies of MAIT cells within the different CD4 count categories (cells/µL). **B)** Frequencies of MAIT cells in individuals with HIV viral loads above and those below 1000 copies/ml. Data presented as scatter plots with lines representing the median and IQR. Blue circles represent individuals with HIV only and red circles, HIV-associated TB.

3.2.8 Correlations between CD4 count, HIV VL and MAIT cell frequencies

3.2.8.1 Correlations between CD4 counts and MAIT cell frequencies

In addition to investigating the effects of different CD4 counts and HIV viral loads on MAIT cell frequencies, we also sought to investigate the relationships between CD4 counts/HIV viral loads and the frequencies and numbers of MAIT cells.

In individuals with HIV only, there were no significant correlations between the CD4 counts and the frequencies and numbers of MAIT cells. In individuals with HIV-associated TB, using the Spearman correlation, we found that there were significant negative correlations between the CD4 count and the frequencies and numbers of MAIT cells (MAIT cell frequencies [p=0.0023] and MAIT cell numbers [p=0.04038]) (Figure A11). There were no significant correlations between the HIV viral loads and the frequencies and numbers of MAIT cells in both HIV groups (Figure A12).

3.2.9 Effect of Anti-Retroviral Treatment (ART) on MAIT cell frequencies

In addition to assessing the impact of the CD4 counts and the HIV viral loads, we assessed the impact of ART on MAIT cell frequencies in the 2 HIV groups (HIV only and HIV-associated TB). To do this, we separated participants from each group into those that were on ART and those who were not on ART. In each group, the frequencies of MAIT cells were similar between participants that were on ART and those not on ART (Figure A10).

3.2.10 Effect of stimulation on MAIT cell frequencies

We next investigated the effects of bacterial stimulation on MAIT cell frequencies. MAIT cell frequencies were compared between unstimulated controls, BCG with MOI of 1 (BCG 1) BCG MOI of 5 (BCG 5) and HK-*M.tb* stimulated samples for each participant, from each group. Figure 3.8 below shows that the stimulation with BCG 1 had no effect on MAIT cell frequencies except in individuals with HIV only where the frequencies of MAIT cells were significantly reduced upon stimulation with BCG 1 (p=0.0214) (Figure 3.8B). For all study groups, stimulation of cells with BCG 5 resulted in significantly higher frequencies of MAIT cells (p<0.0001) (Figure 3.8A-D). The frequencies of MAIT cells with HK-*M.tb* stimulation were found to be similar to those in unstimulated controls in all groups except for healthy controls in which the MAIT cell frequencies were significantly lower (p=0.0006) (Figure 3.8A). Comparisons of cell responses between the different stimulation conditions was done using the Wilcoxon matched pairs rank tests.

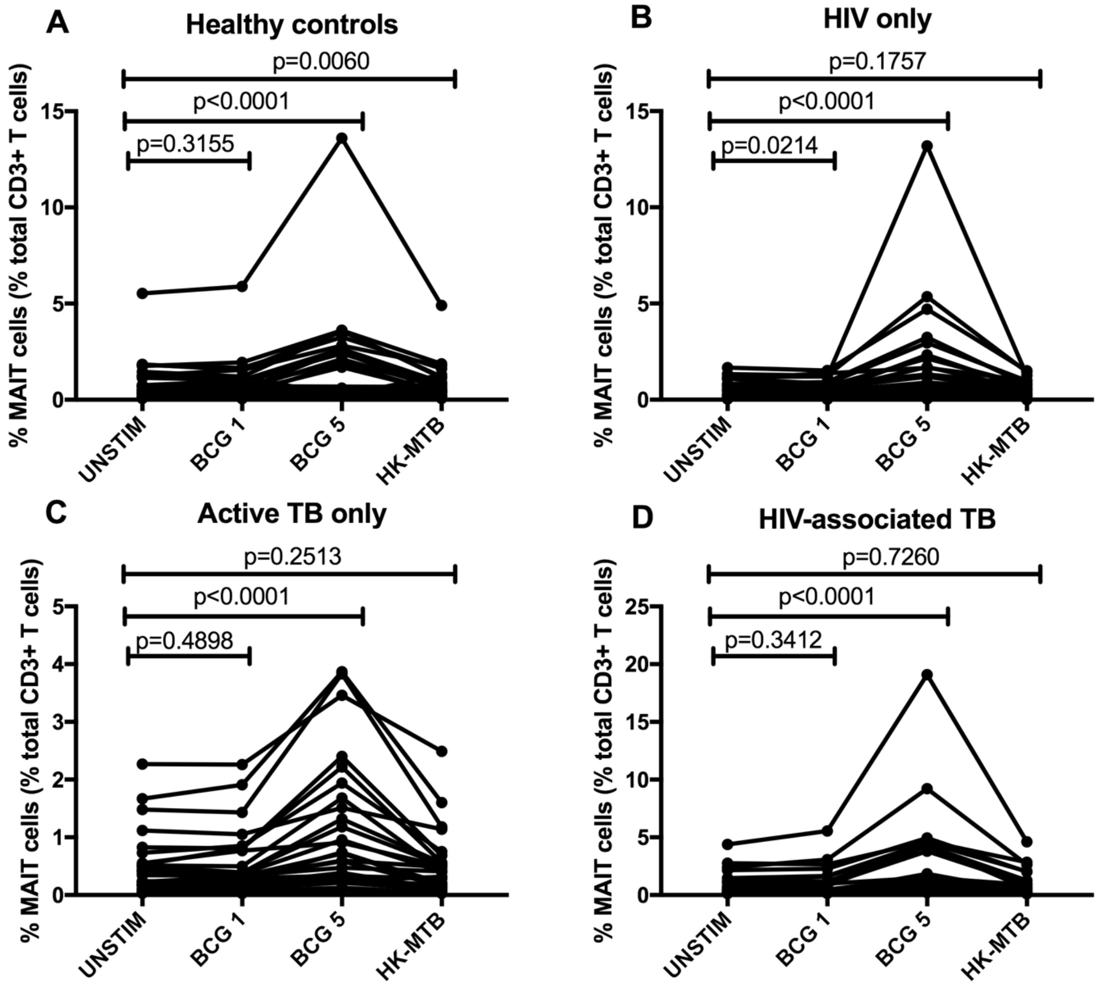


Figure 3.8: Comparisons of MAIT cell frequencies between unstimulated, BCG 1, BCG 5 and HK-*M.tb* stimulated samples. A) Healthy controls. B) HIV only, C) Active TB only, D) HIV-associated TB. P-values represent the Wilcoxon matched-pairs rank tests.

3.2.11 Potential factors which may drive lower frequencies of MAIT cells

3.2.11.1 Cell death

In addition to assessing the frequencies of live cells before and after stimulation, we wanted to determine the impact of the 24-hour incubation period on MAIT cell frequencies and determine if cell death would differ between groups. We therefore gated on dead cells and determined the frequencies of the different T cell subsets (primarily MAIT cells) among these dead cells as shown in Figure 3.9A below.

When frequencies of dead cells were assessed using the Mann-Whitney U test, there was a trend towards lower frequencies of dead MAIT cells from individuals with HIV compared to healthy controls although this was not significant (p=0.0509) while the frequencies of dead MAIT cells from other groups were similar to healthy controls (Figure 3.9B). The frequencies of dead CD4 T cells maintained a similar trend to that observed in live cells in which CD4 T cells from individuals with HIV only and HIV-associated TB were significantly reduced compared to healthy controls (p<0.0001) (Figure 3.9B). No significant differences were observed in the frequencies of dead CD8 T cells.

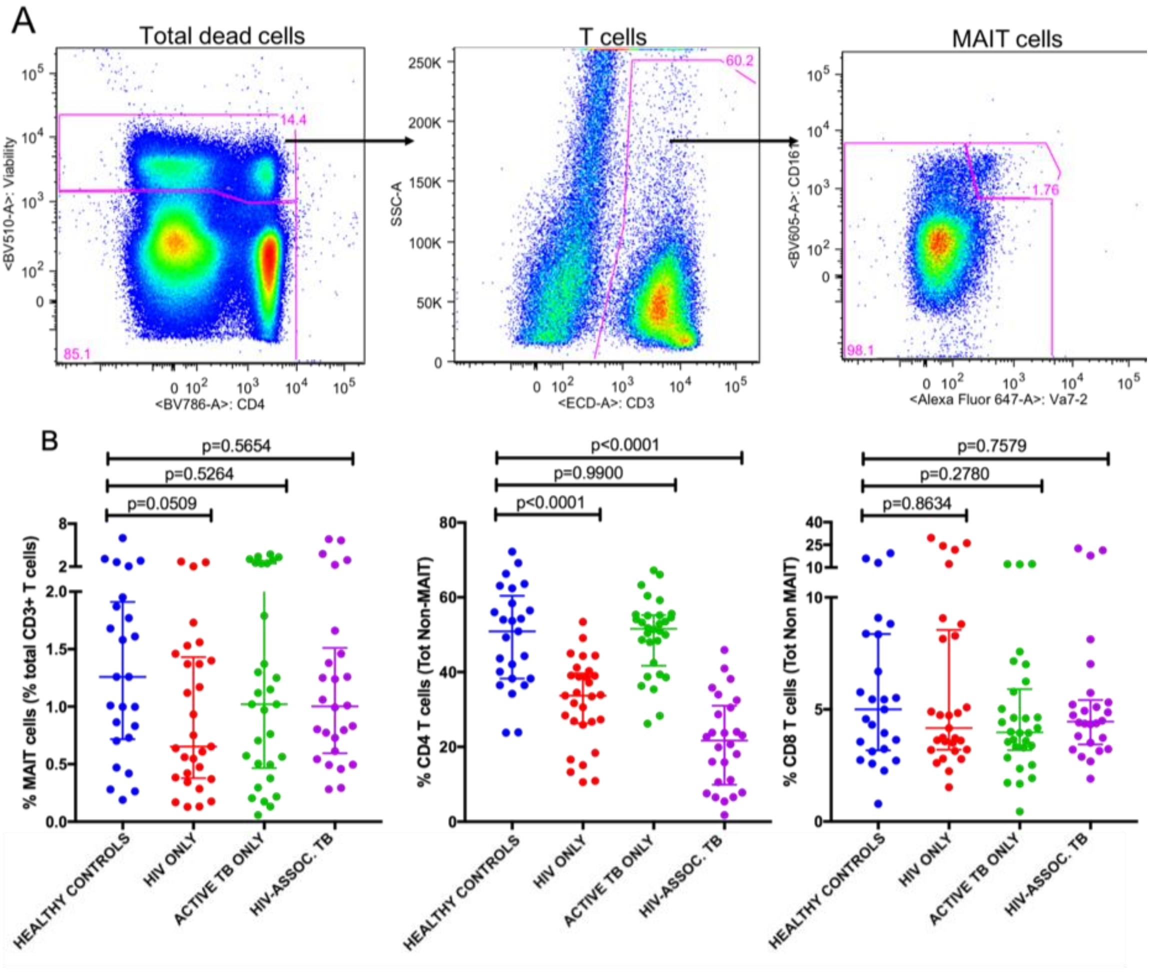


Figure 3.9: The frequencies of T cells among dead cells. **A)** Representative flow cytometry plots showing the gating strategy for the identification of T cells among non-viable T cells. **B), C)** and **D)** show the frequencies of MAIT cells, CD4 T cells, and CD8 T cells, respectively, among healthy controls, individuals with HIV only, active TB only and HIV-associated TB. Data presented as scatter plots with lines representing the median and IQR.

3.2.11.2 Downregulation of CD161

Another possible explanation for the observed lower frequencies of MAIT cells among individuals with HIV and active TB is the downregulation of CD161 which is a marker used to identify MAIT cells. This CD161 downregulation may lead to the decrease in the frequencies of MAIT cells. We therefore sought to determine the frequencies of CD161$^-$Vα7.2$^+$ cells between the different groups. Figure 3.10 below shows the gating strategy used for the identification of CD161$^-$Vα7.2$^+$ cells (Figure 3.10A) and the frequencies of these cells among individuals from different study groups (Figure 3.10B).

From the Mann-Whitney U test, the frequencies of CD161-Vα7.2+ cells were significantly higher in individuals with HIV-associated TB compared to healthy controls (p=0.0132) suggesting a downregulation, while these frequencies were similar between healthy controls and individuals with HIV only and individuals with active TB only (Figure 3.10B).

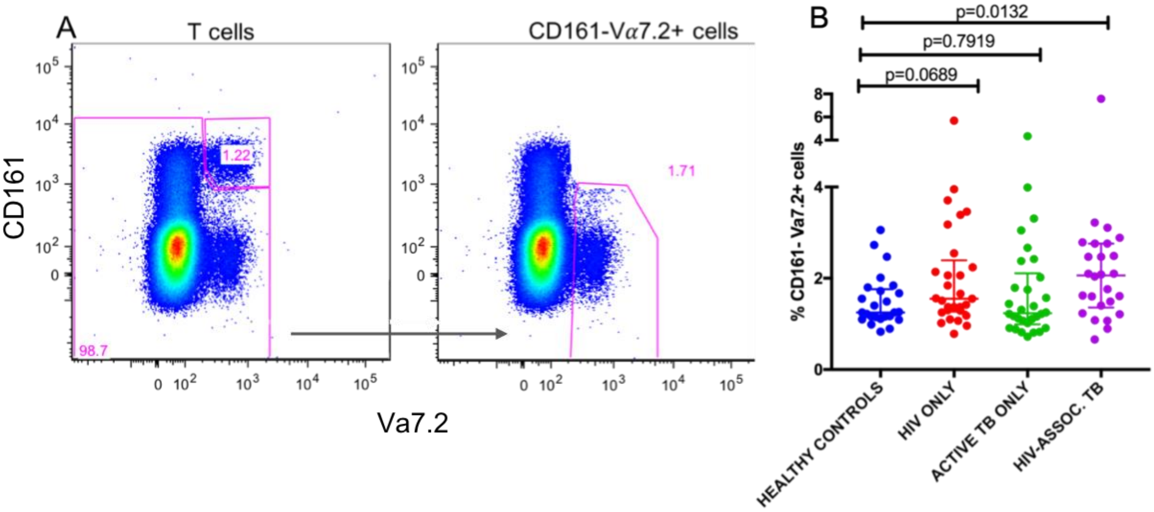


Figure 3.10: Frequencies of CD161$^-$Vα7.2$^+$ cells among individuals with HIV, active TB only and HIV-associated TB. A) Flow cytometry plots showing the gating strategy for the identification of CD161$^-$Vα7.2$^+$ cells. **B)** The frequencies of CD161$^-$Vα7.2$^+$ cells in healthy controls, individuals with HIV only, active TB only and HIV-associated TB. Data presented as scatter plots with lines representing the median and IQR.

3.2.12 Validation of MAIT cell frequencies with antigen loaded MR-1 tetramers

We used MR1 tetramers loaded with 5-OP-RU to validate data obtained with the Vα7.2 antibody. The frequencies of tetramer positive MAIT cells were significantly lower in individuals with active TB only (p=0.0147) while in the 2 HIV groups, the frequencies of MAIT cells were similar to healthy controls (Figure 3.11A). When we evaluated the correlations between the frequencies of MAIT cells identified with Vα7.2 antibody and these frequencies of MAIT cells identified with MR1 tetramer antibody, there was a significant positive correlation between the cell frequencies (Spearman's r=0.7009; p<0.0001) (Figure 3.11B).

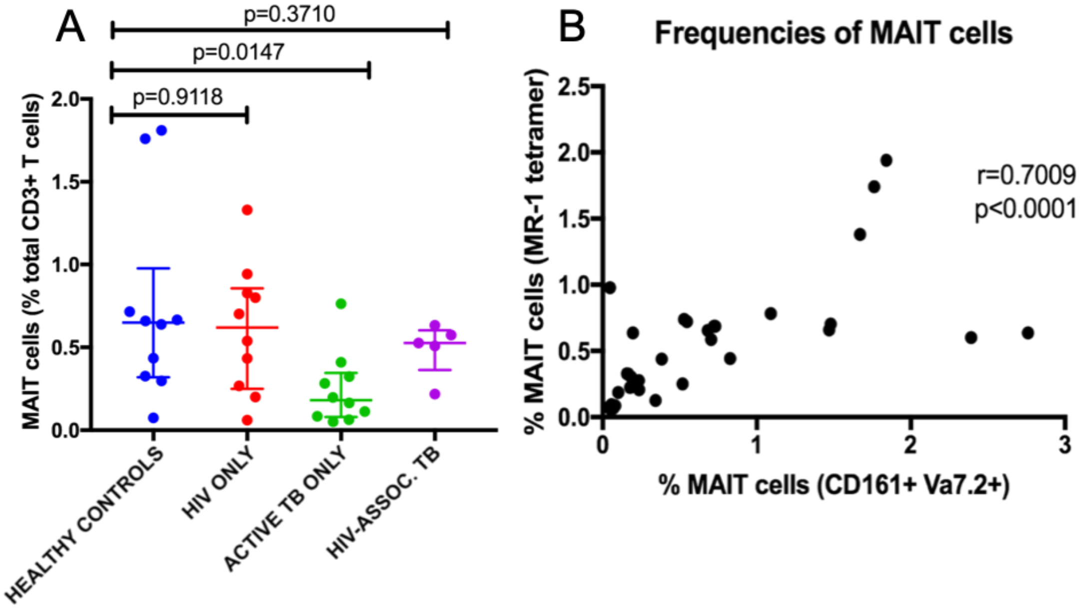


Figure 3.11: The frequencies of MAIT cells as determined by MR1 tetramer staining. A) Frequencies of MAIT cells by MR1 tetramer staining. B) Association between the frequencies of MAIT cells stained/identified by Vα7.2 antibody and MAIT cells identified by MR1 tetramer antibody. Group comparisons were conducted using the Mann-Whitney U test and correlations were done using Spearman rank correlations.

3.2.13 Conclusions

In conclusion, these data show that the frequencies of MAIT cells are depleted in HIV infection and in persons with active TB, but not in HIV/TB. CD161 downregulation and preferential cell death did not account for the lower frequencies of MAIT cells in individuals with HIV and TB.

Due to low number of QFT- people, we could not adequately assess the effect of LTBI on MAIT cell frequencies. In people with HIV, the frequencies of MAIT cells were similar between people on ART and those not on ART.

HIV infection (HIV only and HIV-associated TB) is associated with depleted CD4 T cells (this depletion is more severe in HIV-associated TB) and elevated frequencies of CD8 T cells. There were also increased frequencies of monocytes in people with active TB.

3.3 Association of TB, HIV and HIV-associated TB disease with the cellular immune responses

The effects of disease on cellular responses were investigated after stimulation with bacteria (HK-*M.tb* and BCG-GFP), TLR4 ligand (LPS) and mitogen (PHA).

3.3.1 T cell responses

In order to asses T cell functions, we used IFN-γ and CD107a expression as an indication of the function and degranulation of the cells, respectively. Figure 3.12A shows the representative flow cytometry plots showing expression of IFN-γ and CD107a by MAIT cells. In the unstimulated control (media only) there was low background expression of IFN-γ and CD107a. Following bacterial and PHA stimulation, an increase in expression of both IFN-γ and CD107a was observed. The antigen-specific responses were determined by subtracting background responses from stimulated responses.

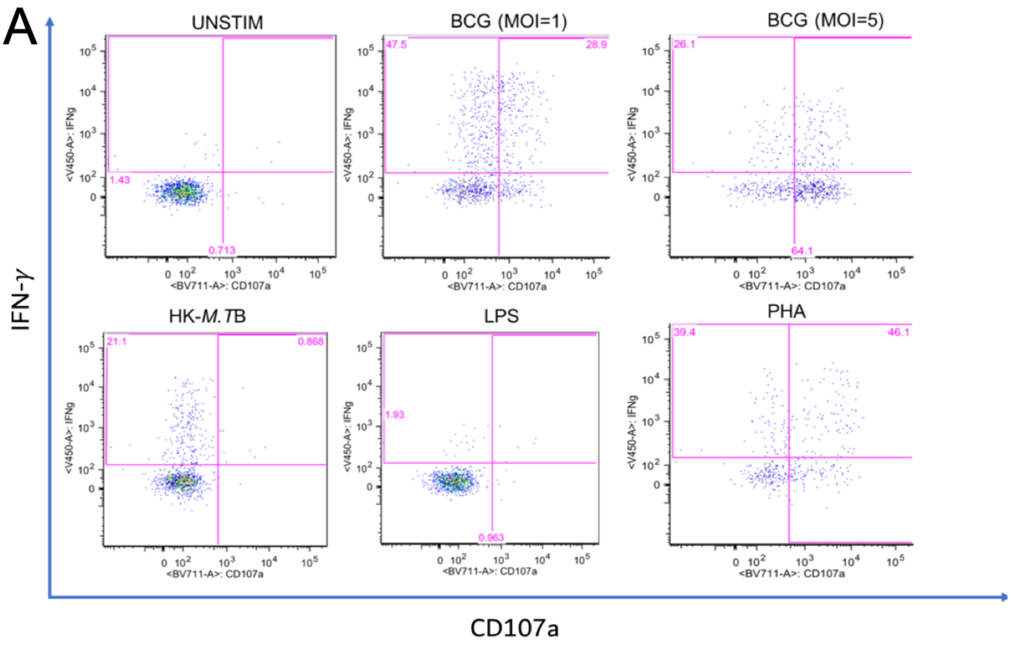
3.3.1.1 MAIT cell responses

In response to stimulation with BCG 1, there were significantly lower frequencies of MAIT cells expressing CD107a (a measure of degranulation/cytotoxicity) (p=0.0041) and IFN-*γ* (a representative T cell effector cytokine) (p=0.0005) in individuals with active TB only (Figure 3.12B). Furthermore, the frequencies of MAIT cells expressing IFN-*γ* were significantly lower in individuals with HIV-associated TB (p=0.0009) compared to healthy controls (Figure 3.12B). Individuals with HIV only had similar frequencies of MAIT cells expressing CD107a and IFN-*γ* compared to healthy controls.

Similar observations were made on MAIT cell responses to BCG 5 stimulation in which the frequencies of MAIT cells expressing CD107a and IFN-*γ* were similar between healthy controls and individuals with HIV only. Furthermore, in individuals with active TB only, the frequencies of MAIT cells expressing CD107a and IFN-*γ* were lower compared to healthy controls (CD107a+ MAIT cells [p=0.0028] and IFN-*γ*+ MAIT cells [p=0.0038]) (Table A2, appendix). However, in individuals with HIV-associated TB; the frequencies of MAIT cells expressing CD107a in response to BCG 5 were similar to healthy controls, and the frequencies of MAIT cells expressing IFN-γ were lower in this same group compared to healthy controls (p=0.0140) (Table A2).

Upon stimulation with HK-*M.tb*, very low frequencies of MAIT cells expressing CD107a were observed for all groups. No differences were observed in the frequencies of MAIT cells expressing CD107a between the groups and healthy controls, in response to HK-*M.tb* stimulation. Additionally, frequencies of MAIT cells expressing IFN-γ were similar between healthy controls and individuals with HIV only. The frequencies of MAIT cells expressing IFN-γ in response to HK-*M.tb* stimulation were significantly lower in individuals with active TB only (p=0.0204) and in individuals with HIV-associated TB (p=0.0109) compared to healthy controls (Figure 3.12C).

As a positive control for T cell activation and stimulation, phytohemagglutinin (PHA) was used to stimulate cells. In response to PHA stimulation, the frequencies of MAIT cells expressing IFN-γ or CD107a were similar to healthy controls in individuals with HIV only and individuals with active TB only. In individuals with HIV-associated TB, the frequencies of MAIT cells expressing CD107a in response to PHA stimulation were significantly lower compared to healthy controls (p=0.0113) and the frequencies of MAIT cells expressing IFN-γ in this same group were similar to healthy controls (Table A2). LPS-induced responses were similar to background responses.



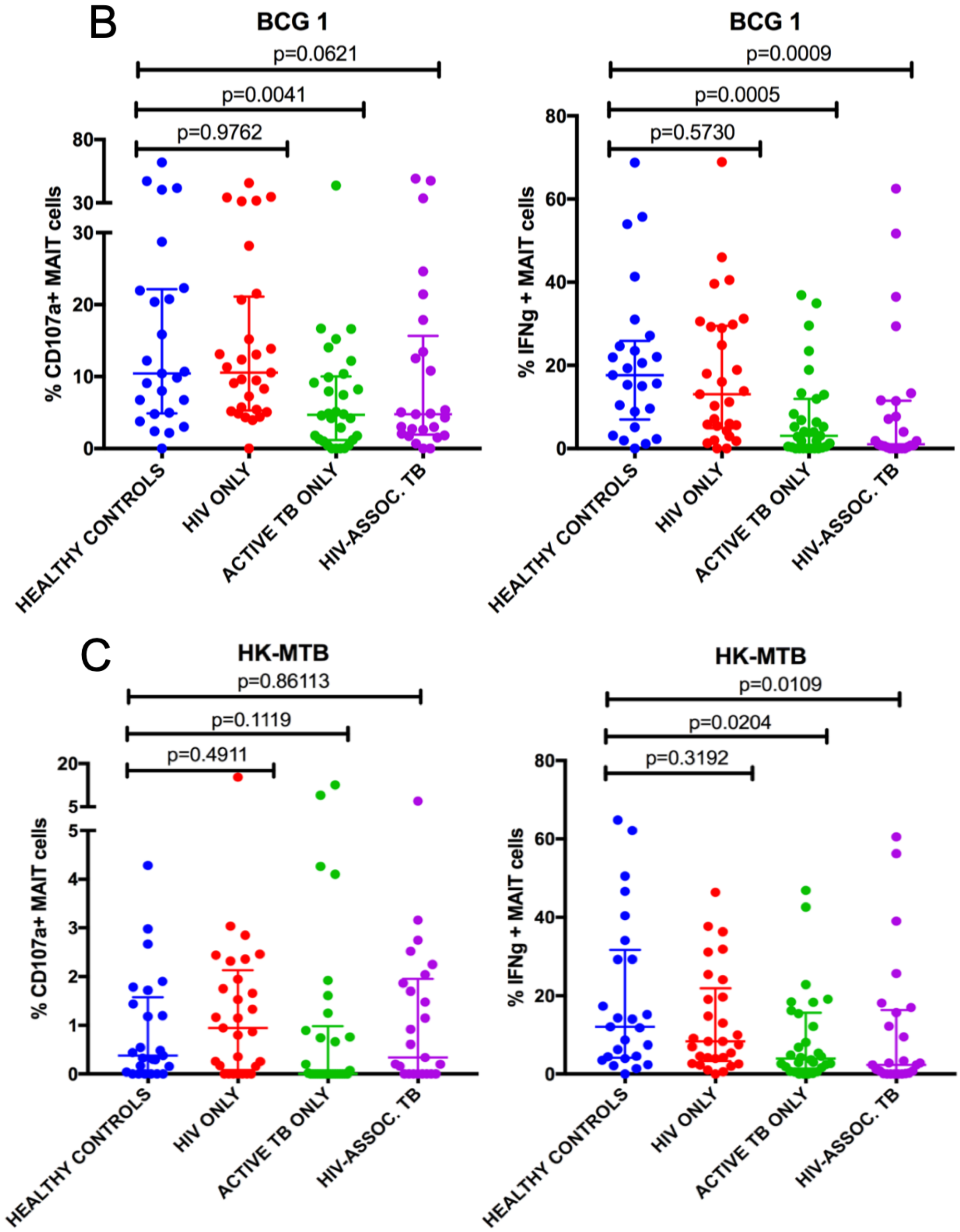


Figure 3.12: MAIT cell responses after bacterial stimulation and the association of HIV, active TB and HIV-associated TB with these responses. A) Representative flow cytometry plots showing IFN-γ and CD107a expression in response to bacterial (BCG and HK-*M.tb*) and antigen stimulation (PHA). The association of HIV, active TB and HIV-associated TB with **B)** BCG-specific and **C)** *M.tb*-specific MAIT cell responses. Data presented as scatter plots with lines representing the median and IQR. P-values represent the Mann-Whitney U test.

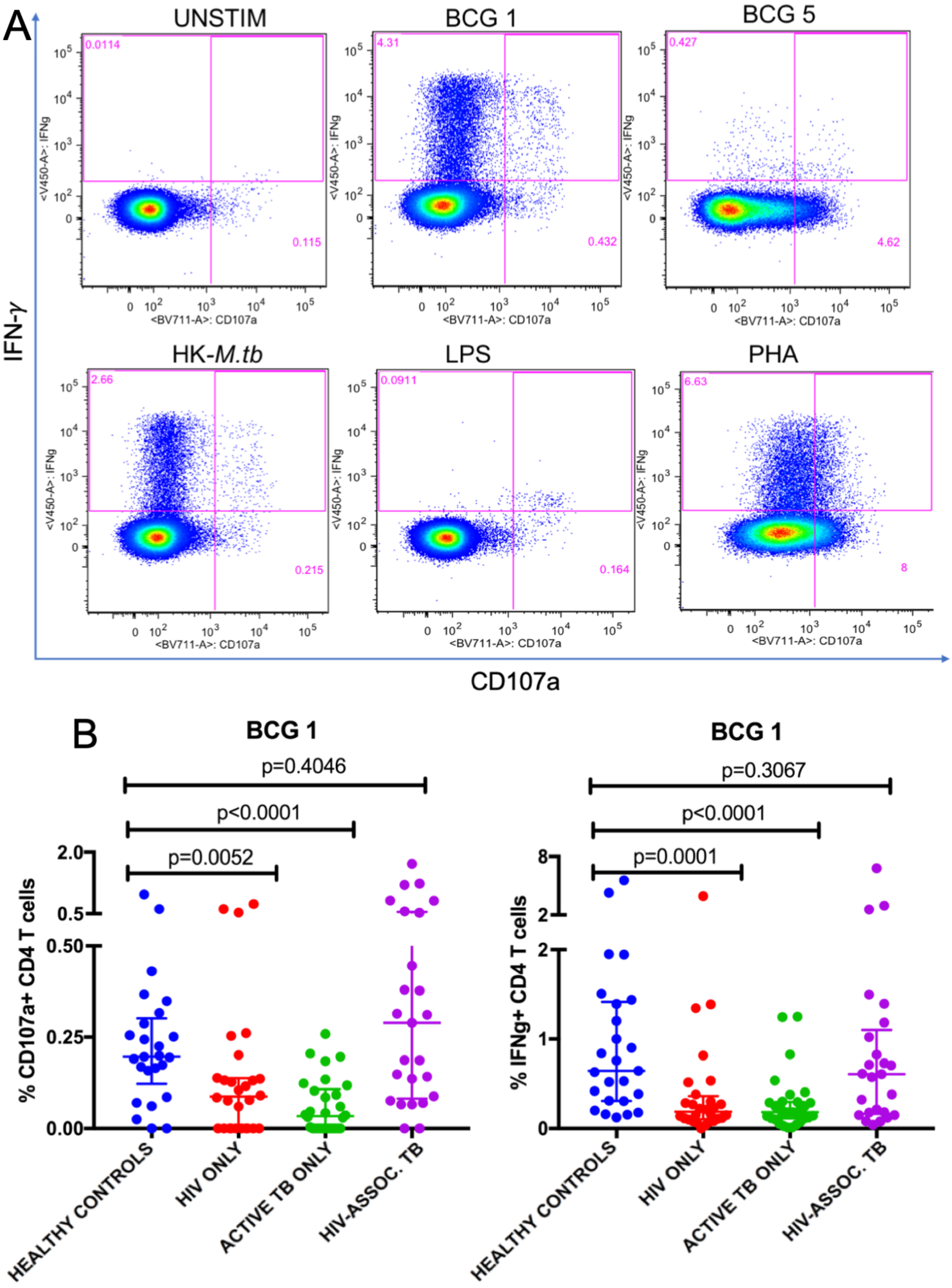
3.3.1.2 CD4 T cell responses

CD4 T cell responses after stimulation with BCG 1 were assessed and we found that the frequencies of CD4 T cells expressing CD107a and IFN-*γ* were significantly lower in individuals with HIV only (CD107a+ CD4 T cells[p=0.0052] and IFN-*γ*+ CD4 T cells [p=0.0001]) and in individuals with active TB only (p<0.0001) compared to healthy controls while individuals with HIV-associated TB had similar frequencies of CD4 T cells expressing CD107a and IFN-*γ* compared to healthy controls (Figure 3.13B).

In response to BCG 5 stimulation, the frequencies of CD4 T cells expressing CD107a were similar to healthy controls in individuals with HIV only and HIV-associated TB. In individuals with active TB only, the frequencies of CD4 T cells expressing CD107a were remarkably lower compared to healthy controls (p=0.0013) (Table A3). Furthermore, the frequencies of CD4 T cells expressing IFN-*γ* in response to BCG 5 stimulation were significantly lower in individuals with HIV only (p=0.0070) and in individuals with active TB only (p=0.0069) while in individuals with HIV-associated TB, the frequencies of CD4 T cells expressing IFN-*γ* were similar to healthy controls (Table A3).

Following stimulation with HK-*M.tb*, the frequencies of CD4 T cells expressing CD107a and IFN-*γ* in individuals with HIV only and individuals with HIV-associated TB were similar to healthy controls. In individuals with active TB, however, the frequencies of CD4 T cells expressing CD107a were significantly lower compared to healthy controls (p=0.0057). The same trend was observed for CD4 T cells expressing IFN-*γ* in which the frequencies were also significantly lower compared to healthy controls (p=0.0078) (Figure 3.13C).

Compared to healthy controls, the frequencies of CD4 T cells expressing CD107a and IFN-*γ* in response to PHA stimulation were similar in the groups. LPS-induced responses were similar to background responses.


A
UNSTIM
BCG 1
BCG 5
HK-*M.tb*
LPS
PHA
0.0114
0.115
4.31
0.432
0.427
4.62
2.66
0.215
0.0911
0.164
6.63
8
<V450-A>: IFNg
<BV711-A>: CD107a
IFN-γ
CD107a
B
BCG 1
p=0.4046
p<0.0001
p=0.0052
% CD107a+ CD4 T cells
2.0
0.5
0.50
0.25
0.00
BCG 1
p=0.3067
p<0.0001
p=0.0001
% IFNg+ CD4 T cells
8
2
2
1
0
HEALTHY CONTROLS
HIV ONLY
ACTIVE TB ONLY
HIV-ASSOC. TB

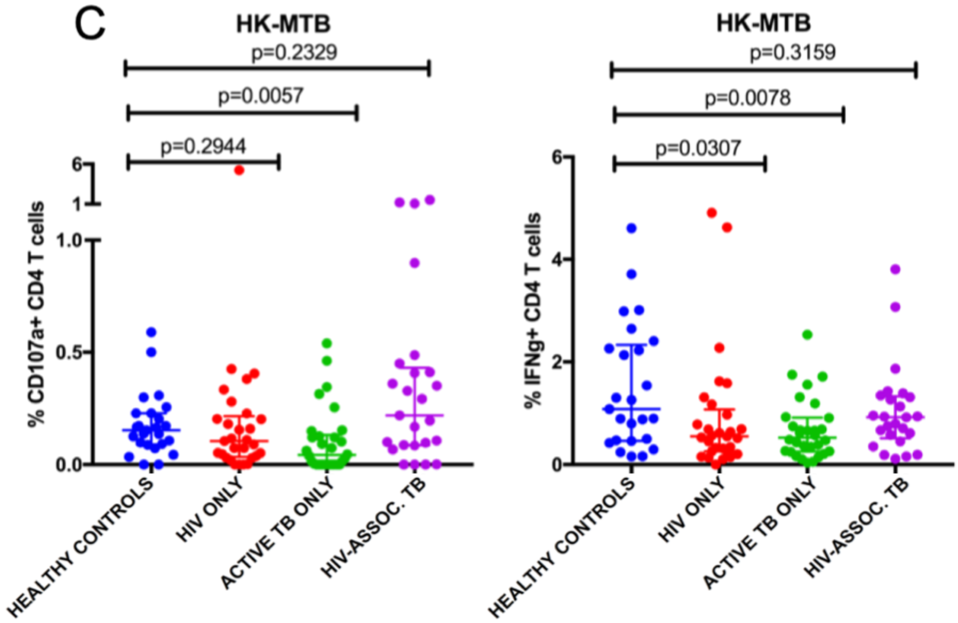


Figure 3.13: CD4 T cell responses after bacterial stimulation and the association of HIV, active TB and HIV-associated TB on these responses. A) Representative flow cytometry plots showing IFN-γ production and CD107a expression in response to bacterial (BCG and HK-*M.tb*) and antigen stimulation (LPS and PHA). The association of HIV, active TB and HIV-associated TB with the **B)** BCG-specific and **C)** *M.tb*-specific CD4 T cell responses. Data presented as scatter plots with lines representing the median and IQR. P-values represent the Mann-Whitney U test.

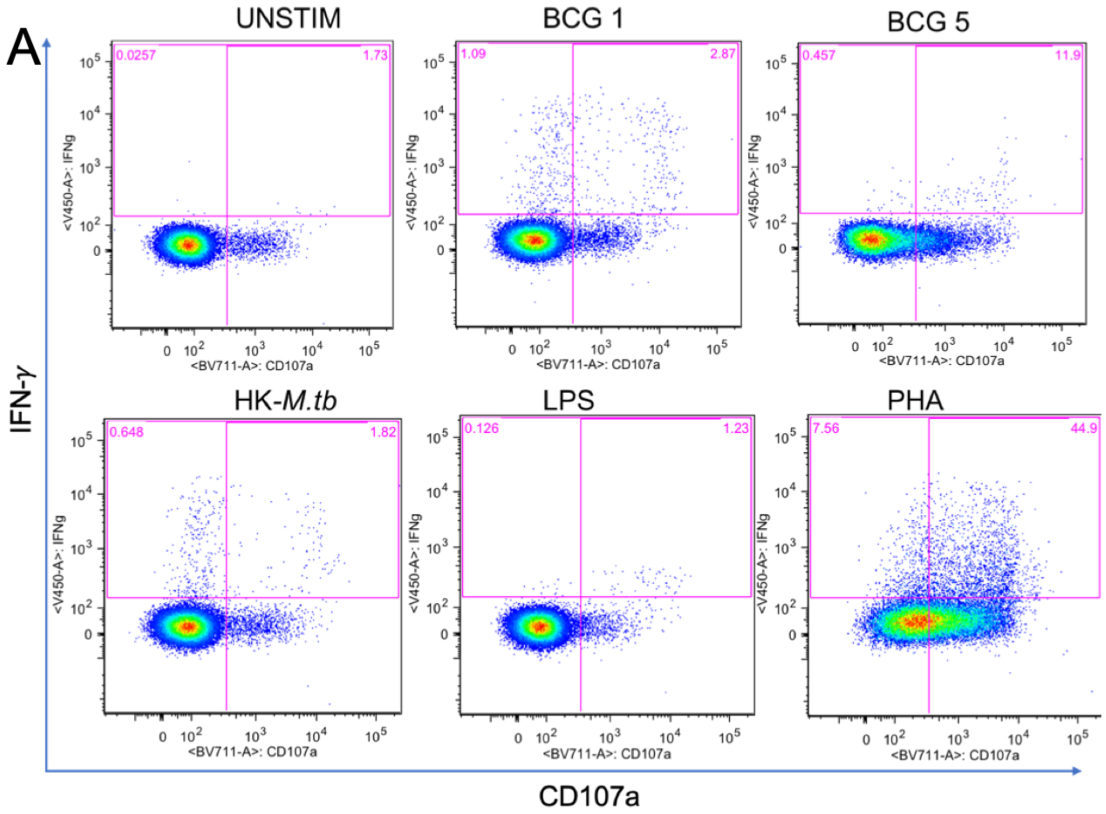
3.3.1.3 CD8 T cells

In response to BCG 1 stimulation, the frequencies of CD8 T cells expressing CD107a were significantly lower in individuals with HIV only (p=0.0178) but were similar to healthy controls in individuals with active TB only and in individuals with HIV-associated TB (Figure 3.14B). However, the frequencies of CD8 T cells expressing IFN-*γ* were significantly lower in all groups compared to healthy controls, (HIV only [p=0.0037], active TB only [p=0.0050], HIV-associated TB [p=0.0080]) (Figure 3.14B).

Compared to healthy controls, the frequencies of CD8 T cells expressing CD107a in response to BCG 5 were only lower in individuals with active TB only (p=0.0202), while individuals with HIV only and individuals with HIV-associated TB had similar frequencies of CD8 T cells expressing CD107a compared to healthy controls (Table A4). The frequencies of CD8 T cells expressing IFN-*γ* were significantly lower in individuals with HIV only (p=0.0070) and individuals with active TB only (p=0.0006) (Table A4).

In response to HK-*M.tb* stimulation, the frequencies of CD8 T cells expressing CD107a were lower in individuals with HIV only (p=0.0032) while individuals with active TB and individuals with HIV-associated TB had similar frequencies of CD8 T cells expressing CD107a compared to healthy controls (Figure 3.14C). Frequencies of CD8 T cells expressing IFN-*γ* were remarkably lower in all groups compared to healthy controls (individuals with HIV only [p=0.0009], active TB only [p=0.0022], HIV-associated TB [p=0.0089]) (Figure 3.14C).

In response to PHA stimulation, no significant differences in the frequencies of CD8 T cells expressing CD107a and IFN-*γ* were observed between the different groups and healthy controls (Table A4). LPS-induced responses were similar to background responses.



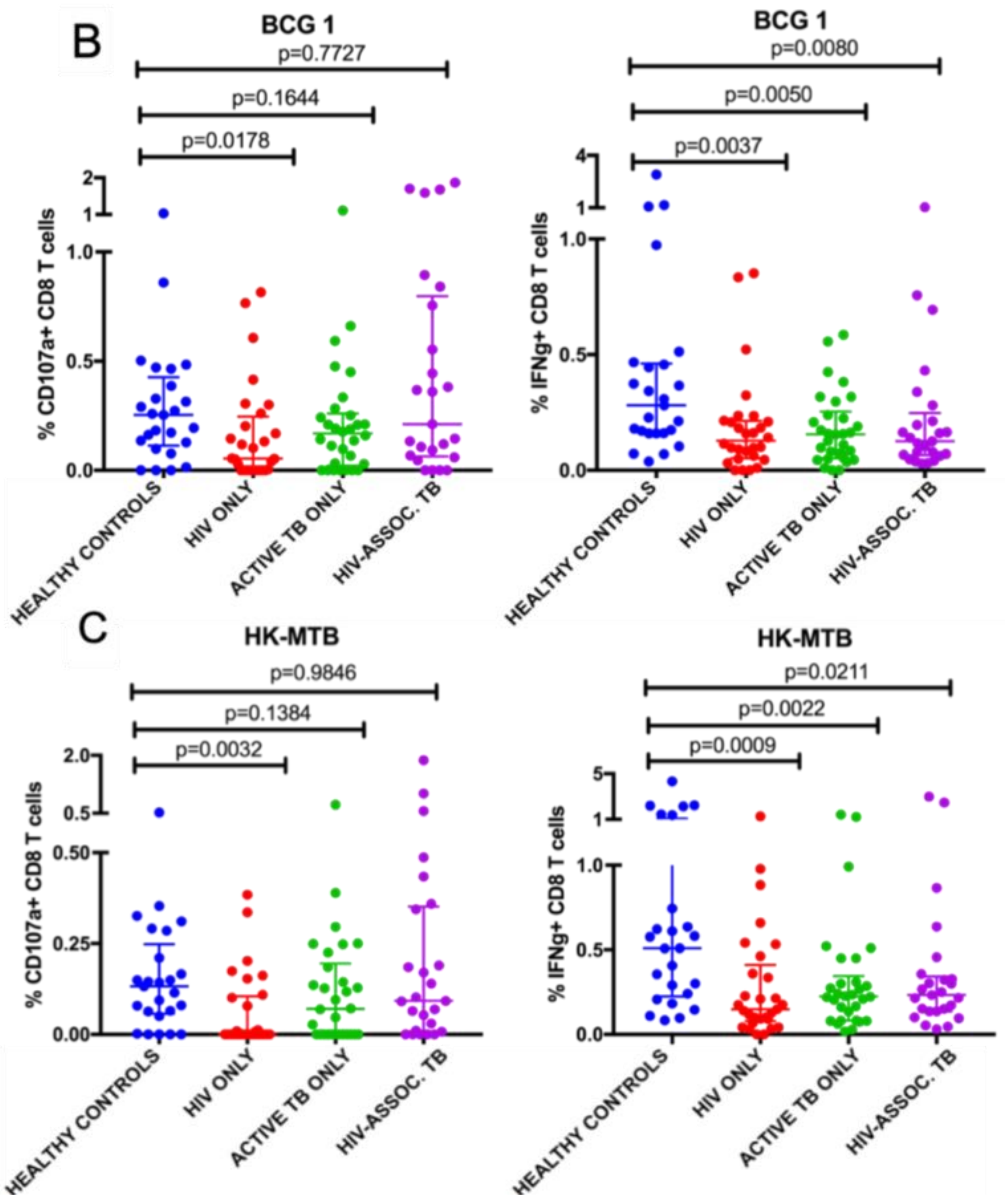


Figure 3.14: CD8 T cell responses after bacterial stimulation and the association of HIV, active TB and HIV-associated TB with these responses. A) Representative flow cytometry plots showing IFN-γ and CD107a expression in response to bacterial (BCG and HK-*M.tb*) and antigen stimulation (LPS and PHA). The association of HIV, active TB and HIV-associated TB with the **B)** BCG specific and **C)** *M.tb* specific CD8 T cell responses. P-values represent the Mann-Whitney U test.

3.3.1.4. T cell mono and bifunctional cytokine profiles

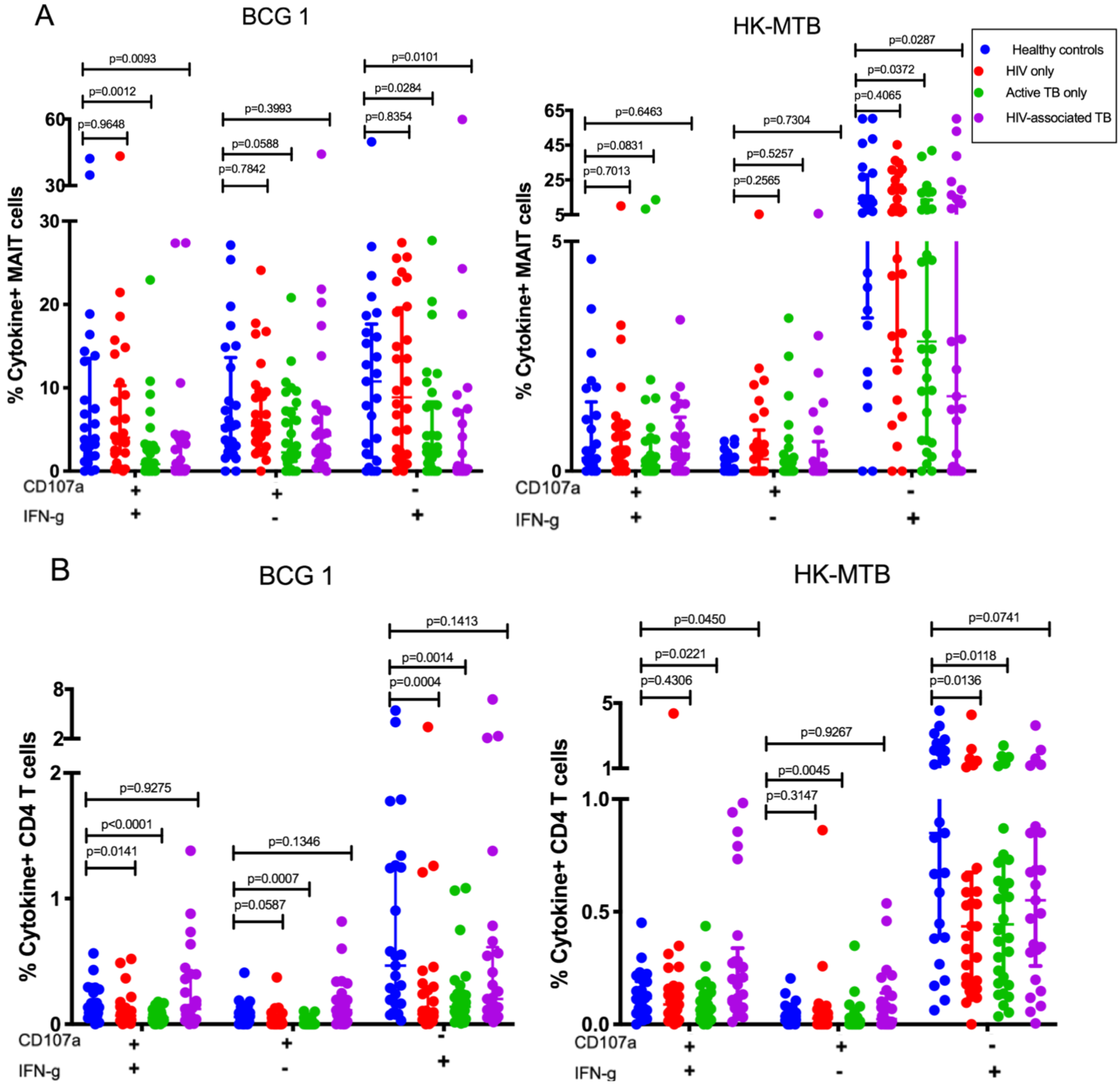
We also assessed the mono- and bifunctional T cell responses on MAIT cells, CD4 and CD8 T cells in response to BCG 1 and HK-*M.tb* stimulation. T cells expressing CD107a only (CD107a monofunctional), IFN-*γ* (IFN-*γ* monofunctional) and both CD107a and IFN-*γ* (bifunctional) were evaluated.

In response to BCG 1 stimulation, there were significantly lower frequencies of bifunctional MAIT cells in individuals with active TB only (p=0.0012) and in individuals with HIV-associated TB (p=0.0093) compared to healthy controls. There were lower frequencies of IFN-*γ* monofunctional MAIT cells in individuals with active TB only (p=0.0284) and individuals with HIV-associated TB (p=0.0101) (Figure 3.15A). In response to HK-*M.tb* stimulation, there were also significantly lower frequencies of IFN-*γ* monofunctional MAIT cells in individuals with active TB only (p=0.0372) and individuals with HIV-associated TB (p=0.0287) compared to healthy controls (Figure 3.15A).

In CD4 T cells, there were significantly lower frequencies of bifunctional CD4 T cells in response to BCG 1 stimulation in individuals with HIV only (p=0.0141) and individuals with HIV-associated TB (p<0.0001) compared to healthy controls. Individuals with active TB only had lower frequencies of CD107a monofunctional CD4 T cells compared to healthy controls (p=0.0007). Finally, the frequencies of IFN-*γ* monofunctional CD4 T cells were lower in individuals with HIV only (p=0.0004) and active TB only (p=0.0014) compared to healthy controls (Figure 3.15B).

In response to HK-*M.tb* stimulation, the frequencies of bifunctional CD4 T cells were significantly lower in individuals with active TB only (p=0.0221) and individuals with HIV-associated TB (p=0.0450) compared to healthy controls. The frequencies of CD107a monofunctional CD4 T cells were also remarkably reduced in individuals with active TB only (p=0.0045), while the frequencies of IFN-*γ* monofunctional CD4 T cells were also significantly lower in individuals with HIV only (p=0.0136) and individuals with HIV-associated TB (p=0.0118) compared to healthy controls (Figure 3.15B).

In response to BCG 1 stimulation, the frequencies of bifunctional CD8 T cells were significantly lower in individuals with HIV only (p=0.0009) and active TB only (p=0.0275) compared to healthy controls, while CD107a monofunctional CD8 T cells were lower in individuals with HIV only (p=0.0246) compared to healthy controls. Finally, the frequencies of IFN-*γ* monofunctional CD8 T cells were lower in all study groups compared to healthy controls (HIV only [p=0.0149], active TB only [p=0.0030], HIV-associated TB [p=0.0014]) (Figure 3.15C).Upon stimulation with HK-*M.tb*, the frequencies of bifunctional and CD107a monofunctional CD8 T cells were lower in individuals with HIV only (p<0.0001 and p=0.0220, respectively). The frequencies of IFN-γ monofunctional CD8 T cells were significantly lower in all study groups compared to healthy controls (HIV only [p=0.0090], active TB only [p=0.0057], HIV-associated TB [p=0.0120]) (Figure 3.15C).



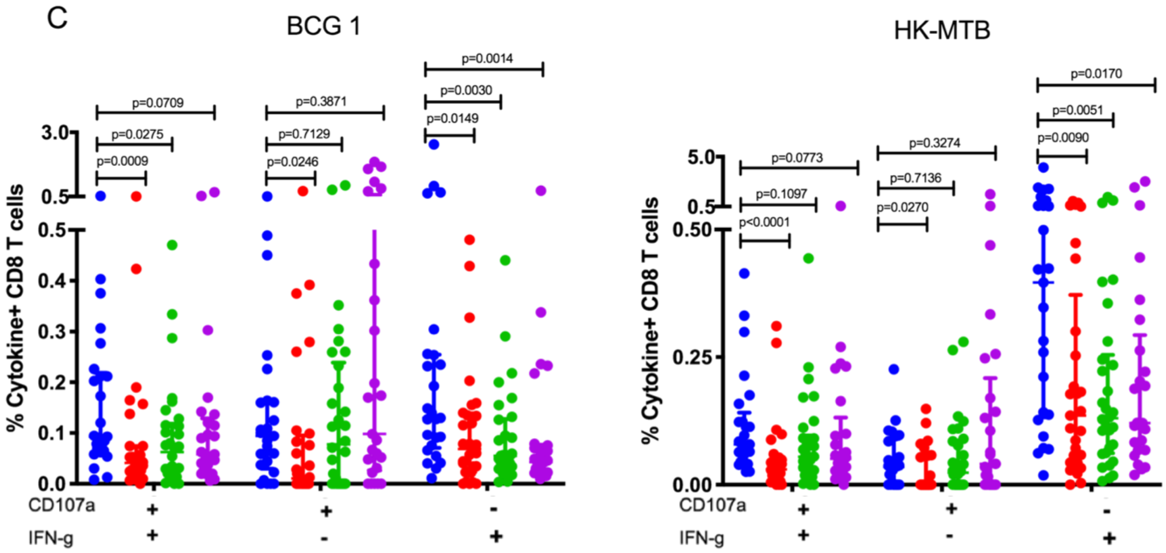


Figure 3.15: Mono and bifunctional T cell profiles after BCG and HK-*M.tb* stimulation. Graphs show the 3 different combinations of CD107a and IFN-γ in response to BCG 1 and HK-*M.tb* stimulation for A) MAIT cells, B) CD4 T cells and C) CD8 T cells. P-values represent the Mann-Whitney U test.

3.3.2 APC Functions

3.3.2.1 APC infection

Detection of GFP positivity in the cells was regarded as uptake of BCG and cells were considered to be infected. For the infection of APCs, there were similar frequencies of GFP+ monocytes between the different groups and healthy controls, with BCG 1 co-culture. In dendritic cells however, frequencies of GFP+ DCs were significantly lower in individuals with HIV-associated TB compared to healthy controls (p=0.0114) (Figure 3.16B). In response to BCG 5 co-culture, the frequencies of GFP+ monocytes were significantly higher in individuals with active TB only (p=0.0409) (Figure 3.16C). In dendritic cells, there were similar frequencies of GFP+ dendritic cells between the groups and healthy controls.

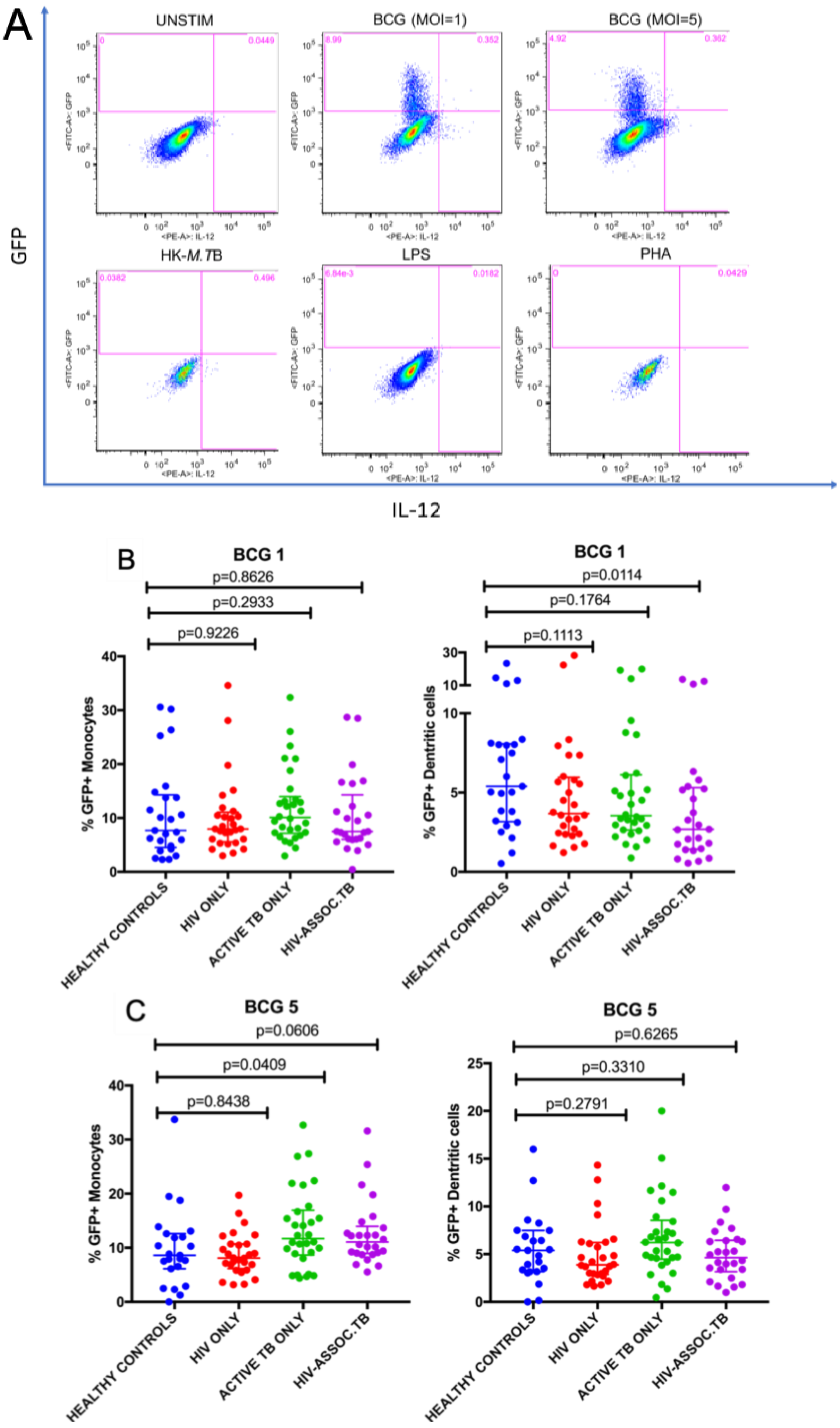

A
UNSTIM
BCG (MOI=1)
BCG (MOI=5)
HK-M.TB
LPS
PHA
0
0.0449
8.99
0.352
4.92
0.362
0.0382
0.496
6.84e-3
0.0182
0
0.0429
<FITC-A>: GFP
<PE-A>: IL-12
GFP
IL-12
B
BCG 1
p=0.8626
p=0.2933
p=0.9226
% GFP+ Monocytes
BCG 1
p=0.0114
p=0.1764
p=0.1113
% GFP+ Dentritic cells
HEALTHY CONTROLS
HIV ONLY
ACTIVE TB ONLY
HIV-ASSOC.TB
C
BCG 5
p=0.0606
p=0.0409
p=0.8438
% GFP+ Monocytes
BCG 5
p=0.6265
p=0.3310
p=0.2791
% GFP+ Dentritic cells
HEALTHY CONTROLS
HIV ONLY
ACTIVE TB ONLY
HIV-ASSOC.TB

Figure 3.16: Association of HIV, active TB, HIV-associated TB with the frequencies of antigen presenting cells (APC) co-cultured with BCG-GFP. A) Representative flow cytometry plots showing APC infection by BCG. B) Frequencies of GFP+ infected APC (monocytes and dendritic cells) in response to BCG 1 co-culture. C) Frequencies of GFP+ infected APCs in response to BCG 5 co-culture. Data presented as scatter plots with lines representing the median and IQR. P-values represent the Mann-Whitney U test.

Next, we assessed the MFI of GFP in APCs as indication of the amount of infection. In response to BCG 1, only monocytes from individuals with active TB only had significantly higher MFI of GFP compared to healthy controls (p=0.0281) while APCs from other groups had similar MFI of GFP compared to healthy controls (Figure 3.17A). There were no significant differences in the MFI of GFP on APCs in response to BCG 5 stimulation.

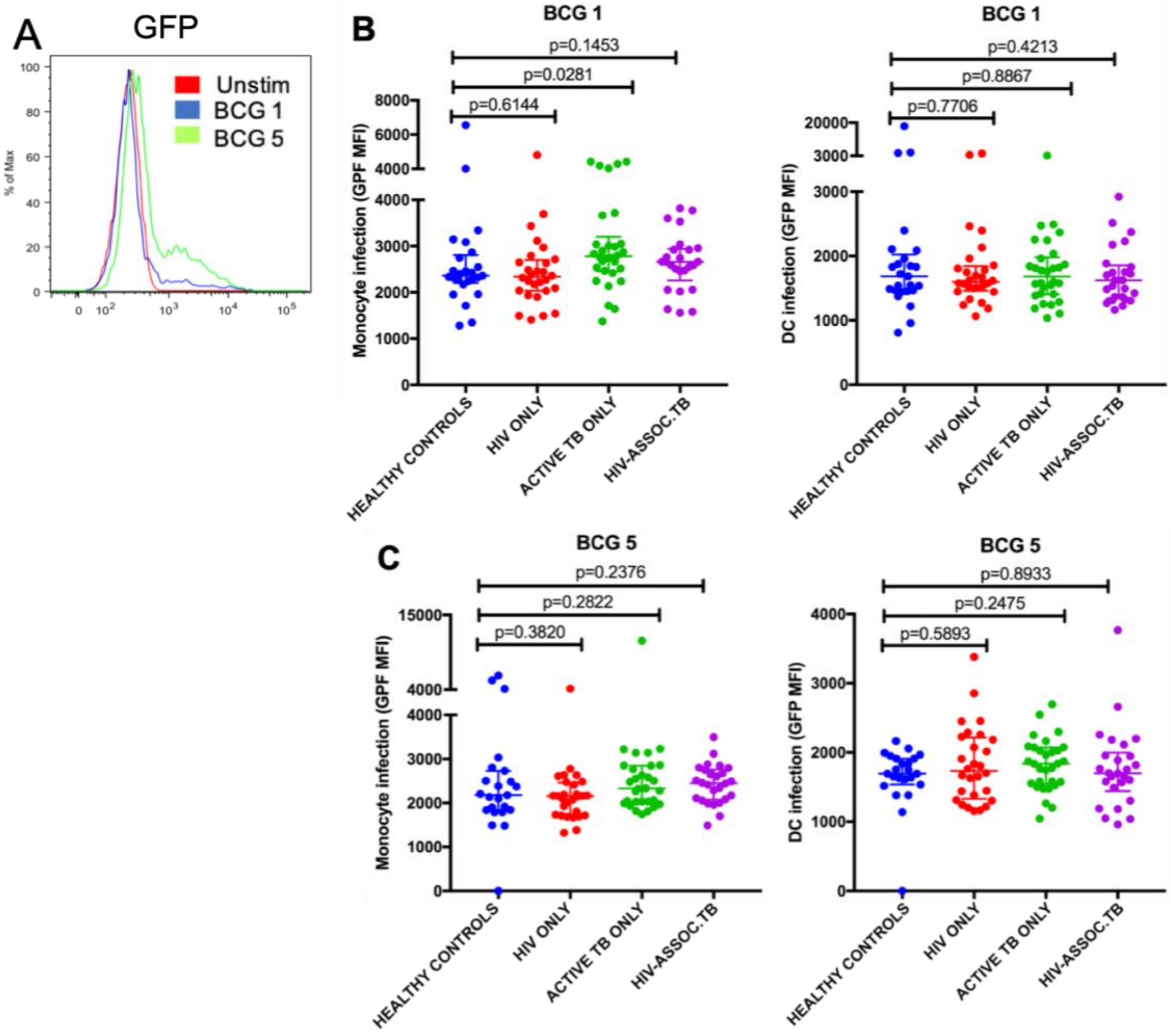


Figure 3.17: MFI of GFP after BCG-GFP+ co-culture. A) GFP+ MFI of cells after BCG 1 and BCG 5 co-culture, **B)** MFI of GFP in response to BCG 1 co-culture and **C)** BCG 5 co-culture. Data presented as scatter plots with lines representing the median and IQR. P-values represent the Mann-Whitney U test.

3.3.3 Effect of CD4 count and HIV viral load on MAIT cell functions

Similar to analyses on MAIT cell frequencies, we conducted analyses on the responses of MAIT cells to stimulation with BCG 1 to evaluate the effects of CD4 count and HIV viral load on the frequencies of MAIT cells expressing IFN-*γ*, CD107a Figure 3.18.

We found that in both HIV groups, the frequencies of MAIT cells expressing CD107a and IFN-*γ* were similar between participants within the different CD4 count.

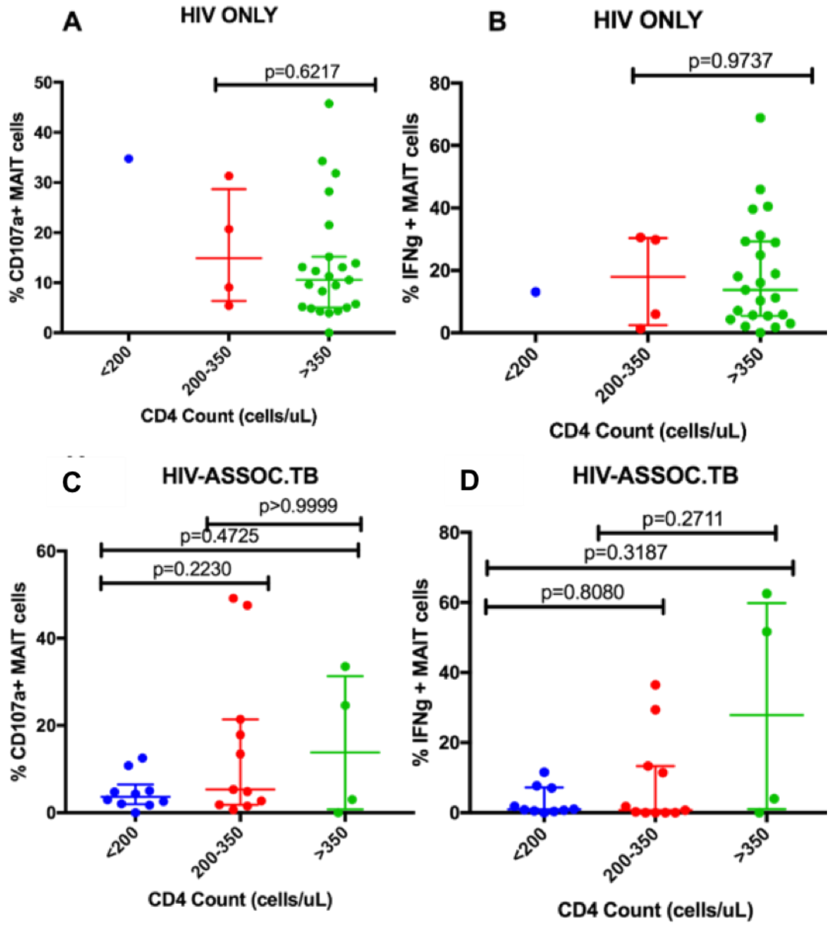


Figure 3.18: The association of CD4 counts with the functional profile and the activation of MAIT cells in individuals with HIV infection. Results presented by CD4 count category for frequencies of **A)** MAIT cells expressing CD107a, **B)** MAIT cells expressing IFN-γ from individuals with HIV only. **C)** and **D)** Frequencies of MAIT cells expressing CD107a, IFN-γ in individuals with HIV-associated TB. Graphs represent MAIT cell responses to BCG 1 stimulation. Data presented as scatter plots with lines representing the median and IQR. P-values represent the Mann-Whitney U test.

When we looked at MAIT cell responses and activation and how these were associated with HIV viral load, we found that in individuals with HIV only, the frequencies of MAIT cells expressing CD107a and IFN-γ were similar between individuals with HIV viral loads below and above 1000 copies/ml. In individuals with HIV-associated TB, the frequencies of MAIT cells expressing CD107a and IFN-γ were similar between individuals with HIV viral loads above and below 1000 copies/ml (Figure 3.19).

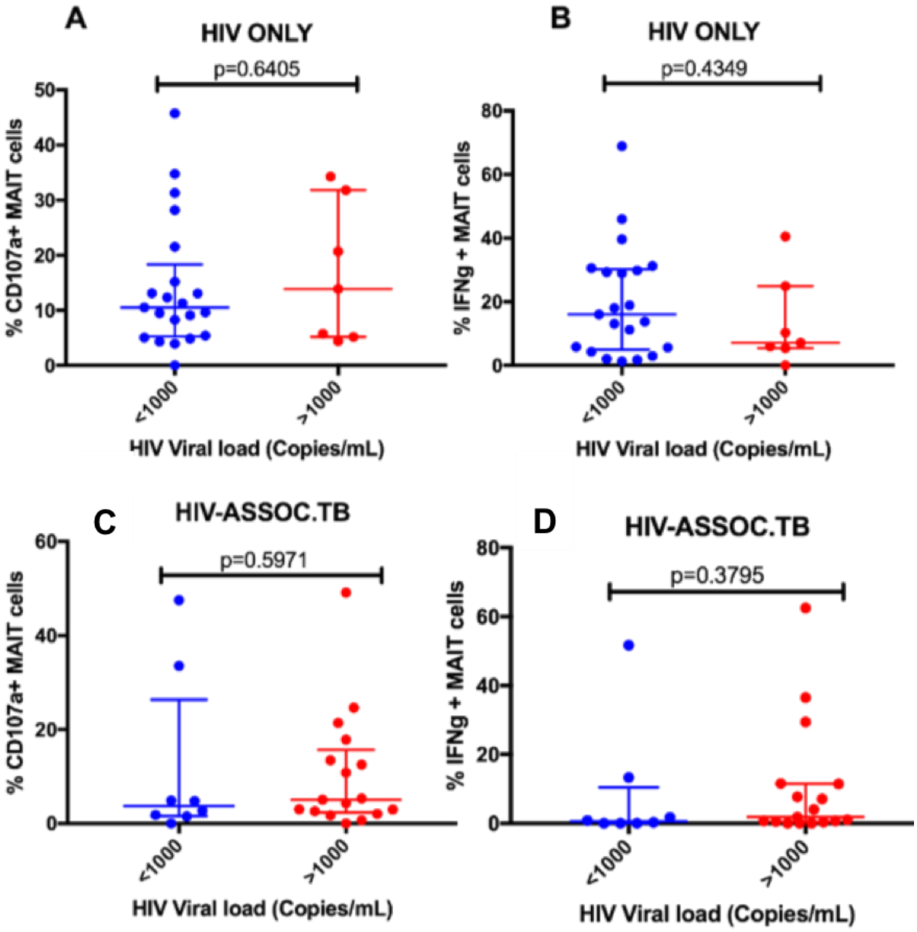


Figure 3.19: The association of HIV viral load with the functional profile and the activation of MAIT cells in individuals with HIV infection. The association of HIV viral load with frequencies of **A)** MAIT cells expressing CD107a, **B)** MAIT cells expressing IFN-γ in individuals with HIV only. **C)** and **D)** Frequencies of MAIT cells expressing CD107a, IFN-γ, in individuals with HIV-associated TB. Graphs represent MAIT cell responses BCG 1 stimulation. Data presented as scatter plots with lines representing the median and IQR. P-values represent the Mann-Whitney U test.

3.3.4 Conclusions

Taken together, these data suggest that MAIT cell function (IFN-γ and CD107a) is impaired in TB disease (active TB and HIV-associated TB) but these functions are retained in people with HIV infection only. HIV only and active TB only are associated with functionally impaired CD4 and CD8 T cells, and HIV-associated TB with impaired CD8 T cell function. In HIV infection, CD4 count <350 and VL>1000 copies/mL were associated with a significant increase in MAIT cell activation.

3.4 Association of TB, HIV and HIV-associated TB with immune activation

The expression of HLA-DR on T cells is used as a marker for T cell activation and as such, we investigated the effect of disease on the activation status of all T cells (MAIT cells, CD4 and CD8 T cells) by assessing the expression of HLA-DR on T cells in the absence of stimulation (unstimulated controls) and upon mycobacterial stimulation.

3.4.1 MAIT cell activation

The frequencies of MAIT cells expressing HLA-DR were similar in all study groups in response to BCG and HK-*M.tb* stimulation, however, upon PHA stimulation, the frequencies of MAIT cells expressing HLA-DR were increased in individuals with active TB only compared to healthy controls (p=0.0202) (Table A5).

The MFI of HLA-DR showed higher values on total MAIT cells for all the different groups compared to healthy controls. These results were however, only significant for individuals with active TB only (p=0.0129) and in individuals with HIV-associated TB (p=0.0012) (Figure 3.20C) in the unstimulated condition. With BCG 1 stimulation, the MFI of HLA-DR were similar between the different study groups and healthy controls. With HK-*M.tb* stimulation, the MFI of HLA-DR was significantly increased in individuals with HIV-associated TB compared to healthy controls (p=0.0308) (Figure 3.20C). Finally, with BCG 5 and PHA stimulation, the MFI of HLA-DR on total MAIT cells was similar between the groups.

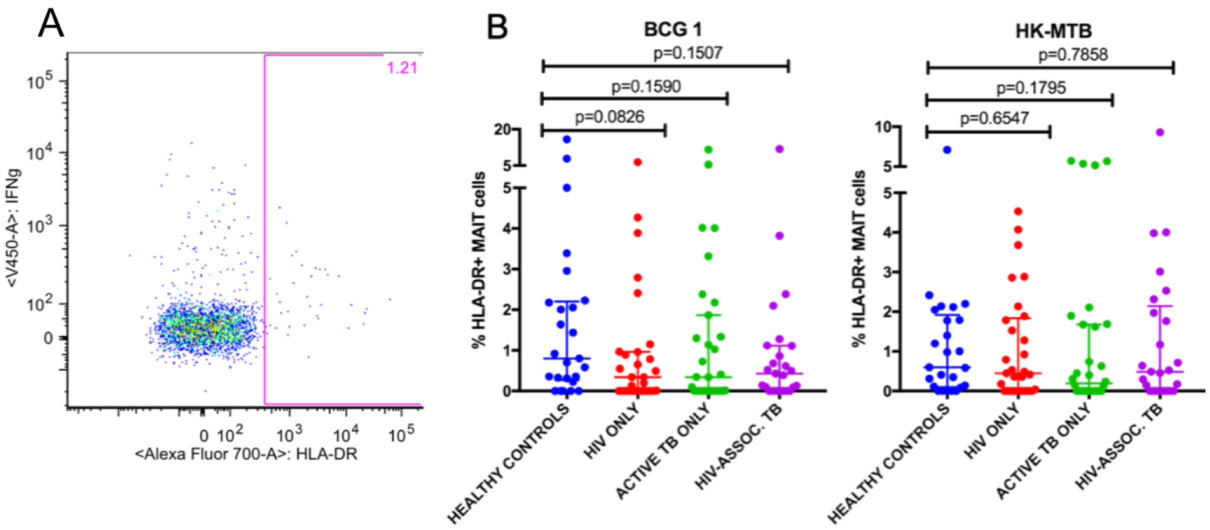
Antigen specific activation of MAIT cells was evaluated by assessing the HLA-DR expression only on MAIT cells expressing IFN-*γ*. Individuals with active TB and individuals with HIV-associated TB had significantly elevated HLA-DR MFI compared to healthy controls (p=0.0068 and p=0.0008, respectively) while MAIT cells from individuals with HIV only had comparable HLA-DR MFI to healthy controls in response to BCG 1 stimulation (Figure 3.20D).

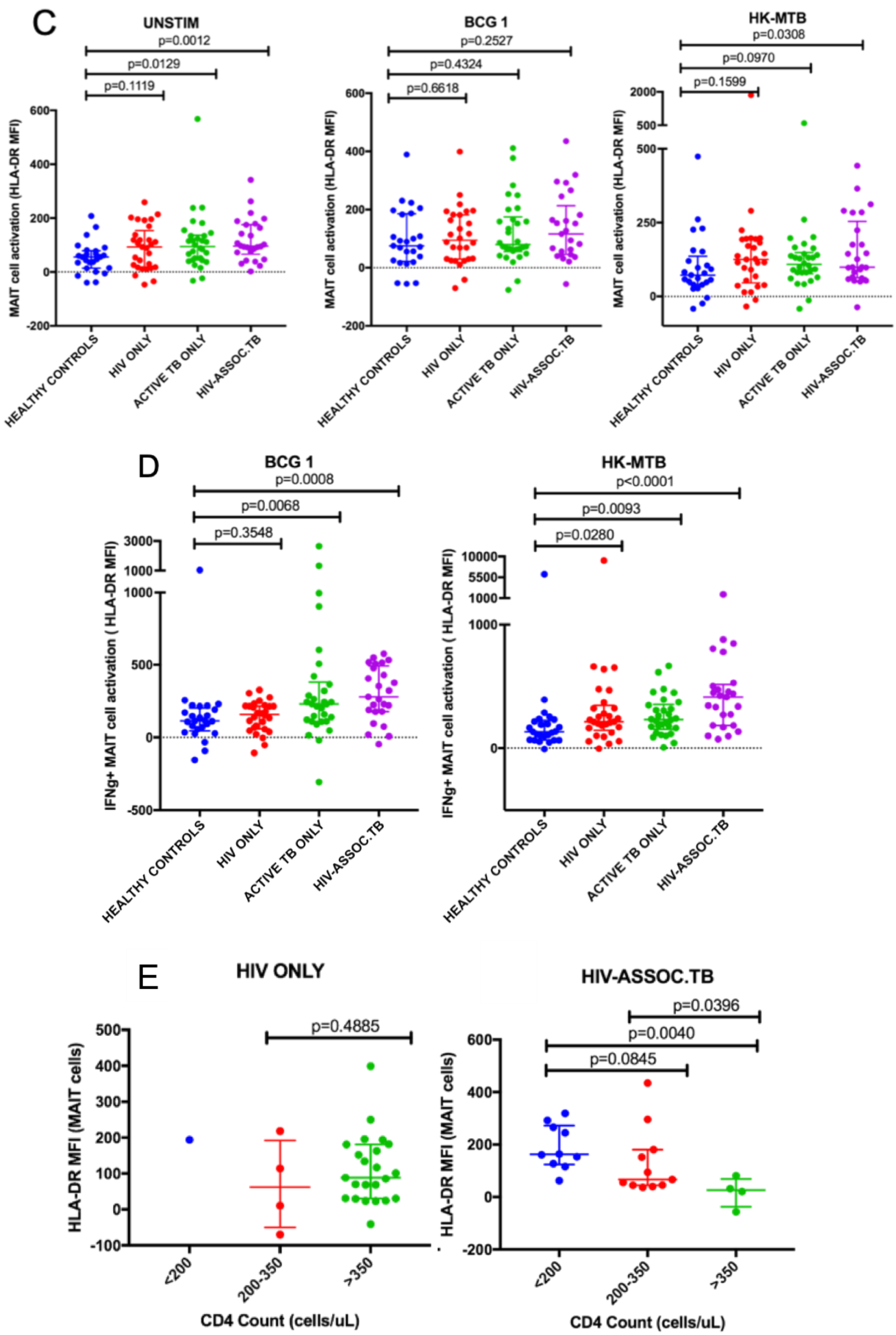
Similar to BCG 1 stimulation, the MFI of HLA-DR on MAIT cells expressing IFN-*γ* was significantly elevated in all groups compared to healthy controls (HIV only [p=0.0280], active

TB only [p=0.0093] and HIV-associated TB [p<0.0001]) (Figure 3.20D) in response to HK-*M.tb* stimulation. In response to BCG 5 stimulation, the MFI of HLA-DR on MAIT cells expressing IFN-γ was only significantly increased in individuals with HIV-associated TB only (p=0.0117) (Table A5).

When we assessed how the activation of MAIT cells was associated with CD4 counts and HIV viral loads in the 2 HIV groups, we found that in individuals with HIV only, the MFI of HLA-DR on MAIT cells in response to BCG 1 were similar between individuals within the different CD4 count categories. In individuals with HIV-associated TB, the MFI of HLA-DR on MAIT cells was 1) similar between individuals with CD4 counts below 200 cells/µl and those with CD4 counts between 200 and 350 cells/µl), 2) lower in individuals with CD4 counts above 350 cells/µl compared to those with CD4 counts below 200 cells/µl (p=0.0040, 3) lower in individuals with CD4 counts above 350 cells/µl compared to those with CD4 counts between 200 and 350 cells/µl (p=0.0396) (Figure 3.20E).

Lastly, when we looked at HIV viral loads, we found that the MFI of HLA-DR on MAIT cells was significantly higher in individuals with viral loads above 1000 copies/ml compared to those with HIV viral loads below 1000 copies/ml (p<0.0001) (Figure 3.19C). In individuals with HIV-associated TB, there was a trend of a higher, although not significant, MFI of HLA-DR on MAIT cells from individuals with HIV viral loads above 1000 copies/ml compared to those with HIV viral loads below 1000 copies/ml (p=0.0571) (Figure 3.20F).




C
UNSTIM
p=0.0012
p=0.0129
p=0.1119
MAIT cell activation (HLA-DR MFI)
600
400
200
0
-200
HEALTHY CONTROLS
HIV ONLY
ACTIVE TB ONLY
HIV-ASSOC.TB
BCG 1
p=0.2527
p=0.4324
p=0.6618
MAIT cell activation (HLA-DR MFI)
600
400
200
0
-200
HEALTHY CONTROLS
HIV ONLY
ACTIVE TB ONLY
HIV-ASSOC.TB
HK-MTB
p=0.0308
p=0.0970
p=0.1599
MAIT cell activation (HLA-DR MFI)
2000
500
500
250
0
HEALTHY CONTROLS
HIV ONLY
ACTIVE TB ONLY
HIV-ASSOC.TB
D
BCG 1
p=0.0008
p=0.0068
p=0.3548
IFNg+ MAIT cell activation (HLA-DR MFI)
3000
1000
1000
500
0
-500
HEALTHY CONTROLS
HIV ONLY
ACTIVE TB ONLY
HIV-ASSOC.TB
HK-MTB
p<0.0001
p=0.0093
p=0.0280
IFNg+ MAIT cell activation (HLA-DR MFI)
10000
5500
1000
1000
0
HEALTHY CONTROLS
HIV ONLY
ACTIVE TB ONLY
HIV-ASSOC.TB
E
HIV ONLY
p=0.4885
HLA-DR MFI (MAIT cells)
500
400
300
200
100
0
-100
<200
200-350
>350
CD4 Count (cells/uL)
HIV-ASSOC.TB
p=0.0396
p=0.0040
p=0.0845
HLA-DR MFI (MAIT cells)
600
400
200
0
-200
<200
200-350
>350
CD4 Count (cells/uL)

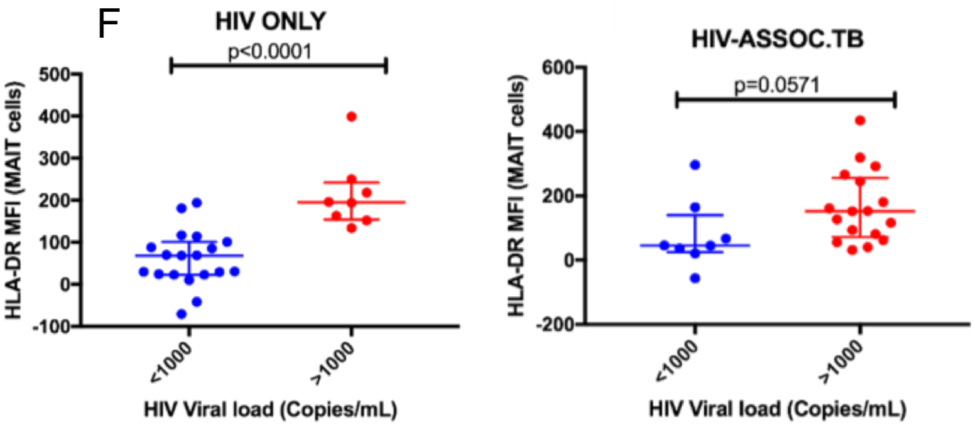


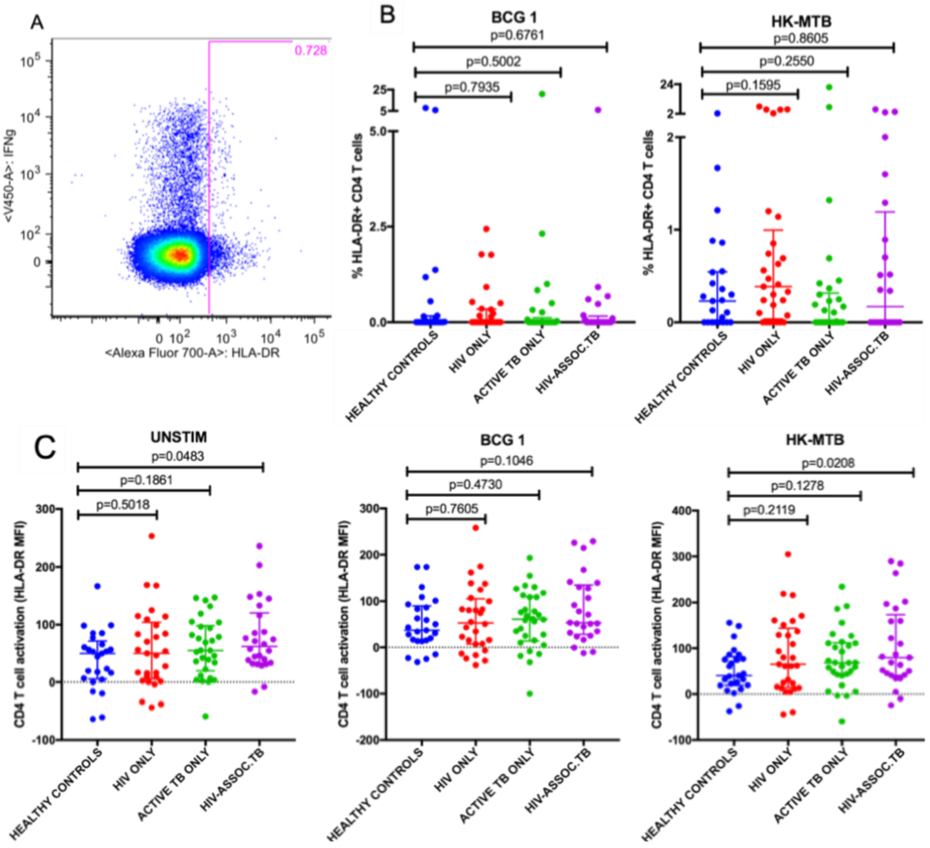
Figure 3.20: Activation of MAIT cells and the association of HIV, active TB and HIV-associated TB with the activation of MAIT cells. A) Representative flow cytometry plots showing the frequencies of MAIT cells expressing HLA-DR. **B)** Frequencies of MAIT cells expressing HLA-DR in response to BCG 1 and HK-*M.tb* stimulation. **C)** MFI of HLA-DR on total unstimulated, BCG 1 and HK-*M.tb* stimulated MAIT cells. **D)** MFI of HLA-DR on MAIT cells expressing IFN-γ in response to BCG and HK-*M.tb* stimulation. **E)** Association between CD4 counts and the MFI of HLA-DR on MAIT cells from people with HIV infection (HIV only and HIV-associted TB). **F)** Association between HIV viral load and the MFI of HLA-DR on MAIT cells from people with HIV infection (HIV only and HIV-associted TB). Data presented as scatter plots with lines representing the median and IQR. P-values represent the Mann-Whitney U test.

3.4.2 CD4 T cell activation

The frequencies of CD4 T cells expressing HLA-DR were similar between the different study groups and the healthy controls in response to BCG 1 and HK-*M.tb* stimulation. In response to BCG 5 stimulation, the frequencies of CD4 T cells expressing HLA-DR were significantly higher in individuals with HIV-associated TB compared to healthy controls (p=0.0034) (Table A6).

The MFI of HLA-DR on total CD4 T cells was significantly higher in individuals with HIV-associated TB compared to healthy controls (p=0.0483) while total CD4 T cells from individuals with HIV only and active TB only had similar HLA-DR MFI to healthy controls in the unstimulated controls (Figure 3.21C). In BCG 1 stimulation, the MFI of HLA-DR on total CD4 T cells in the different study groups were similar to healthy controls. HK-*M.tb* stimulation resulted in a higher HLA-DR MFI in individuals with HIV-associated TB only compared to healthy controls (p=0.0208) (Figure 3.21C). In response to BCG 5 stimulation, total CD4 T cells from individuals with HIV-associated TB were observed to have a significantly elevated HLA-DR MFI compared to healthy controls (p=0.0004) (Table A6). Finally, following PHA stimulation, total CD4 T cells from individuals with HIV-associated TB had significantly elevated HLA-DR MFI compared to healthy controls (p=0.0393) (Table A6).

CD4 T cells expressing IFN-*γ*, the MFI of HLA-DR on CD4 T cells expressing IFN-*γ* were remarkably higher in individuals with active TB only (p=0.0002) and in individuals with HIV-associated TB (p=0.0001) compared to healthy controls (Figure 3.21D) in response to BCG 1. Following HK-*M.tb* stimulation, only individuals with active TB only and individuals with HIV-associated TB had remarkably higher HLA-DR MFI compared to healthy controls (p<0.0001) (Figure 3.21D). Upon BCG 5 stimulation, CD4 T cells expressing IFN-*γ* had significantly elevated HLA-DR MFI in individuals with active TB only (p=0.0159) and in individuals with HIV-associated TB (p=0.0002) compared to healthy controls (Table A6). Finally, CD4 T cells expressing IFN-*γ* from individuals with HIV-associated TB had elevated HLA-DR MFI compared to healthy controls in response to PHA stimulation (p=0.0017) (Table A6).



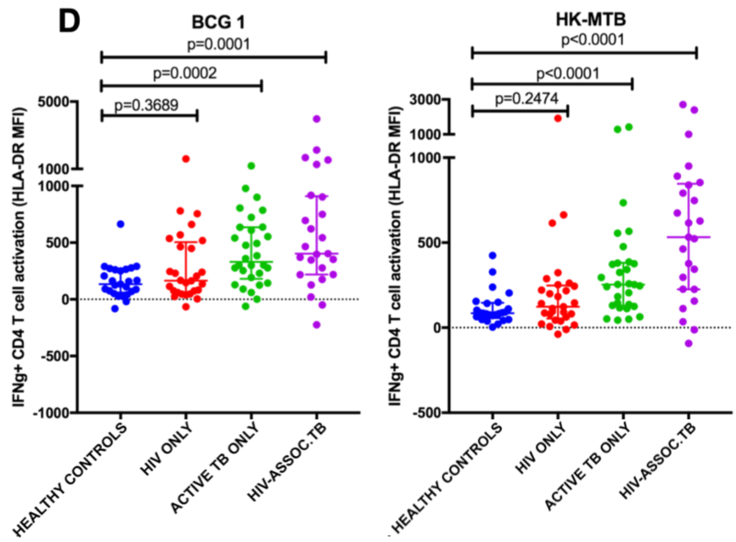


Figure 3.21: Activation of CD4 T cells and the association of HIV, active TB and HIV-associated TB with the activation of CD4 T cells. A) Representative flow cytometry plots showing the frequencies of CD4 T cells expressing HLA-DR. **B)** Frequencies of CD4 T cells expressing HLA-DR in response to BCG 1 and HK-*M.tb* stimulation. **C)** MFI of HLA-DR on total unstimulated, BCG 1 and HK-*M.tb* stimulated CD4 T cells. **D)** MFI of HLA-DR on CD4 T cells expressing IFN-γ in response to BCG and HK-*M.tb* stimulation. Data presented as scatter plots with lines representing the median and IQR. P-values represent the Mann-Whitney U test.

3.4.3 CD8 T cell activation

The frequencies of CD8 T cells expressing HLA-DR were similar between the study groups and healthy controls under BCG, HK-*M.tb* and PHA stimulation. In unstimulated controls, BCG 1 and HK-*M.tb* stimulation, total CD8 T cells from individuals with HIV-associated TB had significantly higher HLA-DR MFI compared to healthy controls (unstimulated controls [p=0.0004], BCG 1 stimulation [p=0.0007], HK-*M.tb* stimulation [p=0.0002]) (Figure 3.22C). With BCG 5 stimulation, the MFI of HLA-DR on total CD8 T cells were increased in individuals with active TB (p=0.0462) and individuals with HIV-associated TB (p=0.0017) compared to healthy controls (Figure 3.22C). Furthermore, only total CD8 T cells from individuals with HIV-associated TB had an elevated MFI of HLA-DR compared to healthy controls (p=0.0181) in response to PHA stimulation (Table A7).

Individuals with active TB only and HIV-associated TB had a significantly elevated HLA-DR MFI on CD8 T cells expressing IFN-*γ* compared to healthy controls (active TB only[p=0.0038], HIV-associated TB [p=0.0048]) in response to BCG 1(Figure 3.22D). Following HK-*M.tb* stimulation, the MFI of HLA-DR on CD8 T cells expressing IFN-*γ* were significantly elevated in individuals with active TB (p=0.0071) and individuals with HIV-associated TB (p=0.0001) while individuals with HIV only had similar MFI of HLA-DR to healthy controls.

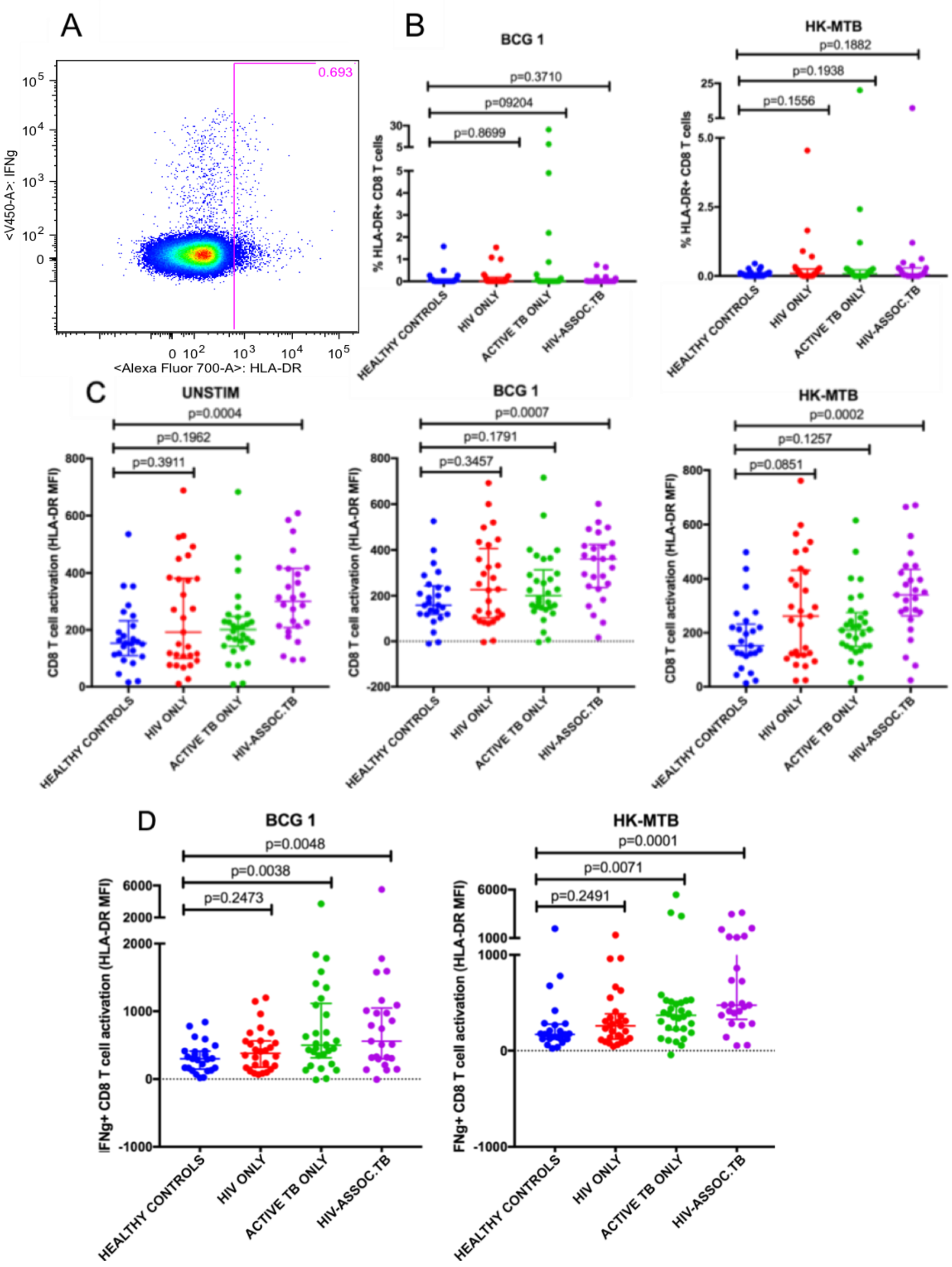


Figure 3.22: Activation of CD8 T cells and the association of HIV, active TB and HIV-associated TB with the activation of CD8 T cells. A) Representative flow cytometry plots showing the frequencies of

CD8 T cells expressing HLA-DR. **B)** Frequencies of HLA-DR expressing CD8 T cells in response to BCG 1 and HK-*M.tb* stimulation. **C)** MFI of HLA-DR on total unstimulated, BCG 1 and HK-*M.tb* stimulated CD8 T cells. **D)** MFI of HLA-DR on CD8 T cells expressing IFN-γ in response to BCG and HK-*M.tb* stimulation. Data presented as scatter plots with lines representing the median and IQR. P-values represent the Mann-Whitney U test.

3.4.4 APC Activation

3.4.4.1 HLA-DR expression

Under the unstimulated condition, the MFI of HLA-DR on monocytes were similar between the groups. DCs from individuals with HIV-associated TB however, were observed to have significantly elevated MFI of HLA-DR compared to healthy controls (p=0.0244) while the other groups had similar HLA-DR MFI to healthy controls (Figure 3.23A).

Upon BCG 1 stimulation, the MFI of HLA-DR was significantly lower in monocytes from individuals with active TB only compared to healthy controls (p=0.0136) and no differences were observed between monocytes from individuals with HIV only nor HIV-associated TB and healthy controls (Figure 3.23B). DCs from the different study groups were also observed to have similar HLA-DR MFI to healthy controls.

Similar to BCG 1 stimulation, the MFI of HLA-DR on monocytes from all groups except those from individuals with active TB were similar to healthy controls upon BCG 5 stimulation. Monocytes from individuals with active TB only were observed to have significantly lower MFI of HLA-DR to healthy controls (p=0.0409) (Table A8). No significant differences were observed in the MFI of HLA-DR on DCs from all study groups compared to healthy controls. Upon stimulation with HK-*M.tb* and LPS, the MFI of HLA-DR on all APCs were similar to those from healthy controls (Table A9).

Furthermore, compared to the unstimulated samples, BCG and HK-M.tb stimulatated samples seem to have a lower MFI of HLA-DR suggesting that stimulation results in the downregulation of HLA-DR Figure 3.23.

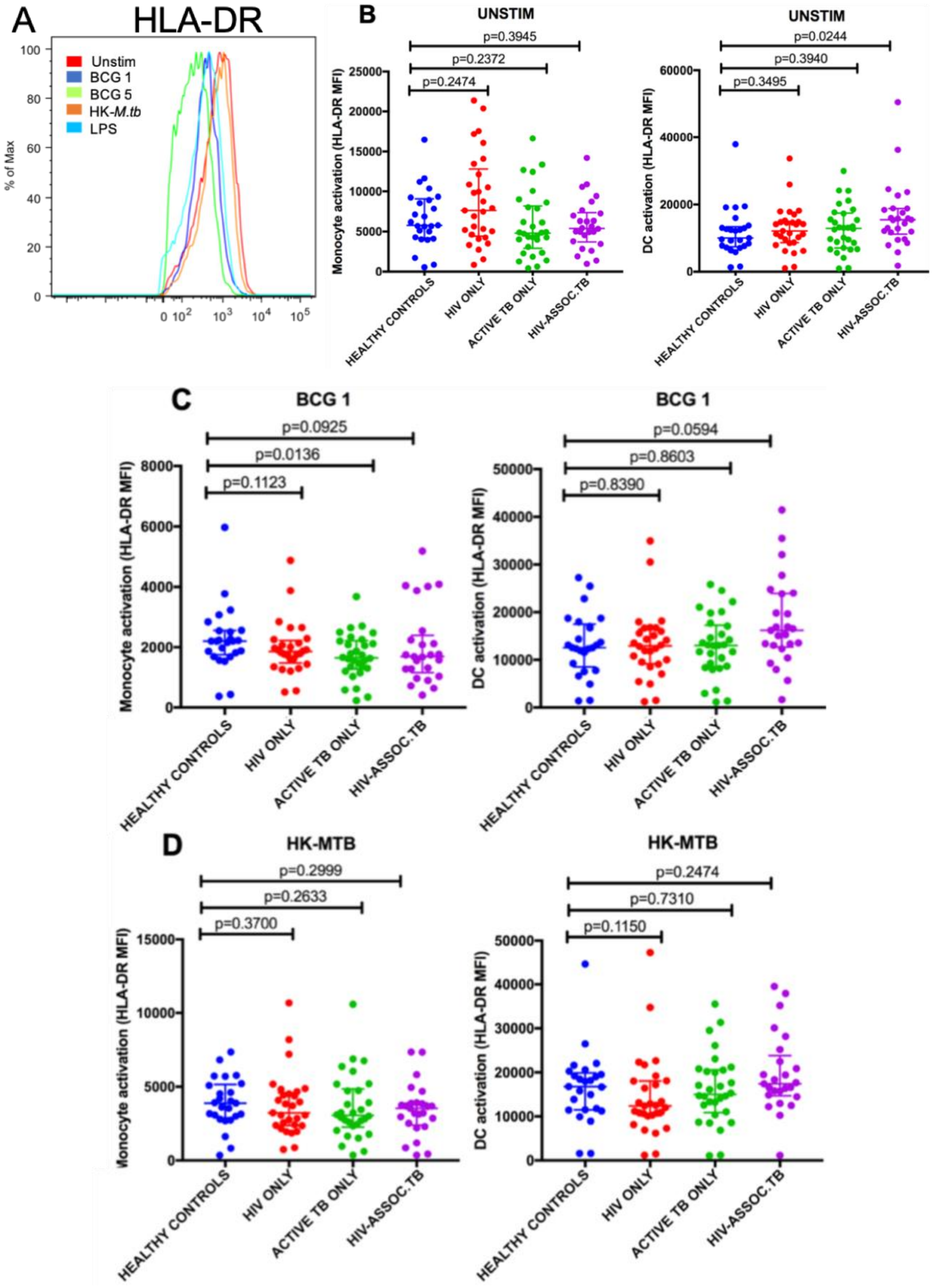


Figure 3.23: MFI of HLA-DR on APCs and the association of HIV, active TB and HIV-associated TB with HLA-DR expression on APCs. **A)** Histogram showing expression of HLA-DR in unstimulated sample (red square) and in response to BCG 1 (dark blue square), BCG 5 (light green square), HK-*M.tb* (orange square) and LPS (Light blue square) stimulated samples. The MFI of HLA-DR under **B)** unstimulated, **C)**

BCG 1 and **D)** HK-*M.tb* stimulated conditions. Data presented as scatter plots with lines representing the median and IQR. P-values represent the Mann-Whitney U test.

3.4.4.3 CD40 expression

CD40 is a cell surface marker that is expressed on the APCs. Interactions between CD40 and its ligand, CD40L, which is expressed in activated T cells leads to strong activation of APCs, (Hernandez *et al*., 2019). Activation of APCs by ligation of CD40 has been reported to increase the stimulatory functions of these cells, resulting in the production of extremely high concentrations of IL-12 which in turn enhances the Th_1 responses in T cells (Celia *et al*., 1996). We therefore sought to assess the levels of CD40 expression as another marker of APC activation in response to stimulation in each of our study groups and compare with healthy controls.

In the unstimulated controls, the MFI of CD40 on APC from all groups were similar to the MFI of CD40 in healthy controls (Figure 3.24A). Upon BCG 1 and BCG5 stimulations, a similar trend was observed in which the MFI of CD40 on APC from all study groups was similar to the MFI of CD40 on APCs from healthy controls (Figure 3.24B). The only exceptions were monocytes from individuals with active TB and DCs from individuals with HIV-associated TB. The monocytes from individuals with active TB only had a significantly lower MFI for CD40 (p=0.0409) (Table A8) and DCs from individuals with HIV-associated TB had significantly lower MFI of CD40 compared to healthy controls (p=0.0051) (Table A9). In response to stimulation with HK-*M.tb* and LPS, no differences in the MFI of CD40 were observed on APCs from different groups compared to healthy controls (Table A8 and A9). In addition, the stimulation with HK-M.tb was also observed to lead to an upregulation of CD40 expression.

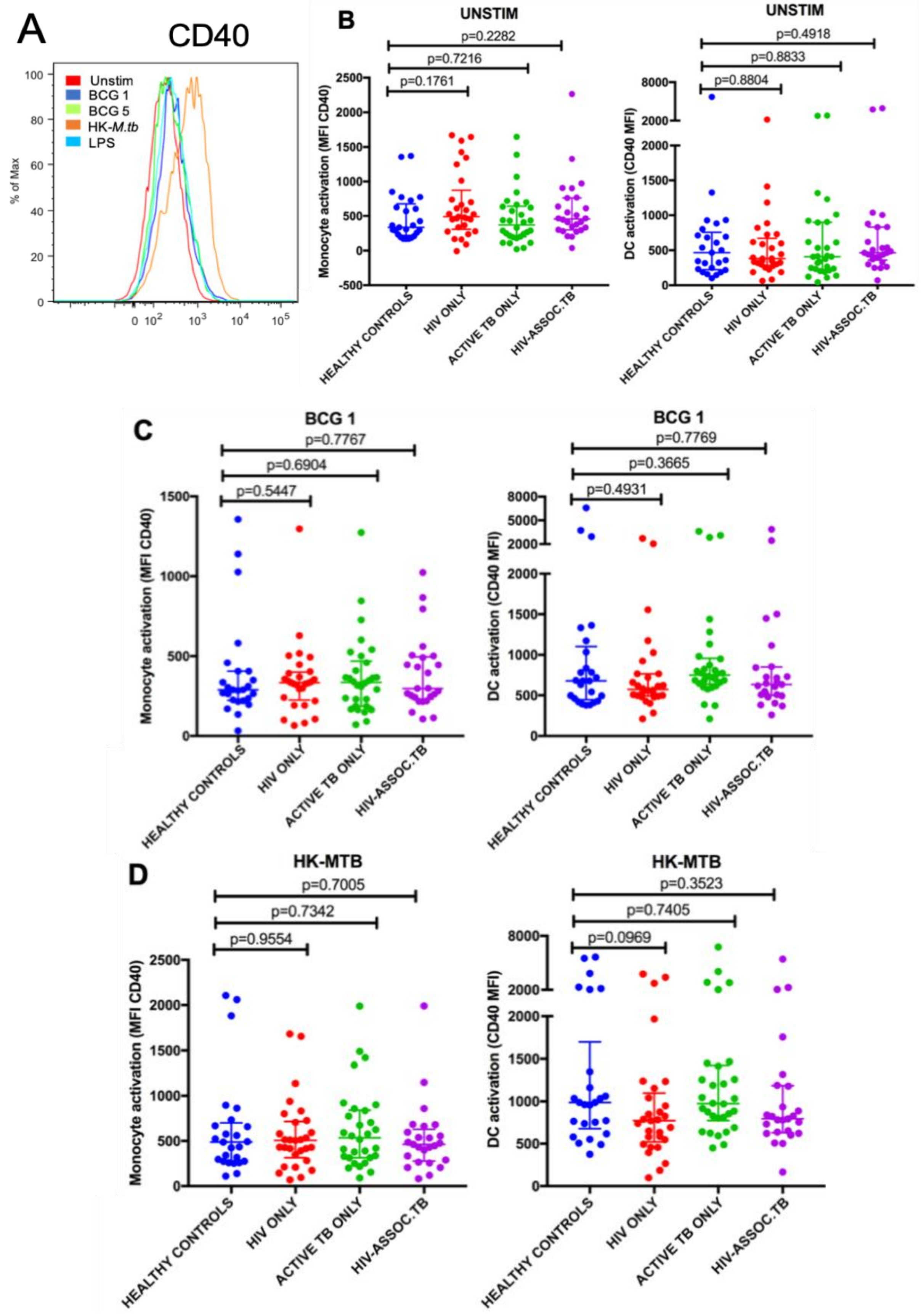


Figure 3.24: MFI of CD40 on APCs in response to bacterial stimulation. **A) Histogram showing** expression of CD40 in unstimulated sample (red square) and in response to BCG 1 (dark blue square), BCG 5 (light green square), HK-*M.tb* (orange square) and LPS (Light blue square) stimulated samples.

B), C) and **D)** represent the MFIs of CD40 in unstimulated, BCG 1 and HK-*M.tb* stimulated conditions, respectively. Data presented as scatter plots with lines representing the median and IQR. P-values represent the Mann-Whitney U test.

3.4.5 Conclusions

In conclusion, these data suggest that there is an increased basal MAIT cell activation and IFN-*γ* specific MAIT cell activation in the context of TB disease (active TB and HIV associated TB), as observed by the increased HLA-DR MFI. In addition, active TB is associated with increased IFN-*γ* specific CD4 and CD8 T cell activation, while in HIV-associated TB there is an increased basal activation and increased IFN-*γ* -specific activation for both the CD4 and CD8 T cells. APC activation was observed to be similar between all groups and healthy controls.

3.5 Association of TB, HIV and HIV associated TB with PD-1 expression

3.5.1 MAIT cells

To evaluate whether the reduced function in TB and HIV-associated TB may be due to immune inhibition, we assessed the expression of PD-1, a marker of inhibition on MAIT cells in a subset of study participants. The frequencies of MAIT cells expressing PD-1 were higher in individuals with active TB only compared to healthy controls (p=0.0232) (Figure 3.25A). Furthermore, the total PD-1 expression was lower in all participant groups compared to healthy controls, but this difference was only significant in individuals with active TB only (p=0.0005) (Figure 3.25B).

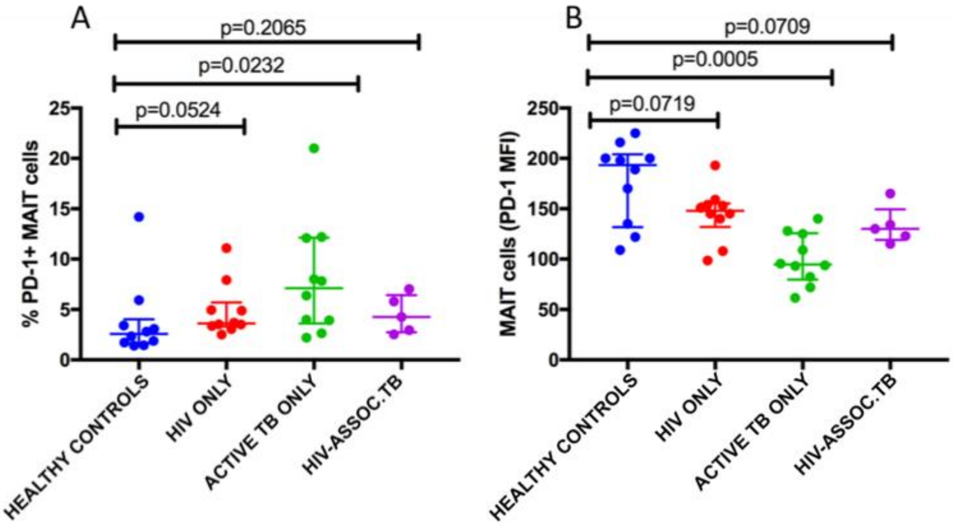


Figure 3.25: The expression of PD-1 on MAIT cells and the association of HIV, active TB and HIV-associated TB with PD-1 expression. A) Graph showing the frequencies of MAIT cells expressing PD-1. B) MFI of PD-1 on MAIT cells. Data presented as scatter plots with lines representing the median and IQR. P-values represent the Mann-Whitney U test.

3.5.2 CD4 T cells

When we also examined CD4 T cells from these same participants, the frequencies of CD4 T cells expressing PD-1 were significantly higher in all 3 groups compared to healthy controls, (individuals with HIV only [p<0.0001], Active TB only [p=0.0433], HIV-associated TB [p=0.0080]) (Figure 3.26A). No significant differences were observed in the MFI of PD-1 among CD4 T cells from different groups compared to healthy controls.

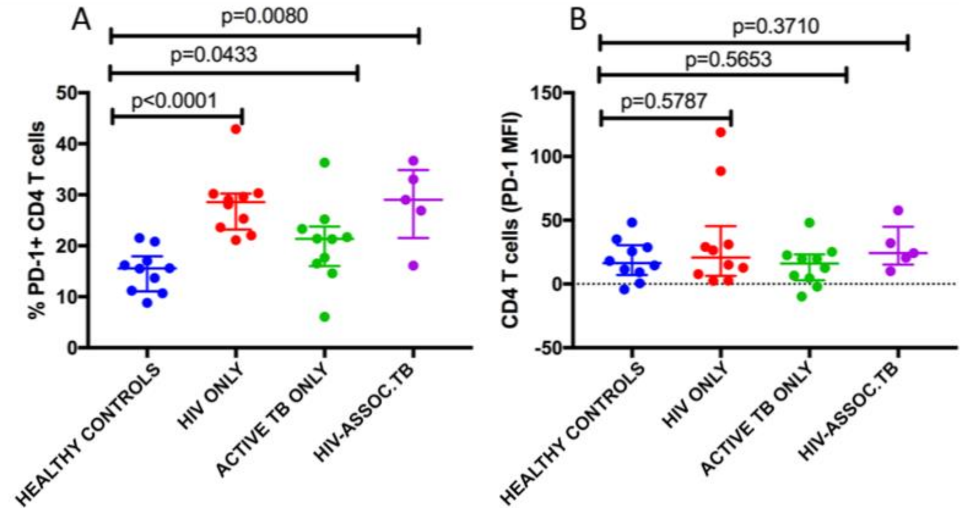


Figure 3.26: The expression of PD-1 on CD4 T cells and the association of HIV, active TB and HIV-associated TB with PD-1 expression. A) Graph showing the frequencies of CD4 T cells expressing PD-1. B) MFI of PD-1 on CD4 T cells. Data presented as scatter plots with lines representing the median and IQR. P-values represent the Mann-Whitney U test.

3.5.3 CD8 T cells

When we looked at PD-1 expression on CD8 T cells, we found that frequencies of CD8 T cells expressing PD-1 were higher in individuals with HIV only compared to healthy controls (p=0.0433), while in individuals with active TB only and those with HIV-associated TB, these frequencies were similar to healthy controls (Figure 3.27A).

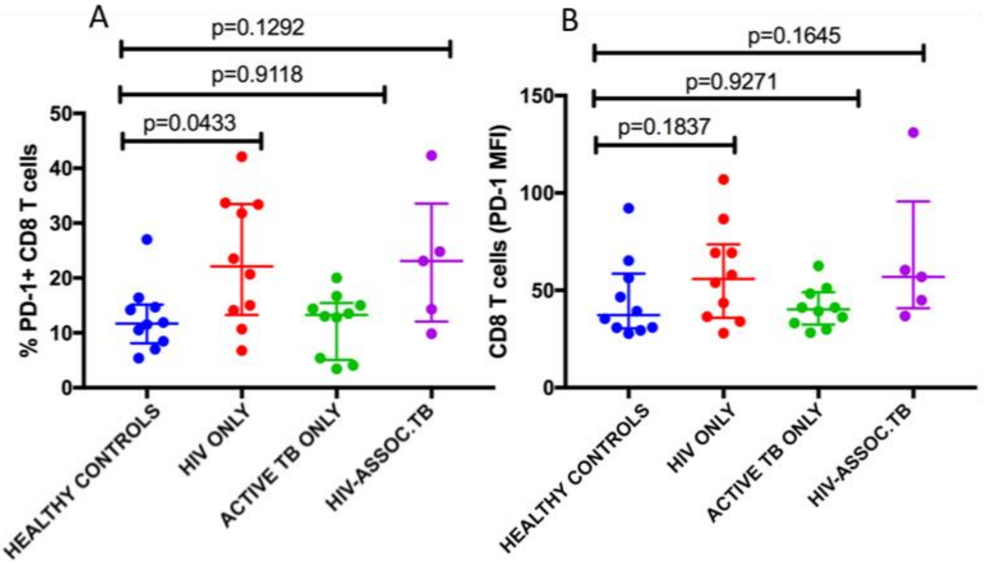


Figure 3.27: The expression of PD-1 on CD8 T cells and the association of HIV, active TB and HIV-associated TB with PD-1 expression. A) Graph showing the frequencies of CD8 T cells expressing PD-1. B) MFI of PD-1 on CD8 T cells. Data presented as scatter plots with lines representing the median and IQR. P-values represent the Mann-Whitney U test.

3.5.4 Monocytes

In addition to assessing the expression of PD-1 on T cells, we also assessed the expression of PD-1 on monocytes and the MFI of PD-1 among these cells and no significant differences were observed in the frequencies of PD-1 expressing monocytes between the groups and healthy controls (Figure 3.28). When the MFI of PD-1 was assessed however, we found that the MFI was significantly lower in all groups compared to healthy controls (healthy controls [p=0.0014], active TB only [p=0.0003] and HIV-associated TB [p=0.0143]) (Figure 3.28B).

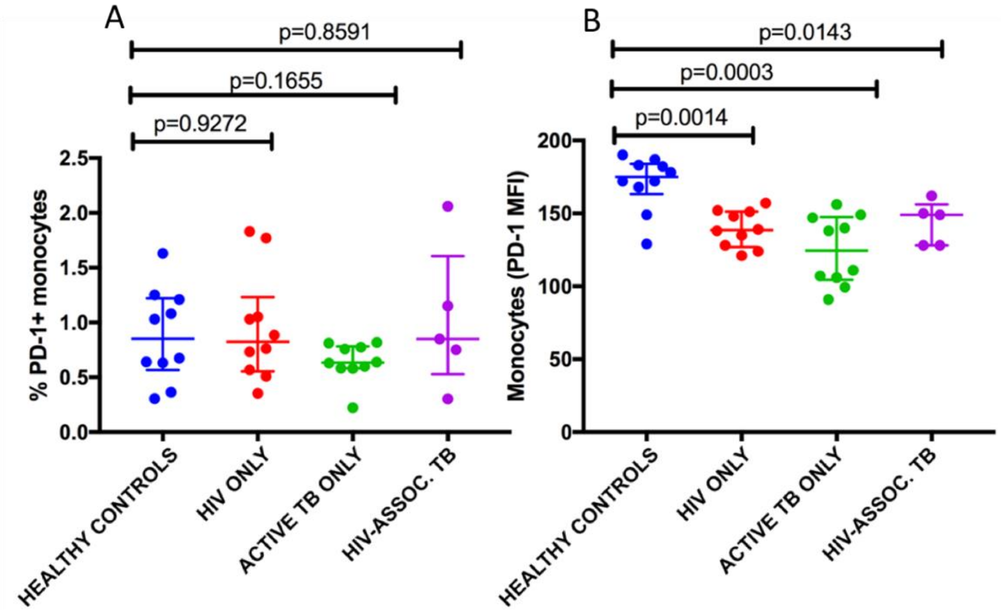


Figure 3.28*: The expression of PD-1 on monocytes and the association of HIV, active TB and HIV-associated TB with PD-1 expression.* A) Graph showing the frequencies of monocytes expressing PD-1. B) MFI of PD-1 on monocytes. Data presented as scatter plots with lines representing the median and IQR. P-values represent the Mann-Whitney U test.

3.5.5 Conclusions

Together, there is an increase in frequencies of MAIT cells expressing PD-1 in persons with active TB, while this was similar between people with HIV infection (HIV only and HIV-associated TB) and healthy controls. Total expression per cell of PD-1 (MFI) was however higher in healthy controls compared to other groups. In addition, there are also increased frequencies of CD4 and CD8 T cells expressing PD-1 in people with HIV and an increase in CD4 T cells expressing PD-1 in people with TB disease (active TB only and HIV-associated TB). The expression (MFI) of PD-1 was reduced in monocytes from persons with HIV infection only, active TB only and persons with HIV-associated TB.

3.6 Soluble cytokine secretion

Following the 18-hour stimulation, we collected supernatants to measure concentrations of cytokines produced in response to stimulation. Luminex assay was used for the quantification of the following cytokines: IL-12p40, IL-12p70, IFN-α2, IFN-γ TNF-α and IL-10. An ELISA assay was used for the quantification of IL-18. For analyses, we divided the cytokines into 2 groups,

1) Cytokines important for MAIT cell activation, IL-12 (IL-12p40 and IL-12p70), IFN-α2 and IL-18; 2) the anti-inflammatory cytokine (IL-10) and T cell cytokines (IFN-γ and TNF-α).

3.6.1 Cytokines involved in MAIT cell activation

IL-12 can be produced as a homodimer (IL-12p40) and a heterodimer (IL-12p70), our panel included both dimers. Figure 3.29 shows the summary data for soluble IL-12p40, IL-12p70, IL-18 and IFN-α2 after BCG 1 stimulation measured from cell supernatants, the values represent background subtracted values in which the concentrations from unstimulated controls were subtracted from the mycobacterial-specific responses. We found no significant differences in the concentrations of soluble IL-12p40 between the groups compared to healthy controls (Figure 3.29A). For IL-12p70, individuals with active TB only produced significantly lower cytokine compared to healthy controls (p=0.0190) while other groups showed similar concentrations of IL-12p70 (Figure 3.29B).

The concentration of soluble IL-18 was significantly higher in individuals with active TB only compared to healthy controls (p=0.0374). The HIV groups had similar concentrations of IL-18 to healthy controls (Figure 3.29C). Significantly lower concentrations of IFN-α2 in individuals with HIV only compared to healthy controls (p=0.0018) were observed while other groups had comparable cytokine concentrations to healthy controls (Figure 3.29D).

In response to HK-*M.tb* stimulation, the concentrations of soluble IFN-α2 were significantly lower in individuals with HIV only (p=0.0154) and individuals with active TB only (p=0.0162) compared to healthy controlsIL-18 concentration was higher in individuals with active TB only (p=0.0317) compared to healthy controls. Similar observations were made for the concentration of IL-18 in which concentrations were higher in this group compared to healthy controls (p=0.0015). Furthermore, IL-12p70 was lower in individuals with active TB (p=0.0167) (Table A10).

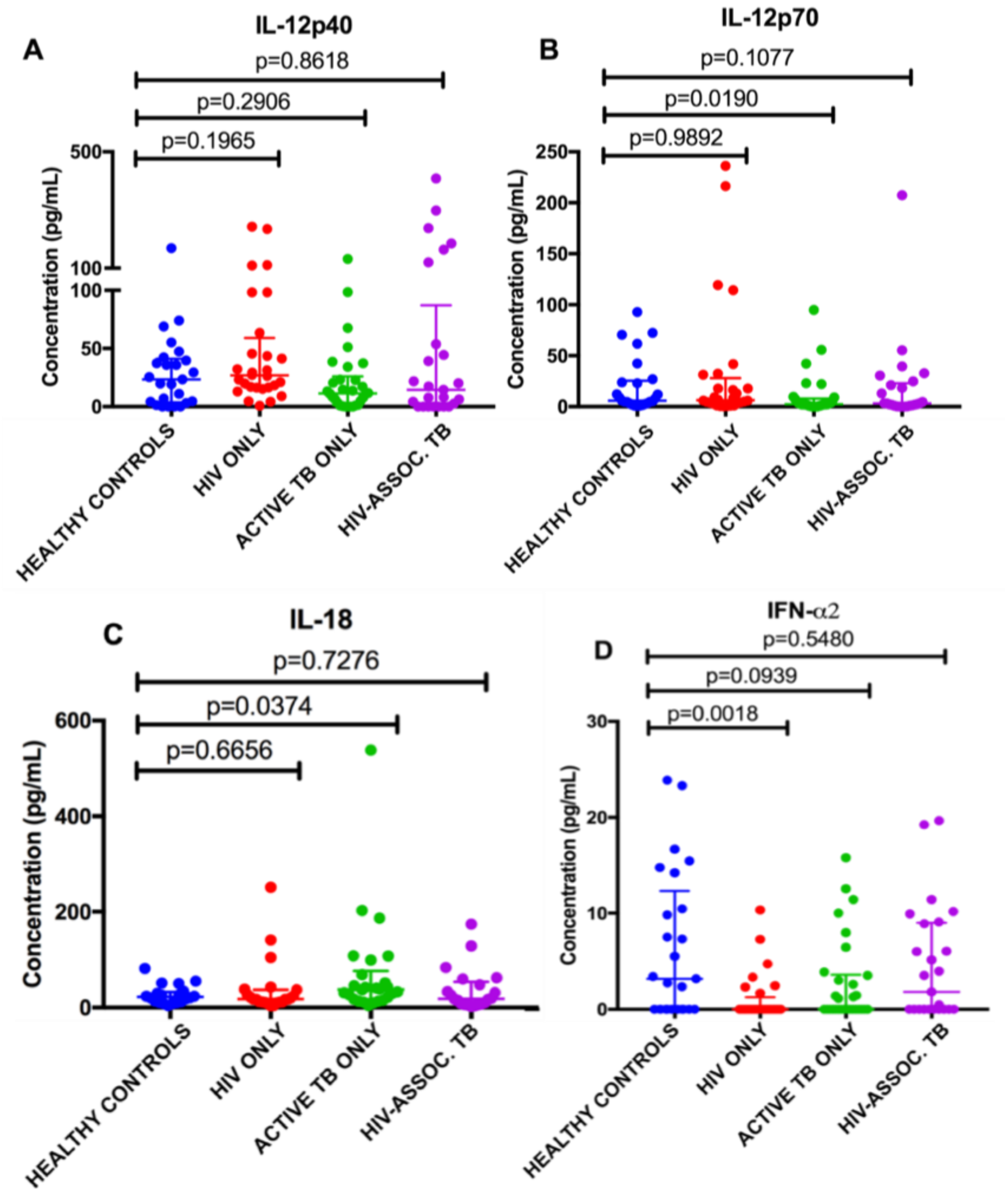


Figure 3.29: Concentrations of MAIT cell activating cytokines in supernatants of PBMCs stimulated with BCG 1, concentrations represent the background subtracted values in which concentrations from unstimulated controls were subtracted from the BCG-specific responses. A) IL-12p40. B) IL-12p70, C) IL-18 and D) IFN-α2. Data presented as scatter plots with lines representing the median and IQR. P-values represent the Mann-Whitney U test.

3.6.2 Anti-inflammatory and T cell cytokines

For the second group of cytokines, the concentrations of soluble IFN-γ were remarkably reduced in all groups compared to healthy controls ($p<0.0001$) in response to BCG 1 stimulation (Figure 3.30B). No significant differences in concentrations of IL-10 and TNF-α were observed between the groups in response to BCG 1 stimulation (Figure 3.30A and C).

In response to HK-*M.tb*, the concentration of soluble IL-10 was significantly higher in individuals with active TB only compared to healthy controls (p=0.0317) while the other 2 groups had similar concentrations to healthy controls (Table A10). Concentrations of IFN-γ were significantly lower in all study groups compared to healthy controls (HIV only ([p=0.0010], active TB only [p=0.0002] and HIV-associated TB [p<0.0001]). Finally, the concentrations of soluble TNF-α was similar in all groups and healthy controls (Table A10).

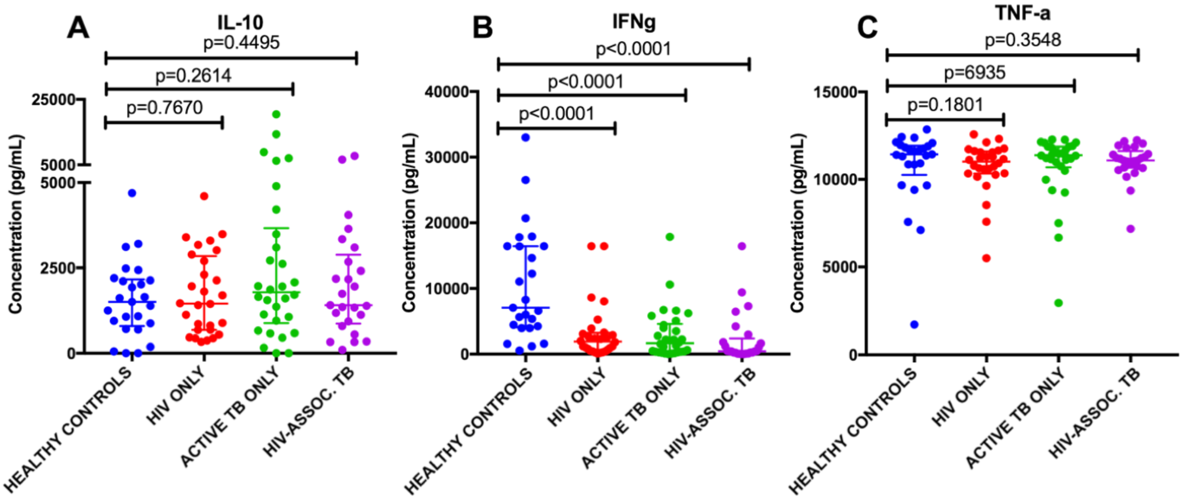


Figure 3.30: Production of soluble cytokines in supernatants of PBMCs stimulated with BCG 1, concentrations represent the background subtracted values in which concentrations from unstimulated controls were subtracted from the BCG-specific responses. A) Soluble IL-10, B) IFN-γ and C) TNF-α. Data presented as scatter plots with lines representing the median and IQR. P-values represent the Mann-Whitney U test.

When we assessed background levels of soluble cytokines in the unstimulated controls, we found that individuals with HIV-associated TB had significantly lower concentrations of IFN-γ (p=0.0062) and IL-12p70 (p=0.0387) compared to healthy controls. However, background concentrations for these cytokines were similar between the other groups (HIV only and active TB only) and healthy controls (Table A11). Furthermore, the background concentrations of all other cytokines assessed were similar in all groups (Table A11).

3.6.3 Conclusions

In conclusion, there were lower concentrations of IFN-α2 and IFN-γ in people with HIV infection. Active TB was associated with lower concentrations of soluble IL-12p70 and IFN-γ, and increased concentrations of soluble IL-18. HIV associated TB was also associated with lower concentrations of soluble IFN-γ.

3.7 Functional interactions between innate cells and MAIT cells

We next sought to understand how the presence of active TB, HIV infection and HIV-associated TB in patients affects the *ex vivo* cellular interactions between the APCs and MAIT cells in samples drawn from these patient groups. To perform these analyses, we used Spearman's correlation to investigate the correlation between 1) APC infection and MAIT cell functional profiles (IFN-γ and CD107a expression) and 2) MAIT activating soluble cytokines and MAIT cell function profiles.

3.7.1 Correlations between infected APC and MAIT cell functions

3.7.1.1 Interactions between infected monocytes and MAIT cells

In response to BCG 1 stimulation, from the Spearman rank correlations, we found that there were significantly correlated relationships between the frequencies of GFP+ APCs and MAIT cell responses in individuals with HIV-associated TB: there was a significant negative correlation between the frequencies of GFP+ monocytes and the frequencies of MAIT cells expressing CD107a (p=0.0017, r=-0.594) (Figure 3.31D).

In response to BCG 1 stimulation, individuals with HIV only exhibited a significant positive correlation between the frequencies of GFP+ monocytes and frequencies of CD4 T cells expressing CD107a (p=0.0297, r=0.4111). Additionally, there was a significant negative correlation between GFP+ monocytes and the MFI of HLA-DR on total CD4 T cells in individuals with HIV-associated TB (p=0.0420, r=0.4095). In CD8 T cells, there was only a strong negative correlation between the frequencies of GFP+ monocytes and the frequencies of CD8 T cells expressing CD107a in individuals with HIV-associated TB (p=0.0012, r=-0.6112) (Table A12).

Upon BCG 5 stimulation, there was a significant negative correlation between the frequencies of GFP+ monocytes and the frequencies of CD4 T cells expressing IFN-*γ* in healthy controls (p=0.0259, r=-0.4632). Furthermore, there was a positive correlation between the frequencies of GFP+ monocytes and the MFI of HLA-DR on total CD4 T cells in individuals with HIV only (p=0.0368, r=0.3963) (Table A12).

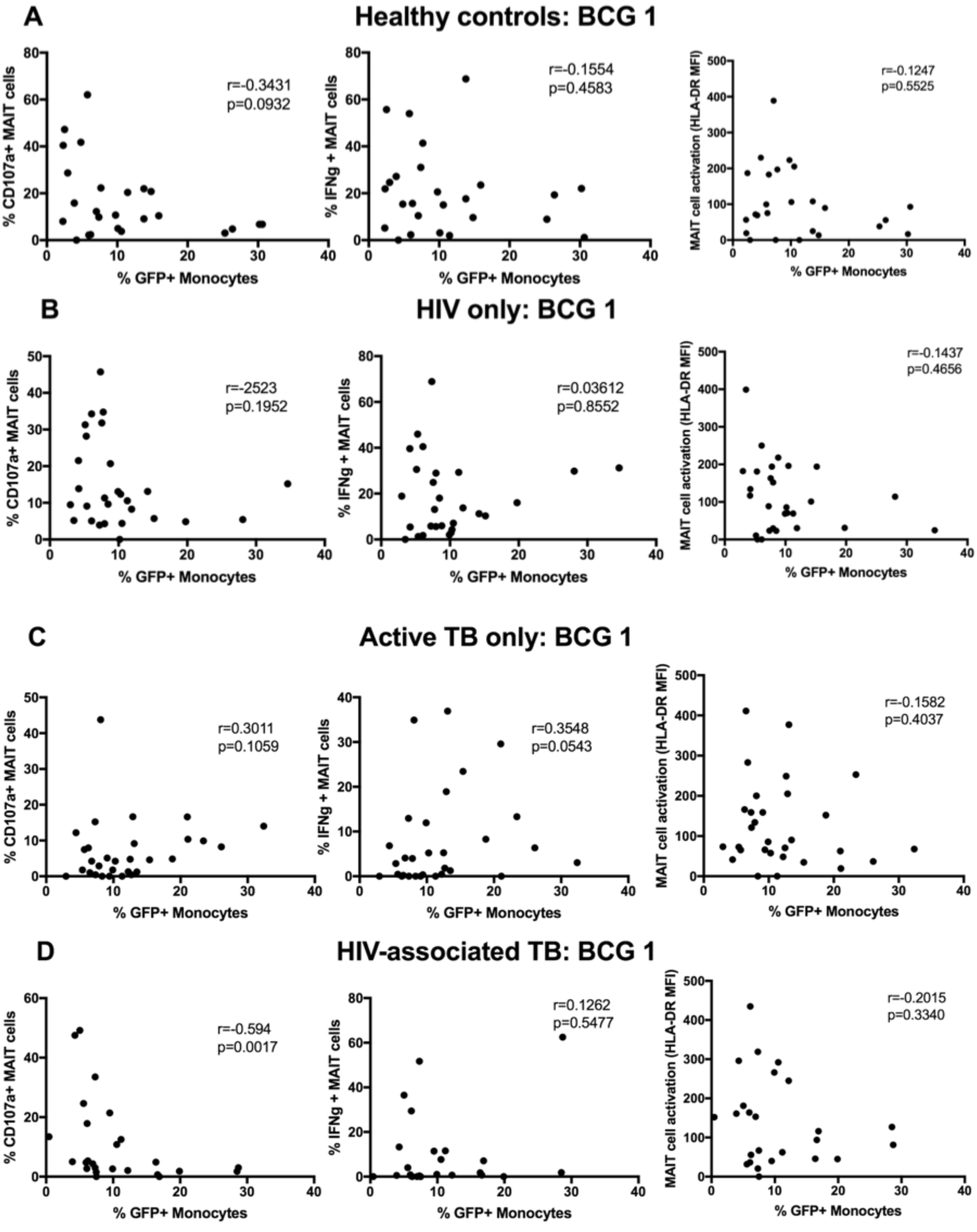


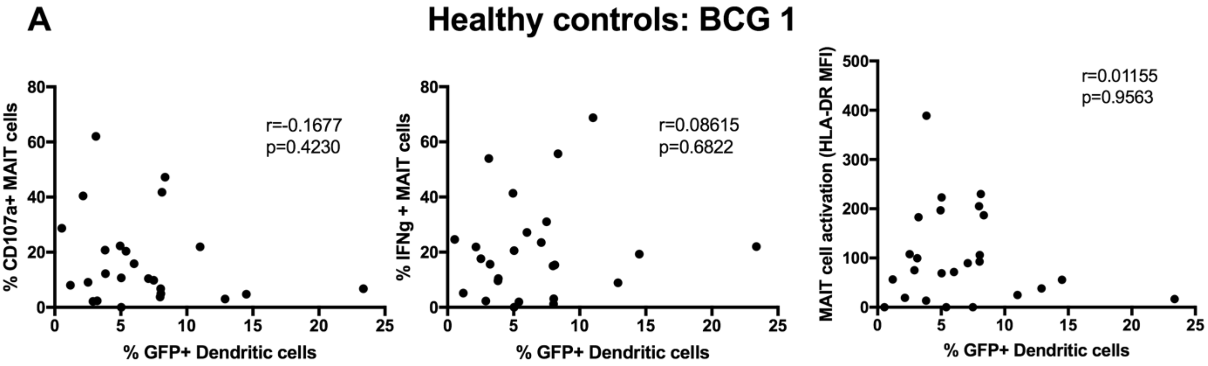
Figure 3.31: Correlation between the proportions of BCG-GFP infected monocytes and MAIT cell functional measures presented for each patient disease category separately. Frequencies of GFP+ monocytes plotted against MAIT cell functional measures (CD107a expression and IFN-γ production) and activation (HLA-DR expression) in A) Healthy controls, B) individuals with HIV only, C) active TB

and D) HIV-associated TB in response to BCG 1 stimulation. Correlations were evaluated using Spearman rank correlation.

3.7.1.2 Interactions between infected DCs and MAIT cells

Similar to monocytes, we only observed a significant positive correlation between the frequencies of GFP+ DCs and the frequencies of MAIT cells expressing IFN-*γ* in individuals with active TB only, using Spearman rank correlations (p=0.0132, r=0.4475) (Figure 3.32C). In addition to this observed correlation, we also observed a significant negative correlation between the frequencies of GFP+ DC and the MFI of HLA-DR in MAIT cells from individuals with HIV only (p=0.0364, r=-0.3972) (Figure 3.32A).

In healthy controls, there was a significant positive correlation between the frequencies of GFP+ DCs and the frequencies of MAIT cells expressing HLA-DR (p=0.0133, r=0.488) in response to BCG 1 (Table A13). In response to BCG 5, individuals with HIV-associated TB showed a significant negative correlation between the frequencies of GFP+ DCs and the MAIT cells expressing CD107a (p=0.0126, r=-0.4824). Additionally, significant positive correlations were evident between GFP+ DCs and the MFI of HLA-DR on MAIT cells expressing IFN-γ in individuals with active TB only (p=0.0193, r=0.4248) and HIV-associated TB (p=0.0042, r=0.5427) (Table A13).



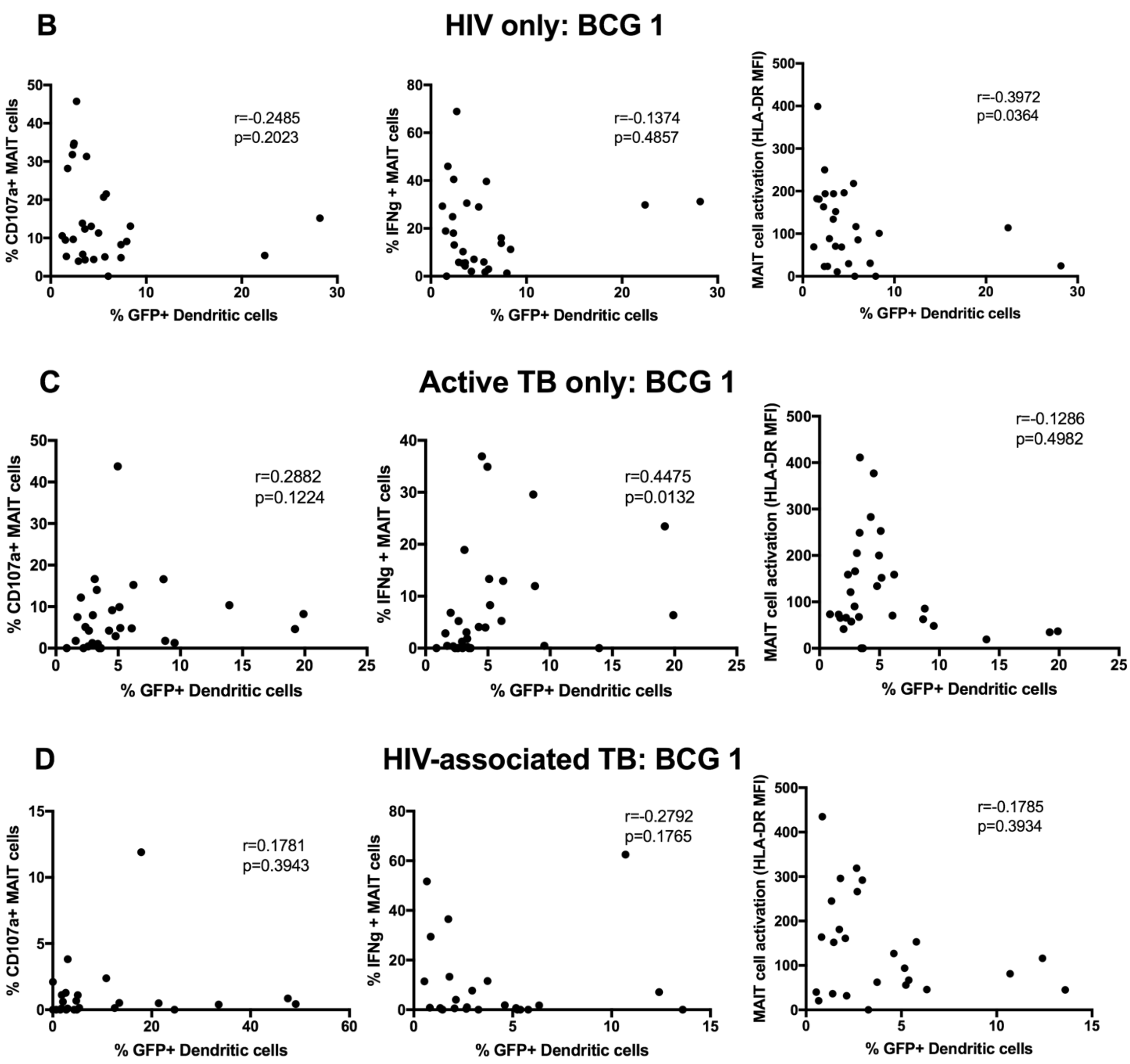


Figure 3.32*: Correlation between the proportions of BCG-GFP infected dendritic cells and MAIT cell functional measures presented for each patient disease category separately*. Frequencies of GFP+ monocytes plotted against MAIT cell functional measures (CD107a expression and IFN-γ production) and activation (HLA-DR expression) in A) Healthy controls, B) individuals with HIV only, C) active TB and D) HIV-associated TB in response to BCG 1 stimulation. Correlations were evaluated using Spearman rank correlation.

3.7.2 Correlations between soluble cytokines and MAIT cell functions

3.7.2.1 IL-12p40

Among healthy controls and individuals with HIV only, there were significant positive correlations between the concentrations of secreted IL-12p40 and the frequencies of MAIT cells expressing IFN-*γ* (healthy controls [p=0.0326, r=0.4285], and HIV only [0.0280, r=0.4152])

(Figure 3.33A and B). In individuals with active TB only, no significant correlations were observed between the concentrations of secreted IL-12p40 and MAIT cell responses. In individuals with HIV-associated TB, significant correlations were observed between the concentrations of secreted IL-12p40 and the frequencies of MAIT cells expressing CD107a (p=0.0048, r=0.5452) and IFN-*γ* (p=0.0180, r=0.469) (Figure 3.33D).

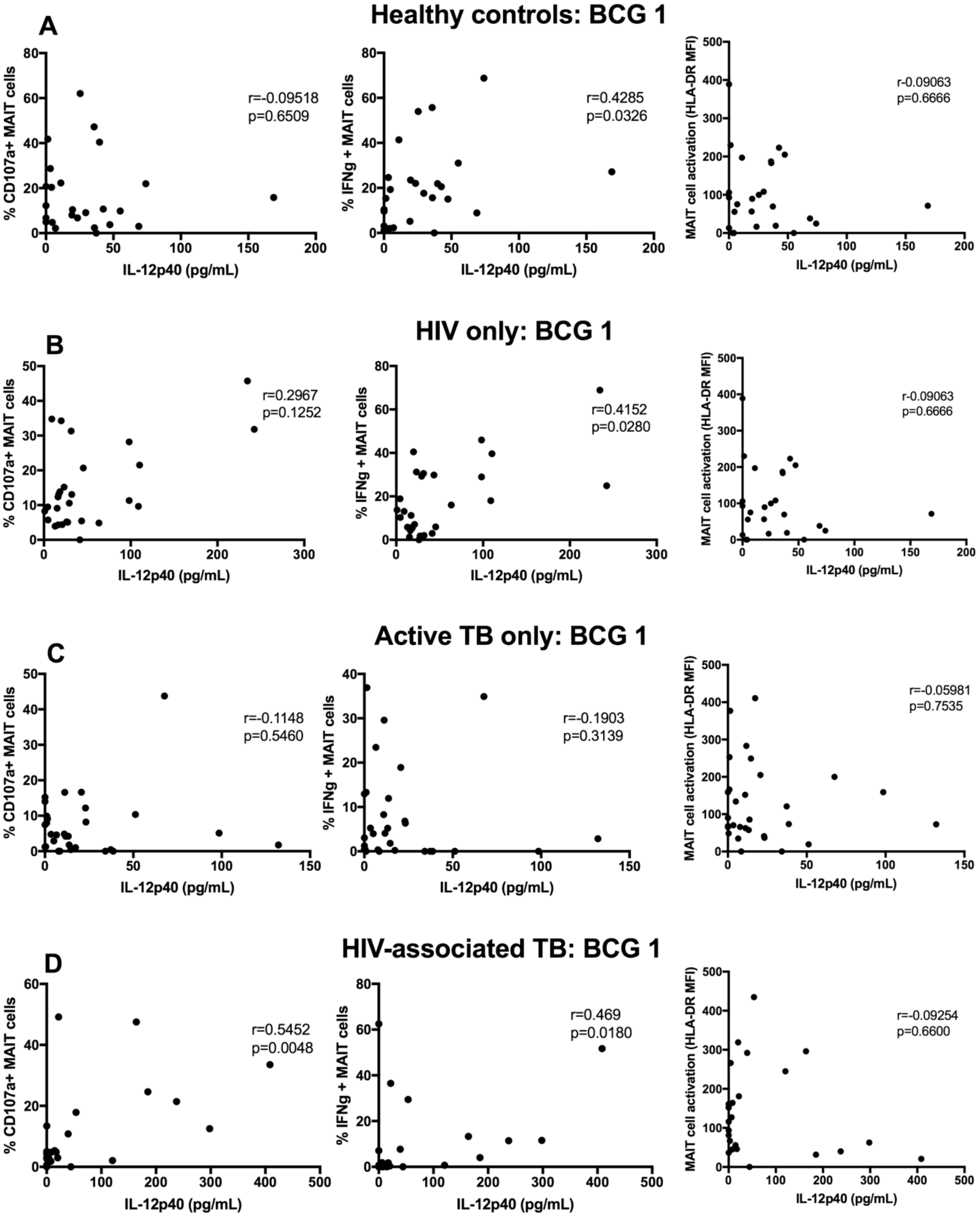
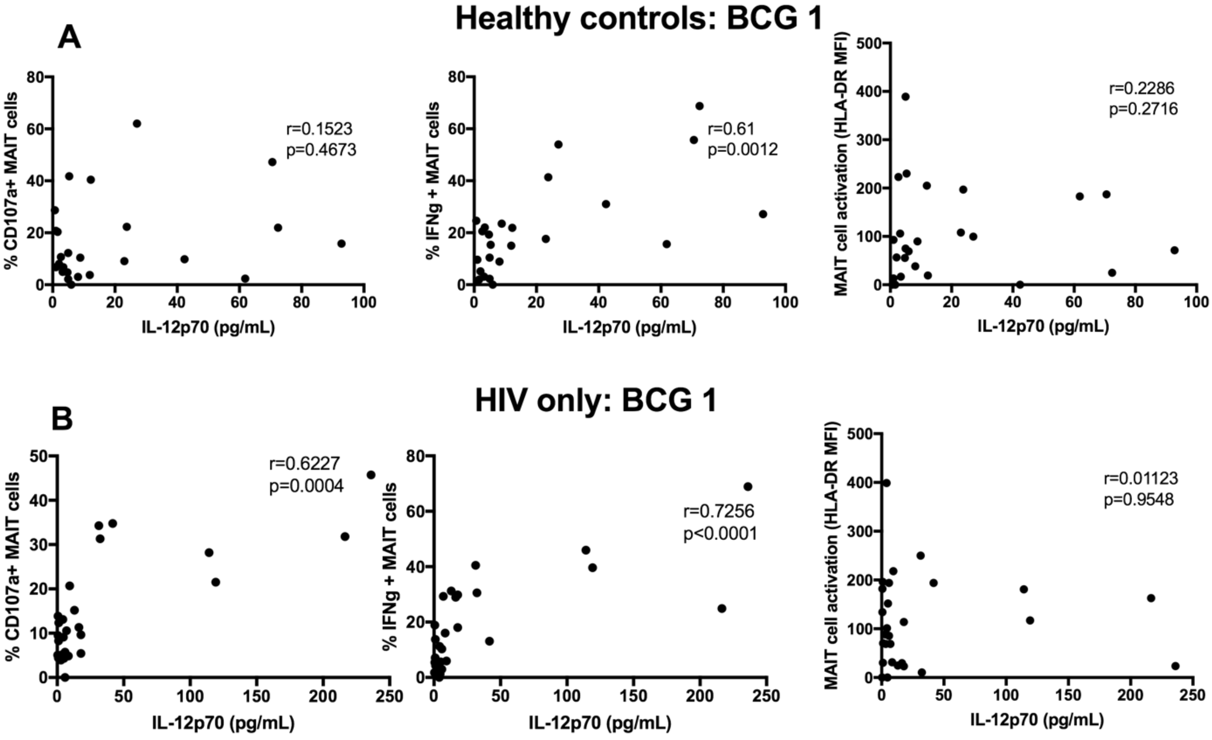


Figure 3.33: Correlations between soluble IL-12p40 and MAIT cell functional measures and activation in response to BCG 1 stimulation in A) Healthy controls, B) HIV only, C) Active TB only, D) HIV-associated TB. Correlations were evaluated using Spearman rank correlation.

3.7.2.2 IL-12p70

A significant positive correlation between the concentration of secreted IL-12p70 and the frequencies of MAIT cells expressing CD107a was observed in healthy controls (p=0.0012, r=0.61) (Figure 3.34A). We also observed significant positive correlations between the concentrations of secreted IL-12p70 and the frequencies of MAIT cells expressing CD107a (p=0.0004, r=0.7256) and IFN-*γ* (p<0.0001, r=0.6227) from individuals with HIV only (Figure 3.34B). Similar observations were made in individuals with HIV-associated TB in which there were significant positive correlations between the concentrations of secreted IL-12p70 and the frequencies of MAIT cells expressing CD107a (p=0.0002, r=0.0002) and IFN-*γ* (p<0.0001, r=0.6724) (Figure 3.34C and D). In individuals with active TB only, no significant correlations between the concentrations of secreted IL-12p70 and MAIT cell responses and activation were observed.



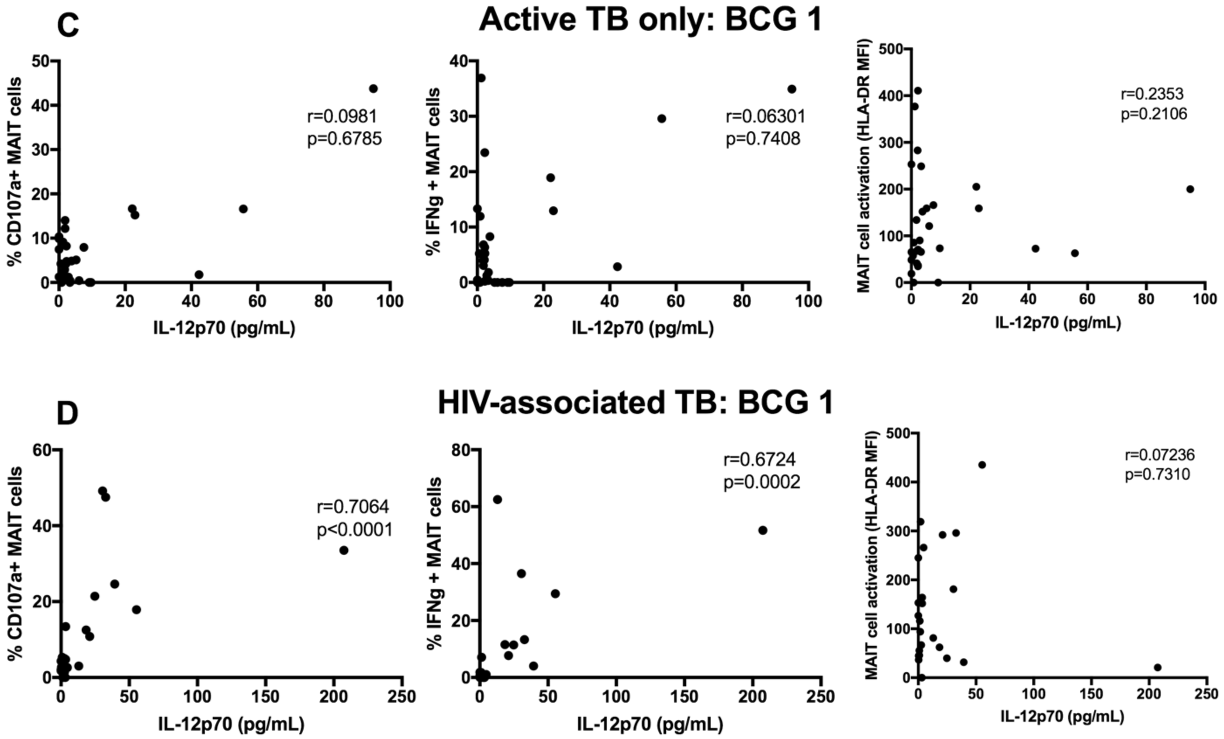
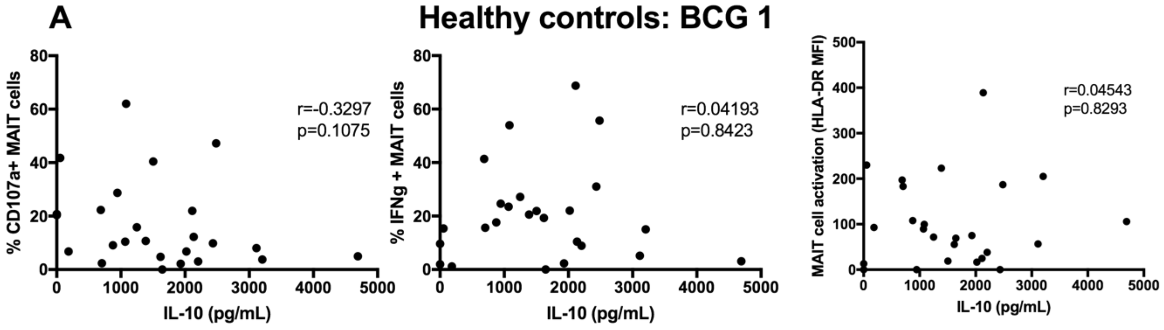


Figure 3.34: Effect of HIV, active TB and HIV-associated TB on the relationship between soluble IL-12p70 and MAIT cell functional measures and activation in response to BCG 1 stimulation A) Healthy controls. B) HIV only. C) Active TB only, D) HIV-associated TB. Correlations were evaluated using Spearman rank correlation.

3.7.2.3 IL-10

In healthy controls and individuals with HIV only, no significant correlations were observed between the concentrations of secreted IL-10 and the magnitude of MAIT cell responses and activation. In individuals with active TB only, there was a significant negative correlation between the concentration of secreted IL-10 and the frequencies of MAIT cells expressing IFN-*γ* (p=0.0408, r=-0.7359) (Figure 3.35C). In individuals with HIV-associated TB, there was a significant negative correlation between the concentration of secreted IL-10 and the frequencies of MAIT cells expressing HLA-DR (p=0.0018, r=-0.5931).



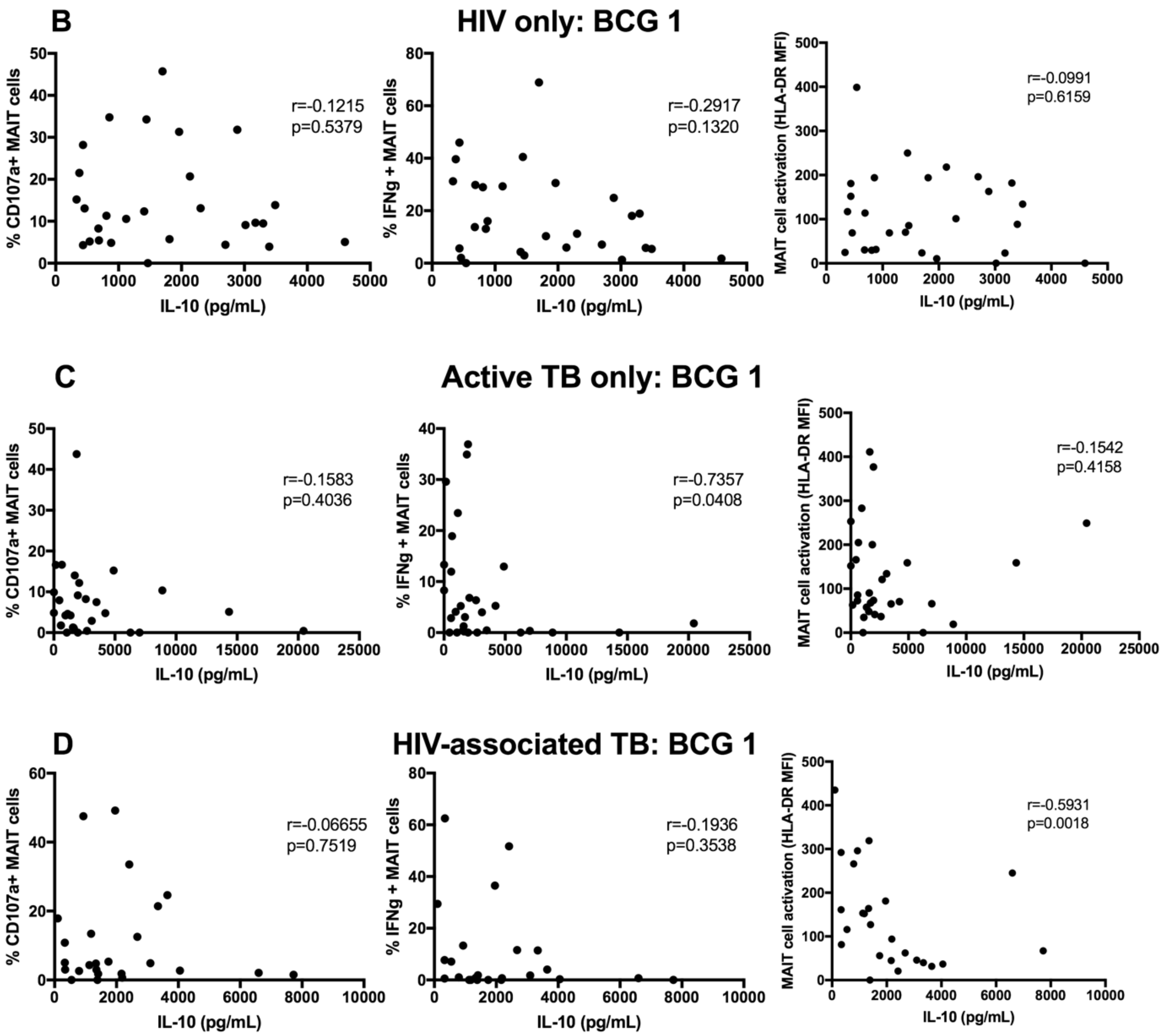


Figure 3.35: Effect of HIV, active TB and HIV-associated TB on the relationship between soluble IL-10 and MAIT cell functional measures and activation in response to BCG 1 stimulation A) Healthy controls. B) HIV only. C) Active TB only, D) HIV-associated TB. Correlations were evaluated using Spearman rank correlation.

In response to HK-*M.tb* stimulation, there were negative correlations between the concentration of IL-10 and the frequencies of MAIT cells expressing CD107a (p=0.0083, r=-0.5159), IFN-*γ* (p=0.0295, r=-0.4347) and HLA-DR (p=0.0275, r=-0.4406) in healthy controls. Positive correlations were observed between IL-10 and the frequencies of MAIT cells expressing HLA-DR in individuals with active TB only (p=0.0079, r=-0.4755) and HIV-associated TB (p=0.0273, r=-0.441) (Table A15). There were significant positive correlation between the concentrations of IL-12p40 and the frequencies of MAIT cells expressing IFN-*γ* in healthy controls (p=0.0110, r=0.4993), HIV only (p=0.0022, r=0.5449) and individuals with HIV-associated TB (p=0.0231, r=0.4527) while a negative correlation was observed between the concentration of IL-12p40 and the MFI of HLA-DR on MAIT cells expressing IFN-*γ* in individuals with HIV only (p=0.0418, r=-0.3803). Furthermore, there were strong positive correlations between the concentrations of IL-12p70 and the frequencies of MAIT cells expressing IFN-*γ* in healthy controls (p<0.0001, r=0.8345), HIV only (p<0.0001, r=0.8757) and HIV-associated TB (p=0.0010, r=0.6171). Finally, there was a negative correlation between the concentrations of IL-12p70 and the MFI of HLA-DR on MAIT cells expressing IFN-*γ* from individuals with HIV only (p=0.0009, r=-0.5812) and individuals with HIV-associated TB (p=0.0115, r=-0.4968) (Table A15).

In CD4 T cells, there was a significant positive correlation between the concentration of IL-12p40 and the frequencies of CD4 T cells expressing IFN-*γ* in healthy controls (p=0.0112, r=0.4983). There also were significant positive correlations between IL-12p70 and the frequencies of CD4 T cells expressing IFN-*γ* in healthy controls (p=0.0355, r=0.3988) and individuals with active TB only (p=0.0266, r=0.4046) (Table A16).

In CD8 T cells, there were significant correlations between the concentrations of IL-12p70 and the frequencies of CD8 T cells expressing CD107a (p=0.0176, r=0.445) and IFN-*γ* (p=0.0133, r=0.462) in individuals with HIV only. CD8 T cells expressing IFN-*γ* from healthy controls had a significantly positive correlation with the concentration of IL-12p70 (p=0.0086, r=0.5138) (Table A16).

In response to HK-*M.tb* stimulation, there was a significant negative correlation between the concentration of IL-10 and the frequencies of CD4 T cells expressing IFN-*γ* in individuals with HIV-associated TB (p=0.0188, r-=-0.4662). There was also a negative correlation between the concentration of IL-10 and the frequency of CD8 T cells expressing HLA-DR in individuals with active TB (p=0.0305, r=-0.3955). (Table A17). Next, there were significant positive correlations between the concentrations of IL-12p40 and the frequencies of CD4 T cells expressing IFN-*γ* in healthy controls (p=0.0435, r=0.407) and individuals with HIV only (p=0.0035, r=5244) and also with the frequencies of CD8 T cells expressing IFN-*γ* (p=0.0042, r=0.5150) in individuals with HIV only. Furthermore, individuals with HIV-associated TB showed a significant negative correlation between the concentrations of IL-12p40 and the frequencies of CD4 T cells expressing HLA-DR (p=0.0161, r=-0.4763) (Table A17).

There were strong positive correlations between the concentrations of IL12p70 and the frequencies of CD4 T cells expressing IFN-γ in healthy controls ($p<0.0001$, $r=0.8083$), HIV only ($p<0.0001$, $r=0.71701$), active TB only ($p=0.0146$, $r=0.4415$) and HIV-associated TB ($p=0.0013$, $r=0.6062$). The same relationship was also evident in CD8 T cells expressing IFN-γ (healthy controls [$p<0.0001$, $r=0.7436$], HIV only [$p<0.0001$, $r=0.7206$], active TB [$p=0.0266$, $r=0.4045$] and HIV-associated TB [$p=0.0003$, $r=0.6664$]) (Table A17).

3.7.3 Conclusions

In conclusion, we found positive correlations between the concentrations of soluble IL-12p40/IL-12p70 and the frequencies of MAIT cells expressing IFN-γ in healthy controls and people with HIV infection (HIV only and HIV-associated TB). However, this correlation was lost in people with active TB only, whose MAIT cell responses negatively correlated with soluble IL-10.

3.8 Evaluation of the mechanisms through which MAIT cells are activated by BCG infected antigen presenting cells

MAIT cells are thought to be activated through MR1-dependent and or MR1-independent (cytokine-dependent) mechanisms. Upon activation, MAIT cells produce effector molecules such as IFN-γ, and degranulate and produce cytotoxic molecules. In order to assess the comparative contributions of the 2 different mechanisms to MAIT cell activation, we used PBMCs from 10 healthy controls and blocked the two pathways involved. Anti-MR1 was used to block MR1 antigen presentation, anti-IL-12/IL-18 was used to block the cytokine-dependent mechanism and a combination of anti-MR1/IL-12/IL-18 was used to block both mechanisms of MAIT cell activation. Figure 3.36A shows the representative flow cytometry plots for the different blocking antibody cocktails used.

From these experiments, blocking of the MR1-dependent mechanism significantly reduced the expression of CD107a on MAIT cells compared to no blocking ($p=0.0078$). Anti-MR1/IL-12/IL-18 also significantly reduced the frequencies of MAIT cells expressing CD107a (p=0.0273) (Figure 3.36B). Blocking with anti-MR1, IL-12/IL-18 and a combination of anti-MR1/IL-12/IL-18 all had no significant effects on the ability of MAIT cells to express IFN-γ.

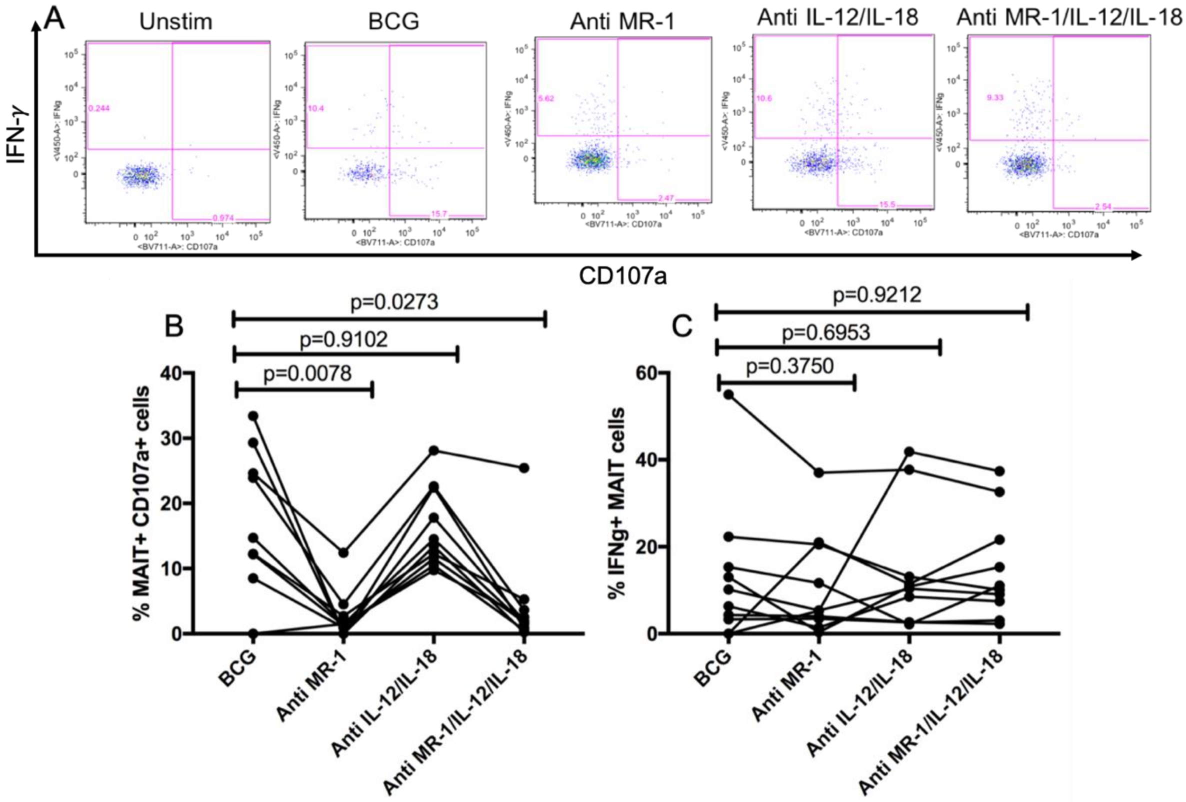


Figure 3.36: Blocking of MAIT cell activation. **A)** Representative flow cytometry plots showing the effects of blocking MAIT cell responses to BCG stimulation with different blocking antibodies **B)** Effect of blocking on CD107a expression. **C)** Effect of blocking on IFN-γ+ production. P-values represent Wilcoxon matched-pairs rank tests.

3.8.1 Conclusions

In summary, MAIT cell degranulation was primarily mediated through the MR1-dependent and not the cytokine-dependent (MR1-independent) mechanisms.

Chapter 4: General Discussion and Conclusion

4.1 Summary of findings

The current study was aimed at evaluating how HIV, active TB and HIV-associated TB affect the frequencies, the functional and activation profiles, as well as the inhibitory potential of MAIT cells. We also sought to validate the frequencies of MAIT cells determined with Vα7.2 antibody, with antigen loaded MR1 tetramer. Furthermore, we investigated the relationship between the magnitude of innate immune responses, as a result of mycobacterial infection, and the magnitude of MAIT cell responses and also how these interactions are affected by HIV, active TB and HIV-associated TB. Lastly, we evaluated the mechanisms through which MAIT cells are activated by BCG infected APCs.

We found that compared to healthy controls, the frequencies of MAIT cells were lower in individuals with HIV only and individuals with active TB only. In individuals with HIV-associated TB however, MAIT cell frequencies were similar to healthy controls without HIV or TB. When evaluated with MR1 tetramers, the frequencies of MAIT cells from individuals with HIV only and with HIV-associated TB were similar to healthy controls, whereas MAIT cell frequencies were lower in individuals with active TB only compared to healthy controls. The frequencies of MAIT cells identified with Vα7.2 antibody were strongly correlated with MAIT cell frequencies identified with MR1 tetramer. We also found that in response to mycobacterial stimulation, MAIT cell responses were significantly impaired in individuals with active TB only and HIV-associated TB and that the total MAIT cells and mycobacterial-specific MAIT cells from individuals with active TB only and HIV-associated TB had higher activation status. The frequencies of MAIT cells expressing PD-1 were also increased in individuals with active TB only compared to healthy controls.

The frequencies of GFP$^+$ DCs and GFP+ monocytes were higher in individuals with HIV-associated TB and active TB only, respectively, compared to healthy controls. We also found that compared to healthy controls, the concentrations of soluble IFN-γ were remarkably lower in individuals with HIV only, active TB only and HIV-associated TB.

When we examined the interactions between MAIT cells and APCs, we found that in healthy controls, individuals with HIV only and individuals with active TB only, there were significant positive correlations between the concentrations of soluble IL-12p40 and IL-12p70, and the frequencies of MAIT cells expressing IFN-γ. There were a moderate to strong negative correlations between the concentration of IL-10 and MAIT cells expressing IFN-γ in individuals with active TB only and the activation of MAIT cells in individuals with HIV-associated TB, respectively. Table 4.1 below provides a summary of the major findings.

Table 4.1: Summary of overall findings from the study showing the association of HIV, active TB and HIV-association with cellular frequencies, functions and interactions between MAIT cells and APCs. Table shows results for study groups in comparison to healthy controls except in cellular interactions.

		Healthy controls	**HIV only**	**Active TB only**	**HIV-associated TB**
Cell frequencies	MAIT cell frequencies	—	Lower	Lower	Similar
	MAIT cell numbers	—	Lower	Similar	Similar
MAIT cell functions		—	Similar	Lower	Similar
	IFN-γ expression	—	Similar	Lower	Lower
MAIT cell activation	HLA-DR expression	—	Similar	Similar	Similar
	MAIT cell HLA-DR MFI	—	Similar	Higher	Higher
	IFN-γ^{+} MAIT cell HLA-DR MFI	—	Similar	Higher	Higher
Inhibitory potential	PD-1 expression	—	Similar	Higher	Similar
Cellular interactions	GFP^{+} Monocytes	NC	NC	NC	GFP^{+} monocytes negatively correlated with CD107a^{+} MAIT cells
	GFP^{+} DCs	NC	GFP^{+} DCs negatively correlated with MAIT cell activation	GFP^{+} DCS positively correlated with IFN-γ^{+} MAIT cells	NC
Interactions between cytokine and MAIT cell functions & activation	IL-12p40/IL-12p70	IL-12 positively correlated with IFN-γ^{+} MAIT cells	IL-12 positively correlated with IFN-γ^{+} MAIT cells	NC	IL-12 Positively correlated with IFN-γ^{+} MAIT cells
	IL-10	NC	NC	IL-10 negatively correlated	NC

				with IFN-γ^+ MAIT cells	
		NC	NC	NC	IL-10 negatively correlated with MAIT cell activation

NC = No significant correlation.

4.2 Cellular frequencies and their association with HIV, active TB and HIV-associated TB

4.2.1 MAIT cell frequencies

We first evaluated the association between HIV, active TB and HIV-associated TB and the frequencies of T cells (MAIT cells, CD4 and CD8 T cells) and APCs (monocytes and DCs). We found that MAIT cells were significantly lower in individuals with HIV only and in individuals with active TB only. These findings are consistent with those observed in other studies such as those conducted by Wong *et al.*, (2013) and Jiang *et al.*, (2014) in which they also found lower frequencies of MAIT cells (defined as CD161^{high} Vα7.2^{high} and CD161^{++} CD8$^+$ T cells, respectively) in individuals with active TB only. The lower frequencies of MAIT cells observed in individuals with HIV only were consistent with results from Cosgrove *et al.*, (2013), Leeansyah *et al.*, (2013) and Eberhard *et al.*, (2014), in which MAIT cells (defined as CD161$^+$ Vα7.2$^+$, CD161^{++} CD8$^+$ cells and CD161$^+$Vα7.2$^+$CD4$^-$ T cells, respectively) were remarkably depleted in the presence of HIV infection. Interestingly, in HIV-associated TB, the frequencies of MAIT cells were similar to healthy controls. This may have been due to the depletion of other CD3 T cells in this particular group (T cells which are much more than MAIT cells), which could have led to a relative enrichment of MAIT cells.

In order to explain the observed decrease of MAIT cell frequencies in individuals with active TB and in those with HIV, several hypotheses have been suggested and these include :
1) lower frequencies of MAIT cells due to activation-induced cell death. Jiang *et al.*, (2014) found that MAIT cells from individuals with active TB had higher expression of annexin V and propidium iodide (PI) which characterize end stage apoptosis and cell death. They therefore proposed that during active TB, there is increased activation-induced cell death of MAIT cells and this may be responsible for the lower frequencies of MAIT cells.

2) downregulation of CD161 on MAIT cells, numerous studies have proposed that the reduction in the frequencies of MAIT cells may be due to the downregulation of CD161 which is one of the surface markers used for the identification of MAIT cells, particularly in HIV infection (Leeansyah *et al.*, 2013; Eberhard *et al.*, 2014).

3) migration of MAIT cells from circulation to site of infection, it has been suggested that during infection, these cells may be recruited to the site of infection so as to contribute to the control of bacterial replication and dissemination, and this may account for the lower frequencies of MAIT cells observed in peripheral blood (Le Bourhis *et al.*, 2010; Meierovics *et al.*, 2013; and Wong *et al.*, 2013).

While we did not analyse markers for apoptosis, we investigated the effects of cell death on MAIT cell frequencies by gating on total dead cells and identifying MAIT cells, CD4 and CD8 T cells from the dead cells. From these we found that the frequencies of MAIT cells were similar between all study groups and healthy controls suggesting that cell death may not be contributing to the lower cell frequencies. It was only for CD4 T cells where we found that there were significantly lower frequencies in the 2 HIV groups compared to healthy controls while CD8 T cells were all similar between all groups and healthy controls.

In order to ascertain if there was any downregulation of CD161, we gated on $CD161^{-}$ $V\alpha7.2^{+}$ cells and compared the frequencies of these cells between study groups: individuals from the HIV groups (HIV only and HIV-associated TB) had a trend of higher $CD161^{-}V\alpha7.2^{+}$ cells although the results were only significant for HIV-associated TB. This suggests that there may be a downregulation of CD161 during HIV infection. For our study, we only collected blood samples and did not collect any tissue samples and therefore we were unable to address the hypothesis of the recruitment of MAIT cells to the site of infection being a contributing factor in the reduction of MAIT cells in the circulation of infected individuals.

When frequencies of MAIT cells were validated with MR1 tetramers, the frequencies of MAIT cells in individuals with HIV only and HIV-associated TB were similar to healthy controls and, only MAIT cells from individuals with active TB only were lower compared to healthy controls. These observations were different from those made for MAIT cells identified with Vα7.2 antibody whereby the frequencies were lower in individuals with HIV only and TB only. It is worth noting that in these 2 different experiments there were different sample sizes which may have contributed to the different observations. MAIT cells identified with Vα7.2 had 26 to 30 participants in each group while MAIT cells identified with MR1 tetramer had 10 participants per group in all groups except for HIV-associated TB, which only had 5 participants. It is also important to note that there was a reasonable positive correlation between frequencies of MAIT cells identified with MR1 tetramers and those identified with Vα7.2 antibody.

Stimulation of PBMCs with BCG 5 resulted in higher frequencies of MAIT cells in all of our study groups while BCG 1 and HK-*M.tb* did not have any profound effects on MAIT cell frequencies. The higher frequencies of MAIT cells observed following BCG 5 stimulation could be due to higher cell death from stimulation in other cell compartments which resulted in a relative enrichment of the MAIT cell compartment.

4.2.2 Conventional T cell frequencies

We found that in individuals from HIV groups (HIV only and HIV-associated TB), the frequencies of CD4 T cells were remarkably reduced compared to healthy controls. This observation was not surprising as it is well known that HIV infection leads to the depletion of CD4 T cells (McCune, 2001; Vijayan *et al*., 2017). This depletion has been proposed to be due to a number of mechanisms such as direct viral killing in which infected CD4 T cells may fuse forming giant multinucleated cells which have a short life-span or as a consequence of infection and viral replication; host cell plasma membranes may be extremely compromised and disrupted as a function of viral budding whereby HIV particles use the cellular membranes to form viral envelopes (Alimonti *et al*., 2003).

Another mechanism which has been proposed for this CD4 T cell depletion is activation-induced cell death whereby there is a hyperstimulation of the immune system by viral gene products. There is also microbial translocation which leads to increased production of pro-inflammatory cytokines and the induction of caspase molecules, leading to apoptosis and pyroptosis (Doitsh *et al.,* 2014; Vijayan *et al.,* 2017). In addition, the numbers of CD4 T cells in peripheral blood are widely used as a marker for HIV disease progression and as a marker of immunodeficiency (Okoye and Picker, 2013; Vijayan *et al.*, 2017).

In contrast to CD4 T cells, the HIV groups had remarkably elevated frequencies of CD8 T cells and this was also unsurprising as this expansion has been described in HIV and although the exact mechanisms which drive this expansion are poorly understood. This expansion of CD8 T cells has been associated with CD8 T cell activation and turnover and is thought to be partially driven by the production of IL-15 during HIV-infection (Okoye and Picker, 2013; Cao *et al.*, 2016; Younes *et al.*, 2016).

4.2.3 Antigen presenting cells

In monocytes, the only differences that we found were in individuals with active TB only where there were higher frequencies and numbers of monocytes. These results are consistent with with previous findings (Janols *et al.*, 2010; Chávez-Galán *et al.*, 2012). Chávez-Galán *et al.*, (2012) in particular, found that there were higher frequencies of monocytes in the context of pulmonary TB. Furthermore, it has also been suggested that the presence of mycobacterial products such as ManLAM and cytokines produced during active TB disease may influence the expansion of new monocytes that facilitate the formation of granuloma (Sánchez et al., 2006).

In the 2 HIV groups (HIV only and HIV-associated TB), the frequencies and numbers of monocytes were similar to healthy controls, and a similar observation was made by McCausland *et al.*, (2015) on people with HIV only. Differences in monocyte frequencies between healthy controls and individuals with HIV infection have been reported by Chen *et al.*, (2017) but these differences were monocyte subset specific. They found that, compared to healthy controls, classical ($CD14^{++}CD16^{-}$) and intermediate ($CD14^{++}CD16^{+}$) monocytes were lower in individuals with acute and chronic HIV-1 infection while there were no differences in the non-classical monocytes ($CD14^{+}CD16^{++}$) (Chen *et al.*, 2017). In the current study however, we could not verify this observation because we only used CD14 to identify monocytes and did not use CD16, therefore could not investigate the differences at monocyte subset level.

4.2.4 Association between LTBI and MAIT cell frequencies

When we assessed the effect of LTBI on MAIT cell frequencies in the groups of individuals with no active TB (healthy controls and individuals with HIV only), we found that the frequencies of MAIT cells in individuals with and without LTBI were similar and this was consistent in both groups. The numbers of individuals without LTBI were very low in both groups and as a result, a larger sample size would likely yield more reliable results. However, our findings (for healthy controls) are consistent with reports by Wong *et* al., (2013) and Jiang *et al*., (2014). A recent study by from Paquin-Proulx *et al.*, (2018) found that in LTBI, the frequencies of MAIT cells were higher in individuals with LTBI compared to those without. The researchers suggested that LTBI may induce the proliferation of MAIT cells due to the constant low exposure of MAIT cells to stimulatory antigens during infection and this may account for the higher MAIT cell frequencies (Paquin-Proulx *et al.*, 2018).

4.2.5 Association between CD4 count/HIV viral load and MAIT cell frequencies

When we compared the association of CD4 counts with the frequencies of MAIT cells from individuals with HIV only, we found that the frequencies of MAIT cells were similar between individuals with CD4 counts of between 200 and 350 and those with higher CD4 counts. From this same group, the frequencies of MAIT cells were similar in individuals with HIV viral loads below 1000 copies/ml and those with HIV viral load above 1000 copies/ml. Secondly, when we assessed the relationships between CD4 counts and HIV viral loads and the frequencies and numbers of MAIT cells, we did not find any significant correlations between CD4 count, HIV viral load and the frequencies and numbers of MAIT cells.

All of these findings are consistent with those from Wong *et al.*, (2013) and Saeidi *et al.*, (2015) who did not find any significant correlations between the frequencies of MAIT cells, defined as CD161^{++}CD8^{+} cells and the CD4 counts and log HIV viral load. However, it is important to note the distinction between these 2 studies and our current study which is the fact that both these studies grouped individuals with HIV only and those with HIV-associated TB in their analyses of the relationships whereas our study separated individuals with HIV only and those with HIV-associated TB into two groups for analysis as the presence of TB and HIV infection may have additive effects on MAIT cells.

No significant correlations were found between the frequencies of CD8+ MAIT cells and the frequencies of CD4 T cells by Khaitan *et al.*, (2016) and Paquin-Proulx, D. *et al.* (2017), who analysed MAIT cells in viral infections other than HIV also did not observe any correlations between the frequencies of MAIT cells and the viral load of Human T lymphotropic virus 1 (HTLV-1).

In individuals with HIV-associated TB, we found that the frequencies and numbers of MAIT cells were higher in individuals with CD4 counts below 200 cells/mL and individuals with higher HIV viral loads (above 1000 copies/mL). Furthermore, there were significant negative correlations between the frequencies and numbers of MAIT cells and CD4 counts. However, in individuals with HIV-associated TB, individuals with lower CD4 counts and higher viral loads had higher frequencies and numbers of MAIT cells.

A possible explanation for the higher frequencies of MAIT cells in patients with lower CD4 counts and higher HIV viral loads could be due to the effects of HIV viral load on CD4 and CD8 T cells. The higher HIV viral loads could be causing the death and decline in the CD4 counts, lowering of T cell numbers, and an increase in the frequencies of CD8 T cells (Lempicki *et al.*, 2000; Alimonti *et al*., 2003; Cummins and Badley, 2010) and MAIT cells may not be affected by this T cell depletion which leads to the relative enrichment of the MAIT cell compartment. A possible reason for why this effect is only observed in the group of individuals with HIV-associated TB and not those with HIV only, could be because this group had higher HIV viral loads and lower CD4 counts (Table 3.1).

4.2.6 Association between ART status and MAIT cell frequencies

In addition, we also investigated the effect of ART on the frequencies of MAIT cells. We found that the frequencies of MAIT cells were similar between individuals on ART and those not on ART. Leeansyah *et al.*, (2013), Wong *et al.*, (2013) and Saeidi *et al.*, (2015) made similar observations in which there was no difference in the frequencies of MAIT cells, defined as CD161^{++}CD8^{+} cells and CD161^{+}Vα7.2^{+} T cells, between individuals pre and post ART, indicating that initiation of ART did not increase or restore frequencies of MAIT cells, at least in the short term. Interestingly, Khaitan *et al.*, (2016) found that in children, ART initiation is able to restore the frequencies of MAIT cells. Taken together with these results, our findings suggest that in adults with HIV and HIV-associated TB, the frequencies of MAIT cells may not be restored by ART. However, ART adherence was not systematically evaluated in our study.

4.3 Cellular responses and their association with HIV, active TB and HIV-associated TB

4.3.1 MAIT cell responses

When we assessed MAIT cell functions (IFN-γ and CD107a expression), we found that the magnitude of expression of CD107a and IFN-γ were similar between MAIT cells from individuals with HIV only and healthy controls in all stimulation conditions (BCG 1, BCG 5, HK-*M.tb*). These findings were similar to results reported by Fernandez *et al.*, (2015) where they found that MAIT cells from individuals with HIV infection (identified with MR1 tetramer and stimulated with 6-hydroxymethyl-8-D-ribityllumazine [6-HM, which is a MAIT activating ligand]) had similar magnitude of IFN-γ expression to healthy controls, and Spaan *et al.*, (2016), who also found that in individuals with HIV infection, the magnitude of IFN-γ expression was similar to MAIT cells from healthy controls stimulated with a combination of IL-12/IL-18 and IFN-α/IL-18.

These results, however, differ from those reported by Leeansyah *et al.*, (2013)., who observed that MAIT cells from individuals with HIV infection have lower expression of IFN-γ compared to healthy controls, when stimulated overnight with *E.coli*. Interestingly, in the same study, when MAIT cells were stimulated for 6 hours with PMA, the expression of IFN-γ was similar to healthy controls which suggests that the nature of the stimulating antigen and the duration of the stimulation may affect MAIT cell responses as these different antigens may affect different signal transduction pathways (Leeansyah *et al.*, 2013; Kwon *et al.*, 2015). In a study by Paquin-Proulx *et al.*, (2017), similar observations to Leeansyah *et al*., (2013) were made in HTLV-1 infection in which MAIT cells from individuals with HTLV-1 infection had lower expression of IFN-γ than healthy controls in response to *E.coli* stimulation.

MAIT cells from individuals with active TB only had lower expression of both CD107a and IFN-γ in response to BCG 1 and BCG 5 stimulation and they also had a lower expression of IFN-γ in response to HK-*M.tb* stimulation. CD107a expression was retained and remained similar to healthy controls in response to HK-*M.tb* stimulation. Our results are consistent with findings by Kwon *et al.*, (2015) and Jiang *et al.*, (2016) who found that IFN-γ expression on MAIT cells from individuals with active TB was lower compared to healthy controls. Interestingly, in an earlier study, Jiang *et al*., (2014) found that when stimulated with live BCG, IFN-γ expression on MAIT cells was higher in TB patients compared to healthy controls (which is in contrast to our findings) but this expression was lower compared to healthy controls in response to *E.coli* stimulation (Jiang *et al.*, 2014).

It is also interesting to note the differences in CD107a expression by MAIT cells in response to BCG and HK-*M.tb* where HK-*M.tb* is associated with a drastic reduction in CD107a expression (Figure 3.16 A, B, C). This could be as a result of the heat-killing process which probably led to the damage of MAIT cell activating ligands that bind and activate MAIT cells through the MR1 pathway, contrary to the live intact whole bacteria such as BCG.

Examination of the co-expression functional profiles of MAIT cells revealed that in active TB and HIV-associated TB, there were lower frequencies of the CD107a$^+$IFN-γ$^+$ bifunctional MAIT cells and lower IFN-γ$^+$ monofunctional MAIT cell subsets. However, the frequencies of CD107a$^+$ monofunctional MAIT cell subsets were similar in all groups compared to healthy controls. These results suggest that TB disease alters MAIT cell IFN-γ production in combination with or independent of CD107a expression.

The cytotoxic profile of MAIT cells in individuals with active TB has also been evaluated by Jiang *et al*., (2016) who found that in patients with TB, the expression of granzyme B was lower compared to the healthy controls. In addition, their RNA sequencing data revealed that genes associated with cytotoxicity (such as genes for granulysin, granzyme B and H) were downregulated in these individuals. We did not use cytotoxic markers in our study and used CD107a, a marker of degranulation, as proxy for cytotoxicity.

The observed functional impairment may be due, in part to the elevated expression of co-inhibitory molecules such as PD-1 which are known to be highly expressed in chronically activated T cells and have been reported to be necessary for the regulation of excessive inflammatory responses in the context of TB and HIV (Lazar-Molnar *et al.*, 2010; Saeidi *et al.*, 2015). To assess this, we stained MAIT cells for PD-1 and found that the frequencies of resting MAIT cells expressing PD-1 were higher in individuals with active TB only compared to healthy controls. These findings support the view that PD-1 expression may be contributing to functional impairment of MAIT cells in the context of active TB and this elevated expression may play a regulatory role against continual activation of MAIT cells due to exposure to mycobacterial antigens. Our results are consistent with other findings that MAIT cells from individuals with active TB also have a higher expression of PD-1 than healthy controls (Jiang *et al.*, 2014; Kwon *et al.*, 2015; Saeidi *et al.*, 2015). Jiang *et al.*, (2014) demonstrated that anti-PD-1 treatment was able to successfully restore the expression of IFN-γ on MAIT cells from individuals with active TB. However, what this means in terms of disease outcome is still unclear as studies have shown that treatment with anti-PD1 possibly reactivates/exarcebates TB in patients with cancer (Barber *et al.*, 2019).

4.3.2 Conventional T cell responses

For CD4 T cells, individuals with HIV only had a lower expression of IFN-γ compared to healthy controls in response to BCG 1, BCG 5 and HK-*M.tb* stimulation. These are in general agreement with results from Sutherland *et al.*, (2006) who observed that in individuals with chronic HIV infection, CD4 T cells have impaired ability to express IFN-γ, and Amelio *et al.*, (2018) who reported that HIV infection impairs the functions of *M.tb*-specific CD4 T cells. It has been shown that this impaired function of CD4 T cells from individuals with HIV infection may be partially due to the regulatory functions of T_{reg} cells which influence IL-10 production by monocytes (Kwon *et al.*, 2012).

CD4 T cells from individuals with active TB only also had lower CD107a and IFN-γ expression in response to BCG 1, BCG 5 and HK-*M.tb* stimulations. These are consistent with a report by Rueda *et al.*, (2010) who also observed that CD4 T cells from individuals with pulmonary TB have lower expression of IFN-γ in response to mycobacterial antigens such as ESAT-6, CFP-10 and PPD. This impairment of function is suggested to be due to the actions of regulatory cytokines, T cell anergy and activation-induced cell death.

Since PD-1 expression may affect T cell function, we also assessed the expression of PD-1 on CD4 T cells and we found that compared to healthy controls there were more CD4 T cells expressing PD-1 in the three study groups compared to health controls, with CD4 T cells from individuals from the HIV groups (HIV only and HIV-associated TB) having remarkably higher expressions of PD-1.

Assessment of CD4 T cell co-expression functional profiles showed that there were lower frequencies of CD107a$^+$ IFN-γ$^+$ bifunctional CD4 T cell subsets in individuals with HIV only and individuals with active TB only compared to healthy controls. In individuals with HIV-associated TB however, there were similar frequencies of the bifunctional CD4 T cell subsets between all groups. The individuals with HIV only and individuals with active TB only had lower frequencies of the IFN-γ$^+$ monofunctional CD4 T cell subset compared to healthy controls. Furthermore, the frequencies of CD107a$^+$ monofunctional CD4 T cell subset were lower in individuals with active TB only compared to healthy controls although there was also a trend of lower frequencies of the CD107a$^+$ monofunctional subset in individuals with HIV only.

These results could mean that TB disease and HIV infection result in the alteration of the functional profile of CD4 T cells by the depletion or anergy of CD107a$^+$IFN-γ$^+$ CD4 T cells, the CD107a$^+$ monofunctional and IFN-γ$^+$ monofunctional CD4 T cell subsets. For the IFN-γ$^+$ subset specifically, this selective reduction has been demonstrated by (Bunjun *et al.*, 2017; Riou *et al.*, 2017) who investigated the polyfunctional profiles of CD4 T cells in individuals with HIV infection and stimulated the cells with ESAT-6/CFP-10, PPD and BCG. These studies are however, different from our studies because they assessed the IL-2, TNF-α and IFN-γ multifunctional CD4 subsets whereas we looked at CD107a and IFN-γ expression only.

For CD8 T cells, we found that individuals with HIV only had lower expression of CD107a and IFN-γ compared to healthy controls, in response to BCG 1, BCG 5 and HK-*M.tb* stimulation. This impairment in function has also been linked to viral replication which leads to chronic stimulation and the exhaustion of T cells during HIV infection (Shankar *et al.*, 2000; Silva *et* *al.*, 2014). CD8 T cells from individuals with active TB only also had lower expression of IFN-γ in response to BCG 1, BCG 5 and HK-*M.tb* stimulation and lower expression of CD107a in response to BCG 5 stimulation. In individuals with HIV-associated TB, we also found that CD8 T cells had lower expression of IFN-γ in response to BCG, BCG 5 and HK-*M.tb* stimulation.

From CD8 T cell co-expression functional profiles, we found that there were lower frequencies of CD107a$^+$IFN-γ$^+$ bifunctional CD8 T cell subsets in individuals with HIV only and individuals with active TB only compared to healthy controls, although, there was also a trend of lower frequencies in HIV-associated TB. There were also lower frequencies of IFN-γ$^+$ monofunctional CD8 T cell subsets in all groups compared to healthy controls and lower frequencies of CD107a$^+$ monofunctional CD8 T cell subsets in individual with HIV only compared to healthy controls. These results could mean that HIV and TB result in the alteration of the functional profile of CD107a$^+$ and IFN-γ$^+$ CD8 T cell subsets.

These results suggest that HIV, TB disease and HIV-associated TB all result in functional impairment of CD8 T cells resulting in decreased capacity of these cells to express IFN-γ while the degranulation of these cells is significantly impaired in the presence of HIV only. Furthermore, CD8 T cells from individuals with HIV only had a higher expression of PD-1 than healthy controls and this expression of inhibitory molecule may be contributing to the dampening of CD8 T cell responses in HIV infection (Trautmann *et al.*, 2006; Sachdeva *et al.*, 2010).

4.3.3 APC responses

The activation of MAIT cell effector function is thought to be partially dependent on the infection of APCs. Once these APCs are infected by organisms such as *M.tb* or BCG, vitamin B metabolites from these organisms are transported to the cell surface by MR1 molecules where they then activate MAIT cells to produce effector cytokines like IFN-γ. This also causes MAIT cells to degranulate, producing cytotoxic molecules which kill the infected cells or activate them for pathogen killing (Shey *et al.*, 2018). We therefore, sought to assess the infection of APCs by BCG-GFP and look at how this infection was affected by HIV, TB and HIV-associated TB.

We found that, compared to healthy controls, individuals with active TB had higher frequencies of GFP+ monocytes and higher levels of GFP (GFP MFI) following co-culture with BCG 5 and BCG 1, respectively. There was also a trend of higher frequencies of GFP+ monocytes in individuals with HIV-associated TB, in response to co-culture with BCG 5.

These results suggest that there were higher levels of BCG infection in monocytes from individuals with active TB compared to healthy controls. These higher levels of infection could mean that there are more antigens to bind to MR1 and thus greater MR1 surface expression/antigen presentation which could result in increased MAIT cell activation and effector function. However, it is not clear whether the greater levels of infection are correlated with the number of bacilli actively metabolizing and producing vitamin B metabolites within these monocytes. In this light, there was a trend towards a positive correlation between GFP+ monocytes and frequencies of MAIT cells expressing IFN-γ in individuals with active TB only.

4.4 Immune activation and the association with HIV, active TB and HIV-associated TB

4.4.1 MAIT cell activation

When we assessed the activation status of MAIT cells using HLA-DR, we did not find any significant differences in the frequencies of MAIT cells expressing HLA-DR in response to BCG 1 and HK-*M.tb* stimulation between healthy controls and other study groups. We found that resting MAIT cells from individuals with active TB and HIV-associated TB had higher activation (HLA-DR expression in unstimulated controls) and only MAIT cells from individuals with HIV-associated TB had higher activation in response to HK-*M.tb* stimulation compared to healthy controls, MAIT cells from other groups had similar activation status to healthy controls. Finally, we found that MAIT cells from individuals with active TB only and HIV-associated TB had remarkably elevated IFN-γ specific (HLA-DR on IFN-γ^+ MAIT cells) activation status in response to BCG and HK-*M.tb* stimulation while those from individuals with HIV only had significantly higher activation status in response to BCG 1 stimulation.

These results contrast with previously published MAIT cell findings. In the HIV only group, we did not find any significant differences between the frequencies of MAIT cells expressing HLA-DR in response to BCG or HK-*M.tb* stimulation and there were also no differences in the levels of HLA-DR on MAIT cells in the unstimulated, BCG and HK-*M.tb*-stimulated conditions, whereas Eberhard *et al.*, (2014) have reported that MAIT cells from individuals with HIV infection (in both viremic and those on ART) had remarkably high activation statuses, defined as CD38+HLA-DR+ expression, especially in those with high viremia. Leeansyah *et al.*, (2013) also found that MAIT cells from individuals with HIV had higher expression of CD38 and HLA-DR compared to healthy controls. In other viral infection such as HTLV-1, significantly higher CD38+ HLA-DR+ MAIT cells have been reported in people with HTLV-1 compared to healthy controls (Paquin-Proulx *et al.*, 2017).

4.4.2 Conventional T cell activation

As with MAIT cells, resting CD4 T cells from individuals with HIV-associated TB had higher activation in unstimulated conditions and also a higher level of activation in response to HK-*M.tb* stimulation and those from individuals with active TB only and HIV-associated TB had significantly higher BCG and HK-*M.tb*-specific activation.

Resting CD8 T cells from individuals with HIV-associated TB had significantly higher activation and were highly activated in response to BCG 1 and HK-*M.tb* stimulation. Furthermore, CD8 T cells from individuals with active TB only and HIV-associated TB exhibited significantly higher IFN-γ-specific activation.

HIV infection is associated with chronic activation of CD4 and CD8 T cells (Paiardini and Müller-Trutwin, 2013; Younas *et al.*, 2016), and the upregulation of markers of T cell activation have been commonly described. For example; Jaspan *et al.*, (2011) and Xia *et al.*, (2018) described an increase in the frequencies of CD4 and CD8 T cells expressing CD38, HLA-DR and co-expressing CD38 and HLA-DR in individuals with HIV infection compared to healthy controls. In their respective studies, Hunt *et al.*, (2012) and Xia *et al.*, (2018) found that the higher T cell activation (particularly in CD4 T cells) was negatively correlated with CD4 counts and therefore individuals with higher CD4 counts have lower levels of T cell activation than those with lower CD4 counts. This might provide an explanation as to why we did not find any significant differences in the activation profiles of T cells in individuals with HIV, while we observed differences in the other HIV group (HIV-associated TB) which had lower CD4 counts than the HIV only group.

4.4.3 APC activation

APCs play an important role in the development of cell-mediated immunity against pathogens such as *M.tb*, among other functions, they are responsible for presenting antigens to T cells, thus activating T cells and being activated by T cells in order to eliminate the infection (Sakhno *et al*., 2015). As a survival mechanism, *M.tb* infection has been demonstrated to inhibit antigen presentation in APCs by disrupting the expression of MHC class II (Chang *et al*., 2005; Hmama *et al.*, 2019). *M.tb* has also been demonstrated to impair the maturation of DCs, thus rendering cells ineffective at antigen presentation and impairing their stimulatory capacity on T cells, and suppressing IL-12 production (Hanekom *et al.*, 2003; Sakhno *et al.*, 2015).

We therefore assessed the expression of HLA-DR, the MHC class II surface receptor molecule on APCs and we assessed how this was associated with HIV, active TB and HIV-associated TB. We found that monocytes from individuals with active TB only has lower expression of HLA-DR compared to healthy controls. This observation was consistent in PBMCs co-cultured with both BCG 1 and BCG 5. In DCs, individuals with HIV-associated TB showed a higher expression of HLA-DR, although this was only significant in the unstimulated controls and under stimulation with BCG 5. When we assessed the activation of APCs, we did not find any significant differences in the expression of CD40 in APCs and in the different stimulation conditions.

The activation status of APCs (CD40 MFI) from the individuals with HIV only, active TB and HIV-associated TB, were similar to healthy controls. However, even though all APCs had similar levels of activation, APCs (specifically monocytes) from individuals with active TB only had lower HLA-DR expression and this could be due to immune de-activation from chronic exposure to antigen and may result in impaired capacity to present antigens. In this same group (individuals with active TB only) the levels of BCG infection were higher than controls which could suggest that there was more antigen within the cells. However, HLA-DR antigen presentation could also be impaired as a results of reduced HLA-DR expression. This would be unsurprising since it has been shown that *M.tb* results in defective antigen presentation in monocytes (Gercken and Pryjma, 1994).

Compared to observations on monocytes, DCs from people with HIV-associated TB showed the opposite trend of HLA-DR expression in the unstimulated controls and after co-culture with BCG 1 and BCG 5, suggesting differential effect of bacteria on antigen presentation by both APC subsets.

4.5 Soluble cytokines

The activation of MAIT cells may occur in an MR1-independent mechanism through activation by cytokines such as IL-12, IL-18 and IFN-α (Van Wilgenburg *et al.,* 2016). IL-12 and IL-18 act synergistically for the initiation of cell mediated immune responses and are crucial for the development of Th_1 cells and the induction of IFN-γ production. Holscher *et al.*, (2001) showed that mice lacking IL-12p40 are highly susceptible to BCG or *M.tb* infection and the function of IL-12p40 is highly dependent on IL-12p70.

Although we did not find any differences in the concentrations of IL-12p40 in cell supernatants in the study groups, the concentration of IL-12p70 was lower in individuals with active TB only and the concentration of IL-18 was higher in the same group. The concentration of IFN-α2 was lower in individuals with HIV only. We also found that the concentrations of IFN-γ were significantly reduced in HIV only, active TB only and HIV-associated TB. All these results can be taken to mean that the production of IFN-γ is impaired by HIV and TB disease while there is defective IFN-α2 production in HIV only (in response to BCG 1 and HK-*M.tb* stimulation) and active TB (in response to HK-*M.tb* stimulation), there is elevated production of IL-10 (in active TB in response to HK-*M.tb* stimulation) and IL-18 in response to BCG 1 and HK-*M.tb* stimulation.

4.6 Functional interactions and the association with HIV, active TB and HIV-associated TB

4.6.1 Interactions between infected APCs and T cells

Assessment of the interactions between infected APCs and MAIT cell responses showed that there were no correlations between monocyte infection and MAIT cell function and activation in all groups except for the individuals with HIV-associated TB where we found a negative correlation between monocyte infection and the expression of CD107a on MAIT cells from this group after BCG 1 co-culture. In response to BCG 5 co-culture, there was a negative correlation between monocyte infection and the expression of IFN-γ on MAIT cells from healthy controls and a positive correlation with the activation of MAIT cells expressing IFN-γ in individuals with active TB.

For DC, there was a negative correlation between DC infection with BCG 1 and the expression of HLA-DR on MAIT cells from individuals with HIV only and a positive correlation between DC infection and the expression of IFN-γ on MAIT cells from individuals with active TB. Furthermore, there was a positive correlation between DC infection and HLA-DR expression on MAIT cells from healthy controls in response to BCG 5 co-culture.

In dendritic cells, the frequencies of infected DCs was negatively correlated with MAIT cell activation in individuals with HIV only and the frequencies of MAIT cells expressing IFN-γ were positively correlated with the frequencies of infected DCs in individuals with active TB only. Since we found that there was a positive correlation between the frequencies of infected DCs and MAIT cells expressing HLA-DR in healthy controls but not in the other groups, this could mean that this relationship was possibly disrupted by infection and or disease in the other groups. This could have been due to inadequate antigen availability, MR1 expression or cytokine production after infection.

4.6.2 Interactions between soluble cytokines and T cells

We also found that there were positive correlations between the concentrations of soluble IL-12p40, IL-12p70 and the expression of IFN-γ on MAIT cells from all groups except in the group with active TB only and these were observed in response to BCG 1 and HK-*M.tb*.

Individuals with HIV only also showed significant correlations between IL-12p70 and the expression of CD107a whereas those with HIV-associated TB showed this trend for both IL-12p40 and IL-12p70.

These results are interesting in that they suggest that in all other groups (apart from the active TB only group), an increase in soluble IL-12p40 and IL-12p70 could result in the increase in the expression of IFN-γ by MAIT cells. This relationship was maintained in these groups whereas in active TB only, this relationship seems to be disrupted which may partially account for the lower MAIT cell responses in the active TB only compared to healthy controls. These may also partially explain why in HIV infection, MAIT cell responses are retained at levels similar to those of healthy controls. However, these do not explain the lower responses observed in the context of HIV-associated TB.

For IL-10, there was a strong negative correlation between the concentration of IL-10 and the expression of IFN-γ on MAIT cells in individuals with active TB in response to BCG stimulation, this correlation was not observed in any of the other study groups. There was also a negative correlation with MAIT cell activation in individuals with HIV-associated TB. These results suggest that in active TB, an increase in the production of IL-10 negatively affects the expression of IFN-γ on MAIT.

For CD4 T cells, we found that the concentration of IL-12p40 produced in response to BCG 1 stimulation positively correlated with the expression of IFN-γ on CD4 T cells from healthy controls. Soluble IL-12p70 had a positive correlation with the expression of IFN-γ on CD4 T cells from healthy controls, individuals with HIV only and individuals with active TB only. IL-12p70 also positively correlated with CD8 T cells from healthy controls and individuals with HIV only. In response to HK-*M.tb* stimulation, the concentration of IL-12p70 showed positive correlation with the expression of IFN-γ by CD4 and CD8 T cells from all the study groups.

Since IL-12p40 production was positively correlated with frequencies of CD4 T cells expressing IFN-γ in healthy controls only, this could mean that in HIV infection, active TB or HIV-associated TB, there was a disruption of these interactions between IL-12p40 produced by APCs and the expression of IFN-γ by CD4 T cells. IL-12 plays an important role in the development of cell-mediated immunity and it contributes to the induction of Th$_1$ responses (Gee *et al*., 2009; Méndez-Samperio, 2010).

Due to the necessity of IL-12 for the induction of IFN-γ production, disruption between soluble IL-12p40 produced by APCs and IFN-γ expressed by T cells in all groups except for active TB may also explain the lower frequencies of T cells (CD4 and CD8) expressing IFN-γ. However, while the positive correlation between IL-12p70 extended from healthy controls to individuals with HIV infection, the effects of IL-12p70 on the expression of IFN-γ by CD4 and CD8 T cells may have been insufficient to compensate for IFN-γ induction, which could be why the frequencies of CD4/CD8 T cells expressing IFN-γ and the concentration of soluble IFN-γ was still lower in these groups.

4.7 Mechanisms of MAIT cell activation by BCG infected APCs

When we assessed the mechanisms by which MAIT cells can be activated, we found that blocking with anti-MR1 antibody and a cocktail of anti-MR1 and anti-IL-12/IL-18 was only able to block the expression of CD107a on MAIT cells and had no effects on IFN-γ production. Blocking with anti-IL-12/IL-18 only had no effects on the expression of CD107a and IFN-γ on MAIT cells.

These results can be taken to mean that in our experimental setting, the cytotoxic function of MAIT cells is dependent on the interactions between APC MR1-antigen complex and the MAIT cell TCR. This is supported by observations made by Jeffery *et al.*, (2016) who studied the interactions between different APCs (macrophages, liver B cells and biliary epithelium cells) and found that MAIT cell degranulation, IFN-γ and CD40L expression were MR1-dependent and cytokine-independent.

We did not find any effects of MR1 or IL-12/IL-18 blocking on IFN-γ expression, this could be due to compensation by other cytokines which are also produced during stimulation. Cytokines such as IFN-α and IFN-β have been shown to affect the capacity of MAIT cells to express IFN-γ (Van Wilgenburg *et al*., 2016). Also in this study, stimulation with live BCG (rich in MR1 ligands) and HK-*M.tb* (deficient in MR1 ligands) resulted in significantly different responses, for example, IFN-γ expression was significantly lower in response to HK-*M.tb* but not in response to BCG, suggesting the importance of the MR1-dependent pathway in the activation and function of MAIT cells. Our results from the blocking experiments are similar in some ways but also differ from other published observations (described in detail in section 1.5.3.2 on Antigen presentation to MAIT cells).

These findings are in line with those by Jeffery *et al.*, 2016, who showed that blocking with α-MR1 on MAIT cells cocultured with biliary epithelium cells led to a significant reduction in the expression of CD107a but blocking with α-IL-12/IL-18 did not. However, they also showed that this blocking with α-MR1 also significantly reduced IFN-g expression whereas we did not.

Another study by Ussher *et al.,(*2014) that assessed IFN-γ expression also showed that blocking either MR1-dependent mechanism or the IL-12/IL-18 mechanisms significantly reduced IFN-g expression on MAIT cells cocultured with THP-1 cells. Together, these studies show that MAIT cell degranulation is particularly dependent on MR1 while IFN-γ expression is more flexible. With respect to IFN-γ in particular, Ussher *et al*., (2014) demonstrated that early IFN-γ expression (‘early activation’), was particularly dependent on MR1 since blocking with α-IL-12/IL-18 had no effect on IFN-γ expression, however, after 20 hours of stimulation, the effector function was dependent on both mechanisms of activation (Ussher *et al.,* 2014).

4.8 MAIT cells in non-communicable diseases

In addition to infectious disease such as TB and HIV, MAIT cells have also been implicated in the pathogenesis of non-communicable diseases such as cancer, asthma and diabetes as reviewed in Shey *et al*., (2018). The decreases in frequencies of circulating MAIT cells have also been reported in people with intestinal cancers, but MAIT cell frequencies have been reportedly similar in people with non-mucosal cancers and healthy controls (Won *et al.*, 2016). Several studies have reported that while the frequencies of MAIT cells were depleted in peripheral blood, they also accumulated in tumor tissues (Zabijak *et al.*, 2015; Won *et al.*, 2016). When colorectal tumors were compared to healthy colons, there were higher frequencies of MAIT cells in colorectal tumors, suggesting that MAIT cells have the ability to migrate and infiltrate tumors or cancerous tissues (Zabijak *et al.*, 2015). There have been variable findings however, about the function of MAIT cells with some studies suggesting a decreased function (Zabijak *et al.*, 2015; Won *et al.*, 2016) and others, a similar functional capacity (Smith *et al.*, 2014; Sundström *et al.*, 2015).

The direct role of MAIT cells in cancers has also been extensively investigated (Ling *et al.*, 2016; Won *et al.*, 2016; Melo *et al.*, 2019). Activated MAIT cells cocultured with cancer cell lines (HCT116, K562 and OE33) produced proinflammatory cytokines such as IFN-γ, TNF-α and IL-17, and the MAIT cells also exhibited an increased degranulation and upregulation of perforin and granzyme B. In addition, the coculture of these cancer cell lines with MAIT cells resulted in the decreased viability of the cell lines (Ling *et al.*, 2016; Won *et al.*, 2016; Melo *et al.*, 2019) and this was in a contact-dependent manner (Ling *et al.*, 2016).

This, coupled with the demonstrated ability of MAIT cells to infiltrate tumors suggests that MAIT cells may also have antitumor function and may possibly play a role in the modulation of tumor microenvironment by maintaining the balance between cytokines suppress and promote tumor growth (Shey *et al.,* 2018).

4.9 Limitations and Future Studies

For most of the analyses in the study, multiple comparisons were conducted. However, we did not correct for multiple comparisons because this study is exploratory and hypothesis generating. Owing to the cross-sectional nature of our study, it is important to note that the findings were observational and thus, we are unable to infer causality. It is also worth noting that a large proportion of our healthy controls were positive for the QuantiFERON test, i.e. latently infected with *M.tb* and as a result, the results observed were from comparisons between disease groups and healthy but latently infected individuals. However, Paquin-

Proulx *et al*., (2018) have recently shown that the frequencies of MAIT cells were higher in LTBI+ (*M.tb* infected; defined as a positive result for either PPD skin test or TB. Spot test) than in LTBI- (M.tb uninfected; defined as a negative PPD skin test or TB-Spot test). Other studies by Jiang *et al*., (2014) and Wong *et al.,* (2013) reported different findings from Paquin-Proulx *et al*., (2018), showing that there were similar frequencies of MAIT cells between healthy controls and people with LTBI.

Most studies use the dual expression of CD38 and HLA-DR for the assessment of T cell activation. In this study however, we only used HLA-DR and thus our results and conclusions regarding MAIT cell (and CD4 and CD8 T cells) activation might have been different if co-expression of CD38 was assessed.Also, because most people were on ART , we were unable to fully evaluate the effect of ART due to the lower numbers of those not on ART. Future longitudinal studies should be able to better assess this effect. Another limitation was that the sample sizes were not adequate for some of the sub-analyses such as assessing the association of MAIT cells with CD4 counts, QFT.

To expand on some of the preliminary observations in this study, we have recruited an additional 30 people with HIV infection in order to increase our sample size and assess and better understand the association of MAIT cells with HIV viral load and CD4 counts. We also longitudinally followed up 19 of the patients in the TB only group and collected samples after 6-10 weeks of TB treatment to assess the effect/impact of TB treatment on MAIT cell frequencies and function. In addition to this, future studies assessing the possible role of MAIT cells in individuals with high exposure to *M.tb* infection would provide a better understanding of the function of MAIT cells in prevention of infection and progression from infection to disease.

The current study assessed MAIT cells in peripheral blood which is important because MAIT cells may circulate between blood and mucosal organs. However, studying MAIT cells in the lungs, bronchoalveolar lavage (BAL), lymph nodes, gut-associated lymphoid tissues (GALT) and mucosal tissues, would be more advantageous as these could shed light on the phenotype and function of MAIT cells at the site of infection. For example, a recent study, Wong *et al*., (2019) showed that although MAIT cells were depleted in peripheral blood, they were enriched in the BAL fluid of people with active TB, suggesting that there is an accumulation of MAIT cells in site of infection in TB disease. Furthermore, Wong *et al*., (2019) also showed that there were functional differences between MAIT cells in blood and in BAL, with MAIT cells from blood having a lower (antigen non-specific) TNF-α expression than MAIT cells in BAL fluid. These results not only demonstrate the phenotypic and functional differences that may exist between different compartments but they highlight the importance of studying MAIT cells at the site of infection.

4.10 Conclusions

The aim of our study was to assess MAIT cell frequencies, functions and activation, and to better understand the interactions between MAIT cells and APCs. We also wanted to assess how these interactions may be altered by HIV, TB and HIV-associated TB. We found that MAIT cell frequencies were significantly lower in individuals with HIV and individuals with active TB. MAIT cell functions were significantly impaired in individuals with active TB and those with HIV-associated TB. Furthermore, in these 2 groups MAIT cells were significantly more activated compared to healthy controls and individuals with HIV infection only who had similar activation status to healthy controls. We also found that compared to healthy controls, cells from all study groups had a diminished capacity to produce IFN-γ, a cytokine known to be important in the immune response against TB and also for activation of APCs for pathogen elimination.

We also found that in all our study groups, soluble IL-12 was positively correlated with the expression of IFN-γ on MAIT cells and this relationship was disrupted in individuals with active TB only. Individuals with active TB only showed a negative correlation between IL-10 and the expression of IFN-γ and this relationship was not found in other groups. Lastly, we found that blocking MAIT cell responses with anti-MR1 was able to successfully reduce MAIT cell degranulation but not the expression of IFN-γ, while blocking the MR1-independent mechanism with anti-IL-12/IL-18 had no significant effects on either of these effector functions, suggesting that in our experimental set-up, MAIT cell cytotoxicity is dependent on MR1-dependent mechanism.

This work is important because it adds to the growing body of MAIT cell research and how these cells are associated with infectious diseases such as HIV and TB. This study has assessed MAIT cell frequencies and functions in these diseases and has assessed how the magnitude of innate cell responses affects the magnitude of MAIT cell responses and how this relationship is affected in HIV, TB and HIV-associated TB. These findings are important because a better understanding of MAIT cell functions and activation may add to knowledge for the development of host-directed therapies/more targeted interventions to prevent or treat these infectious diseases. Since the current TB vaccine candidates are primarily targeted at conventional T cells, a better understanding of the mechanisms of MAIT cell activation may also encourage the augmentation of vaccines to include antigens or attenuated bacteria which can also specifically activate MAIT cells, since these cells recognize different antigens and ligands than conventional T cells. This could in turn, elicit a broader and more effective immune response.

References

Abubakar, I. *et al.* (2013) 'Systematic review and meta-analysis of the current evidence on the duration of protection by bacillus Calmette-Guérin vaccination against tuberculosis', *Health Technology Assessment*, 17(37), pp. 1–4. doi: 10.3310/hta17370.

Alimonti, J. B., *et al.* (2003). Mechanisms of CD4+ T lymphocyte cell death in human immunodeficiency virus infection and AIDS. *Journal of General Virology*, *84*(7), 1649–1661. https://doi.org/10.1099/vir.0.19110-0

Amelio, P., *et al.* (2018). HIV Infection Functionally Impairs *Mycobacterium tuberculosis*-specific CD4 and CD8 T-cell responses. *Journal of Virology*, (December). https://doi.org/10.1128/JVI.01728-18.

Baldwin, K. K. *et al.* (1999) 'Negative Selection of T Cells Occurs Throughout Thymic Development'.

Barber D.L., Sakai S., Kudchadkar R.R., Fling S.P., Day T.A., Vergara J.A., and others, 'Tuberculosis Following PD-1 Blockade for Cancer Immunotherapy', Science Translational Medicine, 11 (2019), 1–8 <https://doi.org/10.1126/scitranslmed.aat2702

Berg, R. E. and Forman, J. (2006) 'The role of CD8 T cells in innate immunity and in antigen non-specific protection', *Current Opinion in Immunology*, 18(3), pp. 338–343. doi: 10.1016/j.coi.2006.03.010.

Boom, W. H. *et al.* (2003) 'Human immunity to M. tuberculosis: T cell subsets and antigen processing', *Tuberculosis*, 83(1–3), pp. 98–106. doi: 10.1016/S1472-9792(02)00054-9.

Le Bourhis, L. *et al.* (2010) 'Antimicrobial activity of mucosal-associated invariant T cells', *Nature Immunology*, 11(8), pp. 701–708. doi: 10.1038/ni.1890.

Le Bourhis, L. *et al.* (2011) 'Mucosal-associated invariant T cells: Unconventional development and function', *Trends in Immunology*. Elsevier Ltd, 32(5), pp. 212–218. doi: 10.1016/j.it.2011.02.005.

Bonilla, F. and Oettgen, H. (2010). Adaptive immunity. *Journal of Allergy and Clinical Immunology*, 125(2), pp.S33-S40. doi:10.1016/j.jaci.2009.09.017

Boyette, L. B. *et al.* (2017) 'Phenotype, function, and differentiation potential of human monocyte subsets', *PLoS ONE*, 12(4), pp. 1–20. doi: 10.1371/journal.pone.0176460.

Canaday, D. H. *et al.* (2001) 'CD4+ and CD8+ T Cells Kill Intracellular Mycobacterium tuberculosis by a Perforin and Fas/Fas Ligand-Independent Mechanism', *The Journal of*

Immunology, 167(5), pp. 2734–2742. doi: 10.4049/jimmunol.167.5.2734.

Brubaker, S. W. *et al.* (2015) *Innate Immune Pattern Recognition: A Cell Biological Perspective*, *Annual Review of Immunology*. doi: 10.1146/annurev-immunol-032414-112240.

Cao, W. *et al.* (2016) 'Elevation and persistence of CD8 T-cells in HIV infection: The Achilles heel in the ART era', *Journal of the International AIDS Society*, 19(1), pp. 1–9. doi: 10.7448/IAS.19.1.20697.

Caruso, A. M. *et al.* (2019) 'Mice Deficient in CD4 T Cells Have Only Transiently Diminished Levels of IFN- γ , Yet Succumb to Tuberculosis'.

Castaño, D., García, L. F. and Rojas, M. (2011) 'Increased frequency and cell death of CD16+monocytes with Mycobacterium tuberculosis infection', *Tuberculosis*. Elsevier Ltd, 91(5), pp. 348–360. doi: 10.1016/j.tube.2011.04.002.

Chackerian, A. *et al.* (2002) 'Activation of NKT Cells Protects Mice from Tuberculosis', 70(11), pp. 6302–6309. doi: 10.1128/IAI.70.11.6302.

Chang, S. T., *et al.* (2005). Multiple mechanisms allow Mycobacterium tuberculosis to continuously inhibit MHC class II-mediated antigen presentation by macrophages.

Chávez-Galán, L., *et al.* (2012). Monocytes from tuberculosis patients that exhibit cleaved caspase 9 and denaturalized cytochrome c are more susceptible to death mediated by Toll-like receptor 2. *Immunology*, *135*(4), 299–311. https://doi.org/10.1111/j.1365-2567.2011.03543.x

Chen, P., *et al.* (2017). Perturbations of monocyte subsets and their association with T helper cell differentiation in acute and chronic HIV-1-infected patients. *Frontiers in Immunology*, *8*(MAR). https://doi.org/10.3389/fimmu.2017.00272

Chen, C. Y. *et al.* (2009) 'A critical role for CD8 T cells in a nonhuman primate model of tuberculosis', *PLoS Pathogens*, 5(4), pp. 1–10. doi: 10.1371/journal.ppat.1000392.

Chien, Y., Meyer, C. and Bonneville, M. (2014) 'γδ T Cells: First Line of Defense and Beyond ', *Annual Review of Immunology*, 32(1), pp. 121–155. doi: 10.1146/annurev-immunol-032713-120216.

Chien, Y., *et al.* (2014). γδ T Cells: First Line of Defense and Beyond . *Annual Review of Immunology*, *32*(1), 121–155. https://doi.org/10.1146/annurev-immunol-032713-120216

Chua, W. J. *et al.* (2012) 'Polyclonal mucosa-associated invariant T cells have unique innate functions in bacterial infection', *Infection and Immunity*, 80(9), pp. 3256–3267. doi: 10.1128/IAI.00279-12.

Churchyard, G. J. *et al.* (2015) 'Xpert MTB/RIF versus sputum microscopy as the initial diagnostic test for tuberculosis: A cluster-randomised trial embedded in South African roll-out of Xpert MTB/RIF', *The Lancet Global Health*. Churchyard et al. Open Access article distributed under the terms of CC BY-NC-ND, 3(8), pp. e450–e457. doi: 10.1016/S2214-109X(15)00100-X.

Collin, M., and Bigley, V. (2018). Human dendritic cell subsets: an update. *Immunology*, *154*(1), 3–20. https://doi.org/10.1111/imm.12888

Collin, M., Mcgovern, N. and Haniffa, M. (2013) 'Human dendritic cell subsets', *Immunology*, 140(1), pp. 22–30. doi: 10.1111/imm.12117.

Constant, S. *et al.* (1995) 'B lymphocytes can be competent antigen-presenting cells for priming CD4+ T cells to protein antigens in vivo', *J Immunol*, 155, pp. 3734–41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7561077.

Corbett, A. J. *et al.* (2014) 'T-cell activation by transitory neo-antigens derived from distinct microbial pathways', *Nature*. Nature Publishing Group, 509(7500), pp. 361–365. doi: 10.1038/nature13160.

Cose, S. *et al.* (2006) 'Evidence that a significant number of naive T cells enter non-lymphoid organs as part of a normal migratory pathway', *European Journal of Immunology*, 36(6), pp. 1423–1433. doi: 10.1002/eji.200535539.

Cosgrove, C., *et al.* (2013). Early and nonreversible decrease of CD161++/MAIT cells in HIV infection. *Blood*, *121*(6), 951–961. https://doi.org/10.1182/blood-2012-06-436436

Couper, K. N., Blount, D. G. and Riley, E. M. (2008) 'IL-10: The Master Regulator of Immunity to Infection', *The Journal of Immunology*, 180(9), pp. 5771–5777. doi: 10.4049/jimmunol.180.9.5771.

Crawford, A. *et al.* (2006) 'Primary T Cell Expansion and Differentiation In Vivo Requires Antigen Presentation by B Cells', *The Journal of Immunology*, 176(6), pp. 3498–3506. doi: 10.4049/jimmunol.176.6.3498.

Cummins, N. W., and Badley, A. D. (2010). Mechanisms of HIV-associated lymphocyte apoptosis: 2010. *Cell Death and Disease*, *1*(11), e99-9. https://doi.org/10.1038/cddis.2010.77

Van Crevel, R. *et al.* (2011) 'Innate immune recognition of mycobacterium tuberculosis', *Clinical and Developmental Immunology*, 2011. doi: 10.1155/2011/405310.

van Crevel, R., Ottenhoff, T. H. M. and van der Meer, J. W. M. (2002) 'Innate immunity to Mycobacterium tuberculosis.', *Clinical microbiology reviews*, 15(2), pp. 294–309. doi:

10.1128/CMR.15.2.294.

Dai, Z., Konieczny, B. T. and Lakkis, F. G. (2000) 'The Dual Role of IL-2 in the Generation and Maintenance of CD8+ Memory T Cells', *The Journal of Immunology*, 165(6), pp. 3031–3036. doi: 10.4049/jimmunol.165.6.3031.

Deckers, J., Hammad, H. and Hoste, E. (2018) 'Langerhans cells: Sensing the environment in health and disease', *Frontiers in Immunology*, 9(FEB), pp. 1–14. doi: 10.3389/fimmu.2018.00093.

Eberhard, J. M. *et al.* (2014) 'CD161+ MAIT Cells Are Severely Reduced in Peripheral Blood and Lymph Nodes of HIV-Infected Individuals Independently of Disease Progression', *PLoS ONE*, 9(11), pp. 6–8. doi: 10.1371/journal.pone.0111323.

Eckle, S. B. G. *et al.* (2015) 'Recognition of Vitamin B precursors and byproducts by mucosal associated invariant T cells', *Journal of Biological Chemistry*, 290(51), pp. 30204–30211. doi: 10.1074/jbc.R115.685990.

Esmail, H. *et al.* (2014) 'The ongoing challenge of latent tuberculosis'.

Fernandez, C. S., *et al.* (2015). MAIT cells are depleted early but retain functional cytokine expression in HIV infection. *Immunology and Cell Biology*, *93*(2), 177–188. https://doi.org/10.1038/icb.2014.91

Flynn, J. A. L. and Chan, J. (2005) 'What's good for the host is good for the bug', *Trends in Microbiology*, 13(3), pp. 98–102. doi: 10.1016/j.tim.2005.01.005.

Flynn, J. L. *et al.* (1992) 'Major histocompatibility complex class I-restricted T cells are.pdf', 89(December), pp. 12013–12017. doi: 10.1111/j.1365-2567.2004.01857.xIMM1857 [pii].

Flynn, J. L. (1993) 'An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection', *Journal of Experimental Medicine*, 178(6), pp. 2249–2254. doi: 10.1084/jem.178.6.2249.

Flynn, J. L. and Chan, J. (2001) 'Imunology of tuberculosis', pp. 93–129.

Ford, N., *et al.* (2017). The evolving role of CD4 cell counts in HIV care. *Current Opinion in HIV and AIDS*, *12*(2), 123–128. https://doi.org/10.1097/COH.0000000000000348

Gasteiger, G. *et al.* (2017) 'Cellular Innate Immunity: An Old Game with New Players', *Journal of Innate Immunity*, 9(2), pp. 111–125. doi: 10.1159/000453397.

Gee, K., *et al.* (2009). The IL-12 Family of Cytokines in Infection, Inflammation and Autoimmune Disorders. *Inflammation & Allergy-Drug Targets*, *8*(1), 40–52. https://doi.org/10.2174/187152809787582507

Gercken, J., and Pryjma, J. (1994). Defective Antigen Presentation by Mycobacterium tuberculosis-Infected Monocytes, 3472–3478.

Gherardin, N. A. *et al.* (2018) ‘Human blood MAIT cell subsets defined using MR1 tetramers’, *Immunology and Cell Biology*, 96(5), pp. 507–525. doi: 10.1111/imcb.12021.

Godfrey, D. I. *et al.* (2015) ‘The burgeoning family of unconventional T cells’, *Nature Immunology*. Nature Publishing Group, 16(11), pp. 1114–1123. doi: 10.1038/ni.3298.

Godfrey, Dale I., Stankovic, S. and Baxter, A. G. (2010) ‘Raising the NKT cell family’, *Nature Immunology*. Nature Publishing Group, 11(3), pp. 197–206. doi: 10.1038/ni.1841.

Godfrey, Dale I, Stankovic, S. and Baxter, A. G. (2010) ‘review Raising the NKT cell family’, *Nature Immunology*. Nature Publishing Group, 11(3), pp. 197–206. doi: 10.1038/ni.1841.

Gold, M. C. *et al.* (2010) ‘Human mucosal associated invariant T cells detect bacterially infected cells’, *PLoS Biology*, 8(6), pp. 1–14. doi: 10.1371/journal.pbio.1000407.

Gold, M. C., Napier, R. J. and Lewinsohn, D. M. (2015) ‘MR1-restricted mucosal associated invariant T (MAIT) cells in the immune response to Mycobacterium tuberculosis’, *Immunological Reviews*, 264(1), pp. 154–166. doi: 10.1111/imr.12271.

Gorski, K. S. *et al.* (2006) ‘Distinct indirect pathways govern human NK-cell activation by TLR-7 and TLR-8 agonists’, *International Immunology*, 18(7), pp. 1115–1126. doi: 10.1093/intimm/dxl046.

Groux, H., *et al.* (1998). Inhibitory and Stimulatory Effects of IL-10 on Human CD8+ T Cells. *The Journal of Immunology*, *160*(7), 3188–3193. Retrieved from http://jimmunol.org/content/160/7/3188.abstract%5Cnhttp://www.jimmunol.org/content/160/7/3188.full.pdf%5Cnhttp://www.jimmunol.org/content/160/7/3188.long

Hanekom, W. A. *et al.* (2003) ‘Mycobacterium tuberculosis Inhibits Maturation of Human Monocyte-Derived Dendritic Cells In Vitro’, 211, pp. 257–266.

Harriff, M. J. *et al.* (2018) ‘MR1 displays the microbial metabolome driving selective MR1-restricted T cell receptor usage.’, *Science immunology*, 3(25), p. eaao2556. doi: 10.1126/sciimmunol.aao2556.

Henderson, R. A., Watkins, S. C. and Flynn, J. L. (2019) ‘Activation of human dendritic cells following infection with Mycobacterium tuberculosis.’

Hernandez, M. G. H., *et al.* (2019). the Absence of CD4 ⍰ T Cell Help 1. https://doi.org/10.4049/jimmunol.180.7.4382

Hoffman, W., Lakkis, F. G. and Chalasani, G. (2016) ‘B cells, antibodies, and more’, *Clinical*

Journal of the American Society of Nephrology, 11(1), pp. 137–154. doi: 10.2215/CJN.09430915.

Holscher, C., *et al.* (2001). A Protective and Agonistic Function of IL-12p40 in Mycobacterial Infection. *The Journal of Immunology*, *167*(12), 6957–6966. https://doi.org/10.4049/jimmunol.167.12.6957

Howson, L. J., Salio, M. and Cerundolo, V. (2015) 'MR1-restricted mucosal-associated invariant T cells and their activation during infectious diseases', *Frontiers in Immunology*, 6(JUN). doi: 10.3389/fimmu.2015.00303.

Huang, S. *et al.* (2008) 'MR1 uses an endocytic pathway to activate mucosal-associated invariant T cells', *The Journal of Experimental Medicine*, 205(5), pp. 1201–1211. doi: 10.1084/jem.20072579.

Huang, S., *et al.* (2009). MR1 antigen presentation to mucosal-associated invariant T cells was highly conserved in evolution. *Proceedings of the National Academy of Sciences*, *106*(20), 8290–8295. https://doi.org/10.1073/pnas.0903196106

Hulspas, R. (2010) 'Titration of fluorochrome-conjugated antibodies for labeling cell surface markers on live cells', *Current Protocols in Cytometry*, (SUPPL.54), pp. 1–9. doi: 10.1002/0471142956.cy0629s54.

Hunt, P. W., *et al.* (2012). Relationship between T Cell Activation and CD4+ T Cell Count in HIV-Seropositive Individuals with Undetectable Plasma HIV RNA Levels in the Absence of Therapy. *Journal of Infectious Diseases*, *197*(March 2007), 126–133. https://doi.org/10.1086/524143.Relationship

Hunter, M. C., Teijeira, A. and Halin, C. (2016) 'T cell trafficking through lymphatic vessels', *Frontiers in Immunology*, 7(DEC). doi: 10.3389/fimmu.2016.00613.

Israeli, E. (2012) 'Capsule: MR1 presents microbial vitamin B metabolites to MAIT cells', *Israel Medical Association Journal*, 14(12), p. 773. doi: 10.1038/nature11605.

Janols, H., *et al.* (2010). Lymphocyte and monocyte flow cytometry immunophenotyping as a diagnostic tool in uncharacteristic inflammatory disorders. *BMC Infectious Diseases*, *10*. https://doi.org/10.1186/1471-2334-10-205

Jaspan, H. B., L *et al.* (2011). Immune activation in the female genital tract during HIV infection predicts mucosal CD4 depletion and HIV shedding. *Journal of Infectious Diseases*, *204*(10), 1550–1556. https://doi.org/10.1093/infdis/jir591

Jeffery, H. C., *et al.* (2016). Biliary epithelium and liver B cells exposed to bacteria activate intrahepatic MAIT cells through MR1. *Journal of Hepatology*, *64*(5), 1118–1127. https://doi.org/10.1016/j.jhep.2015.12.017

Jiang, J. *et al.* (2014) 'Mucosal-associated invariant T-cell function is modulated by programmed death-1 signaling in patients with active tuberculosis', *American Journal of Respiratory and Critical Care Medicine*, 190(3), pp. 329–339. doi: 10.1164/rccm.201401-0106OC.

Jiang, J. *et al.* (2016) 'Enhanced immune response of MAIT cells in tuberculous pleural effusions depends on cytokine signaling', *Scientific Reports*. Nature Publishing Group, 6(September), pp. 1–16. doi: 10.1038/srep32320.

Jiang, J., Y *et al.* (2016). Mucosal-associated invariant T cells from patients with tuberculosis exhibit impaired immune response. *Journal of Infection*, *72*(3), 338–352. https://doi.org/10.1016/j.jinf.2015.11.010

Jeffery, H. C. *et al.* (2016) 'Biliary epithelium and liver B cells exposed to bacteria activate intrahepatic MAIT cells through MR1', *Journal of Hepatology*, 64(5), pp. 1118–1127. doi: 10.1016/j.jhep.2015.12.017.

Joosten, S. A., Sullivan, L. C. and Ottenhoff, T. H. M. (2016) 'Characteristics of HLA-E Restricted T-Cell Responses and Their Role in Infectious Diseases', *Journal of Immunology Research*, 2016, pp. 1–11. doi: 10.1155/2016/2695396.

Kee, S. *et al.* (2012) 'Dysfunction of Natural Killer T Cells in Patients with Active Mycobacterium tuberculosis Infection', 80(6), pp. 2100–2108. doi: 10.1128/IAI.06018-11.

Keller, A. N. *et al.* (2017) 'Drugs and drug-like molecules can modulate the function of mucosal-associated invariant T cells', *Nature Immunology*, 18(4), pp. 402–411. doi: 10.1038/ni.3679.

Khaitan, A. *et al.* (2016) 'HIV-infected children have lower frequencies of CD8+ Mucosal-Associated Invariant T (MAIT) cells that correlate with innate, Th17 and Th22 cell subsets', *PLoS ONE*, 11(8), pp. 1–19. doi: 10.1371/journal.pone.0161786.

Khoshnood, S. *et al.* (2018) 'Novel vaccine candidates against Mycobacterium tuberculosis', *International Journal of Biological Macromolecules*. Elsevier B.V., 120, pp. 180–188. doi: 10.1016/j.ijbiomac.2018.08.037.

Kim, C. H., Johnston, B. and Butcher, E. C. (2019) 'Trafficking machinery of NKT cells : shared and differential chemokine receptor expression among V ␣ 24 ƨ V ␤ 11 ƨ NKT cell subsets with distinct cytokine-producing capacity', 100(1), pp. 11–17. doi: 10.1182/blood-2001-12-0196.Supported.

Kim, H. P., Imbert, J. and Leonard, W. J. (2006) 'Both integrated and differential regulation of components of the IL-2/IL-2 receptor system', *Cytokine and Growth Factor Reviews*, 17(5), pp. 349–366. doi: 10.1016/j.cytogfr.2006.07.003.

Kjer-Nielsen, L. *et al.* (2012) 'MR1 presents microbial vitamin B metabolites to MAIT cells', *Nature*, 491(7426), pp. 717–723. doi: 10.1038/nature11605.

Klein, L. *et al.* (2014) 'Positive and negative selection of the T cell repertoire: What thymocytes see (and don't see)', *Nature Reviews Immunology*. Nature Publishing Group, 14(6), pp. 377–391. doi: 10.1038/nri3667.

Klein, L. *et al.* (2014) 'Positive and negative selection of the T cell repertoire: What thymocytes see (and don't see)', *Nature Reviews Immunology*. Nature Publishing Group, 14(6), pp. 377–391. doi: 10.1038/nri3667.

Klein, L., *et al.* (2014). Positive and negative selection of the T cell repertoire: What thymocytes see (and don't see). *Nature Reviews Immunology*, *14*(6), 377–391. https://doi.org/10.1038/nri3667

Koay, H. F. *et al.* (2016) 'A three-stage intrathymic development pathway for the mucosal-associated invariant T cell lineage', *Nature Immunology*. Nature Publishing Group, 17(11), pp. 1300–1311. doi: 10.1038/ni.3565.

Korb, V. C., Chuturgoon, A. A. and Moodley, D. (2016) 'Mycobacterium tuberculosis: Manipulator of protective immunity', *International Journal of Molecular Sciences*, 17(3). doi: 10.3390/ijms17030131.

Kurioka, A. *et al.* (2015) 'MAIT cells are licensed through granzyme exchange to kill bacterially sensitized targets', *Mucosal Immunology*. Nature Publishing Group, 8(2), pp. 429–440. doi: 10.1038/mi.2014.81.

Kwon, D. S., *et al.* (2012). CD4+ CD25+ Regulatory T Cells Impair HIV-1-Specific CD4 T Cell Responses by Upregulating Interleukin-10 Production in Monocytes. *Journal of Virology*, *86*(12), 6586–6594. https://doi.org/10.1128/JVI.06251-11

Kwon, Y. S. *et al.* (2015) 'Mucosal-associated invariant T cells are numerically and functionally deficient in patients with mycobacterial infection and reflect disease activity', *Tuberculosis*. Elsevier Ltd, 95(3), pp. 267–274. doi: 10.1016/j.tube.2015.03.004.

Lawand, M., Déchanet-Merville, J. and Dieu-Nosjean, M. C. (2017) 'Key features of gamma-delta T-cell subsets in human diseases and their immunotherapeutic implications', *Frontiers in Immunology*, 8(JUN). doi: 10.3389/fimmu.2017.00761.

Lawn, S. D. *et al.* (2012) 'Diagnostic accuracy of a low-cost, urine antigen, point-of-care screening assay for HIV-associated pulmonary tuberculosis before antiretroviral therapy: A descriptive study', *The Lancet Infectious Diseases*. Elsevier Ltd, 12(3), pp. 201–209. doi: 10.1016/S1473-3099(11)70251-1.

Lawn, S. D. and Gupta-Wright, A. (2015) 'Detection of lipoarabinomannan (LAM) in urine is indicative of disseminated TB with renal involvement in patients living with hiv and advanced immunodeficiency: Evidence and implications', *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 110(3), pp. 180–185. doi: 10.1093/trstmh/trw008.

Lazar-Molnar, E. *et al.* (2010) 'Programmed death-1 (PD-1)-deficient mice are extraordinarily sensitive to tuberculosis', *Proceedings of the National Academy of Sciences*, 107(30), pp. 13402–13407. doi: 10.1073/pnas.1007394107.

Leeansyah, E. *et al.* (2013) 'Activation, exhaustion, and persistent decline of the antimicrobial MR1-restricted MAIT-cell population in chronic HIV-1 infection', *Blood*, 121(7), pp. 1124–1135. doi: 10.1182/blood-2012-07-445429.

Lempicki, R. a, *et al.* (2000). Impact of HIV-1 infection and highly active antiretroviral therapy on the kinetics of CD4+ and CD8+ T cell turnover in HIV-infected patients. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(25), 13778–13783. https://doi.org/10.1073/pnas.250472097

Lin, P. L. and Flynn, J. A. L. (2015) 'CD8 T cells and Mycobacterium tuberculosis infection', *Seminars in Immunopathology*, 37(3), pp. 239–249. doi: 10.1007/s00281-015-0490-8.

Ling, L. *et al.* (2016) 'Circulating and tumor-infiltrating mucosal associated invariant T (MAIT) cells in colorectal cancer patients', *Scientific Reports*. Nature Publishing Group, 6(May 2015), pp. 1–10. doi: 10.1038/srep20358.

Loh, L. *et al.* (2016) 'Human mucosal-associated invariant T cells contribute to antiviral influenza immunity via IL-18–dependent activation', *Proceedings of the National Academy of Sciences*, 113(36), pp. 10133–10138. doi: 10.1073/pnas.1610750113.

Louis, S., *et al.* (2010). IL-23 and IL-12p70 production by monocytes and dendritic cells in primary HIV-1 infection. *Journal of Leukocyte Biology*, *87*(4), 645–653. https://doi.org/10.1189/jlb.1009684

Luckheeram, R. V. *et al.* (2012) 'CD4 +T cells: Differentiation and functions', *Clinical and*

Developmental Immunology, 2012. doi: 10.1155/2012/925135.

Madan-Lala, R. *et al.* (2014) ‘Mycobacterium tuberculosis Impairs Dendritic Cell Functions through the Serine Hydrolase Hip1’, *The Journal of Immunology*, 192(9), pp. 4263–4272. doi: 10.4049/jimmunol.1303185.

Maecker, H. T., and Trotter, J. (2006). Flow cytometry controls, instrument setup, and the determination of positivity. *Cytometry Part A*, *69*(9), 1037–1042. https://doi.org/10.1002/cyto.a.20333

Martin, E. *et al.* (2009) ‘Stepwise development of mait cells in mouse and human’, *PLoS Biology*, 7(3), pp. 0525–0536. doi: 10.1371/journal. pbio.1000054.

McCausland, M. R., *et al.* (2015). Altered monocyte phenotype in HIV-1 infection tends to normalize with integrase- inhibitor-based antiretroviral therapy. *PLoS ONE*, *10*(10), 1–19. https://doi.org/10.1371/journal.pone.0139474

McCune, J. M. (2001). The dynamics of CD4+ T-cell depletion in HIV disease. *Nature*, *410*(6831), 974–979. https://doi.org/10.1038/35073648

McWilliam, H. E. G. *et al.* (2016) ‘The intracellular pathway for the presentation of Vitamin B-related antigens by the antigen-presenting molecule MR1’, *Nature Immunology*, 17(5), pp. 531–537. doi: 10.1038/ni.3416.

McWilliam, H. E. G. and Villadangos, J. A. (2017) ‘How MR1 Presents a Pathogen Metabolic Signature to Mucosal-Associated Invariant T (MAIT) Cells’, *Trends in Immunology*. Elsevier Ltd, 38(9), pp. 679–689. doi: 10.1016/j.it.2017.06.005.

McWilliam, H. E. and Villadangos, J. A. (2018) ‘MR1 antigen presentation to MAIT cells: new ligands, diverse pathways?’, *Current Opinion in Immunology*. Elsevier Ltd, 52, pp. 108–113. doi: 10.1016/j.coi.2018.04.022.

Medzhitov, R. and Janeway, C. A. J. (1997) ‘Innate immunity: Minireview the virtues of a nonclonal system of recognition’, *Cell*, 91(3), pp. 295–298. doi: 10.1016/j.immuni.2007.05.022.Toll-like.

Meierovics, A., Yankelevich, W.-J. C. and Cowley, S. C. (2013) ‘MAIT cells are critical for optimal mucosal immune responses during in vivo pulmonary bacterial infection’, *Proceedings of the National Academy of Sciences*, 110(33), pp. E3119–E3128. doi: 10.1073/pnas.1302799110.

Meintjes, G. and Wilkinson, R. J. (2010) ‘Undiagnosed Active Tuberculosis in HIV-Infected Patients Commencing Antiretroviral Therapy’, *Clinical Infectious Diseases*, 51(7), pp. 830–832. doi: 10.1086/656283.

Melo, A. M. *et al.* (2019) 'Mucosal-Associated Invariant T Cells Display Diminished Effector Capacity in Oesophageal Adenocarcinoma', 10(July), pp. 1–17. doi: 10.3389/fimmu.2019.01580.

Méndez-Samperio, P. (2010). Role of interleukin-12 family cytokines in the cellular response to mycobacterial disease. *International Journal of Infectious Diseases*, *14*(5), e366–e371. https://doi.org/10.1016/j.ijid.2009.06.022

Mihret, A. *et al.* (2011) 'Dendritic Cells Activate and Mature after Infection with Mycobacterium tuberculosis', *BMC Research Notes*. BioMed Central Ltd, 4(1), p. 247. doi: 10.1186/1756-0500-4-247.

Mihret, A. (2012) 'The role of dendritic cells in Mycobacterium tuberculosis infection', 3(7), pp. 654–659.

Mildner, A. and Jung, S. (2014) 'Immunity Review Development and Function of Dendritic Cell Subsets', *Immunity*. Elsevier Inc., 40(5), pp. 642–656. doi: 10.1016/j.immuni.2014.04.016.

Mishra, S., *et al.* (2009). Immune Response and Possible Causes of CD4+T-cell Depletion in Human Immunodeficiency Virus (HIV) - 1 Infection. *The Open Nutraceuticals Journal*, *2*(1), 46–51. https://doi.org/10.2174/1876396000902010046

Mittrücker, H. W., Visekruna, A. and Huber, M. (2014) 'Heterogeneity in the Differentiation and Function of CD8$^+$ T Cells', *Archivum Immunologiae et Therapiae Experimentalis*, 62(6), pp. 449–458. doi: 10.1007/s00005-014-0293-y.

Muller, I. *et al.* (1987) 'Impaired Resistance to Mycobacterium tuberculosis Infection after Selective In Vivo Depletion of L3T4 + and Lyt-2 + T Cells', 55(9), pp. 2037–2041.

Napier, R. J. *et al.* (2015) 'The role of mucosal associated invariant T cells in antimicrobial immunity', *Frontiers in Immunology*, 6(JUN), pp. 1–10. doi: 10.3389/fimmu.2015.00344.

Nunes-Alves, C., *et al.* (2014). In search of a new paradigm for protective immunity to TB. *Nature Reviews Microbiology*, *12*(4), 289–299. https://doi.org/10.1038/nrmicro3230

Okoye, A. A. and Picker, L. J. (2013) 'CD4(+) T-cell depletion in HIV infection: mechanisms of immunological failure', *Immunological reviews*, 254(1), pp. 54–64. doi: 10.1111/imr.12066.

Pai, M. *et al.* (2014) 'Gamma interferon release assays for detection of Mycobacterium tuberculosis infection', *Clinical Microbiology Reviews*, 27(1), pp. 3–20. doi: 10.1128/CMR.00034-13.

Pai, M. *et al.* (2016) 'Tuberculosis', *Nature Reviews Disease Primers*, 2, p. 16076. doi: 10.1038/nrdp.2016.76.

Paiardini, M. and Müller-Trutwin, M. (2013) 'HIV-associated chronic immune activation', *Immunological Reviews*, 254(1), pp. 78–101. doi: 10.1111/imr.12079.

Palmieri, F. *et al.* (2002) 'Pulmonary tuberculosis in HIV-infected patients presenting with normal chest radiograph and negative sputum smear', *Infection*, 30(2), pp. 68–74. doi: 10.1007/s15010-002-2062-9.

Palmieri, F., *et al.* (2002). Pulmonary tuberculosis in HIV-infected patients presenting with normal chest radiograph and negative sputum smear. *Infection*, *30*(2), 68–74. https://doi.org/10.1007/s15010-002-2062-9

Paquin-Proulx, D., *et al.* (2018). Latent Mycobacterium tuberculosis infection is associated with a higher frequency of mucosal-associated invariant T and invariant natural killer T cells. *Frontiers in Immunology*, *9*(JUN). https://doi.org/10.3389/fimmu.2018.01394

Paquin-Proulx, D., G *et al.* (2017). MAIT cells are reduced in frequency and functionally impaired in human T lymphotropic virus type 1 infection: Potential clinical implications. *PLoS ONE*, *12*(4), 1–17. https://doi.org/10.1371/journal.pone.0175345

Post, F. A., Wood, R., and Maartens, G. (2012). CD4 and total lymphocyte counts as predictors of HIV disease progression. *Qjm*, *89*(7), 505–508. https://doi.org/10.1093/qjmed/89.7.505

Reantragoon, R. *et al.* (2013) 'Antigen-loaded MR1 tetramers define T cell receptor heterogeneity in mucosal-associated invariant T cells', *The Journal of Experimental Medicine*, 210(11), pp. 2305–2320. doi: 10.1084/jem.20130958.

Reantragoon, R. *et al.* (2016) 'Mucosal-associated invariant T cells in clinical diseases', *Asian Pacific Journal of Allergy and Immunology*, 34(1), pp. 3–10.

Redford, P. S., Murray, P. J., and O'Garra, A. (2011). The role of IL-10 in immune regulation during M. tuberculosis infection. *Mucosal Immunology*, *4*(3), 261–270. https://doi.org/10.1038/mi.2011.7

Rodríguez-Pinto, D. (2005). B cells as antigen presenting cells. *Cellular Immunology*, *238*(2), 67–75. https://doi.org/10.1016/j.cellimm.2006.02.005

Roff, S. R., Noon-song, E. N., and Yamamoto, J. K. (2014). The significance of interferon- γ in HIV-1 pathogenesis , therapy , and prophylaxis, *4*(January), 1–11. https://doi.org/10.3389/fimmu.2013.00498

Rohrer, J. W., and Coggin, J. H. (1995). CD8 T cell clones inhibit antitumor T cell function by secreting IL-10. *The Journal of Immunology*, *155*(12), 5719 LP-5727. Retrieved from http://www.jimmunol.org/content/155/12/5719.abstract

Robertson, B. D. *et al.* (2012) 'Detection and treatment of subclinical tuberculosis', *Tuberculosis*. Elsevier Ltd, 92(6), pp. 447–452. doi: 10.1016/j.tube.2012.06.004.

Rodríguez-Pinto, D. (2005) 'B cells as antigen presenting cells', *Cellular Immunology*, 238(2), pp. 67–75. doi: 10.1016/j.cellimm.2006.02.005.

Rueda, C. M., *et al.* (2010). Characterization of CD4 and CD8 T cells producing IFN-γ in human latent and active tuberculosis. *Tuberculosis*, *90*(6), 346–353. https://doi.org/10.1016/j.tube.2010.09.003

Sachdeva, M. *et al.* (2010) 'Immune exhaustion occurs concomitantly with immune activation and decrease in regulatory T cells in viremic chronically HIV-1-infected patients', *Journal of Acquired Immune Deficiency Syndromes*, 54(5), pp. 447–454. doi: 10.1097/QAI.0b013e3181e0c7d0.

Sada-ovalle, I. *et al.* (2008) 'Innate Invariant NKT Cells Recognize Mycobacterium tuberculosis – Infected Macrophages , Produce Interferon- c , and Kill Intracellular Bacteria', 4(12). doi: 10.1371/journal.ppat.1000239.

Saeidi, A. *et al.* (2015) 'Attrition of TCR Vα7.2+ CD161++ MAIT cells in HIV-tuberculosis co-infection is associated with elevated levels of PD-1 expression', *PLoS ONE*, 10(4), pp. 1–14. doi: 10.1371/journal.pone.0124659.

Saeidi, A. *et al.* (2016) 'Functional role of mucosal-associated invariant T cells in HIV infection', *Journal of Leukocyte Biology*, 100(2), pp. 305–314. doi: 10.1189/jlb.4RU0216-084R.

Sakhno, L. V. *et al.* (2015) 'Impairments of Antigen-Presenting Cells in Pulmonary Tuberculosis', *Journal of Immunology Research*, 2015. doi: 10.1155/2015/793292.

Sampath, P. *et al.* (2018) 'Monocyte Subsets: Phenotypes and Function in Tuberculosis Infection', *Frontiers in Immunology*, 9(July), pp. 1–8. doi: 10.3389/fimmu.2018.01726.

Sasindran, S. J. and Torrelles, J. B. (2011) 'Mycobacterium tuberculosis infection and inflammation: What is beneficial for the host and for the bacterium?', *Frontiers in Microbiology*, 2(JAN), pp. 1–16. doi: 10.3389/fmicb.2011.00002.

Sánchez, M. D., *et al.* (2006). Functional and phenotypic changes in monocytes from patients with tuberculosis are reversed with treatment. *Microbes and Infection*, *8*(9–10), 2492–2500. https://doi.org/10.1016/j.micinf.2006.06.005

Schmaler, M. *et al.* (2018) 'Modulation of bacterial metabolism by the microenvironment controls MAIT cell stimulation article', *Mucosal Immunology*. Springer US, 11(4), pp. 1060–1070. doi: 10.1038/s41385-018-0020-9.

Scorgie, F., *et al.* (2019). Qualitative assessment of South African healthcare worker perspectives on an instrument-free rapid CD4 test. *BMC Health Services Research*, *19*(1), 123. https://doi.org/10.1186/s12913-019-3948-x

Seach, N. *et al.* (2013) 'Double Positive Thymocytes Select Mucosal-Associated Invariant T Cells', *The Journal of Immunology*, 191(12), pp. 6002–6009. doi: 10.4049/jimmunol.1301212.

Shankar, P., *et al.* (2000). Impaired function of circulating HIV-specific CD8(+) T cells in chronic human immunodeficiency virus infection. *Blood*, *96*(9), 3094–3101. Retrieved from www.bloodjournal.org

Shah, D. K. and Zúñiga-pflücker, J. C. (2019) 'An Overview of the Intrathymic Intricacies of T Cell Development'. doi: 10.4049/jimmunol.1302259.

Sharma, P. K. *et al.* (2015) 'High expression of CD26 accurately identifies human bacteria-reactive MR1-restricted MAIT cells', *Immunology*, 145(3), pp. 443–453. doi: 10.1111/imm.12461.

Shey, M. S. *et al.* (2015) 'The role of dendritic cells in driving genital tract inflammation and HIV transmission risk: Are there opportunities to intervene?', *Innate Immunity*, 21(1), pp. 99–112. doi: 10.1177/1753425913513815.

Shey, M. S. *et al.* (2018) 'Contribution of APCs to mucosal-associated invariant T cell activation in infectious disease and cancer', *Innate Immunity*, 24(4), pp. 192–202. doi: 10.1177/1753425918768695.

Siberry, G. K., *et al.* (2014). The future role of CD4 cell count for monitoring antiretroviral therapy. The Lancet Infectious Diseases, 15(2), 241–247. https://doi.org/10.1016/s1473-3099(14)70896-5

Silva, B. D. de S. *et al.* (2014) 'Different phenotypes of CD8+ T cells associated with bacterial load in active tuberculosis', *Immunology Letters*. Elsevier B.V., 160(1), pp. 23–32. doi: 10.1016/j.imlet.2014.03.009.

Sinha, A. *et al.* (2007) 'Mycobacterium tuberculosis and dendritic cells: Recognition, activation and functional implications', *Indian Journal of Biochemistry and Biophysics*, 44(5), pp. 279–288.

Slight, S. R. and Khader, S. A. (2013) 'Chemokines shape the immune responses to tuberculosis', *Cytokine and Growth Factor Reviews*. Elsevier Ltd, 24(2), pp. 105–113. doi: 10.1016/j.cytogfr.2012.10.002.

Smith, D. J. *et al.* (2014) 'Reduced mucosal associated invariant T-cells are associated with increased disease severity and Pseudomonas aeruginosa infection in cystic fibrosis', *PLoS ONE*, 9(10), p. V. doi: 10.1371/journal.pone.0109891.

Soudais, C. *et al.* (2015) 'In Vitro and In Vivo Analysis of the Gram-Negative Bacteria–Derived Riboflavin Precursor Derivatives Activating Mouse MAIT Cells', *The Journal of Immunology*, 194(10), pp. 4641–4649. doi: 10.4049/jimmunol.1403224.

Spaan, M. *et al.* (2016) 'Frequencies of circulating MAIT cells are diminished in chronic hCV, HIV and HCV/ HIV Co-Infection and do not recover during therapy', *PLoS ONE*, 11(7), pp. 1–13. doi: 10.1371/journal.pone.0159243.

Steinman, R. M. *et al.* (1988) 'The sensitization phase of T-cell-mediated immunity. [Review]', *Annals of the New York Academy of Sciences*, 546, pp. 80–90.

Stenger, S. *et al.* (1998) 'An antimicrobial activity of cytolytic T cells mediated by granulysin', *Science*, 282(5386), pp. 121–125. doi: 10.1126/science.282.5386.121.

Sundström, P. *et al.* (2015) 'Human Mucosa-Associated Invariant T Cells Accumulate in Colon Adenocarcinomas but Produce Reduced Amounts of IFN-γ', *The Journal of Immunology*, 195(7), pp. 3472–3481. doi: 10.4049/jimmunol.1500258.

Sutherland, R., *et al.* (2006). Impaired IFN-γ-secreting capacity in mycobacterial antigen-specific CD4 T cells during chronic HIV-1 infection despite long-term HAART. Aids, 20(6), 821–829. https://doi.org/10.1097/01.aids.0000218545.31716.a4

Takaba, H. and Takayanagi, H. (2017) 'The Mechanisms of T Cell Selection in the Thymus', *Trends in Immunology*. Elsevier Ltd, 38(11), pp. 805–816. doi: 10.1016/j.it.2017.07.010.

Théry, C., and Amigorena, S. (n.d.). Downmodulation of internalization in mature dendritic cells, 45–51. Retrieved from https://ac.els-cdn.com/S0952791500001801/1-s2.0-S0952791500001801-main.pdf?_tid=8ac7c6ae-b887-11e7-a207-

Thomas, G. D. *et al.* (2017) 'Human blood monocyte subsets: a new gating strategy defined using cell surface markers identified by mass cytometry Co-Corresponding Authors: HHS Public Access', *Arterioscler Thromb Vasc Biol*, 37(8), pp. 1548–1558. doi: 10.1161/ATVBAHA.117.309145.

Tian, T. *et al.* (2005) ' In Vivo Depletion of CD11c + Cells Delays the CD4 + T Cell Response to Mycobacterium tuberculosis and Exacerbates the Outcome of Infection ', *The Journal of Immunology*, 175(5), pp. 3268–3272. doi: 10.4049/jimmunol.175.5.3268.

Trautmann, L. *et al.* (2006) 'Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction', *Nature Medicine*, 12(10), pp. 1198–1202. doi:

10.1038/nm1482.

Treiner, E. *et al.* (2003) ‘Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1’, *Nature*, 422(6928), pp. 164–169. doi: 10.1038/nature01433.

Ussher, J. E. *et al.* (2014) ‘CD161++CD8+ T cells, including the MAIT cell subset, are specifically activated by IL-12+IL-18 in a TCR-independent manner’, *European Journal of Immunology*, 44(1), pp. 195–203. doi: 10.1002/eji.201343509.

Ussher, J. E., Klenerman, P. and Willberg, C. B. (2014) ‘Mucosal-associated invariant T-cells: New players in anti-bacterial immunity’, *Frontiers in Immunology*, 5(OCT), pp. 1–9. doi: 10.3389/fimmu.2014.00450.

Venkataswamy, M. M. *et al.* (2010) ‘NIH Public Access’, 183(3), pp. 1644–1656. doi: 10.4049/jimmunol.0900858.Incorporation.

Vijayan, K. V. *et al.* (2017) ‘Pathophysiology of CD4+ T-Cell depletion in HIV-1 and HIV-2 infections’, *Frontiers in Immunology*, 8(MAY), pp. 1–8. doi: 10.3389/fimmu.2017.00580.

Voskoboinik, I., Whisstock, J. C. and Trapani, J. A. (2015) ‘Perforin and granzymes: Function, dysfunction and human pathology’, *Nature Reviews Immunology*. Nature Publishing Group, 15(6), pp. 388–400. doi: 10.1038/nri3839.

Walker, N. F. *et al.* (2019) Invariant Natural Killer T cell dynamics in HIV-associated tuberculosis. *Clin Infect Dis.* doi: 10.1093/cid/ciz501.

Wazen, R. M. *et al.* (2014) ‘NIH Public Access’, 8(9), pp. 1385–1395. doi: 10.2217/nnm.12.167.Gene.

Van Wilgenburg, B. *et al.* (2016) ‘MAIT cells are activated during human viral infections’, *Nature Communications*, 7. doi: 10.1038/ncomms11653.

WHO. (2017). *Guidelines for managing advanced HIV disease and rapid initiation of antiretroviral therapy. Geneva: World Healh Organisation; July 2017*.

Won, E. J. *et al.* (2016) ‘Clinical relevance of circulating mucosal-associated invariant T cell levels and their anti-cancer activity in patients with mucosal-associated cancer’, *Oncotarget*, 7(46). doi: 10.18632/oncotarget.11187.

Wong, E. B. *et al.* (2013) ‘Low levels of peripheral CD161++CD8+ Mucosal Associated Invariant T (MAIT) cells are found in HIV and HIV/TB co-infection’, *PLoS ONE*, 8(12), pp. 1–9. doi: 10.1371/journal.pone.0083474.

Wong, E. B., Ndung’u, T. and Kasprowicz, V. O. (2017) ‘The role of mucosal-associated invariant T cells in infectious diseases’, *Immunology*, 150(1), pp. 45–54. doi:

10.1111/imm.12673.

Wong, E., Gold, M., Meermeier, E., Xulu, B., Khuzwayo, S., Sullivan, Z., Mahyari, E., Rogers, Z., Kløverpris, H., Sharma, P., Worley, A., Lalloo, U., Baijnath, P., Ambaram, A., Naidoo, L., Suleman, M., Madansein, R., McLaren, J., Ladell, K., Miners, K., Price, D., Behar, S., Nielsen, M., Kasprowicz, V., Leslie, A., Bishai, W., Ndung'u, T. and Lewinsohn, D. (2019). TRAV1-2+ CD8+ T-cells including oligoconal expansions of MAIT cells are enriched in the airways in human tuberculosis. *Communications Biology*, 2(1).https://doi.org/10.1038/s42003-019-0442-2

Wu, C. *et al.* (2015) 'Antigen-specific human NKT cells from tuberculosis patients produce IL-21 to help B cells for the production of immunoglobulins', 6(30).

Younas, M. *et al.* (2016) 'Immune activation in the course of HIV-1 infection: Causes, phenotypes and persistence under therapy', *HIV Medicine*, 17(2), pp. 89–105. doi: 10.1111/hiv.12310.

Xia, H., *et al.* (2018). Elevated level of CD4+t cell immune activation in acutely HIV-1-infected stage associates with increased IL-2 production and cycling expression, and subsequent CD4+T cell preservation. *Frontiers in Immunology*, *9*(MAR). https://doi.org/10.3389/fimmu.2018.00616

Younes, S. A. *et al.* (2016) 'IL-15 promotes activation and expansion of CD8+ T cells in HIV-1 infection', *Journal of Clinical Investigation*, 126(7), pp. 2745–2756. doi: 10.1172/JCI85996.

Zabijak, L. *et al.* (2015) 'Increased tumor infiltration by mucosal-associated invariant T cells correlates with poor survival in colorectal cancer patients', *Cancer Immunology, Immunotherapy*. Springer Berlin Heidelberg, 64(12), pp. 1601–1608. doi: 10.1007/s00262-015-1764-7.

Ziegler-Heitbrock, L. (2006) 'The CD14+ CD16+ blood monocytes: their role in infection and inflammation', *Journal of Leukocyte Biology*, 81(3), pp. 584–592. doi: 10.1189/jlb.0806510.

Appendix

Antibody titrations.

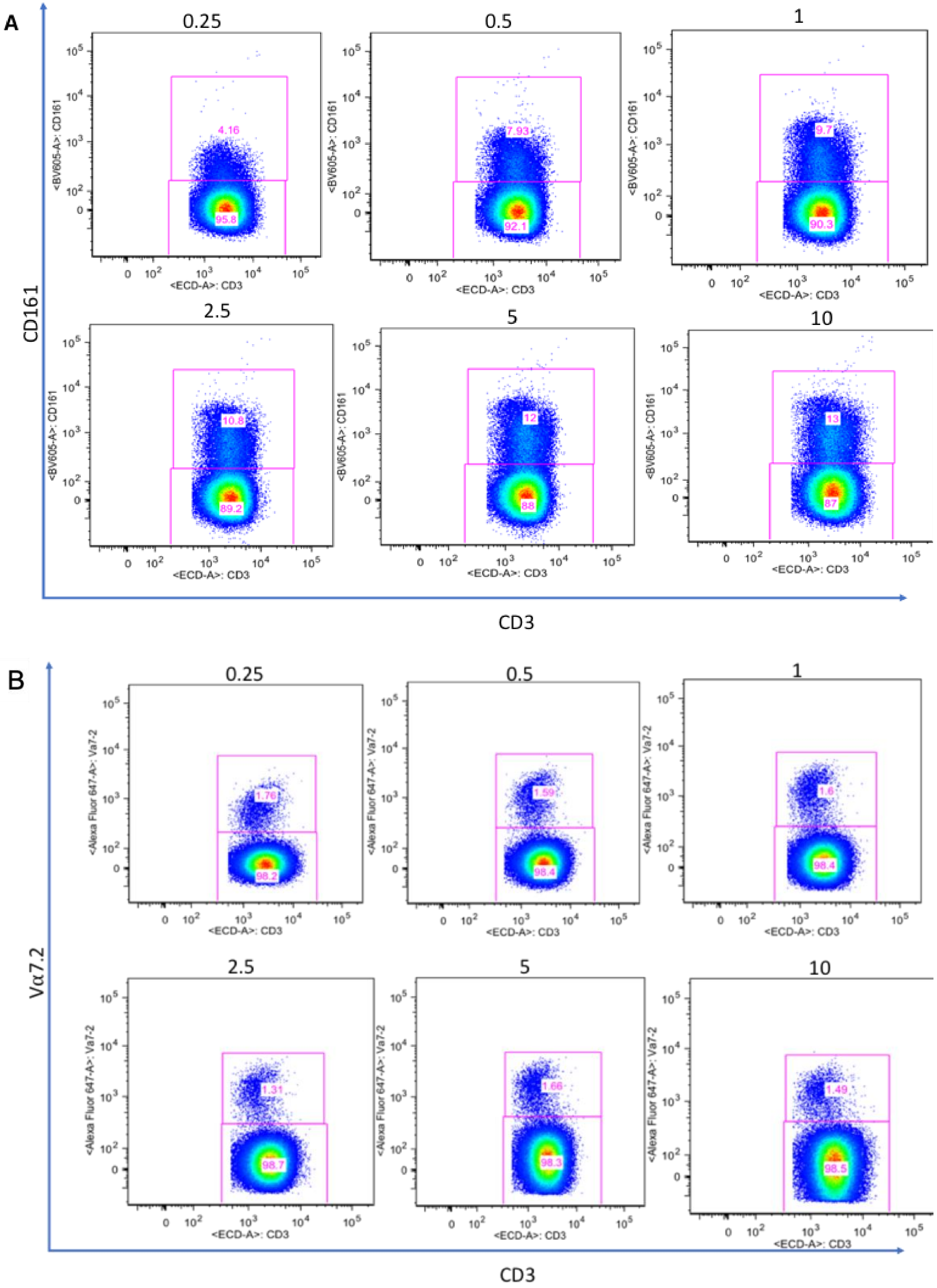
All titrations were conducted using PBMCs isolated from blood obtained from the Western Province Blood Transfusion Services (WPBTS) and these were performed by use of 6 serial dilutions of each of the antibodies with an initial concentration of 2X the recommended concentration by the manufacturer. For surface markers, no stimulation was required. However, the titration of IL-12 and CD40 (produced/expressed by innate cells) required stimulation of PBMCs with LPS and the procedure described in section 2.4.2 was followed.

Optimal antibody titres were determined from the staining indexes which were calculated from the median fluorescence intensities (MFI) of the positive and negative cell populations and the robust standard deviation of the negative population for each antibody as shown below:

$$SI = \frac{\text{median}_{\text{positive}} - \text{median}_{\text{negative}}}{2 \times SD_{\text{negative}}}$$

To obtain the optimal titres, staining indexes were plotted against respective antibody concentrations. Fig. A1 below shows the representative flow cyometry plots for the titrations of CD161 and Vα7.2 antibodies, respectively, with the frequencies of CD161+ and Vα7.2+ cells gated against total CD3+ T cells. It was observed that there was an increase in the frequencies of CD161+ cells with increasing volumes of antibodies from 4.16% at the lowest antibody concentration to 12% at the highest.

Fig. A1.B shows the frequencies of Vα7.2+ cells and although there seems to be no trend of increasing frequencies of Vα7.2+ cells, it was observed that the separation between Vα7.2- and Vα7.2+ cell populations was different between different antibody concentrations. Fig. A1.C and Fig. A1.D are the summary plots for CD161 and Vα7.2 antibodies, respectively, showing the antibody volumes plotted against the staining indexes. For CD161, the staining index was observed to increase with increasing antibody volumes, peaking at 5 µl, followed by a decrease at 10 µl. For Vα7.2, an increase in the staining index was observed from 0.25 µl to 0.5 µl followed by a decrease from 1 µl to 10 µl of antibody. The volume of each antibody with the highest staining index (peak) was selected for the flow cytometry panel. These are indicated by the red squares on each plot.



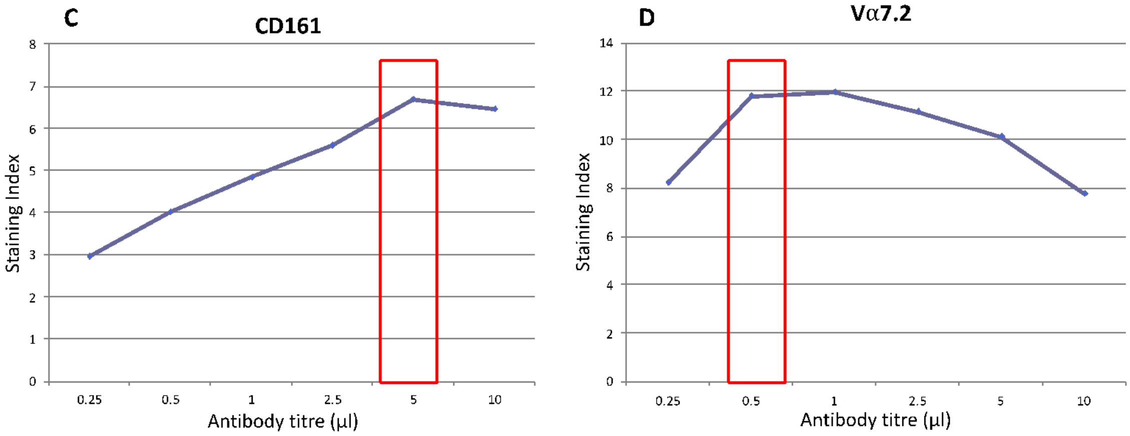


Figure A1: Titration of CD161 and Vα7.2 antibodies for flow cytometry panel. A) and **B)** show the representative flow cytometry plots for the titrations of CD161 and Vα7.2, respectively. Values above each plot represent the final volumes of antibodies used. **C)** and **D)** are the summary plots of the calculated staining index against the volumes of antibodies used in each titre. Titres indicated are in a total of 50 μl staining volume.

Application settings

We developed an application setting with optimal voltages for our panel. This was done after all the antibodies were titrated. To do this, we first prepared 15 FACS tubes, 14 of these tubes were labelled according to the antibody-fluorochrome combination in our flow cytometry panel (including the channels for viability marker and FITC for the GFP), and 1 tube was labelled as the unstained tube. (1 million cells/well) were added into each of the tubes, followed by the corresponding antibody-fluorochrome combination (no antibody was added into the unstained tube). The electronic noise (EN) and the Linearity maximum (Lmax) for each detector required were selected from the baseline performance report. We then calculated 2.5X EN and 3X EN to represent the position for the negative population for each channel. We ran the unstained cells adjusting the voltages until the set robust standard deviations (rSD) of the negative population was between the EN range above for each channel/detector. To set the position for the positive population, we calculated 0.5X Lmax for all required channels/detectors. We then acquired the samples stained with each antibody fluorochrome in the panel (Samples were stimulated if required to detect the marker of interest). The voltages for each specific channel were adjusted appropriately until the rSD of the positive population had the maximum value of 0.5X Lmax for the required channel. If there was discrepancy in the voltages needed to set the negative or positive population, the voltage for the positive population was chosen as it was necessary to have the positive population in range. When all voltages were set, the application setting was saved. Following this, a compensation set up with singly-stained beads was completedto make sure the voltages were appropriate. The application setting with the final voltages was then saved as final and used for all flow cytometry runs. The voltages for the application setting were linked to those of the Fortessa and were automatically appropriately adjusted when the daily QC run for the machine was performed.

The effect of cell culture media on background cellular responses

Monocyte responses were assessed through expression of TNF-α (TNF-α was only used for the testing experiment and not in the final flow cytometry panel, the background responses for both antigen presenting cells (APCs) (myeloid dendritic cells and monocytes) were observed to be lower for Hyclone FBS in comparison to Biochrome FBS. Stimulation with LPS resulted in greater antigen specific responses (TNF-α) with Hyclone FBS compared to Biochrome FBS for both APCs. For MAIT cells, background responses were similar between Biochrome FBS and Hyclone FBS as the frequencies of MAIT cells producing IFN-γ and TNF-α were similar between Biochrome FBS and Hyclone FBS (Fig. A2.C). With PHA stimulation, however, it was observed that frequencies of MAIT cells producing IFN-γ and TNF-α were lower in the cells incubated in Hyclone FBS compared to those under Biochrome FBS (Fig. A2.D).

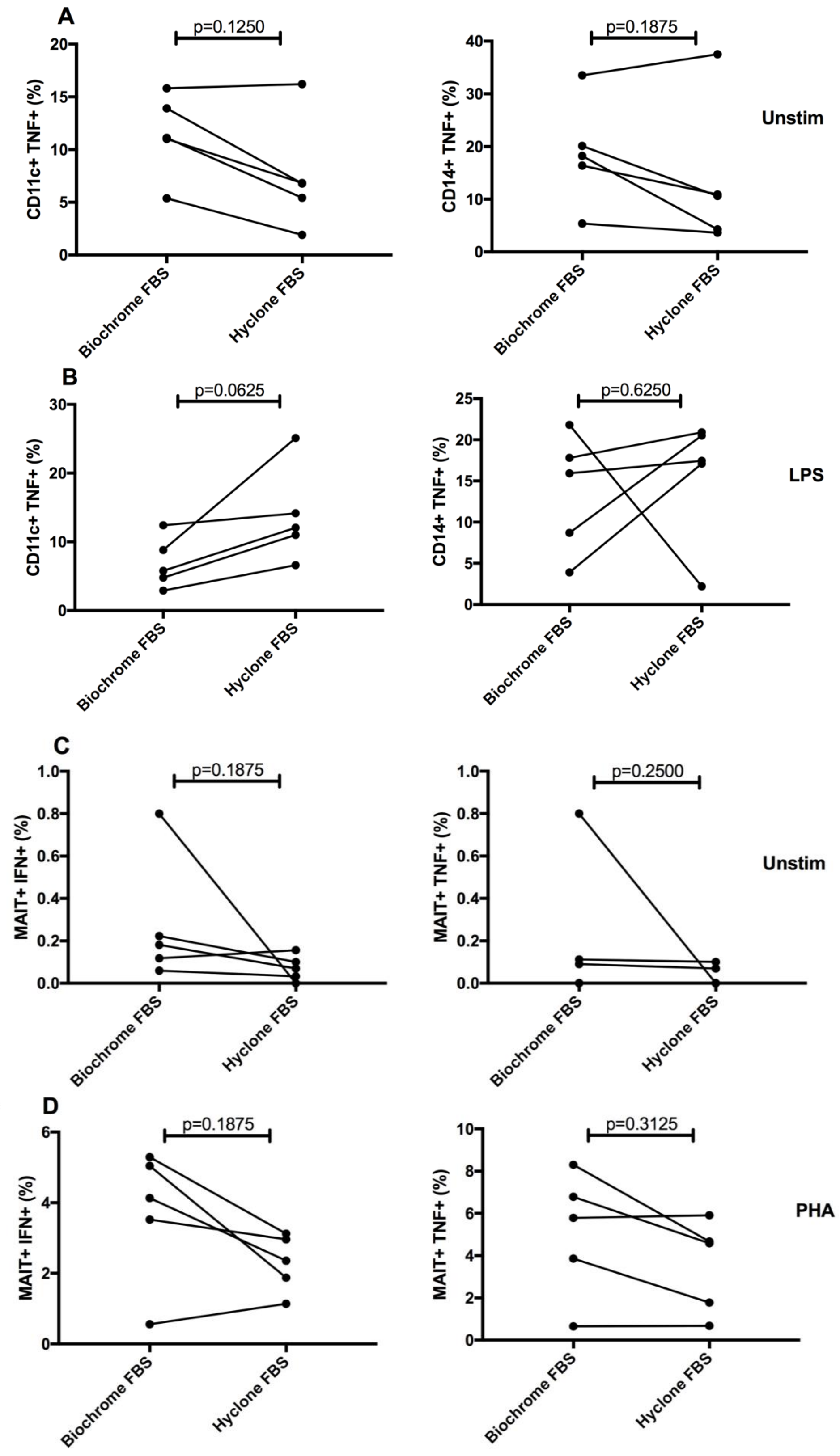


Figure A2: Effects of cell culture medium reconstituted with different sources of FBS on innate cell responses. A) Comparison of innate cell responses of dendritic cells (CD11c+) and monocytes (CD14+) between Biochrome and Hyclone FBS without antigen stimulation. B) Innate cell responses after stimulation with LPS showing antigen specific responses for both dendritic cells and monocytes in cells incubated with R10 media containing Hyclone FBS compared with those in Biochrome FBS. C) and D) represent MAIT cell responses before and after stimulation with PHA in cells incubated with R10 media containing Hyclone FBS compared with those in Biochrome FBS. P-value represents the Wilcoxon matched-pairs rank tests that were conducted for the paired datasets from the APC background and LPS or PHA-specific responses.

Preparation of BCG-GFP stocks.

We prepared new stocks of BCG-GFP by aliquoting BCG-GFP into 50 ml Middlebrook 7H9 media and incubated the culture at 37°C with shaking and we monitored growth over several days by observation of the turbidity of the broth. We included 50 ml of 7H9 media as a negative control for contamination. After 1 week, we transferred 1 ml of broth culture into cuvettes for OD_{600} measurements on a Novespec Plus spectrophotometer (Amersham Biosciences) with 7H9 media as a blank. We then monitored the culture and did OD_{600} measurements until the OD_{600} was observed to be 0.6. At 3 weeks, the culture was removed from incubator and aliquots were prepared in 20% glycerol and stored at -80°C.

BCG titration.

We thawed and rested PBMCs from 3 donors overnight, following which we then counted and adjusted concentrations to 10 million cells/ml. We then stimulated PBMCs from each donor with the different concentrations of BCG for 4 hours and then added golgi-blockers and incubated for an additional 4 hours (the duration of stimulation was only for optimisation purposes and was changed following assessments of IL-12 kinetics). We then stained the samples for flow cytometry to assess the effects of the different BCG concentrations on 1) monocyte infection and responses by assessing GFP^{+} monocytes and TNF-α^{+} monocytes, respectively, 2) MAIT cell responses by assessing IFN-γ and TNF-α expression by MAIT cells.

Monocyte infection

Fig. A3.A shows a representative flow cytometry plots of monocyte infection with BCG of different MOI. Unstimulated condition was used as background control for GFP+ monocytes and TNF-α expression. Stimulation with LPS resulted in an increase in the expression of TNF-α relative to the unstimulated control while the frequencies of GFP+ monocytes was compared to background in unstimulated. Stimulation with live BCG-GFP resulted in both increase in GFP+ monocytes and TNF-α expression by monocytes relative to the unstimulated control. Fig. A3.B shows the summary plots for the GFP+ monocytes and monocyte TNF-α expression for all 3 donors used. From these plots, we found that the frequencies of GFP+ monocytes increased with increasing MOI of BCG. The MOI=5 of BCG was observed to result in higher frequencies of GFP+ monocytes and TNF-α producing monocytes, especially in donor 1 and 2.

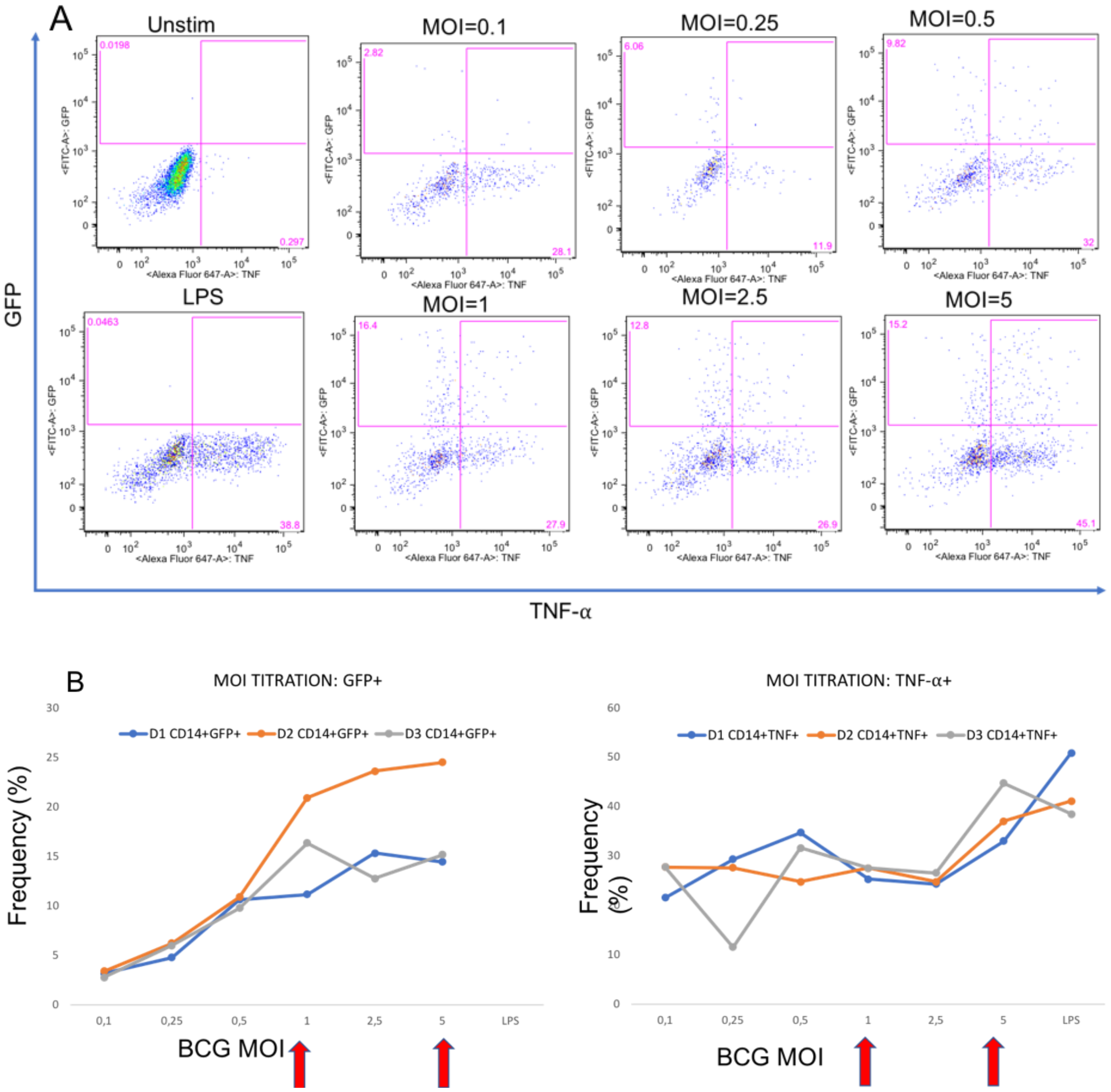
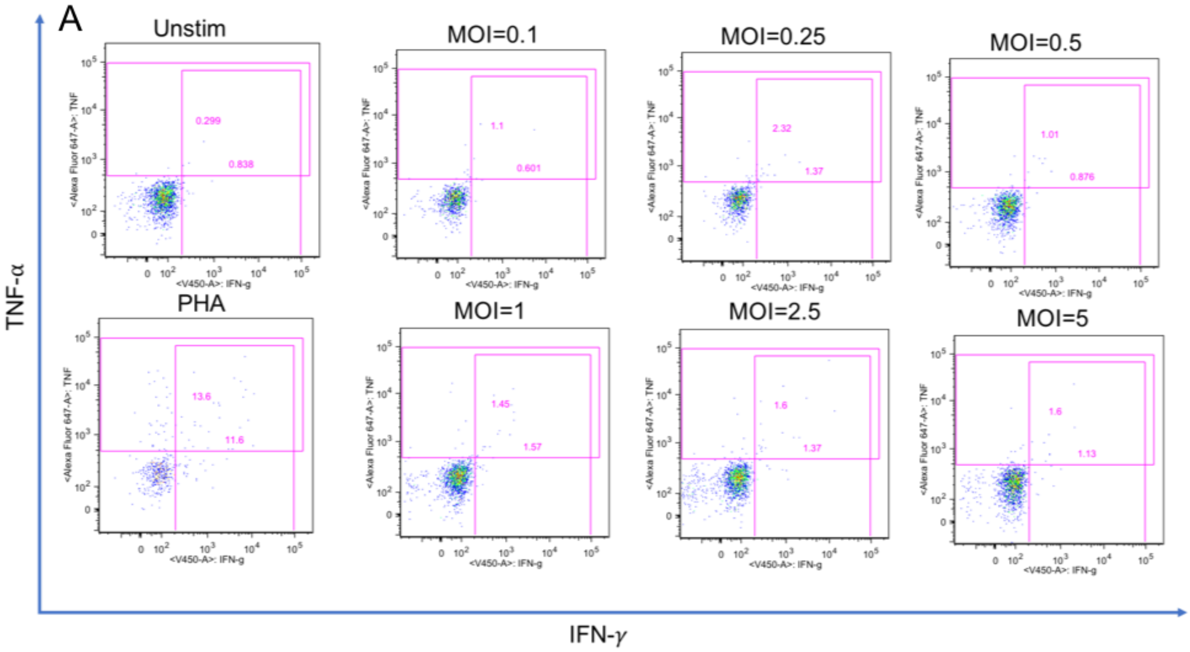


Figure A3 : CD14+ monocyte infection and responses to stimulation with different MOI of BCG. A) Representative flow cytometry plots showing BCG infection and TNF-α expression in response to infection with different MOI of BCG. B) Summary plots showing the frequencies of infected monocytes at different BCG MOI on the left and the frequencies of TNF-α+ monocytes in response to the different MOI of BCG.

MAIT cell responses

Fig. A4 shows the representative flow cytometry plots for IFN-γ and TNF-α expression by MAIT cells in response to stimulation with BCG at different MOI and PHA. Low background responses were observed in unstimulated control. Stimulation with PHA resulted in higher expression of IFN-γ and TNF-α by MAIT cells. Stimulation with BCG at different MOI was also observed to increase the frequencies of MAIT cells expressing IFN-γ and TNF-α compared to unstimulated control and these were lower compared to PHA stimulation.

From the summary plots in Fig. A4, the frequencies of MAIT cells producing IFN-γ and TNF-α were observed to be increased from the BCG MOI=0.25. However, IFN-γ and TNF-α expression were then reduced at MOI=0.5 and highest at MOI=1 for all 3 donors followed by another decrease at higher MOIs. The BCG MOI=1 was observed to result in the highest frequencies of MAIT cells expressing IFN-γ and TNF-α in all 3 donors.



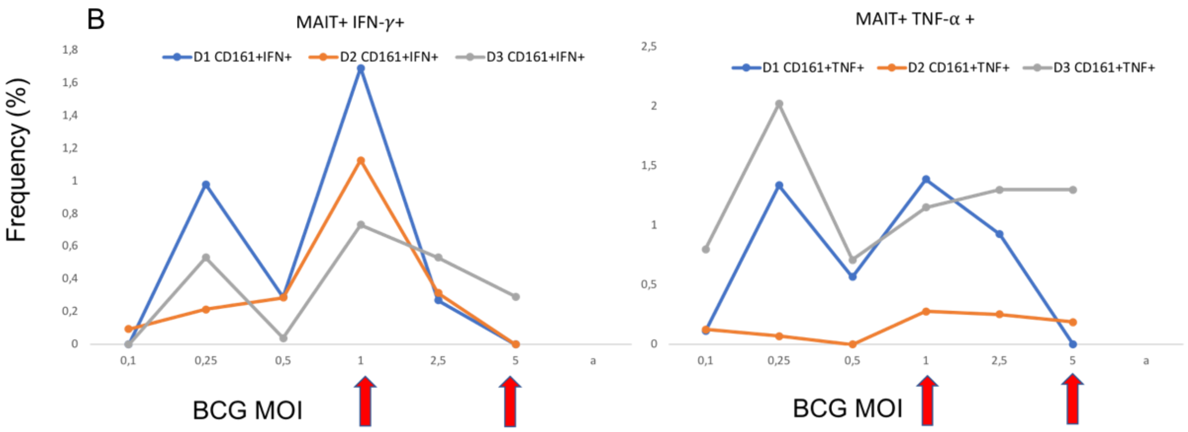


Figure A4 : IFN-γ and TNF-α expression by MAIT cells in response to stimulation with different BCG MOI. A) Representative flow cytometry plots showing expression of IFN-γ and TNF-α by MAIT cells in response to PBMC stimulation with BCG of different MOI. B) Summary plots showing frequencies of MAIT cells expressing IFN-γ and TNF-α against the different BCG MOI.

Assessment of kinetics of IL-12.

At each time point (representing hours of stimulation), supernatants were collected into Sarstedt tubes and stored at -80°C for IL-12 quantification by ELISA. Following this step, samples were re-stimulated with respective antigens for additional 6 hours and stained and acquired in the BD LSR Fortessa flow cytometer. Soluble IL-12 was measured using the Human IL-12/IL-23(p40) Legend Max ELISA kit (Biolegend®). The assay was conducted according to the manufacturer's instructions.

IL-12 ELISA revealed a slight increase in the concentration of IL-12 in response to stimulation with BCG and LPS for 12-16 hours. The concentrations of IL-12 for these stimulations were very low but increased greatly at the last 2 time points (18 and 20 hours) (Fig. A4.A).

The expression of IFN-γ and CD107a by MAIT cells in response to BCG stimulation was also evaluated. Fig. A4.B shows the representative flow cytometry plots for the MAIT cell responses. CD107a expression was observed to decrease sharply between 12-14 hours of stimulation (this trend was consistent in other T cells [data not shown]) following which, CD107a expression increased over time. This same trend was observed for IFN-γ and HLA-DR expression, although the decrease in the frequencies between 12-14 hours was not as pronounced.

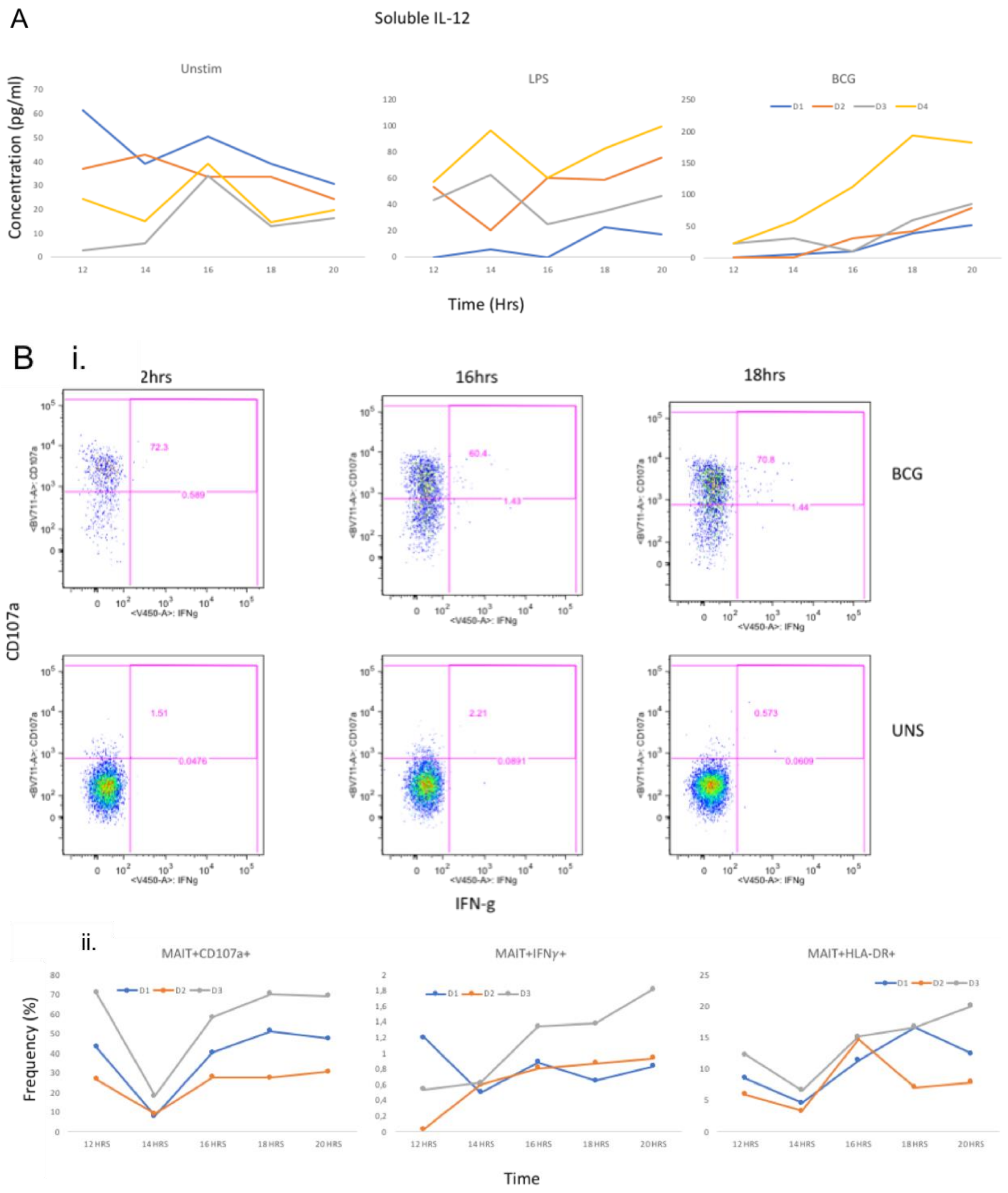
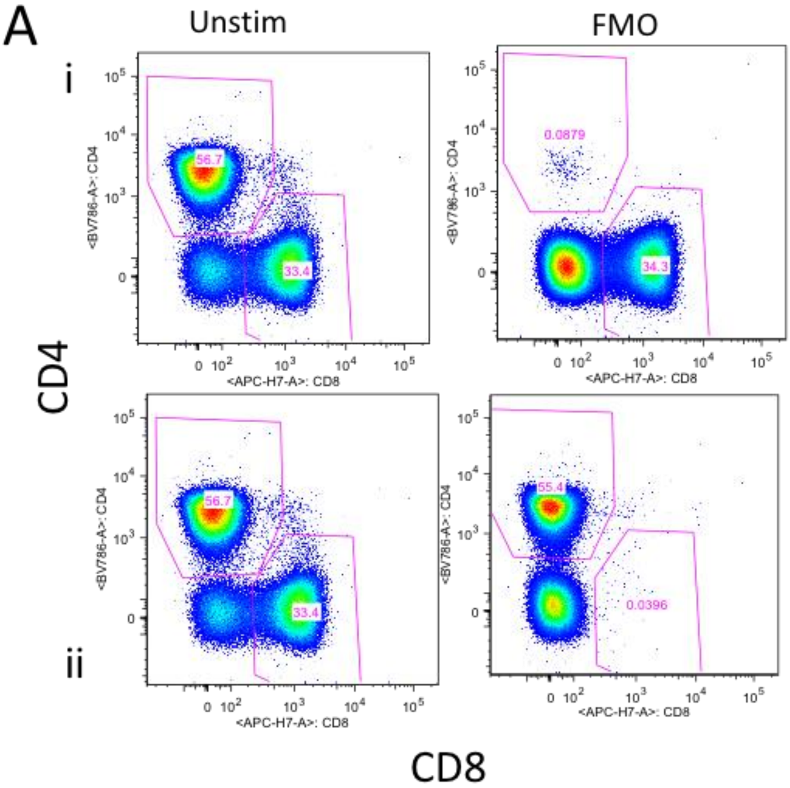
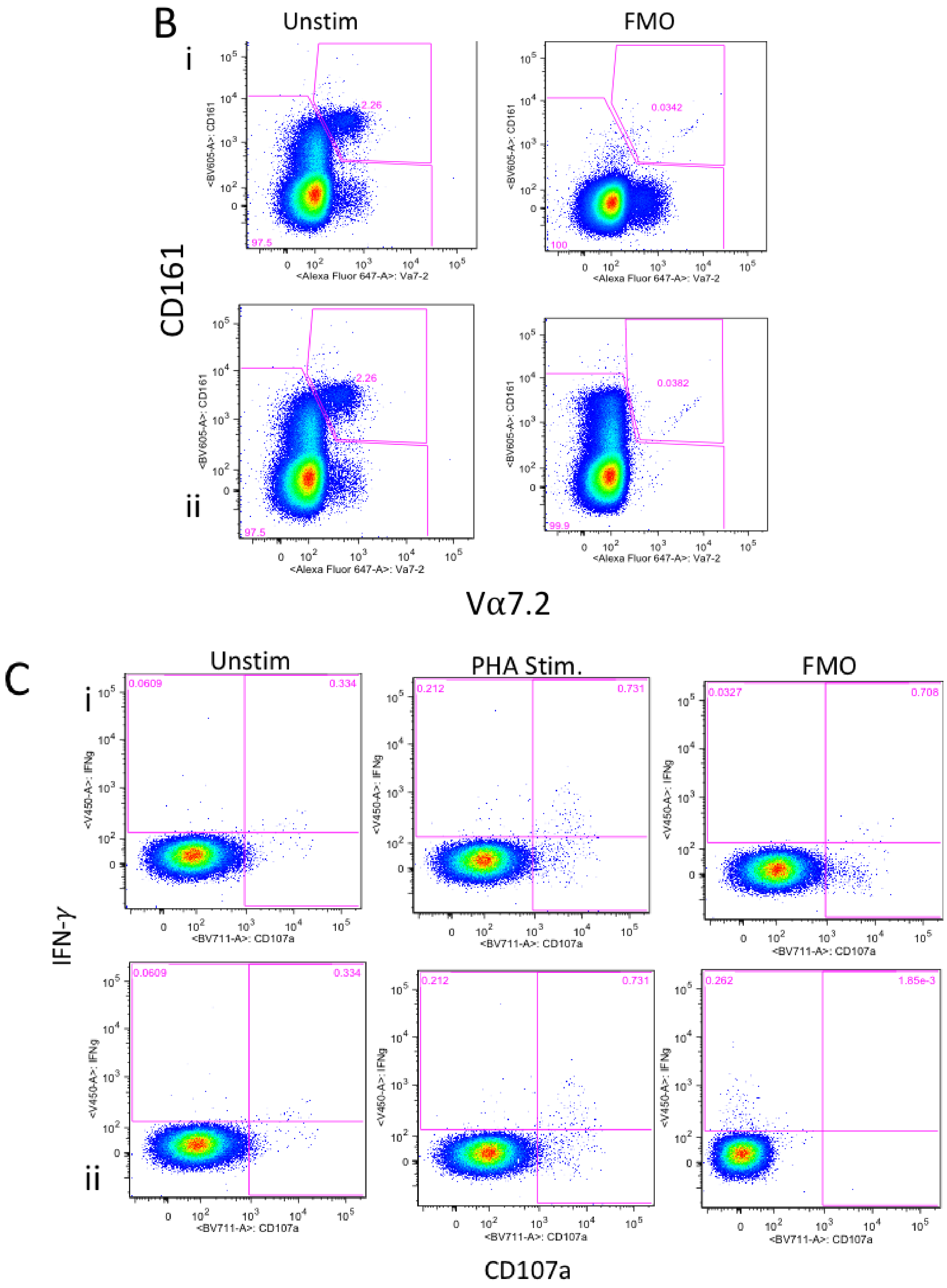


Figure A4: Kinetics of IL-12 production. A) Summary plots showing the concentrations of IL-12 in cell supernatants from samples stimulated with BCG and LPS over time. B) Representative flow cytometry plots and summary plots showing IFN-γ, CD107a and HLA-DR expression by MAIT cells in response to stimulation with BCG over time.

Fig. A5 shows the flow cytometry plots of the FMO controls against non-FMO samples of the same marker in the full flow cytometry panel. For lineage marker FMO controls, distinct cell populations were observed for each marker in the full panel and these populations were absent in the FMO control for each specific marker, although there were minimal frequencies in the compartments probably due to spillover effects or carryover from previous samples. In order to assess expression of intracellular cytokines such as IFN-γ, CD107a and IL-12 and markers of APC infection and activation (GFP and CD40, respectively), we used unstimulated controls to identify background responses and we stimulated with LPS and PHA to assess antigen-specific responses. In FMO controls for each specific marker, we found lower frequencies of cytokines and expression of markers of infection compared to antigen-stimulated samples in the full panel.

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FMO
i
ii
CD161
<BV605-A>: CD161
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97.5
0.0342
100
2.26
97.5
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99.9
Vα7.2
C
Unstim
PHA Stim.
FMO
i
ii
IFN-γ
<V450-A>: IFNg
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0.334
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0.262
1.85e-3
CD107a

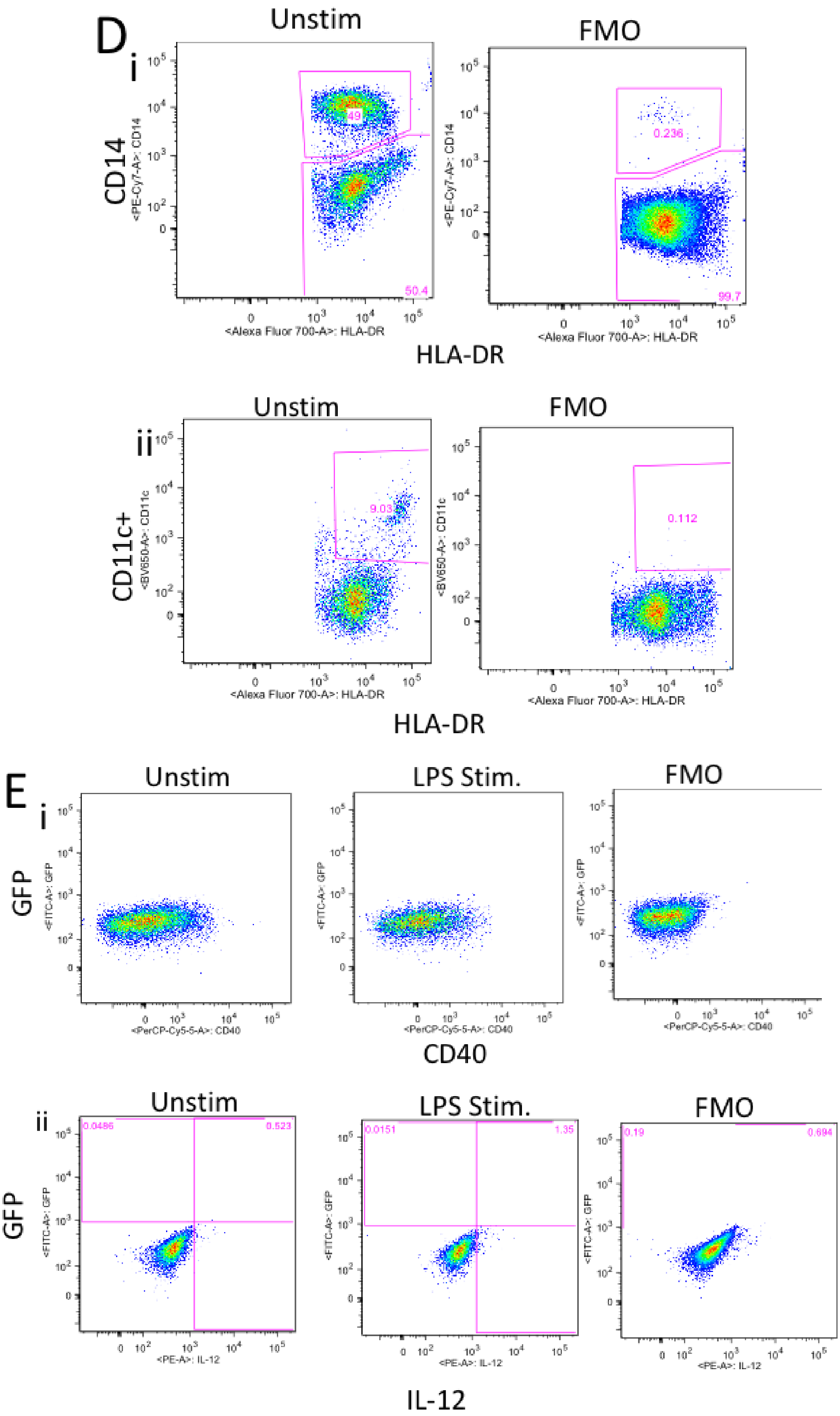


Figure A6:Flow cytometry plots showing FMO controls against samples from the full panel. A) i) and ii) CD4 and CD8 FMOs against CD4 and CD8 in unstimulated controls from full panels, respectively. B)

i) and ii) CD161 and Vα7.2 FMOs against unstimulated samples from the full panel. C) i) and ii) IFN-γ and CD107a FMOs against unstimulated samples and PHA stimulated samples from the full panel. D) i) and ii) CD14 and CD11c FMOs against unstimulated samples in full panel. E) i) and ii) CD40 and IL-12 FMOs against unstimulated and LPS stimulated samples in full panel.

Table A1: Fluorescence minus one analysis of flow cytometry panel. Dark grey areas represent the FMO controls (full panel minus one antibody-fluorochrome)

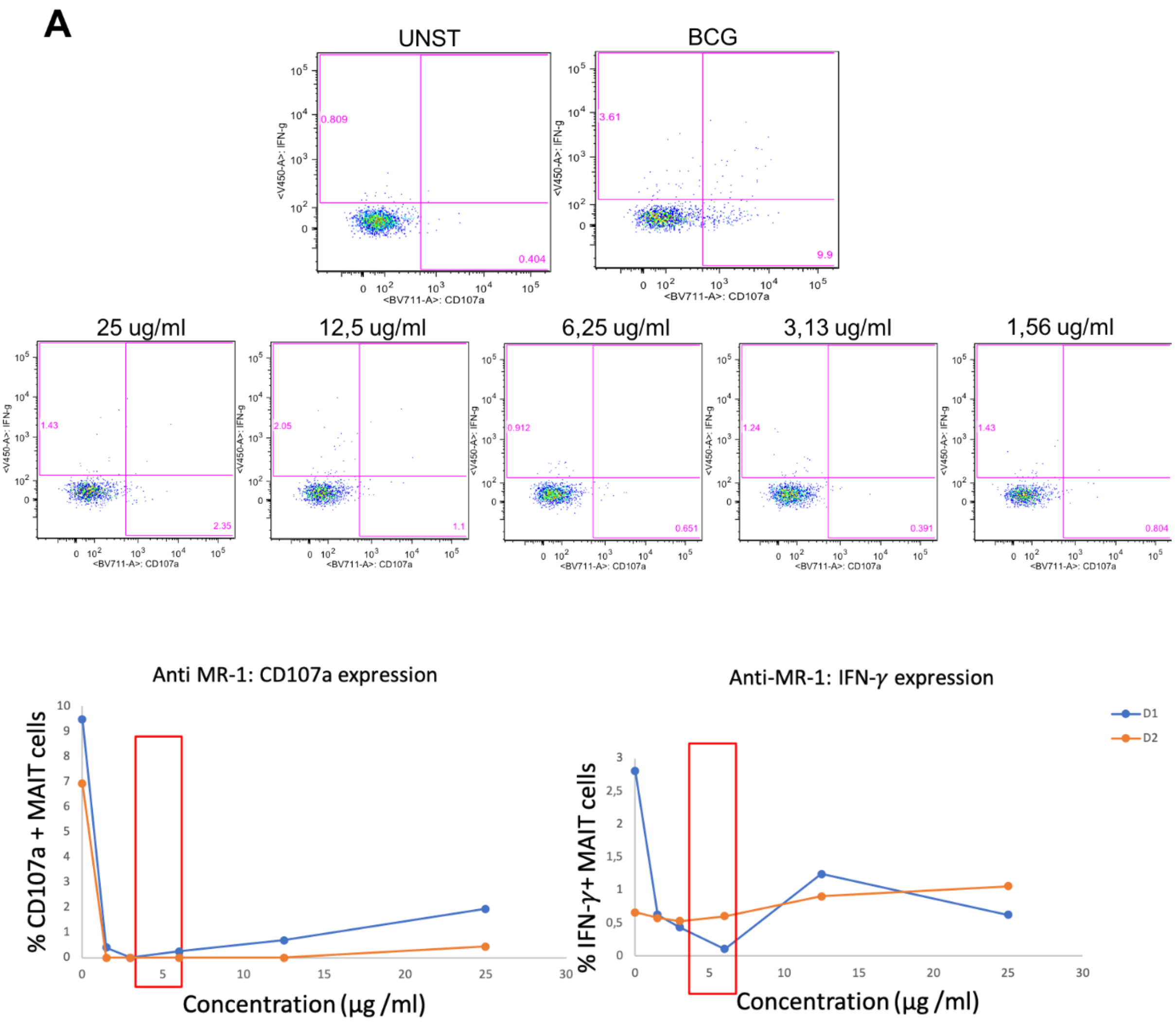
FMO	**BV786**	**APC-H7**	**BV650**	**Pe-Cy7**	**PerCp-Cy5.5**	**BV711**	**BV605**	**V450**	**AF647**	**PE**
CD4	0.09	54.50	54.00	54.70	52.10	52.20	54.90	55.20	54.20	53.10
CD8	34.70	0.03	35.50	34.40	33.70	34.90	33.90	33.30	34.30	36.00
CD11c	59.20	49.80	0.04	49.00	45.80	58.60	44.00	54.20	53.10	54.00
CD14	55.00	46.80	48.60	0.18	46.80	54.20	40.20	50.70	49.70	49.90
CD40	4.47	4.71	4.55	4.76	0.03	6.89	3.73	4.45	4.04	6.54
CD107a	0.23	0.25	0.19	0.22	0.20	0.01	0.20	0.35	0.22	0.51
CD161	12.70	13.40	13.50	13.10	12.50	12.70	0.13	13.20	13.70	13.00
IFN-γ	0.05	0.09	0.07	0.04	0.08	0.15	0.06	0.03	0.04	0.11
Vα7.2	2.15	2.42	2.44	2.54	2.11	2.16	2.78	2.72	0.09	2.51
IL-12	0.12	0.21	0.10	0.01	0.06	0.12	0.06	0.08	0.12	0.12
GFP	0.11	0.29	0.00	1.12	0.27	0.23	0.43	0.22	0.48	4.14
HLA-DR	12.60	10.30	11.30	10.90	11.40	16.70	8.31	9.80	10.10	12.90
CD3	81.00	83.60	83.10	83.30	81.70	78.30	86.00	83.90	84.00	81.50

Blocking antibody titrations.

Fig. A7 shows the representative flow cytometry plots which show the expression of CD107a and IFN-γ by MAIT cells in the unstimulated and BCG stimulated controls, and the expression of CD107a and IFN-γ at varying concentrations of blocking antibodies. In response to BCG stimulation, the expression of IFN-γ and CD107a were increased compared to the unstimulated control (background responses). Blocking of MAIT cell responses reduced the expression of CD107a and IFN-γ by MAIT cells. There was maximal blocking of CD107a expression at the lowest concentrations of anti-MR1 blocking antibody (1-5 µg/ml). A similar trend was observed for the expression of IFN-γ by MAIT cells, the IFN-γ-producing MAIT cells, the expression of IFN-γ decreased at the lowest concentrations of anti-MR1 blocking antibodies with a slight increase at higher concentrations. For both CD107a and IFN-γ expression by MAIT cells, the expression of these cytokines in response to varying concentrations of blocking antibodies remained lower than the BCG stimulated samples without blocking antibodies.

Fig. A7 shows the effect of blocking MAIT cell activation with different concentrations of anti-IL-12 blocking antibodies. The representative flow cytometry plots show the effect of blocking MAIT cell responses with varying concentrations of anti-IL-12 blocking antibodies. The summary plots show that compared to BCG stimulated controls (without blocking antibodies), there was a sharp decrease in the frequencies of MAIT cells expressing CD107a at the lowest concentration of anti-IL-12 (1-6 µg/ml), followed by a gradual decrease at 12 µg/ml, these were then increased at higher concentrations of anti-IL-12. The same trend was observed for IFN-γ expression. This meant that there was optimal blocking of MAIT cell responses at lower concentrations of anti-IL-12 and the effects of blocking decreased at higher concentrations.

For blocking with anti-IL-18 antibodies, Fig. A7.C shows that the frequencies of MAIT cells expressing CD107a and IFN-γ were reduced at the lower concentration of anti-IL-18, this reduction was greatest at 3µg/ml for donor 2. At higher concentrations, the frequencies were slightly higher but did not significantly differ. Donor 1 showed the same decrease in the frequencies of MAIT cells expressing CD107a and IFN-γ, this was followed by a sharp increase at 6 µg/ml of anti-IL-18 and another decrease at higher concentrations of anti-IL-18. This meant that blocking of MAIT cell responses with anti-IL-18 was effective at lower concentrations of anti-IL-18, although there was a slight increase of MAIT responses at 6 µg/ml.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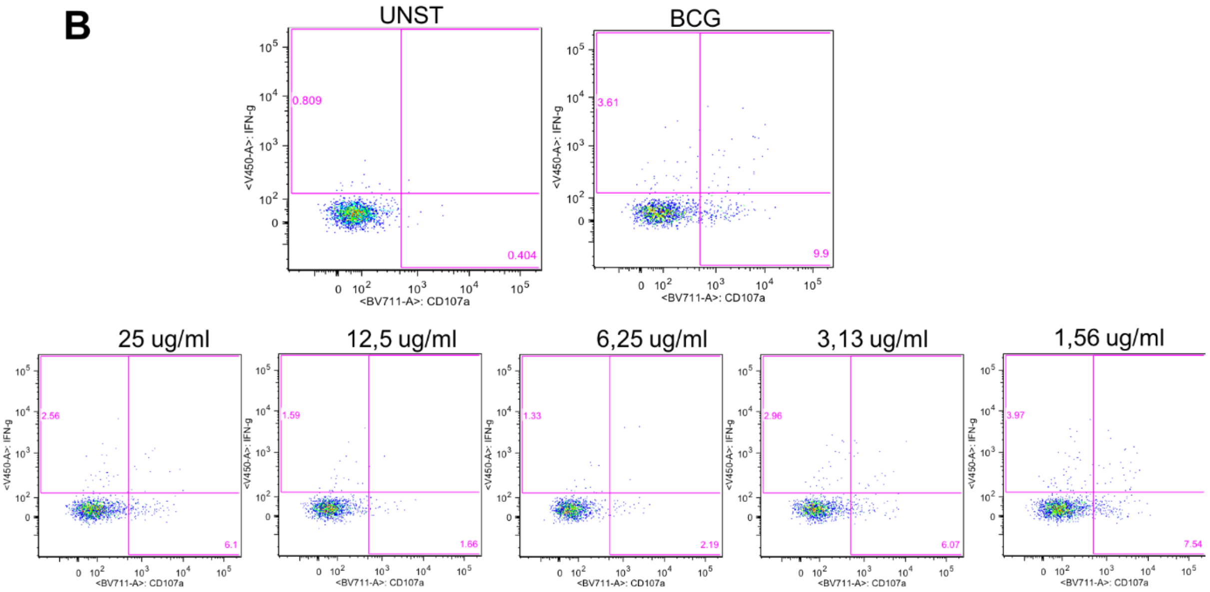
0.404
BCG
3.61
9.9
<V450-A>: IFN-g
<BV711-A>: CD107a
10^5
10^4
10^3
10^2
0
25 ug/ml
1.43
2.35
12,5 ug/ml
2.05
1.1
6,25 ug/ml
0.912
0.651
3,13 ug/ml
1.24
0.391
1,56 ug/ml
1.43
0.804
Anti MR-1: CD107a expression
% CD107a + MAIT cells
0 1 2 3 4 5 6 7 8 9 10
0 5 10 15 20 25 30
Concentration (µg /ml)
Anti-MR-1: IFN-γ expression
% IFN-γ+ MAIT cells
0 0,5 1 1,5 2 2,5 3
0 5 10 15 20 25 30
Concentration (µg /ml)
D1
D2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10^4
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0
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6.1
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1.59
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3.97
7.54

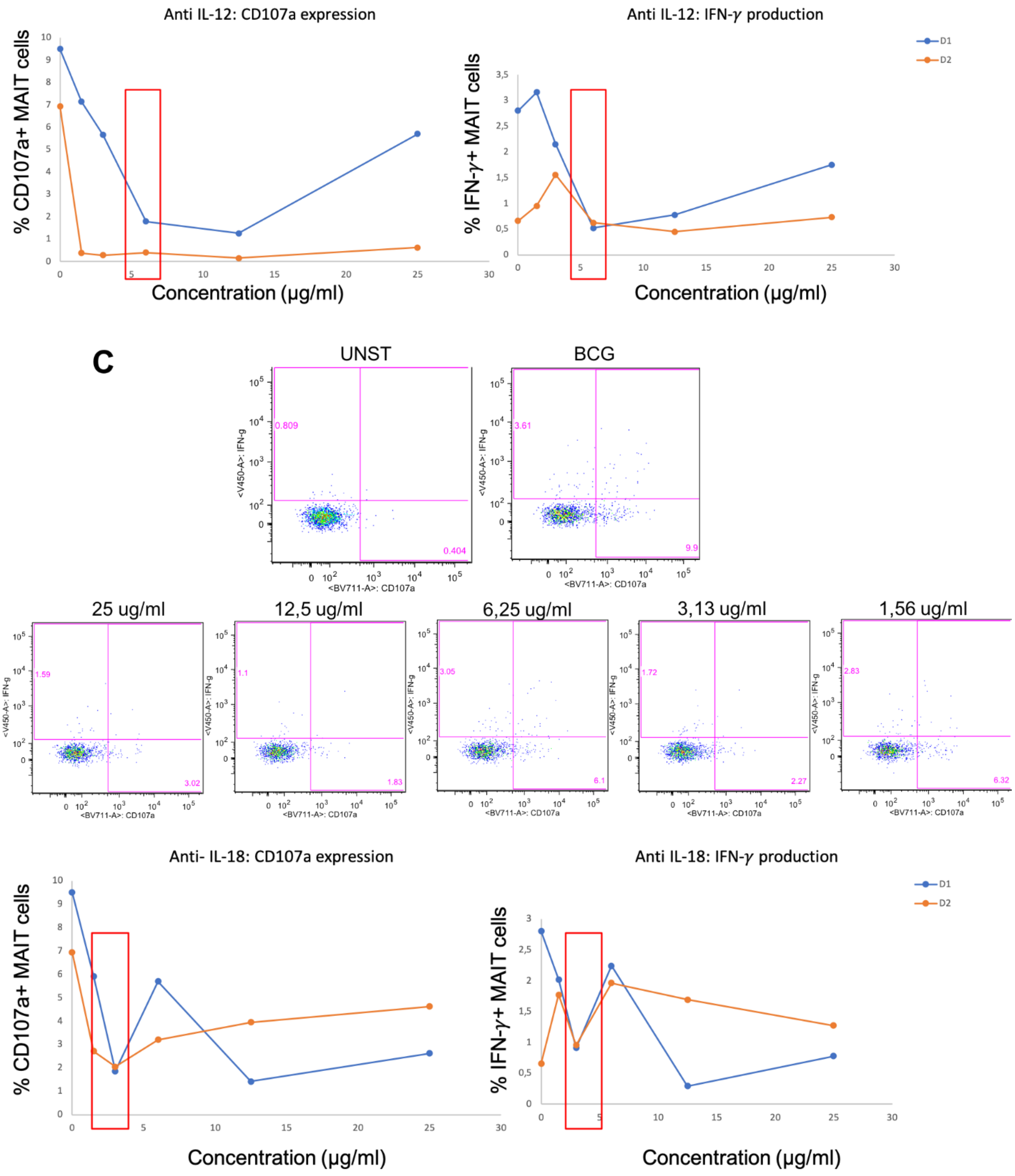


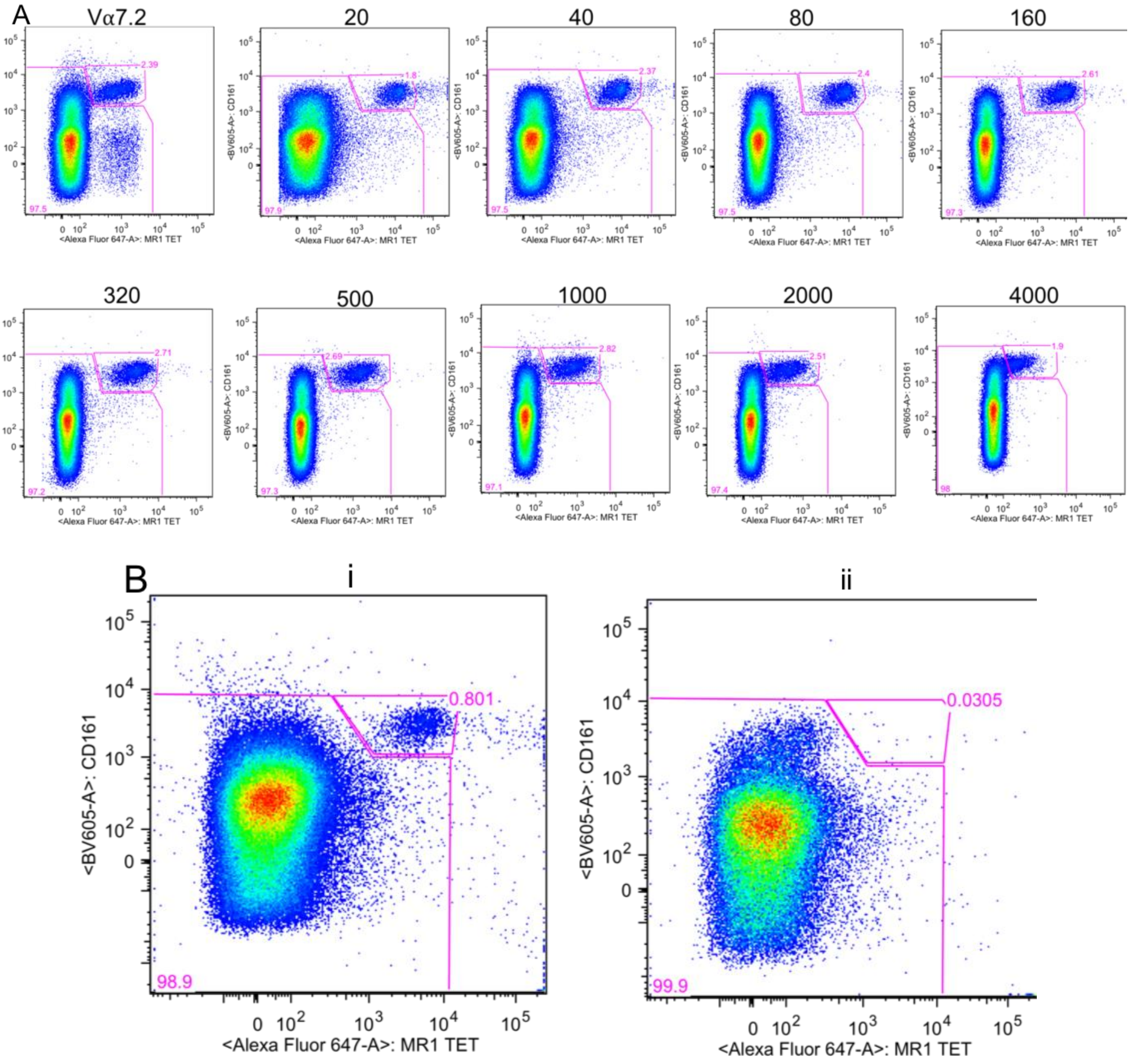
Figure A7: Titration of MAIT cell activation blocking antibodies. Effect of different concentrations of A) anti-MR-1 B) anti-IL-12 and C) IL-18 blocking antibodies on CD107a and IFN-γ expression by MAIT cells. Red boxes in A), B) and C) represent the concentrations of blocking antibodies which were chosen for as they provided optimal blocking.

Tetramer titration

We thawed and rested the PBMCs from 2 donors from WPBTS buffy coats at 37°C for 2 hours, washed and counted the cells and then adjusted cell concentrations to 10 million cells/ml. We then prepared 2 sets of 9 FACS tubes for each donor: 1 set of 8 FACS tubes (with different MR-1 tetramer titers) to incubate at room temperature and another set to incubate at 37°C. An additional control tube for staining with Vα7.2 antibody was included for each donor.

We stained the cells with 50 µl of viability dye cocktail and incubated cells in the dark, at room temperature. Following this step, samples were washed with PBS and we then stained with 50 µl of the MR-1 tetramer cocktails with different tetramer concentrations (with dilutions ranging from 20X to 4000X) into the respectively labelled tubes on both sets. We then incubated the tubes at their respective temperature (room temperature and 37°C) for 30 minutes. Following incubation, we then washed the samples with FACS buffer and stained them with CD3 and CD161 antibodies for 30 minutes in the dark at room temperature. We finally washed all of the samples with FACS buffer and resuspended samples in 1% formaldehyde for flow cytometry acquisition.

The representative flow cytometry plots in Fig. A8.A show the titration of MR1 tetramer. Vα7.2 antibody was used as a control for the frequency of MAIT cells and the separation between Vα7.2$^-$ and Vα7.2$^+$ cell populations was measured. From these plots, it was observed that decreasing concentrations of tetramer antibodies led to the decrease in separation between the Vα7.2$^-$ and Vα7.2$^+$ cell populations although the frequencies were not significantly affected at the lowest concentrations (4000X dilution).


A
Vα7.2
20
40
80
160
320
500
1000
2000
4000
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2.4
97.5
2.61
97.3
2.71
97.2
2.69
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10^3
10^4
10^5

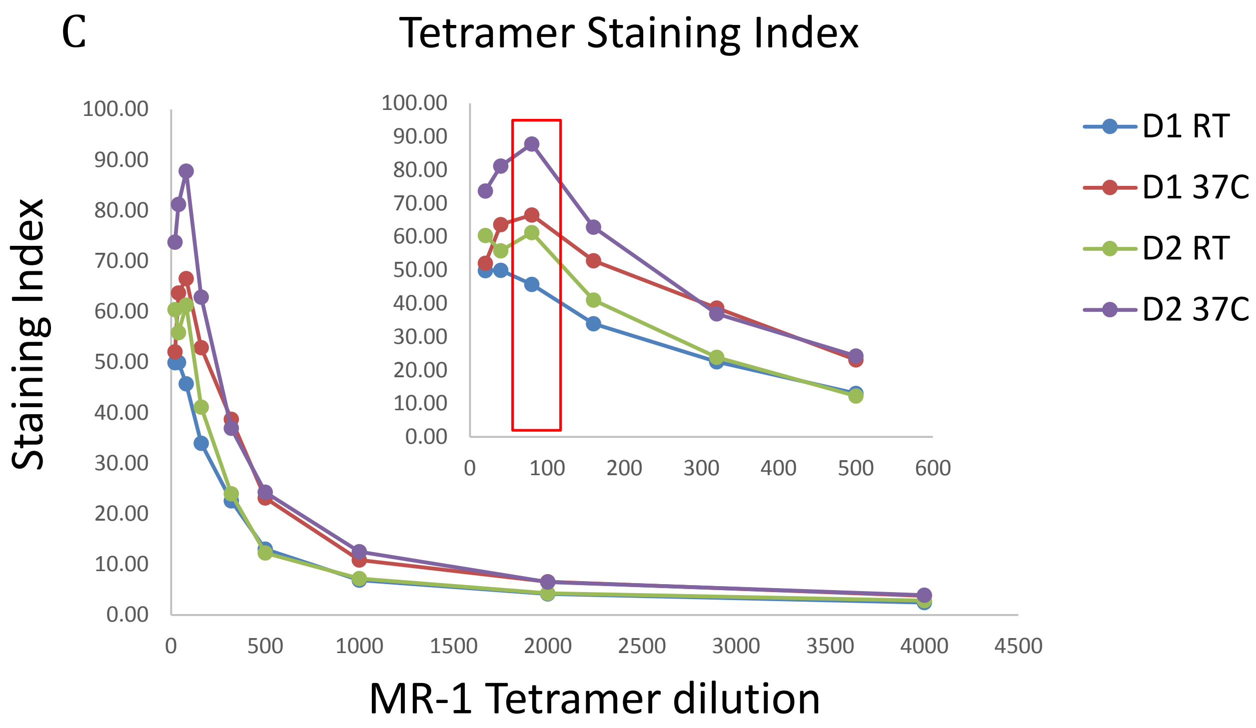


Figure A8: Staining index for the titration of 5-OP-RU MR1 tetramers. A) Representative flow cytometry plots showing different MR1 tetramer titres. The numbers above each plot represent the dilution factor for the MR1 tetramer. B) Representative flow cytometry showing i) MAIT cells identified with 5-OP-RU MR1 tetramer and ii) 6-FP MR1 loaded control tetramer. C) Summary plots showing the MR1 tetramer dilution against the staining index. The red box represents the chosen MR1 tetramer titre.

Association of TB, HIV and HIV-associated TB with cellular frequencies

Correlations between CD4 counts and MAIT cell frequencies

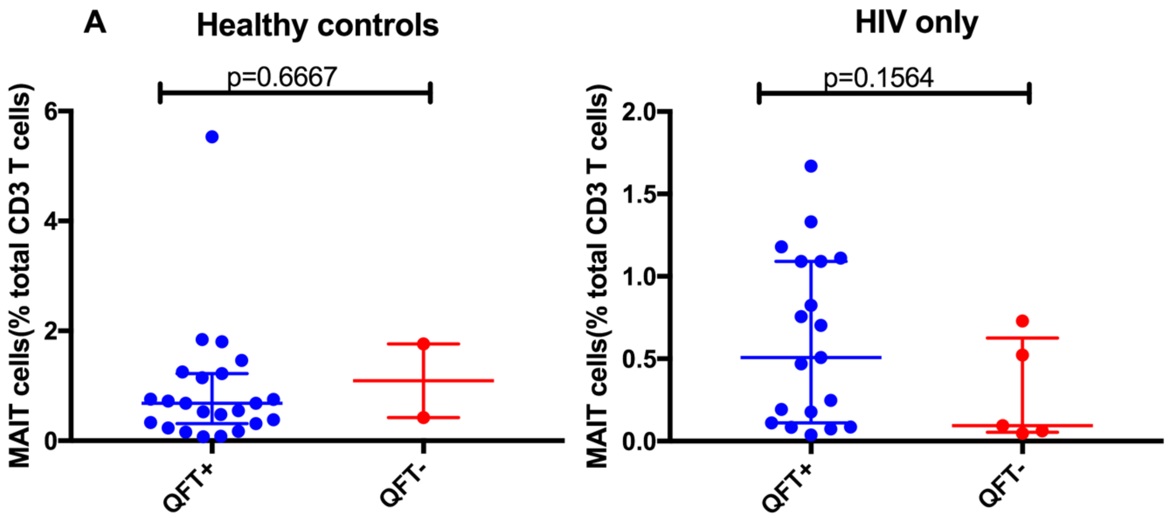


Figure A9 Association between LTBI and the frequencies of MAIT cells in individuals without active TB. A) Frequencies of MAIT cells in healthy controls. B) Frequencies of MAIT cells in individuals with HIV only. Data presented as scatter plots with lines representing the median and IQR.

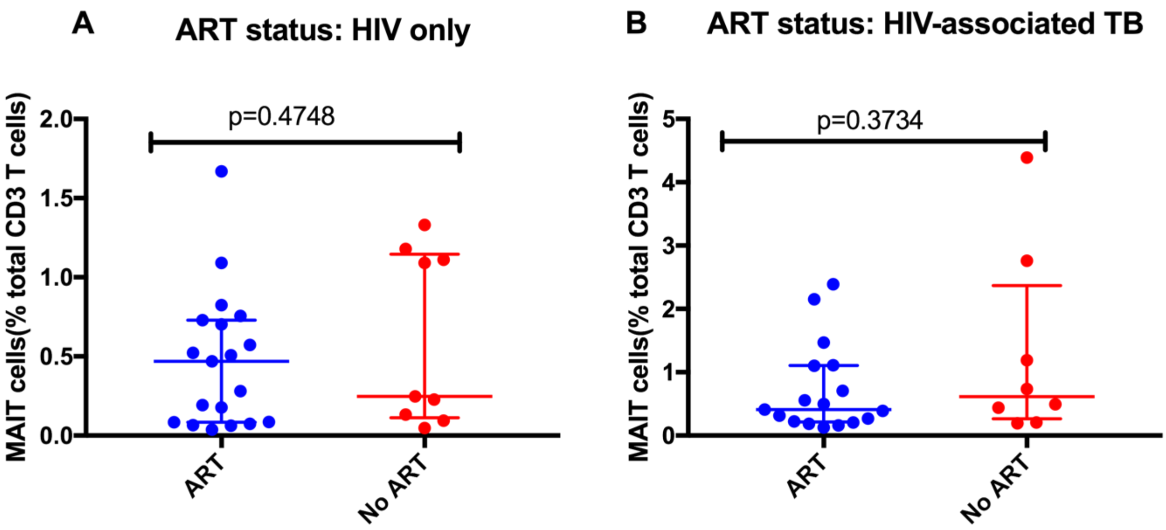
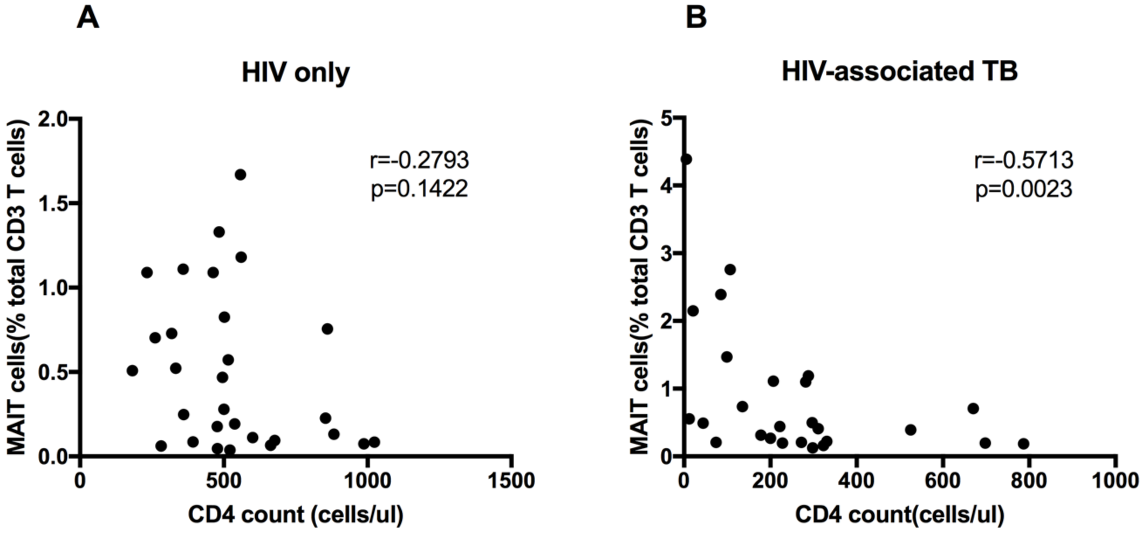


Figure A10: The impact of ART on the frequencies of MAIT cells among individuals with HIV infection. A) and **B)** Graphs showing the comparison of MAIT cells frequencies among individuals on ART and individuals not ART in the groups of individuals with HIV only and individuals with HIV-associated TB, respectively. Data presented as scatter plots with lines representing the median and IQR.



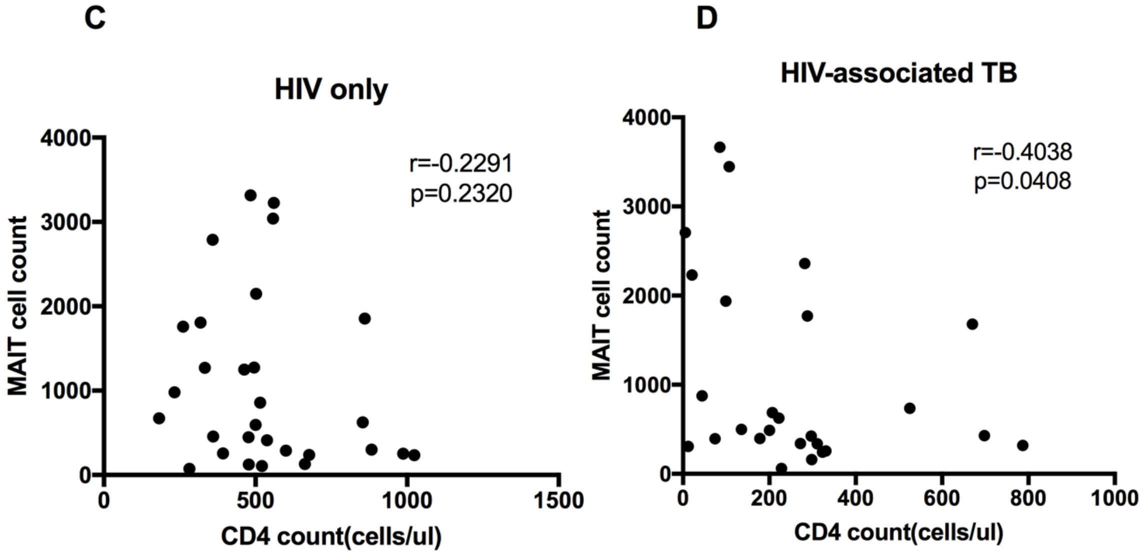
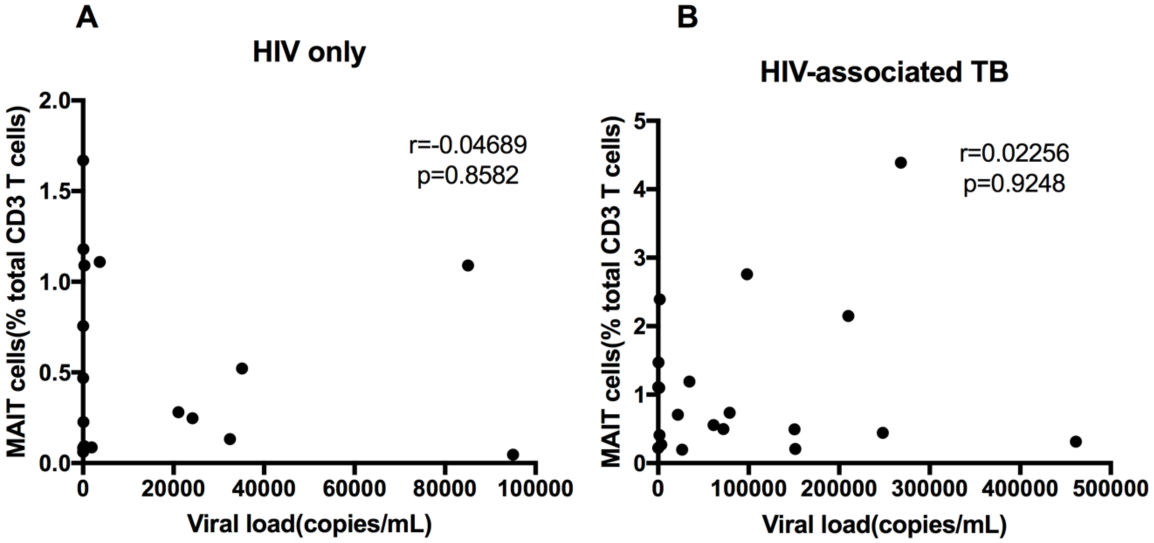


Figure A11: Correlation between CD4 counts and the frequencies and numbers of MAIT cells. The relationship between CD4 counts and the frequencies of MAIT cells in individuals with **A)** HIV only and **B)** HIV-associated TB. The relationship between CD4 counts and the numbers of MAIT cells in individuals with **C)** HIV only and **D)** HIV-associated TB.

Correlations between HIV viral load and MAIT cell frequencies



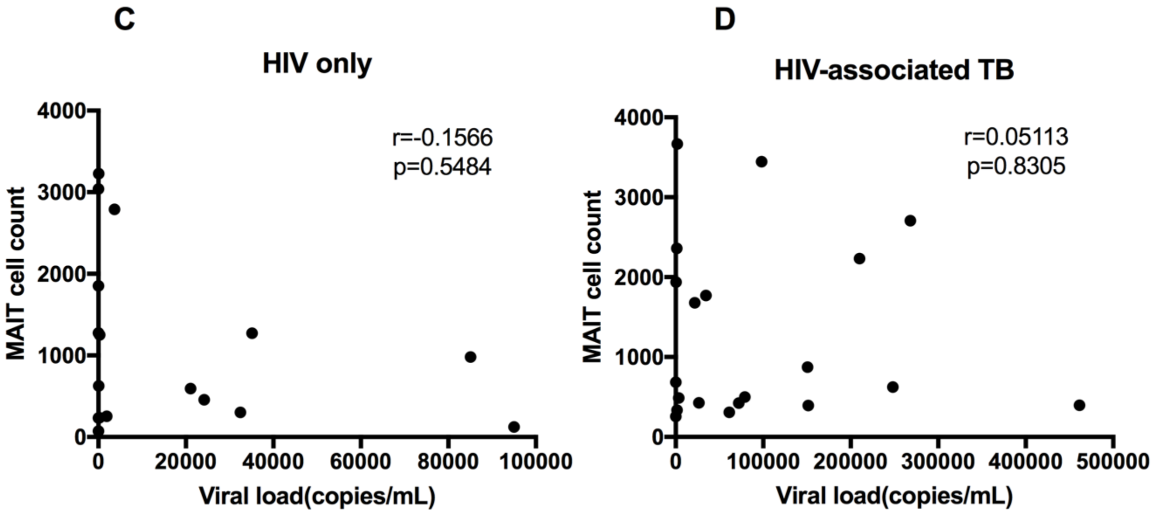


Figure A12: Correlation between HIV viral load and the frequencies and numbers of MAIT cells. The relationship between HIV viral load and the frequencies of MAIT cells in individuals with **A)** HIV only and **B)** HIV-associated TB. The relationship between HIV viral load and the numbers of MAIT cells in individuals with **C)** HIV only and **D)** HIV-associated TB.

Expression of PD-1 on CD4 T cells

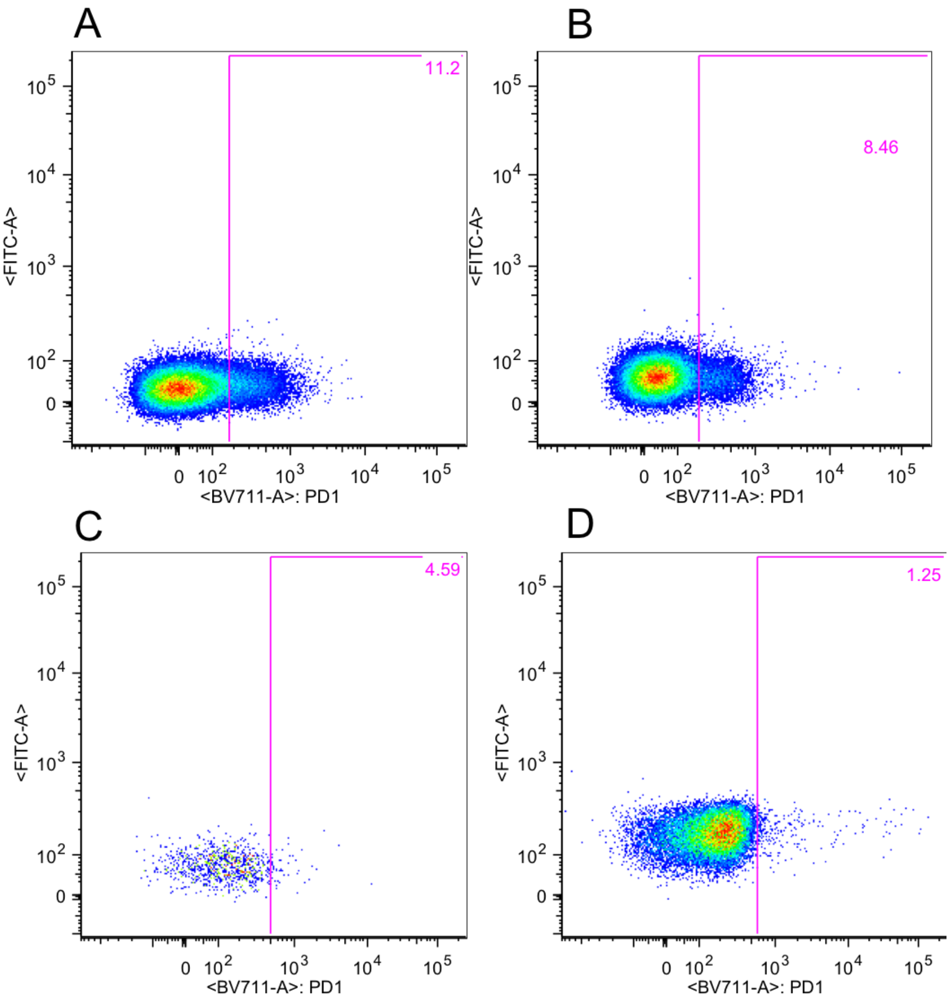


Figure A13: Representative flow plot for the expression of PD-1 on CD4 T cells. A) CD4 T cells, B) CD8 T cells, C) MAIT cells, D) Monocytes

Association of TB, HIV and HIV-associated TB disease with the cellular immune responses

Table A2: Median and Interquartile ranges (IQR) of the frequencies of MAIT cells expressing IFN-γ and CD107a in response to BCG 5 and PHA stimulation among the different study groups and the corresponding p-values of comparisons to healthy controls.

Stimulation	**Cell Subset**	**Healthy controls**	**HIV only**		**Active TB**		**HIV-associated TB**	
		Median (IQR)	**Median (IQR)**	**P-value**	**Median (IQR)**	**P-value**	**Median (IQR)**	**P-value**
BCG 5	**% CD107a+ MAIT cells**	36.05 (22.6-60.23)	42.72 (25.36-53.32)	0.8733	21.45 (10.29-32.72)	0.0028	30.65 (13.96-44.30)	0.1715
	% IFN-γ **+ MAIT cells**	7.46 (2.36-18.19)	7.48 (2.69-11.04)	0.7181	1.64 (0.10-5.28)	0.0038	1.41 (0.03-7.82)	0.0140
PHA	**% CD107a+ MAIT cells**	35.08 (18.28-40.88)	39.08 (25.16-43.94)	0.3604	20.48 (11.77-40.65)	0.3264	21.58 (16.75-28.91)	0.0113
	% IFN-γ + **MAIT cells**	37.05 (25.89-52.06)	35.70 (25.99-50.72	>0.9999	20.98 (13.80-38.03)	0.0437	15.80 (4.13-42.27)	0.0849

Table A3: Median and interquartile ranges (IQR) of the frequencies of CD4 T cells expressing CD107a and IFN-γ in response to BCG 5 and PHA stimulation among the different study groups and the corresponding p-values of comparisons to healthy controls.

Stimulation	**Cell Subset**	**Healthy controls**	**HIV only**		**Active TB**		**HIV-associated TB**	
		Median (IQR)	**Median (IQR)**	**P-value**	**Median (IQR)**	**P-value**	**Median (IQR)**	**P-value**
BCG 5	**% CD107a+ CD4 T cells**	2.87 (1.78-5.08)	2.42 (1.39-3.90)	0.3820	1.35 (0.44-2.08)	0.0013	3.46 (0.98-6.05)	0.9921
	% IFN-γ + CD4 T cells	0.64 (0.31-0.84)	0.29 (0.16-0.49)	0.0070	0.25 (0.17-0.55)	0.0069	1.04 (0.48-2.61)	0.1118

PHA	**% CD107a+ CD4 T cells**	9.09 (5.68-12.77)	10.31 (5.21-17.08)	0.7072	5.39 (2.67-9.30)	0.1072	10.23 (4.75-17.62)	0.7990
	% IFN-γ + CD4 T cells	5.62 (3.36-7.82)	4.22 (2.98-5.74)	0.1675	4.77 (3.15-6.60)	0.6259	4.56 (2.99-7.91)	0.8399

Table A4: Median and Interquartile range (IQR) of the frequencies of CD8 T cells expressing IFN-γ and CD107a in response to BCG 5 and PHA stimulation among the different study groups and the corresponding p-values of comparisons to healthy controls.

Stimulation	**Cell Subset**	**Healthy controls**	**HIV only**		**Active TB**		**HIV-associated TB**	
		Median (IQR)	**Median (IQR)**	**P-value**	**Median (IQR)**	**P-value**	**Median (IQR)**	**P-value**
BCG 5	**% CD107a+ CD8 T cells**	2.13 (0.97-4.83)	1.51 (0.61-3.08)	0.2391	1.18 (0.38-2.16)	0.0202	1.47 (0.75-2.89)	0.2356
	% IFN-γ+ CD8 T cells	0.34 (0.23-0.65)	0.19 (0.08-0.30)	0.0070	0.15 (0.10-0.30)	0.0006	0.30 (0.08-0.53)	0.1715
PHA	**% CD107a+ CD8 T cells**	28.08 (17.34-36.99)	31.07 (25.55-38.76)	0.3820	20.20 (14.77-31.16)	0.1984	24.11 (22.28-30.14)	0.6408
	% IFN-γ+ CD8 T cells	9.86 (5.79-13.90)	11.98 (8.85-15.53)	0.0986	11.07 (7.29-13.08)	0.6901	11.50 (8.00-14.68)	0.2504

Table A5: Median and the IQR of the frequencies of MAIT cells expressing HLA-DR, MFI of HLA-DR on total and MAIT cells expressing IFN-γ in response to BCG 5 and PHA stimulation among the different study groups. Table also includes the corresponding p-values for comparisons to healthy controls.

Stimulation	**Cell Subset**	**Healthy controls**	**HIV only**		**Active TB**		**HIV-associated TB**	
		Median (IQR)	**Median (IQR)**	**P-value**	**Median (IQR)**	**p-value**	**Median (IQR)**	
BCG 5	**% HLA-DR+ MAIT cells**	1.88 (0.82-5.23)	2.4875 (0.69-5.19)	0.6623	0.713 (0.00-2.42)	0.0829	1.628 (0.33-3.96)	0.4579

	MAIT cells (HLA-DR MFI)	73 (31.95-139)	136 (24.53-162.50)	0.2565	97.1 (17.21-144.75)	0.6403	116.5 (59.23-220.00)	0.0987
	IFN-γ+ MAIT cells (HLA-DR MFI)	161 (101.55-229.50)	173.5 (108.70-305.00)	0.4899	242.5 (139.50-430.00)	0.1138	361 (126.00-868.50)	0.0117
PHA	**% HLA-DR+ MAIT cells**	8.71 (2.11-13.35)	4.10 (1.36-11.23)	0.1829	3.57 (0.09-7.07)	0.0202	4.10 (0.67-8.64)	0.2245
	MAIT cells (HLA-DR MFI)	148.5 (59.38-286.00)	210 (90.20-317.00)	0.4375	186.5 (77.83-251.75)	0.8935	188 (96.95-324.00)	0.4435
	IFN-γ + MAIT cells (HLA-DR MFI)	245.5 (156.50-346.75)	232 (150.00-432.00)	0.9894	305 (280.25-360.25)	0.8124	313.5 (181.50-668.25)	0.1562

Table A6: Median and the IQR of the frequencies of CD4 T cells expressing HLA-DR, MFI of HLA-DR on total and CD4 T cells expressing IFN-γ in response to BCG 5 and PHA stimulation among the different study groups. Table also includes the corresponding p-values for comparisons to healthy controls

Stimulation	**Cell Subset**	**Healthy controls**	**HIV only**		**Active TB**		**HIV-associated TB**	
		Median (IQR)	**Median (IQR)**	**P-value**	**Median (IQR)**	**P-value**	**Median (IQR)**	**P-value**
BCG 5	**% HLA-DR+ CD4 T cells**	0.22 (0.0-0.68)	0.28 (0.00-0.98)	0.5392	0.12 (0.00-8.30)	0.8271	1.15 (0.24-8.30)	0.0034
	CD4 T cells (HLA-DR MFI)	25.50 ([-3.98]-50.150)	29.70 (7.71-87.55)	0.6023	48.05 (14.13-86.70)	0.1459	101.00 (46.20-141.00)	0.0004
	IFN-γ+ CD4 T cells (HLA-DR MFI)	259.00 (105.60-340.50)	205.50 (79.13-535.50)	0.7569	385.50 (222.75-765.00)	0.0159	869.50 (421.00-1402.75)	0.0002
PHA	**% HLA-DR+ CD4 T cells**	7.44 (3.35-15.13)	9.54 (3.53-13.25)	0.7430	6.16 (2.29-9.13)	0.2684	4.33 (2.86-10.71)	0.6130

	CD4 T cells (HLA-DR MFI)	140.50 (50.10-197.50)	166.00 (69.70-270.00)	0.2080	150.00 (67.10-199.00)	0.5856	184.50 (91.03-282.75)	0.0393
	IFN-γ+ CD4 T cells (HLA-DR MFI)	229.00 (96.38-333.00)	268.00 (134.00-494.00)	0.2589	269.00 (200.00-372.00)	0.2281	400.50 (222.25-874.00)	0.0017

Table A7: Median and the IQR of the frequencies of CD8 T cells expressing HLA-DR, MFI of HLA-DR on total and CD8 T cells expressing IFN-γ in response to BCG 5 and PHA stimulation among the different study groups. Table also includes the corresponding p-values for comparisons to healthy controls

Stimulation	**Cell Subset**	**Healthy controls**	**HIV only**		**Active TB**		**HIV-associated TB**	
		Median (IQR)	**Median (IQR)**	**P-value**	**Median (IQR)**	**P-value**	**Median (IQR)**	**P-value**
BCG 5	**% HLA-DR+ CD8 T cells**	0.02 (0.00-0.44)	0.00 (0.00-0.32)	0.6036	0.00 (0.00-0.68)	0.8148	0.00 (0.00-0.26)	0.3536
	CD8 T cells (HLA-DR MFI)	181.00 (110.00-242.50)	181.00 (115.00-406.25)	0.2565	234.00 (196.75-302.00)	0.0462	321.00 (197.75-443.00)	0.0017
	IFN-γ**+ CD8 T cells (HLA-DR MFI)**	285.00 (126.20-481.00)	390.50 (196.00-542.00)	0.4666	514.00 (263.25-810.25)	0.0635	395.50 (190.75-1116.00)	0.1249
PHA	**% HLA-DR+ CD8 T cells**	1.65 (0.65-3.63)	2.31 (0.91-2.95)	0.4724	1.06 (0.29-1.73)	0.3175	1.54 (0.47-2.36)	0.4354
	CD8 T cells (HLA-DR MFI)	246.00 (11.75-389.00)	340.00 (151.00-365.00)	0.1273	299.00 (165.00-392.00)	0.3992	423.50 (284.00-543.50)	0.0181
	IFN-γ**+ CD8 T cells (HLA-DR MFI)**	383.00 (94.03-603.50)	463.00 (189.00-723.00)	0.3489	423.00 (208.00-555.00)	0.9118	525.00 (289.25-854.50)	0.0579

Table A8: Median and IQR of the MFI of HLA-DR and CD40 in monocytes in response to BCG 5 and LPS stimulation among the different study groups. Table also includes the corresponding p-values for comparisons to healthy controls.

Stimulation	**Cell Subset**	**Healthy controls**	**HIV only**	**Active TB**	**HIV-associated TB**

		Median (IQR)	Median (IQR)	P-value	Median (IQR)	p-value	Median (IQR)	P-value
BCG 5	**Monocytes (HLA-DR MFI)**	2760.00 (2097.00-2962.50)	2526.50 (1797.75-3342.00)	0.8292	1938.50 (1650.50-2629.50)	0.0409	2510.00 (1541.50-3006.00)	0.4440
	Monocytes (CD40 MFI)	296.00 (249.50-444.00)	325.00 (271.00-400.75)	0.9290	354.50 (243.75-469.50)	0.4956	375.50 (285.00-567.75)	0.1699
LPS	**Monocytes (HLA-DR MFI)**	3455.00 (2751.00-4331.00)	3737.00 (2749-4994.00)	0.3342	2858.00 (2476.00-3643.25)	0.1992	3671.00 (2504.00-4665.00)	0.6443
	Monocytes (CD40 MFI)	329.00 (230.00-616.00)	502.00 (398.00-762.00)	0.0961	387.00 (266.50-804.00)	0.2509	424.00 (325.00-649.00)	0.2332

Table A9: Median and IQR of the MFI of HLA-DR and CD40 in DCs in response to BCG 5 and LPS stimulation among the different study groups. Table also includes the corresponding p-values for comparisons to healthy controls.

Stimulation	**Cell Subset**	**Healthy controls**	**HIV only**	**Active TB**	**HIV-associated TB**	**Healthy controls**	**HIV only**	**Active TB**
		Median (IQR)	**Median (IQR)**	**P-value**	**Median (IQR)**	**Median (IQR)**	**Median (IQR)**	**P-value**
BCG 5	**DCs (HLA-DR MFI)**	26184.00 (14736.50-32169.00)	22383.50 (17244.75-32473.25)	0.8146	28538.00 (22527.00-37691.25)	0.2227	22408.00 (18623.00-25171.00)	0.3760
	DCs (CD40 MFI)	951.00 (675.50-1702.00)	1029.50 (664.25-1460.75)	0.7181	1125.00 (791.00-1624.00)	0.6467	575.50 (455.25-998.75)	0.0051
LPS	**DCs (HLA-DR MFI)**	14985.00 (10535.00-20593.00)	11234.00 (8659.00-19895.00)	0.2926	13177.50 (8953.75-19956.25)	0.6446	16841.00 (13237.00-22701.00)	0.1871
	DCs (CD40 MFI)	882.00 (564.00-1376.00)	665.00 (600-793.00)	0.1994	778.50 (596.00-1387.75)	0.8079	815.00 (492.00-2220.00)	0.6862

Table A10: Median and IQR of the concentrations of soluble cytokines produced in response to HK-M.tb stimulation among individuals in the different study groups. Table includes p-value from comparisons from healthy controls.

Cytokine	Healthy controls	HIV only		Active TB		HIV-associated TB	
	Median (IQR)	Median (IQR)	P-value	Median (IQR)	P-value	Median (IQR)	P-value
IFN-α2	2.33 (0-7.14)	0 (0-1.65)	0.0154	0 (0-1.56)	0.0162	0.52 (0-3.98)	0.2552
IFN-γ	16393.01 (8504.64-16410.97)	4492.60 (2100.87-9831.32)	0.0010	4123.78 (727.36-8529.36)	0.0002	975.84 (221.64-5543.70)	<0.0001
IL-10	4796.77 (2151.42-5050.71)	4796.77 (1947.25-4064.41)	0.7533	4796.77 (2605.55-11669.98)	0.0317	3594.41 (1708.65-6975.51)	0.4948
TNF-α	10979.77 (9517.87-11481.94)	10982.00 (9666.87-11481.94)	0.7178	11135.81 (10532.88-11449.60)	0.7437	10751.62 (10320.47-11340.65)	0.6373
IL-12p40	43.40 (10.12-87.52)	43.07 (24.40-101.03)	0.5553	18.70 (6.38-5134)	0.2471	41.02 (0.97-196.28)	0.9739
IL-12p70	13.04 (3.39-91.46)	19.39 (1.70-38.66)	0.7993	2.88 (0.70-13.83)	0.0167	2.80 (0.28-28.07)	0.0772
IL-18	7.43 (5.14-13.03)	7.92 (4.74-16.30)	0.6841	15.03 (8.64-44.91)	0.0015	8.00 (4.10-12.98)	0.8470

Table 3.11: Median and IQR of the concentrations of soluble cytokines produced in unstimulated control condition among individuals in the different study groups. Table includes p-value from comparisons from healthy controls.

Stimulation	Healthy controls	HIV only		Active TB		HIV-associated TB	
	Median (IQR)	Median (IQR)	P-value	Median (IQR)	P-value	Median (IQR)	P-value
IFN-α2	18.13 (25.88-5.74)	11.84 (24.35-5.79)	0.4667	11.645 (20.38-5.79)	0.6537	13.47 (23.94-5.96)	0.7829
IFN-γ	6.03 (17.02-3.43)	4.56 (9.74-2.25)	0.2527	4.51 (8.69-1.40)	0.1991	2.74 (6.15-1.16)	0.0062

IL-12p40	5.182 (28.59-5.18)	5.182 (12.29-5.18)	0.5554	5.182 (22.05-5.18)	0.5765	5.182 (5.18-5.18)	0.3657
IL-12p70	2.27 (5.77-1.63)	1.89 (5.77-0.73)	0.1423	1.765 (3.805-0.96)	0.1365	1.46 (3.38-0.63)	0.0387
TNF-α	115.86 (569.74-56.27)	225.98 (439.73-51.27)	0.9863	183.77 (405.43-43.97)	0.6812	110.775 (753.54-51.39)	0.6471
IL-10	67.46 (276.42-33)	51.69 (114.46-26.99)	0.1964	69.075 (344.97-18.63)	0.5486	40.99 (226.75-15.2575)	0.2053
IL-18	5.394 (8.26-4.58)	6.4505 (12.86-4.54)	0.5274	7.236 (16.84-4.76)	0.2203	14.3705 (25.73-4.83)	0.2483

Table A12: Correlation between GFP+ monocytes and T cell (MAIT cells, CD4 and CD8 T cells) functional measures (CD107a, IFN-γ) and activation (HLA-DR) in response to BCG 1 and BCG 5 stimulation. Table includes correlation statistics: Spearman r value and p-value.

Stimulation	**Subset**	**Healthy controls**		**HIV only**		**Active TB only**		**HIV-associated TB**	
		r	**p-value**	**r**	**p- value**	**r**	**p-value**	**r**	**p-value**
BCG 5	**% CD107a+ MAIT cells**	-0.2342	0.2821	-0.1505	0.4445	-0.7167	0.0085	-0.4072	0.0390
	% IFN-γ+ MAIT cells	-0.4941	0.0166	0.1242	0.5287	0.1753	0.3542	-0.2233	0.2727
	% HLA-DR+ MAIT cells	-0.01484	0.9464	-0.01807	0.9273	-0.01939	0.9190	-0.01269	0.9509
	HLA-DR (on MAIT cells)	-0.2992	0.1655	0.259	0.1832	0.1046	0.5824	0.01608	0.9379
	HLA-DR MFI (on IFN-γ+ MAIT cells)	-0.04201	0.8491	0.2837	0.1434	0.4951	0.0054	0.2247	0.2699
BCG 1	**% CD107a+ CD4 T cells**	0.3551	0.0816	0.4111	0.0297	0.3196	0.0852	-0.0888	0.6727
	% IFN-γ+ CD4 T cells	-0.1154	0.5828	0.2053	0.2947	0.1386	0.4651	0.04077	0.8466
	% HLA-DR+ CD4 T cells	0.1059	0.6142	0.1124	0.5690	0.04411	0.8170	-0.06799	0.7468
	HLA-DR MFI (on CD4 T cells)	-0.1769	0.3976	-0.308	0.1108	-0.3189	0.0859	-0.4095	0.0420
	% CD107a+ CD8 T cells	-0.0392	0.8522	0.1536	0.4351	0.009179	0.9616	-0.6112	0.0012
	% IFN-γ+ CD8 T cells	0.2123	0.3083	0.2284	0.2425	0.2939	0.1149	-0.01154	0.9563

	% HLA-DR+ CD8 T cells	0.05953	0.7774	-0.06257	0.7518	-0.08987	0.6367	-0.1755	0.4014
	HLA-DR MFI (on CD8 T cells)	0.2004	0.3368	-0.1259	0.5233	0.01446	0.9395	-0.2077	0.3191
BCG 5	**% CD107a+ CD4 T cells**	-0.3804	0.0733	-0.1259	0.5233	-0.2641	0.1585	0.1706	0.4047
	% IFN-γ+ CD4 T cells	-0.4632	0.0259	0.1494	0.4479	-0.05984	0.7534	-0.1385	0.5000
	% HLA-DR+ CD4 T cells	-0.1403	0.5233	0.1286	0.5144	-0.1002	0.5982	0.05455	0.7913
	HLA-DR MFI (on CD4 T cells)	-0.02293	0.09173	0.3963	0.0368	-0.02702	0.8873	0.1152	0.5751
	% CD107a+ CD8 T cells	-0.3103	0.1496	-0.2074	0.2895	-0.2271	0.2274	0.1364	0.5464
	% IFN-γ+ CD8 T cells	-0.3745	0.0783	0.07612	0.7002	0.2044	0.2785	-0.04342	0.8332
	% HLA-DR+ CD8 T cells	0.03928	0.8588	0.2363	0.2261	0.2704	0.8872	0.1081	0.1081
	HLA-DR MFI (on CD8 T cells)	-0.1502	0.4939	-0.03284	0.8682	-0.06319	0.7401	0.02017	0.9221

Table A13: Correlation between GFP+ DCs and MAIT cell functional measures (CD107a, IFN-γ) and activation (HLA-DR) in response to BCG 5 stimulation. Table includes correlation statistics: Spearman r value and p-value.

Stimulation	**Subset**	**Healthy controls**		**HIV only**		**Active TB only**		**HIV-associated TB**	
		r	**p- value**	**r**	**p- value**	**r**	**p- value**	**r**	**p- value**
BCG 1	**% HLA-DR+ MAIT cells**	0.488	0.0133	0.1905	0.9234	0.2297	0.2221	-0.1205	0.5660
	HLA-DR MFI (on IFN-γ+ MAIT cells)	-0.04542	0.8293	-0.2388	0.2210	-0.1796	0.3424	-0.02077	0.9215
BCG 5	**% CD107a+ MAIT cells**	-0.2026	0.3539	-0.2813	0.1470	-0.4659	0.5419	-0.4824	0.0126
	% IFN-γ+ MAIT cells	-0.2401	0.2698	0.005473	0.9779	0.04086	0.0250	-0.3331	0.0963
	% HLA-DR+ MAIT cells	0.2136	0.3277	-0.115	0.5601	0.2776	0.1374	0.272	0.1788
	MAIT cells (HLA-DR MFI)	-0.1377	0.5310	-0.143	0.4679	0.3186	0.0862	0.0934	0.6500

	HLA-DR MFI (on IFN-γ+ MAIT cells)	0.03509	0.8737	0.05505	0.7809	0.4248	0.0193	0.5427	0.0042

Table A14 below shows the summary of the relationships between the MFI of HLA-DR on MAIT cells expressing IFN-*γ* in response to BCG 1 stimulation and soluble cytokine concentrations. No significant correlations were observed.

Table A14: Correlations between the concentrations of soluble cytokine in cell supernatants produced in response to BCG 1 stimulation and MAIT cell activation. Table includes Spearman r value and p-value.

Cytokine	**Subset**	**Healthy controls**		**HIV only**		**Active TB only**		**HIV-associated TB**	
		r	**p- value**	**r**	**p- value**	**r**	**p-value**	**r**	**p-value**
IL-10	**IFN-γ+ MAIT cells (HLA-DR MFI)**	-0.07276	0.7296	-0.1301	0.5094	0.01447	0.9395	-0.3585	0.0785
IL-12p40	**IFN-γ+ MAIT cells (HLA-DR MFI)**	-0.04551	0.8290	-0.2247	0.2503	0.179	0.3439	-0.2325	0.2634
IL-12p70	**IFN-γ+ MAIT cells (HLA-DR MFI)**	0.1409	0.5018	0.063	0.7501	0.303	0.1036	-0.2972	0.1492

Table A15: Correlations between the concentrations of soluble cytokine in cell supernatants produced in response to HK-M.tb stimulation and MAIT cell functional and activation markers. Table includes Spearman r value and p-value.

Cytokine	**Subset**	**Healthy controls**		**HIV only**		**Active TB only**		**HIV-associated TB**	
		r	**p- value**	**r**	**p- value**	**r**	**p-value**	**r**	**p-value**
IL-10	**% CD107a+ MAIT cells**	-0.5159	0.0083	-0.1359	0.4821	-0.3405	0.0656	0.08034	0.7027
	% IFN-γ+ MAIT cells	-0.4347	0.0299	-0.2473	0.1959	-0.2887	0.1218	-0.2664	0.1980
	% HLA-DR+ MAIT cells	-0.4406	0.0275	0.039	0.8408	-0.4755	0.0079	-0.441	0.0273
	MAIT cells (HLA-DR MFI)	-0.02579	0.9026	0.2937	0.1220	-0.08503	0.6551	-0.4239	0.0347
	IFN-γ+ MAIT cells (HLA-DR MFI)	-0.2774	0.1795	0.202	0.2934	-0.1284	0.4990	-0.03193	0.8796

IL-12p40	**% CD107a+ MAIT cells**	0.1027	0.6251	-0.07789	0.6880	0.09543	0.6539	-0.3645	0.0732
	% IFN-γ+ MAIT cells	0.4993	0.0110	0.5449	0.0022	0.09046	0.6345	0.4527	0.0231
	% HLA-DR+ MAIT cells	-0.1796	0.3904	-0.2141	0.2649	-0.1854	0.3267	-0.1229	0.5585
	MAIT cells (HLA-DR MFI)	0.007315	0.9723	0.1386	0.4733	0.1959	0.2996	0.03746	0.8589
	IFN-γ+ MAIT cells (HLA-DR MFI)	-0.3601	0.0771	-0.3803	0.0418	0.2247	0.2325	-0.3263	0.1114
IL-12p70	**% CD107a+ MAIT cells**	0.323	0.1152	-0.02586	0.8941	0.1179	0.5350	-0.1494	0.4759
	%IFN-γ+ MAIT cells	0.8345	<0.0001	0.7857	<0.0001	0.3587	0.0516	0.6171	0.0010
	% HLA-DR+ MAIT cells	0.09428	0.6540	-0.3248	0.0856	0.2021	0.2674	0.04592	0.8275
	MAIT cells (HLA-DR MFI)	0.08937	0.6710	-0.1141	0.5556	0.3797	0.0385	0.3723	0.0669
	IFN-γ+ MAIT cells (HLA-DR MFI)	-0.3333	0.1035	-0.5812	0.0009	-0.1639	0.3868	-0.4968	0.0115

Table A16:Correlations between the concentrations of soluble cytokine in cell supernatants produced in response to BCG 1 stimulation and CD4 and CD8 cell functional and activation markers. Table includes Spearman r value and p-value.

Cytokine	**Subset**	**Healthy controls**		**HIV only**		**Active TB only**		**HIV-associated TB**	
		r	**p-value**	**r**	**p- value**	**r**	**p-value**	**r**	**p-value**
IL-10	**% CD107a+ CD4 T cells**	0.1304	0.5343	-0.1812	0.3561	-0.1047	0.5818	-0.3609	0.0763
	% IFN-γ+ CD4 T cells	-0.04116	0.8451	-0.3684	0.0538	-0.006007	0.9749	-0.2415	0.2448
	% HLA-DR+ CD4 cells	-0.1972	0.3448	-0.1422	0.4703	0.2146	0.2549	-0.4395	0.0279
	CD4 T cells (HLA-DR MFI)	-0.0904	0.6674	-0.1147	0.5612	0.1325	0.4853	-0.2402	0.2475
	IFN-γ+ CD4 T cells (HLA-DR MFI)	0.3655	0.0724	0.07772	0.6942	0.06297	0.7410	-0.4632	0.0197
	% CD107a+ CD8 T cells	0.266	0.1987	-0.1608	0.4136	-0.2895	0.1207	0.006551	0.9752

	% IFN-γ+ CD8 T cells	0.1112	0.5968	-0.3149	0.1027	0.004539	0.8117	0.1231	0.5578
	% HLA-DR+ CD8 T cells	-0.22	0.2906	0.001277	0.9949	0.08576	0.6523	-0.1802	0.3888
	CD8 T cells (HLA-DR MFI)	-0.1079	0.6076	-0.2091	0.2856	-0.05429	0.7757	-0.2892	0.1608
	IFN-γ+ CD8 T cells (HLA-DR MFI)	0.247	0.2340	-0.09141	0.6437	0.00979	0.9591	-0.3785	0.0621
IL-12p40	**% CD107a+ CD4 T cells**	0.04317	0.8377	-0.05462	0.7825	0.0504	0.7914	0.02451	0.9074
	% IFN-γ+ CD4 T cells	0.4983	0.0112	0.3654	0.0559	0.05056	0.7908	0.05521	0.7932
	% HLA-DR+ CD4 cells	0.07744	0.7129	0.2795	0.1497	-0.2755	0.1407	-0.2916	0.1573
	CD4 T cells (HLA-DR MFI)	-0.212	0.3090	0.03493	0.8599	0.09823	0.6056	0.1806	0.3878
	IFN-γ+ CD4 T cells (HLA-DR MFI)	-0.1808	0.3882	-0.1514	0.4420	0.114	0.5485	-0.2858	0.1660
	% CD107a+ CD8 T cells	0.2615	0.2068	0.08183	0.6789	-0.03496	0.8545	0.3636	0.0738
	% IFN-γ+ CD8 T cells	0.3426	0.0937	0.23	0.2389	-0.0793	0.6770	0.1019	0.6280
	% HLA-DR+ CD8 T cells	-0.02108	0.9203	0.02874	0.8846	-0.0487	0.7983	0.1746	0.4038
	CD8 T cells (HLA-DR MFI)	-0.1018	0.6284	0.0531	0.7884	-0.03586	0.8508	0.2924	0.1561
	IFN-γ+ CD8 T cells (HLA-DR MFI)	-0.1094	0.6025	-0.2108	0.2817	0.006236	0.9739	0.01711	0.9353
IL-12p70	**% CD107a+ CD4 T cells**	-0.1821	0.3820	0.02255	0.9093	0.2273	0.2271	0.1078	0.6080
	% IFN-γ+ CD4 T cells	0.6508	0.0004	0.3988	0.0355	0.4046	0.0266	0.1016	0.6289
	% HLA-DR+ CD4 cells	-0.003474	0.9869	0.08355	0.6725	0.2066	0.2737	-0.1312	0.5318
	CD4 T cells (HLA-DR MFI)	-0.1002	0.6336	0.1694	0.3888	0.1949	0.3021	0.2273	0.2746
	IFN-γ+ CD4 T cells (HLA-DR MFI)	-0.2031	0.3302	-0.1193	0.5453	0.06595	0.7291	0.201	0.3354
	% CD107a+ CD8 T cells	0.3634	0.0742	0.445	0.0176	0.1034	0.5867	0.3806	0.0605

	% IFN-γ+ CD8 T cells	0.5138	0.0086	0.462	0.0133	0.1713	0.3655	0.1948	0.3508
	% HLA-DR+ CD8 T cells	0.05863	0.7807	-0.01197	0.9518	0.07247	0.7035	-0.001859	0.9930
	CD8 T cells (HLA-DR MFI)	-0.03424	0.8709	-0.1891	0.3351	0.1021	0.5915	0.1578	0.4512
	IFN-γ+ CD8 T cells (HLA-DR MFI)	0.06462	0.7590	-0.04899	0.8045	0.08467	0.6564	0.1147	0.5851

Table A17: Correlations between the concentrations of soluble cytokine in cell supernatants produced in response to HK-*M.tb* stimulation and CD4 and CD8 cell functional and activation markers. Table includes Spearman r value and p-value.

Cytokine	**Subset**	**Healthy controls**		**HIV only**		**Active TB only**		**HIV-associated TB**	
		r	**p-value**	**r**	**p- value**	**r**	**p-value**	**r**	**p-value**
IL-10	**% CD107a+ CD4 T cells**	-0.06887	0.7436	-0.09315	0.6308	-0.1528	0.4203	-0.321	0.1177
	% IFN-γ+ CD4 T cells	-0.322	0.1165	-0.2517	0.1877	-0.2485	0.1855	-0.4662	0.0188
	% HLA-DR+ CD4 cells	-0.2323	0.2638	-0.1695	0.3793	-0.2527	0.1779	-0.1261	0.5481
	CD4 T cells (HLA-DR MFI)	0.1543	0.4614	0.1867	0.3321	0.1378	0.4677	-0.1093	0.6032
	IFN-γ+ CD4 T cells (HLA-DR MFI)	0.03308	0.8753	0.2232	0.2445	0.1782	0.3461	-0.3104	0.1310
	% CD107a+ CD8 T cells	-0.08749	0.6775	0.1655	0.3910	0.08277	0.6637	0.1506	0.4725
	% IFN-γ+ CD8 T cells	-0.3778	0.0626	-0.2606	0.1721	-0.2298	0.2218	-0.3308	0.1063
	% HLA-DR+ CD8 T cells	-0.2344	0.2595	-0.1051	0.5876	-0.3955	0.0305	-0.1841	0.3782
	CD8 T cells (HLA-DR MFI)	0.006157	0.9767	0.04926	0.7997	-0.117	0.5380	-0.357	0.0798
	IFN-γ+ CD8 T cells (HLA-DR MFI)	-0.09273	0.6593	0.203	0.2910	0.04828	0.8000	0.01577	0.9404
IL-12p40	**% CD107a+ CD4 T cells**	0.3245	0.1135	0.1824	0.3437	-0.0006	0.9976	0.2743	0.1845
	% IFN-γ+ CD4 T cells	0.407	0.0435	0.5244	0.0035	0.05568	0.7701	0.3174	0.1221

	% HLA-DR+ CD4 cells	0.1569	0.4538	0.3016	0.1118	-0.2454	01911	-0.4763	0.0161
	CD4 T cells (HLA-DR MFI)	-0.1008	0.6315	0.06357	0.7432	-0.1593	0.4003	0.1336	0.5243
	IFN-γ+ CD4 T cells (HLA-DR MFI)	-0.17	0.4165	-0.007884	0.9676	0.1176	0.5360	0.06024	0.7748
	% CD107a+ CD8 T cells	0.07342	0.7272	0.03618	0.8522	0.07149	0.9269	-0.3209	0.1178
	% IFN-γ+ CD8 T cells	0.2866	0.0563	0.5159	0.0042	0.1303	0.4926	0.1954	0.3493
	% HLA-DR+ CD8 T cells	-0.2525	0.2234	0.3391	0.0720	-0.03567	0.8516	-0.4047	0.448
	CD8 T cells (HLA-DR MFI)	0.2167	0.2982	0.1527	0.4290	-0.02049	0.9144	0.06024	0.7748
	IFN-γ+ CD8 T cells (HLA-DR MFI)	0.1551	0.4592	-0.090	0.6391	0.1579	0.4046	-0.1325	0.5279
IL-12p70	**% CD107a+ CD4 T cells**	0.114	0.5876	0.3508	0.0621	-0.04714	0.8046	0.2465	0.2349
	% IFN-γ+ CD4 T cells	0.8083	<0.0001	0.7101	<0.0001	0.4415	0.0146	0.6062	0.0013
	% HLA-DR+ CD4 cells	0.3102	0.1313	0.2443	0.2015	0.0136	0.9432	-0.2182	0.2948
	CD4 T cells (HLA-DR MFI)	-0.02233	0.9156	-0.0484	0.8031	0.2268	0.2281	0.3375	0.0989
	IFN-γ+ CD4 T cells (HLA-DR MFI)	0.0385	0.8550	-0.2652	0.1644	0.1646	0.3848	-0.06449	0.07594
	% CD107a+ CD8 T cells	0.03625	0.8634	0.134	0.4885	-0.00507	0.9788	-0.2008	0.3359
	% IFN-γ+ CD8 T cells	0.7436	<0.0001	0.7206	<0.0001	0.4045	0.0266	0.6664	0.0003
	% HLA-DR+ CD8 T cells	0.07252	0.7305	0.2798	0.1416	0.3376	0.0681	-0.09909	0.6375
	CD8 T cells (HLA-DR MFI)	-0.02233	0.9156	-0.0484	0.8031	0.2268	0.2281	0.3375	0.0989
	IFN-γ+ CD8 T cells (HLA-DR MFI)	0.0385	0.8550	-0.2652	0.1644	0.1646	0.3848	-0.06449	0.7594