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Investigating the effect of HIV infection on TCR repertoire diversity and *Mycobacterium tuberculosis*-specific T cell function in Malawian adults

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Abstract

The burden of tuberculosis (TB) is huge, posing a significant health threat worldwide, particularly in HIV-endemic regions such as sub-Saharan Africa. Co-infection with HIV is a major risk factor for development and progression to active *Mycobacterium tuberculosis* (Mtb) disease. People living with HIV (PLHIV) remain at more risk of developing lower respiratory tract infections including TB than HIV-uninfected individuals, despite successful coverage of antiretroviral therapy (ART). HIV depletes and impairs the function of Mtb-specific T cells crucial in controlling Mtb infection. However, the impact of HIV on T cell receptor (TCR) usage in alveolar T cells is incompletely described. Characterisation of TCR repertoire is essential for understanding the mechanisms of recognition and control of Mtb infections by T cell adaptive immunity.

To investigate the Mtb antigen-specific TCR diversity and clonality in the airway and blood and assess the impact of HIV and ART on TCR diversity, peripheral blood and bronchoalveolar lavage (BAL) samples were collected from PLHIV ART–naïve, on ART (≥3 years) and HIV-uninfected adults recruited at Queen Elizabeth Central Hospital, Blantyre, Malawi. Alveolar and peripheral blood lymphocytes were stimulated with Mtb antigens and analysed using flow cytometry and TCR bulk sequencing.

Notably, Mtb-specific TCR repertoires from PLHIV displayed a reduced diversity and clonality compared to HIV-uninfected individuals in both the airway and blood. Moreover, ART was associated with the restoration of TCR clonotypes in PLHIV. Additionally, lower frequencies of Mtb-specific CD4 IFN-γ and TNF-α producing cells were observed in both blood and airway in PLHIV on ART and ART naïve compared to HIV-uninfected individuals. Significant alterations in TCR Vß expression patterns were noted in CD4+ T cells in PLHIV compared to healthy controls. Vß1, Vß7.2, and Vß23 were higher, while Vß9 and Vß18 were lower in blood and airway in PLHIV than in HIV uninfected individuals. In CD8 T cells, no significant differences were found in TCR Vß expression in the PBMC compared to

BAL. However, in the lung, Vβ5.1, Vβ16, and Vβ17 were increased, while Vβ14 was decreased in PLHIV. Furthermore, CDR3 length distribution analysis showed a higher and more diverse distribution of TCR amino acid lengths of Mtb-specific T cells in BAL and PBMCs in HIV-uninfected individuals compared to PLHIV. The elevated TCR Vβ in the lung and blood in PLHIV suggests their potential involvement in HIV immune response whilst depletion of certain TCR Vβ clones in Mtb-specific CD4 and CD8 T cells in the lung and blood may indicate HIV-induced alteration in the repertoire associated with increased TB risk.

These findings suggest a more restricted TCR repertoire in PLHIV compared to healthy controls, with alterations in the frequency of certain families that may impact antigen recognition and specificity. This could lead to a reduced ability to mount protective immune responses against infections, including Mtb, in PLHIV. Identifying highly used and expressed TCR Vβ segments provides insights into mechanisms of host protective immunity in HIV and TB and may offer crucial targets for vaccine development and preventive therapies.

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Author's Declaration

I declare that this thesis is the result of my own work, except where reference is made to the contribution of others and has not been submitted for any other degree at the University of Glasgow or any other institution. The study presented in this thesis was done in conjunction with other studies and, in some instances, the work was shared with colleagues. This research was carried at the Malawi-Liverpool Wellcome Research Programme and Queen Elizabeth Central Hospital, Blantyre, Malawi. My contributions for the reported work were as follows:

Activity	**Responsibility**
Sample collection	Shared
Sample processing	Sole
TCR Vβ amplification	Sole
TCR CDR3 sequencing and data analysis	Shared
Immunophenotyping	Sole
TCR Vβ flow cytometry screening	Sole
TCR RNA extraction	Sole
Flow cytometry data analysis	Sole
Statistical data analysis and presentation	Sole
Thesis preparation and writing	Sole

Signature:

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Abbreviations

αß	Alpha and Beta chain
AEC	Alveolar Epithelial cell
AF700	Alexa Fluor® 700
AIDS	Acquired Immunodeficiency Syndrome
AIRE	Autoimmune regulator
AM	Alveolar macrophage
Ag	Antigen
APC	Antigen presenting cell
APC	Allophycocyanin
APC Cy7	Allophycocyanin-Cyanine
ART	Antiretroviral therapy
Bp	Base pair
BAL	Bronchoalveolar lavage
BCG	Bacille Calmette-Guerin
BCL6	B-cell lymphoma 6
BV 421	Brilliant Violet 421
BV 605	Brilliant Violet 605
CDR3ß	Complementarity determining region 3 of the TCR ß Chain
CCL19	C-C motif ligand 19
CCL21	C-C motif ligand 21
CCR10	C-C motif chemokine receptor 10
CCR4	C-C motif chemokine receptor
CCR5	C-C chemokine Receptor 5
CCR6	C-C chemokine Receptor 6
CCR7	Cysteine Chemokine Receptor 7
CD	Cluster of Differentiation
cRPMI	Complete Roswell Park Memorial Institute
cTEC	Thymic cortical epithelial cells
CTL	Cytotoxic T lymphocytes
CXCL	C-X-C motif Ligand
D	Diversity
DAMP	Danger-associated molecular pattern
DC	Dendritic Cell
dLN	Draining lymph node
DMSO	Dimethyl sulfoxide
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double Positive
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EM	Effector memory
EOMES	Eomesodermin
FoxP3	Forkhead box P3

FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GATA3	GATA Binding Protein 3
GCP	Good Clinical Practice
HIV	Human Immunodeficiency Virus
HEV	High endothelial venule
ICAM-1	Intracellular adhesion molecule
ICOS	Inducible T cell co-stimulator
ICS	Intracellular cytokine staining
IFN-γ	Interferon gamma
IL	Interleukin
ION	Ionomycin
IRF	Interferon regulatory factors
IFN	Interferon
KO	Knockout
ITAM	Immunoreceptor tyrosine activating motifs
LAMP	Lysosome-associated membrane glycoprotein
LFA-1	Lymphocyte function-associated antigen
LIMC	Low - Middle-Income Countries
LN	Lymph Node
LRTI	Lower respiratory tract infection
MadCAM-1	Mucosal vascular addressin cells adhesion molecule
MFI	Mean or Median fluorescence intensity
MHC	Major histocompatibility complex
MLW	Malawi Liverpool Wellcome Programme
MPEC	Memory precursor cells
MR1	MHC-related molecule 1
mRNA	Messenger ribonucleic acid
Mtb	Mycobacterium tuberculosis
NHSRC	National Health Science Research Committee
NO	Nitric oxide
NF-κB	Nuclear factor kappa B
NSO	National Statistical Office
NET	Neutrophil extracellular trap
NK	Natural killer
NP	Nucleoprotein
Nt	Nucleotides
PAMP	Pathogen-associated molecular pattern
PBMCs	Peripheral mononuclear cells
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PD1	Programmed Death Protein 1
PDL1	Programmed Death Ligand 1
Pdpn	Podoplanin
PE	Phycoerythrin

PE CF594	Phycoerythrin-CF594
PE Cy7	Phycoerythrin- Cyanine 7
PerCP Cy 5.5	Peridinin chlorophyll protein-Cyanine 5.5
PLHIV	People living with HIV infection
PMA	Phorbol myristate acetate
PNAd	Peripheral lymph node addressin
PRR	Pathogen recognition receptor
QECH	Queen Elizabeth Central Hospital
RAG	Recombination activating genes
Tregs	Regulatory T cells
RNA-Seq	RNA sequencing
RORγt	Retinoic Acid Receptor (RAR)-Related Orphan Receptor
RSS	Recombination sequences
scRNA-Seq	Single-cell RNA sequencing
STAT	Signal Transducer and Activator of Transcription
TB	Tuberculosis
T-bet	T-box expressed in T cells
TCR	T Cell Receptor
TCM	Central memory T cell
TdT	Terminal deoxynucleotidyl transferase
TEM	Effector memory T cell
Tfh	T follicular helper cell
TGF-β	Transforming Growth Factor-β
Th	T helper
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-alpha
Treg	Regulatory T cell
WT	Wild type
V	Variable
Vβ	Variable region of the TCR β chain
VCAM-1 1	Vascular cell adhesion molecule
ZAP-70	Zeta-chain associated protein kinase

Chapter 1 Introduction

1.1 Synopsis

TB is a chronic inflammatory infectious disease caused by *Mycobacterium tuberculosis* (Mtb). The lack of an effective vaccine, the increasing incidence of multidrug-resistant strains, high rates of human immunodeficiency virus (HIV) infection and the migration of populations from high-to-low-incidence countries exacerbate the situation **(sections 1.7 &1.9).** The synergy between HIV and TB is incompletely understood and remains an area of intense research. Despite long-term antiretroviral therapy (ART), TB remains an important opportunistic infection and one of the leading causes of death in People living with HIV infection (PLHIV), in both low and middle-income countries. HIV increases the risk of active TB by impairing the immune system. Furthermore, despite the availability of potent anti-TB drugs, treatment is hampered by prolonged duration, which often results in poor adherence. Consequently, there is an urgent need to design novel effective therapeutic interventions against TB.

The immune system is composed of innate and adaptive components that work in tandem to protect against pathogens and resolve infection **(section 1.6)**. Cellular immunity mediated by CD4 and CD8 T cells is crucial in providing protective immunity against TB. The hallmark of the adaptive immune system is antigen specificity and recognition of antigens via the T cell receptor (TCR). The TCR is a highly variable heterodimer composed of either alpha and beta chains or gamma and delta chains. The diversity of the TCR is a consequence of the somatic recombination of multiple gene segments such as V, D and J with the TCR chains (8,9). Together, these processes generate a spectrum of TCRs expressed on individual T cells that, following the recognition of antigen, lead to clonal expansion of specific T cells **(section 1.6.3.2).**

In a quest to investigate TCR usage in immune responses to TB, several studies have focused on examining CD4+ T cell diversity and clonality in the peripheral blood of TB patients. However, these studies only offer partial insights into mechanisms underlying localised immune control of infection in the lung that

influence TB outcome. Thus, it is important to understand the impact of HIV on the TCR repertoire in the lung microenvironment, the localised site of Mtb infection and a common site for infectious complications.

PLHIV are more susceptible to lower respiratory tract infections (LTRI), including TB than HIV-uninfected people. To date, no reliable evidence exists regarding host factors that constitute protective immunity against TB following natural infection. While the role of CD4 and CD8 T cells in TB infection has been well established in both animal and human studies, little is known about the impact of TB and/or HIV on T cell receptor repertoire in the lung in humans **(section 1.8).** Additionally, it is not yet clear whether this repertoire is fully restored following successful treatment of either or both infections. Investigating the functional composition of TCR repertoire in HIV-infected individuals will advance the current understanding of host factors that control Mtb infection or favour the progression of the disease. Novel insights into what constitutes protective immunity against Mtb in the lung can inform the development of new interventions to control TB in both HIV-infected and uninfected individuals.

This research aims to provide a detailed analysis of the impact of HIV infection on the diversity and dynamics of CD4+ and CD8+ TCR repertoire and the role of Mtb-antigen-specific immune responses to TB. The data presented here were obtained from paired peripheral venous blood and bronchoalveolar lavage fluid (BAL) from PLHIV on ART, PLHIV not on ART, and HIV-uninfected adults. The findings and conclusions discussed in this thesis aim to advance our understanding and provide new insights into why PLHIV have a higher risk of developing active TB than HIV-uninfected individuals. In addition, this knowledge will enhance the identification of host factors that control susceptibility to and progression of Mtb infection, which may eventually aid in the development of improved vaccines against TB. Furthermore, the identification of the highly used TCR chains may provide prognostic biomarkers and therapeutic targets for HIV and TB, which can inform the early commencement of preventive therapies or vaccines.

1.2 The global burden of TB

TB is one of the top infectious disease killers worldwide. In 2021, over 10.6 million people were infected and progressed to active disease with an annual mortality of 1.7 million people. This makes TB one of the leading causes of death from a single infectious agent slightly below COVID-19 (1). The burden of TB is disproportionally high in Asia and sub-Saharan Africa (SSA) **(Figure 1-1),** powered by unfavourable socio-economic determinants that affect the regions the hardest (2,3). These regions contribute about 70% of TB cases worldwide annually (5). The African continent, in particular, which contains 15% of the world's population, accounts for nearly a third of the global burden of tuberculosis and tuberculosis-related deaths (4,5). According to the World Health Organisation (WHO), Africa is home to 22 out of the 30 high-burden countries (HBCs) for TB/HIV, which collectively represent 97% of the estimated global population affected by both HIV and TB (6). Unfortunately, the implications of this burden are exacerbated by, among other factors, poor access to diagnostics and adequate treatments, and the emergence and spread of multi-drug resistance TB (7). Moreover, the continuing HIV epidemic, overcrowding, poverty and poor adherence to TB treatment continue to drive TB as a cause of morbidity and mortality in these settings.

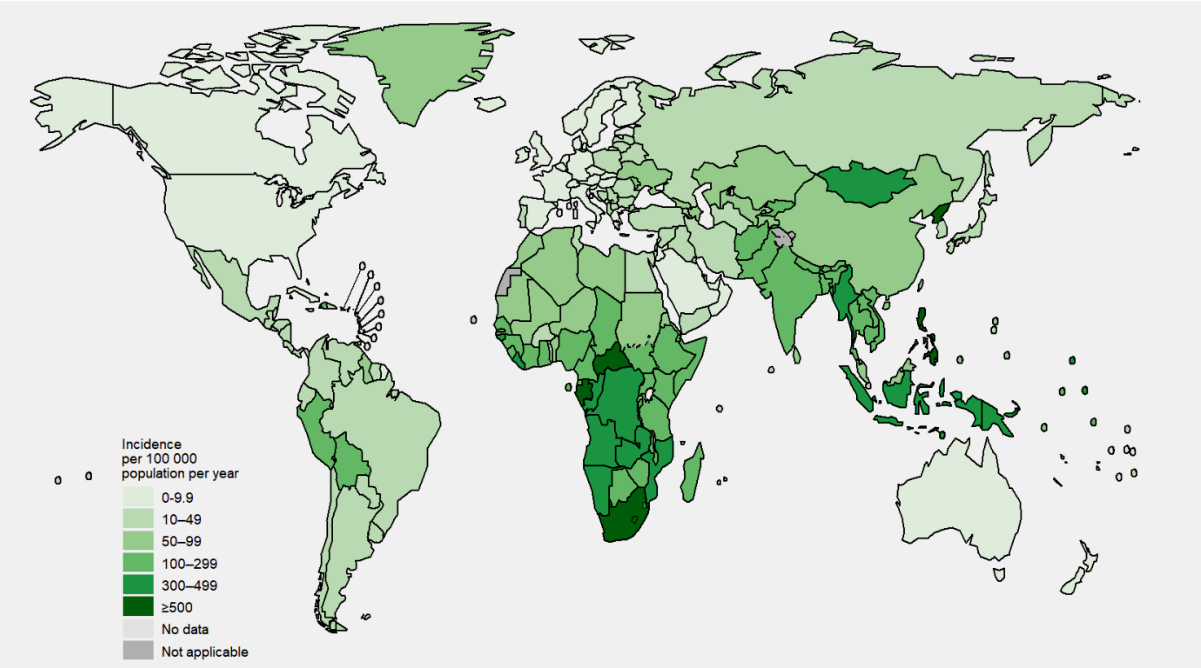


Figure 1-1 Map showing the global distribution of TB.

The map shows the global distribution of TB, with sub-Saharan Africa and Asia bearing most of the TB burden (W.H.O, 2021).

Malawi is a small southeast African country with a population of over 21 million (8) of whom 70-72% live below the poverty line (9). Malawi ranks among the top 20 countries with a significant burden of TB. It has one of the highest estimated numbers of incident TB cases among individuals living with HIV (6). While the incidence of TB in Malawi has decreased from 338 per 100,000 in 2010 to 132 per 100,000 in 2021 (10), the burden of TB persists, aggravating the already fragile health system (11) and compromising efforts to meet the End TB Strategy goals. A deep understanding of the host-pathogen interaction and the immune responses that influence TB outcomes would contribute to achieving the ambitious targets of the WHO End TB Strategy, which aims for a 90% reduction in TB incidence and a 95% reduction in TB-related deaths (from 2015) by 2035.

1.3 The causative agent of TB

Human TB is mainly caused by a tubercle bacillus known as *Mycobacterium tuberculosis* (Mtb). It belongs to a group of closely related organisms—including *M africanum*, *M. bovis*, and *M microti* —in the *M tuberculosis* complex (12).

Although Mtb is the predominant cause of TB in humans, *M bovis* contributes to 0.5% to 7.2% of TB cases in high-income countries and is responsible for about 15% of new cases in low and middle-income countries (LMICs) (13). Mtb and *M bovis* are closely related pathogens with a nucleotide genome identity of 99.95% (14,15). Despite this similarity, differences in phenotypes, virulence, and host tropism distinctly set these pathogens apart. A notable genetic contrast between Mtb and *M. bovis* involves the deletion of 16 specific loci in the *Bacille Calmette-Guérin* (BCG) genome, denoted as RD1 through RD16 (16). Specifically, RD1 was the key deletion during the initial attenuation of M. *bovis* to produce BCG, a strain used in the only licenced TB vaccine (17). Thus, the RD1 genomic region is present in virulent strains of Mtb missing from the vaccine strain *M. bovis* BCG. The RD1 genome in Mtb consists of a total of 9 open reading frames (ORF), which, in conjunction with genes located outside the RD1 locus, form the early secretory antigenic target–6 (ESAT-6) secretion system (ESX-1) (18).

RD1 is primarily believed to play a significant role in encoding and secretion of the key antigenic proteins, namely early secreted antigenic target 6 (ESAT-6) and culture filtrate protein–10 (CFP-10) which are important Mtb virulence factors (19,20). Additionally, Mtb is a slow-growing pathogenic Mycobacterium that exhibits a division rate of 12 to 24 hours and requires up to 21 days for culture growth on agar. The precise mechanisms behind its slow growth remain unclear. However, this may be attributed to the limited nutrient absorption through the highly impermeable cell wall and slow rates of RNA synthesis (21). Mtb is acid-fast, gram-positive bacilli with a size of 1-4×0.4 mm, which is an obligate aerobe and can become a facultative anaerobe depending on the environment (22). Indeed, studies have demonstrated a shift in its metabolism from an aerobic state focused on carbohydrate and lipid metabolism (23). Unlike other bacteria, Mtb has no glycocalyx or adhesion elements such as fimbriae. Nonetheless, its cell wall is composed of membrane-rich lipids called mycolic lipids that contribute to distinct characteristics such as hydrophobicity and resistance to commonly used antibiotics **(Figure 1-2)** (24). Additionally, lipoarabinomannan, a cell wall component, is known to orchestrate host-pathogen interaction to stimulate the immune response (25).

The unique cell wall components of Mtb play a pivotal role in evading the immune system, and they serve as a significant target for numerous anti-TB drugs (26). The cell wall of Mtb primarily consists of several key components, including Peptidoglycan (PG), Arabinogalactan (AG), Trehalose-6,6'-dimycolate (TDM) and Mycolic acid (MA). Mtb's cell membrane features a Peptidoglycan (PG) layer similar to that of gram-positive bacteria, which plays essential roles in cell growth, intercellular communication, and the activation of the host's immune response **(Figure 1-2)** (27). The outer layer consists of highly branched AG, forming the arabinan chain, with the nonreducing end of the glycan chain linked to the Mycolic acid (MA) of the outer layer which restricts fluid permeability into the cells (26). Indeed, the highly lipid-rich outer layer of Mtb is essential for its survival and infectivity. This lipid-rich layer, including components like MA and TDM, not only provides structural integrity to the bacterium but also serves as a formidable defence mechanism against the host immune response. It plays a crucial role in Mtb's ability to persist within the host and evade immune detection, contributing significantly to its pathogenicity. Together these components act as protective barriers, making Mtb resilient and adaptable in the host environment, ultimately facilitating its survival and establishment of infection (28).

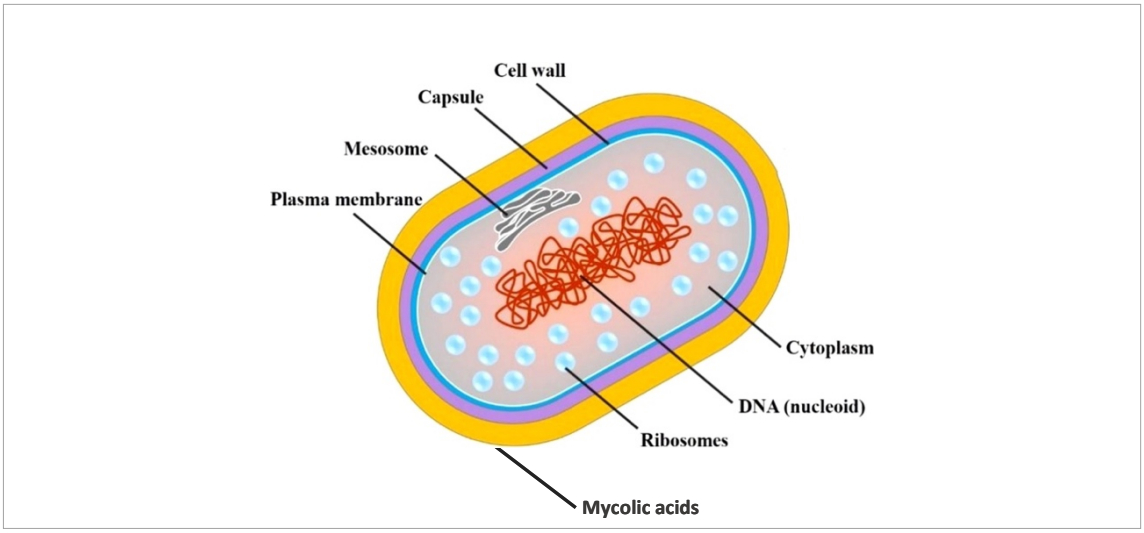


Figure 1-2 The structure of the Mtb organism (adapted from Wang et.al 2022)

1.4 Transmission and Pathogenesis of TB

TB is an airborne disease which is primarily transmitted via inhalation of infected droplet nuclei released by patients with active TB (29). These infectious droplets, about 1– 5 microns in diameter, are often produced when individuals with pulmonary or laryngeal TB disease cough, sneeze, shout, or sing. Depending on

the surrounding conditions, these minuscule particles can stay suspended in the air for several hours (29,30). Currently, there is limited knowledge on the attributes of the transmissible Mtb, particularly whether all the Mtb bacilli within an infected individual are equally capable of causing the disease as well as the bacterial load required to establish the infection. In addition, it remains unclear how the pathogen adapts to different lung microenvironments during its pathogenesis (31). However, it is believed that smaller droplets are more likely to be effective since they are transmitted over a long distance, unlike larger droplets which are often pulled down by gravity (31).

Following inhalation of the infected aerosol, Mtb traverses through several intrinsic host defence mechanisms in the upper respiratory tract such as mucociliary epithelial cells and secretion of mucin which aims at the clearance of the inhaled particulates (32). Successful Mtb migrate to the lower airway where they are deposited in the lung, the primary site of infection. Therefore, lung immunity is crucial in protecting against the development of the disease. The initial interaction between bacilli and immune cells in the lung plays a key role in influencing the outcome of the infection. Thus, determining whether the infection leads to bacterial elimination, containment resulting in asymptomatic infection, or uncontrolled replication leading to active disease. Understanding the intricate interplay between the host immune response and bacterial pathogenic strategies is fundamental in providing insights into disease pathogenesis as well as mechanisms of immune evasion explored by Mtb which will enhance the development of preventive and therapeutic strategies against the pathogen.

1.5 Clinical manifestation of TB

Mtb infection displays a diverse array of clinical outcomes depending on the interactions between the bacilli and the host immune response (33). These range from asymptomatic subclinical infection, known as latent infection (LTBI), to active disease (ATB). Following exposure to TB, over 90% of individuals remain in a latency state characterised by positive Tuberculin skin tests (TST) and Interferon-gamma release assays (IGRA). Of these individuals, only less than 10% develop active disease **(Figure 1-3)** (31). Additionally, in most cases of latent infection, individuals maintain a continuous immune response to Mtb-specific antigens but do not exhibit any signs of illness, essentially remaining in a healthy

state whilst harbouring the infection. Although the risk of reactivation is estimated at 10% at the population level, it remains unclear why some individuals clear the infection whilst others progress to develop active disease. Impaired immunity, for example in uncontrolled HIV, among others, has been shown to accelerate the development and the clinical manifestation of TB (32).

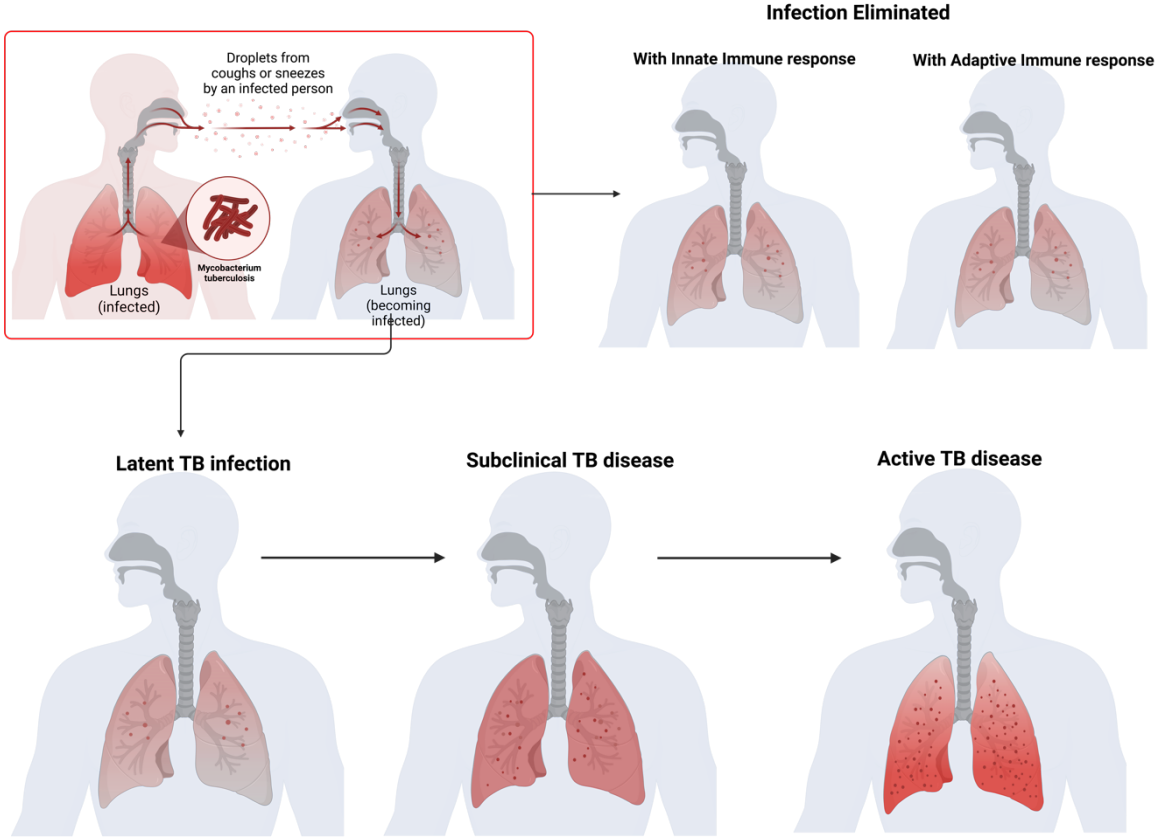


Figure 1-3 The clinical outcomes following Mtb exposure (adapted from *M. Pai et al.2016*)

Infection with Mtb results in 3 clinical outcomes: clearance of the infection (mediated by both the innate and adaptive immune response), latency and progression to active diseases (the increasing red colour in the lung indicates the severity of the TB infection) (Created with BioRender.com).

The clinical spectrum of TB encompasses various forms, each characterised by distinct pathological and symptomatic features. In high TB endemic areas, pulmonary TB is the most common manifestation and accounts for over 60-80% of TB cases in adults (34). Pulmonary TB is often characterised by symptoms such as persistent cough, sputum production, loss of appetite, weight loss, fever, night sweats, and coughing up blood (35). In addition to these widely recognised clinical symptoms, TB has been associated with various secondary systemic complications. These include high oxidative stress, hyponatremia, reduced levels of cholesterol, impaired glucose tolerance, haematological abnormalities, deficiency of vitamin D and alterations in the host's microbiota composition (33).

Diagnosis of TB involves various methods, such as identifying the presence of the infection directly in sputum through smear or culture, utilising nucleic acid amplification techniques like the Gene-Xpert, and detecting signs of TB in chest X-rays. In countries with high TB prevalence, where TB tests are negative, diagnosing the disease often relies on the manifestation of typical symptoms (24). Together, these diagnostic approaches enhance the prompt initiation of effective TB treatment regimens.

Treatment of an active TB infection involves a rigorous antimicrobial regimen, typically combining four medications administered over a six-month period. The standard treatment regimen for drug-susceptible TB comprises an initial intensive phase of rifampicin, isoniazid, Ethambutol, and pyrazinamide (first 2 months), followed by a continuation phase of isoniazid and rifampicin for the remaining 4 months. Ethambutol is added to the treatment regimen as a protective measure to mitigate the risk of unknown resistance to one of the three main drugs (36,37). These drugs are usually supplied in fixed-dose combinations (FDCs), simplifying the prescription, and reducing the likelihood of drug resistance due to episodes of single-drug therapy when the drugs are administered separately (38).

1.6 The host immune response to TB

1.6.1 Mucosal immunity in the airways

The human respiratory tract, encompassing an area of 50-100 square meters, is the second largest mucosal surface area exposed to the external environment, after the skin (39). Given its fundamental function in facilitating gaseous exchange, with approximately 10,000 litres of air passing through them daily, the lungs experience continuous exposure to a diverse spectrum of challenges such as bacteria, viruses and particulate matter (40,41), thus making them a common site of respiratory infections and injury. The host immune responses therefore need to be robust and efficient in the clearance of respiratory pathogens including TB. The maintenance of airway mucosa integrity depends highly on a well-regulated host defence machinery involving both the innate and the adaptive immune response (42). A compromise in the early defence mechanism or evasion of defences by pathogens leads to respiratory infection.

The airway epithelium plays an integral role in lung innate immunity, forming an interface between the internal and the external environment. It is responsible for the binding and transportation of antibodies, bacterial sensing and signal transduction to recruit innate and inflammatory immune cells to the site of inflammation (39). The inner lining of the airway is protected by stratified layers of interconnected epithelial cells, uniquely positioned to provide basal local immunity (43). These epithelial cells can be categorised into bronchial and alveolar cells, each with distinct roles. The lining of the larger airway is mainly composed of bronchial epithelial cells and goblet cells which are crucial in managing ion exchange, regulating gas exchange, producing mucin, orchestrating inflammation, and serving as a physical barrier through tight junctions and desmosomes (44,45). On the other hand, alveolar epithelial cells consist of type I and type II cells. Type I cells facilitate gaseous exchange and detect invading pathogens. Type II cells, also known as type II pneumocytes, contribute to lower airway defence by sensing pathogens through toll-like receptors and secreting antimicrobial substances such as surfactants, lysozymes, lactoferrins, cytokines, and chemokines (45). Furthermore, the antimicrobial and proinflammatory factors produced by the AECs are vital in stimulating other cells of innate immunity. Collectively, this allows a coordinated immune response between the epithelial cells and lung immune cells in response to mycobacteria infection.

The effective establishment of Mtb infection significantly hinges upon its initial interactions with various innate immune cells within the host, including alveolar epithelial cells (AEC), macrophages, dendritic cells (DCs), neutrophils, and natural killer cells **(Section 1.6.2).** During the early phase of the infection in the lung, the bacteria first encounter the airway epithelial cells (AECs) which line the epithelial surface of the alveoli and act as physical barriers. For decades, lung epithelial cells' role has been primarily thought of as physical barriers to preventing the invasion of respiratory pathogens and substances. However, despite being atypical immune cells, these cells have been shown to exhibit several immunological functions within the lung through various mechanisms (46). For instance, the pattern recognition receptors (PRRs), such as epithelial expression of Toll-like receptors (TLRs) and surfactant proteins, play an important role in the recognition and binding of the pathogen and initiating the immune response (47). These TLRs facilitate the interaction between the host and pathogens by detecting pathogen-associated molecular patterns (PAMPs) and initiating signalling before the recruitment of immune cells to the airway.

To date, several TLRs have been identified and are involved in the immune response of AEC against Mtb infection with the expression of TLR-1, TLR-2, TLR-4, TLR-5, TLR-6, and TLR10 associated with the recognition of lipoproteins and bacterial polysaccharides (46). Of these, TLRs such as TLR-2, TLR-4 and TLR-6 are key in initiating the immune response against Mtb. Upon Mtb infection, TLR-2 is activated by the binding of the pathogen's 19 kDa lipoprotein and lipoarabinomannan, leading to downstream signal transduction to produce cytokines, among others (48). TLR-2-dependent activation in AEC is key in the production of proinflammatory cytokines and clearance of TB infection in murine studies. In one study, mice lacking TLR-2 challenged with live Mtb exhibited an increased susceptibility to the infection characterised by high bacterial load, defective granulomas as well as chronic pneumonia. Although, these TLR-2 deficient mice had elevated levels of IFN-γ, TNF-α, and IL-12p40, this alone could not protect them from Mtb infection, indicating the essential role of TLR-2 in the inflammation process (45). Just like TLR-2, TLR4 plays a critical role in the host immune response to Mtb infection in the lung. TLR4 is activated by lipopolysaccharides (LPS) which trigger a cascade of intracellular signalling events

that lead to the production of inflammatory cytokines and the activation of immune cells (48).

The hallmark of Mtb infection relies on the delicate balance between protective immunity as well as tissue damage following an early immune-induced inflammation (49,50). Although the innate immune response provides effective protection for certain individuals, eventually preventing them from becoming infected, this is, however, insufficient in other cases, leading to the initiation of a more targeted immune response (51). Currently, little is known about what influences the initial stages of interactions between the host and pathogen in the human lung, especially regarding the interplay between Mtb and innate immune cells. Moreover, the precise stages of the Mtb infection at which innate immunity contributes to either protective or pathological responses remain to be established (52,53).

1.6.2 Innate immune responses to TB

1.6.2.1 Alveolar macrophages

Human alveolar macrophages (AMs) are the main sentinel cells of the lung, providing the first line of host defence following an early encounter with Mtb (54). These cells comprise the predominant population, exceeding 90% of phagocytic and antigen-presenting cells within the airway, thus making them the natural gatekeepers of the lung. They are also engaged in active surveillance of the lung microenvironment, facilitating the removal of detritus and microbial agents, including Mtb (55). Metabolically, AMs display elevated basal glucose consumption and respiratory rates while exhibiting low respiratory burst activity (56). They are also equipped with weaponry that functions collaboratively to identify and neutralise infiltrating Mtb (57). For instance, AMs sense and respond to their environment via receptors, such as scavenger receptors, mannose receptors and complement receptors, which together aid in recognition and phagocytosis (58). Like many other bacteria, Mtb phagocytosis can be initiated through complement-mediated factors that opsonise bacteria or act in a non-opsonic manner. Indeed, *in vitro* studies have demonstrated that Mtb activates the alternative complement system, leading to binding via complement receptor 3 (CR3), which results in the phagocytosis of the complement-opsonised Mtb (59,60). Nonetheless, non-

opsonised bacterial uptake is crucial in primary TB infection due to the absence of complement factors in the alveolar space.

Like AECs, AMs additionally express a variety of PRRs, including scavenger receptors and TLRs, which play roles in innate immunity signalling and phagocytosis as part of their responsive functions. Stimulation of PRRs within macrophages triggers the activation of antimicrobial defence mechanisms. For instance, TLR2 recognises mycobacterial mannosylated lipoarabinomannan, and the interaction between this receptor and its ligand leads to the activation of NF-κB and the transcription of the inducible nitric oxide synthase (iNOS) gene (61). The expression of iNOS has been associated with the production of nitric oxide (NO) in human macrophages, and a significant increase in macrophage NOS2 mRNA has been reported in the BAL fluid of patients with clinical symptoms of TB compared to healthy controls (62,63). Nevertheless, unlike the findings in mice, where NO provided protection against Mtb (64,65), NO exhibits restricted bactericidal or bacteriostatic effects against Mtb during *in vitro* infection of human alveolar macrophages and primary monocytes following treatment with IFN-γ (66). This suggests that in the presence of IFN-γ, Mtb utilises NO to establish its niche and promote survival within the alveolar macrophages in the lung microenvironment in vivo.

The dynamic interplay between Mtb and AMs is characterised by a high degree of complexity, and many underlying mechanisms remain poorly understood. Nonetheless, it is widely acknowledged that this intricate interaction critically shapes the subsequent course of the infection, ultimately dictating the outcome in terms of infection resolution or disease progression (57,67). Although macrophages play a pivotal role as the first defence mechanism against Mtb infection, the early interactions between macrophages and Mtb tend to favour the pathogen. Consequently, macrophages serve as a primary cellular niche for bacterial replication during the early stages of infection, and they function as reservoirs of persistent bacteria within the latent phase of the infection (57).

1.6.2.2 Alveolar Neutrophils

Neutrophils are the predominant type of immune cells circulating in the systemic circulation, actively patrolling for potential injuries and triggers such as pathogen and damage-associated molecular pattern molecules (PAMP and DAMP), chemokines, cytokines, and lipid mediators (56). They are among the initial innate immune cells to be attracted to the site of infection in a chemotactic process that is coordinated by CXCL1 and CXCL2 (68–70). Neutrophils are quickly trapped in the pulmonary microvasculature and migrate into the lung parenchyma and alveolar spaces, where they carry out various effector functions. However, their effector activities can have adverse effects during acute and chronic inflammation, potentially leading to lung tissue damage (71). To date, the role of neutrophils in Mtb infection has been controversial; while they are considered protective in the early stages of infection, neutrophils can be detrimental in the advanced stage of the disease. For example, it is known that an increase in the neutrophil count in chronic TB infection is associated with lung damage and subsequent progression to severe disease (72,73). Therefore, it is crucial to achieve a delicate balance between recruiting neutrophils and preserving mucosal integrity (74,75). Increasing evidence suggests that neutrophils exhibit phenotypic and functional diversity, demonstrating plasticity and heterogeneity across various disease states, encompassing proinflammatory and anti-inflammatory subsets (74).

Neutrophils possess a diverse array of antimicrobial functions that allow them to clear pathogens effectively. These functions include phagocytosis, antibody-dependent cellular cytotoxicity (ADCC), production of cytokines, degranulation as well as production of reactive oxygen species (ROS) **(Figure 1-4)** (76,77). Alveolar Neutrophils are essential phagocytes in the airway although they are not abundant during homeostasis (78). However, during active TB disease, neutrophils constitute the predominant cell population found in both BAL and sputum among patients diagnosed with active TB (56). During Mtb infection, neutrophils are actively recruited to the site of infection; they engulf the bacteria and release antimicrobial agents from their granules and constitute a potent population of effector cells capable of mediating both anti-Mtb immune response as well as immunopathology (54). This is ascribed to the discharge of cytolytic enzymes such as elastase, collagenase, and myeloperoxidase during the neutrophil's respiratory

burst, causing indiscriminate lysis of both bacterial and host cells (79). Specifically, neutrophils generate superoxide (O2-) through the oxygen-dependent bactericidal pathway using NADPH oxidase. Superoxide and downstream reactive oxygen species (ROS) are subsequently released and directed onto bacteria. Consequently, the ROS degrade the bacteria's DNA, RNA, and proteins (79,80). In a study, Martineau *et al.*, reported an inverse association between the onset of pulmonary TB and the number of neutrophils in the systemic circulation of individuals in contact with patients with active TB (73). However, *in* vitro depletion of these neutrophils from whole blood resulted in insufficient activation of antimicrobial peptides (AMPs) and an inability to control the growth of BCG and Mtb (73) suggesting the potential role of neutrophils in anti-TB immune response. Furthermore, following activation, neutrophils can release their chromatin and histone proteins forming neutrophil extracellular traps (NETs) in a process called NETosis **(Figure 1-4)** (81,82). While NETs have been shown to exert direct microbicidal effects against several pathogens (83,84), recent evidence indicates that these web-like structures only trap the Mtb without killing it, potentially due to the complexity of the its cell wall (85). Indeed, the presence of NETs aids in containing the infection. However, their dysregulation may harm the host by harbouring the Mtb, obstructing immune cell recruitment, and impairing the clearance of infection, while also contributing to the spread of the infection (82). In addition, NETs contain toxic molecules capable of damaging healthy cells and escalating chronic inflammation causing tissue damage (86). Thus, excessive formation of NETs in TB can lead to lung damage and exacerbate the inflammatory response.

1.6.2.3 Dendritic cells

Dendritic cells (DCs) are a type of mononuclear phagocytic cells located in various tissues including the lymphoid organs. They can be found in either an immature or mature state. The immature form is located in peripheral tissues and is highly efficient in endocytosis, allowing it to detect pathogens or damaged host cells. Nevertheless, this form is not efficient in producing peptide-major histocompatibility (MHC) complexes (87). Therefore, to initiate an effective immune response, these immature DCs need to be activated by pathogens leading to their transformation into mature DCs (88). Following their maturation, DCs

migrate to the secondary lymphoid organs where they engage with T cells (89). The mature DCs are characterised by a greater expression of MHC molecules which enable them to produce peptide MHC complexes. Notably, they are efficient at presenting antigens, as they are professional antigen-presenting cells, thus they capture, process, and present antigens on either MHC class I or MHC class II molecules to prime the T cells (90). Upon infection, DCs constitute one of the first cell types to encounter Mtb. They possess a weaponry of receptors expressed on their plasma membrane such as the TLR and intracellular receptors (NOD-like receptors) that are used to detect Mtb pathogen-associated molecular patterns (PAMPs) (87). Thus, DCs' ability to recognise and process antigens is an essential link between innate and adaptive immunity. The role of DCs in the immune response to TB remains a subject of controversy. Significant gaps exist in the current evidence to determine whether these cells enhance cellular immunity or whether their manipulation by Mtb to become a niche can be utilised to reduce specific T cell responses (89).

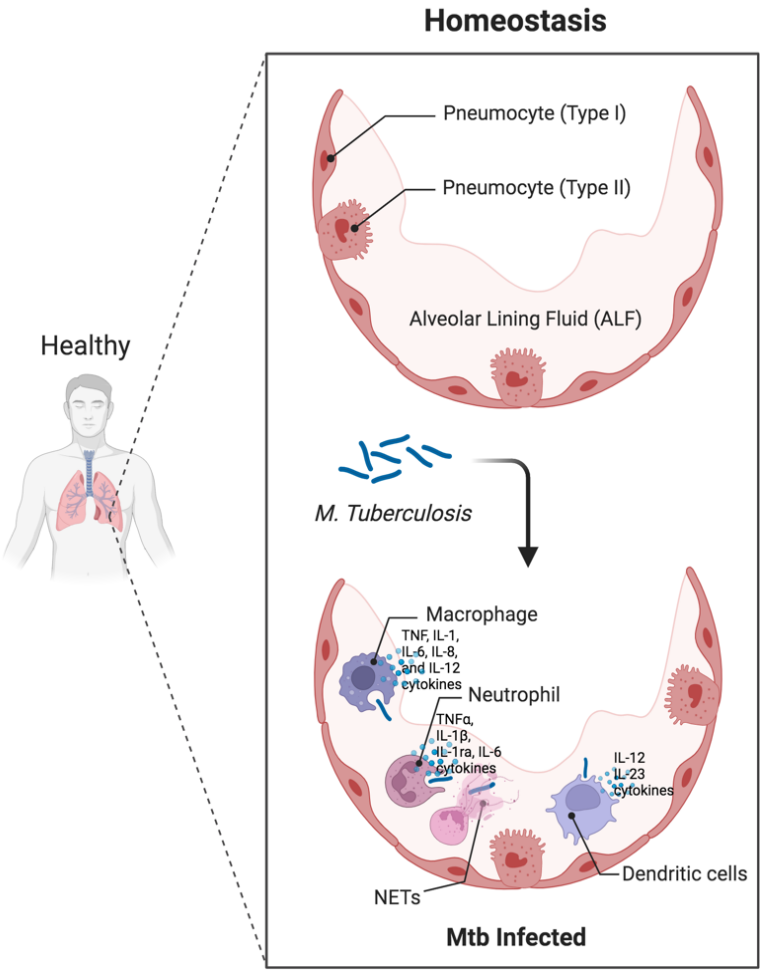


Figure 1-4 The summary of innate immune response to Mtb infection

Overview of the innate immune response to TB infection through alveolar epithelial cell interaction with other innate immune cells such as neutrophils, macrophages and dendritic cells. (Created with BioRender.com)

1.6.3 Adaptive Immunity to TB: Ontogeny, differentiation, and migration of T cells

Adaptive immunity is characterised by its specificity to antigens and the establishment of immunological memory, which allows for a faster and more robust response upon re-exposure to the same pathogen. B and T cells are key components of the adaptive immune system (91,92). These cells are crucial in initiating and sustaining immune responses, coordinating various aspects such as responses to vaccines, allergens, and infections. Both types of lymphocytes can recognise numerous peptides and respond to them using their specific receptors thus giving the adaptive immune system the unique ability to initiate a response against specific antigens. This section will focus on T cells and discuss T cell development, differentiation, and maturation. In addition, I will also cover aspects of the generation of the T cell receptor (TCR), TCR diversity, and the factors that influence this diversity, affecting the level of protection against various infections including TB.

1.6.3.1 T cell development

T cells originate from a common lymphoid progenitor cell derived from the hematopoietic stem cells located in the bone marrow. These precursor cells migrate to the thymus where they undergo differentiation and maturation processes to produce functional self-restricted MHC T cells (93). The thymus is a complex organ with numerous lobes, consisting of the cortex and the medullary region, enclosed in a capsule, which supports the differentiation and selection of T cells (94,95). Lymphoid progenitor cells begin to colonise the cortical area of the thymus at an early stage of embryonic development in mice and during the eighth week of foetal development in humans (96,97). This process involves two distinct pathways: a vasculature-independent pathway that occurs before thymic vascularisation and a vasculature-dependent pathway that occurs after birth. Vasculature-independent colonisation is regulated by the chemotactic attraction

of lymphoid progenitor cells to the thymic primordium in which chemokines such as CCL21 and CCL25 play a critical role (94,98,99). Murine studies with mice that lacked CCL21 or CCR7 (a chemokine receptor for CCL21) showed a reduced proportion of thymocytes compared to wild mice. Similarly, mutant mice deficient in CCR9 (a receptor for CCL25) exhibited a significant decrease in the population of developing thymocytes during embryogenesis (98,99). Collectively, these results suggest a potential role of these chemokines in thymic colonisation. On the other hand, thymic entry of lymphoid progenitor through the vasculature-dependent postnatal pathway is thought to be mediated by a strong interaction between platelet P-selectin and P-selectin glycoprotein ligand 1, expressed on the endothelium of the thymus and lymphoid precursor cells respectively (100,101). In the thymus, the T cell progenitor cells first enter the subcapsular region of the cortex, where they interact with thymic stromal cells, also known as cortical epithelial cells. The cross-communication between the developing thymocytes provides an optimal microenvironment to guide thymocyte movement within and out of the thymus. Particularly, the T cell progenitors interact with numerous ligands for Notch receptors, including growth factors such as Kit-ligand and IL-7 (102). These factors play a crucial role in initiating and supporting the differentiation and proliferation of these cells during the initial stages of T cell development. Additionally, the expression of Notch-1 receptors and their interaction with Delta-like ligands have been shown to suppress the development of non-T cell lineages (103). Within the thymus, developing thymocytes progress through multiple distinct stages marked by changes in their T cell receptor (TCR) genes, TCR expression, and cell surface molecule expression.

The majority (~95%) of the cells generated express a TCR heterodimer comprising an alpha and beta chain, commonly known as the αß T cells. However, only about 5% of the total produced thymocytes bear a gamma-delta TCR denoted as γδ T cell, also known as unconventional T cells (93). The TCRs engage with the cluster of differentiation 3 (CD3) on the cell surface, leading to the production of a functional TCR complex (93,104). The precursor cells that do not express CD4 or CD8 receptors are called double negative (DN) cells. These DN cells are further categorised into four differentiation stages depending on the surface expression of CD44 and CD25, namely DN1 (CD44+ CD25-), DN2 (CD44+, CD25+), DN3 (CD44-,

CD25+), and DN4 (CD44- CD25-) (105,106). CD44 is an adhesion molecule, and CD25 is an interleukin-2 receptor alpha (α) chain. DN3 cells undergo a process called beta-selection, which selects cells that have successfully rearranged their TCR-ß chain locus. The ß chain pairs with a pre-Tα surrogate to generate a pre-TCR, forming a complex with a CD3 molecule (95). The pre-Tα chain consists of a single immunoglobulin-like domain that is structurally distinct from the constant (C) domain of the TCR α-chain. This complex process triggers the proliferation and differentiation of the ß chain loci, causing an increase in CD4 and CD8 expression on the cell surface. This leads to the development of CD4+ and CD8+ double-positive (DP) cells. Subsequently, these DP cells rearrange their TCR-α chain loci to generate αß TCR and undergo maturation into either CD4 or CD8 single-positive T cells (95,105) (**Figure 1-5).**

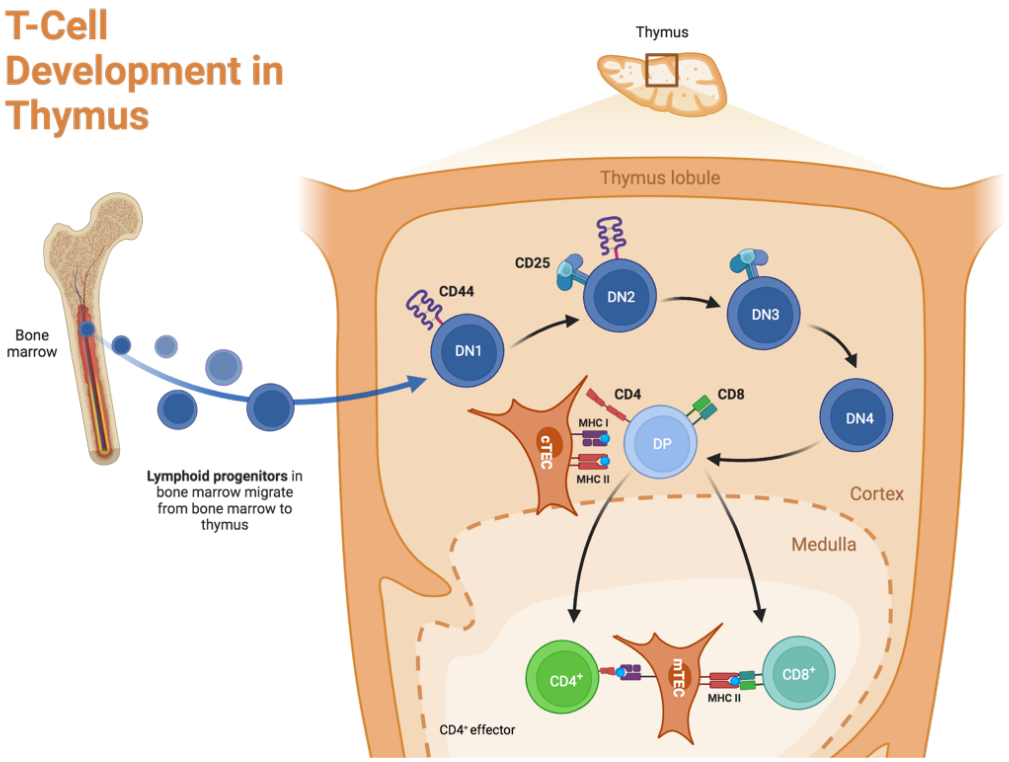


Figure 1-5 T cell development and maturation

The common lymphoid progenitor cell migrates from the bone marrow to the thymus. This movement is facilitated by the cytokines (thymosin, thymotoxin, thymopoetin and thymic factors) through chemotaxis. The earliest developing thymocytes lack the expression of the co-receptors CD4 and CD8 and are called double negative (DN) cells. The DN population can be further subdivided by the expression of CD44 (an adhesion molecule) and CD25 (Interleukin-2 receptor α chain). Cells that lack expression of CD44, but express CD25 (DN3), undergo a process called beta-selection with a consequent production of the TCR-ß chain. The ß chain then pairs with the surrogate chain, pre-Tα, and produces a pre-TCR, which forms a complex with CD3 molecules. This complex leads to survival, proliferation, arrest in further ß chain loci rearrangement, and further differentiation by up-regulation and expression of CD4 and CD8. These cells are termed double-positive (DP) cells. DP cells undergo rigorous positive and negative selection processes to become mature functional αß TCR CD4+ or CD8+ T cells. Cells that do not undergo beta-selection die by apoptosis. (Created with BioRender)

1.6.3.2 Positive and Negative Selection

Following the generation of a full TCR, the DP thymocytes undergo a positive and negative selection to produce functional self-restricted T cells. The fate of the developing thymocytes to be selected depends on their ability to interact with major histocompatibility complex (MHC)-self-peptide complexes presented on the various thymic epithelial cells that are uniquely positioned within the thymic microenvironment (95). In the positive selection, DP cells first encounter thymic cortical epithelial cells (cTECs) that express both MHC-I and MHC-II molecules, also called human leukocyte antigen (HLA) (107). Only thymocytes that interact with a low affinity to self-peptide-MHC complexes receive survival signals and are positively selected to progress in their development, proliferating and upregulating the expression of the TCR (93,108). Furthermore, these cells start to upregulate one co-receptor while downregulating the expression of the other. For instance, cells that encounter and recognise peptides bound to MHC-II increase the expression of CD4 whilst down-regulating the CD8 expression. This process is facilitated by lysosomal proteases cathepsin L (also known as cathepsin L1) and thymus-specific serine protease (TSSP) expressed by cTECs (109). Conversely, CD8 expression persists in thymocytes interacting with peptides presented via the MHC-I complex. Contrary to the CD4 T cell, this process is thought to be mediated by a proteasome catalytic subunit called β5t produced by the cTECs—together known as thymoproteasomes. These proteins are essential in the selection and commitment of the CD8 T cells as mice deficient in thymoproteasomes exhibited a reduced CD8 selection potential (110). DP thymocytes that did not receive survival signals or interact strongly with the MHCs presented by the cTECs are removed through apoptosis (108). Overall, positive selection allows the generation of mature MHC-restricted CD4 and CD8 T cells.

After positive selection, the newly produced single positive CD4 and CD8 T cells migrate towards the thymic medulla where they undergo negative selection. This process ensures the removal of cells that recognise and react strongly with self-peptides. In the medulla, thymocytes are challenged with self-antigens by the medullary thymic epithelial cells (mTECs) (108) **(Figure 1-5).** These epithelial cells express a unique protein known as autoimmune regulator (AIRE), which allows expression of self-peptides from various non-thymic tissues and presents them to

the thymocytes (111). Cells that bind with high affinity to the self-peptides presented by mTECs and other thymic APCs receive apoptotic signals, leading to their elimination. Thus, only thymocyte precursors that survive the rigorous checkpoints of T cell development with subsequent maturation into naïve CD4 and CD8 single-positive T cells exit the thymus and migrate to the periphery where they perform various effector functions. Altogether, negative selection forms a key mechanism of central tolerance which limits the generation of autoreactive T cells, which is the genesis of autoimmune disorders.

Apart from the production of T helper and cytotoxic T cells, the thymus gives rise to regulatory T cells (Tregs) under the same selection conditions. Although Tregs can also be generated in the periphery known as induced Tregs, distinguishing the two remains a hurdle due to the lack of differential markers of discrimination (112). However, previous studies indicate that the majority of regulatory T cells expressing the forkhead box P3 (FoxP3) transcription factor within the thymus are required for immune regulation and prevention of autoimmunity (112).

1.6.4 The T cell receptor (TCR) and TCR diversity

TCR is a transmembrane protein located on the surface of CD4 and CD8 T cells which enables them to recognise a vast array of antigens bound to MHC complexes (113). TCR stimulation leads to a cascade of signalling that consequently results in T cell activation and acquisition of effector functions. Generating a wide range of T cells with a diverse T cell repertoire is critical in protecting the host from the variety of potential pathogens. **Sections 1.6.4.1** to **section 1.6.4.3** discuss the TCR structure and development of a functional TCR.

1.6.4.1 Composition and structure of TCR complex

The TCR is a highly diverse heterodimer composed of the alpha (α) and beta (ß) chains that are linked together by a disulphide bond. T cells expressing this receptor are also known as conventional and are denoted as αß cells which constitute over 95% of the human T cells. A small proportion of TCRs is made up of gamma (γ) and a delta (δ) chain called γδ T cells (114). Although they exhibit

a limited TCR repertoire diversity, γδ T cells recognise a plethora of pathogens due to their exceptional TCR structure and their ability to recognise both protein and non-proteinaceous molecules through MHC-independent mechanisms (115,116). Structurally, the extracellular region of the TCRs contains the variable (V) region, responsible for antigen recognition and the constant(C) region. The variable region of the α chain is composed of variable (V) and joining (J) gene segments with additional diversity (D) segments present within the TCRβ chain (117). On the other hand, the constant region, as the name suggests, remains consistent across different TCRs and is determined by a single C-segment gene for the α chain and two for the β chain (118,119). These TCR gene segments are found on chromosomes 7 and 14. Specifically, the TCRα chain genes are located within the TCRα loci on chromosome 14q11–1 2, while the TCRα comprises 43 Vα and 58 Jα genes, which are positioned on chromosome 7q32–35 (120)(118). The V region of the TCR contains a variety of gene segments (the human TCRβ locus has 42Vβ,2 Dβ and 12Jβ) which are randomly selected to generate a functional variable region in a process known as V(D)J recombination. Together, the recombination of a variable region with a constant gene leads to the formation of a functional TCR transcript that is translated to a full TCR protein.

V(D)J recombination process is a site-specific gene combination within the TCR gene segments flanked by conserved recombination signal sequences (RSS). These conserved signals are identified by recombination activating genes 1 and 2 (RAG1 and RAG2) and non-homologous end joining (NHEJ) proteins to initiate the commencement of the recombination process. RSS are AT-rich palindromic sequences that are separated by non-conserved sequences of either 12 (12-RS) or 23 (23-RS) base pairs (bp) (121). The process of VDJ recombination follows the 12/23 rule, in which recombination only occurs in the RSS segments that have 12bp sequences followed by 23bp sequences (121,122). This ensures that gene rearrangement occurs in an ordered fashion. The RSS are strategically aligned during recombination to uphold an in-frame coding region. This alignment of RSS among various gene segments results in the formation of a hairpin-like structure in the chromosome, which is excised during recombination along with the RSS (122). Subsequently, the variable region undergoes imprecise joining, involving the addition or removal of nucleotides by the terminal deoxynucleotidyl

transferase (TdT) enzyme or exonucleases, respectively. The variable region is then joined by DNA ligase, leaving an exon encoding for the variable region. The newly formed DNA within the V region undergo transcription and splicing to produce a mature mRNA molecule that is translated into a complete α or ß TCR chain protein unique to a particular T cell **(Figure 1-6)** (121–123).

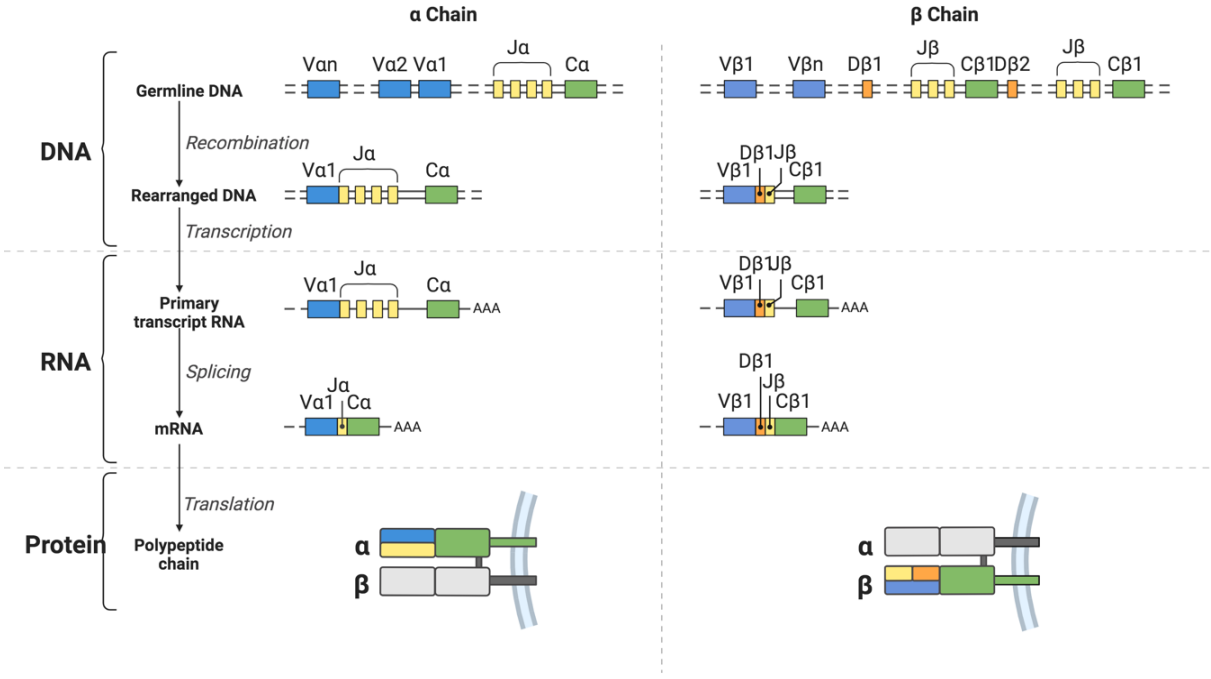


Figure 1-6 Generation of a functional heterodimer αß TCR

During T cell development, gene loci for alpha and beta chains undergo a process of V(D) J recombination in which V-J or V(D) J gene segments rearrange to form a TCR DNA sequence. As a result of random molecular imprecision, nucleotide base pairs are deleted or added within the coding region. Upon successful rearrangement, the mRNA transcript undergoes splicing to remove the genes coding constant (C) region. However, the remaining genes are translated into a complete αß TCR. (Created with BioRender.com, adapted From. Rosati *et al*.2017)

Mature TCRαß chains are made up of three hypervariable regions known as complementarity-determining regions (CDR1-3), responsible for TCR-peptide-MHC interaction (124) CDR1 and CDR 2 are encoded by various V gene segments (42Vα and 46Vß) and are necessary for the TCR's interaction with the MHC complex. CDR3, conversely, is generated following junctional gene recombination between the V and J for TCRα or D and J for TCRß, making it highly variable **(Figure 1-6)**. The CDR3 is key in TCR interaction with the peptide-MHC complex, as it is the region of the TCR that directly contacts the antigen-peptide-MHC complex (125,126), thus CDR3 is important for defining antigen specificity. Assessment of the variability within the CDR3 region is often used to measure the diversity and clonality of T cells within an antigen-selected T cell repertoire.

TCRαß and CD3 proteins together form the TCR complex which is essential for downstream signal transduction for the initiation of T cell responses (127). There are four CD3 proteins namely Delta (δ), epsilon(ε), gamma (γ), and zeta (ζ) chains which are connected to the TCR chains through hydrophobic bonds (128).In contrast to TCRα and ß chains, CD3 proteins do not play a direct role in antigen recognition **(Figure 1-7).** However, their cytosolic domains contain immunoreceptor tyrosine-based activation motifs (ITAMs) responsible for transmitting the TCR signal (128,129).

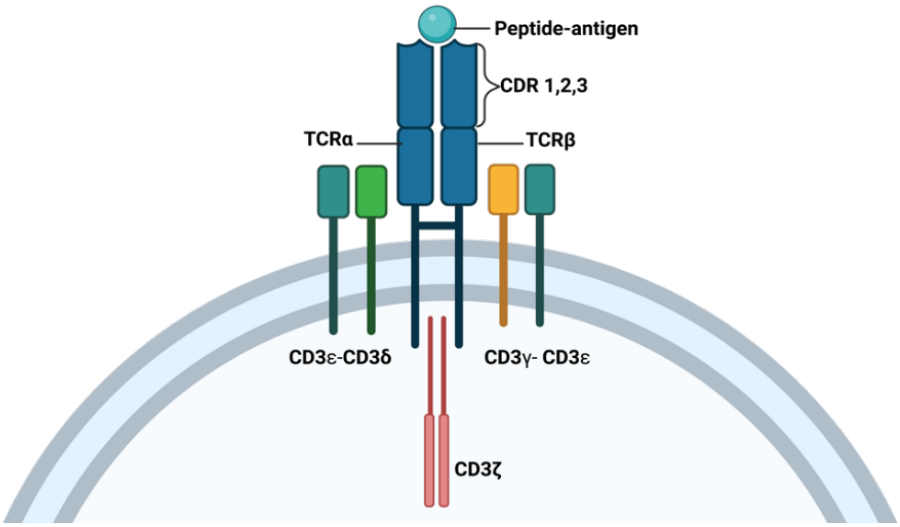


Figure 1-7 The schematic structure of a T cell receptor complex with the TCRα and TCRβ, CD3 and its accessory molecules.

A TCR- complex consists of a functional αß TCR, CDRs and CD3 molecules that work in tandem to initiate a T cell response. The TCR interacts with the peptide MHC complex through hypervariable complementarity determining regions (CDRs), which are encoded within the V gene segments. While CDR1 and 2 originate from different V gene loci, CDR3 is formed through the joining of the VD or VJ genes at the junction region of the germline segments following somatic recombination, thereby increasing the antigen specificity. The αß TCR is anchored by a CD3 molecule with various proteins namely δ, ε, γ and ζ crucial for signal transduction (Created with BioRender.com).

1.6.4.2 Generation of TCR repertoire diversity

Generation of TCR repertoire diversity is crucial in the development of a functional immune system. The diversification allows T cells to recognise and respond to a plethora of pathogens. A diverse functional TCR repertoire is a consequence of three mechanisms (130). Firstly, through a combinatorial diversity in which there is random recombination of various VDJ genes with a constant gene segment. Additional diversity is introduced through imprecise somatic recombination, along with the addition or deletion of non-coding nucleotide bases at the VDJ junction, also known as junctional diversity (128). Lastly, a wide range of unique TCR complexes is created through the pairing of different TCRα and ß chain (117). Together, these processes enable the production of a highly variable TCR repertoire, which ensures the identification of a vast array of pathogens.

The process of the TCR gene rearrangement is thought to produce a theoretical TCR diversity of over 1×10^{15} TCRαß receptors. However, in humans, this is significantly reduced during positive and negative selection in the thymus (131). Thus, ideally, TCR diversity has the potential to generate an extensive TCR repertoire pool capable of recognising virtually any available antigen (131). In reality, studies have demonstrated a limited TCR diversity of naïve T cells which is influenced by the actual number of T cells present in a particular organism. For instance, in humans, TCR-αß diversity is approximately $2x10^7$, which tends to reduce in the memory T cell subset (132). With this massive generation of TCR sequences, the likelihood of genetically identical organisms sharing the same repertoire is relatively low, given the availability of numerous unique sequences. In one study, analysis of naïve T cells from individual mice from the same strain revealed a distinct TCR repertoire with only <30% shared TCR sequence (133). In

the same vein, genetically identical mice exhibited different immune responses following infection with lymphocytic choriomeningitis virus (LCMV) (134). Collectively, this shows that a broad diverse TCR repertoire allows an organism to respond to the target pathogen and reduces the probability of immune evasion by mutated circulating pathogens.

1.6.4.3 Factors influencing TCR diversity

Several factors contribute to TCR diversity and the ultimate immune response. These include thymic involution, antigen exposure, genetic diversity, and environmental determinants. As discussed in the previous sections, the efficiency of the thymic function is key in the generation of T cells with a variable immune repertoire. Nevertheless, the sensitivity of the thymus to pathological and inflammatory insults reduces its T cell output, which results in limited TCR diversity and impaired immune responses (135). Thymic involution caused by age is associated with reduced T cell responses and an overall decreased T cell diversity, which probably explains why the elderly are prone to infections (136). Indeed several studies have reported that this age group has a high risk of developing complications following a pandemic such as COVID-19 or severe acute respiratory syndrome (137,138). Together, this suggests an age-related impairment of T cell immunity characterised by a limited TCR diversity.

Antigen exposure and T cell activation also play a role in shaping the diversity of the naïve T cell repertoire. The presentation and activation of antigens influence the repertoires of effector and memory T cells. Upon encountering their specific antigen, T cells undergo clonal expansion and proliferation, resulting in the preferential growth of certain T cell clones over others. This process skews the overall repertoire to mirror the encountered antigen (139). A limited TCR repertoire diversity has also been reported in chronic viral infections such as HIV, EBV and CMV. Emerging evidence has shown that immunogenetic variants on repertoire diversity are linked to polymorphism at the human leukocyte antigen class I (HLA-I) loci. In one study, Krisna *et al*. demonstrated a strong association between TCR diversity and HLA-I polymorphism (140). This adds to the notion that individuals with one allele for HLA-1 display a broader spectrum of

immunopeptidome, allowing for recognition by cytotoxic T cells (141). Overall, the HLA-I heterozygosity is believed to provide an evolutionary fitness advantage, enhancing TCR diversity. On the contrary, age and CMV infection tend to diminish TCR repertoire diversity, suggesting that genetic factors, along with environmental influences, can shape the diversity of the TCR repertoire.

1.6.4.4 T cell activation

The hallmark of T cell-mediated immunity is the activation of naïve cells following an encounter with an antigen, leading to the development of diverse effector functions. Importantly, T cells do not directly recognise antigens. Instead, they recognise peptides bound to MHC I and MHC II molecules presented on antigen-presenting cells (APC), with CD4 T cells interacting with MHC II and CD8 T cells with MHC I (142). T cells require three signals to become fully activated and acquire effector functions **(Figure 1-8).** These are antigen recognition (TCR: peptide-MHC binding), co-stimulation, and polarising cytokines. The first signal happens when TCR from both CD4 and CD8 T cells binds to the peptide-MHC complex presented on the surface of APC. This induces upregulation of costimulatory molecules such as CD28 on T cells interacting with their receptors B7.1/B7.2 (CD80/86) on the APC. This is called signal two or costimulatory signal. Lastly, signal 3 is provided by polarising cytokines released from APCs which interact with their respective receptors on T cells, influencing the type of effector T cell the activated T cell will develop into (143,144). In the absence of signal two, antigen recognition induces a state of anergy in naive T cells, contributing to the maintenance of tolerance to self-tissue antigens. Taken together, all three signals initiate a signalling cascade inducing an upregulation of T cell activation genes that leads to T cell expansion and differentiation.

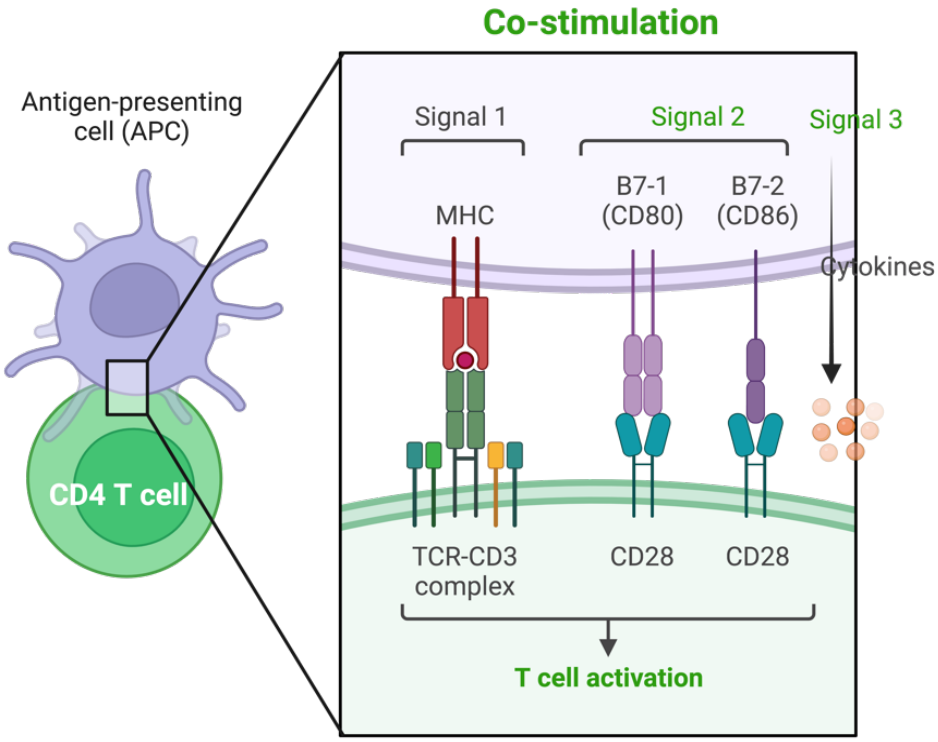


Figure 1-8 Signals required for T cell activation

T cells require 3 signals for complete activation, acquisition of effector function as well as proliferation. Signal 1 occurs when the TCR binds to its specific peptide on the appropriate MHC molecule. The second signal is facilitated by the interaction of the co-stimulatory molecule CD28 on the T cell with CD80/86 on the MHC. Lastly, the third signal is delivered by cytokines released from antigen-presenting cells (APCs), interacting with their corresponding receptors on T cells (Created with BioRender.com).

1. 6. 5 Functional subsets of T cells

Conventional $\alpha\beta$ T cells comprise two main functional subsets namely: T helper (CD4+) and cytotoxic (CD8+) T cells. These different subpopulations display unique expressions of transcriptional factors, profiles of cytokine secretion and effector molecules, and, consequently, specific functions that they employ to fight against pathogens. The following sections **(section 1.6.5.1 & section 1.6.5.2)** will discuss different functional subsets of T cells.

1.6.5.1 T helper cells

CD4 helper T cells play a central, coordinating role in the functioning of the immune system: they help B cells produce antibodies, prime and maintain the responses of CD8 T cells, regulate the function of macrophages, and coordinate immune responses against various pathogens (145). These distinct roles are achieved by functional differentiation of naïve T cells into distinct subunits. Upon activation, T helper (Th) cells further differentiate into various subsets including Th1, Th2, Th9, Th17, and follicular helper (Tfh), T regulatory (Tregs) and Th22 depending on the cytokine milieu of the microenvironment as well as transcription factors (146,147). These T cells can be identified by the patterns of cytokines they secrete and the expression of distinct cell surface molecules.

Th1 cells are involved in the immune responses against intracellular pathogens and are associated with organ-specific autoimmunity. They mainly secrete interferon-gamma (IFN-γ), interleukin (IL-2), and tumour necrosis factor-alpha (TNF-α). IFN-γ is essential in triggering cell-mediated immunity, which involves activating mononuclear phagocytes including macrophages and cytotoxic T cells (CTL). IL-2 promotes proliferation and survival of CD4 and CD8 T cells. Th1 differentiation involves a well-coordinated process with different cytokines and transcription factors at play. Cytokines such as IL-12 and IFN-γ are the key drivers of naïve CD4 T cell differentiation into Th1(148). Particularly, IL-2 produced by the activated APCs induces the production of IFN-γ in natural killer (NK) cells which, in turn, facilitates the downstream signalling cascade to develop Th1 cells in a signal transducer and activator of transcription-4 (STAT4)-dependent manner (149). Several transcription factors work in tandem to induce a complete Th1

differentiation **(Figure 1-9).** Nevertheless, the T-box transcription factor (T-bet) is a master regulator as it ensures the activation of genes responsible for enhancing IFN-γ production whilst suppressing the development of cells from other lineages such as Th2 and Th17 (150). Unlike the IL-12 dependent-STAT4 differentiation, T-bet expression was shown to be highly dependent on IFN-γ signalling and STAT1 activation (151).

Th2 cells predominantly produce IL-4, IL-5, IL-9 and IL-13 with a moderate secretion of TNF-α and IL-9. Initially believed to lack the ability to produce IL-2, Th2 cells have been shown to produce IL-2 in low quantity (146). Th2 subsets play an important role in host immune responses to parasitic and helminth infections, as well as the repairing of tissue damage. In addition, Th2 cells are involved in allergic inflammatory reactions in addition to B cell activation. For instance, a combination of IL-4, IL-5, IL-9 and IL-13, all produced by Th2, has been shown to drive B proliferation and antibody class switching (152). IL-4 is essential in the polarisation of naïve CD4 to differentiate into the Th2 lineage. Through its interaction with STAT6, IL-4 signalling upregulates the expression of the master transcription regulator GATA3 (GATA-binding protein) **(Figure 1-9)** (149,152).GATA3 is thought to promote Th2 differentiation through 3 distinct mechanisms. This includes enhancement of Th2 cytokines such as IL-4, selective expansion of growth factor independent-1 (Gfi-1), and suppression of the Th1 lineage commitment possibly through their unique interaction with T-bet (153). Indeed, GATA3 was shown to inhibit Th1 differentiation by downregulating STAT4-signalling activity in C57BL/6 mice (154). Although IL-4 is a key signal transducer for GATA3 expression, IL-4-independent mechanisms have also been reported to promote Th2 differentiation (155). Moreover, STAT5 activated by IL-2 plays an important role in Th2 lineage commitment(149).

Th9 cells were initially thought to be a subset of Th2 cells. However, growing evidence suggests that these are independent subsets mainly characterised by the secretion of IL-9. Differentiation of Th9 is primarily induced by the synergistic effect of transforming growth factor-beta (TGF-β) and IL-4 (156). Indeed, TGF-β has been demonstrated as a driving factor to divert the differentiation of Th2

towards Th9 lineage commitment. Other transcription factors essential for Th9 development include IFN-regulatory factor 4 (IRF4), which binds directly to the IL-9 promoter region, PU.1 and, in some cases, GATA3 (157). Functionally, IL-9 plays an important role in mediating allergic inflammation as well as in tumour immunity. In addition, IL-9 is critical in limiting helminths and parasitic infections as evidenced by the clearance of worms in mice infected with *Trichuris muris* (158). Apart from mediating proinflammatory function, Th9 cells have been implicated in escalating allergic reactions and are associated with a detrimental role in the pathogenesis of asthma (158).

Th17 cells provide protective immune responses against extracellular bacteria, and fungi, and are responsible for autoimmune disorders (159). They secrete effector cytokines such as IL-7A, IL-17F, IL-21 and IL-22. IL-17 stimulates the production of pro-inflammatory cytokines like IL-6, IL-1, and TNF-α, which are essential in promoting the chemotaxis of inflammatory cells to the site of inflammation (159). IL-6, IL-21, IL-23, and TGF-ß are the primary signalling cytokines responsible for the differentiation of Th17 cells, with retinoic acid receptor-related orphan receptor gamma-T (RORγt) serving as the key transcription factor regulator **(Figure 1-9).** The differentiation process involves 3 stages, starting with the differentiation stage championed by TGF-ß and IL-6, then the IL-21 mediated self-amplification stage and, lastly, the stabilisation stage by IL-23 (146). Although a combination of TGF-ß and IL-6 plays an important role in Th17 lineage commitments, TGF-ß alone, at a high concentration, redirects lineage differentiation towards the development of a suppressive Th subset called induced Tregs (iTregs). Other transcription factors involved in Th17 differentiation include RORα, STAT3 and Runx1 (160).

Follicular helper T cells (Tfh) are characterised by the expression of C-X-C motif receptor-5 (CXCR5+) and are found within the follicular regions of lymphoid tissue, where they contribute to the formation of antigen-specific B-cell responses. In addition, Tfh plays a critical role in the creation of germinal centres, enhancing affinity maturation, producing high-affinity class-switched antibodies and memory B cell formation. The differentiation of Tfh cells is a complex, multistage process

influenced by various factors, with IL-6 and IL-21 as the major cytokines involved (161). Early Tfh differentiation is regulated by IL-6 and an inducible costimulatory (ICOS) under the influence of the transcription factor B cell lymphoma 6 (Bcl6), responsible for early CXCR5 expression (161). The expression of CXCR5, which binds to its ligand C-X-C ligand 13 (CXCL13), allows Tfh cells to migrate and reside in the B cell follicles, where they undergo further developmental processes and form germinal centres. These cells also express programmed cell death 1 (PD-1), and CD40-ligand (CD40L), crucial for B-cell interaction. Although Bcl6 enriches the expression of Tfh-related cytokines, it is also associated with the suppression of Th1, Th2, and Th17 (146). Transcription factors such as STAT3 also play a significant role in the commitment of Tfh lineage. Depending on the cytokine milieu and the predominantly produced cytokine, cells can further be categorised into Tfh1, Tfh2, and Tfh10. Tfh1, which secretes IFN-γ, stimulates the production of Immunoglobulin (Ig) G2a. Tfh2, on the other hand, secretes IL-4, promoting the production of IgG1 and IgE. Tfh10 facilitates IgA secretion through the secretion of IL-10 (162).

Tregs are a specialised subset of T cells that function to suppress the immune response, thereby ensuring homeostasis and self-tolerance. They have been shown to inhibit T cell proliferation and proinflammatory cytokine production, thus playing a crucial role in preventing immunopathology (163). Their primary effector cytokines consist of IL-10, TGF-β, and IL-35 **(Figure 1-9)**. IL-10 is a potent anti-inflammatory cytokine that inhibits the pro-inflammatory response, thereby preventing tissue damage by the inflammatory process. An imbalance or dysregulation of IL-10 can lead to heightened immunopathology in response to infection, as well as increased susceptibility to the development of an exaggerated inflammatory response (164). There are two types of Tregs: the natural Tregs (nTregs) which originate from the thymus already expressing the main transcription factor—Forkhead transcription factor (FOXP3) and induced Tregs (iTregs), which differentiate in the peripheral lymphoid organs following activation of naive T cells (165). These cells are characterised by the expression of FOXP3, CD4 and CD25. TGF-β is the key cytokine responsible for the initiation of the iTregs cell lineage commitment **(Figure 1-9).** FOXP3 is the major lineage-specific transcription factor responsible for the differentiation of Tregs. Moreover,

this transcription triggers downstream signalling of TGF-ß following TCR activation (166). Additionally, Smad2 and Smad3, which are activated through TGF-ß signalling pathways, play a role in the differentiation of iTregs by inducing the expression of FOXP3. Other transcription factors involved in FOXP3 signalling include STAT5 and NFAT (167). The iTregs are further divided into IL-10-secreting Treg 1 (Tr1) and TGF-ß-secreting T helper 3 (Th3). Unlike conventional Tregs, which exert their inhibitory function through cell-to-cell contact and membrane-bound molecules, the latter group caries their function via cytokines such as IL-10 and TGF-ß (168).

Th22 cells, first reported in 2009 by Trifari *et al.*, are identified by their main effector cytokine profile comprised of IL-22, although they also produce IL-13 and TNF-α (169). IL-22 belong to the IL-10 family, which binds to the IL-22R to induce a downstream signalling cascade. Th22 cells express a broad spectrum of chemokine receptors, such as CCR4, CCR6 and CCR10, which allows trafficking to the effector site, where they execute functions such as tissue remodelling, inflammatory processes, and wound healing (170). Like the rest of Th cells, Th22 differentiation is regulated by various factors including cytokines and transcription factors (170). IL-6 and TNF-α, produced by plasmacytoid Dendritic cells (pDCs) and conventional DCs (cDCs), drive naïve Th towards Th22 differentiation. However, the combination of IL-6 and TNF-α demonstrates a more potent promotion of Th22 differentiation compared to IL-6 alone (171). Cytokines such as IL-21, IL-23 and IL-1ß also contribute to the formation of Th22. Specifically, IL-21 activates STAT3, which then stimulates transcription factors aryl hydrocarbon receptor (AhR) and RORγt (RORC in humans), key transcription regulators for Th22 differentiation (169,172) **(Figure 1-9).**

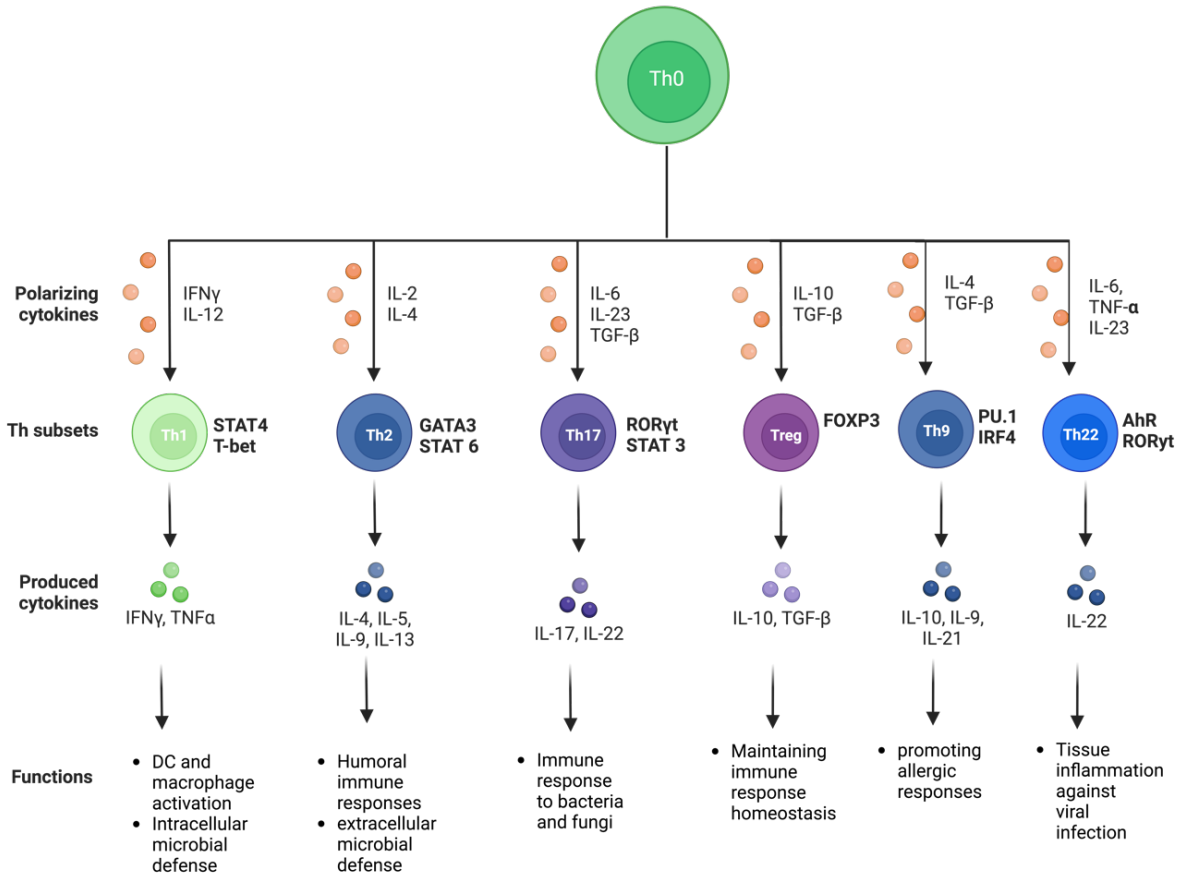


Figure 1-9 Overview of CD4 T cell differentiation into various subsets

Following antigen stimulation, naïve CD4+ T cells differentiate into subtypes such as Th1, Th2, Th17, Treg, Tfh, and Th9, depending on the type of infection. These cells are characterised by the cytokine they produce. Naïve T cell differentiation is mediated by different types of cytokines (enhancing cytokines) and transcription factors. (Created with BioRender.com, adapted from Zhu *et al*.2010)

1.6.5.2 Cytotoxic T cells (CTLs)

CD8 cytotoxic T cells (CTLs) are antigen-specific effector cells of adaptive immunity with the ability to kill infected cells. They primarily eliminate virally infected cells, intracellular pathogens as well as cells bearing tumour antigens. Following priming, CTLs employ three distinct mechanisms of killing, two in a direct cell-to-cell contact-dependent manner between the killer and the target cell. This includes the exocytosis of cytolytic molecules, namely perforin, granulysin and granzyme and the receptor binding of Fas ligand (Fas/APO-L). In the first pathway, the cytoplasmic pore-forming granule, perforin is secreted together with granzyme, which induces death of the target cell (173). The binding of perforin to the membrane creates pores which act as conduits for the penetration of cytolytic proteins such as granzyme and granulysin, ultimately inducing apoptosis. Moreover, perforin triggers an influx of Ca^{2+} which facilitates target cell lysis via osmotic stress (174). In the receptor ligand-mediated pathway, activated CTL express Fas ligand that interacts with the Fas (CD95) receptor on the target cells to trigger a death signal for apoptosis (Figure 1-10) (175). The exact mechanism behind the intracellular downstream induction of Fas-mediated cell death is poorly understood. However, the process is thought to be partially regulated by cytoplasmic proteases such as amin B1, α-fodrin, ß-actin, and topoisomerase 1 (176). The third pathway is mediated by cytokines namely IFN-γ and TNF-α. Not only does IFN-γ inhibit viral replication; but it also stimulates the transcription activation of the MHC class I antigen presentation pathway leading to a heightened presentation of endogenous peptides. This enhances the likelihood of infected cells being identified as targets for Fas-mediated cytotoxic attack (177). On the other hand, ligation of TNF-α to its receptor on the target cell induces the caspase cascade, leading to target cell apoptosis (177) **(Figure 1-10).**

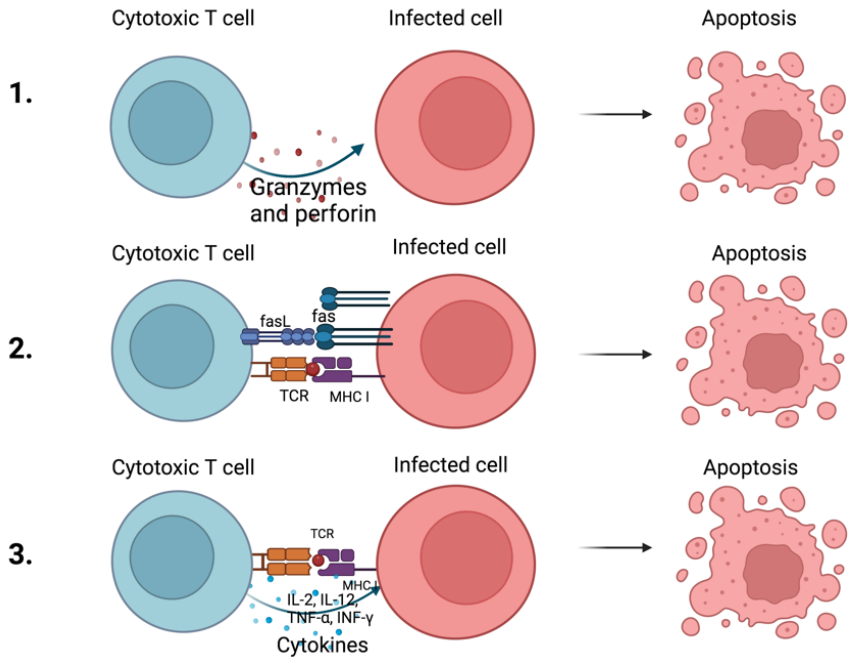


Figure 1-10 CD8 T cell cytolytic mechanisms

(1). CTLs secrete cytolytic granules (perforin and granzyme) through exocytosis which initiates apoptosis of the target cell. Granzymes enter the infected target cell via perforin drilled poles. (2) FasL/Fas killing pathway is mediated by the interaction of FasL on the killer cell to its binding receptor fas on the target cell. This triggers the death signal in the infected cell. (3) The release of cytokines such as IFN-γ and TNF-α enhances the killing of the infected T cells by CTLs. (Created with BioRender.com, adapted from Groscurth *et al.*).

Following activation, CD8+ T cells undergo clonal expansion and contraction leading to effector differentiation and memory formation. Memory cells provide a rapid recall immune response to the same antigen due to their increased frequency and unique localisation to the effector sites (178). CD4 T cells play a crucial role in the generation, proliferation, and maintenance of memory $CD8^+$ CTLs. Recently, a subset of CD4+ T cells exhibiting cytotoxic activity have been identified in diverse immune responses. These cells are distinguished by their capability to release granzyme B and perforin, enabling them to eliminate target cells through an MHC class II-restricted pathway (179). While the mechanisms controlling the development of various CD4+ Th subsets have been described regarding cytokine and transcription factor requirement, the exact factors responsible for CD4 CTL differentiation are incompletely understood. These cells are believed to be associated with Th1 cells that secrete IFN-γ, and their

differentiation is regulated by the transcription factors eomesodermin and/or T-bet (180,181).

Like the CD4 Th subpopulation cells, effector memory $CD8^+$ CTLs further differentiate into Tc1 and Tc2 depending on the cytokine microenvironment and transcription factor expression **(Figure 1-11).** In addition to the production and secretion of cytolytic molecules, Cytotoxic Tc1 also produce IFN-γ and TNF-α, responsible for modulating host immune response against intracellular pathogens. Tc1 cells are activated by cytokines such as IL-2 and IL-12, which are influenced by T-bet (182). Furthermore, Tc1 was reported to promote anti-tumour protective immunity in tumour-induced mice (183). Tc2, on the other hand, produces cytokines such as IL-4, IL-5, IL-10, and IL-13, which propagate Tc2-mediated allergic responses including arthritis. Other alternative $CD8^+$ T cell subsets include Tc9, Tc17, and Tc22 and they produce IL-9, IL-17, and IL-22, IL-2, IL-22, and TNF-α respectively **(Figure 1-11)** (182).

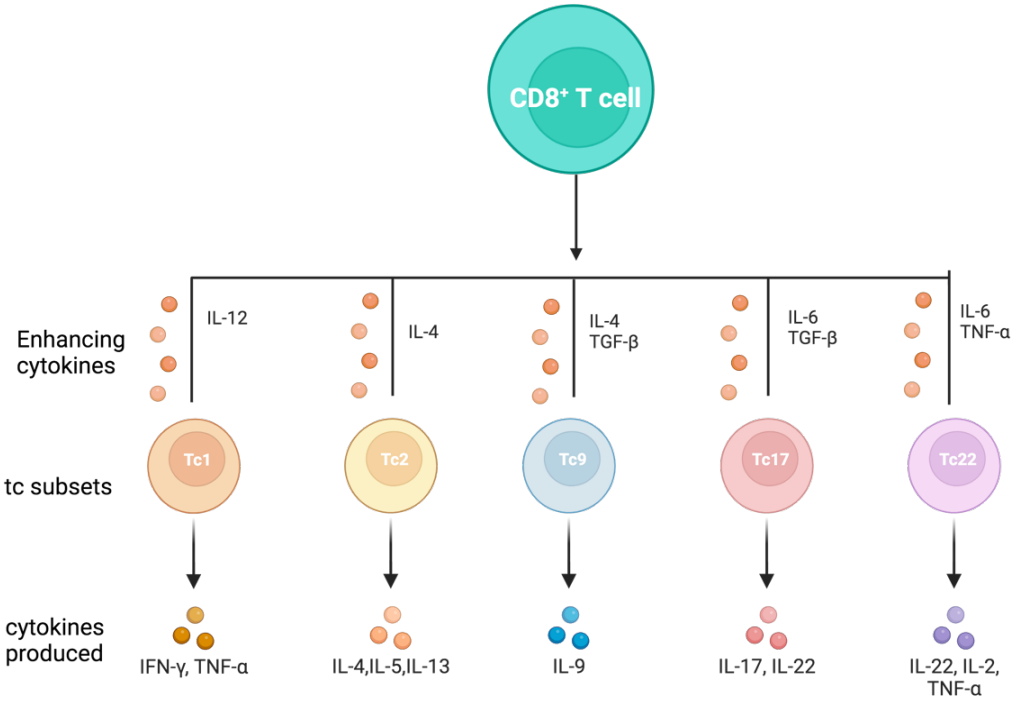


Figure 1-11 CD8 Effector T cell differentiation

CTL CD8+ T cells differentiate into different subtypes including Tc1, Tc2, Tc9, Tc17, and Tc22 depending on the cytokines milieu and the transcription regulatory factors. (Created with BioRender.com with permission from Andersen et al.).

1.6.6 T cell memory differentiation: Naïve, effector and memory

T cells exhibit an extensive diversity in their phenotype and function. The transition of T cells from a naive state to effector and memory cells is essential in driving effective immune responses to resolve infection while retaining memory of previous pathogen exposure. Depending on their cell surface expression pattern, T cells can be differentiated into the following subsets: Naïve, effector, central memory, effector memory, terminally differentiated memory and tissue-resident memory.

1.6.6.1 Naïve T cells

Naïve T cells ($CD45RA^+/CD45RO^-$) are produced in the thymus following successful completion of positive and negative selection processes. They constantly circulate in the blood and the peripheral lymphoid organ searching for their cognate

antigens. Naïve T cells are relatively quiescent and lack effector functions. Phenotypically naïve T cells can be differentiated from memory through the surface expression of chemokine receptor-7 (CCR7), selectin (CD62L), CCR7 and CD45RA. CCR7 and CD62L serve as a homing receptor, facilitating the migration of naïve T cells to the lymph node (184).

1.6.6.2 Effector T cells

Following an encounter with an antigen-presenting cell, naïve T cells activate, undergo clonal expansion, and differentiate into effectors. These cells then migrate to the site of infection to execute effector functions. They are characterised by rapid production of effector cytokines such as IFN-γ, TNF-α, LT-α, IL-4, IL-5, IL-6, IL-10 and IL-13. In addition, they express high levels of CD25 and downregulate CD62L and CCR7, which directs their trafficking to non-lymphoid peripheral sites (185). Effector cells have a short life span. Most of the activated cells undergo apoptosis upon clearance of the infection. However, a small proportion (about 5-10%) revert to a resting state and differentiate into memory cells (178).

1.6.6.3 Memory T cells

Unlike Naïve T cells, memory CD4 and CD8 T cells have lower requirements for activation. These cells provide a heightened immune response to secondary exposure to an antigen due to their increased frequency, low requirements of stimulation as well as strategic localisation at the site of infection (185). To date, the mechanisms underlying the generation of memory T cells and their subsequent characterisation into diverse subsets remain to be completely delineated. However, three possible models are believed to contribute to memory T cell commitment. These include the conventional linear model, the divergent model, and the intersecting/arrest model. In the linear model, activated T cells acquire their memory phenotype directly from the effector cells following a contraction phase probably due to the epigenetic modification **(Figure 1-12)** (185,186). On the contrary, the divergent pathway postulates that following antigen stimulation, memory T cell precursors originate directly from a pool of naïve T cells independent of effector cell differentiation **(Figure 1-13)** (187). By contrast, the

Intersecting model proposes that the acquisition and survival of memory T cells occur in phases and that the phases are driven by the duration of the antigenic stimulation and the strength of the TCR signalling. For instance, suboptimal stimulation of naïve cells generates effector T cells with minimal effector functions while those receiving optimal antigenic stimulation achieve their full effector function potential. In the absence of antigens, these cells can differentiate into central and effector memory respectively **(Figure 1-14)** (187). Moreover, a short duration of antigenic stimulation favours the development of central memory (T_{CM}) cells, whereas a longer duration of stimulation promotes the differentiation of effector memory (T_{EM})T cells (187).

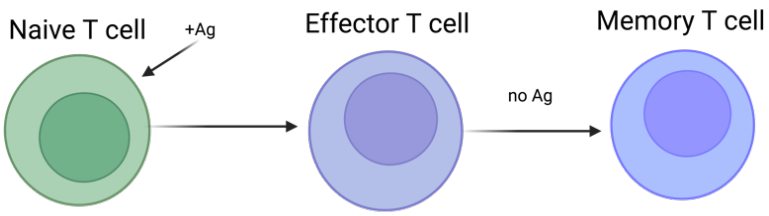


Figure 1-12 The conventional linear of memory T cell generation

Naïve T cells undergo clonal expansion following antigen activation. Upon resolution of the infection, a small proportion of the cells survive the contraction phase and differentiate into long-lasting memory T cells. (Created with BioRender.com, adapted from Forber 2006).

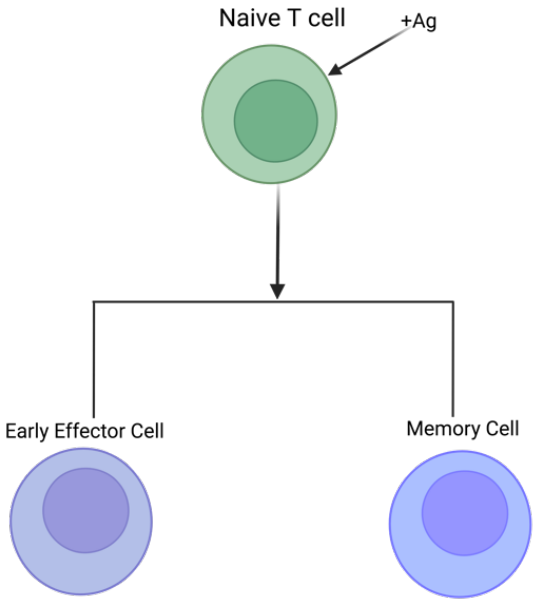


Figure 1-13 The divergent model of memory T cell generation

In this model, memory T cell precursors develop directly from naïve T cells. Upon antigen stimulation, naïve T cells can give rise to daughter cells with either effector or memory phenotype. Thus, naïve T cells can bypass an effector-cell stage and develop directly into memory T cells. (Created with BioRender.com, adapted from Kaech, et al. 2002).

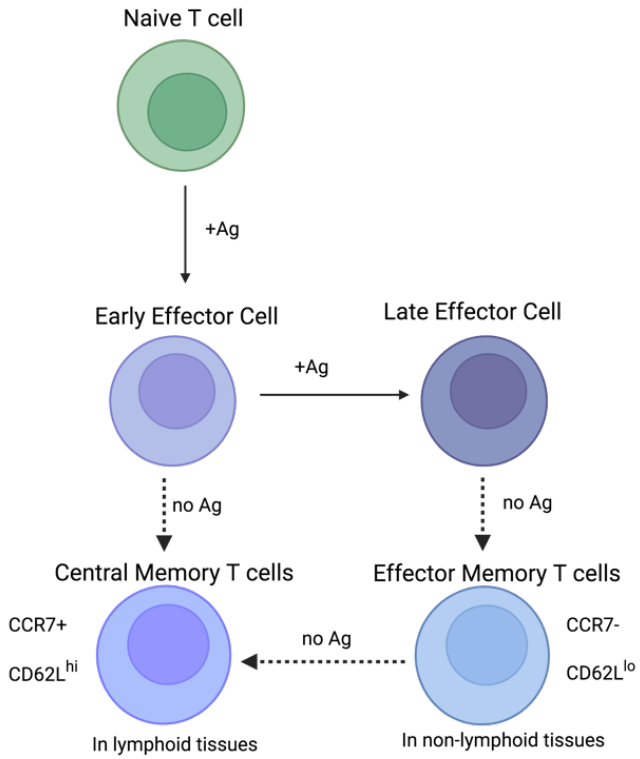


Figure 1-14 Intersect/arrests model of memory T cell generation.

In this model, memory T cell heterogeneity is influenced by the strength of the stimuli and the duration of antigen stimulation. In the former, weaker stimuli result in daughter cells with poor effector (early effector cells) potential compared to stronger stimulation. In the absence of an antigen, these cells differentiate into a subset of central and effector memory cells respectively. In the latter, a short duration of antigenic stimulation favours the development of central memory T cells, whereas a longer duration of stimulation tends to influence the differentiation of effector memory T cells. (Created with BioRender.com, adapted from Kaech, *et al*. 2002 and Farber *et al*.

1.6.6.4 Effector memory (T_{EM})

T_{EM} cells are characterised by their ability to migrate to inflamed peripheral sites. They lack the expression of CD62L and CCR7, and they exhibit a rapid effector function, which clears pathogens at the infection site (188). T_{EM} can perform cytotoxic, helper and regulatory functions. For example, CD8 T_{EM} produce perforin, which targets infected cells to induce apoptosis, and both CD4 and CD8 TEM secrete IFN-γ, IL-4, and IL-5 (173). Moreover, in a population of $T_{EM,}$ the expression of different chemokine receptors has been used to distinguish these cells into either Th1 or Th2 (189). For instance, Th1 cells can be identified using CCR5 and CCR6 while CCR3 and CRTH2 are key chemokine receptors for discriminating Th2 (190). Diverse survival signals play a role in regulating the

persistence of effector T cells within the memory pool. Among these signals, cytokines belonging to the IL-2 family, such as IL-7 and IL-15, and the expression of their corresponding receptors on T cells, are key in sustaining T cell memory (191)

1.6.6.5 Central memory (T_{CM})

Central memory T cells represent a distinctive phenotype characterised by a high expression of lymphoid homing receptors, namely CD62L and CCR7, enabling their migration and guiding their entry into the peripheral lymphoid organs through high endothelial venules (HEV) (188). They are mainly localised within the lymphoid organs owing to their lack of chemokine receptors for trafficking to the peripheral sites. Unlike $T_{EM,}$ T_{CM} cells lack the immediate effector potential such as production of IFN-γ. Nevertheless, they produce IL-2 and can differentiate into a pool of effector memory cells (192). Upon reactivation, T_{CM} cells can stay in the lymphoid organ to help B-cells or traffic to the infection site to directly combat the infection. Since T_{CM} cells are not dedicated to producing any specific effector cytokines, their generation is influenced by the challenging infection under different cytokine microenvironment (193). Circulating T_{CM} Tfh-like cells expressing the CXCR5 receptor have been identified within the B cell follicles. These cells express high levels of Bcl6 and CXCR5, PD1, ICOS, CD40L and IL-21 and help B cell maturation. In addition, they activate B cells, promoting antibody production and isotype class switching (194). The question of whether Tfh cells found in lymphoid organs truly constitute memory T cell populations remains a topic of discussion. Tfh within a T_{CM} have been shown to reside within the tertiary lymph nodes for a long period and they are thought to be sustained by persistent antigen stimulation (195,196). However, relying on antigens suggests that Tfh cells in lymph nodes may not genuinely represent quiescent memory cells (190). Furthermore, T_{CM} can also differentiate into different subsets such as Th1 and Th2 in the absence of enhancing cytokines. For example, T_{CM} expressing chemokine CXCR3 denotes pre-Th1 whilst those expressing CXCR4 denotes pre-Th1 memory effector T cells (192).

1.6.6.6 Terminally differentiated memory (TEMRA)

Terminally differentiated memory T cells are short-lived effector memory T cells that have lost the ability to proliferate, or divide, in response to an antigen (197). While CD45RA expression is linked to naive T cells, TEMRA cells have been observed to re-express CD45RA after antigen stimulation (188). However, the mechanisms behind this phenomenon are yet to be determined. In addition, TEMRA downregulates CCR7 expression, allowing them to migrate to peripheral sites to perform the effector function (198). Indeed, the increase in the frequencies of $CD45RA^{+}$ $CCR7^{-}$ (TEMRA) has been reported to have detrimental effects in viral infections (199,200). In one study, CD8 TEMRA exhibited a low proliferation potential and their increase in peripheral blood was associated with chronic cytomegalovirus (CMV) infection (201) suggesting the role of these cells in prolonged infections. On the contrary, infection with Dengue virus induced highly polarised cytotoxic CD4 $CD45RA^{+}$ $CCR7^{-}$ cells characterised by the massive production of perforin, granzyme B and Eomes, which contributed to protective immunity (198,200). Together, these findings indicate that specific viruses induce terminal effector differentiation, such that the generation of TEMRA cells could be a process attributed to antigen load or persistent infection.

1.6.6.7 Tissue-resident memory (T_{RM})

Tissue-resident memory T cells (T_{RM}) are non-circulating memory T cells that reside in the epithelial mucosal barriers such as the skin, lung and gut. Upon pathogen re-exposure, T_{RM} provides rapid local immunity due to their presence at the site of infection (202). They display a diverse function and phenotype depending on the specific tissue in which they reside. T_{RM} cells in the respiratory mucosa are often identified by key surface markers, such as the high expression of CD69 and CD103. CD69 facilitates the retention of T_{RM} cells in tissues by influencing the expression of a sphingosine-1-phosphate receptor (S1PR). S1PR binds to its ligand S1P, which aids in the migration of cells into lymphatic vessels (203). On the other hand, the binding of CD103 to E-cadherin present on epithelial cells in mucosal tissues promotes tissue retention (204). Although CD69 is expressed by both CD4 and CD8 T_{RM} cells in the nose and the lung in both humans and mice, CD103 is largely expressed on certain subsets of CD8 $T_{RM,}$ with minimal expression on T_{RM} CD4 (202,204). By investigating the phenotype and the

transcription signature of CD69 tissue-resident cells in multiple organs in humans, Kumar *et al.,* showed that $CD69^+$ lung T cells exhibit an increased gene expression profile associated with tissue retention compared to $CD69^-$ T cells. These comprised genes responsible for migration and homing such as CXCR3 and CXCR6 (205). Other surface markers used to identify lung T_{RM} include the expression of the upregulation of adhesion molecules and integrins such as CD11a (LFA-1 and CD49a (VLA-1) used mainly for entry and retention respectively (206).

1.6.7 T cell migration and homing to the lung

Getting T cells to the site of infection or inflammation is paramount for initiating an effective immune response. T cells constantly circulate and migrate to secondary lymphoid organs (SLO), draining various tissues to provide immune surveillance. T cell migration involves a series of coordinated interactions between adhesion molecules on the surface of High Endothelial Venules (HEVs) in SLO and their respective ligands on T cells such as selectins as well as responding via chemokine receptors (207). HEVs exhibit unique characteristics, including the expression of peripheral lymph node addressin (PNAd) and mucosal vascular addressin cell adhesion molecule (MADCAM1). These features play a role in the rolling and tethering of T cells by binding to L selectin and α4ß7 on T cells respectively.

Trafficking of T cells to secondary lymphoid organs (SLO) relies on the presence of specific molecules, including L-Selectin (CD62L), integrin lymphocyte function-associated antigen (LFA-1) composed of integrin alpha L and beta 2 (αLß2), and the expression of the chemokine receptor CCR7 on these cells (208). The adhesion process of T cells involves initial "capture" by endothelial cells (tethering), followed by a phase of loose adhesion (rolling), then an activation step that eventually results in firm arrest (sticking) and the transmigration of lymphocytes into various tissue sites **(Figure 1-15)** (207,208). Since T_{EM} cells lack the expression of L-selectin and/or CCR7 expression, they are unable to migrate back to the lymph node.

Besides L-selectin and CCR7, T_{CM} T cells may express homing receptors associated with extra-lymphoid migration, like effector memory (EM) T cells (209). Thus, the primary functional distinction between central memory T_{CM} and T_{EM} cells lies in the ability of T_{CM} to migrate to lymph nodes, whereas effector memory T_{EM} cells cannot home to these specific lymphoid organs. Tissue-specific homing of activated and memory cells to mucosal sites is, however, imprinted in the mucosal draining lymph node (dLN) during T cell priming (210). Imprinting refers to a process in which Dendritic cells (DCs) from dLN guide activated T cells to home to a specific tissue. For instance, T cell interaction with lung-derived DCs in dLN signals the upregulation of lung homing receptors such as CCR4, CXCR3, CCR5 and LFA-1, which drive T cells to traffic to the lung in a chemokine gradient manner (210,211).

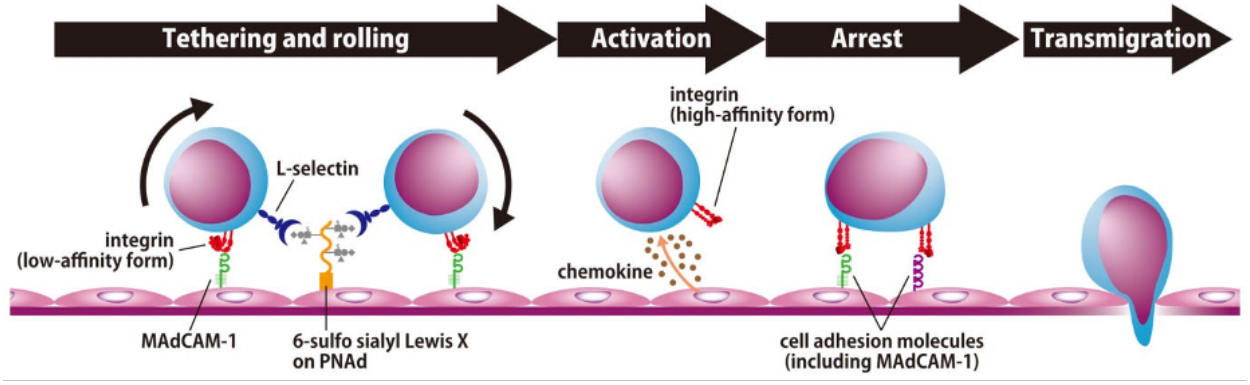


Figure 1-15 Different stages of T cell migration within the HEV.

The initial step involves tethering and rolling, which occurs through the interaction of selectins like P-selectin or L-selectin on endothelial cells with their corresponding ligands, such as P-selectin glycoprotein 1 (PSGL-1) and E-selectin ligand-1 (ESL-1), on T cells. Subsequently, T cell activation leads to the increased expression of integrins (e.g., α4β7), which then bind to their receptors (e.g., MAdCAM-1) on endothelial cells, resulting in firm adhesion. The activation of T cells is facilitated by the interaction between chemokine receptors on T cells, such as CCR9 or CCR4, and their respective ligands (CCL25 and CCL17 respectively) secreted within the specific tissue. After firm adhesion, T cells undergo transendothelial migration, following a chemokine gradient in a process known as chemotaxis, which guides the T cells to the site of infection or the source of chemokine secretion. (Figure produced with permission from Ronald N. Germain *et al*.2012.)

Like all other immune cells, T cell migration into the lung parenchyma involves similar stepwise processes involving selectin interaction, chemokine-induced integrin activation, firm adherence, transmigration, and chemotaxis into the tissue. However, during tethering, unlike L and E selectin, P selectin is crucial in the initial step of T cell migration into the bronchial space. This is followed by the binding of lymphocyte LFA-1 to intracellular adhesion molecule 1 (ICAM-1) and (ICAM-2) important in the adherence to the endothelial vessel. On the other hand, the activation step is mainly facilitated by the binding of CCL21 on HEV to its ligand CCR7 on naïve and T_{CM}. These adhesion molecules play an important role in the recruitment of T cells to the lungs as blocking of the molecules resulted in a reduced T cell migration to the lung (142). Chemokines and their receptors play a crucial role in directing effector and memory T cells to the lungs. Particularly, CCL5 (RANTES) is consistently expressed in the lung, while its associated receptor, CCR5, is prominently present on activated T cells (142).

1.7 The role of T cells in immune protection against TB

CD4 and CD8 T cells are integral in providing protective immunity against TB. CD4 T cells help in priming CD8 T cells. Once activated, they play key roles in the immune response to TB, killing infected cells and producing effector cytokines that recruits other immune cells to the site of infection to form a granuloma (212,213). A TB granuloma is a unique structure composed of a collection of various immune cells, including macrophages, neutrophils, and T cells which restricts the progression of the infection **(Figure 1-16)**. In tuberculous granuloma, Mtb bacilli are primarily located within the caseous necrotic tissues or within epithelioid macrophages close to the core **(Figure 1-16)**. This central area is enclosed by concentric layers of macrophages and lymphocytes in the periphery (212). Evidence from non-human primates challenged with Mtb has demonstrated reduced T cell cytokine responses within the granulomas, which is associated with impaired clearance of Mtb infection (214,215). This may be due to the spatial peripheral localisation of the T cells in the granulomas limiting their access to macrophages for activation (215). Previous studies have shown that T cells within granulomas are heterogeneous, with a distinct T cell repertoire and function (216). Control of Mtb is mainly mediated by IFN-γ and TNF-α producing CD4 T cells (217). IFN-γ and TNF-α activate the microbicidal activities of the recruited immune cells to maintain the stability of granuloma.

The multifaceted role of CD4 T cells in the immune response to TB has been widely discussed in both animal models and human studies. In one study, mice deficient of CD4 T cells demonstrated a higher risk of developing active TB compared to matched controls (218). Similarly, HIV-infected individuals with low CD4 T cell counts and impaired CD8 T cells in peripheral blood showed substantial susceptibility to developing active TB disease (219). Although CD8 T cells were considered less significant than CD4 T cells in anti-Mtb immunity, emerging evidence suggests that these cells are critical in limiting the infection (220). Like CD4 T cells, CD8 T cells produce effector cytokines such as IFN-γ, TNF-α and IL-2, which play fundamental roles during TB infection. Furthermore, these cells induce apoptosis of Mtb-infected cells through Fas-Fas ligand interaction as well as degranulation of cytolytic contents such as perforin, granzyme and granulysin

(221,222). Taken together, this evidence suggests the critical role of T cells in the development and control of Mtb infection.

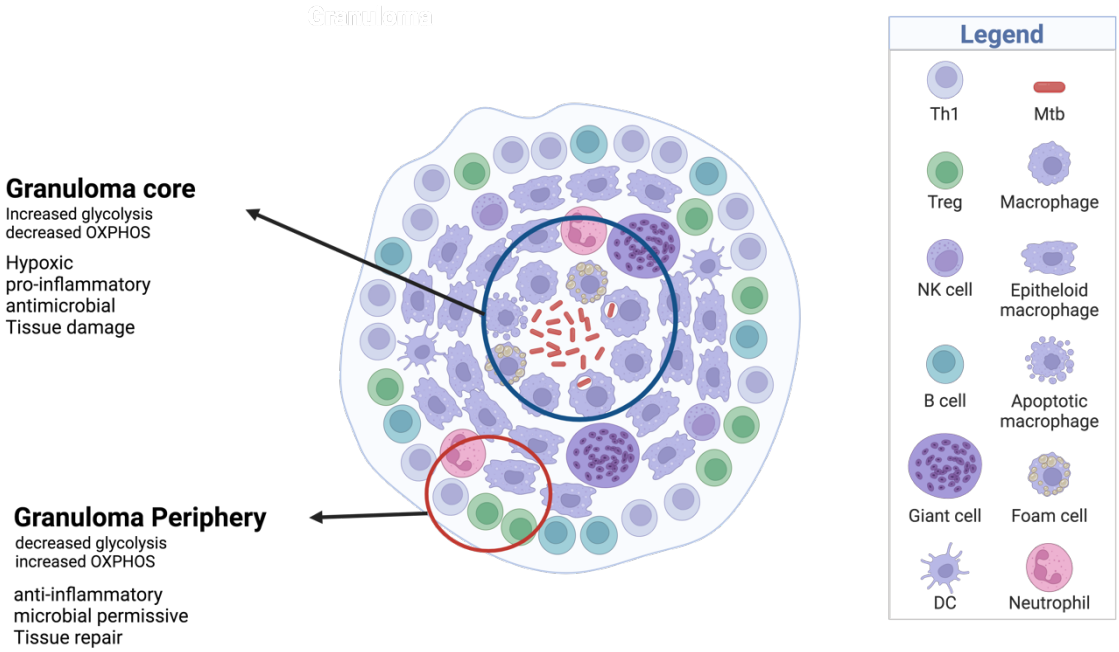


Figure 1-16 Schematic overview presentation of formation of tuberculous granuloma during Mtb infection

Following Mtb infection, immune cell populations are recruited to the site of infection to wall off the bacteria, a mechanism of containing the spread. The cellular composition includes macrophages, neutrophils, dendritic cells, eosinophils, epithelioid cells, and T cells. Layers of the epithelial cells surround the necrotic core together with other macrophage populations including foam cells. A variety of other cell types are also recruited to the granuloma, integrating into the structure both at the periphery and within the epithelioid layers, with T cells mainly localised at the peripheral sites (Created with BioLender.com adapted from Millar *et al.*,).

1.8 HIV and TB coinfection

HIV infection is associated with perturbations of T cell homeostasis and disruption of the immune system by inducing chronic T cell activation (223). This impaired immunity is demonstrated by dysfunction and depletion of CD4 T cells as well as increased levels of markers of T cell activation such as CD38, which indicates disease progression (224). The breadth of the HIV-specific T cell receptor repertoire is the hallmark of HIV infection. While successful antiretroviral therapy (ART) reduces viral replication and prevents attrition of naïve T cells (225,226), it is not clearly known whether ART completely restores the TCR repertoire.

Previous studies have shown that HIV infection changes the usage and normal distribution of TCR repertoire in peripheral blood (227,228).

In addition, a study of PLHIV with persistent opportunistic infections revealed that these individuals tend to lose antigen-specific responses despite reconstitution of absolute CD4 T cell counts following effective therapy (228). Collectively, this, in part, suggests that HIV impairs TCR repertoire diversity, which results in lack of optimal TCR specificities for antigen recognition and clearance of infection. Although previous studies have reported on HIV-induced perturbations of TCR repertoire in peripheral blood (224,229), none have probed into the repertoire usage in the lung microenvironment, a potential reservoir for HIV (230,231). It is well established that HIV infection causes massive alterations in the alveolar compartment of the lung characterised by cellular activation, secretion of pro-inflammatory cytokines and influx of CD8 T cells, resulting in lymphocytic alveolitis (232–234). Consequently, this impairs pulmonary immunity, rendering the host vulnerable to respiratory opportunistic infections such as TB (235,236).

The intricate relationship between HIV and TB has been an area of research since the establishment of the synergy between the two infections (237,238). Despite long-term ART, TB remains the most prevalent opportunistic infection and the leading cause of death in HIV-infected individuals, in both low and middle-income countries (239). HIV predisposes the host to TB by impairing the immune system. This is mainly attributed to fluctuating CD4 T cell function (236). However, HIV's disruption of CD4 T cell function and how this renders the host susceptible to TB is incompletely described. There is a limited understanding of the impact of HIV on the alveolar TCR repertoire and how this influences susceptibility to TB infection.

1.9 The Impact of HIV in shaping TCR repertoire in active pulmonary TB

HIV-infected individuals are more susceptible to lower respiratory tract infections (LRTI), including TB, compared with HIV-uninfected (235,240,241). In high TB transmission regions such as SSA, many TB cases in HIV-infected adults result from recent infections and not reactivation of pre-existing Mtb infections (238). Since Mtb infections are acquired mainly via the respiratory route, the development of

TB disease in HIV-infected individuals represents a failure of the pulmonary immune response to control new Mtb infections. Previous studies, including those from our laboratory, have demonstrated that HIV persists within the lung even in asymptomatic individuals on long-term ART (230,242). Additionally, alveolar CD8 lymphocytosis has been reported in PLHIV, although this does not normalize following successful ART treatment (232,234). Furthermore, HIV depletes CD4 T cells and impairs the cytotoxic potential of CD8 T cells. Consequently, this leads to the formation of dysfunctional granulomas which fail to contain the infection (243). Collectively, this suggests that HIV impairs crucial lung immunity, which increases susceptibility to TB and other LRTI.

1.10 The significance of TCR functional diversity in the control of TB

The interaction between TCR and peptide-MHC (pMHC) complexes plays an important role in the recognition and initiation of T cell-mediated immune responses. A diverse TCR repertoire is key to providing a source of antigen-specific T cell populations required for effective immune control of pathogens including TB. Whilst the role of TCR repertoire has been widely studied in other infectious diseases (244,245), little is known about the influence of Mtb on the diversity of TCR repertoire. In one study, Luo *et al*. observed restrictions in TCR repertoire in the peripheral blood of TB patients, which was associated with the severity of the disease (246). However, our understanding of TCR repertoire diversity in the airway, the natural route of Mtb infection, and how this is impacted by co-infection with HIV, is limited. In addition, the evidence of TCR functional restoration upon successful anti-TB treatment is inconclusive (247). Therefore, this project provides an exciting opportunity to advance current knowledge of the impact of HIV on the diversity on Mtb-specific T cell repertoire the lung, which may inform new insights in host T cell immune responses involved in control of Mtb infection.

1.11 Project rationale and aims

Co-infection with Mtb and HIV is a major risk factor for the development and progression of active TB disease. Despite effective coverage of ART, the burden

of TB remains huge, posing a significant threat to PLHIV. Our ability to manage TB is, however, hampered by a lack of understanding of mechanisms that influence the outcome of Mtb infection. It remains unclear why some individuals control the infection better than others despite being subjected to similar conditions. Antigen-specific MHC-restricted αß T cells play a critical role in controlling Mtb infection. Moreover, effector T cells with the ability to produce multiple cytokines are associated with protective immunity to Mtb. Ironically, HIV depletes and impairs the function of Mtb-specific T cells, thereby predisposing the host to progression of infection and development of active tuberculosis. However, HIV's disruption of CD4 T cell function and how this renders the host susceptible to TB is not fully understood. Specifically, there is a lack of understanding of the impact of HIV on the alveolar TCR repertoire and how this may influence susceptibility to TB infection. Furthermore, it is not clear whether alterations in T cell repertoire due to HIV/Mtb co-infection are restored following successful ART. To reduce the risk of disease and HIV-associated TB mortality, there is a need for a better understanding of mechanisms that constitute protective immunity to TB. Therefore, this thesis aims to investigate the impact of HIV on TCR repertoire diversity and Mtb-specific alveolar T cell function in the airway of adults living with HIV. The hypothesis is that HIV alters the phenotype and function of the Mtb-specific T cells and limits TCR repertoire diversity in the airway and blood, leading to increased host susceptibility to TB **(Figure 1-17)**

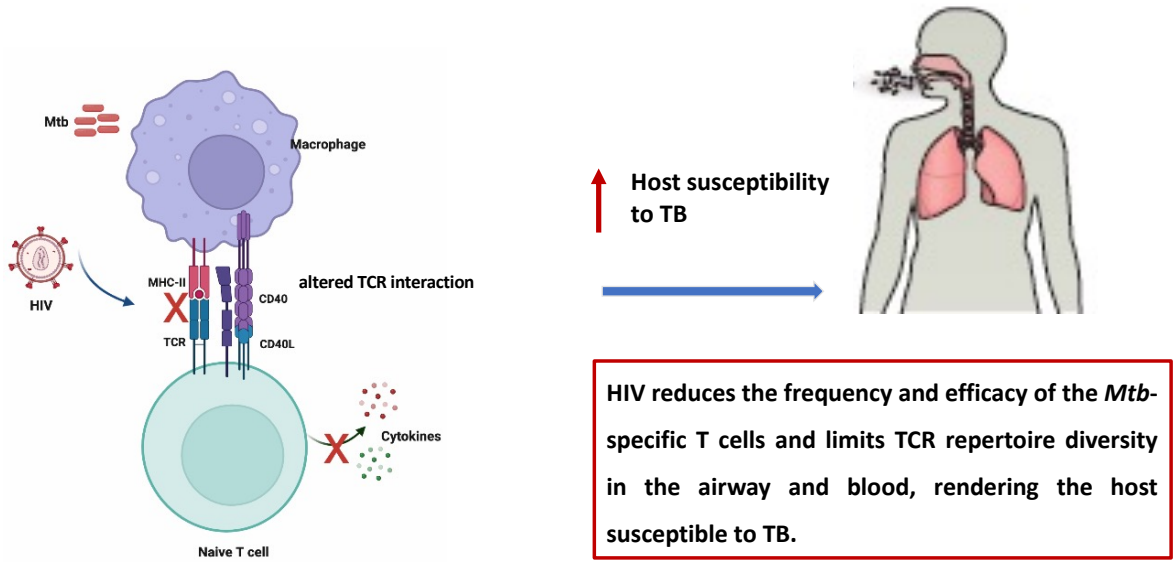


Figure 1-17 Schematic overview of the research hypothesis.

HIV infection reduces the frequency and efficacy of the Mtb- specific T cells and limits TCR repertoire diversity in the airway and blood, rendering the host susceptible to TB (Created with BioRender.com).

1.12 Specific objectives

1. To define the frequency and efficacy of the Mtb-specific TCR repertoire in the airway and peripheral blood of healthy HIV-uninfected adults without TB.

2. To evaluate the impact of HIV on the phenotypic and functional diversity of the Mtb-specific $CD4^+$ and $CD8^+$ TCR repertoire in adult PLHIV without TB.

3. To describe the TCR repertoire associated with HIV and Mtb-specific responses using transcriptome profiling.

By addressing these objectives, the study will advance current knowledge and provide new insights into host factors that influence susceptibility to and progression of Mtb infection. Ultimately, the identification of the highly used TCR chains may inform novel effective vaccine targets and design of long-term host-directed therapy.

Chapter 2 Materials and Methods

This chapter describes the clinical and laboratory methodologies used to generate the data reported in this thesis.

2.1 Study Type

This thesis presents results from a comparative cross-sectional study.

2.2 Study Site

The study discussed in this thesis was conducted at Queen Elizabeth Central Hospital (QECH) and the Malawi Liverpool Wellcome Research Programme (MLW) in Blantyre, Malawi. The study utilised well-established adult TB and HIV services and an experienced bronchoscopy team based at the MLW Clinical Investigation Unit (CIU). MLW is affiliated with the Kamuzu University of Health Sciences (KUHeS) in Malawi. It conducts research on diseases of local relevance that informs health policies. MLW has a robust infrastructure of laboratories equipped with modern equipment and platforms and is strategically located within the QECH campus. Working in collaboration with the QECH-KUHeS provides a unique opportunity for bedside, evidence-based, clinical research activities that aim to improve the quality of patient care. QECH is the largest teaching and tertiary referral hospital in southern Malawi. Established in 1958, the hospital has over 1,000 beds and provides a range of medical services, including general medicine, surgery, paediatrics, obstetrics and gynaecology, orthopaedics, psychiatry, and other specialised services. The hospital is easily accessible from all areas, both rural and urban, around Blantyre. The participants recruited in this study were from in and around Blantyre City.

2.3 Ethical approval

The study was approved in Malawi by the Malawi National Health Science Research Committee (NHSRC) protocol number 20/10/2617. Good clinical practice and ethical guidelines were followed in accordance with the Helsinki Declaration and the local ethics committee, which includes written informed consent.

2.4 Study population

Malawi is one of the countries with a significant burden of TB cases. In 2023, Malawi was ranked among the top 30 high-burden TB countries, with an incidence rate of 125 cases per 100,000 population (WHO, 2023). This burden is further exacerbated by high rates of HIV coinfection and a high prevalence of latent TB, which often reactivates due to factors such as immunosuppression.

The *Mycobacterium tuberculosis* complex consists of multiple lineages, each with distinct geographic distributions and genetic diversity. Common *M. tuberculosis* (Mtb) lineages include Lineage 1 (Indo-Oceanic), prevalent in countries around the Indian Ocean; Lineage 2 (East Asian), which includes the Beijing genotype; Lineage 3 (East African Indian), common in South Asia and parts of Africa; and Lineage 4 (Euro-American), which predominates in Africa, Central America, Europe, and South America.

In Malawi, various *Mtb* strains circulate, but their virulence characteristics remain inconclusive. Studies have demonstrated a diverse range of circulating strains, often geographically confined. The predominant lineage in Malawi is Lineage 4 (Euro-American), accounting for approximately 68% of cases. Lineage 2 (East Asian), including the Beijing genotype, is also present and has shown an increasing prevalence over time. Lineages 1 and 3 are less common but contribute to strain diversity.

Clinical isolates from Blantyre, Malawi, indicate that Lineage 4 is the major circulating strain, while Lineages 2 and 3 are more frequently found in the northern region of the country. This study recruited participants from Blantyre, where exposure to Mtb, particularly Lineage 4, is high which could affect the baseline T cell characteristics.

Study participants were recruited from the Voluntary HIV Counselling and Testing (VCT) and Antiretroviral therapy (ART) clinics at QECH. Participants volunteering to be part of the study provided written informed consent. The process of consenting and disseminating information to the participants was conducted in both Chichewa (local language) and (English). Participants were enrolled from the clinical groups discussed below:

1. Healthy HIV-uninfected individuals with no clinical and laboratory evidence of active respiratory disease. To fully understand perturbations caused by HIV in impairing T cell receptor (TCR) repertoire diversity and function, firstly, the composition of TCR repertoire in healthy HIV-uninfected individuals was determined. This formed the foundation to gain comprehensive insight into the effects of HIV on TCR repertoire.
2. Asymptomatic HIV-infected, ART-naïve (or within seven days of ART initiation) individuals (irrespective of CD4 T cell count) with no clinical evidence of active respiratory disease. This allowed analysis of the impact of HIV on the TCR receptor repertoire. In 2016, Malawi introduced the HIV test and treatment strategy, which involves initiating ART immediately upon HIV diagnosis irrespective of peripheral blood CD4+ T cell count or clinical stage (248). This policy, however, poses challenges in recruiting ART-naïve. For this study, PLHIV not on treatment or on treatment for <7 days were referred to as ART-naïve, and the terminology was used interchangeably.
3. Asymptomatic HIV-infected adults on ART for at least three years with no clinical evidence of active respiratory disease or progression of HIV infection. Plasma HIV viral load results were not available at recruitment for most participants but based on data from the HIV clinic at QECH, approximately 90% of adults on ART for three years have undetectable plasma HIV viremia whilst the remainder have persistent viral load. In this study, PLHIV on ART 3-years were referred to as HIV-infected on long-term ART.

2.4.1 Consenting and recruitment of study participants

Participant recruitment was conducted among potential participants attending the clinic by the study research nurses who had undergone training on the study protocol, Good Clinical Practice, and Human Subject Protection. Potential study participants were sensitised about the study, its aims and objectives. The research nurses disclosed all the information in the Participant Information Sheet, which included the purpose of the study, the possible benefits of their participation in the study, the potential risks that may arise, and their human rights. Additionally, the research nurses emphasised the importance of potential participants making an independent autonomous decision before participating in the study. Participants were made aware that they could withdraw from the study at any

time without any negative consequences to accessing standard medical care. Together, these measures ensured the safeguarding of the participants and the implementation of good clinical research practices. Interested participants had a private and detailed study discussion with the research nurses before consenting to join the study. Only those participants who met the inclusion criteria discussed in **(Table 2-1**) were recruited to take part in the study.

2.4.2 Inclusion criteria

Informed written consent for repeat HIV testing and bronchoscopy was obtained from individuals who voluntarily decided to participate in the study. Only participants with no clinical symptoms and signs of active disease or history of TB treatment in the previous 12 months, able to give informed consent, including consent for HIV testing and bronchoscopy, and physically fit to undergo bronchoscopy were recruited **(Table 2-1)**.

2.4.3 Exclusion criteria

Potential participants were excluded from the study if they were <18 years or >60 years old, febrile (≥37.5°C axillary) or unwell, current cigarette smoker or ex-smoker (<2yrs), significant anaemia (Hb<8 g/dl), severe thrombocytopaenia (platelet count <50 x 10^9/L), patients with arterial oxygen saturation (SaO_2) of <90% while breathing room air, those on corticosteroids, women with known or suspected pregnancy, those with diabetes mellitus, PLHIV on ART for >7days and <3 years, allergy to any medications likely to be used in the study, e.g. lignocaine, midazolam and flumazenil, those with COVID-19 and those with severe immunosuppressive disorder**(Table 2-1)**.

2.4.4 COVID-19 precautionary measures

Prior to recruitment, potential participants were screened and tested for SARS-CoV-2 using the gold standard SARS-CoV-2 qPCR. Similarly, CIU staff were tested for SARS-CoV-2 monthly to ensure participant and staff safety during sample collection. Bronchoscopy was performed on participants who tested negative for SARS-CoV-2 infection up to 72 hours after the initial test. If the potential

participant tested positive, they were advised to adhere to the Ministry of Health isolation policy. To ensure staff safety, Personal Protective Equipment (PPE) was provided during all participant recruitment and sample collection stages. The CIU suite was fitted with COVID-19 posters and signs that emphasised a safe working environment.

Table 2-1 Eligibility criteria for the participant study groups

Inclusion Criteria	Exclusion Criteria	Reason for Exclusion
1. Age 18 and 60 years, 2. Asymptomatic HIV-uninfected with no clinical symptoms or signs of active disease or history of TB treatment in the previous 12 months, 3. Asymptomatic, PLHIV ART-naïve or within 7 days of treatment onset, with no clinical symptoms and signs of active disease or history of TB treatment in the previous 12 months, 4. Asymptomatic PLHIV on ART ≥ 3 years with no clinical symptoms and signs of active disease or history of TB treatment in the previous 12 months 5. Able to give informed consent, including consent for repeat HIV testing and bronchoscopy, 6. Physically fit to undergo bronchoscopy.	1 Age <18 and elderly >60 years old 2 Inability or refusal to give informed consent. 3 Significant anaemia (Hb <8g/dl), 4 Severe thrombocytopaenia (platelet count <50 x 10^9/L), 5 Patients with arterial oxygen saturation (SaO_2) of <90% while breathing room air, 6 Known or suspected pregnancy, 7 Diabetes melitus, 8 Current cigarette smoker or ex-smoker (<2yrs), 9 TB treatment in the past 12 months prior to recruitment, 10 HIV-infected on ART for >7days and <3 years 11 Allergy to any medications likely to be used in the study, e.g., lignocaine, midazolam, and flumazenil 12 Active SARS-COV-2 infection	1 The focus was on young adults 2 Respect for individual's autonomy, 3 High risk of complications during bronchoscopy and BAL, 4 High risk of bleeding during bronchoscopy and BAL, 5 High risk of complications during bronchoscopy and BAL, 6 To avoid stressing the mother and the unborn child, as well as exclude the effect of pregnancy on the immune system, 7 To exclude the influence of cigarette smoke on lung function and immunity, 8 To reduce the incidence of bronchoscopy-associated complications.

2.4.5 Sample size and power calculation

The sample size was calculated based on previous studies demonstrating a limited TCR repertoire diversity in patients with severe active TB (249). Analysis of peripheral blood from these individuals showed that healthy controls had a normally diversified repertoire, whereas TB-infected patients were characterised with a restricted repertoire. To detect a 30% difference in the breadth and

functionality of the Mtb-specific TCR repertoire, and to characterise differences in clonality, diversity index, across the three groups of participants with 80% power and a 95% confidence interval, and a alpha level of 5% assuming a normal distribution of responses, the sample size for each group was determined to be 60. Participant numbers varied depending on the nature of the experimental panels conducted. The formula below was used for sample size calculation:

$$n = \frac{2 \times (Z_{\alpha/2} + Z_{\beta})^2 \times \sigma^2}{d^2}$$

Where:

- n = sample size per group.
- $Z\alpha/_2$ = the Z-score for the desired confidence level (for a 95% confidence level, this is 1.96).
- Zβ = the Z-score corresponding to the desired power (for 80% power, this is 0.84).
- σ^2 = the variance (square of the standard deviation).
- d = effect size.

2.4.6 Participant demographics

Although this study did not characterise the TB latency status of the recruited participants, it was conducted in an environment with repeated Mtb exposure, thus most of the recruited participants were considered to have Mtb-reactive T cells. Previous studies from our lab, including one by Nkhoma-Mitin et al., which recruited from the same cohort, have demonstrated a latent TB burden of approximately 43% in Malawian adults seeking HIV services. The basic demographic characteristics of participants are summarised in (**Table 2-2)**. Although the study did not reach the intended sample size, 82 adult participants were recruited. Of these, 43 were HIV-uninfected (20 females and 23 males, with an average age of 29), 15 were HIV-infected and ART-naïve (8 females and 7 males, with a mean age of 35), and 24 were HIV-infected and on ART (14 females and 10 males, with an average age of 38). All participants were asymptomatic, with no history of COVID-19 or recent airway infections, including TB.

Table 2-2 Participants demographics

Characteristics	HIV-uninfected (N=43)	HIV-infected, ART Naïve (N=15)	HIV-infected ART ≥3yrs (N=24)
Age(years), median (range), Mean	29 (21-52) 29	33 (21-58) 35	36 (26-55) 38
Gender Female Male	 20/43 (47%) 23/43 (53%)	 8/15 (53%) 7/15 (47%)	 14/24 (58%) 10/24 (42%)
CD4, median CD4 cells/ µl count (range), mean	694 (387-1256) 697	409 (116-1078) 557	536 (203-1418) 584
ART Duration(years), mean, (range)	N/A	N/A	7,(3-18)
Viral load copies/ml	N/A	Not detected 10/16 (63%) Detected 6/16 (37%) (<40-1.285x10^6 copies/ml)	Not detected 21/24 (88%) Detected 3/24 (12%) (<40-150 copies/ml)

2.5 Sample collection and processing

2.5.1 Bronchoscopy and bronchoalveolar lavage fluid

Bronchoscopy was performed as previously described by Jambo *et al.*(250) to collect bronchoalveolar lavage fluid (BAL) from participants who tested negative for SARS-COV2. Briefly, participants were asked not to eat or drink for at least 4hrs before bronchoscopy. Pre-bronchoscopy vital signs (blood pressure, heart rate, oxygen saturation and respiratory rate) were recorded. Topical lignocaine gel was then applied to their nasal mucosa while 2% lignocaine was applied to the pharynx, vocal cords and lower airways of participants. A fiber-optic bronchoscope (Olympus, UK) was passed to the level of a sub-segmental bronchus of the right

middle lobe **(Table 2-3)** 200 ml of sterile normal saline at 37 ^{o}C was instilled in four aliquots of 50 ml each and the BAL fluid was retrieved by gentle hand suction. The aspirated BAL fluid, mostly 80-150ml, was placed into 50ml falcon tubes and transferred to the laboratory on ice for processing within 30 minutes of collection **(Figure 2-1)**. Participants were advised not to eat or drink for 1hr after the procedure, and all were reviewed for any immediate complications before discharge. Participants returned for further review 72hrs post-bronchoscopy.

2.5.2 Bronchoalveolar lavage (BAL) fluid processing

The BAL fluid was filtered at the laboratory through a 100µm nylon cell strainer (Corning) to remove debris and mucus before pooling into new sterile 50ml falcon tubes. 5ml of unprocessed whole BAL fluid was stored at -80^{o}C. The remaining BAL fluid was centrifuged at 800g at 4^{o}C for 8 minutes; the supernatant was removed and stored at -80^{o}C. Next, cells were washed with 1X phosphate-buffered saline (PBS) and spun down at 800g for 8 minutes to obtain a cell-rich pellet. Following the wash, the cells were re-suspended in 5ml complete cell culture media (containing RPMI-1640, L-glutamine, penicillin/streptomycin and HEPES) (all from Sigma-Aldrich, UK) with 5% (vol/vol) Fetal Bovine Serum (ThermoFisher). The cell pellet was reconstituted and counted using the KOVA® disposable counting chamber under a bright field microscope (Olympus, Tokyo, Japan). Trypan blue diluted 1:10 1X PBS was used at a 1:1 ratio of cell suspension during cell counting to distinguish between non-viable and live BAL cells. For stimulation experiments, samples were seeded in volumes of 200µl at 1x10^{6} cells per well in 96 well plates and incubated at 37^{0}C in the presence of 5% CO2 **(section 2.5.1).**

2.5.3 Peripheral blood collection and processing

Blood was collected using a vacutainer system, and all aseptic techniques were observed. Blood was collected according to the samples required, and the following blood samples were collected: 4-mls in clot activated SST tubes for serum separation and 4-mls in each of 2 EDTA tubes for HIV rapid test, HIV viral load, full blood count and CD4 count. An additional 4-ml of blood was collected in 4 tubes containing 1ml each for the QuantiFERON®-TB Gold Plus (QFT-Plus) interferon-gamma release assay (IGRA) (Qiagen, Hilden, Germany) according to

the manufacturer's instructions. The tubes comprise a Nil tube (negative control), a Mitogen tube (positive control), a TB1 tube and a TB2 tube. The TB1 tube contains peptides that elicit Mtb-specific CD4+ T-helper cell responses, while the TB2 tube has peptides that elicit Mtb-specific CD4+ T helper cell and CD8+ cytotoxic T cell responses. The carefully labelled blood sample tubes were gently inverted 5-6 times to allow the anticoagulant or clot activator to mix well with the blood and transported in secure biohazard specimen transportation bags and containers to the MLW laboratories. In the laboratory, peripheral CD4+ counts were measured from EDTA tubes upon reaching the laboratory. The remaining sample from EDTA tube was spun at 2,000g for 10 minutes in a refrigerated centrifuge. Plasma samples from PLHIV was harvested and used for viral load testing.

Table 2-3 Consumables for sample collection

Name	Manufacturer	Product code
Venous blood sample collection tubes, Sodium Heparin, plastic	Becton Dickinson	KFK279
QuantiFERON®-TB Gold Plus (QFT-Plus) collection tubes	Qiagen, Hilden, Germany	622536
Venous blood sample tube, K2EDTA, Plastic	Becton Dickinson	KFK042
Lidocaine hydrochloride	Hameln Pharmaceutical	PL01502/0021R
50ml polypropylene falcon tubes	Corning	352070
50ml Syringe	BD plastic pack	300866
Neovac IV cannula with wings and injection port	Neovac	
BD Vacutainer Safety -Lock Blood Collection set with pre-attached holder (21g)	BD bioscience	368657
Syringe, 10ml hypodermic eccentric luer	Terumo	FWC068
Syringe, 50ml hypodermic eccentric luer	Terumo	613-3926
IV cannula	Biomatrix Healthcare	IT0101-20

2.5.4 Peripheral blood mononuclear cell Isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density centrifugation using Lymphoprep™ (Axis-shield, Norway) per the manufacturer's instructions. The procedure exploits differences in density between various leukocytes and the density of the gradient medium. Briefly, a whole blood sample was first diluted with an equal volume (1:1 ratio) of Phosphate Buffered Saline (PBS). The diluted blood was then layered on top of the Lymphoprep™ in a 50ml Falcon centrifuge tube and spun for 25 minutes at 800g with brakes off. Following centrifugation, due to their higher density, red blood cells and granulocytes passed through the media and settled at the bottom of the tube. In contrast, the mononuclear cells accumulated at the plasma-ficoll

boundary and were harvested using a Pasteur pipette. PBMCs were washed twice with PBS by spinning at 500g for 8 minutes and resuspended in cell culture complete media **(Table 2-4)**. The PBMCs were then counted using a hemocytometer and 0.1% trypan blue dye exclusion method to assess cell viability. The method measures the cell viability by assessing cell membrane integrity (251). It is based on the principle that viable cells have an integral cell membrane that inhibits penetration of dyes into the cells, unlike dead cells. Non-viable cells possess a compromised porous membrane, which allows intracellular staining and cells appear blue under the light microscope. Thus, dead cells take up the trypan blue stain and have a blue cytoplasm, while the live cells visually have a clear cytoplasm. The cell counts ranged from 9×10^6 to 15×10^6 cells per individual and were divided and seeded in a 96-well plate according to the experimental design.

Table 2-4 Consumables for tissue culture sample processing

Name	Manufacturer	Product code
Transfer Pasteur pipettes	Corning (Falcon)	357575
Nunc 1.8ml Cryovials	ThermoFisher Scientific	368632
15ml Polypropylene Conical Tube,17X20mm style, non-pyrogenic	Corning Science Mexico	352097
50ml polypropylene falcon tubes	Corning	352070
Cell culture plate, 96 well round	Costar	2721040
FACS tubes, polypropylene with lids,12X75mm	Greiner Bio-one (From SLS	GD115261
Gibco RPMI 1640 ™ 1X	Gibco	2192977
Trypan blue 0.4%	Gibco	15250-061
100µm nylon cell strainer	Corning	352360
Kovac Glasstic Slide with Grid	KOVA	87144
Hemacytometer		
EDTA Ultrapure 0.5M EDTA pH8.0	Invitrogen	15575-038
UltraPure™ DNase/RNase-free Distilled water	ThermoFisher Scientific	10977015
Phosphate buffered saline pH 7.2 (1X) 500ml	Gibco	20012-019
Hi-FBS	ThermoFisher Scientific	10-082-139
Pen/Strep	Fisher Scientific	MT30001CI
BD Golgi Stop™ Protein transport inhibitor (containing Monensin)	BD Biosciences	51-2097KZ (Sold as554724)
BD Golgi Plug™ Protein transport inhibitor (containing Brefeldin A)	BD Biosciences	B1-2301KZ(Sold as 555029)
Invitrogen™ Fixation Permeabilization Concentrate	eBioscience	5123-43
Invitrogen™ Fixation Perm Diluent	eBoscience	00-5223-56
Aqua fluorescent reactive dye	Le Technologies	1878891

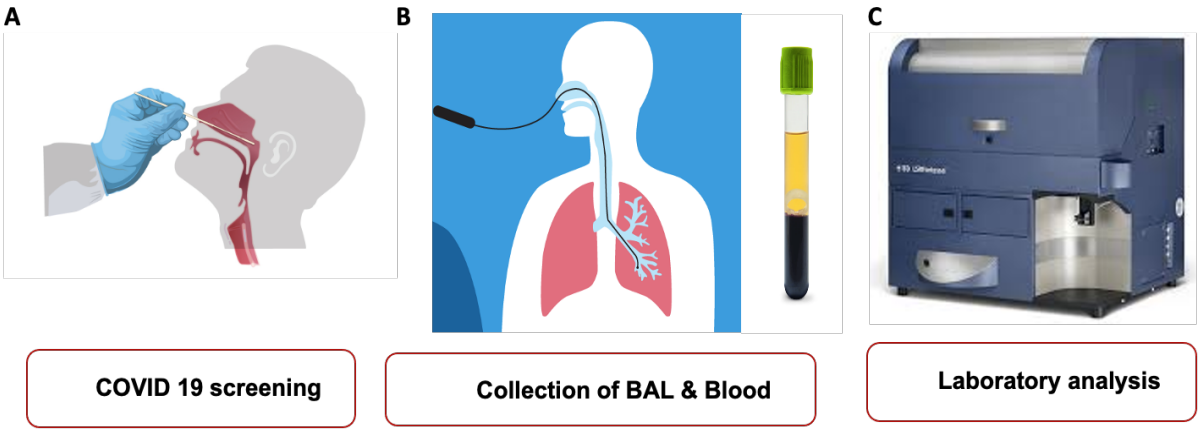


Figure 2-1 schematic representation of the methodology

(A) COVID-19 screening of potential participants (B), Collection of BAL and Blood and (C) Acquisition of samples on a flow cytometer. *(Created with BioRender.com)*

2.6 Laboratory experiments

This section details all the laboratory experiments and preparations of antigens used to address various research questions within the study. As summarised in **(Figure 2-2),** this project used two main techniques; Flow cytometry and TCR bulk sequencing which will be discussed in the subsequent sections.

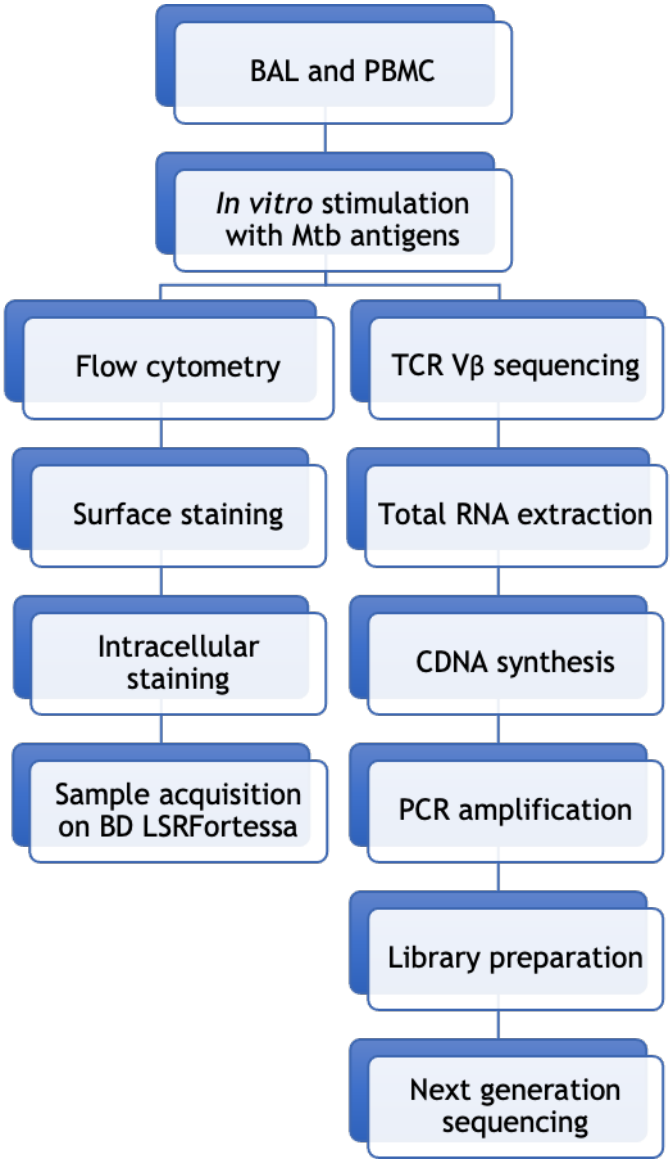


Figure 2-2 shows a flow diagram summarising laboratory techniques and procedures used in the project.

2.6.1 Antigens

2.6.1.1 Early secreted antigenic target-6 (ESAT-6)

A recombinant form of ESAT-6 (10 ug/ml) from Mtb CDC1551 strain (NR-49424, BEI Resources, USA) was used to stimulate the T cells. **(Table 2-5)**. A final working concentration was derived by diluting 1mg/ml stock solution with sterile PBS in a ratio of 1:4. ESAT-6 is a low molecular weight protein which members of pathogenic *Mycobacterium tuberculosis* complex, including Mtb, produce. Located within the RD1 (Region of Difference 1) loci, ESAT-6 is encoded by the *Esx* gene in the chromosomes of the pathogenic bacteria genome (20,252). The protein

sequence consists of amino acid residues 1 to 103, including a hexa-histidine tag at the C-terminus. It is then exported via the ESX secretion pathway, which interacts with major cluster differentiation (MHC) to activate the T cells, making it a potent antigen for T cell activation(253).

2.6.1.3 *Mycobacterium tuberculosis* whole cell lysate

Mtb lysate, strain CDC1551, whole cell lysate (NR-14823) obtained from Bei resources was prepared by diluting 10mg with sterile PBS in a 1:4 ratio to make a working concentration of 10ug/ml. Whole Mtb lysate refers to the preparation of bacterial proteins derived from the lysis of Mtb cells. The lysate, normally obtained by mechanical and chemical disruption, contains a complex mixture of proteins, lipids and carbohydrates present within the bacterial cell (254). Collectively, this provides a broad range of antigenic targets with the potential to elicit a diverse immune response. Additionally, the whole Mtb lysate contains cytoplasmic mixtures of antigens that are present in the natural infection. The antigens are key in developing anti-TB immune responses and may be extrapolated to clinical TB infection.

2.6.1.4 Phorbol 12-myristate 13-acetate (PMA) and Ionomycin

PMA and Ionomycin are often used synergistically to stimulate T cells to secrete cytokines. PMA is a potent compound that activates a set of protein kinases, including protein kinase C (PKC), a vital signalling factor in T cell activation (255,256). Interaction of PMA with PKCs stimulates a downstream signalling cascade that leads to activation of nuclear factor κB (NF-κB). Stimulation of NF-κB leads to the activation of transcription factors that regulate the expression of various cytokines, including TNF-α, IL-2, and IL-8, among others. Unlike other superantigens, PMA/Ionomycin diffuses into the cell and activates the PKC in a TCR-independent manner (257). A cell activation cocktail containing PMA/Ionomycin (1ug/ml) without brefeldin was used to induce non-specific T cell responses.

Table 2-5 Antigens for stimulation

Antigen	Manufacturer	Cat Number
ESAT-6 (CDC1551)	BEI Resources	NR-49424
CFP-10 (CDC1551)	BEI Resources	NR-14826
Mtb lysate Whole cell lysate (CDC1551)	BEI Resources	NR-14823
PMA/Ionomycin	BD Bioscience	

2.6.2 *In vitro* T cell stimulation

Processed BAL and PBMC samples were seeded in 96 well plates at 1 × 10^6 cells per well suspended in 200µl. To identify Mtb specific T cells, samples were stimulated with Mtb antigens discussed in **Section 2.6.1** above. All stimulation experiments were performed in sterile complete RPMI media with 5% each of Penicillin, Streptomycin and HEPES in 96 well plates. BAL and PBMCs were stimulated with 10 µg/ml Mtb whole cell, ESAT-6 10ug/ml, CFP-10 and PMA/Ionomycin 1 µg/ml for 6hrs detailed in **sections 2.6.1.1 and 2.6.1.2** respectively. Golgi plug (Transport inhibitor) and Monensin (1µl) (BD Bioscience, UK) were added at 6hrs, and the cells were incubated at 37^0C, 5% CO_2 for a further 12hrs.

2.6.3 Harvesting cells

Following stimulation, cells were harvested and transferred into well-labelled sterile FACS tubes. Briefly, cell suspension from each well in the plate was

transferred to the labelled FACS tubes. 0.5ml of cold PBS was added to the cells and pipetted up and down to remove all non-adherent cells. The process was repeated 3 times, and cells were washed with PBS, spun down for 8 minutes at 500g and later resuspended in 100µl PBS ready for staining.

2.7 Flow cytometry assays

Flow cytometry is a technique that analyses single cells as they flow past lasers while suspended in a fluid suspension. Cells scatter and emit light of specific wavelength as they pass through the laser beam. This allows multiparametric analysis of cells, including their physical and chemical properties. For example, the scattered light may be used to determine the size and granularity of the cells. The resulting data is then used to identify types of cells and quantify the number of cells expressing a particular protein or markers.

2.7.1 Cell surface staining

To characterise T cell phenotype and subsets, 50µl BAL and PBMC with 1 $X10^6$ cells were incubated with titrated and optimised fluorochrome conjugated antibodies: Anti-CD3 allophycocyanin-H7 (APC-H7) anti-CD4 BV605, anti-CD8 PerCP-CY5.5, anti-CD69 APC, anti-CD45RA FITC, and anti-CCR7 AF700 (all antibodies from BD Bioscience, UK) were used to characterise: naive ($CD45RA^+CCR7^+$), central memory ($CD45RA^-CCR7^+$), effector memory ($CD45RA^-CCR7^-$) and terminal effector T cells ($CD45RA^+CCR7^-$) detailed in **(Table 2-6)**. In addition, 2.5µl Violet viability dye (LIVE/DEAD® Fixable Dead Cell Stain kit, Invitrogen, UK) was used as per manufacturer's instructions to distinguish live cells from dead cells. The mixture was incubated in the dark at room temperature for 15 minutes. Cells were then washed by adding 1ml PBS and spun at 500g for 8 minutes. The supernatant was discarded, and the pellet was resuspended in 500µl of PBS for acquisition on BDLSR Fortessa flow cytometer.

Table 2-6 Flow cytometry panel for characterising T cell subsets (Differentiation, activation, and maturation)

Marker	Fluorochrome	Clone	Manufacturer	Cat. No	Rationale
CD3	APC-CY7	SK7	BioLegend	300318	T cells
CD4	BV605	SK3	BioLegend	344646	Helper T cells
CD8	PerCP-CY5.5	SK1	BioLegend	344710	Cytotoxic T cells
CCR7	AF700	G043H7	BioLegend	353244	Differentiating central and effector memory
CD69	APC	FN50	BioLegend	310910	T cell activation
CD45RA	FITC		BD Pharmingen	555488	Differentiating central and effector memory

Helper CD4 T cell subsets were identified using a panel of antibodies shown in **Table 2.6**. Briefly, fresh BAL and PBMC samples were resuspended in 50µl ($1x10^{6)}$ of PBS. Cells were stained with Anti-CD3 PerCP-CY5.5, anti-CD4 allophycocyanin-Cyanine H7 (APC-H7,) anti-CXCR3 BV605 to identify Th1 cells, anti-CRTH2 AF647 to identify Th2, CCR6 AF700 for Th17 and CD103 FITC to detect tissue resident cells. In addition, a combination of Anti-PD1 PE-CY7, CXCR5 BV421, and BCL-6 PE (all antibodies from BioLegend) was used to classify T follicular helper cells described in **(Table 2-7)**. Cells were stained with a cocktail of the appropriate antibodies and incubated for 15 minutes at room temperature in the dark. Cells were then washed with 1ml of 1X PBS and centrifuged for 8 minutes at 500g. The supernatant was decanted, and tubes were blotted on a paper towel. Next, 100 µl of Cyotofix/Cytoperm solution (BD Bioscience) was added to the cells and incubated for 20 minutes at $4^{O}C$. This enables fixation and permeabilisation of the cells to facilitate penetration of antibodies into the cytosol. Following incubation, samples were then washed in 500µl Perm/Wash buffer (BD Bioscience) and centrifuged at 500g for 8 minutes. The supernatant was discarded, and the pellet resuspended in 100µl of perm wash buffer. The cells were then stained with the antibody for the transcription factor BCL-6 and incubated for 30 minutes in the dark as per the manufacturer's instructions. Ultimately, cells were washed with

500µl of 1x Perm Wash buffer (BD Bioscience, UK) and resuspended in 500µl of PBS ready for Flow cytometry acquisition.

Table 2-7 Flow cytometry panel for the classification of CD4 helper T cell subsets (Th1, Th2, Th17 and Tfh)

Marker	Fluorochrome	Clone	Manufacturer	Cat. No	Rationale
CD3	PerCP-CY5.5	HIT3a	BioLegend	300328	T cells
CD4	APC-CY7	RPA-T4	BD Pharmingen	560251	Helper T cells
CXCR3	BV605	G025H7	BioLegend	353728	Th1
CRTH2	AF647	BM16	BioLegend	350104	Th2
CCR6	AF700	G034E3	BioLegend	353434	Th17
PD1	PE-CY7	EH12.2H7	BioLegend	329918	Tfh
CXCR5	BV421	J252D4	BioLegend	356920	
BCL-6	PE	IG91E/A8	BioLegend	648304	
CD103	FITC	Ber-ACT8	BioLegend	350204	Tissue-resident cells

2.7.2 Intracellular cytokine staining (ICS)

To detect cytokine-producing cells following stimulation, samples were fixed and permeabilised using 100µl of Cyotofix/Cytoperm solution (BD Bioscience) for 20 minutes at 4ºC, then washed in 500µl Perm/Wash buffer (BD Bioscience) and spun at 500g for 8 minutes. The supernatant was discarded, and the pellet resuspended in 100µl of Perm/Wash buffer. The cells were then stained with fluorochrome conjugate antibodies against IFN-γ, TNF-α and IL-2, detailed in **(Table 2-8)** (all BD Bioscience, UK) and incubated for 30 minutes in the dark at 4ºC. Lastly, cells were washed with 500µl of 1x Perm Wash buffer (BD Bioscience, UK) and resuspended in 500µl of PBS and acquired on BD LSRFortessa flow cytometer.

Table 2-8 Flow cytometry panel for identifying antigen-specific T cells

Marker	Fluorochrome	Clone	Manufacturer	Cat. No	Rationale
CD3	APC-CY7	SK7	BioLegend	300318	T cells
CD4	BV605	SK3	BioLegend	344646	Helper T cells
CD8	Percpcy5.5	SK1	BioLegend	344710	Cytotoxic T cells
IFN-γ	BV421	4S. B3	BioLegend	502532	IFN-γ producing cells
TNF-α	PE-CY7	MbA111	BioLegend	502930	TNF-α producing cells
IL-2	PE-DAZZLE	MQ1-17H12	BioLegend	500344	IL-2 producing cells
Aqua viability dye			Life Technologies	1878891	Live/Dead

2.7.3 Flow cytometry assessment of T cell receptor repertoire (Beta Mark Vß repertoire)

IOTest Beta Mark (Beckman Coulter) Multi-analysis Flow cytometry kit was used to analyse the TCR Vß repertoire in BAL and PBMC quantitatively. The kit is composed of 8 vials containing mixtures of conjugated TCR Vß antibodies corresponding to 24 different specificities, representing about 70% coverage of normal human TCR Vß repertoire. Each tube contains 3 antibodies conjugated to 2 different fluorochromes: one TCR Vß antibody is conjugated to FITC, another one to PE and the third to both FITC and PE, detailed in **(Table 2-9**).

To determine the TCR-Vß repertoire of the total T cells and Mtb-specific IFN-γ and TNF-α producing T cells, fresh alveolar Lymphocytes and PBMCs were stimulated with Mtb antigens and stained with CD3 APC-CY7, CD4 BV605, CD8 PerCY-Cy5.5 (all from BD bioscience) (summarised in **Table 2-9**) and a cocktail of contents IOTest Beta Mark's Tube A, Tube B, Tube C, Tube D, Tube E, Tube F, Tube G and Tube H, each conjugated with either PE, FITC and FITC+ PE. Following stimulation, cells were harvested and transferred to well-labelled FACs tubes (Labelled A -H). Cells were then pelleted by centrifugation at 500 for 8 minutes at 4^{o}C and resuspended in 100µl of PBS. Next, the cells were stained for extracellular markers and receptors of interest. Briefly, 20µl of contents from each Tube A, Tube B, Tube C, Tube D, Tube E, Tube F, Tube G and Tube H were added to the corresponding tube as per the manufacturer's instruction. In addition, titrated surface antibodies such as CD3 APC-CY7, CD4 BV605, and CD8 PerCP--Cy 5.5 (summarised in **Table 2-10**) were added to identify T cells. The mixture was incubated for 20 minutes in the dark, washed with 1 ml of PBS, and spun for 8 minutes at 500g. Lastly, samples were resuspended in 500µl of PBS and acquired on BD LSR Fortessa flow cytometer.

Table 2-9 List of TCR Vß antibody conjugates and the Nomenclature

Tube	Clone	Fluorochrome	V beta Wei Nomenclature	V beta (IMGT
A	3D11 ZOE CH92	PE FITC+PE FITC	VB 5.3 VB 7.1 VB 3	TRBV5-5 TRBV4-1, TRBV4-2, TRBV4-3 TRBV28
B	FIN9 E17.5F3.15.13 TAMAYA1.2	PE FITC+PE FITC	VB 9 VB 17 VB 16	TRBV3-1 TRBV19 TRBV14
C	BA62.6 IMMU157 ELL1.4	PE FITC+PE FITC	VB 18 VB 5.1 VB 20	TRBV18 TRBV5-1 TRBV30
D	IMMU222 JU74.33 56C5.2	PE FITC+PE FITC	VB 13.1 VB 13.6 VB 8	TRBV6-5, TRBV6-6, TRBV6-9 TRBV6-6 TRBV12-3, TRBV12-4
E	36213 MPB2D5 VER2.32.1.1	PE FITC+PE FITC	VB 5.2 VB 2 VB 12	TRBV5-6 TRBV20-1 TRBV10-3
F	AF23 BL37.2 IG125	PE FITC+PE FITC	VB 23 VB 1 VB 21.3	TRBV13 TRBV9 TRBV11-2
G	C21 IMMU546 CAS1.1.3	PE FITC+PE FITC	VB 11 VB 22 VB 14	TRBV25-1 TRBV2 TRBV27
H	H132 WJF24 ZIZOU4	PE FITC+PE FITC	VB 13.2 VB 4 VB 7.2	TRBV6-2 TRBV29-1 TRBV4-3

2.7.4 Flow cytometry data acquisition and analysis

PMT voltages were set using unstained cells and single stained compensation controls, ensuring no fluorescent overlap on other channels. Multiparameter flow cytometry data were analysed using FlowJo version 10 (Treestar). Briefly, lymphocytes were selected using Forward Scatter-Area (FSC-A)/Side Scatter Area (SSC-A) dot plot and single cells selected by FSC-A/FSC-H plot, removing doublets. Live cells and lymphocyte-specific cells were determined by excluding dead cells. CD4+ and CD8+ T cells were plotted from the CD3+ cells. Combinations of cytokine-producing cells were determined using Boolean gating in FlowJo.

Table 2-10 summary of the list of antibodies used for Flow cytometry assays

Marker	Fluorochrome	Clone	Manufacturer	Cat. No
CD4	BV605	SK3	BioLegend	344646
CD4	APC-CY7	RPA-T4	BD Pharmingen	560251
CD8	AF-700	Sk1	BioLegend	344724
CD8	PerCP-Cy5.5	SK1	BioLegend	344710
CD3	APC-CY7	SK7	BioLegend	300318
CD3	PerCP-Cy5.5	HIT3a	BioLegend	300328
CCR7	AF700	G043H7	BioLegend	353244
CD69	APC	FN50	BioLegend	310910
CD45RA	FITC	NA	BD Pharmingen	555488
CD103	FITC	Ber-ACT8	BioLegend	350204
CD1O3	PE-CY7	Ber-ACT8	BioLegend	350212
CXCR5	BV421	J252D4	BioLegend	356920
CCR6	AF700	G034E3	BioLegend	353434
CXCR3	BV605	G025H7	BioLegend	353728
CRTH2	APC	BM16	BioLegend	350104
PD1	PE-CY7	EH12.2H7	BioLegend	329918
BCL-6	PE	IG91E/A8	BioLegend	648304
IFN-γ	PE		BD Pharmingen	554701
IFN-γ	FITC	4S.B3	BioLegend	502515
IFN-γ	PE-CY7	4S.B3	BioLegend	502527
IFN-γ	BV421	4S.B3	BioLegend	502532
TNF-α	PE-CY7	MbA111	BioLegend	502930
TNF-α	PE	6401.1111	BD Fastimmune™	340512
IL-2	PE-DAZZLE	MQ1-17H12	BioLegend	500344
IL-17A	PE	BL168	BioLegend	512306
PD-1	APC-CY7	Eh12.2h7	BioLegend	329918
CD38	PE-DAZZLE	HIT2	BioLegend	303538
EOMES	Eff60	WD1928	Invitrogen	50-4877-42
Perforin	PE	B-D48	BioLegend	353303
Granzyme B	PE-CF594	GB11	BD Biosciences	562462
TBET	PE	eBio4B10(4B10)	Invitrogen	2196245

2.7.5 Statistical analysis

Statistical analyses and graphical presentations were carried out using GraphPad Prism 9 software (San Diego, CA). This allowed determination of the frequency and function of Mtb specific CD4 and CD8 T cells in both BAL and PBMC. Differences between groups were compared by ANOVA, where more than two groups are

indicated, as illustrated in figure legends. Results are given as mean with ranges for all data sets; p-value of ≤ 0.05 were considered statistically significant.

2.8 CDR3β sequencing

TCR VB CDR3 sequencing for BAL and PBMC samples was performed by a commercial vendor iRepertoire Inc. (Huntsville, AL, USA). Overall, TCR sequencing involved several processes which were performed both at MLW and iRepertoire laboratories, including RNA extraction, cDNA synthesis, PCR amplification, library preparation and next-generation sequencing (NGS) detailed below.

2.8.1 Total RNA extraction

Total RNA was extracted from PBMC, alveolar lymphocytes and distilled water (Negative control) using the Qiagen RNeasy Micro Kit (cat. no. 74004) per the manufacturer's instruction. The RNeasy Micro technology utilises a silica-based membrane with microspin technology to selectively bind RNA molecules longer than 200 nucleotides. Biological samples are first lysed and homogenised in a highly denaturing guanidine-isothiocyanate–containing buffer and ethanol to create optimal binding conditions. The RNeasy MinElute spin column is then used to isolate RNA by binding it to the silica membrane and removing traces of copurifying DNA through DNase treatment. Contaminants are then washed away, and high-quality total RNA is eluted in RNase-free water, as depicted in **(Figure 2-3)**.

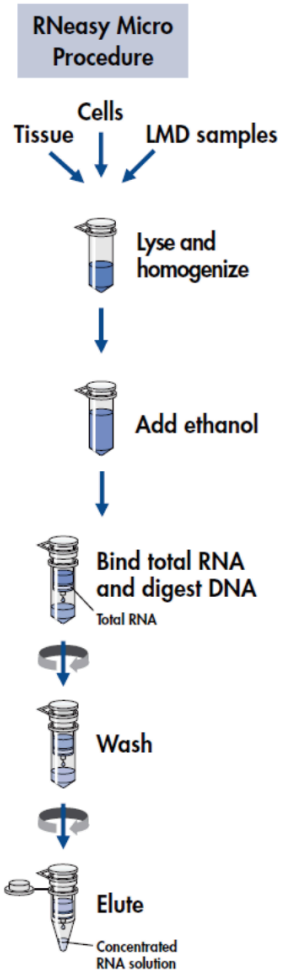


Figure 2-3 RNeasy Micro RNA extraction procedure modified from the RNeasy Micro RNA Handbook as provided by Qiagen.

To isolate RNA, BAL and PBMC cells (1x 10^5) were first treated with 350µl RLT buffer and vortexed for 30 secs and homogenised on a sample homogeniser to lyse and homogenise the cells. Cell lysis/disruption is a key step as it ensures the release of all the nucleic acid contained in the sample. Partial lysis often results in low RNA yield. Homogenisation, on the other hand, reduces the viscosity of the lysates produced during cell lysis. The process of homogenisation removes the high-molecular-weight genomic DNA of above 200 nucleotide and other high-molecular-weight cellular components to create a consistent lysate. Insufficient homogenisation often leads to ineffective binding of RNA to the RNeasy MinElute spin column membrane, resulting in significantly decreased RNA yields.

Following homogenisation, 1ml of 70% ethanol was added to the sample and mixed gently for RNA purification. The samples, including precipitates, were then transferred to an Rneasy MiniElute spin column in a 2ml collection tube, centrifuged for 15 seconds at ≥8000 x g and discarded the flow-through. Next, 350µl Buffer RW1 was added to the Rneasy MiniElute spin columns. Lids were closed gently, and the tubes were spun for 15 seconds at ≥8000 x g (≥ 10,000rpm) to wash the spin column membranes. The flow-through was discarded, leaving the RNA-bound membrane. Thereafter, the DNase 1 incubation mix was prepared by adding 10µl DNase I stock solution to 70µl Buffer RDD; the combination was mixed gently by inverting the tube. DNase incubation mix allows enzymatic digestion and removal of DNA traces in RNA samples. Following preparation, the DNase I incubation mix (80µl) was added directly to the RNeasy MinElute spin column membrane. The MinElute spin column membranes were then incubated at room temperature (20–30^{O}C) for 15 min.

Next, 350µl Buffer RW1 **(Table 2-11)** was added to the RNeasy MinElute spin columns. Lids were closed gently, followed by a quick spin of 15 seconds at ≥8000 x g. The collection tubes were discarded immediately after spin, and the RNeasy MinElute spin column was placed in new labelled 2 ml collection tubes. Next, 500µl of 80% ethanol (prepared using RNase free water) was added to wash the RNeasy MinElute spin columns. Lids were secured, and tubes were centrifuged for 2 minutes at ≥8000 x g. The spin columns were transferred to the new 2 ml collection tubes whilst the used collection tubes were discarded. The spin columns were then centrifuged at a high speed for 5 minutes with lids open to dry the membrane and to ensure no alcohol carried over during RNA elution. At this point, the spin columns were retained whilst the flow-through and the collection tubes were discarded.

Lastly, the spin columns were transferred to new 1.5 ml collection tubes. 14µl RNase-free water was then added directly to the centre of the spin column membrane. The lids were closed gently and centrifuged for 1 minute at full speed to elute the RNA. The isolated total RNA concentration and integrity were measured using Qubit (Invitrogen). The platform uses two fluorochrome dyes that

selectively bind to RNA molecules and emit fluorescence when excited by a light source of a specific wavelength. One dye binds to structured, large, intact RNA, whilst the other binds to small, degraded RNA. The fluorescence intensity is then correlated with the RNA concentration within the sample.

Table 2-11 Reagents for RNA extraction

RNeasy Micro Kit contents	Storage condition	Rationale
Buffer RLT	2-8^{0}C.	Cell lysis and homogenisation
Buffer RW1	2-8^{0}C.	Cell lysis and homogenisation
RNase free water	2-8^{0}C.	RNA degradation
RNeasy MinElute® Spin Columns	2-8^{0}C.	Selective column binding of RNA
Collection Tubes (2 ml)	15-25^{0}C	Collection of Flow-through
Collection Tubes (1.5 ml)	15-25^{0}C	Collection of the eluted RNA
RNase-Free DNase Set RNase-Free DNase I (lyophilised) Buffer RDD RNase-Free Water	2-8^{0}C.	DNA degradation
Ethanol	15-25^{0}C	RNA purification

2.8.2. TCR amplification and library preparation

Library preparation involved PCR amplification of the TCRVβ region, cleaning the amplified products, quantifying the PCR products, library clean-up, pooling the PCR products into libraries, pre-sequencing Quality Control and illuminating sequencing, as indicated in **(Figure 2-4).**

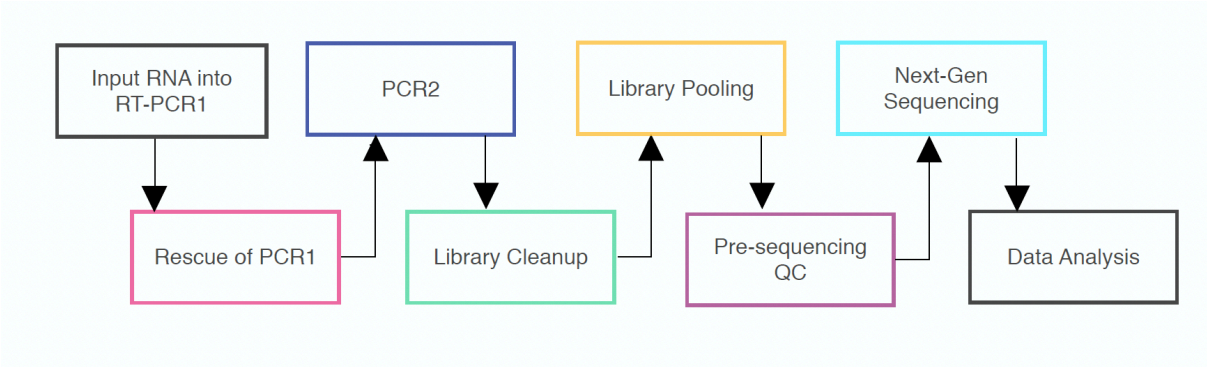


Figure 2-4 shows a flow diagram of an overview of steps for TCR amplification, library preparation and sequencing (adapted from the iRepertoire RNA dual index amplification user manual).

2.8.2.1 TCR PCR amplification

The arm-PCR (amplicon rescued multiplex PCR) technology (iRepertoire, Patent No. 7,999,092) was used to amplify the TCR immune repertoire. The technique utilises two-step PCR reactions: PCR1 synthesis, which involves reverse transcription to synthesise the cDNA, and PCR 2 for library preparation. Products of PCR1 amplification are used as starting material for the second round PCR, which involves the addition of dual index primers and unique molecular identifiers (UMIs) detailed in **(Figure 2-5).** In the first round of PCR, specific nested gene primers are used to target V and either -C or -J genes (depending on whether the sample is gDNA or RNA compatible) in a primer system-dependent manner. The forward primers (Fo and Fi) are in the V genes, while the reverse primers (Ro and Ri) are in either the -C or -J genes and include partial sequencing adaptors B and A for the Illumina platforms. The Ri primers additionally contain barcodes between sequencing primer A and the -C or -J gene-specific primers. The second round of PCR employs dual index adaptor primers B and A, which correspond to a specific dual index pair. After magnetic bead clean-up or gel purification, the product is ready for high throughput sequencing with Illumina's next-generation sequencing platforms without requiring any additional enzymatic steps. The first round of PCR introduces an internal sample barcode and partial sequencing primers into the PCR products, while the exponential phase of the amplification is achieved by the dual index Illumina primers in the second round of PCR, resulting in even and semi-quantitative amplification of the entire repertoire.

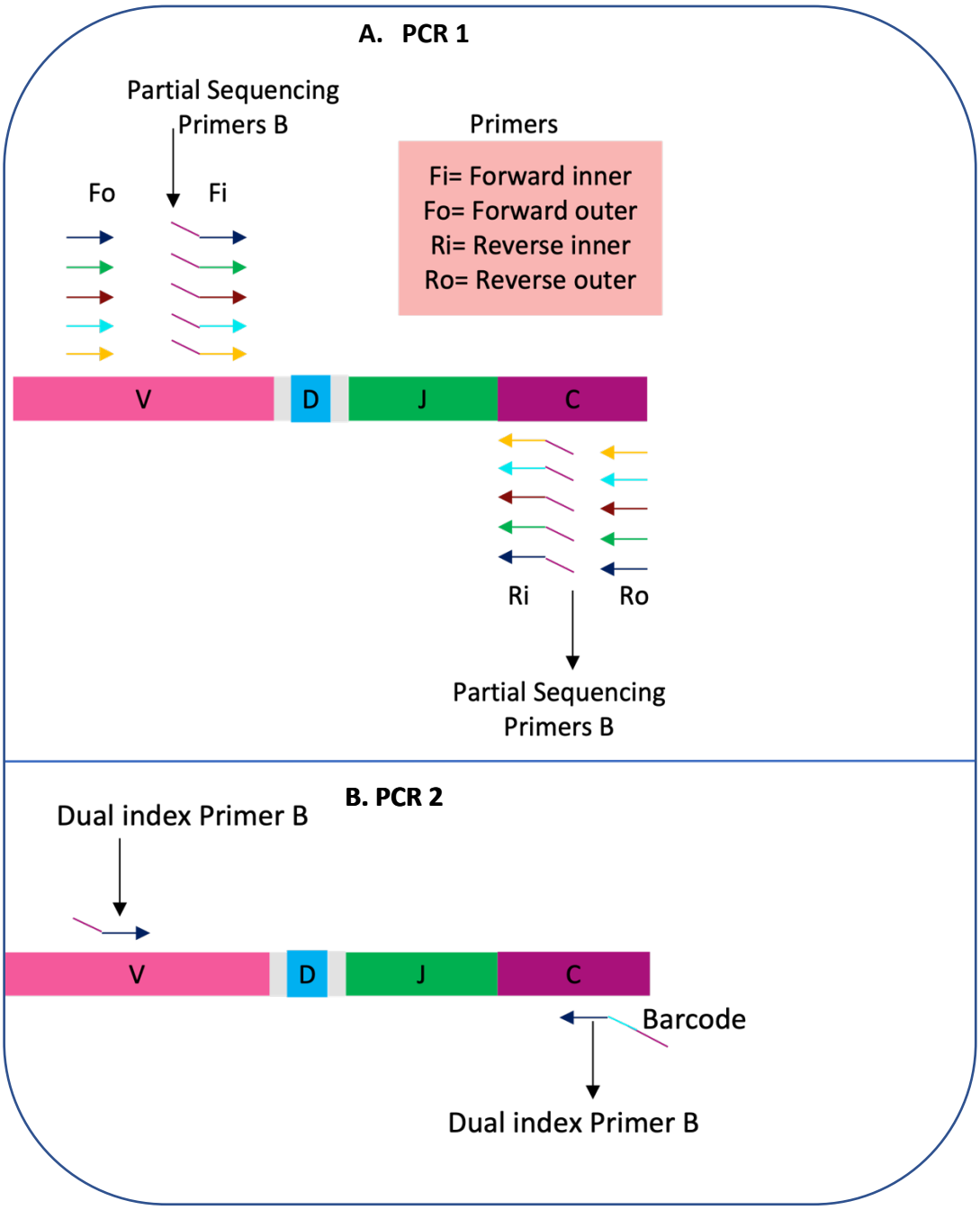


Figure 2-5 Illustrates the basic principle of the arm PCR Technology

A. RT-PCR is performed using multiplex primers that attach to V-genes. This is followed by the addition of internal adaptors at the priming sites. **B.** In the second round of PCR, external dual index primers are introduced during the exponential phase, leading to the complete amplification of the TCR repertoire. (Modified from the iRepertoire RNA dual index amplification User manual).

2.8.2.2 Procedures for PCR 1

Reverse transcription amplification of the extracted total RNA from BAL and PBMCs samples was performed according to the manufacture's instruction (iRepertoire), including nuclease-free water as a negative control and a provided control RNA sample (Positive control). Before amplification, 2µl (~ >50ng/ml) of the template was added to the 11.25µl nuclease-free water to make a 13.25µl template-water mix. Next, a Master mix (MM) was prepared for 12 samples (run in a batch) by adding iR-RT-PCR1 Buffer Mix (86.25µl), iR-RT-PCR1 Enzyme mix (13.8µl), iR-RT-PCR1 Primers (55.2µl) detailed in **(Table 2-12).** A 15% extra volume of each component of the MM was added to account for pipetting errors. A total of RT-PCR 1 MM of 155.25µl was aliquoted in a 1.5 ml sterile microcentrifuge tube. Thereafter, 11.25µl of the freshly prepared RT-PCR 1 was added to each of the well-labelled PCR Tubes. To each tube, 14µl of the template—water mix and a negative control—were added and mixed gently to avoid the generation of bubbles. The tubes were then vortexed and centrifuged using a mini spin to remove air from the air bubbles. The final PCR 1 volume of 25µl per reaction was loaded on the Thermocycler with the conditions indicated in **(Table 2-13)**.

Table 2-12 RT-PCR 1 Reaction Master Mix set up for 12 samples.

RT-PCR1 Master Mix (Reagents)	Amount (µl)/Reaction	Master Mix (µl)	Number of Reactions
iR-RT-PCR1 Buffer Mix	6.25	86.25	12
iR-RT-PCR1 Enzyme mix	1	13.8	12
iR-RT-PCR1 Primers	4	55.2	12
Template- water mix	13.75 (Added separately)		
Total Reaction volume	25	Total MM = 155.25µl	
		For each tube = 11.25µl	

Table 2-13 RT-PCR TCR Thermocycler conditions

Step	Temperature (^{0}C)	Rationale	Time	# Cycles
1	50 90	Initial denaturation and activation of Taq Polymerase	60min 15min	1 1
2	94 60 72	Cycling (Denaturation, Annealing & Extension)	30 sec 5 min 45 sec	 10
3	94 72	Exponential amplification	30 sec 3 min	10
4	72 4	Final extension and stabilisation	15min Infinity	1 Hold

2.8.2.3 Procedures for RT-PCR1 product rescue

Following the PCR 1 run, products were recovered using the selection beads, which bind to cDNA. Briefly, 25 nuclease-free water was loaded into each well of the new PCR strip. To these wells, 25µl of the RT-PCR products and 35µl of the selection beads (Human Long Read) were added. This was then incubated for 3 minutes at room temperature. This was followed by bead clean-up using the magnetic strip. Upon incubation, the strips were put on the magnetic strip stand for 3 minutes to allow bead collection until a clear supernatant. The supernatant was then discarded, and 160µl of 85% ethanol was added to the strips whilst on the magnetic strand and incubated for 5 minutes to wash off the beads. Finally, the supernatant and residual ethanol were discarded, and the beads were left to dry.

2.8.2.4 PCR 2 (library preparation)

A Master Mix (MM) for 12 well reaction PCR 2 was prepared by adding 345µl of iR-RT-PCR2 mix and 234.6µl of nuclease-free water. In addition, 8µl of each dual index primer were added to the PCR strip tubes matching with the internal

barcodes to ensure unique labelling. The PCR 2 MM was then vortexed and spun down in a mini spin. Thereafter, 42µl of PCR 2 MM was transferred into each strip tube to make a final PCR reaction volume of 50 µL. To the dried RT-PCR1 beads discussed in **section 2.8.2.3**, 50µl of the MM was added. Lastly, the PCR strips were loaded on the thermocycler with the conditions listed in **(Table 2-14)**.

Table 2-14 PCR 2 TCR Thermocycler conditions

Step	Temperature (^{0}C)	Time	# Cycles
1	95	15 min	1
2	94 72	30 sec 2 min	30
3	72	5 min	11

2.8.2.5 Library clean-up

PCR library clean-up is a procedure that is used to remove impurities such as primers, nucleotides, and other contaminants from a PCR product. The bead method described in **Section 2.8.2.3** was used to rescue the PCR2 products. 35µl of well mixed beads was transferred into new PCR strips. Upon completion of the PCR 2 reaction, the strips were put on the magnetic strip stand for 5 minutes until a clear supernatant was to enable the collection of beads. The library-rich supernatant was then added to the strip containing the selection beads and mixed thoroughly by pipetting evenly. This was followed by a 5-minute incubation of the beads at room temperature. After incubation, the strips were put on a magnetic stand for 5 minutes to allow sedimentation and re capture of the library template to the beads. After the 5-minute incubation, liquid aspirates from the strips were discarded and 160µl of freshly prepared 85% ethanol was added whilst the PCR strips were still on the magnetic strand.

Once the beads were separated from the supernatant, the supernatant was carefully discarded, and the remaining alcohol was removed. The PCR strips were

then removed from the magnetic stand. Afterwards, the bead pellets were left to air dry in the hood at room temperature for 5 minutes. Once air-dried, the library template was then eluted from the beads. To each bead pellet, 37µl was added and gently mixed by pipetting up and down to resuspend the beads. The beads were then vortexed and spun down on a mini spin. This was followed by a 2-minute incubation of the beads with nuclease-free water. The strips were later placed back on the magnetic strip for 3 minutes or until the supernatant had cleared to allow the collection of beads **(Figure 2-6).** Finally, the clear supernatant containing the amplified library was transferred to the new set of well-labelled PCR strips ready for sequencing.

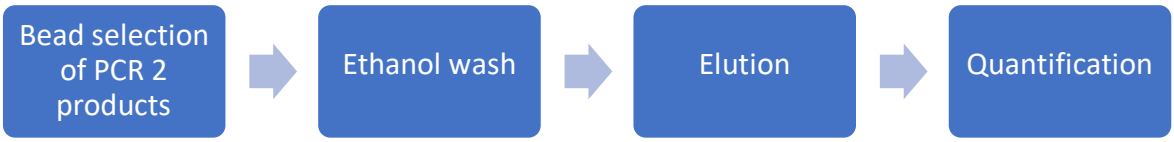


Figure 2-6 Illustrates a summary of major steps of library clean-up

2.8.2.6 Library quantification

Analysis of the cleaned amplified DNA concentration is crucial in determining the actual volumes to be pooled together to form a uniform library. The size and quality of the cleaned amplified PCR products were determined using Tape Station (Agilent 2200 Tape Station system, High sensitive D1000). A Tape station is an automated electrophoresis system that utilises precast gel cartridges and an electrophoresis instrument to measure the quantity and size of the library to ensure equal distribution of the amplicon before sequencing (258,259). Briefly, a ladder (positive control) was prepared by pipetting 2µl high sensitivity D1000 sample buffer and 2µl high sensitivity D1000 ladder. The mixture was loaded on position A1 in a tube strip. Next, the sample buffer and DNA sample, 2µl each, were mixed and vortexed on a KA vortexer for 1 minute. The samples and the ladder were then centrifuged at 2,000 rpm for 1 minute and later loaded on a tape station machine for analysis. A run was considered a success if the concentration of the amplicons were greater than, or equal to, 10 ng/µl when eluted in 37µl of nuclease-free water; otherwise, anything less than or equal to 4 ng/µl was taken as unsuccessful.

2.8.2.7 Library pooling

Uniquely barcoded amplified samples were pooled in an equivalent equimolar amount to ensure a homogenised representation of the samples. This enabled sequencing data to be tracked back to a specific sample. To determine the sample volume required to be pooled together for the library, the highest and lowest sample concentrations in the library were determined; these were used to decide the amount of DNA from each sample to be added to make up the final equimolar mix. The required volume for each sample was calculated using the following formula: Volume (µl) = Total DNA in the most concentrated sample (ng)/individual sample concentration (ng/µl). Frozen pooled samples were shipped to iRepertoire Inc. on dry ice and sequenced using the Illumina Next seq platform.

2.9 Data analysis

Basic data analysis was conducted by iRepertoire (Huntsville, AL, USA) and data was available on iRepertoire's data web viewer iRweb. Raw sequencing reads underwent initial quality control by the sequencing service provider (iRepertoire inc), with filtering based on Phred quality scores (Q scores) to retain high-quality reads. The Phred score is calculated as $Q = -10 \log_{10}(e)$, where e represents the probability of an incorrect base call. Higher Q scores indicate a lower probability of sequencing errors, while lower Q scores suggest a higher likelihood of unusable reads. Reads with a mean Q score below 30 (Q30), corresponding to a base call accuracy of 99.9%, were discarded to minimize sequencing errors. Quality assessment was performed using FastQC, and further processing, including adapter trimming and removal of low-quality bases, was carried out using BBduk. Only reads meeting the Q30 threshold were retained for downstream analyses to ensure high-confidence variant calling and transcript quantification. Data was also prepared for further analysis using MiXCR as described by Bolotin *et al*. (260) and analysed using the tcR R package.

2.9.1 Preparation of sequencing data by MiXCR

The sequencing data was downloaded onto Malawi Liverpool Wellcome Research Programme servers. The reads were checked for quality using FastQC software and the poor-quality reads were trimmed using the BBDduk tool using the following code:

```
bbduk.sh threads=8 in=SAMPLE_R1.fastq.gz in2=SAMPLE_R2.fastq.gz out=bbduk_fastqs/SAMPLE_R1_bbduk.fastq.gz out2=bbduk_fastqs/SAMPLE_R2_bbduk.fastq.gz ktrim=r k=23 mink=11 hdist=1 tbo tpe qtrim=r trimq=20 minlength=50
```

The trimmed reads were prepared for analysis using MiXCR using the following Linux code:

```
mixcr analyze iRepertoire-human-rna-xcr-repseq-sr SAMPLE_R1_bbduk.fastq.gz SAMPLE_R1_bbduk_mixcr
```

This code aligns, assembles, and creates. clns (clone) files. .clns and .txt files for subsequent analysis using the Immunarch R package.

2.9.2 Analysis of sequencing files with the tcR R package

The Immunarch R package is uniquely made to analyse sequencing data generated from different software including the MiXCR software. The package was used in the R statistical software framework and the code detailed below was used for visualising and analysing TCR sequencing data.

2.9.3 Arrangement of data

Individual text files were compiled into a list of data frames from their directory using the code below:

```
#path to the folder with data
```

```
file_path <- paste0(system.file(package="immunarch"), "/Tcer_data/io/")
Tcer_data <- repLoad(file_path)
```

2.10.4 Top proportion charts

The following code was used to generate figures showing the clonal proportion

```
Tcer_top <- repClonality(Tcer_data$data, .method = "top")
Tcer_top
```

2.9.5 Heat maps showing CDR3β Sequence Overlap

```
Tcer_overlap <- repOverlap(Tcer_data$data, .method = "public", .verbose = F)
vis(Tcer_overlap)

```

2.10.6 Plots showing kmer profiles

The following code was used to identify kmers

```
Kmers <- getKmers(Tcer_data$data, 5)
kmer_profile(Kmers,, “self”) %>% vis()

```

2.9.7 Plots showing spectra types of the amino acids

```

vis(spectratype(Tcer_data$data[[1]], .quant = "count", .col = "aa+v"))
```

Chapter 3 Phenotypic profiling of Mtb-specific cytokine-producing CD4 and CD8 T cells in the lungs of people living with HIV infection.

3.1 Introduction

Human immunodeficiency virus (HIV) infection predisposes individuals to active TB, resulting in a global HIV/TB coinfection epidemic that disproportionately affects individuals in resource-limited regions with high prevalence of HIV infection, particularly Sub-Saharan Africa (SSA) (261). People living with HIV (PLHIV) are more susceptible to lower respiratory tract infections, including active TB, compared to those who are HIV-uninfected (262). In regions with a high prevalence of TB, such as SSA, most TB cases among PLHIV are due to recent infections rather than the reactivation of pre-existing *Mycobacterium tuberculosis* (Mtb) infections (263). However, the risk of developing TB disease increases throughout the course of untreated HIV infection (261,264), and it is estimated to be over 20-fold higher in PLHIV than in a cohort without HIV -1comorbidity (265).

The increased susceptibility to TB in HIV-infected individuals suggests an altered lung immune function that occurs as a result of HIV infection. Previous studies, including those from our laboratory, have demonstrated that HIV persists within the lung in asymptomatic PLHIV, even after immune reconstitution due to effective long-term ART (250,266). Collectively, this indicates that HIV impairs pulmonary immunity, rendering the host vulnerable to respiratory opportunistic infections, including TB.

Progressive loss of CD4 T cells and increased susceptibility to opportunistic infections, including TB, are the hallmarks of HIV infection (267,268). HIV is associated with impairment of host adaptive immunity by depleting and impairing T cell function, particularly the CD4 T cells (265,269). Mtb-specific T cells play a crucial role in controlling Mtb infection. Both CD4 and CD8 T cells produce effector cytokines such as IFN-γ, TNF- α and IL-2, which are critical in activating microbicidal functions of alveolar macrophages responsible for controlling the

progression of intracellular infections (270). Early murine studies and evidence from HIV infection in humans have established the integral role of T cells in the control of Mtb infection. In one study, Caruso *et al*. reported that CD4 T-cell-depleted mice had a higher risk of developing active TB compared with age-matched controls (271). Similarly, HIV-infected individuals with low CD4 T cell counts and impaired CD8 T cells in peripheral blood showed substantial susceptibility to developing TB disease (13), indicating that HIV triggers a massive alteration in these cell subsets.

3.2 Chapter aims and summary.

Although the role of CD4 and CD8 T cells has been well described, the impact of HIV on Mtb antigen-specific T cell cytokine production and how that affects host susceptibility to TB disease has been poorly characterised. Moreover, there is a limited understanding of the role of different helper CD4 T cell subsets in mediating immune response against TB. This chapter assesses Mtb antigen-specific CD4 and CD8 T cell cytokine production in the host's immune response to mycobacterial antigens. It further investigates whether HIV infection is associated with depletion and impairment of Mtb-specific CD4 and CD8 T cells in both lung and peripheral blood.

Here, flow cytometry and Mtb in-vitro stimulation assays were used to probe the impact of HIV infection on Mtb-specific CD4 and CD8 T cell responses in BAL and PBMCs (**Chapter 2, section 2.6 & 2.7).** Peripheral blood and bronchoalveolar lavage (BAL) samples were collected from PLHIV who were ART-naïve, on ART and HIV-uninfected adults recruited at Queen Elizabeth Central Hospital, Blantyre, Malawi. Alveolar and peripheral blood lymphocytes were stimulated with Mtb antigens and analysed for cytokine response using flow cytometry. The number of sample groups varied depending on the nature of the experiment and the type of analysis conducted to address specific research questions.

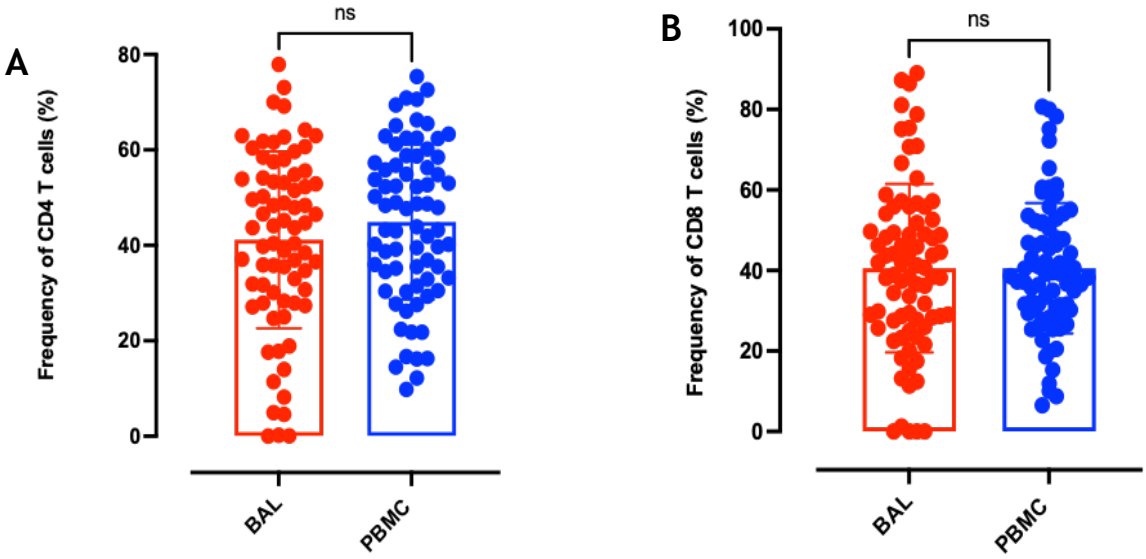
Overall, the findings reveal a low frequency of Mtb-specific CD4 T cells and CD8 lymphocytosis in both blood and lung in HIV-infected individuals. Furthermore, there is a preferential depletion of a subset of Mtb-specific CD4 and CD8 IFN-γ producing T cells in PLHIV in both BAL and PBMCs compared to HIV-uninfected

individuals. These findings suggest altered lung immune function following HIV infection, potentially contributing to increased TB susceptibility in PLHIV.

3.3 Results

3.3.1 HIV infection is associated with the depletion of CD4 T cells and alveolar CD8 T lymphocytosis in BAL and PBMC

To test the hypothesis that HIV infection alters immune cell composition in the airway, BAL and blood samples were obtained from healthy HIV-uninfected and asymptomatic PLHIV adults either on ART or ART naive. Subsequently, flow cytometry was used to measure the frequency of CD4 and CD8 T cells in both Mtb stimulated and unstimulated samples. There was no notable difference in the percentage of CD4 T cells between BAL and PBMC in a mixed population regardless of HIV status **(Figure 3-1 A)**. Similarly, the proportion of CD8 T cells was comparable in both BAL and PBMCs **(Figure 3-1 B**). Following this, we assessed the impact of HIV infection on the frequency of CD4 and CD8 T cells in the lung and PBMCs. As expected, the frequencies of CD4+ T cells were significantly lower in both PBMCs and BAL of PLHIV both on ART and those not on treatment compared to HIV-uninfected individuals ($P< 0.003$) ($P< 0.009$) respectively **(Figure 3-1 C)**.In addition, the frequencies of CD4 T cells were comparable between PLHIV treatment naïve and those on treatment in both compartments. However, this was higher in HIV-uninfected individuals compared to PLHIV on ART in both PBMC ($P< 0.0011$) and BAL ($P< 0.0011$) **(Figure 3-1 C)**. Furthermore, the percentage of CD8+ T cells was higher in both BAL and PBMCs of PLHIV, both not on ART and those on ART compared to HIV-uninfected individuals **(Figure 3-1 D)**. These findings align with previous results reporting CD8 T cell lymphocytosis in HIV-infected individuals (232,233). Taken together, these findings suggest that HIV infection causes significant perturbations in CD4 and CD8 T cells in peripheral blood but not in the alveolar compartment.


A
Frequency of CD4 T cells (%)
80
60
40
20
0
ns
BAL
PBMC
B
Frequency of CD8 T cells (%)
100
80
60
40
20
0
ns
BAL
PBMC

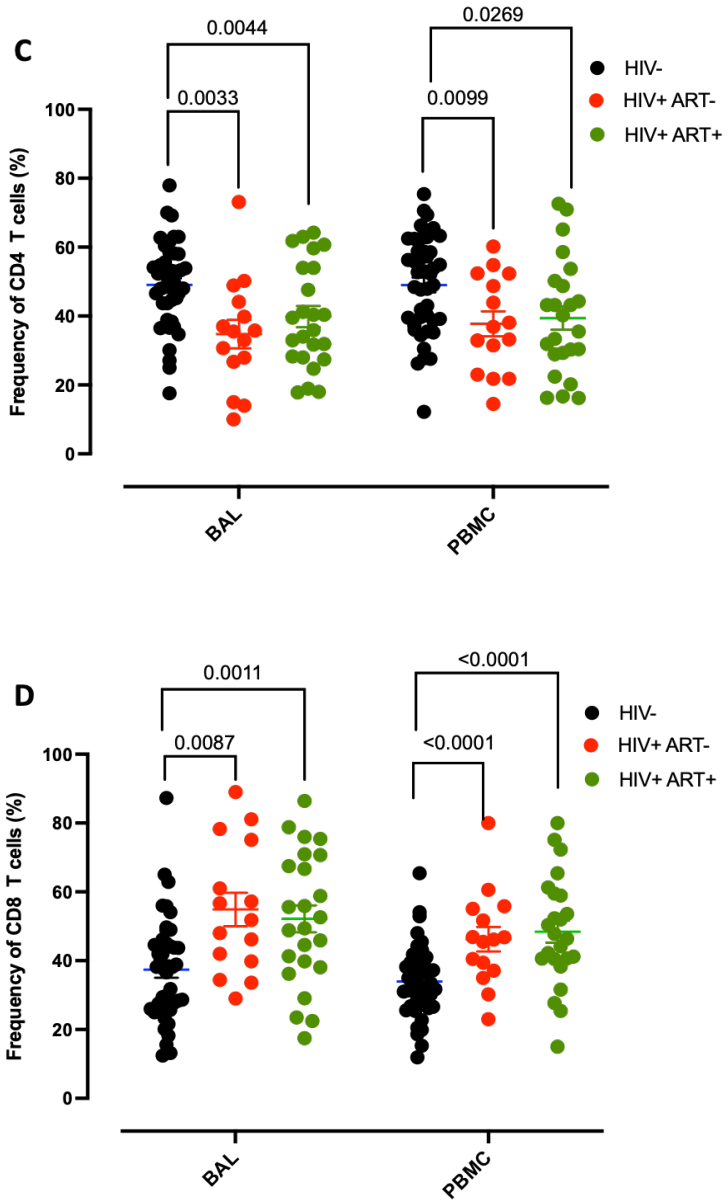


Figure 3-1 Lower frequencies of CD4+ T cells and higher frequencies of CD8+ T cells in BAL and PBMCs of HIV-infected individuals compared to HIV-uninfected individuals.

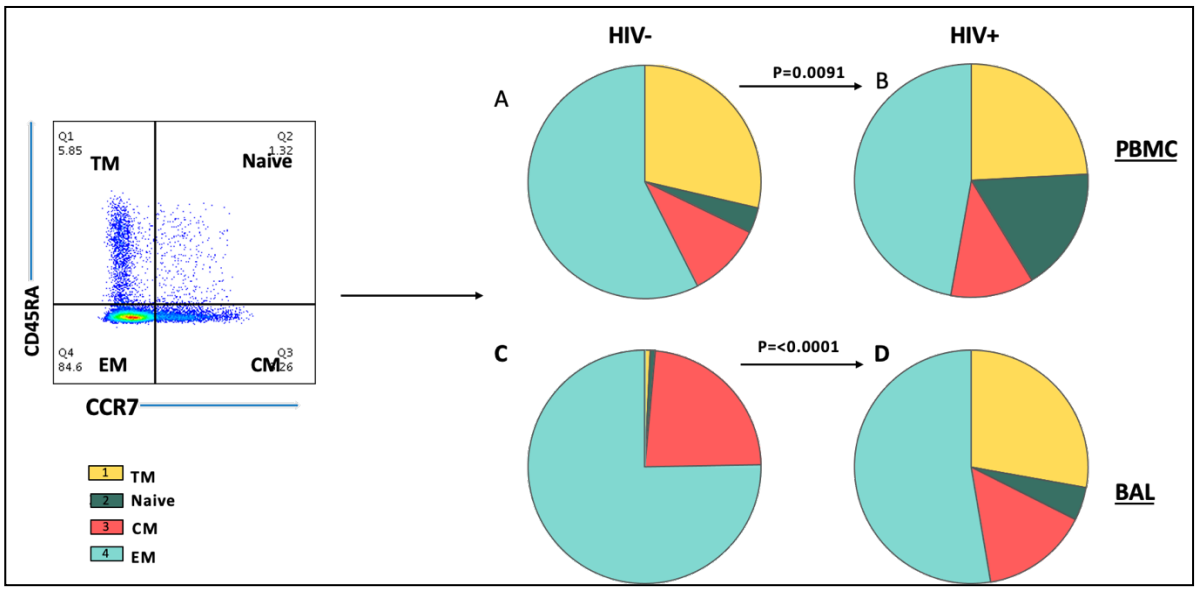
Alveolar and peripheral T cells were stained with fluorochrome-conjugated antibodies. (A) Frequency of CD3+CD4+ and (B) CD3+CD8+ T cells in BAL fluid and PBMC (n=82). (C) Lower frequencies of Frequency of CD3+CD4+ in both HIV+ART- and HIV+ART- compared to HIV- (C). A higher frequency of CD8+ T cells in BAL and peripheral blood of HIV-infected individuals both ART+ and ART naive compared to HIV-uninfected adults **(D)** Each data point represents an individual, and the horizontal bars show the mean SEM of the group. Data were analysed using Mann Whitney U test, and where necessary, one-way ANOVA was used, followed by Tukey's multiple comparison test (*: P< 0.05) where * means statistically significant. Non statically significant comparisons are not show on the graphs (HIV- n=43, HIV+ART- n=15 and HIV+ART+ n=24).

3.3.2 The proportion of naive and memory T cell subsets varies between BAL and peripheral blood and undergoes changes during HIV infection.

To assess the impact of HIV infection on T cell subsets, CD45RA and CCR7 expression on CD4 and CD8 T cells were measured in BAL and PBMC samples. A Boolean gating strategy was used **(inserted in Figure 3-2)** to identify the following subsets: naive (CD45RA+CCR7+), central memory (CD45RA-CCR7+), effector memory (CD45RA-CCR7-), and terminally differentiated memory T cells (CD45RA+CCR7-). Intriguingly, the majority of CD4 and CD8 T cells in both BAL and PBMC samples exhibited an effector memory phenotype (CD45RA−CCR7−). Both BAL and PBMC CD4 and CD8 T cells were predominantly of effector memory phenotype ($CD45RA^{-}CCR7^{-}$) followed by terminal effector T cells (CD45RA+CCR7-) **(Figure 3-2 (i) & (ii) A-D)**. This observation is in contrast with existing studies by Jambo *et al.*, who reported a higher proportion of effector memory cells in BAL CD4 and CD8 T cells than in peripheral blood (272). This inconsistency could be attributed to differences in markers used to identify these cell subsets as well as differences in the cohort of participants. The predominant presence of effector memory cells in the lung mucosa was not surprising as the respiratory tract is in close proximity to the external environment with constant exposure to pathogenic organisms and normal flora, which would drive recruitment and retention of activated cells (266,273).

Next, we investigated alterations in effector memory subsets following HIV infection. HIV-infected individuals exhibited decreased proportions of CD4 and CD8 effector memory phenotype subsets in both compartments compared to HIV-uninfected individuals.

(i) CD4 T cells



(ii) CD8 T cells

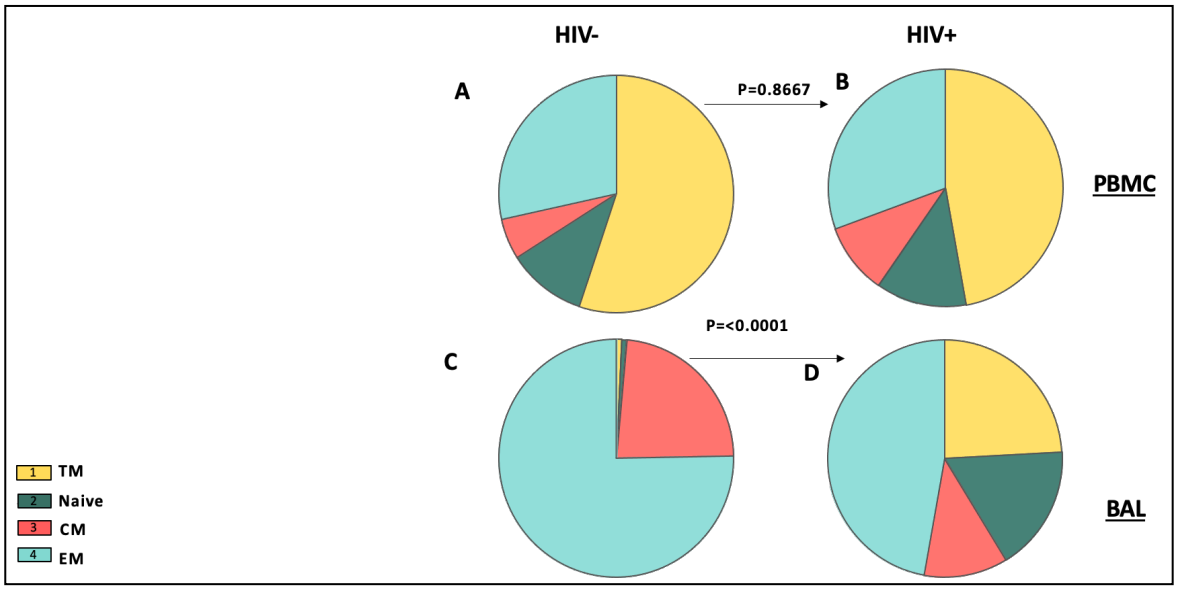


Figure 3-2 **HIV is associated with depletion of CD4 effector memory T cells in the lung**

T lymphocytes obtained from BAL and peripheral blood were stimulated with Mtb antigens and stained with anti-CD3, anti-CD4, anti-CD8, anti-CD45RA and anti-CCR7 antibodies to investigate the impact of HIV on different T cell memory subsets. (A) A representative flow cytometry plot showing the gating strategy for terminal effector (CD45RA+CCR7-), naïve (CD45RA+CCR7+), central memory (CD45RA-CCR7+), and effector memory (CD45RA-CCR7-) T cells. In (i), the pie charts compare the mean proportion of different CD4 memory T cell subsets (colour-coded) in PBMCs (A & B) and BAL (C & D) between HIV- and HIV+ individuals. In (ii), the pie charts present the proportions of CD8 memory cell subsets in PBMCs and BAL, comparing HIV- and HIV+ individuals. P values

were calculated using SPICE and PEStle with a Student's t-test comparing HIV-uninfected to HIV-infected individuals for each memory subset, with $P < 0.05$ considered statistically significant. Non-significant results are not shown. (HIV- n = 22, HIV+ ART+ n = 15)

3.3.3 Higher expression of CD69 on $CD4^+$ and $CD8^+$ T Cells in lung compared with peripheral blood.

CD69 serves as an early activation marker on T cells, indicating their initial response to Mtb infection. It is rapidly upregulated on the cell surface upon antigen recognition, indicating T cell activation (274). To assess immune activation, we analysed CD69 expression on CD4 and CD8 T cells from BAL and PBMCs after stimulation with various Mtb antigens namely CFP-10, PPD and Mtb lysate detailed described in the Methods). Our analysis showed that CD69 expression was significantly lower on CD4 and CD8 T cells in PBMCs compared to BAL following Mtb stimulation. Interestingly, CD69 was constitutively expressed in BAL in both CD4 and CD8 T cells, which was not affected by antigenic stimulation **(**

Figure 3-3 **A & B)**. These findings suggest that CD69 is constitutively expressed in lung-resident T cells, consistent with its role as a marker of tissue residency. This difference in expression between BAL and PBMCs underscores the unique activation and tissue-resident characteristics of lung T cells during Mtb infection.

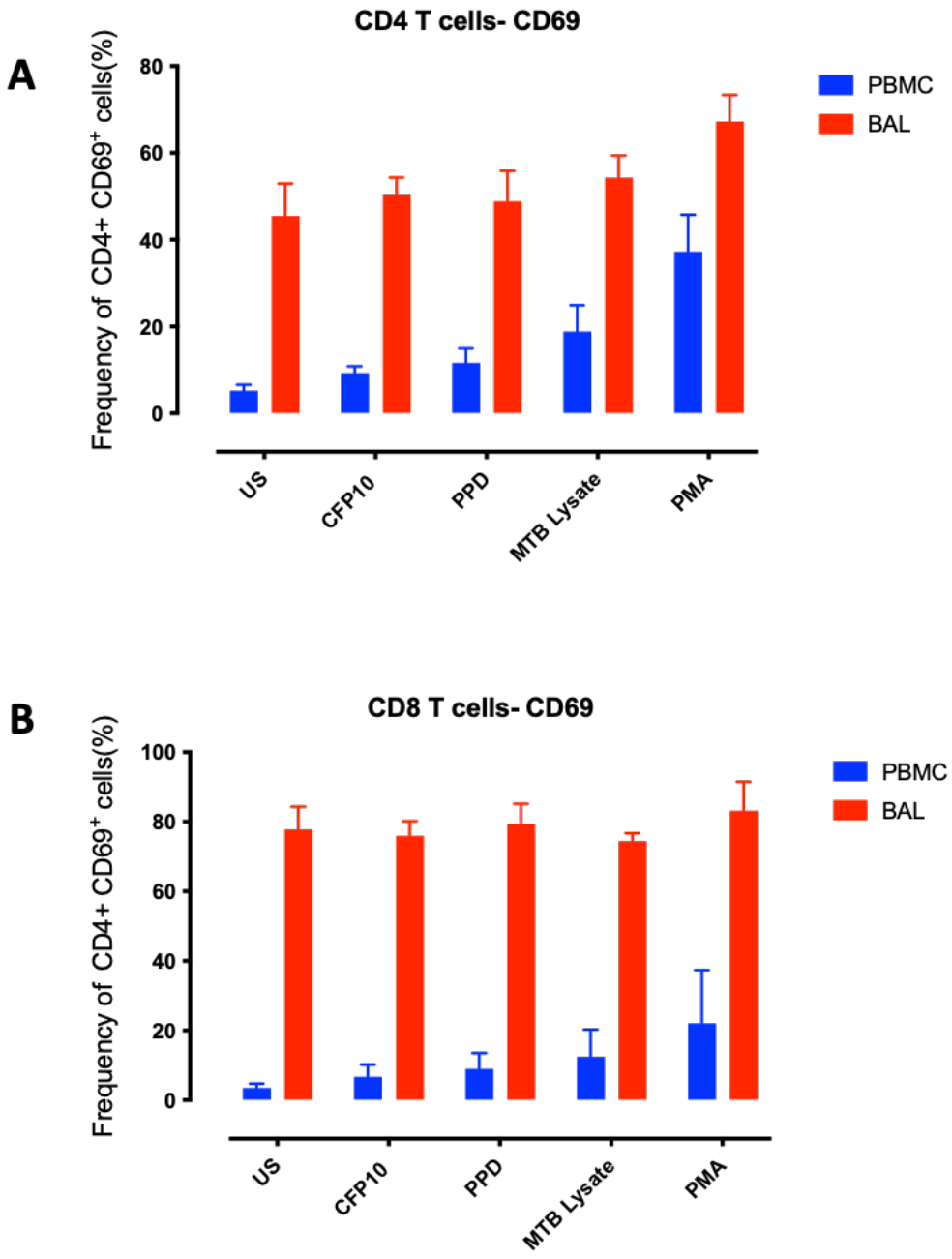


Figure 3-3 CD69 expression profile in BAL and PBMC

T cells were stimulated with various Mtb antigens *in vitro* for 18 hours and were stained with anti-CD3, anti-CD4, anti- CD8 and anti-CD69 (A) Frequency of CD69 expressing cells within the CD4 T cell population in BAL and PBMC. (B) Frequency of CD69 expressing cells in CD8 population in BAL and PBMC. The bar graphs show summarised data of individuals, and the error bars show mean SEM. Data were analysed using the Mann-Whitney U test (*: P< 0.05) where * means statistically significant, non-significant results are not shown. (n=12).

3.3.4 Lower frequency of Mtb specific IFN-γ CD4 T cells in PLHIV

T cells exert their function through the production of effector cytokines namely IFN-γ, TNF-α and IL-2 which activates other cells involved in clearance of infection. To investigate Mtb antigen-specific T cell responses in BAL and peripheral blood, the frequency and magnitude of the $CD4^+$ T cell response to Mtb antigens such as Mycobacteria lysate (CDC1551), Early secretory-antigen-6, and PMA/Ionomycin was measured using intracellular cytokine staining upon 18-hour incubation. The study was based on the hypothesis that HIV reduces the frequency and impairs the function of Mtb antigen-specific T cell cytokine production. Antigen-specific CD4 and CD8 T cells producing IFN-γ and TNF-α against Mtb antigens were detected in both BAL and PBMC using the gating strategy illustrated in **(Figure 3-4).** Next, the frequency and magnitude of the CD4 and CD8 cytokine-producing cells were compared between HIV-uninfected, PLHIV treatment naïve and PLHIV on treatment cohorts. For cytokine assay analysis, background cytokine production in the negative control of intracellular cytokine staining (ICS) assays was subtracted from each antigen-stimulated condition. In HIV uninfected individuals, the frequency and magnitude of CD4 T cells producing IFN-γ against Mtb lysate were higher in both PBMCs (P< 0.0456) and BAL (P< 0.0118) compared to the PLHIV not on treatment **(Figure 3-5 i A & B**). Furthermore, IFN-producing CD4 T cells in both PBMCs and BAL from the PLHIV not on treatment were lower compared to those on treatment (P< 0.0369) and (P< 0.0292) respectively **(Figure 3-5 i A-D).** A trend for a lower frequency of IFN-producing CD8 T cells in PLHIV not on treatment was also observed following ESAT-6 stimulation **(Figure 3-5 iii B & D) (Figure 3-5 iii A & C).** Specifically, the frequency of these cells was lower in BAL from PLHIV ART naive compared to both HIV-uninfected individuals (P< 0.0108) and PLHIV on ART (P< 0.0213) **(Figure 3-5 B & D).** This frequency was also lower than in PLHIV on ART. However, the frequencies were comparable between HIV-uninfected individuals and those HIV-infected on treatment.

By contrast, no significant differences were observed in Mtb-specific CD4 TNF-α expressing cells across all the study groups **(Figure 3-5 ii A-D)** in both compartments. However, the frequency of Mtb specific TNF-α producing CD8 T cells was higher in PBMC in HIV-uninfected individuals compared to HIV-uninfected who were not on treatment (P< 0.0507) (P< 0.0352) **(Figure 3-5 iv A & B).**

Collectively, these data suggest that Mtb antigen-specific CD4 and CD8 portray distinct cytokine expression profiles in BAL and PBMC. In addition, the impairment in cell cytokine production in PLHIV is consistent with the altered lung immune function that occurs as a result of HIV infection. Furthermore, while the frequencies of Mtb-specific cytokine-producing cells is restored following ART, the magnitude (quality of the response) remains impaired.

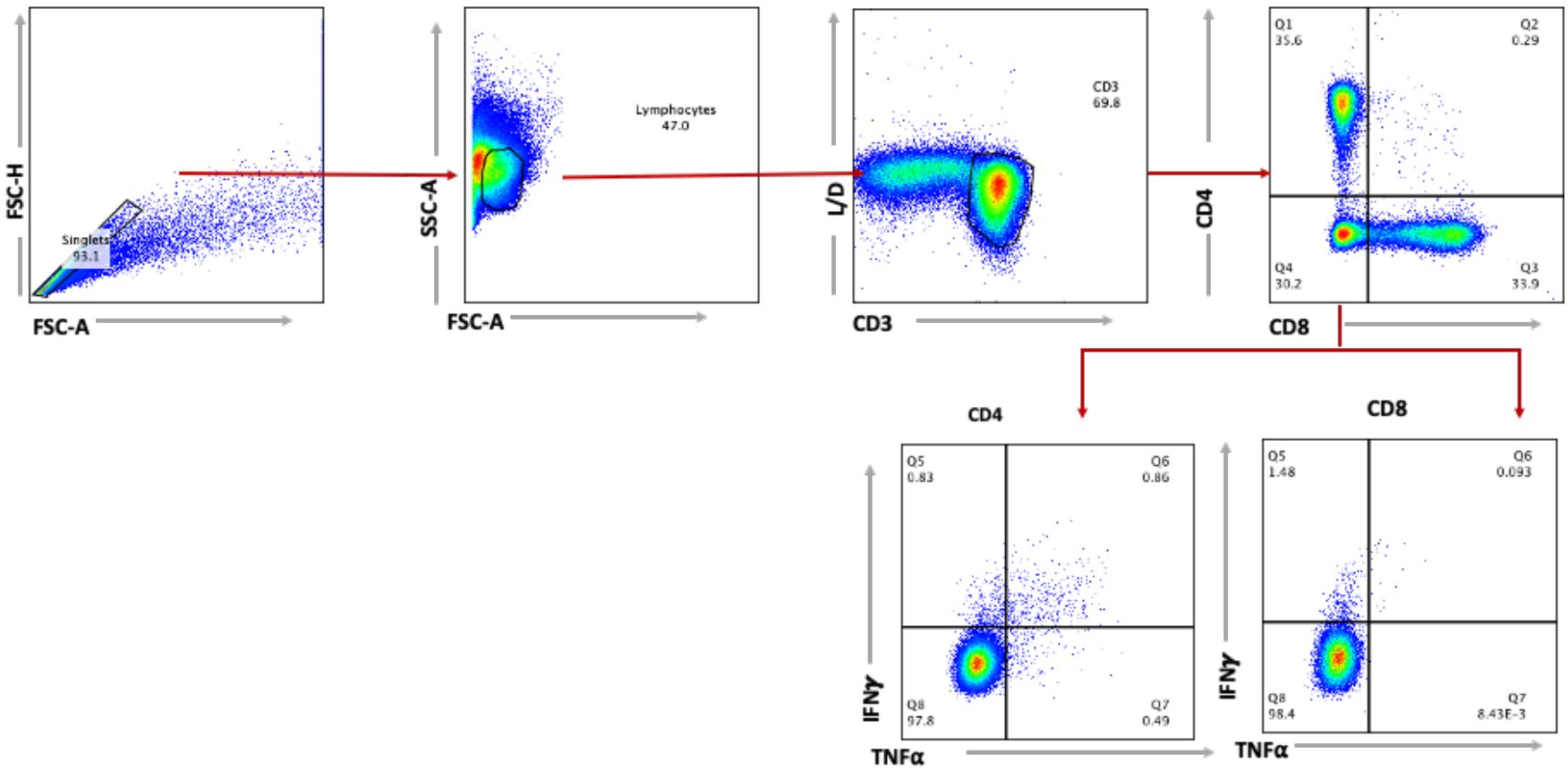
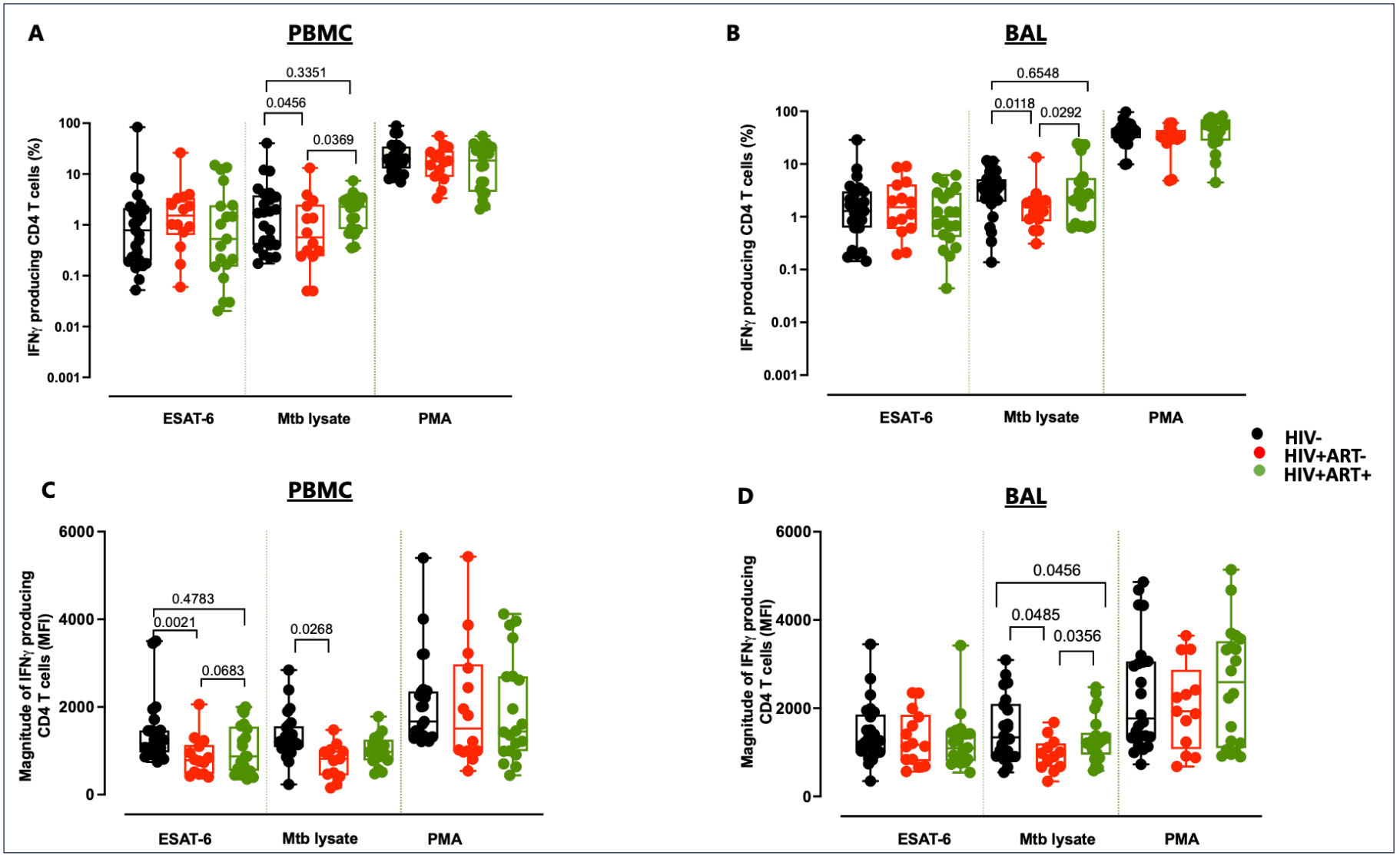


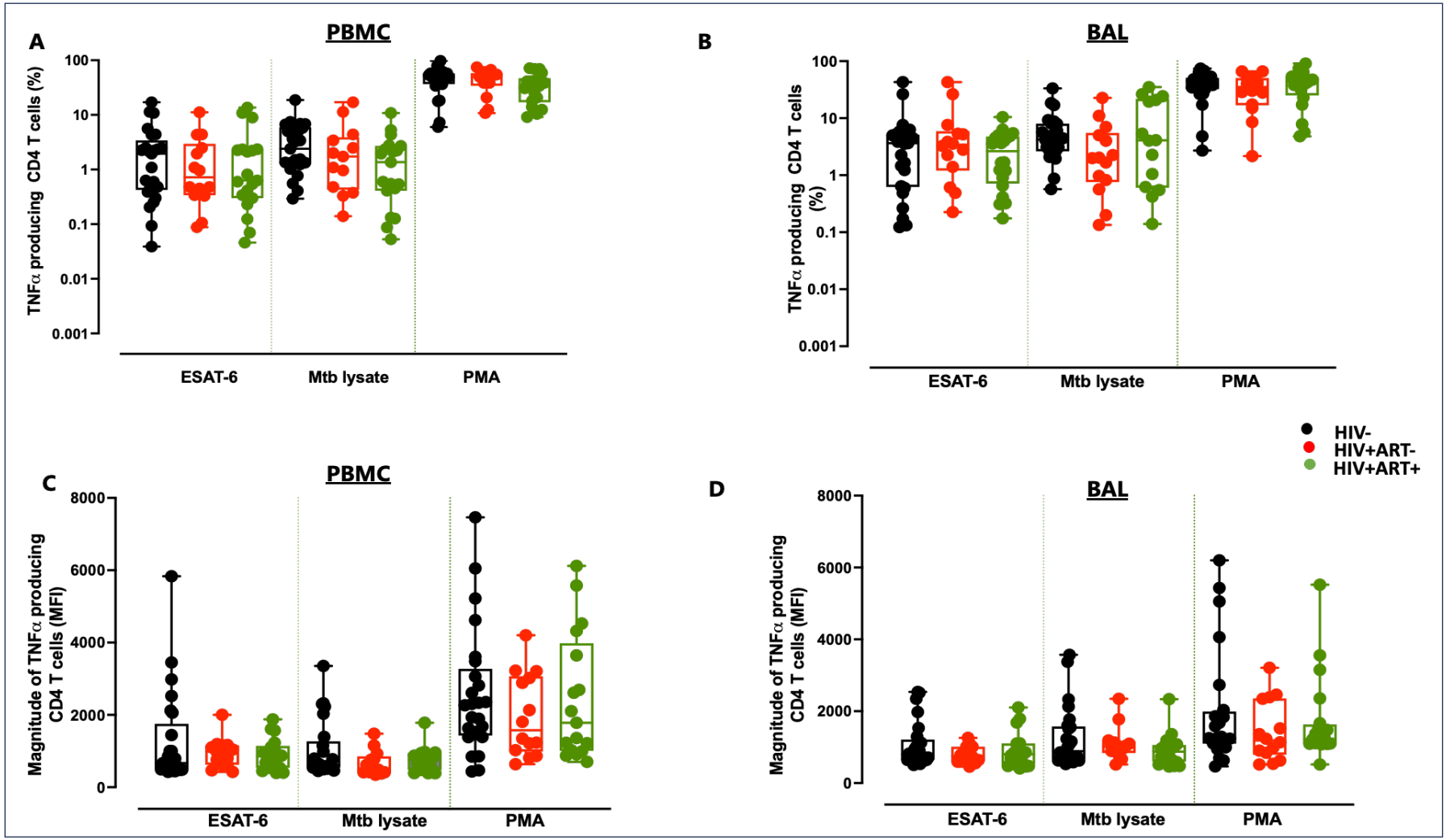
Figure 3-4 Gating strategy for T cell cytokine response

BAL and PBMCs were stimulated in vitro with PMA/Ionomycin, Mycobacteria TB lysate of CDC 1551 strain and ESAT-6 for 18 hours. Cells are gated on singlets, then lymphocytes, and on CD3 cells. CD4+ and CD8+ T cells are gated on a population that expresses CD3. Cytokine expression profiles are determined by plotting the cytokine IFN-γ and TNF-α.

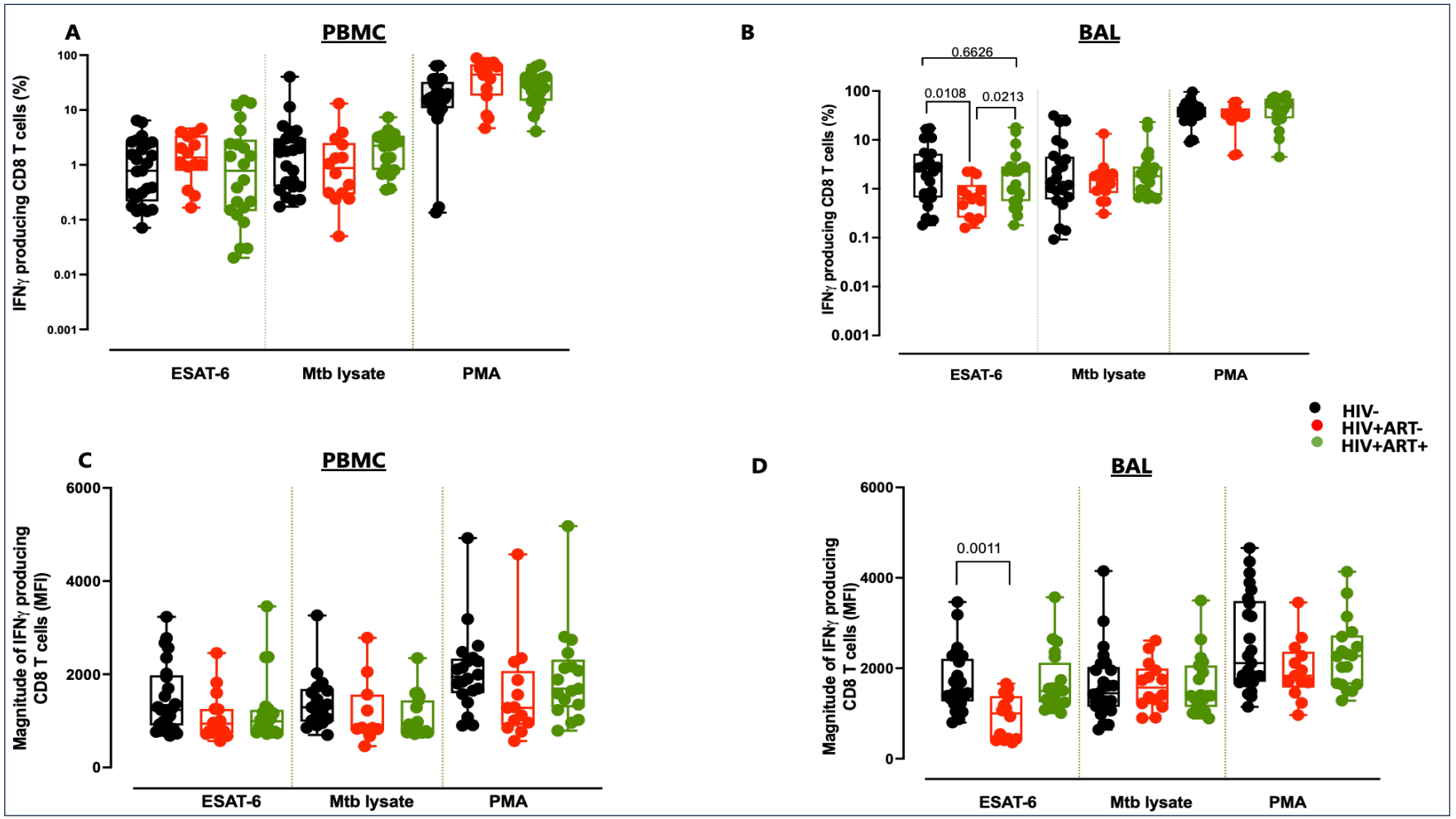
(i) CD4 IFN-γ +


A
PBMC
IFNγ producing CD4 T cells (%)
100
10
1
0.1
0.01
0.001
0.3351
0.0456
0.0369
ESAT-6
Mtb lysate
PMA
B
BAL
IFNγ producing CD4 T cells (%)
0.6548
0.0118
0.0292
ESAT-6
Mtb lysate
PMA
HIV-
HIV+ART-
HIV+ART+
C
PBMC
Magnitude of IFNγ producing CD4 T cells (MFI)
6000
4000
2000
0
0.4783
0.0021
0.0683
0.0268
ESAT-6
Mtb lysate
PMA
D
BAL
Magnitude of IFNγ producing CD4 T cells (MFI)
0.0456
0.0485
0.0356
ESAT-6
Mtb lysate
PMA

(ii) CD4 TNF- α +


A
PBMC
TNFα producing CD4 T cells (%)
100
10
1
0.1
0.01
0.001
ESAT-6
Mtb lysate
PMA
B
BAL
TNFα producing CD4 T cells (%)
100
10
1
0.1
0.01
0.001
ESAT-6
Mtb lysate
PMA
HIV-
HIV+ART-
HIV+ART+
C
PBMC
Magnitude of TNFα producing CD4 T cells (MFI)
8000
6000
4000
2000
0
ESAT-6
Mtb lysate
PMA
D
BAL
Magnitude of TNFα producing CD4 T cells (MFI)
8000
6000
4000
2000
0
ESAT-6
Mtb lysate
PMA

(iii) CD8 IFN-γ +


A
PBMC
IFNγ producing CD8 T cells (%)
100
10
1
0.1
0.01
0.001
ESAT-6
Mtb lysate
PMA
B
BAL
0.6626
0.0108
0.0213
IFNγ producing CD8 T cells (%)
ESAT-6
Mtb lysate
PMA
HIV-
HIV+ART-
HIV+ART+
C
PBMC
Magnitude of IFNγ producing CD8 T cells (MFI)
6000
4000
2000
0
ESAT-6
Mtb lysate
PMA
D
BAL
0.0011
Magnitude of IFNγ producing CD8 T cells (MFI)
ESAT-6
Mtb lysate
PMA

(iV) CD8 TNF- α +

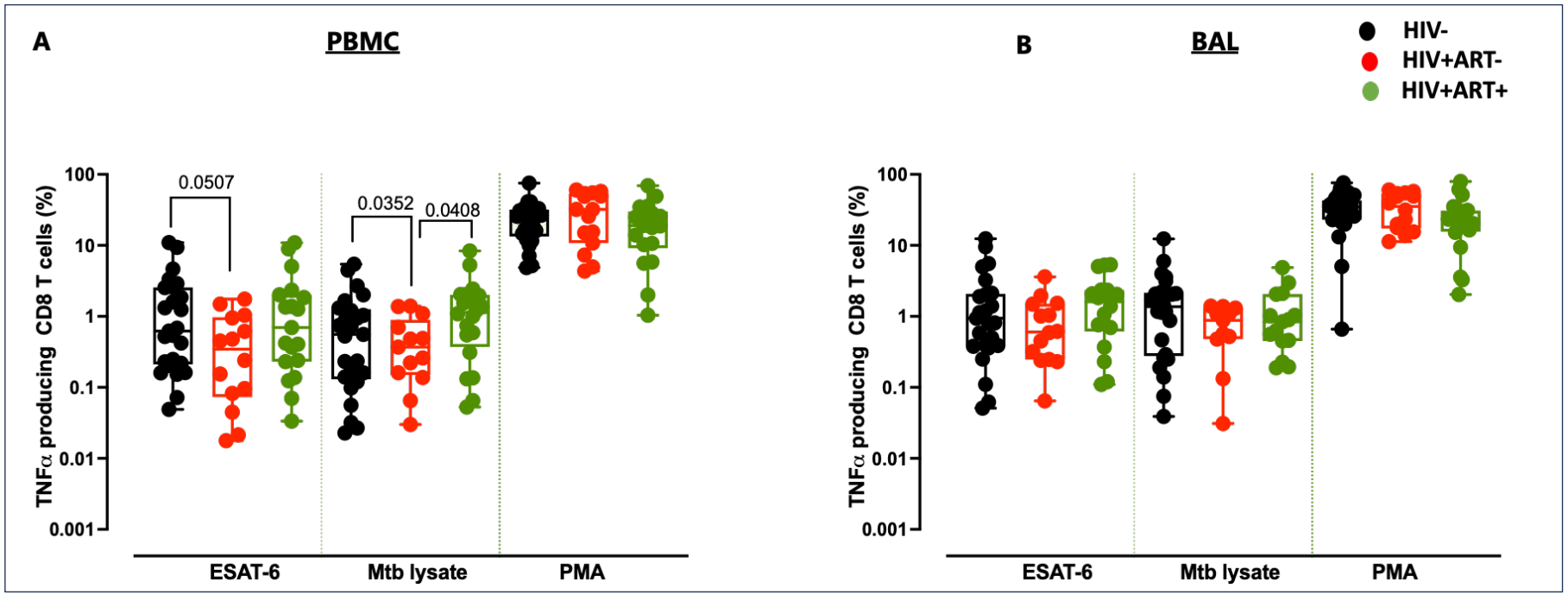


Figure 3-5 Cytokine expression by mycobacterial specific T cells

Lymphocytes from BAL and PBMCs were stimulated with various Mtb antigens in vitro for 18 hours (i) Proportion and magnitude of IFN-producing CD4 in both BAL fluid and PBMC. (ii) Percentage and Median Fluorescent intensity (MFI) of TNF-α producing CD4+ T cells (iii) The frequency of CD8 and MFI of IFN-producing cells in BAL and PBMC. (iv) The frequency of TNF- α -producing CD8+ T cells in BAL and PBMC. (n= 25 HIV-ve,14 HIV+ART- and 20 HIV+ ART+). P< 0.05 indicates statistically significant, non-significant results are not shown.

3.3.5 Analysis of helper CD4 T cell subsets in the airway

Airway T helper cell responses such as Th1 and Th17 cells are critical for immunity against TB (27,28). However, Th1 immunity alone does not fully correlate with protection (29,30), suggesting that additional CD4+ T cell subsets contribute to TB immunity. We hypothesised that HIV infection preferentially depletes Mtb antigen-specific CD4+ helper T cell subsets, thereby compromising immune responses in both the airway and blood. To investigate this, we characterised helper CD4+ T cell subsets following Mtb antigen stimulation and assessed the impact of HIV on these responses. To measure the composition of helper CD4 T cells, alveolar and peripheral blood lymphocytes were stimulated with Mtb and analysed using flow cytometry. Subsets were identified based on a defined gating strategy **(Figure 3-6),** allowing for a comparative analysis between the lung and peripheral blood in HIV-infected and uninfected individuals. The composition of helper CD4 T cell subsets varied between the lung and blood in HIV-uninfected individuals, with specific clusters predominantly present in BAL but not in PBMC **(Figure 3-7 A & B)**. AS expected, among the assessed subsets, CD103+ cells were more enriched in the mucosal airway than in the periphery (275) **(Figure 3-7 C & D)**. However, HIV infection led to a decreased population of CD103+ CD4 T cells in the BAL compared to uninfected individuals **(Figure 3-7 E & F)**. Together, these findings suggest that HIV reduces the frequency of CD103+ CD4 T cells in the airway, potentially impairing local immunity against Mtb.

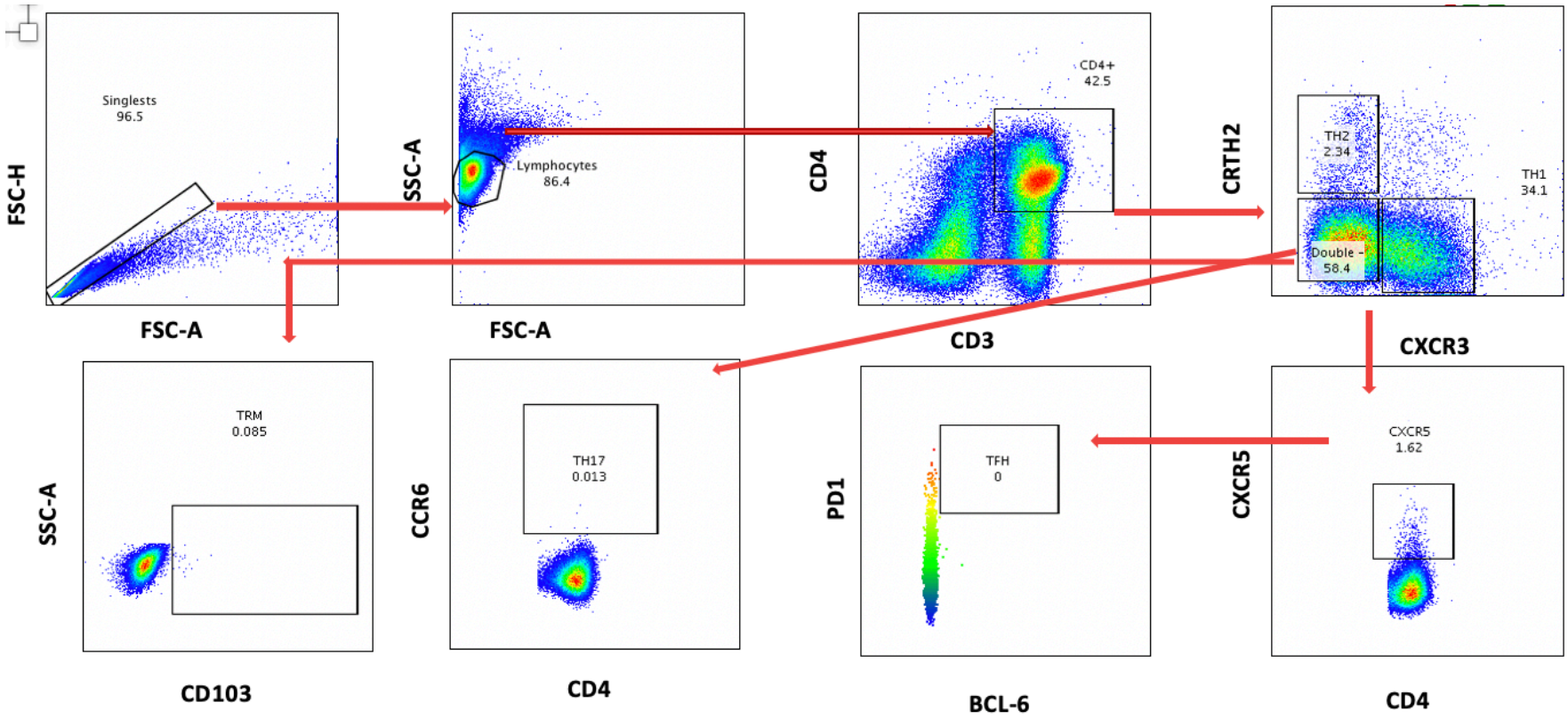


Figure 3-6 Gating strategy for CD4 T helper cell subsets

BAL and PBMCs were stimulated in vitro with PMA/Ionomycin, mycobacteria TB lysate of CDC 1551 strain and ESAT-6 for 18 hours. Cells are gated on singlets, then lymphocytes, and on CD4 T cells. Th1 and Th2 cells were identified via expression of CXCR3 and CRTH2 respectively. Tfh cell subsets were identified from cells expressing CXCR5 and those expressing transcription factor BCL-6. Th17 and tissue-resident cells (Trm) are identified by expression of CCR6 and CD103 respectively from the Th1 and Th2 double negative populations.

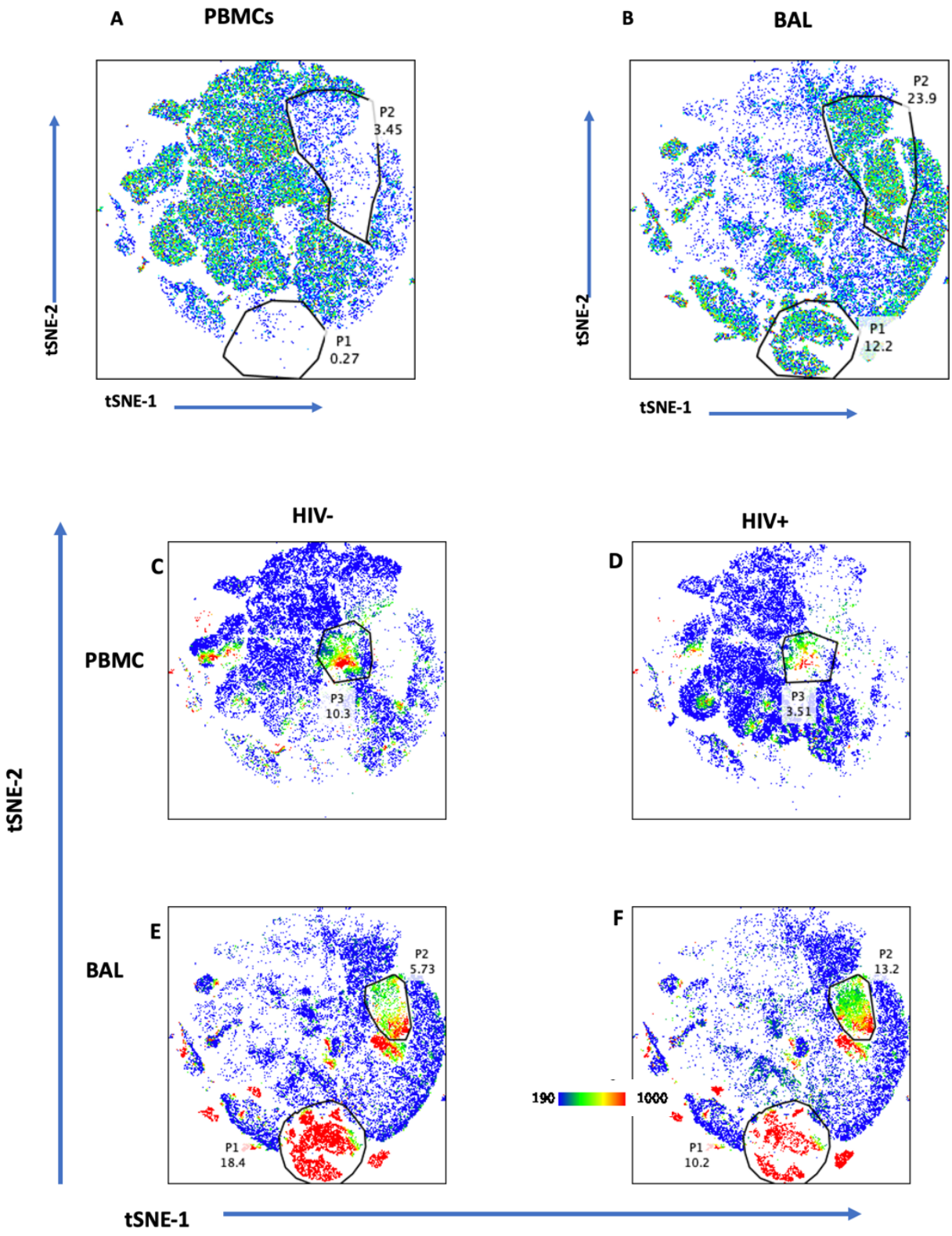


Figure 3-7 Differential composition of helper CD4+T cell subsets in BAL and PBMC and the impact of HIV infection

BAL and PBMCs were stimulated with Mtb-specific antigens *in vitro* for 18 hours. Cells were stained with appropriate titrated antibodies to determine different helper T cell subsets. t-SNE plots show the composition of CD4 helper T cell subsets in the (A) PBMC and BAL (B) in HIV uninfected individuals. Panels (C-D) illustrate the impact of HIV on CD103+ T cell subsets in the blood, while (E-F) show the effect of HIV in the lung. The

colour intensity represents expression levels, with red indicating higher expression (P1 = CD103 high, P2 = CD103 low). (HIV−: n=10; HIV+: n=10).

3.3.6 HIV is associated with a decrease in the frequency of CD103 cells in the BAL of HIV-infected individuals compared to HIV-uninfected ones.

We further characterised the phenotype of the CD103-expressing cells in the lung by measuring different CD4 helper T cell subsets within this population, namely Th1, Th2, Th17, and Tfh cells. We hypothesise that HIV reduces the frequency of Mtb-specific CD4+ helper T cell subsets in the lung, impairing the host's immune response and increasing susceptibility to developing TB infection. Consistent with the t-SNE analysis, the proportion of CD4 T cells expressing CD103 was higher in BAL compared to PBMC (P< 0.0001) **(Figure 3-8 A).** This suggests that although most CD103+ T cells are tissue-resident in the lung, a subset of CD103- exists in the lung that may represent recirculating or exiting cells.

Next, the frequency of CD103+ and CD103- was compared between HIV uninfected, HIV-infected treatment naïve and HIV infected individuals on treatment. In BAL, CD4 T cell frequencies of CD103- were lower in treatment-naive HIV-infected individuals compared to HIV-uninfected individuals (P< 0.0417) **(Figure 3-8 B).** Conversely, the frequencies of CD103+ were comparable across all the study groups. We also examined whether HIV alters the phenotype of CD4 helper T cell subsets (Th1, Th2, Th17, and Tfh) in the lung. While the proportions of airway Th1, Th2, Th17, and Tfh cells were not different among groups in CD103- cells **(Figure 3-8 C),** the frequency of Th17 cells was lower in HIV-infected individuals not on treatment compared to both HIV-uninfected individuals (P< 0.0427) and HIV-infected individuals on treatment **(Figure 3-8 D).** Collectively, these data show that HIV affects the composition of CD4 helper T cell subsets, including Th17, in the lung.

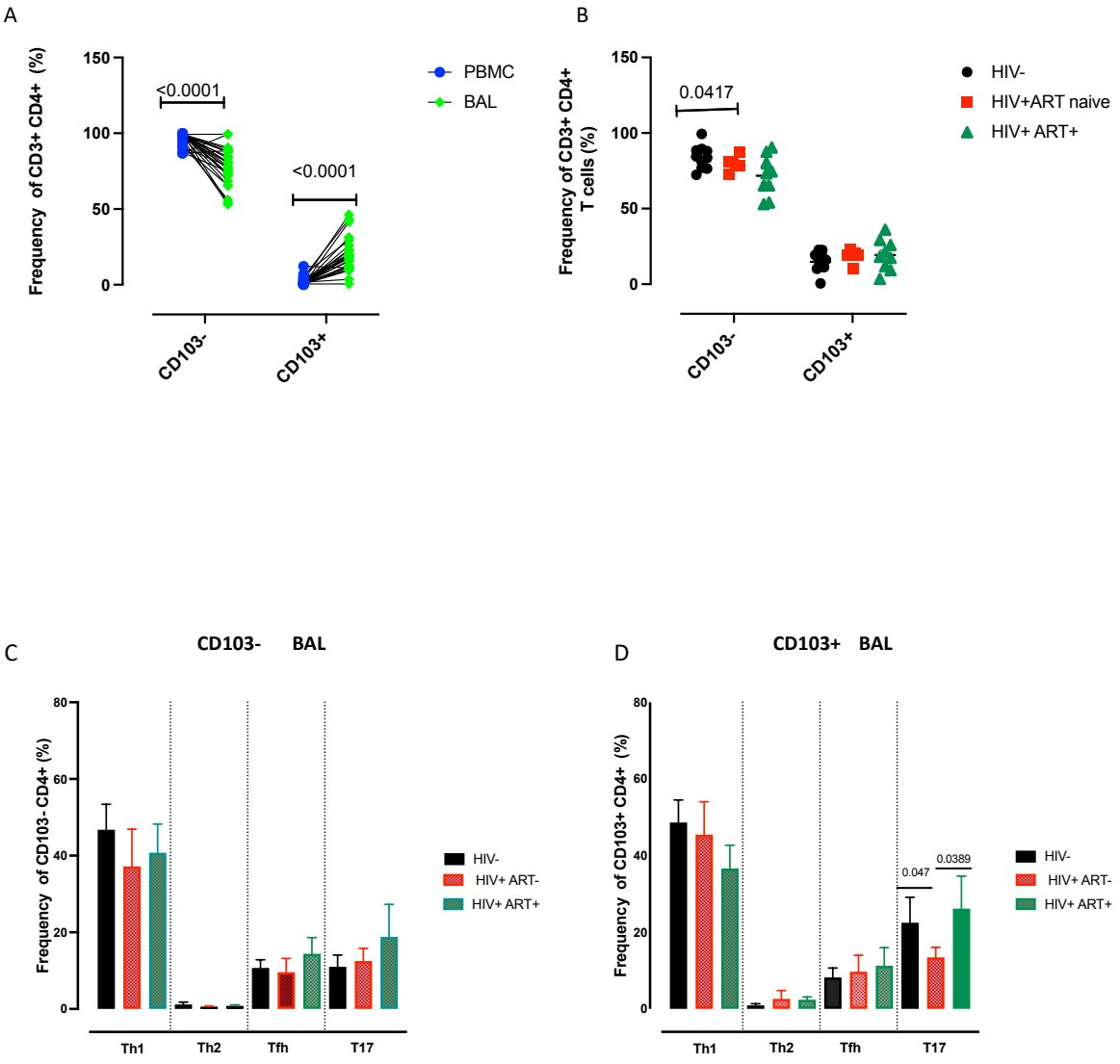


Figure 3-8 The impact of HIV on CD103+ CD4 helper T cell subsets

BAL and PBMCs were stimulated with Mtb antigens in vitro for 18 hours. Cells were stained with appropriate titrated antibodies to determine different helper CD3+ CD4+ T cell subsets. (A) Shows the frequency of the CD103+ CD4+ T cells in PBMCs and BAL. In (B), the data shows the percentage of CD103+ and CD103- in BAL in HIV-, HIV+ ART- and HIV+ART+. Each data point represents an individual (A&B) within the group. The impact of HIV on the frequency of different CD4 T cell subsets in BAL in CD103- (C) and CD103+ (D). The bar graphs show summarised data of the sample groups. Data were analysed using Mann Whitney U test and, where necessary, one-way ANOVA was used, followed by Turkey's multiple comparison test (*: P< 0.05), where * means statistically significant (HIV- n=11, HIV+ART- n=5 and HIV+ART+ n=11).

3.4 Discussion

Co-infection with Mtb and HIV is a major risk factor for progression to active TB disease. HIV infection predisposes individuals to tuberculosis (TB) by causing depletion and dysfunction of CD4 T cells. However, the precise mechanisms by which HIV affects CD4 and CD8 T cell function and the resulting susceptibility to TB remain incompletely understood. This chapter reports the broad impact of HIV infection on Mtb antigen-specific T cell immunity characterised by impaired CD4 and CD8 T cell immune responses in the lung.

Compared with the peripheral blood, we show differences between CD4 and CD8 T cell immune responses to Mtb antigens in the bronchoalveolar space. These include a higher frequency of Mtb antigen-specific CD4 T cells producing IFN-γ and TNF-α in BAL than in PBMCs. Consistent with other groups, our data has demonstrated a reduced frequency of CD4 T cells in PBMCs of HIV-infected individuals and a well-characterised CD8 T cell lymphocytosis in both the lung and periphery (276). In addition, we show that HIV is associated with impairment of Mtb-specific T cells producing IFN-γ in both the airway and periphery. Although there was no difference in the frequency of Mtb-specific TNF-α producing CD4 T cells across all the study groups, HIV-infected individuals not on treatment had a significantly lower frequency of TNF-α producing CD8 T cells in the blood compared to HIV -uninfected individuals, thus suggesting an HIV-driven immune response. The differences in the cytokines of Mtb-specific CD4 and CD8 T cells preferentially affected by HIV in this study versus studies elsewhere by Kalsorf *et al*. (277) could be attributed to the difference in study cohorts, the biological effect of the newly introduced HIV test and treat procedures as well as technical variations in the assay used to measure the Mtb specific T cells. Nonetheless, throughout our analysis, it remained consistent that antigen-specific T cell subsets, particularly those producing IFN-γ are prone to preferential depletion in people living with HIV compared with their matched controls. Collectively, this suggests that specific characteristics of Mtb-specific T cells may render them more susceptible to depletion. It will be interesting to establish whether activation could be a potential reason HIV targets these cells.

Indeed, previous studies have reported that there is an overall reduction of the frequencies and impairment of the function of Mtb-specific CD4 T cells in people

living with HIV compared to HIV-uninfected individuals, which are not fully reconstituted following ART (272,277–280). Here, we build upon these prior findings to further delineate the subsets and phenotype of Mtb CD4 and CD8 T cells and assess their roles in anti-TB immune responses. Apart from their ability to produce cytokines, antigen-specific T cell subsets can be identified by the simultaneous expression of surface molecules. These molecules serve to refine their profiles related to memory T cell differentiation, activation status, and functional programmes. To evaluate the impact of HIV infection on the differentiation status of T cells, the expression of CD45RA and CCR7 was measured on CD4 and CD8 T cells. This combination of two receptors allows the identification of cells with varying lymphoid homing potential effector functions, subsequently providing insights into the differentiation and maturation of the cell (281). In this study, the majority of the Mtb-specific CD4 and CD8 T cells were predominately of effector memory phenotype (CD45RA-CCR7) in both the airway mucosa and periphery. Nevertheless, in the context of HIV infection, there was a reduced proportion of these cells, indicating that HIV might selectively affect this subset to facilitate its own persistence. HIV infection disrupts the effector memory T cell ($CD45RA^{-}CCR7^{-}$) compartment through multiple mechanisms. Chronic immune activation drives excessive T cell differentiation, leading to an imbalance in TEM populations and accelerated immune senescence, which reduces their proliferative capacity and functional longevity Additionally, persistent antigen stimulation contributes to T cell exhaustion, impairing cytokine production and cytotoxic function, ultimately weakening immune responses. HIV also skews T cell differentiation, particularly in $CD8^{+}$ T cells, leading to an accumulation of dysfunctional effector memory subsets. These disruptions collectively impair immune surveillance, increasing susceptibility to opportunistic infections and reducing the effectiveness of pathogen-specific immune responses.

While we characterise the presence of different T cell subsets in BAL and PBMCs, it is somewhat surprising that most CD4 and CD8 T cells are of effector phenotype with a small proportion of naïve T in PBMCs compared to BAL. Other studies have reported a higher proportion of effector memory cells in BAL CD4 and CD8 T cells compared to peripheral blood (272,277). This discrepancy could be due to differences in markers used to identify these cell subsets as well as differences in the cohort of participants. We observed a higher frequency of CD69 expressing CD4 and CD8 T cells in the BAL fluid than PBMCs. For decades, CD69 has been

known as an early activation marker on T cells following engagement with TCR/CD3 (274,282). In addition, CD69 also plays an essential role in regulating lymphocyte egress from the tissues by binding to Sphingosine-1-phosphate receptor 1 (S1PR1)(283). There was a low expression of CD69 on both CD4 and CD8 T cells in peripheral blood compared to BAL. Interestingly, CD69 was constitutively expressed in BAL in both CD4 and CD8 T cells unaffected by stimulation. This suggests that CD69 expression may not necessarily be a marker of activation in the lung but could instead be a marker of tissue residency, hence the use of CD103, a mucosal retention receptor often used to differentiate tissue-resident from infiltrating cells (202,284).

We observed a significant increase in CD103+ cells in BAL compared to blood. This was anticipated as these are mucosal cells and have been reported to be key in anti-TB immunity (289). Furthermore, we show that HIV is associated with a decrease in the frequency of CD4 helper T cell subsets, particularly Th17 cells, in the airway in HIV infected individuals not on ART compared to both healthy controls and HIV infected individuals on treatment. A possible explanation for this could be due to the duration of ART therapy. HIV-infected participants recruited in this study had been on ART for an average of 4 years. It is well established that HIV persists even after immune reconstitution owing to effective long-term ART-mediated viral suppression (8). The role of CD4 T cells in protection against the development and progression of TB has been widely discussed (217,222,235). However, significant gaps remain regarding the impact of HIV infection on CD4 helper T cell subsets. Th1 and Th17 cells that produce IFN-γ and IL-17 are key in providing immunity to TB (285,286). However, Th1 immunity alone is not conclusive as a correlate of immune protection induced by BCG vaccination against TB in animal models (287,288). Additionally, there have been reports of IFN-independent mechanisms through which CD4 T cells control Mtb infection (275). Therefore, it is likely that additional subsets of CD4 T cells, apart from Th1 cells, are crucial for protecting against TB.

Although the role of Mtb antigen-specific CD4 and CD8 T cell responses has been characterised, significant questions still remain; to what extent does HIV impact Mtb-specific T cell receptor repertoire diversity and dynamics? How do the changes in the TCR repertoire contribute to TB susceptibility in people living with

HIV infections? What is the role of ART on the function restoration of these repertoire changes? A deeper understanding of the functional composition of TCR repertoire in HIV-infected individuals will advance our current knowledge and provide new insights into host factors that control susceptibility to, and progression of, Mtb infection. Investigating the impact of HIV on *Mtb*-specific TCR repertoire diversity and dynamics is crucial for advancing our understanding of immunological mechanisms underlying TB susceptibility and progression in HIV-infected individuals. HIV-induced immune dysregulation, characterised by chronic activation, immune exhaustion, and altered T cell differentiation, likely impairs the diversity and functionality of the TCR repertoire, particularly in CD4+ and CD8+ T cells. These changes may result in a narrowed antigen-specific response to *Mtb*, thereby increasing risk to infection and influencing the progression from latent TB to active disease. Furthermore, understanding how HIV influences the clonality and functional composition of the TCR repertoire could elucidate critical host factors, such as T cell exhaustion or anergy, that modulate TB immunity. Assessing the effects of ART on restoring TCR repertoire diversity and functionality could provide insights into the potential for immune restoration in HIV-infected individuals, with implications for improving TB outcomes. These findings may contribute to the development of novel immunomodulatory approaches, targeted therapies, and diagnostic tools that incorporate TCR repertoire characteristics as biomarkers for TB risk, infection, and progression advanced HIV diseases.

3.5 Conclusion

The findings reveal phenotypic and functional differences between CD4 and CD8 T cells in the lung and periphery, indicating compartmentalisation within the immune system. Specifically, BAL T cells display a more effector phenotype and are more responsive to Mtb antigen stimulation compared to peripheral T cells, likely due to higher frequencies of antigen specific T cells. This differential phenotype and function highlight the importance of studying immunity at localised sites of infection. Additionally, the study demonstrates that HIV infection alters the distribution of Mtb-specific CD4 helper T cell subsets and impairs cytokine production in the lung. While the impact of HIV on T cell function is established, a deeper understanding of the mechanisms of Mtb antigen recognition by T cells is necessary to probe their effector functions. Characterising TCR repertoires and usage patterns is crucial for understanding how T cell adaptive immunity recognises and controls Mtb infection.

Chapter 4 Flow cytometry analysis of the *Mtb*-specific T cell receptor Vβ usage in the lung and peripheral blood

4.1 Introduction

Numerous studies have investigated TCR repertoire diversity in both bacterial and viral infections. However, conflicting data exist regarding the changes of TCR Vβ expressing T cells in chronic diseases such as HIV and TB. For instance, HIV has been associated with $CD4^+$ and $CD8^+$ T cells expressing TCR Vβ14, Vβ15, Vβ16 and Vβ17 in the peripheral blood (290). Nevertheless, a clonal expansion of some TCR Vβ families has been reported in acute T cell immune responses to HIV such as TCR Vβ2, suggesting heterogeneity of the immune response (291). Indeed, limited TCR diversity has also been reported in other chronic infections like TB, where the proliferation of some TCRs was shown to contribute to a unique repertoire. These variances in TCR repertoires can be used to probe the composition and function of T cells (292). For instance, studies have demonstrated that the adoptive transfer of *in vitro* TCR transduced T cells can generate antigen-specific responses *in vivo*, contributing to anti-tumour immune responses in patients with melanoma and lymphoma. Furthermore, the analysis of TCR diversity within this population could be crucial for monitoring treatment and assessing the risk of developing lymphoma (293,294).

While the diversity of the TCR repertoire Vβ usage has been extensively studied in various chronic inflammatory diseases, including Cancer and Pulmonary sarcoidosis (245,295), little is known about the impact of HIV and TB on the TCR usage pattern in the lung, the localised primary site of TB infection. Previous studies have reported compartmentalised differences in TCR Vβ sequences found in the lung compared to those from peripheral blood (296,297). This suggests that the TCR Vβ expression in the lung may be distinct from those in the peripheral blood, indicating potential differential functions of T cell populations in these sites. From BAL studies, it is well-known that the accumulated lung immune cells are activated in individuals with active and latent TB infection suggesting that the differential Vβ usage could be due to retention of activated cells in the lung

(298,299). Moreover, an altered TCR Vβ usage of culture filtrate protein 10 (CFP-10)-specific CD4+ and CD8+ T cells with a biased preference for TCR Vβ9, Vβ12, or Vβ7.2, was observed in patients with tubercular effusion (300). However, on a molecular level, patients with pleural effusion exhibited clonal expansion of CD4 and CD8 T cells expressing the TRBV4-1 (Vβ4) gene segment. This expansion correlated with Th1 and cytotoxic signatures, indicating that these cells are important in providing protective immunity against TB (301). Characterisation of TCR repertoire usage patterns in patients' lung and peripheral blood can offer valuable insights into disease-related mechanisms and processes. Analysis of the TCR repertoire provides an insight into the diversity and visibility of the potential expansion of a specific clone, essential in providing an effective immune response (302).

4.2 Chapter aims and summary.

Several techniques are currently available for measuring TCR repertoire, enabling detailed investigations into its composition and diversity (303). These include molecular biology-based methods such as quantitative PCR, TCR spectratyping and sequencing. These methods are often expensive, require T cell sorting, and do not provide functional data.

Here, we utilised a flow cytometry and Mtb *in vitro* stimulation assay to comprehensively examine the Mtb-specific CD4 and CD8 TCR usage in BAL and PBMCs. Specifically, we used the IOTest Flow cytometry analysis kit that allows quantitative analyses of the TCR Vβ repertoire of 24 different TCR Vβ specificities representing about 70% coverage of normal human TCR Vβ repertoire (304). By examining TCR usage patterns in BAL and PBMC, we aimed to understand the distribution and composition of TCRs in the lung microenvironment compared to systemic circulation. Additionally, we focused on assessing the specific TCR repertoire associated with immune responses to Mtb antigens, particularly in CD4 and CD8 T cells. This analysis provided insights into the diversity and specificity of TCRs involved in the immune response against Mtb infection and could be combined with intracellular cytokine analysis to provide functional detail of antigen specific T cells.

Our results revealed a notable increase in TCR Vß chain usage frequency in both BAL and PBMC following stimulation with Mtb lysate, suggesting an antigen-driven response. Furthermore, we observed a distinct selection of specific TCR Vß chains in CD4 and CD8 T cells, emphasising the specificity of the immune response against Mtb. Moreover, the analysis unveiled an expansion of the TCR Vß repertoire coverage within Mtb-specific CD4 and CD8 T cells producing the cytokines IFN-γ and TNF-α upon stimulation. Intriguingly, in the lung microenvironment, a majority of cytokine-producing CD4 with the highly utilised TCRs exhibited a greater tendency to produce both IFN-γ and TNF-α, in contrast to the blood compartment. These findings provide compelling evidence for selective TCR Vß chain usage in Mtb-specific T cell responses within both BAL and PBMC. Besides, the observed discrepancies in cytokine production patterns between the lung and blood compartments suggest potential functional differences in the immune response against Mtb infection.

4.3 Results

4.3.1 T cell stimulation reveals antigen expanded TCR Vß chains

Engagement of the TCR receptor with the MHC-peptide complex is the hallmark of T cell antigen recognition and initiation of the T cell responses. *In vitro* Mtb-antigen, stimulation has been widely used to assess antigen-specific T cell responses to Mtb (305,306). We sought to determine the impact of Mtb antigen stimulation on the TCR repertoire of T cells in the airway and periphery. We compared the frequency of total TCR Vß usage in BAL and PBMC in both Mtb lysate-stimulated and unstimulated conditions in HIV-uninfected individuals. TCR Vß chains were identified using the gating strategy shown in **(Figure 4-1).** Overall, there was a significant increase in the frequency of the TCR Vß chains in Mtb-stimulated CD4 T cells in both PBMC ($P<0.008$) **(Figure 4-2 A)** and BAL ($P<0.0125$) **(Figure 4-2 B)**. Similarly, there was an increase in the frequency of the TCR Vß chain usage in CD8 T cells in both PBMC ($P<0.0077$) and BAL ($P<0.0092$) (**Figure 4-2 C** and **4-2 D** respectively). These results suggest that T cell stimulation increases the proportion of T cell TCR Vß chains in both BAL and PBMCs.

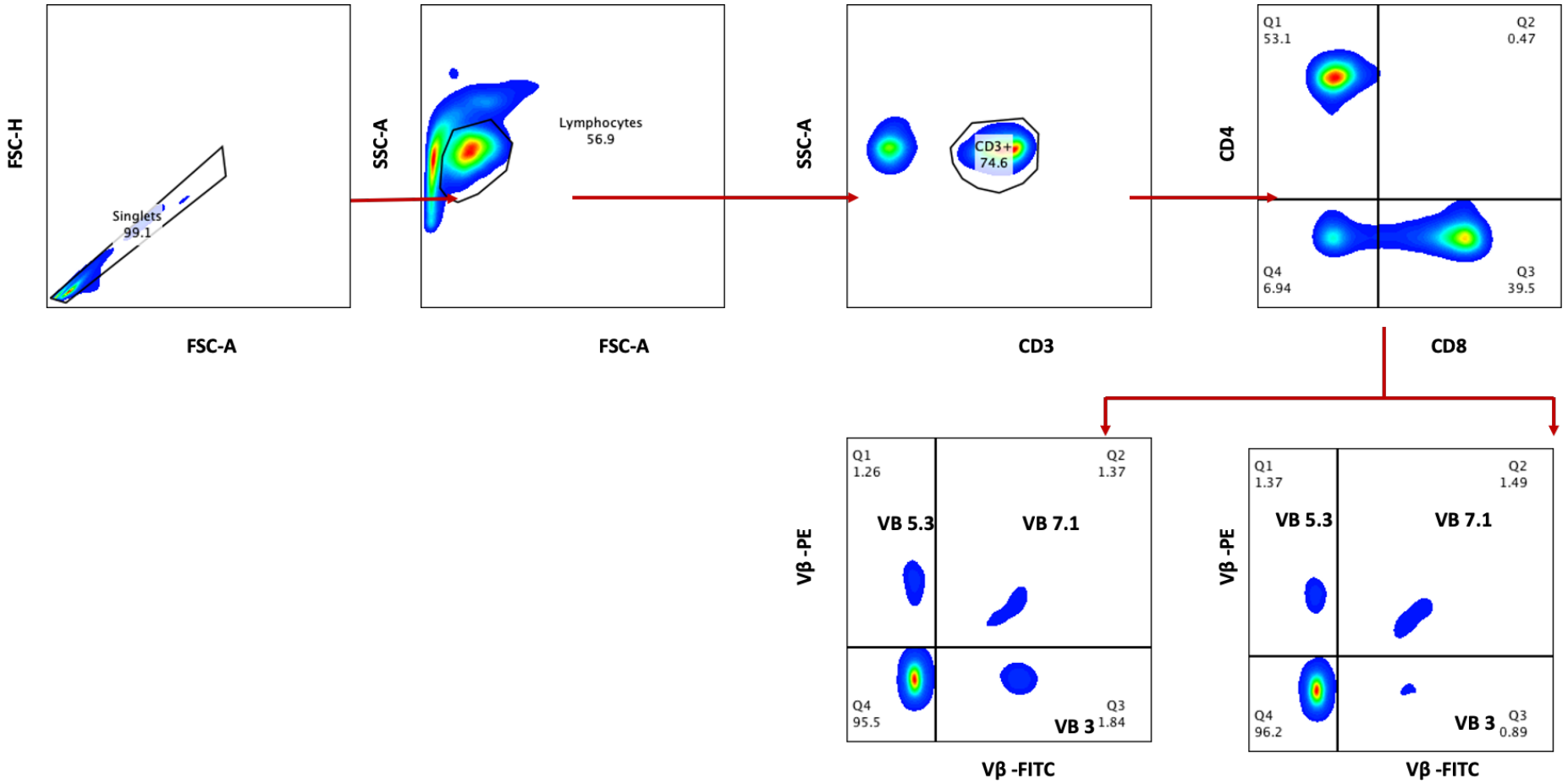
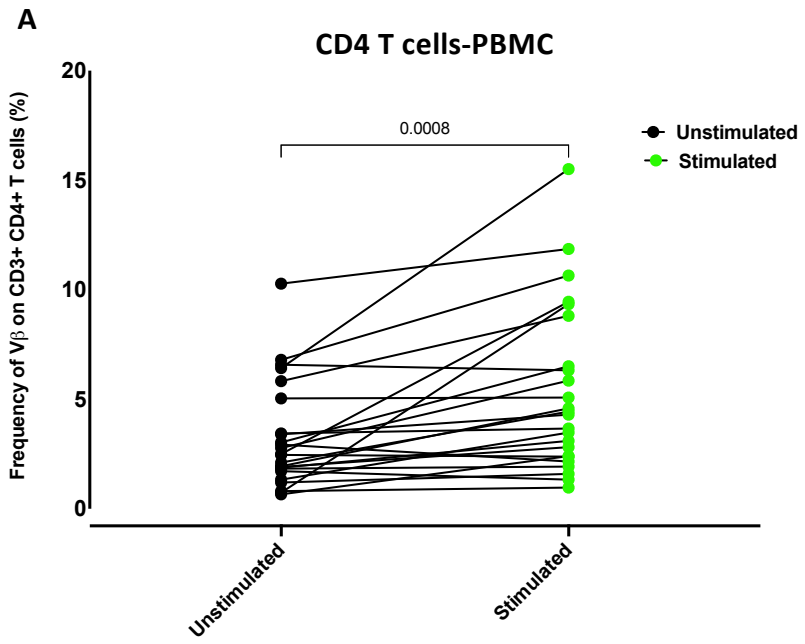
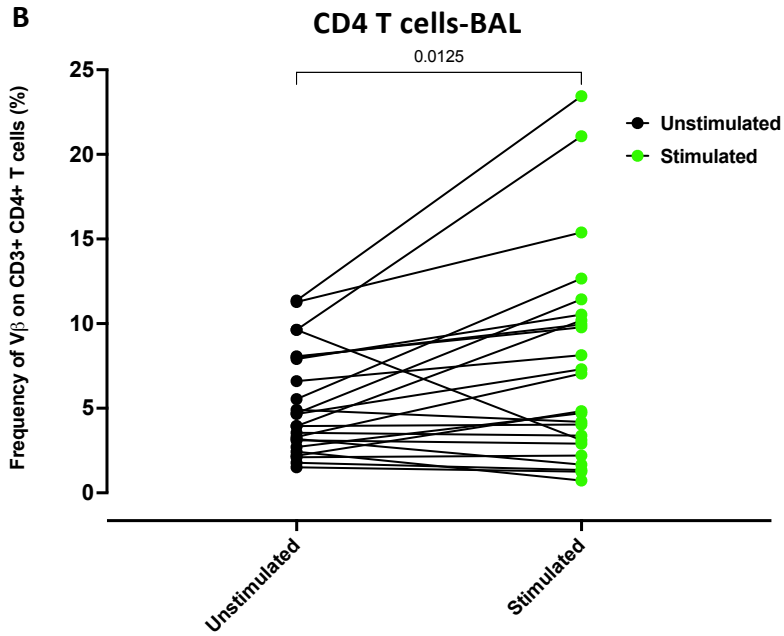
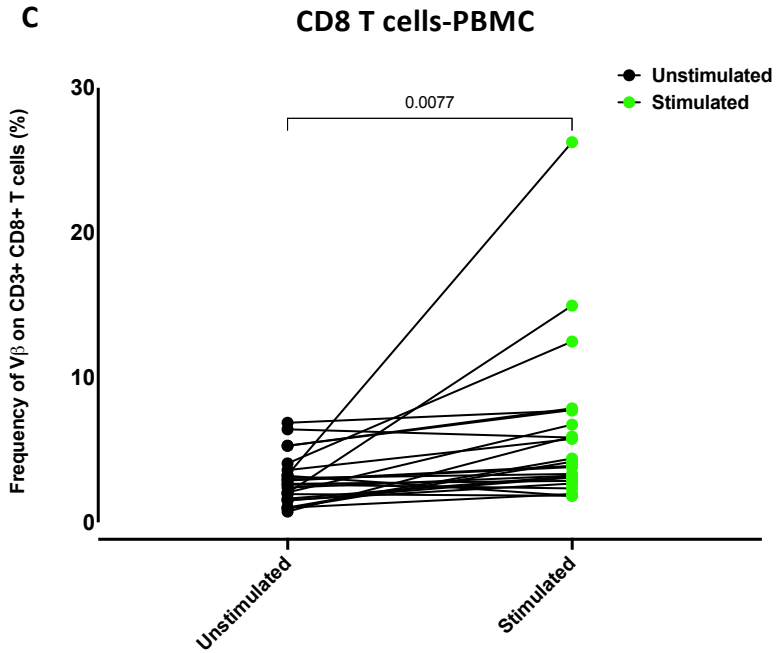


Figure 4-1 Gating strategy for TCR Vß repertoire gated on PBMC

Mtb-stimulated and unstimulated PBMCs and BAL cells were stained with appropriate antibodies. Cells were gated on lymphocytes, CD3, then CD4 and CD8, respectively. The Vß chains were gated on PE, PE+FITC and FITC to identify Vß chains according to the conjugates present in every tube (A-H) per the manufacturer's instructions.


A
CD4 T cells-PBMC
Frequency of Vβ on CD3+ CD4+ T cells (%)
0.0008
Unstimulated
Stimulated
Unstimulated
Stimulated


B
CD4 T cells-BAL
Frequency of Vβ on CD3+ CD4+ T cells (%)
0.0125
Unstimulated
Stimulated
Unstimulated
Stimulated


C
CD8 T cells-PBMC
Frequency of Vβ on CD3+ CD8+ T cells (%)
0.0077
Unstimulated
Stimulated
Unstimulated
Stimulated

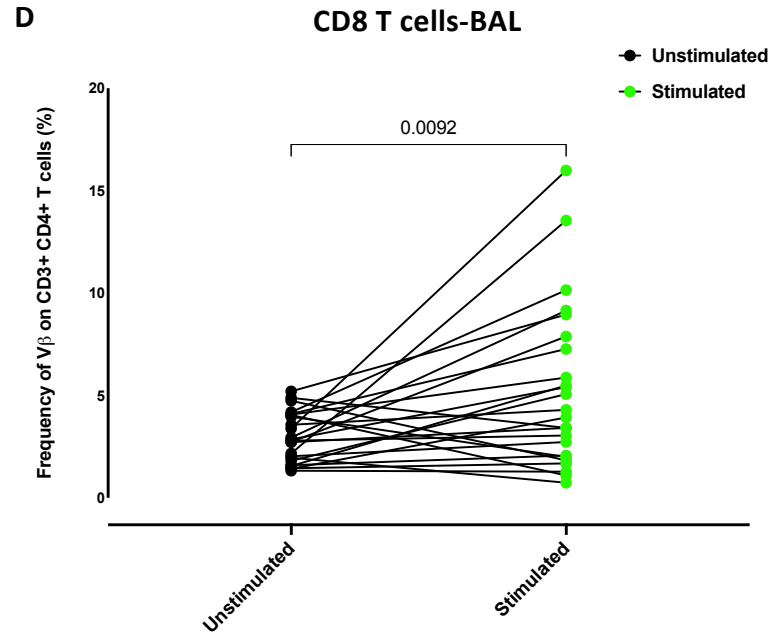

D
CD8 T cells-BAL
Frequency of Vβ on CD3+ CD4+ T cells (%)
0.0092
Unstimulated
Stimulated
Unstimulated
Stimulated

Figure 4-2 Usage Patterns of TCR Vβ chains of the CD4 TCR Repertoire

BAL cells and PBMCs were stimulated with whole Mtb lysate and stained with fluorochrome-conjugated antibodies for different TCR Vβ chains. Frequency of CD4 TCR usage in unstimulated and stimulated conditions in (A) PBMC and (B) BAL fluid. (C) and (D) shows the frequency of CD8 TCR usage in PBMC and BAL, respectively. Each data point corresponds to the frequency of a TCR Vβ chain expressed by either CD3+CD4+ or CD3+CD8+ T cells. Black dots denote unstimulated conditions, while green dots represent conditions following Mtb stimulation in the pooled samples. Statistical analysis was performed using the Mann-Whitney U test, with a significance threshold of $P < 0.05$ (n=13).

4.3.2 T cell receptor repertoire analysis reveals a selective usage of TCR Vβ chains following stimulation.

Next, we investigated the specific TCR Vβ chains that were influenced by stimulation. The proportions of the individual TCR Vβ chains detected by the IOTest kit were compared between Mtb-stimulated and unstimulated conditions in BAL and PBMC. Out of the 24 specific TCR Vβ chains examined, only four (Vβ3, Vβ5.1, Vβ20, and Vβ23) demonstrated an increase in proportion within CD4 TCRs upon stimulation with Mtb lysate in PBMCs **(Figure 4-3 A)**. Whilst in BAL, more than half of the analysed TCR specificities demonstrated an increased proportion of $CD4^+$ TCR Vβ chains under the stimulated condition compared to the unstimulated condition. These specificities included Vβ1, Vβ5.1, Vβ7.1, Vβ8, Vβ12, Vβ13.2, Vβ14, Vβ16, Vβ18, Vβ20, and Vβ23 **(Figure 4-3 B)**. A similar trend was also observed in CD8 TCRs, in which there was a higher frequency of the TCR Vβ chains in stimulated condition than in unstimulated in both PBMC and BAL **(Figure 4-3 C & D)**. The observed increase in TCRs could be attributed to either TCR Vβ proliferation or activation-induced cell death, particularly for those with a low frequency. Additionally, even in the absence of stimulation, certain TCR chains displayed a higher frequency in BAL compared with blood, observed in both CD4 and CD8 T cells **(Figure 4-3 B & D)**. This may be attributed to non-specific TCR stimulation and/or retention of T cells in the respiratory tract, which is close to the external environment and constantly exposed to pathogenic organisms and normal flora, activating the cells (202,273,307). Intriguingly, upon Mtb stimulation, both PBMC and BAL samples consistently exhibited an increase in CD4 and CD8 TCR Vβ5.1 **(Figure 4-3 A-D)**. TCR Vβ5.1 has been linked to the immune response against Mtb. Previous studies have demonstrated that T cells expressing TCR Vβ5.1 are expanded in the blood and lungs of individuals with active TB disease, indicating their involvement in the immune response against Mtb.

Moreover, these T cells have been shown to recognise multiple Mtb antigens, including the ESAT-6 peptide (306). Taken together, these findings suggest a selective TCR usage in both BAL and PBMC in response to Mtb-specific antigens.

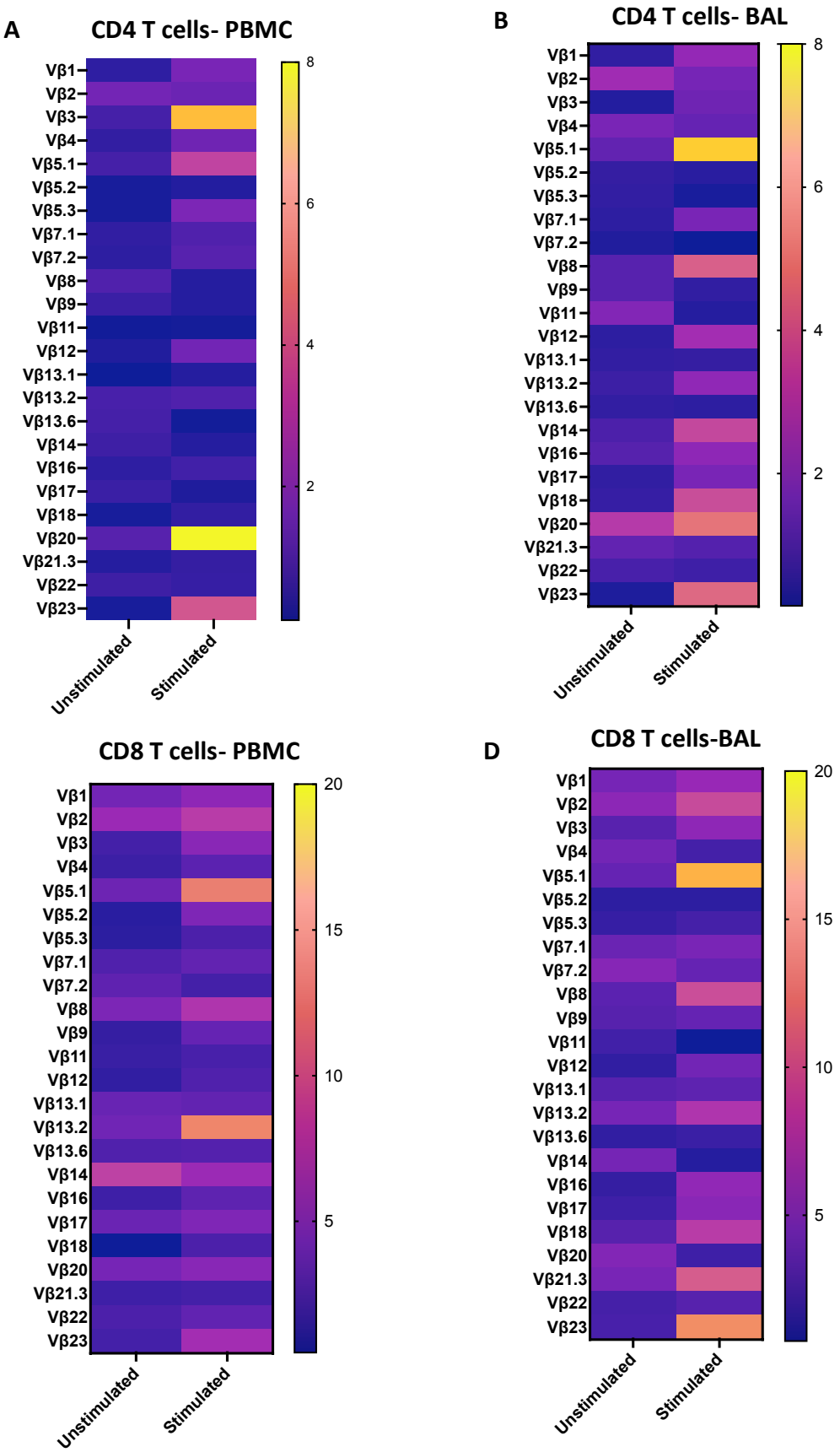


Figure 4-3 Effect of Mtb stimulation on TCR Vß chain usage in CD4 and CD8 T cells from BAL and PBMC

BAL and PBMC Alveolar cells and PBMCs were stimulated for 18 hours with whole Mtb lysate and stained with fluorochrome-conjugated antibodies for different TCR Vß chains. The Heatmaps compare the frequencies of 24 TCR chains in Mtb lysate-stimulated and unstimulated conditions. Differential CD4 TCR Vß chain usage in PBMC (A) and BAL (B). CD8 TCR Vß expression pattern in PBMC (C) and BAL (D). Blue (darker shades) indicates a lower frequency while yellow- orange represents higher frequency, (n=13).

4.3.3 The highly used TCR Vß chains are more likely to cluster together within compartments.

Having observed a differential expression of the TCR Vß chains following Mtb stimulation between CD4 and CD8 T cells in both PBMC and BAL, we then assessed the effect of the highly used chains in the T-cell-mediated immune response to Mtb. Through correlation analysis, we tested the hypothesis that antigen-specific T cells with high expression of TCR Vß chains cluster together within compartments. Overall, in PBMCs, most CD4+ T cells expressing high frequencies of TCR Vß chains clustered together compared to the CD4+ T cells with low expression **(Figure 4-4 A & B**). These include: Vß3.6, Vß4, Vß5.1, Vß5.2, Vß5.3, Vß7.2, Vß17 and Vß23. Similarly, a comparable trend was observed in BAL samples, albeit with distinct coverage of TCR specificities, such as Vß2, Vß11, Vß13.6, Vß13.1, Vß7.2, and Vß21.3 **(Figure 4-4 B).** Likewise, there was a differential clustering of $CD8^+$ T cells expressing the frequently utilised TCR Vß chains in both PBMC and BAL **(Figure 4-4 C** and **D**), with more chains observed in the BAL than in PBMC **(Figure 4-4 D).** Together, these results show that $CD4^+$ and $CD8^+$ T cells expressing high levels of specific TCR Vß chains tend to aggregate within the blood and the lung to provide a broad range of immune coverage in response to Mtb antigens.

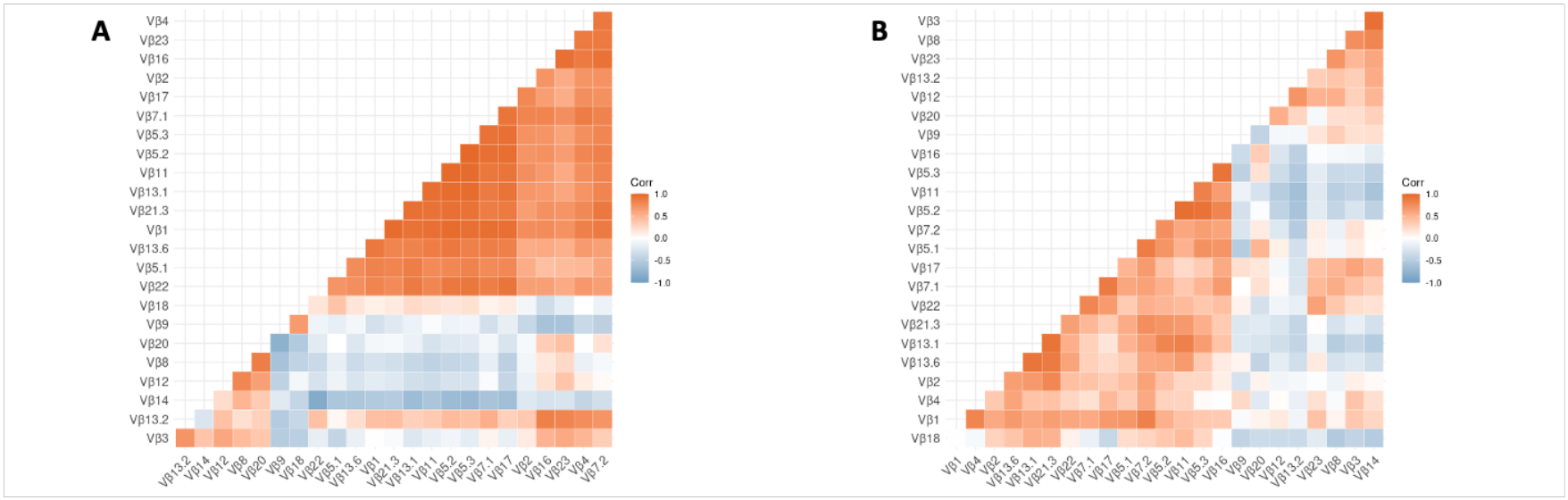

CD4 T cells-PBMC
A
Vβ4
Vβ23
Vβ16
Vβ2
Vβ17
Vβ7.1
Vβ5.3
Vβ5.2
Vβ11
Vβ13.1
Vβ21.3
Vβ1
Vβ13.6
Vβ5.1
Vβ22
Vβ18
Vβ9
Vβ20
Vβ8
Vβ12
Vβ14
Vβ13.2
Vβ3
Corr
1.0
0.5
0.0
-0.5
-1.0
CD4 T cells-BAL
B
Vβ3
Vβ8
Vβ23
Vβ13.2
Vβ12
Vβ20
Vβ9
Vβ16
Vβ5.3
Vβ11
Vβ5.2
Vβ7.2
Vβ5.1
Vβ17
Vβ7.1
Vβ22
Vβ21.3
Vβ13.1
Vβ13.6
Vβ2
Vβ4
Vβ1
Vβ18
Corr
1.0
0.5
0.0
-0.5
-1.0
CD8 T cells-PBMC
C
Vβ7.2
Vβ16
Vβ2
Vβ13.1
Vβ13.2
Vβ3
Vβ5.3
Vβ4
Vβ22
Vβ14
Vβ23
Vβ12
Vβ20
Vβ13.6
Vβ11
Vβ9
Vβ7.1
Vβ5.2
Vβ5.1
Vβ18
Vβ17
Vβ21.3
Vβ1
Corr
1.0
0.5
0.0
-0.5
-1.0
CD8 T cells-BAL
D
Vβ4
Vβ18
Vβ12
Vβ13.1
Vβ1
Vβ22
Vβ21.3
Vβ13.6
Vβ2
Vβ5.3
Vβ5.1
Vβ16
Vβ17
Vβ5.2
Vβ11
Vβ20
Vβ23
Vβ8
Vβ14
Vβ13.2
Vβ3
Vβ9
Vβ7.1
Corr
1.0
0.5
0.0
-0.5
-1.0

Figure 4-4 Comparison of TCR Vβ expression within the lung and blood

Lymphocytes from BAL and PBMCs were stimulated with Mtb lysate in vitro for 18 hours. Heatmaps illustrate the clustering of $CD4^+$ and $CD8^+$ TCR Vβ chain frequencies in PBMC and BAL, highlighting differences in their distribution. Differential correlation of $CD4^+$ TCR Vβ-expressing cells is shown in (A) for PBMC and (B) for BAL, while (C) and (D) depict the correlation of $CD8^+$ TCR Vβ-expressing cells in PBMC and BAL, respectively. Stronger correlations are represented by darker orange shades, whereas weaker correlations appear as lighter blue (n=13).

4.3.4 TCR Vβ expression by Mtb-specific IFNγ+ and TNF-α+ T cells

Subsets of CD4 and CD8 T cells that produce IFNγ+ and TNF- α and are crucial in immune responses to HIV and TB have been described (278,308). We characterised Mtb-specific IFNγ+ and TNF-α+ CD4 and CD8 T cells after antigen stimulation and assessed the relative contribution of the TCR Vβ to the production of these effector cytokines. This was done to test the hypothesis that T cells in the blood and lung bearing different TCR Vβ exhibit distinct variations in cytokine expression profiles. We measured the frequency of IFNγ+ and TNF-α+ cells for each TCR chain using the gating strategy **(Figure 4-5)** and compared them between BAL and PBMC samples. Notably, there was a higher frequency of certain CD4 TCR Vβ chains associated with the production of IFNγ+ in BAL compared to PBMCs **(Figure 4-6 A).** Specifically, these chains were Vβ2, Vβ7.1, and Vβ20. Conversely, a higher proportion of TCR Vβ chains associated with TNF-α+ production was found in PBMCs compared to BAL **(Figure 4-6 B)**. These findings collectively suggest that TCRs in BAL samples are more likely to be associated with IFNγ+ production, while TCRs in PBMCs are more prone to be involved in TNF-α+ production.

By contrast, most CD8 IFNγ+ TCRs showed no disparity between BAL and PBMC, except for Vβ8, which exhibited higher levels of IFN-γ in PBMC than in BAL **(Figure 4-6 C)**. A similar trend was observed in CD8 TNF-α+ cells, where the majority of TCRs did not display any distinction. However, there was a notable increase in Vβ1 and Vβ23 in PBMC compared to BAL **(Figure 4-6 D)**. These findings collectively suggest that CD8 TCRs are more inclined to generate either IFN-γ or TNF-α in PBMC rather than in BAL.

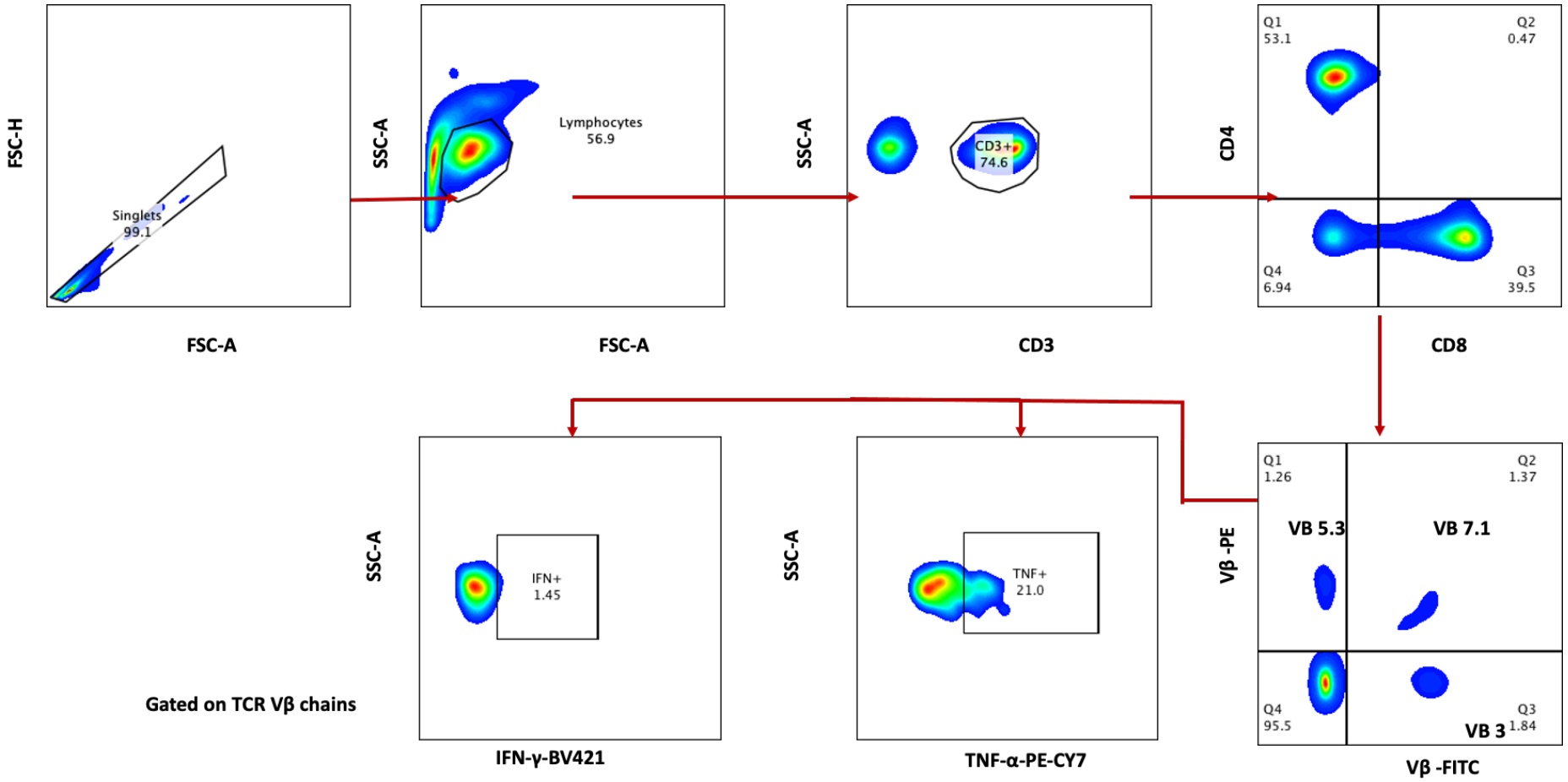


Figure 4-5 Gating strategy for TCR Vß repertoire gated on PBMC

Stimulated PBMCs and BAL cells were stained with appropriate antibodies. Cells were gated on lymphocytes, CD3, then CD4 and CD8 respectively. IFN-γ and TNF-α producing T cells were then gated on Vß chains expressing PE, PE+FITC and FITC to identify Vß chains according to the conjugates present in every tube (A-H) as per the manufacturer's instruction.

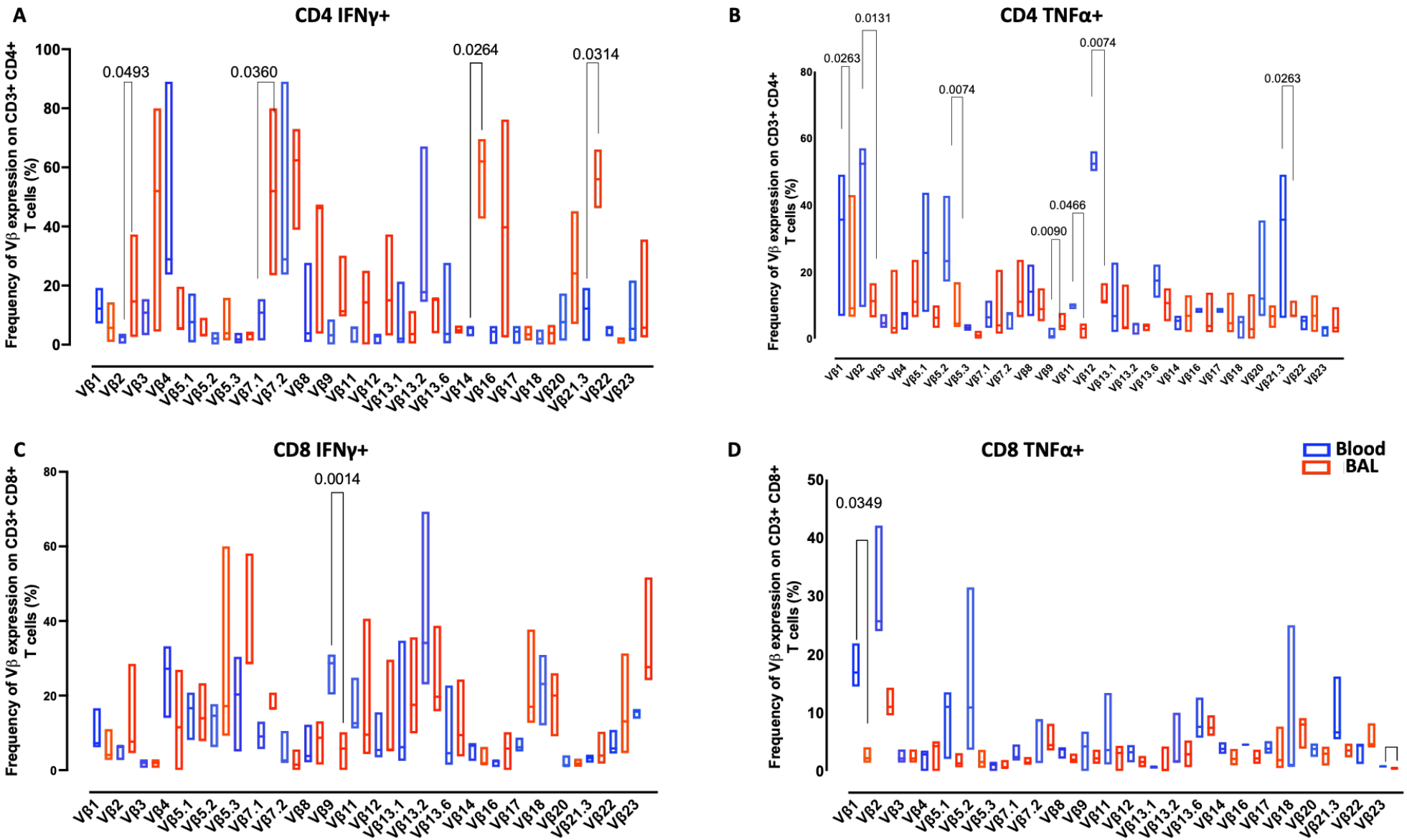

A
CD4 IFNγ+
Frequency of Vβ expression on CD3+ CD4+ T cells (%)
0.0493
0.0360
0.0264
0.0314
B
CD4 TNFα+
Frequency of Vβ expression on CD3+ CD4+ T cells (%)
0.0263
0.0131
0.0074
0.0074
0.0466
0.0090
0.0263
C
CD8 IFNγ+
Frequency of Vβ expression on CD3+ CD8+ T cells (%)
0.0014
D
CD8 TNFα+
Frequency of Vβ expression on CD3+ CD8+ T cells (%)
0.0349
Blood
BAL
Vβ1 Vβ2 Vβ3 Vβ4 Vβ5.1 Vβ5.2 Vβ5.3 Vβ7.1 Vβ7.2 Vβ8 Vβ9 Vβ11 Vβ12 Vβ13.1 Vβ13.2 Vβ13.6 Vβ14 Vβ16 Vβ17 Vβ18 Vβ20 Vβ21.3 Vβ22 Vβ23

Figure 4-6 TCR Vß repertoire of IFN-γ and TNF-α producing T cells.

BAL cells and PBMCs were stimulated with *Mtb* lysate in vitro for 18 hours and stained for various TCR Vß chains. CD4+ and CD8+ T-cell cytokine responses were assessed using intracellular cytokine staining. (A) Frequency of IFN-γ and TNF-α-producing CD4+ T cells across different TCR Vß chains in BAL and PBMCs. (B) Proportion of TNF-α-secreting CD4+ and (C) CD8+ T cells in BAL and PBMCs. Bar graphs summarize individual data, with error bars representing SEM (n=10). Statistical significance ($P < 0.05$) was determined using the Mann-Whitney U test; non-significant comparisons are not shown.

4.3.5 A strong network of TCR Vß chains in $CD4^+$ and CD8 T cells is associated with IFN-γ and TNF-α production in both the airway and peripheral blood.

Next, we assessed whether the inter-relationships among different TCR Vß chains were associated with cytokine production by constructing a TCR Vß network analysis comparing CD4+ T cell Vß expression in the lung and peripheral blood. Notably, the airway exhibited a more robust network of CD4 TCR Vß chains linked to the generation of IFN-γ and TNF-α compared to the blood. Specifically, Vß1, Vß2, Vß3, Vß4, Vß5.1, Vß5.2, Vß5.3, Vß7.1, Vß7.2, Vß8, Vß11, Vß13.6, Vß16, Vß17, Vß21.3, Vß22 and Vß23 **(Figure 4-7 A)** showed strong correlations ($r \geqslant 0.80$, $p<0.05$) with IFN-γ production in the blood, while a distinct set of Vß expressions was associated with IFN-γ production in the airway, including Vß2, Vß3, Vß8, Vß9, Vß12, Vß13.2, Vß14, and Vß23 **(Figure 4-7 B).** By contrast, out of the 24 CD4 TCR Vß expression networks analysed, a few chains were notably associated with TNF-α production, namely Vß3, Vß4, Vß8, Vß9, Vß12, Vß13.2, Vß13.6, and Vß14 **(Figure 4-7 C).** Conversely, 18 TCRs exhibited a stronger correlation with TNF-α production in the airway than in the periphery. These included Vß1, Vß2, Vß3, Vß4, Vß5.1, Vß5.2, Vß5.3, Vß7.1, Vß7.2, Vß11, Vß13.1, Vß13.6, Vß16, Vß17, Vß21.3, and Vß23 **(Figure 4-7 D).** These results indicate that a tight network of CD4 TCR Vß chains exists, which is associated with IFN-γ and TNF-α production in the lung and blood.

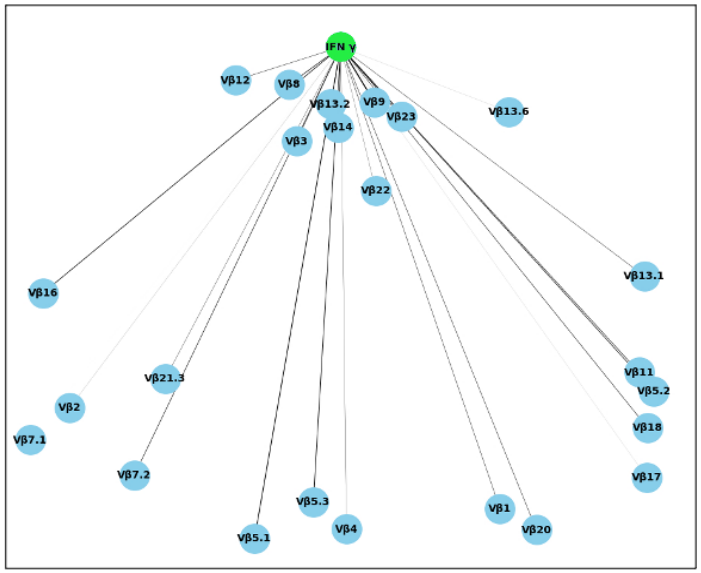
A similar analysis of TCR Vß chain networks was also performed in the $CD8^+$ T cells in the airway and blood. Overall, few distinct Vß chains showed a strong association of IFN-γ production in the lung and blood. These were Vß1, Vß4,Vß5.1, Vß5.2, Vß5.3, Vß8, Vß11, Vß13.1 and Vß21.3 in PBMC and Vß2, Vß3,Vß8, Vß9, Vß13.2, Vß12, and Vß23 **(Figure 4-7 E & F)**. However, most of the Vß in $CD8^+$ T cells displayed a strong TCR network, which was associated with TNF-α production

in both PBMC and BAL **(Figure 4-7 G & H)**. Altogether, these data suggest that variations in the TCR Vβ integrated networks between the periphery and airway suggest differences in T cell specificity in these compartments.

CD4 T cells

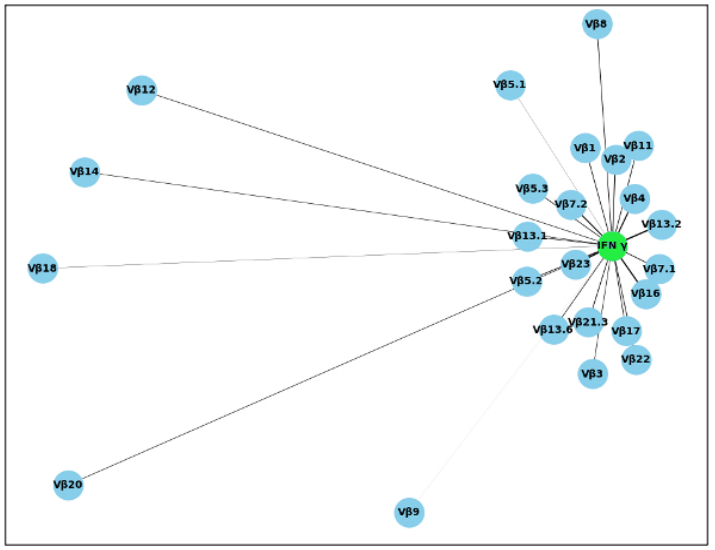
A

Periphery



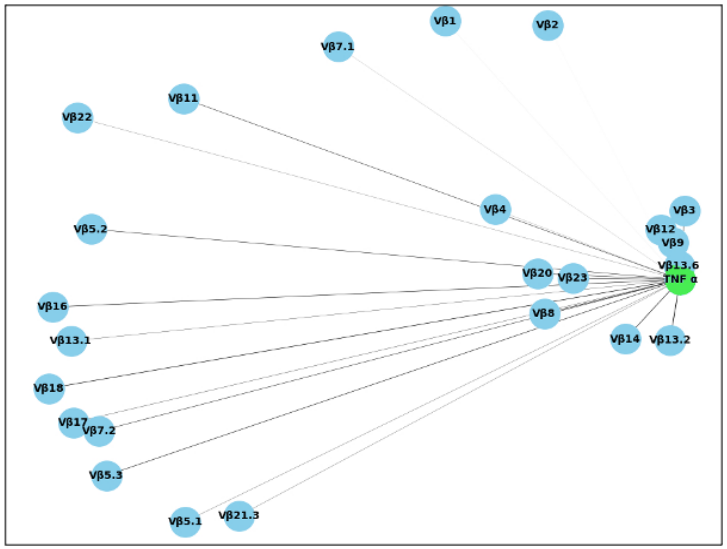
B

Airway

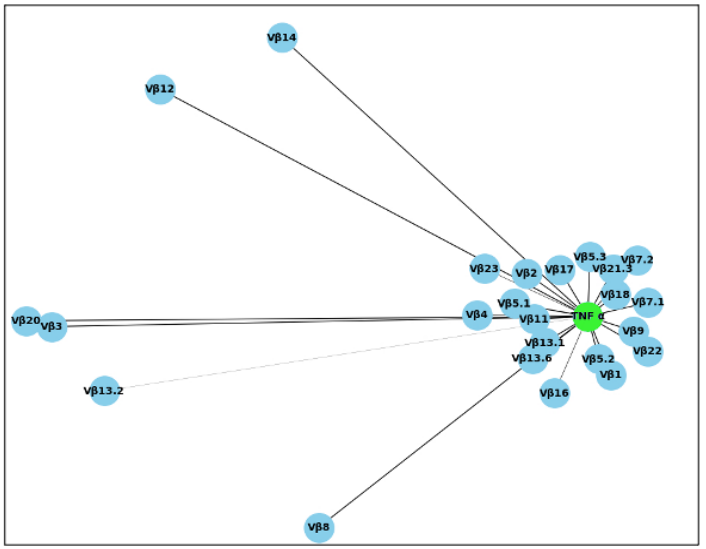


IFN-γ

C



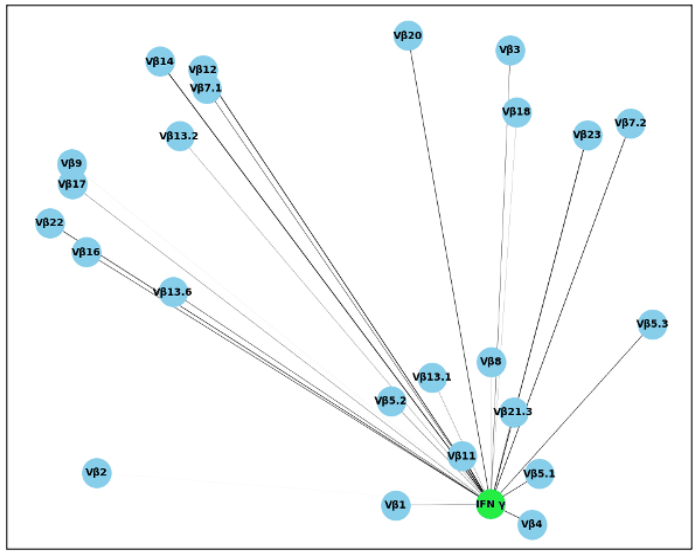
D



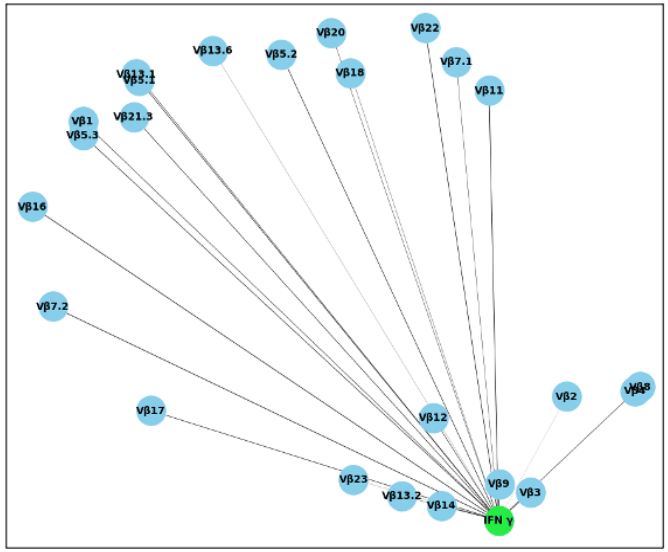
TNF- α

CD8 T cells

E **Periphery**

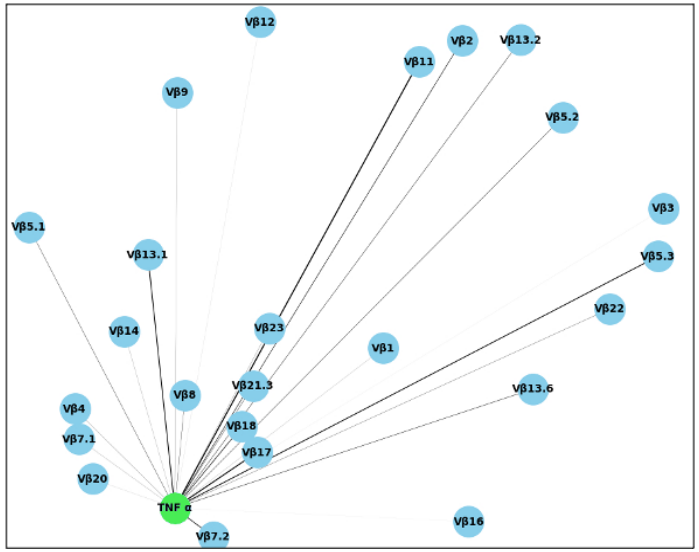


F **Airway**

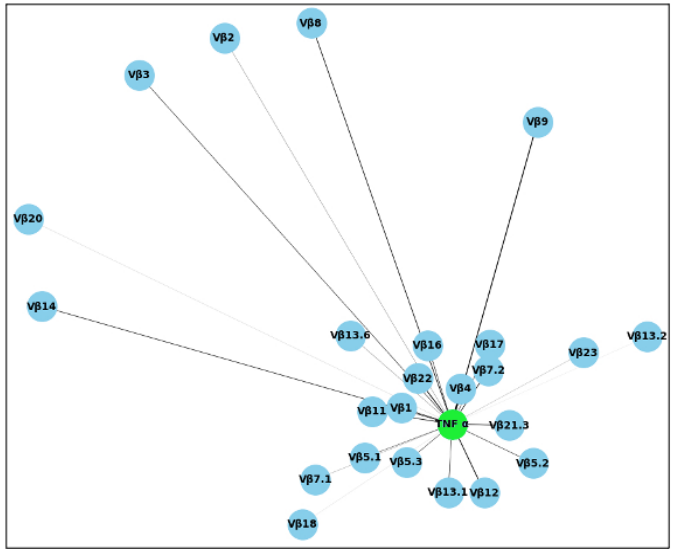


IFN-γ

G



H



TNF- α

Figure 4-7 TCR Vß cytokine network analysis of Mtb specific CD4 and CD8 T cells in HIV-uninfected individuals

Alveolar lymphocytes from BAL and PBMCs were stimulated in vitro with Mtb lysate for 18 hours and stained for various TCR Vß chains. Network graphs illustrate the association of CD4+ TCR Vß chains with IFN-γ production in (A) PBMCs and (B) BAL, as well as TNF-α production in (C) PBMCs and (D) BAL. Similarly, the contribution of TCR Vß chains to IFN-γ production in CD8+ T cells is shown for (E) BAL and (F) PBMCs, and for TNF-α in (G) BAL and (H) PBMCs (n=13). The strength of the correlation is indicated by the length of the connecting lines between nodes, with shorter lines representing stronger correlations ($r \geqslant 0.80$, $p<0.05$). These were considered as strong correlations.

4.3.6 TCR Vß are more likely to be multifunctional in the lung than in the blood.

To further characterise the phenotype of cytokine-producing TCR Vßs and investigate their association with cytokine production, we calculated the percentage of maximum response. The role of multifunctional T cells in clearing intracellular pathogens, including TB, has been well-established (309–311). Our analysis revealed that a significant proportion of CD4 T cells expressing TCRs in PBMC produced either IFNγ+ or TNF-α+ **(Figure 4-8 A)**. In BAL samples, out of the 24 specific CD4 TCR Vß chains examined, only three (Vß7.2, Vß13.6, Vß14) exhibited an increased proportion of IFNγ+ TNF-α+ double producers **(Figure 4-8 B)**. These findings suggest that the majority of cytokine+ CD4 TCRs in the blood primarily produce either IFN-γ or TNF-α, while the TCRs highly utilised in the lung are more associated with T cells that produce both IFN-γ and TNF-α+ (*$p < 0.05$; **$p < 0.01$).

In contrast, CD8+ bearing TCR Vß in both PBMC and BAL displayed an increased proportion of either IFNγ+ or TNF-α+ production **(Figure 4-8 C& D)**. Together, these results indicate that Mtb-specific CD8 cytokine+ TCRs are associated with the production of either IFN-γ or TNF-α.

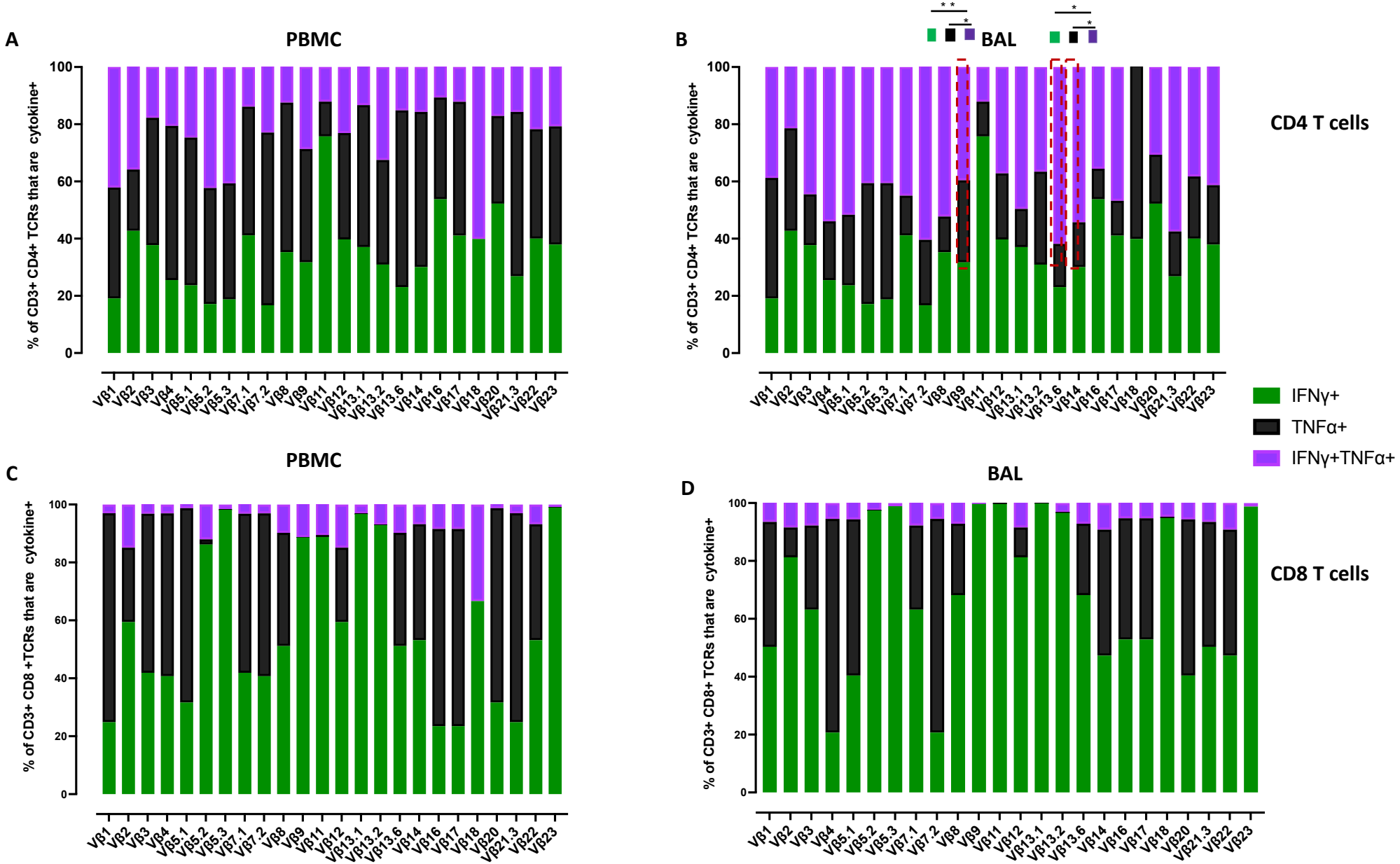

A
PBMC
% of CD3+ CD4+ TCRs that are cytokine+
100
80
60
40
20
0
Vβ1 Vβ2 Vβ3 Vβ4 Vβ5.1 Vβ5.2 Vβ5.3 Vβ7.1 Vβ7.2 Vβ8 Vβ9 Vβ11 Vβ12 Vβ13.1 Vβ13.2 Vβ13.6 Vβ14 Vβ16 Vβ17 Vβ18 Vβ20 Vβ21.3 Vβ22 Vβ23
B
BAL
* *
*
*
*
% of CD3+ CD4+ TCRs that are cytokine+
CD4 T cells
IFNγ+
TNFα+
IFNγ+TNFα+
C
PBMC
% of CD3+ CD8 +TCRs that are cytokine+
D
BAL
% of CD3+ CD8+ TCRs that are cytokine+
CD8 T cells

Figure 4-8 Analysis of TCR Vß expression and cytokine production following Mtb antigen stimulation.

BAL and PBMC lymphocytes were stimulated with Mtb lysate in vitro. The percentages of IFN-γ+ CD4 and CD8 T-cells expressing TNF-α were determined following 18 hours of stimulation. The (A) proportion of TCR Vß chain usage of cytokine-producing CD4 in (A) PBMC and (B) BAL fluid. Percentage of CD8+ TCRs that are cytokine+ in (C) PBMC and (D) in BAL. The bar graphs show summarised data of individuals, and the error bars are SEM n=8. Samples were analysed by ANOVA followed by Tukey's multiple comparison test. *p < 0.05; **p < 0.01

4.3.7 HIV infection is associated with a differential expression of TCR Vß in Mtb-specific CD4+ and CD8+ T cells.

It is well known that HIV depletes Mtb-specific T cells and impairs their function. However, the mechanism behind this is poorly understood. We sought to investigate the impact of HIV on the frequency of Mtb-specific T cells expressing various TCRs Vß chains. The frequencies of TCR Vß chains in CD4 and CD8 T cells were compared between HIV-uninfected and people living with HIV (PLHIV) on ART, as ART naïve PLHIV were not included due to limited sample availability. Significant alterations in the frequencies of some TCR Vß expressions were observed in CD4+ and CD8+ T cells in PLHIV compared to healthy controls. Specifically, CD4 T cells expressing Vß1, Vß7.2, and Vß23 were significantly increased ($p < 0.05$) in PBMCs of HIV-infected individuals compared to healthy controls **(Figure 4-9 A)**. Conversely, CD4 T cells expressing Vß9 and Vß18 showed a lower frequency in PLHIV than in HIV-uninfected individuals **(Figure 4-9 A)**. In CD4+ BAL T cells, however, Vß13.1 expression was significantly higher in PLHIV than in healthy controls, whilst Vß14 showed a decreased frequency of expression in HIV-infected individuals than in HIV-uninfected **(Figure 4-9 B)**. Together, these findings suggest that HIV infection may affect the diversity and distribution of TCR Vß chains in both blood and lung.

Similar to the analysis conducted in CD4+ cells, TCR Vß expression was examined in CD8+ T cells. No statistically significant differences were found in the TCR Vß specificities in the blood of both PLHIV and healthy controls **(Figure 4-9 C)**. However, in the lung, three Vß families (Vß5.1, Vß16, and Vß17) showed increased expression, while one family (Vß14) displayed decreased expression in HIV-infected individuals compared to HIV-uninfected **(Figure 4-9 D)**. This indicates

that HIV infection affects $CD8^+$ TCR Vβ expression differently in different tissue compartments.

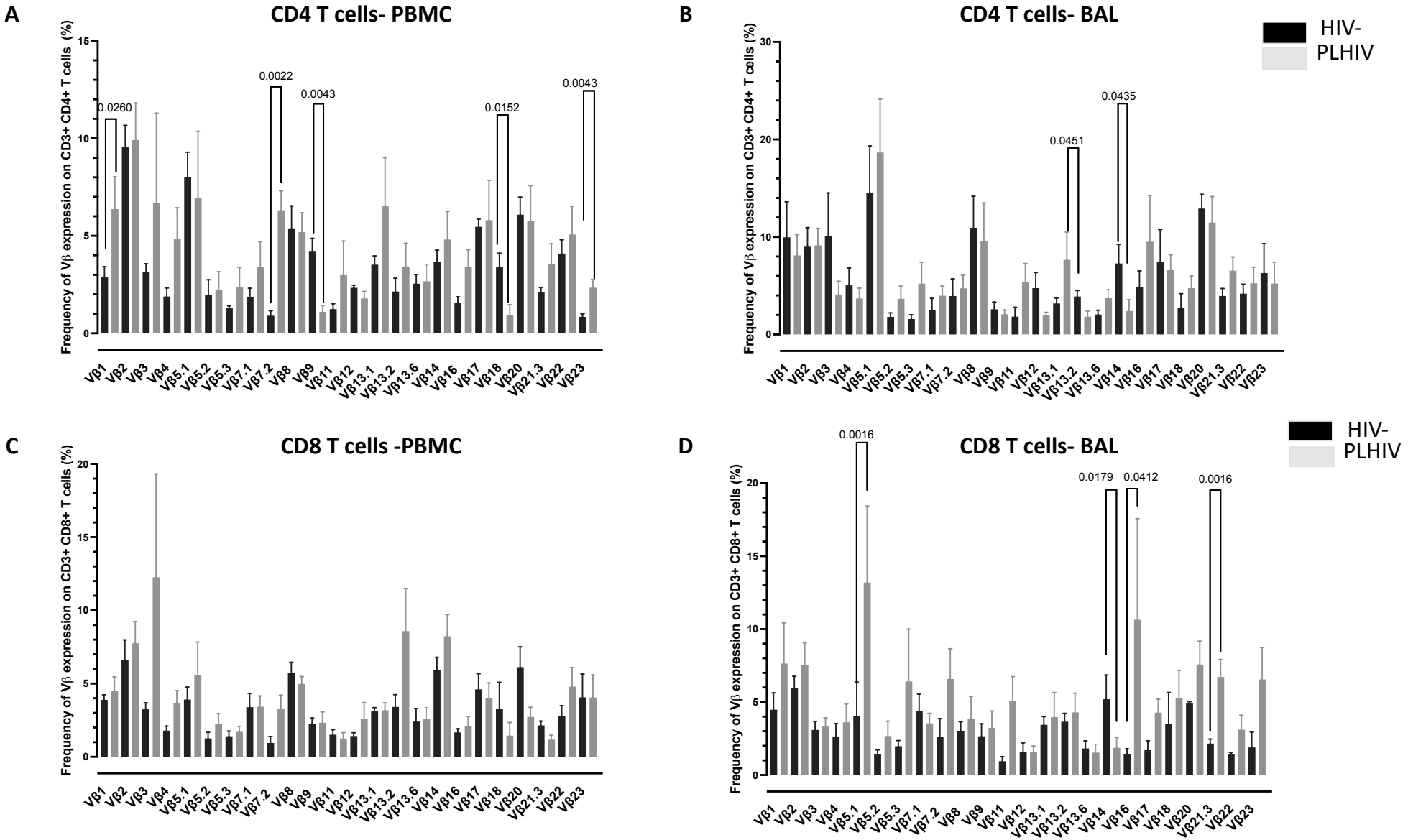

A
CD4 T cells- PBMC
Frequency of Vβ expression on CD3+ CD4+ T cells (%)
0.0260
0.0022
0.0043
0.0152
0.0043
B
CD4 T cells- BAL
Frequency of Vβ expression on CD3+ CD4+ T cells (%)
0.0451
0.0435
HIV-
PLHIV
C
CD8 T cells -PBMC
Frequency of Vβ expression on CD3+ CD8+ T cells (%)
D
CD8 T cells- BAL
Frequency of Vβ expression on CD3+ CD8+ T cells (%)
0.0016
0.0179
0.0412
0.0016
HIV-
PLHIV
Vβ1 Vβ2 Vβ3 Vβ4 Vβ5.1 Vβ5.2 Vβ5.3 Vβ7.1 Vβ7.2 Vβ8 Vβ9 Vβ11 Vβ12 Vβ13.1 Vβ13.2 Vβ13.6 Vβ14 Vβ16 Vβ17 Vβ18 Vβ20 Vβ21.3 Vβ22 Vβ23

Figure 4-9 The impact of HIV-infection on CD4 and CD8 TCR Vß répertoire in BAL and PBMCs in PLHIV

Lymphocytes from BAL and PBMCs were stimulated with Mtb lysate in vitro for 18hrs. Frequency of CD4 TCR Vß chain usage in HIV+ and HIV-ve in (A) Periphery (B) Airway. TCR Vß expression in CD8+ T cells(C) PBMC and (B) BAL. Bar graphs summarize individual data, with error bars representing SEM (n=10). Statistical significance ($P < 0.05$) was determined using the Mann-Whitney U test; non-significant comparisons are not shown.

4.3.8 HIV infection is associated with heterogeneous expression of TCR Vß networks in Mtb-specific IFNγ+ and TNF-α+ producing T cells in both blood and the lung.

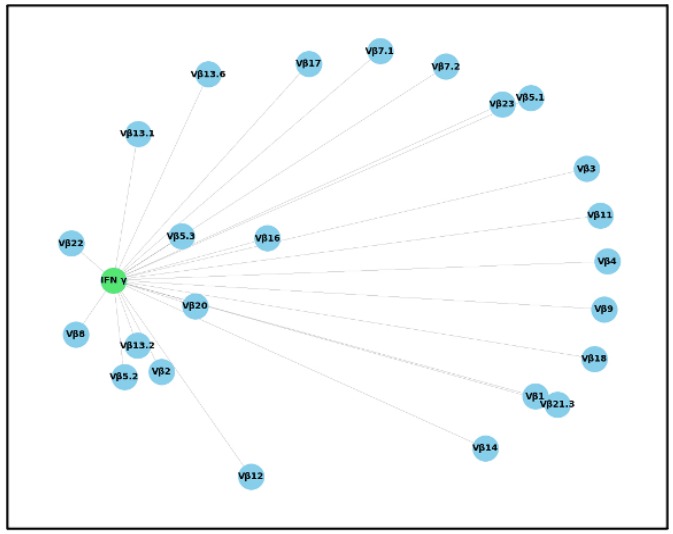
Lastly, the impact of HIV infection on the TCR Vß network and the relative contribution of cells bearing these Vß to the production of IFN-γ and TNF-α was assessed in blood and the lung. To explore the relationship between TCR Vß in CD4+and CD8+ T cells and the likelihood of producing cytokines, the distance between various TCR Vß segments was measured and created networks connecting chains to the cytokine (either IFN-γ or TNF-α) **(Figure 4-10)**, in which the length was associated with a correlation. Overall, a weaker network of CD4 TCR Vß chains associated with the production of IFN-γ and TNF-α was observed in the lung of PLHIV compared to the blood. In the blood, most TCR Vß chains in CD4 T cells showed a weak correlation with IFN-γ production ($r<0.40$). Notably, among the 24 TCR Vß chains analysed, strong associations were observed only in a few chains, specifically Vß2, Vß5.2, Vß5.3, Vß13.1, Vß, Vß20, and Vß22 **(Figure 4-10 A)**. A similar pattern was seen in the lung, where a close network of Vß in CD4 T cells was associated with IFN-γ. These included Vß3, Vß8, Vß9, Vß12, Vß13.2, Vß14, Vß22, and Vß23 **(Figure 4-10 B)**. On the contrary, most TCR Vß in CD4 cell T cells in PLHIV were associated with the production of TNF-α in blood, which was not observed in the lung (**Figure 4-10C & D).** Collectively, these findings suggest that HIV has a varying effect on the TCR networks in relation to cytokine production. The strong associations observed in certain TCR Vß chains suggest a potential role for these chains in HIV-associated inflammation mediated by both IFN-γ and TNF-α. In comparison, the weak association may indicate that cells expressing these Vß are less involved or targeted by HIV infection.

A comparable examination of the TCR Vß chain networks was conducted in CD8+ T cells within the airway and bloodstream of PLHIV. Overall, only a limited number

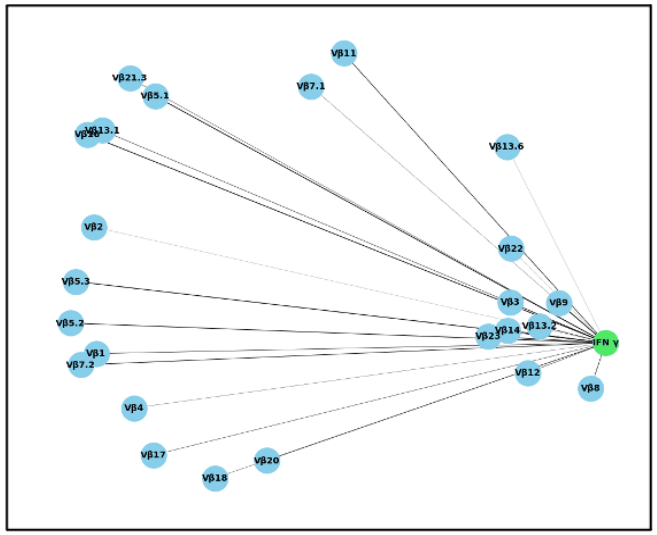
of distinct Vβ chains exhibited a pronounced association with IFN-γ production in the lung and blood compartments. These included Vβ1, Vβ3, Vβ5.1, Vβ5.2, Vβ7.1, Vβ8, Vβ9, Vβ11, and Vβ21.3 in PBMCs, Vβ12, Vβ13.2, and Vβ13.6 (as illustrated in **Figure 4-10 E & F**). However, the majority of the Vβ chains in CD8+ T cells exhibited a robust TCR network linked with TNF-α production in both PBMCs and BAL **(Figure 4-10 G & H)**. Taken together, these findings suggest that differences in the TCR Vβ integrated networks between the peripheral blood and airway in PLHIV indicate alterations induced by HIV infection, which distinctly affect cytokine production.

CD4 T cells

A **Periphery**

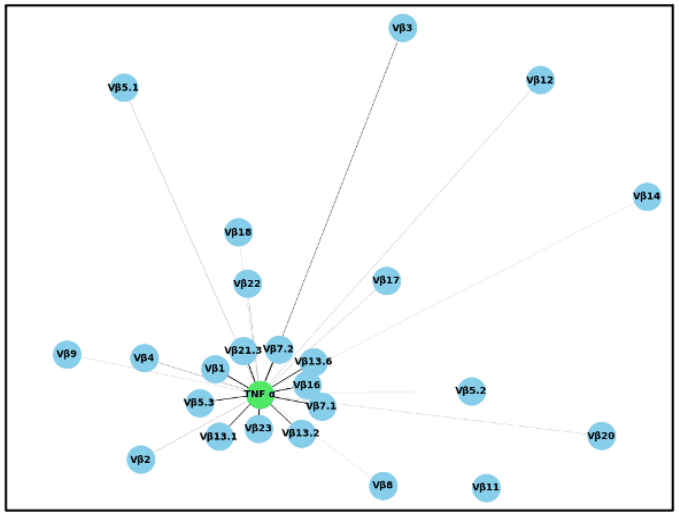


B **Airway**

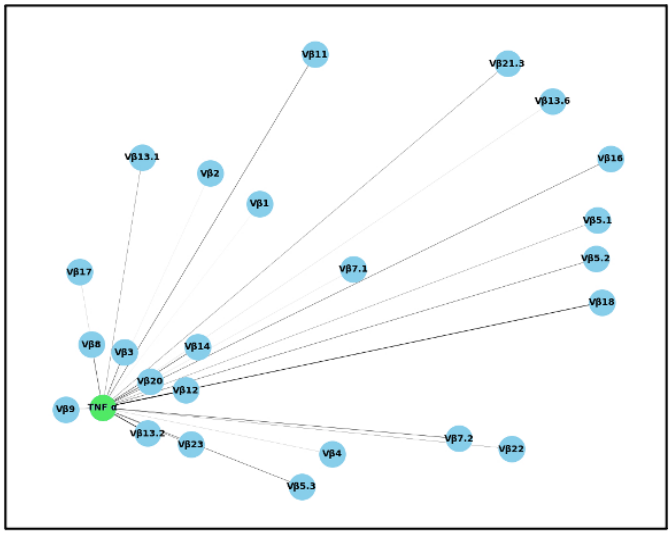


IFN-γ

C



D

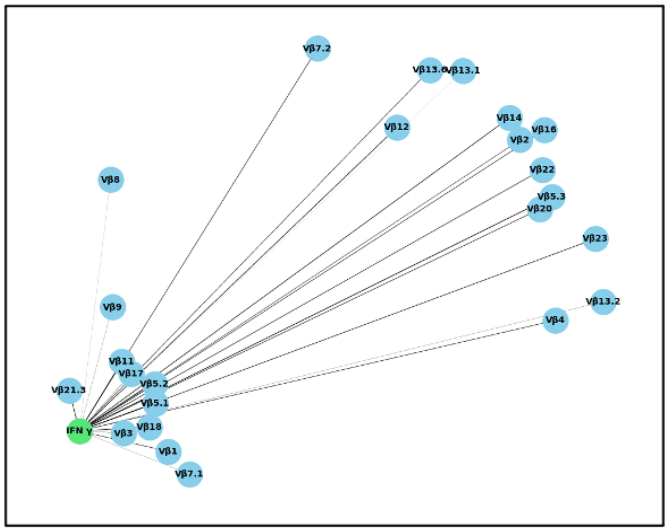


TNF- α

CD8 T cells

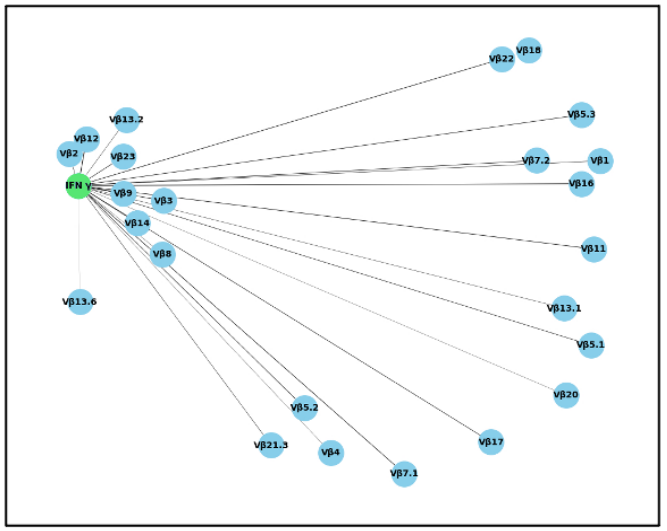
Periphery

E



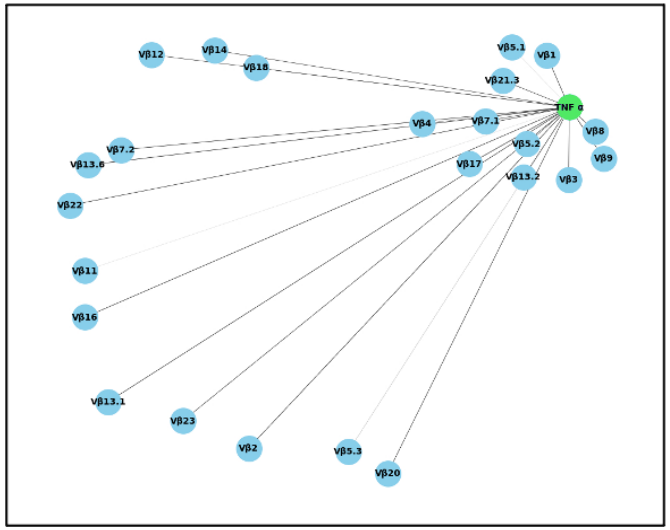
Airway

F

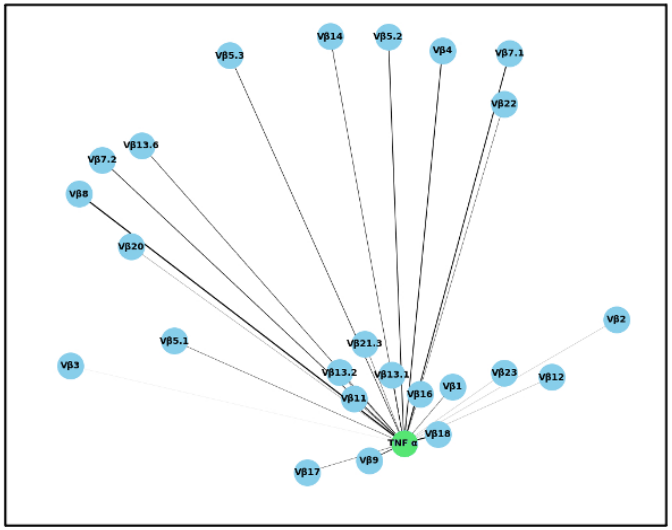


IFN-γ

G



H



TNF- α

Figure 4-10 TCR Vß network analysis of Mtb specific CD4 and CD8 T cells in PLHIV

Alveolar lymphocytes from BAL and PBMCs were stimulated with Mtb lysate in vitro for 18hrs and stained for different TCR Vß chains. Network graphs of $CD4^+$ TCR Vß chain associating with IFN-γ production in (A) PBMC, (B) BAL as well as TNF-α (C) & (D) respectively. TCR Vß chain contribution to IFN-γ production in CD8 T cells (E) in BAL, (F) PBMC and TNF-α in (G). & (H) n=10. The shorter the connecting line between the nodes, the stronger the correlation.

4.4 Discussion

A diverse TCR repertoire, plays a crucial role in the control of infections, including Mtb and HIV, by ensuring clonal selection of antigen specific T cell clones. However, limited evidence exists on the diversity and phenotype of antigen specific T cells in TB and the impact of HIV infection on these parameters. This chapter provides novel insights into the usage of TCR Vβ chains in both the lung and blood, highlighting their functional capacity and compartmentalisation. Furthermore, it reveals a preferential selective utilisation of specific TCR Vβ chains on T cells responding to Mtb antigens in both PLHIV and healthy HIV-uninfected individuals. Additionally, the study further describes the phenotype of IFN-γ and TNF-α producing CD4 and CD8 T cells within the lung and periphery in PLHIV.

Assessment of the TCR Vβ expression is commonly used to identify changes in T cell repertoires, spanning from broad diversity to the proliferation of individual T cells. A better understanding of the patterns of TCR chain usage can provide valuable insights into the pathogenic processes implicated in diseases, including HIV and TB. Additionally, such knowledge can contribute to unravelling the complex mechanisms underlying cellular-mediated immune responses against these diseases. Previous studies have consistently demonstrated reduced TCR diversity during chronic infection (11,29). For instance, a study conducted by Luo *et al*. observed a significant decline in TCR diversity (246), which corresponded to more severe clinical outcomes in individuals with active TB. This finding suggests a potential link between diminished TCR diversity and the progression or severity of chronic infections.

In line with the observation that T-cell stimulation with mitogen alters the TCR Vβ usage in blood (313), our findings reveal that stimulation with Mtb lysate leads to an increased proportion of certain TCR Vβ chains in both BAL and PBMCs as compared to unstimulated conditions. Specifically, we demonstrate a selective usage of certain TCR Vβ chains following stimulation. These include a higher proportion of TCR Vβ3, Vβ5.1, Vβ20, and Vβ23 within CD4 TCRs in peripheral blood in stimulated compared to unstimulated samples. Indeed, our analysis revealed that the elevated percentages of specific TCR Vβ chains following stimulation were not necessarily the same clones that exhibited an increase in the BAL

samples, except for Vβ5.1. In BAL, we observed a distinct set of TCR specificities that displayed an increased proportion of CD4 TCR Vβ chains under the stimulated condition, which did not entirely overlap with those observed in PBMCs. The elevated chains in stimulated BAL were Vβ1, Vβ5.1, Vβ7.1, Vβ8, Vβ12, Vβ13.2, Vβ14, Vβ16, Vβ18, Vβ20, and Vβ23. The same pattern was observed for CD8 TCRs, where the specific clones exhibiting a higher frequency of TCR Vβ chains varied between PBMCs and BAL. However, the underlying mechanism behind the observed increase in the frequency of TCR usage remains unclear. It is not known whether the increase is attributed to the proliferation of TCR Vβ chains or activation-induced cell death, especially for those with a low TCR Vβ frequency (**summarised in Figure 4-11).** In addition, it remains to be established whether the increase in frequency could be due to in situ expansion of T-cells in the lung or as a result of increased tissue retention. Further investigation is warranted to determine whether TB reactive T-cells receive signals that promote their survival and proliferation or if they undergo apoptosis.

Previous studies have reported a notable biased pattern of TCR usage, particularly with a preferential expression of TCR Vβ4 in TB pleural effusion (301). However, it is important to note that these studies primarily focused on gene expression analysis, which may have restricted the scope of their findings and could potentially account for discrepancies with the data reported in this study. Indeed, gene transcripts and proteins are distinct molecules with unique roles and regulatory mechanisms in cells. Transcripts, derived from DNA, serve as templates for protein synthesis, while proteins execute various cellular functions. Analysis of transcripts and proteins can differ due to factors such as post-transcriptional modifications, protein degradation, and variations in translation efficiency (314). Understanding these mechanisms would provide valuable insights into the dynamics of the immune responses against TB. Together, these findings suggest that the response to Mtb lysate stimulation is compartmentalised, and the usage of TCR Vβ chains may differ between the lung and peripheral blood.

The observed differences in TCR Vβ usage between these compartments imply potential distinct immune responses and dynamics involved in controlling Mtb infection at these specific sites. Although the expansion of TCR Vβ chains was anticipated under stimulated conditions, it was somewhat unexpected to observe

an increase in TCR chain expression even without stimulation in BAL. This disparity could be attributed to non-specific TCR stimulation occurring in the respiratory tract, which is proximal to the external environment. The respiratory tract is continuously exposed to various pathogenic organisms and normal flora. Consequently, these environmental factors may activate the T cells, increasing TCR chain expression even without specific stimulation (307).

We have further identified distinct subsets of highly utilised Mtb-specific CD4 and CD8 T cell TCR Vβ families that are specifically associated with the production of IFNγ+ in BAL samples or TNF-α+ in PBMC. It is well known that Mtb-specific CD4 and CD8 T cells that produce effector cytokines such as IFN-γ, TNF-α and IL-2 play a crucial role in immunity against TB (315,316). Moreover, emerging evidence suggests that Mtb-specific CD4 Th1/Th17 and NK cells are potential correlates of protection conferred by the BCG vaccine (317,318). However, the precise antigens being recognised and the specific TCR chains involved in these immune responses remain poorly understood. The observed increase in the frequency of CD4 TCR Vβ chains in BAL associated with IFNγ+ could indicate their essential role in mounting a protective immune response against particular Mtb antigens. However, it is unclear why the majority of highly expanded CD4 T cells expressing TCR Vβ chains incline towards the production of TNF-α in PBMC than in BAL. Further investigations are required to unravel these TCR chains' underlying mechanisms and functional significance in TB immunity.

It is well established that multifunctional T cells play a critical role in the clearance of intracellular pathogens, including Mtb (311,319,320). While we confirm that the presence of specific TCR Vβ chains is associated with the production of both IFN-γ and TNF-α, little is known about the precise factors contributing to the likelihood of being multifunctional in the lung compared to PBMC. Interestingly, we did not observe a similar trend with CD8 TCRs, suggesting that CD4 T cell responses exhibit greater cytokine production diversity than CD8 T cells. This study primarily focused on characterising multifunctional cells based on their cytokine production. Therefore, we lack knowledge regarding their potential for other effector functions, such as cytotoxicity. By incorporating high-throughput methods, such as scRNAseq, we can gain insights into the gene expression profiles associated with multifunctionality (245,303).

The observed increase in the proportion of TCR Vβ7.2, Vβ13.6, and Vβ14 chains associated with the potential for double cytokine production suggests two plausible explanations. Firstly, these specific TCR Vβ chains might play a pivotal role in anti-TB immunity and are preferentially selected to proliferate to generate a robust immune response. Alternatively, there might be alterations in the proportions of other T cell subsets, where a decrease in one subset results in an increase in another, contributing to the observed changes in cytokine-producing potential. A significant challenge in further investigating these possibilities is the absence of surface markers that can distinguish between TCR Vβ IFN-γ/TNF-αsingle cytokine-producing cells and multifunctional cytokine+ cells. To overcome this limitation, it is crucial to prioritise the identification of assays or markers that can accurately track and differentiate these specific cell subsets. By developing such tools, we can obtain definitive evidence and better understand the functional heterogeneity within T cell populations.

To understand the link between TCR Vβ expression and the production of IFN-γ and TNF-α, a TCR cytokine network analysis was constructed, correlating various Vβ specificities to either IFN-γ or TNF-α production. The observation of robust TCR Vβ networks in CD4 T cells associated with IFN-γ and TNF-α production in the airway than in the periphery leads to speculation that T cells bearing these Vβ repertoires might be specifically selected to respond to TB antigens in the lung. On the other hand, cells with weakly correlated TCR Vβ may be less likely to produce key effector cytokines using these TCR affinities. In CD8, however, the study demonstrates preferential expansion of cells expressing a strong network of TCR Vβ associated with TNF-αproduction in blood compared to the lung. Collectively, these findings indicate a coordinated site-specific cytokine immune response involving a wide array of CD4 and CD8 TCR Vβ chains. The differences observed in the integrated TCR Vβ networks between the peripheral blood and airway may be attributed to variations in T cell specificity, as evidenced by TCR Vβ usage in these tissues.

To date, the Mtb-specific TCR Vβ repertoire in PLHIV and the phenotypic characterisation of these cells has been poorly described. Moreover, it remains unclear why some individuals living with HIV infection progress rapidly and are

more prone to lower respiratory tract infection than others within the same population. This study, consistent with findings by Smith *et al.*, identified skewed TCR Vß usage in people living with HIV (PLHIV), with varying Vß expression observed in both CD4 and CD8 T cells in the lung and blood (321). Specifically, increased frequencies of Vß1, Vß7.2, and Vß23 were noted in CD4 T cells in PBMCs, while Vß13.2 was elevated in the lung, suggesting potential involvement in HIV response. Conversely, depletion of certain TCR Vß clones in Mtb-specific CD4 and CD8 T cells in the lung and blood may indicate HIV-induced alteration in the repertoire. HIV is known to target Mtb antigen-activated T cells selectively. Early studies in HIV-infected individuals have reported dysregulated heterogeneous TCR Vß repertoires in both CD4 and CD8 T cells, characterised by clonal expansion and depletion of specific TCR Vß clones (322–324). It raises questions about similar observations in TCR Vß in CD8 T cells and their contribution to effector cytokine production. Differences were noted in the frequency and usage of TCR Vß in CD8 T cells between HIV-uninfected and PLHIV individuals, with more significant disparities seen in the lung, where an expansion of Vß5.1, Vß16, and Vß21.3 was observed in PLHIV compared to controls. The accumulation of functionally impaired CD8 T cells, known as lymphocytic alveolitis, due to an influx of CD8 T cells in the lungs of HIV-infected individuals, may play a role in the observed increase in certain TCR Vß segments noted in this study (325). It remains unclear whether this lymphocytic alveolitis of CD8 T cells in the lung influences the increase in some TCR Vß observed in this study.

Furthermore, we demonstrate that HIV infection correlates with diverse and dysregulated expression of TCR Vß networks in Mtb-specific T cells producing IFN-γ and TNF-α in the blood and the lung. Both strong and weak TCR chain networks were observed to be associated with IFN-γ and TNF-α production. This suggests that HIV has a varying effect on the TCR networks with cytokine production. The strong associations observed in certain TCR Vß chains suggest a potential role for these chains in HIV-associated inflammation mediated by both IFN-γ and TNF-α. Whereas the weak association may indicate that T cells expressing these Vß are less involved or targeted by HIV infection. Overall, these findings suggest that, in contrast to healthy controls, HIV+ patients displayed a more restricted TCR repertoire, with alterations in the frequency of some families that may be important in antigen recognition and specificity. This effect, consequently, may

infer a decreased ability for protection in those patients. This, in part, might explain why PLHIV have an increased risk of developing lower respiratory tract infections, including TB, compared to their matched controls.

Although this study has provided insights into the diversity of Mtb antigen-specific CD4 and CD8 TCR Vß usage in the airway and blood, several significant questions remain unanswered. It is unclear how changes in the TCR repertoire contribute to the susceptibility of individuals living with HIV to natural TB infection. Gaining a deeper understanding of the functional composition of the TCR repertoire in HIV-infected individuals with pulmonary TB will advance our current knowledge and provide novel insights into the host factors that influence susceptibility and progression of Mtb infection. Such knowledge is crucial for developing more effective strategies for controlling and managing TB in individuals living with HIV.

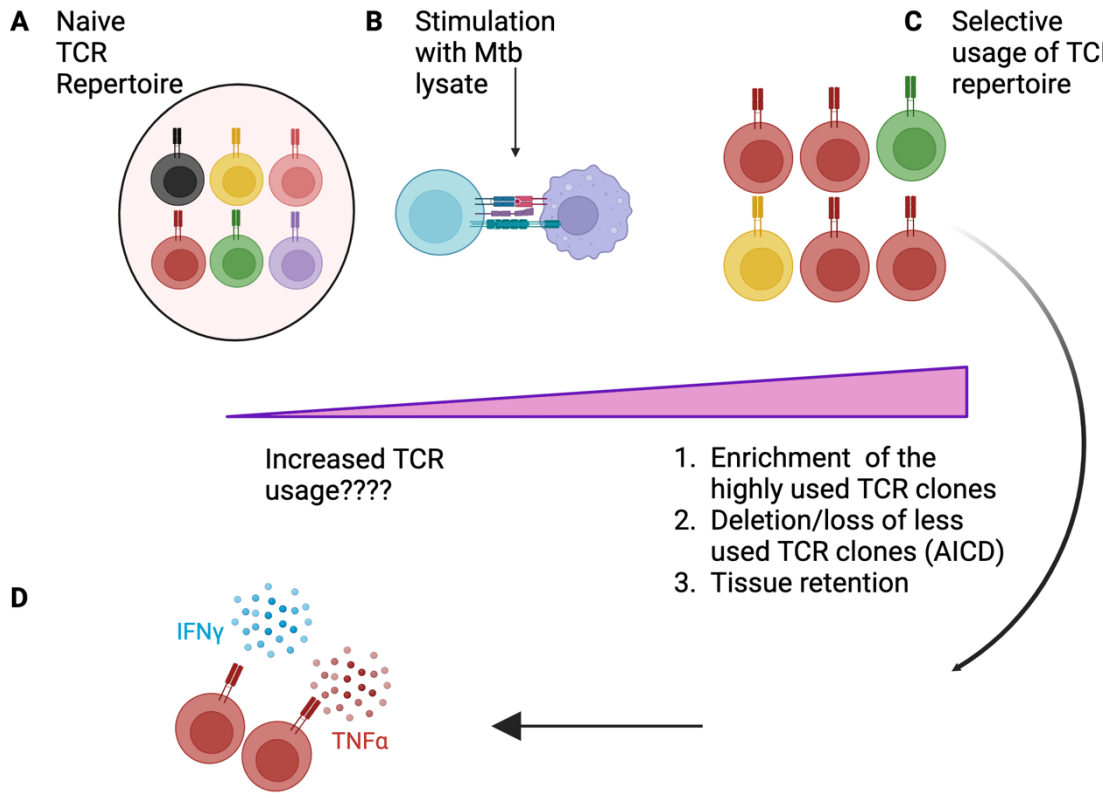


Figure 4-11 A summary of possible theories that contribute to the selection of Mtb-specific T cells with distinct TCR Vß specificities.

The schematic represents a summary of potential mechanisms that may influence changes in TCR Vß usage following stimulation with Mtb antigens (created with BioRender.com). (A) T cells from blood and lung are stimulated *in vitro* with Mtb antigens (B) to induce an Mtb-specific immune response. This leads to a skewed distribution of T cells bearing specific TCR Vß chains, characterised by the expansion of certain clones and the loss of others (C). This may result from the enrichment of frequently used TCR clones or the

deletion of less involved clones through activation-induced cell death (AICD), as part of the immune response to Mtb antigens. The selected T cells bearing specific TCR Vβ chains are associated with the production of key proinflammatory cytokines (D) (IFN-γ and TNF-α), indicating their potential role in the host immune response to TB.

4.5 Conclusion

Our findings demonstrate that T cell stimulation with Mtb antigens goes beyond the simple identification of highly utilised TCR chains. It also leads to a selective preferential usage of TCRs by Mtb-specific CD4 and CD8 T cells in both the airway and peripheral compartments. Moreover, CD4 and CD8 TCRs exhibit distinct patterns of cytokine expression profiles in the systemic circulation and BAL samples. Interestingly, within the same airway compartment, certain TCR chains are associated with a higher capacity for T cells to produce two cytokines compared to others. These variations in functional profiles between blood and lung TCRs reflect the specific immune responses taking place in these tissues. In addition, the findings suggest a site-specific antigen-driven alteration of the TCR Vβ usage which HIV may influence. These novel findings provide valuable insights into antigen recognition in BAL and PBMCs and the host immune responses to TB.

One potential limitation of the study is the lack of consideration for ethnic diversity in analysing TCR Vβ repertoire usage and its impact on immune responses to Mtb and HIV. Ethnic and genetic differences, such as variations in HLA haplotypes, can influence TCR diversity, antigen recognition, and cytokine production (140), potentially affecting susceptibility to TB and HIV progression. The absence of ethnic diversity analysis limits the generalisability of the findings. Future studies should incorporate ethnicity as a variable to better understand how genetic and environmental factors shape TCR repertoire diversity and immune responses in different populations. This would provide a more comprehensive understanding of the mechanisms underlying Mtb specific T cell responses in PLHIV and improve the development of targeted interventions for diverse populations.

Chapter 5 Assessment of the impact of HIV on Mtb-specific TCR diversity in the airway and peripheral blood by CDR3 ß sequencing

5.1 Introduction

Analysing TCR usage, particularly with a focus on CDR3 and the TRBV family, is vital for elucidating mechanisms involved in autoimmunity and infectious diseases including HIV and TB (326–328). As discussed in Chapter 1, Section 6.4, the hypervariable regions of the T cell receptor (TCR) beta chain, especially the CDR3 region, substantially contribute to T cell diversity (328,329). Within the CDR3 loop, random deletions and insertions of sequences facilitate interactions and recognition of various peptides presented by MHC molecules. These variations in CDR3 sequence determine the structure and specificity of the TCR repertoire (126,330). Consequently, the assessment of CDR3 sequences enables the identification of antigen-specific TCR repertoires within the same cell.

Given that the TCR repertoire reflects the specificity of the host immune response, characterising its features has been an area of investigation in chronic HIV infection to understand disease progression and potential correlates of protection (331–333). Previous studies have reported reduced systemic TCR diversity and a skewed repertoire distribution during HIV infection, but little is known about the immune repertoire in the lung (223,227,324). One study found that despite successful ART, patients with recurrent opportunistic infections showed an impaired antigen-specific response irrespective of improved CD4 counts (334). Evidence like this highlights the influence of HIV on the loss of the TCR specificities necessary for recognising and mounting an effective immune response against opportunistic infections including TB (335). Effective T cell immune response to Mtb relies on a diverse TCR repertoire capable of recognising various antigenic peptides. Although the significance of CD4 and CD8 T cells in TB and HIV immunity is established, the relationship between alterations in the T cell repertoire and disease presentation remains inadequately characterised in the airway mucosa.

In the previous chapter, CD4 and CD8 TCR receptor usage in peripheral blood and airway was assessed using flow cytometry techniques to identify Vβ regions of the TCR. The findings provided evidence supporting the preferential usage of particular TCR Vβ following stimulation with Mtb-specific antigens. This observation suggests that these TCR Vβ families may play a crucial role in immune responses to TB by recognising and responding to antigens associated with the pathogen. However, flow cytometry assessment of Vβ only detects major Vβ changes within the repertoire, leaving out under-represented Vβ families (303). Additionally, it does not capture the broad range of CDR3 sequences within each Vβ family that contribute to specificity. By contrast, next-generation sequencing (NGS) techniques offer a more comprehensive and unbiased approach, providing a detailed analysis of the entire TCR repertoire, TCR Vβ expression, and clonal expansion. In this chapter, NGS was employed to leverage these advantages, enabling a deeper understanding of the impact of HIV on Mtb-specific clonality and diversity in the lung and peripheral blood. By sequencing the CDR3 of the TCR β region, clonal distribution, CDR3 length distribution, and the frequency of TRBV gene segments were characterised.

5.2 Chapter aims and summary

While HIV is known to alter TCR repertoire distribution and reduce TCR diversity in peripheral blood, its effect on alveolar Mtb-specific T cells is not fully described. Examining changes in the TCR Vβ repertoire can provide insights into the impact of HIV infection on the T cell-mediated immune response in the lung, the primary site of TB infection. This chapter aims to describe the Mtb antigen-specific TCR diversity and clonality in the airway and blood and assesses the impact of HIV and ART on TCR diversity.

TCR bulk sequencing and CDR3 spectratyping were used to describe the TCR diversity of Mtb-specific T cells in BAL and PBMCs **(Chapter 2, sections 2.8–2.9)**. Peripheral blood and bronchoalveolar lavage (BAL) samples were collected from PLHIV ART-naïve, PLHIV on ART (<3 years), and HIV-uninfected adults recruited at Queen Elizabeth Central Hospital, Blantyre, Malawi. Alveolar and peripheral blood

lymphocytes were stimulated with Mtb lysate and analysed using the immunarch and tcR R packages.

Overall, the results demonstrate that Mtb-specific TCR repertoires from HIV-uninfected individuals displayed increased diversity compared to PLHIV in both the airway and blood. Additionally, airway T cells exhibited a more restricted repertoire compared to peripheral blood. The Mtb-specific T cell repertoire in BAL and PBMCs showed a higher and more diverse distribution of TCR CDR3 sequences in HIV-uninfected individuals compared to PLHIV. The elevated TRVß in the lung and blood in PLHIV suggests their potential involvement in HIV immune response, while the depletion of certain TRVß clones in Mtb-specific T cells in the lung and blood may indicate HIV-induced alteration in the repertoire. Furthermore, a low clonal count was observed in PLHIV not on ART compared to those on long-term ART, suggesting restoration of Mtb specific CDR3 clones with long-term ART.

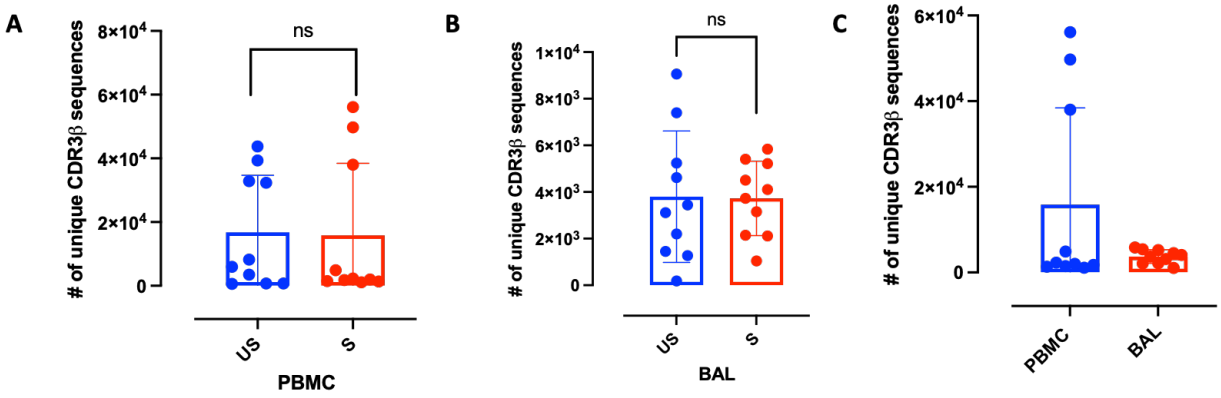
Together, these findings suggest a more restricted TCR repertoire in PLHIV compared to healthy controls, with alterations in the frequency of certain clonotypes that may impact antigen recognition and specificity. This effect could lead to a decreased ability to protect against infections, including TB, in PLHIV. Identifying the most abundant TRVß segments involved in protective immunity in HIV and TB may offer crucial targets for vaccine development and preventive therapies, potentially improving outcomes for individuals living with HIV and reducing the risk of lower respiratory tract infections, including TB.

5.3 Results

5.3.1 T cell repertoire in PBMC and BAL comprise a comparable number of unique CDR3 sequences.

To assess the TCR clonality in PBMC and BAL, the TCR repertoire was first assessed at the nucleotide sequence level by examining the CDR3 motifs. Overall, a total of 53x10^6 reads were obtained from 10 paired samples, each with two conditions (Mtb stimulated and unstimulated). On average, 9.7 x10^6 reads were collected per individual sample **(Appendix Table 1)**. From this, about 9.3 x10^3 unique

CDR3 sequences per sample were obtained, amounting to a total of 40x10^6 unique CDR3 **(Appendix Table 1).** In this project, the unique CDR3, also known as a clone, is defined as the number of unique CDR3 sequences within the sample. Clonality was characterised by the number of unique CDR3β sequences present in the sample, as described by Al Khabouri et al. (22,23) **(Appendix Figure 1).** The number of unique CDR3β was assessed across the sample types in both stimulated and unstimulated conditions to describe their composition and to examine how stimulation with Mtb antigens affects the TCR repertoire **(Figure 5).** There was no difference in the number of unique CDR3β sequences between unstimulated and Mtb-lysate stimulated in both PBMC (P = 0.99, Mann- Whitney test) and BAL (P = 0.85, Mann-Whitney test) (**Figure 5-1 A & 5-1 B).** However, there was a trend towards a higher number of unique CDR3βs in PBMC compared to BAL (P = 0.073) Mann- Whitney test) **(Figure 5-1 C).** In addition, the clonal repertoire composition in stimulated and unstimulated conditions was comparable across all the study groups (HIV-, HIV+ART- and HIV+ART+) in both PBMC **(Figure 5-1 D & 5-1 E)** and BAL **(Figure 5-1 F & 5-1 G).** Together, these results suggest an even distribution and representation of clones in both PBMC and BAL irrespective of stimulation and HIV infection.



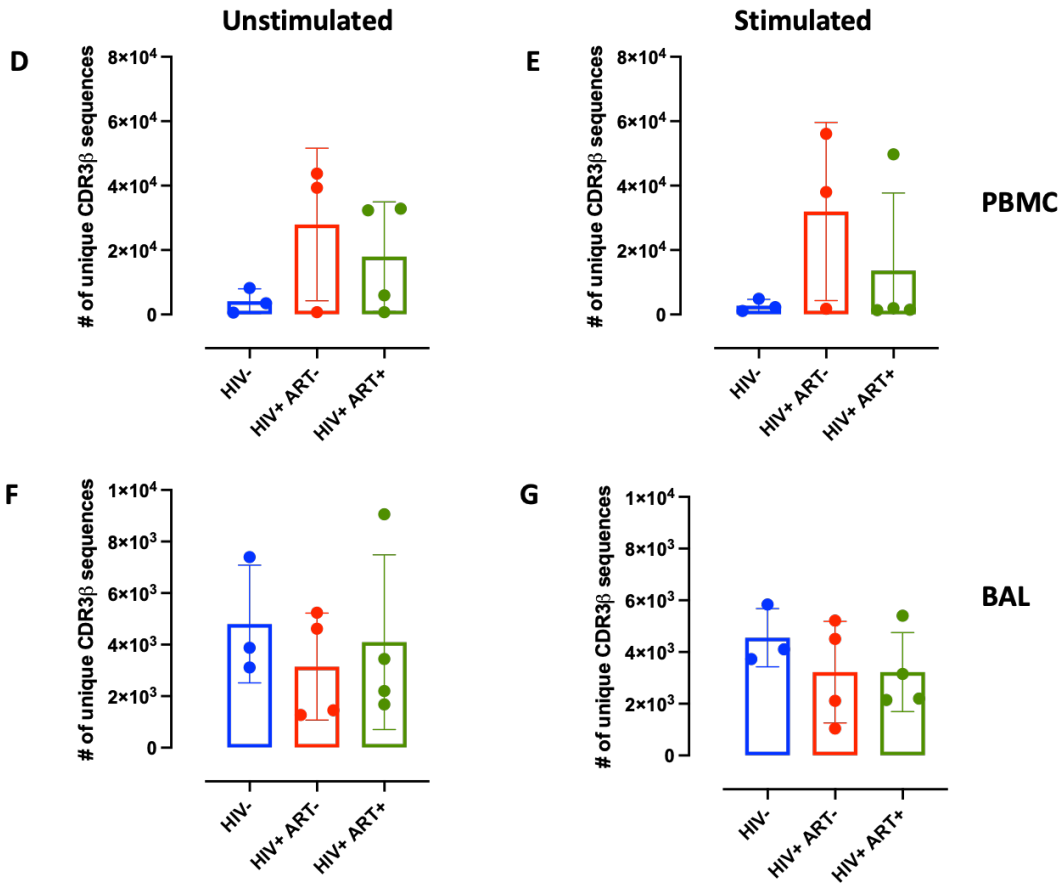


Figure 5-1 Comparison of unique CDR3 sequences across the group

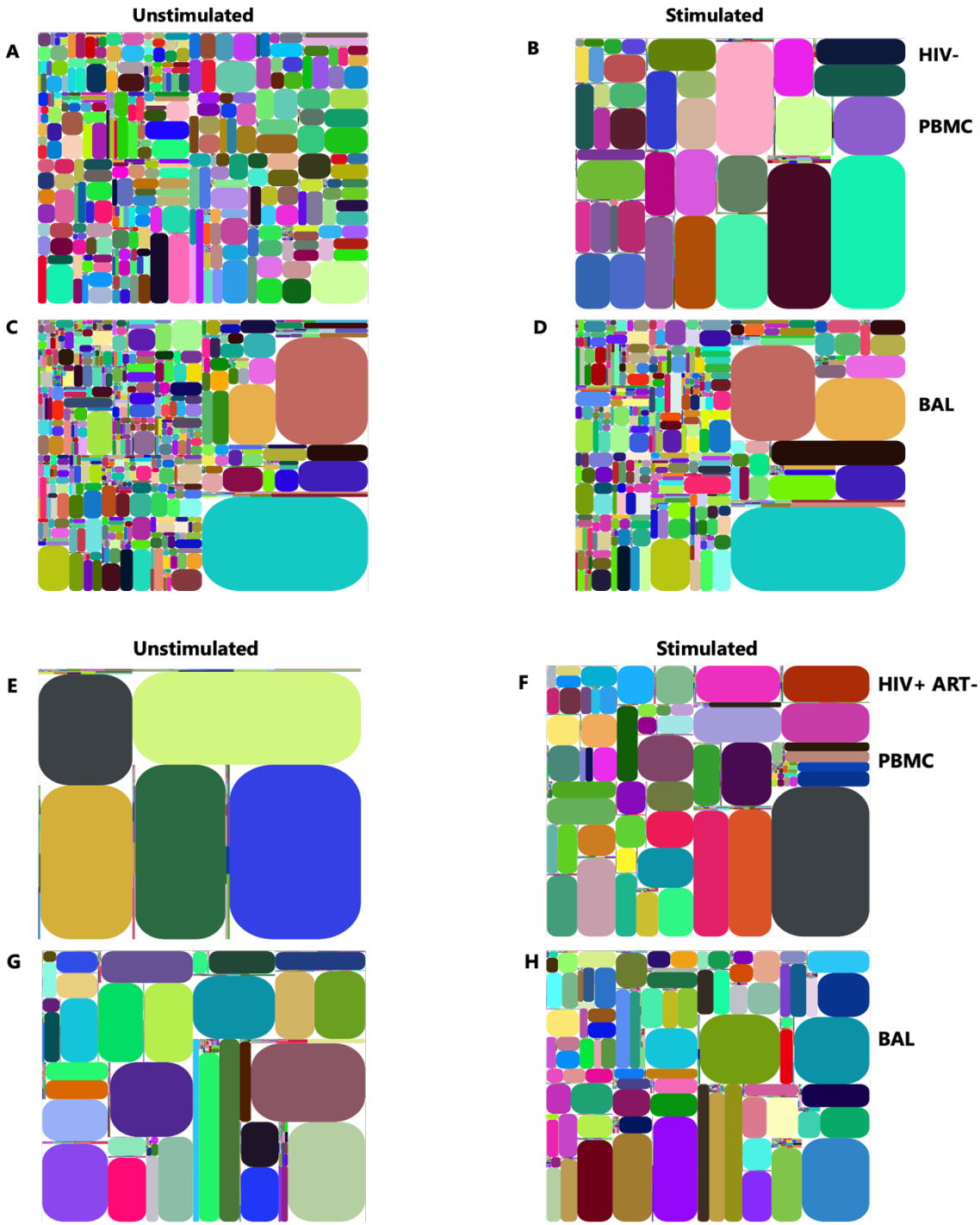
The number of unique CDR3 sequences within the sample (also known as unique CDR3) was obtained from the pool of CDR3s using an iRepertoire analysis pipeline. Number of unique CDR3 sequences in Mtb stimulated vs unstimulated condition in PBMC(A) and BAL (B). (C) comparison of unique CDR3s between PBMC and BAL. The impact of HIV on the distribution of unique CDR3 in unstimulated(E) and stimulated (F) PBMC. G & F, number of unique CDR3s unstimulated and stimulated BAL respectively. Data were analysed using an unpaired Student's t-test and data was presented as mean ± SD. (*: P< 0.05). P< 0.05 is considered statistically significant while ns= means no statistical significance. Graphs represent 1 experiment from paired BAL and PBMC samples, n=10 (HIV+ART-n=4, HIV+ART+ n=4 and HIV-, n=3)

5.3.2 Mtb-specific T cells in BAL display a restricted repertoire compared to PBMC irrespective of HIV status.

Next, a tree map was used to investigate and illustrate Mtb-specific TCR diversity following stimulation. A tree map is a bioinformatics data analysis tool used for the visualisation of complex hierarchical data sets in the form of rectangles (336). TCR clonotype frequencies were compared between PBMC and BAL in HIV-, HIV+ART-, and HIV+ART+ groups. TCR diversity was defined as the relative contribution of each unique CDR3β sequence to the total population of CDR3s. In the tree maps below, each rounded, coloured rectangle signifies a unique V-

uCDR3β entry, where the size of a spot represents the relative clonal frequency. The unevenness of squares reflects areas of clonal expansion within the sampled immune repertoire, revealing underlying biases. In HIV-negative samples, a more heterogeneous and diverse set of clonotypes was observed in unstimulated **(Figure 5-2 A)** compared to Mtb-stimulated PBMC **(Figure 5-2 B).** Intriguingly, while there was a reduction or near absence of certain TCR clones following stimulation, some TCRs were observed at higher proportions. In contrast to the altered repertoire observed after antigen stimulation of PBMC, fewer differences in clonal composition were noted between unstimulated and stimulated conditions in BAL **(Figure 5-2 C & 5-2 D, respectively)**. A similar trend was observed in HIV-infected individuals on ART, where various clonotypes were present in unstimulated conditions **(Figure 5-2 I),** with subsequent selective expansion of some clones following stimulation **(Figure 5-2 J).** Conversely, no sizeable differences in the distribution of clones were observed in BAL in both unstimulated **(Figure 5-2 H)** and stimulated conditions **(Figure 5-2 K)**, suggesting a more stable repertoire.

In HIV-infected ART-naive individuals, fewer sets of clonotypes were observed at high frequencies in unstimulated PBMC **(Figure 5-2 E).** Following stimulation, the repertoire space was predominantly occupied by the expanded clones that were previously observed at lower frequencies in the unstimulated condition. **(Figure 5-2 F).** In BAL, selected clonotypes with high frequency were observed in both unstimulated **(Figure 5-2 G)** and stimulated **(Figure 5-2 H)** conditions. However, there was limited expansion and reduced frequency of some TCR clonotypes in BAL **(Figure 5-2 H)** which indicates that BAL TCR clones are less dynamic and more diverse. Together, these results show a broad range of TCR repertoire diversity between PBMC and BAL, with a selective Mtb-antigen-driven clonal expansion. Additionally, BAL repertoires are less diverse compared to PBMC.


Unstimulated
Stimulated
A
B
HIV-
PBMC
C
D
BAL
Unstimulated
Stimulated
E
F
HIV+ ART-
PBMC
G
H
BAL

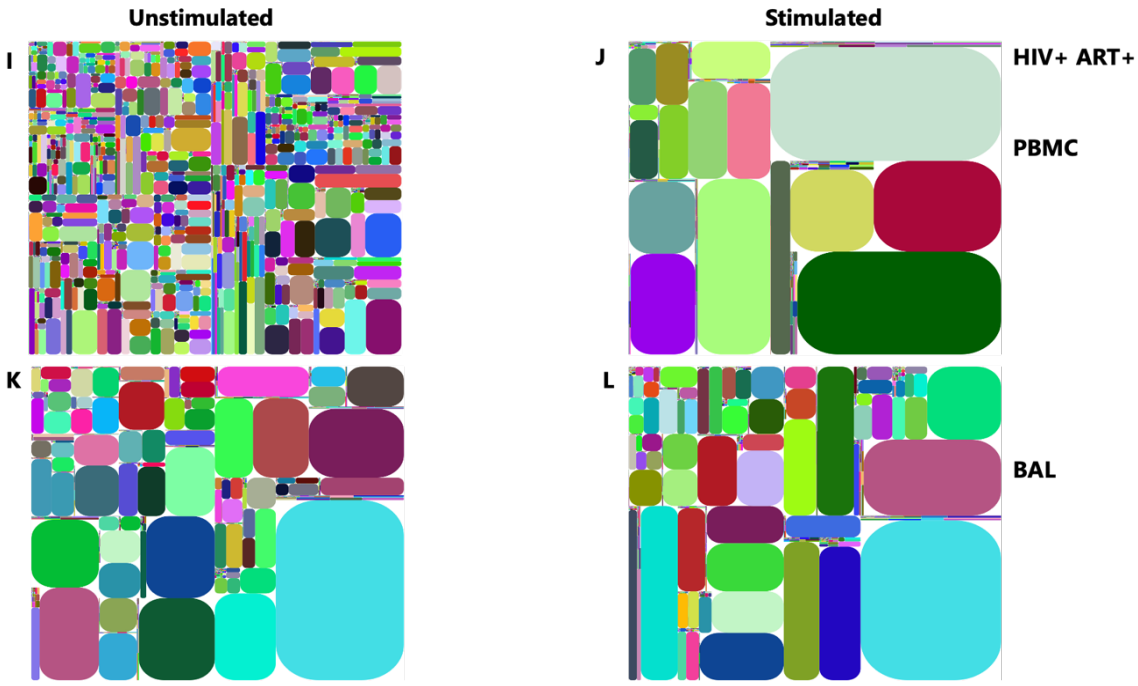


Figure 5-2 Illustration of TCR clonal types in HIV-, HIV+ART- and HIV+ART+ using tree maps.

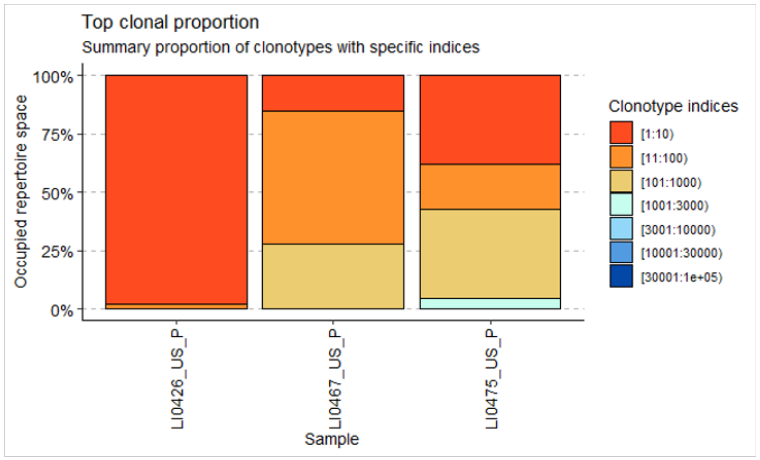
BAL and peripheral blood T cells were stimulated with Mtb lysate *in vitro* for 18 hours followed by bulk RNA sequencing. The representative plots of *tree maps* compare TCR clonotypes between PBMC and BAL in HIV-, HIV+ART- and HIV+ART+ in Mtb lysate-stimulated and unstimulated conditions. In a tree map, each rounded rectangle represents a unique entry: V-J-uCDR3, where the size of a spot denotes the relative frequency. The irregular sizes and shapes of the squares reflect areas of clonal expansion within the immune repertoire sampled, thus each shape represents a clonal expansion. Comparison of the frequency of the clonotypes in stimulated vs unstimulated conditions between PBMC and BAL in HIV- (A-D), HIV+ ART- (E—H) and HIV+ART+ individuals (I- L). Data were analysed using the iRepertoire data analysis pipeline. Graphs represent 1 experiment with n=10

5.3.3 Mtb-specific T cells exhibit a distinct composition and expression of TRVB genes in Blood and lung in HIV-negative individuals.

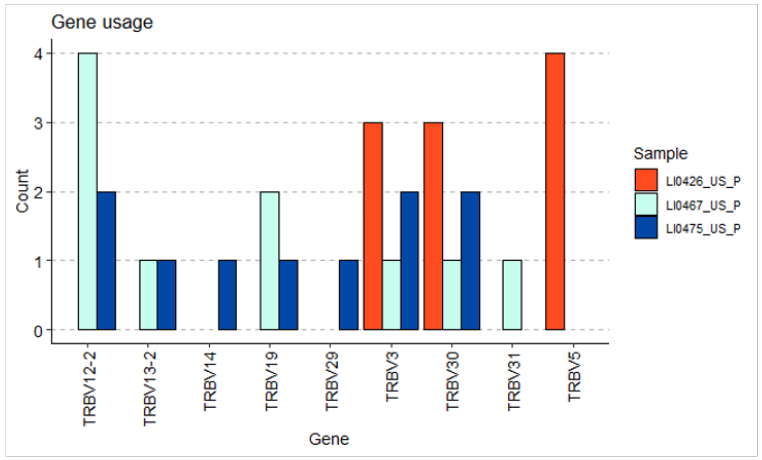
Having observed a distinct TCR clone distribution in PBMC and BAL, we next sought to determine the degree to which clonotypes contributed to the composition of the repertoire space in the population. A clonotype is defined by its unique CDR3 sequence, which characterises a T-cell receptor. Overall, the repertoire space occupied by the top 10 clones was higher in stimulated T cells in PBMCs **(Figure 5-3 C)** than in unstimulated cells **(Figure 5-3 A)**, indicating clonal expansion in response to antigen. However, one sample showed a repertoire predominantly comprised of the top 10 clones even in the unstimulated condition. Given the heterogeneous composition of clonal repertoire occupancy, we next asked whether the top clones comprised similar TRBV gene segments by tracking the clonotype counts **(Figure 5-3 B & D)**, Clonal tracking revealed a distinct and diverse composition of TRBV segments across the samples in both stimulated and unstimulated conditions. The top clones in unstimulated PBMCs had high expression of TRBV12-2, TRBV3, TRBV30, and TRBV5 **(Figure 5-3 B)**, while TRBV12-2, TRBV13-2, TRBV30, TRBV31, and TRBV4 were highly expressed in stimulated PBMCs **(Figure 5-3 D)**. Moreover, the major difference was the individual variation in the top clonal contribution, such that the increase in expression of specific TRBV segments in samples did not necessarily correspond with an increase in other samples. In contrast to the observation from PBMC, the distribution of the top clones was comparable in BAL in both unstimulated and stimulated conditions **(Figure 5-3 E & G, respectively)**. Additionally, the top 10 clones in both conditions largely expressed TRBV12-2, TRBV13 and TRBV31 **(Figure 5-3 F & H)**, indicating a conserved expression pattern of these TRBV genes. The data suggest that while stimulation induces significant changes in the clonal distribution and TRBV gene expression in PBMCs, the BAL TCR repertoire remains restricted.

Top clonal proportions in the PBMC in HIV-ve

A

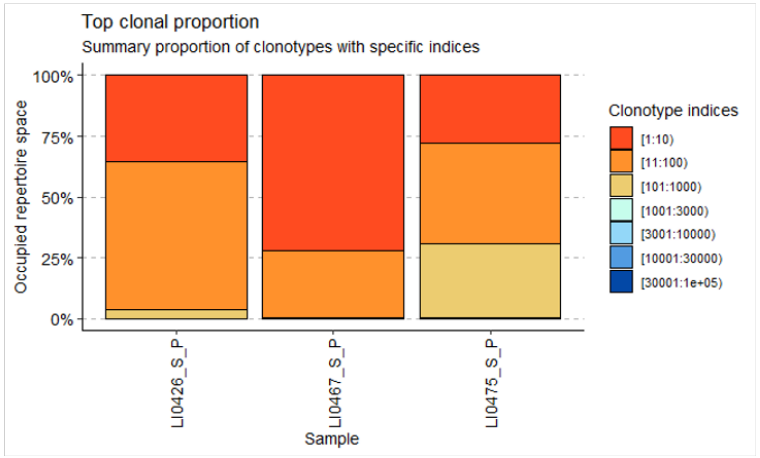


B

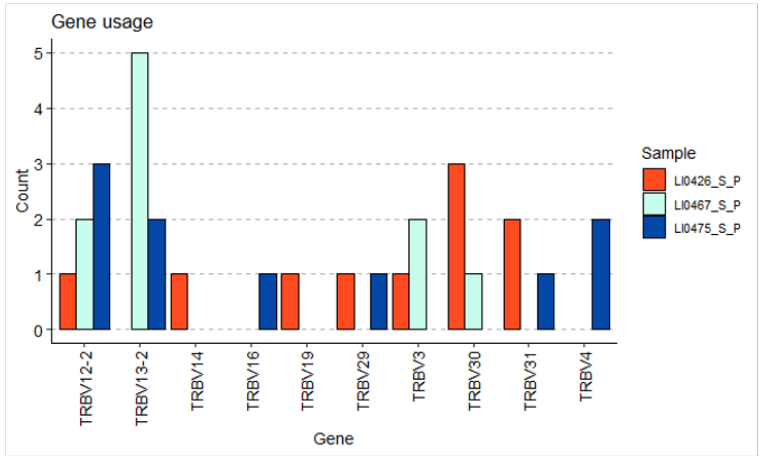


Unstimulated

C



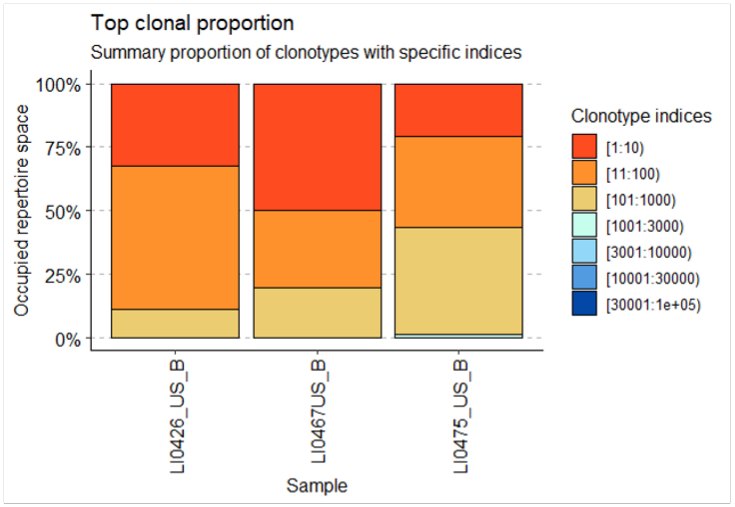
D



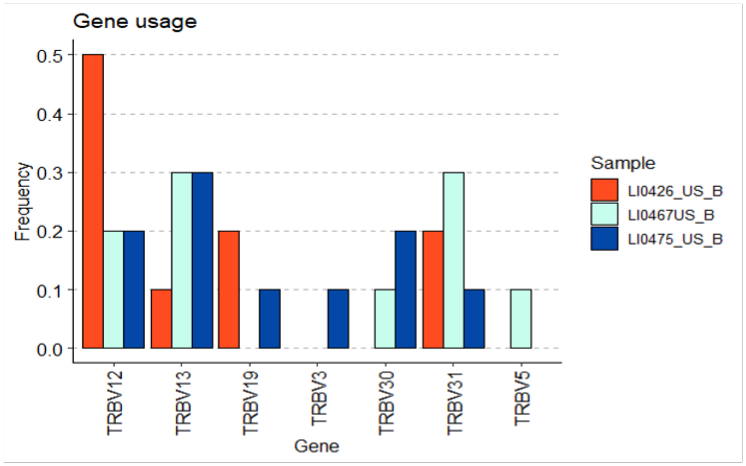
Stimulated

Top clonal proportions in the BAL in HIV-ve

E

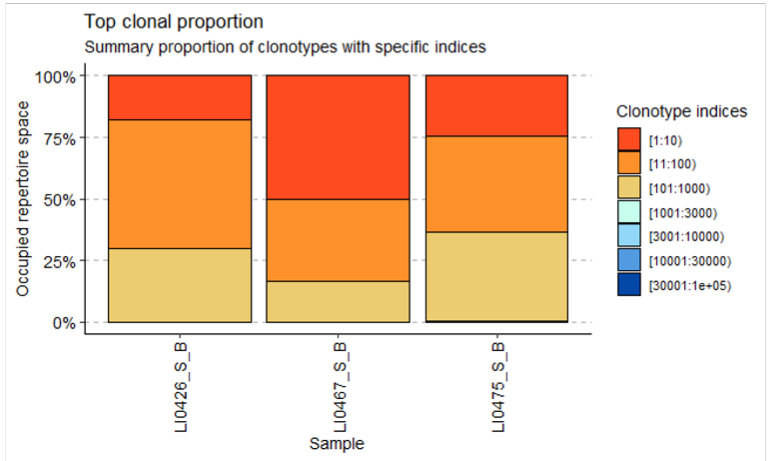


F

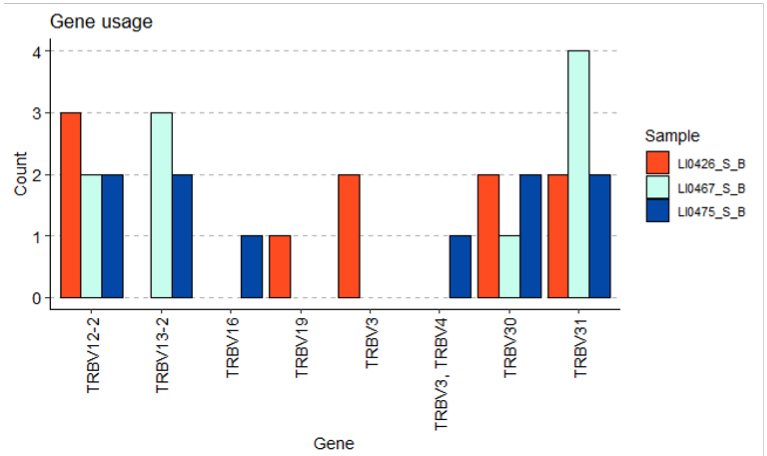


Unstimulated

G



H



Stimulated

Figure 5-3 Distribution of T cell clones in the TCR repertoire space in PBMC and BAL in HIV- negative individuals

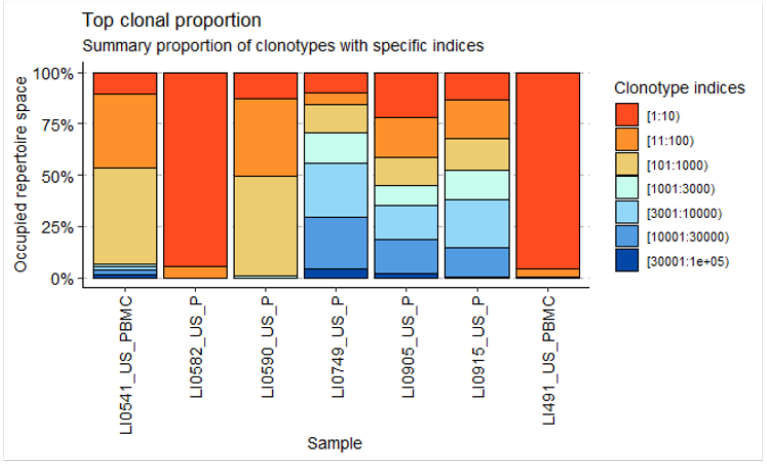
The stacked bars show the proportion of the top 10 most abundant clones, followed by the next 100, 1000, 3000,10,000, 30,000 and 100,000 most frequent clones occupying the T cell repertoire space in BAL and PBMCs. The colours represent different clonotype indices. A & C in Mtb lysate unstimulated and stimulated PBMCs respectively. B & D, the clonotype tracking bar plots display the frequency of different TRBV segment samples from the top 10 clones in unstimulated and stimulated conditions, respectively. E & D, proportions of the most frequently occurring clones in unstimulated and stimulated BAL. The bar plots show clonotype tracking in BAL in unstimulated (F) and stimulated conditions (H). The TCR repertoire was analysed by the immunarch and tcR R package. Graphs represent 1 experiment from paired BAL and PBMC samples, n=10, 3 HIV- and 7 HIV+.

5.3.4 In PLHIV, BAL T cells display a higher frequency of selected clones that occupied the repertoire spaces than PBMC in HIV-infected individuals.

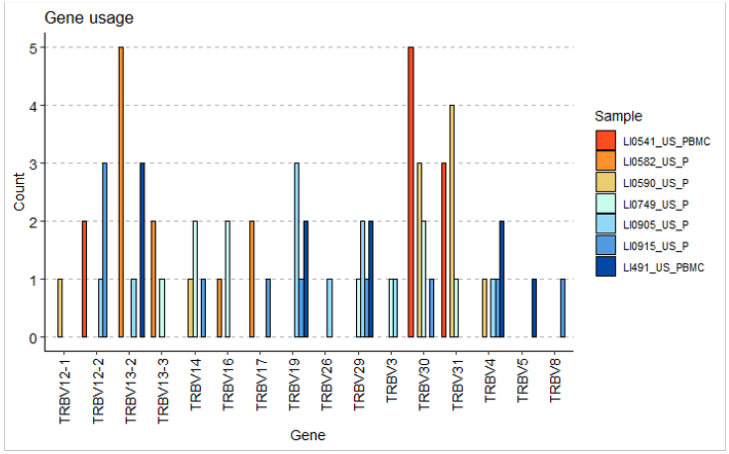
A similar analysis was conducted to assess the impact of HIV on the distribution of clonal repertoire composition in PBMC and BAL. The top clonal proportions in PBMCs were highly variable across different samples in unstimulated **(Figure 5-4 A)** compared with stimulated PBMC **(Figure 5-4 C)**. This was followed by a diverse expression of TRBV segments, with TRBV12-2, TRBV3, TRBV30, and TRBV5 being prominently expressed **(Figure 5-4 B)**. A noticeable shift in the clonal proportions was observed in Mtb-stimulated PBMCs, with the top 10 clones occupying a larger repertoire space **(Figure 5-4 C).** This repertoire space was mainly characterised by high expression of TRBV12-2, TRBV13-2, TRBV30, TRBV31, and TRBV4 **(Figure 5-4 D).** In contrast, the clonal distribution in BAL T cells remained comparable between unstimulated and stimulated conditions. The top 10 clones in both conditions largely expressed TRBV12-2, TRBV13, and TRBV31, demonstrating a stable pattern of TRBV gene usage **(Figure 5-4 E & F)**. This stability suggests that the lung compartment maintains a consistent clonal composition regardless of stimulation and irrespective of HIV infection.

Top clonal proportions in the PBMC in HIV+ individuals

A

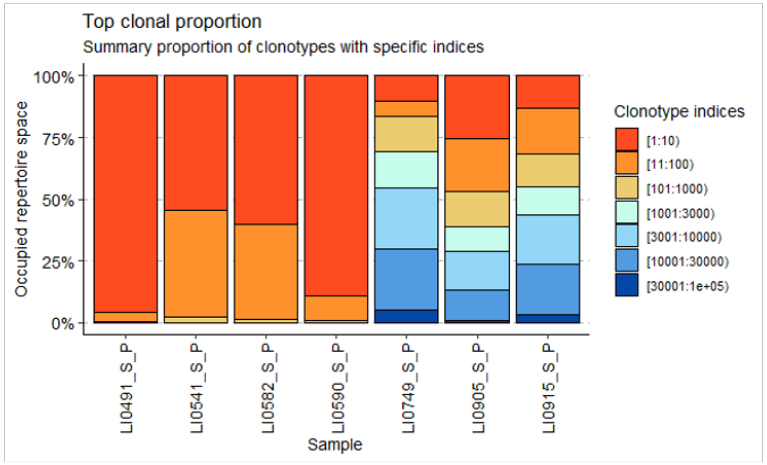


B

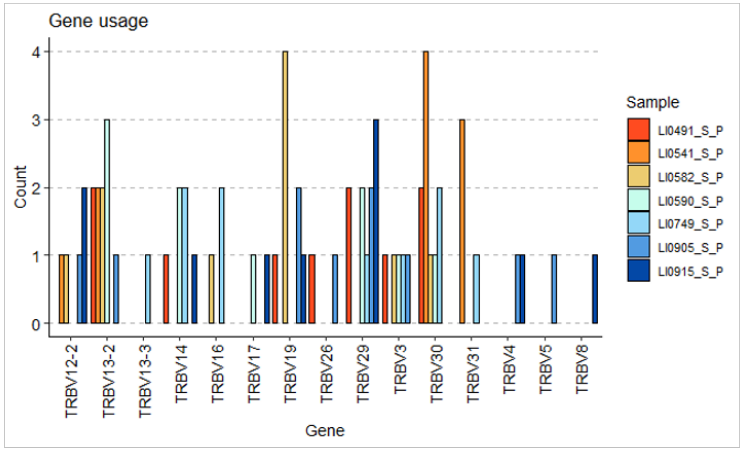


Unstimulated

C



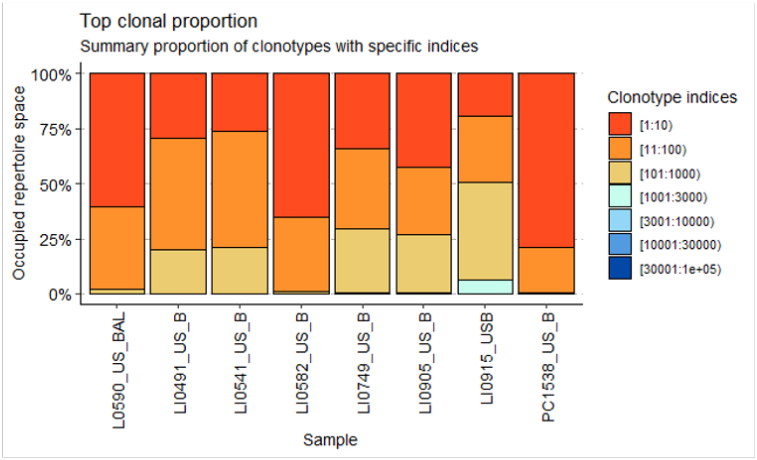
D



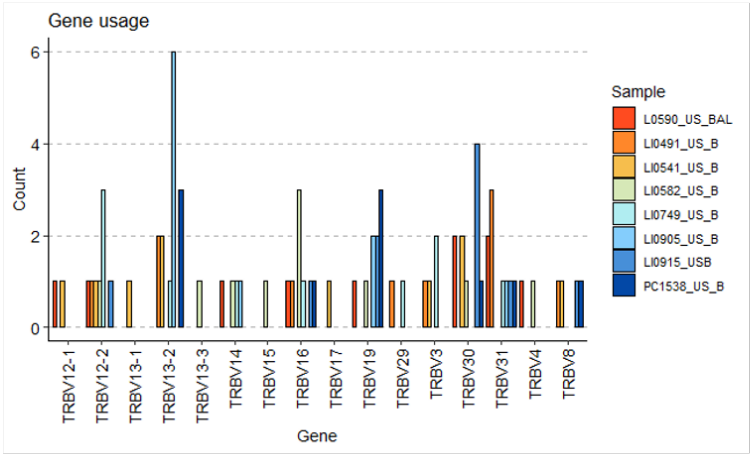
Stimulated

Top clonal proportions BAL in HIV+ individuals

E

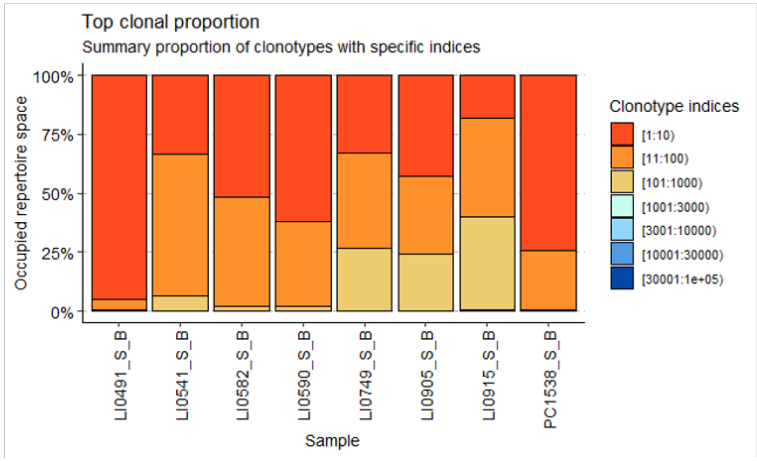


F

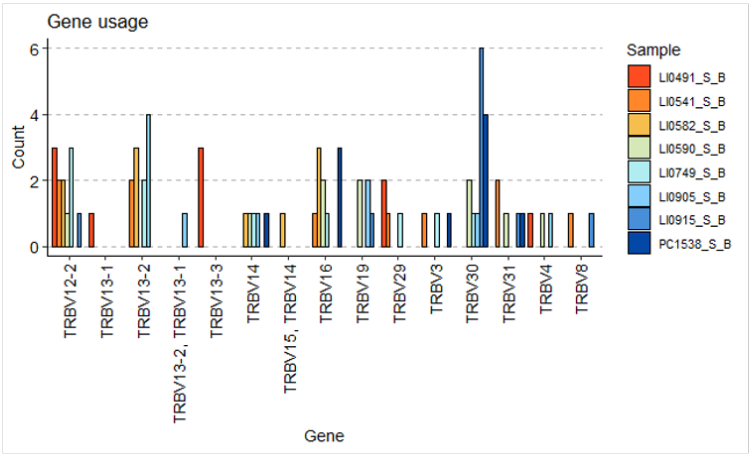


Unstimulated

G



H



Stimulated

Figure 5-4 Distribution of T cell clones in the TCR repertoire space in BAL and PBMC in HIV-positive individuals

Alveolar and peripheral blood T cells were stimulated with Mtb lysate in vitro for 18hrs followed by bulk RNA sequencing. The stacked bars show the proportion of the top 10 most abundant clones, followed by the next 100, 1000, 3000,10,000, 30,000 and 100,000 most frequent clones occupying the T cell repertoire space in BAL and PBMCs. The colours represent different clonotype indices. A & C in Mtb lysate unstimulated and stimulated PBMCs respectively. B & D, the clonotype tracking bar plots display the frequency of different TRBV segment samples from the top 10 clones in unstimulated and stimulated conditions, respectively. E & D, proportions of the most frequently occurring clones in unstimulated and stimulated BAL. The bar plots show clonotype tracking in BAL in unstimulated (F) and stimulated conditions (H). The TCR repertoire was analysed by the immunarch and tcR R package. Graphs represent 1 experiment from paired BAL and PBMC samples, n=7.

5.3.5 A higher frequency of TCR clonotypes in PLHIV on ART compared to ART naïve.

To assess the impact of HIV and ART on the TCR repertoire clonality, proportions of clonotypes were compared in PBMC and BAL in HIV-uninfected individuals, HIV infected not on treatment and HIV infected individuals on long-term ART for an average of 12 years. In the BAL samples, the ART-naïve group exhibited a lower proportion of clonotypes, while the ART-treated group showed a significant increase in clonotype frequency, with almost similar levels observed in the HIV - uninfected (control group). Likewise, in PBMCs, the ART-treated group displayed a markedly higher frequency of clonotypes compared to ART-naïve individuals **(Figure 5-5).** No significant differences in clonal frequencies were observed between the ART-treated group and controls. Additionally, there was no significant difference in TCR repertoire diversity across the study groups in both BAL and PBMC **(Appendix Figures 1 & 2)** These findings suggest that HIV is associated with reduced TCR clonality in both BAL and PBMC, which is nearly restored to control levels following long-term ART.

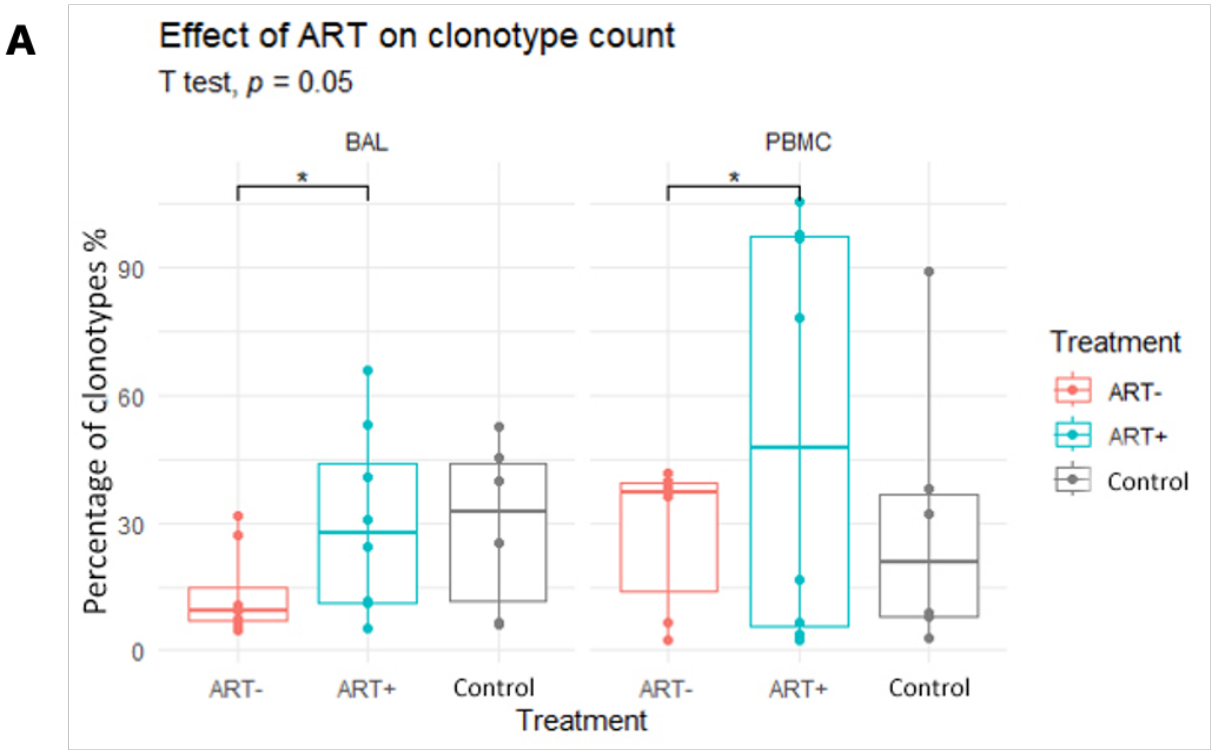


Figure 5-5 HIV is associated with a reduced TCR repertoire in BAL and PBMCs

Comparison of proportions of TCR clonotypes among the study groups: HIV+ART-(Red), HIV+ART+ (light blue) and HIV- (black). Data were analysed using an unpaired Student's t-test and data was presented as mean ± SD. (*: P< 0.05). Graphs represent 1 experiment from paired BAL and PBMC samples, (HIV+ART-, n=6, HIV+ART+, n=7 and HIV-, n=5).

5.3.6 PBMC is associated with a higher and more diverse distribution of TCR CDR3 lengths in PLHIV compared to HIV uninfected.

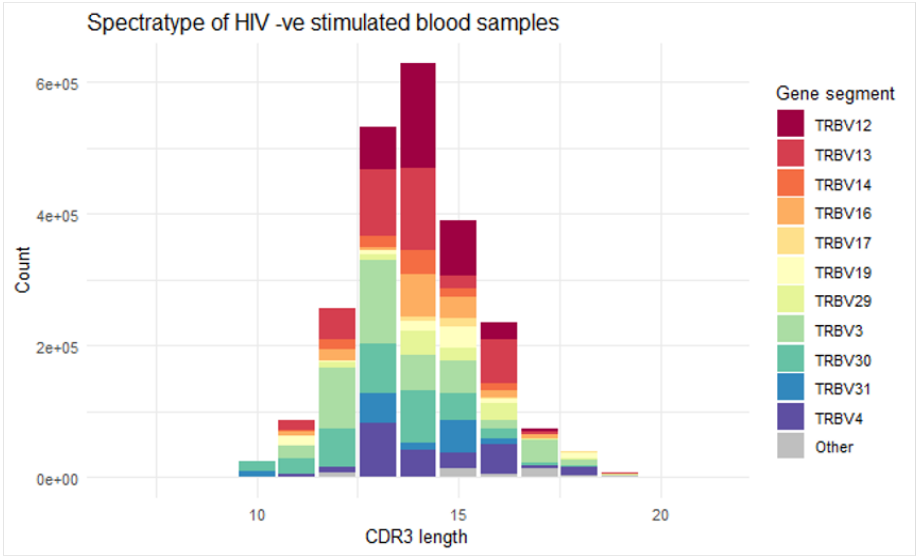
Next, the length and TRBV composition of the CDR3 loop in BAL and PBMC HIV-infected and uninfected individuals was examined using spectratyping. Spectratyping is a technique that Identifies the distribution pattern of the CDR3 lengths within each TRBV subfamily encoded by the corresponding genes (337). The amino acid sequence length ranged between 10-20 amino acid across the analysed samples. In this analysis, we focused on the middle portion of the CDR3 length as it is the key determinant of the TCR diversity owing to its ability to reach into narrow antigenic sites (338). The CDR3 length distribution in stimulated blood in HIV-infected individuals showed a longer and more varied range compared to HIV uninfected individuals with a dominance of TRBV12 followed by TRBV13 and

TRBV14 and TRBV3 observed between 14-16 amino acid sequence length **(Figure 5-6 A & B)**.

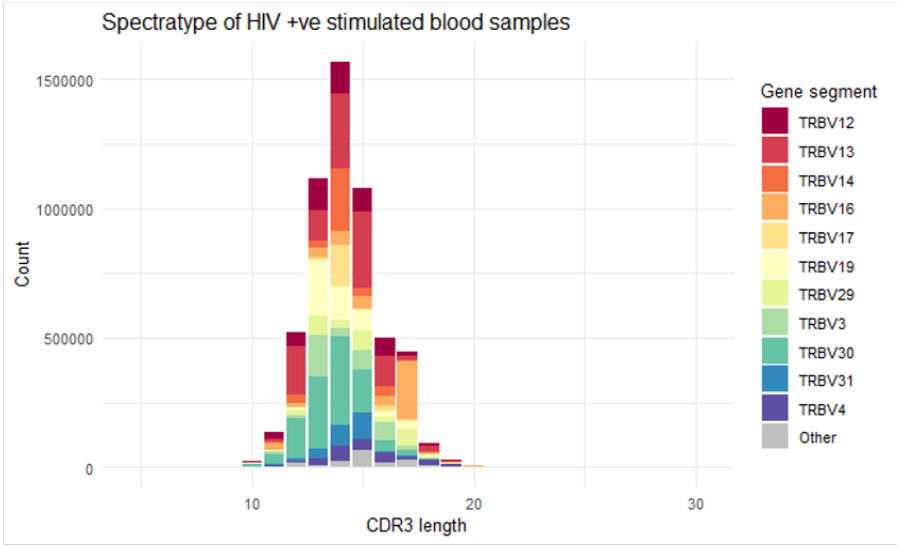
Similarly, the CDR3 length distribution in stimulated BAL samples in HIV-infected individuals displayed a higher and a more diverse range of amino acid sequences than in uninfected individuals characterised by expansion of TRBV12 and TRBV13 gene segments **(Figure 5-6 C & D)**. Together, the findings suggest that HIV infection leads to a more heterogeneous T cell response with a potential selection of different T cell clones compared with healthy controls. Moreover, a preferential expansion of longer amino acid sequences in the CDR3, could suggest their critical role in antigen recognition.

CDR3 length distribution in PBMC

A



B



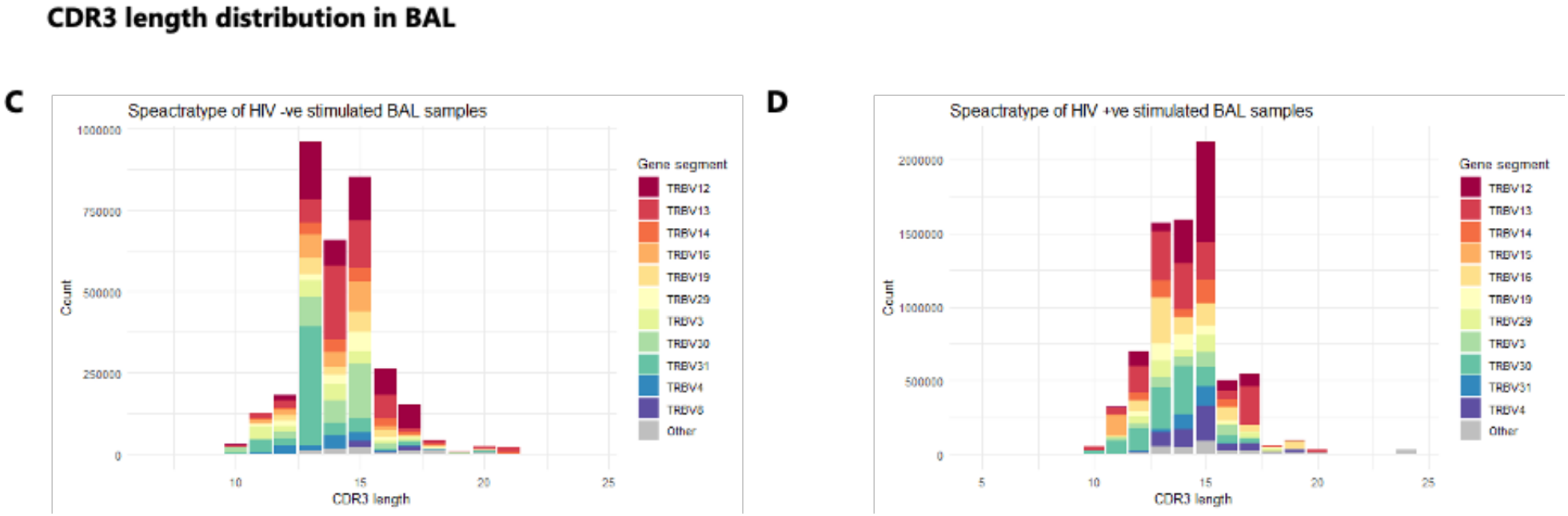


Figure 5-6 CDR3 length distribution across samples

Spectratyping was used to determine the CDR3 length distribution and TRVB expression. The X-axis denotes the length of amino acid sequences from 10 to 20. The Y-axis shows the cumulative frequency of the indicated size. The stacked coloured bars represent the TRBV expression associated with each length. A & B display CDR3 lengths in blood in the HIV uninfected group in Mtb unstimulated and stimulated conditions, respectively. C & D shows CDR3 length distribution in BAL PLHIV in unstimulated and Mtb stimulated conditions. The Y-axis displays the sum productive frequency for the residues of the indicated length from a given sample. This frequency is reported as counts of the filtered CDR3 sequence leads. Samples, n=10 (HIV- n=3 PLHIV, n+7).

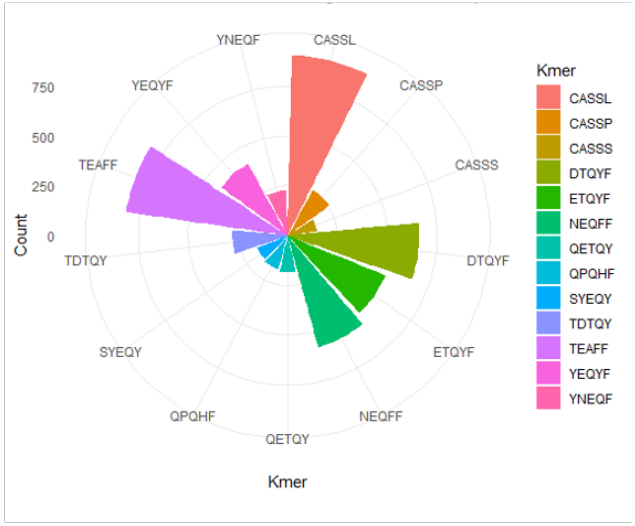
5.3.7 The most frequently occurring amino acid sequences in both PBMC and BAL are associated with the highest levels of TRVβ expression.

To identify amino acid substring sequences that were over- or under-represented within the CDR3 sequence distribution, I conducted a motif analysis. This involved breaking down the TCR CDR3 amino acid sequences into overlapping short sequence fragments of a specific length, known as k-mers. This analysis compared k-mers from PBMC and BAL in HIV-uninfected and HIV-infected individuals to identify enriched motifs within a repertoire associated with key TRBV gene segments that contribute to the specificity of a TCR **(Figure 5-7).** Notably, four k-mers, namely CASSL, NEQFF, TEAFF and NEQF, were the most frequently occurring CDR3 amino acid motif within both the PBMC **(Figure 5-7 A & 7B)** and BAL **(Figure 5-7 C & 7 D)** repertoires. Intriguingly, these k-mers showed consistent frequencies in both compartments irrespective of HIV status, indicating that their abundance in PBMC and BAL may not be significantly affected by HIV infection.

Next, we examined the relationship between commonly identified CDR3 amino acid motifs (CASSL, NEQFF, TEAFF, and DRQW) and TRVB gene expression. This analysis aimed to elucidate how specific motifs are associated with the expression of particular TRVB genes, providing insights into the Mtb-specific TCR specificity. The CAS9L motif was associated with a high expression of TRBV12-2 and TRBV16 **(Figure 5-7 E).** Whilst high expression of TRBV 30, TRBV12-2 and TRBV 13-2 were more prominent in NEQFF, TEAFF, and DRQW motifs **(Figure 5-7 F, G & H)**. Together, the consistent expression of these selected TRBV gene segments associated with the common motifs indicates their potential role in immune responses to Mtb and HIV infection. Moreover, the high expression of TRBV 12-2 and TRBV 13-2 gene segments in CD8 T cells was reported in patients with active TB compared to controls, suggesting their involvement in TB pathogenesis (339).

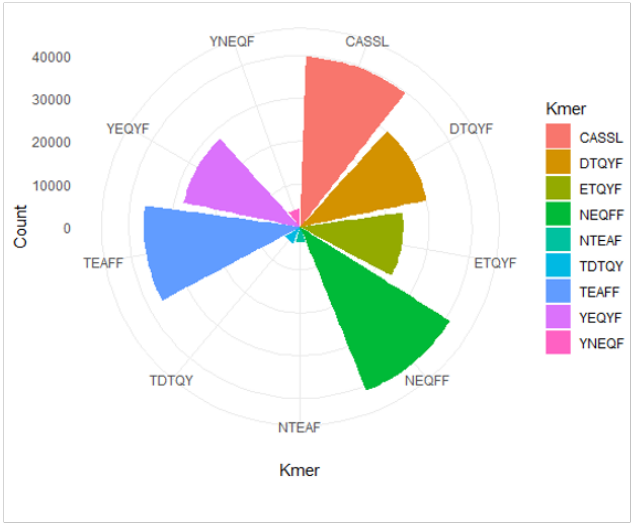
A

HIV-



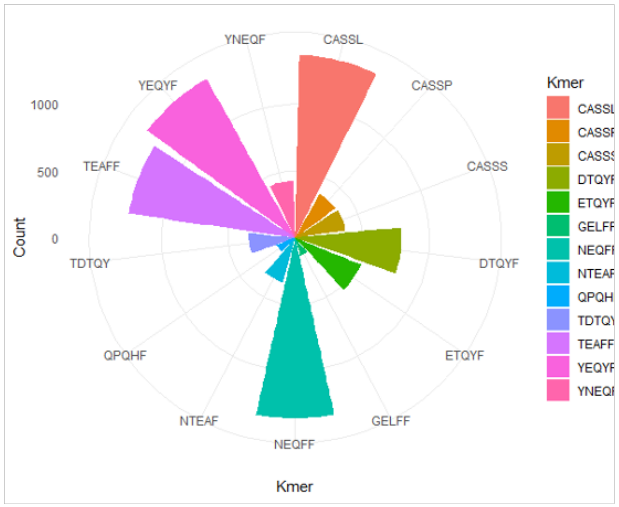
B

HIV+

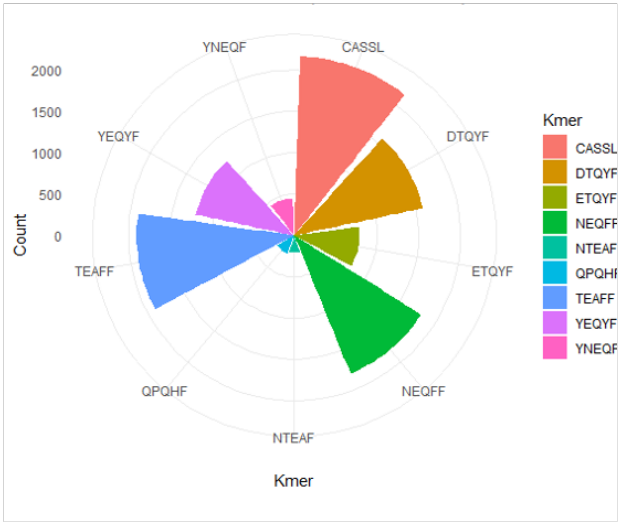


PBMC

C

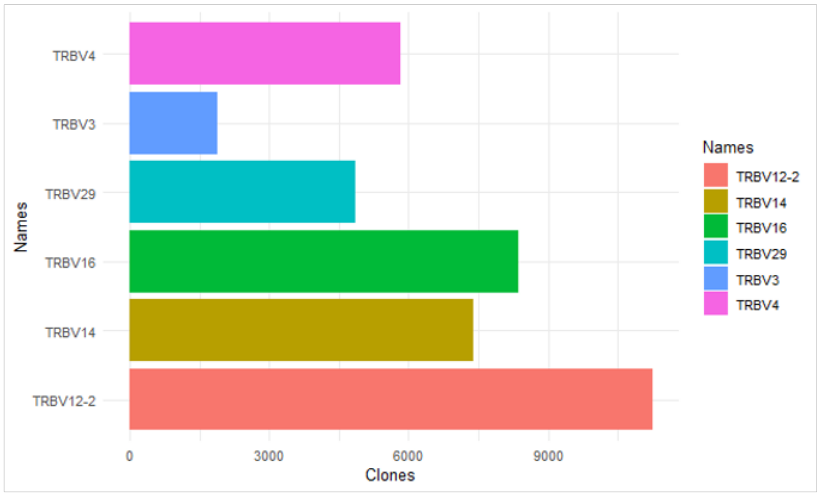


D

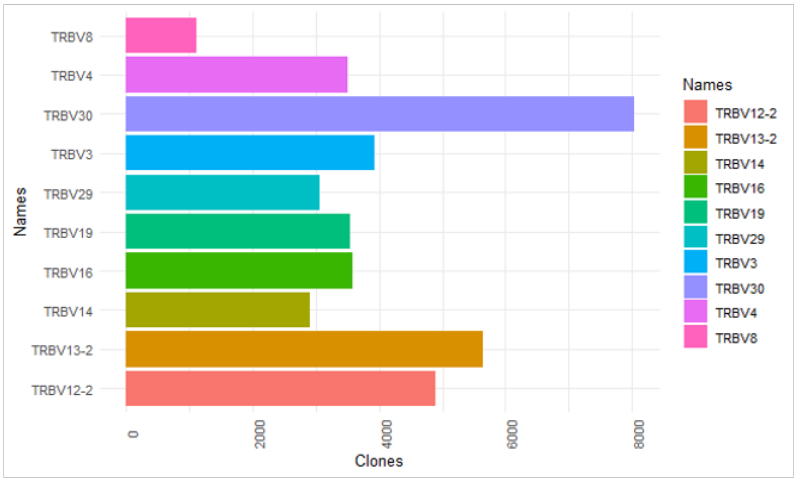


BAL

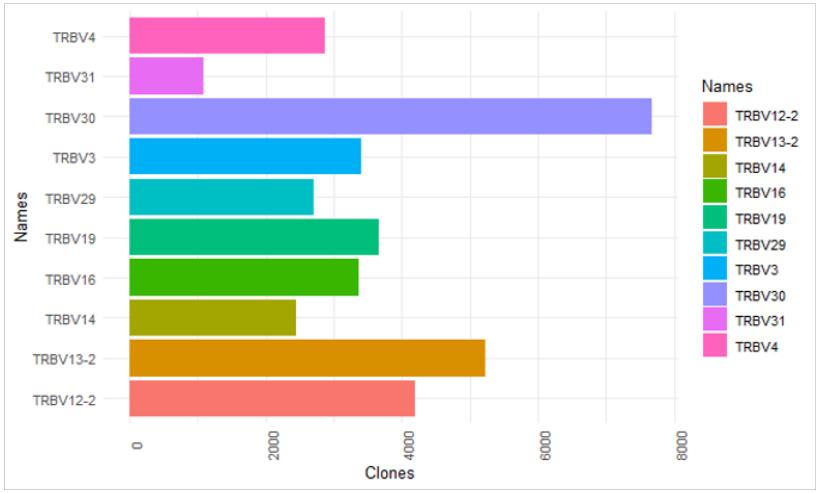
E **CASSL**



F **NEQFF**



G **TEAFF**



H **DTQYF**

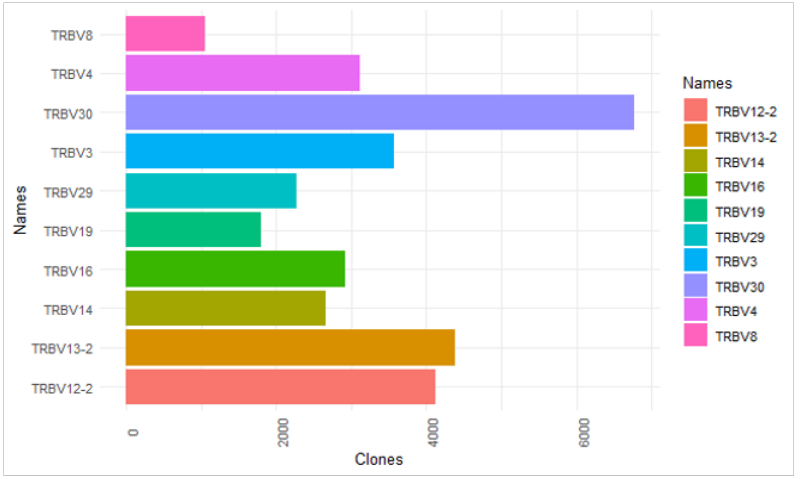


Figure 5-7 A detailed analysis of common CDR3 amino acid motifs and TRVB expression

K-mers (aa sequences) were obtained and analysed from the sequencing data, followed by the identification of TRBV expression. A & B show PBMC k-mer expression in HIV uninfected and PLHIV respectively. C & D shows k-mer expression in BAL in HIV uninfected and PLHIV. The colours represent different aa sequences. The bar plots (E-F) show the TRVB clone count from the top 4 most highly expressed k-mers.

5.4 Discussion

A stable diverse TCR repertoire indicates a T cell population capable of recognising a broad range of antigens (324). However, dynamic changes in the TCR repertoire are common in a variety of conditions including chronic HIV infection. HIV infection is known to induce CD4 and CD8 T cell immune defects, leading to susceptibility to opportunistic infections including TB (278). Moreover, there is compelling evidence that HIV reduces TCR diversity and clonality in the periphery, with partial recovery following long-term ART (331,340). This suggests that persistent immune defects may contribute to the high risk of lower respiratory tract infections (LRTI) in PLHIV (331). Although the effect of HIV on TCR clonality has been described in blood, little is known about its impact on lung Mtb-specific TCR repertoire and how the clonotypes change following ART in adults from HIV and TB endemic areas. Understanding these changes in the TCR Vβ repertoire will enhance our knowledge of host T cell factors involved in the immune response to TB in PLHIV, potentially aiding in the design of more effective vaccines to reduce the risk of TB and other LRTI in vulnerable populations. Here, TCR bulk sequencing was used to describe Mtb-specific TCR repertoire and assess the impact of HIV on the TCR repertoire diversity and clonality in PBMC and BAL in PLHIV.

The composition of unique CDR3 sequences was found to be similar between BAL and PBMC regardless of stimulation, indicating an even distribution and representation of T cell clones in both compartments. However, differences were observed in the relative frequencies of each clonotype, characterised by the expansion and shrinkage of some clones. This suggests an Mtb-antigen-driven clonal expansion or deletion of Mtb-specific T cells expressing specific CDR3 sequences, which may impact the immune response to Mtb infection. This is supported by the observed heterogeneity of TCR clonotype frequencies between PBMC and BAL in both HIV-infected and uninfected individuals following stimulation. The repertoire composition between unstimulated and Mtb-stimulated conditions was distinct in PBMC, with the Mtb-stimulated condition showing a less diverse repertoire mostly composed of high-frequency clones, suggesting a skewed repertoire. This indicates that T cells bearing these TCRs in PBMCs are more likely to expand following stimulation with Mtb antigens compared to the unstimulated condition. By contrast, the TCR repertoire in the

BAL displayed minimal or no change in the frequency of clonotypes under Mtb antigen-driven stimulation in both HIV-infected and uninfected individuals, including those on ART, indicating a stable repertoire. Altogether, this shows that while TCR clones in PBMC tend to be dynamic, Mtb-specific T cells in BAL exhibit a more restricted repertoire regardless of HIV infection. This may reflect compartmentalisation of the immune system and variations in antigen-specific responses.

Unravelling the mechanisms and factors affecting the observed Mtb-specific T cell clonal expansion between PBMCs and BAL was beyond the scope of this study. However, after stimulation, we noted a differential composition of the top 10 most abundant clones in the repertoire space. Clonal tracking analysis revealed distinct TRBV gene segments in these compartments: TRBV12-2, TRBV3, TRBV30, and TRBV5 were more abundant in PBMCs, while BAL predominantly expressed TRBV12-2, TRBV13, and TRBV31. These variations in the expanded ranges of TRBV genes may explain their role in Mtb antigen recognition and specificity, contributing to the observed diversity and clonality. Interestingly, while there were changes in the repertoire space in HIV-infected individuals in PBMCs, there was no difference in the top 10 highly expanded clonal compositions in BAL. This indicates the presence of an antigen-specific immune response. Progressive loss of systemic CD4 T cells is a hallmark of HIV infection and has been reported by many studies (219,224). It could, therefore, be possible that this contributes to the dynamic repertoire changes observed in PBMCs in HIV-infected individuals. If this were the case, one would expect changes in clonotypes to correlate with CD4 T cell count. Surprisingly, we did not observe any relationship, possibly due to the considerably high CD4 count in our study participants (average of 619 cells/µl). This potentially reflects that other factors may contribute to this phenomenon. It will be interesting to probe whether the restricted repertoire observed in the BAL is due to tissue retention of activated T cells or repopulation of the more restricted repertoire.

Our results further demonstrate that HIV infection is associated with reduced TCR clonality in both PBMC and BAL, which is restored following long-term ART. This

aligns with other studies that report HIV-induced alterations in TCR repertoire diversity, leading to decreased clonal T cell expansion in blood (229,341,342). The impact of ART on TCR repertoire is still debated. Some investigators report partial recovery of TCR diversity (331) (340) while others suggest a complete restoration, particularly in children and adolescents (223). For instance, a longitudinal study in PLHIV adults on long-term ART (mean of 6 years) using pre- and post-ART blood samples found that despite a reduced viral load, TCR diversity remained significantly lower compared to healthy controls (340). This suggests persistent immune dysregulation, potentially explaining increased morbidity in these populations. Conversely, Yin *et al*. reported normalised TCR diversity in children and teenagers following combined ART, possibly due to drug-induced thymic output (223). While this study reports reduced TCR clonotypes in BAL and PBMCs with a potential for repertoire restoration following long-term ART, its cross-sectional nature limits the generalisability of the findings. Furthermore, unlike Andrea *et al.*, who studied PLHIV for an average of 6 years, our participants had been on ART for a mean of 12 years, this could therefore contribute to the observed restored clonality in this cohort. Altogether, the study has shown that while HIV decreases the proportion of TCR clonotypes in both BAL and PBMC, the potential to maintain a diverse repertoire remains.

Additionally, the analysis of CDR3 length distribution in this study revealed a greater and more diverse range of TCR amino acid lengths in the Mtb-specific T cell repertoire in BAL and PBMCs from HIV-uninfected individuals compared to PLHIV. Specifically, the highest CDR3 peak lengths in PBMCs, characterised by TRBV12 and TRBV14, were observed between 14 and 16 amino acids. In BAL, peaks in CDR3 lengths ranged from 12-15 amino acids, with a dominance of TRBV12, TRBV13, and TRBV14. Interestingly, we observed consistent expression of certain TRBV gene segments associated with common CDR3 motifs. This consistent expression suggests a significant role for these TRBV segments in the immune response to Mtb and HIV infection. Indeed, elevated expression of TRBV12-2 and TRBV13-2 gene segments in CD8 T cells has been reported in patients with active TB compared to controls, suggesting their involvement in TB pathogenesis (339).

Despite the important insights demonstrated in this study, the data need to be interpreted with caution because of the small sample size which might not be transferable to a wider population. Furthermore, the use of unsorted T cells limited the scope of the contribution of individual T cell subsets to the overall repertoire diversity and clonality in this cohort. It is important to note that the possible biases in the observed repertoire changes could be a result of an expansion or deletion of either Mtb-specific CD4 or CD8 T cells of certain phenotypes. Lastly, because this was not a longitudinal study, we can only infer that ART reverses the changes observed in HIV-infected individuals. Moreover, most of the participants recruited in this study were not severely immunocompromised. Further work with a large sample size that will follow up with participants for an extended period is therefore required to test the generality of the findings and allow multivariate analysis of other potential confounding factors such as age, genotype, and sex.

5.5 Conclusion

In summary, this study demonstrates a dynamic, Mtb antigen-driven clonal expansion in the blood but not in the lung, characterised by differential composition and expression of TRVβ segments. Furthermore, HIV infection reduces TCR repertoire clonality, which is restored with long-term ART. Collectively, these findings enhance our understanding of site-specific antigen-specific T cell responses in the blood and lung and how they are influenced by HIV infection. Identifying highly expressed TRVβ segments provides insights into the mechanisms of recognition and antigen specificity in host protective immunity against HIV and TB, potentially offering crucial targets for vaccine design and immunomodulatory therapies.

Chapter 6 General Discussion

6.1 Introduction

The synergy between HIV and TB infection poses a public health threat globally. TB remains one of the leading causes of morbidity and mortality from a single infection in PLHIV (6). Despite successful ART, the risk of developing TB in PLHIV remains higher than in HIV-uninfected individuals, suggesting an impaired immune response. Efforts to manage TB are, however, affected by a lack of understanding of immune-mediated correlates of protection that are at play and influence the disease outcome. It remains to be established why some individuals control the infection better than others despite similar exposure. Antigen-specific T cells with a diverse TCR repertoire are key in controlling Mtb infection (339). Moreover, multifunctional T cells with the ability to produce multiple effector cytokines are associated with protective immunity to TB. Ironically, HIV infection does not only cause the progressive loss and impairment of Mtb-specific CD4-specific T cells but also results in a limited TCR repertoire in the blood, thereby predisposing the host to opportunistic infections including TB (331). To date, few studies have explored lung immunity to assess the impact of HIV and ART on the alveolar Mtb-specific TCR repertoire. To reduce the risks of disease severity and HIV-associated TB mortality, there is a need for a better understanding of mechanisms that constitute protective immunity to TB. Therefore, this thesis investigated the impact of HIV on TCR repertoire diversity and Mtb-specific alveolar T cell function in the airway in PLHIV. The thesis tested the primary hypothesis that Mtb-specific T cells are functionally and phenotypically distinct between the airway and peripheral blood. The study also assessed the impact of HIV on Mtb-specific CD4 and CD8 T cell repertoire. Lastly, the study tested the hypothesis that HIV alters the TCR repertoire diversity and clonality of Mtb-specific T cells, which are restored following long-term ART. Overall, this thesis has generated major conclusions that address the impact of HIV on Mtb-specific T cell repertoire clonality and function in the lung and blood.

6.2 Summary of key findings

The first aim of this study was to assess the impact of HIV on the phenotype and function of Mtb antigen-specific CD4 and CD8 T cells in the airway and peripheral blood. To achieve this, we collected peripheral blood and bronchoalveolar lavage (BAL) samples from PLHIV Naïve, PLHIV on ART and HIV-uninfected recruited adults. PBMCs and BAL were stimulated with Mtb antigens and analysed for cytokine response using flow cytometry. A distinct phenotype and function of Mtb-specific CD4 and CD8 T cells between PBMC and blood was observed, with the predominance of effector memory phenotype (CD45RA-CCR7-) in BAL **(Chapter 3).** We also observed that HIV was associated with a lower frequency and number of Mtb-specific CD4 T cells and a CD8 lymphocytosis in both blood and lung. In addition, there was a preferential depletion of a discrete subset of Mtb-specific IFN-producing cells in PLHIV compared to HIV-uninfected individuals. Consistent with previous work by Kalsdorf *et al*. (277) and Jambo *et al* .(272), this study reports that *Mtb*–specific CD4 and CD8 cytokine production is impaired in the lung of adults living with HIV.

The study also investigated the composition and usage of patterns of Mtb-specific CD4 and CD8 TCR repertoire in BAL and PBMC and how it is influenced by HIV infection. Indeed, a differential usage of TCR repertoire was observed between CD4 and CD8 T cells in immune responses to Mtb antigens between PBMC and BAL. Additionally, diverse patterns of TCR Vβ expressions were observed in CD4+ and CD8+ T cells in PLHIV compared to healthy controls, characterised by significantly higher frequencies of certain TCR specificities such as Vβ1, Vβ7.2, and Vβ23 expressions **(Chapter 4).** Collectively, we show that Mtb -specific T cells exhibit a differential TCR Vβ chain usage in the airway and blood of adults living with HIV.

Lastly, analysis of CDR3β sequencing of Mtb-specific TCR repertoires from PLHIV displayed reduced clonality compared with HIV-uninfected individuals in both the airway and blood. Additionally, a low frequency of clonotypes was observed in PLHIV not on ART compared to those on long-term ART, suggesting restoration of repertoire clonality with long-term ART **(Chapter 5).** Furthermore, by combining TCR Vβ CDR3 length analysis with the determination of TCR Vβ frequencies and usage by flow cytometry, we demonstrate an HIV-driven alteration in the TCR repertoire, mostly in blood but not in the lung. This difference may reflect

functional variation in Mtb-specific T cells from these sites. Overall, combining these approaches provides a platform to objectively define and identify TCR VB families involved in immune responses to Mtb across the study groups.

6.3 Implications of the findings

The results reported in this thesis have a number of potential implications for understanding T cell biology, vaccine development and clinical management. These implications are discussed below.

6.3.1 Implications on understanding T cell immunity against TB

Firstly, by showing phenotypic and functional differences in Mtb-specific T cells isolated from the airway and periphery, the findings from this study contribute to our knowledge of how the TCR repertoire responds to Mtb-specific antigens in different tissue compartments in the presence of HIV. The results identified the key TCRs that are over and underrepresented in these conditions. These findings can be used to inform the development of vaccines that target key T cell immune responses. Additionally, identifying unique TCR sequences associated with the whole Mtb lysate antigen response could lead to biomarkers for immune status and disease susceptibility, providing a foundation for developing diagnostic tools based on TCR repertoire profiles. Insights from TCR diversity analysis can also guide the design of TCR-based immunotherapies, such as TCR transgenic -T cells for targeted treatment (343). Furthermore, understanding TCR dynamics may help optimise stimulation protocols in adoptive cell therapy. Methodological refinements, driven by the limitations highlighted in this study, can encourage the adoption of single-cell RNA sequencing for more detailed paired TCR chain repertoire studies and the development of more sensitive and accurate bioinformatics tools for TCR analysis. Overall, the study's results contribute to the broader field of immunology by enhancing the understanding of how TCR repertoires evolve in response to antigens. This may reveal new aspects of T cell biology, such as clonal expansion dynamics and TCR evolution, under Mtb antigenic pressure.

6.3.2 Implications for vaccine development

The lung is the primary site of TB infection and a potential reservoir for HIV infection (266). Therefore, it is crucial to acquire knowledge on airway immune responses to TB, the distribution, and characteristics of responsive cells. Such knowledge is key to identifying target pathways and biomarkers for the discovery of immunomodulatory therapies and effective vaccines. This thesis has outlined clear differences in CD4 and CD8 T cell cytokine-producing profiles between the airway mucosa and the periphery. The study demonstrated that Mtb-specific IFN-γ and TNF-α CD4-producing T cells were higher in the airway than in the peripheral blood, suggesting that these cells are preferentially retained and localised in the lung. Moreover, we show an increase in frequencies of CD4 T cells bearing TCR specificities such as TCR Vβ7.2, Vβ13.6, and Vβ14 chains, which were likely to produce double cytokines (IFN-γ and TNF-α) in BAL, indicating that these specific TCR Vβ chains might play a pivotal role in anti-TB immunity and are preferentially selected to proliferate and generate a robust immune response.

Indeed, evidence suggests that multifunctional (cells with the ability to produce two or more cytokines) T cells are key in the eradication of intracellular pathogens such as Mtb (344). For decades, airway CD4 T cells capable of simultaneous production of IFN-γ, TNF-α and IL-2 have, along with other cells, have been considered as potential correlates of protection in both natural infection and T cell-induced vaccines such as BCG in non-human primates (317,345). On the other hand, an investigation of vaccine-induced antigen-specific T cell responses to novel Mtb vaccine candidates, including M72/AS01$_{E,}$ in South African adolescents showed a greater magnitude of response characterised by higher frequencies of CD4 T cell responses producing Th1 cytokines with limited functional characteristics (346), highlighting the need to find effective correlates of immune protection. Antigen-specific CD4 T cells with a diverse repertoire in the lung may offer protective immunity at the site of infection. Targeting the most abundant TCR specificities and enhancing naturally acquired antigen-specific CD4 T cell immunity against Mtb in lungs represents a promising target for future vaccine development.

Although antigen-specific CD4 T cells in the lungs may offer protective immunity at the infection site, creating a potent and long-lasting T-cell-based vaccine for TB remains a challenge (347). Additionally, selecting suitable immunodominant vaccine antigens to elicit effective T cell responses poses a potential hurdle for many vaccine candidates. It is well known that Mtb is capable of modulating the host immune response to favour its progression (348). Indeed previous studies have demonstrated that TCR epitopes of most Mtb immunodominant peptides are highly conserved, indicating that host recognition of these peptides might be beneficial to the Mtb (349).

Another challenge in developing an effective T cell based vaccine is identifying the optimal combination of antigenic peptides to achieve significant breadth of T cell responses (347,349,350). To navigate these challenges, other groups have suggested the use of potent adjuvants such as $ASO1_E$ to heighten the immune response and promote antigen-specific T cell cross-immunity against Mtb, thereby reducing the immune escape (351,352). However, adjuvants need to be used with caution to ensure safety, as they are often strong immune system inducers that can potentially trigger unwanted immune responses and lead to excessive immunopathology (352,353). Therefore, it is important to maintain a balance between increasing immunogenicity and minimising toxicity.

In addition to adjuvant, the mode of vaccine delivery has also been shown to improve vaccine-induced T cell responses. For instance, by comparing intravenous rather than intradermally administered BCG vaccine, Darrah *et al*. demonstrated that intravenous vaccination elicited a high frequency of CD4 and CD8 antigen-responsive T cells in the lung. This was associated with a favourable outcome in susceptible Mtb-infected macaques, suggesting a possible effective mechanism of vaccine-induced immunity against TB (354). The success stories of vaccine-induced CD4 and CD8 Mtb-specific antigen T cell responses following the Mtb challenge in animal models indicate that it is possible to induce effective T cell-based vaccines in humans.

6.3.3 Clinical implications

This thesis provides evidence of an altered frequency, phenotype, and functional impairment of Mtb-specific CD4+ T cells in the lung, accompanied by an influx of CD8+ T cells, in PLHIV adults. This likely contributes to increased susceptibility to lower respiratory tract infections, including TB, associated with HIV. HIV infection causes profound immune dysregulation, characterised by a progressive loss of the ability to mount an effective T cell response to respiratory pathogens and other opportunistic infections **(Chapter 3)** (277,278,289). Given that most changes in effector immune cells are driven by HIV, it is expected that ART therapy would have a profound beneficial effect. While ART is associated with a rapid reduction in viraemia and the reconstitution of CD4+ T cells (355), we and others have previously reported a persistent functional impairment of Mtb-specific CD4+ T cells even with successful ART, suggesting an incomplete immune functional reconstitution (280,356).

The data further demonstrate that HIV preferentially depletes tissue- resident T cells (CD103+ cells) in the lung. Additionally, we show that HIV was associated with a decrease in the frequency of CD4 helper T cell subsets, particularly Th17 cells in the lung mucosa but not in the blood. The role of Th17 in TB immunity has been a subject of debate, with some investigators providing evidence of TB pathology characterised by neutrophilic inflammation leading to lung damage (357) while others have provided evidence of immune protection against TB. Indeed, Th1 and Th17 cells that produce IFN-γ and IL-17 are key in providing immunity to TB (285,286). In this study, we show an increase in Th1 and Th17 subsets in the lung compared to the blood, along with a significant reduction of these subsets in PLHIV. **(Chapter 3).**

For decades, Th1 cells have been a key player in cellular-mediated immunity to TB. Emerging evidence from animal studies suggests that Th1 immunity alone is not conclusive as a correlate of immune protection induced by BCG vaccination against TB (287,288). Additionally, there have been reports of IFN-independent mechanisms through which CD4 T cells control Mtb infection (275). Therefore, it

is likely that additional subsets of CD4 T cells, apart from Th1 cells such as Th17, are crucial in protecting against TB.

The thesis further described the usage and composition of the Mtb-specific TCR Vß repertoire in HIV-uninfected individuals and PLHIV. This study, consistent with findings by Smith *et al.*, identified differential TCR Vß usage in PLHIV with varying Vß chain expression observed in both CD4 and CD8 T cells in the lung and blood (321). Specifically, Vß1, Vß7.2, and Vß23 expression were significantly higher in CD4+ T cells from PBMCs of HIV-infected individuals compared with healthy controls. Conversely, CD4 T cells expressing Vß9 and Vß18 showed a lower frequency in PLHIV than in HIV-uninfected individuals. In CD4 BAL cells, however, Vß13.1 expression was significantly higher in PLHIV than in healthy controls, whilst Vß14 showed a decreased frequency of expression in HIV-infected individuals than in HIV-uninfected. No differences were found in the TCR Vß specificities in the blood of both PLHIV and healthy controls in CD8 T cells. However, in the lung, three Vß families (Vß5.1, Vß16, and Vß17) showed increased expression, while one family (Vß14) displayed decreased expression in HIV-infected compared to HIV-uninfected individuals.

The elevated TCR Vß in the lung and blood in PLHIV suggests their potential involvement in HIV immune response whilst depletion of certain TCR Vß clones in Mtb-specific CD4 and CD8 T cells in the lung and blood may indicate HIV-induced alteration in the repertoire **(Chapter 4).** Identifying highly expressed TCR chains associated with key pro-inflammatory cytokine production may offer valuable biomarkers for disease diagnosis and prognosis. Furthermore, correlating the elevation of T cells bearing certain TCR specificities with clinical manifestation may identify people at high risk of TB, which could be overly beneficial. In one study, Musvosvi *et al.* found distinct repertoires associated with either control or progression of active TB disease in blood (321). Identifying common antigens recognised by similar TCRs can provide an exciting window for diagnosis and clinical prognosis.

Lastly, the data from bulk RNA sequencing showed that Mtb-specific TCR repertoires from PLHIV individuals displayed reduced diversity compared with HIV-

uninfected in both the airway and blood. Additionally, a low frequency of clonotypes was observed in PLHIV not on ART compared to those on long-term ART, suggesting restoration of repertoire clonality with long-term ART. CDR3 length distribution analysis showed a higher and more diverse distribution of TCR amino acid lengths of Mtb-specific T cell repertoire in BAL and PBMCs in HIV-uninfected individuals compared to PLHIV. Particularly the highest CDR3 peak lengths were observed between 14 and 16 aa in PBMC characterised by TRBVß12 and TRBVß14 while BAL displayed peaks in CDR3 lengths 12-15 with a dominance of TRBVß12, TRBVß 13, and TRBVß14 suggesting their potential involvement in antigen recognition and specificity. The clonally expanded clones within the CDR3 regions indicate that HIV elicits a distinct immune response in the BAL **(Chapter 5)**. Evidence such as this, which describes changes in repertoire, has the potential to provide insights into clinical management. However, clinical use of the CDR3 sequence has been limited mainly by enormous variation in CDR3 sequences, resulting in high inconsistency, even among healthy individuals, creating uncertainty in the theoretical value of CDR3 quantity (358).

6.5 Limitations and challenges

While this thesis has generated valuable insights, several limitations must be acknowledged, as they impact the scope and generalizability of the findings. The primary limitation was the insufficient sample size, particularly with the HIV samples. This resulted from study time constraints caused by two main factors. Firstly, my research timeline experienced a significant disruption for nearly a year, from March 2020 to April 2021, due to the COVID-19 pandemic. During this period, the Malawi Liverpool Wellcome Programme (MLW) temporarily closed its laboratory facilities to any non-COVID-19-related work. Additionally, I encountered challenges obtaining ethics approval from the regulatory body, which was only allowing COVID-19-related studies. Given the nature of my bronchoscopy-based study, it was classified as high-risk, and there were many misconceptions about COVID-19 and my project. Despite these obstacles, through community engagement and sensitisation campaigns, we managed to recruit a substantial number of participants. However, participant recruitment was significantly affected by the recurrent waves of COVID-19, and Malawi experienced three major waves. Consequently, recruitment could not resume until it was considered safe.

These challenges forced me to modify the project from a longitudinal study to a cross-sectional one. This change impacted my plans to collect samples from TB patients to explore the diversity and dynamics of the TCR repertoire in natural TB infection and assess the impact of anti-TB drugs on the repertoire composition. Nevertheless, by using stimulation with Mtb antigens to probe T cell function and TCR repertoire, this thesis provided a detailed overview of Mtb-specific T cell responses in the lung and blood.

Secondly, the COVID-19 pandemic disrupted global supply chain systems and shipping, leading to significant challenges in sourcing reagents and consumables for the project. As MLW is affiliated with the University of Liverpool, all our laboratory reagents are centrally purchased from Liverpool. Consequently, critical experiments were not conducted as planned or on time. One such experiment was the TCR sequencing, which was conducted by iRepertoire Inc. in the USA.

The study used bulk RNA sequencing using the MiSeq Illumina technology to explore the TCR repertoire diversity in the study population. Unlike single cell RNA sequencing, bulk RNA sequencing aggregates signals from all cells within a sample, combining results from several cells. The utilisation of bulk RNA sequencing to study TCR repertoire diversity and repertoire clonality has some limitations. One major limitation is the loss of single-cell resolution in the results obtained, thereby masking the heterogeneity of T cell subsets (359). Therefore, we cannot fully elucidate which gene segments are more expressed in CD4 and CD8 T cell subsets when subjected to these conditions and in different compartments. As a result, there is emphasis on the importance of single cell resolution in the understanding of the complexity of T cell receptor repertoire diversity and clonality. Additionally, bulk RNA sequencing's sensitivity might be limited, leading to the incomplete detection of lowly expressed TCR transcripts. This is corroborated with recent advances in ultra-sensitive TCR detection methods that aim to address these limitations (359).

Furthermore, while BAL is a valuable tool for studying lung immune responses, it has limitations, particularly in human Mtb infection studies. As an airway wash rather than a direct sampling of lung parenchyma, BAL primarily reflects immune

cells lining the airways or in transit rather than tissue-resident populations at the site of infection. This limitation affects the interpretation of findings, as airway responses may not fully represent immune dynamics within the lung parenchyma. Additionally, BAL samples contain a mix of recruited and circulating cells, complicating the distinction between transient and resident immune populations. Therefore, while BAL provides insights into the airway immune environment, its findings should be interpreted with caution, and complementary approaches, such as lung tissue biopsies or imaging studies, may be necessary for a more comprehensive understanding of pulmonary immunity in Mtb infection.

Lastly, an 18-hour stimulation period may not capture all dynamic changes in TCR expression, potentially missing early or late response genes. Furthermore, the strength and consistency of stimulation with the whole antigen could vary, affecting the measurement of TCR repertoire diversity. Reliance on existing TCR reference databases might limit the discovery of novel TCR variants or underrepresented sequences. Though the sample size was not enough to fully address all research questions, the thesis has provided preliminary data that can be utilised in designing future mucosal studies.

6.6 Future work

Based on the findings reported in this thesis, some points came up that warrant further investigations to fully understand the immune landscape in the lung and the potential role of Mtb-specific T cells bearing different TCR specificity in controlling Mtb infections.

6.6.1 Further investigating the roles of Mtb-specific T cells expressing various TCR Vβ chains in the immune response to TB

In this study, the frequency of cytokine-producing cells was used as a measure of Mtb-specific T cell function. It was observed that CD4 and CD8 T cells with certain TCR specificities were more associated with cytokine production, suggesting that highly expressed TCR Vβ may play a key role in the immune response to TB. However, cytokine production represents only one aspect of T cell function. It is possible that cells not highly expressing these TCR chains may be essential for

other functions, such as cytotoxic potential, providing help to innate cells, and interacting with B cells. Therefore, assessing other functional roles of T cells expressing these TCRs would be desirable and could provide a deeper understanding of T-cell-mediated host immunity in the lung.

6.6.2 Identification of TCR specificities responsible for immunodominant epitope recognition

The data presented in this thesis show that Mtb-specific T cells in the lung and blood exhibit a heterogeneous TCR Vß repertoire, characterised by the high expression and decrease of certain TCR Vß specificities. This suggests their potential role in antigen recognition. However, further investigation into this hypothesis was not possible due to limited resources. Ideally, experiments using MHC peptide-specific tetramers could provide a more detailed analysis of antigen-specific T cells recognising particular epitopes (360). Moreover, use of advanced methods such as GLIPH (grouping of lymphocyte interactions by paratope hotspots) could allow identification and clustering of antigen-specific T cells with similar characteristics responsible for recognising specific peptides, thus determining the TCR specificity (361,362). Combining these methods with *Ex-vivo* Mtb infection assays to assess the role of cells expressing common TCRs in controlling Mtb infection could help identify key antigenic epitopes and T cell subsets with relevant functional differences. In this approach, T cells with specific TCRs for particular epitopes can be co-cultured with macrophages infected with Mtb reporter strains and assessed at different time points: 4 hours, 24 hours, 48 hours, and 72 hours (363). This will improve the study of the kinetics and dynamics of T cell-mediated Mtb control at both early and later time points. Ultimately, this knowledge might inform targets for immune boosting and vaccine development.

6.6.3 Describing the TCR repertoire in a natural TB infection

The findings reported in this thesis used stimulation with Mtb antigens as a surrogate to assess antigen specific immune responses to TB in both PBMC and BAL in PLHIV. We described evidence suggesting an impaired T cell response, selective expansion of TCR Vß genes and a reduced TCR repertoire clonality following stimulation. However, the distribution and repertoire usage in the lungs of

patients with active TB disease remain inconclusive. It would be clinically relevant to conduct a longitudinal study to assess TCR repertoire diversity and dynamics in natural TB infection and to probe the impact of the anti-TB drugs on repertoire restoration. Luo et al. found a negative correlation between reduced repertoire diversity and clinical severity of the disease in the blood of pulmonary TB patients before starting treatment. However, this study did not assess the effect of TB on the TCR repertoire in the lungs post-treatment (246). I propose a comparative study recruiting recently diagnosed pulmonary TB patients and healthy adult controls. This study will collect serial sputum, blood, and BAL samples at four-time points: baseline (before starting treatment), 2 weeks post-treatment, 2 months, and 8 months upon treatment completion. Treatment efficacy will be monitored by sputum smear microscopy and sputum culture. Antigen-specific T cells will be measured using the laboratory techniques employed in this project, and TCR repertoire analysis will be conducted using both bulk and scRNA-seq sequencing to improve sensitivity.

6.6.4 Kinetics and stability of TCR repertoire diversity following ART

The data presented in this thesis show that Mtb-specific T cells in the lung and blood exhibit a heterogeneous TCR Vß repertoire, which is altered by HIV infection. We also observed reduced TCR diversity and clonal frequencies in these compartments, which is restored following ART. However, the study could not establish the kinetics of repertoire restoration following ART or correlate it with clinical outcomes. The applicability of the findings is limited by the study's cross-sectional nature and sample size. Therefore, further research is required, involving a larger clinical study where participants are followed up to assess the long-term effects of repertoire recovery and stability. This, combined with functional assessments, would allow for the determination of potential correlates of protection.

6.7 Final conclusions

The research findings presented in this thesis provide a detailed analysis of the effects of HIV on the TCR repertoire diversity and Mtb-specific function in the lung

and peripheral blood. The data suggests that T cell immunity is compartmentalised, with a more restricted TCR repertoire observed in the airway than in the blood. Additionally, the results show a site-specific HIV-associated impairment of Mtb-specific CD4 and CD8 cytokine production, with diverse distributions of TCR Vβ usage. The impaired Mtb-specific T cell responses and reduced TCR diversity observed in PLHIV may contribute to the increased risk of developing and progressing to active TB disease in susceptible populations. There is a need for further clinical research to determine if the reduced TCR diversity in PLHIV can be a valuable tool for assessing the immune response to TB and predicting a patient's risk of disease progression. This knowledge may provide prognostic biomarkers and therapeutic targets for HIV and TB, informing the early commencement of preventive therapies or vaccines. The work conducted for this thesis has enhanced the current understanding of cellular T cell immune responses in the airway mucosa. It has important implications for future mucosal immunity studies and the design of novel long-lasting vaccines.

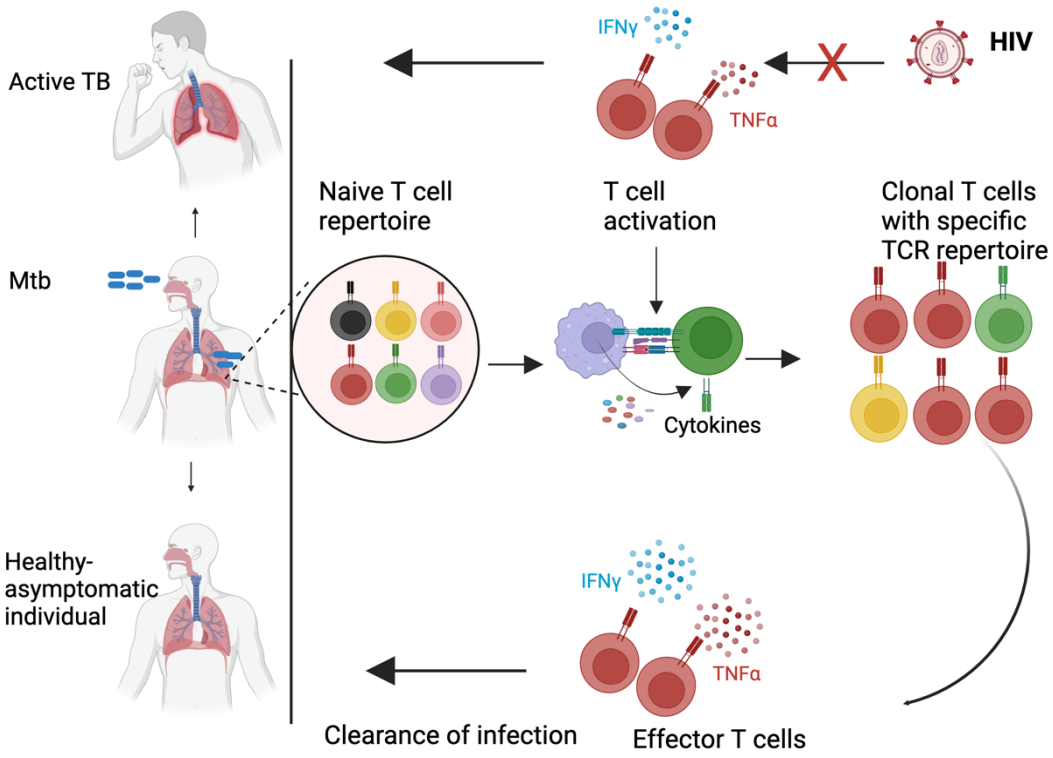


Figure 6-1 Graphical summary of major findings highlighting the impact of HIV on TCR Mtb-specific T cell function and TCR repertoire diversity in PMBC and BAL

Schematic representation of HIV-mediated impairment of Mtb-specific T cell function and TCR diversity (Created with BioRender.com). HIV infection is a major risk factor for the progression and reactivation of TB disease. Infection with Mtb results in three clinical outcomes: clearance of the infection, latency, or progression to active disease. However,

the exact mechanisms influencing control and progression to active disease are incompletely understood. Antigen-specific T cells play a critical role in containing TB infection by recognising Mtb and mounting an effective immune response. HIV depletes these Mtb-specific T cells and impairs their cytokine-producing functions, particularly in the lung, where the frequency of IFN-γ and TNF-α producing cells is reduced. Additionally, Mtb stimulation preferentially selects T cells bearing specific TCR Vβ variants, which are associated with the production of multiple cytokines essential for TB control. This process is disrupted by HIV, which is associated with reduced TCR clonality and diversity. These findings suggest a more restricted TCR repertoire in PLHIV compared to healthy controls, with alterations in the frequency of certain TCR families potentially impacting antigen recognition and specificity. This effect may reduce the ability to protect against infections, including TB, in PLHIV, contributing to their increased susceptibility to active TB. This knowledge provides new insights into why HIV-infected individuals have a higher risk of developing active TB compared to healthy controls.

Chapter 7 References

1. Glaziou P, Floyd K, Raviglione MC. Global Epidemiology of Tuberculosis. Semin Respir Crit Care Med. 2018;39(3):271–85.

2. Tuberculosis Reports. Vol. 188, The Lancet. 1916. 1120–1121 p.

3. Rook GAW, Dheda K, Zumla A. Immune responses to tuberculosis in developing countries: Implications for new vaccines. Nat Rev Immunol. 2005;5(8):661–7.

4. World Health Organization [WHO]. High Burden Countries for Tuberculosis | Stop TB Partnership [Internet]. 2019. Available from: https://www.stoptb.org/securing-quality-tb-care-all/high-burden-countries-tuberculosis

5. Bassett I V., Wang B, Chetty S, Giddy J, Losina E, Mazibuko M, et al. Intensive tuberculosis screening for HIV-infected patients starting antiretroviral therapy in Durban, South Africa. Clin Infect Dis. 2010;51(7):823–9.

6. WHO. WHO Releases New Global Lists Of High-Burden Countries For TB, HIV-Associated TB And Drug-Resistant TB. WhoInt [Internet]. 2021;(June):4–7. Available from: https://www.who.int/news/item/17-06-2021-who-releases-new-global-lists-of-high-burden-countries-for-tb-hiv-associated-tb-and-drug-resistant-tb

7. Millet JP, Moreno A, Fina L, Baño L Del, Orcau A, De Olalla PG, et al. Factors that influence current tuberculosis epidemiology. Eur Spine J. 2013;22(SUPPL.4).

8. National Statistical Office. MALAWI POPULATION AND HOUSING CENSUS REPORT-2018 2018 Malawi Population and Housing Main Report. 2019;(May). Available from: http://www.nsomalawi.mw/images/stories/data_on_line/demography/census_2018/2018 Malawi Population and Housing Census Main Report.pdf

9. World Bank. Key Indicators International Poverty Line(%). 2023;(April):1–2. Available from: www.worldbank.org/poverty

10. Moshinsky M. Global Tuberculosis Report 2022. Vol. 13, Nucl. Phys. 1959. p. 104–16.

11. World Health Organization. WHO releases new global lists of high-burden countries for TB, HIV-associated TB and drug-resistant TB [Internet]. Who.Int. 2021. p. 4–7. Available from: https://www.who.int/news/item/17-06-2021-who-releases-new-global-lists-of-high-burden-countries-for-tb-hiv-associated-tb-and-drug-resistant-tb

12. Addo K., Owusu-darko K, Yeboah-manu D, Caulley P, Minamikawa M, Bonsu F, et al. Mycobacterial species causing pulmonary tuberculosis At the korle bu teaching hospital, Accra,. Ghana Med J. 2010;41(2):52–7.

13. Pinsky BA, Banaei N. Multiplex real-time PCR assay for rapid identification of Mycobacterium tuberculosis complex members to the species level. J Clin Microbiol. 2008;46(7):2241–6.

14. Mc, Craik C, Adam Moser and Darrin M. York KR. 基因的改变NIH Public Access. Bone. 2008;23(1):1–7.

15. Garnier T, Eiglmeier K, Camus JC, Medina N, Mansoor H, Pryor M, et al. The complete genome sequence of Mycobacterium bovis. Proc Natl Acad Sci U S A. 2003;100(13):7877–82.

16. Behr MA, Wilson MA, Gill WP, Salamon H, Schoolnik GK, Rane S, et al. Comparative Genomics of BCG Vaccines by Whole-Genome DNA Microarray Published by : American Association for the Advancement of Science Stable URL : http://www.jstor.org/stable/2898945 REFERENCES Linked references are available on JSTOR for this article : Sci New Ser. 1998;284(5419):1520–3.

17. Hsu T, Hingley-Wilson SM, Chen B, Chen M, Dai AZ, Morin PM, et al. The primary mechanism of attenuation of bacillus Calmette-Guérin is a loss of secreted lytic function required for invasion of lung interstitial tissue. Proc Natl Acad Sci U S A. 2003;100(21):12420–5.

18. Brodin P, Rosenkrands I, Andersen P, Cole ST, Brosch R. ESAT-6 proteins: protective antigens and virulence factors? Trends Microbiol. 2004

Nov;12(11):500–8.

19. Kurenuma T, Kawamura I, Hara H, Uchiyama R, Daim S, Dewamitta SR, et al. The RD1 locus in the Mycobacterium tuberculosis genome contributes to activation of caspase-1 via induction of potassium ion efflux in infected macrophages. Infect Immun. 2009;77(9):3992–4001.

20. Guinn KM, Hickey MJ, Mathur SK, Zakel KL, Grotzke JE, Lewinsohn DM, et al. Individual RD1 -region genes are required for export of ESAT-6/CFP-10 and for virulence of Mycobacterium tuberculosis. Mol Microbiol. 2004;51(2):359–70.

21. Harshey RM, Ramakrishnan T. Rate of ribonucleic acid chain growth in Mycobacterium tuberculosis H37R(v). J Bacteriol. 1977;129(2):616–22.

22. Larsen MH, Dao DN, Baughn AD, Jalapthy K, Jacobs WR. Pathogenesis of Mycobacterium Tuberculosis. 4th Ed Virulence Mech Bact Pathog. 2022;104:31–50.

23. BLOCH H, SEGAL W. Biochemical differentiation of Mycobacterium tuberculosis grown in vivo and in vitro. J Bacteriol. 1956 Aug;72(2):132–41.

24. Sakamoto K. The pathology of Mycobacterium tuberculosis infection. Vet Pathol. 2012 May;49(3):423–39.

25. Daffé M, Draper P. The envelope layers of mycobacteria with reference to their pathogenicity. Adv Microb Physiol. 1998;39:131–203.

26. Kalscheuer R, Palacios A, Anso I, Cifuente J, Anguita J, Jacobs WR, et al. The Mycobacterium tuberculosis capsule: A cell structure with key implications in pathogenesis. Biochem J. 2019;476(14):1995–2016.

27. Wang Y, Shi Q, Chen Q, Zhou X, Yuan H, Jia X, et al. Emerging advances in identifying signal transmission molecules involved in the interaction between Mycobacterium tuberculosis and the host. Front Cell Infect Microbiol. 2022;12(July):1–11.

28. Arcos J, Sasindran SJ, Fujiwara N, Turner J, Schlesinger LS, Torrelles JB. Human lung hydrolases delineate Mycobacterium tuberculosis-macrophage interactions and the capacity to control infection. J Immunol. 2011

Jul;187(1):372–81.

29. Gupta KB, Atreja A. Transmission of tuberculous infection and its control in health care facilities. NTI Bull. 2006;42:63–7.

30. Farhat MR, Shapiro BJ, Kieser KJ, Sultana R, Jacobson KR, Victor TC, et al. Genomic analysis identifies targets of convergent positive selection in drug-resistant Mycobacterium tuberculosis. Nat Genet. 2013;45(10):1183–9.

31. Torrelles JB, Schlesinger LS. Integrating Lung Physiology, Immunology, and Tuberculosis. Trends Microbiol [Internet]. 2017;25(8):688–97. Available from: http://dx.doi.org/10.1016/j.tim.2017.03.007

32. Mendelsohn SC, Fiore-Gartland A, Awany D, Mulenga H, Mbandi SK, Tameris M, et al. Clinical predictors of pulmonary tuberculosis among South African adults with HIV. eClinicalMedicine. 2022;45.

33. Luies L, Preez I du. The echo of pulmonary tuberculosis: Mechanisms of clinical symptoms and other disease-induced systemic complications. Clin Microbiol Rev. 2020;33(4):1–19.

34. Loddenkemper R, Lipman M, Zumla A. Clinical aspects of adult tuberculosis. Cold Spring Harb Perspect Med. 2016;6(1):1–26.

35. Lawn SD, Zumla AI. Tuberculosis. Lancet (London, England). 2011 Jul;378(9785):57–72.

36. Hodapp T, Sester U, Mack U, Singh M, Meier T, Wiech E, et al. Massive monoclonal expansion of CD4 T-cells specific for a Mycobacterium tuberculosis ESAT-6 peptide. Eur Respir J. 2012;40(1):152–60.

37. Zumla A, Chakaya J, Centis R, D'Ambrosio L, Mwaba P, Bates M, et al. Tuberculosis treatment and management--an update on treatment regimens, trials, new drugs, and adjunct therapies. Lancet Respir Med. 2015 Mar;3(3):220–34.

38. Organization WH. Guidelines for Treatment of Tuberculosis and Patient Care [Internet]. Vol. 1, Who. 2017. 85–97 p. Available from: http://www.tandfonline.com/doi/full/10.1586/17476348.1.1.85

39. Kyd JM, Foxwell AR, Cripps AW. Mucosal immunity in the lung and upper airway. Vaccine. 2001;19(17–19):2527–33.

40. Nochi T, Kiyono H. Innate immunity in the mucosal immune system. Curr Pharm Des. 2006;12(32):4203–13.

41. Kumar V. Pulmonary Innate Immune Response Determines the Outcome of Inflammation During Pneumonia and Sepsis-Associated Acute Lung Injury. Front Immunol. 2020;11(August):1722.

42. Bals R, Hiemstra PS. Innate immunity in the lung: How epithelial cells fight against respiratory pathogens. Eur Respir J. 2004;23(2):327–33.

43. Sato S, Kiyono H. The mucosal immune system of the respiratory tract. Curr Opin Virol [Internet]. 2012;2(3):225–32. Available from: http://dx.doi.org/10.1016/j.coviro.2012.03.009

44. Rose MC, Nickola TJ, Voynow JA. Airway mucus obstruction: Mucin glycoproteins, MUC gene regulation and goblet cell hyperplasia. Am J Respir Cell Mol Biol. 2001;25(5):533–7.

45. Eisele NA, Anderson DM. Host Defense and the Airway Epithelium: Frontline Responses That Protect against Bacterial Invasion and Pneumonia. J Pathog. 2011;2011:1–16.

46. Li Y, Wang Y, Liu X. The role of airway epithelial cells in response to mycobacteria infection. Clin Dev Immunol. 2012;2012.

47. de Martino M, Lodi L, Galli L, Chiappini E. Immune Response to Mycobacterium tuberculosis: A Narrative Review. Front Pediatr. 2019;7(August):1–8.

48. Fitzgerald KA, Kagan JC. Toll-like Receptors and the Control of Immunity. Cell [Internet]. 2020;180(6):1044–66. Available from: https://doi.org/10.1016/j.cell.2020.02.041

49. Ehlers S, Mielke ME, Hahn H. Progress in TB research: Robert Koch's dilemma revisited. Vol. 15, Immunology today. England; 1994. p. 1–4.

50. Ehlers S. Immunity to tuberculosis: A delicate balance between protection and pathology. FEMS Immunol Med Microbiol. 1999;23(2):149–58.

51. Nomula K, Ale H, Chang C. Innate and Adaptive Immunity. Absol Allergy Immunol Board Rev. 2022;19(6):1–11.

52. Korbel DS, Schneider BE, Schaible UE. Innate immunity in tuberculosis: myths and truth. Microbes Infect [Internet]. 2008;10(9):995–1004. Available from: http://dx.doi.org/10.1016/j.micinf.2008.07.039

53. Van Crevel R, Ottenhoff THM, Van Der Meer JWM. Innate immunity to Mycobacterium tuberculosis. Adv Exp Med Biol. 2003;531(2):241–7.

54. Sia JK, Georgieva M, Rengarajan J. Innate Immune Defenses in Human Tuberculosis: An Overview of the Interactions between Mycobacterium tuberculosis and Innate Immune Cells. J Immunol Res. 2015;2015.

55. Hussell T, Bell TJ. Alveolar macrophages: Plasticity in a tissue-specific context. Nat Rev Immunol. 2014;14(2):81–93.

56. Hartl D, Tirouvanziam R, Laval J, Greene CM, Habiel D, Sharma L, et al. Innate Immunity of the Lung: From Basic Mechanisms to Translational Medicine. J Innate Immun. 2018;10(5–6):487–501.

57. Cohen SB, Gern BH, Delahaye JL, Adams KN, Plumlee CR, Winkler JK, et al. Alveolar Macrophages Provide an Early Mycobacterium tuberculosis Niche and Initiate Dissemination. Cell Host Microbe [Internet]. 2018;24(3):439-446.e4. Available from: https://doi.org/10.1016/j.chom.2018.08.001

58. Chatterjee S. The Lung Immune Niche in Tuberculosis : Insights from Studies on Human Alveolar Macrophages. 2015;49–53.

59. Schlesinger LS, Horwitz MA. Phagocytosis of leprosy bacilli is mediated by complement receptors CR1 and CR3 on human monocytes and complement component C3 in serum. J Clin Invest. 1990;85(4):1304–14.

60. Ernst JD. Macrophage Receptors for Mycobacterium tuberculosis. Infect Immun. 1998;66(4):1277–81.

61. Brightbill HD, Libraty DH, Krutzik SR, Yang RB, Belisle JT, Bleharski JR, et al. Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. Science (80-). 1999;285(5428):732–6.

62. Jung JY, Madan-Lala R, Georgieva M, Rengarajan J, Sohaskey CD, Bange

FC, et al. The intracellular environment of human macrophages that produce nitric oxide promotes growth of mycobacteria. Infect Immun. 2013;81(9):3198–209.

63. Nicholson S, Bonecini-Almeida MDG, Lapa E Silva JR, Nathan C, Xie QW, Mumford R, et al. Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis. J Exp Med. 1996;183(5):2293–302.

64. Chan J, Tanaka K, Carroll D, Flynn J, Bloom BR. Effects of nitric oxide synthase inhibitors on murine infection with Mycobacterium tuberculosis. Infect Immun. 1995 Feb;63(2):736–40.

65. Scanga CA, Mohan VP, Tanaka K, Alland D, Flynn JL, Chan J. The inducible nitric oxide synthase locus confers protection against aerogenic challenge of both clinical and laboratory strains of Mycobacterium tuberculosis in mice. Infect Immun. 2001 Dec;69(12):7711–7.

66. Jung J-Y, Madan-Lala R, Georgieva M, Rengarajan J, Sohaskey CD, Bange F-C, et al. The intracellular environment of human macrophages that produce nitric oxide promotes growth of mycobacteria. Infect Immun. 2013 Sep;81(9):3198–209.

67. Srivastava S, Ernst JD, Desvignes L. Beyond macrophages: the diversity of mononuclear cells in tuberculosis. Immunol Rev. 2014 Nov;262(1):179–92.

68. Sawant K V., Poluri KM, Dutta AK, Sepuru KM, Troshkina A, Garofalo RP, et al. Chemokine CXCL1 mediated neutrophil recruitment: Role of glycosaminoglycan interactions. Sci Rep. 2016;6:4–11.

69. Sawant K V., Sepuru KM, Penaranda B, Lowry E, Garofalo RP, Rajarathnam K. Chemokine Cxcl1-Cxcl2 heterodimer is a potent neutrophil chemoattractant. J Leukoc Biol [Internet]. 2023;114(6):666–71. Available from: https://doi.org/10.1093/jleuko/qiad097

70. Capucetti A, Albano F, Bonecchi R. Multiple Roles for Chemokines in Neutrophil Biology. Front Immunol. 2020;11(July):1–9.

71. Mortaz E, Alipoor SD, Adcock IM, Mumby S, Koenderman L. Update on neutrophil function in severe inflammation. Front Immunol.

2018;9(OCT):1–14.

72. Gaffney E, Murphy D, Walsh A, Connolly S, Basdeo SA, Keane J, et al. Defining the role of neutrophils in the lung during infection: Implications for tuberculosis disease. Front Immunol. 2022;13(September):1–14.

73. Martineau AR, Newton SM, Wilkinson KA, Kampmann B, Hall BM, Nawroly N, et al. Neutrophil-mediated innate immune resistance to mycobacteria. J Clin Invest. 2007 Jul;117(7):1988–94.

74. Margraf A, Perretti M. Immune Cell Plasticity in Inflammation: Insights into Description and Regulation of Immune Cell Phenotypes. Cells. 2022;11(11).

75. Ng LG, Ostuni R, Hidalgo A. Heterogeneity of neutrophils. Nat Rev Immunol. 2019 Apr;19(4):255–65.

76. Burn GL, Foti A, Marsman G, Patel DF, Zychlinsky A. The Neutrophil. Immunity. 2021;54(7):1377–91.

77. Remijsen Q, Berghe T Vanden, Wirawan E, Asselbergh B, Parthoens E, De Rycke R, et al. Neutrophil extracellular trap cell death requires both autophagy and superoxide generation. Cell Res. 2011;21(2):290–304.

78. Jambo KC, Mwale A, Hummel A, Mvaya L, Kamng'ona R, Chimbayo E, et al. B cell, CD8+ T cell and gamma delta T cell infiltration alters alveolar immune cell homeostasis in HIV-infected Malawian adults. Wellcome Open Res. 2018;2:1–22.

79. Kobayashi SD, Malachowa N, DeLeo FR. Neutrophils and Bacterial Immune Evasion. J Innate Immun. 2018;10(5–6):432–41.

80. Corleis B, Korbel D, Wilson R, Bylund J, Chee R, Schaible UE. Escape of Mycobacterium tuberculosis from oxidative killing by neutrophils. Cell Microbiol. 2012;14(7):1109–21.

81. Ramos-Kichik V, Mondragón-Flores R, Mondragón-Castelán M, Gonzalez-Pozos S, Muñiz-Hernandez S, Rojas-Espinosa O, et al. Neutrophil extracellular traps are induced by Mycobacterium tuberculosis. Tuberculosis (Edinb). 2009 Jan;89(1):29–37.

82. Cavalcante-Silva LHA, Almeida FS, Andrade AG de, Comberlang FC,

Cardoso LL, Vanderley SER, et al. Mycobacterium tuberculosis in a Trap: The Role of Neutrophil Extracellular Traps in Tuberculosis. Int J Mol Sci. 2023;24(14).

83. Guimarães-Costa AB, Nascimento MTC, Froment GS, Soares RPP, Morgado FN, Conceição-Silva F, et al. Leishmania amazonensis promastigotes induce and are killed by neutrophil extracellular traps. Proc Natl Acad Sci U S A. 2009 Apr;106(16):6748–53.

84. Liang C, Lian N, Li M. The emerging role of neutrophil extracellular traps in fungal infection. Front Cell Infect Microbiol. 2022;12(August):1–14.

85. Filio-Rodríguez G, Estrada-García I, Arce-Paredes P, Moreno-Altamirano MM, Islas-Trujillo S, Ponce-Regalado MD, et al. In vivo induction of neutrophil extracellular traps by Mycobacterium tuberculosis in a guinea pig model. Innate Immun. 2017 Oct;23(7):625–37.

86. Delgado-Rizo V, Martínez-Guzmán MA, Iñiguez-Gutierrez L, García-Orozco A, Alvarado-Navarro A, Fafutis-Morris M. Neutrophil extracellular traps and its implications in inflammation: An overview. Front Immunol. 2017;8(FEB):1–20.

87. Mellman I. Dendritic cells: master regulators of the immune response. Cancer Immunol Res. 2013 Sep;1(3):145–9.

88. Mellman I, Steinman RM. Dendritic cells: Specialized and regulated antigen processing machines. Cell. 2001;106(3):255–8.

89. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998;392(6673):245–52.

90. Lugo-Villarino G, Cougoule C, Meunier E, Rombouts Y, Vérollet C, Balboa L. Editorial: The mononuclear phagocyte system in infectious disease. Front Immunol. 2019;10(JUN).

91. Cruse JM, Lewis RE, Wang HBT-IG, editors. 1 - MOLECULES, CELLS, AND TISSUES OF IMMUNITY. In San Diego: Academic Press; 2004. p. 1–15. Available from: https://www.sciencedirect.com/science/article/pii/B978012198382650025X

92. Yatim KM, Lakkis FG. A brief journey through the immune system. Clin J Am Soc Nephrol. 2015;10(7):1274–81.

93. Wilson CB. The ontogeny of T lymphocyte maturation and function. J Pediatr. 1991;118(3):4–9.

94. Takahama Y. Journey through the thymus: Stromal guides for T-cell development and selection. Nat Rev Immunol. 2006;6(2):127–35.

95. Kumar B V., Connors TJ, Farber DL. Human T Cell Development, Localization, and Function throughout Life. Immunity [Internet]. 2018;48(2):202–13. Available from: https://doi.org/10.1016/j.immuni.2018.01.007

96. Haynes BF, Heinly CS. Early human T cell development: analysis of the human thymus at the time of initial entry of hematopoietic stem cells into the fetal thymic microenvironment. J Exp Med. 1995 Apr;181(4):1445–58.

97. Owen JJ, Ritter MA. Tissue interaction in the development of thymus lymphocytes. J Exp Med. 1969;129(2):431–42.

98. Liu C, Ueno T, Kuse S, Saito F, Nitta T, Piali L, et al. The role of CCL21 in recruitment of T-precursor cells to fetal thymi. Blood. 2005 Jan;105(1):31–9.

99. Bleul CC, Boehm T. Chemokines define distinct microenvironments in the developing thymus. Eur J Immunol. 2000 Dec;30(12):3371–9.

100. Rossi FMV, Corbel SY, Merzaban JS, Carlow DA, Gossens K, Duenas J, et al. Recruitment of adult thymic progenitors is regulated by P-selectin and its ligand PSGL-1. Nat Immunol. 2005;6(6):626–34.

101. Foss DL, Donskoy E, Goldschneider I. The importation of hematogenous precursors by the thymus is a gated phenomenon in normal adult mice. J Exp Med. 2001;193(3):365–74.

102. González-García S, García-Peydró M, Alcain J, Toribio ML. Notch1 and IL-7 receptor signalling in early T-cell development and leukaemia. Curr Top Microbiol Immunol. 2012;360:47–73.

103. Taghon T, Waegemans E, Van de Walle I. Notch signaling during human T

cell development. Curr Top Microbiol Immunol. 2012;360:75–97.

104. Zúñiga-pflücker JC. Zuñiga_2004_Revisio-T-development. 2004;4(January).

105. Budd RC, Fortner KA. T-cell development: The Thymus. Clin Immunol Princ Pract Expert Consult Online Print. 2008;127–37.

106. Kang J, Fehling HJ, Laplace C, Malissen M, Cado D, Raulet DH. T cell receptor γ gene regulatory sequences prevent the function of a novel TCRγ/pTα pre-T cell receptor. Immunity. 1998;8(6):713–21.

107. Bousso P, Bhakta NR, Lewis RS, Robey E, Bousso P, Bhakta NR, et al. Linked references are available on JSTOR for this article : Dynamics of Thymocyte-Stromal Cell Interactions Visualized by Two-Photon Microscopy. 2016;296(5574):1876–80.

108. Klein L, Kyewski B, Allen PM, Hogquist KA. Positive and negative selection of the T cell repertoire: What thymocytes see (and don't see). Nat Rev Immunol. 2014;14(6):377–91.

109. Nakagawa T, Roth W, Wong P, Nelson A, Farr A, Deussing J, et al. Cathepsin L: critical role in Ii degradation and CD4 T cell selection in the thymus. Science. 1998 Apr;280(5362):450–3.

110. Florea BI, Verdoes M, Li N, Van Der Linden WA, Geurink PP, Van Den Elst H, et al. Activity-based profiling reveals reactivity of the murine thymoproteasome-specific subunit β5t. Chem Biol [Internet]. 2010;17(8):795–801. Available from: http://dx.doi.org/10.1016/j.chembiol.2010.05.027

111. Pinto S, Michel C, Schmidt-Glenewinkel H, Harder N, Rohr K, Wild S, et al. Overlapping gene coexpression patterns in human medullary thymic epithelial cells generate self-antigen diversity. Proc Natl Acad Sci U S A. 2013;110(37):3497–505.

112. Caramalho Í, Nunes-Cabaço H, Foxall RB, Sousa AE. Regulatory T-cell development in the human thymus. Front Immunol. 2015;6(JUL):1–7.

113. William Harvey Research Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, Charterhouse

Square, London EC1M 6BQ, UK, 2 Section of Immunobiology, Department of Medicine, Imperial College London, Hammersmith H. 2013;1:422–40.

114. Mahe E, Pugh T, Kamel-Reid S. T cell clonality assessment: Past, present and future. J Clin Pathol. 2018;71(3):195–200.

115. Shah K, Al-Haidari A, Sun J, Kazi JU. T cell receptor (TCR) signaling in health and disease. Signal Transduct Target Ther. 2021;6(1).

116. Kaufmann SHE. γ/δ and other unconventional T lymphocytes: What do they see and what do they do? Proc Natl Acad Sci U S A. 1996;93(6):2272–9.

117. Rosati E, Dowds CM, Liaskou E, Henriksen EKK, Karlsen TH, Franke A. Overview of methodologies for T-cell receptor repertoire analysis. BMC Biotechnol [Internet]. 2017;17(1):61. Available from: https://doi.org/10.1186/s12896-017-0379-9

118. Warr GW, Clem LW, Söderhäll K. The international imMunoGeneTics database IMGT. Dev Comp Immunol. 2003;27(1):1.

119. van Boxel GI, Holmes S, Fugger L, Jones EY. An alternative conformation of the T-Cell receptor α constant region. J Mol Biol [Internet]. 2010;400(4):828–37. Available from: http://dx.doi.org/10.1016/j.jmb.2010.05.053

120. Hodges E, Krishna MT, Pickard C, Smith JL. Diagnostic role of tests for T cell receptor (TCR) genes. J Clin Pathol. 2003 Jan;56(1):1–11.

121. Bassing CH, Swat W, Alt FW. The mechanism and regulation of chromosomal V(D)J recombination. Cell. 2002;109(2 SUPPL. 1):45–55.

122. Lescale C, Deriano L. V(D)J Recombination: Orchestrating Diversity without Damage. Encycl Cell Biol. 2016;3(D):550–66.

123. Lin SG, Ba Z, Alt FW, Zhang Y. Chapter Three - RAG Chromatin Scanning During V(D)J Recombination and Chromatin Loop Extrusion are Related Processes. In: Alt F, editor. Academic Press; 2018. p. 93–135. (Advances in Immunology; vol. 139). Available from: https://www.sciencedirect.com/science/article/pii/S0065277618300282

124. Garboczi DN, Ghosh P, Utz U, Fan QR, Biddison WE, Wiley DC. Structure of

the complex between human T-cell receptor, viral peptide and HLA-A2. Nature. 1996;384(6605):134–41.

125. Chothia C, Boswell DR, Lesk AM. The outline structure of the T-cell alpha beta receptor. EMBO J. 1988;7(12):3745–55.

126. Turner SJ, Doherty PC, McCluskey J, Rossjohn J. Structural determinants of T-cell receptor bias in immunity. Nat Rev Immunol. 2006;6(12):883–94.

127. Thaventhiran T. T Cell Co-inhibitory Receptors-Functions and Signalling Mechanisms. J Clin Cell Immunol. 2013;01(S12).

128. Borst J, Coligan JE, Oettgen H, Pessano S, Malin R, Terhorst C. The delta- and epsilon-chains of the human T3/T-cell receptor complex are distinct polypeptides. Nature. 1984 Nov;312(5993):455–8.

129. Letourneur F, Klausner RD. Activation of T cells by a tyrosine kinase activation domain in the cytoplasmic tail of CD3 epsilon. Science. 1992 Jan;255(5040):79–82.

130. Turner SJ, Doherty PC, McCluskey J, Rossjohn J. Structural determinants of T-cell receptor bias in immunity. Nat Rev Immunol [Internet]. 2006;6(12):883–94. Available from: https://doi.org/10.1038/nri1977

131. Nikolich-Žugich J, Slifka MK, Messaoudi I. The many important facets of T-cell repertoire diversity. Nat Rev Immunol. 2004;4(2):123–32.

132. Arstila TP, Casrouge A, Baron V, Even J, Kanellopoulos J, Kourilsky P. A direct estimate of the human alphabeta T cell receptor diversity. Science. 1999 Oct;286(5441):958–61.

133. Bousso P, Casrouge A, Altman JD, Haury M, Kanellopoulos J, Abastado JP, et al. Individual variations in the murine T cell response to a specific peptide reflect variability in naive repertoires. Immunity. 1998 Aug;9(2):169–78.

134. Lin MY, Welsh RM. Stability and diversity of T cell receptor repertoire usage during lymphocytic choriomeningitis virus infection of mice. J Exp Med. 1998;188(11):1993–2005.

135. Cardinale A, De Luca CD, Locatelli F, Velardi E. Thymic Function and T-

Cell Receptor Repertoire Diversity: Implications for Patient Response to Checkpoint Blockade Immunotherapy. Front Immunol. 2021;12(November):1–12.

136. Qi Q, Liu Y, Cheng Y, Glanville J, Zhang D, Lee JY, et al. Diversity and clonal selection in the human T-cell repertoire. Proc Natl Acad Sci U S A. 2014;111(36):13139–44.

137. Peiris JSM, Chu CM, Cheng VCC, Chan KS, Hung IFN, Poon LLM, et al. Clinical progression and viral load in a community outbreak of coronavirus-associated SARS pneumonia: a prospective study. Lancet (London, England). 2003 May;361(9371):1767–72.

138. Jean CM, Honarmand S, Louie JK, Glaser CA. Risk factors for West Nile virus neuroinvasive disease, California, 2005. Emerg Infect Dis. 2007 Dec;13(12):1918–20.

139. Coates DR, Chin JM, Chung STL. 基因的改变NIH Public Access. Bone [Internet]. 2011;23(1):1–7. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3624763/pdf/nihms412728.pdf

140. Krishna C, Chowell D, Gönen M, Elhanati Y, Chan TA. Genetic and environmental determinants of human TCR repertoire diversity. Immun Ageing. 2020;17(1):1–7.

141. Chowell D, Morris LGT, Grigg CM, Weber JK, Samstein RM, Makarov V, et al. Patient HLA class I genotype influences cancer response to checkpoint blockade immunotherapy. Science (80-). 2018;359(6375):582–7.

142. Kohlmeier JE, Woodland DL. Memory T cell recruitment to the lung airways. Curr Opin Immunol. 2006;18(3):357–62.

143. Bromley S. (s.d.). 1999;90–1.

144. Dustin ML. The immunological synapse. Cancer Immunol Res. 2014;2(11):1023–33.

145. Zhu X, Zhu J. CD4 T helper cell subsets and related human immunological disorders. Int J Mol Sci. 2020;21(21):1–26.

146. Zhu J, Yamane H, Paul WE. Differentiation of effector CD4+ T cell populations. Annu Rev Immunol. 2010;28:445–89.

147. Golubovskaya V, Wu L. Different subsets of T cells, memory, effector functions, and CAR-T immunotherapy. Cancers (Basel). 2016;8(3).

148. Trinchieri G, Pflanz S, Kastelein RA. The IL-12 family of heterodimeric cytokines: New players in the regulation of T cell responses. Immunity. 2003;19(5):641–4.

149. Luckheeram RV, Zhou R, Verma AD, Xia B. CD4 +T cells: Differentiation and functions. Clin Dev Immunol. 2012;2012.

150. Djuretic IM, Levanon D, Negreanu V, Groner Y, Rao A, Ansel KM. Erratum: Transcription factors T-bet and Runx3 cooperate to activate Ifng and silence Il4 in T helper type 1 cells (Nature Immunology). Nat Immunol. 2007;8(2):145–53.

151. Afkarian M, Sedy JR, Yang J, Jacobson NG, Cereb N, Yang SY, et al. T-bet is a STATI-induced regulator for IL-12R expression in naïve CD4+ T cells. Nat Immunol. 2002;3(6):549–57.

152. Walker JA, McKenzie ANJ. TH2 cell development and function. Nat Rev Immunol. 2018;18(2):121–33.

153. Zhu J, Yamane H, Cote-Sierra J, Guo L, Paul WE. GATA-3 promotes Th2 responses through three different mechanisms: Induction of Th2 cytokine production, selective growth of Th2 cells and inhibition of Th1 cell-specific factors. Cell Res. 2006;16(1):3–10.

154. Usui T, Nishikomori R, Kitani A, Strober W. GATA-3 Suppresses Th1 Development by Downregulation of Stat4 and Not through Effects on IL-12R2 Chain or T-bet particularly in relation to its ability. Immunity. 2003;18:415–28.

155. Justin I. Odegaard, Chawla A. 基因的改变NIH Public Access. Bone. 2008;23(1):1–7.

156. Veldhoen M, Uyttenhove C, van Snick J, Helmby H, Westendorf A, Buer J, et al. Transforming growth factor-ß “reprograms” the differentiation of T

helper 2 cells and promotes an interleukin 9-producing subset. Nat Immunol. 2008;9(12):1341–6.

157. Staudt V, Bothur E, Klein M, Lingnau K, Reuter S, Grebe N, et al. Interferon-Regulatory Factor 4 Is Essential for the Developmental Program of T Helper 9 Cells. Immunity. 2010;33(2):192–202.

158. Huang M, Dong J. Critical Roles of Balanced T Helper 9 Cells and Regulatory T Cells in Allergic Airway Inflammation and Tumor Immunity. J Immunol Res. 2021;2021.

159. Annunziato F, Cosmi L, Santarlasci V, Maggi L, Liotta F, Mazzinghi B, et al. Phenotypic and functional features of human Th17 cells. J Exp Med. 2007;204(8):1849–61.

160. Gaffen SL. Structure and signalling in the IL-17 receptor family. Nat Rev Immunol [Internet]. 2009;9(8):556–67. Available from: http://dx.doi.org/10.1038/nri2586

161. Crotty S. T Follicular Helper Cell Differentiation, Function, and Roles in Disease. Immunity [Internet]. 2014;41(4):529–42. Available from: http://dx.doi.org/10.1016/j.immuni.2014.10.004

162. Gong F, Zheng T, Zhou P. T Follicular Helper Cell Subsets and the Associated Cytokine IL-21 in the Pathogenesis and Therapy of Asthma. Front Immunol. 2019;10(December):1–12.

163. Kondělková K, Vokurková D, Krejsek J, Borská L, Fiala Z, Ctirad A. Regulatory T cells (TREG) and their roles in immune system with respect to immunopathological disorders. Acta Medica (Hradec Kralove). 2010;53(2):73–7.

164. Iyer SS, Cheng G. Role of interleukin 10 transcriptional regulation in inflammation and autoimmune disease. Crit Rev Immunol. 2012;32(1):23–63.

165. Wang YM, Ghali J, Zhang GY, Hu M, Wang Y, Sawyer A, et al. Development and function of Foxp3+ regulatory T cells. Vol. 21, Nephrology. 2016. 81–85 p.

166. Chen WJ, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, et al. Conversion of Peripheral CD4+CD25- Naive T Cells to CD4+CD25+ Regulatory T Cells by TGF-β Induction of Transcription Factor Foxp3. J Exp Med. 2003;198(12):1875–86.

167. Yoshimura A, Muto G. TGF-β function in immune suppression. Curr Top Microbiol Immunol. 2011;350:127–47.

168. Jonuleit H, Schmitt E. The Regulatory T Cell Family: Distinct Subsets and their Interrelations. J Immunol. 2003;171(12):6323–7.

169. Trifari S, Kaplan CD, Tran EH, Crellin NK, Spits H. Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from TH-17, TH1 and TH2 cells. Nat Immunol. 2009;10(8):864–71.

170. Hossein-Khannazer N, Zian Z, Bakkach J, Kamali AN, Hosseinzadeh R, Anka AU, et al. Features and roles of T helper 22 cells in immunological diseases and malignancies. Scand J Immunol. 2021;93(5):1–14.

171. Duhen T, Geiger R, Jarrossay D, Lanzavecchia A, Sallusto F. Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. Nat Immunol. 2009;10(8):857–63.

172. Yeste A, Mascanfroni ID, Nadeau M, Burns EJ, Tukpah AM, Santiago A, et al. IL-21 induces IL-22 production in CD4+ T cells. Nat Commun. 2014;5(May).

173. Groscurth P, Filgueira L. Killing Mechanisms of Cytotoxic T Lymphocytes. Physiology [Internet]. 1998;13(1):17–21. Available from: https://doi.org/10.1152/physiologyonline.1998.13.1.17

174. Lowin B, Peitsch MC, Tschopp J. Perforin and granzymes: crucial effector molecules in cytolytic T lymphocyte and natural killer cell-mediated cytotoxicity. Curr Top Microbiol Immunol. 1995;198:1–24.

175. Trapani JA, Smyth MJ. Functional significance of the perforin/granzyme cell death pathway. Nat Rev Immunol. 2002;2(10):735–47.

176. Patel T, Gores GJ, Kaufmann SH. The role of proteases during apoptosis.

FASEB J Off Publ Fed Am Soc Exp Biol. 1996 Apr;10(5):587–97.

177. Andersen MH, Schrama D, Thor Straten P, Becker JC. Cytotoxic T cells. J Invest Dermatol [Internet]. 2006;126(1):32–41. Available from: http://dx.doi.org/10.1038/sj.jid.5700001

178. Williams MA, Bevan MJ. Effector and memory CTL differentiation. Annu Rev Immunol. 2007;25:171–92.

179. Takeuchi A, Saito T. CD4 CTL, a cytotoxic subset of CD4+ T cells, their differentiation and function. Front Immunol. 2017;8(FEB):1–7.

180. Glimcher LH, Townsend MJ, Sullivan BM, Lord GM. Recent developments in the transcriptional regulation of cytolytic effector cells. Nat Rev Immunol. 2004 Nov;4(11):900–11.

181. Brown DM, Lampe AT, Workman AM. The differentiation and protective function of cytolytic CD4 T cells in influenza infection. Front Immunol. 2016;7(MAR):1–8.

182. Mittrücker HW, Visekruna A, Huber M. Heterogeneity in the Differentiation and Function of CD8+ T Cells. Arch Immunol Ther Exp (Warsz). 2014;62(6):449–58.

183. Kemp RA, Ronchese F. Tumor-Specific Tc1, But Not Tc2, Cells Deliver Protective Antitumor Immunity. J Immunol. 2001;167(11):6497–502.

184. Selin LK, Welsh RM. Plasticity of T Cell Memory Responses to Viruses. Immunity. 2004;20(1):5–16.

185. Moulton VR, Farber DL. Committed to memory: lineage choices for activated T cells. Trends Immunol. 2006;27(6):261–7.

186. Kaech SM, Wherry EJ, Ahmed R. Effector and memory T-cell differentiation: implications for vaccine development. Nat Rev Immunol. 2002 Apr;2(4):251–62.

187. Farber DL. Cutting Edge Commentary: Differential TCR Signaling and the Generation of Memory T Cells. J Immunol. 1998;160(2):535–9.

188. Sallusto F, Lenig D, Förster R, Lipp M, Lanzavecchia A. Two subsets of

memory T lymphocytes with distinct homing potentials and effector functions. Nature. 1999 Oct;401(6754):708–12.

189. Sallusto F, Lanzavecchia A, Mackay CR. Chemokines and chemokine receptors in T-cell priming and Th1/Th2-mediated responses. Immunol Today. 1998 Dec;19(12):568–74.

190. Hale JS, Ahmed R. Memory T follicular helper CD4 T cells. Front Immunol. 2015;6(FEB):1–9.

191. Omilusik KD, Goldrath AW. Remembering to remember: T cell memory maintenance and plasticity. Curr Opin Immunol. 2019;58:89–97.

192. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: Function, generation, and maintenance. Annu Rev Immunol. 2004;22:745–63.

193. Gray JI, Westerhof LM, MacLeod MKL. The roles of resident, central and effector memory CD4 T-cells in protective immunity following infection or vaccination. Immunology. 2018;154(4):574–81.

194. Kalia V, Sarkar S, Gourley TS, Rouse BT, Ahmed R. Differentiation of memory B and T cells. Curr Opin Immunol. 2006;18(3):255–64.

195. Fazilleau N, Eisenbraun MD, Malherbe L, Ebright JN, Pogue-Caley RR, McHeyzer-Williams LJ, et al. Lymphoid reservoirs of antigen-specific memory T helper cells. Nat Immunol. 2007;8(7):753–61.

196. Marriott CL, Dutton EE, Tomura M, Withers DR. Retention of Ag-specific memory CD4+ T cells in the draining lymph node indicates lymphoid tissue resident memory populations. Eur J Immunol. 2017;47(5):860–71.

197. Milner JJ, Nguyen H, Omilusik K, Reina-Campos M, Tsai M, Toma C, et al. Delineation of a molecularly distinct terminally differentiated memory CD8 T cell population. Proc Natl Acad Sci U S A. 2020;117(41):25667–78.

198. Tian Y, Babor M, Lane J, Schulten V, Patil VS, Seumois G, et al. Unique phenotypes and clonal expansions of human CD4 effector memory T cells re-expressing CD45RA. Nat Commun [Internet]. 2017;8(1). Available from: http://dx.doi.org/10.1038/s41467-017-01728-5

199. Libri V, Azevedo RI, Jackson SE, Di Mitri D, Lachmann R, Fuhrmann S, et al. Cytomegalovirus infection induces the accumulation of short-lived, multifunctional CD4+CD45RA+CD27- T cells: The potential involvement of interleukin-7 in this process. Immunology. 2011;132(3):326–39.

200. Weiskopf D, Bangs DJ, Sidney J, Kolla R V., De Silva AD, De Silva AM, et al. Dengue virus infection elicits highly polarized CX3CR1+ cytotoxic CD4+ T cells associated with protective immunity. Proc Natl Acad Sci U S A. 2015;112(31):E4256–63.

201. Larbi A, Fulop T. From "truly naïve" to "exhausted senescent" T cells: When markers predict functionality. Cytom Part A. 2014;85(1):25–35.

202. Ogongo P, Zachary Porterfield J, Leslie A. Lung Tissue Resident Memory T-Cells in the Immune Response to Mycobacterium tuberculosis. Front Immunol. 2019;10(MAY).

203. Mackay LK, Braun A, Macleod BL, Collins N, Tebartz C, Bedoui S, et al. Cutting Edge: CD69 Interference with Sphingosine-1-Phosphate Receptor Function Regulates Peripheral T Cell Retention. J Immunol. 2015;194(5):2059–63.

204. Zheng MZM, Wakim LM. Tissue resident memory T cells in the respiratory tract. Mucosal Immunol [Internet]. 2022;15(3):379–88. Available from: http://dx.doi.org/10.1038/s41385-021-00461-z

205. Kumar B V., Ma W, Miron M, Granot T, Guyer RS, Carpenter DJ, et al. Human Tissue-Resident Memory T Cells Are Defined by Core Transcriptional and Functional Signatures in Lymphoid and Mucosal Sites. Cell Rep [Internet]. 2017;20(12):2921–34. Available from: http://dx.doi.org/10.1016/j.celrep.2017.08.078

206. Turner DL, Bickham KL, Thome JJ, Kim CY, D'Ovidio F, Wherry EJ, et al. Lung niches for the generation and maintenance of tissue-resident memory T cells. Mucosal Immunol [Internet]. 2014;7(3):501–10. Available from: http://dx.doi.org/10.1038/mi.2013.67

207. Agace WW. Tissue-tropic effector T cells: Generation and targeting opportunities. Nat Rev Immunol. 2006;6(9):682–92.

208. Career THE, Cell OFAT. AND Two Sides of the Same Coin. N Engl J Med. 2000;

209. Mora JR, von Andrian UH. T-cell homing specificity and plasticity: new concepts and future challenges. Trends Immunol. 2006 May;27(5):235–43.

210. Mikhak Z, Strassner JP, Luster AD. Lung dendritic cells imprint T cell lung homing and promote lung immunity through the chemokine receptor CCR4. J Exp Med. 2013;210(9):1855–69.

211. Abramo JM, Reynolds A, Crisp GT, Weurlander M, Söderberg M, Scheja M, et al. Individuality in music performance. Assess Eval High Educ [Internet]. 2012;37(October):435. Available from: http://books.google.com/books?id=_DDwCqx6wpcC&printsec=frontcover&dq=unwritten+rules+of+phd+research&hl=&cd=1&source=gbs_api%255Cnpapers2://publication/uuid/48967E01-55F9-4397-B941-310D9C5405FA%255Cnhttp://medcontent.metapress.com/index/A65RM03P4874243N.p

212. Millar JA, Butler JR, Evans S, Mattila JT, Linderman JJ, Flynn JAL, et al. Spatial Organization and Recruitment of Non-Specific T Cells May Limit T Cell-Macrophage Interactions Within Mycobacterium tuberculosis Granulomas. Front Immunol. 2021;11(January):1–15.

213. Chen CY, Huang D, Wang RC, Shen L, Zeng G, Yao S, et al. A critical role for CD8 T cells in a nonhuman primate model of tuberculosis. PLoS Pathog. 2009;5(4):1–10.

214. Mattila JT, Maiello P, Sun T, Via LE, Flynn JL. Granzyme B-expressing neutrophils correlate with bacterial load in granulomas from Mycobacterium tuberculosis-infected cynomolgus macaques. Cell Microbiol. 2015;17(8):1085–97.

215. Joslyn LR, Renardy M, Weissman C, Grant NL, Flynn JL, Butler JR, et al. Temporal and Spatial Analyses of TB Granulomas to Predict Long-Term Outcomes. In: Vodovotz Y, An G, editors. Complex Systems and Computational Biology Approaches to Acute Inflammation: A Framework for Model-based Precision Medicine [Internet]. Cham: Springer International Publishing; 2021. p. 273–91. Available from:

https://doi.org/10.1007/978-3-030-56510-7_15

216. Flynn JL, Chan J, Lin PL. Macrophages and control of granulomatous inflammation in tuberculosis. Mucosal Immunol. 2011;4(3):271–8.

217. Serbina N V, Lazarevic V, Flynn JL. CD4 + T Cells Are Required for the Development of Cytotoxic CD8 + T Cells During Mycobacterium tuberculosis Infection. 2019;

218. Caruso AM, Serbina N, Klein E, Bloom BR, Flynn JL, Caruso AM, et al. Mice Deficient in CD4 T Cells Have Only Transiently Diminished Levels of IFN- γ , Yet Succumb to Tuberculosis. 2019;

219. Geldmacher C, Ngwenyama N, Schuetz A, Petrovas C, Reither K, Heeregrave EJ, et al. Preferential infection and depletion of Mycobacterium tuberculosis – specific CD4 T cells after HIV infection. 2010;(December).

220. Lin PL, Flynn JL, Genetics M. HHS Public Access. 2016;37(3):239–49.

221. Stenger S, Hanson DA, Teitelbaum R, Dewan P, Niazi KR, Froelich CJ, et al. An Antimicrobial Activity of Cytolytic T Cells Mediated by Granulysin. 1998;282(October):121–6.

222. Grotzke JE, Lewinsohn DM. Role of CD8 + T lymphocytes in control of Mycobacterium tuberculosis infection. 2005;7:776–88.

223. Yin L, Kou ZC, Rodriguez C, Hou W, Goodenow MM, Sleasman JW, et al. Antiretroviral Therapy Restores Diversity in the T-Cell Receptor V $^{N}_{L}$ Repertoire of CD4 T-Cell Subpopulations among Human Immunodeficiency Virus Type 1-Infected Children and Adolescents . 2009;16(9):1293–301.

224. Hazenberg MD, Otto SA, van Benthem BHB, Roos MTL, Coutinho RA, Lange JMA, et al. Persistent immune activation in HIV infection is associated with progression to AIDS. AIDS [Internet]. 2003 Sep 5;17(13):1881–8. Available from: https://www.ncbi.nlm.nih.gov/pubmed/12960820

225. Conrad JA, Ramalingam RK, Duncan CB, Smith RM, Wei J, Barnett L, et al. Antiretroviral Therapy Reduces the Magnitude and T Cell Receptor Repertoire Diversity of HIV-Specific T Cell Responses without Changing T

Cell Clonotype Dominance. 2012;4213–21.

226. Oxenius A, Price DA, Easterbrook PJ, Callaghan CAO, Kelleher AD, Whelan JA, et al. Early highly active antiretroviral therapy for acute HIV infection preserves immune function of CD8 ⁺ and CD4 ⁺ T lymphocytes. 1999;

227. Baum PD, Young JJ, Schmidt D, Zhang Q, Hoh R, Busch M, et al. Blood T-cell receptor diversity decreases during the course of HIV infection, but the potential for a diverse repertoire persists. Blood [Internet]. 2012/02/27. 2012 Apr 12;119(15):3469–77. Available from: https://www.ncbi.nlm.nih.gov/pubmed/22371879

228. Gea-banacloche JC, Weiskopf EE, Quiro B De, Hallahan C, Lo JC, Flanigan M, et al. Progression of Human Immunodeficiency Virus Disease Is Associated with Increasing Disruptions within the CD4 / T Cell Receptor Repertoire. :579–85.

229. Heather JM, Best K, Oakes T, Gray ER, Roe JK, Miles JJ, et al. Dynamic Perturbations of the T-Cell Receptor Repertoire in Chronic HIV Infection and following Antiretroviral. 2016;6(January):1–15.

230. Almodovar S. The Complexity of HIV Persistence and Pathogenesis. 2014;27(5):186–99.

231. Wang B. HIV reservoirs in vivo and new strategies for possible eradication of HIV from the reservoir sites. 2010;103–22.

232. Neff CP, Chain JL, Mawhinney S, Martin AK, Linderman DJ, Flores SC, et al. Lymphocytic Alveolitis Is Associated with the Accumulation of Functionally Impaired HIV-Speci fi c T Cells in the Lung of Antiretroviral Therapy – Naive Subjects. 2015;191(4):464–73.

233. HIV-associated disruption of lung cytokine networks is incompletely restored in asymptomatic HIV-infected Malawian adults on antiretroviral therapy. 2017;

234. Manuscript A. NIH Public Access. 2014;34(2):155–64.

235. Raj C, Rijal ON, Palpasa KCKK. Lower respiratory tract infections among HIV positive and control group in Nepal. VirusDisease. 2015;26(1):77–81.

236. Smith I. Mycobacterium tuberculosis Pathogenesis and Molecular Determinants of Virulence. 2003;16(3):463–96.

237. Mwandumba HC, Squire SB, White SA, Nyirenda MH, Kampondeni SD, Rhoades ER, et al. Association between sputum smear status and local immune responses at the site of disease in HIV- infected patients with pulmonary tuberculosis. 2008;58–63.

238. Houben RMGJ, Crampin AC, Ndhlovu R, Sonnenberg P, Haas WH, Engelmann G, et al. Human immunodefi ciency virus associated tuberculosis more often due to recent infection than reactivation of latent infection. 2011;15(1):24–31.

239. Dravid A, Natarajan K, Medisetty M, Gawali R, Mahajan U, Kulkarni M, et al. Incidence of tuberculosis among HIV infected individuals on long term antiretroviral therapy in private healthcare sector in Pune , Western India. 2019;1–12.

240. Mayaud C, Parrot A, Cadranel J. Pyogenic bacterial lower respiratory tract infection in human immunodeficiency virus-infected patients. 2002;28–39.

241. Peno C, Banda DH, Jambo N, Kankwatira AM, Malamba RD, Allain TJ, et al. Alveolar T-helper 17 responses to streptococcus pneumoniae are preserved in ART-untreated and treated HIV-infected Malawian adults. J Infect [Internet]. 2018;76(2):168–76. Available from: https://doi.org/10.1016/j.jinf.2017.10.013

242. Jambo KC, Banda DH, Kankwatira AM, Sukumar N, Allain TJ, Heyderman RS. Small alveolar macrophages are infected preferentially by HIV and exhibit impaired phagocytic function. Mucosal Immunol. 2014;7(5).

243. Ahmed A, Rakshit S, Vyakarnam A. ORIGINAL ARTICLE HIV – TB co-infection : mechanisms that drive reactivation of Mycobacterium tuberculosis in HIV infection. 2016;22:53–60.

244. Liu J, Liu Z, Zhu Y, Dong B, Cai Z, Liang Q, et al. T Cell Receptor Immune Repertoires Are Promptly Reconstituted After Methicillin-Resistant Staphylococcus aureus Infection. 2019;10(August):1–9.

245. Paula A De, Sousa A, Johnson KR, Ohayon J, Zhu J, Muraro PA, et al.

Comprehensive Analysis of TCR- ß Repertoire in Patients with Neurological Immune-mediated Disorders. 2019;(October 2018):1–10.

246. Luo W, Su J, Zhang X, Yang Z, Zhou M, Jiang Z, et al. Limited T Cell Receptor Repertoire Diversity in Tuberculosis Patients Correlates with Clinical Severity. 2012;7(10):1–10.

247. Cheng C, Wang B, Gao L, Liu J, Chen X, Huang H. Next generation sequencing reveals changes of the γδ T cell receptor repertoires in patients with pulmonary tuberculosis. 2018;(February):1–13.

248. Chigona RK, Mipando ALN. "There Are Just Too Many Rooms Here!" Perception of Clients and Health Care Workers on the Implementation of Test and Treat Strategy at Area 25 Health Center in Lilongwe, Malawi. J Int Assoc Provid AIDS Care. 2023;22.

249. Manuscript title: "Airway HIV-specific CD8 + T Cells are Enriched for Tissue Resident Memory and Exhibit Limited Cytolytic Potential." 2020;(April):876444.

250. Jambo KC, Banda DH, Afran L, Kankwatira AM, Malamba RD, Allain TJ, et al. Asymptomatic HIV-infected individuals on antiretroviral therapy exhibit impaired lung CD4+ T-cell responses to mycobacteria. Am J Respir Crit Care Med. 2014;190(8):938–47.

251. Strober W. Trypan Blue Exclusion Test of Cell Viability. Curr Protoc Immunol. 2015;111(1):A3.B.1-A3.B.3.

252. Stanley SA, Johndrow JE, Manzanillo P, Cox JS. The Type I IFN Response to Infection with Mycobacterium tuberculosis Requires ESX-1-Mediated Secretion and Contributes to Pathogenesis . J Immunol. 2007;178(5):3143–52.

253. Day CL, Abrahams DA, Harris LD, Rooyen M Van, Kock M De, Hanekom WA, et al. HHS Public Access. 2018;199(6):2069–80.

254. Dobos K. Product Information Sheet for NR-14824 Mycobacterium tuberculosis, Strain HN878, Whole Cell Lysate [Internet]. 2010. Available from: www.beiresources.org.

255. Ai W, Li H, Song N, Li L, Chen H. Optimal method to stimulate cytokine production and its use in immunotoxicity assessment. Int J Environ Res Public Health. 2013;10(9):3834–42.

256. Majowicz A, van der Marel S, te Velde AA, Meijer SL, Petry H, van Deventer SJ, et al. Murine CD4+CD25- cells activated in vitro with PMA/ionomycin and anti-CD3 acquire regulatory function and ameliorate experimental colitis in vivo. BMC Gastroenterol. 2012;12.

257. Abdurrahman G, Schmiedeke F, Bachert C, Bröker BM, Holtfreter S. Allergy—A New Role for T Cell Superantigens of Staphylococcus aureus? Toxins (Basel). 2020;12(3):1–21.

258. Interpretation of Tapestation Traces.

259. Note A. Genomic DNA Analysis with the Agilent 2200 TapeStation System and Agilent Genomic DNA ScreenTape.

260. Bolotin DA, Poslavsky S, Mitrophanov I, Shugay M, Mamedov IZ, Putintseva E V, et al. MiXCR: software for comprehensive adaptive immunity profiling. Vol. 12, Nature methods. United States; 2015. p. 380–1.

261. Infection HI V, Impairs F. crossm.

262. Kwan CK, Ernst JD. HIV and Tuberculosis : a Deadly Human Syndemic. 2011;24(2):351–76.

263. Houben RMGJ, Dodd PJ. The Global Burden of Latent Tuberculosis Infection : A Re-estimation Using Mathematical Modelling. 2016;5:1–13.

264. Bell LCK, Noursadeghi M. Pathogenesis of HIV - 1 and Mycobacterium tuberculosis co - infection. Nat Publ Gr [Internet]. 2017;16(2):80–90. Available from: http://dx.doi.org/10.1038/nrmicro.2017.128

265. Hanna LE. Pathophysiology of CD4 + T-Cell Depletion in HIV and Hiv-2 infections. 2017;8(May):1–8.

266. Costiniuk CT, Salahuddin S, Farnos O, Orlova M, Olivenstein R, Schurr E, et al. HIV persistence in mucosal CD4 R T cells within the lungs of adults receiving long-term suppressive antiretroviral therapy. 2018;0(April):2279–89.

267. Lawn SD, Butera ST, Shinnick TM. Tuberculosis unleashed: the impact of human immunodeficiency virus infection on the host granulomatous response to Mycobacterium tuberculosis. Microbes Infect. 2002 May;4(6):635–46.

268. Badri M, Wilson D, Wood R. Effect of highly active antiretroviral therapy on incidence of tuberculosis in South Africa: a cohort study. Lancet (London, England). 2002 Jun;359(9323):2059–64.

269. Beck JM. NIH Public Access. 2014;34(2):143–53.

270. Arlehamn CSL, Lewinsohn D, Sette A, Lewinsohn D. in Tuberculosis. 2014;1–15.

271. Caruso AM, Serbina N, Klein E, Bloom BR, Flynn JL, Caruso AM, et al. Mice Deficient in CD4 T Cells Have Only Transiently Diminished Levels of IFN- γ , Yet Succumb to Tuberculosis. 2021;

272. Jambo KC, Sepako E, Fullerton DG, Mzinza D, Glennie S, Wright AK, et al. Bronchoalveolar CD4 + T cell responses to respiratory antigens are impaired in HIV-infected adults. 2011;375–82.

273. Woodland DL, Scott I. T Cell Memory in the Lung Airways. 2005;2:126–31.

274. Chen Z, Wang L, Gu L, Qu R, Lowrie DB, Hu Z, et al. Decreased Expression of CD69 on T Cells in Tuberculosis Infection Resisters. 2020;11(August):1–11.

275. Lu LL, Smith MT, Yu KKQ, Luedemann C, Suscovich TJ, Grace PS, et al. IFN-γ-independent immune markers of Mycobacterium tuberculosis exposure. Nat Med. 2019 Jun;25(6):977–87.

276. Iii HLT, Soliman DM, Day RB, Knox KS, Anderson RJ, Wilkes DS, et al. Viral Load , and Outcome in Human Immunodeficiency Virus Infection. 1993;

277. Kalsdorf B, Scriba TJ, Wood K, Day CL, Dheda K, Dawson R, et al. HIV infection impairs the bronchoalveolar T-cell response to mycobacteria. Am J Respir Crit Care Med. 2009;180(12):1262–70.

278. Day CL, Abrahams DA, Harris LD, van Rooyen M, Stone L, de Kock M, et al. HIV Infection Is Associated with Depletion and Functional Impairment of

Mycobacterium tuberculosis –Specific CD4 T Cells in Individuals with Latent Tuberculosis Infection . J Immunol. 2017;199(6):2069–80.

279. Crothers K, Thompson BW, Burkhardt K, Morris A, Flores SC, Diaz PT, et al. HIV-associated lung infections and complications in the era of combination antiretroviral therapy. Proc Am Thorac Soc. 2011;8(3):275–81.

280. Jambo KC, Banda DH, Afran L, Kankwatira AM, Malamba RD, Allain TJ, et al. Asymptomatic HIV-infected individuals on antiretroviral therapy exhibit impaired lung CD4+ T-cell responses to mycobacteria. Am J Respir Crit Care Med. 2014;190(8):938–47.

281. Sallusto F, Lenig D, Förster R, Lipp M, Lanzavecchia A. Western blots for IP 3 R1. 1999;401(October). Available from: www.nature.com

282. Ogongo P, Porterfield JZ, Leslie A. Lung Tissue Resident Memory T-Cells in the Immune Response to Mycobacterium tuberculosis. 2019;10(May):1–11.

283. Shiow LR, Rosen DB, Brdičková N, Xu Y, An J, Lanier LL, et al. CD69 acts downstream of interferon-α/ß to inhibit S1P 1 and lymphocyte egress from lymphoid organs. Nature. 2006;440(7083):540–4.

284. No Title.

285. Zielinski CE. T helper cell subsets: diversification of the field. Eur J Immunol. 2023;1–12.

286. Boom WH. The role of T-cell subsets in Mycobacterium tuberculosis infection. Infect Agents Dis. 1996 Mar;5(2):73–81.

287. Mittrücker H-W, Steinhoff U, Köhler A, Krause M, Lazar D, Mex P, et al. Poor correlation between BCG vaccination-induced T cell responses and protection against tuberculosis. Proc Natl Acad Sci U S A. 2007 Jul;104(30):12434–9.

288. Majlessi L, Simsova M, Jarvis Z, Brodin P, Rojas M-J, Bauche C, et al. An increase in antimycobacterial Th1-cell responses by prime-boost protocols of immunization does not enhance protection against tuberculosis. Infect Immun. 2006 Apr;74(4):2128–37.

289. Murray LW, Satti I, Meyerowitz J, Jones M, Willberg CB, Ussher JE, et al.

Human Immunodeficiency Virus Infection Impairs Th1 and Th17 Mycobacterium tuberculosis – Specific T-Cell Responses. 2018;217.

290. Imberti L, Sottini A, Bettinardi A, Puoti M, Primi D. Selective depletion in HIV infection of T cells that bear specific T cell receptor V beta sequences. Science. 1991 Nov;254(5033):860–2.

291. Hodara VL, Jeddi-Tehrani M, Grunewald J, Andersson R, Scarlatti G, Esin S, et al. HIV infection leads to differential expression of T-cell receptor V beta genes in CD4+ and CD8+ T cells. AIDS. 1993 May;7(5):633–8.

292. Yang J, He J, Huang H, Ji Z, Wei L, Ye P, et al. Molecular characterization of T cell receptor beta variable in the peripheral blood T cell repertoire in subjects with active tuberculosis or latent tuberculosis infection. 2013;1–10.

293. Charles J, Mouret S, Challende I, Leccia M-T, De Fraipont F, Perez S, et al. T-cell receptor diversity as a prognostic biomarker in melanoma patients. Pigment Cell Melanoma Res. 2020 Jul;33(4):612–24.

294. Allam A, Kabelitz D. TCR trans-rearrangements: biological significance in antigen recognition vs the role as lymphoma biomarker. J Immunol. 2006 May;176(10):5707–12.

295. Ahlgren KM, Ruckdeschel T, Eklund A, Wahlström J, Grunewald J. T cell receptor-V ß repertoires in lung and blood CD4 + and CD8 + T cells of pulmonary sarcoidosis patients. 2014;

296. Li D, Gao G, Li Z, Sun W, Li X, Chen N, et al. Profiling the T-cell receptor repertoire of patient with pleural tuberculosis by high-throughput sequencing. Immunol Lett. 2014 Nov;162(1 Pt A):170–80.

297. Soudeyns H, Rebai N, Pantaleo GP, Ciurli C, Boghossian T, Sékaly RP, et al. The t cell receptor vß repertoire in HIV infection and disease. Semin Immunol. 1993;5(3):175–85.

298. Mwale A, Hummel A, Mvaya L, Kamng'ona R, Chimbayo E, Phiri J, et al. B cell, CD8 + T cell and gamma delta T cell infiltration alters alveolar immune cell homeostasis in HIV-infected Malawian adults [version 2; referees: 1 approved, 2 approved with reservations]. Wellcome Open Res.

2017;2.

299. Mwale A, Hummel A, Mvaya L, Kamng'ona R, Chimbayo E, Phiri J, et al. B cell, CD8+ T cell and gamma delta T cell lymphocytic alveolitis alters alveolar immune cell homeostasis in HIV-infected Malawian adults. Wellcome Open Res. 2017;2:105.

300. Qiao D, Li L, Guo J, Lao S, Zhang X, Zhang J, et al. Mycobacterium tuberculosis culture filtrate protein 10-specific effector/memory CD4+ and CD8+ T cells in tubercular pleural fluid, with biased usage of T cell receptor Vß Chains. Infect Immun. 2011;79(8):3358–65.

301. Shao MM, Yi FS, Huang ZY, Peng P, Wu FY, Shi HZ, et al. T Cell Receptor Repertoire Analysis Reveals Signatures of T Cell Responses to Human Mycobacterium tuberculosis. Front Microbiol. 2022;13(February):1–11.

302. Pilch H, Höhn H, Freitag K, Neukirch C, Necker A, Haddad P, et al. Improved assessment of T-cell receptor (TCR) VB repertoire in clinical specimens: Combination of TCR-CDR3 spectratyping with flow cytometry-based TCR VB frequency analysis. Clin Diagn Lab Immunol. 2002;9(2):257–66.

303. Rosati E, Dowds CM, Liaskou E, Kristine E, Henriksen K, Karlsen TH, et al. Overview of methodologies for T-cell receptor repertoire analysis. 2017;1–16.

304. Ezzouaouy B, Scifo C, Coulter B, France M. Assessment of T-cell receptor repertoire by flow cytometry using the Beta Mark TCR Vß repertoire kit and Kaluza analysis software. :1–6.

305. Mpande CAM, Rozot V, Mosito B, Musvosvi M, Dintwe OB, Bilek N, et al. Immune profiling of Mycobacterium tuberculosis-specific T cells in recent and remote infection. EBioMedicine. 2021 Feb 1;64.

306. Marei A, Ghaemmaghami A, Renshaw P, Wiselka M, Barer M, Carr M, et al. Superior T cell activation by ESAT-6 as compared with the ESAT-6-CFP-10 complex. Int Immunol. 2014;4(7):1–16.

307. Yamasaki K, Van Eeden SF. Lung macrophage phenotypes and functional responses: Role in the pathogenesis of COPD. Int J Mol Sci. 2018;19(2):1–

12.

308. Makatsa MS, Omondi FMA, Bunjun R, Wilkinson RJ, Riou C, Burgers WA. Characterization of Mycobacterium tuberculosis –Specific Th22 Cells and the Effect of Tuberculosis Disease and HIV Coinfection . J Immunol. 2022;ji2200140.

309. Westerhof LM, McGuire K, MacLellan L, Flynn A, Gray JI, Thomas M, et al. Multifunctional cytokine production reveals functional superiority of memory CD4 T cells [Internet]. European Journal of Immunology. 2019. p. eji.201848026. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1002/eji.201848026

310. Darrah PA, Patel DT, De Luca PM, Lindsay RWB, Davey DF, Flynn BJ, et al. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. Nat Med [Internet]. 2007;13(7):843–50. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17558415

311. Arroyo L, Rojas M, Franken KLMC, Ottenhoff THM, Barrera LF. Multifunctional T Cell Response to DosR and Rpf Antigens Is Associated with Protection in Long-Term Mycobacterium tuberculosis-Infected Individuals in Colombia. Clin Vaccine Immunol. 2016;23(10):813–24.

312. Pannetier C, Even J, Kourilsky P. T-cell repertoire diversity and clonal expansions in normal and clinical samples. (Table 1).

313. Wong FS, Hibberd ML, Wen L, Millward BA, Demaine AG. The human T cell receptor Vβ repertoire of normal peripheral blood lymphocytes before and after mitogen stimulation. Clin Exp Immunol. 1993;92(2):361–6.

314. Ghazalpour A, Bennett B, Petyuk VA, Orozco L, Hagopian R, Mungrue IN, et al. Comparative analysis of proteome and transcriptome variation in mouse. PLoS Genet. 2011;7(6).

315. Sakai S, Mayer-Barber KD, Barber DL. Defining features of protective CD4 T cell responses to Mycobacterium tuberculosis. Curr Opin Immunol. 2014;29(1):137–42.

316. Jasenosky LD, Scriba TJ, Hanekom WA, Goldfeld AE. T cells and adaptive immunity to Mycobacterium tuberculosis in humans. Immunol Rev. 2015

Mar;264(1):74–87.

317. Darrah PA, Zeppa JJ, Maiello P, Hackney JA, Wadsworth MH, Hughes TK, et al. Prevention of tuberculosis in macaques after intravenous BCG immunization. Nature [Internet]. 2020;577(7788):95–102. Available from: http://dx.doi.org/10.1038/s41586-019-1817-8

318. Irvine EB, O'Neil A, Darrah PA, Shin S, Choudhary A, Li W, et al. Robust IgM responses following intravenous vaccination with Bacille Calmette–Guérin associate with prevention of Mycobacterium tuberculosis infection in macaques. Nat Immunol. 2021;22(12):1515–23.

319. Westerhof LM, McGuire K, MacLellan L, Flynn A, Gray JI, Thomas M, et al. Multifunctional cytokine production reveals functional superiority of memory CD4 T cells. Eur J Immunol. 2019;49(11):2019–29.

320. Kannanganat S, Ibegbu C, Chennareddi L, Robinson HL, Amara RR. Multiple-Cytokine-Producing Antiviral CD4 T Cells Are Functionally Superior to Single-Cytokine-Producing Cells. J Virol. 2007;81(16):8468–76.

321. Musvosvi M, Huang H, Wang C, Xia Q, Rozot V, Krishnan A, et al. T cell receptor repertoires associated with control and disease progression following Mycobacterium tuberculosis infection. Nat Med [Internet]. 2023; Available from: http://www.ncbi.nlm.nih.gov/pubmed/36604540

322. Helbert M. T-cell receptor variable gene products and early HIV infection. Vol. 339, Lancet (London, England). England; 1992. p. 1299–300.

323. Bostik P, Stephenson ST, Lynch RM, Cardona A, Ansari AA. Maintenance of CD4+ T cell TCR Vß repertoire heterogeneity is characteristic of apathogenic SIV infection in non-human primate model of AIDS. Virology. 2007;369(2):324–8.

324. Kharbanda M, McCloskey TW, Pahwa R, Sun M, Pahwa S. Alterations in T-cell receptor Vß repertoire of CD4 and CD8 T lymphocytes in human immunodeficiency virus-infected children. Clin Diagn Lab Immunol. 2003;10(1):53–8.

325. Neff CP, Chain JL, MaWhinney S, Martin AK, Linderman DJ, Flores SC, et al. Lymphocytic alveolitis is associated with the accumulation of

functionally impaired HIV-specific T cells in the lung of antiretroviral therapy-naive subjects. Am J Respir Crit Care Med. 2015 Feb;191(4):464–73.

326. Balamurugan A, Ng HL, Yang OO. Rapid T Cell Receptor Delineation Reveals Clonal Expansion Limitation of the Magnitude of the HIV–Specific CD8+ T Cell Response. J Immunol. 2010;185(10):5935–42.

327. Payne RP, Branch S, Kløverpris H, Matthews PC, Koofhethile CK, Strong T, et al. Differential Escape Patterns within the Dominant HLA-B*57:03-Restricted HIV Gag Epitope Reflect Distinct Clade-Specific Functional Constraints. J Virol. 2014;88(9):4668–78.

328. Hirokawa M, Matsutani T, Saitoh H, Ichikawa Y, Kawabata Y, Horiuchi T, et al. Distinct TCRAV and TCRBV repertoire and CDR3 sequence of T lumphocytes clonally expanded in blood and GVHD lesions after human allogeneic bone marrow transplantation. Bone Marrow Transplant. 2002;30(12):915–23.

329. Gorski J, Yassai M, Zhu X, Kissela B, Kissella B [corrected to Kissela B], Keever C, et al. Circulating T cell repertoire complexity in normal individuals and bone marrow recipients analyzed by CDR3 size spectratyping. Correlation with immune status. J Immunol. 1994 May;152(10):5109–19.

330. Pymm P, Tenzer S, Wee E, Weimershaus M, Burgevin A, Kollnberger S, et al. Epitope length variants balance protective immune responses and viral escape in HIV infection. Cell Rep. 2022;38(9).

331. Li G, Li J, Zhang H, Zhang Y, Liu D, Hao Y, et al. Partial recovery of disturbed V-J pairing profiles of T-cell receptor in people living with HIV receiving long-term antiretroviral therapy. Sci China Life Sci. 2021;64(1):152–61.

332. Gantner P, Pagliuzza A, Pardons M, Ramgopal M, Routy JP, Fromentin R, et al. Single-cell TCR sequencing reveals phenotypically diverse clonally expanded cells harboring inducible HIV proviruses during ART. Nat Commun [Internet]. 2020;11(1):1–9. Available from: http://dx.doi.org/10.1038/s41467-020-17898-8

333. Yang J, Chen J, He J, Xie Y, Zhu Y, Cao H, et al. Profiling the repertoire of T-cell receptor beta-chain variable genes in peripheral blood lymphocytes from subjects who have recovered from acute hepatitis B virus infection. Cell Mol Immunol. 2014;11(4):332–42.

334. Komanduri K V., Feinberg J, Hutchins RK, Frame RD, Schmidt DK, Viswanathan MN, et al. Loss of cytomegalovirus-specific CD4+T cell responses in human immunodeficiency virus type 1-infected patients with high CD4+T cell counts and recurrent retinitis. J Infect Dis. 2001;183(8):1285–9.

335. Wilson BJDK, Ogg GS, Allen RL, Goulder PJR, Kelleher A, Sewell AK, et al. Oligoclonal Expansions of CD8. 1998;188(4):785–90.

336. Kolomeets M, Desnitsky V, Kotenko I, Chechulin A. Graph Visualization: Alternative Models Inspired by Bioinformatics. Sensors. 2023;23(7).

337. Fozza C, Barraqueddu F, Corda G, Contini S, Virdis P, Dore F, et al. Study of the T-cell receptor repertoire by CDR3 spectratyping. J Immunol Methods. 2017 Jan;440:1–11.

338. Hou X, Wang M, Lu C, Xie Q, Cui G, Chen J, et al. Analysis of the repertoire features of TCR beta chain CDR3 in human by high-throughput sequencing. Cell Physiol Biochem. 2016;39(2):651–67.

339. Fu Y, Li B, Li Y, Wang M, Yue Y, Xu L, et al. A comprehensive immune repertoire study for patients with pulmonary tuberculosis. Mol Genet Genomic Med. 2019;7(7):1–23.

340. Towlerton AMH, Ravishankar S, Coffey DG, Puronen CE, Warren EH. Serial Analysis of the T-Cell Receptor ß-Chain Repertoire in People Living With HIV Reveals Incomplete Recovery After Long-Term Antiretroviral Therapy. Front Immunol. 2022;13(May):1–14.

341. Baum PD, Young JJ, Schmidt D, Zhang Q, Hoh R, Busch M, et al. Blood T-cell receptor diversity decreases during the course of HIV infection, but the potential for a diverse repertoire persists. Blood. 2012;119(15):3469–77.

342. Bodman-Smith MD, Williams I, Johnstone R, Boylston A, Lydyard PM, Zumla

A. T cell receptor usage in patients with non-progressing HIV infection. Clin Exp Immunol [Internet]. 2002 Sep 19 [cited 2022 Dec 14];130(1):115–20. Available from: https://academic.oup.com/cei/article/130/1/115/6461199

343. Mazzotti L, Gaimari A, Bravaccini S, Maltoni R, Cerchione C, Juan M, et al. T-Cell Receptor Repertoire Sequencing and Its Applications: Focus on Infectious Diseases and Cancer. Int J Mol Sci. 2022;23(15):1–25.

344. Lewinsohn DA, Lewinsohn DM, Scriba TJ. Polyfunctional CD4+ T cells as targets for tuberculosis vaccination. Front Immunol. 2017;8(OCT).

345. Darrah PA, Zeppa JJ, Wang C, Irvine EB, Bucsan AN, Rodgers MA, et al. Airway T cells are a correlate of i.v. Bacille Calmette-Guerin-mediated protection against tuberculosis in rhesus macaques. Cell Host Microbe [Internet]. 2023;31(6):962-977.e8. Available from: https://www.sciencedirect.com/science/article/pii/S1931312823001993

346. Rodo MJ, Rozot V, Nemes E, Dintwe O, Hatherill M, Little F, et al. A comparison of antigen-specific T cell responses induced by six novel tuberculosis vaccine candidates. PLoS Pathog. 2019;15(3):1–20.

347. Zhuang L, Ye Z, Li L, Yang L, Gong W. Next-Generation TB Vaccines: Progress, Challenges, and Prospects. Vaccines. 2023;11(8).

348. Panda S, Kearns K, Cheng C, Lindestam Arlehamn CS. From antigens to immune responses: Shaping the future of TB detection and prevention. Int J Infect Dis. 2024;141.

349. Comas I, Chakravartti J, Small PM, Galagan J, Niemann S, Kremer K, et al. Human T cell epitopes of Mycobacterium tuberculosis are evolutionarily hyperconserved. Nat Genet. 2010 Jun;42(6):498–503.

350. Kim H, Choi HG, Shin SJ. Bridging the gaps to overcome major hurdles in the development of next-generation tuberculosis vaccines. Front Immunol. 2023;14(August):1–24.

351. Coccia M, Collignon C, Hervé C, Chalon A, Welsby I, Detienne S, et al. Cellular and molecular synergy in AS01-adjuvanted vaccines results in an early IFNγ response promoting vaccine immunogenicity. npj Vaccines

[Internet]. 2017;2(1). Available from: http://dx.doi.org/10.1038/s41541-017-0027-3

352. Enriquez AB, Izzo A, Miller SM, Stewart EL, Mahon RN, Frank DJ, et al. Advancing Adjuvants for Mycobacterium tuberculosis Therapeutics. Front Immunol. 2021;12(October):1–15.

353. Schijns VEJC, Lavelle EC. Trends in vaccine adjuvants. Expert Rev Vaccines. 2011 Apr;10(4):539–50.

354. Darrah PA, Zeppa JJ, Maiello P, Hackney JA, Wadsworth MH 2nd, Hughes TK, et al. Prevention of tuberculosis in macaques after intravenous BCG immunization. Nature. 2020 Jan;577(7788):95–102.

355. Twigg HL, Knox KS. Impact of Antiretroviral Therapy on Lung Immunology and Inflammation. 2013;

356. Schluger NW, Perez D, Liu YM. Reconstitution of immune responses to tuberculosis in patients with HIV infection who receive antiretroviral therapy. Chest. 2002 Aug;122(2):597–602.

357. Lyadova I V, Panteleev A V. Th1 and Th17 Cells in Tuberculosis: Protection, Pathology, and Biomarkers. Mediators Inflamm. 2015;2015:854507.

358. n, Eunsung Mouradian MM, Michael R. Bronsert, William G. Henderson, Robert Valuck, Patrick Hosokawa and KH. 基因的改变NIH Public Access. Bone. 2008;23(1):1–7.

359. Valkiers S, de Vrij N, Gielis S, Verbandt S, Ogunjimi B, Laukens K, et al. Recent advances in T-cell receptor repertoire analysis: Bridging the gap with multimodal single-cell RNA sequencing. ImmunoInformatics [Internet]. 2022;5(January):100009. Available from: https://doi.org/10.1016/j.immuno.2022.100009

360. Sims S, Willberg C, Klenerman P. MHC-peptide tetramers for the analysis of antigen-specific T cells. Expert Rev Vaccines. 2010;9(7):765–74.

361. Dayanand K, Olivia E. Atherton1, Jennifer L. Tackett2, Emilio Ferrer1 and RWR. 乳鼠心肌提取 HHS Public Access. Physiol Behav [Internet]. 2018;176(5):139–48. Available from:

http://europepmc.org/backend/ptpmcrender.fcgi?accid=PMC5604322&blobtype=pdf

362. Huang H, Wang C, Rubelt F, Scriba TJ, Davis MM. Analyzing the Mycobacterium tuberculosis immune response by T-cell receptor clustering with GLIPH2 and genome-wide antigen screening. Nat Biotechnol [Internet]. 2020;38(10):1194–202. Available from: http://dx.doi.org/10.1038/s41587-020-0505-4

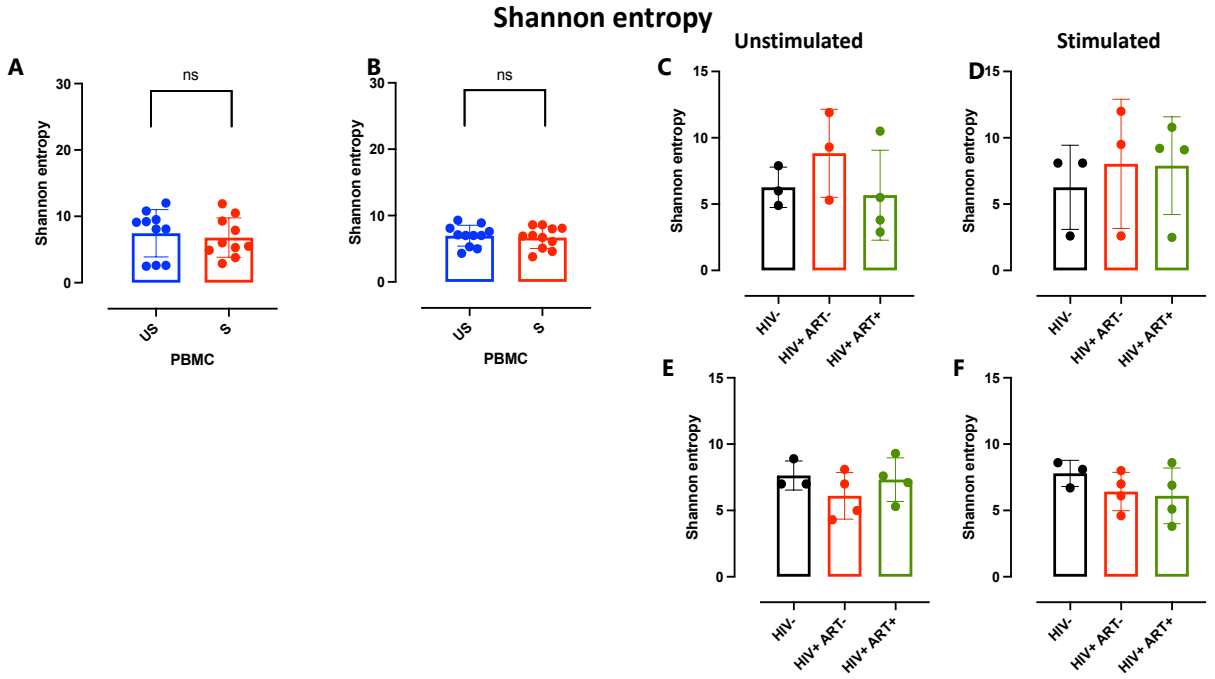
363. Patankar YR, Sutiwisesak R, Boyce S, Lai R, Lindestam Arlehamn CS, Sette A, et al. Limited recognition of Mycobacterium tuberculosis-infected macrophages by polyclonal CD4 and CD8 T cells from the lungs of infected mice. Mucosal Immunol [Internet]. 2020;13(1):140–8. Available from: http://dx.doi.org/10.1038/s41385-019-0217-6

Chapter 8 Appendices

Appendix Table 1 TCR CDR3

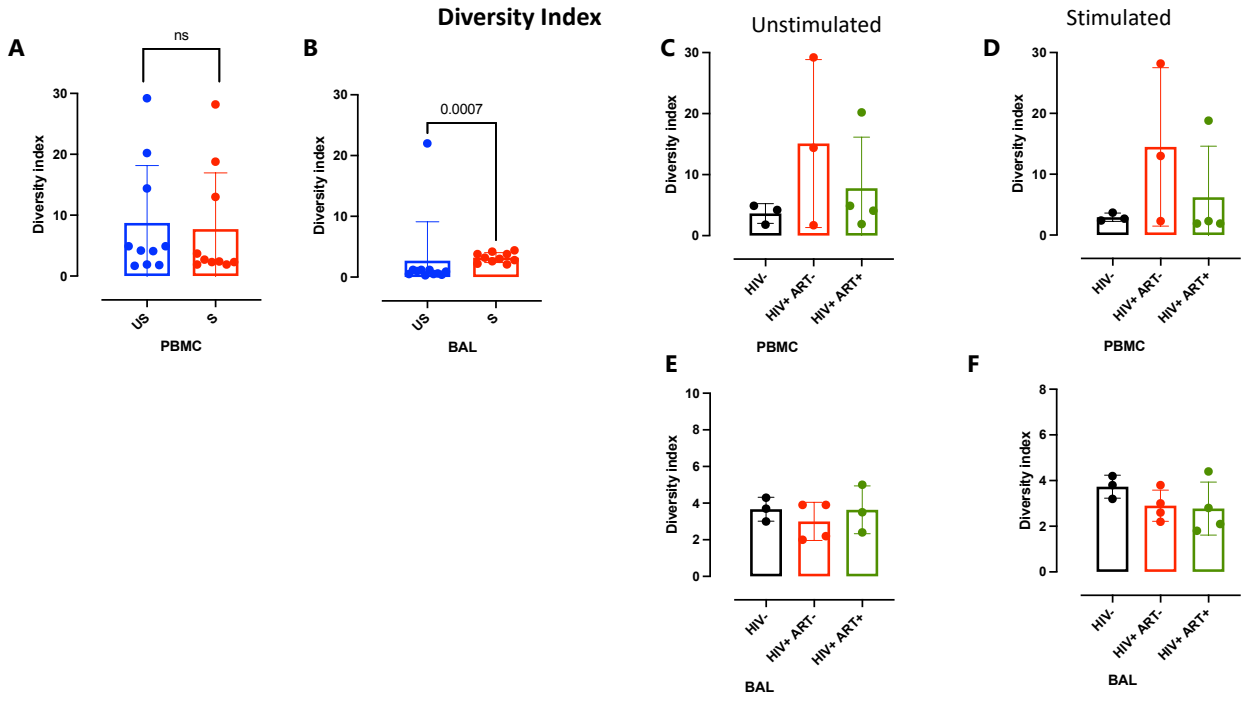
Sample	compartment	Stimulation	HIV	ART	ART durati	CDR3	Unique CDR3
PostControl_9						694652	178676
PostControl_11						619362	169487
PostControl_10						620911	175148
PC1538_S_B	B	S	HIV+	ART-		998934	1044
PC1538_US_B	B	US	HIV+	ART-		1078707	1273
LI0590_S_P	P	S	HIV+	ART+	15yrs	1026440	1335
LI0491_S_B	B	S	HIV+	ART+	16yrs	1547265	2198
LI0467_S_P	P	S	NR			909367	1120
LI0426_S_B	B	S	NR			1750384	4103
LI0590_US_P	P	US	HIV+	ART+	15yrs	1387986	5967
LI0491_US_B	B	US	HIV+	ART+	16yrs	1171875	3444
LI0467_US_P	P	US	NR			1339509	3512
LI0426_US_B	B	US	NR			1842702	3114
LI0905_S_B	B	S	HIV+	ART-		2383271	5214
LI0582_S_B	B	S	HIV+	ART-		1316503	2118
LI0491_S_P	P	S	HIV+	ART+	16yrs	835206	1503
LI0426_S_P	P	S	NR			1270197	2285
LI491_US_P	P	US	HIV+	ART+	16yrs	472156	715
LI0905_US_B	B	US	HIV+	ART-		1251503	4616
LI0582_US_B	B	US	HIV+	ART-		1155215	1456
LI0426_US_P	P	US	NR			625524	607
LI0905_S_P	P	S	HIV+	ART-		1144242	38027
LI0582_S_P	P	S	HIV+	ART-		1207486	1769
LI0541_S_B	B	S	HIV+	ART+	3yrs	1539917	3153
LI0475_S_B	B	S	NR			1723341	5835
LI0905_US_P	P	US	HIV+	ART-		567347	39316
LI0582_US_P	P	US	HIV+	ART-		944293	737
LI0541_US_B	B	US	HIV+	ART+	3yrs	753	186
LI0475_US_B	B	US	NR			1888762	7397
LI0915_S_B	B	S	HIV+	ART+	11yrs	1340541	5406
LI0749_S_B	B	S	HIV+	ART-		1262265	4509
LI0541_S_P	P	S	HIV+	ART+		1096076	1992
LI0475_S_P	P	S	NR			1645143	4899
LI0915_USB	B	US	HIV+	ART+	11yrs	1915948	9059
LI0749_US_B	B	US	HIV+	ART-		1529410	5244
LI0541_US_P	P	US	HIV+	ART+		1554162	32367
LI0475_US_P	P	US	NR			1758305	8242
LI0915_S_P	P	S	HIV+	ART+		1333432	49721
LI0749_S_P	P	S	HIV+	ART-		1468368	56084
LI0590_S_B	B	S	HIV+	ART+	15yrs	1396744	2145
LI0467_S_B	B	S	NR			1451725	3730
LI0915_US_P	P	US	HIV+	ART+		1190435	32854
LI0749_US_P	P	US	HIV+	ART-		485242	43771
LI0467US_B	B	US	NR			1336736	3880
L0590_US_BAL	B	US	HIV+	ART+	15yrs	1186990	1685
Sum						53330407	407632
Average						**1269771.595**	9705.52381

NB: US= Unstimulated, S= Stimulated, P= PBMC, B= BAL, NR= Non-reactive,



Appendix Figure 1 TCR diversity across the group (Measured by Shannon index)

TCR diversity measured by Shannon entropy was compared between Mtb stimulated vs unstimulated condition in all samples in PBMC(A) and BAL (B). The impact of HIV on TCR diversity in unstimulated(C) and stimulated (D) PBMC. G & F, diversity index in unstimulated and stimulated BAL respectively. Data were analysed using an unpaired Student's t-test and data was presented as mean ± SD. (*: P< 0.05). P< 0.05 is considered statistically significant while ns= means no statistical significance. Graphs represent 1 experiment from paired BAL and PBMC samples, n=10 (HIV+ART-, n=4, HIV+ART+, n=4 and HIV-, n=3).



Appendix Figure 2 TCR diversity across the group (Measured by D50)

Diversity index was compared between Mtb stimulated vs unstimulated condition in all samples in PBMC(A) and BAL (B). The impact of HIV on TCR diversity in unstimulated(C) and stimulated (D) PBMC. G & F, diversity index in unstimulated and stimulated BAL respectively. Data were analysed using an unpaired Student's t-test and data was presented as mean ± SD. (*: $P< 0.05$). $P< 0.05$ is considered statistically significant while ns= means no statistical significance. Graphs represent 1 experiment from paired BAL and PBMC samples, n=10 (HIV+ART-, n=4, HIV+ART+, n=4 and HIV-, n=3).