Studies on CD4+ T cell subsets in *Mycobacterium tuberculosis* immunity during HIV co-infection

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List of abbreviations

° Degrees
Ag85A Antigen 85A
AIDS Acquired Immunodeficiency Syndrome
APC Antigen presenting cell
APC-Cy7 Allophycocyanin-Cy7
ART Antiretroviral therapy
AUC VL Area under the curve viral load (cumulative viral burden)
BAL Bronchoalveolar lavage
BCG Bacille Calmette Guérin
BEI Biodefense and emerging infections research resources repository
BFA Brefeldin-A
BV Brilliant violet
CAPRISA Centre for the AIDS Programme of Research in South Africa
CCL Chemokine (C-C motif) ligand
CCR Chemokine (C-C motif) receptor
CD Cluster of differentiation
CFP-10 Culture filtrate protein 10
CMV Cytomegalovirus
CO2 Carbon dioxide
CXCR Chemokine (C-X-C motif) receptor
DC Dendritic cells
DC-SIGN Dendritic cell-specific intracellular adhesion molecue-3 grabbing non-integrin
DNAse Deoxyribonuclease
ED Early differentiated
ELISA Enzyme-linked immunosorbent assay
ESAT-6 6kDa Early Secretory Antigen
FACS Fluorescence activated cell sorter
FCS Foetal calf serum
FITC Fluorescein isothyocyanate
FMO Fluorescence minus one
Foxp3 Forkhead box p3
FSC Forward scatter
HIV Human Immunodeficiency virus
HLA Human leukocyte antigen
HREC Human Research Ethics Committee

ICS Intracellular cytokine staining

IDM Institute of Infectious Disease and Molecular Medicine

IFN-γ Interferon gamma

IGRA Interferon gamma release assays

IL Interleukin

IQR Interquartile range

IRIS Immune reconstitution inflammatory syndrome

LD Late differentiated

LDL Lower detection limit

LTBI Latent tuberculosis infection

M.tb *Mycobacterium tuberculosis*

MDR-TB Multi-drug resistant tuberculosis

MFI Median Fluorescent Intensity

MHC Major histocompatibility complex

min Minutes

MIP Macrophage inflammatory protein

mL Millilitre

mRNA messenger ribonucleic acid

MVA85A Modified vaccinia ankara expression Adenovirus expressing antigen 85A

NHP Non-human primate

NK Natural killer

NOD Nucleotide-binding oligomerisation domain

PBMC Peripheral blood mononuclear cells

PBS Phosphate buffered saline

PCR Polymerase chain reaction

PD-1 Programmed cell death protein-1

PE Phycoerythrin

PE-Cy5 Phycoerythrin-Cy5

PErCP-Cy5.5 Peridinin chlorophyll-Cy5.5

PPD Purified protein derivative

QFT Quantiferon

RD-1 Region of difference 1

RNA Ribonucleic acid

RORγt RAR-related orphan receptor gamma t

RPMI Roswell Park Memorial Institute cell culture medium

RR-TB Rifampicin-resistant tuberculosis

RT Room temperature

SSC Side scatter

TB Tuberculosis

TCR T cell receptor

TD Terminally differentiated

TEM Effector memory T cell

Th T helper

TLR Toll-like receptor

TNF-α Tumor necrosis factor alpha

Treg Regulatory T cell

TST Tuberculin skin test

ViViD LIVE/DEAD Fixable Violet Dead Cell Stain

VL viral load

WHO World Health Organisation

XDR-TB Extensively drug-resistant tuberculosis

μg Microgram

μL Microliter

μM Micromolar

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Abstract

Tuberculosis (TB) is a global pandemic which resulted in 5.8 million disease cases and approximately 1.5 million deaths worldwide in 2020. TB disease is the leading cause of death worldwide from a single infectious agent, *Mycobacterium tuberculosis* (*M.tb*). It is estimated that a one fourth of the world's population is latently infected with TB and of these 5-10% will go on to develop active TB in their lifetime. Human immunodeficiency virus (HIV) infection is the greatest risk factor for developing active TB, with people living with HIV (PLWH) up to 19 times more likely to develop active TB compared to HIV uninfected individuals. Epidemiological studies show that there is increased risk of developing active TB (either reactivation of latent TB or new *M.tb* infection) throughout the course HIV infection, with the highest risk observed within the first year of infection, even when CD4+ T cell counts are still high. Previous studies have demonstrated that *M.tb*-specific CD4+ T cell responses are depleted early after HIV infection, within 3-12 months. This early defect in *M.tb*-specific CD4+ T cells may explain the elevated risk of TB even within the first year of HIV infection, prior to substantial immunosuppression.

Antiretroviral therapy (ART) is an important strategy in preventing TB in PLWH. Earlier studies done in sub-Saharan Africa show that ART is associated with a 70-90% reduction in TB risk and a 52% decrease in TB related mortality. Several studies have also shown the importance of early ART initiation for HIV pathogenesis and transmission. However, despite ART intervention studies in sub-Saharan Africa, as well as Europe and North America, show that in these regions rates of TB remain high in PLWH. These high TB rates are most likely linked to the incomplete restoration of TB immune responses during ART.

Overall, this thesis focused on characterising *M.tb*-specific CD4+ T cell responses during the course of HIV infection, from the acute phase of infection to post ART initiation. In Chapter 3, we aimed to confirm and extend previous findings that show there is a rapid depletion of *M.tb*-specific CD4+ T cells soon after HIV infection. We examined the kinetics of *M.tb*-specific CD4+ T cell responses over the course of HIV infection in two cohorts, a cross sectional cohort (n=58) and a longitudinal cohort (n=17). Consistent with previous findings, our results showed that there is a significant decrease in the frequency of *M.tb* responders 3 months after HIV infection. However, not all participants experienced *M.tb*-specific CD4+ T cell loss after HIV infection, with half the cohort losing 50% or more of their responses and the other half maintaining their responses. In the group that maintained their *M.tb* responses after HIV infection, the majority had very little fluctuations in their responses during the acute and chronic phase of infection. When comparing the clinical characteristics and the function and phenotype of *M.tb*-specific CD4+ T cells between individuals who maintained their *M.tb*-specific response and those who don’t, no significant differences were found.

In Chapter 4, to investigate the impact of early ART on *M.tb* immunity, we characterised *M.tb*-specific CD4+ T cell responses in individuals who initiated ART at an early stage of HIV infection (median 7.5 months post infection, n=16) in comparison to those who started ART during the chronic phase of HIV infection (median 66 months post infection, n=22). In this study, we examined multiple parameters between the two groups including their clinical characteristics, as well as the magnitude, function and phenotype of their *M.tb*-specific CD4+ T cells. We show that 2 years after ART initiation (irrespective of its timing) induced a significant immune reconstitution, marked by an increase in CD4 T cell count and restoration of the C4/CD8 ratio, but had no significant impact on the magnitude, function or phenotype of *M.tb*-specific CD4+ T cells.

In Chapter 5, we sought to characterise *M.tb*-specific T cell responses in a small (n=13) cohort of participants who developed active TB during the CAPRISA 002 study. We performed a descriptive study examining the magnitude and phenotype of *M.tb*-specific T cells longitudinally over the course of TB treatment: before TB treatment/diagnosis, during treatment and after successful treatment. Despite the limited number of participants in this sub-study, we showed that after TB treatment there was a decreasing trend of activated *M.tb*-specific CD4+ T cells coincident with an increase in IFN-γ+ IL-2+ dual functional cells.

Overall, our data confirms previous findings that there is an early depletion of *M.tb*-specific CD4+ T cells within a year HIV infection. However, in our study we found that not all HIV infected individuals lose their *M.tb*-specific responses. Instead, there was a variation in *M.tb*-specific responses after HIV infection, with some individuals maintaining their responses and others completely losing them. Results of our study also showed there were no major differences between participants who initiated ART early and those who initiated later during chronic HIV infection. To our knowledge this is the first study which looks at the impact of ART timing on *M.tb*-specific immunity in PLWH. The study therefore provides further insight, for future research, into whether early ART preserves TB immunity and therefore reduce the early, elevated risk of TB in HIV infected individuals.

CHAPTER 1

Literature review

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1.1 The Global burden of TB

Tuberculosis (TB) is a global pandemic which resulted in over 5.8 million disease cases and 1.6 million deaths worldwide in 2021 (WHO Global TB report, 2022). It is estimated that 1/4 of the world's population has latent TB infection (LTBI) and of these 5-10% will go on to develop active TB in their lifetime (Bloom and Murray, 1992; Vynnycky and Fine, 2000; Bhatt *et al*., 2015). Although TB is considered a global health issue, the disease incidence varies within countries depending on geographical location, socioeconomics, and prevalence of co-morbidities. According to WHO, the majority of the TB cases reported in 2021 were located in South-East Asia (45%), Africa (23%) and the Western Pacific (18%) (WHO Global TB report, 2022). **Figure 1.1** shows the estimated global TB incidence in 2020. Eight countries accounted for 2/3 of the total global TB cases reported in 2021, including India (28%), Indonesia (9.2%) China (7.4%), the Philippines (7%), Pakistan (5.8%), Nigeria (4.4%), Bangladesh (3.6%) and Democratic Republic of the Congo (2.9%) (WHO Global TB report, 2022). In order to mitigate the global TB burden, the WHO has established the "End TB strategy" (67th World Health Assembly, 2014). This strategy aims to reduce TB deaths by 90% and TB incidence by 80% by the year 2030. Today, only 6 of the high TB burden countries are currently on track to achieve the 2020 milestone to reduce TB incidence by 20%: Ethiopia, Kenya, Myanmar, Namibia, South Africa and the United Republic of Tanzania (WHO Global TB report, 2022). Furthermore, while there is currently a worldwide decrease in TB incidence rate (at about 2% per year), the global reduction in TB incidence between 2015 and 2021 was 10%, only halfway of the 2020 WHO milestone (WHO Global TB report, 2022). It is quite apparent today that the global prevalence of the human immunodeficiency virus (HIV), as well as the lack of an effective vaccine for adult pulmonary TB (the driver of the pandemic), has greatly impaired the control of TB disease worldwide.

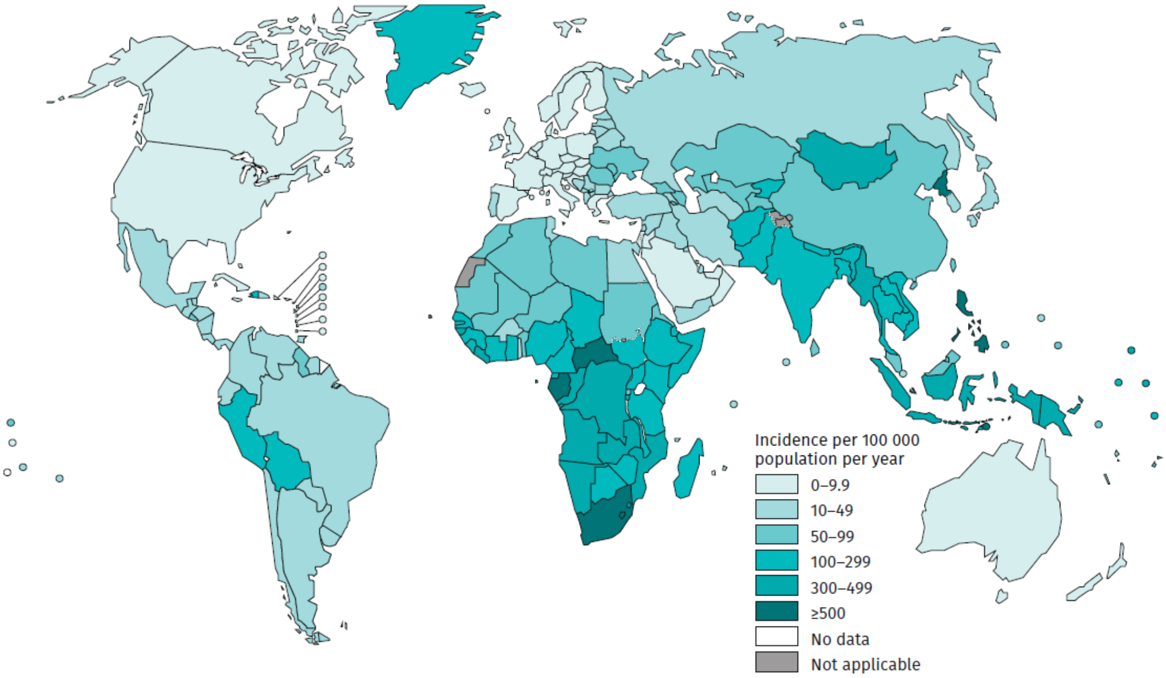


Figure 1.1: The estimated worldwide tuberculosis incidence rate in 2020. Taken from the Global Tuberculosis Report 2021, World Health Organisation.

1.2 Impact of HIV on the TB pandemic

HIV infection is the greatest risk factor for developing active TB. People living with HIV (PLWH) are up to 16 times more likely to develop active TB, compared to those who are not infected (WHO Global TB Report, 2022). Epidemiological studies have shown that there is increased risk of developing active TB throughout the course HIV infection. Surprisingly, the most dramatic increase in TB development was observed within first year of HIV infection, even when CD4+ T cell counts are still high (Kauffman and McMichael, 2005; Sonnenberg *et al*., 2005; Getahun *et al*., 2010). Today, this deadly synergy between HIV and TB has become a major global health crisis. Of the 1.6 million TB death cases reported in 2021, it is estimated 12% (187, 000cases) were

HIV co-infected (WHO Global TB report, 2022). Most concerning, these numbers are most likely underestimated, as a post-mortem study of HIV-infected persons done in sub-Saharan Africa shows that 45.8% (ranging from 32.6 to 59.1%) of confirmed TB cases were undiagnosed at the time of death (Gupta *et al*., 2015). Such findings highlight the current shortfall in TB testing for populations at heighten risk, and advocate for the development of novel tools for the accurate and rapid diagnostic TB, particularly for PLWH.

The global burden of the HIV/TB pandemic has had its biggest impact in developing countries. Of the 16 high TB burden countries, 14 of these are also HIV endemic (WHO Global TB Report, 2021). Consequently, a large proportion of HIV-related TB cases reported worldwide in 2021 were from African regions, with more than 50% of these from southern African countries (WHO Global TB Report, 2022). Fortunately, the provision of antiretroviral therapy (ART) and isoniazid preventative therapy (IPT) has had a significant impact on HIV-related TB mortality rates in these regions. Studies show that ART decreases TB risk by 54–90% (Lawn *et al*., 2011), and IPT by 32% in ART-naïve patients and 37% in ART-established patients (Akolo *et al*., 2010; Rangaka *et al*., 2014). The expanded access to ART has substantially decreased TB incidences in Africa by a rate of 3.8% per year, with several countries in southern Africa showing a decrease of 4 to 8% per year (WHO Global TB Report, 2019).

1.3 TB diagnosis

Traditional TB diagnosis relies on the clinical presentation of TB disease, such as chronic cough, night sweats and weight loss. These symptoms, however, are often shared with other diseases such pneumonia and lung cancer, therefore making TB diagnosis difficult solely on clinical presentation (Storla *et al*., 2008). Several tests are available for TB diagnostic. Today, in very limited resource setting, the most accessible diagnostic test in TB-endemic countries is sputum smear microscopy (Golleti *et al*., 2018). Unfortunately, this test has several limitations as it tends to have poor sensitivity (Steingart *et al*., 2006; Mase *et al*., 2007). The current gold standard for TB diagnosis is the isolation and confirmation of *Mycobacterium tuberculosis* (*M.tb*) culture using body fluid, such as sputum or samples from the site of disease in the occurrence of extrapulmonary TB (e.g, cerebrospinal, pleural, pericardial or ascitic fluids, lymph node aspirate) (Engku *et al*., 2016; Zijenah, 2018). While *M.tb* culture is more sensitive than smear microscopy, it has a long turnaround time (up 6 weeks), which may delay TB treatment initiation (WHO Early detection of Tuberculosis, 2011; Engku *et al*., 2016). The introduction of the PCR-based GeneXpert Mtb/RIF in 2010 has revolutionized TB diagnostic (Boehme *et al*.,2011). This real-time PCR assay can simultaneously detect *M.tb* and rifampicin resistance with high sensitivity and specificity (Helb *et al*., 2010; Boehme *et al*.,2011). However, the major drawback of GeneXpert Mtb/RIF is that it is quite expensive and therefore not as accessible as the other tests in resource poor countries. In addition to this, the GeneXpert Mtb/RIF test cannot distinguish between viable and non-viable bacterial cells and therefore is not always reliable in diagnosing active disease (Golleti *et al*., 2018). A major challenge TB diagnostic tests is their sub-optimal sensitivity in immunosuppressed patients, such as HIV-infected individuals. PLWH tend to have a high proportion of disseminated TB and negative sputum smear tests (Read *et al*., 2009). In addition to this, immunosuppression in PLWH is often associated with a reduction in sputum production and TB cavitation, thereby reducing the sensitivity of sputum-based assays (Esmail *et al*., 2018). This therefore highlight the current need for the development of new TB diagnostic tests, that are not only rapid and accurate, but are also easily accessible in limited-resource setting and communities with high HIV prevalence.

1.4 TB prophylaxis and treatment

Isoniazid preventive therapy (IPT) is a TB preventative regimen that has been recommended by the WHO as a strategy to reduce elevated risk of TB in HIV-endemic settings (WHO Three I's Meeting Report, 2008). Isoniazid (INH) is an antibiotic that is used in combination with other drugs to treat active TB but can also be used on its own as a prophylaxis for PLWH. INH is a pro-drug that is activated by the *M.tb* KatG enzyme(Johnson *et al*., 1997). INH inhibits the production of mycolic acids in the bacillus, disrupting the biosynthesis of the bacterial cell wall (Johnson *et al*., 1997). Bacilli without an intact cell wall are therefore exposed the host's immune cells, resulting in its recognition and clearance (Wilkinson *et al*., 2006). Although IPT is an effective prophylaxis against TB, there are several clinical cases were patients either receive inadequate therapy or are over-treated, resulting in failure of treatment and/or potentially increase the levels of drug-resistant infection (Wilkinson *et al*., 2006; Sauzullo *et al*., 2016). Previous studies that used interferon-gamma release assays IGRA to monitor IPT treatment response have had varying results, with either decrease/increase or no change in the immune response after treatment (Pai *et al*., 2006; Wilkinson *et al*., 2006; Higuchi *et al*., 2008). Therefore, there is a need for the development of reliable biomarkers to monitoring patients' response to IPT.

Currently in South Africa, the standard treatment for TB consists of 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol (RHZE), followed by 4 months of isoniazid and rifampicin (RH) (National Tuberculosis Management Guidelines, 2014).

This regimen is usually very effective in curing drug-susceptible TB, with a treatment success rate of 85% (WHO Global TB report, 2019; 2021). However, the global emergence of drug-resistant TB has greatly hampered the successful treatment of TB patients. Multidrug-resistant TB (MDR-TB) is TB resistant to both rifampicin and isoniazid, extensively drug-resistant TB (XDR-TB) is MDR-TB plus resistance to one fluoroquinolone and a second-line injectable agent cases (WHO Global TB report, 2019). In 2020, a total of 157,903 cases of drug-resistant TB were reported, among these were 132,222 patients with multidrug-resistant/rifampicin-resistant TB (MDR/RR-TB) and 25,681 with XDR-TB (WHO Global TB report, 2021).

A major challenge for TB control efforts has been the monitoring of TB treatment. Current recommendations rely on sputum and culture conversion (Golleti *et al*., 2018). However, there are reports of individuals developing recurrent TB despite negative sputum culture results (Gillespie *et al*., 2014). Recurrent TB or relapse can be attributed to several factors which are dependent on both the host and pathogen characteristics (WHO Global TB report, 2019; Mirsaeidi *et al*., 2018). There is a widely acknowledged need for novel tools that not only predict successful TB treatment outcome but can also determine which individuals are at risk of relapse. To date, several promising markers are under investigation, including i) pathogen-based biomarkers that measure *M.tb* physiological state and bacterial burden (Burman, 2003; Li *et al*., 2010; Ottenhoff *et al*, 2012; Sloan *et al*., 2015; Chengalroyen *et al*., 2016), ii) host-based biomarkers focusing on the transcriptomic and proteomic profiles in the blood of relapsing versus non-relapsing patients (Ottenhoff *et al*, 2012; De Groote *et al*., 2013; Cliff *et al*., 2016; Danelishvili *et al*, 2017), and iii) biomarkers assessing the characteristics of the innate and adaptive immune response (Su *et al*., 2010; Ugarte-Gil *et al*., 2013; Hong *et al*., 2014; Kamada *et al*., 2017).

1.5 TB pathogenesis

1.5.1 Early events in *M.tb* infection

During *M.tb* infection, bacilli are mainly transmitted through airborne droplets, from an infected individual into the respiratory system of a new host. The pathogen predominately causes disease in the lungs (pulmonary TB), however it can also disseminate to other organs (such as lymph nodes, bone or meninges) causing extrapulmonary TB (Mukesh *et al*., 2000; O'Garra *et al*., 2013). The primary target of infecting *M.tb* bacilli are alveolar macrophages. Once inside these immune cells, *M.tb* can alter the environmental conditions in their phagosomes, through transcriptional reprogramming, inducing anaerobic respiratory pathways and allowing the bacilli to use cholesterol as a carbon source (Schnappinger *et al*., 2003). Studies show that *M.tb* can also escape degradation in the macrophages by disrupting the fusion of phagosomes with lysosomes and thereby inhibiting normal phagosome maturation and acidification (Rohde *et al*., 2007). This immune escape mechanism has been shown to be driven by multiple *M. tb* virulence factors, several of which are secreted by the ESAT-6 secretion system 1 (ESX-1 system) (Tan *et al*., 2006; Vergne *et al*., 2014; Peddireddy *et al*., 2017). The ESX-1 system, encoded by the *M.tb* RD1 locus, is one of the five subtypes of *M.tb* type VII secretion systems (Wang *et al*., 2020). Secretions of these systems play an essential role in assisting *M.tb* bacilli to escape phagosomal degradation and trigger macrophage cell death (Simeone *et al*., 2012). This macrophage induced cell death ultimately benefits the pathogen, as the process releases the bacteria into the extracellular space allowing them to disseminate to new host cells (Simeone *et al*., 2012).

1.5.2 Granuloma formation

M.tb infection is characterised by the formation of histopathological lesions known as granulomas. These are organised structures of immune cells forming in the lungs or lymph nodes of the host in response to persistent *M.tb* infection (Gideon *et al*., 2015). TB granulomas usually consist of an initial structure of *M.tb*-infected macrophages surrounded by dendritic cells, neutrophils, foamy macrophages and multi-nucleated giant cells, with lymphocytes on the periphery to form a highly organised immune structure (Ndlovu and Marakalala, 2016). **Figure 1.2** shows the structure of a TB granuloma. Initially, it was believed that TB granulomas were immunological barriers that “wall off” infecting bacteria and thereby preventing bacterial growth and dissemination (Saunders, 2000; Ndlovu and Marakalala, 2016). However, it is now suggested that TB granulomas may act as a “double-edged sword”: providing *M.tb* with an environment where it can thrive and protect itself from the host’s immune response (Gideon *et al*., 2015; Ndlovu and Marakalala, 2016). The outcome of *M.tb* infection within a granuloma is therefore dependent on the ability of the surrounding immune cells to sufficiently contain the bacteria within. T cells, at periphery of granulomas, play an essential role in the control of *M.tb* infection. These immune cells produce a milieu of pro-inflammatory, such as TNF-α and regulatory cytokines that assist in containing infection and thereby preventing bacteria from spreading to other host cells (Gideon *et al*., 2015).

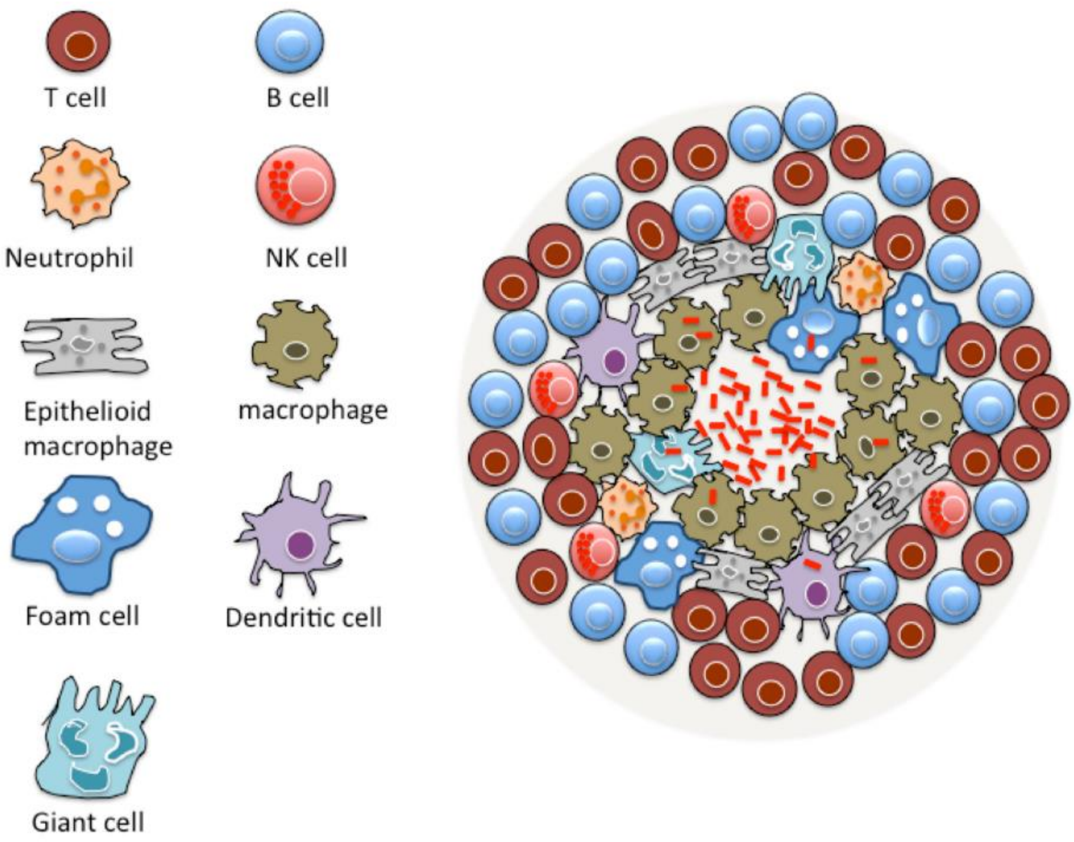
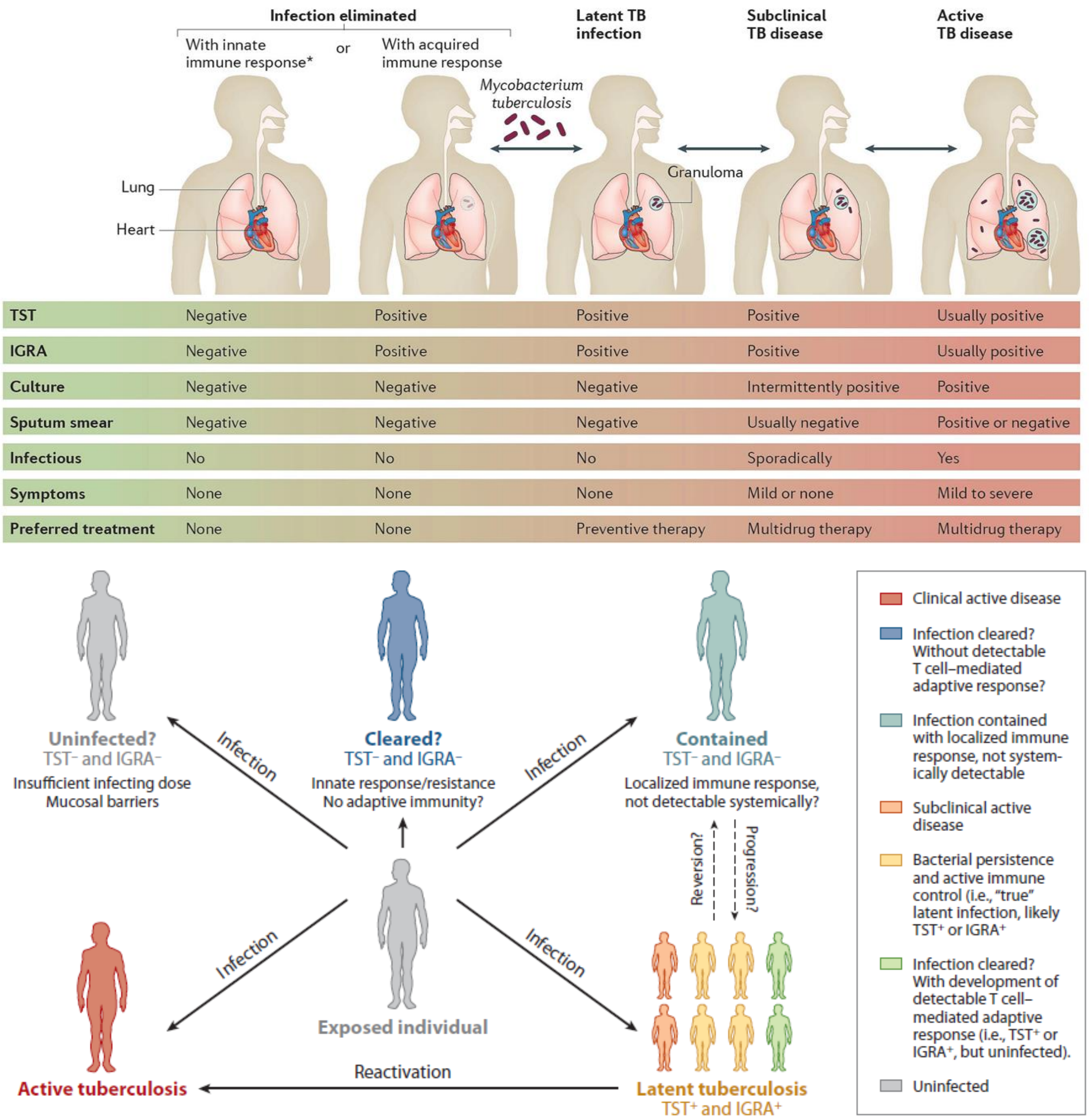


Figure 1.2: The TB granuloma. An organised immunological structure consisting of innate and adaptive immune cells aggregated around *M.tb*-infected macrophages at the centre. Reproduced from Ndlovu and Marakalala, 2016.

1.5.3 The spectrum of TB disease

M.tb infection has been historically classified as a binary model, with individuals who progress to active disease after initial infection (primary TB) and those that contain the infection and are asymptomatic (latent TB). However, recent findings suggest that the TB extends beyond the classical active versus latent TB and should be appreciated as a spectrum or continuum (Barry *et al*., 2009; Delogu and Goletti, 2014; Cadena *et al*., 2017). These reports show that latent TB in effect is not a single state but rather represents a spectrum of *M.tb* infections (**Figure 1.3**), with different degrees of bacterial replication, host immune response and clinical outcomes. Similarly, TB disease itself is a continuum, ranging from smear-negative pulmonary TB to severe disseminated form of the disease (Barry *et al*., 2009; Delogu and Goletti, 2014; Cadena *et al*., 2017).

Most *M.tb* infections result in latency, where the infection is controlled by the host's immune response, hence individuals show no clinical signs of disease (Gomez and Mckinney, 2004 ;O' Garra *et al*., 2013). In these latently infected individuals, TB may reactivate many years after initial infection, defined as post-primary TB (O'Garra *et al*., 2013). This risk of TB reactivation is highest among individuals with suppressed immune responses, such as HIV-infected individuals (Corbett *et al*., 2003). Consequently, accurate diagnosis of latent TB infection is quite difficult. A positive TST or IGRA test could represent more than one clinical outcome: those with contained *M.tb* infection hence have an active adaptive immune response to the bacillus (true latency), those who have cleared the infection but still retain an adaptive immune response and those with subclinical disease (O'Garra *et al*., 2013). Furthermore, a negative TST and IGRA test result does not always mean individuals were not exposed to *M.tb*, as negative results can also be present in individuals who were exposed but did not result in infection or those that cleared the infection without mounting an adaptive immune response (O' Garra *et al*., 2013, **Figure 1.3**).



	With innate immune response*	With acquired immune response	Latent TB infection	Subclinical TB disease	Active TB disease
TST	Negative	Positive	Positive	Positive	Usually positive
IGRA	Negative	Positive	Positive	Positive	Usually positive
Culture	Negative	Negative	Negative	Intermittently positive	Positive
Sputum smear	Negative	Negative	Negative	Usually negative	Positive or negative
Infectious	No	No	No	Sporadically	Yes
Symptoms	None	None	None	Mild or none	Mild to severe
Preferred treatment	None	None	Preventive therapy	Multidrug therapy	Multidrug therapy

Figure 1.3: TB heterogeneity after *M.tb* exposure. Latent TB infection results in a spectrum of immune responses to *M.tb* infection and hence diverse clinical outcomes in the host. Reproduced from O' Garra *et al*., 2013 and Pai *et al*., 2016.

1.5.4 Correlates of TB risk

An important hurdle in the field of TB research today is the lack reliable correlates of immune protection and biomarkers to identify individuals at risk of progression to TB disease (Getahun *et al*., 2015; Lönnroth *et al*., 2015). It is essential to correctly identify such individuals to target them with preventive intervention (Getahun *et al*., 2015;

Lönnroth *et al*., 2015). It is now evident that TST and IGRA tests are not sufficient, as they are unable to differentiate latent from active TB and have low accuracy in immunosuppressed individuals (Rangaka *et al*., 2012; Goletti *et al*., 2014). Therefore, better prognostic tests are required. Recently, many studies focusing on identifying correlates of TB risk showed promising results. These include studies assessing whole blood transcriptomics profile of TB patients, where authors showed that gene signatures in *M.tb*-infected individuals can predict TB progression with a moderate sensitivity (~66-88%) and specificity (~75-80%) (Maertzdorf *et al*., 2015; Zak *et a*l., 2016). In addition to this, there are also reports that suggest elevated peripheral T cell activation profiles (Tameris *et al*., 2013; Fletcher *et al*., 2016) and whole blood differential count (e.g., elevated monocyte/lymphocyte ratio) as potential correlates of TB risk (Baldridge *et al*., 2011; Naranbhai *et al*., 2014).

1.6 Immune response to TB

1.6.1 Innate Immunity

In healthy individuals, *M.tb* infection is usually contained by the host's immune system and remains asymptomatic, referred to as latent TB. Both the innate and adaptive immune response play essential roles in host defence against *M.tb* infection. Early during infection, there is an influx of multiple populations of innate cells into the lungs, such as the macrophages, neutrophils, dendritic cells and natural killer cells (Sia *et al*., 2015). These innate cell subsets express pattern recognition receptors (Toll-like, NOD-like and C-type lectin receptors) that assist in the uptake of *M.tb* bacilli and initiate immune responses such as phagocytosis, autophagy and apoptosis (Killick *et al*., 2013; Lerner *et al*., 2015; Khan *et al*, 2016). The successful control of *M.tb* early during infection is therefore largely depends on the interactions of pathogen with the innate arm of the immune system (Liu *et al*., 2017).

Macrophages

Macrophages are the first line of defence during *M.tb* infection. They use multiple mechanisms to eliminate *M.tb*, after phagocytosis including autophagy, production of oxygen and nitrogen components, and production of cytokines and antimicrobial peptides (Fabri *et al*., 2011; Liu *et al*., 2017). Phagocytosis is mediated by a diverse array of binding receptors such as the complement and mannose receptors (Berrington and Hawn, 2007, Kleinnijenhuis *et al*., 2011), surfactant molecules and DC-SIGN (dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin) (Berrington and Hawn, 2007, Kleinnijenhuis *et al*., 2011). Once *M.tb* is phagocytosed by a macrophage, a phagosome is formed around the ingested bacillus (Armstrong and Hart, 1971). Thereafter, phagosome maturation increases the acidification inside the phagosome through autophagy, a process whereby the *M.tb* bacilli in the cytoplasm is internalised by a autophagosome and delivered to the lysosome for degradation (Gutierrez et al., 2004; Colombo et al., 2006). Alternatively, ingestion of *M.tb* by macrophages may result in two other outcomes. Cell lysis, which allows *M.tb* to exit the macrophage and spread to other cells, or apoptotic cell death where the plasma membrane remains intact and is associated with diminished pathogen viability (Behar *et al*., 2011; O’Garra *et al*., 2013). *M.tb*-infected macrophages can also be ingested by other macrophages through a process known as efferocytosis (Liao *et al*., 2012). In this process, the engulfed *M.tb*-infected macrophage fuses with a lysosome which then results in the degradation of the *M.tb* bacilli (O’Garra *et al*., 2013). Additionally, CD4+ and CD8+ T cells may also stimulate macrophages, through the production of IFN-γ, to start producing reactive oxygen and nitrogen species which assist in inhibiting *M.tb* growth (van Crevel *et al*., 2002).

Neutrophils

There is evidence that suggests that neutrophils can have both a protective and detrimental role to play during *M.tb* infection. In mouse models, it has been shown that mice that are susceptible to *M.tb* had higher numbers of neutrophils recruited into the lungs compared to the resistant mice (Keller *et al*., 2006). Furthermore, the elimination of neutrophils in these susceptible mice led to enhanced protection from *M.tb* infection and prolonged survival (Keller *et al*., 2006). In contrast, other reports show a protective role for neutrophils during *M.tb* infection, likely mediated through the initiation of the adaptive immune response. In these studies there was a decrease in *M.tb* bacterial loads when neutrophils were recruited to the airways of mice prior to *M.tb* infection (Pedrosa *et al*., 2000; Sugawara *et al*., 2004). Similarly in humans, a study done by Martineau and colleagues showed that *in vitro* depletion of neutrophils in the whole blood of TB contacts led to a failure to control *M.tb* growth (Martineau *et al*., 2007). Neutrophils can initiate the adaptive immune response during *M.tb* infection, as illustrated in a study by Blomgran and colleagues, where infection with a proapoptotic *M.tb nuoG* mutant was compared to infection with the virulent *M.tb* H37Rv (Blomgran *et al*., 2012). Notably, the virulent H37Rv inhibited apoptosis of neutrophils, resulting in a delay in the activation of naïve CD4+ T cells (Blomgran *et al*., 2012). In contrast, the *nuoG* mutant resulted in accelerated uptake of *M.tb* by dendritic cells and therefore faster priming of naïve CD4+ T cells (Blomgran *et al*., 2012). These findings suggest that the protective or detrimental role of neutrophils after *M.tb* infection may, in part, be dependent on the *M.tb* infecting strain (Keller *et al*., 2006; Blomgran *et al*., 2012; O'Garra *et al*., 2013).

Dendritic cells

Dendritic cells (DCs) are important as the bridge between the innate and adaptive immune response (Liu *et al*., 2017). DCs also phagocytose *M,tb* and become activate, and thereafter transport the bacilli to the draining lymph nodes where the antigens are processed and presented to naïve T cells which become activated in turn (Wolf *et al*., 2008). For this interaction to occur successfully, appropriate antigen presentation molecules such as major histocompatibility complex (MHC), costimulatory molecules and cytokines are required (Sia and Rengarajan, 2019). Given their ability to modulate adaptive immune responses, DCs have been considered as adjuvants for TB vaccines. In a study by Griffiths and colleagues, the authors showed that transfer of activated, *M.tb* antigen-loaded DCs into the lungs of bacillus Calmette-Guérin (BCG) vaccinated mice, early after *M.tb* exposure, enhanced vaccine-induced CD4+ T cell immunity (Griffiths *et al*., 2016). Similarly, there are reports that suggest that the efficacy of TB vaccines can be enhanced by augmenting DC autophagy where the stimulation of DC autophagy in BCG-vaccinated mice (using rapamycin) enhanced CD4+ T cell protection in subjects during *M.tb* challenge(Jagannath *et al*., 2009).

Natural killer cells

Natural killer (NK) cells can directly control *M.tb* infection through cytotoxic mechanisms mediated by perforin, granulysin and granzyme, leading to the elimination of infected macrophages (Allen *et al.,* 2015; Liu *et al*., 2017) or indirectly by secreting cytokines that stimulate the activation of macrophages and CD8+ T cells

(Vankayalapati *et al*., 2004; Allen *et al*., 2015). NK cells can also enhance phagolysosomal fusion (fusion of phagosome and lysosome) of *M.tb*-containing phagosomes within macrophages by secreting IL-22 and IFN-γ and inhibiting *M.tb* growth (Dhiman *et al*., 2009; Liu *et al*., 2017). In addition to this, these cells can also induce IL-15 and IL-18 production by macrophages and these cytokines in turn enhance the ability of CD8+ T cells to lyse *M.tb*-infected monocytes (Vankayalapati *et al*., 2004). NK cells have also been shown to play an important role regulating a T cell independent resistance to *M.tb* infection through the production of IFN-γ (Feng *et al*., 2006; Venkatasubramanian *et al*., 2017). In these studies, IFN-γ secreted by NK cells mediated *M.tb* protection in T cell deficient mice by inducing the microbicidal activity of macrophages (secretion of NO synthase type 2) and regulating inflammation in lungs early during *M.tb* infection (Feng *et al*., 2006).

1.6.2 Adaptive Immunity

The adaptive immune system includes both humoral and cell-mediated immunity. This immune response is mediated by primed memory T and B cells. These antigen-experienced cells induce a highly specific immune response that is effective but much more slow acting than the innate immunity (Kirman *et al*., 2016).

CD4+ T cells

Although CD4+ T cells are essential in the control of *M.tb* infection, their specific attributes necessary for protection is still elusive. Studies in mouse models have shown that mice with defective CD4+ T cells are susceptible to *M.tb* infection (Flory et al., 1992; Srivastava and Ernst, 2013). In humans, HIV-infected individuals with low CD4+ T cell counts are more susceptible to TB compared to uninfected individuals

(Pape *et al*., 1983). Table 1.1 summarises the role of CD4+ T helper (Th) subsets in TB protection. It is now well known that Th1 cells, producing IFN-γ, play an important role in *M.tb* immunity. Mouse model studies also show that mice that are unable to secrete IFN-γ were unable to control bacterial growth and had disseminated TB (Flynn *et al*., 1993; Cooper *et al*., 1993). Similarly in a study by Green and colleagues, they observed that knock-out mice (where all cells except CD4+ T cells could produce IFN-γ) lost their ability to effectively control *M.tb* growth and were therefore more susceptible to *M.tb* infection (Green *et al*., 2013). These findings therefore suggests that IFN-γ derived from CD4+ cells is necessary for *M.tb* immunity. However, studies show that although crucial in TB immune protection, IFN-γ on its own is not a sufficient for *M.tb* control. In an adoptive transfer study, Gallegos and colleagues showed that Th1-primed TCR transgenic cells from IFN-γ deficient mice protected mice during *M.tb* infection just as well as cells from the wild-types mice (Gallegos *et al*., 2011). This seems to be also the case for humans. Previous studies in humans show that individuals with mutations in IFN-γ pathways or secreting neutralising antibodies to IFN-γ were more susceptible to TB disease (Filipe-Santos *et al*., 2006; van de Vosse *et al*., 2013; Browne, 2014). Moreover, while the MVA85A vaccine (a modified vaccinia virus Ankara expressing the *M.tb* antigen 85A, which was the first TB vaccine to reach clinical efficacy testing), induced a robust and durable IFN-γ T cell response, this vaccine did not show any enhanced protection against TB infection and disease (Tameris *et al*., 2014; Kirman *et al*., 2016). Hence, the lack of efficacy of the MVA85A vaccine suggests that robust IFN-γ responses are not sufficient to ensure protective *M.tb* immunity in humans. This advocates for the measurement of immune response beyond IFN-γ to elucidate TB correlates of protection. Overall, these results show that protective immunity to *M.tb* is more complex and suggest that other arms of the immune system and/or IFN-γ-independent T cell response may play a prominent role for TB control.

There are reports that suggest that polyfunctional CD4+ T cells that secrete IFN-γ, IL-2 and tumor necrosis factor α (TNF-α) simultaneously, may have a role to play in TB protection. Previous studies have shown that, compared to IFN-γ alone, polyfunctional CD4+ T cells show a greater association with protective immunity in several infectious diseases such as *Leishmania major* (Darrah *et al*., 2007), Cytomegalovirus (Kannanganat *et al*., 2007; Snyder *et al*., 2016), hepatitis C virus (Ciuffreda *et al*., 2008) and HIV (Duvall *et al*., 2008). In the case of *M.tb* infection, studies, using BCG-vaccinated mouse models, have shown that there was a positive correlation between protective immunity and polyfunctional CD4+ T cell responses (Lindenstrøm *et al*., 2009; Derrick *et al*., 2011). In humans, it is still unclear what role polyfunctional CD4+ T cells play in TB protection. Previous studies have shown that although necessary polyfunctional *M.tb*-specific CD4+ T cell responses did not correlate with TB protection in infants vaccinated with BCG or a booster dose of MVA85A (Kagina *et al*., 2010; Tameris *et al*., 2013). Recently, emerging evidence suggests an IFN-γ-independent mechanism of TB protection. A study by Gallegos and colleagues showed that adoptive transfer of *M.tb*-specific Th1 cells from IFN-γ-deficient mice protected the hosts from *M.tb* infection independently of IFN-γ and TNF-α production (Gallegos *et al*., 2011). Using a similar method, they also showed that adoptive transfer of *M.tb*-specific Th17 cells partially inhibited bacterial growth in the lungs of *M.tb*-infected mice (Gallegos *et al*., 2011). Th17 cells, producing IL-17 and IL-22, are a subset of CD4+ T cells that have been identified both in human TB patients (Peng *et al*., 2008) and in mouse models (Lockhart *et al*., 2006; Khader *et al*; 2007). Studies in mice show that

IL-17 responses are important in the early stages of *M.tb* infection, where they facilitate the establishment of CD4+ T cells in the lungs through the induction of the chemokines CXCL9, CXCL10 and CXCL11 (Khader *et al*., 2007). In a study by Gopal and colleagues, the authors showed that in BCG-vaccinated mice, Th17 cells localised to the lungs and assisted in the recruitment of Th1 cells into the lungs during *M.tb* infection (Gopal *et al*., 2012). Other studies have also shown that mice deficient in IL-17A receptor had impaired long-term control of *M.tb* infection and this impairment was due to a decrease in the recruitment of neutrophils in the early stages of infection (Freches *et al*., 2013). In humans, the role that *M.tb*-specific Th17 cells play in TB protection is still unclear. However, an increase in *M.tb*-specific CD4+ T cells producing IL-17 has been reported in the blood individuals with latent TB compared to those with active TB (Scriba *et al*., 2008; Loxton *et al*., 2012; Perreau *et al*., 2013). Furthermore, there are also reports showing that genetic mutations in the canonical Th17/Th22 lineage-defining factor, RORγt is associated with increased susceptibly to TB in humans (Okada *et al*., 2015; Wang *et al*., 2016).

The role that IL-22 plays in TB immunity is still elusive, however accumulating evidence suggests that it may have a protective function. IL-22 is a member of the IL-10 cytokine family, and a pro-inflammatory cytokine previously described in mice as being co-produced by Th17 cells (Dumoutier *et al*., 2000). In humans, however, IL-22 appears to be produced mainly by the ‘Th22’ cell subset, a novel lineage of T cells, that unlike Th17 cells, do not co-express IL-17 with IL-22 (Duhen *et al*., 2009; Trifari *et al*., 2009) and exert distinct functions from Th17 cells (Aujla *et al*., 2008; Eyerich *et al*., 2009). In a study by Treerat and colleagues, IL-22 deficient mice (IL-22$^{-/-}$) infected with a clinical *M.tb* strain, HN878, and not the lab strain H37Rv, had higher bacterial burden in their lungs during chronic stages of infection (Treerat *et al*., 2017). HN878-infected IL-$22^{-/-}$ mice were also reported to have a discontinuous epithelial barrier in their lungs and a decrease in the recruitment of macrophages into site of *M.tb* infection (Treerat *et al*., 2017). These results suggest a protective function for IL-22 during chronic stages of *M.tb* infection where it plays a role in maintaining the structure of the epithelial barrier and in macrophage recruitment to the lung. Studies in humans show that individuals with mutations in the IL-22 promotor region and the Th22 transcription factor had a greater risk of developing TB (Zhang *et al*., 2011; Okada *et al*., 2015). Scriba and colleagues reported that individuals with active TB had greater concentrations of IL-22 in their bronchoalveolar lavage fluid, suggesting that during active TB, IL-22 producing cells are recruited from the peripheral blood to the lung, where they may participate in TB protection (Scriba *et al*., 2008). Recently our research group has shown that *M.tb*-specific CD4+ Th22 cells are significantly depleted in PLWH when compared to HIV-uninfected individuals (Bunjun *et al*., 2021; Makatsa *et al*., 2022). Overall, these findings therefore suggest that Th22 cells may have an important role to play in TB immunity.

CD8+ T cells

CD8+ T cells recognise *M.tb* antigens presented by the MHC class I molecules on antigen presenting cells (APCs) (Lin and Flynn, 2015). They can control *M.tb* growth either indirectly through the production of Th1 cytokines (specifically IFN-γ) leading to macrophage activation or directly by eliminating of *M.tb*-infected cells through cytotoxic pathways mediated by cytotoxic molecules (such as perforin, granzyme, and granulysin) (North and Jung, 2004; Lu *et al*., 2014; Kirman *et al*., 2016). Several studies have shown the importance of CD8+ T cells in TB immunity. In one such study, Wang *et al.* showed that BCG-activated CD8+ T cells contributed to TB protection in the absence of CD4+ T cells (Wang *et al*., 2004). BCG-vaccinated CD4+ T cell-deficient mice were able to mount a protective immune response during *M.tb* infection and had a significantly lower bacterial burden in their lungs 6 weeks after vaccination when compared to unvaccinated controls (Wang *et al*., 2004). Similarly, Mangalakumari *et al*. reported that immunisation of mice by AdAg85A (Ad–based antigen 85A TB vaccine) generated *M.tb*-specific CD8+ T cells with an effector memory phenotype in the lungs; and these vaccinated mice were protected during *M.tb* infection in the absence of CD4+ T cells (Mangalakumari *et al*.2010). Moreover, depletion of CD8+ T cells in mice during the chronic/latent phase of *M.tb* infection caused an increase in bacterial burden in the lungs, while this had no effect during the acute phase of infection (van Pinxteren *et al*., 2000). These results highlight the potential role of CD8+T cells for the long-term control of *M.tb* infection. Although data on mouse models support a protective role for CD8+ T cells against *M.tb*, in humans, their contribution to TB immunity is still unclear. Previous reports have shown that there is an increase in the *M.tb*-specific CD8+ T cell response in individuals with active TB compared to those with LTBI (Day *et al*., 2011; Rozot *et al* 2014; Lancioni *et al*., 2019). However, this could be simply related to bacterial burden. Indeed, a study by Lewinsohn and colleagues suggest that in humans, the role of CD8+ T cells during *M.tb* infection may be that of immune surveillance, where *M.tb*-specific CD8+ T cells preferentially recognise and lyse heavily infected APCs (Lewinsohn *et al*., 2003). These findings therefore suggest that, unlike LTBI, active TB reflects a state of impaired immune surveillance where containment of *M.tb* growth is disrupted (Lewinsohn *et al*., 2003). Similarly, data from non-human primates has also shown that depletion of CD8+ T cells led to decreased protection after subjects were challenged with a high dose of *M.tb* (Chen *et al*., 2009). In this study, BCG-vaccinated rhesus macaques lost effective immune control of *M.tb* replication in their lung tissue, suggesting that CD8+ T cells may have a role play a role in vaccine-induced TB protection (Chen *et al*., 2009).

Antibody responses

The role of humoral immunity and more specifically, antibodies, in *M.tb* immunity remains controversial. Studies in mouse models show that mice with impaired antibody production or defects in their B cells were more susceptible to *M.tb* infection when compared to the controls (Maglione *et al*., 2007; Torrado *et al*., 2013). In humans, previous reports suggest that *M.tb*-specific antibody responses associate with disease severity, with serum immunoglobulin G (IgG) responses to *M.tb* antigens significantly lower in children with disseminated TB when compared to those with localised infection (Bothamley *et al*., 1988; Castello *et al*., 1992). Similarly, a case-control study on infants by Fletcher and colleagues showed an association between increased Ag85A-specific IgG responses and a reduction in TB risk (Fletcher *et al*., 2016). Mechanistically, it has been suggested that that antibodies can enhance macrophage phagolysosomal maturation, promoting the killing *M.tb*-infected cells (Chen *et al*., 2016). Contrasting these previous findings, there are also studies that show *M.tb*-specific antibody responses having no significant impact on *M.tb* protection (Casadevall, 2017; Li and Javid, 2018). In one such study, depletion of B cells (using an anti-CD20 antibody, rituximab) in non-human primates early after *M.tb* infection, altered cytokine responses by T cells in the granulomas, but had no overall effect in disease progression and outcome (Phuah *et al*., 2016).

Table 1.1: The role of different CD4+T helper subsets in TB protection.

	Th1	**Th17**	**Th22**
Transcriptional factor	T-bet	RORγt	RORγt
Cytokines produced	IFN-γ, TNF-α, IL-2	IL-17	IL-22
Effector function	Intracellular infections	Extracellular infections	Extracellular infections
Role in TB protection	Mouse model: Control of *M.tb* growth and dissemination (Green *et al*., 2013). Humans: Increase TB risk in individuals with mutations in IFN-γ pathways or secreting neutralising antibodies (Filipe-Santos *et al*., 2006; van de Vosse *et al*., 2013; Browne, 2014).	Mouse model: Recruitment of Th1 cells and neutrophils into the lungs (Gopal *et al*., 2012,; Freches *et al*., 2013). Humans: Increase TB risk in individuals with RORγt mutations (Okada *et al*., 2015; Wang *et al*., 2016).	Mouse model: Recruitment of macrophages into the lungs Treerat *et al*., 2017). Humans: Increase TB risk in individuals with RORγt mutations (Zhang *et al*., 2011; Okada *et al*., 2015)

1.7 The effect of HIV on TB immunity

1.7.1 Impairment of innate immunity

The innate immune response, particularly the alveolar macrophages, form the first line of defence during *M.tb* infection and therefore play an important role in TB protection. Several studies showed that HIV infection impairs the innate immune response to *M.tb* by interfering with macrophage function. *In vitro* co-infection of human macrophages with HIV and *M.tb* reduced cell viability and therefore the ability of macrophages to effectively control and kill *M.tb* (Pathak *et al*., 2010). Moreover, TNF-α-mediated apoptosis of *M.tb*-infected macrophages was significantly reduced in PLWH compared to HIV-uninfected individuals. Such defects were associated with an enhanced production of IL-10, inhibiting TNF-α secretion and its proapoptotic activity (Patel *et al*., 2009). Similarly, other studies have shown that the HIV negative factor (Nef) protein reduces macrophage phagocytosis and apoptosis, during *M.tb* infection, by inhibiting the recruitment of endosomes required phagosome biogenesis (Mazzolini *et* *al*., 2010). Mechanistically, the HIV Nef protein blocked the maturation of autophagosomes by interacting directly with the autophagy regulatory factor (Beclin 1) and/or through the impairment of TNF-α promoter activation and mRNA stability (Kyei *et al*., 2009; Kumawat *et al*., 2010). Additionally, a recent study showed that innate cells derived from PLWH had impaired anti-mycobacterial activity, due to alterations in pattern recognition receptors (Toll-like receptors) and IL-27 signalling pathways, compared to HIV-uninfected individuals (Namdev *et al*., 2021).

1.7.2 The impact of HIV-associated lymphopenia

HIV infection and replication is characterised by a progressive depletion of CD4+ T cells, that if left untreated often leads to opportunistic infections and acquired immune deficiency syndrome (AIDS) (Okoye and Picker, 2013). During HIV infection, there is an increased risk of TB morbidity and mortality as the absolute CD4+ T cell counts decreases due to HIV-associated lymphopenia (Ellies *et al*., 2017; Aung *et al*., 2019). Although the risk of developing TB is highest among individuals with prolonged untreated HIV infection (when CD4+ T cells are severely depleted), the risk of TB is also increased during the first year of HIV infection, even when CD4+ T cell counts are still maintained (Kauffman and McMichael, 2005; Sonnenberg *et al*., 2005). In fact, in countries where there is a high HIV and TB burden, such as South Africa, PLWH are at higher risk for developing TB at all stages of HIV infection: during untreated HIV infection, shortly after treatment (e.g., TB- Immune reconstitution inflammatory syndrome, TB-IRIS) and even after successful viral suppression compared to HIV-uninfected persons (WHO Global TB report, 2021; Xue *at al*., 2020; Lawn *et al*., 2006). These findings therefore suggest that HIV-infection can lead to loss of TB protection due immune defects not always linked to HIV-induced lymphopenia. Indeed, a study by Gelmacher and colleagues showed that there is a rapid depletion of *M.tb*-specific CD4+ T cells early within the first year of HIV infection. This study suggest that elevated expression of CCR5 (the primary HIV co-receptor) on *M.tb*-specific CD4+ T cells enhanced their susceptibility to HIV infection, leading to their preferential depletion (Geldmacher *et al*., 2008). Indeed, a follow up study by the same research group showed that *M.tb*-specific CD4+ T cells from HIV co-infected individuals harboured a significantly higher level of the HIV *gag* DNA in vivo when compared to total memory CD4+ T cells (Geldmacher *et al*., 2010). Moreover, when compared to cytomegalovirus-specific CD4+ T cells (an opportunistic pathogen that only causes disease late in HIV infection), *M.tb*-specific CD4+ T cells produced significantly lower levels of the CCR5 ligand, MIP-1β (Geldmacher *et al*., 2010). As cells expressing high levels MIP-1β are able to block HIV replication (Cocchi *et al*., 1995, Kinter *et al*., 1996), these findings suggest that the high infectivity potential of *M.tb*-specific CD4+ T cells was most likely due to their phenotypic and functional attributes, expressing elevated CCR5 and secreting limited amount of MIP-1β Geldmacher *et al*., 2008; 2010). Moreover, *M.tb*-specific CD4+ T cells from PLWH were characterized by: i) a reduced capacity to secrete IFN-γ+TNF-α+IL-2+ polyfunctional cells (Kalsdorf *et al*., 2009; Jambo *et al*., 2011; Wilkinson *et al*., 2016; Day *et al*., 2017; Amello *et al*., 2019), ii) a reduced capacity to secrete IL-17 and IL-10 co-expressing regulatory cells, promoting a more inflammatory response (Rakshit *et al*., 2020) and iii) an impaired proliferative ability (Day *et al*., 2017). Overall, these results suggest that, beyond HIV-associated loss of *M.tb*-specific CD4+ T cells, HIV-induced alterations to *M.tb*-specific T cell functions can potentially drive TB incidence.

1.8 The impact of HIV treatment on TB immunity

1.8.1 ART and TB risk

ART reduces HIV viral load, increasing CD4+ T cell count and therefore enabling immune recovery (Bisset *et al*., 1998; Gras *et al*., 2007). Studies done in sub-Saharan Africa show that ART is associated with a 65-90% reduction in TB incidence and a 52% decrease in TB-related mortality (Badri *et al*., 2002; Moore *et al*., 2007; Suthar *et al*., 2012). Several studies have highlighted the importance of early ART as strategy to control TB incidence in high burden countries. When initiated early after HIV diagnosis, ART resulted in an 89% reduction in HIV transmission and a 2.5-fold decrease in TB incidence, when compared to those with deferred treatment until CD4+ T cell count of 200 cells/µL (Cohen *et al*., 2011; Collins *et al*., 2015). Additionally, the benefits of ART initiated at high CD4 counts is further highlighted by two randomised controlled trials, the START and TEMPRANO studies, showing that PLWH who started ART at CD4 counts above 500cells/µL had a 44-72% reduction in serious AIDS-related illnesses (including TB) when compared to those who differed ART (INSIGHT START Study Group, 2015; TEMPRANO Study Group, 2015). When initiated early, ART results in a reduction in chronic T cell activation and a reduced HIV reservoir size, therefore increasing the likelihood of CD4+ T cell count recovery (Bisset *et al*., 1998; Jain *et al*., 2013; Le *et al*., 2013; Chevalier *et al*., 2016).

1.8.2 The effects of ART on TB immunity

It is still unclear what effect ART has on *M.tb*-specific T cell responses as previous reports have shown varying data. Firstly, in individuals with LTBI, ART restored *M.tb*-specific responses in the peripheral blood, however this restoration was delayed by more than 8 months and did not normalize to levels observed in HIV-uninfected individuals, particularly in individuals with chronic HIV infection (Schluger *et al*., 2002; Sutherland *et al*., 2006). In contrast, a study Jambo and colleagues showed that there was no significant differences in the frequency of cytokine-producing *M.tb*-specific CD4+ T cells in the peripheral blood of HIV-infected patients on ART (irrespective of duration of treatment) and healthy HIV-uninfected individuals (Jambo *et al*., 2014). Moreover, when TB responses were examined in the bronchoalveolar lavage (BAL), the frequency of M.tb-specific CD4+ T cells in individuals who were on ART, for less than 4 years, was a significantly lower compared to healthy HIV uninfected individuals (Jambo *et al*., 2014). Additionally, our research group reported that while ART increased the absolute number of *M.tb*-specific CD4+ T cells in the peripheral blood in PLWH (mirroring the reconstitution of the overall CD4 compartment), no significant changes in the frequency, the functional capacity or the memory phenotype of these cells was observed (Riou *et al*., 2015). Importantly, this study show that the restoration of *M.tb*-specific CD4+ T cells after ART was dependent of their memory phenotype, where cells with an early differentiated phenotype were restored at a higher capacity when compared to those with late differentiated phenotype (Riou *et al*., 2015).

1.9 Aims, study rationale and objectives

As stated before, the greatest risk factor of developing active TB is HIV infection (WHO Global TB report, 2022). PLWH are susceptible to TB at all phases of infection, even before immunosuppression or after CD4 reconstitution (Sonnenberg *et al*., 2005; Lawn *et al*., 2006; Getahun *et al*., 2010; Gopal *et al*., 2017). A better understanding of the effect of HIV infection on the quantity and quality *M.tb*-specific T cell responses may shed light on the mechanism by which HIV increases TB susceptibility and assist in the understanding of the specific attributes of TB immunity required for a protective response to *M.tb*.

In this thesis, we aimed to characterise *M.tb*-specific CD4+ T cell responses during HIV co-infection. We undertook this study using a cohort of HIV infected South African women from the CAPRISA 002 study: a longitudinal cohort following PLWH from HIV seroconversion to up to 5 years after ART initiation. We assessed *M.tb*-specific CD4+ T cell responses during the course of HIV infection: pre HIV infection, acute and chronic infection, and up to 2 years post antiretroviral therapy (ART) initiation. In addition, we also investigated whether immunity to TB is better preserved following early initiation of ART compared delayed ART administration during chronic infection.

Aim 1: The kinetics of *M.tb*-specific CD4+ T cell responses during HIV infection

Rationale: Previous studies have demonstrated that *M.tb*-specific CD4+ T cell responses are depleted rapidly within 3 to 12 months after HIV infection (Geldmacher *et al*., 2008; 2010). This early defect targeting the immunological memory response to TB may partly explain the elevated risk of TB even within the first year of HIV infection, prior to substantial immunosuppression (Kauffman and McMichael, 2005; Sonnenberg *et al*., 2005). We aimed to confirm and extend these findings in a larger cohort of individuals from the CAPRISA 002 study. We examined *M.tb*-specific CD4+ Th1 responses over the course of HIV infection. In addition, to ascertain whether observed changes in immune responses over time were attributable to HIV infection, we examined the natural fluctuation of *M.tb* responses in a sub-group of this cohort prior to HIV infection.

Aim 2: The impact of early ART on immunity to *Mycobacterium tuberculosis* immunity

Rationale: Early ART is associated with reduced mortality and a lower risk of developing TB compared to late ART (TEMPRANO Study Group, 2015; Insight START Study Group, 2015). Given the rapid depletion of *M.tb*-specific CD4+ cells soon after HIV infection, early ART could be an important step in preserving *M.tb* immunity. In this study, a comparative examination of *M.tb*-specific CD4+ T cell responses was conducted between individuals who initiated ART early (< 1yr after infection) and those who started later on during chronic HIV infection (>2yrs after infection). We hypothesise that early ART preserves the specific immune response to *M.tb* thereby reducing the risk of TB.

Aim 3: The impact of TB treatment on *M.tb*-specific CD4+ T cells

Rationale: To assess the kinetics of *M.tb*-specific responses in active TB infection, we examined a small cohort of participants who developed TB during the CAPRISA 002 study. In this sub-group, we assessed longitudinally the evolution of *M.tb*-specific T cell responses at three timepoints: prior to TB diagnosis, during TB treatment, and after successful completion of treatment.

CHAPTER 2

Materials and Methods

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2.1 Study Participants

2.1.1 Study cohort

Three hundred and four archived peripheral blood mononuclear cell (PBMC) samples from the Centre of AIDS Programme of Research in South Africa (CAPRISA) 002 and 004 cohorts were used for this study. CAPRISA 002, previously described by van Loggerenberg *et al*. (2008) and Mlisana *et al*. (2014), is an HIV acute infection study consisting of HIV-infected women followed from HIV seroconversion until 5 years on ART treatment. The CAPRISA 004 study was a clinical trial of a vaginal microbicide (1% tenofovir) gel for the prevention of HIV infection (Abdool Karim *et al*., 2010), that recruited women aged 18 to 40 years, and any participants who seroconverted were approached for recruitment into the CAPRISA 002 study and followed longitudinally in the way. Participants from both the CAPRISA 002 and 004 studies were recruited from clinics in KwaZulu Natal, South Africa. For each participant, HIV viral loads (VLs) were determined using a PCR-based diagnostic assays. The assays used to determine VL changed overtime for the CAPRISA 002 and 004 studies. Initially the Roche AMPLICOR HIV-1 Monitor test (v1.5, lower detection limit (LDL) of 400 RNA copies/mL) was used, thereafter, from June 2010 to December 2011, the Roche 42 Taqman (v1.0, LDL of 40 RNA copies/mL) was used, and since January 2012 HIV VL were measured using the Roche Taqman (v2.0, LDL of 20 RNA copies/mL). To determine absolute blood CD4+ and CD8+ T-cell counts the FACSCalibur TruCOUNT method (BD Biosciences) was utilised.

The CAPRISA 002 and CAPRISA 004 study were approved by the Biomedical Research Ethics Committee at the University of KwaZulu-Natal (HREC BE178/150) and the University of Cape Town’s Faculty of Health Sciences Human Research Ethics

Committee (HREC 025/2004 and HREC 711/2018). All participants provided written informed consent.

2.2 Assays

2.2.1 Antigens and mitogens

MTB300 is a peptide megapool consisting of 300 *M.tb*-derived 15mer peptides, (Arlehamn *et al*., 2016). The peptide megapool consist of 66 highly immunodominant epitopes identified from Bacille Calmette-Guerin (BCG) vaccine and Interferon-Gamma Release Assay (IGRA) antigens. Stimulation with MTB300 induces robust cytokine responses (IFN-γ, IL-2, IL-22 and TNF-α) equivalent in frequencies to that of heat-killed H37Rv *M.tb* lysate. The 300 peptides can be grouped into the following protein categories: cell wall and cell processes (106), PE/PPE (104), conserved hypotheticals (25), lipid metabolism (24), intermediary metabolism and respiration (23), insertion sequences and phages (8), information pathways (6) and virulence, detoxification and adaptation (4). Peptides in the cell wall and cell processes (including ESAT-6 and CFP-10) category are the most immunogenic, followed by those in the PE/PPE category.

2.2.2 Thawing, resting and stimulation of PBMC

Cryopreserved PBMC samples were thawed in a water bath at 37˚C and added to a 50 mL centrifuge tube. Ten mL of RPMI 1640 medium (Sigma) supplemented with 1% heat inactivated foetal bovine serum (FBS) (R1 medium) was added dropwise to the thawed cells. The cell suspension was then topped up to 25 mL with R1 medium and centrifuged (288 x g for 10 min at room temperature (RT). After centrifuging, the supernatant was discarded, and the cell pellet was resuspended in 500 µL of R1 medium containing 0.002% DNAse. The cell suspension was incubated for 5 min at RT to enable the DNAse to work and reduce clumping of cells after thawing. Twenty-five mL of R1 medium was added and the sample was then centrifuged (288 x g) for 10 min at RT. The supernatant was discarded, and cells were resuspended in 2 mL RPMI 1640 supplemented with 10% heat inactivated FBS and penicillin-streptomycin (50 U/mL) (R10 medium). Trypan Blue exclusion staining was used to count the cells. Briefly, 10 µL of cell suspension was mixed with 10 uL of Trypan Blue (Invitrogen) and counted using a TC20 Cell Counter and slides (Bio-Rad). The cell suspension was topped up to 5 mL with R10 medium (2 to 5 million cells/mL) and rested for 4 h at 37˚C in a 5% CO_2 incubator. After resting, cells were seeded in a 96 well v-bottom plate at 2 $x10^6$ cells/well and stimulated with *M.tb*-specific peptide pools MTB300 (1 µg/mL) and unstimulated samples were included as negative controls. Co-stimulatory antibodies anti-CD28 and anti-CD49d were used at a final concentration of 1 µg/mL each and BFA at 10 µg/mL. Samples were then incubated for 16 h at 37˚C in a 5% CO_2 incubator.

2.3 Flow Cytometry

2.3.1 Surface and Intracellular cytokine staining (ICS)

After stimulation, cells were centrifuged (883 x g rpm) for 5 min. The supernatant was discarded, and the cell pellet was washed with 200 µL phosphate-buffered saline (PBS). Cells were then resuspended in 50 µL of PBS containing 1 µL of a 1:40 dilution of a viability dye (‘ViViD’, Molecular Probes) for 20 min at RT. After staining, the cell suspension was topped up to 200 µL with FACS wash buffer (1% FBS in PBS), centrifuged (883 x g rpm) for 5 min and the supernatant was discarded. This wash step was repeated. Cell pellets were then resuspended in 50 µl of a surface antibody mix in FACS wash buffer (1% FBS in PBS) and incubated in the dark for 20 min at RT. The cells were then washed twice with 200 µL of FACS wash buffer, resuspended in 100 µL of Cytofix/Cytoperm (BD Biosciences) and incubated in the dark for 15 min at RT. Cells topped up to 200 µL with Permwash (BD Biosciences), centrifuged at (883 x g) for 5 min and the supernatant was discarded. The cell pellets were resuspended in 50 µL of an intracellular antibody staining mix in Permwash and incubated in the dark for 30 min at RT. After staining, the cells were washed twice in Permwash and resuspended in 150µL of CellFix (BD Biosciences). For acquisition, the cells were transferred to cluster tubes (Corning). Detailed surface and intracellular antibody staining mixes used in the study are presented below (**Table 2.1**).

2.3.2 Antibodies and panel optimisation

The antibodies used in this study were titrated to determine the volume for optimal staining and separation. The titer for each antibody was selected based on the frequency, median fluorescence intensity (MFI) and the signal/noise ratio. Antibody titrations were performed for every new lot of antibodies obtained. The details of the antibodies used in this study are described in **Table 2.1.** To determine whether the fluorescence detected in each channel was a true signal and not a result of spectral overlap from other channels, the antibody panel was optimised as shown in **Figure 2.1**. Starting with a previously optimised core panel (**Figure 2.1A**), antibodies were added sequentially to the panel to measure their effect on the frequency of lineage markers (CD3, CD4 and CD8) and three cytokines (IFN-γ, TNF-α and MIP-1β) (**Figure 2.1B-D**). No significant spectral overlap was detected with the final antibody panel used. Moreover, additional controls (FMO: fluorescence minus one) were performed to further validate the antibody panel**.** FMO controls correspond to a sample stained with all the fluorophores included in the final panel, minus one of them. They are used to define the upper boundary for background signal on the omitted antibody, and thus properly identify positive populations in multicolour experiments. **Figure 2.2** shows flow cytometry plots of FMO controls and setting of the positive gates for two memory markers (CD27 and CD45RA).

Table 2.1: 13-color antibody panel used for flow cytometry

Marker	Fluorophore	Clone	Manufacture	Function	Staining
Viability marker	Pacific Blue	-	Invitrogen	Exclude dead cells	Surface
CD14	Pacific Blue	M5E2	Biolegend	Exclude monocytes	Surface
CD19	Pacific Blue	6D5	Biolegend	Exclude B cells	Surface
CD3	BV650	OKT3	Biolegend	T cell Lineage	ICS
CD4	PerCP-Cy5.5	L200	BD Biosciences	T cell Lineage	Surface
CD8	BV510	RPA-T8	Biolegend	T cell Lineage	Surface
IFN-γ	AF700	B27	BD Biosciences	Cytokine	ICS
TNF-α	BV786	MAb11	Biolegend	Cytokine	ICS
IL-2	PE Dazzle	MQ1	Biolegend	Cytokine	ICS
MIP-1β	FITC	24006	R&D Systems	Cytokine	ICS
CD153	PE	116614	R&D Systems	CD30 Ligand	ICS
HLA-DR	APC-Cy7	L243	Biolegend	Activation	Surface
IL-21R	Alexa 647	2G1-K12	Biolegend	IL-21 receptor	Surface
CD45RA	BV570	HI100	Biolegend	Memory	Surface
CD27	PE-Cy5	1A4CD27	Beckman Coulter	Memory	Surface

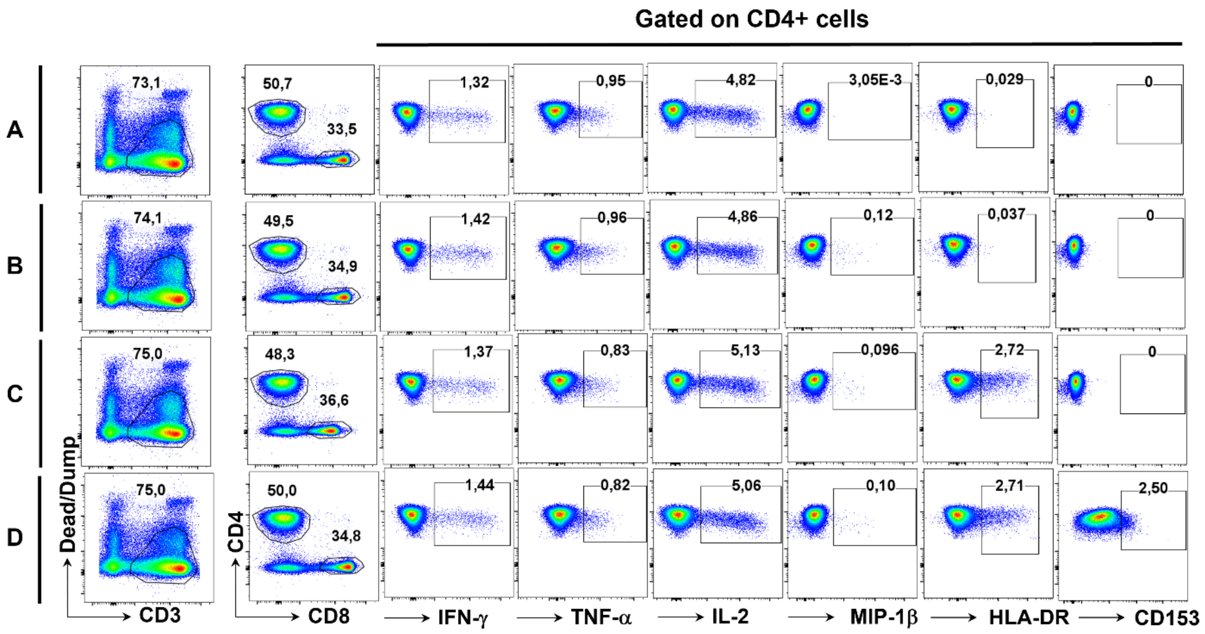


Figure 2.1: **Antibody panel optimisation.** Flow plots depicting staining of lineage markers (1st and 2nd columns), cytokines produced by CD4+ T cells (3rd to 6th columns), HLA-DR (7th column) and CD153 (8th column) when antibodies were added sequentially to the core panel. **(A)** Core panel (Dead/Dump, CD3, CD4, CD8, IFN-γ, TNF-α and IL-2), **(B)** Core panel plus MIP-1β, **(C)** Core panel plus MIP-1β and HLA-DR and **(D)** Core panel plus MIP-1β, HLA-DR and CD153.

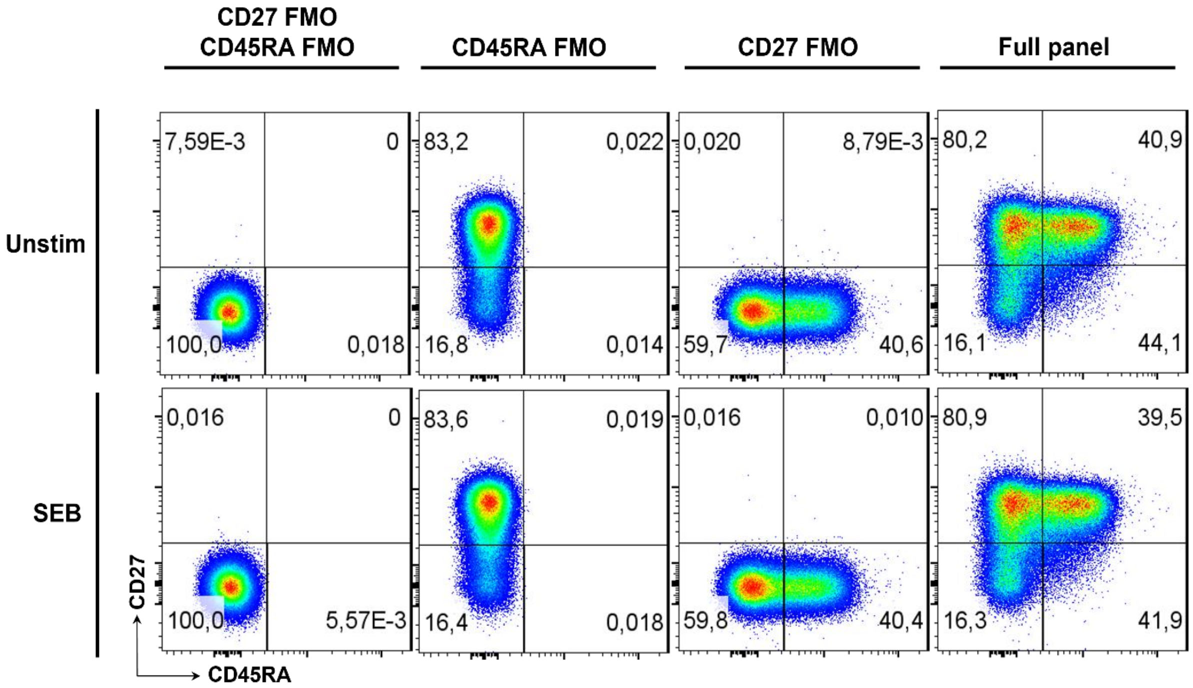


Figure 2.2: **Fluorescence minus one (FMO) controls.** CD27 and CD45RA FMO (1st column), CD45RA FMO (2nd column) and CD27 FMO (3rd column). The 4th column shows the expression profile of CD27 and CD45RA in samples stained with the final antibody panel (13 colours, 15 markers). Cells are gated on live lymphocytes that are CD3+CD4+ and either unstimulated (top panel) or after Staphylococcus enterotoxin B (SEB) simulation (bottom panel).

2.3.3 Compensation

A compensation control was prepared for each antibody used in the flow cytometry panel. A single drop of compensation beads (BD Biosciences) was incubated with antibody (at optimal titer) in a 5 mL polystyrene tube for 10 min at RT. Anti-mouse Ig-κ or anti-rat Ig-κ beads were used, depending on the species used to generate the antibodies. The stained beads were then washed with 2 mL FACS wash and centrifuged (512 x g) for 8 min. The supernatant was discarded, and the beads resuspended in 200 µL of Cellfix.

2.3.4 Acquisition and data analysis

Samples were acquired on a BD Fortessa (**Figure 2.3**) using FACSDiva software. Flow cytometry data were analysed using FlowJo v10.8.0 (Treestar, USA). **Figure 2.4** illustrates the gating strategy used in this study. A time gate was used to exclude inconsistencies in the fluorescent intensity, and this was then followed by sequential gating for singlets, lymphocytes and live CD3+ cells. When gating for live CD3+ cells, CD14 (monocytes) and CD19 (B cells) were also excluded (dumped), as these two markers are detected in the same channel as dead cells (ViViD). CD4+ and CD8+ T cells were gated from live CD3+ cells and thereafter cytokines (IFN-γ, TNF-α, IL-2 and MIP-1β) and CD153 on CD4+ T cells. A positive cytokine response was defined as twice the cytokine frequency of the unstimulated samples and at least 10 events in the stimulated samples. To define the phenotype of cytokines-producing cells, a cut-off of 40 events was used. Memory phenotypes were then delineated on CD4+ T cells using CD45RA and CD27, and HLA-DR was used to identify activated cells. IL-21R was also gated on CD4+ T cells, however after analysis, this marker was excluded from the study due to suboptimal expression on cells.

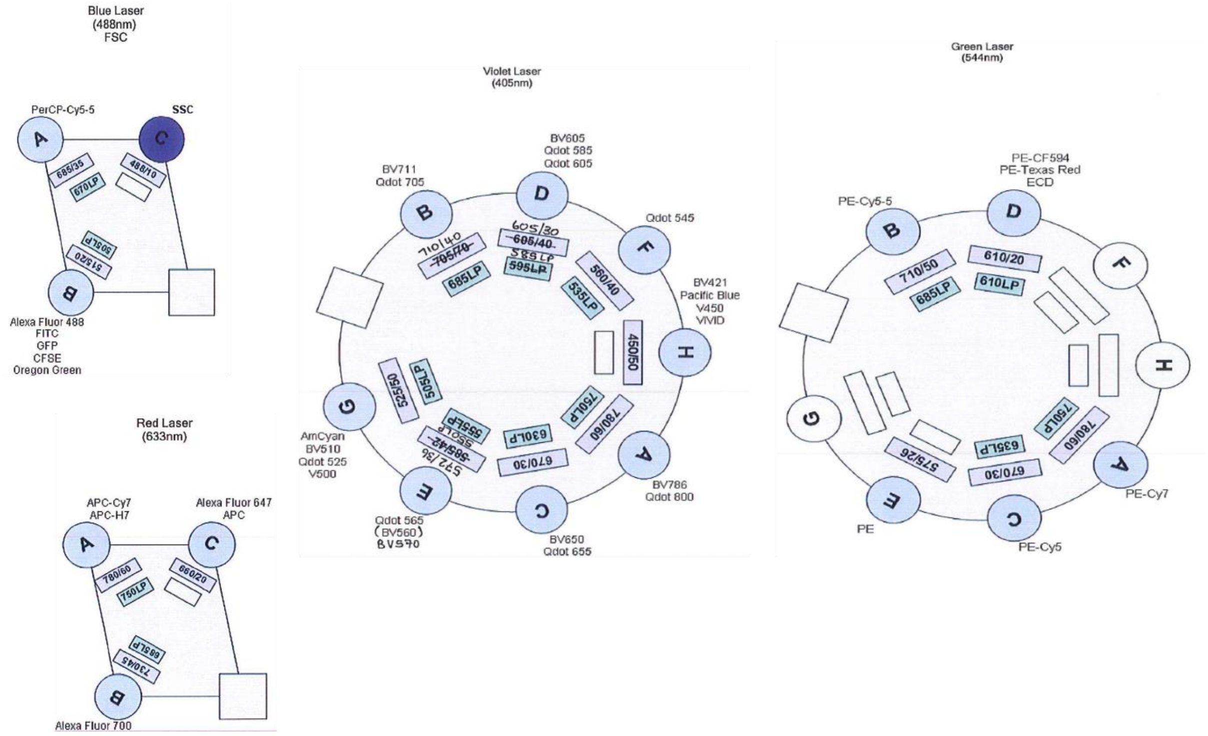


Figure 2.3: **The filter configuration of the BD Fortessa.** The filter arrangement is shown for each of the lasers, with two trigons (blue and red lasers) and two octagons (violet and green lasers).

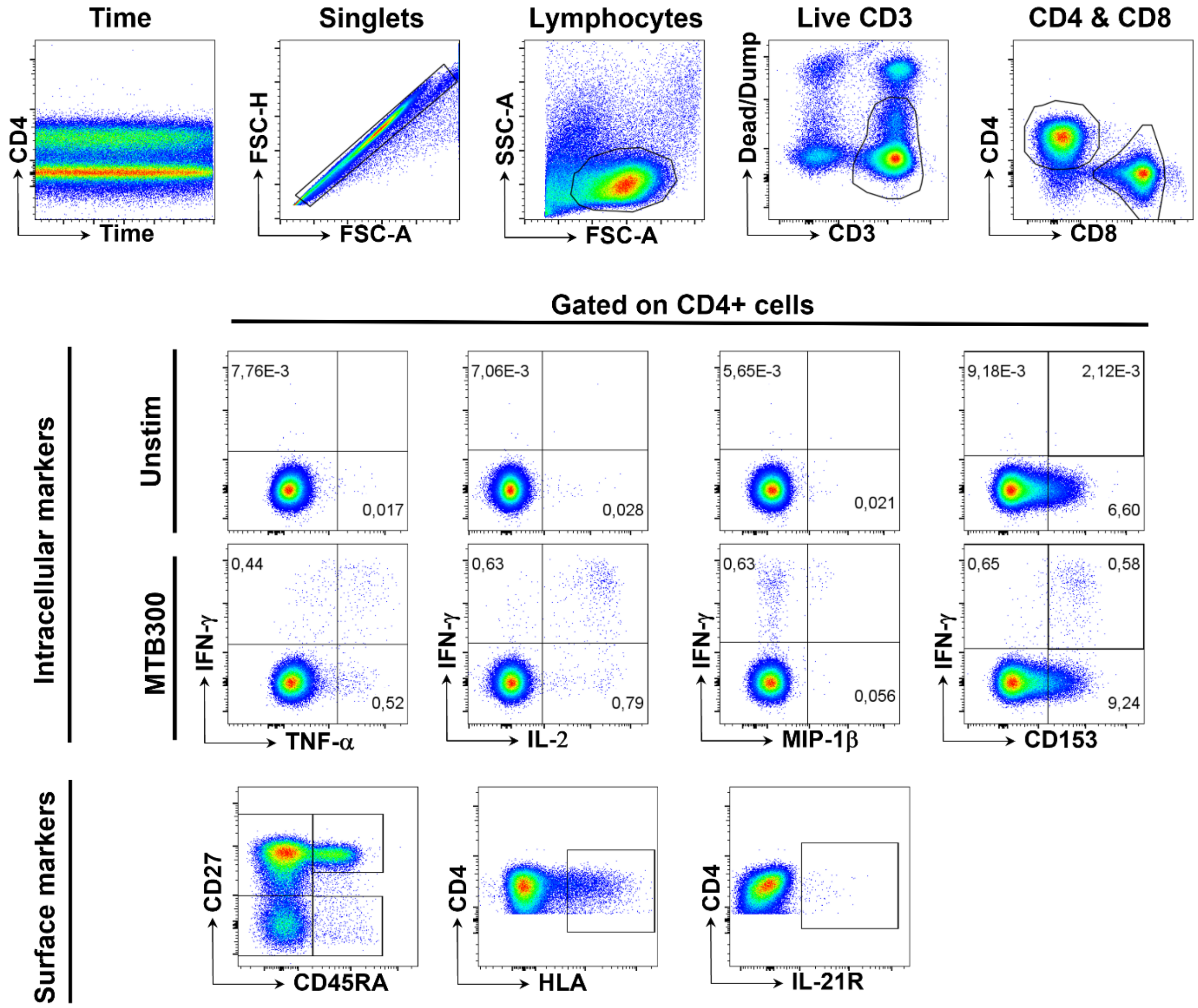


Figure 2.4: **Gating strategy**. Representative plots of the time, singlets, lymphocytes, live CD3+ cells, CD4+ and CD8+ T cell gates. Intracellular markers (IFN-γ, TNF-α, IL-2, MIP-1β and CD153) and surface markers (CD27, CD45RA, HLA-DR and IL-21R) were gated on CD4+ T cells.

2.3.5 Quality control (QC) sample

PBMCs were isolated from a single blood donor (WP8314) obtained from the Western Cape Blood Service. The PBMCs were then cryopreserved to be used later as QC samples. A QC sample was used in every run to monitor the consistency of cell staining between runs for the lineage populations (Live CD3+ cells, CD4+ and CD8+ T cells). The frequencies of lineage populations were plotted over different runs on a Levey-

Jennings Control Chart (**Figure 2.5**), showing minimal variations for the frequencies of live CD3+ cells, CD4+ and CD8+ T cell populations (Coefficient of variation: 3.55%, 2.63% and 7.04%, respectively).

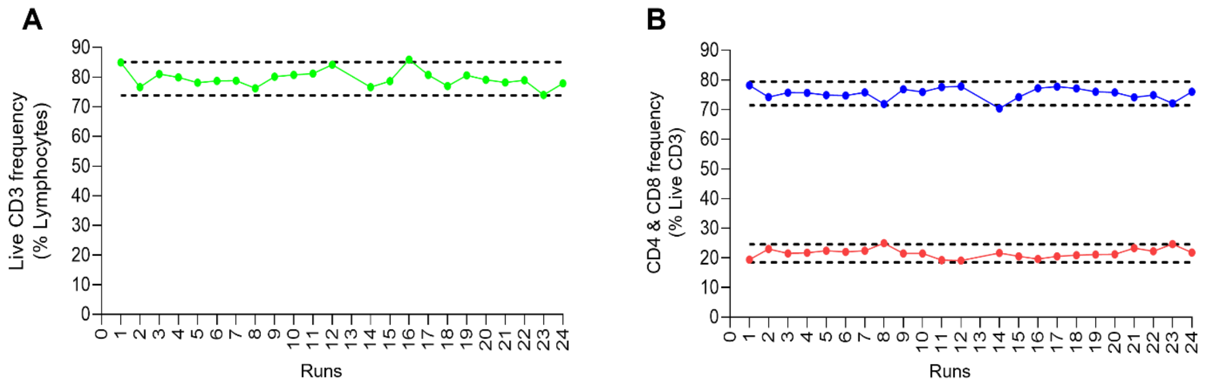


Figure 2.5: Frequency of lineage populations for QC sample WP8314. **(A)** Live CD3+ cells (green) and **(B)** CD4+ (blue) and CD8+ (red) T cells. Dotted lines represent the mean plus one standard deviation (SD) (top line) and mean minus one SD (bottom line).

2.3.6 QC of flow cytometry data

To monitor for staining and/or gating issues a preliminary analysis of all markers in the panel was performed on total CD4+ and CD8+ T cells. A CD4+ T cell event cut off of 15000 events was used in order obtain a sufficient number of cells to accurately detect rare-cell events such as cytokine producing CD4+ T cells (Roederer, 2008). Flow plots of samples with less than 15000 CD4+ T cells were visually inspected. Cytokine-producing CD4+ T cells were backgated and gates were adjusted accordingly. Every applied gate was visually inspected and where necessary lineage and memory gates were adjusted . Where cytokine gates were adjusted the same gates were set for the stim and unstim of each sample.

2.4 Statistical analyses

Statistical tests were performed using Prism v8 (GraphPad). The Mann-Whitney U test and Wilcoxon matched pairs test were used for unmatched and paired samples, respectively, and nonparametric tests were used for all comparisons. Prism v8 was used to calculate the area under the curve (AUC) after generating the viral load trajectories of participants. All correlations were tested using the non-parametric Spearman's Rank test. SPICE v6.1 was used to analyse the co-expression profiles of antigen-responding CD4+ T cells. Pestle v2 was used for background subtraction and generating SPICE data. Linear regression models were performed on IBM SPSS Statistics v28.0 to determine an association between the predictor and outcome. For regressions with multiple co-variables, the "stepwise" method was used to correct for multiple comparisons and the "enter" method to generate β coefficients for variables excluded from the model. For all statistical analyses, a P value of < 0.05 was considered statistically significant.

CHAPTER 3

The kinetics of *M.tb*-specific CD4+ T cell responses during HIV infection

Table of Contents

3.1 Introduction

Studies in mouse models of TB and human clinical studies show that CD4+ T cells, specifically those secreting IFN-γ, are essential in TB immunity (Green *et al*., 2013; van de Vosse *et al*., 2013; Browne, 2014). Understanding the events that alter CD4+ T cell responses during HIV co-infection favouring TB disease may be important to illuminate key components of TB immunity. Using a cohort of five participants, a longitudinal study by Geldmacher and colleagues showed a rapid depletion of *Mycobacterium tuberculosis* (*M.tb*)-specific CD4+ T cells within the first year of HIV infection (Geldmacher *et al*., 2008). When compared to total memory CD4+ T cells, there was a higher proportion of *M.tb*-specific CD4+ T cells expressing the HIV co-receptor CCR5, suggesting that these cells may be more susceptible to HIV infection, with possible preferential depletion (Geldmacher *et al*., 2008). A follow-up study revealed that *M.tb*-specific CD4+ T cells were indeed directly targeted for depletion during HIV infection, demonstrated by the fact that they harboured a significantly higher level of HIV *gag* DNA *in vivo* compared to total memory CD4+ T cells (Geldmacher *et al*., 2010). Moreover, it was also reported that *M.tb*-specific cells produced significantly less CCR5 ligand MIP-1β, and instead produced high levels of IL-2, when compared to CD4+ T cells of the opportunistic pathogen cytomegalovirus (CMV). These findings suggested that the high infectivity of the *M.tb*-specific cells was most likely due to their low expression of MIP-1β, since cells expressing high levels MIP-1β can block HIV replication (Cocchi *et al*., 1995, Kinter *et al*., 1996). In addition to these previous reports, there are several cross-sectional studies that provide evidence that in PLWH *M.tb*-specific CD4+ T cells are depleted not only in the peripheral blood but also at sites of TB disease. In one such study, there was a significant decrease the frequency of IFN-γ–secreting *M.tb*-specific CD4+ T cells in the bronchoalveolar lavage (BAL) of antiretroviral therapy (ART)-naïve PLWH compared to HIV-uninfected individuals (Kalsdorf *et al*., 2009). Furthermore, PLWH had lower *M.tb*-specific responses in BAL compared to peripheral blood. Similarly, in a study conducted in our group we reported that, despite well-maintained CD4+ T cell counts (>400 cells/µL), ART-naïve PLWH showed a significant decrease in TB responses (Bunjun *et al*., 2017). In this study, *M.tb*-specific CD4+ Th1 responses (IFN-γ, TNF-α and IL-2) were 2-fold lower in peripheral blood and 15-fold lower in the BAL of PLWH when compared to HIV-uninfected individuals (Bunjun *et al*., 2017).

In addition to affecting the quantity of *M.tb*-specific CD4+ T cells, HIV infection may also impair the functional capacity (‘quality’) of these cells. In a study of 72 participants, Day and colleagues reported that ART-naïve PLWH with LTBI had a decrease of IFN-γ+TNF-α+ double positive *M.tb*-specific CD4+ T cells compared to HIV-uninfected people (Day *et al*., 2017). Most notably, the proliferative ability of *M.tb*-specific cells in PLWH was 45-fold lower compared to HIV-uninfected individuals, with cytokine production significantly reduced in the remaining proliferating cells. Additionally, there are several other studies that also show that in comparison to HIV-uninfected individuals, there is a significantly lower proportion of IFN-γ+TNF-α+IL-2+ polyfunctional *M.tb*-specific CD4+ T cells in peripheral blood and BAL of PLWH (Kalsdorf *et al*., 2009; Jambo *et al*., 2011; Wilkinson *et al*., 2016; Amello *et al*., 2019). In contrast, we have shown that HIV infection had no significant impact on the proportion IFN-γ+TNF-α+IL-2+ polyfunctional *M.tb*-specific CD4+ T cells (Riou *et al*., 2016; Bunjun *et al*., 2017). Instead in these studies, ART-naïve PLWH demonstrated a lower proportion of IFN-γ+ single positive and IFN-γ+IL-2+ double positive *M.tb*-specific CD4+ T cells in their peripheral blood compared HIV-uninfected individuals

(Riou *et al*., 2016; Bunjun *et al*., 2017). Moreover, this decrease in IFN-γ+ single positive cells was inversely associated with HIV plasma viral loads, suggesting that the depletion of these cells was linked to HIV replication (Riou *et al*., 2016).

The mechanisms by which HIV infection impairs the functional profile of *M.tb*-specific CD4+ T cells is still poorly understood. However, our recent studies suggest that HIV infection may skew the lineage-defining transcriptional profiles of these cells. We showed that PLWH had a lower proportion of *M.tb*-specific CD4+ T cells co-expressing the transcriptional factors Foxp3 (Treg cells) and T-bet^{high} (Th1 cells) compared to HIV-uninfected individuals (Riou *et al*., 2016). Furthermore, the proportion of these T-bet^{high} Foxp3+ *M.tb*-specific CD4+ T cells was inversely correlated with HIV viral loads and TNF-α concentrations in the plasma. This suggests that the decrease of these cells in PLWH is related to HIV viral replication as well as the inflammatory status of the individuals (Riou *et al*., 2016). Related to this, a study by Amello and colleagues showed that HIV infection altered the cytokine profile of *M.tb*-specific CD4+ T cells from a polyfunctional Th1/Th2/Th17 profile into a Th1-restricted profile (Amello *et al*., 2019). PLWH had a decrease in *M.tb*-specific CD4+ T cells expressing the transcription factors Gata-3 (Th2) and RORγt (Th17) and instead an increase in cell expressing of T-bet (Th1), compared to HIV-uninfected individuals.

The function of T cells is linked to their phenotype. Not much is known about how HIV infection affects the activation and phenotype of *M.tb*-specific CD4+ T cells. Wilkinson and colleagues reported that in PLWH, more IFN-γ-secreting *M.tb*-specific cells expressed the activation marker HLA-DR than HIV-uninfected individuals, and this expression was further increased in PLWH with active TB (Wilkinson *et al*., 2016). This study demonstrated that in PLWH with LTBI, the activation of *M.tb*-specific CD4+ T cells mirrored the activation of overall CD4+ T cell compartment (Wilkinson *et al*., 2016). This suggests that the high activation of *M.tb*-specific cells in these individuals was most likely a consequence of HIV-associated systemic activation. It is not clear how HIV infection affects the memory profile *M.tb*-specific CD4+ T cells. To date, several studies focusing on TB responses in the peripheral blood have reported no differences in the memory profile of *M.tb*-specific CD4+ T cells between PLWH and HIV-uninfected people (Riou e*t al*., 2016; Riou *et al*., 2017; Day *et al*., 2017). However, a prior study by Matthews and colleagues reported that HIV infection is associated with an altered memory profile of *M.tb*-specific CD4+ T cells at the site of TB disease in individuals with extrapulmonary TB (Matthews *et al*., 2012). In that study, PLWH had a higher proportion of less differentiated (CD28+CD45RA-) *M.tb*-specific cells in pericardial fluid compared to HIV-uninfected people. In addition, PLWH had higher HIV viral loads in pericardial fluid compared to peripheral blood, and this was inversely correlated with the frequency of *M.tb*-specific CD4+ T cells at this site. Thus, HIV and *M.tb* replication at the site of disease may have skewed the memory differentiation profile of *M.tb*-specific CD4+ T cells.

In this study, we aimed to confirm and extend the ‘early depletion’ findings by Geldmacher *et al.* (2008) in a larger cohort of PLWH. To do this, we performed a detailed analysis of the dynamics of *M.tb*-specific CD4+ T cell responses longitudinally over the course of HIV infection. Additionally, in order to ascertain whether the observed changes in immune responses over time were attributable to HIV infection, we also examined the natural variation in *M.tb*-specific responses prior to HIV infection.

3.2 Materials and methods

All materials and methods used in this study are described in Chapter 2.

3.3 Results

3.3.1 Natural variation in M.tb-specific CD4+ T cell responses

We first wished to ascertain the degree of natural variation of *M.tb*-specific T cell responses over time in healthy, HIv-uninfected people, in order to interpret any changes we might observe during HIV infection. We examined the natural fluctuation of *M.tb*-specific T cell responses longitudinally in a subgroup of nine participants prior to HIV infection. We used cryopreserved PBMC from South African women enrolled in the CAPRISA 004 study from Durban, KwaZulu-Natal. PBMC were stimulated with a pool of peptides based on dominant T cell targets from *M.tb* ('MTB300', Arlehamn *et al*., 2016). As expected from *M.tb* exposure/latent TB infection, we rarely detected CD8+ T cell responses to *M.tb,* and thus only present *M.tb*-specific CD4+ T cell responses throughout this Chapter. In this HIV-uninfected subgroup, we assessed two timepoints, denoted as T1 and T2; T1 was measured at a median of 11 months (IQR 6-17) prior to HIV infection and T2 at a median of 6 months (IQR 2-9.5) (**Figure 3.1A**). T1 and T2 were a median of 3 months apart (IQR 3-8). **Figure 3.1B** shows a representative example of the frequency of *M.tb*-specific CD4+ T cells, producing IFN-γ, TNF-α, IL-2 or MIP-1β, at T1 and T2 in one participant. All 9 participants had a detectable *M.tb*-specific response (measured as total cytokine response) at both time points. For most participants the frequency of total *M.tb*-specific CD4+ T cells remained comparable between T1 and T2, from a median of 0.29% (IQR 0.16-0.35) to 0.25% (IQR 0.08-0.25) (**Figure 3.1C**). Only 2/9 participants had either a decrease or an increase of ≥ 50%. Of note, although not statistically significant, there was a marginal association between the length of time between T1 and T2 and the percentage change in responses between the two timepoints (p=0.0635) (**Figure 3.1D**). However, there was some variation in individual cytokine responses within the participants (**Figure 3.1E**). While the majority (8/9) of participants had IFN-γ and TNF-α responses at both T1 and T2, there were a few participants who either completely lost or regained their IL-2 responses between the two timepoints. Taken together, these results indicate that there is some natural variation in *M.tb*-specific CD4+ T cell responses in the absence of HIV infection, and this variation appears independent of the length of time between the two measured timepoints.

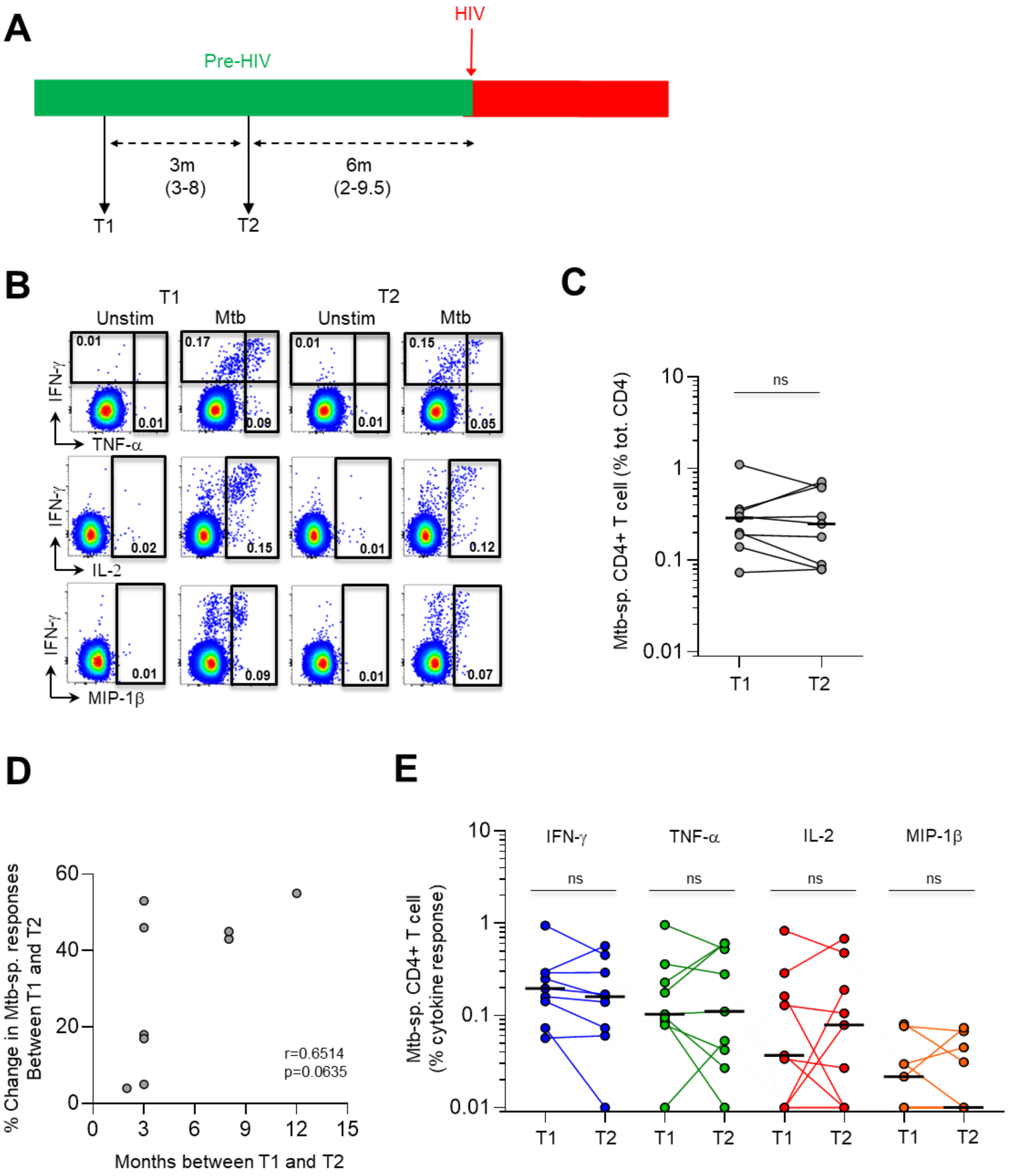
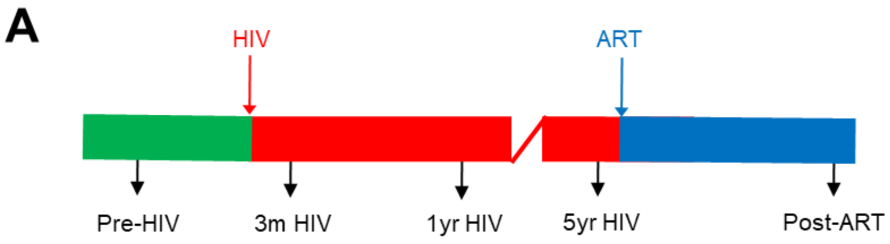


Figure 3.1: Natural variation in *M.tb*-specific CD4+ T cells prior to HIV infection. (A) A study schema showing the two timepoints assessed (T1 and T2) prior to HIV infection (green). **(B)** Representative flow cytometry plots showing IFN-γ, TNF-α, IL-2 and MIP-1β responses at T1 and T2 after stimulation with *M.tb* peptides in a single donor. **(C)** Frequency of total *M.tb*-specific CD4+ T cells at timepoints T1 (n=9) and T2 (n=9). **(D)** Spearman correlations assessing the relationship between the length of time (in months) between T1 and T2 and percentage change in *M.tb*-specific responses between the two timepoints. **(E)** Frequency of IFN-γ, TNF-α, IL-2 and MIP-

1β–producing *M.tb*-specific CD4+ T cells in participants at timepoints T1 and T2. Black horizontal bars indicate the median values. Statistical significance was calculated using Wilcoxon matched-pairs. Statistically non-significant P-values (P>0.05) are annotated as ns.

3.3.2 The impact of HIV infection on M.tb-specific CD4+ T cells

To characterise the impact of HIV on TB T cell immunity we performed a cross-sectional analysis of *M.tb*-specific responses in 58 participants (**Figure 3.2**). Participants were examined at five timepoints, namely prior to HIV infection (median: 6.5 months, IQR 10-4), soon after HIV infection (median: 3 months, IQR 3-3.75), 1 year after infection (median: 12 months, IQR 11-12), at chronic infection (median: 60 months, IQR 40-71) and 2 years after ART initiation (median: 57.5, IQR 31-98.5) (**Figure 3.2A**). **Figure 3.2B** shows the clinical characteristics of these participants. To note, not all participants had samples available at all timepoints. Of the 30 HIV-infected participants we examined prior to HIV infection, 77% had a *M.tb*-specific CD4+ T cell response (pie charts, **Figure 3.2C**). In contrast, in individuals with acute and chronic HIV infection, although not significantly different, the frequency of *M.tb* responders was lower (58-59%) when compared to HIV-uninfected participants. Interestingly, at the post-ART timepoint there was a similar frequency of *M.tb* responders as HIV-uninfected individuals (77%). When we assessed the magnitude of the T cell response, there were no significant differences in the median frequencies of total *M.tb*-specific CD4+ T cells between HIV-uninfected participants and those in the acute and chronic phases



B

	Pre HIV	3m post HIV	1yr post HIV	5yr post HIV	2yr post ART
n	30	44	12	27	43
Gender (Female)	100%	100%	100%	100%	100%
Age (median, IQR)	23.5 (22-26)	25 (22.25-28)	28 (24.25-30)	30 (27-34)	30 (27.75-34)
Months after HIV infection (median, IQR)	-6.5 (-10--4)	3 (3-3.75)	12 (11-12)	60 (40-71)	57.5 (31-98.5)

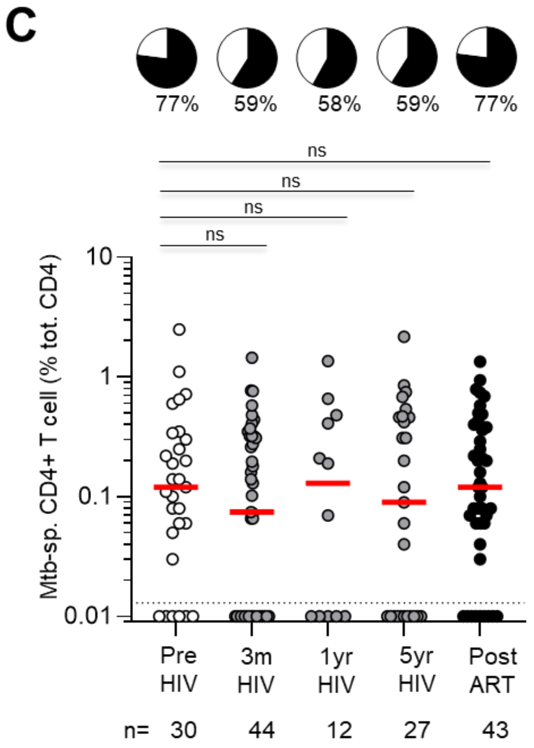


Figure 3.2: Cross-sectional analysis of *M.tb*-specific CD4+ T cells prior to and after HIV infection. (A) Study schema showing timepoints prior to HIV infection (green), after HIV infection (red) and post ART initiation (blue). **(B)** Clinical characteristics of the study groups at the different timepoints assessed. **(C)** Frequency of total *M.tb*-specific CD4+ T cells in participants at timepoints prior to and after HIV infection. Pies and percentages show the frequency of *M.tb* responders at each

timepoint. Red horizontal bars represent the medians. Statistical significance was calculated using one-way ANOVA with Dunn’s correction. Statistically non-significant P-values (P>0.05) are annotated as ns.

of HIV infection. Of note, the were no significant differences in the median viral load (VL) and absolute CD4+ T cell count of participants with acute and chronic HIV infection (**Figure 3.3A and 3.3B**). However, as expected, in participants on ART, the median VL was lower when compared to those with acute HIV infection (9,854 copies/mL [IQR 1,770-36,159] *vs.* undetectable, p= 0.0390), with the majority (35/43) achieving VL suppression. Likewise, the median absolute CD4+ T cell count of participants on ART was significantly higher compared to those with acute HIV infection (563 cells/µL [IQR 461-727] *vs.* 735 cells/µL [IQR 595-856], p=<0.0001). Together, these cross-sectional analyses indicate that while we did not detect lower frequencies of *M.tb*-specific T cells during HIV infection, 20% fewer individuals had detectable *M.tb*-specific responses during HIV infection, compared to pre-HIV and post-ART timepoints.

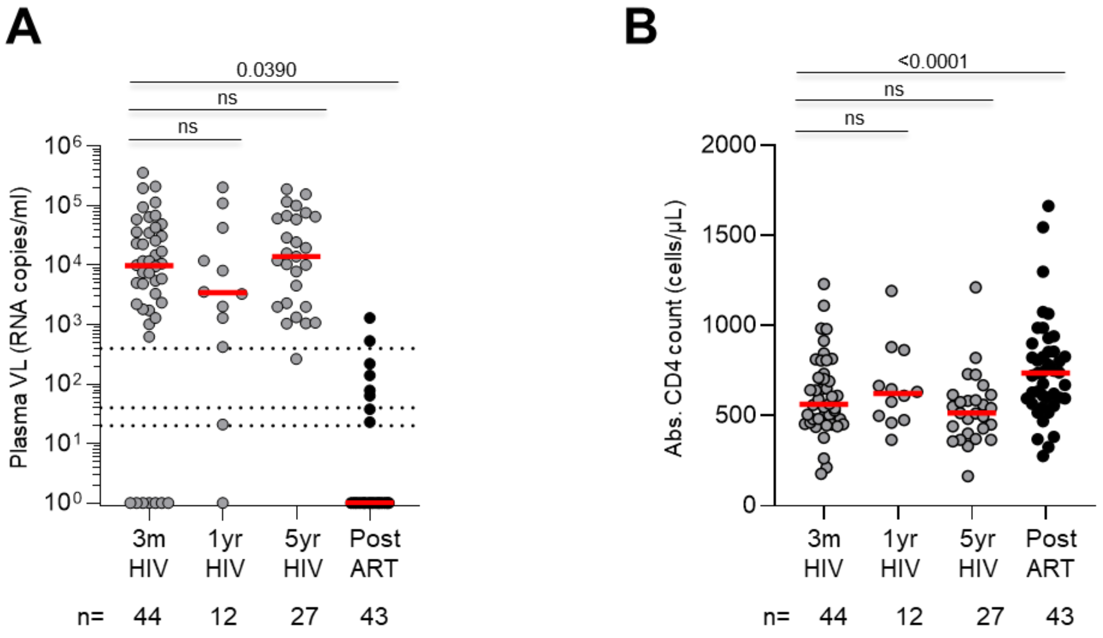


Figure 3.3: Clinical characteristics of study participants. (A) Plasma viral load and **(B)** absolute CD4+ T cell count of participants after HIV infection. The horizontal dashed lines indicate the detection limits of the assay, which differed over the years of the study. Red horizontal bars indicate the median values. Statistical significance was calculated using one-way ANOVA with Dunn's correction . Statistically non-significant P-values (P>0.05) are annotated as ns.

Next we examined whether HIV infection had any effect on the functional profile of *M.tb*-specific CD4+ T cells. We compared the polyfunctional profiles of *M.tb*-specific CD4+ T cells of HIV-uninfected participants and individuals with acute HIV infection (median: 3 months) using a Boolean gating strategy (**Figure 3.4)**. The polyfunctional profiles *M.tb*-specific CD4+ T cells were comparable between HIV-infected and uninfected participants, with responses consisting mainly of cells co-expressing three cytokines (IFN-γ, TNF-α and IL-2; ~30%), IL-2+TNF-α+ or IFN-γ+IL-2+ dual functional cells or IFN-γ+ cells (~10% each) (**Figure 3.4A**). Similar results were observed when we compared HIV-uninfected participants to those on ART (**Figure 3.4B**).

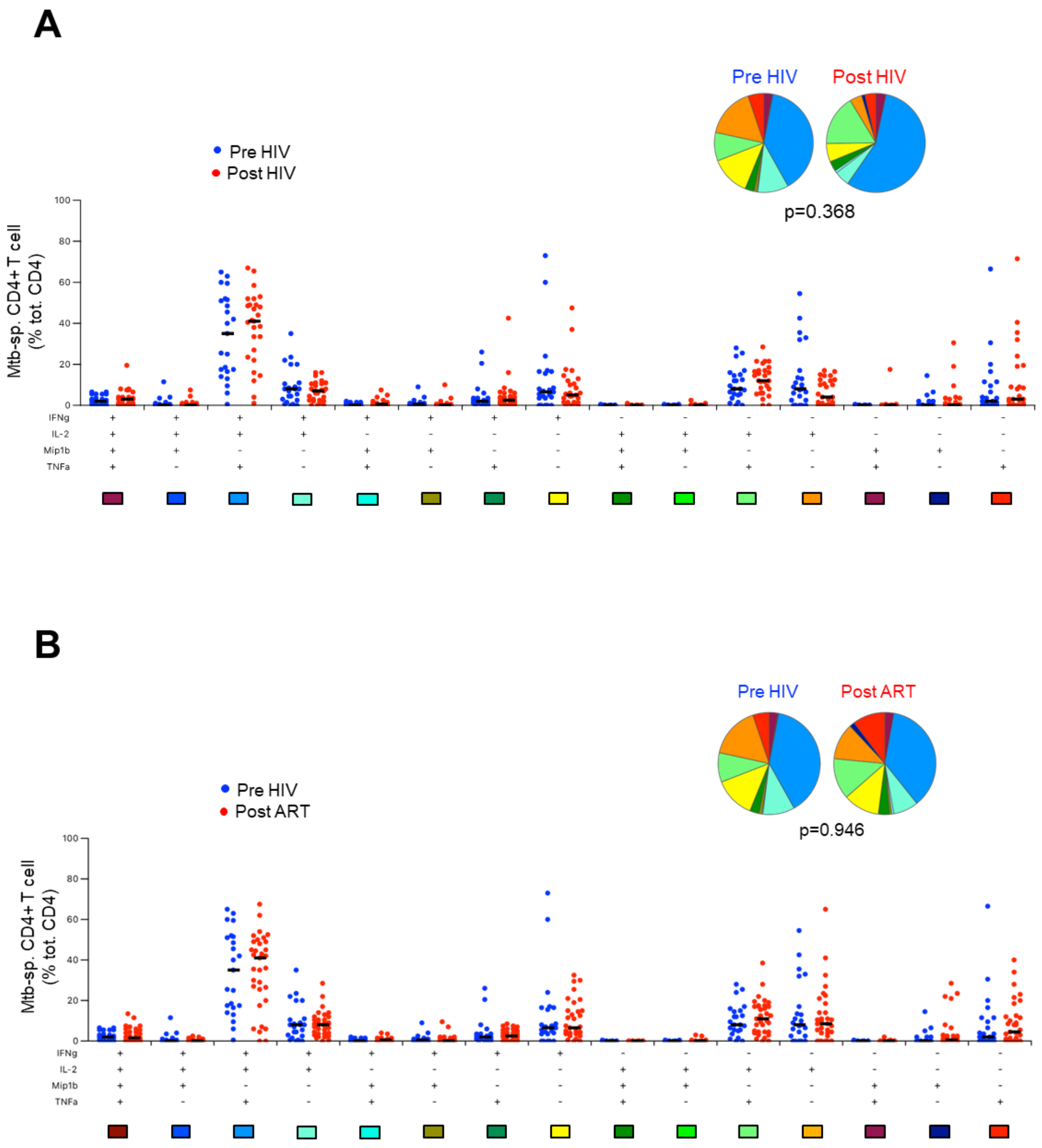


Figure 3.4: **The polyfunctional profile of *M.tb*-specific CD4+ T cells.** Proportion of different combinations of IFN-γ, TNF-α, IL-2 and MIP-1β responses in **(A)** HIV-uninfected participants (blue, n=30) and those infected for a median of 3 months (red, n=44) and **(B)** HIV-uninfected participants (blue, n=30) and participants on ART (red, n=43). The coloured bars (underneath) and pie chart slices represent different cytokine combinations produced by *M.tb*-specific CD4+ T cells (e.g. a light blue bar/slice is cells producing IFN-γ, IL-2 and TNF-α, in the absence of MIP-1β).

Next, to determine the phenotypic characteristics of *M.tb*-specific CD4+ T cells after HIV infection, we measured the activation marker HLA-DR (**Figure 3.5**) and the memory differentiation markers CD45RA and CD27 (**Figure 3.6**). Analysis of HLA-DR expression showed no significant differences in the frequencies of activated *M.tb*-specific CD4+ T cells between HIV-infected and uninfected participants (**Figure 3.5A**). In contrast, when we analysed the overall CD4 compartment, we observed a higher frequency of total HLA-DR+ CD4+ T cells in participants during acute HIV infection compared to HIV-uninfected individuals (median: 2.4% [IQR 2-3.2] vs 1.5% [IQR 1.2-2.2] respectively, p=0.0209) and in those with chronic HIV infection (median: 3.6% (IQR 2.9-5), p=<0.0001) (**Figure3.5B**). Of note, there was a positive correlation between the frequency of HLA-DR+ *M.tb*-specific CD4+ T cells and total HLA-DR+ CD4+ T cells (p= 0.0067, r=0.518). This suggested that the profile of HLA-DR expression on *M.tb*-specific CD4+ T cells mirrors that of total CD4+ T cells. Measurement of CD45RA and CD27 enabled the detection of four memory subsets, namely early differentiated (CD45RA-CD27+), late differentiated (CD45RA-CD27-), terminally differentiated (CD45RA+CD27-) and naïve (CD45RA+CD27+) T cells (**Figure 3.6**). The frequency of *M.tb*-specific CD4+ T cells in **Figure 3.6B-E** is overlaid onto the frequencies of the memory subsets found in total CD4+ T cells, indicated as light grey bars. *M.tb*-specific CD4+ T cells in HIV-infected and uninfected participants had a predominately early differentiated (ED) (~60%) profile at all timepoints assessed (**Figure 3.6B**). Overall, there were no differences in the memory phenotype of total CD4+ T cells in HIV-infected and uninfected individuals (**Figure 3.6A-E**).

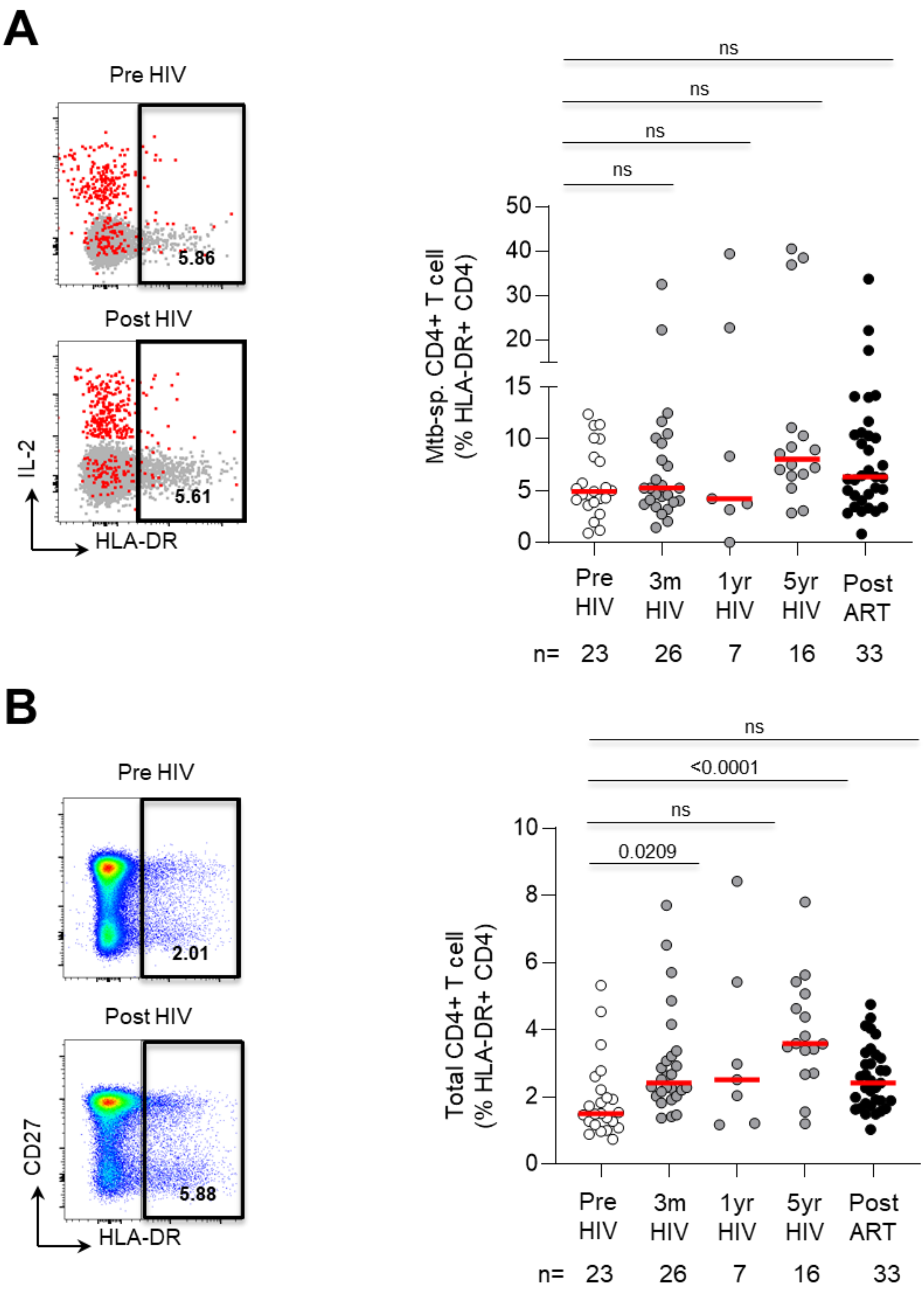


Figure 3.5: **Frequency of HLA-DR+ CD4+ T cells. (A)** Representative flow cytometry plots and the frequency of HLA-DR+ total (grey dots) and *M.tb*-specific (red dots) CD4+ T cells in participants at timepoints prior to and after HIV infection. **(B)** Representative flow cytometry plots and the frequency of total HLA-DR+ CD4+ T cells in participants at timepoints prior to and after HIV infection. Red horizontal bars indicate the median values. Statistical significance was calculated using one-way

ANOVA with Dunn's correction. Statistically non-significant P-values (P>0.05) are annotated as ns.

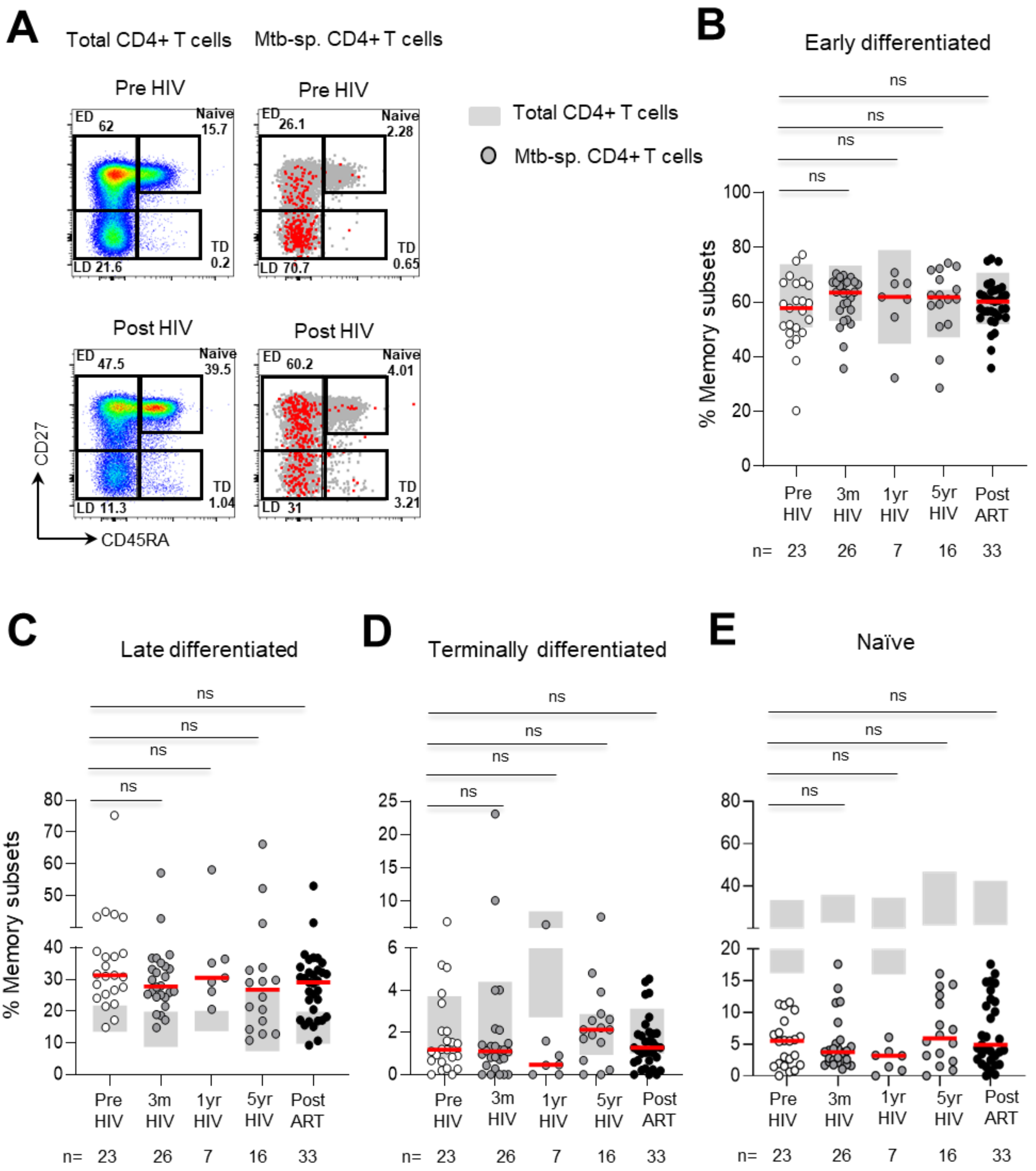


Figure 3.6: The memory differentiation profiles of CD4+ T cells. (A) Representative overlay plots showing memory subsets in total (grey dots) and *M.tb*-specific (red dots) CD4+ T cells prior to and after HIV infection. Frequency of **(B)** early differentiated (ED: CD27+CD45RA-), **(C)** late differentiated (LD: CD27-CD45RA-), **(B)** terminally differentiated (TD: CD27-CD45RA+) and **(E)** Naive (CD27+CD45RA+) *M.tb*-specific (dark grey dots) and total CD4+ T cells (light grey floating bars indicating

interquartile ranges) at timepoints prior to and after HIV infection. Red horizontal bars indicate the median values. Statistical significance was calculated using one-way ANOVA with Dunn's correction. Statistically non-significant P-values ($P>0.05$) are annotated as ns.

3.3.3 The kinetics of M.tb-specific CD4+ T cell responses during HIV infection

To measure *M.tb*-specific responses longitudinally prior to and after HIV infection, we selected out the 17 participants who had paired timepoints. We first examined the participants at two timepoints, prior to and 3 months after HIV infection, at a median of 10 months (IQR 8-14.5) apart (**Figure 3.7A**). Three months after HIV infection there was a significant decrease in the frequency of total *M.tb*-specific CD4+ T cells, from a median of 0.20% (IQR 0.09-0.47) to 0.11% (IQR 0.01-0.28), p=0.0429 (**Figure 3.7D**). Importantly, of the 17 participants examined, 6 participants lost their peripheral *M.tb*-specific responses ~3 months after HIV infection, decreasing the frequency of responders from 100% before HIV infection to 65% (p=0.007). Based on the percentage change in *M.tb*-specific responses, participants were grouped into two, namely those who maintained their *M.tb*-specific responses after HIV infection (% change of <50%) and those who lost 50% or more of their responses (**Figure 3.7B and 3.7C**). **Figures 3.7E-G** show the clinical characteristics of these two groups. There were no significant differences in median plasma VL, absolute CD4 counts or CD4/CD8 ratios of participants after HIV infection. Interestingly, four participants had undetectable VL at the 3 month timepoint, despite being untreated, and these were enriched in the group who maintained their *M.tb*-specific responses.

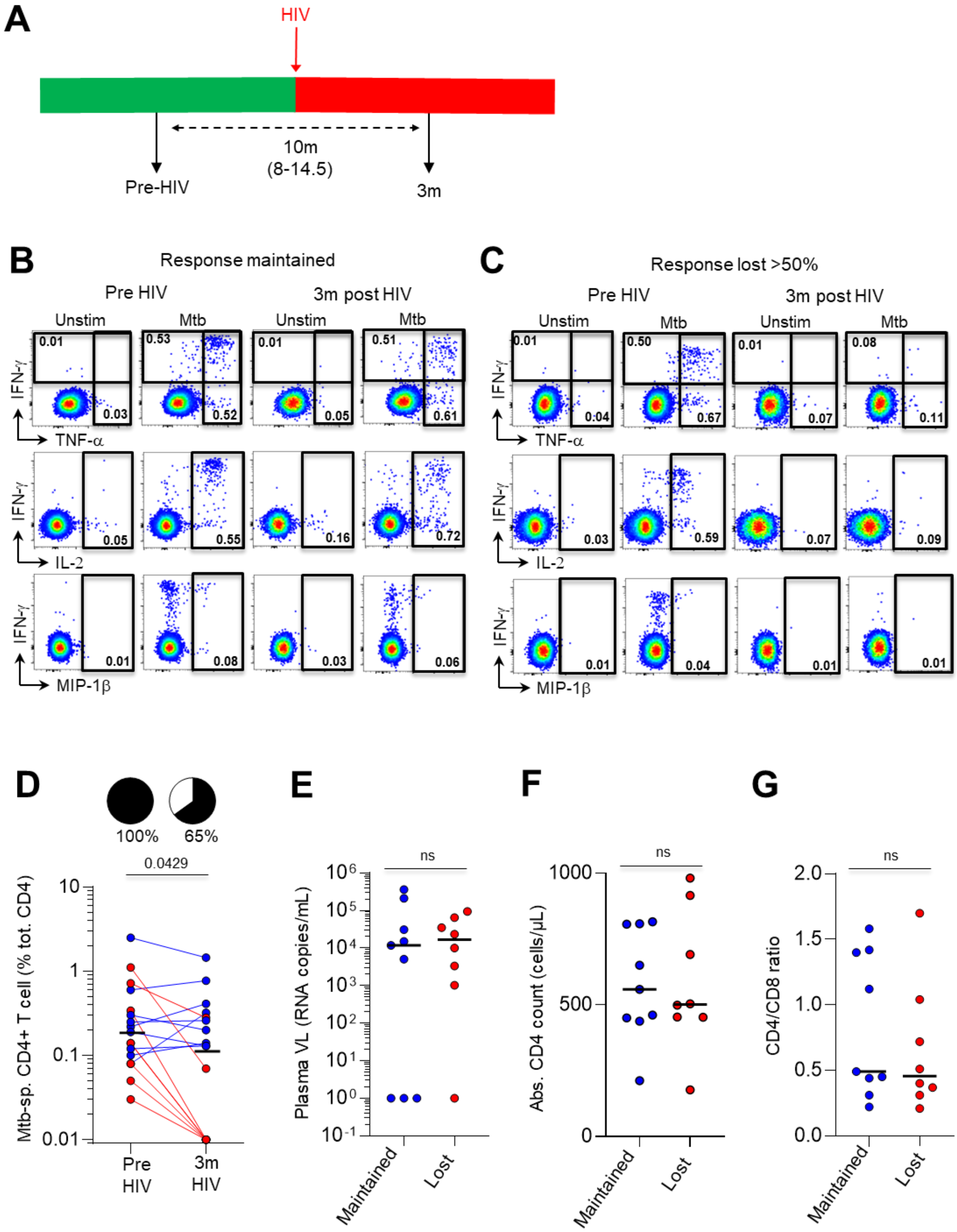


Figure 3.7: **Frequency of *M.tb*-specific CD4+ T cells prior to and after HIV infection in paired samples**. **(A)** A study schema showing two timepoints prior to (green) and 3 months after HIV infection (red). Representative flow cytometry plots showing IFN-γ, TNF-α, IL-2 and MIP-1β responses prior to and 3 months after HIV infection in participants who **(B)** maintained their *M.tb*-specific response and **(C)** lost

more than 50% of their response. **(D)** Frequency of total *M.tb*-specific CD4+ T cells in paired participants prior to and 3 months after HIV infection (n=17). Participants who maintained their *M.tb*-specific responses are indicated in blue (n=9) and those who lost 50% or more of their responses in red (n=8). Pies and percentages show the frequency of *M.tb* responders at each timepoint. **(E)** Plasma viral load, **(F)** absolute CD4+ T cell count and **(G)** CD4/CD8 ratios at 3 months after HIV infection in participants within the two groups. Black horizontal bars indicate the median values. Statistical significance was calculated using Wilcoxon matched-pairs and Mann-Whitney U tests. Statistically non-significant P-values (P>0.05) are annotated as ns.

Previous reports show that *M.tb*-specific CD4+ T cells secrete significantly lower levels of the CCR5 ligand MIP-1β and instead produced high levels of IL-2 (Geldmacher *et al*., 2008; 2010). **Figure 3.8A** shows the frequency of IFN-γ, TNF-α, IL-2 and MIP-1β-producing *M.tb*-specific CD4+ T cells, prior to HIV infection, in participants who maintained their *M.tb*-specific responses and those who lost 50% or more of their responses. We found no significant differences in frequencies of IFN-γ, TNF-α, IL-2 and MIP-1β-producing *M.tb*-specific CD4+ T cells between the two groups. In fact, only a minority of the participants (5/17) produced MIP-1β in response to *M.tb* stimulation. Next, to determine whether the maintenance of *M.tb*-specific responses was linked to a particular function or phenotype prior to HIV infection, we compared the polyfunctional and phenotypic profile of *M.tb*-specific CD4+ T cells between the two groups. The polyfunctional profiles (**Figure 3.8B**) and memory phenotypes (**Figure 3.9A-D**) did not differ between participants who maintained their *M.tb*-specific responses and those who lost 50% or more of their responses.

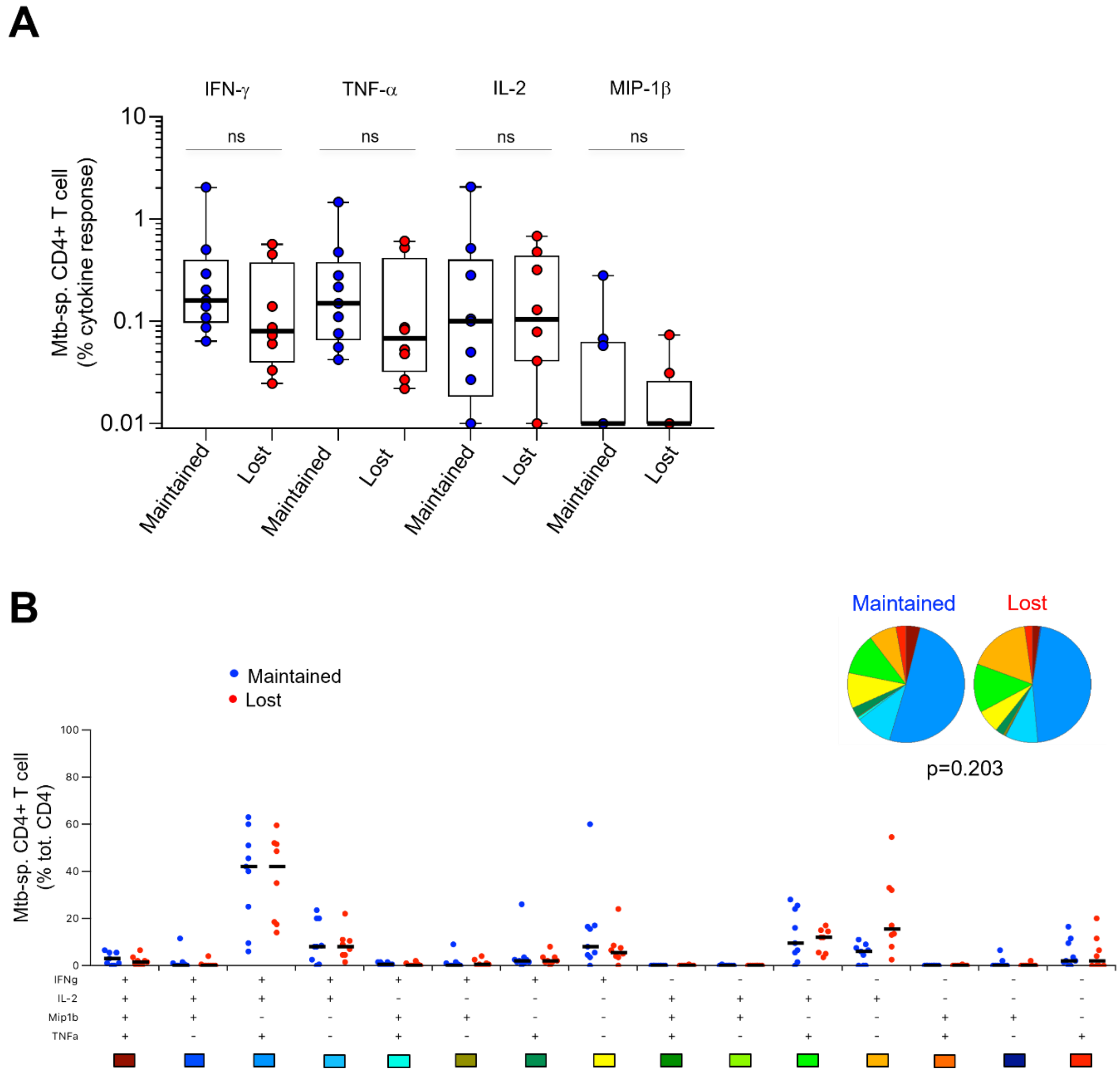


Figure 3.8: **The function of *M.tb*-specific CD4+ T cells.** **(A)** Frequency of IFN-γ, TNF-α, IL-2 and MIP-1β-producing *M.tb*-specific CD4+ T cells measured prior to HIV infection in participants who maintained responses (blue, n=9) and those who lost 50% or more of their responses (red, n=8) 3 months after HIV infection. **(B)** Proportion of different combinations of IFN-γ, TNF-α, IL-2 and MIP-1β responses prior to HIV infection in the same participants. Coloured bars underneath the graph and pie chart slices represent different cytokine combinations produced by *M.tb*-specific CD4+ T cells (e.g. a light blue bar/slice is cells producing IFN-γ, IL-2 and TNF-α, in the absence of MIP-1β). Black horizontal bars indicate the median values. Statistical

significance was calculated using a Mann-Whitney U test. Statistically non-significant P-values (P>0.05) are annotated as ns.

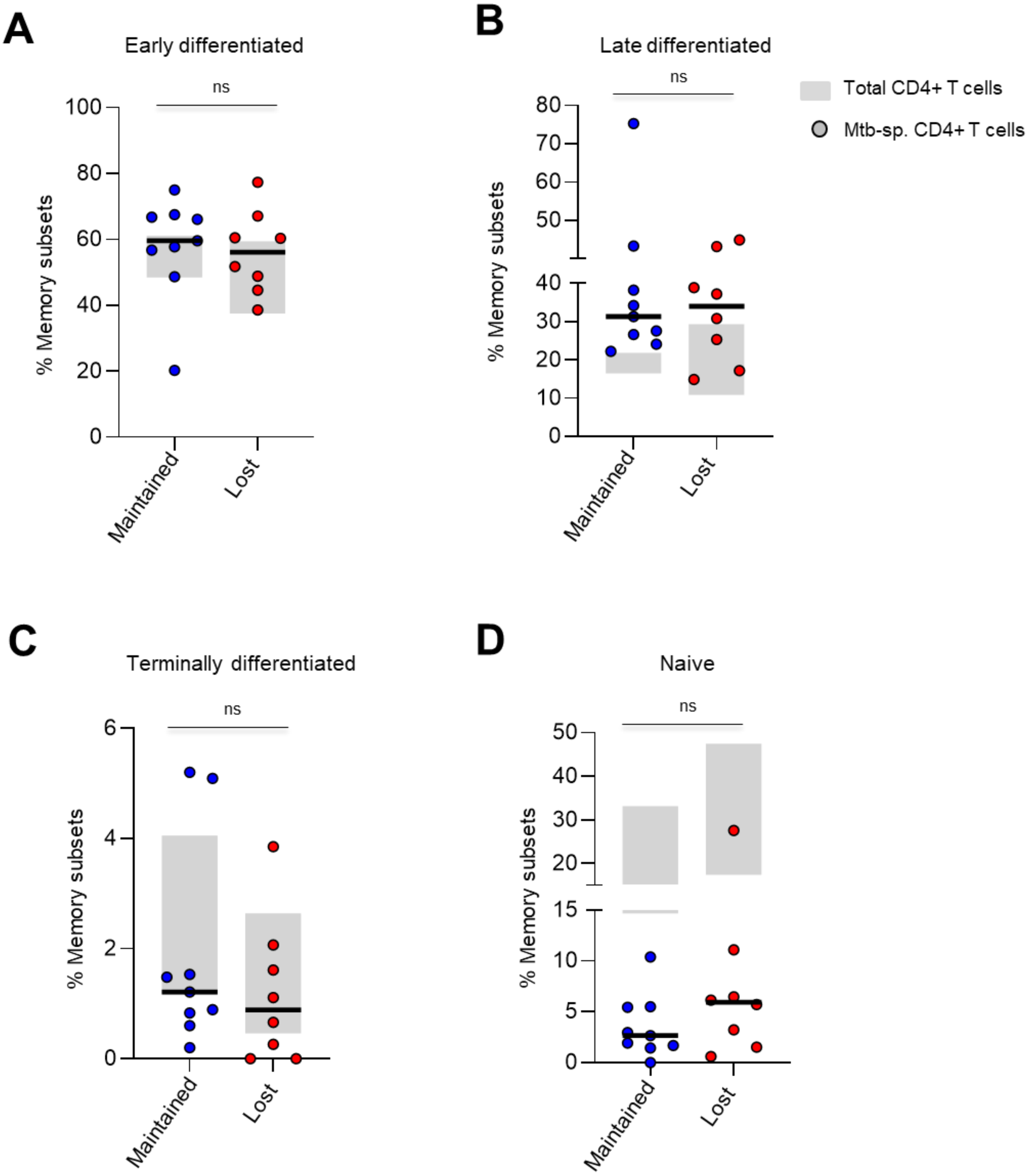


Figure 3.9: **Memory differentiation profiles in those who maintained or lost their of *M.tb*-specific CD4+ T cells responses 3 months after HIV infection.** Frequency of **(A)** early differentiated (ED: CD27+CD45RA-) and **(B)** late differentiated (LD: CD27-

CD45RA-), **(C)** terminally differentiated (TD: CD27-CD45RA+) and **(D)** Naive (CD27+CD45RA+) *M.tb*-specific (coloured dots) and total CD4+ T cells (light grey floating bars indicating interquartile ranges) prior to HIV infection in participants who maintained responses (blue, n=9) and those who lost 50% or more of their responses (red, n=8) at 3 months post HIV infection. Black horizontal bars indicate the median values. Statistical significance was calculated using the Mann-Whitney U test. Statistically non-significant P-values (P>0.05) are annotated as ns.

Lastly, within the two groups, we measured the kinetics of *M.tb*-specific responses longitudinally over the five timepoints (**Figures 3.10A and 3.10B).** In participants who maintained their responses, the frequencies of *M.tb*-specific CD4+ T cells remained relatively unchanged throughout acute and chronic HIV infection (**Figure 3.10A**). For only one participant in this group *M.tb*-specific responses became undetectable 1y post HIV infection but re-emerged during the chronic phase of infection. Of the six participants who lost their *M.tb*-specific response after infection, four participants restored their responses at later timepoints to frequencies similar to or greater than that prior to HIV infection (**Figure 3.10B**). This mostly occurred prior to ART initiation. Taken together, these data indicate that early after HIV infection there is a decrease in the frequency *M.tb* responders. Notably, not all participants lost their responses after HIV infection, and there is a variation in *M.tb*-specific responses.

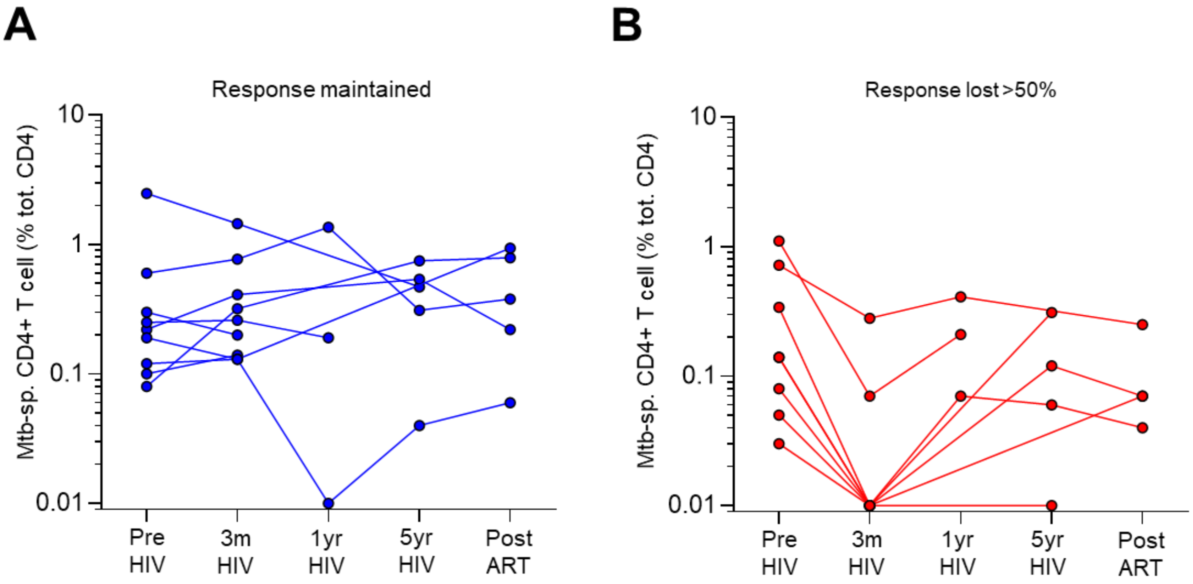


Figure 3.10: **Longitudinal analysis of *M.tb*-specific CD4+ T cells prior to and after HIV infection**. Frequency of total *M.tb*-specific CD4+ T cells in participants who **(A)** maintained *M.tb*-specific responses (n=9) and **(B)** lost 50% or more of responses (n=8) 3 months after HIV infection. Responses were measured at various timepoints after HIV infection and 2 years after ART initiation, where samples were available.

3.4 Discussion

HIV infection is the greatest risk factor for developing active tuberculosis (TB), with studies showing an increased risk of developing active TB even during the first year of infection when CD4+ T cell counts are still high (Kauffman and McMichael, 2005; Sonnenberg *et al*., 2005; Getahun *et al*., 2010). Previous longitudinal studies suggested that depletion of *M.tb*-specific CD4+ T cells in the first year of HIV infection may contribute to the early onset of TB in PLWH (Geldmacher *et al*., 2008; 2010). In this study, in order to confirm and extend this "early depletion" hypothesis, we performed a detailed analysis of the dynamics of *M.tb*-specific CD4+ T cell responses longitudinally over the course of HIV infection. Our main findings show that there is a significant decrease in the frequency of *M.tb* responders and the magnitude of *M.tb*-specific CD4+ T cells 3 months after HIV infection.

Our findings are consistent with prior reports by Geldmacher and colleagues where, in a small longitudinal cohort, they showed that in peripheral blood there was rapid depletion of *M.tb*-specific CD4+ T cells during acute HIV infection (Geldmacher *et al*., 2008; 2010). In these studies, of the five participants they examined, four had a decrease in *M.tb*-specific responses within a year of HIV infection, with 3/5 (60%) completely losing all their responses. In our study, we found that half (8/17) of our participants in the longitudinal analysis had a decrease in the frequency of *M.tb*-specific CD4+ T cells 3 months after HIV infection, with 6/17 (35%) completely losing their responses. Several factors may account for the difference in frequency of responders after HIV infection between our study and these earlier reports. The main contributing factor is likely the size of our cohort, where we examined a larger cohort of participants (n=17). Additionally, while the previous study examined *M.tb*-specific responses to two separate peptide pools ESAT-6 and CFP-10 as well as tuberculin purified protein derivative (PPD), in our study we used a larger peptide pool consisting of 300 *M.tb*-derived peptides. This may have influenced our results as it has been previously reported that the magnitude *M.tb*-specific T cell responses and the frequency of *M.tb* responders can be influenced by the type of mycobacterial antigens used in a stimulation (Day *et al*., 2008). It is also important to note that some of the 300 peptides used in our study are also found in the TB vaccine Bacille Calmette-Guérin (BCG) which is given to all infants at birth in South Africa. This may suggest that in our study we had a broader spectrum of CD4+ T cell epitopes cross-reactive to *M.tb* compared to the Geldmacher *et al.* (2008) study.

In our cross-sectional analysis, we found that 77% of our HIV-uninfected cohort had a *M.tb*-specific CD4+ T cell response prior to HIV infection. These results are in accordance with earlier findings that show that ~80% of South Africans have an *M.tb*-specific CD4+ T cell response (Rangaka *et al*., 2007), reflecting the high endemicity of *M.tb* in our setting. In contrast to our longitudinal data, we found no significant differences in the frequency of responders between HIV-infected and uninfected participants. However, we did observe an approximately 20% lower frequency of *M.tb* responders during acute and chronic HIV infection when compared to HIV uninfected participants and participants on ART. This discrepancy in our cross-sectional and longitudinal data may be due the fact that the data obtained from longitudinal analysis is more robust as we were able to follow the same individuals over two timepoints. Similar to our study, a previous cross-sectional study measuring blood responses showed a 28% decrease in the frequency of *M.tb* responders in PLWH when compared to between HIV-uninfected individuals (Geldmacher *et al*., 2008). In contrast, other studies have shown that despite there being a significant decrease in *M.tb*-specific CD4+ T cell responses in the peripheral blood of PLWH, all participants still had an *M.tb*-specific response irrespective of HIV status (Jambo *et al*., 2011 Bunjun *et al,* 2017). Interestingly, Bunjun *et al*., (2017) showed that when *M.tb*-specific CD4+ T cells responses were measured in the BAL of participants instead of the peripheral blood, the frequency of *M.tb* responders was lower (50%) in PLWH when compared to HIV-uninfected individuals (79%).

To examine the impact of HIV infection on the magnitude of TB responses we measured the frequency of *M.tb*-specific CD4+ T cells over the course of HIV infection. Results of our longitudinal analysis showed a significant decrease in the frequency of *M.tb*-specific CD4+ T cells 3 months after HIV infection. In this analysis half of the cohort had a decrease of 50% or more in *M.tb*-specific responses after HIV infection. To define these participants as having a decrease in responses we measured longitudinally the natural fluctuations in *M.tb*-specific responses prior to HIV infection in a small sub-group of participants. We found that for the majority of participants in this group, *M.tb*-specific responses were well maintained, with only two participants having a decrease of approximately 50%. In our study, of the 8/17 participants that had a decrease *M.tb*-specific responses 3 months after HIV infection, 6 completely lost their responses, while the other two retained their responses but with decrease of more than 50%. Interestingly, these two participants who didn’t completely lose their *M.tb*-specific responses, their median frequency of *M.tb*-specific CD4+ T cells prior to HIV infection were 8-fold higher when compared to the other six participants. This suggests that these two participants may have retained some of their *M.tb*-specific responses during acute HIV infection because they had fairly high responses prior to infection. In our cross-sectional study, although not significantly different, we also found that the median frequency of total *M.tb*-specific CD4+ T cells (IFN-γ, TNF-α, IL-2 and MIP-1β) in participants with acute HIV infected (median of 3 months) was 1.5-fold lower in comparison to the HIV-uninfected individuals. In a previous cross-sectional study in our lab we have shown that the frequency of total *M.tb*-specific CD4+ T cells (IFN-γ, TNF-α and IL-2) was 2-fold lower in the peripheral blood and 15-fold lower in the BAL of PLWH when compared to HIV-uninfected individuals (Bunjun *et al*., 2017). Geldmacher and colleagues also showed that in comparison to HIV-uninfected individuals, IFN-γ-producing *M.tb*-specific CD4+ T cells were 9-fold lower in peripheral blood of PLWH (Gelmacher *et al*., 2008). In another cross-sectional study, it was reported that while there were no differences in the frequency of total *M.tb*-specific CD4+ T cells (INF-γ and TNF-α) in the peripheral blood of HIV-infected and uninfected individuals, BAL responses in PLWH were 11-fold lower (Jambo *et al*., 2011). Similarly, Kalsdorf and colleagues also reported that in BAL the frequency of IFN-γ- producing *M.tb*-specific CD4+ T cells was 3.3-fold lower in PLWH compared with HIV-uninfected individuals (Kalsdorf *et al*.,2009). Taken together these reports suggest that during HIV infection depletion of *M.tb*-specific CD4+ T cells is more severe in the site of TB disease (lungs) compared to the periphery.

To determine the kinetics of *M.tb*-specific CD4+ T cell responses over the course of HIV infection we measured TB responses longitudinally over five timepoints. We found that of the group (9/17) who maintained their *M.tb*-specific responses 3 months after HIV infection, 8 of these had very little change in their responses throughout the five timepoints. Only one participant in this group had major variations in responses during the acute and chronic phase of HIV infection. However, there were very few changes in the clinical characteristics of this individual (VL, CD4 count and CD4/CD8) during HIV infection. In the group (8/17) who lost 50% or more of their *M.tb*-specific responses, six participants completely lost their responses after 3 months of HIV infection. Of these, four participants regained their responses at later timepoints at frequencies similar to or greater than prior to HIV infection. Interestingly, in all four of these participants, *M.tb*-specific responses were restored prior to ART initiation. This indicates that in these participants *M.tb*-specific CD4+ T cells were replenished even during chronic HIV infection. A likely cause of this could be re-exposure to *M.tb* antigens either from outside the host (*M.tb* infection) or from within the host (*M.tb* re-activation, although no TB disease was reported in these individuals during the study period). Given that South Africa has a high prevalence of TB (WHO Global TB Report, 2019; 2021; Rangaka *et al*., 2007), may have been exposed to *M.tb* during the course of HIV infection.

Finally, we wanted to determine if the maintenance of *M.tb*-specific responses was linked a to particular functional characteristic. Previously, Geldmacher and colleagues reported that when compared to the opportunistic pathogen CMV, *M.tb*-specific CD4+ T cells produced lower levels of the CCR5 ligand MIP-1β and this may contribute to their high infectivity by HIV (Gelmacher *et al*., 2008, Cocchi *et al*., 1995, Kinter *et al.,* 1996). In our study, we observed no differences in the frequency of MIP-1β-producing *M.tb*-specific CD4+ T cells prior to HIV infection, between participants who maintained their *M.tb*-specific response and those who lost 50% or more of their responses. However, it was interesting to note that in the group that lost 50% or more of their responses, the only two participants that had a MIP-1β response prior to HIV infection were those who retained their *M.tb*-specific responses after HIV infection. We also examined a variety of clinical characteristics (VL, VL AUC, absolute CD4 count and CD4/CD8 ratios), and the function and phenotypic profiles of their *M.tb*-specific CD4+ T cells prior to HIV infection. There were no significant differences in the clinical characteristics of the two groups or the polyfunctional and phenotypic profiles of their *M.tb*-specific CD4+ T cells. However, although not significantly different, we observed that prior to HIV infection the median frequency of *M.tb*-specific CD4+ T cells in the participants who maintained their responses was 1.6-fold higher than those participants who lost 50% or more of their responses. This suggests that participants who maintained their *M.tb*-specific CD4+ T cells during acute HIV infection had relatively higher responses prior to infection. Further studies confirming this observation are warranted.

Our study had several limitations. Firstly, we did not have clinical data, such absolute CD4 and CD8 counts, for our participants prior to HIV infection. This means that we were unable to examine how CD4 counts and CD4/CD8 ratios changed from prior to HIV infection and how this may have influenced *M.tb*-specific responses in our participants. Another limitation is that our study only focused on *M.tb*-specific responses in the peripheral blood and not at sites of TB disease. This may account for why we saw no significant differences in frequencies of *M.tb*-specific responses in participants in our cross-sectional analysis. Lastly, our sample size for our longitudinal study was small, n=17. In future studies it would be important to conduct longitudinal studies comparing the effects of HIV infection on TB responses in both the periphery and tissue sites using a larger cohort. In our study, we measured Th1 cytokines (IFN-γ, TNF-α, IL-2 and MIP-1β). Earlier studies, including those in our group, have shown that other *M.tb*-specific CD4+ T cells such as Th22 and Th17 cells are also depleted in PLWH when compared to HIV-infected individuals (Murray *et al*., 2018; Bunjun *et al*., 2021; Makatsa *et al*., 2022). For future studies it would be essential to compare the impact of acute and chronic HIV infection on different *M.tb*-specific CD4+ T cell subsets and their proliferative ability. Lastly, we did not include chemokine receptors such as CCR5, CXCR3 and CCR6. These markers may have assisted us in better characterising *M.tb*-specific CD4+ T cells that are depleted during HIV infection, as earlier reports have shown that CD4+ T cells that express these markers are preferential targets of HIV (Geldmacher *et al*., 2008; 2010, Gosselin *et al*., 2010).

Overall, our data confirm previous findings that there is an early depletion of *M.tb*-specific CD4+ T cells within a year HIV infection. In our study we found that approximately half of HIV-infected individuals lost their peripheral *M.tb*-specific T cell responses, and this did indeed occur as early as 3 months after HIV infection. However, these responses were reconstituted over the course of untreated infection, prior to ART. This depletion of *M.tb*-specific responses early after HIV infection may explain the elevated risk of TB in PLWH even within the first year of HIV infection in some individuals, before substantial immune suppression.

CHAPTER 4

The impact of early ART on immunity to *Mycobacterium tuberculosis*

Table of Contents

4.1 Introduction

Antiretroviral therapy (ART) is an important strategy in preventing TB in PLWH. Earlier studies performed in sub-Saharan Africa show that ART is associated with a 70-90% reduction in TB risk (Badri *et al*., 2002) and a 52% decrease in TB-related mortality (Moore *et al*., 2007). A systematic review and meta-analysis of data from sub-Saharan Africa, South America, Asia and the Caribbean showed that ART reduces TB incidence by 65% regardless of the CD4 count of a patient (Suthar *et al*., 2012). Moreover, several studies have shown the importance of ART timing for HIV pathogenesis and transmission. When 1763 serodiscordant couples (with one of the pair living with HIV) from nine countries were followed for a period of 4 years, participants who initiated ART immediately after diagnosis had an 89% reduction in HIV transmission when compared to those who deferred treatment until CD4 counts declined to 250 cells/µL (Cohen *et al*., 2011). Participants who initiated early ART also had a 41% reduction in the number of acquired immunodeficiency syndrome (AIDS)-related events, including pulmonary TB, bacterial infections, and death. Similarly, a study by Collins and colleagues showed that in a cohort of 816 HIV-infected individuals, there was 2.5-fold decrease in TB incidence in individuals who went on ART immediately after diagnosis compared to those initiating treatment when their CD4 count was below 200 cells/µL (Collins *et al*., 2015). Moreover, two randomised controlled trials, the START and TEMPRANO studies, have shown the benefits of ART initiated at high CD4 counts. In the START study, participants who started ART at a CD4 count > 500 cells/µL, had a 72% reduction in serious AIDS-related illnesses, including TB, when compared to those who deferred ART until CD4 counts dropped to less than 350 cells/µL (INSIGHT START Study Group, 2015). In a similar study by the TEMPRANO study group, immediate ART, in addition to isoniazid preventive therapy (IPT), was associated with a 44% reduction in AIDS-related illnesses as well as 35% decrease in risk of death, in comparison to deferred ART with no IPT (TEMPRANO Study Group, 2015).

Despite ART intervention studies in sub-Saharan Africa, as well as Europe and North America, rates of TB remain high in PLWH in these regions (Girardi *et al*., 2005; Bonnet *et al*., 2006; Lawn *et al*., 2006). These high TB rates are most likely linked to the incomplete restoration of TB immunity during ART. Indeed, a study by Schluger and colleagues reported that in HIV-infected people with latent TB infection (LTBI), ART restored *Mycobacterium tuberculosis* (*M.tb*)-specific responses, however the restoration was delayed by more than 8 months and did not reach levels observed in healthy, HIV-uninfected people (Schluger *et al*., 2002). Furthermore, this delay in responses in HIV-infected subjects was more apparent with the proinflammatory cytokine IFN-γ, which is known to play an important role in TB immunity. Interestingly, partial restoration of TB responses has been observed both in individuals with acute and chronic HIV infection (Sutherland *et al*., 2006). In this report, despite long-term ART and CD4 count recovery, *M.tb* CD4+ IFN-γ responses in HIV-infected individuals were greatly reduced compared to HIV-uninfected subjects. This impairment was more severe in individuals with chronic HIV infection, and was independent of the patient's CD4 count or the duration of ART.

Several factors may contribute to defective TB immune responses during HIV infection. Previous reports have shown that there is a rapid depletion of *M.tb*-specific CD4+ T cells early after HIV infection (Geldmacher *et al*., 2008; 2010). In these studies, *M.tb*-specific CD4+ T cells were shown to be preferential targets of HIV, as they produced low levels of MIP-1β, a ligand of the HIV co-receptor CCR5 (Geldmacher *et al*., 2010). Similarly, a study by Singh and colleagues reported that HIV altered the phenotype of *M.tb*-specific CD4+ T cells from one that is protective against *M.tb* to an immune suppressive one. Indeed, HIV-*M.tb* co-infected dendritic cells impaired the proliferation of *M.tb*-specific CD4+ T cells, upregulated co-inhibitory molecules (PD-1, CTLA-4 and BLIMP-1) and downregulated co-stimulatory molecule expression (CD40L, CD28, ICOS) on *M.tb*-specific CD4+ T cells (Singh *et al*., 2019). In other reports HIV infection was shown to skew the cytokine profile of *M.tb*-specific CD4+ T cells from a polyfunctional Th1/Th2/Th17 response to a Th1-restricted profile, by impairing the expression of transcription factors Gata-3 (Th2 cells) and RORγt (Th17 cells) (Amello *et al*., 2019). These reports may explain the reduced capacity of *M.tb*-specific CD4+ T cells to control *M.tb* from early on during HIV infection.

Surprisingly, in the context of ART very little known about the impact of the timing of ART initiation on *M.tb*-specific responses. In order to address this, we sought to characterise *M.tb*-specific immunity in individuals who initiated ART at an early stage of HIV infection (<12 months, “early group”) in comparison to those who started ART during the chronic phase of HIV infection (>24 months, “late group”). We reasoned that in comparison to late treatment, early ART preserves the specific immune response to *M.tb* thereby reducing the early, elevated risk of TB in HIV-infected individuals.

4.2 Materials and methods

All materials and methods used in this study are described in Chapter 2.

4.3 Results

4.3.1 Clinical characteristics of the early and late ART groups

Archived PBMC from 38 participants were selected from the CAPRISA 002 study, a longitudinal cohort following of PLWH from HIV seroconversion to up to 5 years after ART initiation. A summary of the demographic and clinical characteristics of the early and late ART groups is presented in **Table 4.1** and details for each participant included in this study is presented in **Table 4.2** and **4.3**. The cohort consisted of 16 PLWH who initiated ART at an early stage of the HIV infection (median: 7.5 months after HIV infection, IQR 3-9, Early ART group) and 22 who initiated ART later during the chronic HIV infection (median: 66 months after HIV infection, IQR 46.5-77.3, Late ART group). **Figure 4.1** shows the study schema for the early and later ART groups, illustrating the different timepoints at which blood was collected. First, HIV plasma viral load (VL), absolute CD4 T cell counts and CD4/CD8 ratios were compared pre- and post-ART initiation within each group and between groups. **Figure 4.2A** shows the HIV plasma VL of the early and late ART groups prior to ART initiation (median: 3 months, IQR 1-6)] and 2 years after ART (median: 24 months, IQR 22-28.3)]. Two years after ART initiation, most participants achieved HIV viral suppression in both groups. For the early ART group, the median VL decreased from 19,941 RNA copies/ml (IQR 5,599-66,284) to undetectable level ($p < 0.0001$). Likewise, in the late ART group the median VL also decreased from 14,961 (IQR 2,010-70,529) to undetectable level ($p < 0.0001$). Whilst the median VL after treatment was undetectable, there were a few participants in both the early and late ART groups having VL above the limit of detection (3/16 and 3/22, respectively). A comparison of the two groups showed no significant difference in VL before or after ART initiation ($p = 0.7591$ and 0.8951, respectively). Since we expected a greater difference in VL between the two groups, we examined VL trajectories in the late ART group in more detail (**Supplementary Figure 4.1**). Of note, 7/22 (around 32%) of participants in the late ART group could be regarded as VL 'controllers', who we defined as participants who consistently had an average VL below 15,000 copies/mL and a CD4 count above 500 cells/µL after the first year of HIV infection. In these participants, the average VL before ART initiation was less than 15,000 copies/mL. In addition, in 6 of these participants, the absolute CD4 T cell counts after the first year of HIV infection remained above 500 cells/µL. We then calculated the area under the curve (AUC) VL of the two ART groups (**Figure 4.2B**). In the late ART group, the median AUC VL (6 months copies/mL, IQR 5.6-6.4) was significantly higher than the early ART group (5.1 months copies/mL, IQR 4-6), (p = 0.0035). This therefore suggests that, despite the presence of VL controllers, participants in the late ART group generally had an overall higher VL burden compared to the early ART group, even though this difference was not evident at the specific timepoints examined in our study.

Table 4.1: A summary of the clinical characteristics of participants in the early and late ART groups

	Early ART group (median, IQR)	**Late ART group (median, IQR)**	**P value**
n	16	22	-
Age Pre-ART timepoint[1]	25 (24-29)	31 (27-34)	0.0087
Age Post-ART timepoint[1]	28 (26-31)	33 (30-36)	0.0046
Time after HIV infection Pre-ART timepoint (months)	3 (2.2-3.8)	61.5 (44.5-77.2)	<0.0001
Time after HIV infection Post-ART timepoint (months)	31 (21.7-34.5)	97 (68.5-102.3)	<0.0001
Time before ART initiation Pre-ART timepoint (months)	3.5 (1-5.7)	3 (1.7-6)	0.9473
Time after ART initiation Post-ART timepoint (months)	23 (18.5-24)	24 (23-29)	0.1645
ART initiation (months)	7.5 (3-9)	66 (46.5-77.2)	<0.0001
CD4 count Pre-ART timepoint (cells/µL)	493.5 (393-609)	532 (392.5-629.3)	0.6878
CD4 count Post-ART timepoint (cells/µL)	611.5 (573.3-849)	746 (624.3-865.5)	0.2372
CD4/CD8 ratio Pre-ART timepoint	0.59 (0.41-0.96)	0.48 (0.36-0.64)	0.2369
CD4/CD8 ratio Post-ART timepoint	1.24 (0.97-1.58)	0.82 (0.69-1.1)	0.0023
VL Pre-ART timepoint (copies/mL)	19941 (5599-66284)	14961 (2010-70529)	0.7591
VL Post-ART timepoint (copies/mL)	ND	ND	-
Log10 (AUC VL) (months.copies/mL)	5.1 (3.9-5.9)	6 (5.6-6.4)	0.0035

[1] "Pre-ART timepoint and "post-ART timepoint' refer to the points when blood sampling took place and at which immunological analyses were performed. ND = not detectable

Table 4.2: Detailed clinical characteristics of participants in the early ART group

		Age		**Time after HIV infection (months)**		**Time after ART initiation (months)**		**ART initiation (months)**	**CD4 count (cells/µL)**		**CD4/CD8 ratio**		**VL (copies/mL)**		**Log10 (AUC VL) (months.copies/mL)**
PID	**ART group**	**Pre ART**	**Post ART**	**Pre ART**	**Post ART**	**Pre ART**	**Post ART**		**Pre ART**	**Post ART**	**Pre ART**	**Post ART**	**Pre ART**	**Post ART**	
100345	Early	27	29	4	31	7	20	11	212	517	0.45	1.00	213000	ND	7.1
100353	Early	22	25	3	44	9	32	12	377	828	0.57	1.25	26100	ND	5.7
100358	Early	25	28	3	35	10	22	13	177	325	0.21	0.65	1020	ND	5.4
100410	Early	28	30	4	31	4	23	8	480	566	0.55	1.54	115360	ND	5.9
100427	Early	25	28	3	38	4	31	7	263	382	0.23	0.73	68629	38	6
100428	Early	34	36	3	31	5	23	8	548	931	0.29	0.96	199334	79	6.1
100434	Early	28	30	3	33	6	24	9	441	622	0.43	1.23	59248	ND	5.8
100436	Early	24	25	2	20	1	17	3	610	856	1.06	1.20	43272	140	5.1
100438	Early	25	27	3	32	5	24	8	566	670	1.00	1.60	5474	ND	5.2
100444	Early	29	31	3	21	0	18	3	704	987	0.83	1.39	7596	ND	4.2
100446	Early	25	28	6	45	3	36	9	643	1299	0.70	1.76	22683	ND	5.1
100447	Early	26	27	2	21	1	18	3	482	601	1.28	1.72	1830	ND	3.7
100458	Early	34	36	4	31	3	24	7	605	596	0.61	1.49	2222	ND	4.4
100460	Early	23	25	1	24	0	23	1	446	595	0.41	0.90	7469	ND	3.9
100461	Early	24	26	2	26	1	23	3	505	601	0.70	1.19	5972	ND	3.4
100467	Early	30	32	3	21	0	18	3	1111	782	1.09	2.00	17199	ND	3.9
Median		**25**	**28**	**3**	**31**	**3.5**	**23**	**7.5**	**493.5**	**611.5**	**0.59**	**1.24**	**19941**	**ND**	**5.1**
IQR		**24-28**	**26-31**	**2.2-3.7**	**21.7-34**	**1-5.7**	**18.5-24**	**3-9**	**393-608.8**	**573.3-849**	**0.4-0.96**	**0.97-1.6**	**5599-66284**	**-**	**4-6**

ND = not detectable, AUC VL = Area under the curve viral load.

Table 4.3: Detailed clinical characteristics of participants in the late ART group

		Age		Time after HIV infection (months)		Time after ART initiation (months)		ART initiation (months)	CD4 count (cells/μL)		CD4/CD8 ratio		VL (copies/mL)		Log10 (AUC VL) (months.copies/mL)
PID	**ART group**	**Pre ART**	**Post ART**	**Pre ART**	**Post ART**	**Pre ART**	**Post ART**		**Pre ART**	**Post ART**	**Pre ART**	**Post ART**	**Pre ART**	**Post ART**	
100284	Late	34	37	104	132	11	17	115	820	900	0.81	1.09	1342	ND	5.8
100291	Late	33	35	76	100	0	24	76	583	801	0.46	1.12	10214	<20	5.9
100295	Late	31	34	86	114	3	25	89	616	806	0.40	0.82	12142	ND	5.9
100297	Late	30	33	91	121	6	24	97	550	854	0.40	0.69	19693	23	6
100311	Late	25	28	71	98	3	24	74	560	823	0.83	1.06	2007	ND	5.1
100315	Late	28	30	70	100	6	24	76	482	826	0.47	1.22	15902	ND	6.2
100324	Late	29	31	86	115	0	29	86	730	743	0.77	0.73	24701	ND	5.6
100330	Late	26	29	77	106	0	29	77	615	749	0.62	0.70	76653	1302	5.9
100335	Late	32	36	64	100	6	30	70	400	517	0.33	0.54	10413	ND	6.1
100336	Late	22	24	32	56	1	23	33	164	547	0.08	0.80	191139	ND	6.4
100342	Late	28	30	63	85	4	18	67	366	554	0.54	0.69	65478	<20	6.5
100349	Late	32	34	47	72	2	23	49	365	677	0.24	0.44	157617	220	7.1
100351	Late	34	36	27	59	7	25	34	370	276	0.25	0.77	29160	<20	6.1
100352	Late	40	42	22	53	3	28	25	451	722	0.41	1.05	14020	ND	6
100355	Late	34	36	56	82	2	24	58	429	629	0.37	0.62	1065	ND	5.6
100356	Late	27	30	57	98	7	34	64	441	735	0.54	0.89	2293	ND	6.9
100368	Late	34	36	78	101	0	23	78	727	988	0.71	0.82	2011	ND	5.6
100371	Late	27	30	55	96	10	31	65	514	610	0.57	0.92	1045	ND	4.4
100372	Late	25	27	40	64	2	22	42	357	739	0.50	1.18	118267	ND	6.3
100379	Late	31	33	46	70	2	22	48	585	1664	0.54	0.66	68487	ND	6.6
100382	Late	32	34	60	92	2	30	62	669	1545	0.33	1.45	101278	<20	6.7
100415	Late	34	36	36	62	3	23	39	1213	1066	1.73	1.88	1087	ND	4.2
Median		**31**	**33**	**61.5**	**97**	**3**	**24**	**66**	**532**	**746**	**0.48**	**0.82**	**14961**	**ND**	**6**
IQR		**27-34**	**30-36**	**44.5-77.2**	**68.5-102**	**1.7-6**	**23-29**	**46.5-77.3**	**392.5-629.3**	**624.3-865.5**	**0.36-0.64**	**0.69-1.1**	**2010-70529**	-	**5.6-6.4**

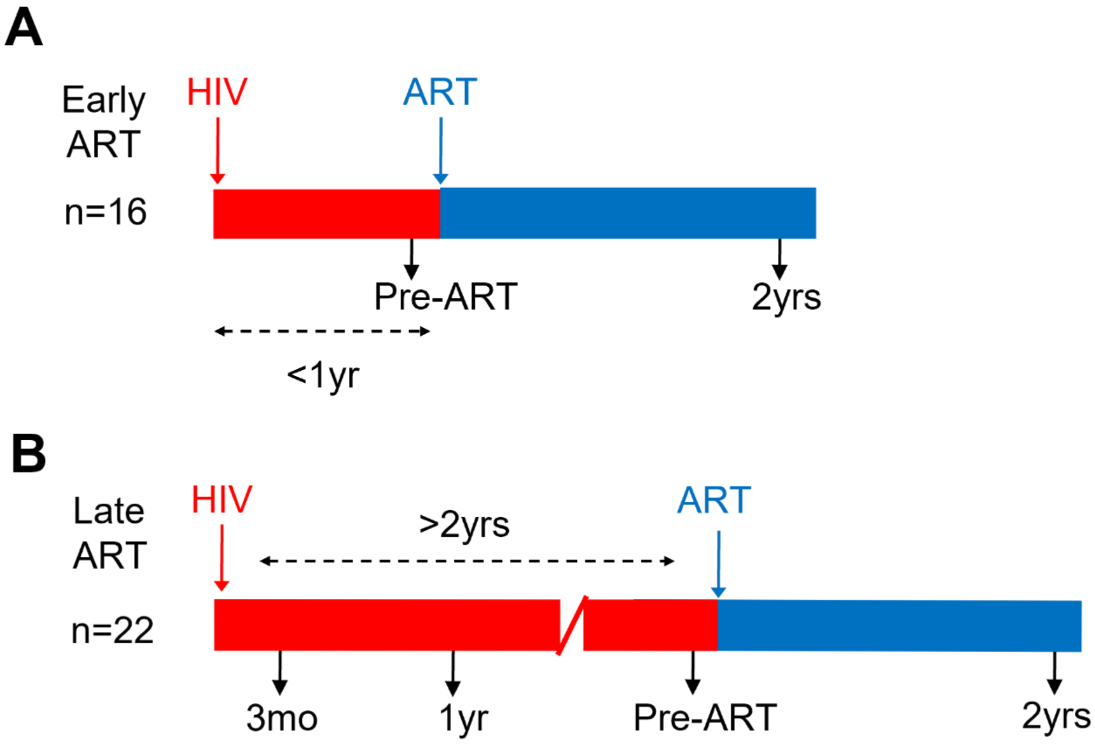


Figure 4.1: Study schema. An outline of the study showing timepoints before ART initiation (red) and after ART (blue) in the **(A)** early ART and **(B)** late ART groups. Arrows depict time at which blood was sampled.

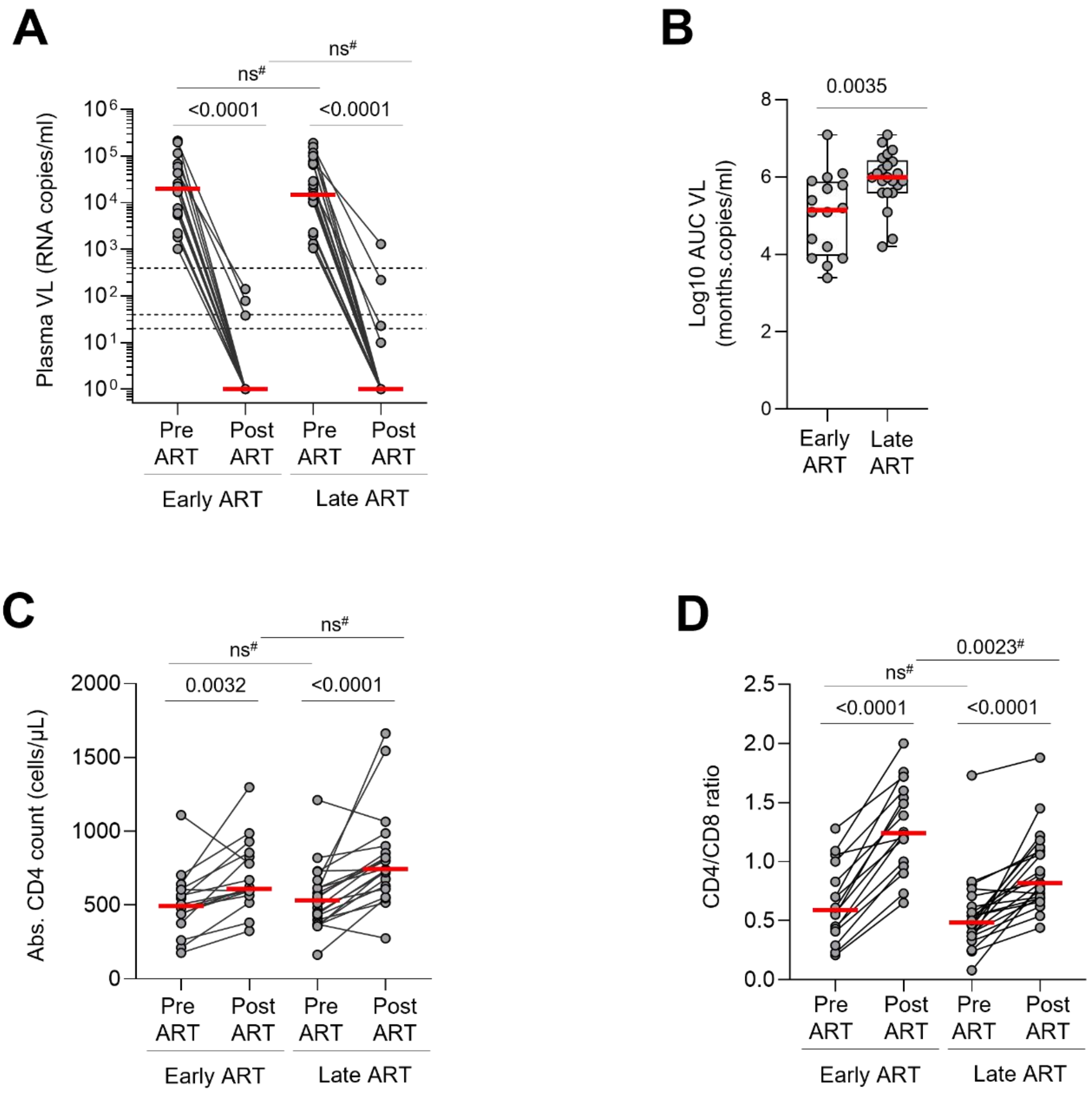


Figure 4.2: Clinical characteristics of participants before and after ART initiation. (A) Plasma viral load, **(B)** area under the curve (AUC) VL, **(C)** absolute CD4 T cell count and **(D)** CD4/CD8 ratio in participants before ART initiation and at two years after treatment in early ART (n=16) and late ART groups (n=22). The horizontal dashed lines indicate the detection limit of the assays. Red horizontal bars indicate the median values. Statistical significance was calculated using a Wilcoxon matched-pairs test and Mann-Whitney U test (indicated as #). Statistically non-significant P-values (P>0.05) are annotated as ns.

Figure 4.2C shows the absolute CD4 T cell counts in the early and late ART groups. An increase in the median CD4 count was observed after ART initiation in the early

ART group, from 494 cells/µL (IQR 393-610) to 612 cells/µL (IQR 573-849) (p = 0.0032) (**Figure 4.2C**). Similarly, in the late ART group, CD4 counts also increased from a median 532 cells/µL (IQR 392-629) to 746 cells/µL (IQR 624-866) ($p < 0.0001$). Moreover, when the two groups were compared there were no significant differences in their CD4 counts before and after ART (p = 0.6878 and 0.2372, respectively). The CD4/CD8 ratios, another measure of immune reconstitution, increased after ART in the early ART group, from a median of 0.59 (IQR 0.41-0.96) to 1.24 (IQR 0.97-1.58) ($p < 0.0001$). A similar fold-change was observed in the late ART group, from a median 0.48 (IQR 0.36-0.64) to 0.82 (IQR 0.69-1.1) ($p < 0.0001$) (**Figure 4.2D**). Comparing the two groups, the median CD4/CD8 ratio in the late ART group was significantly lower after ART (0.82, IQR 0.69-1.1) in comparison to the early ART group (1.24, IQR 0.97-1.6) (p = 0.0023). Overall, these data show that in both groups, ART led to immune reconstitution. However, when ART was initiated at a later stage, while CD4 counts did not differ from the early ART group when measured 2 years after ART, incomplete restoration of CD4/CD8 ratios was observed, suggesting that immune reconstitution or normalisation was only partial upon ART deferral, and some immune defects may have persisted.

4.3.2 ART timing does not affect the magnitude M.tb-specific CD4+ T cells

To better understand the dynamics of *M.tb*-specific CD4+ T cell responses upon ART, we first measured *M.tb*-specific responses before and after 2 years of ART in the early and late ART groups. We used intracellular staining and flow cytometry, and measured cytokines in response to stimulation with a pool of peptides from *M.tb,* MTB300 (**Figure 4.3A**). **Figure 4.3B** shows the frequencies of total *M.tb*-specific CD4+ T cells (producing IFN-γ, TNF-α, IL-2 and MIP-1β) in both groups. In the early ART group, the frequency *M.tb*-specific CD4+ T cells remained the same overall after ART initiation, from a median 0.15% (IQR 0.03-0.37) before ART to 0.16% (IQR 0.02-0.48) 2 years after ART (p = 0.6147). Similarly, in the late ART group, there was no significant difference in the median frequency *M.tb*-specific CD4+ T cells after ART, from 0.09% (IQR 0.01-0.48) to 0.12% (IQR 0.03-0.37) (p = 0.3838). When looking at the frequency of *M.tb* responders, they also remained similar after ART in both the early ART (75% to 75%) and late ART groups (64% to 73%, p= 0.517) (**Figure 4.3B**).

Next, we examined the impact of absolute CD4 T cell counts on *M.tb*-specific CD4+ T cell responses. Using CD4 counts before ART initiation, participants with a CD4 count > 500 cells/µL were grouped as high CD4 count (median: 612.5 cells/µL, IQR 561.5-721.3) and a CD4 count of < 500 cells/µL as low CD4 count (median: 388.5, IQR 333.5-447.3). **Figure 4.3C** shows the frequencies of total *M.tb*-specific CD4+ T cells when the data are divided into these two groups. Two years after ART, there were no differences in the overall frequency of *M.tb*-specific CD4+ T cells in the high and low CD4 count groups (p= 0.1991 and 0.9661, respectively). When the two groups were compared there were also no significant differences in responses before and after ART (p=0.3196 and 0.6247, respectively). Additionally, the frequency of *M.tb* responders remained similar after ART in both the high (65% to 70%) and low CD4 count groups (72% to 78%) (**Figure 4.3C**).

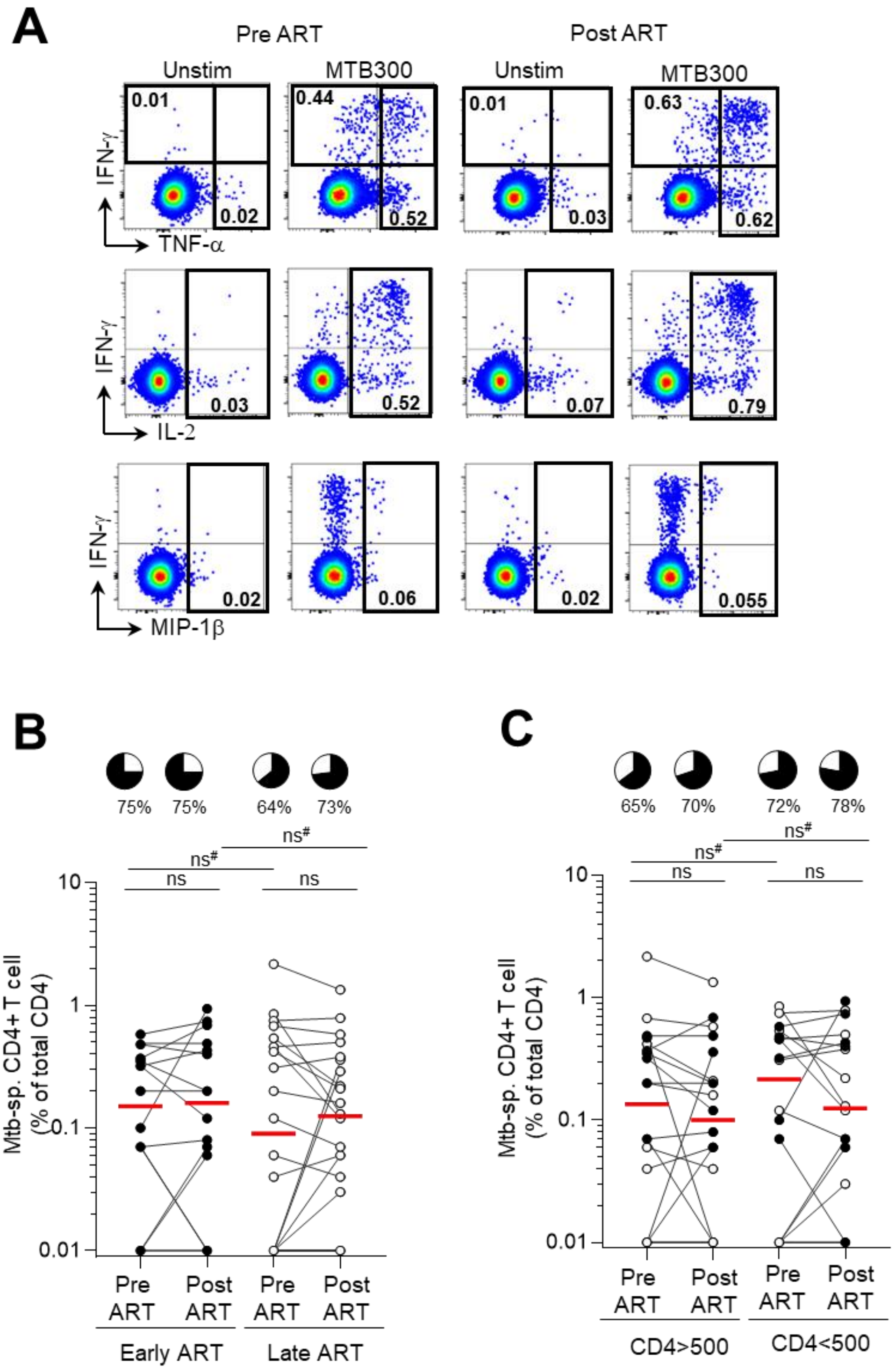


Figure 4.3: Frequency of *M.tb*-specific CD4+ T cells. (A) Representative flow cytometry plots showing total IFN-γ, TNF-α, IL-2 and MIP-1β responses after stimulation with MTB300. Frequency of total *M.tb*-specific CD4+ T cells in participants before ART initiation and at two years after treatment in **(B)** the early ART (black dots, n=16) and late ART groups (white dots, n=22), and **(C)** CD4 count > 500 (n=20) and

CD4 count < 500 groups (n=18) when participants were divided according to their CD4 count prior to ART initiation. Pies and percentages show the frequency of *M.tb* responders at each timepoint. Red horizontal bars indicate the median values. Statistical significance was calculated using a Wilcoxon matched-pairs test and Mann-Whitney U test (indicated as #). Statistically non-significant P-values (P > 0.05) are annotated as ns.

Lastly, to assess the relationship between *M.tb*-specific CD4+ T cell responses and the clinical characteristics of participants (such as CD4 count, VL, CD4/CD8 ratios, time of ART and AUC VL), spearman correlations and linear regression were performed (**Figure 4.4**). After correcting for multiple comparisons, linear regression models showed a positive association between *M.tb*-specific responses before ART initiation and 2 years post-ART (p < 0.001) (**Figure 4.4A**). Similarly, spearman correlations showed a positive association between *M.tb*-specific CD4 responses before and after ART (<0.0001, r=0.7468, **Figure 4.4B**). There was no significant association between *M.tb*-specific responses and any of the clinical parameters tested. Together these results demonstrate that 2 years of ART, irrespective of when it was initiated, induced immune reconstitution marked by an increase in CD4 T cell count and improvement of the CD4/CD8 ratio. However, there was no overall difference in the frequency of peripheral *M.tb*-specific CD4+ T cells.

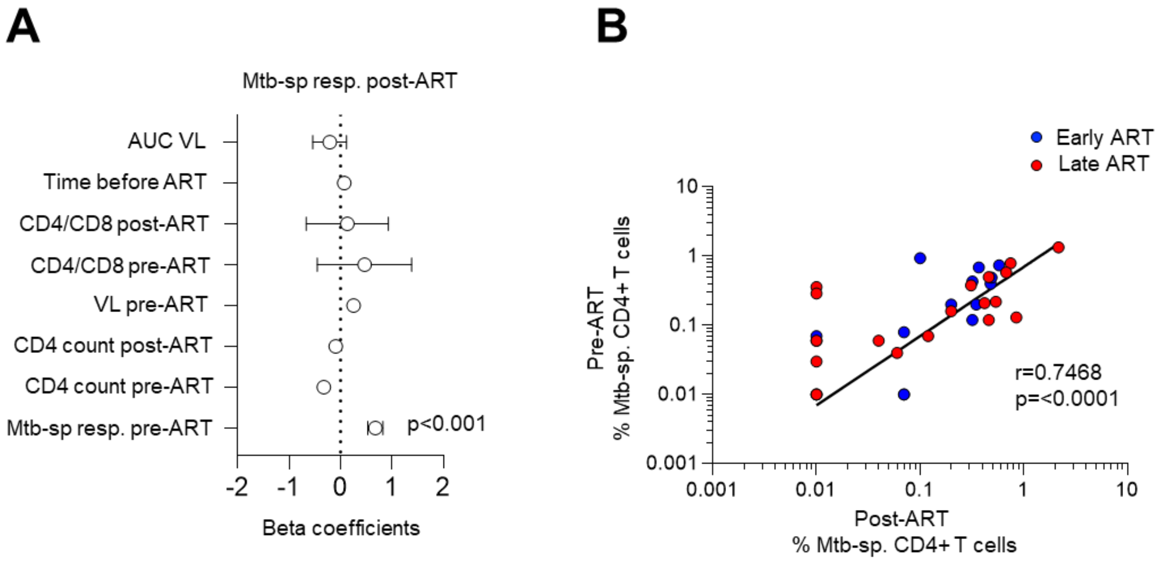


Figure 4.4: Associations between *M.tb*-specific CD4+ T cell responses and the clinical characteristics of participants. (A) Stepwise multivariate linear regression showing the associations between post-ART *M.tb*-specific CD4+ T cell responses and area under the curve (AUC) VL (months copies/mL), time before ART initiation (months), pre- and post-ART CD4/CD8 ratios, pre-ART VL (copies/mL), pre- and post-ART CD4 count (cells/µL) and pre-ART *M.tb*-specific CD4+ T cell responses. Each association is shown as a β-coefficient and the error bars are 95% confidence intervals. **(B)** Spearman correlations assessing the relationship between the frequency of total *M.tb*-specific CD4+ T cells pre- and post-ART for the early (blue dots) and late (red dots) ART groups. P-values and Spearman r coefficients are displayed in the bottom right corner of each graph. Only statistically significant associations are shown ($p \leq 0.05$).

To take into account the variation in absolute CD4 T cell counts among participants due to HIV infection and ART reconstitution, we calculated the absolute number of *M.tb*-specific CD4+ T cells (**Figure 4.5A** and **B**). In the early ART group, the median absolute number of *M.tb*-specific CD4+ T cells increased after ART, from 0.83 cells/µL (IQR 0.06-2.1) to 1.1 cells/µL (IQR 0.07-3.5), however this was not significantly different (p=0.2734) (**Figure 4.5A**). In the late ART group, the absolute number of

M.tb-specific CD4+ T cells remained the same after ART, median 0.47 cells/µL (IQR 0.01-2.6) to 0.6 cells/µL (IQR 0.17-2.5), (p= 0.4801). Of note, although absolute number of *M.tb*-specific CD4+ T cells in the late ART group was almost 2-fold lower after treatment compared to the early ART group, this was not significantly different (p=0.4645). When participants were grouped according to CD4 counts pre-ART, no statistical difference in the absolute number of *M.tb*-specific CD4+ T cells pre- and post-ART was observed either for the CD4 > 500 group (median: 0.8 cells/µL vs 0.9 cells/µL, respectively, p = 0.8040) or the CD4 < 500 group (median: 0.67 cells/µL vs to 0.61 cells/µL, p = 0.1876) (**Figure 4.5B)**

To further assess the dynamics of *M.tb*-specific responses, we examined the fold change in frequencies of *M.tb*-specific CD4+ T cells after ART initiation (**Figure 4.5C** and **D**). In the late ART group, the majority of participants (41%) had a decrease the frequencies of *M.tb*-specific CD4+ T cells after ART (fold change < 0.8) (**Figure 4.5C**). In contrast, in the early ART group a similar percentage of participants (31%) had either an increase (fold change >1.2) or decrease (fold change < 0.8) in the frequencies after ART. Interestingly, in the late ART group a large percentage of participants (27%) had undetectable responses before and after ART initiation when compared to the early ART group (13%). When we grouped participants according to absolute CD4 counts, the fold changes in responses were comparable in high and low CD4 count groups (**Figure 4.5D**

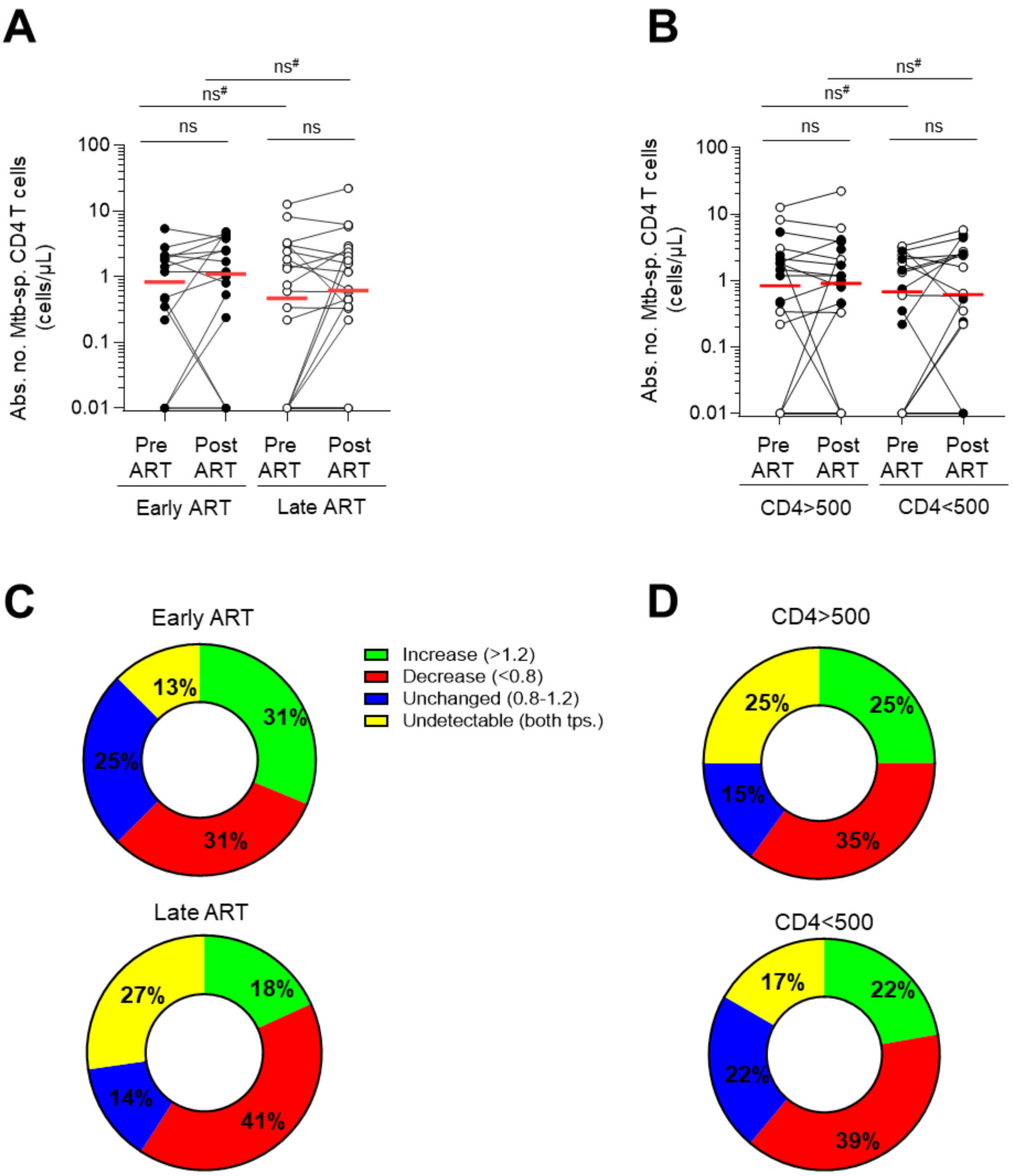


Figure 4.5: The magnitude of *M.tb*-specific CD4+ T cells. The absolute number of *M.tb*-specific CD4+ T cells in participants before ART initiation and at 2 years after treatment in **(A)** the early ART (black dots, n=16) and late ART groups (white dots, n=22), and **(B)** CD4 count > 500 (n=20) and CD4 count < 500 groups (n=18). Fold change in the frequency of *M.tb*-specific CD4+ T cells in the **(C)** early ART and late ART groups, and **(D)** CD4 count > 500 and CD4 count < 500 groups. Red horizontal bars indicate the median values. Statistical significance was calculated using a

Wilcoxon matched-pairs test and Mann-Whitney U test (indicated as #). Statistically non-significant P-values (P > 0.05) are annotated as ns.

4.3.3 ART timing has no impact on the functional capacity of M.tb-specific CD4+ T cells

Having demonstrated that ART did not impact *M.tb*-specific CD4+ T cells quantitatively in our study cohort, we next examined whether it had an effect on their functional profiles. We compared the polyfunctional profile of *M.tb*-specific CD4+ T cells pre- and post-ART using a Boolean gating strategy (**Figure 4.6)**. In both groups (early and late ART), *M.tb*-specific CD4+ T cell polyfunctional capacity was comparable pre- and post-ART, with CD4+ T cell responses consisting mainly (~50%) of cells co-expressing three cytokines (IFN-γ, TNF-α and IL-2) and IL-2+ TNF-α+ dual functional cells (~20%) . When we compared the two groups, there was no significant difference in the cytokine profiles of *M.tb*-specific C4+ T cells (**Figure 4.7A**). When participants were grouped according to CD4 counts, there was also no difference in cytokine profiles post-ART between patients who started ART at a high or low CD4 count (p= 0.18) (**Figure 4.7B**).

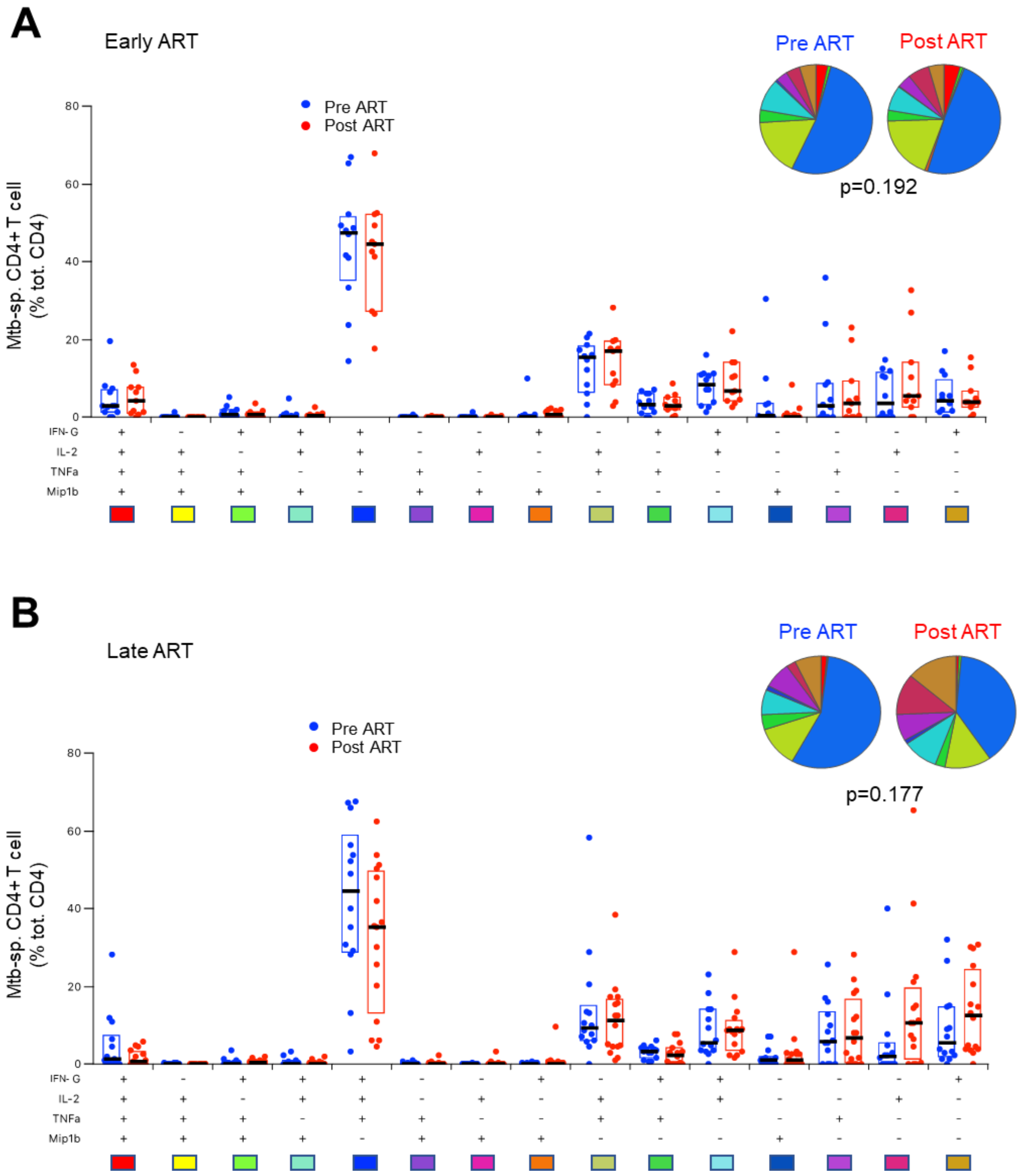


Figure 4.6: The polyfunctional profile of *M.tb*-specific CD4+ T cells. Proportion of different combinations of IFN-γ, TNF-α, IL-2 and MIP-1β in participants before ART initiation (blue dots) and at two years after treatment (red dots) in the **(A)** early ART (n=16) and **(B)** late ART (n=22) groups. The coloured bars (underneath) and pie chart slices represent different cytokine combinations produced by *M.tb*-specific CD4+ T cells (e.g. a blue bar/slice are cells producing IFN-γ, IL-2 and TNF-α, in the absence of MIP-1β).

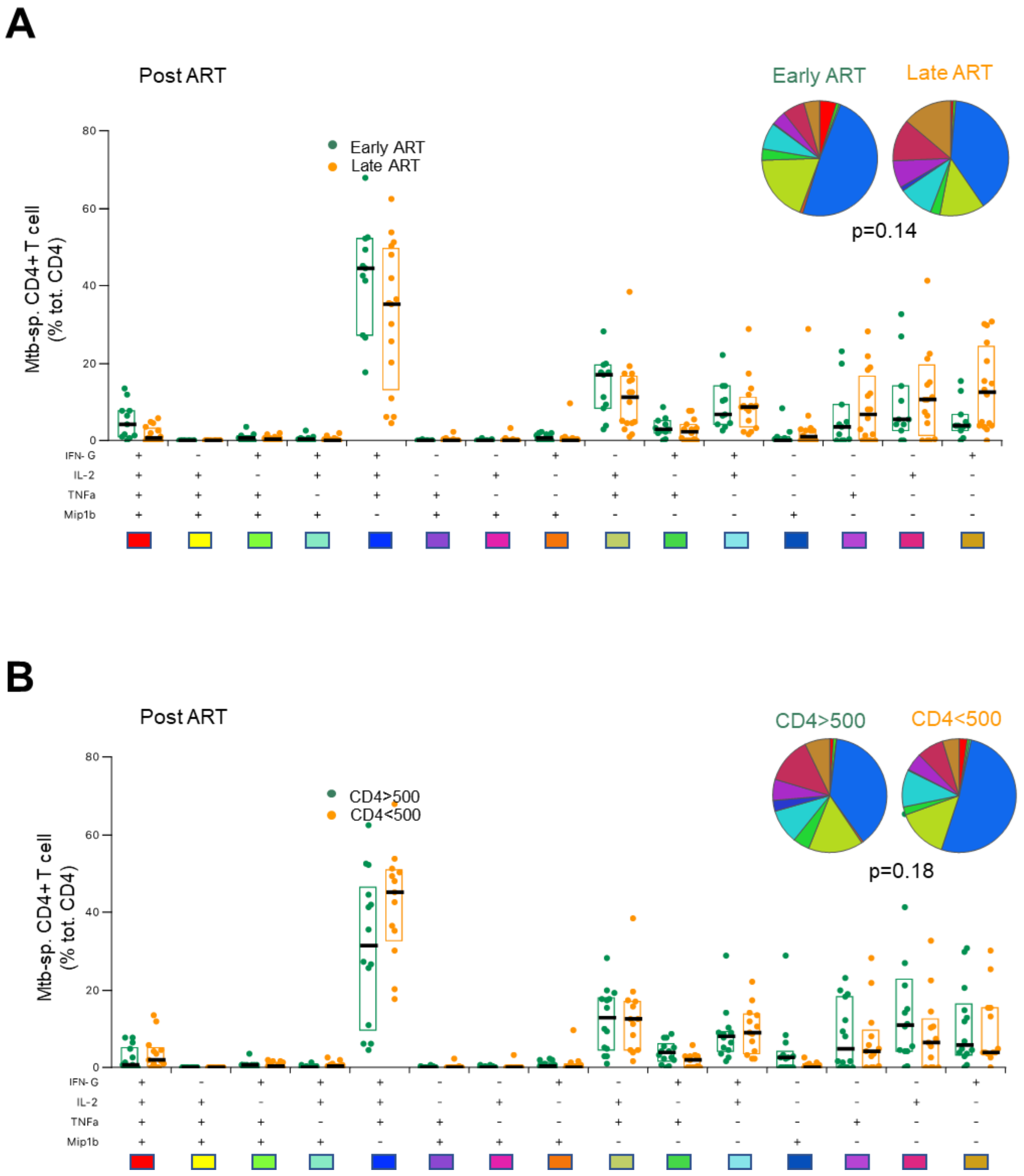


Figure 4.7: The polyfunctional profile of *M.tb*-specific CD4+ T cells. Proportions of different combinations of IFN-γ, TNF-α, IL-2 and MIP-1β in participants 2 years after treatment in **(A)** the early ART (green dots, n=16) and late ART (orange dots, n=22) groups and **(B)** CD4 count >500 (green dots, n=20) and CD4 count <500 (orange dots, n=18) groups. The coloured bars (underneath) and pie chart slices represent different cytokine combinations produced by *M.tb*-specific CD4+ T cells (e.g. a blue bar/slice are cells producing IFN-γ, IL-2 and TNF-α, in the absence of MIP-1β).

We next examined the expression of CD153 on *M.tb*-specific CD4+ T cells. CD153 (TNFSF8, CD30L) is a CD30 ligand expressed on activated T cells, which has a co-stimulatory function assisting in T cell proliferation and the secretion of IFN-γ, TNF-α and IL-2 (Marin and Garcia, 2017). Previous studies in mouse models have shown that CD153, through its expression on CD4+ T cells, is able to control *M.tb* infection in the lungs and hence limit pulmonary bacterial loads (Sallin *et al*., 2018). Additionally, in humans, CD153+ *M.tb*-specific CD4+ T cells were more abundant in individuals with controlled LTBI compared to those with active TB (Sallin *et al*., 2018). In this study, analysis of this marker on *M.tb*-specific CD4+ T cells showed no significant differences in the frequencies of these cells pre-and post-ART in both the early and late groups and between the groups. (**Figure 4.8A**). Notably, participants in both the early and late ART groups had elevated CD153 expression (median: ~60%) on *M.tb*-specific CD4+ T cells and this is consistent with frequencies previously observed in individuals during LTBI (Du Bruyn *et al.,* 2021). Of interest, there was a positive correlation between CD153+ *M.tb*-specific CD4+ T responses before ART and responses after treatment (p = 0.0074, r = 0.5431; **Figure 4.8B**).

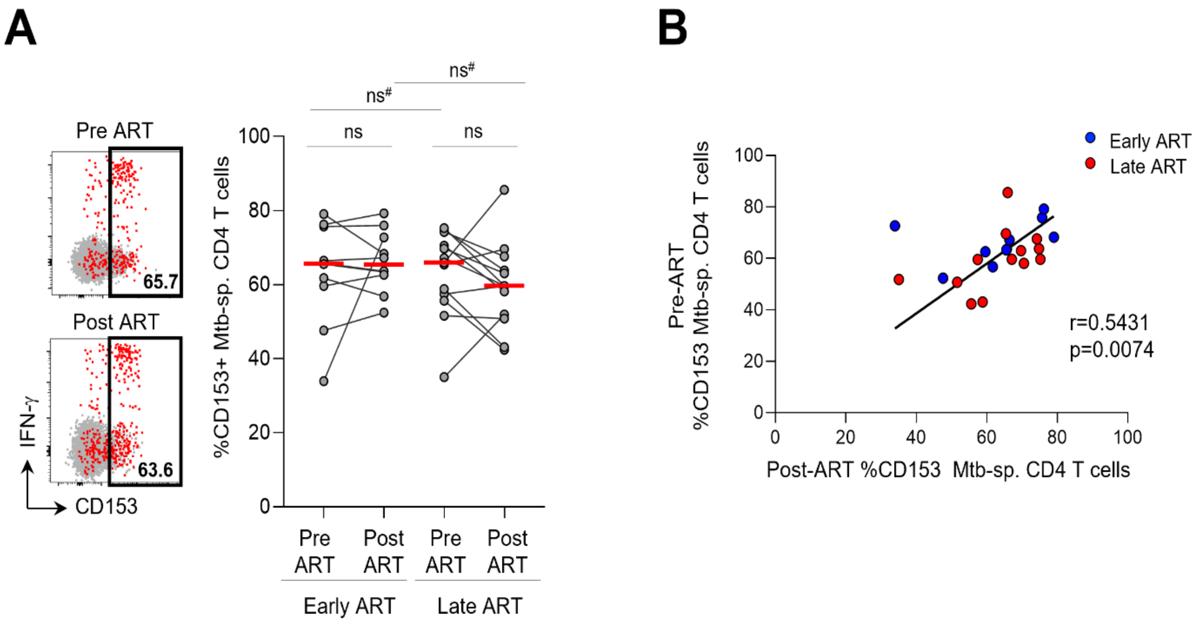


Figure 4.8: Frequency of CD153+ CD4+ T cells. (A) Representative flow cytometry plots and **f**requency of CD153+ *M.tb*-specific CD4+ T cells in participants before ART initiation and at two years after treatment in early ART (n=10) and late ART groups (n=14). Red horizontal bars indicate the median values. Statistical significance was calculated using a Wilcoxon matched-pairs test and Mann-Whitney U test (indicated as #). Statistically non-significant P-values (P > 0.05) are annotated as ns. **(B)** Spearman correlation assessing the relationship between the frequency of CD153+ *M.tb*-specific CD4+ T cells in the early ART (blue, n=10) and late ART (red, n=14) groups pre- and post-ART. P-values and Spearman r coefficients are displayed in the bottom right corner of the graph. Associations of significance (P < 0.05) were also fitted with a best-fit line.

4.3.4 ART timing does not alter the phenotype of M.tb-specific CD4+ T cells

To determine the phenotypic characteristics of *M.tb*-specific CD4+ T cells, a comparative analysis of their memory profiles was performed. The measurement of memory differentiation markers CD45RA and CD27 enabled the detection of four memory subsets, namely naïve (CD45RA+CD27+), early differentiated (CD45RA-CD27+), late differentiated (CD45RA-CD27-) and terminally differentiated (CD45RA+CD27-) (**Figure 4.9A**). *M.tb*-specific CD4+ T cells in the early and late ART groups exhibited primarily an early differentiated (ED) profile both before and after ART (approximately 60% of the response) (**Figure 4.9B**). The remainder of the response was made up of late differentiated (LD), naïve-like and terminally differentiated (TD) cells (approximately 30%, 5% and 1.5%, respectively). Notably, ART did not markedly alter the memory phenotype of *M.tb*-specific CD4+ T cells in the early and late ART group for any of the subsets examined (**Figure 4.9 B-E**). A comparison of the two groups showed a minor enrichment of the TD subset before ART initiation in the late ART group, in comparison to the early ART group, medians 2.18% (IQR 0.57-2.91) and 1.17% (0.63-1.37), respectively (p=0.0340). When we analysed these memory subsets in the total CD4 compartment, there were no differences in the memory phenotypes of total CD4+ T cells after ART in the early and late ART groups or when the two groups were compared before and after ART.

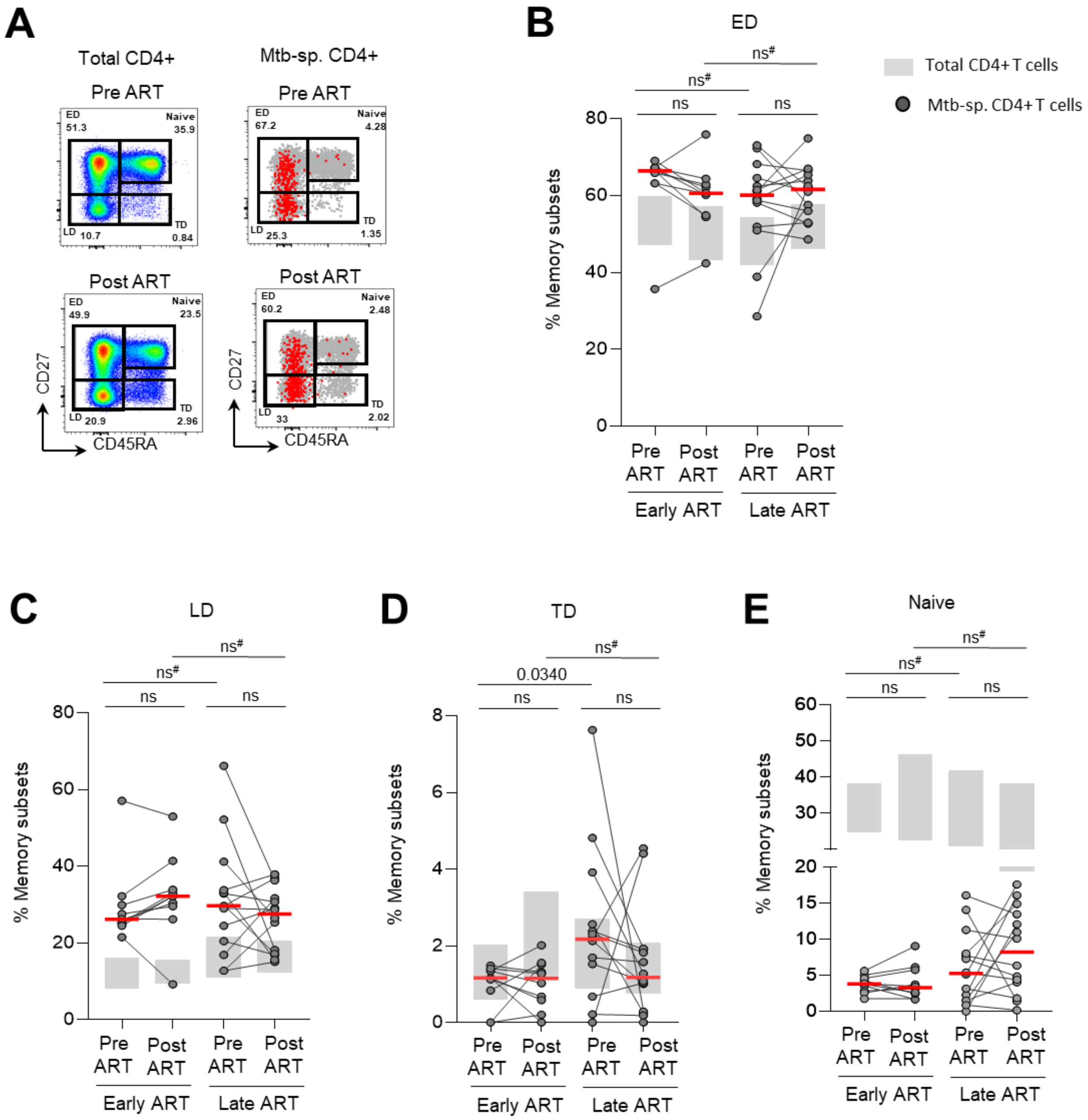


Figure 4.9: The memory differentiation profiles of CD4+ T cells. (A) Representative overlay plots showing memory subsets in *M.tb*-specific CD4+ T cells and total CD4+ T cells before and 2 years after ART. Frequency of **(B)** early differentiated (ED: CD27+CD45RA-), **(C)** late differentiated (LD: CD27-CD45RA-), **(D)** terminally differentiated (TD: CD27-CD45RA+) and **(E)** Naive (CD27+CD45RA+) memory subsets in participants before and after ART in the early ART (n=10) and late ART (n=14) groups. *M.tb*-specific CD4+ T cells are represented in dark grey dots and total CD4+ T cells in light grey floating bars (indicating the interquartile ranges). Red horizontal bars indicate the median values. Statistical significance was calculated

using a Wilcoxon matched-pairs test and Mann-Whitney U test (indicated as #). Statistically non-significant P-values (P > 0.05) are annotated as ns.

Lastly, we examined the activation profile of *M.tb*-specific CD4+ T cells (**Figure 4.10**). Analysis of HLA-DR in the overall CD4 compartment showed that ART led to a significant decrease, as expected, in the median frequency of total HLA-DR+ CD4+ T cells in both the early ART group (2.7% (IQR 2.2-3.6) vs 2.1% (1.6-2.6) (p = 0.00590) and late ART group (3.6% (IQR 3-5.5) vs 2.8% (IQR 2.2-3.3) (p = 0.0245). However, pre-ART the frequencies of HLA-DR+ CD4+ T cells were comparable regardless of the timing of ART initiation (p=0.1083) (**Figure 4.10A**). Next, we analysed HLA-DR on *M.tb*-specific CD4+ T cells (**Figure 4.10B**). While there was no significant differences in the expression of HLA-DR on *M.tb*-specific CD4+ T cells pre-and post-ART in both groups, HLA-DR expression was variable among participants, with some participants in the early ART group (5/10) and late ART group (3/14) showing a decrease in the frequency of activated *M.tb*-specific cells after ART. Furthermore, there was a positive association between frequency of HLA-DR+ *M.tb*-specific CD4+ T cells and the VL of participants before ART in participants both in the early and late ART groups (p= 0.0008, r=0.6367) (**Figure 4.10C**). When we compared the two ART groups, the median frequency of HLA-DR+ *M.tb*-specific CD4+ T cells in the late ART group, compared to the early ART group, was higher before (9.8% vs 5.25%, respectively) and after ART (8% vs 5.7%, respectively), however this was not significantly different (**Figure 4.10B**). Interestingly, there was a positive correlation between total HLA-DR+ CD4+ T cells and HLA-DR+ *M.tb*-specific CD4+ ART before ART initiation (p = 0.0095, r = 0.519) (**Figure 4.11A**). Given that *M.tb*-specific CD4+ T cells exhibited primarily an ED memory profile (**Figure 4.9B**), we repeated the same analysis for total ED CD4+ T cells (**Figure 4.11C** and **D**). Results showed that there was a moderate positive association between activated *M.tb*-specific CD4+ T cells and activated total ED CD4+ T cells before and after ART (p = 0.0013 and 0.0003, r = 0.6164 and 0.6783, respectively). These data therefore demonstrate that the profile of HLA-DR expression on *M.tb*-specific CD4+ T cells mirrors that of total CD4+ T cells , a likely consequence of HIV-associated systemic activation.

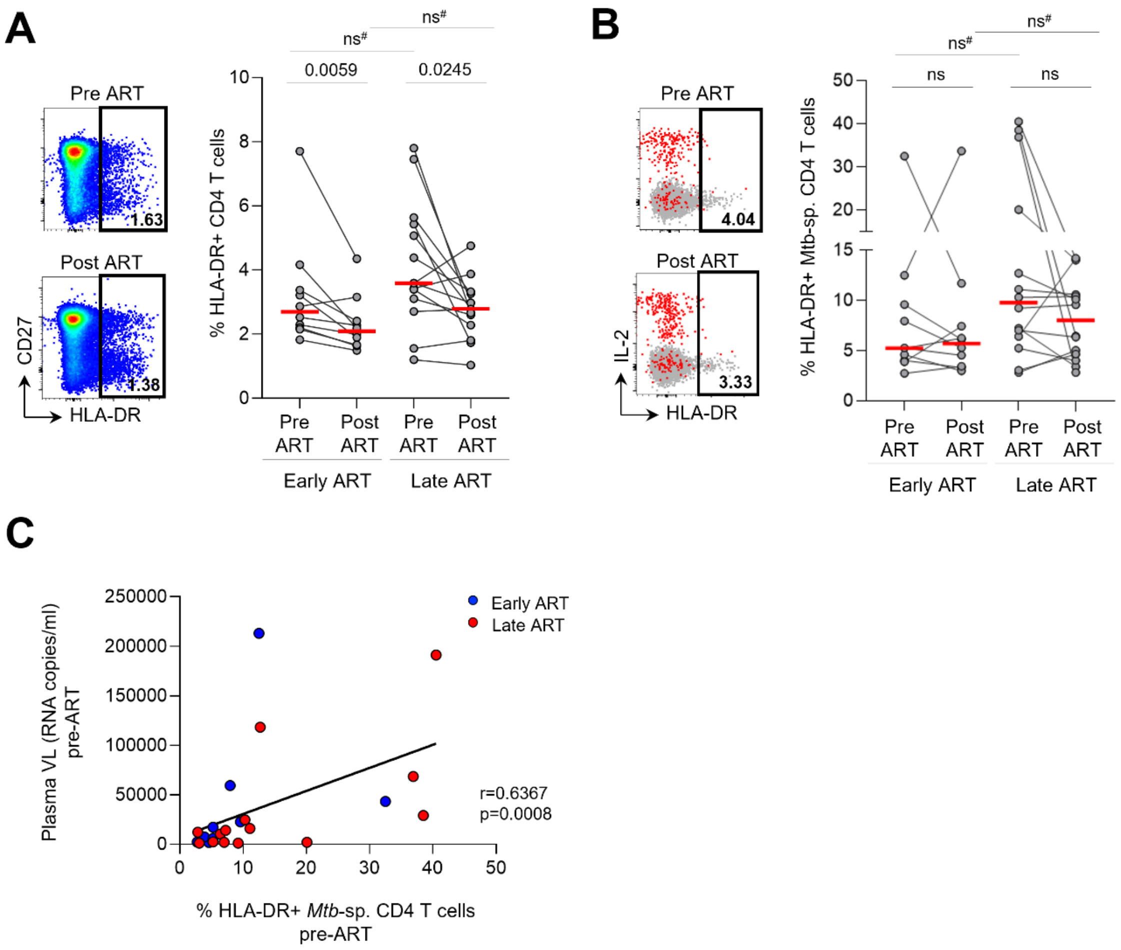


Figure 4.10: Frequency of HLA-DR + CD4+ T cells. (A) Representative flow cytometry plots and frequency of total HLA-DR+ CD4+ T cells in participants before ART initiation and at 2 years after treatment in early and late ART groups. **(B)** Representative flow cytometry plots and frequency of HLA-DR+ *M.tb*-specific CD4+ T cells in participants before ART initiation and at 2 years after treatment in early ART (n=10) and late ART groups (n=14). Red horizontal bars indicate the median values. Statistical significance was calculated using a Wilcoxon matched-pairs test and Mann-Whitney U test (indicated as #). Statistically non-significant P-values (P > 0.05) are

annotated as ns. (**C**) Spearman correlation assessing the relationship between the frequency HLA-DR+ *M.tb*-specific CD4+ T cells before ART and VL pre-ART in the early ART (blue, n=10) and late ART (red, n=14) groups. Associations of significance (P < 0.05) were also fitted with a best-fit line.

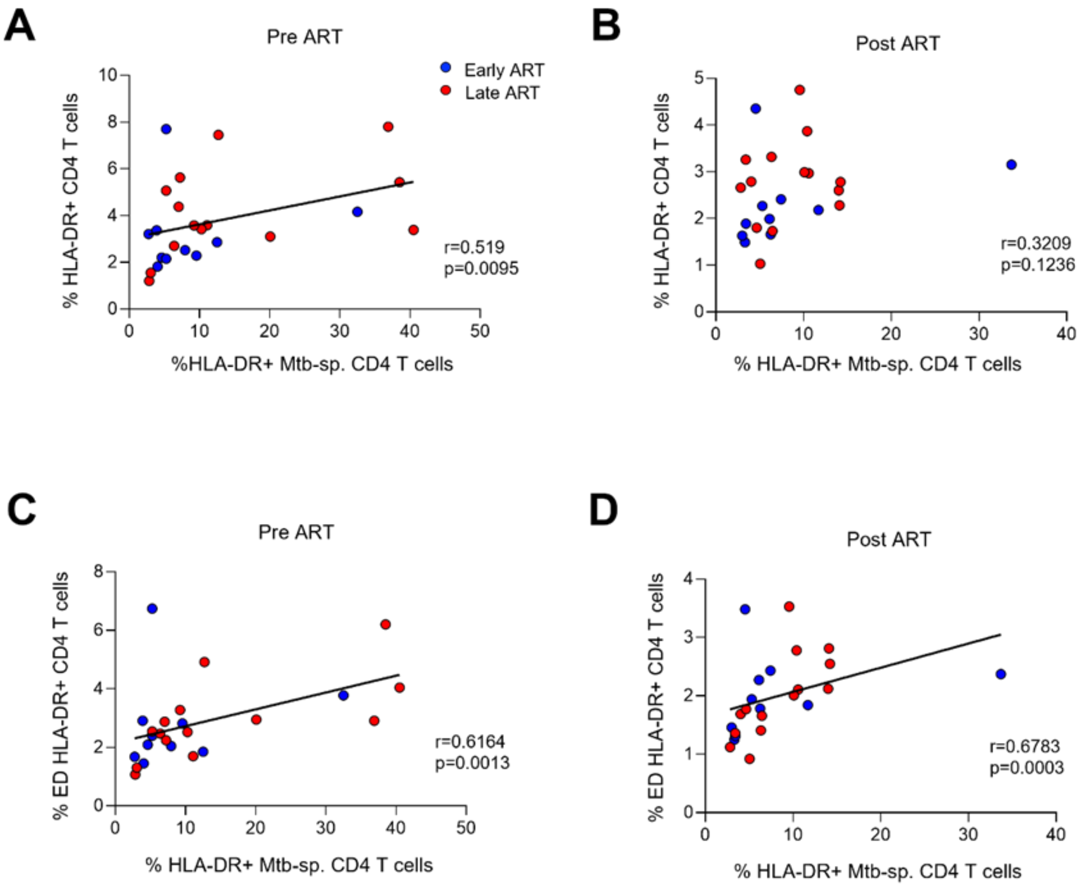


Figure 4.11: Association between frequency of HLA-DR+ *M.tb*-specific CD4+ T cells and total HLA-DR+ CD4+ T cells. Spearman correlations assessing the relationship between the frequency HLA-DR+ *M.tb*-specific CD4+ T cells and total HLA-DR+ CD4+ T cells in the early ART (blue, n=10) and late ART (red, n=14) groups **(A)** before and **(B)** 2 years after ART initiation, and between the frequency HLA-DR+ *M.tb*-specific CD4+ T cells and early differentiated (ED) total HLA-DR+ CD4+ T cells **(C)** before and **(D)** 2 years after ART initiation. P-values and Spearman r coefficients are displayed in the bottom right corner of each graph. Associations of significance (P <0 .05) were fitted with a best-fit line.

4.4 Discussion

ART is an important strategy in preventing TB in PLWH, resulting in a decrease in TB incidence and TB-related mortality (Moore *et al*., 2007; Suthar *et al*., 2012). When initiated early, ART is associated with a 2.5 fold decrease in TB incidence (Collins *et al*., 2015), making early ART a crucial step in TB protection for PLWH. In this study, to better understand the mechanism behind this decreased incidence of TB, impact of ART timing on TB responses, we characterised *M.tb*-specific immunity in individuals who initiated ART early and compared them to those who started later on during chronic HIV infection. Our main findings were that early ART initiation had no significant impact on the magnitude, function or phenotype of *M.tb*-specific CD4+ T cells 2 years after ART, when compared to late ART.

To our knowledge this is the first study which looks at the impact of ART timing on *M.tb*-specific immunity in PLWH. Prior studies that have looked at the effect of ART on TB immunity did not consider ART timing as a variable to consider. To date only one study, performed in our laboratory, has characterised *M.tb*-specific immune response in a cohort similar to ours. In this prior study, TB responses were examined in a group of HIV-infected women who initiated ART more than 2 years after infection, at a median of 4.9 years (Riou *et al*., 2015). That study showed that ART increased the absolute number of *M.tb*-specific CD4+ T cells (as a result of CD4 reconstitution), but had no significant impact on the frequency, the functional capacity or memory phenotype of these cells. Similarly, in our current study, we observed that in participants who initiated ART more than 2 years after infection (late ART group), ART had no significant impact on the function or phenotype of *M.tb*-specific CD4+ T cells. In contrast to the previous report, ART initiation in the late ART group did not significantly alter the absolute number of *M.tb*-specific CD4+ T cells. This may be due to cohort differences; in our late ART group the median absolute CD4 count before ART initiation was much higher (532 cells/µL) when compared to the previous study (289 cells/µL).

Previous reports show that early ART initiation reduces the viral burden that is associated with prolonged HIV infection and normalises CD4/CD8 ratios (Serrano-Villar *et al*., 2014; Rajasuriar *et al*., 2015). Consistent with these previous reports, in our study we found that participants in the early ART group had a significantly lower HIV viral burden (inferred by AUC VL) and a more normalised CD4/CD8 ratios after ART, when compared to late ART starters. Of the 22 participants we analysed in the late ART group, only 36% had CD4/CD8 ratios > 1 after ART, compared to 75% in the early ART group. This suggests that late ART initiation delays T cell normalisation. Recently, a study performed in sub-Saharan Africa showed that in PLWH, incomplete restoration of CD4/CD8 ratios increases TB risk, with significantly higher TB incidence rates observed in HIV-infected participants with a CD4/CD8 ratio of less than 0.3 (Wolday *et al*., 2020). Most importantly in this study, a low CD4/CD8 ratio was associated with an increased risk of TB, irrespective of viral suppression after ART. This suggests that despite viral suppression, sub-optimal T cell subset normalisation plays a role in increased risk of TB. It must be noted that in our study, none of the participants analysed in early and late ART groups developed active TB at any of the timepoints analysed. In fact, of the 22 participants analysed in the late ART group, only two had a CD4/CD8 ratio of less than 0.3 before ART, which later increased to more than 0.45 after treatment.

Studies performed in mouse models (Flynn *et al*., 1993; Cooper *et al*., 1993) and in humans (Vosse *et al*., 2013; Browne, 2014) show that IFN-γ plays a role in protective immunity against TB. However, it has been shown that while crucial in *M.tb* immunity, IFN-γ on its own is not sufficient for TB protection (Mittrücker *et al*., 2007; Tameris *et al*., 2014). Polyfunctional T cells, producing IFN-γ, TNF-α and IL-2, show greater association with protective immunity in infectious diseases such as *Leishmania major* (Darrah *et al*., 2007), cytomegalovirus (Kannanganat *et al*., 2007; Snyder *et al*., 2016), hepatitis C virus (Ciuffreda *et al*., 2008) and HIV (Duvall *et al*., 2008), than IFN-γ alone. In the case of HIV infection, previous studies show that HIV non-progressors express high levels of polyfunctional CD4 T cells (Duvall *et al*., 2008). Similarly, *M.tb*-specific CD4+ T cells that co-express IFN-γ, TNF-α and IL-2 have been shown play a role in *M.tb* immunity. Day and colleagues showed that individuals with active TB had a decrease in polyfunctional IFN-γ+ TNF-α+ IL-2+ *M.tb*-specific CD4+ T cells compared to those with LTBI (Day *et al*., 2011). Moreover, in individuals with active TB there was an increase in polyfunctional *M.tb*-specific CD4+ T cells following a decrease in bacterial load due to TB treatment. Thus, in our study to determine the effects of ART timing on the qualitative aspect of *M.tb* immunity, we examined the ability of *M.tb*-specific CD4+ T cells to co-express four cytokines (IFN-γ, TNF-α, IL-2 and MIP-1β). We found that a large majority of *M.tb*-specific CD4+ T cells (40%) were polyfunctional cells, co-expressing IFN-γ, TNF-α and IL-2. We also showed that the timing of ART initiation, whether early or late, did not significantly alter the polyfunctional profile of these cells after two years of treatment. It is important to acknowledge that although in this study we focused on the expression of Th1 cytokines as a measure of TB immunity, these responses do not encompass all Th subsets involved in TB protection. Indeed, recent studies show that several other T helper subsets such as Th22 and

Th17 cells may have a role to play in TB immunity. Evidence from mouse studies show that IL-22 deficient mice infected with a clinical *M.tb* strain, HN878, had higher bacterial burden in their lungs during chronic stages of infection (Treerat *et al*., 2017). In humans, individuals with mutations in essential IL-22 pathways, such as the IL-22 promotor region (Zhang *et al*., 2011) and the Th17/Th22 transcription factor, RORγt (Okada *et al*., 2015) had a greater risk of developing TB. In the case of Th17 cells, Freches *et al.* showed that mice deficient in IL-17A receptor had impaired long-term control of *M.tb* infection (Freches *et al*., 2013). This impairment was due to a decrease in the recruitment of neutrophils in the early stages of infection. Thus, further investigation is needed to define the impact of ART on T helper subsets and potential mechanisms of protection beyond Th1 immunity.

In our study, to further assess the function of *M.tb*-specific responses we examined CD153, a CD30 ligand that mediates the secretion of Th1 cytokines in *M.tb*-specific CD4+ T cells (Marin and Garcia, 2017). Recent studies in mice and humans have highlighted this marker as a potential correlate of TB immunity. A study by Sallins and colleagues showed that CD153 knock-out mice develop high mycobacterial loads in the lungs and had increased death after *M.tb* infection compared to wildtype mice (Sallins *et al*., 2018). In a more recent study in humans, the proportions CD153+ *M.tb*-specific CD4+ T cells were lower in individuals with active TB compared to those with LTBI (Du Bruyn *et al*., 2021). Furthermore, the proportion of CD153+ *M.tb*-specific CD4+ T cells in the periphery was inversely associated with bacterial load in sputum of individuals with TB (Du Bruyn *et al*., 2021). In our cohort, we found that a large proportion of *M.tb*-specific CD4+ T cells express CD153 (~60%) and CD153 expression was similar in the early and late ART groups and was not affected after 2 years of ART. Such expression levels are in keeping with the latent TB status of the participants included in our study.

Previous reports have shown that there is a rapid depletion of *M.tb*-specific CD4+ T cells early after HIV infection (Geldmacher *et al*., 2008; 2010). In this study, they showed that *M.tb*-specific Th1 responses were depleted within a year of HIV infection in participants with LTBI. Notably in the five participants examined in this prior study, three of these showed decreases in responses within the first 6 months of infection. In our study, the median time from ART in the early ART group was 7.5 months, with the majority of participants (63%) starting treatment more than 6 months after HIV infection. This suggests that in our early ART group some of the participants may have already lost their *M.tb*-specific responses before ART initiation, as demonstrated in Chapter 3. This may account for why in our study we observed no differences between the early and late ART starters in terms of greater preservation of *M.tb*-specific responses.

Our study had several limitations. Around 32% of participants in our late ART group could be classified as VL controllers. We defined VL controllers as participants who consistently had an average VL below 15,000 copies/mL and a CD4 count above 500 cells/µL after the first year of HIV infection. In fact, we may have inadvertently enriched our late ART group with those who controlled their HIV infection and survived for longer periods in the absence of ART. Our selection of participants for study was based upon years of infection without ART and sample availability in the biorepository. Consequently, this may have had a substantial effect on our results. We do note however that in our study CD4 counts and VL were not identified as associating with

M.tb-specific responses using multivariate regression models. In fact, results of the model showed that it is only the participants' *M.tb*-specific CD4+ T cell responses before ART initiation, and not any of the clinical characteristics we assessed, that determined *M.tb*-specific responses after treatment. Another factor our study did not account for is the effect of sex. Given that our study consisted of an all-female cohort, we note that results obtained from this cohort may not be generalisable to males. Lastly, our study had a limited number of participants in our early (n=16) and late (n=22) ART groups. There was clear heterogeneity among the participants within groups, and larger numbers may enable a more detailed examination of different patterns of responses. Thus, to better understand the effects of ART timing on *M.tb*-specific responses, a larger cohort is warranted with more careful selection to avoid selection biases. Another possible interpretation for why we saw no significant differences in TB responses between the two ART groups in our study is that the high TB rates, in the setting of HIV , a likely due to impairments in the innate immune system and not necessarily due to defects in *M.tb*-specific CD4+ T cells. This may explain why certain individuals are still at high risk of TB even when on ART or with maintained *M.tb*-specific CD4+ T cells.

In conclusion, this is the first study that examined the impact of ART timing, whether early or late, on *M.tb*-specific responses. We showed no overall differences in the function or phenotype of *M.tb*-specific CD4+ T cell responses between participants who initiated ART early and those who initiated later during chronic HIV infection. The study provides important insights to guide future research into whether early ART preserves TB immunity and reduces the early, elevated risk of TB in HIV-infected individuals.

CHAPTER 5

The impact of TB treatment on *M.tb*-specific CD4+ T cells

Table of Contents

5.1 Introduction

The risk of developing TB is increased during HIV infection, with the risk greatest during untreated HIV infection, when CD4 counts are low and viral loads are high, often leading to opportunistic infections (Daley *et al*., 1992; Lawn *et al*., 2005; WHO Global TB report, 2021). People living with HIV (PLWH) who are highly immunocompromised are also at high risk of developing TB shortly after starting antiretroviral therapy (ART) due to a clinical condition known as TB-associated immune reconstitution inflammatory syndrome (IRIS) (Lai *et al*., 2015; Gopal *et al*., 2017; Xue *at al*., 2020). This is an acute inflammatory response that is often accompanied by the worsening or development of new TB disease in patients already on ART or TB treatment (Lanzafame and Vento, 2016; Walker *et al*., 2018). Additionally, PLWH are also a risk of TB even after successful viral suppression and CD4 reconstitution through ART (Girardi *et al*., 2005; Bonnet *et al*., 2006; Lawn *et al*., 2006). Currently the standard treatment for TB in South Africa consists of 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol (RHZE), followed by 4 months of isoniazid and rifampicin (RH) (National Tuberculosis Management Guidelines, 2014). Despite this effective TB treatment regimen TB is very far from being under control, with the incidence of new TB cases caused by drug-resistant *M.tb* remaining high globally (WHO Global TB report, 2021). There is therefore an urgent need for an effective TB vaccine, especially for PLWH, who tend to have poorer TB treatment outcomes compared to HIV-uninfected individuals (Bastard *et al*., 2018; Fekadu *et al*., 2020).

Currently the only available TB vaccine, bacille Calmette-Guerin (BCG) is not effective against pulmonary TB in adults, with the efficacy of the vaccine ranging from 0 to 80%

(Andersen and Doherty, 2005; de Gijsel and Reyn, 2019). The current lack of an effective vaccine for TB is mainly due to the fact that our knowledge on what constitutes protective immunity to *M.tb* is still limited. While it is clear that CD4+ T cells are essential for TB protection (Caruso *et al*., 1999; Mogues *et al*., 2001; Lin *et al*., 2012), the mechanism that results in loss of TB immunity or *M.tb* reactivation is still unknown in humans. It is well established from mouse models deficient in IFN-γ and related genes (Flynn *et al*., 1993; Cooper *et al*., 1993; Green *et al*., 2013) and patients with mutations in IFN-γ pathways (Filipe-Santos *et al*., 2006; Vosse *et al*., 2013), that this cytokine plays an important role in TB immunity. However, several studies have now shown that IFN-γ alone is not sufficient as a correlate for TB protection (Winkler *et al*., 2005; Caccamo *et al*., 2010; Mattila *et al*., 2011). There are reports that suggest that polyfunctional CD4+ T cells, producing multiple cytokines in combination with IFN-γ, may have a role to play in TB protection. In these studies, BCG vaccine induced immunity to *M.tb* in mice, which correlated to polyfunctional CD4+ T cell responses (Lindenstrøm *et al*., 2009; Derrick *et al*., 2011). However, studies in humans show that although necessary, polyfunctional CD4+ T cell responses are not sufficient against TB. In one such study by Kagina and colleagues, polyfunctional *M.tb*-specific CD4+ T cell responses did not correlate with TB protection in infants vaccinated with BCG (Kagina *et al*., 2010). Similarly, when BCG vaccinated infants were given a booster dose of MVA85A there was no enhancement in TB protection even though the booster dose induced significantly higher proportions of polyfunctional CD4+ T cells compared to BCG alone (Tameris *et al*., 2013).

Recently, evidence emerged to suggest IFN-γ independent protective immunity against TB by CD4+ T cells. A study by Gallegos and colleagues showed that adoptive transfer of *M.tb*-specific Th1 cells from IFN-γ deficient mice protected the hosts from *M.tb* infection independently of IFN-γ and TNF-α production (Gallegos *et al*., 2011). Using a similar method, they also showed that adoptive transfer of *M.tb*-specific Th17 cells partially inhibited bacterial growth in the lungs of *M.tb* infected mice (Gallegos *et al*., 2011). Similarly, a study by Gopal and colleagues showed that in BCG-vaccinated mice, antigen-specific Th17 cells localised to the lungs and assisted in the recruitment of Th1 cells into the lungs during *M.tb* infection (Gopal *et al*., 2012). In another study by the same group, they also reported that the role of Th17 cells in TB protection was strain dependent. In this report, they showed that in mice IL-17 was protective against the hypervirulent strain HN878 but not the lab strain H37Rv (Gopal *et al*., 2014). In humans it is still poorly understood what role *M.tb*-specific Th17 cells play in TB protection. However, there are reports that show that genetic mutations in Th17 cells in humans is associated with susceptibly to TB (Okada *et al*., 2015; Wang *et al*., 2016).

To date, methods for TB diagnosis and monitoring of treatment are mainly based on microbiological, clinical, and radiographic examinations. However, these methods all have their limitations. For example, the gold standard of monitoring TB treatment outcome is sputum culture conversion, which takes up to 3 or 6 weeks (Parrish *et al*., 2011). Immunological tests that look at *M.tb*-specific T cell responses would be an ideal method to distinguish between LTBI (latent TB infection) and active TB and for monitoring responses to treatment. While tests like IFN-γ release assays (IGRAs) are useful in detecting *M.tb* infection, they have proven to be inadequate in discriminating between LTBI and active TB, as well as monitoring treatment outcomes (Meier *et al*., 2005; Diel *et al*., 2008; Pinto *et al*., 2012, Nikitina *et al*., 2012; Denkinger *et al*., 2013). A number of studies have highlighted the potential of using functional and phenotypic characterisation of *M.tb*-specific CD4+ T cells. In one such study they showed that there was an association between the quantity and quality of *M.tb*-specific CD4+ T cells and the bacterial load in the lungs of patients with TB (Day *et al*., 2011). When compared to individuals with LTBI, TB patients had significantly lower IL-2 production and polyfunctional (INF-γ+ IL-2+ TNF-α) *M.tb*-specific CD4+ T cells. In addition, *M.tb*-specific T cells in patients with TB had lower proliferative responses compared to those with LTBI. Following a reduction in bacterial load by TB treatment, there was an increase in polyfunctional *M.tb*-specific CD4+ T cell responses (Day *et al*., 2011). In another study, Nikitina and colleagues showed that while individuals with LTBI and TB could not be discriminated by the frequency of IFN-γ-producing *M.tb*-specific CD4+ T cells, they could instead be distinguished by the degree of differentiation of these cells (Nikitina *et al*., 2012). TB Patients had a higher frequency of *M.tb*-specific CD27^{low} CD4+ T Cells in their peripheral blood compared to those with LTBI. More importantly, after TB treatment there was a decrease in the frequency of *M.tb*-specific CD27^{low} CD4+ T Cells in TB patients and this was associated with a reduction in tissue damage in the lungs (Nikitina *et al*., 2012). Similarly, a study by Saharia and colleagues also reported that while TB treatment had no significant effect on the frequency of total *M.tb*-specific CD4+ T cells in both HIV-infected and uninfected individuals, there was a higher proportion of IFN-γ+IL-2+TNF-α+ polyfunctional and IFN-γ+IL-2+ dual functional *M.tb*-specific CD4+ T cells after treatment (Saharia *et al*., 2016). This change in cytokine profile *M.tb*-specific CD4+ T cells after TB treatment was related to the maturation state of cells and also in a decrease in the expression of the inhibitory markers PD1 and CTLA-4 (Saharia *et al*., 2016). More recently, there are reports that show that individuals with TB have a high frequency of activated *M.tb*-specific CD4+ T cells compared to those with LTBI. In these reports, individuals with TB had higher frequencies of *M.tb*-specific CD4+ T cells expressing the activation markers CD38, HLA-DR, and Ki-67 compared to those with LTBI (Adekambi *et al*., 2017; Ahmed e*t al*., 2018). They showed that the expression of these three activation markers inversely correlated with a successful response to TB treatment, with both studies showing a marked decline in activated *M.tb*-specific CD4+ T cells 2 months after treatment (Adekambi *et al*., 2017; Ahmed e*t al*., 2018).

In this chapter, to characterise *M.tb*-specific responses in active TB infection, we performed a descriptive study examining the dynamics of *M.tb*-specific CD4+ T cell responses longitudinally during active disease. To do this we selected 13 participants with sufficient PBMC samples from a small cohort of individuals who developed TB during the CAPRISA 002 study. We assessed the function and phenotype of *M.tb*-specific CD4+ T cells before, during and after TB treatment, in the context of treated and untreated HIV. We hypothesize that following TB treatment there is a modification in both the magnitude and phenotype of *M.tb*-specific CD4+ T cells.

5.2 Materials and methods

All materials and methods used in this study are described in Chapter 2

5.3 Results

5.3.1 Clinical characteristics of participants during TB treatment

The CAPRISA 002 study is a longitudinal cohort following PLWH from HIV seroconversion to up to 5 years after ART initiation. In this study, 10% (25/250) of the participants developed TB over the course of HIV infection and treatment. From these 25 participants, 13 participants had with sufficient cryopreserved PBMC samples for study. The PBMC samples were stimulated with a pool of peptides (MTB300) based on dominant T cell targets from *M.tb* (Arlehamn *et al*., 2016). A summary of the demographic and clinical characteristics of these participants are presented in **Table 5.1**. **Figure 5.1** shows the study schema for the 13 participants, illustrating the different timepoints at which blood was collected for immunological analyses. The cohort consisted of 13 participants who developed TB at a median of 57 months (IQR 26-74) after HIV infection. Of these 13 participants, three developed TB and completed TB treatment prior to ART initiation, 6 participants developed TB prior to ART initiation and completed TB treatment during ART, and 4 participants developed TB during ART and received TB treatment. Firstly, we examined the clinical characteristics of the cohort over the course of TB treatment. To do this we compared the HIV plasma viral load (VL), absolute CD4+ T cell counts and CD4/CD8 ratios of the participants prior to (median: 8 months before TB treatment [IQR 3-16]), during (median: 2 months after start of TB treatment [IQR 1-5]), and after TB treatment (median: 19 months after TB treatment [IQR 10-22]). Prior to TB treatment only a few (4/12) participants were on ART compared to during (6/9) and after (8/10) TB treatment (**Figure 5.1A-C**). As a consequence, the median HIV plasma VL of participants before TB treatment was higher compared to after treatment (median: 34,956 RNA copies/mL [IQR 31-171,250] vs undetectable, respectively, p = 0.0159) (**Figure 5.1A**). Only one participant on ART had a high VL (25,053 RNA copies/ml) after TB treatment. Similarly, because of more individuals on ART, participants had a significantly higher CD4/CD8 ratio after treatment compared to before treatment (median: 0.43 [IQR 0.33-0.89] vs 0.78 [IQR 0.53-1.46] respectively, p = 0.0286) (**Figure 5.1B**). In contrast, there was no significant difference in the absolute CD4+ T cell count after TB treatment (**Figure 5.1C**).

Table 5.1: Demographics and clinical characteristics of participants with TB

PID	Age at TB diagnosis	TB prior to ART initiation	TB after ART initiation	TB diagnosis (Months after HIV infection)	End of TB treatment (Months after HIV infection)	TB diagnosis* (Months after ART initiation)	End of TB treatment (Months after ART initiation)	TB treatment successful	HIV VL at TB treatment start (RNA copies/mL)	CD4 count at TB treatment start (cells/µL)	HIV VL after TB treatment (mRNA copies/mL)	CD4 count after TB treatment (cells/µL)
100037	26	Yes		65	71	-2	4	Yes	40449	302	1	704
100065	46	Yes		79	88	-1	8	Yes	41106	223	1	426
100088	29	Yes		57	63	-4	2	Yes	177000	633	1	1047
100188	39		Yes	75	82	18	25	Yes	121	350	1	370
100222	28		Yes	102	109	29	36	Yes	ND	742	1	548
100274	28	Yes		74	81	-21	-14	Yes	29463	507	31876	565
100290	24	Yes		25	32	-4	3	Yes	N/A	N/A	1	677
100314	23	Yes		26	36	-1	9	Yes	10100	310	N/A	N/A
100325	26	Yes		31	38	-45	-38	Yes	154000	417	9522	494
100329	26		Yes	58	78[#]	40	60	Yes	ND	456	1	645
100361	21	Yes		23	30	-45	-38	Yes	265000	492	N/A	N/A
100377	23	Yes		26	32	-2	5	Yes	507905	301	25053	671
100454	31		Yes	39	45	5	11	Yes	ND	891	N/A	N/A
Median	26	N/A	N/A	57	63	2	5	N/A	34956	436	ND	605
IQR	23-30	N/A	N/A	26-74	31-81	-12-11	-6-18	N/A	31-171250	304-601	ND	477-683

[#] This participant had TB treatment for 19 months according to clinical records, but no further details are available; *TB treatment was initiated within the same week as TB diagnosis, ND = not detectable.

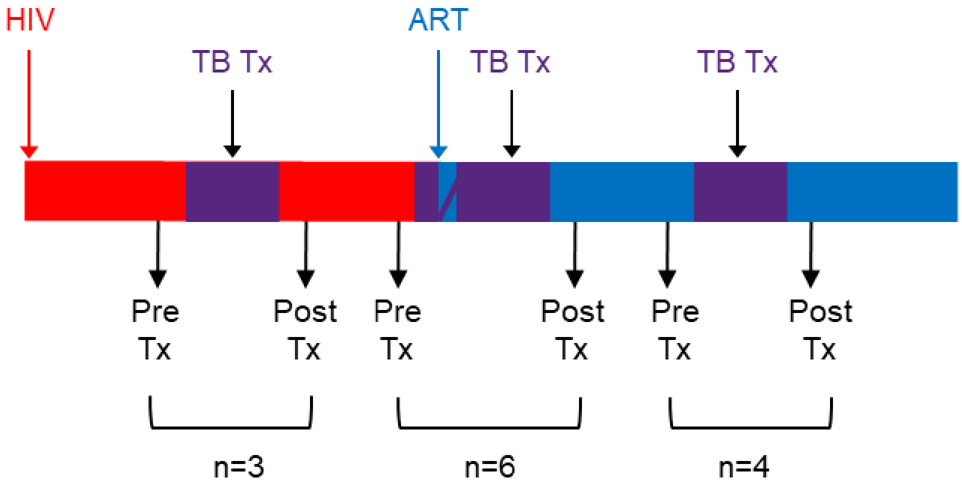


Figure 5.1: Study schema of the TB cohort. An outline showing sampling timepoints (indicated by arrows) during HIV infection (red), ART initiation (blue) and TB treatment (purple) for 13 participants who developed TB during the CAPRISA 002 study. The black arrows indicate timepoints at which blood was collected.

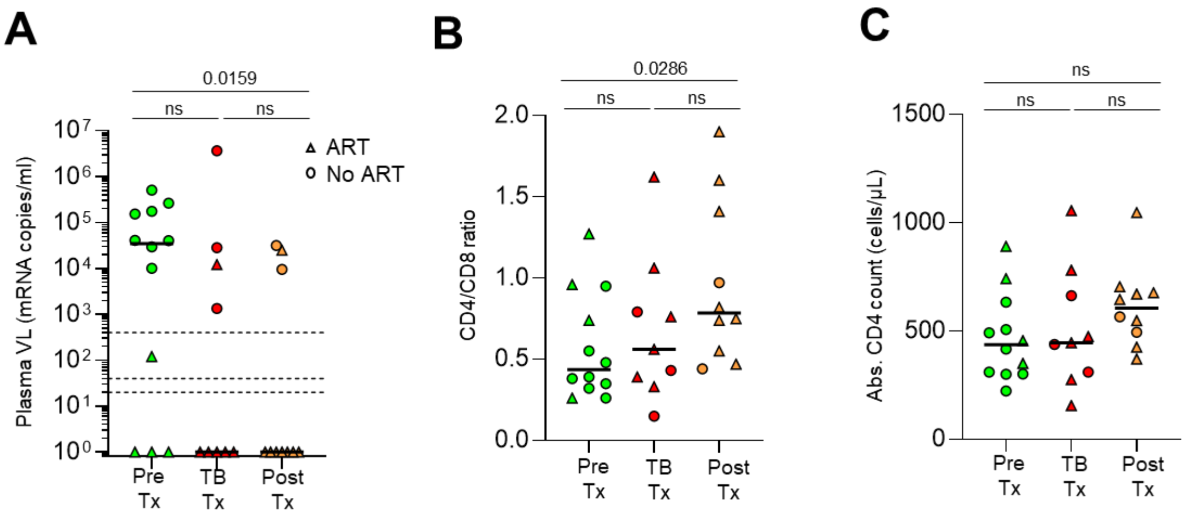


Figure 5.2: Clinical characteristics of participants during TB treatment. (A) Plasma HIV viral load, **(B)** CD4/CD8 ratio and **(C)** absolute CD4+ T-cell count for participants (n=13) before (green), during (red) and after (orange) TB treatment. Triangles represent participants who are on ART and circles are individuals who have not initiated ART. The horizontal dashed lines indicate the detection limit of the assays. Black horizontal bars indicate the median values. Statistical significance was

calculated using a Mann-Whitney U test. Statistically non-significant P-values (P>0.05) are annotated as ns.

5.3.2 The dynamics of M.tb-specific T cell responses during TB treatment

We assessed the effects of TB treatment on *M.tb*-specific T cell responses. **Figure 5.3A and 5.3B** show the frequencies of *M.tb*-specific CD4+ T cells producing IFN-γ, TNF-α, IL-2 or MIP-1β in two participants prior to, during and after TB treatment. *M.tb*-specific CD4+ T cell responses were readily detected throughout the course of TB treatment in the majority of our cohort. To characterise the effects of TB treatment on *M.tb*-specific CD4+ T cells we performed a cross-sectional analysis of TB responses in the 13 participants (**Figure 5.4A**), as well as a longitudinal analysis of eight participants who had paired timepoints (**Figure 5.4B**). Assessment of both the cross-sectional and longitudinal analyses showed no significant differences in the median frequencies of *M.tb*-specific CD4+ T cells over the course of TB treatment (**Figure 5.4A and 5.4B**). However, we did observe that in the cross-sectional analysis half (6/12) of the participants had a high frequency of *M.tb*-specific CD4+ T cells (above 0.8%) prior to TB treatment (**Figure 5.4A**). This suggest that these individuals may have had TB prior to TB treatment/diagnosis. Given that some of the participants in our cohort were on ART during the course of TB treatment, we also examined the impact of absolute CD4 T cell counts on *M.tb*-specific CD4+ T cell responses. We found no significant differences in the absolute number of *M.tb*-specific CD4+ T cells both in the cross-sectional and longitudinal analyses (**Figure 5.4C and 5.3D**). To assess if there is a relationship between *M.tb*-specific CD4+ T cell responses and the duration of TB treatment, we performed spearman correlations (**Figure 5.4E and 5.5F**). We found no significant association between *M.tb*-specific responses (during

TB treatment) and the months after TB initiation or between responses (after TB treatment) and the months after the end of treatment. Compared to *M.tb*-specific CD4+ T cell responses, CD8+ T cell responses were of a lower magnitude, with only a few individuals having responses before (3/12), during (1/9) and after (2/10) TB treatment (**Figure 5.5A**). There were no significant differences in the frequency of *M.tb*-specific CD8+ T cell responses over the course of TB treatment (**Figure 5.5A and 5.5B**). Taken together, these data suggests that in our cohort overall, TB treatment had no significant effect on the magnitude of *M.tb*-specific T cells.

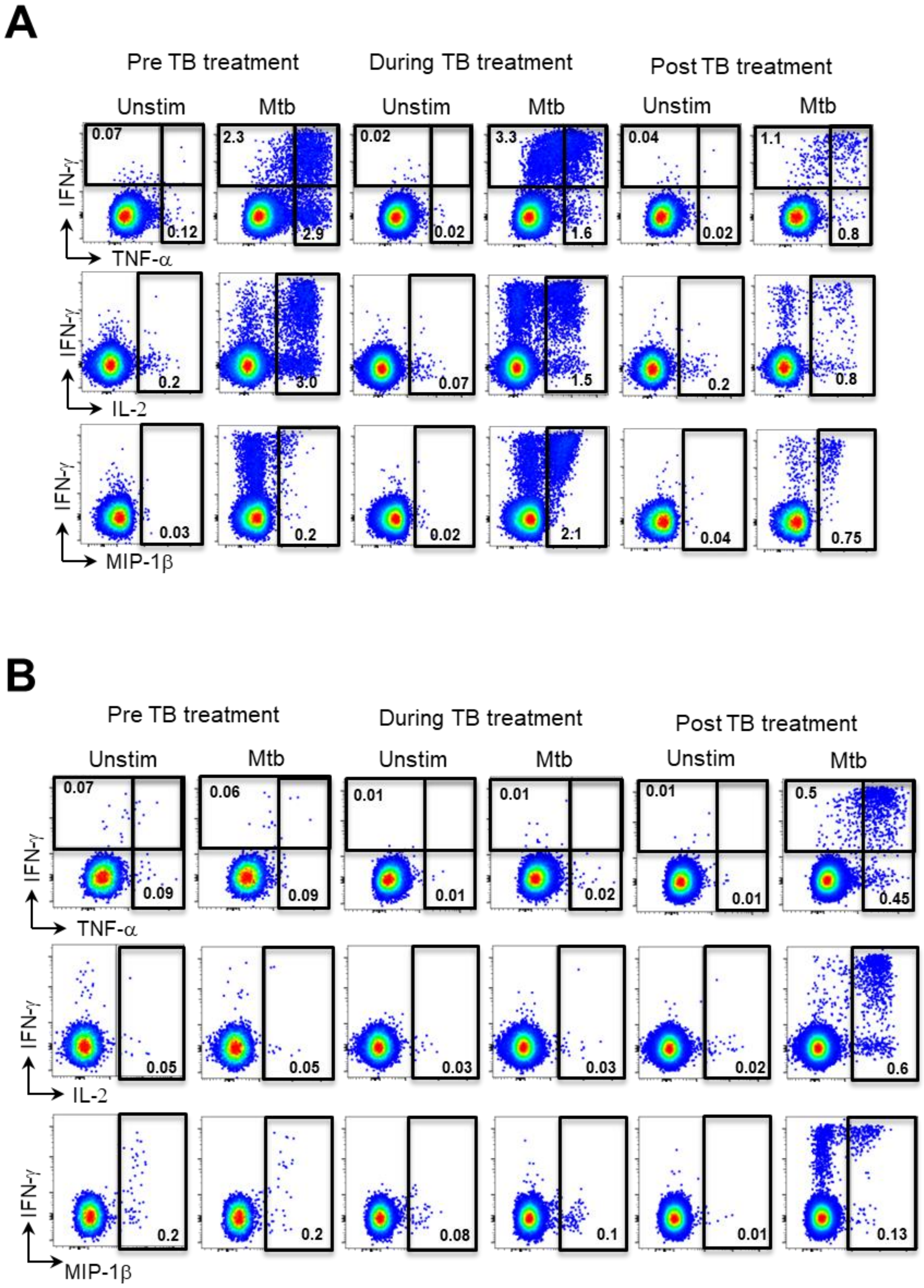


Figure 5.3: Flow cytometry plots showing IFN-γ, TNF-α, IL-2 and MIP-1β responses in *M.tb*-specific CD4+ T cells after stimulation with MTB300 in **(A)** a participant whose responses were readily detected throughout the course of TB treatment and **(B)** a participant who only had a detectable response after TB treatment.

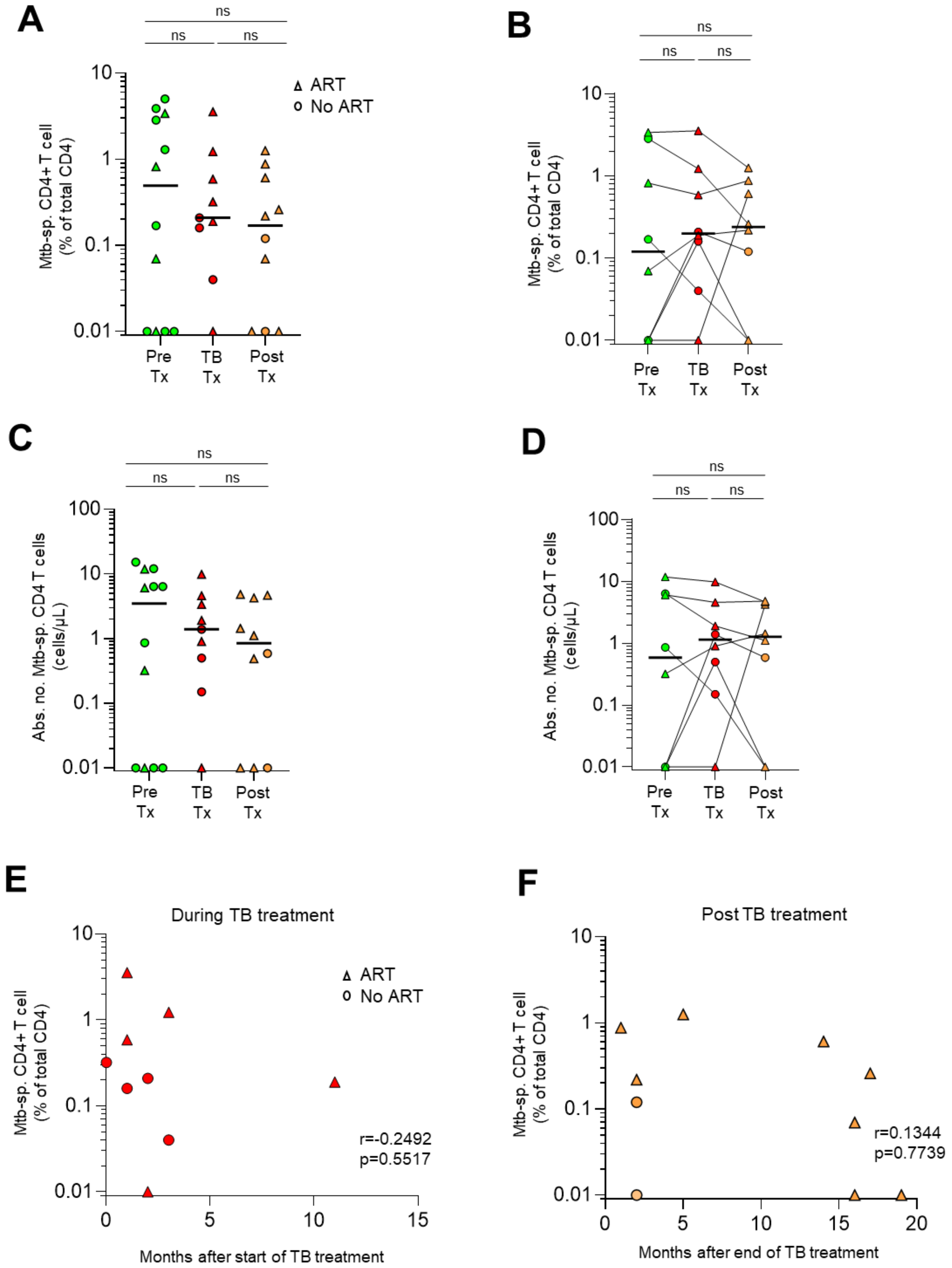


Figure 5.4: The magnitude of *M.tb*-specific CD4+ T cell responses. (A) Cross-sectional (n=13) and **(B)** longitudinal (n=8) analyses of the frequency total *M.tb*-specific CD4+ T cells in participants before (green), during (red) and after (orange) TB treatment. The absolute number of *M.tb*-specific CD4+ T cells in participants in the **(C)**

cross-sectional (n=13) and **(D)** longitudinal (n=8) analyses during the course of TB treatment. Black horizontal bars indicate the median values. Statistical significance was calculated using a Mann-Whitney U test and Wilcoxon matched-pairs test. Non-significant P-values (P > 0.05) are annotated as ns. Spearman correlations assessing the relationship between **(E)** the frequency of total *M.tb*-specific CD4+ T cells during TB treatment and months after the start of treatment and **(F)** responses after TB treatment and months after the end of treatment. P-values and Spearman r coefficients are displayed in the bottom right corner of each graph.

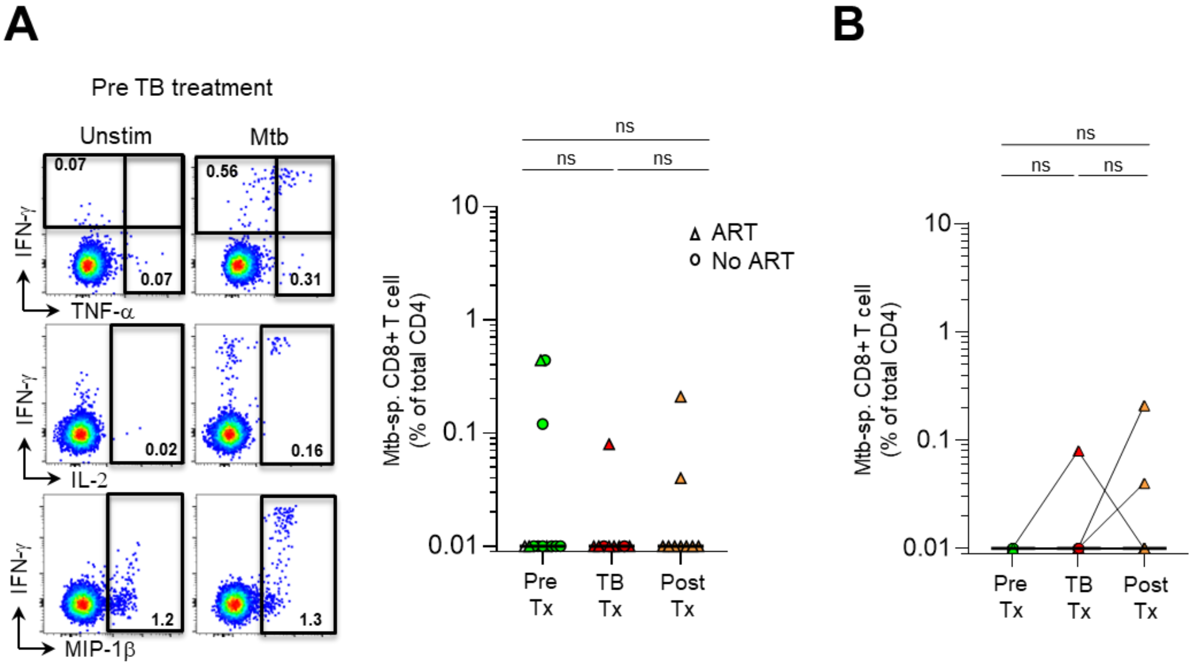


Figure 5.5: The magnitude of *M.tb*-specific CD8+ T cell responses. (A) Flow cytometry plots of CD8+ cytokine responses in one donor pre-TB treatment (left panel) and cross-sectional (n=13) analyses (right panel) of the frequency of total *M.tb*-specific CD8+ T cells in participants before (green), during (red) and after (orange) TB treatment. **(B)** Longitudinal (n=8) analyses of the frequency of total *M.tb*-specific CD8+ T cells. Black horizontal bars indicate the median values. Statistical significance was calculated using a Mann-Whitney U test and Wilcoxon matched-pairs test. Statistically non-significant P-values (P > 0.05) are annotated as ns.

Next we wanted to assess the kinetics of *M.tb*-specific CD4+ T cell responses longitudinally over the course of TB treatment. To do this, we examined the trajectory of the *M.tb*-specific CD4+ T cell responses in eight participants with at least three longitudinal samples (**Figure 5.6**). We found that in participants (4/8) who had two timepoints prior to TB diagnosis there was a significant increase in the frequency of *M.tb*-specific CD4+ T cells at the timepoints closer to treatment initiation (median: 5 months before TB treatment initiation [IQR 3–9]) (**Figure 5.6A-D**). These results likely reflect active bacterial replication triggering the expansion of *M.tb*-specific CD4+ T cells, months before TB diagnosis. We also observed that in the majority (5/8) of participants *M.tb*-specific responses decrease after TB treatment (median: 5 months after TB treatment [IQR 2–17]), with only one participant showing a major increase in responses after treatment (**Figure 5.6H**). Overall, these data suggests that most participants had similar kinetics of *M.tb*-specific responses, showing an increase in the frequency of *M.tb*-specific CD4+ T cell responses at timepoints closer to TB diagnosis and a contraction of these response after successful TB treatment.

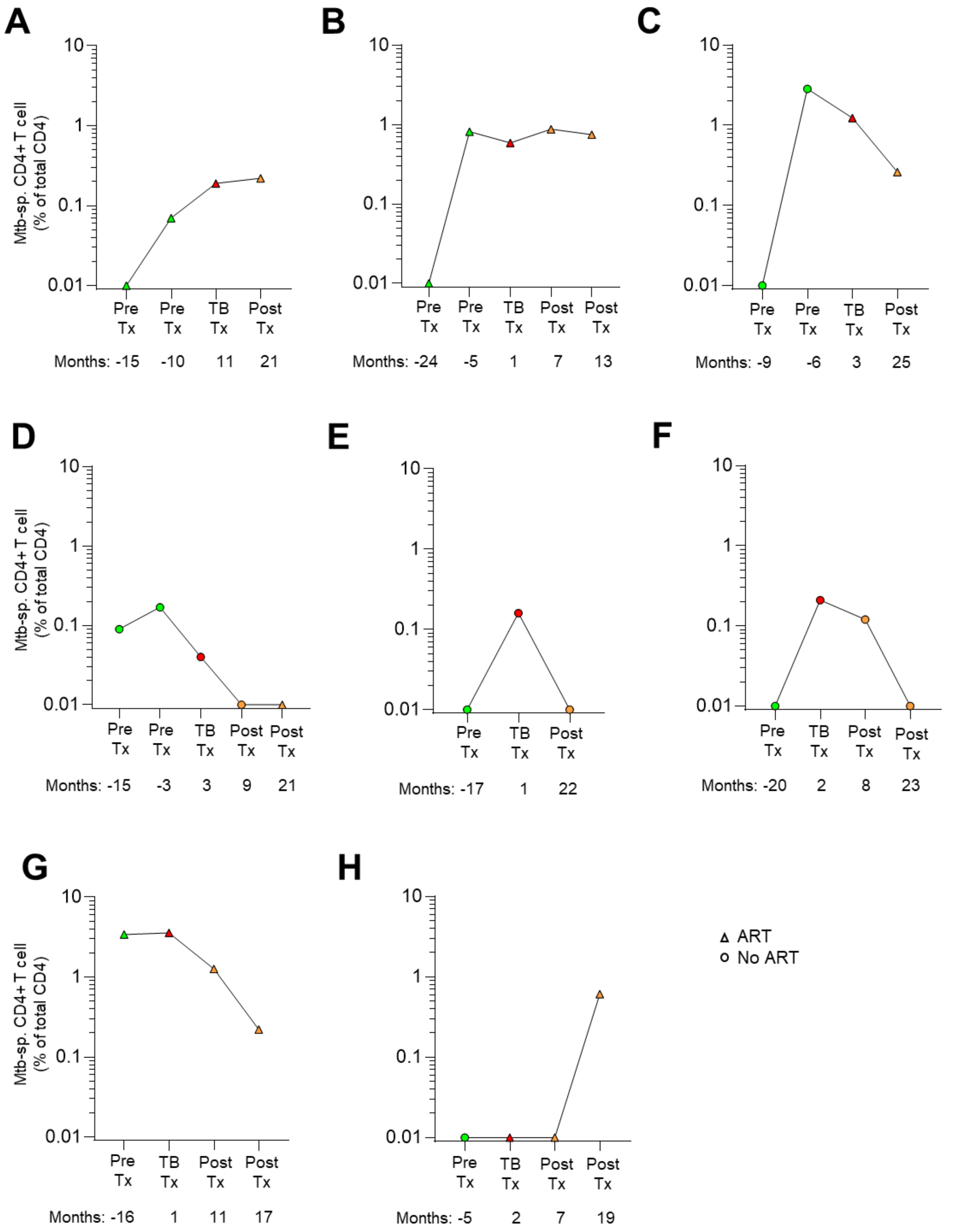


Figure 5.6: The kinetics of *M.tb*-specific CD4+ T cells during TB treatment. Longitudinal analysis showing the trajectories of *M.tb*-specific CD4+ T cell responses in participants (n=8) before (green), during (red) and after (orange) TB treatment.

Months shows the time relative to TB treatment initiation. Some participants had two timepoints available for study prior to or after TB treatment.

5.3.3 The impact of TB treatment on the function and phenotype of M.tb-specific CD4+ T cells

Finally, we wished to determine whether TB treatment had an impact on the functional and phenotypic profile of *M.tb*-specific CD4+ T cells. We performed a cross-sectional comparison of the polyfunctional profiles of *M.tb*-specific CD4+ T cells of participants at different phases of TB treatment using a Boolean gating strategy (**Figure 5.7A**). Polyfunctional profiles of *M.tb*-specific CD4+ T cells were comparable over the course of TB treatment, with responses consisting mainly of cells co-expressing three cytokines (IFN-γ, IL-2 and TNF-α; ~40%), IL-2+TNF-α+ or IFN-γ+IL-2+ dual functional and IL-2+ cells (~8% each), as well as polyfunctional cells expressing all four cytokines (~6%). While there were no significant differences overall, we did observe a trend towards a higher proportion of IFN-γ+IL-2+ dual functional cells after TB treatment compared to before treatment (median: 7.5% [IQR 3.9-10.7] vs 11.1% [IQR 10.8-18.6] respectively, p = 0.058). Next, we examined the expression of CD153 (TNFSF8, CD30L) on *M.tb*-specific CD4+ T cells longitudinally during TB treatment (**Figure 5.7B**). Previous findings show that CD153+ *M.tb*-specific CD4+ T cells were more abundant in individuals with LTBI compared to those with active TB (Sallin *et al*., 2018). We found that participants with TB had elevated CD153 expression (median: ~60%) on *M.tb*-specific CD4+ T cells, although this was similar to frequencies we previously observed in individuals with LTBI in Chapter 3. In addition, there were no significant differences in the frequencies of CD153+ *M.tb*-specific CD4+ T cells over the course of TB treatment. Overall, these data indicate that there are only minor changes in the cytokine profile of *M.tb*-specific CD4+ T cells after TB treatment.

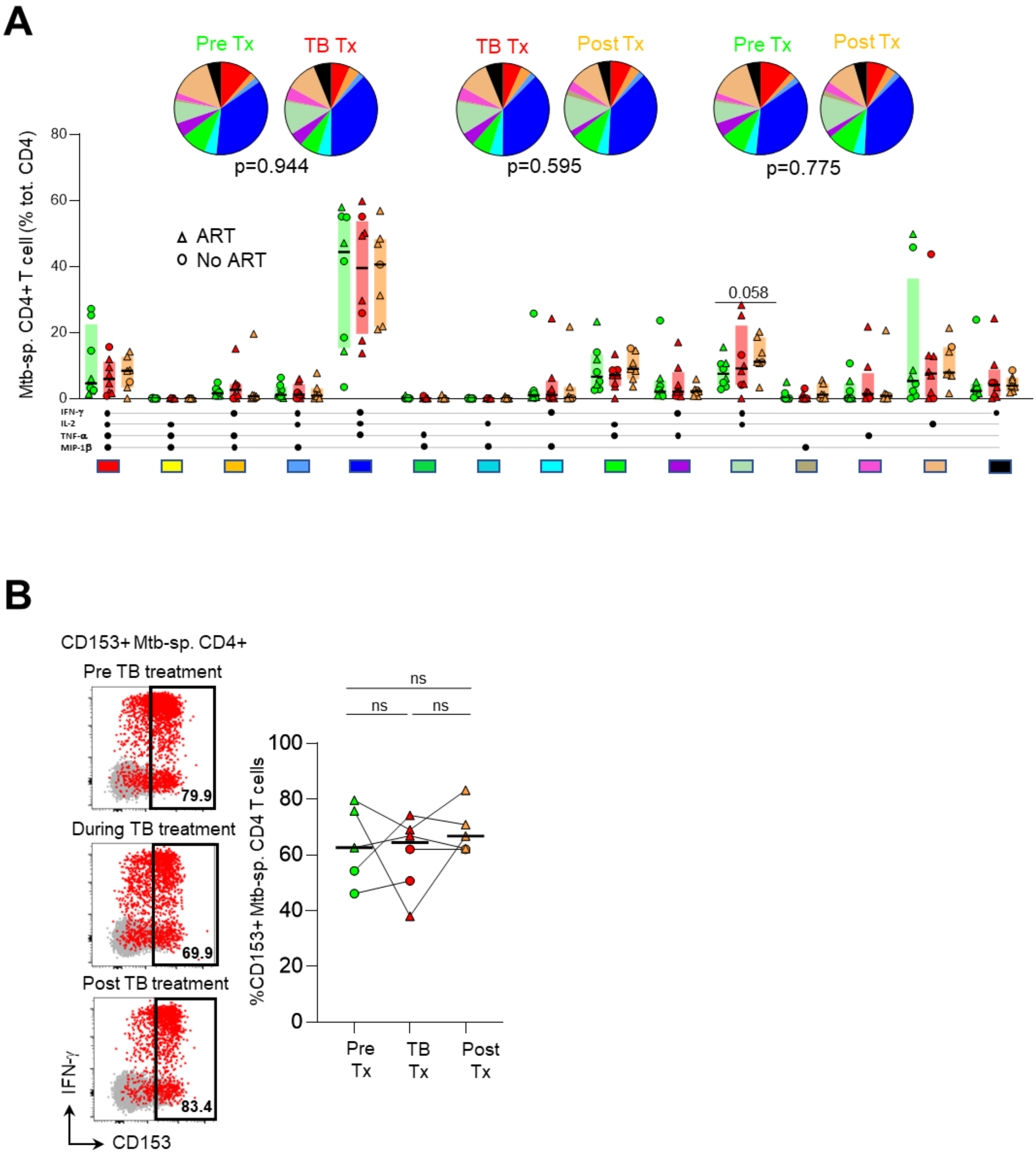


Figure 5.7: **The function of *M.tb*-specific CD4+ T cells. (A)** Proportion of different combinations of IFN-γ, TNF-α, IL-2 and MIP-1β responses in participants (n=13) before (green), during (red) and after (orange) TB treatment. Coloured bars underneath the graph and pie chart slices represent different cytokine combinations produced by *M.tb*-specific CD4+ T cells (e.g. a red bar/slice is cells producing all four cytokines IFN-γ, IL-2, TNF-α and MIP-1β). **(B)** Flow cytometry plots and longitudinal analysis showing the frequency of CD153+ *M.tb*-specific CD4+ T cells in participants before (green, n=5), during (red, n=6) and after (orange, n=5) TB treatment. Black horizontal bars indicate the median values. Statistical significance was calculated

using a Mann-Whitney U test and Wilcoxon matched-pairs test. Statistically non-significant P-values (P > 0.05) are annotated as ns.

To determine the activation status of *M.tb*-specific CD4+ T cells during TB treatment, we measured the expression of the T cell activation marker HLA-DR (**Figure 5.8**). Several studies have highlighted the potential of using phenotypic characterisation of *M.tb*-specific T cells as a way of differentiating between active TB and LTBI and to monitor TB treatment outcome (Adekambi *et al*., 2017; Riou *et al*., 2017; Ahmed *et al*., 2018). Analysis of the frequency of total CD4+ HLA-DR+ cells determined that there was no overall difference in the frequencies of activated CD4+ T cells over the course of TB treatment (**Figure 5.8A**). In contrast, when we analysed HLA-DR expression on *M.tb*-specific CD4+ T cells, there was trend towards a decrease in the frequency of HLA-DR+ *M.tb*-specific CD4+ T cells after TB treatment compared to during treatment (median: 2.4% [IQR 2-3.2] vs 1.5% [IQR 1.2-2.2] respectively, p = 0.0625) (**Figure 5.8B**). These results are consistent with published studies that show that after TB treatment there is a significant decrease in activated *M.tb*-specific CD4+ T cells (Adekambi *et al*., 2017; Ahmed *et al*., 2018). Of note, in the cross-sectional analysis the majority (5/8) of participants had a very high (> 40%) frequency of activated *M.tb*-specific CD4+ T cells, as is typical of active TB (**Figure 5.8C**). When we compared our TB cohort to participants LTBI described in previous Chapters we observed that the majority of participants with LTBI had frequencies of HLA-DR+ *M.tb*-specific CD4+ T cells below 40% (**Figure 5.8D**). Of note, we saw no significant association between the frequency of HLA-DR+ *M.tb*-specific CD4+ T cells and the time (months) before TB diagnosis (**Figure 5.9**). These results suggest that the elevated activation of *M.tb*-

specific CD4+ T cells before TB treatment is not related to the length of time before TB diagnosis.

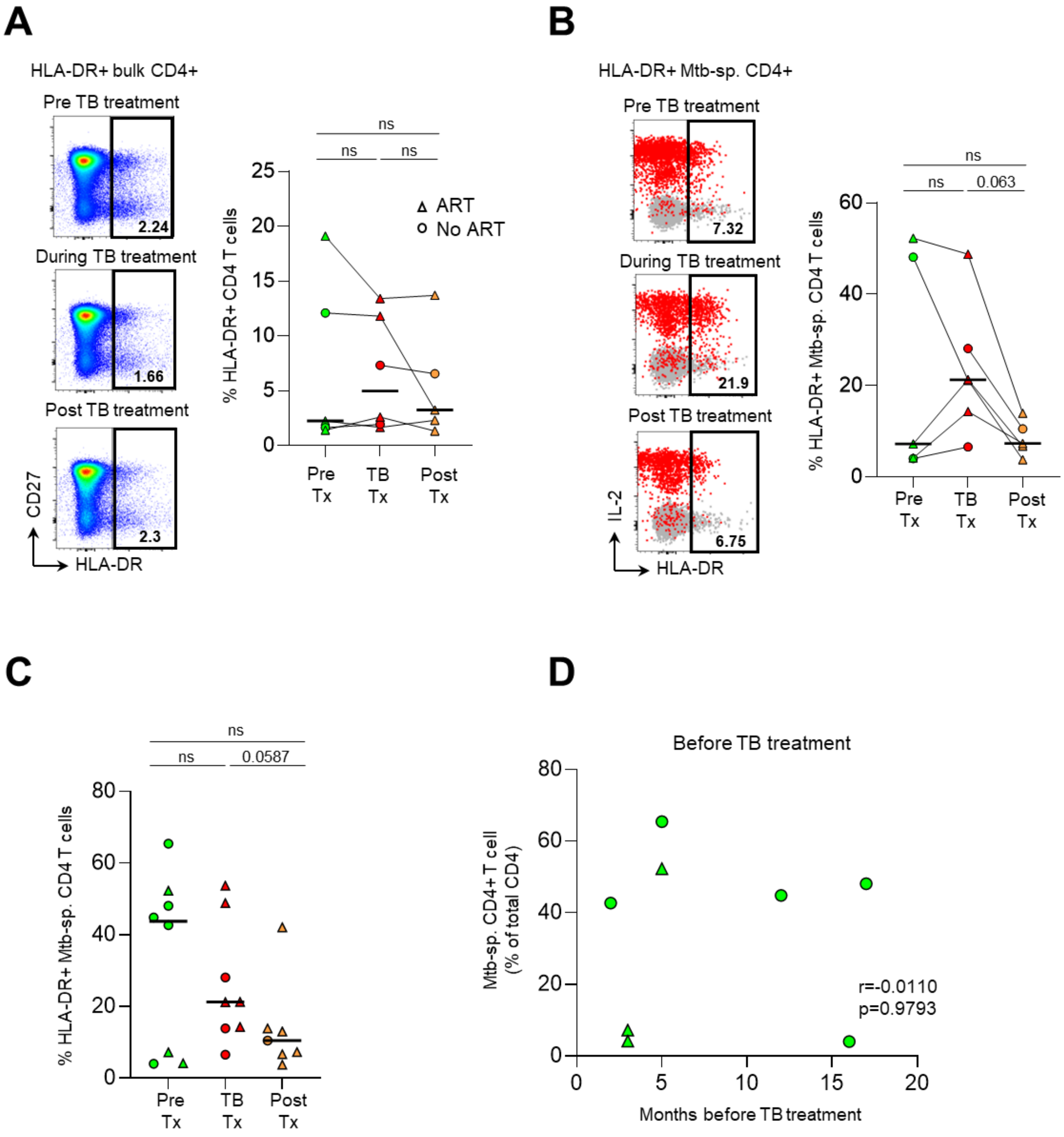


Figure 5.8: **The activation phenotype of *M.tb*-specific CD4+ T cells. (A)** Flow cytometry plots and longitudinal analysis showing the frequency of total HLA-DR+ CD4+ T cells and **(B)** HLA-DR+ *M.tb*-specific CD4+ T cells in participants before (green, n=5), during (red, n=6) and after (orange, n=5) TB treatment. **(C)** A cross-sectional analysis showing the frequency of HLA-DR+ *M.tb*-specific CD4+ T cells in

participants over the course of TB treatment. Black horizontal bars indicate the median values. Statistical significance was calculated using a Mann-Whitney U test Wilcoxon matched-pairs test. Statistically non-significant P-values (P > 0.05) are annotated as ns. **(D)** Spearman correlation assessing the relationship between frequency of HLA-DR+ *M.tb*-specific CD4+ T cells in participants before TB treatment and months before start of treatment. P-values and Spearman r coefficient are displayed in the bottom right corner of the graph.

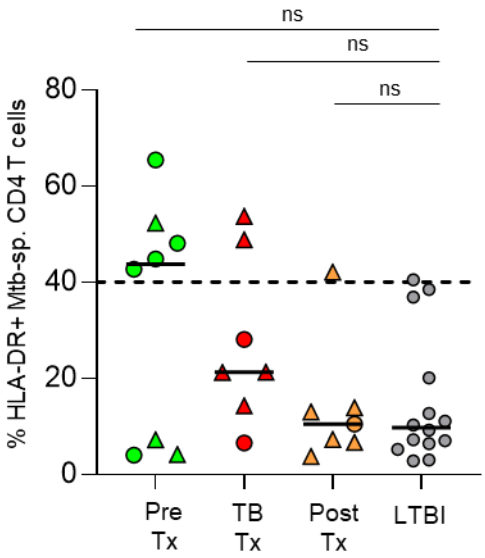


Figure 5.9: **The frequency of HLA-DR+ *M.tb*-specific CD4+ T cells in active TB and LTBI.** HLA-DR+ *M.tb*-specific CD4+ T cells in TB participants before (green, n=8), during (red, n=7) and after (orange, n=7) TB treatment and in participants with LTBI (grey, n=14) prior to ART initiation. The horizontal dashed line indicates the highest frequency of HLA-DR+ *M.tb*-specific CD4+ T cells in individuals with LTBI. The black horizontal bars indicate the median values. Statistical significance was calculated using a one-way ANOVA with Dunn's correction. Statistically non-significant P-values (P > 0.05) are annotated as ns.

5.4 Discussion

PLWH are at higher risk for developing TB at all stages of HIV infection: during untreated HIV infection, shortly after treatment (*e.g.* TB-IRIS) and even after successful viral suppression (WHO Global TB report, 2021; Xue *at al*., 2020; Lawn *et al*., 2006). Currently, we do not know what the correlates of TB protection are and hence why certain individuals are at higher risk for TB. In this study, to determine the dynamics of *M.tb*-specific T cell responses during active TB in the context of HIV infection, we analysed a small cohort PLWH who developed TB during the CAPRISA 002 study. In this cohort we assessed the magnitude and phenotype of *M.tb*-specific T cells before, during and after TB treatment. This chapter is a descriptive study of a small cohort of thirteen individuals. As such, the number of samples available to us limited our capacity to assess significant differences in the cohort. Although this is a major limitation of our study, data generated from this chapter is valuable and can be used to design a larger study to explore the dynamics of *M.tb*-specific T cell responses during active TB and TB treatment in a more robust manner.

In our study, analysis of total *M.tb*-specific CD4+ T cells (IFN-γ, TNF-α, IL-2 and MIP-1β) showed no significant differences in the frequency of these cells before, during and after TB treatment. However, we did observe that in half of the participants in our cohort there was a high frequency *M.tb*-specific CD4+ T cells even before TB diagnosis. Interestingly, while there were no overall significant differences in the cytokine profiles of *M.tb*-specific CD4+ T cells during the course of TB treatment, there was a trend towards a higher proportion of IFN-γ+IL-2+ dual functional cells after treatment. These results suggest that following TB treatment in our cohort there was a minor modification in the cytokine profile, and not the magnitude, of *M.tb*-specific

CD4+ T cells. Our findings are consistent with prior reports by Saharia and colleagues, who showed in a cohort of 21 HIV-infected individuals with TB that TB treatment had no significant effect on the frequency of total purified protein derivative (PPD)-specific CD4+ T cells expressing IFN-γ, TNF-α, IL-2 and MIP-1β (Saharia *et al*., 2016). Instead, TB treatment altered the cytokine profile of PPD-specific CD4+ T-cells, with TB patients showing a higher proportion of IFN-γ+IL-2+TNF-α+ polyfunctional and IFN-γ+IL-2+ dual functional PPD-specific CD4+ T cells, coincident with a decrease in subsets producing MIP-1β after treatment (Saharia *et al*., 2016). Similarly, a study by Day *et al*. has also shown that there is an increase in polyfunctional *M.tb*-specific CD4+ T cells following reduction in bacterial load by TB treatment (Day *et al*.,2011). In this study in a cohort of 54 HIV-uninfected individuals, TB treatment resulted an increase in the proportion of PPD-specific polyfunctional (IFN-γ+IL-2+TNF-α+) and dual functional IL-2+ TNF-a+ CD4+ T cells, and a decrease in IFN-γ+TNF-α+ and TNF-α+ cells (Day *et al*., 2011).

To assess the kinetics of *M.tb*-specific CD4+ T cell responses over the course TB treatment we examined the individual trajectories of eight participants in our cohort. An individual descriptive analysis of these participants showed that half of participants in this cohort had marked increase in the frequency of *M.tb*-specific CD4+ T cells at timepoints close to TB treatment/TB diagnosis. These participants had increased HLA-DR expression which typical of active TB months before their TB diagnosis, which likely signifies subclinical TB. Subclinical TB is defined as an infection with *M.tb* where, although there are no clinical symptoms of active TB, there are other abnormalities that can be detected by radiological and microbiological methods (Drain *et al*., 2018). Recently several studies have suggested the use of immunological biomarkers as alternatives to sputum-based methods to improve the diagnosis of subclinical TB and for monitoring treatment response (Weiner 3rd *et al*., 2018; Darboe *et al*., 2019; Penn-Nicholson *et al*., 2020; Duffy *et al*., 2021). Changes in these immunological biomarkers have been shown to be associated with asymptomatic pre-clinical TB and hence can assist in the identification of individuals at risk of TB, in order to enable timely interventions. These biomarkers include the use of whole blood transcriptomic correlate of risk (COR) signatures for TB comprising of 16 interferon stimulated gene (ISG) (Darboe *et al*., 2019), a PCR-based host-blood transcriptomic signature, RISK6 (Penn-Nicholson *et al*., 2020), IFN-γ–based immunomonitoring methods such TruCulture and enzyme-linked immunosorbent assay (ELISA) (Duffy *et al*., 2021), as well as monitoring of metabolic signatures in the blood TB exposed individuals (Weiner 3rd *et al*., 2018).

Of note, despite participants in our study having active TB, only a few individuals had *M.tb*-specific CD8+ T cell responses. Compared to *M.tb*-specific CD4+ T cell responses, CD8+ T cell responses in our cohort were of a lower magnitude and there was also a lower frequency of CD8+ T cells responders compared to those with a CD4+ responses (25% vs 67%, respectively). In addition, analysis of *M.tb*-specific CD8+ T cells in our cohort showed no significant differences in the frequencies of these cells over the course of TB treatment. Our results are inconsistent with previous reports that show that there is an increase *M.tb*-specific CD8+ T cell responses in individuals with active TB compared to those with LTBI, irrespective of HIV status (Day *et al*., 2011; Rozot *et al* 2015; Lancioni *et al*., 2019). In these studies they showed that while not as robust as *M.tb*-specific CD4+ T cell responses, CD8+ T cell responses could distinguish between individuals with TB and LTBI, with TB subjects having a higher frequency *M.tb*-specific CD8+ T cells in response to *M.tb* peptides ESAT-6 and CFP-10 (Day *et al*., 2011; Rozot *et al* 2015; Lancioni *et al*., 2019). Moreover, in these previous reports the frequency of *M.tb*-specific CD8+ T cell responders in TB individuals was higher (~60%) when compared to those with LTBI (~20%) (Day *et al*., 2011; Rozot *et al* 2014). A likely cause for the discrepancy in our study may the small sample size, increasing the cohort size may detect a greater number of *M.tb* CD8+ T cell responder.

Previous studies show that assessment of T cell activation markers on *M.tb*-specific CD4+ T cells can be used to distinguish between individuals with LTBI and TB, as well as assist in monitoring TB treatment outcome. In a cohort of 49 HIV-uninfected individuals, Adekambi and colleagues reported that while there was no significant differences in the frequency of IFN-γ producing *M.tb*-specific CD4+ T cells between subjects with TB and LTBI, individuals with TB had a higher frequency of *M.tb*-specific CD4+ T cells expressing the activation markers CD38, HLA-DR, and Ki-67 (Adekambi *et al*., 2017). In that study, by using cut-off values for the frequency of activated IFN-γ *M.tb*-specific CD4+ T cells (60% for HLA-DR, 18% for CD38, and 5% for Ki-6), they were able to distinguish between subjects with LTBI and those with active TB (Adekambi *et al*., 2017). Similarly, other studies have also shown that there is a significant difference in the frequency activated *M.tb*-specific CD4+ T cells between individuals with TB and those with LTBI, with cut-off values of 54% for HLA-DR in individuals with HIV infection (Riou *et al.,* 2017), 31.55% for CD38 and 3.7% for Ki67 in HIV-uninfected individuals (Ahmed *et al*., 2018). In a more recent study, Mpande and colleagues showed that HLA-DR median fluorescent intensity (MFI) of *M.tb*-specific CD4+ T cells could discriminate between TB non-progressor and progressors, (Mpande *et al*., 2021). In this study, *M.tb*-specific CD4+ T cells of TB progressors had a significantly higher HLA-DR MFI when compared to non-progressors even before symptom onset (Mpande *et al*., 2021). Consistent with these previous findings, in our study the majority of TB participants had a high (> 40%) frequency of HLA-DR+ *M.tb*-specific CD4+ T cells before treatment. These frequencies were higher than those seen in participants with LTBI reported in Chapter 3 and 4 Interestingly, although there were no overall significant differences in the frequency of HLA-DR+ *M.tb*-specific CD4+ T cells in our cohort, there was a trend towards a decrease in activated cells after successful TB treatment. These results suggest that in our cohort, TB treatment resulted in minor alterations in the activation profile of *M.tb*-specific CD4+ T cells. These data are in line with previous findings that show that the frequencies of activated *M.tb*-specific CD4+ T cells in individuals with clinically resolved TB are lower when compared to untreated TB (Adekambi *et al*., 2017; Ahmed *et al*., 2018). In these studies they showed that there was a marked decline in activated *M.tb*-specific CD4+ T cells even as early as 2 months after the start of treatment (Ahmed *et al*., 2018). Moreover, individuals with successful TB treatment had frequencies of activated cells approaching the cut-off discriminating between active TB and LTBI (Adekambi *et al*., 2017).

We also examined the frequency of CD153+ *M.tb*-specific CD4+ T cells in individuals with TB. CD153 is a CD30 ligand that mediates T cell proliferation and associates with the production of IFN-γ, TNF-α and IL-2 in *M.tb*-specific CD4+ T cells (Marin and Garcia, 2017). A previous study by Du Bruyn and colleagues showed that CD153+ *M.tb*-specific CD4+ T cells are significantly lower in HIV-infected individuals with active TB compared to LTBI (Du Bruyn *et al*., 2021). They also showed that in those with active TB, the proportion of CD153+ *M.tb*-specific CD4+ T cells in peripheral blood was inversely associated with bacterial load in the lungs. Our results are inconsistent with these reports, where we found that the median frequencies of CD153+ *M.tb*-specific CD4+ T cells in our TB cohort was approximately 60% before TB treatment, similar to frequencies seen in individuals with LTBI described in Chapters 3 and 4. Likely reason for the discrepancies between our results and the previous reports could be differences in the immune characteristics of the cohorts. Despite having active TB disease, participants in our cohort had a relatively healthy immune system before TB treatment with median CD4 count of 416 cells/µL. In comparison, TB patients in the previous study by Du Bruyn *et al* had much lower median CD4 count of 273 cells/µL.

Our study had several limitations. We examined only a small number of participants, and this study can rather be regarded as a case series to inform larger studies, given the heterogeneity in patterns of responses observed longitudinally. In addition, in a few (2/8) participants in our cohort the post TB treatment timepoints were a long time (~16 months) after the end of TB treatment and therefore might not be a true indication of the dynamics of TB responses immediately post treatment. Also, the clinical data we obtained about the participants did not include treatment adherence. It would be important to examine whether the immune characteristics observed in this study were influenced by treatment adherence. Furthermore, we did not include a control group (HIV-uninfected individuals with TB) to show the effect of treatment in this group compared with PLWH. Another limitation of our study is that we only assessed TB responses in the peripheral blood and not at the site of TB infection, hence our study is a limited reflection of what is occurring in the airways and lungs where *M.tb*-specific T cells would likely have migrated. Lastly, we acknowledge that the antigens used in this study, MTB300, may not be optimal for measuring CD8 T cell responses. MTB300 is made up of peptides that are 15 amino acids in length, while peptides of 8–11 amino acids in length would be more optimal to detect CD8+ T cell responses. While this study may have several limitations, the data reported here is still of importance in assessing the dynamics of *M.tb*-specific T cell responses in individuals with active TB. Findings from this study could be used as a framework for future more detailed and larger studies. Recently, Riou and colleagues described the frequency of CD4+ T cell responses to *M.tb* in a cohort of 95 participants with active TB (Riou *et al*., 2020). They compared *M.tb*-specific CD4+ T cell responses at two timepoints, at the start of TB treatment and 6 months after treatment. TB treatment had a significant impact on both the frequency and polyfunctional profile of *M.tb*-specific CD4+ T cells in HIV-infected and uninfected individuals. Interestingly, while there was an overall decrease in the frequency of activated *M.tb*-specific CD4+ T cells after successful TB treatment in both HIV-infected and uninfected individuals in their study, these frequencies still remained higher compared to individuals with LTBI (Riou *et al*., 2020). Overall, the findings of that study showed that analysis of activation and maturation markers on *M.tb*-specific CD4+ T cells can be used as biomarkers of disease extent and treatment monitoring of TB in both HIV infected and uninfected individuals.

In conclusion, despite the small number of participants in our cohort, data generated in this study data is somewhat consistent with previous findings, and show that following TB treatment there is a modification in both the cytokine and activation profile of *M.tb*-specific CD4+ T cells. Findings from this study will assist in future research to better understand dynamics of *M.tb*-specific responses during active TB and hence help us better understand why PLWH are at higher risk of developing TB.

CHAPTER 6

Discussion

Despite significant progress in the past decades, the efforts to curb the TB pandemic remains hindered by a lack of efficient tools such as new vaccines, reliable assays to diagnose and monitor TB treatment and novel treatments. Thus, a better understanding of the properties and evolution of *M.tb*-specific T cell responses, especially in high-risk populations (such as people living with HIV, PLWH) may help in the development of new tools to combat TB. Currently, the only licenced TB vaccine, Bacille Calmette-Guerin (BCG), was introduced almost 100 years ago (Luca and Mihaescu, 2013). While BCG vaccination provides protection against severe forms of TB in infants (i.e., meningeal and miliary disease), BCG efficacy wanes in adolescents and offers little to no protection against pulmonary TB in adults in high TB incidence settings (Andersen and Doherty, 2005; Mangtani *et al*., 2014; Gijsel and Reyn, 2019). Moreover, to date reliable correlates of TB immunity are still unclear. While it is apparent from experimental and clinical studies that CD4+ T cells producing IFN-γ are important for TB protection (Cooper *et al*., 1993; Filipe-Santos *et al*., 2006; Green *et al*., 2013; Vosse *et al*., 2013), this alone is not sufficient (Winkler *et al*., 2005; Caccamo *et al*., 2010; Mattila *et al*., 2011). Recently, several studies have highlighted that polyfunctional CD4+ T cells (Lindenstrøm *et al*., 2009; Derrick *et al*., 2011; Tameris *et al*., 2013), IFN-γ–independent CD4+ T cell subsets (Gallegos *et al*., 2011; Gopal *et al*., 2012; Okada *et al*., 2015; Wang *et al*., 2016), as well innate immune responses (Koeken *et al*., 2019; Ferluga *et al*., 2020; Zhou *et al*., 2021) could play a major role in TB protection. However, as most of these studies are done in murine models, it still remains unclear whether these immune responses are relevant in protection from human TB. The greatest risk factor for developing active TB is HIV infection (WHO

Global TB report, 2021). PLWH are susceptible to TB at all phases of infection, even before immunosuppression or after CD4 reconstitution (Sonnenberg *et al*., 2005; Lawn *et al*., 2006; Getahun *et al*., 2010; Gopal *et al*., 2017). An understanding of what defects occur in TB immunity early after HIV infection can help give us insight into correlates of TB protection, which was the motivation for the studies we conducted.

In this thesis we aimed to characterise *Mycobacterium tuberculosis* (*M.tb*)-specific CD4+ T cell responses during HIV co-infection. We undertook this study using a cohort of HIV-infected South African women from the CAPRISA 002 study: a cohort following PLWH from HIV seroconversion to up to 5 years after ART initiation. Using available samples from this cohort, we assessed longitudinally *M.tb*-specific CD4+ T cell responses prior to HIV infection, during the acute and chronic phase of infection, and up to 2 years after antiretroviral therapy (ART) initiation. Our study focused on the quantity and quality (polyfunctional, activation and memory differentiation) of *M.tb*-specific CD4+ T cells, and we examined the kinetics and attributes of *M.tb*-specific CD4+ T cell responses during the course of HIV infection. We also conducted a detailed assessment of the impact of early and late ART initiation on TB immunity. Lastly, in a small cohort of individuals who developed TB during CAPRISA 002 study, we examined the effects of TB treatment on *M.tb*-specific T cell responses in the context of treated and untreated HIV.

Previous studies by Geldmacher and colleagues have suggested that there is a rapid depletion of *M.tb*-specific CD4+ T cells within a year of HIV infection (Geldmacher *et al*., 2008; 2010). In these studies, they showed that HIV co-infection was associated with preferential and early depletion of a specific Th1 *M.tb*-specific CD4+ T cell subset, producing limited amounts of MIP-1β (a ligand of the HIV co-receptor CCR5), (Geldmacher *et al*., 2010). This may partially explain the increased TB susceptibility in PLWH even before profound CD4 loss. In Chapter 3 of this thesis, we aimed to confirm these previous findings by Geldmacher *et al*. by examining the quantity of *M.tb*-specific CD4+ T cells over the course of HIV infection both cross-sectionally (n=58) and longitudinally (n=17). Results of our longitudinal analyses were consistent with previous reports by Geldmacher and colleagues, showing a significant decrease in the frequency of *M.tb* responders 3 months after HIV infection. However, in our study not all participants experienced *M.tb*-specific CD4+ T cell loss after HIV infection. While half of the participants in our cohort lost 50% or more of their *M.tb*-specific response early after infection, the other half maintained their responses. Moreover, in the participants who maintained their responses, the majority had very little fluctuation in their responses during the acute and chronic phase of HIV infection. Interestingly, we also observed that in our cohort *M.tb*-specific CD4+ T cell depletion was not exclusively link to poor MIP-1β production or an increase in IL-2 production, as previously reported by Geldmacher *et al*. Overall, these results suggest that there might be substantial heterogeneity in the generation and maintenance of *M.tb*-specific CD4+ T cells in individuals. As our cohort was recruited from a TB endemic country, South Africa, the regular exposure to *M.tb* may also play a role in promoting maintenance of *M.tb*-specific CD4+ T cells.

In addition to affecting the quantity of *M.tb*-specific CD4+ T cell responses, HIV infection has also been shown to have an impact of the quality (functional capacity) of these responses. While it is still not known what the mechanisms underlying these defects are, previous findings suggest that HIV infection skews the lineage transcription factors and the phenotype (memory and activation profiles) of *M.tb*-specific CD4+ T cells (Mathews *et al*., 2012; Riou *et al*., 2016; Amello *et al*., 2019). Studies in humans (Beveridge *et al*., 2007; Day *et al*., 2011) and mouse models (Lindenstrøm *et al*., 2009; Derrick *et al*., 2011) show that polyfunctional CD4+ T cells, producing multiple cytokines in combination with IFN-γ, may have a role to play in TB protection. Additionally, the memory differentiation profile of *M.tb*-specific CD4+ T cells may also have an impact on the maintenance of TB immunity. Riou *et al.* showed that *M.tb*-specific CD4+ T cells with an early differentiated phenotype were replenished faster in PLWH after ART initiation, as cells with this phenotype tend to be longer lived (Riou *et al*., 2015). Thus, to further explore the immune parameters that could explain the maintenance or loss of *M.tb*-specific CD4 response after HIV infection, we compared the functional and phenotypic characteristics of these cells. Surprisingly, we found no significant differences in the immune characteristics of *M.tb*-specific CD4+ T cells between the two groups. Overall, our results indicate that in some individuals there is an early defect in the quantity, but not the quality, of *M.tb*-specific CD4+ T cells after HIV infection. Future studies could examine the effects of HIV infection on other potential correlates of TB protection, as we only looked at Th1 cytokines, and limited phenotypic characterstics, namely activation (HLA-DR) and memory marker (CD27 and CD45RA) profiles. Previous studies show that several other T helper subsets such as Th22 and Th17 cells may have a role to play in TB immunity. In these reports it was observed that individuals with mutations in essential Th17/Th22 pathways had a greater risk of developing TB (Zhang *et al*., 2011; Okada *et al*., 2015). Moreover, in our group we recently showed that *M.tb*-specific CD4+ Th22 cells are significantly depleted in PLWH when compared to HIV-uninfected individuals (Bunjun *et al*., 2021; Makatsa *et al*., 2022).

ART is an essential strategy in preventing TB in PLWH (Badri *et al*., 2002; Moore *et al*., 2007), with several studies showing the importance of early ART initiation in decreasing TB incidence (Collins *et al*., 2015; INSIGHT START Study Group, 2015; TEMPRANO Study Group, 2015). However, despite ART interventions, TB risk still remains high in PLWH, especially in sub-Saharan African (Girardi *et al*., 2005; Bonnet *et al*., 2006; Lawn *et al*., 2006). Several factors may contribute to these high TB rates, including incomplete restoration of TB immune responses after ART (Schluger *et al*., 2002; Sutherland *et al*., 2006), defects in TB immunity early after HIV infection (Schluger *et al*., 2002; Geldmacher *et al*., 2008; 2010; Amello *et al*., 2019; Singh *et al*., 2019) as well as residual systemic hyperactivation and immune damage that persists even after CD4 reconstitution under ART (Vanham *et al*., 1996; Wilkinson *et al*., 2016). In Chapter 4, we sought to gain better insight into whether early ART preserves TB immunity and therefore reduces the early, elevated risk of TB in PLWH. To do this, we examined *M.tb*-specific CD4+ T cell responses in individuals who initiated ART at an early stage of HIV infection (< 12 months, n=16) in comparison to those who started ART during chronic infection (> 24 months, n=22). We compared multiple immune parameters between these two groups, including their clinical characteristics, as well as the magnitude, function and phenotype of their *M.tb*-specific CD4+ T cells. As expected, our results showed that 2 years of ART (irrespective of timing) induced a significant global immune reconstitution, marked by an increase in CD4 counts and restoration of the CD4/CD8 ratio. However, we observed no significant impact on the magnitude, function or phenotype of *M.tb*-specific CD4+ T cells. Surprisingly, our findings are inconsistent with previous data from our group that show that, as a result of CD4 reconstitution, there is an increase in the absolute number of *M.tb*-specific CD4+ T cells after ART (Riou *et al*., 2015). The reason for this discrepancy could be that participants in our late ART group (despite long years of untreated HIV) showed only modest immunosuppression, with the majority of participants having a CD4 count above 500 cell/µL before ART initiation. This is a limitation of our study, as sample availability in the CAPRISA 002 study did not enable us to look at participants starting treatment at very low CD4 counts. The CAPRISA 002 study is a cohort of HIV-infected women with regular follow-up from HIV infection to 5 years after treatment. This means that due to these regular check-ups ART was made immediately available to participants according to South African policies at that time. Hence, severely immunosuppressed participants were underrepresented in such a well-followed cohort. While the ART groups in our study may not be ideal, the data generated from this study provides an essential framework for future study designs to better understand if early ART preserves TB immunity.

As stated earlier, HIV is a heightened risk factor for TB, with PLWH susceptible at all stages of HIV infection (WHO Global TB report, 2021; Xue *at al*., 2020; Lawn *et al*., 2006). In the CAPRISA 002 study, 10% (25/250) of the cohort developed TB during the study. This is consistent with previous reports that show that approximately 5-10% of individuals with LTBI will develop active TB in their lifetime (Bloom and Murray, 1992; Vynnycky and Fine, 2000; Bhatt *et al*., 2015). In Chapter 5, we had the opportunity to examine the dynamics of *M.tb*-specific T cell responses in 13 of those participants who had sufficient samples available. In this small TB cohort, we assessed the magnitude and phenotype of *M.tb*-specific T cells longitudinally over the course of TB treatment: before TB treatment/diagnosis, during treatment and after successful treatment; in the context of treated and untreated HIV. Despite the limited number of participants in this sub-study, we showed that after TB treatment there was a decreasing trend of activated *M.tb*-specific CD4+ T cells coincident with an increase in IFN-γ+ IL-2+ dual functional cells. These results are consistent with previous reports showing that functional and phenotypic markers can be used to monitor treatment outcome in TB patients (Adekambi *et al*., 2017; Ahmed *et al*., 2018; Riou *et al*., 2020). In these studies they showed that activation markers (particularly HLA-DR) expressed by *M.tb*-specific CD4+ T cells are a robust measure to delineate LTBI from active TB (including extrapulmonary TB) (Adekambi *et al*., 2017; Riou *et al*., 2017; Ahmed *et al*., 2018). Moreover, these activation markers could be used to monitor TB treatment response in both PLWH and HIV-uninfected persons (Adekambi *et al*., 2017; Ahmed *et al*., 2018; Riou *et al*., 2020). Overall, these studies suggest that by examining changes in the quality and phenotype of *M.tb*-specific CD4+ T cell responses that occur in individuals with active TB, when compared to those with LTBI, we can get better insight into what immune parameters constitute protective immunity to TB.

The studies described in this thesis had several limitations. Due to limited availability of PBMC samples from the CAPRISA cohort, our study was underpowered and only trends were observed in the cohort who had active TB. Also, we measured *M.tb*-specific T cell responses in peripheral blood and not in the lungs. Most *M.tb*-specific T cells may home to the lungs during active TB, it would be important to confirm if the responses we examined in the blood are a true reflection of what is occurring at the site of TB infection. Future study designs could further examine why there is a variation in the loss of *M.tb*-specific CD4+ T cells during HIV infection, by examining other potential correlates of TB protection: other CD4+ T cell subsets such as Th22 and Th17 cells, and the expression of chemokine receptors that mediate homing.

Moreover, new studies could also source samples from patients starting ART at low CD4 counts; samples where late ART is also based on the degree of immunosuppression and not just time before ART initiation. Such study designs with a broader description of early and late ART may assist us in better understanding the impact of ART timing on TB immunity.

Overall, our study confirms previous reports that there is a depletion in *M.tb*-specific CD4+ T cell responses early after HIV infection. We extend these reports and emphasise the heterogeneity of immune reactions – certainly not all PLWH lose their TB response during acute HIV infection. Studying the dynamics of *M.tb*-specific responses is challenging in TB endemic countries such as South Africa, as it is possible that regular environmental exposure to the pathogen may influence responses. Our results therefore warrant further study to compare *M.tb* evolution in low burden countries, to determine whether our findings are consistent even in conditions of low TB exposure. As stated earlier, a major impediment in TB research is that correlates of TB protection still remain an enigma, with some studies suggesting that immune correlates of TB protection may differ during *M.tb* infection, disease progression and reactivation (Bhatt *et al*., 2015). Adding to this level of complexity, we now understand TB to be not a bi-modal disease (LTBI vs active TB) but a spectrum of disease (Barry *et al*., 2009; Delogu and Goletti, 2014; Cadena *et al*., 2017), and it is still unclear how *M.tb* immune responses evolve along this continuum. Further studies are needed to answer these important gaps in our knowledge of TB immunity and hence to assist with the development of more effective tools to prevent and control TB. During the COVID-19 pandemic, SARS-CoV-2 overtook all other infectious pathogens as the leading global cause of death. However, even when COVID-19 has subsided, TB will remain, and take its place again as the leading global infectious disease. The need for an effective TB vaccine, and the studies that will inform its development, are more urgent than ever.

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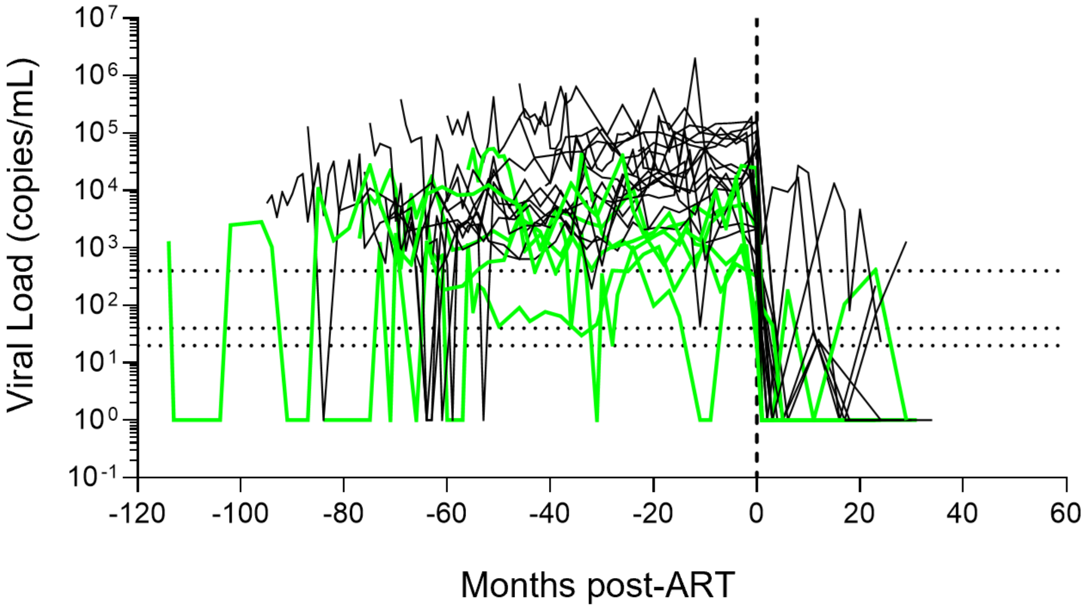
APPENDIX



Supplementary Figure 4.1: Viral load (VL) trajectories of participants in the late ART group. VL for participants in the late ART group. The horizontal dotted lines indicate detection limits of the assay and the vertical dashed line ART initiation. The VL ‘controllers’ in this group are indicated in green. They were defined as those participants who consistently had an average VL below 15,000 copies/mL and a CD4 count above 500 cells/µL after the first year of HIV infection.