LUNG TISSUE RESIDENT MEMORY T AND B CELLS IN THE IMMUNE RESPONSE TO *MYCOBACTERIUM TUBERCULOSIS* IN HUMANS

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Thesis submitted in fulfilment of the requirements of the degree of Doctor of Philosophy (Medical Microbiology) in the school of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal.

2019

DECLARATION

I, Paul Ogongo, declare that this is my original work, where others have made contributions it has been acknowledged in the thesis. The work presented in this thesis has not been submitted in any other form to another University. The experimental work described in this thesis was performed at Africa Health Research Institute (Durban Research Campus), Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa under the supervision of Dr. Alasdair Leslie

Paul Ogongo

Signature:  Date: 22nd March 2019

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Signature:  Date: 22nd March 2019

As the candidate's supervisor, I agree to the submission of this thesis.

Dr. Alasdair Leslie

Signature:  Date: 22nd March 2019

PLAGIARISM DECLARATION

I, Paul Ogongo, declare that:

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

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

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DEDICATION

To James Robert Onyango for ensuring the candle of education lit in me by my parents never went off; and to my wife Martha Bitta and son Ersendyck Luke Ogongo for their love and perseverance while I was away.

PUBLICATIONS AND CONFERENCES

First author manuscripts

1. **Ogongo P,** Porterfield J.Z and Leslie A (2019). Lung tissue resident memory T cells in the immune response to *Mycobacterium tuberculosis*. *Front. Immunol.* 10:992. doi: 10.3389/fimmu.2019.00992
2. **Ogongo P,** Steyn AJC, Karim F, Dullabh KJ, Awala I, Madansein R, Leslie A and Behar SM (2019). Differential skewing of donor-unrestricted and γδ T-cell repertoires in tuberculosis-infected human lungs. Journal of Clinical Investigations. Under review
3. **Ogongo P,** Nhamoyebonde S, Ardain A, Ramsuran D, Madansein R, Singh A, Ng'oepe A, Karim F, Arlehamn CL, Osaretin E, Steyn AJ and Leslie A. Human lung Th17 tissue resident memory T-cells are enriched in TB infected human lung and correlate with disease severity. Manuscript in preparation
4. **Ogongo P,** Grace PS, Muema D.M, Karim F, Madansein R, Steyn AJ, Galit A and Leslie A**.** Lung tissue resident B-cells are present and may play a role in human TB infection. Manuscript in preparation
5. **Ogongo P,** Nhamoyebonde S, Ardain A, Ramsuran D, Singh A, Ng'oepe A, Karim F, Madansein R, Steyn AJ, Behar S and Leslie A**.** Unique TCR-clonotypes are enriched in TB infected lung tissue. Manuscript in preparation

Co-author manuscripts

1. Attaf M, Malik A, Severinsen MC, Roider J, **Ogongo P**, Buus S, Ndung'u T, Leslie A, Kløverpris HN, Matthews PC, Sewell AK and Goulder P (2018) Major TCR repertoire perturbation by immunodominant HLA-B∗44:03-restricted CMV-specific T cells. *Front. Immunol.* 9:2539. doi: 10.3389/fimmu.2018.02539
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Conferences

1. **Paul Ogongo**, R. Madansein, P. Ramdial, A. Ardain, S. Nhamoyebonde, D. Ramsuran, E. Osaretin, S. Behar, A. Steyn, and A. Leslie. *Tuberculosis specific human lung tissue resident memory T cells are predominantly TNF-α monofunctional and express FoxP3.* Poster presentation at the Keystone Symposium: Tuberculosis: Mechanisms, Pathogenesis and Treatment (A3). 17th – 21st January 2019: Banff, Canada
2. **Paul Ogongo**, R. Madansein, P. Ramdial, A. Ardain, S. Nhamoyebonde, S. Behar, A. Steyn, and A. Leslie. *HIV coinfection impacts recruitment of human lung tissue resident T-cells during TB disease.* Oral presentation at the 10th Federation of African Immunological Societies (FAIS). 3rd – 7th December 2017: Hammamet, Tunisia.
3. **Paul Ogongo**, R. Madansein, P. Ramdial, A. Ardain, S. Nhamoyebonde, A. Steyn, and A. Leslie. *Lung tissue resident T cell immune responses to Mycobacterium tuberculosis infection in humans*. Oral and poster presentation at 1st Kenya Biology of Pathogens, Pathogenesis and Parasitism Workshop. 19th – 24th September 2016: Kilifi, Kenya.
4. **Paul Ogongo,** R. Madansein, P. Ramdial, A. Steyn and A. Leslie. *Lung tissue resident T cell immune responses to Mycobacterium tuberculosis infection in humans*. Oral presentation at the University of KwaZulu Natal, College of Health Sciences Research Symposium 8th – 9th September 2016: Durban, South Africa.
5. **Paul Ogongo,** R. Madansein, P. Ramdial, A. Steyn and A. Leslie. *Lung tissue resident T cell immune responses to Mycobacterium tuberculosis infection in humans*. Oral presentation at the 16th International Congress of Immunology, 21st – 28th August 2016: Melbourne, Australia.
6. **Paul Ogongo,** R. Madansein, P. Ramdial, A. Steyn and A. Leslie. *Lung tissue resident T cell immune responses to Mycobacterium tuberculosis infection in humans*. Poster presentation at the TB Preconference at 21st International AIDS Conference 2016:16th – 22nd July 2016: Durban, South Africa.

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To our study participants who consented for the study, your contribution is making us learn more and better about tuberculosis disease. You are the ones that count the most!
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Above all, I thank the Almighty God for His enduring love and mercy without which I could not have made a single step in the quest for knowledge.

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ABBREVIATIONS

AFB	Acid fast bacilli
Ag85/A	85kDa Antigen A/B of *M. tuberculosis*
AHRI	Africa Health Research Institute
APC	Antigen presenting cell
ART	Anti-retroviral therapy
BAL	Bronchoalveolar lavage
BCR	B cell receptor
BREC	Biomedical Research Ethics Committee
CCR5	C-C motif chemokine receptor type 5
CD1a	Cluster of differentiation 1a
CD1b	Cluster of differentiation 1b
CD1c	Cluster of differentiation 1c
CD1d	Cluster of differentiation 1d
CDR	Complementarity determining region
CDR3α	Complementarity determining region 3 – alpha
CFP10	10 kDa culture filtrate antigen of *M. tuberculosis*
CFU	colony forming unit
CMV	Cytomegalovirus
CXCL13	chemokine (C-X-C motif) ligand 13
CXCR3	C-X-C motif chemokine receptor 3
CX3CR1	CX3C chemokine receptor 1
DC	Dendritic cell
DJβ	Diversity Joining region beta
DNA	Deoxyribonucleic acid
DURT	Donor unrestricted T cell
EBV	Epstein-Barr virus
ELISA	Enzyme linked immunosorbent assay
ELISPOT	Enzyme linked immunospot
ESAT-6	6 kDa early secretory antigenic target of *M. tuberculosis*
FACS	Fluorescence-activated cell sorting
FcR	Fragment crystallizable receptor

GEM	Germline-encoded mycolyl lipid
GM-CSF	Granulocyte – macrophage colony stimulating factor
GMM	Glucose monomycolate
HCV	Hepatitis C virus
HCWs	Health care workers
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HLA-I	Human leukocyte antigen class I
HLA-II	Human Leukocyte antigen class II
HLA-A	Human leukocyte antigen A
HLA-B	Human leukocyte antigen B
HLA-C	Human leukocyte antigen C
HLA-DR	Human leukocyte antigen DR
HLA-E	Human leukocyte antigen E
HPV	Human papilloma virus
HSV	Herpes simplex virus
Hsp	heat-shock proteins
H_2O_2	Hydrogen peroxide
IFN-γ	Interferon gamma
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IGRA	Interferon gamma release assay
IL-1β	Interleukin 1 beta
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-17	Interleukin 17
IL-17F	Interleukin 17F
IMGT	International Immunogenetics database
iNKT	invariant Natural Killer T cell
IR	Infrared

JS	Jensen Shannon
kDa	kilo Dalton
KLRG1	Killer Cell Lectin Like Receptor G1
LAM	Lipoarabinomannan
LN	Lymph nodes
LTBI	Latent tuberculosis infection
mAbs	monoclonal antibodies
MAIT	Mucosa associated invariant T cell
MDR	multi-drug resistant
MHC	Major histocompatibility complex
MHC-I	Major histocompatibility complex class I
MHC-II	Major histocompatibility complex class II
MR1	MHC-related protein 1
Mtb	Mycobacterium tuberculosis
NHPs	Non-human primates
PAMPs	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PMA	phorbol 12-myristate 13-acetate
PPD	Purified protein derivative
PRRs	Pattern recognition receptors
RD-1	**Region of difference 1**
TB	Tuberculosis
TCM	Central memory T cells
TCR	T cell receptor
TCRA/D	T cell receptor alpha diversity segment
TEM	Effector memory T cells
TCRα	T cell receptor - alpha
TCRβ	T cell receptor – beta
TCRδ	T cell receptor – delta
TCRγ	T cell receptor – gamma
Th17	T helper 17

TLRs	Toll like receptors
TNF- α	Tumor necrosis factor – alpha
TRAJ	T Cell Receptor Alpha Joining
TRAV	T Cell Receptor Alpha Variable
TRBV	T Cell Receptor Beta Variable
TRM	Tissue resident memory T cells
TST	Tuberculin skin test
UMAP	Uniform Manifold Approximation and Projection
VDJ	Variable diversity joining regions
VαJα	Variable- alpha Joining- alpha region
Vβ	Variable beta region
WHO	World Health Organization
αβ	alpha-beta
γδ	gamma-delta

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ETHICAL APPROVAL

The research project from which this thesis was written is registered with the Biomedical Research Ethics Committee (BREC) of the University of Kwa-Zulu Natal and was given approval reference number; **BREC reference: BE019/13** for the Lung study (participants from this cohort are referred to as 'Lung Cohort' in the thesis).
In the lung study, participants are defined as Active TB and/or Previous TB based on results of GeneXpert, *Mycobacterium tuberculosis* culture and auramine tests, as well as determination by cardiothoracic surgeons based on X-ray results, patient clinical history data and their professional experience. Cancer control participants are defined as TB negative (based on the set criteria mentioned) as well as being on treatment for, or investigated for suspected malignancy.

For healthy control blood donors, participants were enrolled in a different study: Collection of Sputum, Urine and Blood Samples (CUBS) for research at AHRI registered by BREC: **BREC reference: BE022/13**

The Academic Leader for Research at the School of Laboratory Medicine and Medical Sciences granted approval of the research project towards studying for a PhD degree.

ABSTRACT

Tuberculosis (TB), caused by *Mycobacterium tuberculosis (Mtb)* is the main cause of death from a single infectious agent. Although a quarter of the world population is estimated to be infected with TB, only 5–10% of these individuals will develop active disease during their lifetime, suggesting that the immune system is highly effective at containing *Mtb* infection in most people. Extensive analysis of T-cells in blood has, however, failed to identify correlates of protection in humans, which is a prerequisite for rationale vaccine design. A likely explanation for this failure is that most memory T-cells in lung tissue do not recirculate in blood. Indeed, adoptive transfer of lung T-cells from infected mice protects recipients from *Mtb* challenge whereas transfer of blood T-cells does not. While the crucial role of T-cells in controlling *Mtb* infection is well appreciated, the role of B cells and humoral immunity in TB disease is unclear. Furthermore, human studies that directly compare the B-cell involvement in TB between infected lung tissue and matched peripheral blood are lacking. Therefore, this study was based on the premise that studying the human adaptive immune responses to *Mtb* at the site of infection would provide new findings that are not present in the systemic circulation. Thus, we hypothesised that the lung T and B-cell responses to TB in humans are fundamentally different from those in the blood, because they involve cells that do not recirculate (particularly T-cells). The overall aim was to determine the phenotype, function and TB specificity of adaptive immune responses (T and B-cells) at the site of TB disease and to understand how they correlate with the responses detectable in circulation. To achieve this, a cohort of study participants undergoing medical lung resection for tuberculosis related lung pathologies was established

The results presented in this thesis show that lung T-cells are different from peripheral blood T-cells in terms of phenotype, function, and specificity. Lung tissue contains a distinct population of effector memory T-cells expressing canonical markers of Tissue Resident Memory (Trm) T-cells, including CD69 and CD103, which are expanded during active TB disease. These Trm are highly functional, are enriched for IL-17 and IFN-γ secreting cells, and are depleted by HIV co-infection, compared to matched blood samples. In addition, they are highly enriched for TB-specific T-cells, including TB-specific Th17 and regulatory T-cell subsets. Th17 TB-specific T-cells in the lung negatively correlate with plasma cytokine markers of inflammation, suggesting an important role for this subset in human lung in limiting disease severity. Interestingly, although CD103 expressing T-cells produce the highest levels of cytokine by non-specific stimulation, TB-specific T-cells in the lung lack

CD103 expression. Finally, detailed phenotyping of lung Trms was conducted using Mass cytometry and a panel of 40 T-cell markers. This confirmed patterns of expression of markers that are consistent with published data, including CXCR3, PD1, KLRG1 and other integrins.

The existence of Trms in TB infected lung tissue was also investigated by global TCR sequencing. This revealed a significant clonal expansion of T-cells in the lung tissue compared to peripheral blood, consistent with a non-recirculating tissue resident memory population. When T-cells from blood and lung tissue were pre-sorted by flow cytometry before TCR sequencing, the results clearly demonstrated that the lung tissue is made of up to 20% clonally expanded T-cell clones that do not recirculate. This approach was also used to investigate the potential contribution of non-classical, donor unrestricted T-cells to lung immunity. By flow cytometry and TCR sequencing, we show that unconventional T-cells such as mucosal associated invariant T-cells (MAIT), germline-encoded mycolyl-reactive (GEM) T-cells and γδ T-cells are all depleted in peripheral blood of TB infected individuals compared to healthy controls while iNKT cells are not. However, this is unlikely to be due to recruitment to the site of disease, as no subset was consistently expanded in the lung, GEMs were rarely detected and γδ T-cells were present at a much lower frequency than in matched blood. Indeed, this study demonstrates the presence of unique and expanded γδ T-cells in TB infected lung that are absent in circulation, suggesting some DURTs may also exist as non-recirculating lung resident memory cells.

Analysis of B-cells in individuals with TB revealed skewing of blood B-cell phenotypes in both active and latent TB infection and an enrichment of total B-cells in the lung compared to matched blood. Lung B-cells were mainly of memory B-cell phenotype including plasmablasts, and, like T-cells, were enriched for the expression of the Trm marker CD69, suggesting that B-cells in the lung probably do not recirculate. This potential Trm B-cell population was enriched for B-cell activation markers, CD95 and HLA-DR, suggesting involvement at the site of disease. However, using TB-specific BAITs, the frequency of TB specific B-cells in the lung tissue was found to be lower compared to TB specific B-cells in blood. The functional role of B-cells in TB disease warrants further investigation.

These results provide clear evidence of compartmentalization of the adaptive immune responses to *Mtb* in the human lung, which maybe important when interpreting data from human PBMC for correlates of protection or vaccine response.

CHAPTER 1: OVERVIEW OF ADAPTIVE IMMUNE RESPONSES TO TUBERCULOSIS

1.1 Tuberculosis disease

Despite tuberculosis (TB) being a disease of antiquity (Dubos and Dubos 1952), it still causes more deaths (1.7 million in 2016) than HIV (http://www.who.int/tb/en/) (WHO 2017). *Mycobacterium tuberculosis (Mtb)*, the bacteria that causes TB, is estimated to have infected 23% of the current human population (Houben and Dodd 2016) and progressed to cause active disease in 10.4 million people in 2016 (figure 1) (WHO 2017). Many years of coevolution of *Mtb* with humans has resulted in an infection that induces partial immunity, where the host survives most of the time and so does the pathogen (Ernst 2018). Indeed, most people that encounter *Mtb* do not progress to active TB disease and are considered to have latent TB infection (LTBI) (O'Garra et al 2013; Jeyanathan et al 2018; Ernst 2018). Although only 5% - 10% of people progress to active TB disease, a person with active TB is estimated to transmit TB to an average of 10 other people per year, super spreaders (Kline, Hedemark, and Davies 1995; Curtis et al. 1999; Ypma et al. 2013) may infect as many as 200, so progression from LTBI to active TB occurs at a rate sufficient to sustain the global epidemic (Ernst 2018).

South Africa remains one of the highest burdened countries in all three WHO-defined tuberculosis categories: tuberculosis, multi-drug resistant (MDR) tuberculosis, and tuberculosis/HIV coinfection cases (Ismail et al. 2018). Moreover, sputum-positive TB prevalence in South Africa was 5% among symptomatic participants who remained unidentified by the health system (Claassens et al. 2013), mainly due to low rates of TB screening and testing (Kweza et al. 2018). Of additional concern, is the fact that active TB among healthcare workers (HCWs) in South Africa was 1565 per 100,000 in 2015 - more than double the notification rate in the general adult population (WHO 2016). Although mine workers are estimated to contribute a disproportionately large number of *Mtb* infections in South Africa on a per-capita basis, mine work contribute only a small fraction of overall *Mtb* infections in South Africa (Chang et al. 2018). This disproportionate infection rate in the population requires that control efforts should be intensified.

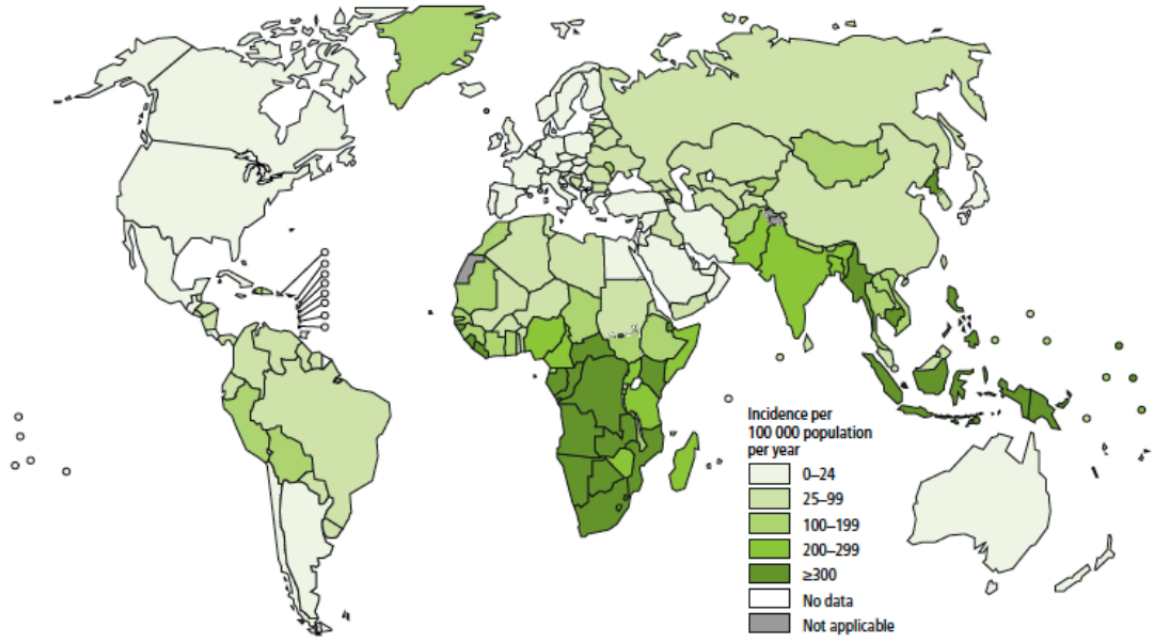


Figure 1: Estimates TB incidence rates, 2016. Source, WHO Global Tb report 2017.

In high burden settings, like South Africa, TB has its peak incidence in early adulthood, affecting the most economically productive age-groups (Heemskerk et al. 2015). Infection with HIV (figure 2) greatly increases the chances of an individual developing active TB following exposure (primary TB), or of having reactivation of latent disease (post primary TB), with the probability increasing as immunosuppression advances (Lin and Flynn 2010). In general, HIV coinfection increases the chance of developing active TB disease from 10% lifetime risk in individuals with latent TB to a 10 % annual risk (Światowa Organizacja Zdrowia 2008). Other risk factors for developing active TB include smoking, occupational lung damage, social networking pattern, diabetes and other co-morbidities (Heemskerk et al. 2015).

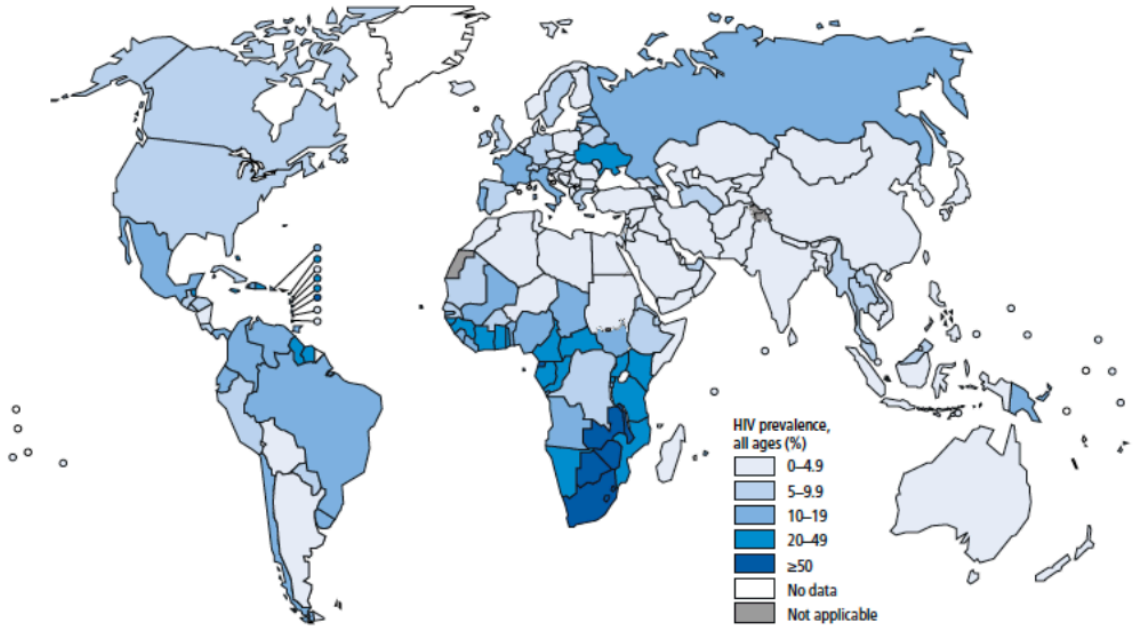


Figure 2: Estimated HIV prevalence in new and relapse TB cases, 2016. Source, WHO Global TB report 2017.

TB is spread through the air by people with active pulmonary disease, so mechanisms that promote release of bacteria from the lungs benefit the bacteria, and TB is unique in its ability to exploit adaptive immune responses (through inflammatory lung tissue damage) to promote its transmission (Heemskerk et al 2015; Cadena et al 2017; Ernst 2018). TB is curable, but despite the availability of anti-TB drugs, there were an estimated 500,000 new cases of drug-resistant TB in 2016 (WHO 2017), suggesting that TB treatment could have been inadequate or that there were high mutation rates of Mtb bacilli. The inadequacy of drug treatment can also result from a combination of factors such as the patient inability to access drugs due to lack of time, money, stock-outs, shut downs or the drug doses may be inappropriate due to the presence of comorbidities. Thus, alternative TB control strategies like improved vaccine and vaccination strategies are warranted. Cure rates are lower with resistant strains, the drugs are more costly and more toxic, and drug-resistant *Mtb* can be transmitted to other individuals (Shah et al. 2017). Given that approximately 90% of people who are infected with *Mtb* do not develop active TB disease proves that the immune system is capable of containing the disease and provide sufficient premise that a vaccination strategy has a realistic chance of TB disease eradication. This is greatly supported by a recent clinical trial of a new subunit TB vaccine from Glaxo Smithkline, M72/AS01E, which showed a 54% efficacy in preventing

progression to active TB in infected adults (Van Der Meeren et al. 2018). However, there clearly remains room for improvement and understanding the mechanisms of protective immunity to TB are key to this. Specifically, more data is crucially needed on the immunological processes that occur at the site of TB disease in humans since correlates of protection identified from studies of systemic immune signatures vary depending on the age group studied (Fletcher et al 2016) while other blood signatures reveal correlates of tuberculosis disease risk (Petruccioli et al 2016).

1.2 Initial immune events upon TB infection

Among the earliest immune cells that respond against *Mtb* are the alveolar macrophages and dendritic cells. Macrophages, dendritic cells and other innate immune cells recognize mycobacterial structures, termed pathogen associated molecular patterns (PAMPs), via membrane associated pattern recognition receptors (PRRs), of which the most studied are the Toll-like receptors (TLR2, TLR4, TLR9) (Mittal et al. 2018; Blanc et al. 2017; Schurz et al. 2015). On interaction with the TLRs, signalling pathways are activated which lead to the production of predominantly proinflammatory cytokines, such as TNF, IL-1β, IL-12 and nitric oxide (Kleinnijenhuis et al. 2009; van Crevel, Ottenhoff, and van der Meer 2002).

Mycobacteria are phagocytosed by these innate immune cells and should be destroyed through phagosome-lysosome fusion and acidification (by H_2O_2 and other reactive oxygen intermediates). However, *Mtb* appears to have the ability to subvert this process and evade destruction (Sullivan et al. 2012). Essentially, early immune response mediated through macrophages can have three major results; (1) cell necrosis, (2) apoptosis and, (3) survival of the infected macrophages. If the cell undergoes necrosis, mycobacteria are released and may infect new macrophages or disseminate, whereas an apoptotic cell membrane is not compromised and the bacteria are destroyed with the macrophage (Heemskerk et al. 2015). Survival of infected macrophages, on the other hand, enables the mycobacteria to persist and even proliferate before the adaptive immune response is activated. This happens when *Mtb* antigen specific T-cells have been selected in the regional lymph nodes and generally occurs only 2 – 6 weeks (depending on host) after primary infection (Sáenz et al. 2013; Grace and Ernst 2016). This process is initiated by dendritic cells, important mediators between the innate and adaptive immune response, which phagocytose in the lung and present mycobacterial antigens to naïve T-cells after migrating to regional lymph nodes (Pancholi,

Steinman, and Bhardwaj 1992; Baird et al. 1995; Shafiani et al. 2013, Enamorado et al 2018). Following priming in lymph nodes, activated CD4+ T-cells migrate to the lungs to impede mycobacterial progressive growth, in part through activation of macrophages. IFN-γ is key to this process and it's production by T-cells is essential in macrophage activation and intracellular mycobacterial killing (Flynn et al. 1993). Other cytokines play important roles in the immune response to TB, including TNF-α, produced by macrophages, dendritic cells and T-cells, which is central to granuloma formation, macrophage induction and has immunoregulatory properties (Singh et al. 2011). The important role TNF-α cytokine has been clearly demonstrated by the observation of increased risk of tuberculosis in patients treated with all TNF-α antagonists for major conditions including rheumatoid arthritis, ulcerative colitis, ankylosing spondylitis, psoriatic arthritis among other conditions (Bruns et al. 2009; Miller and Ernst 2009; Zhang et al. 2017). Data on T-cell functionality and specificity assays in the human tuberculous lung in this thesis are robustly measured by TNF-α production after intracellular cytokine staining of simulated cells. Other cytokines including IFN-γ, IL-2 and IL-17 were also measured.

1.3 Tuberculosis granuloma

Most TB infections are localized in the lung, resulting in pulmonary TB disease. Some patients present with a more lethal phenotype of disseminated extra-pulmonary TB disease such as TB meningitis (Thwaites and Tran 2005). However, this study is focused on pulmonary TB, particularly immune responses within the lung, which is therefore the emphasis of this review. Following inhalation of virulent *Mtb* - containing aerosol droplets into the alveoli of the lung, the bacteria are phagocytosed by resident alveolar macrophages (Russell 2007). This creates an early influx of phagocytic cells, including more alveolar macrophages, neutrophils, and dendritic cells (DCs) that arrive to this infection focus which marks the beginning of the formation of a granuloma (Flynn and Chan 2001; O'Garra et al. 2013). Cytokines, chemokines and other inflammatory mediators produced and secreted by these cells, mainly by macrophages, recruit additional leukocytes from the circulation to the site of infection (Ramakrishnan 2012). The resulting granulomas are highly cellular structures that contribute to limiting the spread of *Mtb* (Reece and Kaufmann 2012; Silva Miranda et al. 2012; Cadena, Fortune, and Flynn 2017). Observations in *M. marinum* zebrafish model helped to characterize the earliest cells that are recruited to the infection site and found that macrophages, and not neutrophils, were the most dominant phagocytosing

cells in the first 4 days post infection (Clay et al. 2007). In a different animal model, studies with both *Mtb* (Wolf et al. 2007) and *M. bovis* BCG (Humphreys et al. 2006) in the mouse suggested that there is early infection of alveolar macrophages, various populations of DCs, and neutrophils (Tsai et al. 2006). The use of reporter strains of *Mtb*, for example green fluorescent protein (GFP)-labelled *Mtb*, have further revealed not only the diversity but also the dynamic interplay between host cells and bacteria, as studied in animal models especially in the mouse (Wolf et al. 2007; Sukumar et al. 2014). These observations demonstrate the diverse range of early phagocytic responder cells to *Mtb* infection and implicate their initial influence on the progression of early disease.

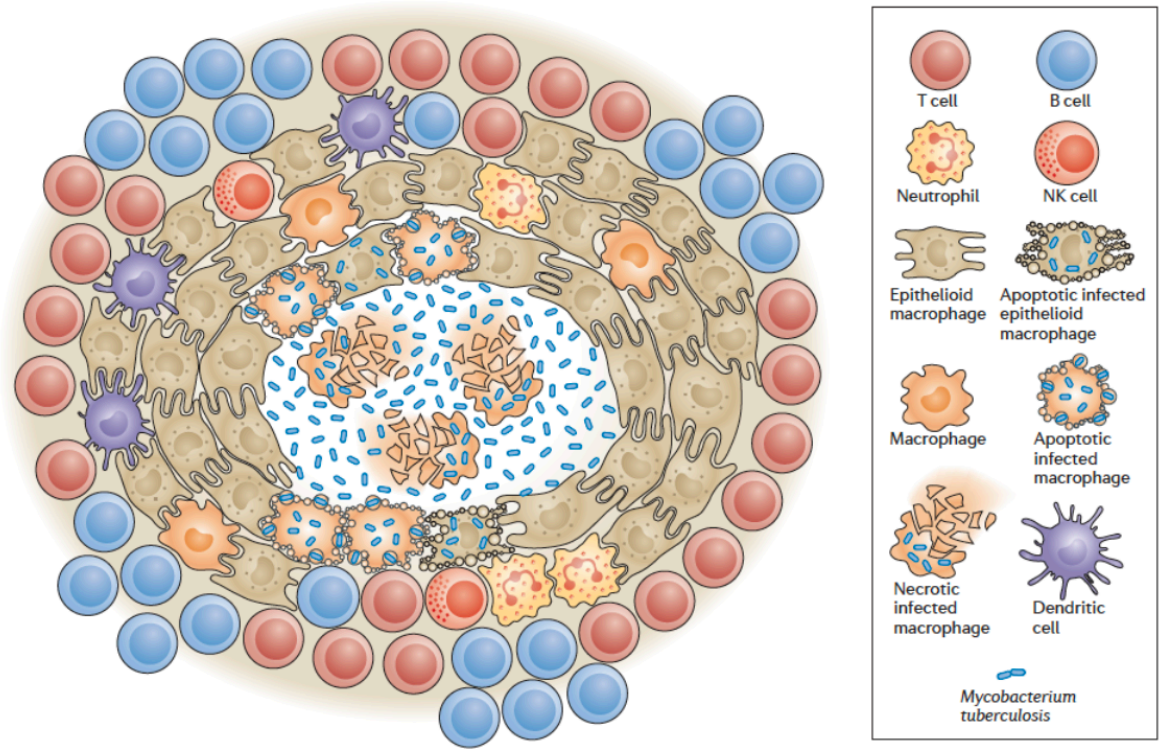


Figure 3: Schematic representation of tuberculosis granuloma. Cellular organization of the pathological hallmark of TB disease showing arrangement of immune cells around *Mtb* bacilli. Image reproduced from Cadena et al 2017

TB granulomas are highly heterogenous in nature and, the lungs usually contain multiple infection foci (Subbian et al. 2015; Gideon et al. 2015; Cadena, Fortune, and Flynn 2017). Of

note, granulomas in *Mtb*-infected human lungs mature and evolve independently of each other, determined primarily by local immunity and bacteria-derived factors (Fukusumi et al. 2013; Hunter 2011; Ulrichs et al. 2005). In most cases, at the centre of the granuloma, heavily infected macrophages that are unable to control bacillary growth undergo necrosis, releasing *Mtb* as well as host cell contents into the extracellular milieu (Subbian et al. 2015). These bacilli can be phagocytosed by newly arriving macrophages, resulting in further recruitment and activation of immune cells, and the formation of larger and more differentiated granulomas (Russell et al. 2009; Dannenberg 2006). In some granulomas, the necrotic centre may undergo caseation and liquefaction, ultimately leading to cavity formation (Kim et al. 2010; Cardona 2011). Cavitation facilitates dissemination of the infecting bacilli from the granuloma via the airways to the external environment (Kaplan et al. 2003; Grosset 2003). The mechanisms that define controlling granulomas, whose global effect would result in latency, are less clearly defined (Marakalala et al. 2016), and this is further complicated by the observation in non-human primate studies where macaques with active TB as well as those with latent TB had sterile granulomas (Lin et al. 2014). Data suggest that controlling granulomas (sometimes to the level of lesional sterilization) is a consequence of the level of bacterial killing in the individual lesions and this happens at the induction of adaptive immune responses (Lin et al. 2014). Lin et al found that fibrocalcific lesions contain less viable bacteria and represent sites of bacterial control whereas caseous sites are associated with poor control (Lin et al. 2014).

Evidence from non-human primates and humans suggest that at the granuloma level, there exists an association between the maturation state of a lesion and the level of immune-stimulation (Kim et al. 2010; Lin et al. 2014; Gideon et al. 2015; Cadena, Fortune, and Flynn 2017). In general, cavitary lesions with numerous acid-fast bacilli (AFB) appear to be immunologically more active, while the fibrotic nodules and other lesions with scanty or no AFB appear less active (Subbian et al. 2015). Consequently, granulomas play a crucial role in the control of *Mtb* infection and establishment of latency (LTBI) as well as in the spread of the disease (active TB). These data support the hypothesis that the outcome of *Mtb* infection is determined at the granuloma level and the sum total of granulomas are collectively responsible for disease outcome at the total host level (Gideon et al. 2015).

Thus, the TB granuloma is the pathological hallmark of pulmonary TB disease. Complete granuloma formation requires the adaptive immune responses, orchestrated mainly by T-cells

(Flynn et al. 2015; Marakalala et al. 2016; Kauffman et al. 2018; Cadena, Fortune, and Flynn 2017). Upon the development of adaptive immunity, granulomas acquire a more intact structure with the macrophage-rich centre surrounded by CD4+ and CD8+ T-cells and B-cells resulting in a lymphocytic cuff at the periphery of the structures (Marakalala et al. 2016; Kauffman et al. 2018; Cadena, Fortune, and Flynn 2017; Gautam et al. 2018). Heterogeneity of granuloma is not only limited to the cell composition but also to the functionality of the cells. Functional dynamics of T-cells with respect to cytokine profile and bacterial burden of individual granulomas in the macaque model showed that each granuloma is independent with respect to total cell numbers, frequency of T-cells, patterns of cytokine response and bacterial burden within an individual host and among animals (Gideon et al. 2015). Therefore, the complex organization of granulomas, with their populations of macrophages and lymphocytes suggest that there is significant cross-talk between cell types, and that for granulomas to function properly there needs to be coordination between these cell types (Flynn et al. 2015; Kauffman et al. 2018).

1.4 T-cells: beyond the granuloma formation

The essential role of T-cells in controlling *Mtb* infection has been demonstrated by several lines of evidence, in both animal models and human infections. Data from knock out studies show that *Mtb* infection of mice deficient in CD4+ T-cells results in higher bacterial burdens in the lung and other tissues and in shortened survival compared with infection of immunocompetent mice (Flory, Hubbard, and Collins 1992; Ladel et al. 1995; Caruso et al. 1999; Mogues et al. 2001; Green, Difazio, and Flynn 2013; Sakai et al. 2016). Non-human primates depleted of CD4+ T-cells are also highly susceptible to *Mtb* infection (Flynn and Chan 2001; Yao et al. 2014). During latent infection in macaques, depleting CD4+ T-cells with antibody for up to 14 weeks resulted in half of the animals developing reactivation of TB (Lin et al. 2012; Yao et al. 2014). In humans coinfected with TB and HIV, CD4+ T-cell deficiency leads to poor granuloma formation, a higher frequency of progression to active TB, a higher frequency of disseminated extrapulmonary disease, and higher mortality (Selwyn et al. 1989; Chaisson et al. 1987; Perlman et al. 1997; Grace and Ernst 2016; Murray et al. 2018).

Data suggests that the protective role of T-cells during *Mtb* infection goes beyond just the frequency of T-cells but crucially the quality and type of immune molecules they produce. In

the lung, T-cells are required for their macrophage-activating and cytolytic activities, which are essential for control of not only initial *Mtb* infection but also persistence of the infection (Cooper and Khader 2008; Flynn and Chan 2001; Flynn and Ernst 2000; O'Garra et al. 2013). T-cells are significant sources of pro-inflammatory cytokines IFN-γ and TNF-α, that are necessary for macrophage activation and protection. Other T-cell produced cytokines such as IL-2 or IL-17 have been reported to stimulate proliferation, activate local cells, or recruit cells from the periphery to the primary site of *Mtb* infection (Algood, Lin, and Flynn 2005; Cooper and Khader 2008; O'Garra et al. 2013; Brighenti and Andersson 2012; Khader and Gopal 2010; Millington et al. 2007). On the other hand, anti-inflammatory/regulatory cytokines such as IL-10 are also produced by T-cells at the site of infection (Redford, Murray, and O'Garra 2011). These anti-inflammatory cytokines may be important in reducing pathology, although this remains controversial. Studies indicate that additional T-cell cytokines, such as GM-CSF, may play a direct role in *Mtb* control within the lung, although the details are less clear (Rothchild et al. 2014, 2017). Finally, lung lesion associated T-cells also express cytolytic and antimicrobial factors including perforin, granzyme B, and granulysin, which may have direct anti-Mtb activity (Flynn et al. 2015).

Although T-cells produce different kinds of cytokines during *Mtb* infection, available data indicate that Th1 polarized effector responses play a central role in host resistance to TB (Sáenz et al. 2013; Grace and Ernst 2016). For example, in non-human primates, it has been observed that despite the loss of CD4+ T-cells, control of infection did not correlate with decreased IFN-γ production, suggesting that the loss of CD4+ T-cells may result in suboptimal CD8+ T-cell responses (Lin et al. 2012; Yao et al. 2014). Finally, humans with inborn errors in genes involving IFN-γ signalling, and mice deficient in IFN-γ signalling or T-bet are all extremely susceptible to *Mtb* infection (Cooper et al. 1993; Flynn et al. 1993; van Crevel, Ottenhoff, and van der Meer 2002; Filipe-Santos et al. 2006; North and Jung 2004; Sullivan et al. 2005).

Improvements in techniques of studying immune cells in infected tissues have increased our understanding of the T-cell responses to *Mtb* infection in the lung. Data from mouse model has elegantly demonstrated that in tuberculosis infected lung, the CD4+ T-cell response against *Mtb* is composed of two major subsets that either enter the lung parenchyma or reside within the vasculature (Sakai et al. 2014) allowing investigators to tease out the contribution

of each subset, within the same tissue, in *Mtb* control. Unfortunately, the same group did not observe the same phenomenon in non-human primate model of tuberculosis in a later study (Kauffman et al 2017). From these two incongruent results in two different animal models, it is unclear which of the two models mimic events that occur in the human lung during TB disease. On the basis of cell surface markers, the parenchymal effectors express CXCR3 and are PD-1^{hi}/$CD69^{hi}$, likely reflecting their access to antigen within the tissue. In contrast, most of the vasculature CD4+ T-cells express CX3CR1 and have a more terminally differentiated phenotype (KLRG1^{hi}/T-bet^{hi}). By studying the granuloma, it has been shown that the important role of CD4+ T-cells in controlling *Mtb* infection is dependent on direct contact between the CD4+ T-cells and infected antigen presenting cells (Srivastava and Ernst 2013; Kauffman et al. 2018). Thus, it is likely that the enhanced protective capacity of the parenchymal-homing subset (Sakai et al. 2014) is principally due to its ability to gain access to the infected cells in the granuloma; conversely, the relative ineffectiveness of the CX3CR1+ subset is likely due to its inability to enter the lung tissue.

The role of PD-1 expressing CD4+ T-cells during TB infection continues to be appreciated but whether these cells are protective (especially in humans) is not yet clear. Lázár-Molnár et al demonstrated protective role of PD-1 in mouse model following aerosol *Mtb* infection (Lázár-Molnár et al. 2010). PD-1–deficient mice showed dramatically reduced survival compared with wild-type mice. They showed that the lungs of the PD-$1^{-/-}$ mice showed uncontrolled bacterial proliferation and focal necrotic areas with predominantly neutrophilic infiltrates, but a lower number of infiltrating T and B-cells. Proinflammatory cytokines, including TNF-α, were significantly increased in the lung and sera of infected PD-$1^{-/-}$ mice, consistent with an aberrant inflammation. Additionally, microarray analysis of lungs infected with *Mtb* between PD-$1^{-/-}$ and wildtype mice showed that the PD-1 pathway is required to control excessive inflammatory responses after *Mtb* infection in the lungs (Lázár-Molnár et al. 2010). Thus, PD-1 is essential for containment *Mtb* infection and prolonged survival. Although IFN-γ is important for controlling *Mtb*, excessive production by parenchymal CD4+ T-cells exacerbates lung infection and leads to the early death of the host (Sakai et al. 2016). Sakai and colleagues demonstrated that PD-1 facilitates host resistance to *Mtb* by preventing the detrimental over-production of IFN-γ by CD4+ T-cells by specifically suppressing the parenchymal accumulation of, and pathogenic IFN-γ production by the CXCR3+KLRG1-CX3CR1- subset of lung-homing CD4 T-cells that otherwise mediates control of *Mtb* infection. Mucosal BCG vaccination induced antigen-specific CD4+ T-cells

that were PD-1+ KLRG1− were recently associated with enhanced protection against *Mtb* infection (Bull et al. 2018), most importantly, these cells were exclusively present within the lung parenchyma and airways. However, these lung parenchymal PD-1+ KLRG1− CD4+ T-cells decreased with time after mucosal BCG vaccination.

Whereas animal models show protective role for PD-1 in TB, human studies show association of PD-1 with high bacterial burden and disease progression. PD-1 expression on CD4+ or Th1 lymphocytes was associated with a high extent of disease, as defined by a higher radiographic score of active TB patients implying that the percentage of PD-1 is induced by TB bacilli, and that it increases with disease progression (Shu et al. 2017). Furthermore, *ex vivo* stimulation of peripheral blood lymphocytes with PMA and ionomycin showed a significantly higher expression of PD-L1 on CD4+IFN-γ+ and CD4+IL-17+ T-cells in tuberculosis infected subjects than healthy controls. Shen and colleagues reported increased expressions of PD-1 and its ligands on CD4+ T-cells among patients with active tuberculosis (Shen et al. 2016). By blocking PD-1/PD-L pathway, there was an enhanced proliferation of *Mtb* -specific CD4+ T-cells *in vitro* and inhibition of the PD-1/PD-L pathway helped to control *M. bovis* bacillus Calmette-Guerin (BCG) replication in macrophages co-cultured with CD4+ T-cells *in vitro*. Thus, the PD-1/PD-L pathway inhibits not only *Mtb*-specific CD4+ T-cell-mediated immunity but also innate immunity, such as phagocytosis of macrophages in active TB. Most recently, Day et al observed higher levels of PD-1 on Th1 cytokine-producing *Mtb*-specific CD4+ T-cells from patients with smear-positive TB, compared with smear-negative TB and LTBI, which decreased after completion of anti-TB treatment. By *in vitro* stimulation of PBMC with *Mtb* antigens, they demonstrated that PD-1 is induced on proliferating *Mtb*-specific CD4+ T-cells and that Th1 cytokine production capacity is preferentially maintained within PD-1+ proliferating CD4+ T-cells, compared with proliferating *Mtb*-specific CD4+ T-cells that lack PD-1 expression (Day et al. 2018). Thus, expression of PD-1 on *Mtb*-specific CD4+ T-cells was indicative of mycobacterial antigen exposure and treatment response in human TB. However, cases of patients developing acute pulmonary tuberculosis during immune checkpoint inhibitor treatment does show that continued abrogation of PD-1 expression in humans can be detrimental. One such case was a patient with relapsed Hodgkin's lymphoma who was receiving pembrolizumab (2 mg/kg) and in whom fever, and unintentional weight loss developed after the fifth cycle. Tuberculosis infection was confirmed by *Mtb* growth on a sputum culture (Lee, Chan, and Tang 2016). In yet another case, active TB (positive for acid-fast bacilli and positive

polymerase chain reaction) developed in a patient with advanced non-small cell lung cancer treated with third-line nivolumab after the eighth cycle of nivolumab (Fujita, Terashima, and Mio 2016).

Beyond the conventional T-cells, are the donor unrestricted T-cells (mucosa associated invariant T-cells - MAIT, invariant natural killer T-cells – iNKT, Germline encoded T-cells – GEM and γδ T-cells) that also have been shown to have a protective role in tuberculosis. MAIT-cells respond to cells infected with bacterial pathogens without prior exposure to that pathogen, produce cytokines and are cytotoxic (Sharma et al. 2015; Wong, Ndung'u, and Kasprowicz 2017). In MR-1 deficient murine models, MAIT-cells were associated with early protection against bacterial pathogens, including mycobacteria (Gold, Napier, and Lewinsohn 2015). Thus, these cells may act as early sensors of *Mtb* infection in airways and provide early cytokines to activate macrophages against infection. *Mtb*-reactive MR1-restricted T-cells were found in the peripheral circulation of humans including those with no evidence of previous exposure to *Mtb* (Gold et al. 2010). Interestingly, they were found at much lower levels in individuals with active tuberculosis. The few MAIT-cells that remain in circulation during active pulmonary tuberculosis have diminished functional capacity, producing less IFN-γ upon stimulation and decreased transcription of effector genes (IFN-γ, TNF-a, IL-17F, granulysin and granzyme B) (Jiang et al. 2014; Kwon et al. 2015). CD1b-restricted T-cell numbers including GEM T-cells are enriched in peripheral blood and at the site of infection during active TB, expand and contract markedly according to pathogen burden, and therefore can be markers of disease severity (Montamat-Sicotte et al. 2011). Activated iNKT-cells enhance host resistance to *Mtb* and when cocultured with *Mtb*-infected macrophages, iNKT-cells do inhibit intracellular *Mtb* replication (Rothchild et al. 2014). In addition, iNKT produce GM-CSF that is essential for immunity to *Mtb* in the lung. A recent study reported significant increase in the frequency of MAIT and iNKT-cells in individuals with latent *Mtb* infection compared with active *Mtb* infection, or uninfected controls (Paquin-Proulx et al. 2018). The role of γδ T-cells in the immune response to *Mtb* was first reported in 1989 (Janis et al. 1989) and additional work has demonstrated that the activation of γδ T-cells appear rapidly following *Mtb* infection, and γδ T-cells provide protective immunity against *Mtb* infection with the combined properties of both innate and adaptive immunity (Casetti and Martino 2008; Holtmeier and Kabelitz 2005; Davey et al. 2011).

In summary, it is clear that different types of T-cells are involved in the control of tuberculosis. While the conventional T-cell roles are more studied, increasing data show that donor unrestricted T-cells act as a link between innate and adaptive immunity to tuberculosis. The impact of immune checkpoint blockade therapy in control of other conditions like cancer brings into focus the role played by T-cell exhaustion markers in containment of *Mtb* infection.

1.5 Peripheral blood signatures predict TB disease progression

Due to the ease of access to blood samples, our understanding of human immune responses to tuberculosis have come from studies of systemic circulation. Recent efforts including unbiased approaches employing "omics" technologies, as well as more directed, hypothesis-driven approaches have identified correlates of TB risk (reviewed in detail by Petruccioli et al 2016). Indeed, there have been correlates of TB risk including commercial or traditional tests for LTBI diagnosis that use RD1-specific immune response in IGRA and immune sensitization to PPD in TST that have been validated in children and adults (Sester et al 2011; Rangaka et al 2012; Ottenhoff et al 2012). Unbiased molecular tests revealed that mRNA expression signature of 16 interferon response genes was a correlate of TB risk in adolescent African population (Zak et al 2016). T-cell activation markers such as HLA-DR-expressing CD4+ T-cells was associated with an increased risk of TB in infants (Tameris et al 2013; Fletcher et al 2016). Furthermore, blood cell counts, for instance, elevated monocyte/lymphocyte ratio in African adult population have also been shown to be a correlate of TB risk (Naranbhai et al 2014; Baldridge et al 2010). Thus, although only a handful of studies have identified biomarkers of progression to TB, the available data point to the common biological processes, including immune activation, IFN responses and changes in peripheral blood myeloid and lymphoid cells which have been clinically validated and are associated with risk of TB. Several other biomarkers are being explored as correlates of TB risk and their validation is an area of ongoing research (reviewed in Petruccioli et al 2016).

While the studies highlighted above have identified correlates of TB risk, other studies have identified biomarkers associated with protection. BCG IFN-γ ELISPOT and Ag85A IgG were identified as correlates of protection following vaccination of infants with BCG (Fletcher et al 2016). Host blood-based biomarkers (CD38, HLA-DR, and Ki-67–expressing) on Mtb-specific CD4+ T cells were shown to discriminate between ATB and LTBI. Furthermore,

treatment success was associated with decreased frequencies of CD38+IFN-γ+, HLA-DR+IFN-γ+, and Ki-67+IFN-γ+CD4+ T cells, which mirrored culture conversion, providing a set of tools for monitoring treatment response and cure (Adekambi et al 2015). A recent multi-cohort study showed that CD16 and NK cells play a key role in maintaining LTBI state and the frequency of these cells were shown to go down during ATB, and NK cells are restored after successful treatment suggesting that these cells can predict TB outcomes (Chowdhury et al 2018).

It is evident that the correlates identified from blood perform differently based on age group and region studied. For example, while biomarkers based on T-cell activation predict progression of disease (Tameris et al 2013; Fletcher et al 2016), the same biological process was shown to discriminate between ATB and LTBI and can be used to monitor treatment outcomes (Adekambi et al 2015). Studying immune responses to TB in humans at the primary site of disease, the lung, could potentially provide a clearer picture or provide additional evidence of reliable correlates of disease control.

1.6 T-cell immune responses to TB in blood versus lung

Majority of the vaccine studies in humans have been done using blood samples, with results indicating responses in circulation and not at the site of disease (Gideon et al. 2015; Flynn et al. 2015). Crucially, to date these studies have failed to consistently identify a correlation between the circulating immune response and protection (Beverley et al. 2014), and reliable correlates of protection for TB disease are still lacking. Until the time of induction of adaptive immunity, *Mtb* grows logarithmically, but the appearance of T-cells in the lungs correlates with inhibition of growth, indicating their importance in controlling infection (Beverley et al. 2014). Thus, the containment of challenge mycobacteria may not be due to an influx of cells from the periphery but rather expansion of T-cells already present in the lung tissue (Connor et al. 2010). From the mouse model, although systemic immunity may confer some degree of containment, it does not prevent establishment of infection as *Mtb* continues to grow unchecked for greater than 10 days (Beverley et al. 2014). Thus, the control of the early phase of *Mtb* growth is likely to require local lung immune responses and therefore a better understanding of mucosal immunity is vital in order to design effective mucosal vaccines.

Lung granulomas provide the most direct measure of the actual responses induced by *Mtb* at the site of disease/infection. It is, however, acknowledged that the relationship between T-cell responses in blood and lung tissues are complex (Flynn et al. 2015). Even bronchoalveolar lavage (BAL) T-cell responses, which are considered to be a closer match to the lung T-cell responses, differ from the responses in the monkey tuberculous granuloma (Lin et al. 2006). Differences in the T-cell responses might be further complicated by the existence of a spectrum of infection at both local and systemic levels in animals and humans (Flynn et al. 2015). Animal studies have reported a substantial range of bacterial burden between different granuloma within each animal, reiterating that the extent of immune containment varies even at the primary site of TB infection (Flynn et al. 2015).

In contacts of active TB patients and those with paucibacillary pulmonary disease, *Mtb* Ag85-specific T-cell responses are detected in the BAL but not in the peripheral blood, indicating compartmentalization of Ag85-specific cells to the lungs (Schwander et al. 2000). The sample size used in this study was relatively small making it hard to draw major conclusions from the results. However, this observation suggests that sampling of blood would miss some individuals who already have active TB and remain potential source of transmission. In general, however, active TB patients with higher bacterial loads have antigen specific CD3+ T-cell responses in both the BAL and blood, although responses are generally higher in the BAL near the site of infection, than the peripheral blood (Schwander et al. 1996; Boras et al. 2007). Reports from non-human models of *Mtb* suggest that locally initiated immune responses have an important role in the early control of *Mtb* infection after exposure, although definitive evidence for their role in mediating protection in humans is yet to be ascertained (Beverley et al. 2014).

1.7 Tissue resident memory T-cells

Following antigen encounter in lymph nodes (LNs), naïve T-cells undergo rapid proliferation, giving rise to differentiated effector T-cells and long lived memory T-cells that are distributed more broadly throughout the body (Beura et al. 2015). On re-exposure, memory T-cells are able to mount a more rapid and robust response to the antigen, (Cauley and Lefrançois 2013; Mueller et al. 2013), which is the basis of vaccine efficacy. There is growing evidence that this memory response is most effective if it is positioned at the site of pathogen infection. This is possible as a subset of memory cells, called Tissue Resident

Memory (Trm) cells (Sakai et al. 2014), can persist in tissue for an extended period without recirculating in blood, ready to rapidly respond to a new infection.

Activated T-cells migrate into inflamed tissue under the guidance of chemokines, integrins and adhesion molecules (David Masopust and Schenkel 2013). T-cells that encounter their specific antigen at the infection site expand and receive tissue-specific cues that influence their function and memory potential (Gray, Westerhof, and MacLeod 2018). Following pathogen clearance, some of these effector memory T-cells remain within the tissue site and form a Trms population ready for the next pathogen encounter. Trms have been described in many non-lymphoid tissues, including the skin, gut, female genital tract and the lungs, typically at the sites of initial infectious exposure and often at a mucosal surface. As a result of this positioning, Trms can play a critical role in subsequent immune challenge by accelerating clearance of local reinfections at these mucosal surfaces (Masopust et al. 2001; Schenkel and Masopust 2014; Gebhardt and Mackay 2012; Park and Kupper 2015; Mueller and Mackay 2016). A direct consequence of this tissue tropism, is that Trms are typically absent from blood (Teijaro et al. 2011; Hogan, et al. 2001), which can have profound implications for their study in humans, in whom peripheral blood mononuclear cells (PBMCs), are often the main substrate for studying immune responses.

Trm cells vary in phenotype and function, depending on the tissue they reside in (Gebhardt et al. 2013; Cauley and Lefrançois 2013; Mackay et al. 2013a; Perdomo et al. 2016). The study of the biology and role of Trms is complicated by the need to differentiate cells truly isolated from the lung parenchyma from circulating cells contaminating the sample. Much of the biology of Trm has been worked out in mice and has relied on several novel approaches; notably, intravenous labelling of circulating cells just prior to sacrifice to distinguish cells in circulation (labelled) to those in tissue (unlabelled); or the use of Fingolimod (FTY720), a drug which prevents T-cell egress from lymph nodes and thus blocks new recruitment of memory cells into tissue sites (Anderson et al. 2012, 2014; Knudson et al. 2016; Gray, Westerhof, and MacLeod 2018; Mohammed and Harikumar 2017; Connor et al. 2010; Sawicka et al. 2003; David Masopust and Soerens 2019). Although these techniques are not applicable to humans, they have confirmed that many of the cell surface markers used in animal models are shared in humans, notably high CD69 expression together with CD103 (Kumar et al. 2017; Thome et al. 2014). While CD69 is expressed by the majority of Trms, CD103 is mostly expressed on a subset of Trm, primarily CD8+ T-cells (Kumar et al. 2017).

Numerous other cell surface markers have been associated with Trm cells, including the upregulation of CD49a (an adhesion molecule), CXCR6, CD101, PD-1, and loss of CD62L and the chemokine receptor CX3CR1. Although there does not appear to be lung specific Trm marker(s) per se, those that have been associated with T-cell residency within the lung are highlighted in table 1.

Table 1: Cell surface markers of Trms in humans validated in animal models.

Marker	***Tissue measured in***	***Pathogen/condition studied***	***Reference***
CD103(integrin αE which combines with integrin β7 to form αEβ7 heterodimer)	Upregulated in: Skin, Lung, FRT, Kidney, Gut	*Mycobacterium tuberculosis, Influenza*, HSV, LCMV,	(Sathaliyawala et al. 2013; Mackay et al. 2012; Beura et al. 2015; Gebhardt et al. 2018)
CD11a (ITGAL-1: integrin alpha L which combines with β2 to form LFA-1)	Upregulated in: Lung, Skin	*Influenza, Asthma, Allergy, Leishmania major*	(Neyt, GeurtsvanKessel, and Lambrecht 2016; Trischler et al. 2016; Knight et al. 2014)
CD49a (VLA-1: integrin α subunit that combines with integrin β1 subunit to form α1β1 heterodimer)	Upregulated in: Lung	*Mycobacterium tuberculosis*, *Influenza*	(Perdomo et al. 2016; Haddadi et al. 2017; Ray et al. 2004; McMaster et al. 2018)
VLA-4(α4β1: integrin dimer composed of α4 (CD49d) subunit and β1(CD29 subunit)	Upregulated in: Lung	*Mycobacterium tuberculosis*	(White et al. 2015; Walrath and Silver 2011; Taylor et al. 2008)
CD69(Binds to and downregulates S1PR1)	Upregulated in: Skin, Lung, FRT, Kidney, Gut	*Mycobacterium tuberculosis, Influenza*, HSV, LCMV,	(Sathaliyawala et al. 2013; Mackay et al. 2013; Masopust and Picker 2012; Beura et al. 2018)
CD101	Upregulated in: Skin, intestines, Liver, Lung	*Plasmodium*	(Bagot et al. 1997; Schey et al. 2016; Fernandez-Ruiz et al. 2016; Kumar et al 2017)
CD44	Upregulated in: Lung	*Influenza*	(Topham and Reilly 2018; Hogan et al. 2001)
CD62L	Downregulated in: Gut, Skin, Lung, Lymph Node	HIV, Vaccinia virus	(Sathaliyawala et al. 2013; Brinkman et al. 2013; Zaric et al. 2017)

1.7.1 Formation and maintenance of tissue resident memory T-cells

Understanding the signals driving the formation and maintenance of Trm cells is crucial if they are to be exploited by novel vaccination strategies. Trm cells derive from precursors entering tissues during the effector phase of immune responses and remain positioned within these compartments (Schenkel and Masopust 2014; Turner and Farber 2014). For this to occur, they must adapt to local survival cues, resist shedding into the lumen at mucosal epithelial surfaces, and ignore egress signals (Masopust and Schenkel 2013; Mueller et al. 2013). The transition of recruited T-cells to Trm under normal conditions appears to require

simultaneous tissue damage and TCR signaling on antigen exposure (Khan et al. 2016; Takamura et al. 2016; McMaster et al. 2018). Thus, whilst on-going inflammation may recruit non-specific T-cells from circulation, only those that encounter their cognate antigen should set up tissue residency. However, this is not always the case (Casey et al. 2012; Mackay et al. 2012) and may vary according to location. Flu-specific Trms in the upper respiratory tract, but not in lung tissue, for example, can develop independently of local cognate antigen recognition (Pizzolla et al. 2017), possibly as a consequence of localized production of cytokines such as IL-15 (Mackay et al. 2013; Schenkel et al. 2016). Nonetheless, any infection in non-lymphoid tissue that is antigenic and causes pathology has the potential to establish a Trm population at that site.

One consequence of TCR activation in general, is temporary upregulation of surface CD69 (Ferenczi et al. 2000; González-Amaro et al. 2013; Cibrián and Sánchez-Madrid 2017), the canonical Trm marker, which in turn interacts with sphingosine-1-phosphate receptor 1(SIPR1) and downregulates its expression (Cyster and Schwab 2012; Matloubian et al. 2004; Shiow et al. 2006; Schober et al. 1999; Skon et al. 2013) . In tissue this is crucial, as it prevents cells from following the SP1 chemokine gradient back into circulation, and therefore results in prolonged retention and local memory formation (Mackay et al. 2015). However, it is not clear how CD69 expression is sustained in cells that become Trm, and whether all CD69+ve T-cells identified in tissue at any given time are actually Trm. Indeed, although it is widely accepted as a good marker, there is some evidence that not all Trm constitutively express CD69 (Schenkel and Masopust 2014). Another molecule that may help retain Trm in tissue is the integrin αE (CD103) (Casey et al. 2012; Mackay et al. 2013; Wakim, Woodward-Davis, and Bevan 2010), which forms a heterodimer with the integrin ß7 and binds E-cadherin (Cepek et al. 1994), a glycoprotein constitutively expressed by epithelial cells. Therefore, CD103 is thought to be particularly important for retaining Trm at the epithelial surfaces of mucosal barriers such as the lung. Other integrins thought to be involved in retention of Trm in the lung and other non-lymphoid tissue sites are highlighted in Table 1.

The mechanistic details of how Trm responsiveness and longevity is maintained remains an area of much on-going research. In central memory T-cell populations, enhanced lifespan and proliferative capacity is associated with a shift in metabolism towards endogenous lipids and

oxidative phosphorylation (Buck et al. 2017). Similarly, skin CD8+ Trm appear to rely on fatty acid β-oxidation to support their long-term survival, although via exogenous lipid uptake (Pan et al. 2017). In addition, RNA sequencing of Trms in both animal models and humans shows there is a distinct transcriptional profile associated with tissue residency (Hombrink et al. 2016; Oja et al. 2017). Thus, transcriptional and metabolic reprogramming probably underlie the formation and maintenance of Trm within their tissue niches. Two recent publications have elegantly shed light on the dynamics of Trm cell formation and maintenance *in situ*. First, using intra-vital imaging of the female genital tract revealed that CD8+ Trm cells continually patrol the tissue, but pause and rapidly expand after LCMV challenge (Beura et al. 2018). These data are important, as they remove the image of Trm as a sedentary cell waiting for its pathogen to land and provide a clear mechanism by which a relatively small number of Trm can effectively protect a large organ or mucosal barrier. In this study, LCMV infection also triggered the recruitment of recirculating memory T-cells, but the expansion of pre-existing Trm, was independent of this process and far outweighing that of memory cells recruited from circulation (Beura et al. 2018). In a separate study, Park and colleagues demonstrated that newly recruited T-cells could establish a Trm population in the skin without displacement of the pre-existing Trm pool. This is also important as it demonstrates that multiple Trm cell specificities can be stably maintained within the tissue (Park et al. 2018). Together these studies provide a clear mechanistic rationale for how vaccine induced Trm could possibly protect a large organ such as the lung and establish against the backdrop of Trm to other respiratory pathogens that are likely to already be present (Purwar et al. 2011).

From the perspective of *Mtb* infection, it is unfortunate that the majority of work on the biology of Trm has focused on tissues other than the lung. Moreover, there is evidence that Trm populations are not as stable in lung tissue as they are in organs such as the skin (Slütter et al. 2017; Wu et al. 2014). However, observations of long-lived Trms against respiratory pathogens in the lungs of both mice and humans have been made (Turner et al. 2014; Teijaro et al. 2011; Purwar et al. 2011). Although more work is needed, it seems reasonable to assume that life time exposure to lung pathogens, should build up a repertoire of Trms that patrol the lung and are poised to rapidly respond to new re-infection or re-activation of latent disease, as may be the case in TB. Indeed, regularly low level exposure to *Mtb* antigen, either through transitory blip in latency (Kiazyk and Ball 2017), or exposure to *Mtb* or other

environmental mycobacteria might be expected to facilitate TB-specific Trm maintenance in the human lung.

1.7.2 Trafficking of T-cells to the lung during tuberculosis.

Direct contact between CD4+ T-cells and antigen presenting cells is required for a robust T-cell response to *Mtb* infection (Srivastava and Ernst 2013). T-cells within the lung can be divided into parenchymal and intravascular subsets. In TB infected mice, T-cells that cross the lung vasculature into the parenchyma express CXCR3 and are negative for Killer Cell Lectin Like Receptor G1 (KLRG1), while intravascular CD4+ T-cells express CX3CR1 and expressed a more terminally differentiated, KLRG1^{hi} /T-bet^{hi}, phenotype (Sakai et al. 2014). Importantly, when adoptively transferred to naïve mice, CXCR3+ve KLRG1-ve CD4+ T-cells from the lung parenchyma of TB infected mice provided better immune protection than their blood counterpart (Sakai et al. 2014; Reiley et al. 2010). Although this observation was not reported in non-human primate model of TB (Kauffman et al 2017), it does suggest, at least in the mouse, that the parenchyma derived CD4+ T-cells express the markers that allow them to home directly back to the lung and contact *Mtb* infected macrophages. Upregulation of CXCR3 is likely to be key, as increased levels of CXCR3 ligands, such as IP-10, in the lung are required for T-cell lung recruitment (Jeyanathan et al. 2017). Indeed IP-10, along with other CXCR3 ligands (MIG and I-TAC) are all expressed in the granulomas of TB-infected lungs and contribute to their formation (Seiler et al. 2003). This suggests a model whereby primed T-cells are recruited to the lung parenchyma upon TB infection in a CXCR3-dependent manner and are positioned within granulomas along a chemokine gradient (Sakai et al. 2014; Woodworth et al. 2017). Once at the granuloma the combination of TCR triggering and signaling from tissue damage should provide the conditions to establish a population of Trm. The classic granuloma structure is defined by a “cuff” of T-cells surrounding a core of infected phagocytes. Moreover, granuloma associated T-cells have been described in the structure of inducible bronchioalveolar lymphoid tissue (iBALT) thought to be protective in TB infection (Ulrichs et al. 2004; Khader et al. 2011). Whether these T-cells are TB-specific Trm cells is not known, but, given these conditions, it is certainly biologically plausible.

1.7.3 Evidence of Trm activity against Tuberculosis

Although there are relatively few studies that have directly investigated the role of Trms in TB infection, the evidence they provide is compelling. In an early study, Connor et al used FTY720, to negate recall responses from lymph nodes, and found that administration of the drug during infection but after vaccination (with BCG), had no effect on vaccine efficacy (Connor et al. 2010). Giving FTY720 prior to vaccination, however, diminished protection, showing recruitment of vaccine specific T-cells to the lung from secondary lymphoid organs was essential to protection. Mice in this study were all vaccinated sub-cutaneously with no adjuvant, showing that lung Trm formation is possible under these circumstances, although it was unlikely to have been optimal. Again exploiting the properties of FTY720, Flórido et al. 2018 recently showed that mice intranasally vaccinated with a recombinant influenza A virus vaccine expressing an immunodominant *Mtb* epitope from antigen 85A (PR8.p25), were protected from subsequent infection with *Mtb* when further T-cell recruitment to the lung was blocked (Flórido et al. 2018). Importantly, treatment of unvaccinated mice with FTY720 significantly increased lung mycobacterial burden upon infection, confirming that, in the absence of pathogen specific T-cell population in the lung, recruitment of newly primed T-cells from circulation is required. In both studies, however, once priming has occurred, further recruitment from circulation is not needed, implying the protective T-cells are already at the site of disease in the lung.

Rather than blocking peripheral T-cell recruitment as a way of demonstrating the importance of tissue resident T-cells, other groups have sought to specifically boost lung Trm via novel vaccination routes. Intratracheal injection of BCG, for example, has been shown to improve vaccine activity, and reduce lung burden of *Mtb* by nearly 2 logs at the highest dose, compared to sub-cutaneous BCG (Aguilo et al. 2014). This was associated with a much higher frequency of TB-specific CD4+ T-cells in lung homogenate, although the responding cells were not phenotyped for Trm markers. Copland and colleagues sought to enhance Trm formation by boosting BCG vaccinated mice with intranasal application of *Bacillus subtilis* spores coated with TB antigens (Copland et al. 2018). This approach improved protection over BCG vaccination alone and was associated with an expansion of CD69+CD103+ Trm cell type in the lungs that was not seen with either BCG alone or uncoated spores. Boosting BCG vaccinated mice via intranasal or intratracheal administration of nanoparticles coated with *Mtb* antigens was also found to induce CD69+CD103+ TB-specific lung Trm compared to subcutaneous BCG alone or uncoated nanoparticle (Hart et al. 2018). In both studies, the

vaccine induced expansion of lung Trms was associated with improved protection from subsequent TB challenge. However, in neither case was the activity of these cells formally tested by adoptive transfer or other approaches. Intranasal boosting with a Sendai virus vectored TB vaccine, SeV85AB, was also found to elicit antigen-specific CD103+ CD4+ and CD8+ T-cell responses in the lung and was associated with a reduction of mycobacterial load in lung and spleen compared to subcutaneous BCG alone (Hu et al. 2017). Interestingly, in this case protection was exclusively mediated by CD8+ T-cells as protection was lost when these cells were depleted, which was not the case in the BCG vaccinated group.

Perdomo and colleagues found that both intratracheal and intranasal administration of BCG enhanced the generation of both CD4+ and CD8+ CD69+ Trms, that expressed high levels of CD103 and CXCR3, and improved protection against subsequent *Mtb* challenge, compared to subcutaneous BCG (Perdomo et al. 2016). These authors went a step further, by adoptively transferring airway T-cells, via intratracheal injection, into naïve mice, demonstrating they can mediate protection compared to control mice receiving only PBS. However, these finding are complicated by the fact that transferred cells were derived from BAL fluid rather than lung parenchyma. The protection offered by cells with a CD69+ Trm phenotype, was not different from those lacking this marker. In addition, the authors did not test T-cells from either unvaccinated or subcutaneous BCG vaccinated mice by adoptive transfer to demonstrate any superiority of Trm generated by airway vaccination. Novel vaccine candidates have also been used in the mouse model to generate lung homing T-cells. Using a prime boost vaccination approach, Woodworth et al observed that subcutaneous vaccination (three times) with a cationic adjuvanted protein-based TB vaccine (H56/CAF01) generated circulating CD4+ T cells with accelerated lung-homing properties post-*Mtb* exposure (Woodworth et al. 2017). In this study, H56 vaccinated mice had low mycobacterial burden in the lungs, and TB-specific CD4+ T-cells in the lung were multifunctional.

All the above studies were done in mice, which, although highly informative, lacks many of the features of human pulmonary TB infection, including the generation of caseating lung granuloma. Non-Human Primate (NHP) models are generally considered to more closely reflect the pathology of human TB but studying Trm in these animals comes with additional challenges. Intravenous injection of BCG in NHPs has been shown by several groups to provide superior protection to standard intradermal inoculation (Sharpe et al. 2016), and surprisingly, vaccination by this route can boost lung Trm responses (Kaveh et al. 2011).

Others have used mucosal vaccination with BCG or subunit vaccines to significantly boost TB-specific lung T-cell responses (White et al. 2015; Darrah et al. 2014) with better disease outcome following *Mtb* challenge although this was due more to a reduction in lung damage than an effect on mycobacterial burden (Wong et al. 2016). Kaushal and colleagues examined the effect of mucosal vaccination with BCG or the attenuated SigH *Mtb* mutant vaccine strain (Kaushal et al. 2015). Interestingly, in their hands BCG had little impact on the lung T-cell response and only offered minor protection. However, the SigH mutant greatly boosted lung T-cell responses and induced the formation of inducible bronchioalveolar lymphoid tissue (iBALT) thought to be protective in TB infection (Ulrichs et al. 2004; Khader et al. 2011). Importantly, this was associated not only with reduced lung pathology, but a significant reduction in lung mycobacterial burden and survival of all animals from an otherwise lethal infection (Kaushal et al. 2015). More recently, mucosal vaccination with BCG, but not intradermal, was found to protect NHP from repeated low does exposure through the induction of IL-17 producing T-cell response in the airway (Sharpe et al. 2016). Taken together these studies in mice and NHP suggest that TB vaccination strategies that induces lung or airway Trm are significantly more protective than those that do not.

As mentioned, one of the key challenges in these experiments is distinguishing parenchymal Trm cells from contaminating PBMCs in lung homogenate (Schenkel and Masopust 2014). Sakai et al. directly addressed this issue when they used intravenous infusion of fluorescently labelled anti-CD45 antibody immediately prior to removing the lungs, which labels blood leucocytes but the label does not have time to penetrate tissue (Sakai et al. 2014). This system was then used to study the activity of lung resident T-cells by aerosol infecting mice with *Mtb*, removing their lungs at 30 days and FACs sorting the parenchymal and blood T-cells from lung homogenate based on the presence of anti-CD45 fluorescent antibody. Adoptive transfer of these cells, to naïve, TCR$\alpha^{-/-}$ transgenic mice, prior to *Mtb* infection, demonstrated parenchymal T-cells, which expressed the Trm CXCR3+KLRG1- phenotype, had far greater activity than circulating T-cells (18 fold vs 4 fold reduction in lung bacterial burden) (Sakai et al. 2014). This observation was confirmed in a follow-up study from the same group, where it was found that IFN-gamma production by CXCR3+KLRG1- lung T-cells was not a major component of this protective effect (Sakai et al. 2016). Indeed, expression of the inhibitory T-cell surface molecule PD-1 was critical to limit IFN-gamma production and prevent lethal lung pathology. This highlights the fact that Trm activity, though associated with a protective effect, can have pathological consequences when not

regulated. Parallel observations were made by Moguche and colleagues, who found that TB specific CXCR3+KLRGI-PD1+ CD4+ T-cells preferentially homed to the lung parenchyma and rapidly expanded on antigen re-encounter (Moguche et al. 2015). Adoptive transfer of this subset into TCR KO mice resulted in 10-fold greater reduction in lung CFU compared to KLRG1+PD1- CD4+ T-cells, supporting the superior antimycobacterial activity of the Trm-like subset. Importantly, the authors also demonstrated this subset had characteristics of long-lived memory cells, as adoptively transferred KLRG1-PD1+ CD4+ T-cells persisted in lung parenchyma in the absence of antigen stimulation, whilst the KLRG1+ subset did not. In this study, the authors noted intriguing similarities between the protective Trm-like subsets and Follicular Helper T-cells (Tfh), which both appear to rely on the transcription factor BCL6. Torrado et al. also found KLRG1-PD1+ T-cell preferentially homed to the lung parenchyma and provided superior protection from *Mtb* infection when adoptively transferred to TCR KO mice (Torrado et al. 2015) and found the protective Trm subset was preferentially generated by knocking out the IL27 receptor, implicating this signaling pathway in inhibiting Trm development. Both studies also highlight an important role for IL-2 in maintaining protective lung Trm. Although adopting differing approaches, these studies all provide direct experimental evidence of the importance of T-cells that readily enter the lung parenchyma and establish persistent memory populations in controlling *Mtb* infection. Importantly, taken together these data also demonstrate two key factors. First, that non-protective T-cells phenotypes that do not migrate into the lungs are generated by TB infection, and possibly by vaccination. Secondly, that vaccine strategy can affect the proportion of Trms cells in the lung. This provides both a potential reason for why circulating TB specific T-cells do not generally correlate with immune protection and hope that human TB vaccine efficacy may be improved through novel vaccination strategies.

As highlighted above, the generation of lung Trms by vaccination depends on a number of factors. TB specific Trms can be generated by initial mucosal vaccine administration (Hart et al. 2018; Perdomo et al. 2016; Kaushal et al. 2015b); as prime boost strategy to improve efficacy of BCG vaccine (Z. Hu et al. 2017; Copland et al. 2018; Hart et al. 2018) or multiple boosts subcutaneously (Woodworth et al. 2017). Furthermore, the vaccine vector can also influence the type of T-cells (CD4+ or CD8+) that will confer the greatest protective immunity (Hart et al. 2018; Copland et al. 2018; Z. Hu et al. 2017). The role of Trm in TB protection still needs further experimental confirmation, particularly in NHPs, but induction

of this subset has the potential to improve early control of infection and maybe even provide sterilizing immunity.

1.8 Impact of HIV coinfection on T-cells during TB disease

Although a substantially increased risk of TB and its extra-pulmonary dissemination is strongly correlated with CD4+ T-cell depletion in individuals infected with HIV-1 (Bell and Noursadeghi 2018), the precise mechanisms involved are less clear, particularly at the primary site of TB disease - the lung. T-cell depletion is evident in peripheral blood, in the respiratory tract and at the site of tuberculin skin test (TST) challenge (Bell et al. 2016; Kalsdorf et al. 2009; Geldmacher et al. 2010), although absolute cell counts can be preserved in well controlled HIV infection (Bunjun et al. 2017). End stage HIV is associated with severely reduced levels of CD4+ T-cells that are a primary source of IFN-γ critical in the control of *Mtb* infection in mice, and likely in humans (Cooper et al. 1993; Flynn et al. 2015b). Importantly, *Mtb*-specific CD4+ T-cells are depleted from the periphery early after HIV infection in humans. Indeed, HIV may preferentially target *Mtb*-specific memory CD4+ T-cells (Geldmacher et al. 2010, 2008).

TB disease enhances CCR5+ expression and/or the frequency of CCR5+CD4+ T-cells in the absence and presence of HIV co-infection (Juffermans et al. 2001; Wolday et al. 2005; Kabue et al. 2013; Haridas et al. 2015), probably as a result of chronic immune activation, and these cells are highly susceptible to infection by HIV. A strong CD4+ T-cell response to *Mtb* might directly lead to enhanced HIV replication. In *in vitro* studies, a primary subtype AE HIV-1 isolate replicated to a greater extent in CD4+ T-cells from PPD-responsive donors compared to CD4+ T-cells from PPD-anergic donors after PPD stimulation (Ranjbar et al. 2004). Supporting this, higher viral loads are consistently detected in TB/HIV co-infected patients versus patients singly infected with HIV who exhibit similar levels of immunosuppression (Day et al. 2004; Barnabas et al. 2011; Haridas et al. 2015).

In comparison to uninfected controls, HIV-infected individuals showed contraction of *Mtb*-specific CD4+ T-cells co-expressing lineage transcription factors FoxP3 and T-bet upon *in vitro* proliferation, which was inversely associated with HIV viral load and plasma TNF concentrations (Riou et al. 2016). HIV infection was also associated with a contraction of *Mtb*-specific CD4+ T-cells expressing the Th1 phenotype CXCR3+CCR5+CCR4-, a

major subset contributing to *Mtb*-specific responses in HIV-uninfected individuals with asymptomatic *Mtb* infection (Strickland et al. 2017). From these studies, it is suggestive that T-cell polarization and differentiation of *Mtb*-specific T-cells are negatively affected by HIV infection and the associated pro-inflammatory milieu.

Other T-cell effector functions, long-term memory capacity and tissue-homing potential may also be important in protection against TB and can be affected by HIV co-infection (Nemes, Scriba, and Hatherill 2018). HIV infection was associated with increased *in vivo* turnover of *Mtb*-specific CD4+ T-cells, *Mtb* antigen-induced cell death *ex vivo* and decreased *in vitro* proliferation (Day et al. 2017). *Mtb*-specific CD8+ T-cell *in vitro* proliferation as well as cytotoxic potential are also affected negatively by HIV coinfection (Kalokhe et al. 2015). Thus, these observations suggest that HIV infection may affect long-term memory potential of *Mtb*-specific T-cells.

HIV-1-infected T-cells may also contribute to the increased risk of TB in early HIV-1 disease before substantial depletion of peripheral blood CD4+ T-cell counts (Geldmacher et al. 2010). HIV-1 DNA was detected more frequently in *Mtb*-specific T-cells. These T-cells produced high levels of IL-2, which made them more permissive to HIV-1 infection. In comparison to the total memory T-cell population or memory T-cells that specifically recognize human cytomegalovirus, *Mtb*-specific T-cells were preferentially depleted in early HIV-1 infection (Bell et al. 2016). These data suggest that the depletion of these cells is a direct result of HIV-1 infection. However, the functional relevance of the reduced T-cell recruitment was not clear, as comparable levels of IFN-γ inducible gene expression were found in the groups with and without HIV-1. Thus, IFN-γ activity as a surrogate of robust CD4+ T-cell responses to mycobacterial antigens was preserved in early HIV-1 disease, despite previous reports of preferential depletion of *Mtb*-specific T-cells. Therefore, these data suggest that the increased risk of TB in patients infected with HIV-1 is not solely mediated by T-cell depletion, as has been reported in literature (Nemes, Scriba, and Hatherill 2018; Singh et al. 2016; Moir and Fauci 2009).

Increased immunosuppression in HIV positive patients with LTBI infection is associated with a loss of polyfunctional *Mtb*-specific CD4+ T-cell responses, which were restored following ART (Sutherland et al. 2010). BAL fluid from HIV positive patients with LTBI showed a reduced *Mtb*-specific polyfunctional as well as bi- and mono-functional CD4+ T-cell

frequencies compared to those from patients without HIV (Kalsdorf et al. 2009; Jambo et al. 2011).

As $CD4^+$ T-cell protection against *Mtb* is conferred by the pro-inflammatory cytokines that they produce, the proportions of polyfunctional *Mtb*-reactive T-cells, which produce the pro-inflammatory cytokines IFN-γ, TNF and IL-2, are also depleted in individuals infected with HIV-1 (Kalsdorf et al. 2009). Thus, HIV-1 depletes T-cell populations that are likely to be functionally important for protection against TB. Viral load in HIV patients with LTBI inversely correlated with *Mtb* - specific IL-2-expressing CD4+ T-cells that were positive for TNF-α or IFN-γ, while the *Mtb*-specific CD8+ T-cell population did not have inverse correlation with viral load (Day et al. 2008). This data suggests preferential depletion or impaired function of IL-2–secreting *Mtb*-specific CD4+ T-cells under conditions of high levels of HIV-1 viremia. Transcriptional profiling of biopsy samples taken from the site of the TST challenge in humans confirmed that T-cell recruitment and IFN-γ activity were both substantially reduced in patients co-infected with HIV-1 and TB, with blood $CD4^+$ T-cell counts of <200 or >200 per mL, indicative of advanced HIV-1 disease (Bell et al. 2016).

A functional role for Th17 and Th22 T-cell populations in protection against TB, primarily in mouse models is based on their role in the recruitment of phagocytic cells including macrophages (Cruz et al. 2010; Nandi and Behar 2011; Treerat et al. 2017). The depletion or impairment of these T-cell subtypes during primary HIV-1 infection (Brenchley et al. 2008; Kim et al. 2012; Murray et al. 2018b) may therefore contribute to differences in the immune response to *Mtb* in patients co-infected with HIV-1 compared with patients not infected with HIV-1 (Bell and Noursadeghi 2018).

MAIT-cells are also depleted in HIV-1 infection (Saeidi et al. 2016). MAIT-cells are activated by *Mtb* and are enriched at the site of TB disease (Jiang et al. 2014). Therefore, their depletion in HIV-1 infection may attenuate a component of host immune responses to *Mtb*. MAIT-cells are not infected by HIV-1 and their depletion is thought to be caused indirectly by immune activation (Bell and Noursadeghi 2018). Compared to the general population, the risk of active TB remains higher in patients infected with HIV-1 even after becoming established on effective ART (Gupta et al. 2012). Thus, the failure of ART to

restore the T-cell repertoire, including MAIT-cells (Heather et al. 2015; Cosgrove et al. 2013), may also be an important factor in the persistently elevated risk of TB.

1.9 Role of B-cells in TB disease

While the initial aim of the project was to look at T-cell immunity to TB in the lung, I observed (in different assays) that granulomatous TB lung was unexpectedly rich in B-cells, and the frequency was enriched in lung tissue compared to blood. Thus, I also sought to investigate the role played by B-cells in TB infected human lung. Whereas multiple lines of evidence support the importance of cellular immunity, the role of humoral immunity in TB is unclear (Kozakiewicz et al. 2013; Achkar and Casadevall 2013). For example, active TB has variously been associated with no changes in circulating B-cell frequencies (Barcelos et al. 2006), an increase (Wu et al. 2009) or a decrease (Corominas et al. 2004; Hernandez et al. 2010) compared to healthy controls. Also compared to healthy donors, LTBI individuals are reported to have decreased B-cell frequencies (Corominas et al. 2004), whereas those successfully treated for TB had increased B-cell frequencies (Barcelos et al. 2006). Furthermore, patients with MDR TB were found to have decreased frequencies of unswitched, IgD+CD27+ B-cells and decreased plasma cell frequencies, which are frequently observed during chronic inflammation (Abreu et al. 2014).

Although B-cell and antibody deficiencies are not risk factors for human TB disease (Glatman-Freedman and Casadevall 1998), mice lacking B-cells or the ability to secrete antibodies are more susceptible to infection (Maglione, Xu, and Chan 2007; Torrado et al. 2013; Vordermeier et al. 1996), and depletion of B-cells in non-human primates (NHPs) resulted in increased lesional bacterial burden (Phuah et al. 2016). Elevated Ag85Aspecific IgG titers was identified as a correlate of lower risk of TB disease in a post hoc analysis of the MVA85A vaccine trial (Fletcher et al. 2016) and Fc-mediated antibody effector function in *Mtb* control was recently reported (Lu et al. 2016) suggesting a possible role for antibodies in *Mtb* protective immunity.

B-cells can act as professional antigen presenting cells, provide co-stimulatory signals, produce cytokines and can exert immunoregulatory properties (Joosten et al. 2016). Antibodies can exert their functions in mycobacterial infection in two ways: by the direct blocking of host cell invasion and neutralization of bacterial products, or indirectly through

Fc-mediated complement and cell activation mechanisms through Fc receptors (Ravetch and Clynes 1998). Antibodies, plasma cells, and antibody-responsive innate immune cells bearing Fc receptors (FcR) have been reported in animal model TB granulomas (Phuah et al. 2012; Tsai et al. 2006), suggesting that they may play a role in the antimicrobial response.

Fc-mediated antibody functions have been reported in tuberculosis (Maglione et al. 2008; Sutherland et al. 2014; Machado et al. 2013) and other infections such as influenza (DiLillo et al. 2014), HIV (Bournazos et al. 2014; Hessell et al. 2007), Ebola (Olinger et al. 2012; Schmaljohn and Lewis 2016), and malaria (Bergmann-Leitner et al. 2006; Pleass 2009). These Fc effector functions are regulated immunologically via two features of the antibody Fc domain: (1) through Fc class-switch recombination selecting different isotypes (i.e., immunoglobulin G [IgG], IgM, IgA, IgD, and IgE) and/or subclasses (e.g., IgG1, 2, 3, 4) and (2) the posttranslational addition of distinct glycan species on the Fc domain of antibodies, specifically at asparagine 297 on IgG (Vidarsson, Dekkers, and Rispens 2014; Lu et al. 2016). Antibody glycosylation varies with age, sex, disease state, treatment, infection, and vaccination, which likely reflect the highly sensitive and dynamic processes that actively alter antibody effector function during an inflammatory response (Ackerman et al. 2013; Gardinassi et al. 2014; Mahan et al. 2016; Parekh et al. 1989), like tuberculosis.

Recent data have shown that some individuals exposed to *Mtb* with evidence of LTBI are able to mount protective antibody responses to prevent additional active infection (Lu et al. 2016; Li et al. 2017). Plasmablast antibody response to *Mtb* infection in humans generates functionally active antibodies and anti-TB B-cell response spreads from the lung to the periphery independently of active TB disease in humans (Zimmermann et al. 2016). Furthermore, general B-cell function is impaired during active TB and LTBI and this B-cell dysfunction compromises cellular host immunity during *Mtb* infection (Joosten et al. 2016).

Mtb-infected B-cell-deficient mice lungs display exacerbated inflammation, with enhanced neutrophil recruitment (Maglione, Xu, and Chan 2007; Kozakiewicz et al. 2013), which is associated with excessive lung pathology and with poor bacillary control in genetically susceptible mice (Eruslanov et al. 2005; Keller et al. 2006). B-cells and the humoral immune response modulate T-cell immunity including the development of memory T-cell responses during infection (Lund and Randall 2010; Maglione and Chan 2009) and vaccine-induced protection against secondary challenge with other intracellular pathogens such as Chlamydia

(Igietseme et al. 2004) and Francisella (Rawool et al. 2008). B-cell-depletion with rituximab treatment revealed that B-cells may enhance or diminish the IL-17/Th17 response (Hamel et al. 2008; Matsushita et al. 2008). Experimental evidence suggests that humoral immunity plays a role in regulating the Th1 response in TB (Maglione et al. 2008; Kozakiewicz et al. 2013) and a subset of B-cells in the blood of humans with tuberculosis infection can suppress Th17 response (Zhang et al. 2012; Kozakiewicz et al. 2013). The inflammation regulatory role of B-cells during *Mtb* infection can be strain and infection phase specific (Maglione, Xu, and Chan 2007; Bosio, Gardner, and Elkins 2000; Kozakiewicz et al. 2013).

At the site of TB disease, B-cells are a conspicuous cellular component of the lung granulomatous response in tuberculous mice (Slight et al. 2013; Tsai et al. 2006; Maglione, Xu, and Chan 2007; Gonzalez-Juarrero et al. 2001; Turner et al. 2001; Khader et al. 2009), nonhuman primates (Slight et al. 2013; Phuah et al. 2012), and humans (Slight et al. 2013; Tsai et al. 2006; Ulrichs et al. 2004, 2005). B-cells form prominent aggregates with characteristic features of the germinal center (Slight et al. 2013; Maglione, Xu, and Chan 2007; Khader et al. 2009; Ulrichs et al. 2004, 2005). However, the mechanistic involvement of these cells in the granulomatous response remains unclear, as does much of the detail. For example, whilst *Mtb* specific antibodies are readily detected in blood (Li et al. 2017; Lu et al. 2016), the specificity of granuloma associated B-cells, in humans at least, is completely unknown. Overall, it is clear that there is a B-cell response to human TB infection, however, more work is needed to better understand the nature of this response and what role it may play in TB immunopathogensis.

1.10 Hypothesis

In this study, it is hypothesized that adaptive immune responses to *Mtb* infection at the site of disease are different from the immune responses in circulation and these responses at the site of infection determine the outcome of TB disease.

1.11 Study aims and objectives

Most of what is known on human immune responses to *Mtb* infection has been learnt from studies done in blood, yet TB is primarily a disease of the lung tissue. Furthermore, these studies have failed to provide correlates of protection in TB disease to date. A cohort of study participants undergoing medical lung resection for tuberculosis related lung pathologies was

established to learn the phenotype, function and TB specificity of adaptive immune responses at the site of TB disease and to understand how they correlate with the responses detectable in circulation. Specifically, the objectives of the study were:

1. To characterize the phenotype of T and B-cells in human lung tissue during TB disease and compare with those in systemic circulation.

2. To determine the functionality of lung tissue resident memory T-cells in active TB disease and previous treated TB cases.

3. To determine specificity of and magnitude of response of TB specific lung T-cells during TB disease and compare with systemic responses.

4. To characterize the phenotype of B-cells and evaluate B-cells function in TB infected human lung tissue.

5. To determine the impact of HIV co-infection on the feature of lymphocytes within the lung.

CHAPTER 2: CHARACTERIZATION OF T-CELLS IN TUBERCULOSIS INFECTED HUMAN LUNG

2.1 Introduction

The literature review provided in chapter 1 has identified the research gaps that exist in understanding how the human body responds to *Mtb* infection especially in the lung, the primary site of disease. There are very limited human studies that look at tissue resident T-cell responses to *Mtb* in the lung tissue itself. Where attempts have been made to study *Mtb* at the primary site of infection, such studies have been limited to studying the alveolar space by analysing bronchoalveolar lavage (BAL) fluid. Thus, a cohort of study participants undergoing medically indicated lung resections for TB related pathologies was set up at AHRI to investigate the T-cell immune responses to *Mtb* in fresh lung tissue and compare these findings to responses measured in peripheral blood.

T-lymphocytes have been shown to be critical for the prevention of primary disease on initial *Mtb* infection, and the development of post primary TB once latent infection has been established (Torrado and Cooper 2010; Cooper and Flynn 1995; Flynn 2004; Flynn and Chan 2005; O'Garra et al. 2013). However, in humans, responses are generally measured in blood so very little is known about T-cell function in lung tissue, the site of infection (Sathaliyawala et al. 2013; Gideon et al. 2015). Despite having plenty of data generated from studies on systemic immune responses, the correlates of protection in tuberculosis are still unknown. Many questions remain unanswered regarding the functional characteristics of T-cells in TB-infected human lung, including the extent to which systemic T-cell responses predict the T-cell responses in the lung. Much of what is known about T-cell function in tuberculous lungs comes from animals models, none of which develop the full spectrum of lung involvement seen in human tuberculosis (Gideon et al. 2015).

Due to the recruitment of activated T-cells to the site of disease, T-cell responses are thought to be enriched in the lung when compared to the systemic circulation (Gideon et al. 2015). These concepts are supported by studies from small animal models where whole lung homogenates were studied (Jafari et al. 2009; Jafari et al. 2008), and from studies using cells from pleural TB (Wilkinson et al. 2005) or bronchoalveolar lavage (BAL) from active TB patients (Brighenti and Andersson 2012; Forbes et al. 2008) and non-human primates (Fuller,

Flynn, and Reinhart 2003). Overall, animal studies have shown that responses to TB measured in circulation do not exactly mirror the responses at the site of the disease (Gideon et al. 2015; Gordon et al. 2017).

After initial encounter with an infection, non-recirculating memory T-cells, here referred as tissue resident memory T-cells (Trms), remain positioned at common portals of re-infection, including barrier tissues such as the mucosae and skin (Gebhardt et al. 2009; Skon et al. 2013; Masopust et al. 2010; Wakim et al. 2013; Hofmann and Pircher 2011; Jiang et al. 2012). Due to their position, Trms can confer quick response against homologous re-infections (Teijaro et al. 2011; Shin and Iwasaki 2012; Mackay et al. 2012; Hogan et al. 2001; T. Wu et al. 2014b). Mucosal vaccination against TB confers superior protection compared to parenteral vaccination (Connor et al. 2010; Perdomo et al. 2016) suggesting existence of tissue specific compartmentalization of response to TB following vaccination, and that these responses can be protective. This is expected as other respiratory viral infections are known to induce resident memory T-cells that persist in the lung tissue and the lung airways following infection clearance (Hogan et al. 2001; Teijaro et al. 2011; Turner et al. 2014). Understanding the biology of TB specific T-cells will support efforts to design optimal immunological interventions. Here, a detailed study of lung Trms in participants with TB related lung pathologies was performed to characterize T-cell immunity to TB at the site of disease.

2.2 Materials and methods

2.2.1 Participants

TB infected lung tissue samples and blood were obtained from participants undergoing surgical resections due to severe lung complications, including haemoptysis, bronchiectasis, shrunken or collapsed lung or non-responsive infection, at the King Dinuzulu hospital in Durban, KwaZulu-Natal and Inkosi Albert Luthuli Central Hospital (IALCH) in Durban, KwaZulu-Natal. TB negative control samples were obtained as healthy tissue margins from lung cancer resections or inflammatory lung disease. All participants provided written informed consent and the study obtained ethical approval from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal, the Department of health and the hospital management. All assays were conducted with approval of the AHRI biosafety committee. Detailed demographics data of the study participants is provided in table 2. TB

and HIV infection status was confirmed by a number methods including self-reporting (for HIV) by participants at enrolment followed by a confirmatory rapid test on arrival of samples in the laboratory; chest X-ray results analysed by dedicated cardio-thoracic surgeons and pathologist, in addition clinical history data documented on individual's previous TB episodes and anti-TB medication administered. Furthermore, histology reports of lung tissue collected on the day of resection together with Gene X-pert and/or microbiology culture results was provided for the participants.

2.2.2 PBMC isolation

Blood was collected in BD vacutainer (Sodium heparin) tubes (BD, Plymouth, UK) and transported to the laboratory and processed upon arrival. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using the Ficoll-Histopaque (Sigma) density gradient centrifugation and used fresh in assays or frozen in liquid nitrogen in freezing media (90% FBS containing 10% DMSO) for later use. Approximately 30ml of whole blood was layered onto 10ml of Ficoll-Histopaque and centrifuged for 20 minutes at 2000 rpm at 18^{o}C (with breaks off). The buffy coat was gently removed using a Pasteur pipette and transferred to a new 50ml centrifuge tube and topped up to 50ml with PBS and washed by spinning at 2000 rpm for 5 minutes at 18^{o}C. After washing step, the cell pellet dislodged by pipetting up and down then washed one more time with 30ml of PBS. Cells were resuspended in 10ml of PBS and 10µl mixed with 10µl of Trypan blue dye and cell count determined in an automated cell counter (BioRad). Cells were then used fresh or frozen until when needed.

2.2.3 Lung Processing

Lung tissue of approximately 3 cm^3 was isolated from different areas of resected lungs, corresponding to the most diseased (A), intermediate (B), and healthiest tissue (C). The operating surgeon classified the tissue based on their experience and the pre-operative radiological data. Each sample was washed in multiple changes of HBSS (BioWhittaker) and then diced into approximately 1 mm^3 pieces and re-suspended in 8mls of pre-warmed digestion media (R10 (RPMI supplemented with 10% FCS, 2 mM L-glutamate, 100 U/ml Penstrep), containing 0.5 mg/ml collagenase D (Roche) and 40 U/ml DNaseI (Roche), and transferred to GentleMACS C-tubes (Miltenyi) for mechanical digestion as per the manufacturer's instructions, using the lung disassociation programme. The suspension was incubated for 30 minutes at 37^{o} C, subjected to an additional mechanical digestion step

followed by another 30-minute incubation step at 37° C. The resulting suspension was strained through a 70 µ m cell strainer, washed twice in HBSS, and cell viability and count determined using Automatic cell counter (Bio-Rad).

2.2.4 Cell surface, Intracellular cytokine and transcription factor staining

Fresh lung cells and PMBCs or frozen PBMCs cells were plated in each well of a round bottom 96-well plate and incubated in the presence of MTB300 peptide (2ug/ml). MTB300 is an MHC class II restricted mega pool of 300 epitopes of *Mtb* proteins spanning both the cell wall and secretory proteins (Lindestam Arlehamn et al. 2013). Where frozen cells were used, the cells were thawed and washed with R10 containing 40 U/ml DNase I, then resuspended in R10 medium and rested in the incubator for at least four hours before start of stimulation. Incubation in the presence of mixture of PMA(0.5uL/200uL) and Ionomycin (0.3uL/200uL) or in the absence of stimuli were used as positive and negative controls, respectively. Cells were incubated for 1 h at 37° C, at which point Golgi Stop solution and Golgi Plug (BD Biosciences) was added to each well for the remaining 4 h. Cells were collected after the 5 h stimulation and then surface stained with the antibody panel (see appendix I), followed by intracellular cytokine staining for IFN-γ, TNF-α, IL-2 and IL-17 using BD Permwash Kit (BD Pharmingen, CA, USA) as per manufacturer's instructions. For measurement of transcription factors, the eBioscience Fixation/Permeabilization kit was used for intracellular staining of cells and blocked with 20% goat serum for 20 mins prior to antibody staining. Data acquisition was performed using Aria Fusion or Aria III cytometers (BD) and analyzed using FlowJo Software v.9.9 (Treestar Inc, Ashland, OR). In cases when stimulation was not performed, single cell suspensions from lung and PBMC were incubated with antibody mix for 20 minutes in the dark followed by washing twice with PBS before fixation in 2% PFA prior to acquisition on FACS Aria Fusion.

2.2.5 Plasma Cytokine Analysis

Plasma samples were collected from whole blood and frozen at –80°C for later use. Analyte levels were quantified using a multi-plex high-sensitivity Milliplex Map Kits (Millipore) on a Bio-Plex 200 system (Bio-Rad) using manufacturer's instructions. The panel included the following analytes: IFN-α2, IL-1β, IL-2, TNF-α, IL-12 (p70), IL-12 (p40), IL-6, IL-10, IL-4, IL-17a, IL-22, IL-21, TGF- β1 and IL-13.

2.2.6 Mass cytometry staining

Frozen lung cells and PBMC (isolated as outlined in 2.2.2 and 2.2.3) were thawed and washed with FACS buffer containing 40 U/ml DnaseI. Lung cells were counted and stained in 100µl containing APC-CD45 (1:20) in a 96-well round bottom plate and incubated on ice for 20 mins. 100µl of cold FACS buffer was added and the plate centrifuged for 1500rpm for 3 minutes at 4ºC, the supernatant was discarded, and the pellet resuspended in 100µl cold cyFACS buffer. Thawed PBMCs were counted in 1ml of cold cyFACS buffer and $3x10^6$ live cells resuspended in 100µl of cold cyFACS buffer. Both lung cells and PBMC were kept on ice while antibody cocktail was being prepared.

A calculated amount of cyFACS buffer was added into 0.1µm Ultrafree® centrifugal filter unit followed by appropriate volume of each antibody into the filter unit. The antibody cocktail was then centrifuged at 14000 rpm for 3 minutes at 4ºC. The flow through antibody cocktail was used to stain the cells. 100µl APC-CD45 positive lung cells was mixed with 100µl of PBMC from the same individual in a 96-well round bottom plate. The total number of cells stained varied between 3 – $6x10^6$. The mixed samples were then centrifuged at 1500rpm for 3 minutes at 4ºC and supernatant discarded. The cells were resuspended in 100µl of freshly diluted cisplatin (1:1500 in cyFACS buffer) and incubated on ice for 5 minutes. 100µl of cyFACS was added to the cells to quench the reaction then centrifuged at 1500rpm for 3 minutes at 4ºC and supernatant discarded. Cells were resuspended in 50µl of cyFACS buffer containing anti-human CD294 CRTH2 (diluted 1:10) and anti-human Yδ TCR (dilute 1:25) primary antibody. Cells were incubated at 37ºC for 15 minutes followed by addition of 150µl of cyFACS buffer and centrifuged at 1500rpm for 3 minutes at 4ºC.After discarding the supernatant, 200µl of cyFACS buffer was added followed by an additional washing step. Cells were resuspended in 50µl of antibody cocktail panel solution, incubated on ice for 30 minutes. After addition of 150µl of cyFACS buffer, cells were washed, supernatant discarded followed by another washing step in cyFACS buffer. Cells were further washed once in 200µl of PBS then resuspended in 150µl of freshy reconstituted 2% PFA. The plate was sealed with an adhesive sealing film and stored overnight at 4ºC. The plate was centrifuged at 1800 rpm for 3 minutes at 4ºC, supernatant discarded followed by one more wash in 200µl of cyFACS buffer. The cells were then resuspended in 150µl of cold freezing media and stored at -80ºC until later for CyTOF acquisition.

After thawing, cells were washed in 1x permeabilization buffer and each sample was incubated with 50µl of intracellular antibody in 1x permeabilization buffer for 30 min on ice. Cells were washed twice, and each sample was barcoded with a unique combination of two distinct palladium barcodes for 30 min on ice. After washing, cells were resuspended in 250 nM iridium intercalator (DNA staining) in 2% paraformaldehyde/PBS at RT. Cells were washed and adjusted to 0.5 million cells per ml H_2O together with 1% equilibration beads (EQ Four element calibration beads, Fluidigm) for acquisition on a CyTOF® Helios system.

2.2.6.1 CyTOF Data analysis

Signals for each parameter were normalized based on EQ beads added to each sample. Any zero values were randomized using a custom Rscript that uniformly distributes values between minus-one and zero. Each sample was manually de-barcoded followed by gating on DNA+ cells. Immune cells were identified by gating on live (cisplatin-) CD45+ cells and tissue and PBMCs from the same donor were further identified according to the tissue-specific live cell barcode tag (APC-CD45). Subset identification followed a traditional gating cascade according to the lineage markers using FlowJo (Tree Star Inc) software. High dimensional data analysis was performed using immunoSCAPE's cloud-based analytical pipeline tool Cytographer®. For the visualization of high dimensionality data, Uniform Manifold Approximation and Projection (UMAP) as dimensionality reduction technique (Becht et al, 2018) was used. Phenotypic dissection was performed using the PhenoGraph clustering algorithm (Levine et al. 2015). Marker expression intensities were represented as heatmaps and expression plots. Dot plots and UMAP plots were displayed using Flowjo.

2.2.7 Statistical analysis

All statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad Software, Inc.). Comparisons of two groups were done with a paired or unpaired student's t-test (Wilcoxon test or Mann-Whitney test respectively). Comparisons of more than two groups were done using two-way ANOVA. P- value of 0.05 and below were considered statistically significant. Where appropriate, correction for multiple comparison was carried out using the Bonferroni method.

2.3 Results

The characteristics of the study population is shown in table 2 below. Participants were defined as previous TB cases based on chest x-ray results and documented clinical history of previous TB episodes and when the individuals indicated they had finished their TB therapy months before the date of lung resection surgery. Histology reports were also used to define participants as either active TB or previous TB. Majority of the TB cases were drug susceptible although there were few cases of MDR TB and a single case of XDR TB. Main comorbidities included consolidation/nodules, Cavitary lung, interstitial lung disease, bronchiectasis and mycetoma. Over 90% of the participants coinfected were on ART treatment and those who could not remember their ART regimen were marked as 'unknown' although they indicated they were on antiretroviral medication. Approximately 70% of HIV infected participants had no detectable virus in plasma. Due to the difficulty of extracting single cell suspension from the lung tissue, particularly the most diseased samples, the data presented here are only from those samples in which % live cells was >50% of total lymphocytes. In addition lung tissue samples in which %CD45+ cells was less than 10% were not included in the analysis.

Table 2: Demographic information of the study participants

Characteristics of study participants	Active TB	Previous TB	Cancer Control
Characteristic	(n = 43)	(n = 26)	(n = 9)
Sex, No. (%)			
Female	14(32.6)	13(50)	5 (55.6)
Male	29(67.4)	13 (50)	4 (44.4)
Age (y), Median (range)			
All Subjects	40 (20 -66)	37 (20 - 65)	50 (27 - 63)
Female	40 (20 - 60)	35 (20 -53)	54 (27 - 63)
Male	41 (20 - 66)	38 (20 -65)	37 (32 - 59)
HIV, No. (%)			
Positive	29 (67.4)	13 (50)	0 (0)
CD4 Count; Mean (range)[μ]	484.4 (166 - 814)	300 (30 - 785)	
Viral Load[#]			
Suppressed, i.e, <40 copies/ml (No, (%))	20 (68.9)	10(76.9)	
Unsuppressed, i.e, >40 copies/ml; (No, (%))	670 - 15489; 9 (31)	450 - 1041; 3 (23)	
Antiretroviral Therapy regimen	Odiumune/Atrioza/Tribuss		
Negative	14 (32.6)	13 (50)	9 (100)
Other lung pathologies[ʒ], No. (%)			
Cavitary lung	22 (51.2)	4 (15.4)	3 (33)
Bronchiectasis	12 (27.9)	9 (34.6)	
Mycetoma	8 (18.6)	0	
Fibrosis	0	4 (15.4)	
Aspergilloma	0	4 (15.4)	
Interstitial lung disease	19 (44.2)	0	
Consolidation/Nodules	21 (48.8)	0	
Smoking history, No. (%)			
Smokers	10 (23)	4 (15.4)	
Non-smokers	33 (77)	22 (84.6)	
TB treatment outcomes, No. (%)			
Drug susceptible	35 (81.4)	26 (100)	
MDR TB	3 (6.9)		
XDR TB	5 (11.6)		
Treatment regimen	Combination therapy[Ω]		

Notes:
μ = CD 4 count unavailable for three HIV infected individuals (both active TB)
= Viral load value for two active TB not available
ʒ = Some individuals had more than one lung pathology
Ω = Combination of any two or three of these drugs:
Rifafour/Pyradoxine/Streptomycin/Rifinah/Tenzidone/Pyrazinamide/Isoniazid/Kanamycin/Moxifloxacin/ Clofazimine

2.3.1 Phenotype of T cells in TB infected human lung

2.3.1.1 Recruitment of T cells to the lung in TB cases is dysregulated by HIV co-infection

Due to the nature of lung homogenate it is not possible to perform a full blood count to obtain relevant HIV metrics such as CD4 count. Therefore, we determined the ratio of CD4:CD8 T-cells in fresh lung tissue of study participants, as skewing of the CD4:CD8 ratio in blood is a hallmark of HIV infection. Overall, and in non-TB controls, the CD4:CD8 in lung tissue appears to be lower than in the blood (approximately 1:1 vs 2:1 in the blood of healthy control), although all subjects examined had some existing lung condition (either active or previous TB or cancer), which may have impacted this ratio. None-the-less we observe a significantly lower CD4:CD8 ratio in the lung of HIV co-infected patients when compared to patients without HIV, regardless of whether they have active TB or had a previous TB infection (figure 1a). Interestingly, this difference is most striking in the subjects with pervious TB, perhaps exacerbated by reduced ongoing recruitment of T-cells to the lungs of these individuals. A similar trend was observed in the PBMC, although the differences between HIV+ and –ve subjects did not reach statistical significance (figure 1b). However, in comparison to healthy control PBMCs, there was a significantly low CD4:CD8 ratio in active TB and previous TB co-infected with HIV. All HIV positive subjects were on ART, and thus HIV co-infection leads to a significant skewing of the lung CD4:CD8 ratio that persists with antiretroviral therapy.

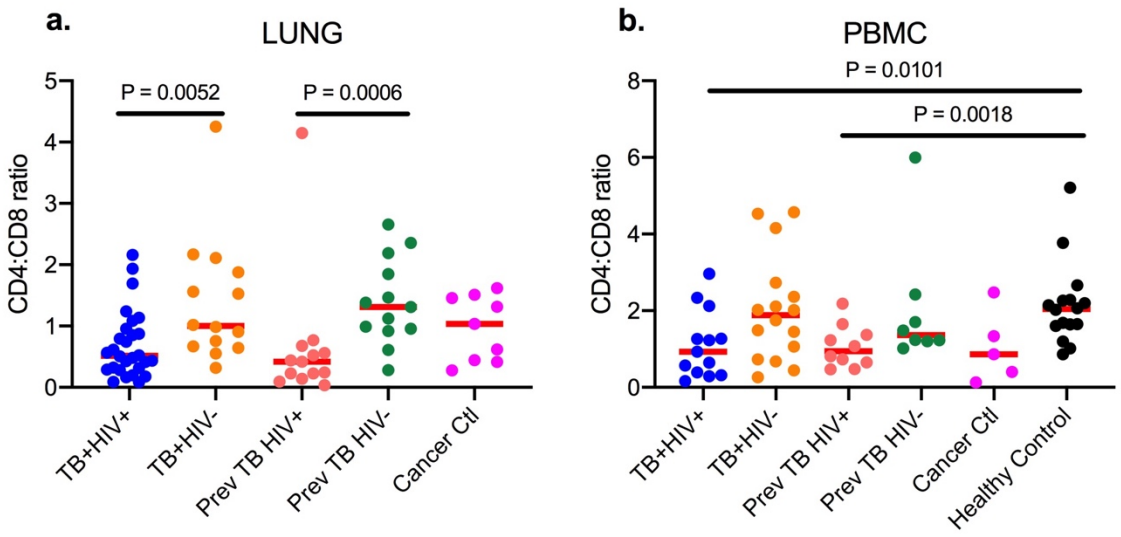


Figure 2.1: Ratio of CD4:CD8 T-cells in (a) lung and (b) PBMCs. HIV+ participants are on ART and control samples are participants with no history of TB and are HIV negative. Statistically significant non-parametric t-test (Mann-Whitney tests) p values are shown.

2.3.1.2 Identification of lung tissue resident memory T-cells

Human lung is a highly vascularized organ and cells isolated from the lung will always contain cells from the systemic circulation that were present in the lung at the time of tissue resection. In an attempt to distinguish non-recirculating cells in the lung tissue, we stained lung homogenate with three common markers for tissue resident T-cells, CD69, CD103 and CD62L. CD103 has been routinely used in studying virus specific responses in human lung tissue (Hombrink et al. 2016; Gordon et al. 2017) while CD69 has been reported in the murine study of tuberculosis (Sakai et al. 2014) and several other human studies (Sathaliyawala et al. 2013; Schenkel and Masopust 2014b; Steinert et al. 2015; Beura et al. 2018). It has also been reported that tissue resident cells down regulate their expression of CD62L in previous studies (Schenkel and Masopust 2014; Kløverpris et al. 2016) making it a suitable marker to distinguish T-cells in the two compartments. The utility of these markers is made clear by flow cytometry staining, which shows that T-cells within lung homogenate are highly enriched for CD69 and CD103, and downregulate the expression of CD62L in comparison to blood T-cells (figure 2.2a).

The proportion of cells within homogenate vary between subjects (figure 2.2b), which may reflect disease state or level of blood contamination. However, overall CD69 and CD103 expression was significantly higher ($P<0.0001$) in both CD4+ and CD8+ cells in the lung than PBMC (figure 2.2c). The median of lung CD4+CD69+ T-cells was 47.89% (1.58 – 69.2) versus 1% (0.07 – 8.9) in PBMCs; and lung CD8+CD69+ T-cells 49.9% (2.9 – 78.1) versus 2.1% (0.15 – 7.75). Lung CD4+ expressing CD103+ had a median frequency of 10.6% (0.17 – 40.3) versus 0.57% (0.07 – 8.78) in PBMC; lung CD8+CD103+ T-cells 21.94% (3.8 – 67.7) versus 1.6% (0.26 – 9.71). Conversely, the expression of CD62L was reduced in tissue compared to PBMC ($p = 0.0026$ for CD4+CD62L+; $p < 0.0001$ for CD8+CD62L+) (figure 2.2c). Lung CD4+CD62L+ median 25.5% (0.3 – 47.6) versus PBMC CD4+CD62L+ median 57.29% (8.45 – 84.84); Lung CD8+CD62L+ median 7.05% (0.04 – 27.05) versus PBMC CD8+CD62L+ median 26.85% (3.26 – 63.04). Consistent with the importance of CD69 expression in the biology of Trm, the majority of CD103 T-cells co-express this marker.

The similarity in expression levels of CD69 on CD4 and CD8 T-cells from lung homogenate is in line with the overall CD4:CD8 ratio of 1:1 reported above. There was a significant higher expression of CD103 in CD8+ T cells than in CD4+ T-cells in the lung (figure 2.2d).

This is expected, given previous reports of CD103 expression on lung T-cells (Sathaliyawala et al. 2013). However, it is clear that a CD103+ve CD4+ T-cell population is present in TB infected lung, which is important, as this subset in particular is thought to be the most biologically active during tuberculosis within this tissue (Yang et al. 2018). Finally, the presence of CD103+ and CD69+ T-cells in the lung was confirmed by immunohistochemistry staining of fixed lung tissue (figure 2.2e). Taken together, these markers appear to reliably identify T-cells in the lung that are not in circulation and were therefore used to distinguish these cells in further analyses.

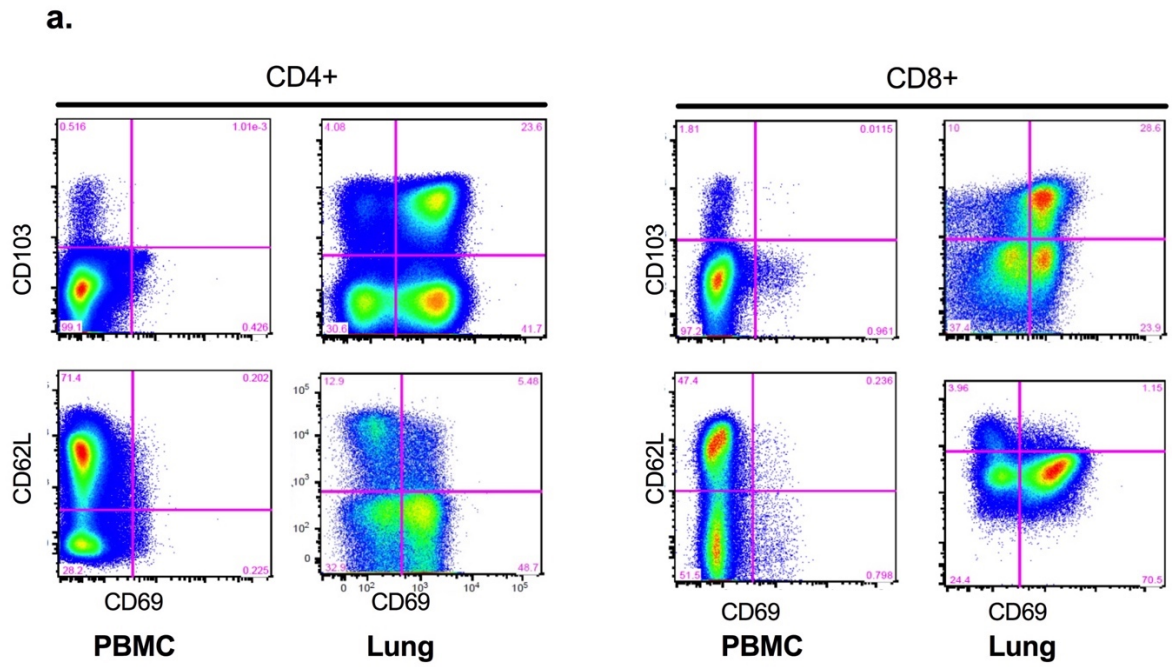


Figure 2.2: Identification of tissue resident T-cells. a) Representative flow cytometry plots showing staining of CD103, CD69 and CD62L on CD4+ and CD8+ T-cells in PBMC and lung tissue.

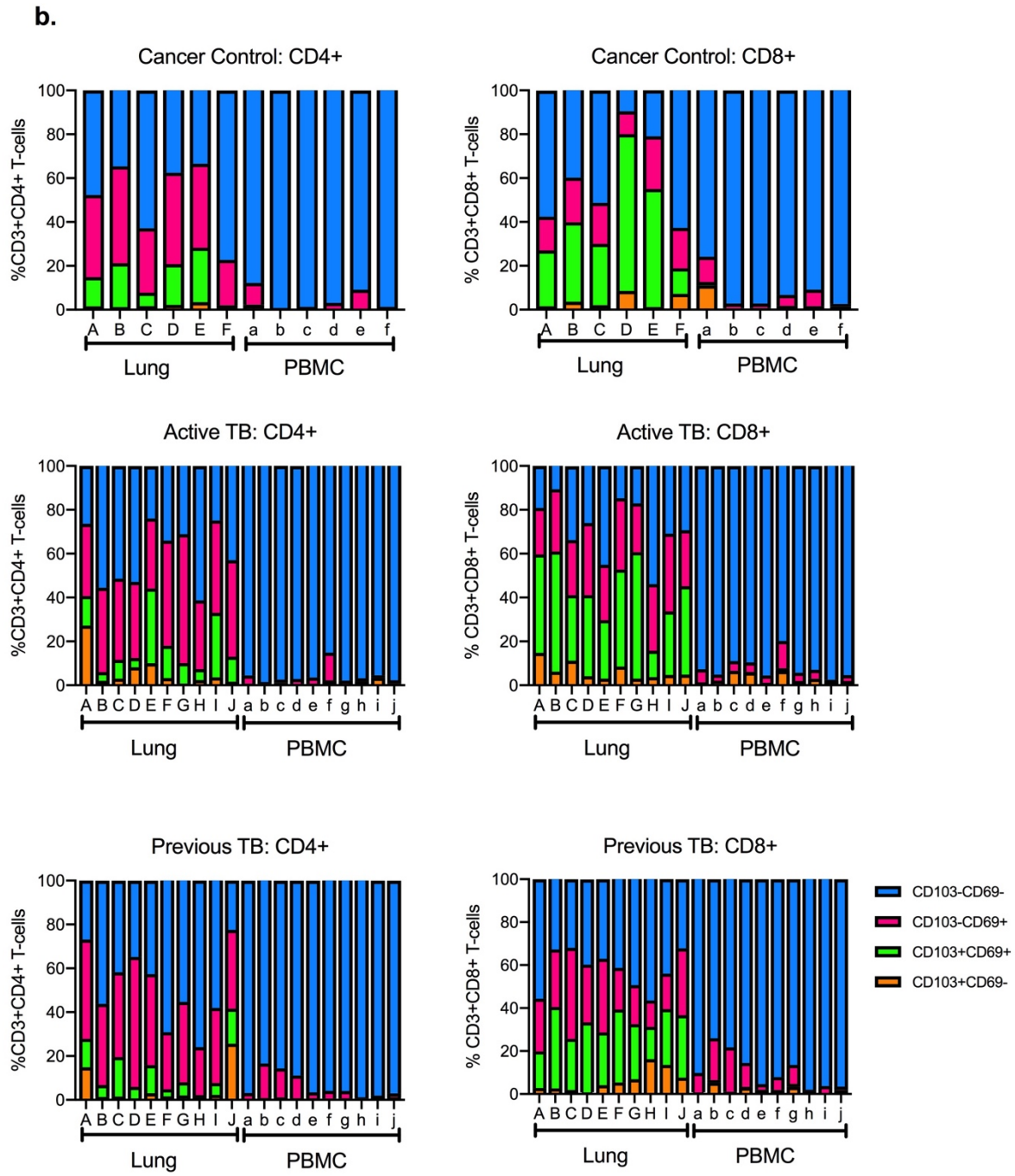


Figure 2.2: Identification of tissue resident T-cells. b) Stacked bar graphs of CD103 and CD69 co-staining of CD4+ and CD8+ from lung tissue and PBMC in cancer control, previous TB and active TB samples.

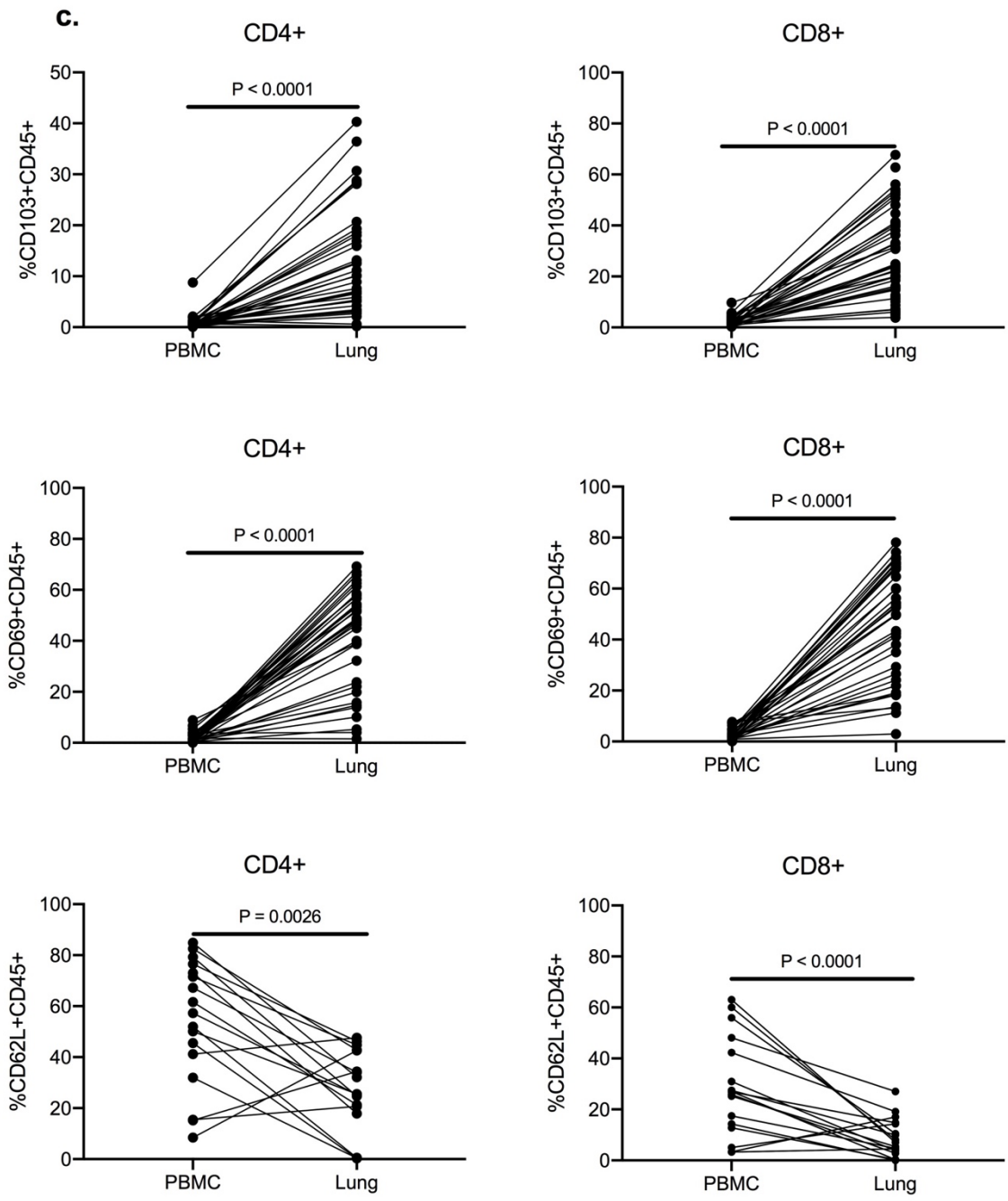


Figure 2.2: Identification of tissue resident T-cells. c) cumulative frequency of CD103, CD69 and CD62L in matched PBMC and lung tissue. Non-parametric T (Wilcoxon test) p values are shown.

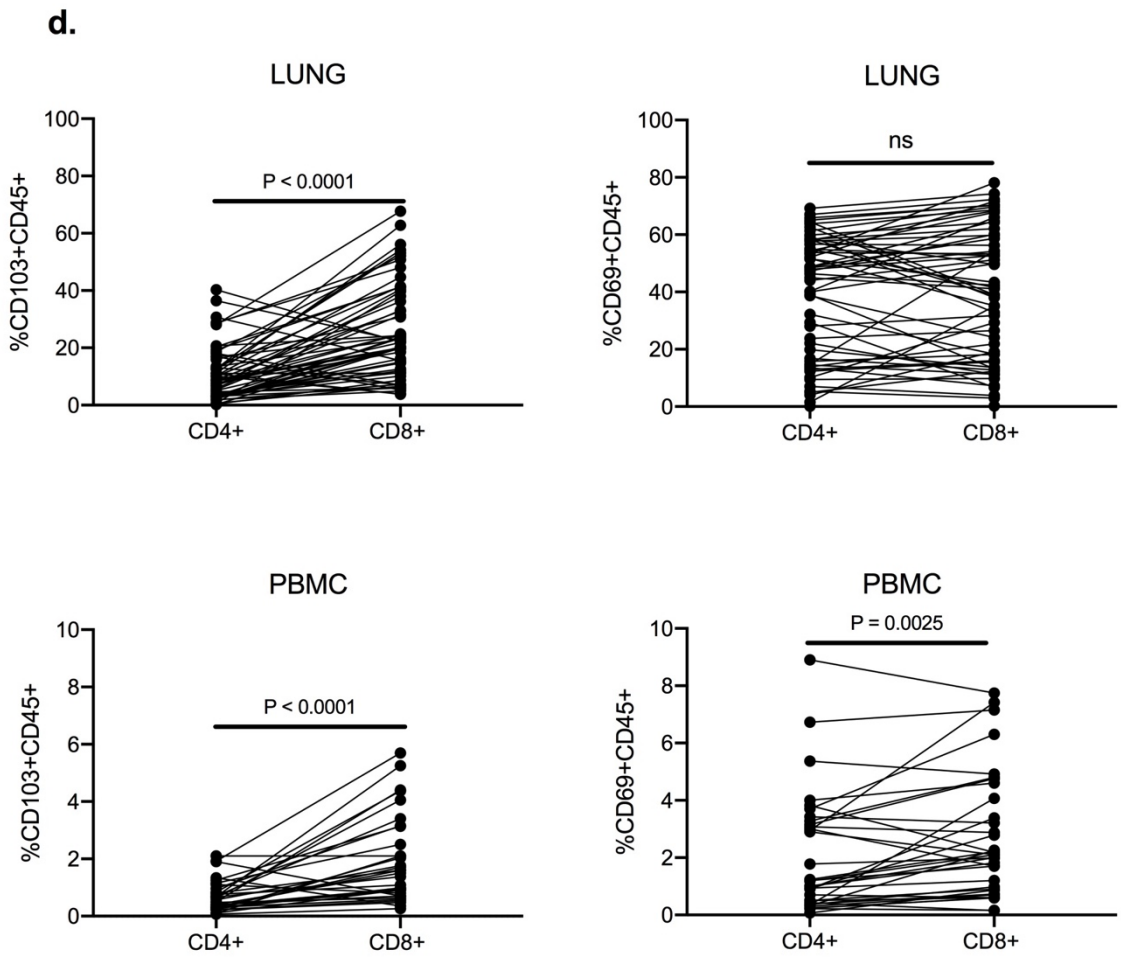


Figure 2.2: Identification of tissue resident T-cells. d) Frequency of CD103 and CD69 on lung and peripheral blood CD4+ and CD8+ T-cells during TB disease. Non-parametric T (Wilcoxon test) p values are shown.

e.

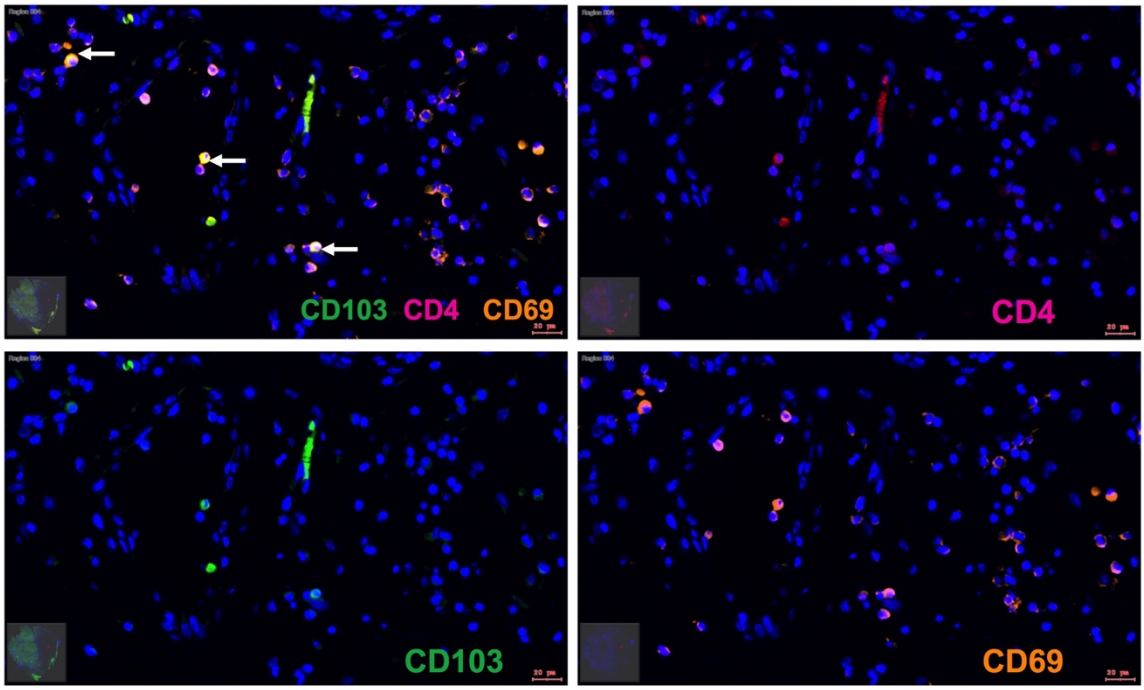


Figure 2.2: Identification of tissue resident T-cells. e) Immunohistochemistry staining of formalin fixed lung tissue, white arrows show cells staining for three markers (CD4, CD69 and CD103).

2.3.1.3 Tissue resident memory T-cells are expanded in tuberculosis infected human lung

We next examined whether active TB and/or HIV coinfection has an impact on the frequency of CD103+ and CD69+ T-cells within the lung. Samples were defined as active TB or previous TB as explained in sections 2.2.1 and 2.3 above. As stated, the majority of individuals (70%) in our lung cohort are HIV+ve, therefore we first sought to determine whether or not HIV+ and HIV- subjects could be analysed together. Overall, HIV co-infection appears to have no impact on the frequency of CD103+ve CD4 T-cells, irrespective of TB status (figure 2.3a). However, the frequency of CD8+CD103+ tended to be higher in HIV positive than HIV negative, although the difference did not reach statistical significance (figure 2.3a). CD69 expression, was consistently higher on both CD4 and CD8 T-cells in lung homogenate from HIV+ individuals, significantly in the previous TB group (figure 2.3a). Consequently, impact of on-going TB infection was analysed separately in regardless of HIV status (figure 2.3b). In HIV infected individuals, the frequency of CD103+ve and CD69 +ve CD4 and CD8 T-cells was consistently higher in those with active TB disease compared to those with previous TB (figure 2.3c), reaching significance for CD103+ve CD8 T-cells. The same trend was observed in HIV uninfected subjects, (figure 2.3a), with CD103 and CD69 expression being highest in subjects with active compared to those with previous TB, and those with no history of TB (cancer controls; all of whom are HIV-ve) (figure 2.3b). In contrast, there were no differences in the frequencies of CD103 and CD69 expression in PBMC of the participants with the different disease states (figure 2.3d). Taken together, these data suggest that Trm, as defined by the expression of CD103 and/or CD69 are expanded in the lung during active TB infection. As expected, this population declines following the resolution of disease, but a detectable population of both CD69+ and CD103+ CD4+ and CD8+ T-cells persist, consistent with a resident memory T-cell population. The impact of HIV co-infection is not entirely clear, however, it certainly does not deplete CD4+ve Trm, as might have been predicted. The apparent expansion of CD8+ Trms, although not consistent across all subsets, is in line with clinical descriptions of HIV caused CD8 T-cell alveolitis (Neff et al. 2015).

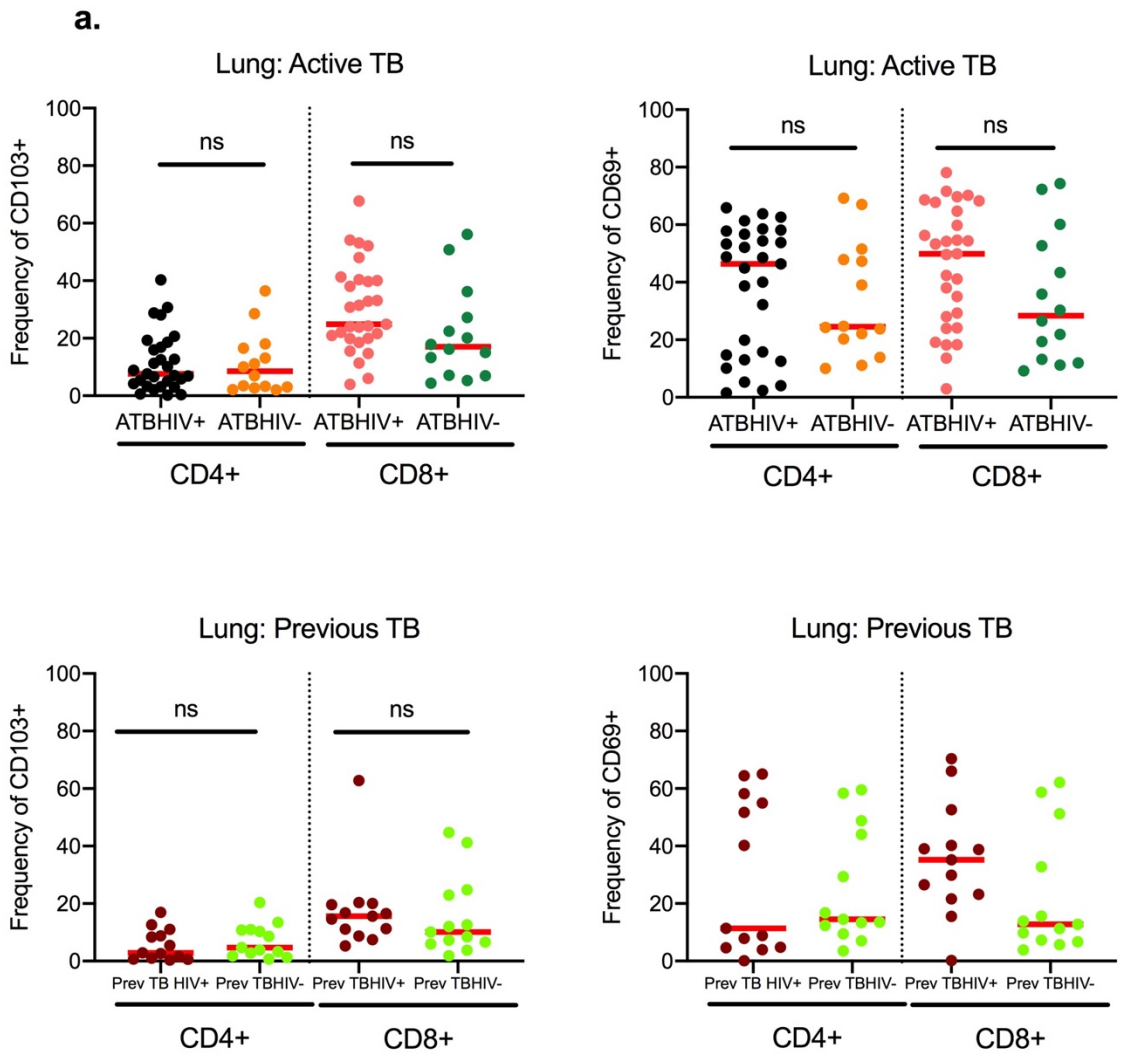


Figure 2.3: Lung tissue resident memory T-cells during TB disease (a) Frequency of CD103 and CD69 on lung CD4+ and CD8+ T-cells in active and previous TB with or without HIV. No significant differences observed (non-parametric Mann-Whitney t-test).

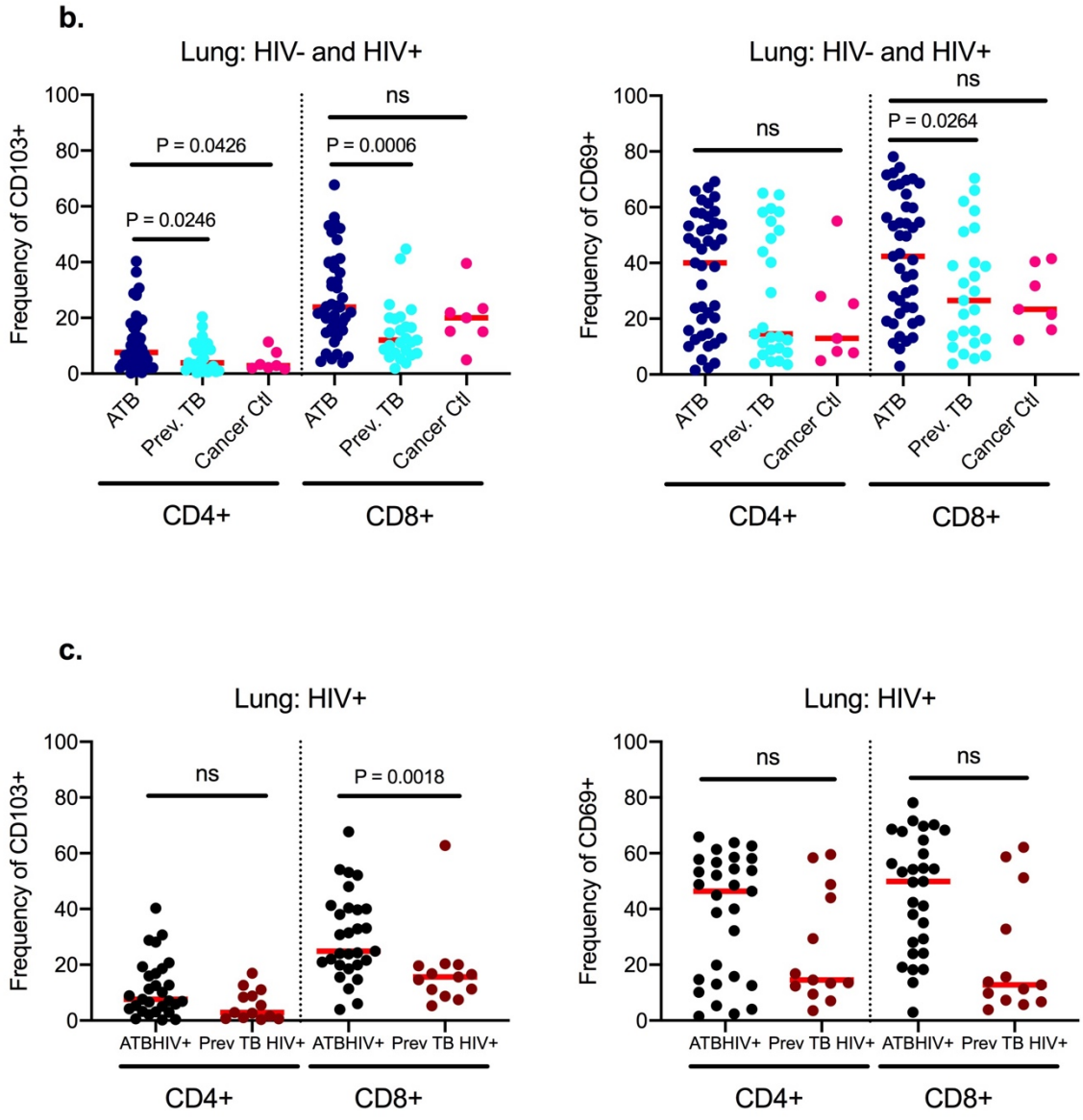


Figure 2.3: Lung tissue resident memory T-cells during TB disease (b - c) Frequency of CD103 and CD69 in the lung of TB cases and cancer control participants showing effect of HIV coinfection. Non-parametric Mann-Whitney t-test p values are indicated.

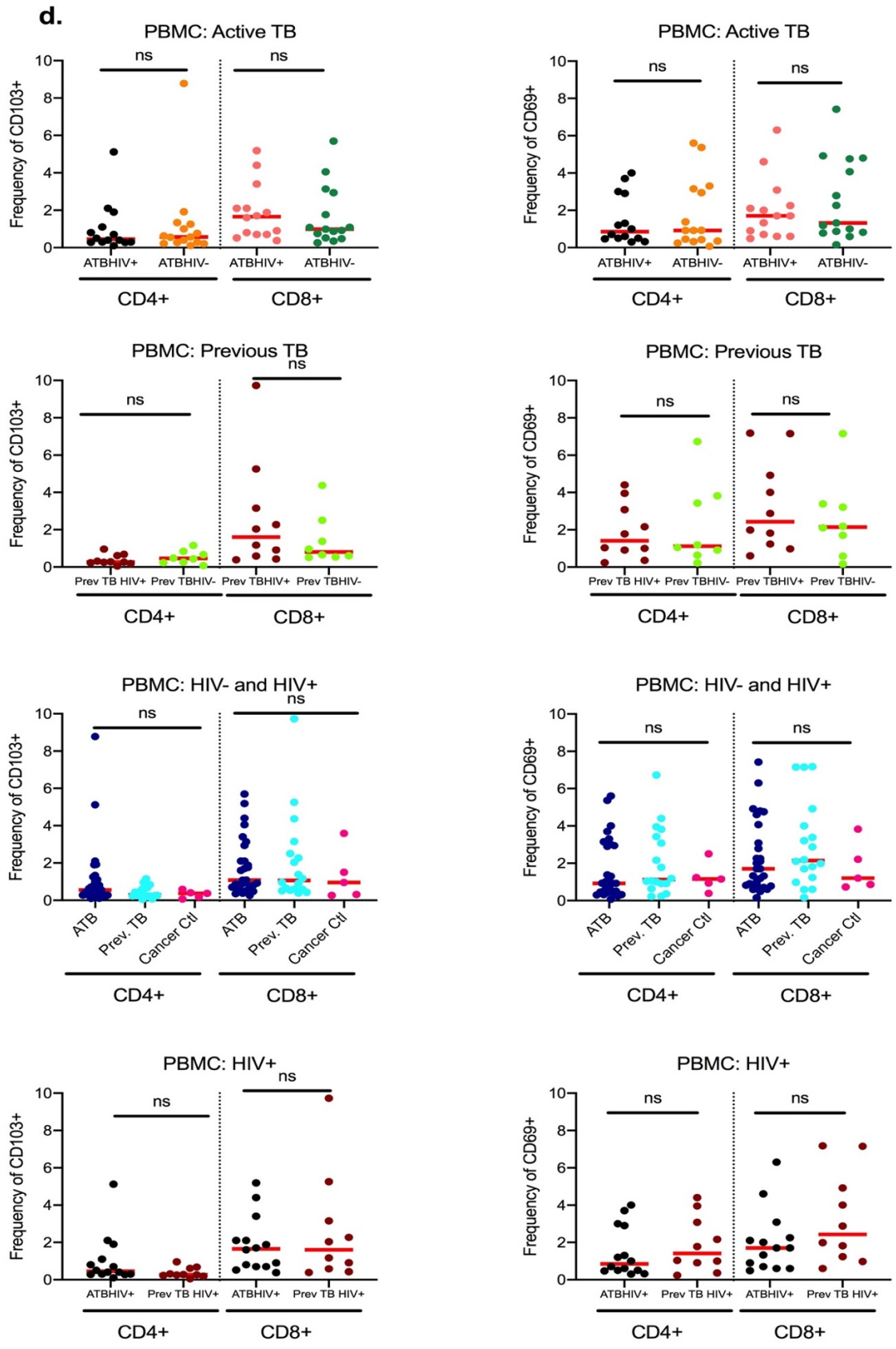


Figure 2.3d: Frequency of CD103 and CD69 in systemic circulation in TB cases and cancer control participants. No significant difference in cell frequencies regardless of the disease status. Non-parametric Mann-Whitney t-test p values are indicated.

2.3.1.4 Lung T-cells are predominantly effector memory

To further confirm the existence of Trms within TB infected human lung tissue, we next examined the memory profile of T-cells within lung homogenate. T-cell memory subsets and their diverse distribution in lymphoid, mucosal and other non-lymphoid sites are integral to their protective capacity and long-term maintenance (Bevan 2011; Masopust and Picker 2012; Sheridan and Lefrançois 2011; Teijaro et al. 2011). However, in humans, studies on T-cell activation and memory have been largely limited to T-cells isolated from blood, and there are limited studies regarding human T-cell activation and differentiation in tissue sites (Sathaliyawala et al. 2013; Hombrink et al. 2016; Gordon et al. 2017).

To investigate differentiation phenotype of T-cells in the lung, CD4 and CD8 T-cells were further classified on the basis of co-expression of CD45RA and CCR7 into Naïve (CD45RA+CCR7+), Central Memory (CD45RA-CCR7+), Effector Memory (CD45RA-CCR7-) and Terminally differentiated effector memory (CD45RA+CCR7-) phenotypes. As expected for Trm, the majority of CD4+ lung T-cells displayed an effector memory phenotype (median 81%), followed by central memory (median 11.45%; figure 2.4a). Naïve and terminally differentiated phenotypes where much less frequent, with a median of 2.6% and 4.3% respectively (figure 2.4a). Again, as expected, matched PBMC samples were dominated by naive CD4+ T-cells (median 37.6%), followed by effector memory (28.2%), central memory (17.3%) and terminally differentiated cells 10.2% (figure 2.4a). A similar pattern was observed for lung CD8+ T-cells, although less dominated by effector memory cells (median 56.3%) and with higher levels of terminally differentiated effector cells (24.7%), compared to central memory (8.2%) and naïve cells (7.34%) (figure 2.4a). Again, this was strikingly different from PBMC, which were similarly dominated by naïve T-cells. The fact that we observe such a profound skewing of memory phenotypes within the lung homogenate strongly supports the enrichment of distinct CD4 and CD8 Trm populations within the lung homogenate.

To further confirm this, we next examined the expression of these markers on the CD69+ and CD103+ and – subsets respectively (figure 2.4 b-c). As one would predict, Lung CD4+CD69+ and CD8+CD69+ cells, contained significantly more effector memory cells and fewer of all other memory subsets (figure 2.4b). CD103+ CD8 T-cells are also highly enriched for effector memory T-cells whereas CD103+ and CD103- CD4 T-cells had similar differentiation phenotypes (figure 2.4c). Overall these data confirm that CD69 and CD103

positive T-cells in lung homogenate represent a distinct Trm subset, that is highly enriched for cells with effector memory phenotype

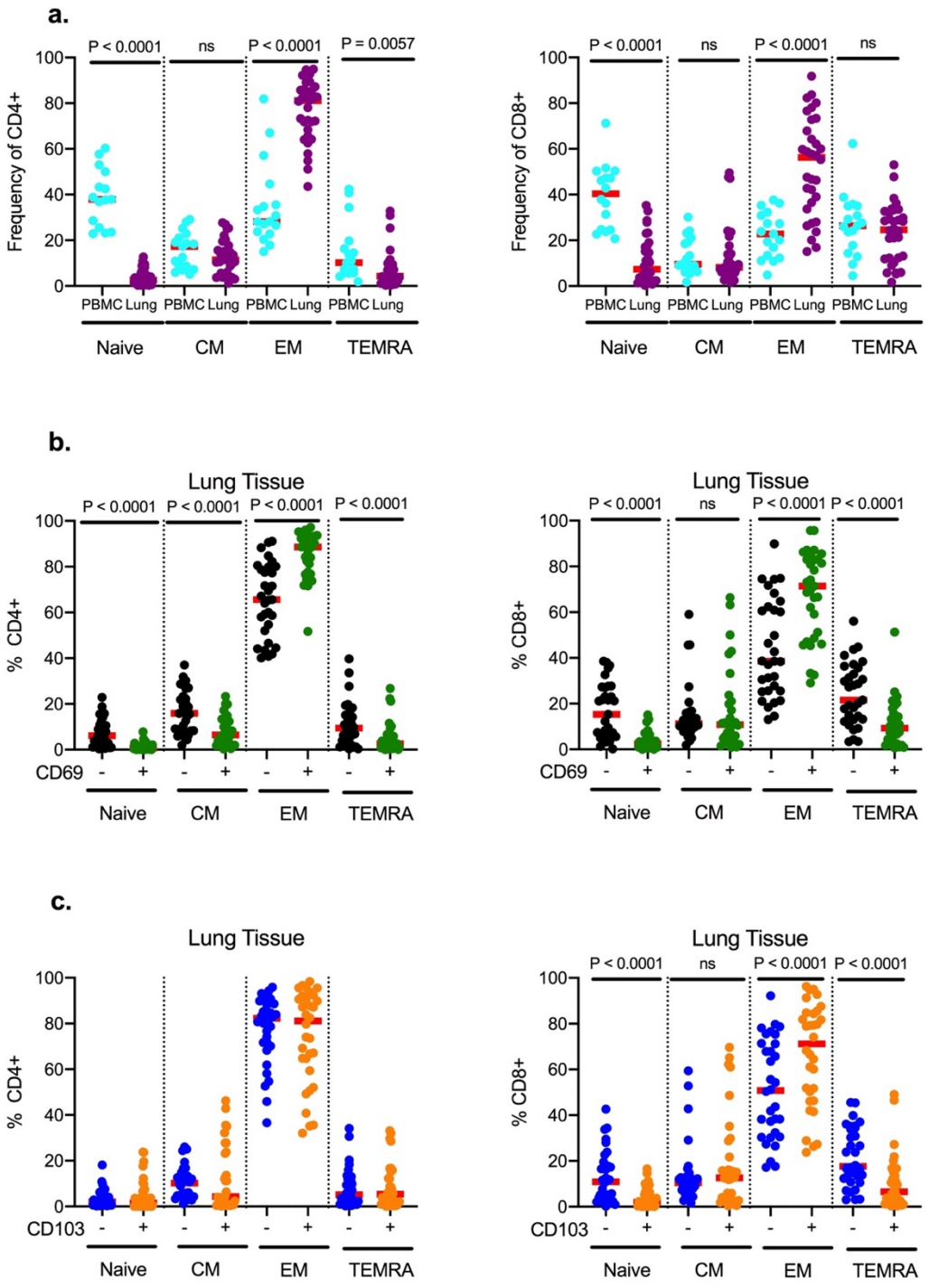


Figure 2.4: Differentiation of T-cells on the basis of co-expression of CCR7 and CD45RA. a) Comparison between PBMC and lung tissue (b) CD69+ and CD69- lung tissue (c) CD103+ and CD103- lung tissue. Statistics; non-parametric Mann-Whitney t-test for PBMC vs lung and Wilcoxon t-test for lung tissue subsets .

2.3.1.5 CD69+ lung T cells express high levels of T cell exhaustion marker PD-1

During chronic viral infections T cells become exhausted and express more PD-1 inhibitory receptor (Yi, Du, and Zajac 2009; Fröhlich et al. 2009). Higher frequencies of PD-1+ T cells are observed in systemic circulation of patients with active pulmonary tuberculosis and PD-1 blockade in vitro can enhance TB-specific IFN-γ production (Singh et al. 2013). In mice, PD-1 deficiency boosts *Mtb*-specific T_H1 cell expansion and effector functions, but rather than leading to enhanced bacterial control, the increased production of IFN-γ by CD4+ T-cells drives large necrotic lesions and leads to accelerated death of the host (Barber et al. 2011; Sakai et al. 2016) suggesting that uncontrolled production of IFN-γ is harmful during *Mtb* infection and that particularly PD-1 is important in keeping IFN-γ production under check. Indeed, it was reported recently that two cancer patients who were on long-term anti-PD-1 blockade had reactivated *Mtb* infection one of whom died from TB related complications (Barber et al. 2019).

Since there is a high TB antigen load in the lung compared to systemic circulation, the expression of T-cell exhaustion markers, PD-1, CTLA-4 and TIM-3, were measured in T-cells from lung and PBMC. PD-1 expression was variable in both lung and PBMC, although this appeared to be more pronounced in the lung (figure 2.5a). Overall, PD-1 expression in the lung was not significantly higher than in matched PBMC in either CD4+ or CD8+ T-cells (CD4+ T-cells median 9.2% (0.07 – 55.7) vs 9.7% (2.6 – 27.8), and lung CD8+ median 11% (0.1 – 85.7) vs median 7.1 (0.5 – 29.2). However, some individuals had markedly increased expression of PD-1 in the lung (figure 2.5a). Again, in an attempt to focus on Trm, the expression of PD-1 on CD69+ or CD69- T-cells was compared in lung tissue and peripheral blood. Regardless of the site measured, PD-1 expression was higher in CD69+ T-cells than CD69- T-cells (figure 2.5b), reflecting the dual role of PD-1 as an activation marker as well as a marker of tissue residency (Torrado et al. 2015; Moguche et al. 2015). Thus, the impact of HIV coinfection on PD-1 expression was measured, but only on CD69+ T-cells. Interestingly in the lung tissue, PD-1 expression was higher in HIV- participants than HIV coinfected individuals in both CD4+ and CD8+ T-cells (figure 2.5c) although fewer individuals were used for this analysis and it does not reach statistical significance. The frequency of PD-1 in peripheral blood CD69+ T-cells was similar between HIV- and HIV+ participants or marginally higher in HIV+ (figure 2.5c).

In contrast to PD-1, the frequency of other markers of immune exhaustion, CTLA-4 and TIM-3, were higher in circulation than in the lung tissue (figure 2.5d - e), although only TIM-3+CD8+ difference between PBMC and lung reached statistical significance. In addition, frequency of CTLA-4 and TIM-3 tended to be higher in peripheral blood of HIV+ than HIV-, although the difference was not statistically significant, while their frequency in the lung tissue was not affected by HIV coinfection (figure 2.5 d-e), the small sample size in each subgroup does not allow definitive conclusions to eb drawn from this observations. Unfortunately, the flow cytometry panels used at the time of study did not allow co-staining of PD-1 and cytokines, and so information is unavailable on PD-1 expression levels on TB-specific T-cells. However, these data do suggest variable PD-1 expressing T-cells in the lungs of individuals with active TB infection, and furthermore, that this is affected by HIV.

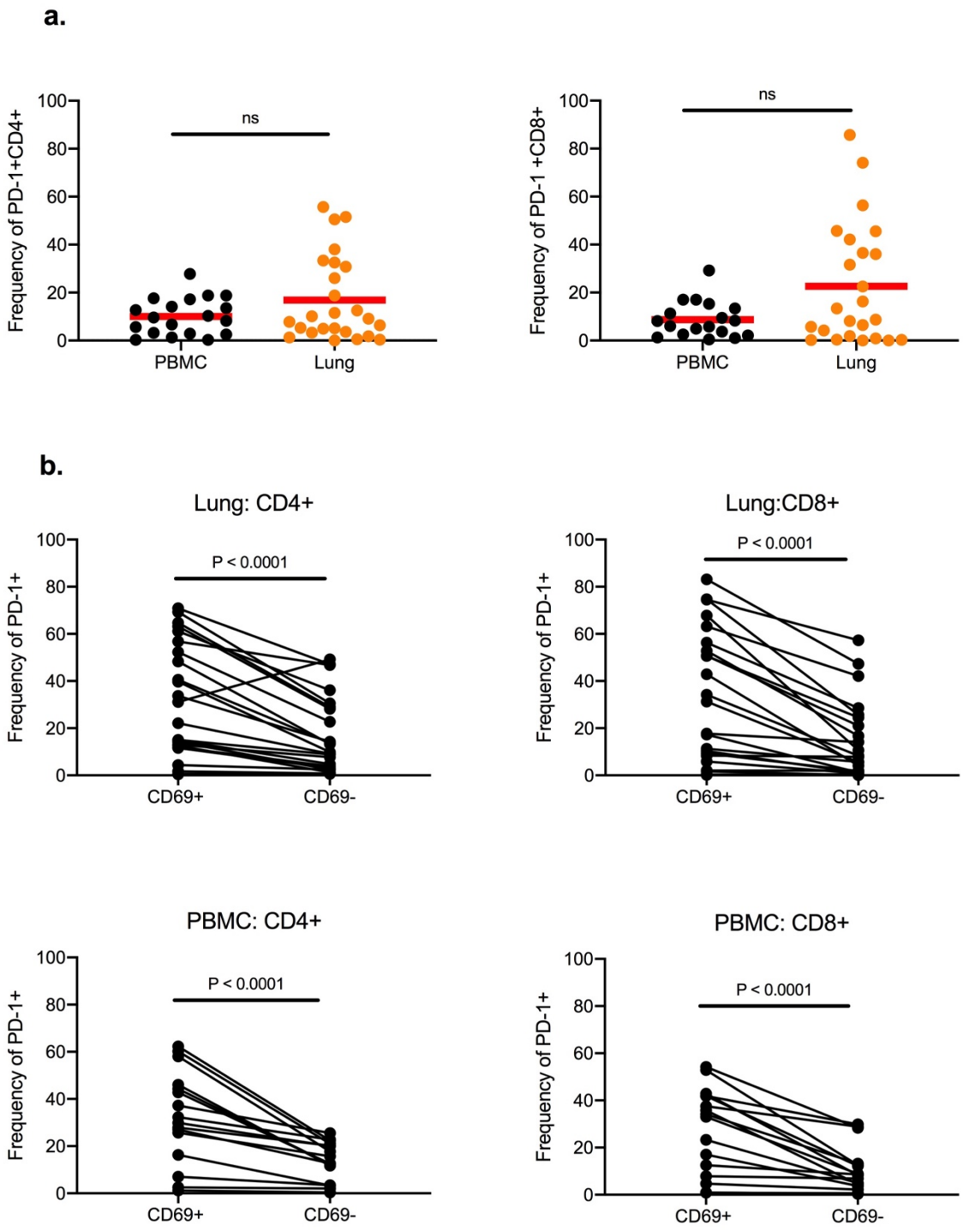


Figure 2.5: T-cell exhaustion markers. a) Frequency of PD-1 on CD4 and CD8 T-cells in circulation and in the lung in participants with TB cases coinfected with or without HIV coinfection. b) Frequency of PD-1 on CD69+ or CD69- T-cells in lung tissue and peripheral blood. Statistics; non parametric Mann-Whitney t-test (PBMC vs Lung) and Wilcoxon t-test (CD69+ vs CD69-).

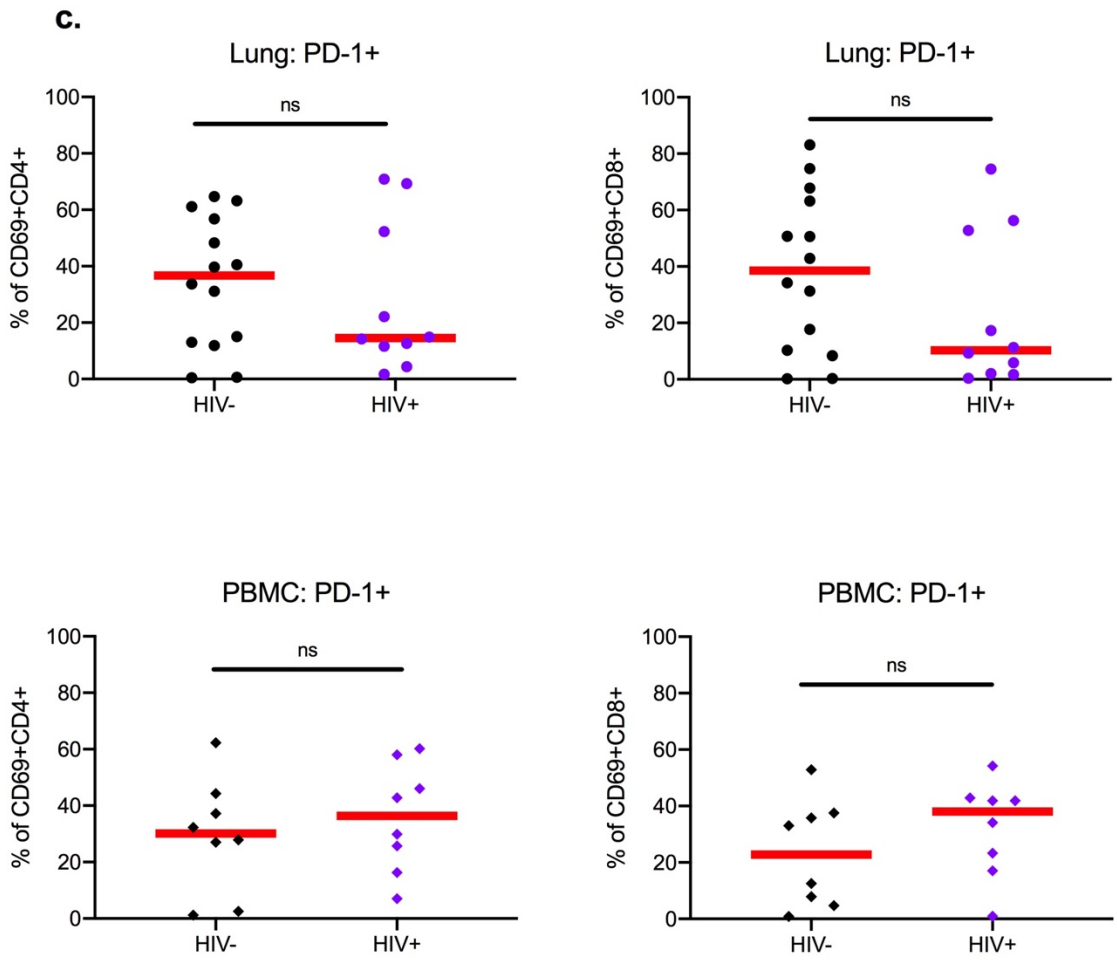


Figure 2.5: T-cell exhaustion markers. c) PD-1 frequency on CD69+ T-cells stratified on participants based on HIV status. Statistics; non parametric Mann-Whitney t-test.

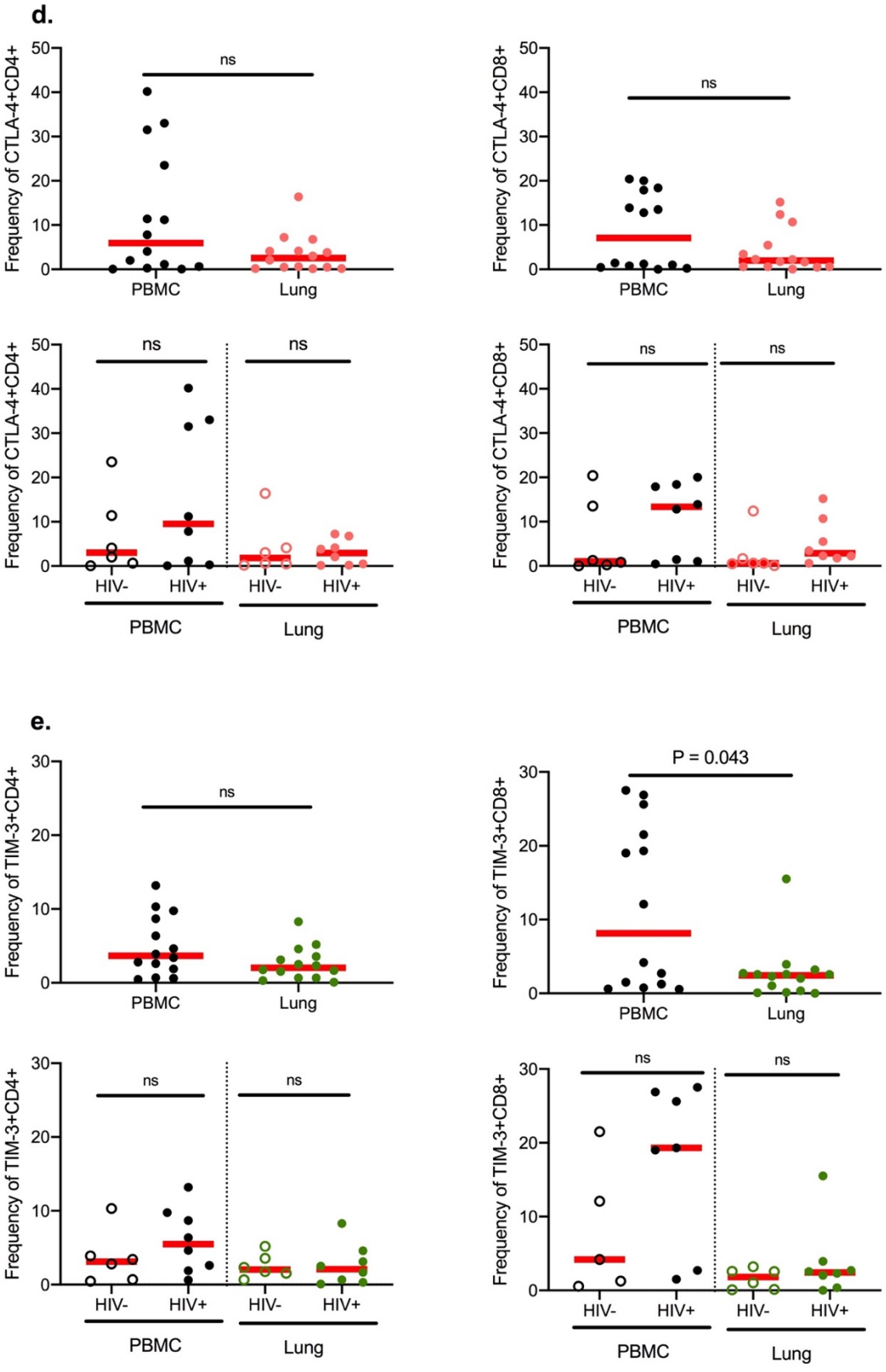


Figure 2.5: T-cell exhaustion markers. (d) Frequency of CTLA-4 and (e)TIM-3 on CD4 and CD8 T-cells in circulation and in the lung in participants with TB cases coinfected with HIV or HIV negative participants. Statistics; non parametric Mann-Whitney t-test.

2.3.2 Lung T cells are superior cytokine producers than T cells in circulation

The hallmark of memory T cells is their rapid functional recall within hours of stimulation (Sathaliyawala et al. 2013; Gideon et al. 2015). First, because CD69 is also a marker of T-cell activation, we first sought to understand the effect of *ex vivo* stimulation with PMA + ionomycin on CD103 and CD69 expression in the lung homogenate. The frequency of CD69 expressing T-cells was significantly increased following 5hr simulation but CD103 expression was not altered (figure 2.6a).

Important roles of T-cell produced cytokines such as IFN-γ, IL-2, TNF-α, and IL-17 during TB have been demonstrated in animal model studies, with a subset of these cytokines demonstrated to be critical in humans as well (Flynn and Bloom 1996; O'Garra et al. 2013; Algood, Lin, and Flynn 2005; J. Lee and Kornfeld 2010; Lin et al. 2010; Green, Difazio, and Flynn 2013) . Using matched lung cells and PBMC, TNF-α, IL-2, IFN-γ and IL-17 were measured by intracellular cytokine staining. Overall, lung CD4 T-cells produced higher levels of all cytokines than PBMCs, reaching significance for TNF-α, IFN-γ and IL-17 (figure 2.6b). The same trend was observed for CD8 T-cells, significant for IFN-γ and IL-17. The presence of IL-17 producing T-cells in the lung is particularly notable, and suggestive of a Th-17 subset. For CD4 T-cells, CD69+ subset contained the highest frequency of cytokine positive cells, although this may have been a bystander effect of stimulation, as shown above. However, the CD69+CD103+ subset contained the highest frequency of cytokine positive cells for all 4 cytokines (figure 2.6c); suggesting these T-cells are the most highly functional in lung tissue, as CD103 expression itself is not affected by stimulation. This difference is less pronounced in CD8 T-cells, in which the CD69-CD103- subset appear equally functional (figure 2.6d). In general, the CD103+CD69- subset appear to be the least functional. To further investigate the difference between CD103+ and CD103- T-cells in the lung we examined the memory subset make up of these 2 populations. No difference was observed for CD4 T-cells except for TEMRA that was significantly higher in CD103+, but CD103+ CD8 T-cells tended to be enriched for central memory cells and CD103- for naïve and TEMRA cells (figure 2.6 e).

Next, the effect of HIV co-infection on cytokine production was determined (figure 2.6f-g). In total lung homogenate there was a trend for lower production of TNF-α and IL-2 by CD4s (figure 2.6f) and TNF-α by CD8s (figure 2.6g) in individuals with HIV co-infections,

reaching significance for CD8s. However, for CD4+ T-cells, these differences which were not observed in matched blood (figure 2.6f-g), were much more striking when focusing on Trms (figure 2.6h). Production of TNF-α , IL-2 and IL-17 were all significantly lower in CD69+ CD4+ T-cells from HIV+ individuals. Interestingly, IFN-γ production was unaffected. Only TNF-α production was affected in Trms of the CD8+ T-cells (figure 2.6i). This reveals significant reduction in the ability of CD69+ T-cells from HIV co-infected lungs to produce TNF-α, IL-2 and IL-17 and in the ability of CD8 T-cells to make TNF-α.

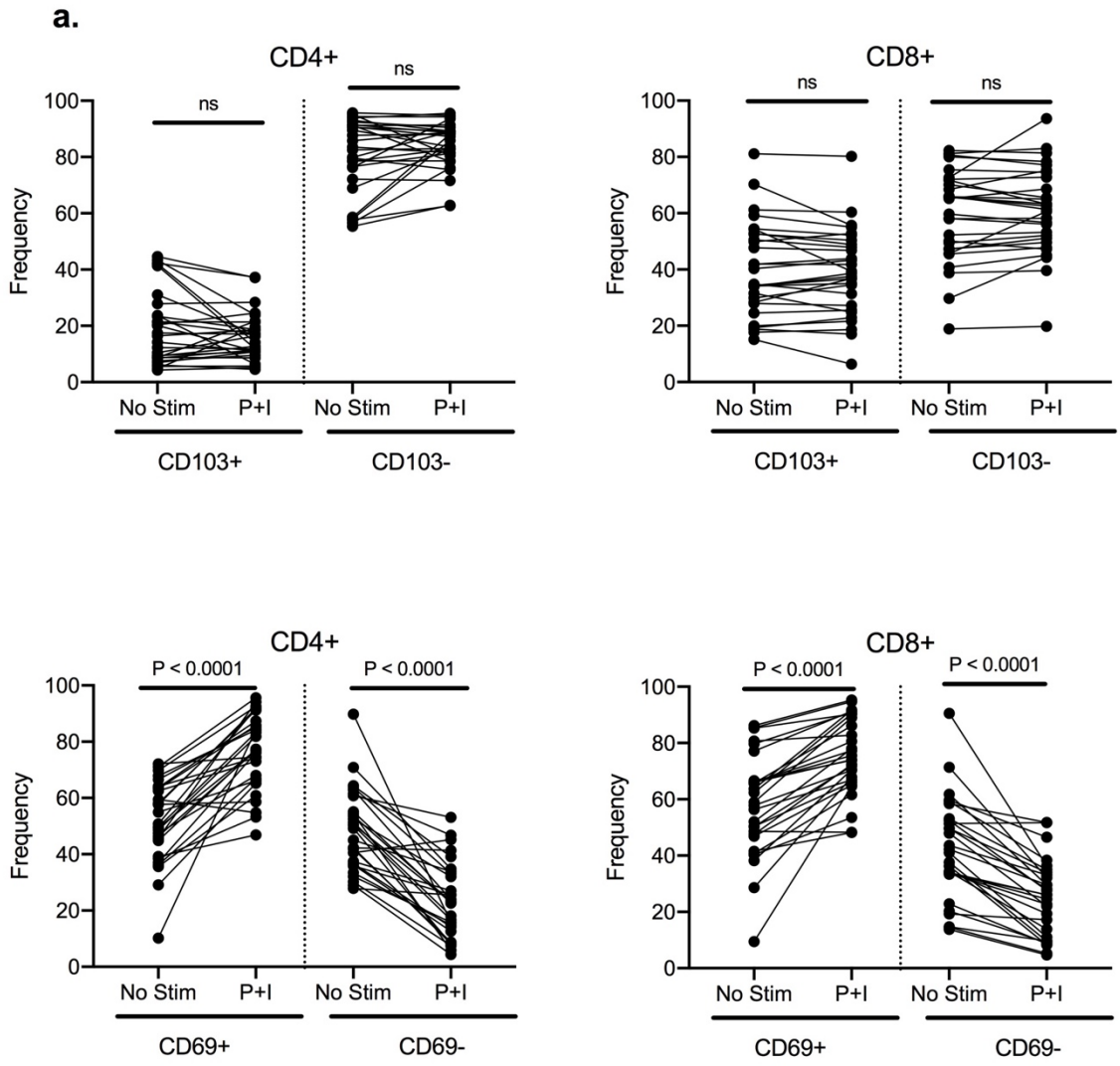


Figure 2.6: Functionality of lung T-cells. a) Effect of *ex-vivo* stimulation of lung T-cells on expression of CD103 and CD69. Statistics; non-parametric Wilcoxon t-test.

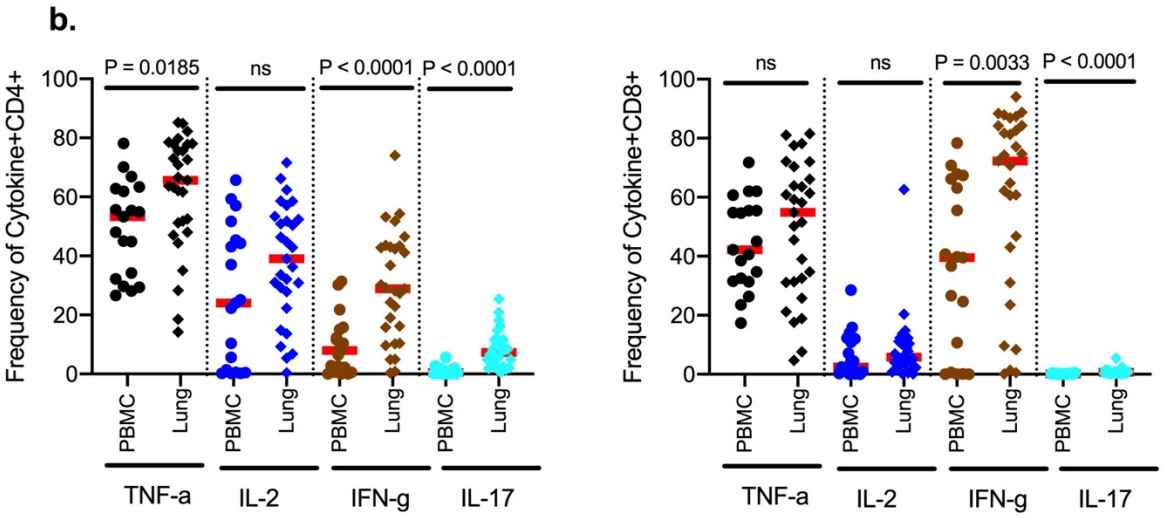


Figure 2.6: Functionality of lung T-cells. (b) production of cytokines by lung and PBMC CD4+ and CD8+ T-cells after 5hr stimulation with PMA + ionomycin. Statistics; non-parametric Mann-Whitney t-test.

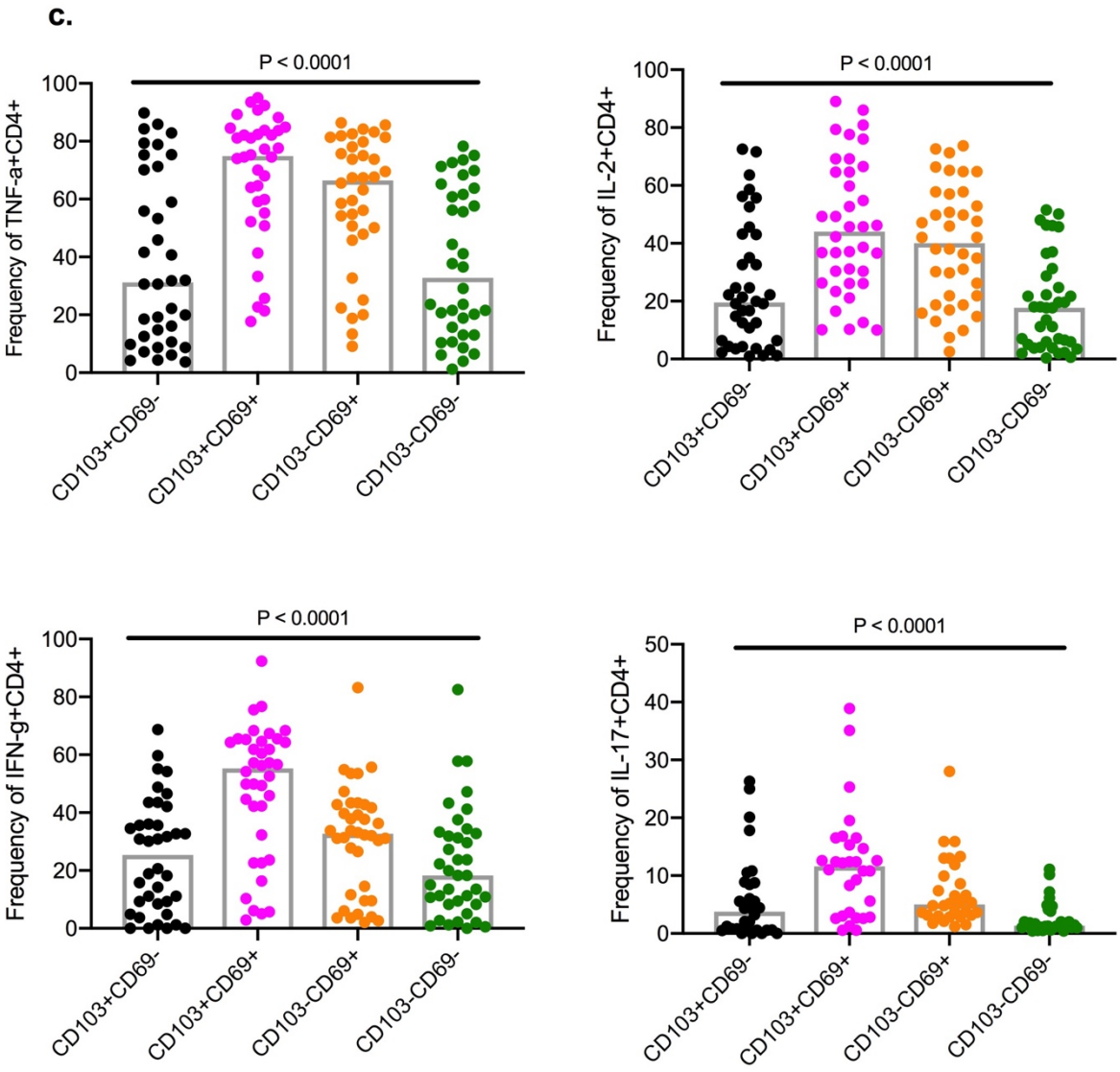


Figure 2.6: Functionality of lung T-cells. (c) frequency of cytokines produced by lung tissue resident memory T-cells in CD4+ lung T-cells. Statistics; one-way ANOVA

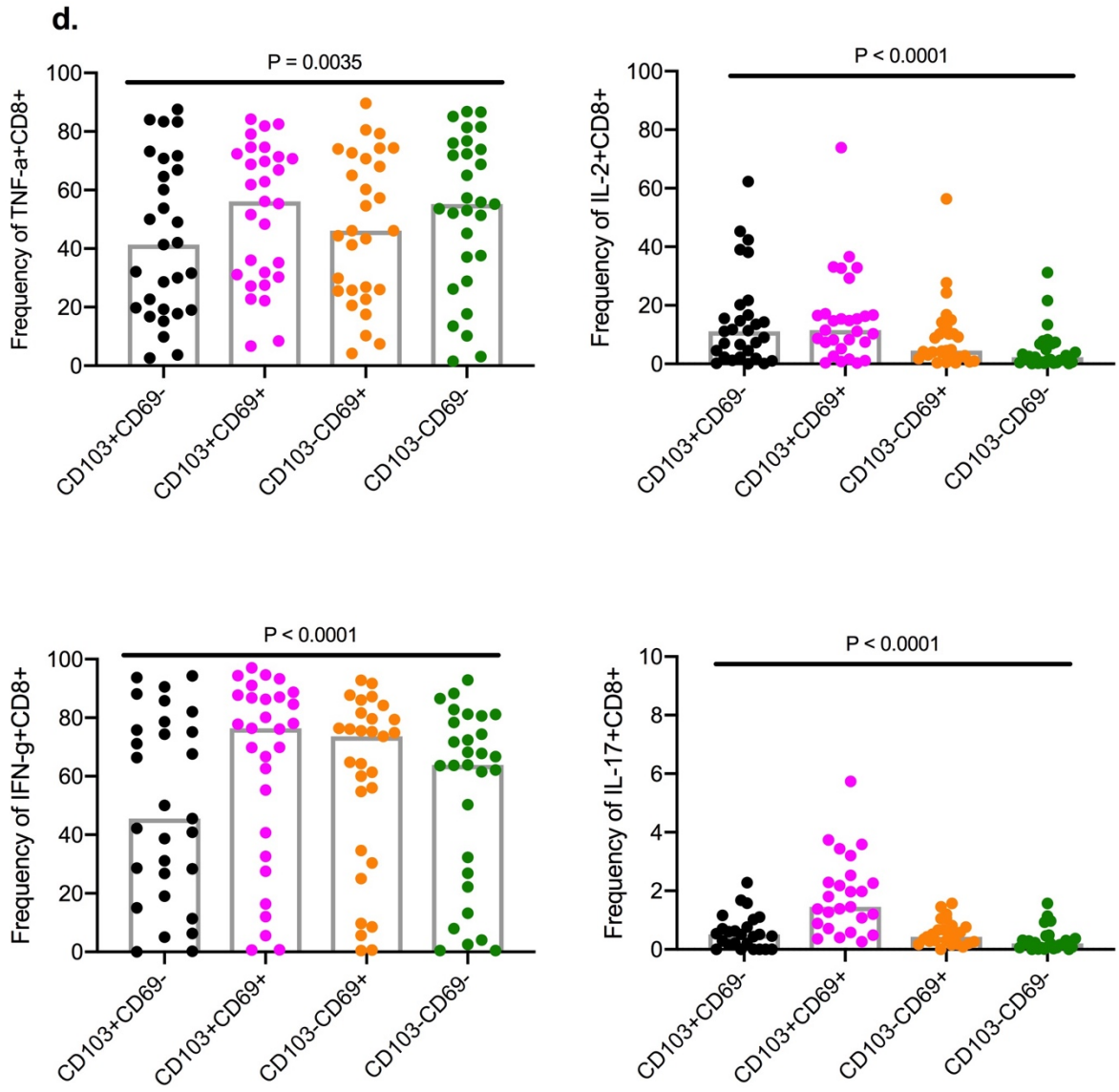


Figure 2.6: Functionality of lung T-cells. (d) frequency of cytokines produced by lung tissue resident memory T-cells in CD8+ lung T-cells. Statistics; one-way ANOVA

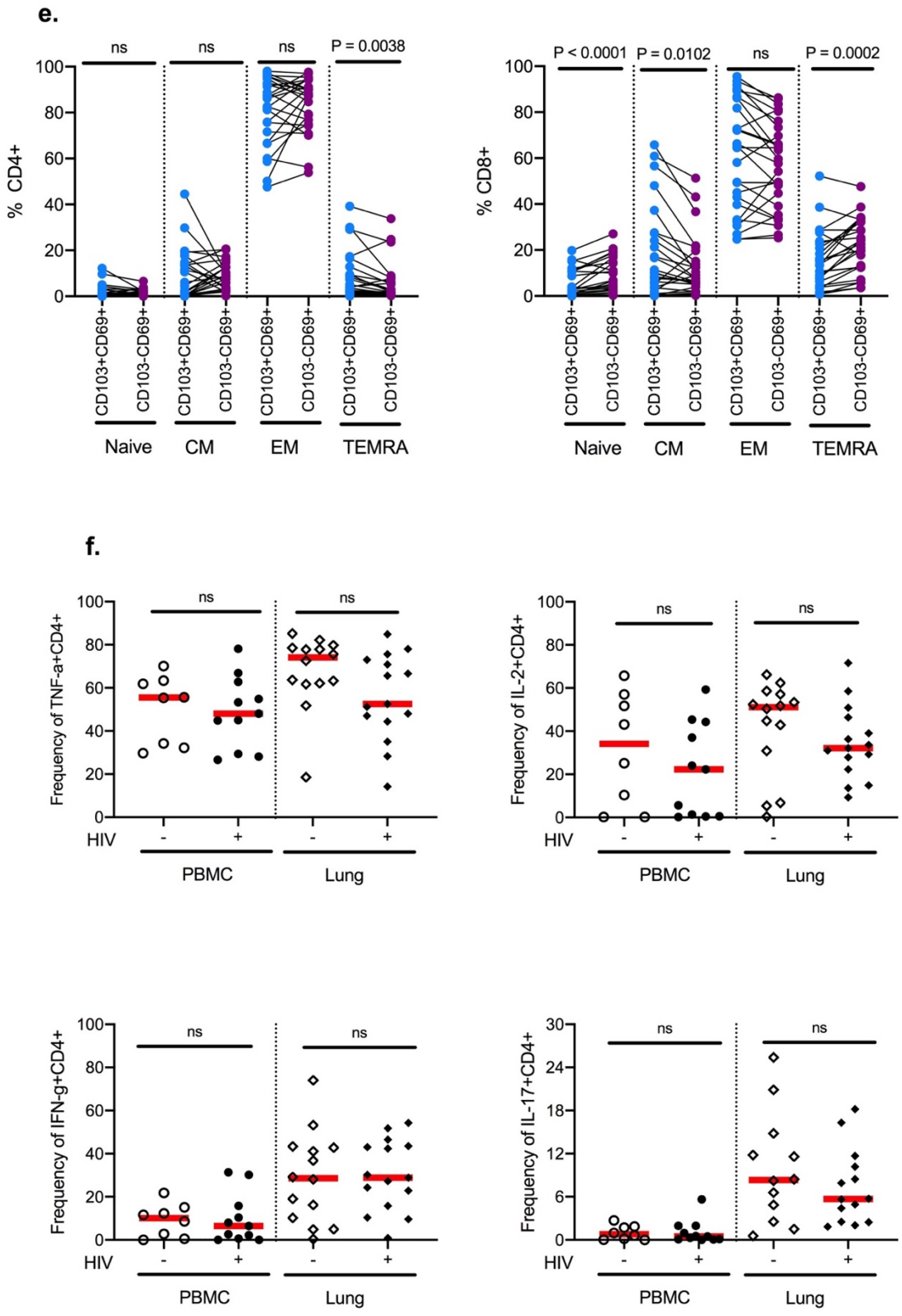


Figure 2.6: Functionality of lung T-cells. (e) frequency of cytokine production by tissue resident memory T-cells in the lung. (f) effect of HIV on cytokine production by CD4+ and T-cells in circulation and in the lung. Statistics; non-parametric Wilcoxon t-test for paired comparisons and Mann-Whitney for unpaired comparisons.

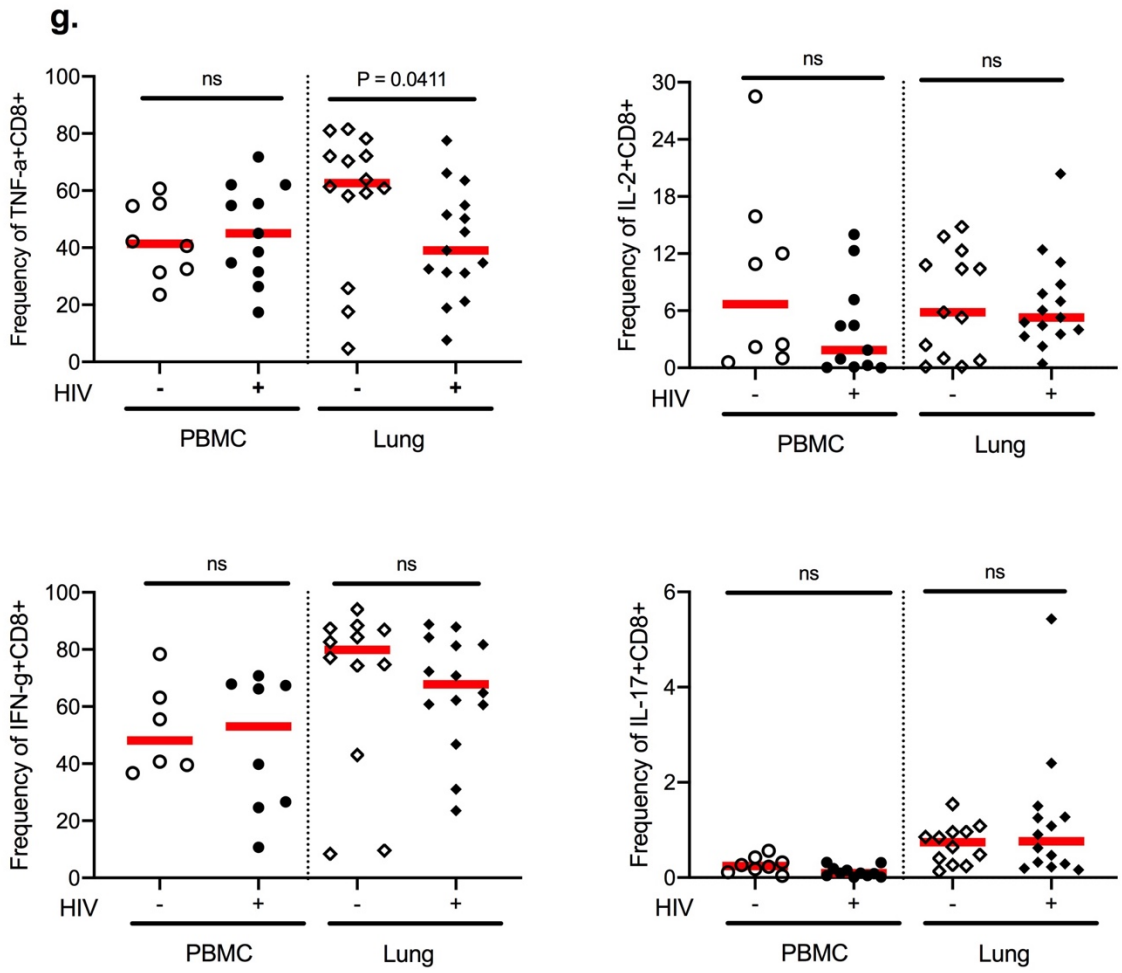


Figure 2.6: Functionality of lung T-cells. (g) Effect of HIV on cytokine production by CD8+ and T-cells in circulation and in the lung. Statistics; non-parametric Mann-Whitney t-test.

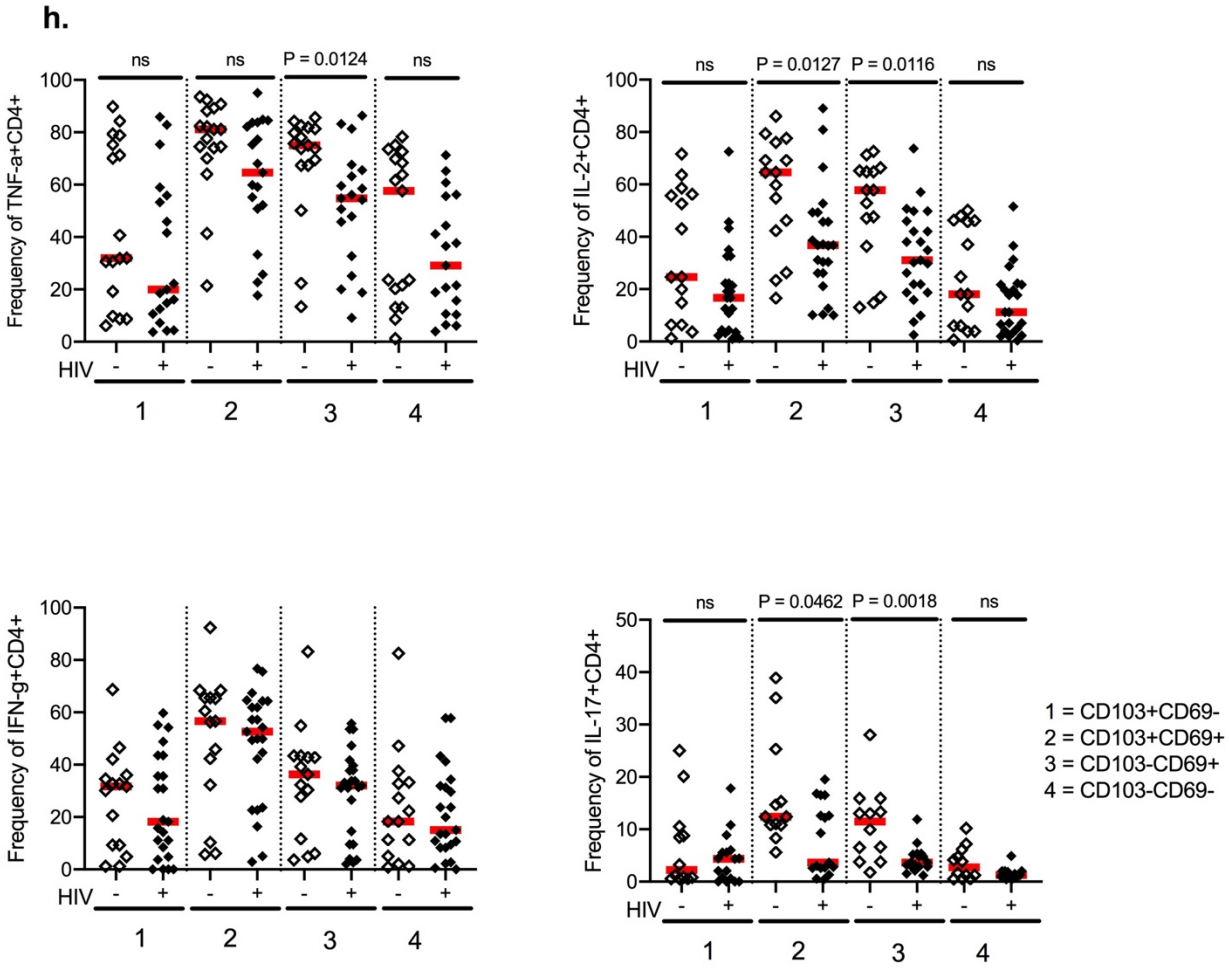


Figure 2.6: Functionality of lung T-cells. (h) cytokine production by tissue resident memory CD4+ T-cells after non-specific stimulation with PMA+ ionomycin. Statistics; non-parametric Mann-Whitney t-test.

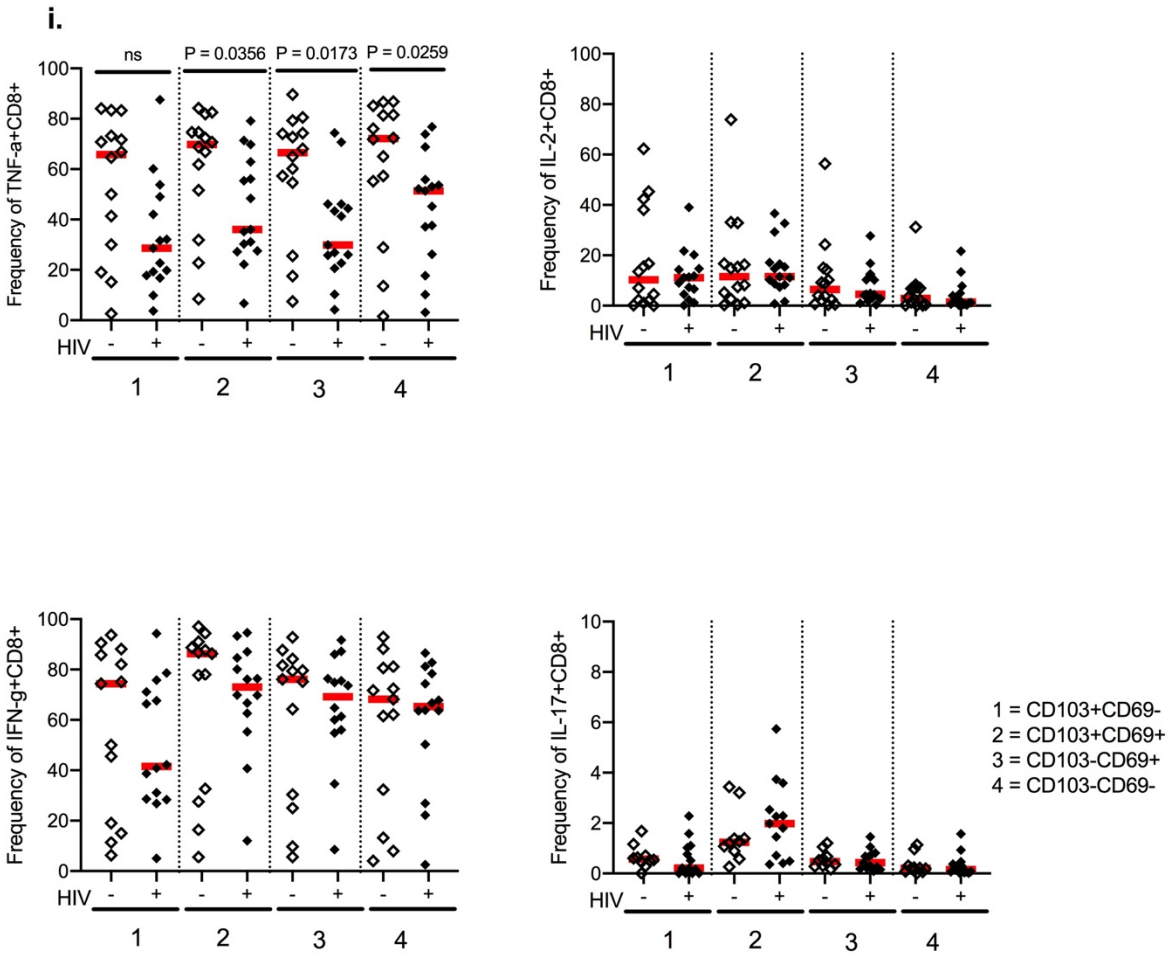


Figure 2.6: Functionality of lung T-cells. (i) cytokine production by tissue resident memory CD8+ T-cells after non-specific stimulation with PMA+ ionomycin. Statistics; non-parametric Mann-Whitney t-test.

2.3.3 Specificity of lung T cells in tuberculosis

2.3.3.1 TB specific T cell responses are enriched at the site of infection

Next, the frequency of TB specific T-cells in lung homogenate and matched PBMC was measured by intracellular cytokine staining after stimulation with a mega pool of 300 TB peptides (MTB300) (Lindestam Arlehamn et al. 2013). The data showed here are values after subtraction of cells with no stimulant added; negative values after subtraction were assigned an arbitrary value of zero. T-cell responses producing TNF-α, were most abundant and frequent in both lung and blood, being detected in all but one subject studied (figure 2.7a). TB specific cells producing the other cytokines were generally of lower frequency and entirely absent in some individuals. Examining TNF-α and IL-2 producing cells alone, we observe a highly significant enrichment of TB-specific CD4 T-cells in the lung both in comparison to matched blood samples (figure 2.7a) and between all lung and blood samples analysed from the lung cohort (matched PBMC not always available; figure 2.7b). The median frequency of TB T-cell in lung homogenate was compared to blood was X vs Y for TNF-α (median blood = 0.4835 vs median lung = 2.399) and X vs Y for IL-2 (median blood = 0.05 vs median lung = 0.503) (p=0.0002 and P=0.002, respectively). Although less frequent, TB-specific Th-17 T-cells (producing IL-17), were also more often detected in the lung homogenate compared to matched blood (15/20 vs 6/17; P = 0.022 (Fisher's exact test)). Surprisingly, the same pattern was however not observed for IFN-γ, expression of which was highly variable, and no overall significant difference were observed. In stark contrast to data generated using non-specific stimulation, there was no difference in TB-specific cytokines between HIV coinfected and HIV negative participants, although the median values were marginally higher in the HIV negative groups (figure 2.7c).

Next, TB-specific cytokine production was analysed in relation to the Trm markers utilised. Again, surprisingly considering the data from non-specific stimulation, CD103+ T cells, irrespective of CD69 expression, produced very little or no cytokine in response to TB peptide stimulation (Fig.2.7d). This may be consistent with the lack of PD-1 expression on the CD103+ subset, as PD-1 is known to be upregulated in response to antigen stimulation. The frequency of cytokine producing cells in the CD103-ve fraction are roughly evenly divided between CD69+ and CD69- cells for TNF-α and IL-2. However, in CD69+ cells there is a trend for lower production of IFN-γ and higher production of IL-17. This is consistent with a general enrichment of Th17 in the lung. In general, and again contrary to

non-specific stimulation, HIV coinfection did not appear to affect the ability of lung Trm cells to produce TB-specific cytokines (figure 2.7d). As expected, TB-specific cytokine positive CD4+ lung T cells were mainly effector memory phenotype (figure 2.7e).

Finally, the polyfunctionality of TB specific T cell responses in the lung was analysed and found that majority of the response was TNF-α mono-functional particularly in active TB and previous TB cases (figure 2.7f). However bifunctional T cells producing, TNF-α+IL-17+ or TNF-α+IL-2+ are present and appear to be enriched in participants with active TB (figure 2.7f). In addition, TB-specific T cell responses in the lung are not dysregulated by HIV coinfection and that the TB-specific responses are mainly effector memory lung T cells.

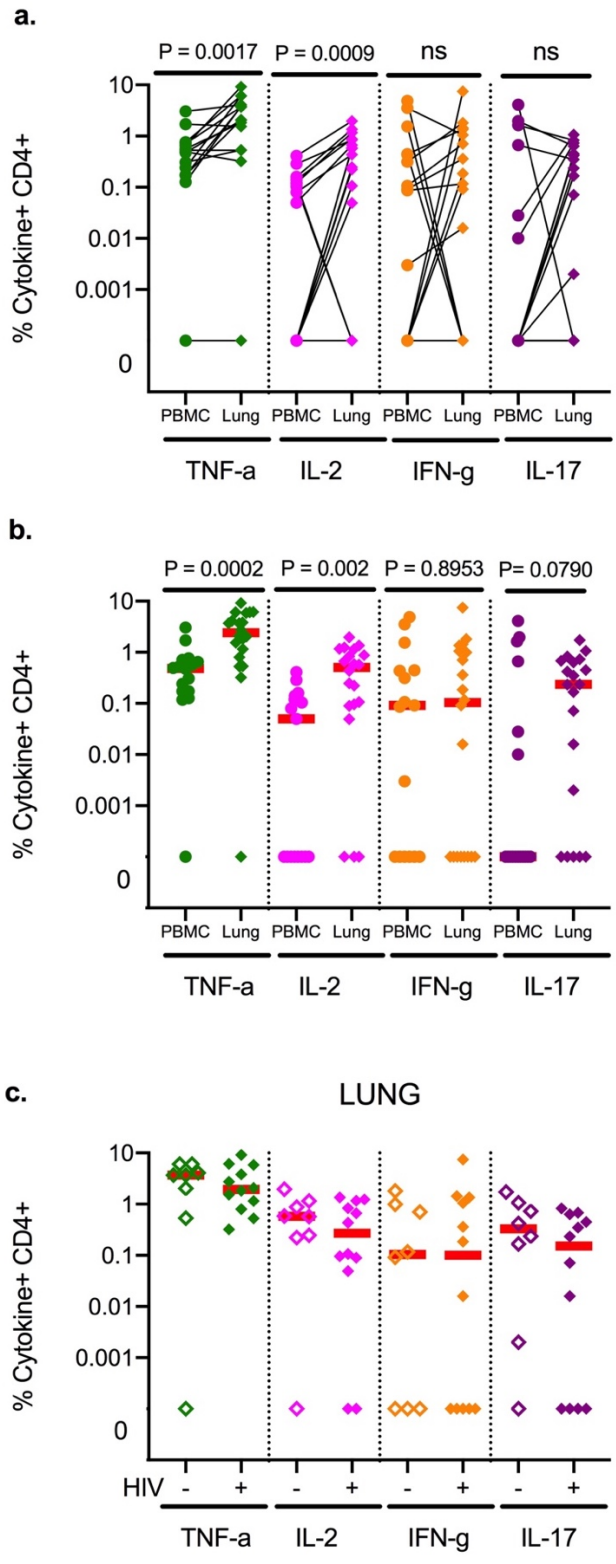


Figure 2.7: *M. tuberculosis* specific T cells in the lung. Frequency of CD4+ cytokine-producing T-cells in peripheral blood and lung tissue after stimulation with MTB-300 peptide pool, matched blood and lung (a) and all blood and lung samples (b). (c) impact of HIV co-infection on TB-specific cytokine production by lung CD4+ T-cells; Statistics; non parametric Wilcoxon t-test for paired comparisons and Mann-Whitney for unpaired comparisons.

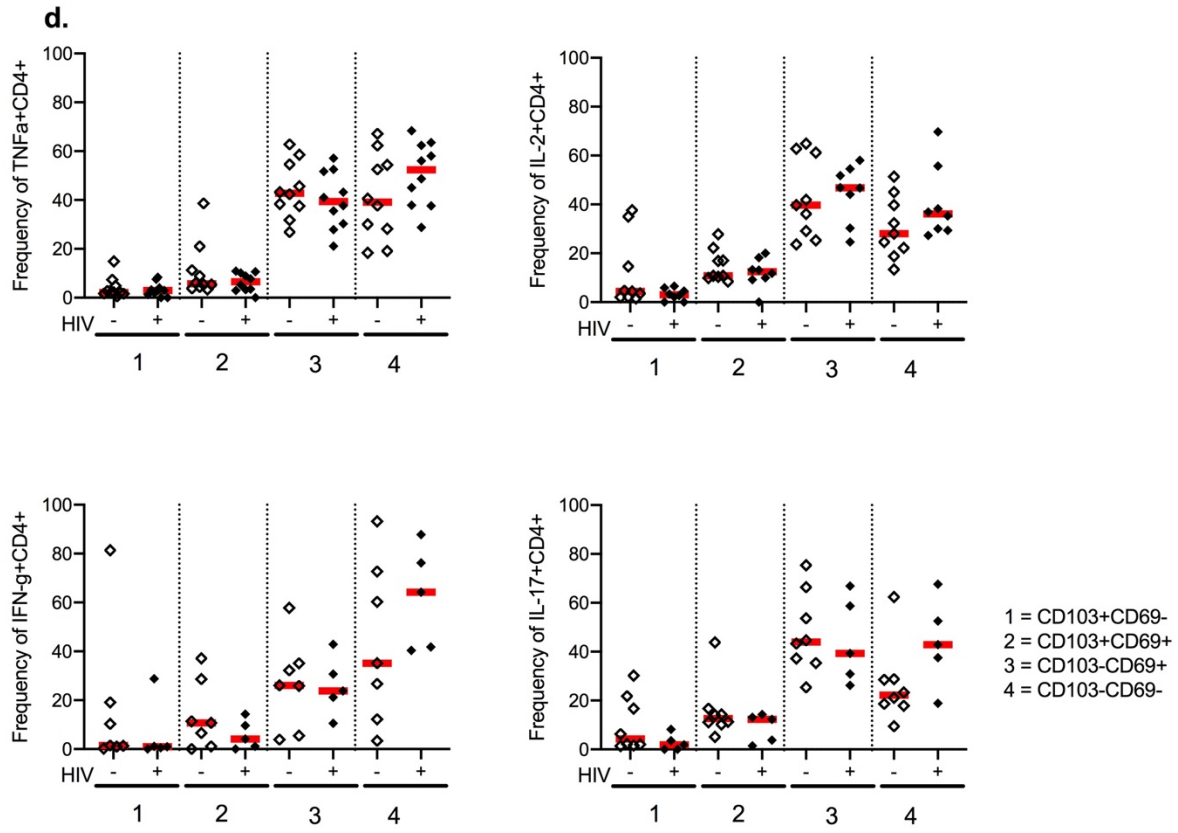


Figure 2.7: *M. tuberculosis* specific T-cells in the lung. d) lung tissue resident memory T-cells TB specific cytokine production. HIV coinfection does not affect TB-specific cytokine production by lung Trms.

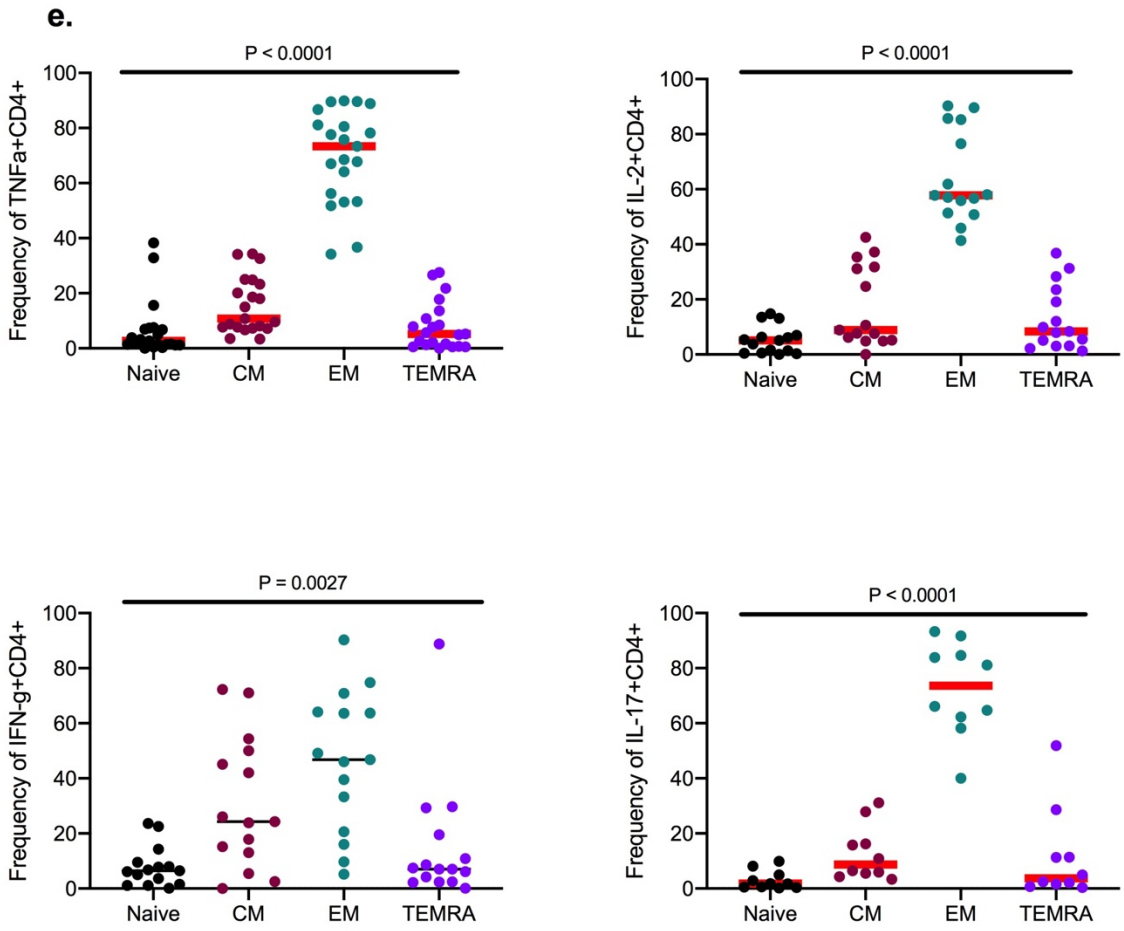
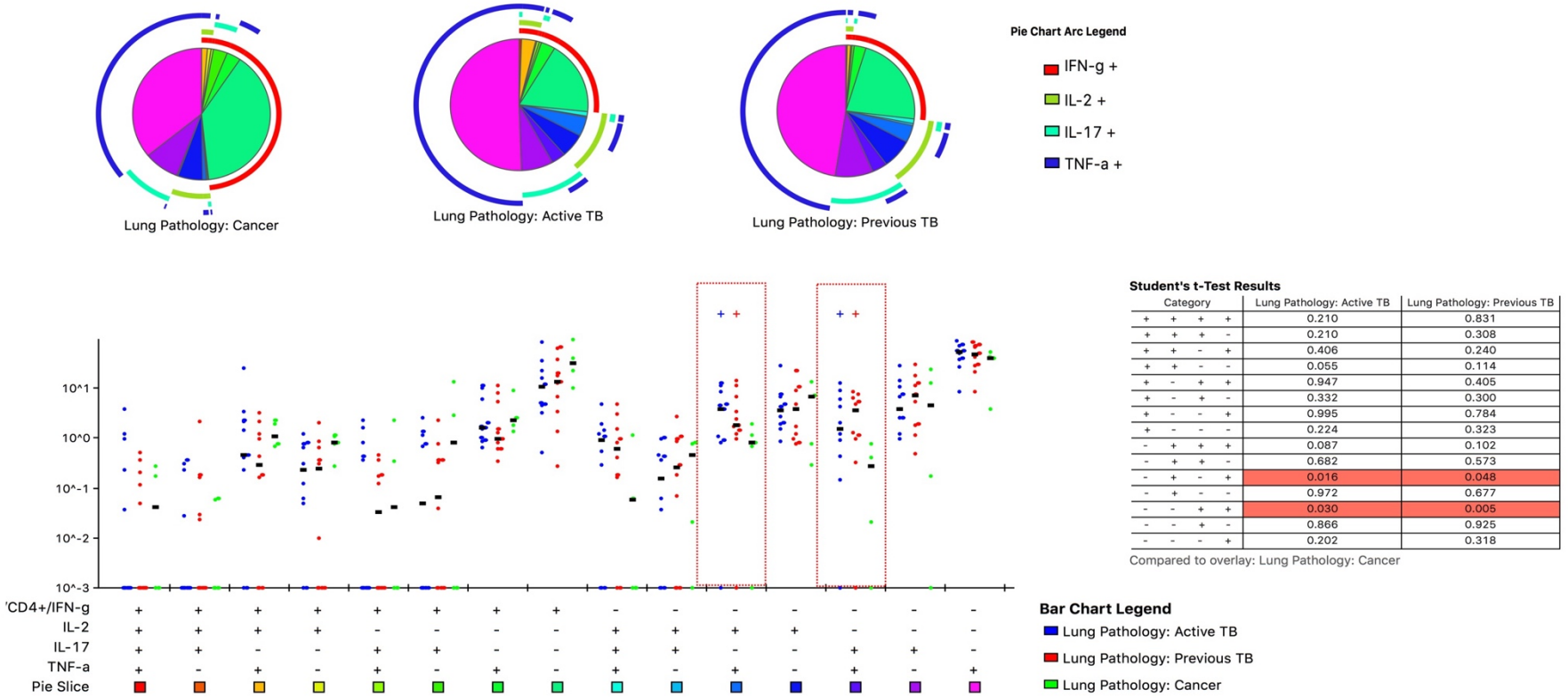


Figure 2.7: *M. tuberculosis* specific T-cells in the lung. (e) Phenotype of TB-specific cytokine producing lung CD4+ T cells. Statistics; one-way ANOVA

f.



Student's t-Test Results

Category				Lung Pathology: Active TB	Lung Pathology: Previous TB
+	+	+	+	0.210	0.831
+	+	+	-	0.210	0.308
+	+	-	+	0.406	0.240
+	+	-	-	0.055	0.114
+	-	+	+	0.947	0.405
+	-	+	-	0.332	0.300
+	-	-	+	0.995	0.784
+	-	-	-	0.224	0.323
-	+	+	+	0.087	0.102
-	+	+	-	0.682	0.573
-	+	-	+	0.016	0.048
-	+	-	-	0.972	0.677
-	-	+	+	0.030	0.005
-	-	+	-	0.866	0.925
-	-	-	+	0.202	0.318

Compared to overlay: Lung Pathology: Cancer

Figure 2.7: *M. tuberculosis* specific T-cells in the lung. (f) Multiple TB specific cytokine production analysis of lung T cells between cancer control, active TB and previous TB

2.3.3.2 TB specific regulatory T-cells in the lung

In several diseases, regulatory T-cells (T-regs) play a central role in the prevention of autoimmunity and in the control of immune responses by down-regulating the effector function of CD4+ or CD8+ T-cells (Semple et al. 2013). The expression of the forkhead box P3 transcription factor, Foxp3, on T-cells is fundamental for the development and function of these suppressive cells (Fontenot et al. 2005; Hori, Nomura, and Sakaguchi 2003). Unfortunately, given the multiple parameters measured simulateneously, our antibody panel did not contain CD25 antibody and ,thus, the expression of T-regs in the lung and circulation was analysed by measuring the frequency of FoxP3 on CD4+ T-cells. Overall, there was a higher frequency of FoxP3+ cells in circulation than in the lung (figure 2.8a), suggesting regulatory cells are not enriched in the lung in general, even during active disease. Moreover, the frequency of FoxP3 was not affected by HIV co-infection in both circulation and at the site of disease (figure 2.8a).

Analysis of expression of FoxP3 on CD103 and CD69 T-cells in the lung showed that CD103+ cell in particular lack FoxP3 (figure 2.8b). However, the frequency of TB-specific Tregs in the lung was highly enriched compared to matched blood (figure 2.8c). Thus, although FoxP3 expression in the lung tends to be lower in circulation, TB-specific T regs are highly enriched in the lung. We did not measure the canonical regulatory cytokine IL-10, but we did detect varying levels of TNF-α, IL-2, IFN-γ, and IL-17 by FoxP3 positive TB-specific T-cells in the lung (median frequency of 6.4%, 2.9%, 4% and 7.5% respectively)(figure 2.8d). Taken together, these data show an enrichment of TB-specific responses in the lung compared to blood, which although largely TNF-α mono-functional, are also skewed towards Th17 and regulatory subsets compared to matched blood. In addition, they suggest that, although CD103 lung T-cells are highly functional, they are unlikely to be participating directly in the TB immune response as they do not make cytokines in response to TB stimulation, are not regulatory and do not express PD-1 which would be indicative of exhausted T-cells.

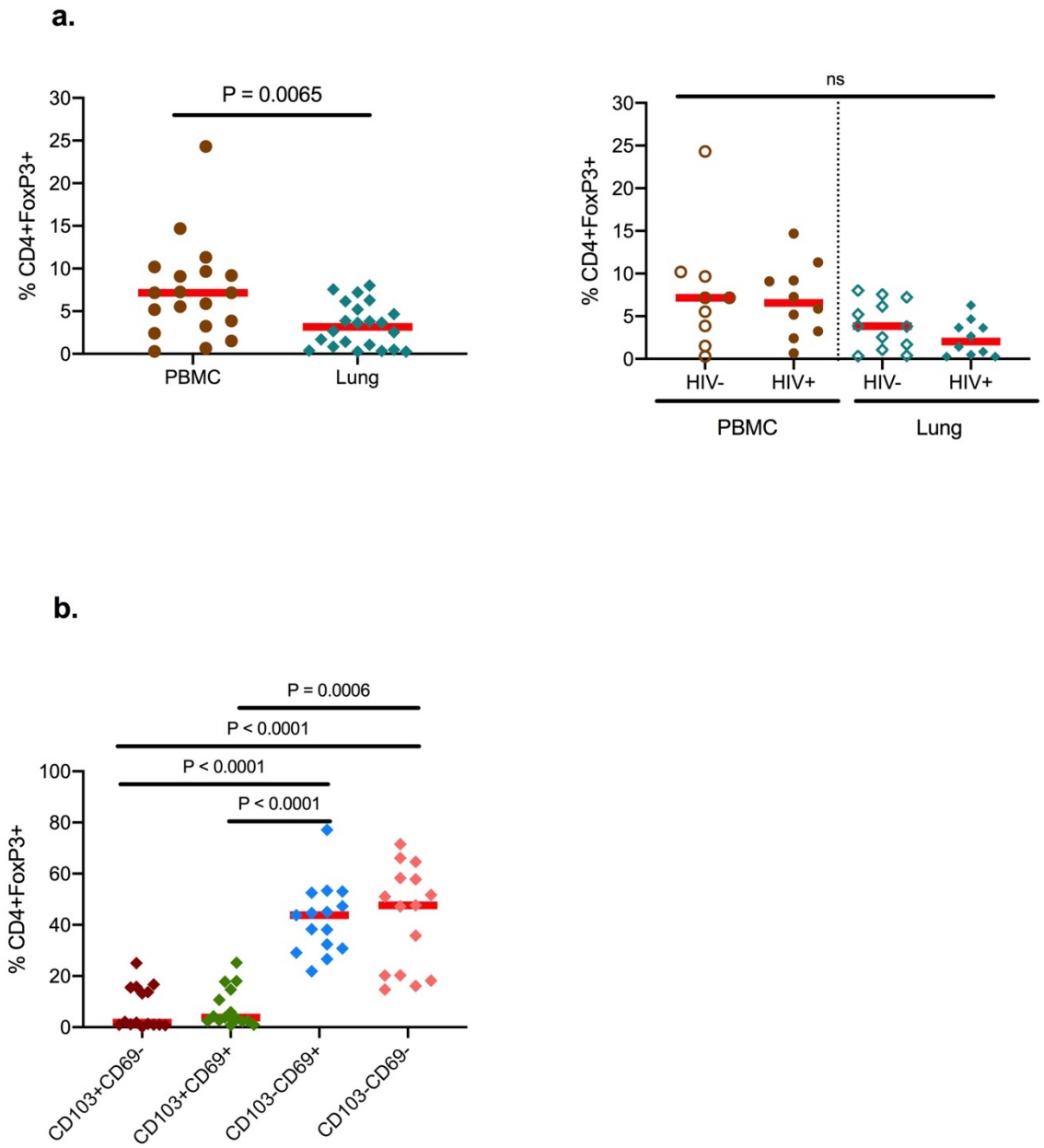


Figure 2.8: Regulatory T cells in the lung. a) Comparison of FoxP3+ expression on CD4+ T-cells in systemic circulation and lung. b) FoxP3+ levels in tissue resident memory T-cells. Statistics; non parametric Mann-Whitney t-test

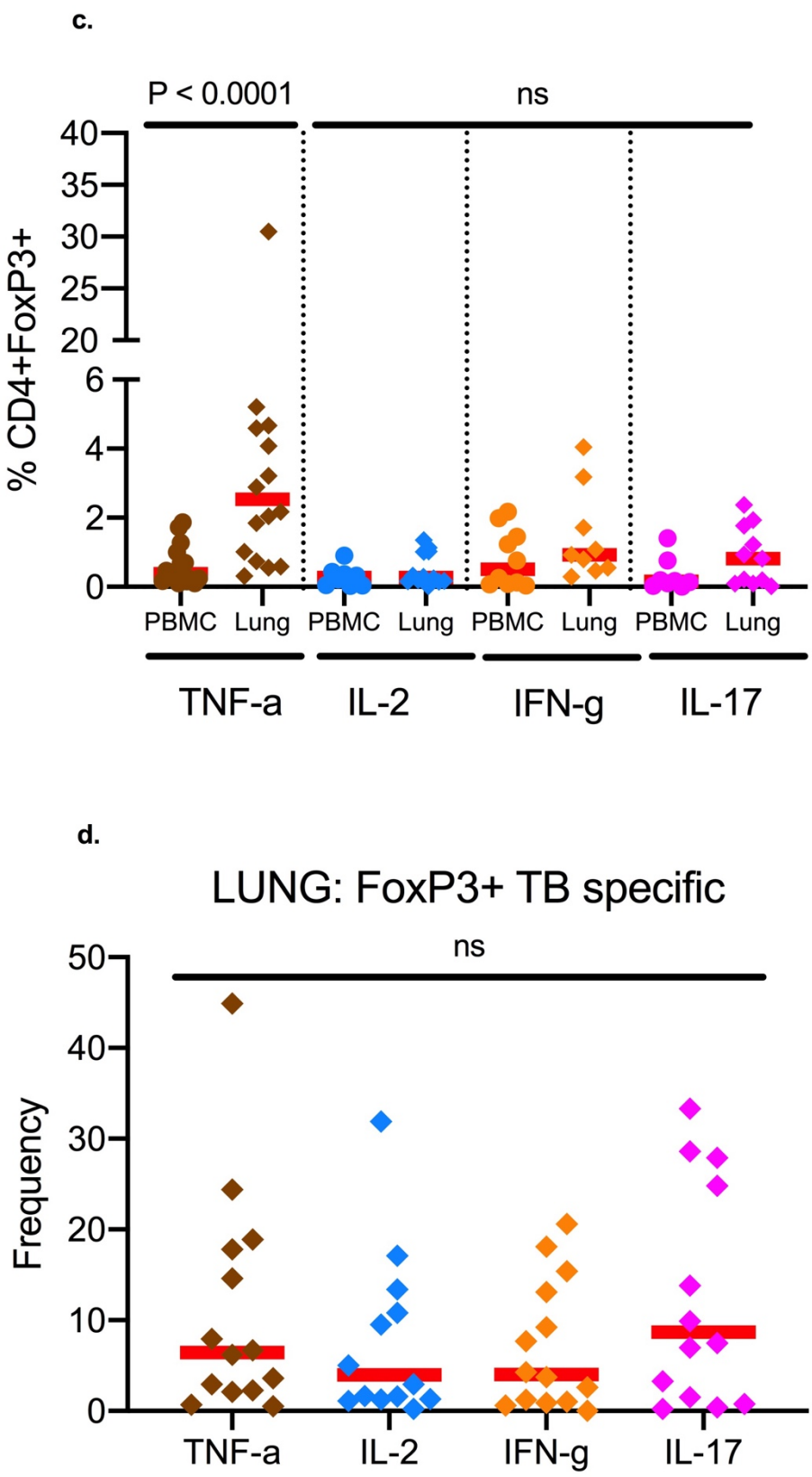


Figure 2.8: Regulatory T cells in the lung. (c) Comparison of cytokine positive cells that are FoxP3 expressing in peripheral blood and lung (d) Frequency of TB-specific cytokines production by FoxP3+ CD4+ T-cells in the lung. Statistics; non parametric Mann-Whitney t-test for two group comparisons.

2.3.4 Plasma cytokines and TB disease at site of infection and systemic circulation

Plasma cytokine levels have been used in TB to understand different aspects of the disease progression including response to treatment, the difference between latent and active TB and predict likelihood of relapse following treatment (Walzl et al. 2011; Mihret et al. 2014; Djoba Siawaya et al. 2009). Plasma cytokine levels of 14 different analytes were measured by Luminex assay and levels between active TB, previous TB and cancer control participant groups compared.

First, to determine the effect of treated HIV on plasma cytokine levels, active and previous TB participants were grouped into those with or without HIV. No statistically significant difference between HIV groups for all the analytes measured were observed (figure 2.9a). We therefore ignored HIV status and grouped individuals into those with ATB, previous TB and non-TB controls. Surprisingly, we detected no significant differences in cytokine levels between active and previous TB participants (figure 2.9b), and only for IL-4 did we detect a significant difference between subjects with ATB and non-TB cancer controls, ($p=0.0099$; figure 2.9b). However, although not significant, IL-10 was undetectable in 7/18 subjects with ATB compared to 1/21 subjects with either previous TB or non-TB cancer controls ($P = 0.0149$ Fisher's exact test). Interleukin -10 is a known regulator of immune response and has been reported in most infectious diseases including TB (Fiorentino et al. 1991; Redford, Murray, and O'Garra 2011). Therefore, to test if this indicated a subset on subjects with ATB in whom systemic disease was more prevalent, we next correlated IL-10 levels with other circulating cytokines. There was no correlation between IL-10 with any of the cytokines in the previous TB group. However, there was a significant positive correlation between IL-10 and the other cytokines except for TGF-β, IL-12(p70) and IL-13 in active TB group (figure 2.9c), supporting the hypothesis that some individuals in the ATB group have more extensive disease than others. In light of this, we next correlated plasma cytokine levels with lung and peripheral blood CD4:CD8 ratio. We observe a strong positive correlation between IL-10, IL-1β and IL-4 and lung CD4:CD8 in active TB group (figure 2.9 d), but not the previous TB group. No correlations were observed between any cytokine and peripheral CD4:CD8 count. In a small subset of participants (active TB and previous TB) in whom FoxP3+ T-cells were measured by flow cytometry, a correlation of the frequency of regulatory T-cells

(CD4+FoxP3+) in lung and peripheral blood was done. Lung T reg cells correlated positively with IL-12 (p40) while peripheral T reg cells positively correlated with IFN-α2 (figure 2.9e).

Finally, we asked whether TB specific T-cell responses in the lung correlated with plasma cytokine levels in a subset of participants with active TB or previous TB. The frequency of lung CD4+ cytokine producing cells after stimulation with MTB300 peptide compared with plasma cytokine levels. There were no correlations between TNF-a and IL-2 in the lung tissue with any of the plasma cytokines measured. However, we observed a negative correlation between IFN-γ in the lung and plasma IL-4 (figure 2.9f), and also a negative correlation between IL-17 in the lung and plasma IL-2 and IL-1ß (figure 2.9g). Although this analysis was done on a small number of the study participants such that active and previous TB cases could not be separated, the trend is generally the same between these two patient groups (figure 2.9 f-g). This observation suggests a role of lung Trm IL-17 and IFN-γ producing T-cells in the lung against TB disease.

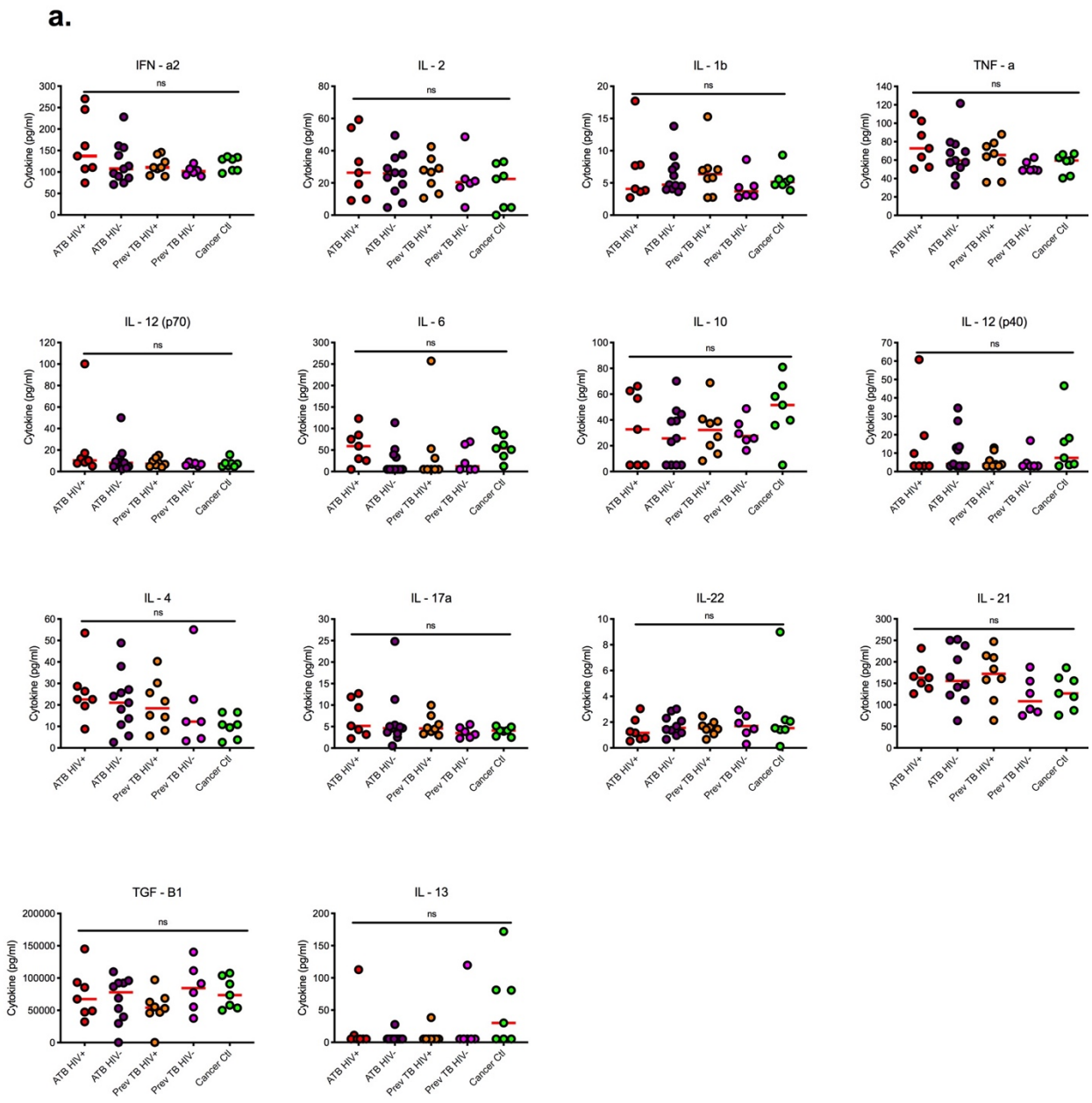


Figure 2.9: Plasma cytokine levels. HIV coinfection does not have an effect on plasma analytes in active TB and previous TB cases (a) Statistics; non parametric Mann-Whitney t-test for two groups comparisons.

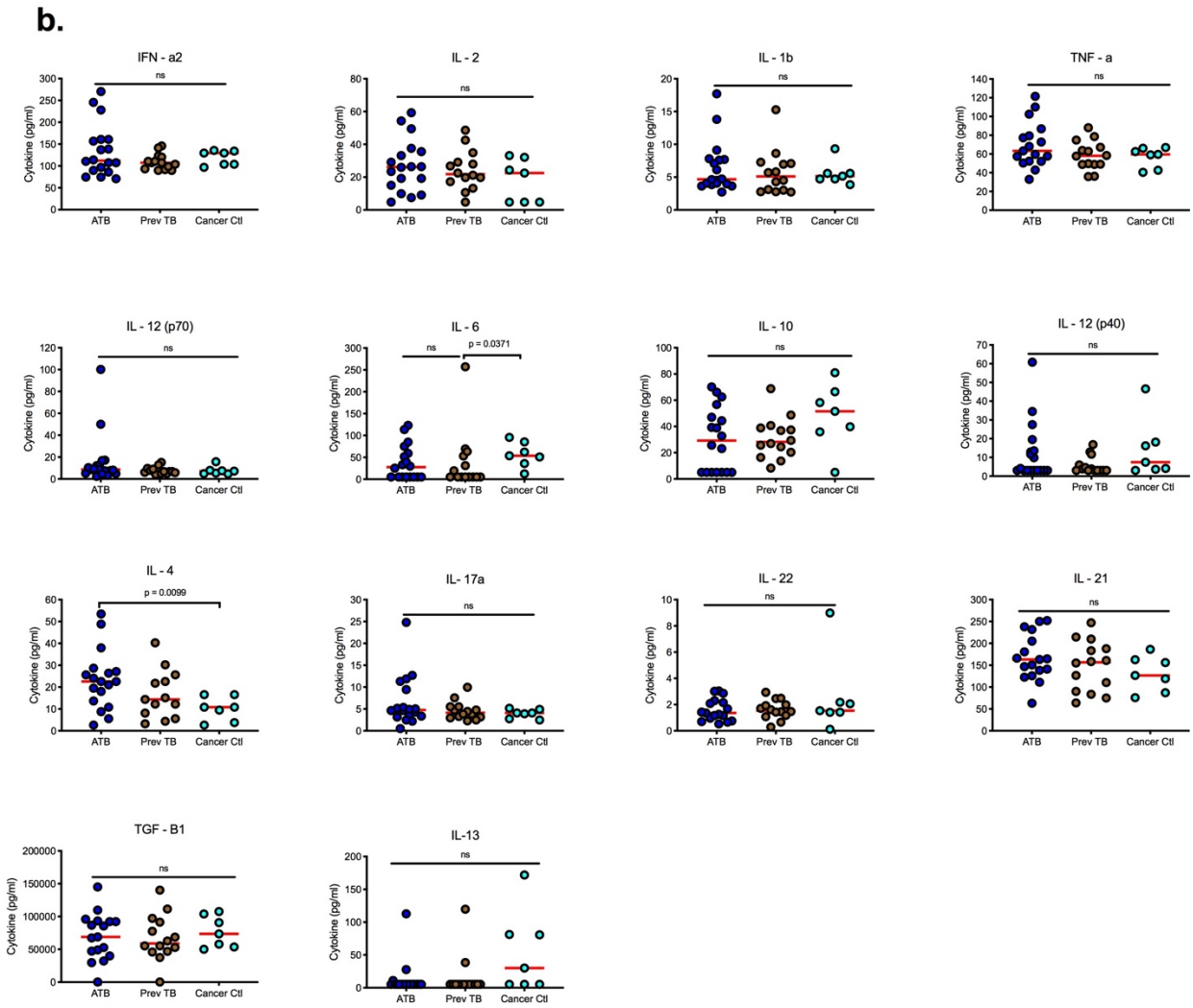


Figure 2.9: Plasma cytokine levels. No differences in most plasma cytokine levels between active TB and previous TB compared to cancer controls except for IL-6 and IL-4 (b). Statistics; non parametric Mann-Whitney t-test for two groups comparisons.

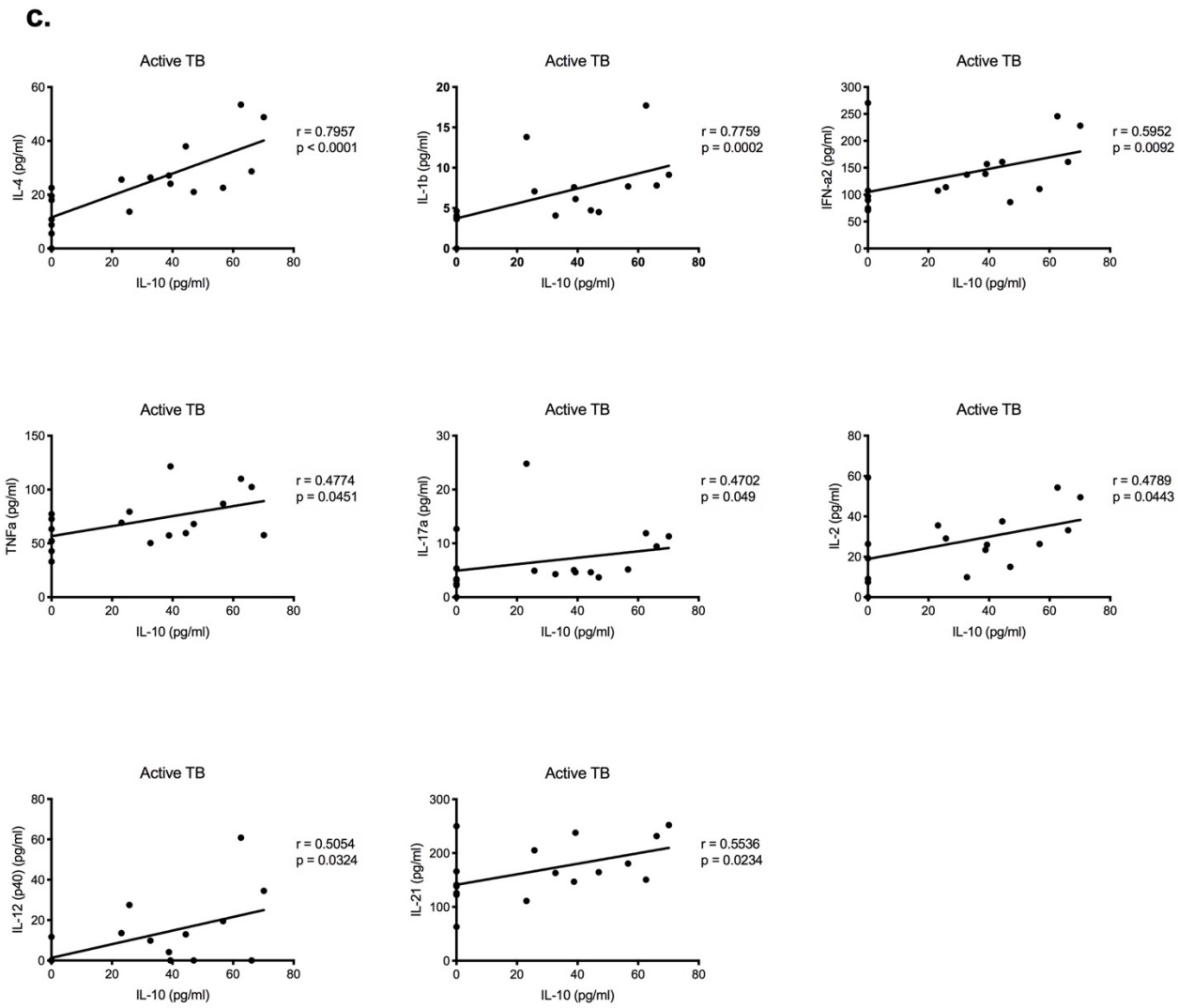


Figure 2.9: Plasma cytokine levels. (c) Positive Correlation of plasma cytokines with IL-10 for 8 of the 13 analytes measured.

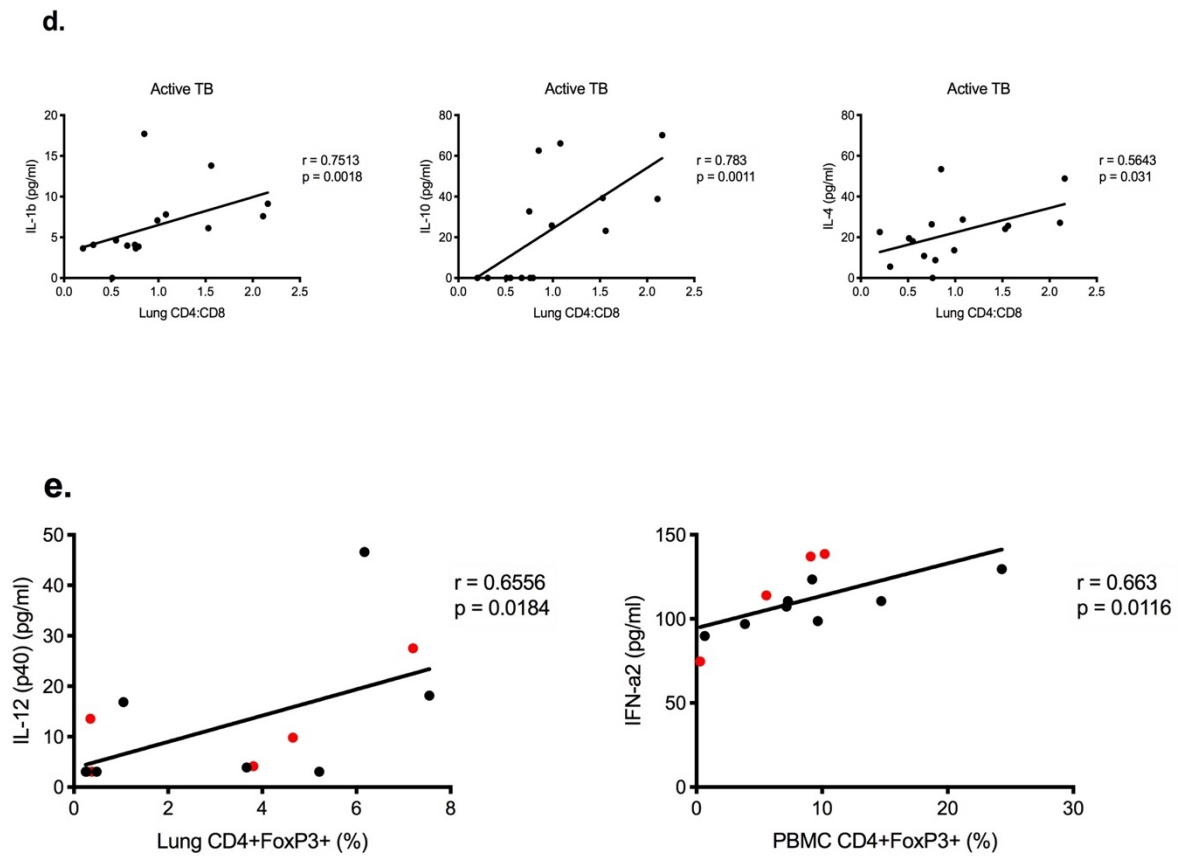


Figure 2.9: Plasma cytokine levels. Correlation of plasma cytokines with CD4:CD8 ratio in the lung of active TB participants (d) and FoxP3+ (e). Red data points (active TB), black data points previous TB.

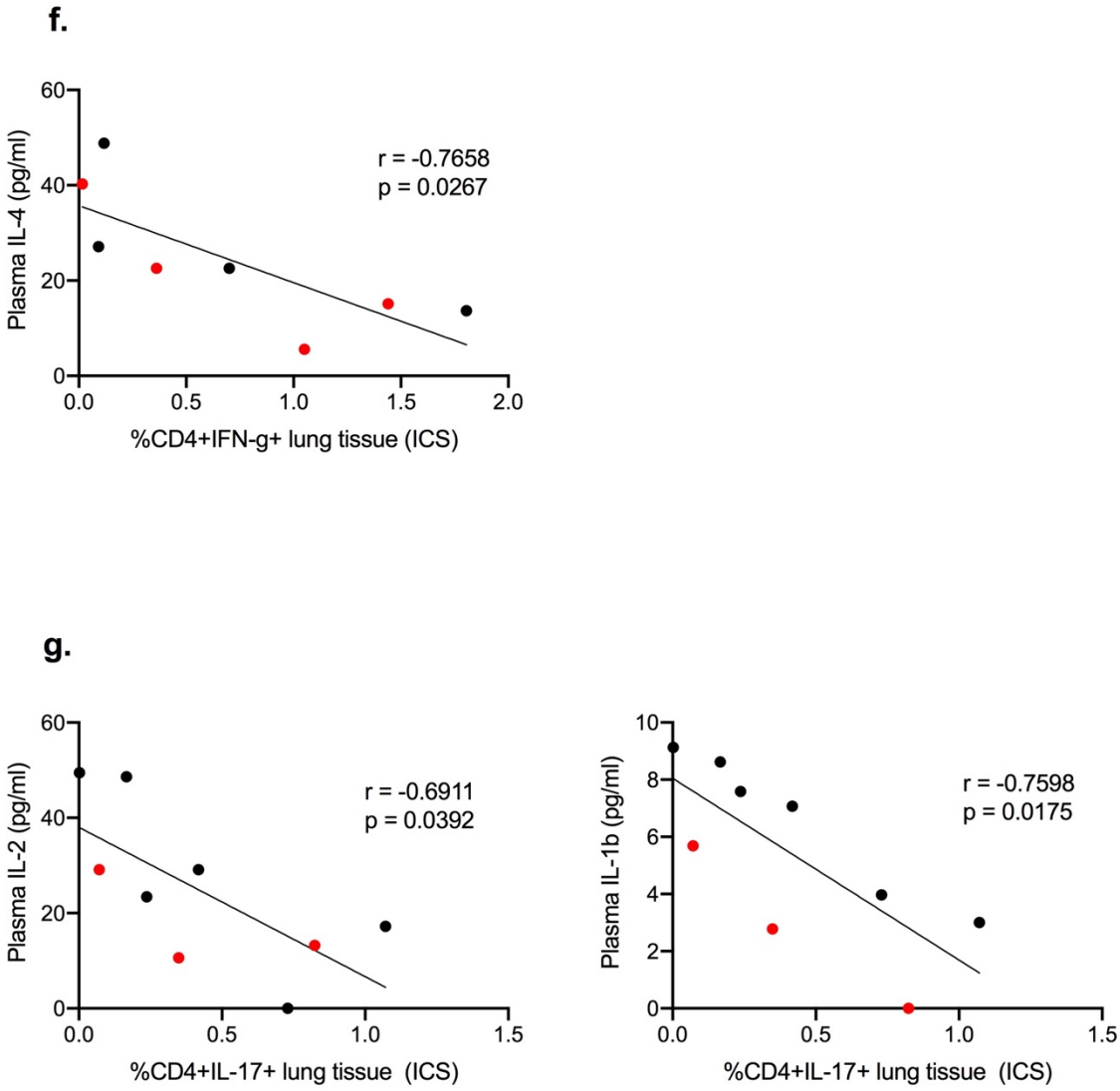


Figure 2.9: Plasma cytokine levels. (f-g) correlation of plasma cytokines with *Mtb*-specific CD4+ T-cells in the lung, IFN-γ producing CD4+ T-cells (f) and IL-17 producing CD4+ T-cells (g). Red data points (active TB), black data points previous TB.

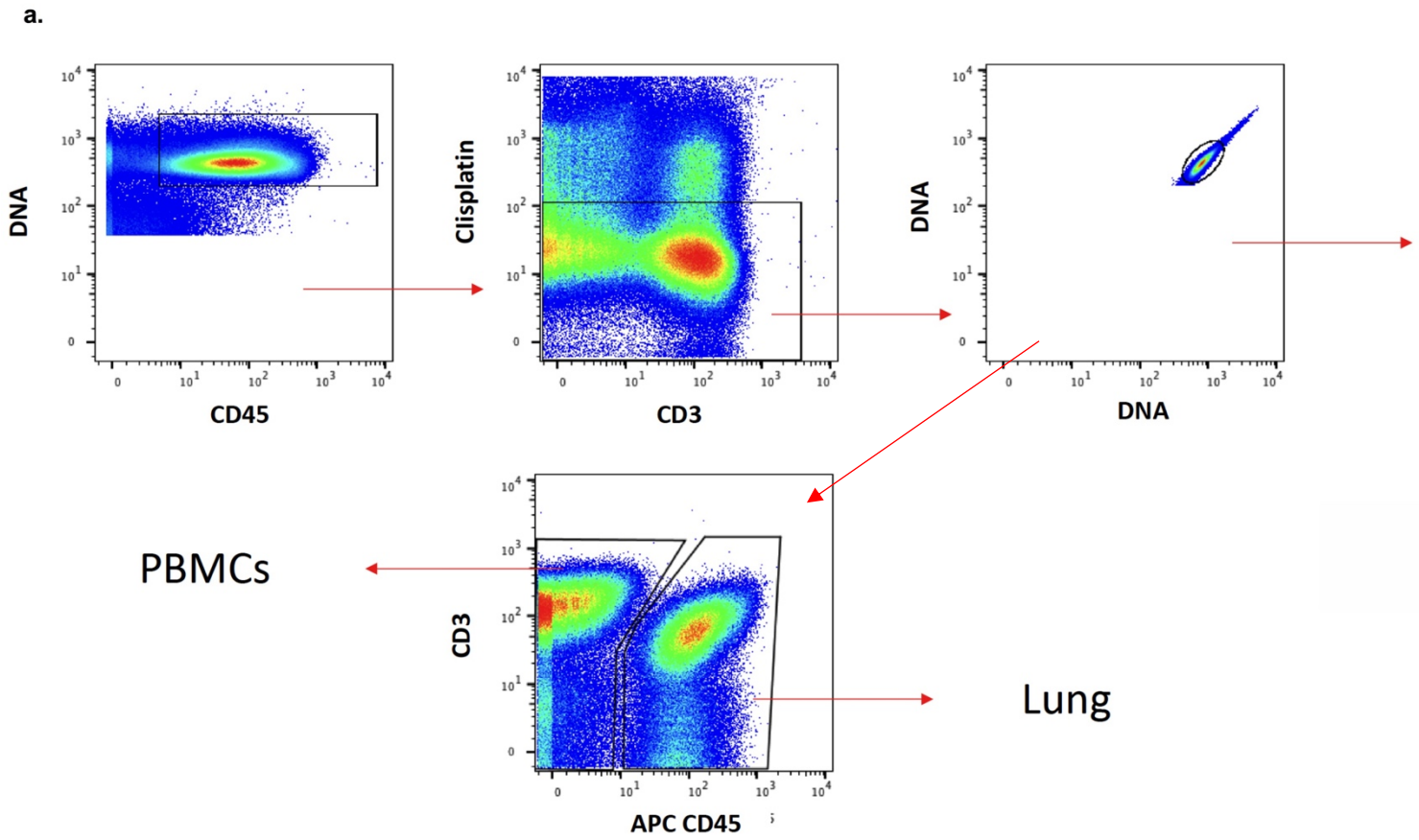
2.3.5 High-dimensional mass cytometry (CyTOF) immune profiling of lung tissue T-cells

All the data we have presented above looking at lung Trms have been generated on the basis of their identification by expression of CD103 and CD69 on T-cells as measured flow cytometry staining. However, these markers are not perfect and do not identify all the Trms. Indeed, although it is widely accepted as a good marker, there is some evidence that not all Trms constitutively express CD69 (Skon et al 2013; Schenkel and Masopust 2014). In addition, CD103 (Integrin αE) is just one of the many integrins that can interact with cadherins on the epithelial surfaces (Cepek et al 1994; Wakim et al 2010; Casey et al 2012).

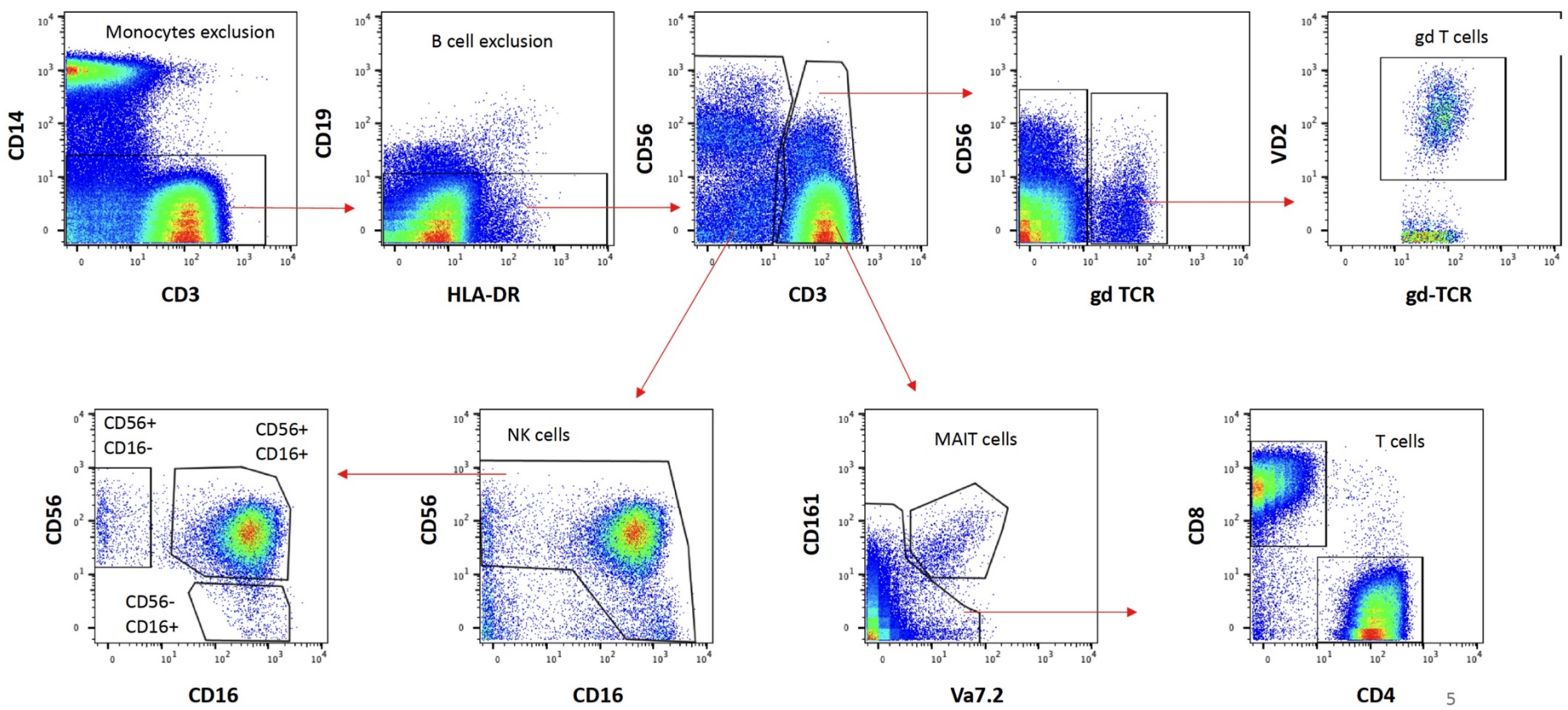
With these limitations in mind, we performed a pilot study on a few lung samples using a different approach to phenotype T-cells in the lung by mass cytometry (CyTOF). In CyTOF, antibodies are labelled with heavy metal ion tags (Bendall et al 2011; Finck et al 2013; Zunder et al 2015), and this is particularly important as it gets around the problem of autofluorescence which is a serious limitation, especially for lung cells. Furthermore, because fluorochrome spectral overlap is not an issue with CyTOF, it allows for staining of many markers in one single run than most flow cytometers can allow. The number of cells recovered from lung tissue can be a limiting factor at times due to the disease state of the patients. We therefore devised a method in which lung cells were pre-tagged with a fluorescent labelled CD45 antibody before mixing with autologous PBMC to bulk up the numbers to efficiently run on CyTOF. Once data is acquired, cells from the two compartments can be easily identified based on fluorescent labelled CD45 staining (figure 2.10a). Figure 2.10b shows the gating strategy used for cell subset identification based on respective lineage markers.

Using UMAP as dimensionality reduction technique (Becht et al. 2018), we show clustering of subsets of lung tissue CD4+ and CD8+ T-cells (figure 2.10 c-d). In agreement with the flow cytometry data, CD69 cells are clustered together with CD103 cells, and CD69 also clusters together with other integrins (ITGβ7 and CD49a), tissue homing chemokines (CCR5, CCR6) and does not cluster with CD62L, suggesting that CD69+ve is a marker for T-cells in the tissue and not circulation. Marker expression and intensities are shown as heatmaps with phenograph plots for lung tissue CD4+ and CD8+ T-cells (figure 2.10 e-d). Again considering CD69 expression, CD69+ve cells (identified as high intensity staining) also appears in high intensity staining for markers that have been associated with tissue

specific T-cells, such as integrins (CD103, ITGβ7 and CD49a), markers of tissue residency and/or activation (PD-1), tissue homing markers (CCR5 and CCR6) and low intensity expression of CD62L. In the context of TB disease, markers that have been associated with a protective T-cell phenotype such as CXCR3, PD-1, ICOS (Sakai et al 2014 and 2016, Moguche et al 2015, Bull et al 2018) also showed high intensity with CD69+ve cells. Similarly, markers associated with poor outcomes in Mtb infection (KLRG1) lowly expressed on CD69+ve cells (Sakai et al 2014). From the heatmaps, it is evident that the lung tissue has very low levels of NK cells based on CD16 and CD56 staining intensity. Thus, with all caveats in place, such as cell loss during tissue processing, CD69 in combination with CD103 that we used in the flow cytometry panel identified most of the lung Trms as confirmed by CyTOF as an alternative staining approach.


a.
DNA
CD45
Clisplatin
CD3
DNA
DNA
CD3
APC CD45
PBMCs
Lung

b.


Monocytes exclusion
CD14
CD3
B cell exclusion
CD19
HLA-DR
CD56
CD3
CD56
gd TCR
gd T cells
VD2
gd-TCR
CD56+
CD16-
CD56+
CD16+
CD56-
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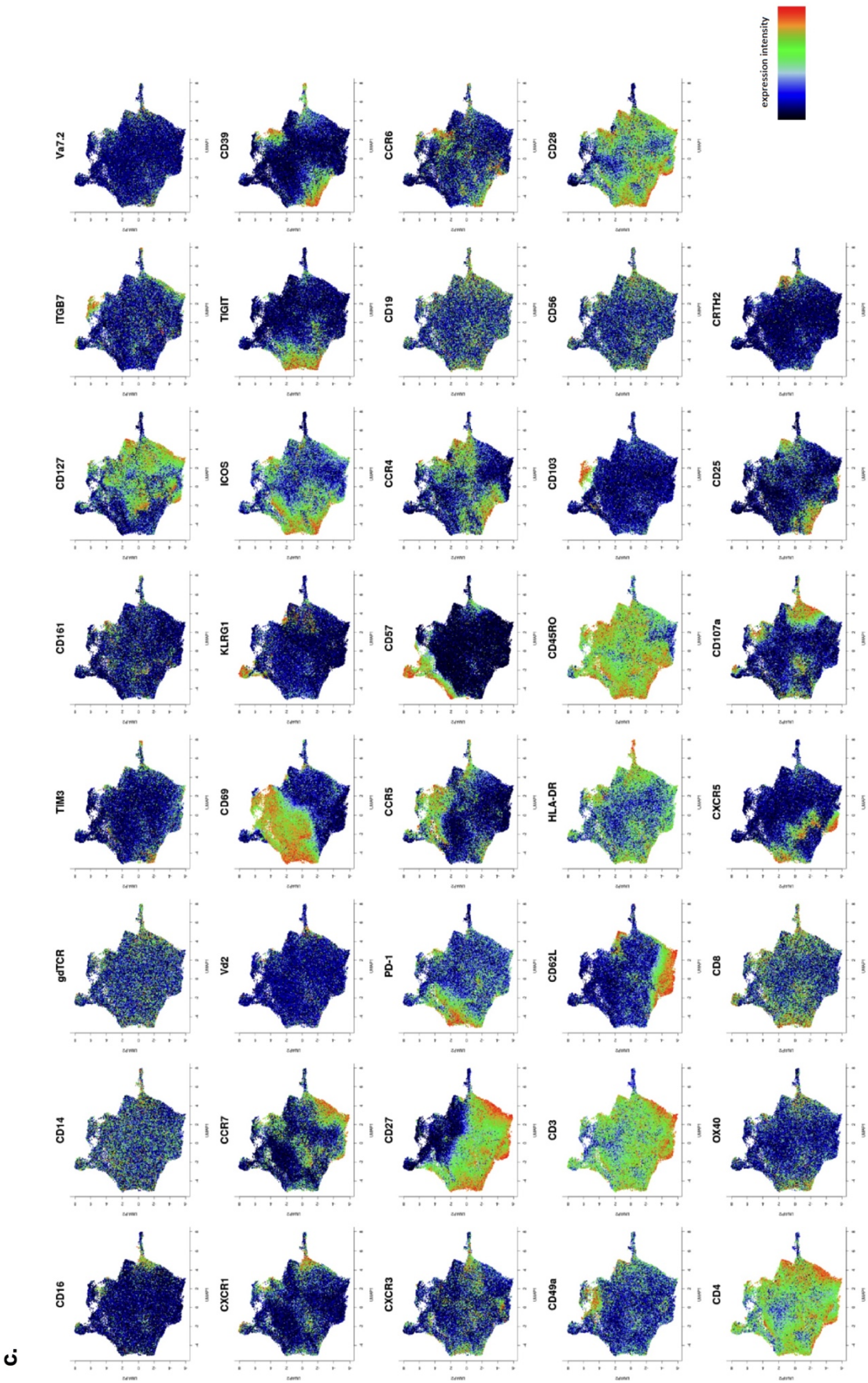
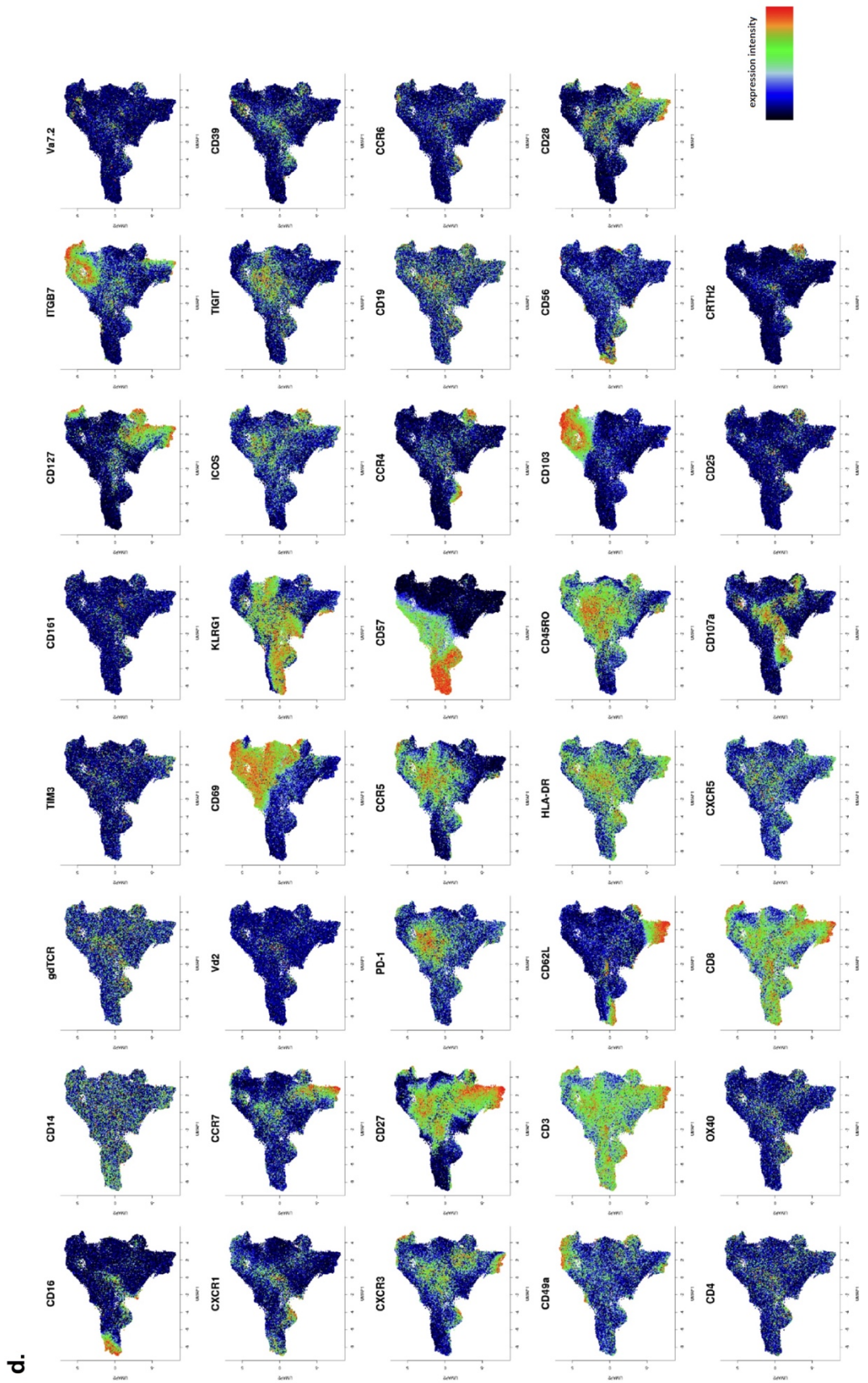
CD56
CD16
NK cells
CD56
CD16
MAIT cells
CD161
Va7.2
T cells
CD8
CD4


c
CD16
CXCR1
CD49a
CD4
CD14
CCR7
CXCR3
CD27
CD3
OX40
gdTCR
Vd2
PD-1
CD62L
CD8
TIM3
CD69
CCR5
HLA-DR
CXCR5
CD161
KLRG1
CD57
CD45RO
CD107a
CD127
ICOS
CCR4
CD103
CD25
ITGB7
TIGIT
CD19
CD56
CRTH2
Va7.2
CD39
CCR6
CD28
expression intensity


d.
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CD14
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TIM3
CD161
CD127
ITGB7
Va7.2
CXCR1
CCR7
Vd2
CD69
KLRG1
ICOS
CD39
CD4
CXCR3
CD27
PD-1
CCR5
CD57
CCR4
TIGIT
CCR6
CD49a
CD3
CD62L
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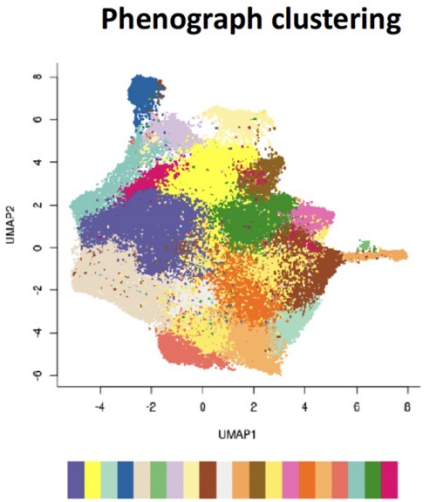
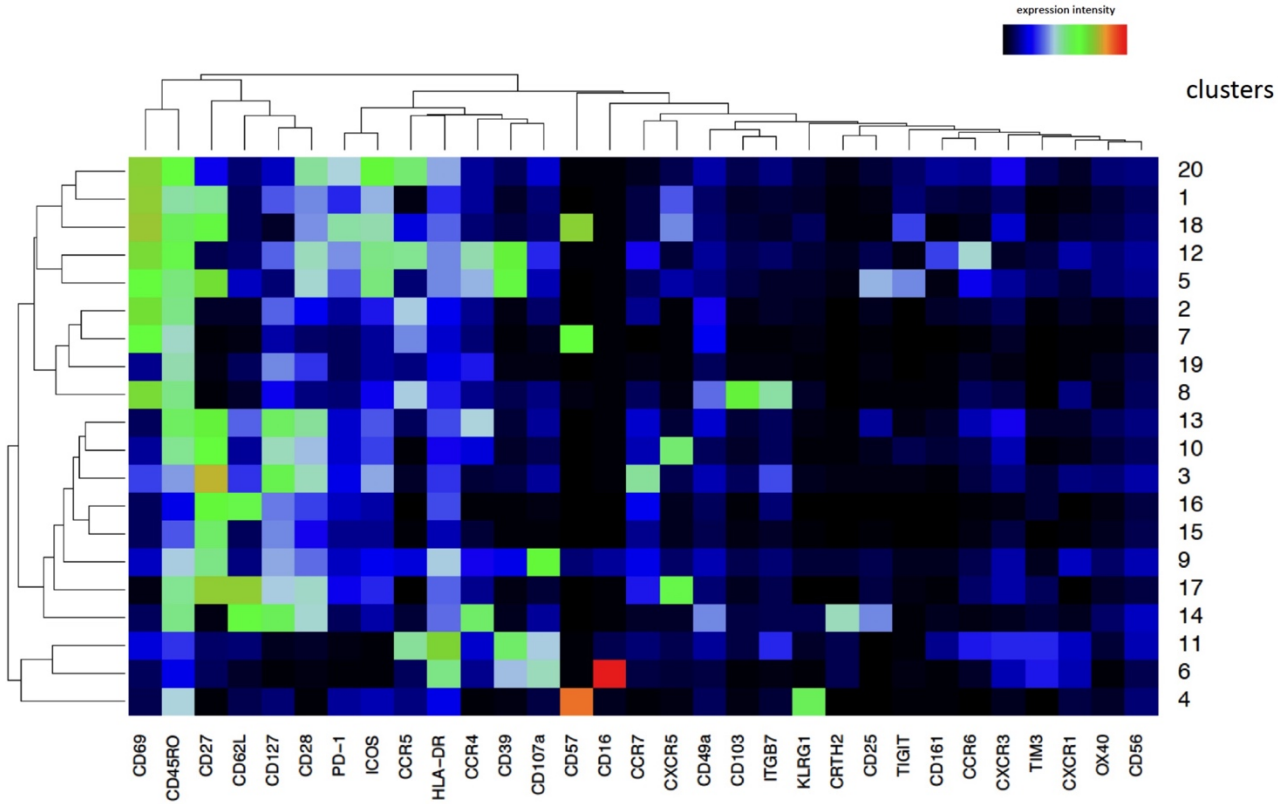
CD25
CD56
CRTH2
expression intensity

e.


Phenograph clustering
UMAP2
UMAP1
Cluster 1-20


expression intensity
clusters
20
1
18
12
5
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19
8
13
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16
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9
17
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11
6
4
CD69
CD45RO
CD27
CD62L
CD127
CD28
PD−1
ICOS
CCR5
HLA−DR
CCR4
CD39
CD107a
CD57
CD16
CCR7
CXCR5
CD49a
CD103
ITGB7
KLRG1
CRTH2
CD25
TIGIT
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CCR6
CXCR3
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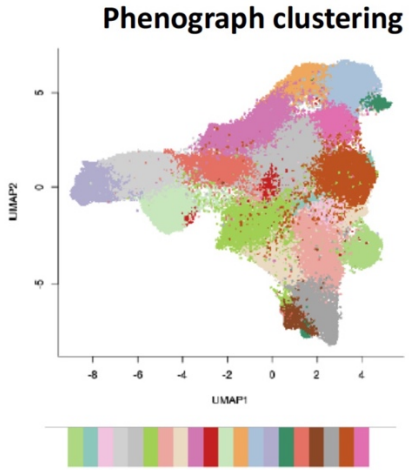
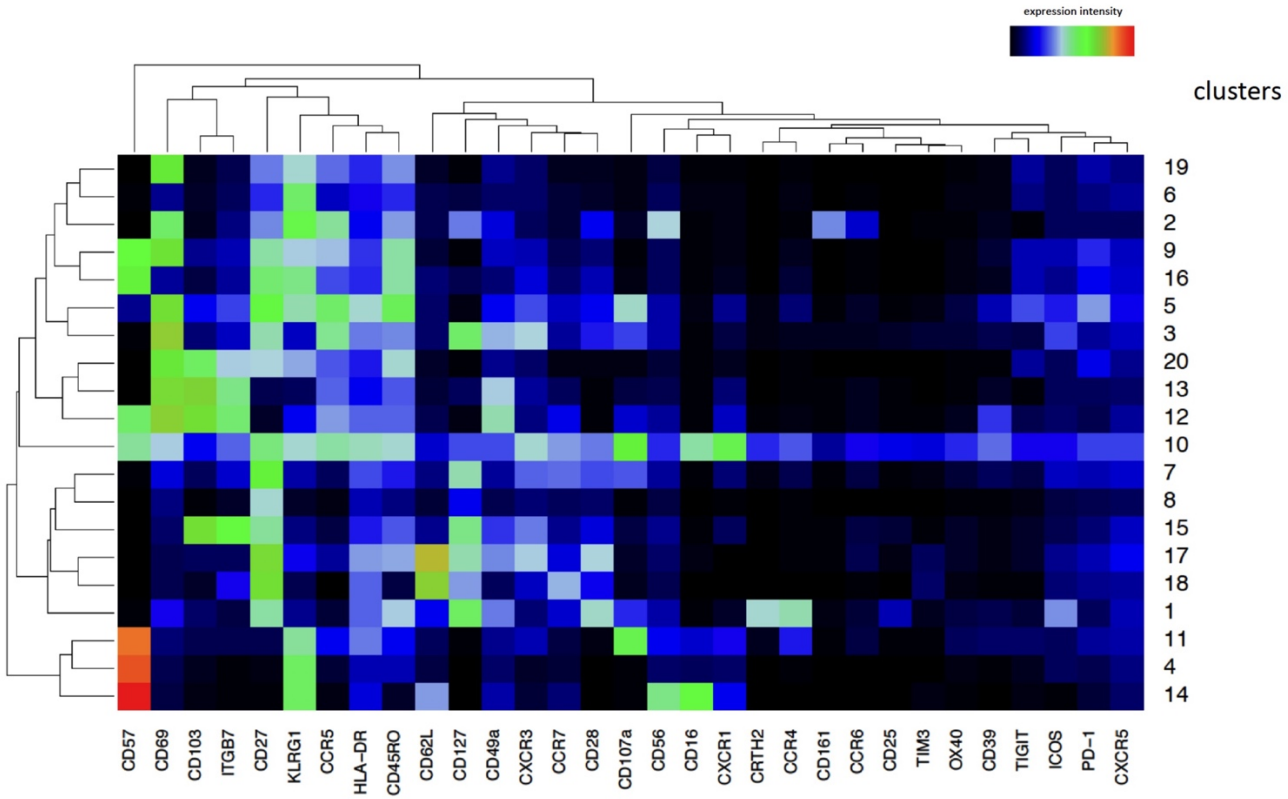
CXCR1
OX40
CD56

f.


Phenograph clustering
UMAP2
UMAP1
Cluster 1-20


expression intensity
clusters
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6
2
9
16
5
3
20
13
12
10
7
8
15
17
18
1
11
4
14
CD57
CD69
CD103
ITGB7
CD27
KLRG1
CCR5
HLA−DR
CD45RO
CD62L
CD127
CD49a
CXCR3
CCR7
CD28
CD107a
CD56
CD16
CXCR1
CRTH2
CCR4
CD161
CCR6
CD25
TIM3
OX40
CD39
TIGIT
ICOS
PD−1
CXCR5

Figure 2.10: Mass cytometry (CyTOF) high dimensional phenotyping of lung T-cells. (a) Representative plots for live cell barcoding strategy and exclusion of PBMC from lung tissue cells based on APC-CD45 staining. (b) Representative plots for lineage markers classical gating strategy to identify cells subsets. (c- d) Lung T-cell subset composition – high density visualization based on UMAP plotted as UMAP1 (x-axis) vs UMAP2 (y-axis) for each cell type. Lung tissue CD4+ T-cells (c) and Lung tissue CD8+ T-cells (d). (e-f) Heatmap of staining intensity of T-cell subsets. Lung tissue CD4+ T-cells (e) and CD8+ T-cells (f), phonograph clustering (left) identified 20 clusters (cluster 1-20).

2.4 Discussion

Our knowledge about human T-cell immune responses during TB disease has been generated from studies done on peripheral blood. Despite this vast data from peripheral blood, these studies have not shown consistent and reliable correlates of protection against *Mtb* infection, a key pre-requisite for an effective vaccine. With this limitation, it is imperative that more studies in the human lung, the primary site in *Mtb* infection, are conducted to identify potential correlates of protection. In this study we have provided a detailed analysis of T-cell phenotype, functionality and specificity in fresh human lung tissue mononuclear cells in comparison with T-cells in circulation in participants with TB disease with or without HIV coinfection. As a control, we also analyzed lung tissue and blood samples from lung cancer participants. The definition of participant groups is outlined in the methodology section.

To study cells in the lung tissue, we had to first identify T-cells that are resident in the lung which do not recirculate in blood. Although there are different techniques of identifying tissue specific T-cells mostly worked out in mouse experiments, such as parabiosis, intravenous labelling of circulating cells just prior to sacrifice to distinguish cells in circulation (labelled) from those in tissue (unlabelled), and the use of Fingolimod (FTY720), a drug which prevents T-cell egress from lymph nodes and thus blocks new recruitment of memory cells into tissue sites, these techniques are not applicable in humans. However, they have confirmed that many of the cell surface markers used in animal models are shared in humans, notably high CD69 expression together with CD103 (Thome et al 2014; Kumar et al 2017). Thus, we used these two cell surface markers of tissue resident T-cells together with CD62L to identify lung Trms as previously reported by others (Hombrink et al 2016, Oja et al 2017, Pizzolla et al 2017, Sathaliyawala et al 2013).

In comparison to peripheral blood, lung T-cells can be divided into two subsets based on their CD69 expression; CD69- and CD69+. In the tissue, CD69+ cells also express CD103 resulting in a tissue specific subset of CD103+CD69+ T-cells. Furthermore, lung CD69+ T-cells down regulate the expression of CD62L. Tissue-resident memory T-cells are derived from precursors that entered tissues during the effector phase of immune responses and remained positioned within this compartment (Schenkel and Masopust 2014). Once in the tissues, recruited T-cells must resist tissue egress cues to remain at these sites for extended periods and establish residency. One mechanism of egress from tissues depends on gradual T-cell sensitization to sphingosine-1 phosphate receptor 1 (S1PR1)-dependent chemotactic

gradients (Arnon et al. 2011; Lo et al. 2005; Shannon et al. 2012). Thus, T-cells in tissue downregulate S1PR1 by transiently reducing expression of the transcription factor kruppel-like factor 2 (KLF2) (Preston et al. 2013; Skon et al. 2013; Grayson et al. 2001). S1PR1 downregulation is concomitantly associated with upregulation of CD69 expression (Cyster and Schwab 2012; Matloubian et al. 2004; Schober et al. 1999; Shiow et al. 2006; Skon et al. 2013). It is noteworthy that tissue egress being a continuous process would explain the variability in CD69 expression in the lung tissue as reported here. Direct comparison of T-cells based on CD69 expression in circulation and tissue show that T-cells in these two compartments are different suggesting that tissue resident T-cell phenotypic signatures are acquired only after migration to destination tissues of residence as proposed by previous literature (Schenkel and Masopust 2014). Indeed, CD69 is induced on tissue resident memory T-cells after they migrate to sites of residence (Mackay et al. 2013; Masopust et al. 2006; Skon et al. 2013).

On the other hand, CD103 expression is associated with intestinal immune cell populations and tissue infiltrating effector CD8+ T-cells in mice (Schön et al. 1999; Sheridan and Lefrançois 2011) and epithelial barrier of human lung (Hombrink et al. 2016; Oja et al. 2017). We have shown that there is up to ten-fold enrichment of CD103 expression on lung tissue T-cells compared to circulation suggesting that most of the lung CD103+ cells are not recirculating. It has also been reported that the majority of putative tissue resident memory cells in many organs do not express CD103 (Casey et al. 2012; Schenkel, Fraser, and Masopust 2014; Beura et al. 2018). Thus, while CD103 expression is indicative of residence, it is likely that many memory tissue resident T-cells do not depend on, nor even express CD103.

As expected, lung tissue T-cells down regulate the expression of CD62L compared to T-cells in circulation. CD62L (L-selectin) belongs to the family of cell adhesion molecules which contribute to leukocyte tethering and rolling along the luminal surface of venules (Shenkel and Masopust 2014). When T-cells enter tissues, they downregulate the expression of CD62L to prevent re-entry into the circulation thereby establishing residency.

One of the ways in which tissue resident T-cells are maintained at the site of infection is when the memory T-cells that enter the infection site from circulation engage their cognate antigen (Schenkel and Masopust 2014; von Andrian and Mackay 2000; Ulrich H. von

Andrian and Mempel 2003; Beura et al. 2015). The observation that total lung CD4+ and tissue resident T-cells are higher in active TB cases than previous TB cases or cancer control participants agrees with this hypothesis since a fraction of the total CD4+ T-cells that enter the lung would include memory recirculating T-cells that engage the cognate antigen in the lung. This would lead to downregulation of S1PR1that occurs concomitantly with increased CD69 expression.

Previously, in a nonhuman primate TB model, it was reported that T-cells are continuously recruited to the non-sterile granulomas, resulting in increased total cell count, T-cell number and increase size of the granuloma, while the sterile granulomas are maintained with the minimum required cell numbers for the continued maintenance (Gideon et al. 2015). It is probable that this is the case for previous TB participants who had eliminated TB antigens by chemotherapy resulting in less recruitment of cells to the lung.

Apart from the cell surface markers, we used T-cell differentiation to identify phenotypic differences between T-cells in the lung and those in peripheral blood. We reasoned that T-cells in lung tissue would be more activated by the pathogens they encounter in the tissue. Therefore, the expression of the lymph node homing receptor CCR7 together with CD45RA was analyzed to define the functional subsets of T-cells (Rufer et al. 2003; Sallusto, Geginat, and Lanzavecchia 2004). In comparison with peripheral T-cells that are mainly of naïve phenotype, lung tissue T-cells have predominantly effector memory phenotype. This observation is in agreement with an earlier study where cells from different tissues – spleen, lung, ileum, jejunum, colon and lung lymph node – were mainly effector memory while blood cells were majorly of naive phenotype (Sathaliyawala et al. 2013). These results demonstrate that the differentiation and maintenance of CD4+ and CD8+ T-cells within an individual is compartmentalized with T-cells in lung tissue being more differentiated most likely in response to the pathogens encountered in the airway. Importantly, we also observed that within the lung, CD69+ T-cells were more effector memory than CD69- T-cells, again suggesting that CD69+ T-cells in the lung are different from cells that are CD69- which mostly consist of blood contaminating cells from circulation.

Functionality of T-cells is often measured by their ability to produce cytokines upon *in vitro* stimulation. T-cells secreting IFN-γ, TNF-α and IL-17 are generally assumed to be necessary for activation of macrophages and initiation of antimicrobial activity (O'Garra et al. 2013; Algood, Lin, and Flynn 2005; Peters and Ernst 2003). Thus, we measured the production of TNF-α, IL-2, IFN-γ and IL-17 by lung tissue and peripheral blood T-cells after non-specific stimulation with PMA + ionomycin. In general, lung tissue T-cells had a higher median frequency of cytokines TNF-α, IL-2, IFN-γ and IL-17 than peripheral blood T-cells. The marked enrichment of Th17 (IL-17 producing) cells in the lung tissue compared to peripheral blood is worth mentioning as these cells have been reported to be involved in early control *Mtb* infection (Cruz et al. 2010; Nandi and Behar 2011; Treerat et al. 2017). This increased functionality of lung tissue T-cells can be explained in part by the fact that they are mostly effector memory while peripheral blood is of naive phenotype. *Ex-vivo* stimulation of lung T-cells revealed that CD69+ cells but not CD103+ cells were upregulated suggesting the role of CD69 as an activation marker (Meng et al. 2016; Chawansuntati et al. 2015) in addition to being a marker of tissue residency. Within the lung tissue, most cytokine production came from bona fide Trm cells, that is, CD69+CD103+ and/or CD69+CD103- T-cell subsets.

Having established that lung T-cells were superior cytokine producers, polyfunctional and enriched for Th17 cells, we addressed the issue of their TB specificity. To do this, lung tissue cells and PBMCs were stimulated with an MHC class II restricted peptide pool of TB antigens – MTB300 (Lindestam Arlehamn et al. 2013) for 5 hours. We hypothesized that TB-specific T-cell responses are enriched at the site of infection compared to circulation as it has also been reported that responses to tissue specific diseases at the site of infection are not necessarily mirrored in circulation (Gideon et al. 2015; Gordon et al. 2017). We have shown that TB-specific responses are enriched at the site of infection than in circulation for TNF-α, IL-2 and IL-17 cytokines but surprisingly not for IFN-γ. Again, the enrichment of TB-specific Th17 cells in the lung is commendable. Our results show that it is more likely to detect IL-17 production in the lung than in peripheral blood, suggesting that these cells are probably absent in circulation during chronic TB or they do exist in much lower frequency in blood that they are often missed. Previous study looking at BAL TB-specific cytokine responses also reported enriched cytokine production at the site of disease (BAL) than PBMC (Jambo et al. 2011). Unlike in non-specific stimulation, *Mtb*-specific lung tissue CD4+ T-cells were mainly CD103-CD69+ and CD103-CD69- subsets. This observation is in line with

the suggestion that lung homogenate consists of two populations of memory T-cells; recirculating and localized memory T-cell subsets as reported in mouse models. However, even the CD103-CD69- cells in the lung are not the same as blood T-cells as we observed lower frequency of these cytokines except IFN-γ in blood than lung. Previous studies in the mouse model reported that the pulmonary CD4 T-cell response against *Mtb* is comprised of two major subsets that either enter the lung parenchyma or reside within the vasculature (Reiley et al. 2010; Sakai et al. 2014).

Multiple cytokine production by antigen specific T-cells is an important measurement of their functionality, and is thought to be associated with antigen load (Harari et al. 2005; Millington et al. 2007). As to which cytokine is the most important contributor on its own to the control of *Mtb*, the available literature is divided in their conclusion. Whereas studies done in human peripheral blood suggest that IFN-γ is the key cytokine produced by CD4+ T-cells (Green, Difazio, and Flynn 2013; Tameris et al. 2013; Jasenosky et al. 2015; Urdahl 2014) it was reported that protective lung pulmonary CD4+ T-cells produce less IFN-γ compared to non-protective lung CD4+ T-cells in the mouse model (Sakai et al. 2014). In our hands, compared to cancer control lung, there was a higher frequency of TNF-α+IL-2+ and TNF-α+IL-17+ bifunctional T-cells in active TB participants suggesting important roles for IL-2 and IL-17 cytokines. It was only TNF-α+IL-17+ bifunctional T-cells that was different between cancer control and active TB in systemic circulation. Given that we used an MHC class II restricted peptide pool to test the specificity of T-cells in the lung, we were unable to pick any CD8+ T-cell responses and we cannot rule out the contribution of this T-cell subset immune response in TB at the site of disease in humans.

Next, we investigated other T-cell features that have impact on T-cell immune responses to infections such as immune regulation and exhaustion. Regulatory T-cells play a central role in the prevention of autoimmunity and in the control of immune responses by down-regulating the effector function of T-cells (Semple et al. 2013). Thus, we measured the level FoxP3 on T-cells in lung tissue and peripheral blood. Contrary to our expectation, we found a high expression of FoxP3+ in circulation than at the site of TB disease. Our findings are in contrast with an earlier study in which T-regs at the site of disease were higher than in the blood of TB participants (Geffner et al. 2014). The difference between our study and Geffner et al could be explained by the difference in samples studied (lung tissue vs pleural fluid)

and/or the stage of TB (chronic TB vs early diagnosed TB pleurisy). From studies in peripheral blood, available data is inconclusive. Higher percentage of CD4+FoxP3+ T-cells in the blood of TB participants compared to healthy controls (Chen et al. 2007; Guyot-Revol et al. 2006; Hougardy et al. 2007), no difference in the frequency of CD4+CD25+FoxP3+ T-regs between TB participants and healthy controls (Chiacchio et al. 2009), and a lower frequency of T-regs in TB participants than healthy controls living in a TB endemic area (Roberts et al. 2007) have been reported. Within the lung tissue, CD69+ T-cells were enriched for FoxP3+ compared to CD103+ cells. Indeed, CD69 has been reported to control T-cell differentiation (de la Fuente et al. 2014). However, and of particular interest, we observed that the TB specific T-cells at the site of TB disease were enriched for T-regs than TB specific T-cells in circulation suggesting that too much immune regulation of *Mtb*-specific T-cells occurs in the lung, and probably explains the ineffectiveness of enriched T-cell responses in the lung to control the infection. A possible limitation of our definition of regulatory T-cells is the lack of CD25 in our antibody staining panel due to many parameters we measured simultaneously and the limitation of number of colours our flow cytometer could accommodate. We argued, however, that measuring transcription factor, FoxP3, was a better indicator regulatory cells than using CD25 alone which is also expressed by activated T-cells.

In chronic infections, including TB, T-cells can become exhausted (Egen et al. 2011; Fenhalls et al. 2000; Theron et al. 2012). Therefore, we measured the expression of T-cell exhaustion markers – PD-1, CTLA-4 and TIM-3 – in lung homogenate and peripheral blood T-cells. The role of PD-1 in *Mtb* infection is particularly important as it has been shown in mouse studies that PD-1 is essential to prevent immune pathology from excessive IFN-γ production (Sakai et al 2016), mice lacking PD-1 are highly susceptible to *Mtb* infection (Lázár-Molnár et al. 2010), and protective role of PD-1+ve T-cells in non-human primates has also been reported (Moguche et al 2015). In humans, individuals receiving PD-1 blockade immunotherapy for cancer treatment are at an increased risk of reactivating TB disease (Barber et al 2019). We observed that PD-1 expression was variable in both lung tissue and peripheral blood, although more variable in the lung. Within the lung, PD-1 expression was predominantly higher in CD69+ T-cells than CD69- T-cells, in agreement with the fact that PD-1 can also be a marker of activated T-cells. There was a general trend of CTLA-4 and TIM-3 lower expression in the lung tissue compared to peripheral blood but we

did not detect T-cells that co-expressed both PD-1 with either TIM-3 or CTLA-4 as was observed by others in the mouse model (Jayaraman et al 2016; Booty et al. 2016).

Immune activation and tissue destruction in the lung can be inferred from the analytes measured in circulation. Thus, we measured cytokines in plasma by Luminex. The cytokine levels between active and previous TB were identical except for TGF-1β, IL-4 and IL-6 where the median levels were less in the previous TB group. To a large extent, these findings do not agree with previous studies, in which there were more clear differences between TB groups studied (Mihret et al. 2014; Djoba Siawaya et al. 2009; Walzl et al. 2011). However, our results need to be put into context in comparison to earlier reports. First, majority of the participants in our TB groups have documented history of long-term lung tissue destruction from other conditions like bronchiectasis, aspergilloma and shrunken lung tissue all of which would contribute to immune activation. Second, active TB group participants are on anti-TB therapy at the time of lung resection thus the effect of treatment on plasma cytokines cannot be ruled out. Thirdly, although cancer control group have no documented history of TB, immune activation from metastasis of lung cells could probably have an impact on the analytes measured in circulation.

Our cytokine results in conjunction with tissue specific measurements described earlier allowed us to make interesting observations and revealed important differences. First, since IL-10 is a well-known immune regulator (Fiorentino et al. 1991; Couper, Blount, and Riley 2008; Redford, Murray, and O'Garra 2011), IL-10 levels was correlated with the rest of the cytokines and only in active TB group was there correlation with cytokines IL-4, IL-1β, IFN-α2, TNF-α, IL-17a, IL-2, IL-21, IL-12 (p40) and IL-12(p70). This observation suggests that more tissue destruction of the lung during active TB results in production of inflammatory cytokines that are released into the general circulation. Secondly, only in active TB did IL-1β, IL-10 and IL-4 correlate positively with CD4:CD8 ratio in the lung and not peripheral circulation, no such association was observed in the previous TB group. Thirdly, frequency of FoxP3+ cells in lung correlated with IL-12 (p40) while peripheral blood T-regs correlated positively with IFN-α2. These two observations highlight the differences in the impact of TB infection on lung tissue and blood. Surprisingly, there was no correlation between IL-10 and CD4+FoxP3+ suggesting that plasma IL-10 could be produced by other immune cells, for example, monocytes and macrophages are a major source of IL-10, during TB disease.

Indeed, dendritic cells have been shown to produce IL-10 during *Mycobacterium* infection (Demangel, Bertolino, and Britton 2002; Madura Larsen et al. 2007; Roque et al. 2007; Couper, Blount, and Riley 2008). Lastly, and most importantly, we were able to compare the level of *Mtb*-specific T-cells in lung tissue with plasma cytokine markers of inflammation as an indicator of disease severity. We found a negative correlation between plasma IL-4 and CD4+ IFN-γ+ lung tissue T-cells, and negative correlation of both IL-2 and IL-1β with CD4+ IL-17+ lung tissue T-cells. The association with IL-17 producing T-cells in the lung is noteworthy as this subset of cells was also less frequent in peripheral circulation.

Loss of CD4+ T-cells through HIV coinfection exacerbates TB susceptibility and reactivation of latent TB (Day et al 2008; Geldmacher et al 2010; Nemes et al 2018; Bell and Noursadeghi 2018). We simultaneously investigated the impact of HIV coinfection on Trm phenotype, function and specificity. We observed that HIV coinfection modulates recruitment of T-cells to the site of disease during *Mtb* infection by dysregulating recruitment of CD4+ T-cells to the lung of during TB. In the absence of HIV, frequency of tissue resident T-cells in lung expands in active TB disease which is diminished after clearance of TB antigens. We found a decreased production of TNF-α, IL-2 and IL-17 in participants coinfected with HIV than those without HIV after non-specific stimulation of T-cells. However, in contrast to nonspecific stimulation, *Mtb*-specific lung CD4+ T-cell responses were unaffected by HIV coinfection. This observation is different from a previous study in which reduced TB-specific polyfunctional as well as monofunctional CD4+ T-cells was reported in BAL cells of HIV coinfected participants compared to HIV naive (Kalsdorf et al. 2009; Jambo et al. 2011). This difference can be explained by the type of samples studied. We found that PD-1 expression was higher in lung tissue T-cells of TB participants without HIV coinfection than those co-infected with HIV, while PD-1 levels in blood was comparable between the groups. This observation is important in light of literature showing that PD-1 expression on *Mtb*-specific T-cells has a protective role and provides a probable role through which HIV exacerbates TB disease. However, we did not measure PD-1 expression on TB-specific T-cells to confirm this suggestion. We found no significant differences in the levels of plasma cytokines between participants with treated HIV or without HIV regardless of TB disease status. These results are in agreement with an earlier study where levels of pro-inflammatory cytokines (IFN-γ, IL-12 (p40) and TNF-α) and anti-

inflammatory cytokines (IL-4 and IL-10) in TB participants with or without HIV were similar (Mihret et al. 2014).

In summary, results presented here suggest that T-cell immune responses to *Mtb* are compartmentalised between lung tissue and peripheral blood. The differences in immune responses between these two sites can be explained in part by the existence of tissue specific T-cells (Trms) that do not recirculate. IL-17 cells in the lung are of particular interest as they appear to be the most depleted in circulation and also correlated negatively with plasma cytokine markers of inflammation as indicators of disease severity. Identification of Trms in human tissue is still a challenge as it relies on cell surface markers that in themselves are not perfect. However, with high dimensional immune phenotyping of T-cells in the lung by CyTOF, we conclude that CD69 and CD103 identified majority of lung Trms and thus reliable markers for studying Trms in human lung.

CHAPTER 3: T-CELL RECEPTOR REPERTOIRES IN HUMAN LUNG TISSUE AND BLOOD DURING TUBERCULOSIS DISEASE

3.1 Introduction

In the previous chapter I characterized Trm cells in TB infected lung tissue and provided evidence to support the existence of TB specific T-cell functional subsets in the lung that are not well represented in circulation. However, these could result from compartment specific functional differences and not due to the existence of non-recirculating TB-specific Trm. An alternative question is to ask whether T-cells in the lung and blood of TB infected subjects target the same antigens, which was not tested using antigen pool such as MTB300. Unfortunately, the lung tissue sections available did not provide sufficient material to conduct a traditional antigen screen, especially given the size of the TB proteome. Moreover, relevant antigen might include non-peptide ligands seen by "unconventional" T-cells. Therefore, in this chapter I examined the TCR repertoires in blood and matched lung tissue to look for evidence of TB-specific Trm within infected human lung tissue.

Recognition of foreign antigens by T-cells is a central part of the immune response. Mediating this recognition are T-cell receptor (TCR) heterodimers, which like antibodies, have the ability to create vast numbers of different variable (V) region sequences through DNA rearrangement and other mechanisms (Davis et al. 1998). The adaptive immune system can generate an estimated 10^{14} unique alpha beta TCRs, providing the capacity to recognize an enormous range of distinct antigens (Davis and Bjorkman 1988; Venturi et al. 2008; Jenkins et al. 2010; Plumlee et al. 2013; Nunes-Alves et al. 2015). However, thymic selection constraints ensure that unique TCRs in any individual are far fewer than the number of potential TCRs (Venturi et al 2008, Jenkins et al 2010, Nunes-Alves et al 2015). The diversity of the TCR arises from three principal mechanisms: 1) V, D, and J segments generate combinatorial diversity; 2) imprecise recombination and insertion of non-templated 'N' sequences at the Vβ D, DJβ and Vα Jα junctions; and 3) random assortment between TCRα and TCRβ chains (Qi et al. 2014). In majority of cases, alpha beta TCRs binds its cognate peptide antigen in the context of specific cell surface major histocompatibility complex (MHC) proteins which are encoded by the highly polymorphic human leukocyte antigen (HLA) loci *HLA-A*, *HLA-B*, and *HLA-C* class II alleles, thus, a TCR's antigen specificity is further modulated by HLA context (Emerson et al. 2017).

The size of the T-cell response generally correlates with the abundance of antigen-specific T-cells in the naïve repertoire (that is, precursor frequency) (Obar, Khanna, and Lefrançois 2008; Kotturi et al. 2008; Jenkins and Moon 2012). Paradoxically, pathogen-specific T-cell responses are often focused on a small number of the available antigenic epitopes and use a narrow TCR repertoire. This phenomenon is termed "immunodominance" (Nunes-Alves et al. 2015). If the number of antigen-specific T-cells in the naïve repertoire is limiting, clonotypic dominance could arise by a "founder" effect in which few T-cells are primed and expand, leading to T-cell populations of restricted diversity (Nunes-Alves et al 2015). Regardless, upon antigen recognition, activated T-cells proliferate by clonal expansion and some become part of the memory compartment, where they can reside for many years as clonal populations of cells with identical TCR rearrangements by virtue of their descent from a common naive T-cell (Arstila et al. 1999; Neller et al. 2013; Robins et al. 2009).

Distinct from the conventional αβ T-cells, are the γδ T-cells containing TCRγ and TCRδ chains with diverse structural and functional heterogeneity (Zhao, Niu, and Cui 2018). Whereas peptide antigen recognition by conventional αβ T-cells is strictly under the control of the MHC complex, γδ T-cells recognize their antigens in different ways and the rules governing how γδ T-cells recognize antigens are less clear. In general, γδ T-cells described to date target lipid-based antigens bound to MHC-like surface molecules of the CD1 family. In addition, γδ T-cells can be stimulated by certain stress or heat-shock proteins (Hsp) generated by a variety of cells during stress (Shinnick, Vodkin, and Williams 1988).

In addition to conventional αβ-T-cells, that recognize antigens in the context of polymorphic MHC complexes resulting in high restriction between individuals, 'donor unrestricted' alpha beta T-cells also exist (Van Rhijn and Moody 2015). That is, certain T-cells recognize antigens presented by non-polymorphic proteins that are present in all donors and are thus not "restricted" by the genotype of the donor. These non-polymorphic molecules include CD1a, CD1b, CD1c, CD1d, MR1, and HLA-E, all of which are expressed as protein heterodimers on the surface of APCs in a similar or identical form among nearly all humans (van Rhijn and Moody 2015). Among the currently well described donor unrestricted T-cells (DURTS), mucosa associated invariant T (MAIT) and invariant NKT (iNKT) recognize antigens presented by MR1 and CD1d respectively. The essential defining feature of iNKT and MAIT-cells is the expression of invariant TCRs with two key properties: expansion of T-cells with many similar, but nonidentical, TCR sequences (intradonor conservation) and

recapitulation of these patterns among nearly all humans (interdonor conservation) (van Rhijn and Moody 2015). iNKT-cells use invariant TCR α-chains of TRAV10 joined to TRAJ18 (Turner et al. 2006) while iNKT β chain almost always uses TRBV25-1 (Porcelli et al. 1993; Lantz and Bendelac 1994), but without a preferred J gene or a fixed length, (Turner et al. 2006). MAITs express TCRs with α-chains that use TRAV1-2 joined to TRAJ33 that pair with TRBV20-1 or TRBV6-1 (Porcelli et al. 1993; Tilloy et al. 1999). TRAJ20 and TRAJ12 have also been associated with MAITs. The fact that MAIT and iNKT-cells are activated or expanded in all humans, even at a very young age (van Der Vliet et al. 2000; Martin et al. 2009; Gold et al. 2013), suggests that selecting antigens are widespread and most likely are not limited to one pathogen. CD1b present antigens to donor unrestricted germline-encoded mycolyl lipid-reactive (GEM) T-cells (Chancellor et al. 2017; Van Rhijn et al. 2014, 2013; Van Rhijn and Moody 2015). Both GEM T-cells and MAIT-cells express TRAV1-2, but they use different joining genes and different TCR β-chains to recognize their antigens without cross-reactivity (van Rhijn and Moody 2015).

Although DURTS are to a large extent defined by their expression of shared TCRs between all or most individuals within a population, conventional T-cell TCRs can also be shared between different individuals, though typically much less frequently and only between subjects that express the same restricting MHC molecule. These TCRs are referred to as public T-cell receptors, in which a particular antigen is targeted by the same TCR sequence in multiple individuals. Although the mechanisms are not entirely clear, they may result when the space of potential high-avidity TCR chains that could bind to a particular antigen–MHC complex includes one or more TCR chains that also have a high likelihood of existing in the naive repertoire at any given time (Venturi et al. 2008; Li et al. 2012). TCR sequences associated with a public T-cell response to a particular antigen will only be intermittently observed in the naive compartment of subjects who have not been exposed to that antigen (Emerson et al. 2017). However, T-cells carrying these TCR sequences undergo clonal expansion upon antigen encounter, which increases the probability that these sequences will be detected in the repertoire of exposed subjects (Li et al. 2012; Emerson et al. 2017). Public T-cell responses have been reported in infectious diseases, such as cytomegalovirus (CMV), Epstein–Barr virus (EBV), *Clostridium tetani*, parvovirus, herpes simplex virus (HSV), HIV, and influenza, as well as in malignancies and autoimmunity (Venturi et al. 2008; Hanjie Li et al. 2012).

3.1.1 TCR expansion as a measure of immune response to infectious diseases

Measures of T-cell activity, like cytokine production, in response to invading infections are the consequence of T-cell activation which is triggered by TCR engaging antigen ligand in the right context of presenting molecule. Ideally, only the clones that are specific to a given antigen expand when the TCR repertoire is engaged by the cognate antigen resulting in a highly specific response. However, T-cell cross-reactivity is an essential characteristic of antigen recognition (Gras et al. 2012; Mason 1998), with some estimates proposing that a single T-cell can recognize up to a million different peptide-MHC complexes (Wooldridge et al. 2012). Analysis of the TCR expansions during infections provides an opportunity to identify and study in detail antigen specific T-cells and this information can guide vaccine design approaches.

Identification of signatures of CMV exposure history on the T-cell repertoire by immunosequencing revealed that only a few of the CMV-reactive TCRβ sequences previously reported in the literature were either seen more often in CMV+ subjects or were more abundant in CMV+ subjects than in CMV− subjects (Emerson et al. 2017). This observation could be explained by the stringent criteria used by Emerson et al to define CMV-reactive TCRβ sequences that may have excluded other previously described public TCRs for CMV. Also, it has been established that a large majority of T-cell responses are private rather than public (Venturi et al. 2008).

Apart from host factors, T-cell responses are also governed by the type of infectious agent. For pathogens that rapidly mutate, a diverse T-cell response could benefit the host by efficiently detecting escape mutants, while a biased response could be detrimental. For slowly replicating pathogens that encode numerous antigens, the relation between diversity and protection is less clear (Nunes-Alves et al. 2015). Immunodominant T-cell responses in patients with tuberculosis have been suggested to be both a correlate of protection and a marker of disease progression (Tully et al. 2005; Luo et al. 2012; Jacobsen et al. 2007; Du et al. 2010) suggesting, in part, that during the course of TB disease, there are differences in antigenic insults that the T-cells must contend with.

In a mouse model of *Mtb*, TCR bias was found to emerge soon after T-cell priming in the lymph node and becomes more extreme during chronic infection (Nunes-Alves et al 2015). In

the same model, it was observed that TCR affinity significantly affects immunodominance (Nunes-Alves et al. 2015; Carpenter et al. 2016) but was not the only contributor. Inflammatory environment and tissue-specific cues influence the fate of individual T-cells during infection (Plumlee et al. 2013) and all these factors may contribute to the establishment of immunodominant T-cell responses during tuberculosis (Nunes-Alves et al 2015). Although there was a high precursor frequency (a large naive repertoire pool), only a few of these T-cell clones were significantly represented in the final immune response following challenge of mice with *Mtb* indicating that a high precursor frequency is not a prerequisite for immunodominance during chronic infection (Nunes-Alves et al 2015).

Spectratyping of peripheral blood T-cells from tuberculosis patients revealed TCR skewing compared to healthy controls (Jacobsen et al. 2007; Yang, Xu, et al. 2013). Extreme TCR bias was noted primarily in the setting of severe clinical disease, raising the possibility that TCR bias is associated with disease progression (Luo et al. 2012; Jacobsen et al. 2007). Counter to this interpretation is the presence of highly skewed TCR repertoires in lung granulomas from patients with latent tuberculosis (Tully et al. 2005). Clonal T-cell expansions in the lungs of patients with active disease compared to the frequency of T-cells in the peripheral blood of normal donors has also been reported. Within TB infected lungs, TCR bias at the level of the granuloma may be driven by heterogeneity in bacteria and bacterial antigens, as well as the persistent immune response. Therefore, the links between TCR bias, functional capacity of T-cells and protection during tuberculosis remain incomplete (Nunes-Alves et al 2015). The above studies have primarily focused on tracking the T-cell response to TB via conventional TCR receptor expansion, which, as discussed are complicated by limited publicity. In contrast, the shared nature of DURT TCRs would appear to make them ideal for tracking by this method.

3.1.2 Donor unrestricted T-cells in tuberculosis infection

Although the criteria for definition of cells that make up the DURTs is expanding, four types of cells have been described as DURTs: MAITs, iNKTs, GEM T-cells and γδ T-cells. MAITs respond to cells infected with bacterial pathogens without prior exposure to that pathogen, produce cytokines and are cytotoxic (Sharma et al. 2015; Wong, Ndung'u, and Kasprowicz 2017). MAIT-cells are uniquely activated by small molecule vitamin B metabolites presented by the ubiquitously expressed and non-polymorphic MHC-class 1-like

molecule, MR1 (Kjer-Nielsen et al. 2012; Reantragoon et al. 2012; López-Sagaseta et al. 2013; Corbett et al. 2014). In MR-1 deficient murine models, MAIT-cells were associated with early protection against bacterial pathogens, including mycobacteria (Gold, Napier, and Lewinsohn 2015). Thus, these cells may act as early sensors of *Mtb* infection in airways and provide early cytokines to activate macrophages against infection.

Mtb-reactive MR1-restricted T-cells were found in the peripheral circulation of humans including those with no evidence of previous exposure to *Mtb*. Interestingly, they were found at much lower levels in individuals with active tuberculosis (compared with those with no history of exposure, or those with evidence of latent infection) (Gold et al. 2010). It has also been confirmed by other studies that MAIT-cell frequencies are decreased during active tuberculosis (and non-tuberculosis mycobacterial lung disease) in diverse geographic settings (Sharma et al. 2015; Le Bourhis et al. 2010; J. Jiang et al. 2014; Kwon et al. 2015). Indeed, peripheral depletion of MAIT-cells does not appear to be disease specific and low levels of peripheral MAIT-cells found in individuals with cystic fibrosis experiencing pulmonary exacerbations due to *Pseudomonas aeruginosa* (Smith et al. 2014), non-streptococcal sepsis (Grimaldi et al. 2014) and cholera (Leung et al. 2014). In TB, the degree of peripheral MAIT depletion correlates with disease severity and may be reversed with antibiotic treatment during recovery from illness (Sharma et al 2015, Kwon et al 2015).

The few MAIT-cells that remain in circulation during active pulmonary tuberculosis have diminished functional capacity, indicated by reduced IFN-γ production upon stimulation and decreased transcription of effector genes (IFN-γ, TNF-a, IL-17F, granulysin and granzyme B) (Jiang et al. 2014; Kwon et al. 2015). In addition, these functionally impaired MAIT-cells express higher levels of pro-apoptotic markers and higher levels of PD-1 than MAIT-cells from non-tuberculous patients (Jiang et al 2014, Kwon et al 2015). *In vitro* blockade of PD-1 can increase IFN-γ production in circulating MAIT-cells from patients with tuberculosis (Jiang et al 2014).

One potential reason for the loss of MAIT cells from the circulation during disease is that these cells migrate to the site of infection. Indeed, as suggested by the name, MAITs were originally associated with the mucosal immune response. *Mtb*-reactive MAIT-cells have been identified in healthy uninfected human lung tissue (Gold et al. 2010), in two *Mtb*- infected

human lungs, and were enriched in the ascites fluid from a single patient with tuberculosis peritonitis (Le Bourhis et al. 2010) . However, in contrast and in a larger study, Jiang et al. 2014 found diminished frequencies of MAIT-cells in pleural fluid from patients with tuberculosis pleuritis and no expansion of MAIT-cells in the peritoneal fluid of patients with tuberculosis peritonitis. Thus, it remains unclear whether MAIT-cell depletions in bacterial infections are due to redistribution to mucosal sites.

CD1b-restricted T-cell numbers, including GEMs, are reported to be enriched in peripheral blood and at the site of infection during active TB, expand and contract markedly according to pathogen burden, and therefore can be markers of disease severity (Montamat-Sicotte et al. 2011). By histology, CD1b expression was confirmed in lung biopsies from patients with active pulmonary TB (Chancellor et al. 2017) and a protective role for this molecule has been reported in leprosy (Sieling et al. 1999; Inkeles et al. 2016). Thus, T-cell responses directed to bacterial lipids presented by CD1b are important for infection containment, including *Mtb*.

The role of γδ T-cells in the immune response to *Mtb* was first reported in 1989 (Janis et al. 1989) and additional work has demonstrated that the activation of γδ T-cells appear rapidly following *Mtb* infection, and γδ T-cells provide protective immunity against *Mtb* infection with the combined properties of both innate and adaptive immunity (Casetti and Martino 2008; Holtmeier and Kabelitz 2005; Davey et al. 2011). However, it has also been reported that γδ T-cells in peripheral blood are present in increased proportions in a fraction of patients with tuberculosis or sarcoidosis (Balbi et al. 1993) and some clonotypic γδ T-cells exhibited clonal expansion during BCG infection and re-infection (Chen and Letvin 2003). A subset of γδ T-cells, Vγ9Vδ2 T-cells, play an important role in the protective immune responses to *Mtb* infection (Chen 2016). It was shown that they can expand in response to the stimulation of *Mtb* antigens *in vitro* and *in vivo* (Shen et al. 2002; Esin et al. 1996; Huang et al. 2012). Thus, γδ T-cells are clearly altered during TB disease and respond to *Mtb* infection.

The iNKT cells antimicrobial function was originally based on the recognition of microbial lipid or glycolipid molecules presented by the MHC-like molecule CD1d, and activated iNKT cells can enhance host resistance to *Mtb* (Rothchild et al. 2014). Administration of αGalCer, which activates iNKT cells *in vivo*, significantly prolongs the survival of susceptible mouse strains following *Mtb* infection and this effect is synergistic with antibiotics (Chackerian et al. 2002; Sada-Ovalle et al. 2010). αGalCer activates human iNKT

cells to lyse *Mtb*-infected macrophages and kill intracellular bacteria (Gansert et al. 2003). Additionally, BCG vaccination is more effective when it is conjugated with αGalCer (Venkataswamy et al. 2009), probably by activating iNKT cells and enhance bacterial killing. iNKT cells can also restrict bacterial growth without αGalCer influence. For instance, iNKT cells cultured with *Mtb*-infected primary macrophages stimulate antimicrobial activity that restricts bacterial growth and adoptive transfer of iNKT cells limits bacterial growth *in vivo* (Sada-Ovalle et al. 2008). In clinical studies, a decrease of iNKT cells in the periphery is a marker of active disease compared to latent infection or healthy controls (Im et al. 2008; Montoya et al. 2008; Sutherland et al. 2009). A recent study reported that the frequency of iNKT cells was increased in subjects with latent Mtb infection compared with uninfected individuals or subjects with active infection, but HIV coinfection had a deleterious impact on these cells (Paquin-Proulx et al. 2018). Despite these findings of iNKT cell activation leading to enhanced *Mtb* control, iNKT cells are dispensable in the murine model of chronic tuberculosis infection (Behar et al. 1999; Sugawara et al. 2002; Sousa et al. 2000).

In this chapter, I examine both the conventional and unconventional alpha beta and gamma delta TCR repertoire of matched blood and lung tissue from subjects with active TB to look for differences between the circulating and lung resident T-cell compartments. In addition, this provides the opportunity to examine whether HIV co-infection effects the lung TCR repertoire. TCR sequencing was done using Adaptive Biotechnologies Corp. (Seattle, WA) ImmunoSEQ assay (http://www.immunoseq.com) providing direct comparison of TCR from blood and lung tissue.

3.2 Methods

The study participants are described in section 2.2.1 of the thesis.

3.2.1 DNA extraction

DNA was extracted from snap frozen lung tissue, PBMC and sorted lung cells using DNeasy Blood and Tissue kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer instructions. For whole lung tissue, different areas of resected lungs, were removed by the operating surgeon, corresponding to their assessment of the most diseased (A), intermediate (B), and healthiest tissue (C); based on their experience and the pre-operative radiological data. Homogenate was added to a 2ml tube containing 100µL Zarconia beads (BioSpec) and then bead beaten 5 times (7000rpm, 60s) with 30s on ice between intervals in MagNA Lyser (Roche Diagnostics GmbH, Germany). The concentration and purity of DNA was determined using NanoDrop Lite Spectrophotometer (Thermo Scientific).

3.2.2 Cell sorting

Lung cells and PBMC (obtained as indicated in chapter 2) were stained with surface antibodies and a live dead cell marker (near IR, Invitrogen) for 20 minutes at room temperature, washed twice with PBS and sorted using BD FACS ARIA. Cells were directly sorted into RLT buffer (Qiagen) + 1% β-Mercaptoethanol. Lysates were snap frozen on dry ice and stored at -80°C until when needed for DNA extraction. The following antibodies were used in the panel: anti-CD45 V500 Horizon clone HI30 (BD Biosciences), anti-CD3 Brilliant Violet 785 clone OKT3 (Biolegend), anti-CD4 Brilliant Ultra Violet 496 clone SK3 (BD Bioscience), anti-CD8 Brilliant Violet 605 clone RPA-T8 (Biolegend), anti-CD69 Brilliant Ultra Violet 395 clone FN50 (Brilliant Horizon)

3.2.3 Immunosequencing

For TCR-beta and alpha high-throughput sequencing, known quantities of purified genomic DNA were sequenced by Adaptive Biotechnologies Corp. (Seattle, WA) using the ImmunoSEQ assay (http://www.immunoseq.com) as previously described (Lepore et al 2014). Due to differences in sample input, DNA from PBMCs was sequenced using the deep sequencing option while lung tissue DNA was sequenced using the survey option. Raw HiSeq sequence data were pre-processed to remove errors in the primary sequence of each read and to compress the data. To remove both PCR and sequencing errors, a nearest-

neighbour algorithm was used to collapse the data into unique sequences by merging closely related sequences. After data clean-up, analysis was done using the ImmunoSEQ analyser toolset.

3.2.4 Definition of donor unrestricted T-cells

To determine the frequency of iNKT, GEM, MAIT and γδ T cell subsets, we searched for TCR features that are indicative of the different T cell subsets (Appendix III). The TCR sequences that we used to define the DURTs have been reported in literature (Greenaway et al 2013, Gras et al 2016, van Rhijn and Moody 2015). For iNKT we used the invariant combinations of TRAV10/TRAJ18 for a consensus sequence CVVSDRGSTLGRLYF with a CDR3α length of 15 amino acids and there was only one unique CDR3α sequence. For GEM T cells an invariant combination of TRAV01-02/TRAJ9 with a CDR3α length of 13 amino acids with a consensus sequence of CAVRNTGGFKTIF was used. In addition, we extended the definition of GEMS to CAVRxTGGFKTIF or CAVLxTGGFKTIF where 'x' is any amino acid substitution at position five of the sequence. There were 40 unique CDR3α sequences identified for all the GEM T-cells. With regards to MAIT cells, a combination of TRAV01-02/TRAJ33 with a CDR3α length of 12 amino acids with a consensus sequence CAVRDSNYQLIW was used to define 'classic' MAIT cells. With the same TRAV/TRAJ combination, we expanded the MAIT sequence to 'extended MAIT sequence of CAVxDSNYQLIW where x = R,A,K,L,M,S,T,V amino acid substitution at position four of the classic sequence but with same CDR3α length. We also broadened the definition of MAIT cells to the same TRAV/TRAJ combination but with a CDR3α length of 11 -15 amino acids. We called this 'MAIT-like' TCRs. There was a total of 47 CDR3α sequences identified for all the MAIT classes.

As for γδ-TCRs, we used V genes of Vδ1,2,3,4 (TRAV14), 5(TRAV23), 6(TRAV29), 7(TRAV36) and 8 (TRAV38) in combination with Jδ1,2,3,4. The CDR3 length for the γδ-T cells was variable. There were multiple numbers of unique CDR3α sequences for the γδ T-cells.

3.2.5 Statistics

A two-tailed student's t-test was used for normally-distributed data to compare two groups. One-way or Two-way ANOVA were used to compare more than two groups (where applicable), followed by Bonferroni or Sidak post-tests. A p value < 0.05 was considered to be statistically significant. Statistical analyses were performed using Prism V8 (GraphPad Software, San Diego, CA).

3.3 Results

3.3.1 Population characteristics and sequence quality control.

Sequencing of TCRA/D and TCRB loci was done from a total of 37 individuals in a cohort of participants with either active or previous tuberculosis or non-TB controls as described previously. In addition, PBMC from twelve healthy control participants (HIV negative TB negative) was sequenced in a similar manner. The details of these study subjects are outlined in Table 3. All the participants who were coinfected with HIV were on antiretroviral therapy while active TB participants were on anti-TB therapy combination of any two of the drug regimens shown in table 3. Definition of participants into TB groups was as explained in section 2.2.1

Table 3: Characteristics of chronic tuberculosis participants

Table 1. Demographics of Study Participants	Active TB	Previous TB	Cancer Control	Healthy Controls
Characteristic	n = 22	n = 6	n = 3	n = 12
Sex, No. (%)				
Female	8 (36)	4(66.7)	1 (33.3)	8 (66.7)
Male	14(64)	2 (33.3)	2 (66.7)	4 (33.3)
Age (y), Median (range)				
All Subjects	37.5 (18 - 55)	41 (32 - 68)	61 (58 - 66)	35 (22 - 55)
Female	35.5 (22 - 55)	49 (37 - 68)	58 (58 - 58)	33 (22 - 55)
Male	37.5 (18 - 55)	33 (32 - 34)	63.5 (61 - 66)	37.5 (25 - 42)
HIV, No. (%)				
Positive	7(32)	4(66.7)	0 (0)	0 (0)
CD4 Count; Mean (range)[μ]	484.4 (166 - 814)	300 (30 - 785)		
Viral Load[#]				
Suppressed , i.e, <40 copies/ml (No, (%))	4(57)	4(100)		
Unsuppressed, i.e, >40 copies/ml; (No, (%))	15489 and 670; 2(29)	0		
Antiretroviral Therapy regimen	Odiumune/Atrioza/Tribuss			
Negative	15 (68)	2 (33.3)	3 (100)	12 (100)
Other lung pathologies[ℑ], No. (%)				
Cavitary lung	11 (50)	1 (17)	1 (33)	n/a
Bronchiectasis	6 (27)	2 (33.3)		n/a
Mycetoma	4 (18)	0		n/a
Fibrosis	0	1 (17)		n/a
Aspergilloma	0	1 (17)		n/a
Intestitial lung disease	9 (41)	0		n/a
Consolidation/Nodules	10 (45)	0		n/a
Smoking history, No. (%)				
Smokers	5 (23)	1 (17)		1 (8)
Non-smokers	15 (77)	5 (83)		11 (92)
TB treatment outcomes, No. (%)				
Drug susceptible	15 (68)	6 (100)		
MDR TB	3(14)			
XDR. TB	1 (4)			
Treatment naïve	3 (14)			
Treatment regimen	Combination therapy[Ω]			

Notes:
μ = CD 4 count unavailable for two HIV infected individuals (both active TB)
= Viral load value for one active TB not available
ℑ = Some indindividuals had more than one lung pathology
Ω= Combination of any two or three of these drugs: Rifafour/Pyradoxine/Streptomycin/Rifinah/Tenzidone/Pyrazinamide/Isoniazid/Kanamycin/Moxifloxacin/Clofazimine

Deep sequencing of blood and/or PBMC and survey level sequencing of tissue samples generated enough templates for further TCR analysis (table 4a and b). Samples that did not meet the threshold were not included in the downstream analysis. In total, 79 samples of PBMC, blood and multiple tissue samples were analysed.

Table 4 a & b: Sequencing products after quality control check. Deep sequencing (blood and PBMC) and survey sequencing (lung tissue). a - TCRA/D; b - TCRB

a.

Sample type	# of templates (total T cells)	# of rearrangements (unique T cells)	Clonality	Max Frequency	Input DNA (ng)
Blood	180152 (45806-486194)	76222 (31489-141354)	0.137 (0.053-0.286)	2.615 (0.736-6.546)	4240 (673-5999)
PBMC	334352 (220248-561580)	147171 (32718-275006)	0.199 (0.086-0.405)	4.528 (1.12-18.892)	1935 (1663-1998)
Lung Tissue	110980 (6465-364269)	29516 (2219-82137)	0.237 (0.111-0.458)	4.903 (0.618-15.505)	2610 (1029-2667)

Mean (min – max)

b.

	# of templates (total T cells)	# of rearrangements (unique T cells)	Clonality	Max Frequency	Input DNA (ng)
Tissue	57581.438 (1288–183690)	21641.125 (999–60578)	0.139 (0.036–0.317)	4.478 (0.913–24.309)	2378.445 (450.4–2675.15)
CD8posCD69pos	19238 (10370–36268)	4513.4 (2255–7563)	0.205 (0.124–0.286)	5.667 (1.703–12.199)	258.018 (179.61–416.334)
CD8posCD69neg	20437.5 (6251–36939)	8967.7 (1403–24490)	0.226 (0.088–0.362)	10.369 (3.063–20.926)	248.195 (151.812–393.27)
CD4posCD69pos	29114.4 (5017–45678)	13765 (2899–27245)	0.091 (0.035–0.159)	1.595 (1.049–2.441)	311.533 (133.176–410.532)
CD4posCD69neg	41075.6 (6822–62624)	30965.5 (5157–52586)	0.038 (0.013–0.103)	2.204 (0.339–7.391)	352.846 (242.07–452.532)
Blood	112357.647 (9552–236718)	74865.471 (8362–149729)	0.093 (0.017–0.213)	3.26 (0.89–10.119)	2261.123 (305.67–6008.55)

Mean (min – max)

3.3.2 Donor unrestricted T-cells are depleted in peripheral circulation in tuberculosis

First, due to the ease of identifying DURT TCR sequences, we examined the impact of TB on MAIT-cells, iNKT-cells, GEM T-cells and gamma-delta T-cells in peripheral blood of healthy control and chronic TB participants. Furthermore, frequency of these cells was also measured in lung tissue T-cells from chronic TB participants.

In line with other reports, by flow cytometry, there was a significant reduction in MAIT-cells frequency in TB cases compared to healthy controls (figure 3.1a). There was no significant difference in total γδ T-cells between TB cases and controls (figure 3.1b) but staining for γδ T-cell subsets revealed significant reduction in the frequency of Vδ2 and Vγ9Vδ2 in TB cases compared to healthy controls. Vδ1 T-cells, on the other hand, were significantly higher in TB cases than healthy controls (figure 3.1 c – e). Consistent with this, bulk TCR sequencing showed reduction in the frequency of MAIT-cells and total γδ in circulation of TB cases compared to healthy controls although reduction in total γδ T-cells did not reach significance (figure 3.1 f-h). In addition, the frequency of GEM TCRs was significantly lower in TB cases, which was not assessed by flow cytometry as it required CD1b tetramers. No differences in the frequency of iNKTs was observed by TCR sequencing, again not assessed by flow cytometry, for the same reason.

Next, by TCR sequencing, we examined DURT frequency in lung tissue from TB subjects. Within the TB cases, the frequency of MAIT-cells was marginally higher in the lung than peripheral circulation, but this did not reach significance (figure 3.1f) but was significantly lower than in the blood of healthy donors. Total γδ T-cells were significantly lower in lung tissue compared to circulation for both TB cases and controls (figure 3.1g), while GEM T-cells frequency was similar between lung tissue and circulation for TB cases (figure 3.1h). Finally, the frequency of iNKT-cells was similar between all groups (figure 3.1i).

Sequencing techniques may sometimes be affected by primer bias resulting into unequal amplification. To ensure that TCR sequencing results were a true reflection of DURT cell frequency, a comparison of frequency of T-cells by flow cytometry and TCR sequencing was done. A positive and significant Spearman correlation is shown between frequencies of MAIT-cells, total γδ T-cells, Vδ1 T-cells and Vδ2 T-cells as measured by flow cytometry and TCR sequencing (figure 3.1j-m). This observation supports the validity of global TCR sequencing as method for enumerating T-cell frequency.

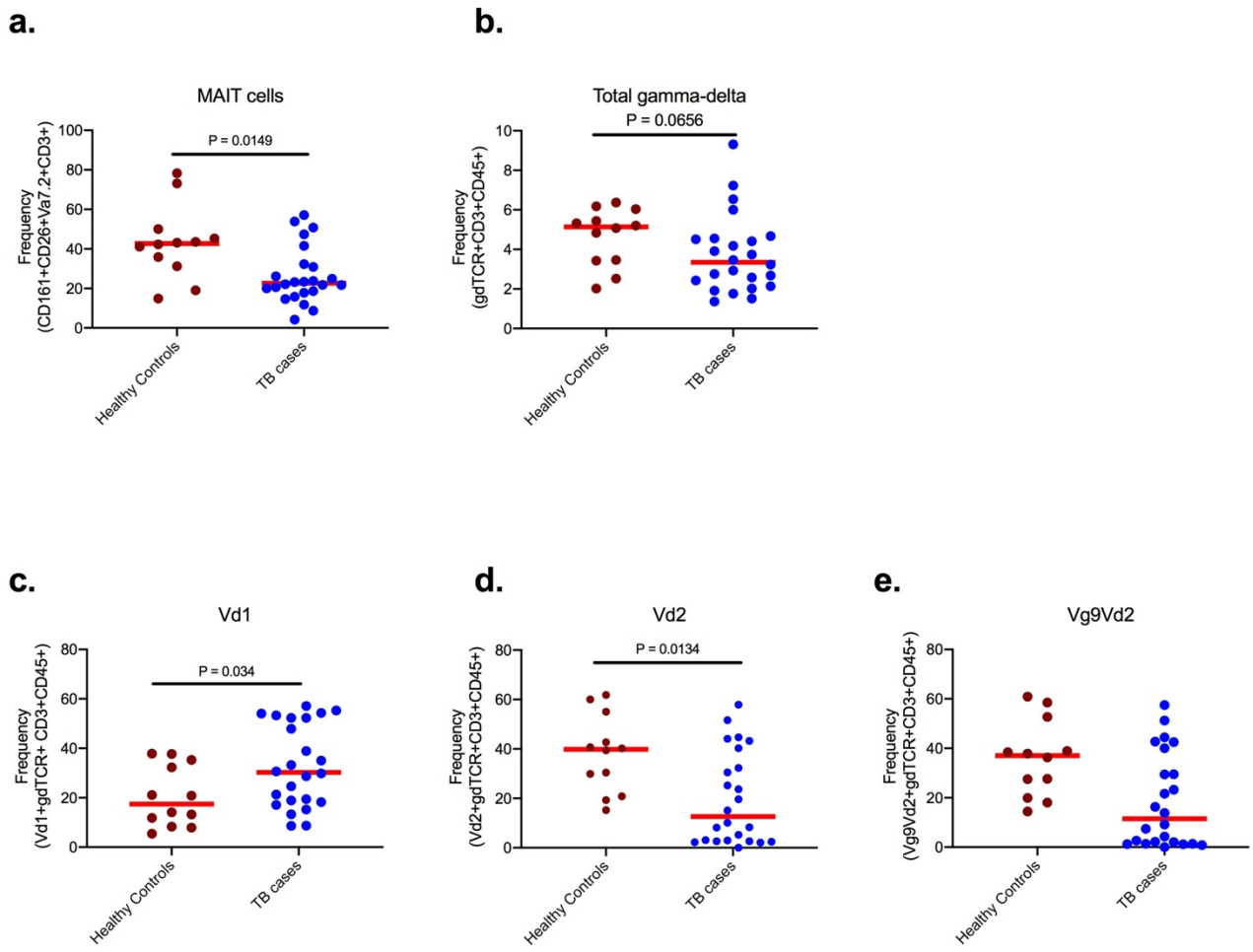


Figure 3.1: Frequency of donor unrestricted T-cells (DURTs). DURT-cells in peripheral blood of healthy controls and TB cases measured by flow cytometry (a – e). Statistics; non-parametric Mann-Whitney t-test

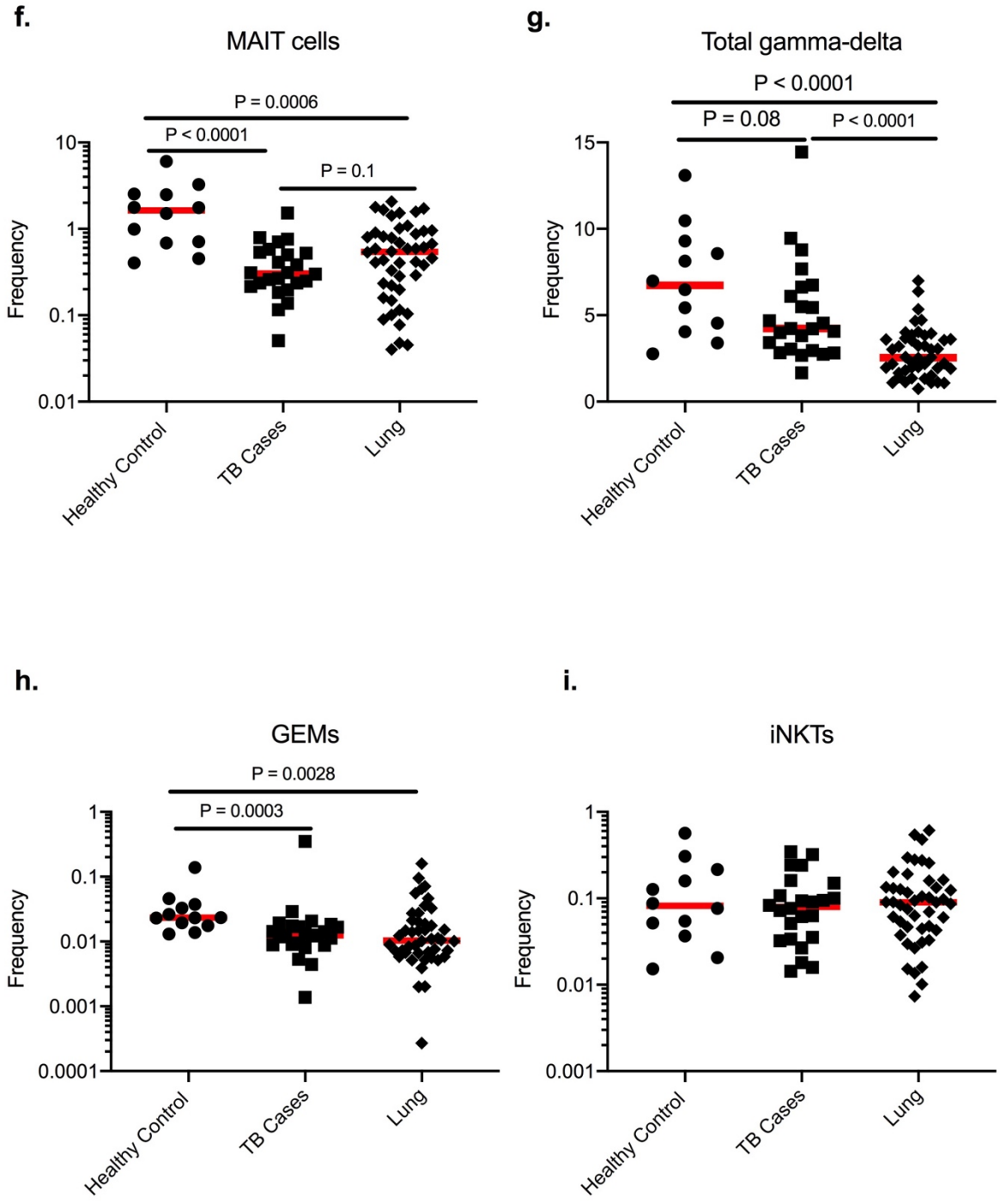


Figure 3.1 Frequency of donor unrestricted T-cells (DURTs). DURT-cells in peripheral blood (healthy control and TB cases) and lung tissue (Lung) as measured by TCR sequencing (f – i). Statistics; non-parametric Mann-Whitney t-test

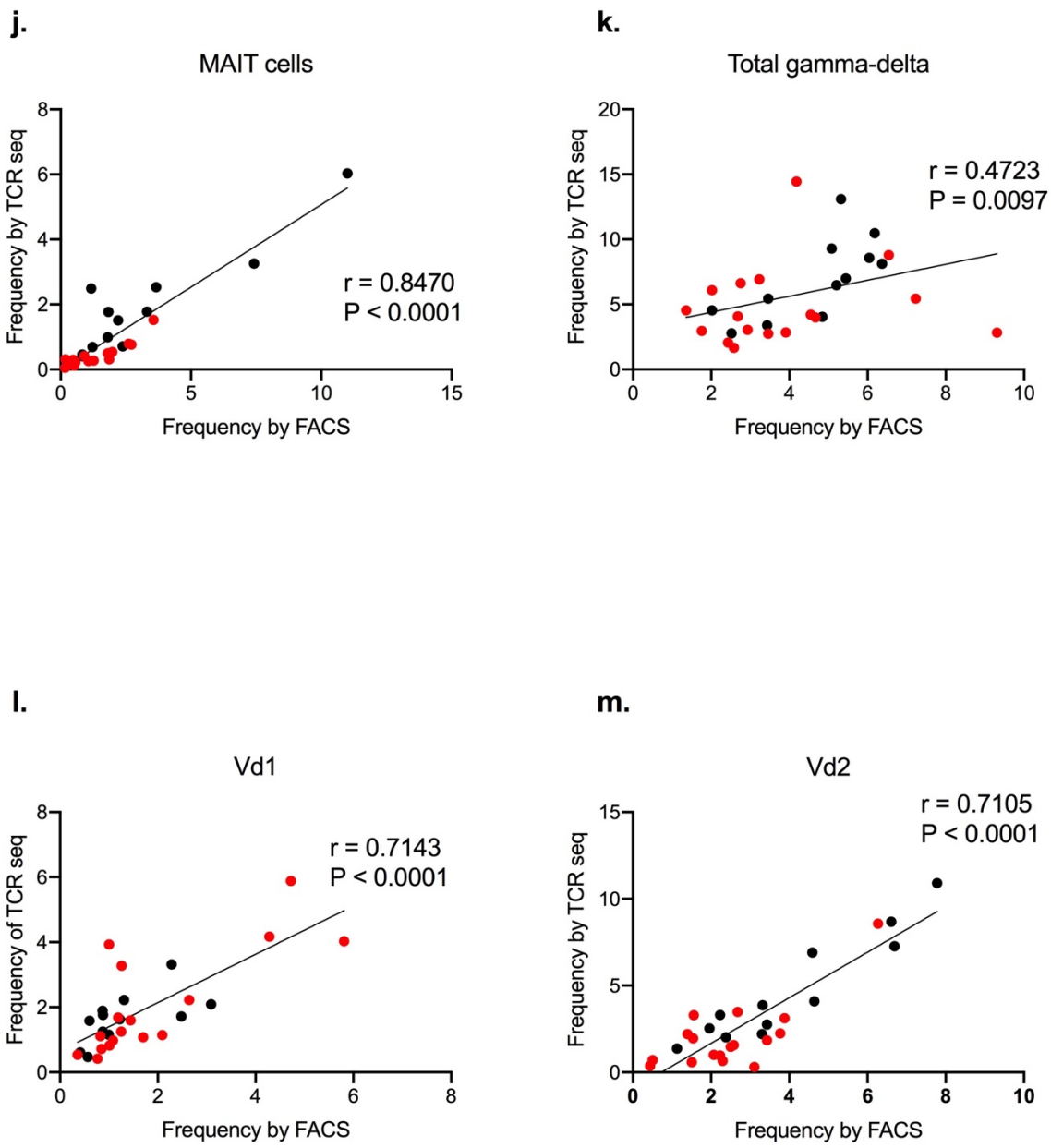


Figure 3.1 Frequency of donor unrestricted T-cells (DURTs). Spearman correlation of frequency of DURTs as measured by flow cytometry and TCR sequencing (j – m), black circles (healthy controls), red circles (TB cases).

3.3.3 Peripheral blood CD8+ MAIT-cells are the most depleted in tuberculosis

MAIT-cells are defined as CD3+CD8+ or CD3+ double negative for CD4 and CD8 T-cells that have T-cell receptors encoded by the TRAV1-2 genes and are restricted by non-classical molecule MR-1 (Morris et al 2016). By both flow cytometry and TCR sequencing, MAIT-cells were depleted in peripheral circulation of TB cases compared to healthy controls (figure 3.1a, f &j). However, TCR sequencing does not distinguish between CD8 and CD4 T-cell subsets. Therefore, to determine which subset was most affected using flow cytometry γδ-CD3+ T-cells were divided into four populations based on expression of CD4 and CD8 (figure 3.2a) then frequency of MAIT cells (Va7.2+CD161+CD26+ cells) analysed in each compartment for healthy controls and TB cases (figure 3.2b). Using this classification, the majority of MAIT cells were CD3 double negative (CD4-CD8-) in both healthy controls and TB cases (figure 3.2b). However, MAIT-cells in this T-cell sub population were not significantly depleted in active TB (figure 3.2c). All other MAIT subsets were significantly lower in TB cases compared to controls, with the most striking effect observed in the CD3+CD8+ T-cells subset (figure 3.2c).

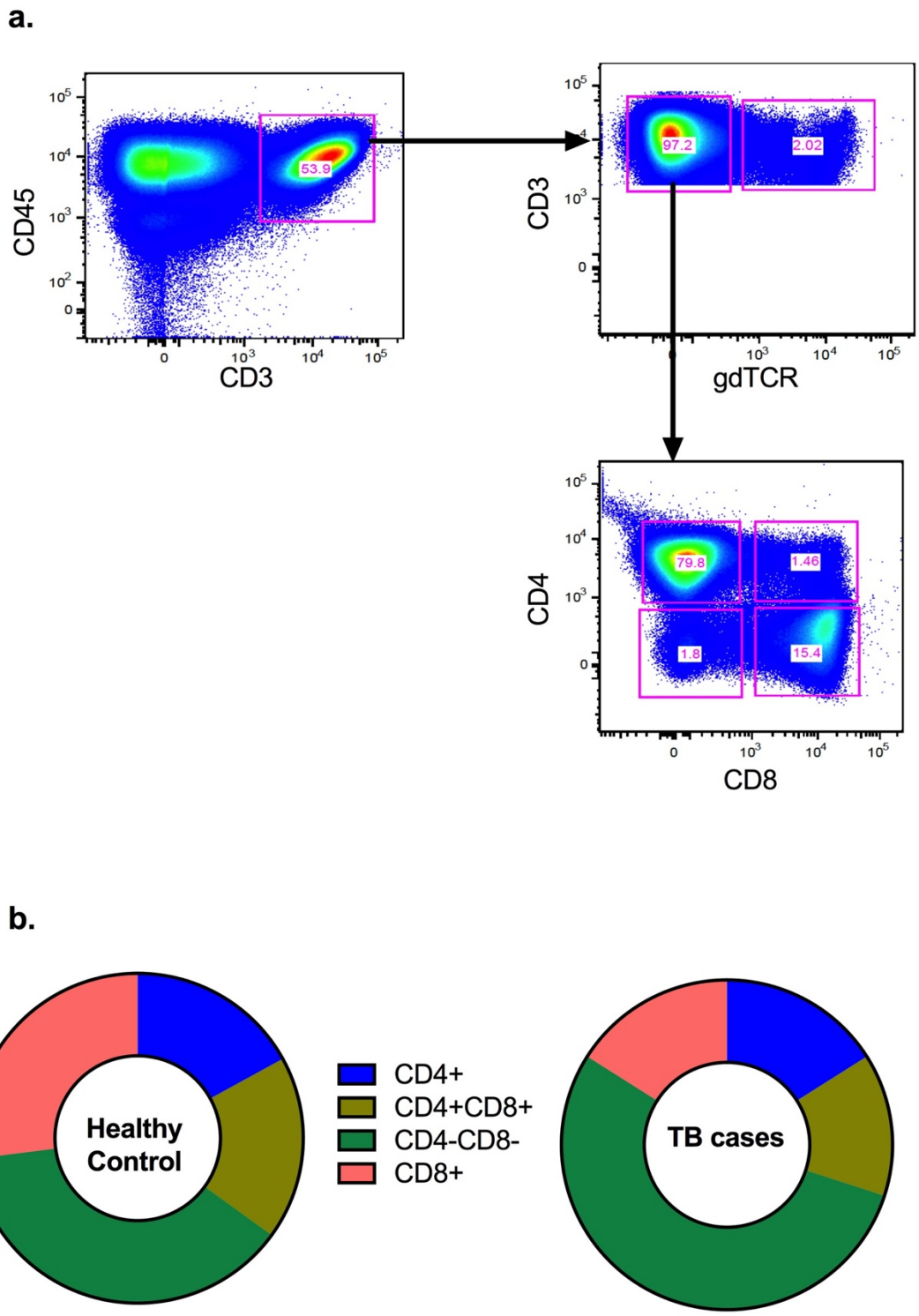


Figure 3.2: MAIT-cell frequency in T-cell subsets. (a) Representative FACS plots of expression of CD4 and CD8 on γδ-CD3+ T-cells. (b) The frequency of MAIT cells in subpopulation of γδ-CD3+ T-cells.

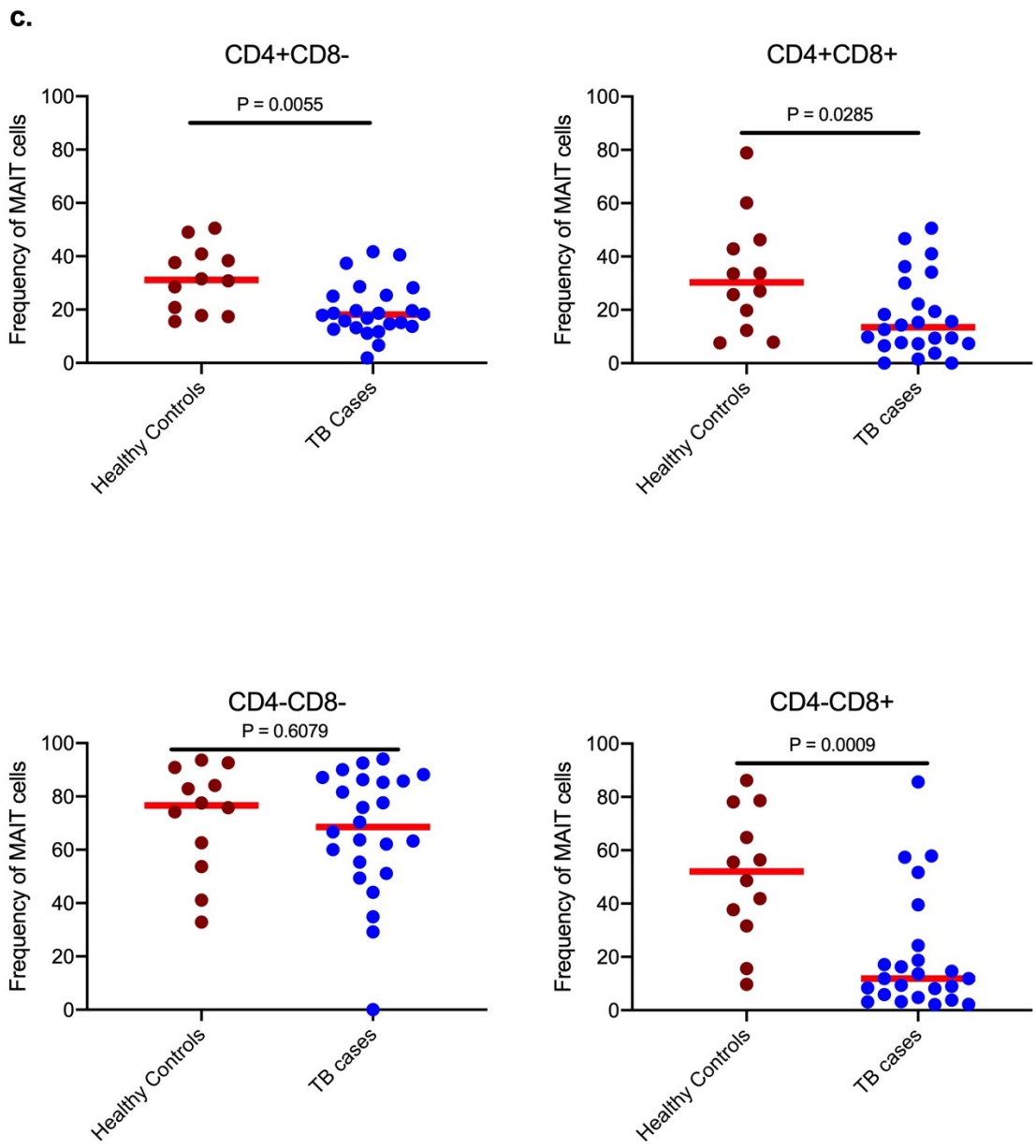


Figure 3.2: MAIT-cell frequency in T-cell subsets. (c) impact of tuberculosis on MAIT-cells in different γδ-CD3+ T-cells subsets. Statistics; non parametric Mann-Whitney t-test.

3.3.4 Clonality and relatedness of T-cells in blood and lung tissue

Having focused on DURT cells to validate this approach we next examined the total TCR repertoire in blood and matched lung samples. First, quantitation of the extent T-cell expansion was done by comparing the clonality score for each sample. The clonality score is a measure of degree of TCR expansion within a repertoire that is robust to sampling depth and normalizes for differences in deep and survey sampling. The clonality score values range from 0 – 1, in which a value of 1 would represent a monoclonal population. Clonality score were calculated using the Adaptive on-line analysis tool as described. Multiple lung tissue samples were taken from each individual to examine heterogeneity in the TCR repertoire within the lung. Figure 3.3a shows all subjects sampled, with the red dots indicating individual tissue sections and the blue dots PBMC. Not all individuals have data from both matched blood and lung and the number of separate lung sections from each individual varies between 1 and 3. However, in general, tissue samples from the same individual have a similar clonality and the tissue clonality is higher than in matched blood (figure 3.3a). Cumulatively, there is a significantly higher clonality score in lung tissue samples than in periphery while there is no difference in clonality between lung pieces (figure 3.3 b). Clonality correlation between tissue and blood was done by plotting the average tissue clonality against peripheral blood clonality score. Although the tissue clonality is higher than blood clonality, the two are marginally correlated (figure 3.3c). Overall this suggest a significant clonal expansion of T-cells within TB infected lung tissue compared to matched blood, consistent with the existence of an expanded memory T-cell pool within this organ.

An alternative global measure of TCR repertoire characteristics is the Morisita index, which quantifies the overall similarity between repertoires; where Morisita's score of 0 represents no overlap and 1 represents perfect correlation of clonal overlap and frequencies. An example of this is shown in figure 3.3d, were two different tissue samples from the same individual have very similar TCR repertoire, generating a high Morisita's index, while matched tissue and blood can have quite different TCR repertoire, giving a low Morisita index value (figure 3.3d). In these plots each dot represents a different TCR sequence, and in the second comparison, dots on either the X or Y axis therefore represent TCRs that were not seen in either the blood or lung. In total, there was a higher Morisita's index in lung-vs-lung than periphery-vs-lung (figure 3.3e) demonstrating that T-cell repertoires from lung tissues are more similar than T-cell repertoires from lung and blood in the same individual. Further, there is no difference in Morisita's index within lung pieces (figure 3.3e). Thus, lung tissue

from TB infected individuals is both clonally expanded and distinct from that of matched blood. However, these data do not support the existence of a high degree of TCR heterogeneity within the same lung.

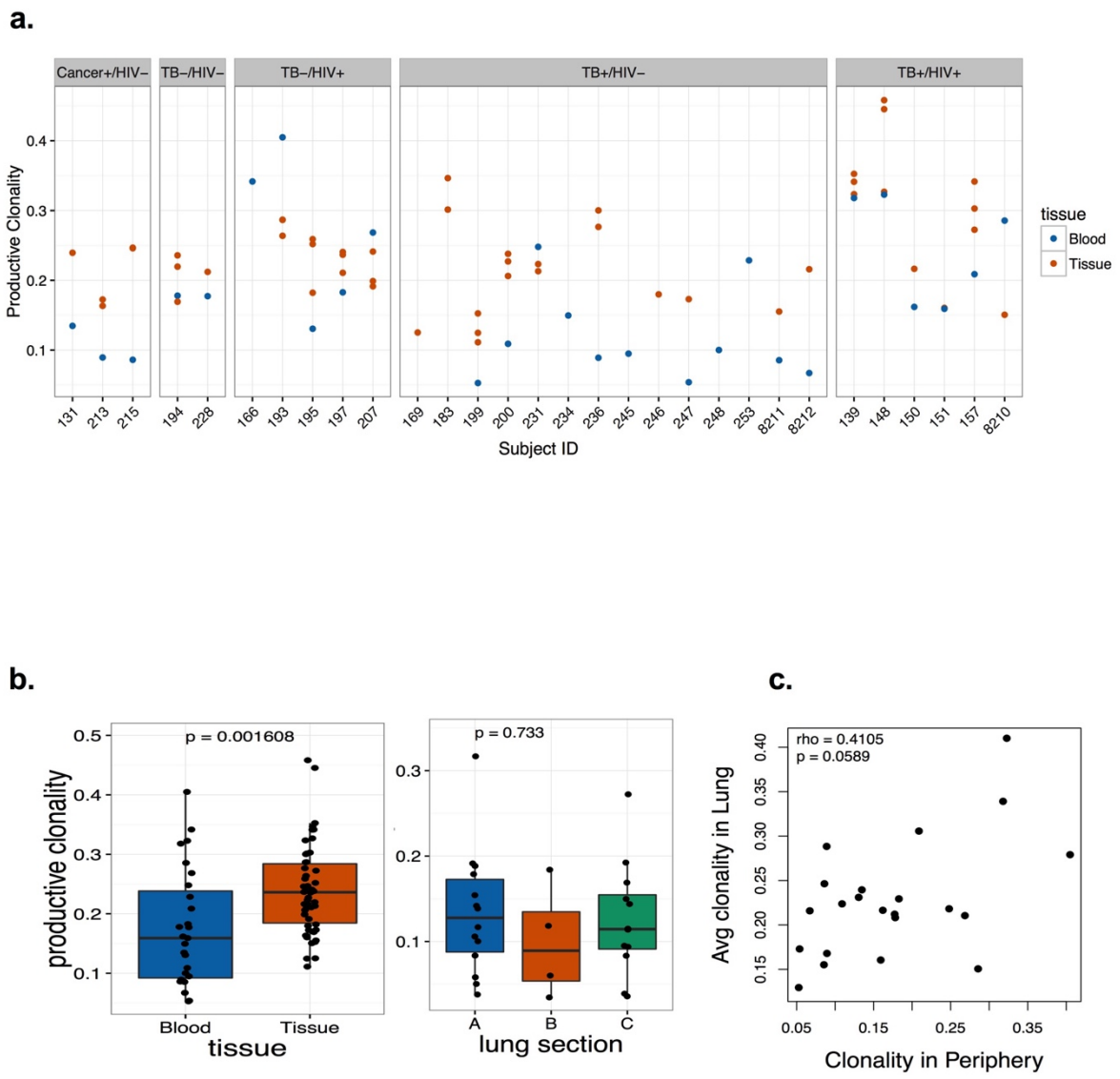


Figure 3.3: T-cell receptor repertoire measurement. a) Representative clonality values of participants showing matched blood and lung tissue samples with different disease states. (b) Cumulative clonality between blood and lung tissue, and between lung tissue pieces. (c) Correlation between clonality in circulation and tissue. Statistics; non-parametric Mann-whitney t-test and one way ANOVA.

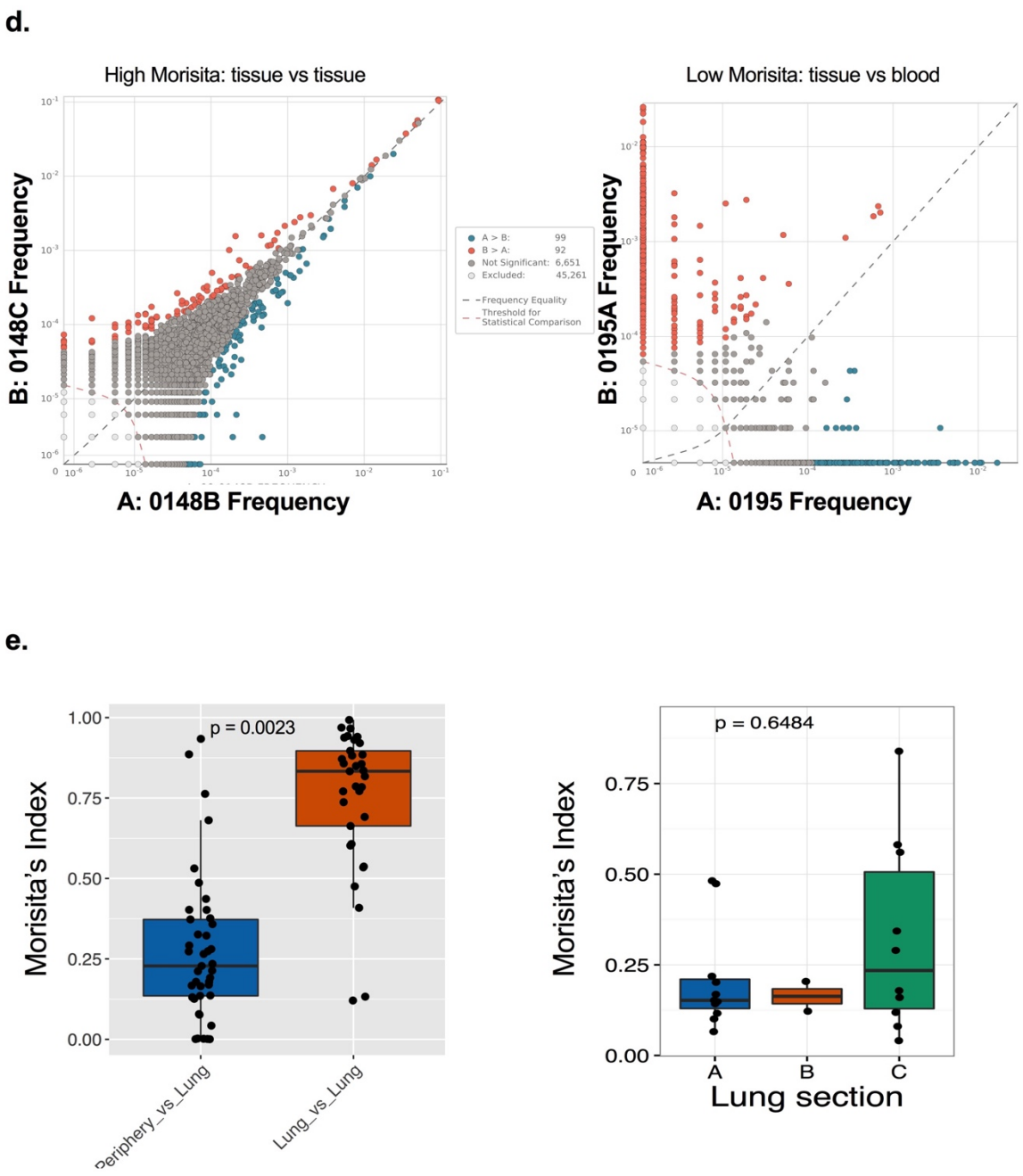


Figure 3.3: T-cell receptor repertoire measurement. (d) Representative Morisita's index plot between two lung pieces of the same individual (left) and between lung tissue and blood of the same individual (right). (e) Cumulative comparison of Morisita's index between peripheral circulation and lung (left) and within lung tissue pieces (right). Lung section – most diseased (A); moderate pathology (B) and least diseased (C). Statistics; non-parametric Mann-whitney t-test and one-way ANOVA.

3.3.5 Effect of active TB disease and HIV on T-cell clonality in blood and tissue

As discussed in the previous chapter the TB lung cohort is divided into those subjects with evidence of active TB disease, those with previous TB disease presumed to no longer be active and non-TB control. Thus, we next examined effect of TB disease status, in addition to HIV co-infection on the TCR repertoire. Overall, with the caveat that numbers in some groups are small, we find no significant differences in clonality score between any of the patient groups (figure 3.4a). HIV coinfection has an effect on TCR clonality in tissue, but there was no difference between clonality in tissue of TB negative and TB positive participants (figure 3.4a - c). In PBMC, by comparison, although TB had no difference in clonality of TCR in the peripheral blood when compared with previous TB, participants coinfected with HIV had a significantly higher clonality of TCR in circulation than participants without HIV (figure 3.4d - f). This effect can also be observed when plotting the paired clonality scores for PBMC and lung tissues (averaged across all lung pieces sequenced for that subject). Overall, clonality is higher in lung tissue, but this only true in subjects without HIV co-infection (figure 3.4g - i).

To explore the effect of HIV further, we treated every lung tissue piece as an independent sample and stratified on the basis of HIV status. By analyzing the data this way, we found there were fewer productive rearrangements (43,000 ± 4,000 vs. 28,000 ± 2,400, p=0.0024), and higher clonality (0.240 ± 0.08 vs 0.208 ± 0.06, p=0.0022) in the lung lesions of HIV positive subjects than in seronegative participants (figure 3.4k-l). This data suggests there are fewer CD3 T-cells within the lungs of HIV co-infected subjects but a higher clonal expansion, which could be consistent with the relative loss of CD4 T-cells and expansion of CD8 T-cells observed in the lungs of HIV co-infected subjects reported in the previous chapter.

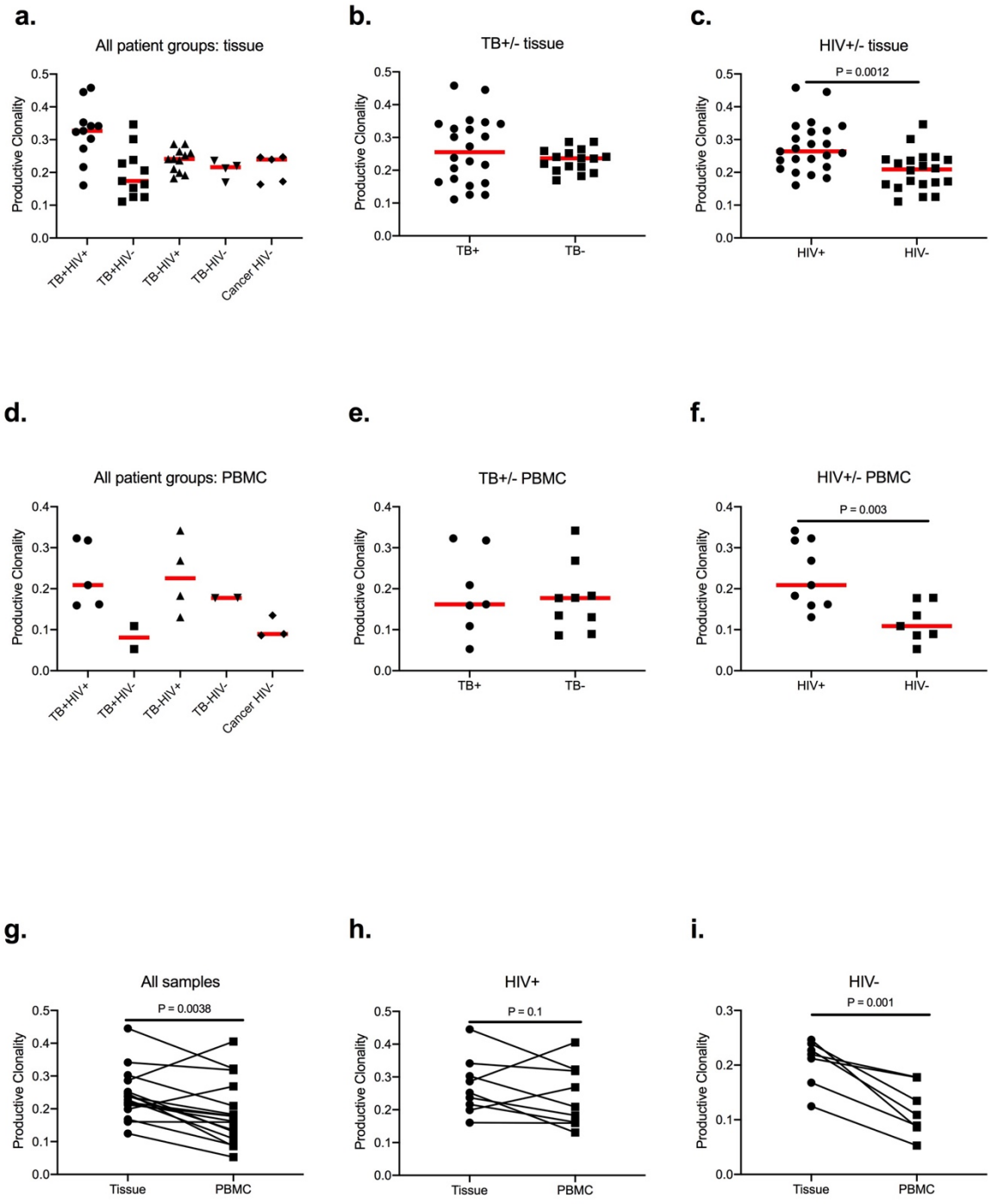


Figure 3.4: Effect of Active TB and HIV on T-cell clonality in tuberculosis. (a-c) TCR clonality in lung tissue. (d-f) TCR clonality in peripheral blood. (g - i) Paired TCR clonality in paired peripheral blood and lung tissue. Statistics, Non-parametric T-test (unpaired samples - Mann-Whitney), paired samples (Wilcoxon test).

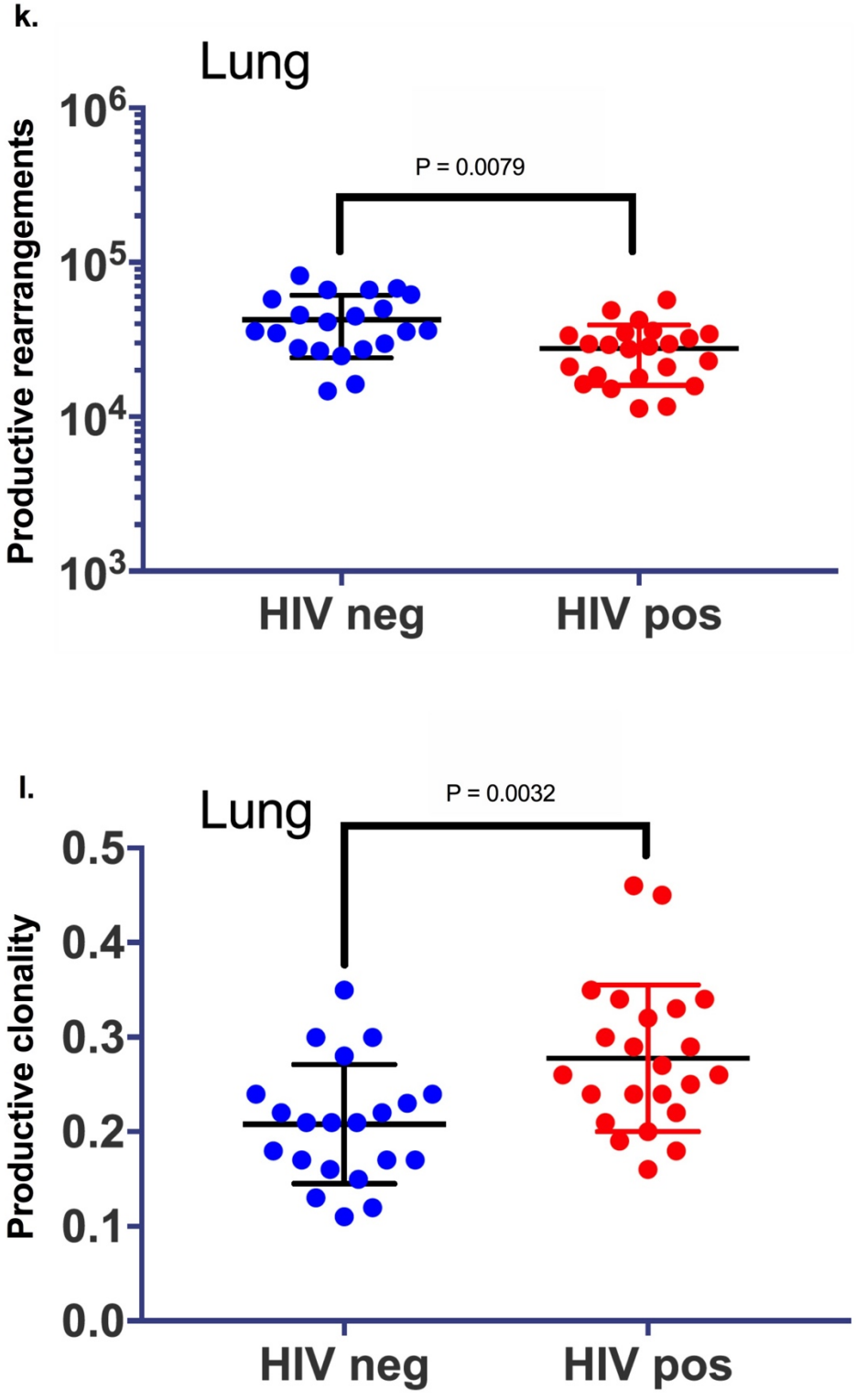


Figure 3.4: Effect of HIV on T-cell clonality in tuberculosis. (k-l) Productive rearrangements and clonality of T-cells in lung tissue by HIV co-infection. Statistics, Non-parametric Mann-Whitney t-test.

3.3.6 Distribution of unique T-cell clones

Next to further explore the contribution of CD4 and CD8 T-cells to the TCR repertoire in the lung we identified an additional 5 subjects with suspected active TB but no HIV co-infection and FACS sorted lung T-cell subsets for TCR sequencing. For this subset of participants, we sequenced the alpha chain of the TCR.

Based on FACS data discussed in chapter 2, CD4 and CD8 T-cells were sorted based on the expression of CD69, with the hypothesis that CD69+ve T-cells should be enriched for Trm subsets whilst CD69-ve T-cells would be more like circulating blood samples. DNA was extracted from sorted T-cells and sequenced using the Adaptive platform as before.

Figure 3.5a-b shows the data from all 5 subjects sequenced, and clearly shows that the clonal enrichment observed in the lung is likely to derive primarily from CD4+ve T-cells. In all subjects, clonality score was higher in lung T-cells than matched blood and was generally higher in the CD69+ compared to CD69-ve fraction in the lung (figure 3.5a). However, this was not the case for CD8 T-cells, in which differences in clonality were less consistent (figure 3.5b). This is clearly seen when pooling the data from all 5 subjects, where CD69+ CD4+ T-cells sorted from the lung collectively have a significantly higher clonality score than PBMC (figure 3.5c); but in the same comparison for CD8 T-cells the clonality score is not different (figure 3.5d). Overall these data suggest that the clonal expansion observed in TB infected lung tissue, in the absence of HIV, is driven by CD4 T-cells. This is in contrast to observations from viral infection of the lung, which have generally focused on CD8+ve T-cells but is consistent with the importance of CD4+ve T-cells to the TB immune response.

To further drill down on the differences between CD69+ve and CD69-ve T-cells, we searched each fraction for the presence of unique TCR clonotypes that were clonally expanded. An example of this is shown in figure 3.5e, where clonally expanded cells are represented by any dots outside the red dotted line, with unique TCRs those on the X or Y-axis. Just comparing lung cells, in all individuals there were more unique and clonally expanded TCR clonotypes in the CD69+ fraction than the CD69-ve fraction, consistent with non-recirculating Trm clonotypes. In addition, and again consistent with the above observation, this was far more apparent in CD4+ T-cells than CD8+ve T-cells (figure 3.5 f&i). The same trend is seen when comparing CD69+ve T-cells with matched blood, which

shows there is an average of 200 unique and clonally expanded in CD69+ve CD4 lung T-cells but almost none in the blood (figure 3.5g). Again, the same trend is seen in CD8 T-cells, but to a lesser extent (figure 3.5j). Interesting, unique clonally expanded TCR clonotypes are also seen in the CD69-ve lung T-cells compared to matched blood (figure 3.5 h&k). As expected, they are less frequent than in the CD69+ve fraction. However, there presence does suggest the potential existence of non-recirculating T-cell clones in the lung that lack CD69 expression. This is biologically plausible, as data from animal models does suggest that some Trms lack CD69 expression and Trms can develop even in CD69 knock mice (Schenkel and Masopust 2014). Taken together these data strongly support the existence of clonal expanded T-cells in the lung that are not found in circulation and may include TB specific Trm. This is further supported by the fact that, in these TB infected subjects, this is primarily seen in the CD4 compartment, which is known to be central to the TB adaptive immune response.

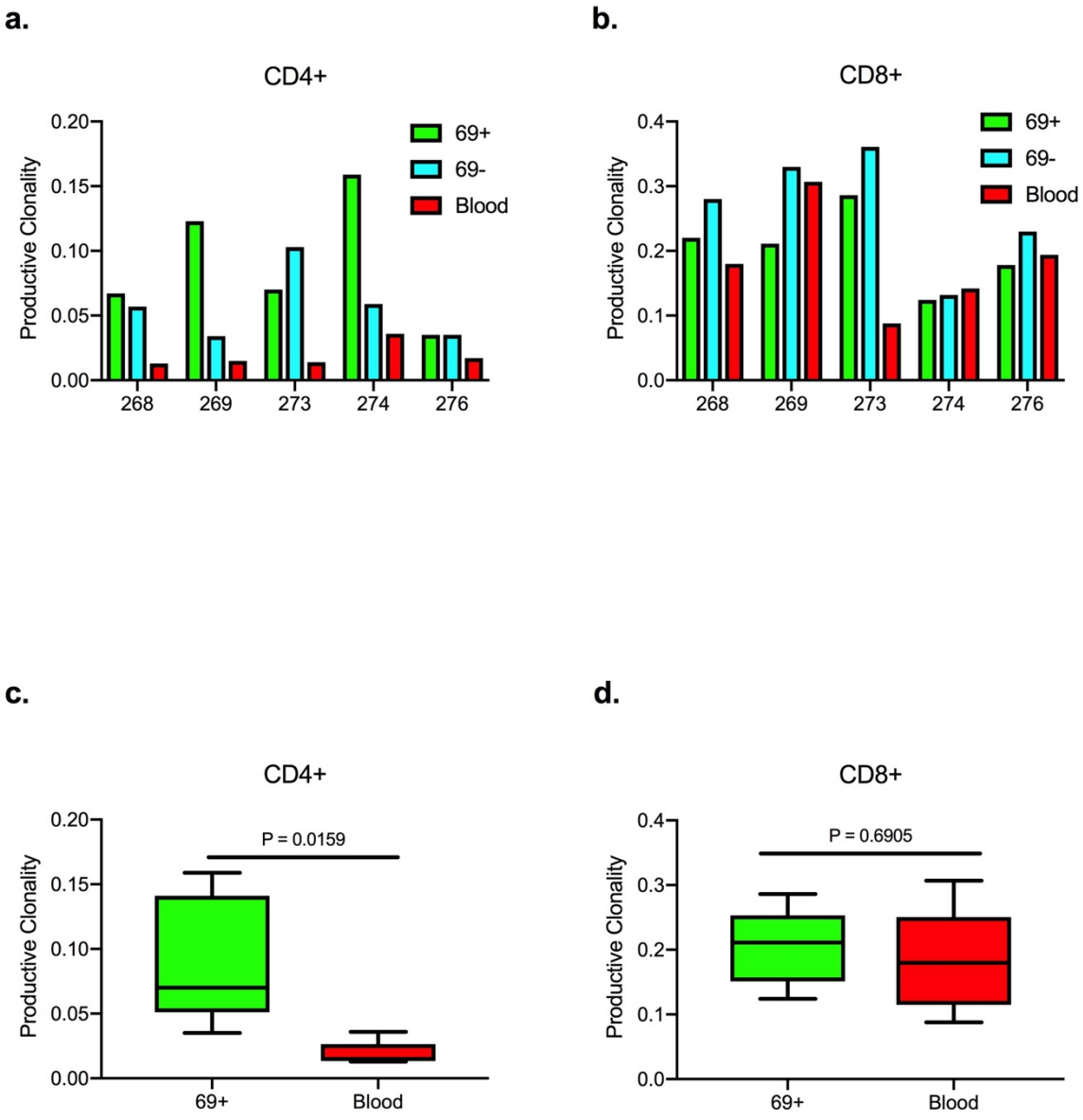


Figure 3.5: Distribution of T-cell clones. (a- d) Clonality of TCR in sorted CD4 and CD8 T-cells from systemic circulation and lung tissue. All samples from HIV negative participants and pre-FACS sorted before DNA extraction. Numbers on the x-axis (in a&b) refer to participant identification number. Statistics, non-parametric Mann-Whitney t- test.

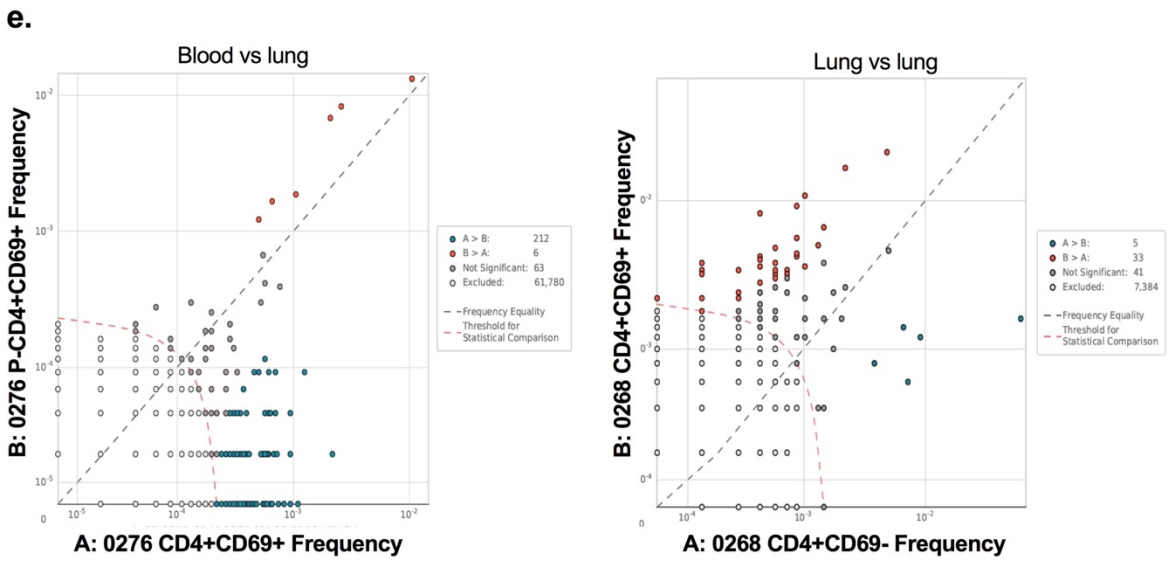


Figure 3.5: Distribution of T-cell clones. (e) Representative distribution of unique T-cell clones within CD69+ and CD69- lung T-cells and CD69- blood T-cells.

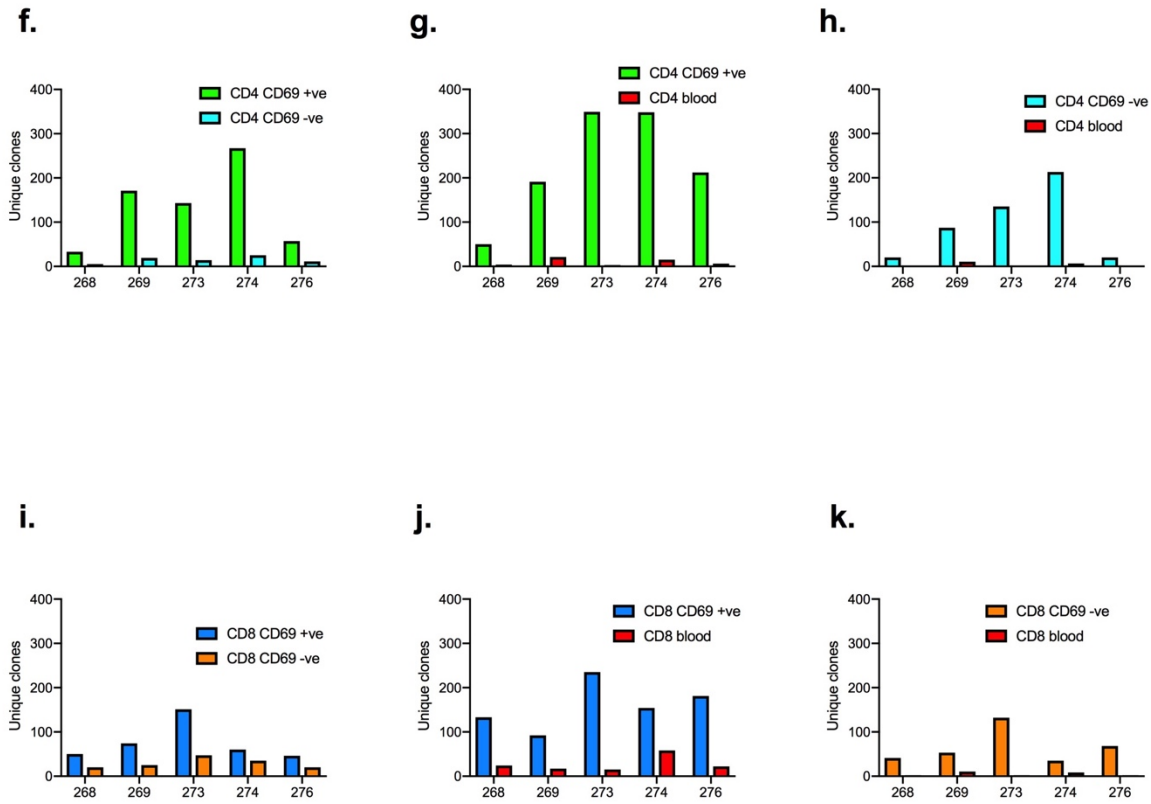


Figure 3.5: Distribution of T-cell clones. (f-k) Cumulative data of unique clones in sorted lung T-cells and blood T-cells.

3.3.7 Contribution of unconventional T-cells on TCR repertoire clonality

As DURTs such as MAITs are have a limited TCR repertoire and are present at a high frequency, we were interested to test to what extent they had an impact on TCR clonality. Using an extended definition of donor unrestricted T-cells (DURTs) (Appendix table III), conventional T-cell clonality was compared to overall clonality to determine if overall clonality was skewed by the unconventional T-cells. First, the clonality of on each sample was calculated after removing DURT TCRs. Surprisingly, the results show that clonality appeared to be unaffected by the exclusion of unconventional T-cells in all subjects (figure 3.6a). Next, using this combined definition of DURTS we compared DURT frequency in healthy controls with TB cases found an overall decline in frequency of DURTs in blood of TB cases compared to healthy controls, consistent with data show earlier (figure 3.1a-e). Examining the data this way, clearly shows that γδ T-cells are the most abundant DURT TCR, followed by MAITS, iNKTs and GEMS (figure 3.6b), and the frequency of these different DURT subsets does not appear to vary between lung different lung tissue fractions in the same lung (figure 3.6c). Taken together, the total T-cell clonality is not affected by unconventional T-cells present in the sequenced sample and γδ T-cells is the most frequent of the DURTs in both blood and lung tissue.

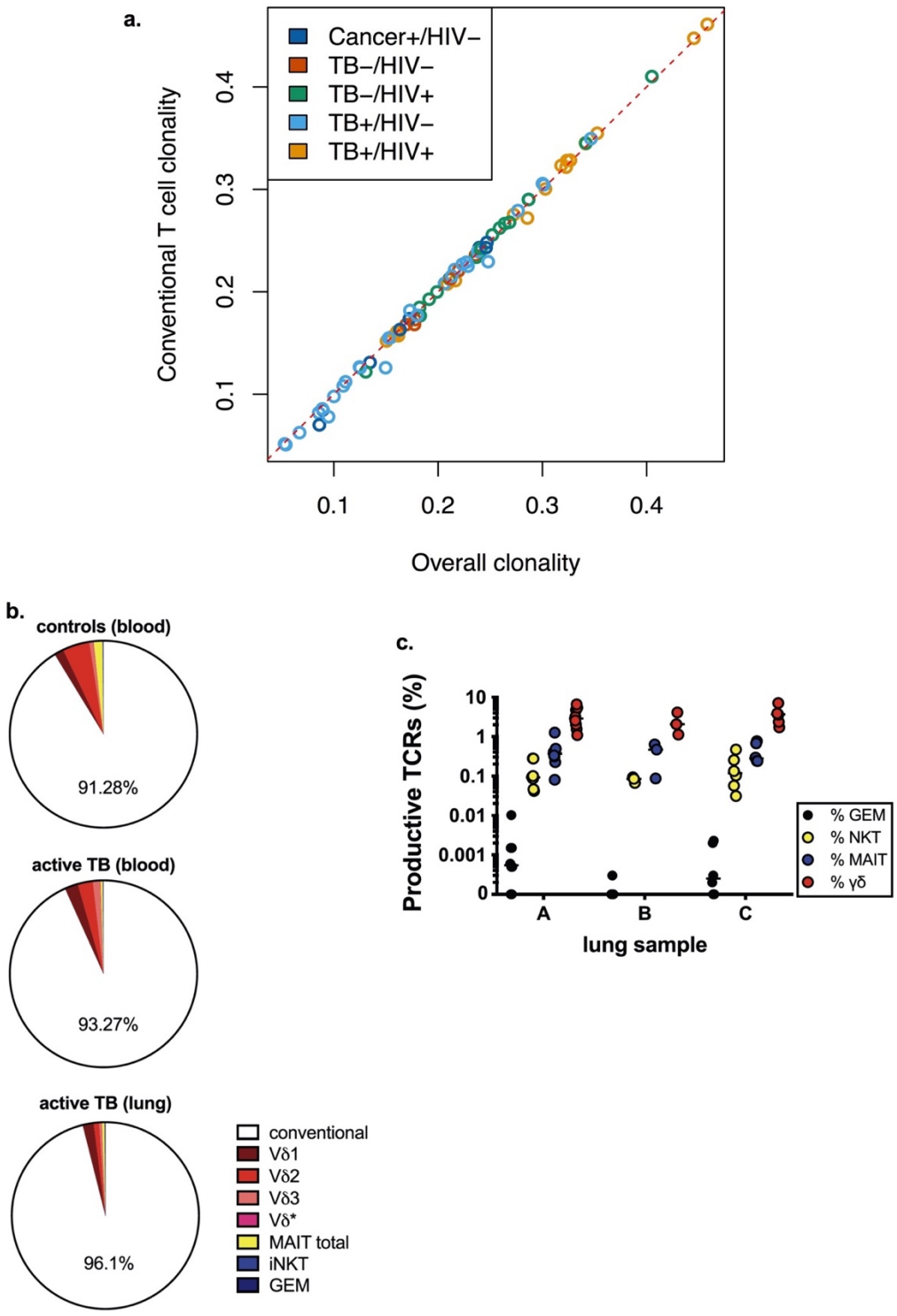


Figure 3.6: Contribution of DURTS on the clonality of T-cells. (a) Correlation of conventional T-cell clonality with the overall clonality. Conventional T-cell clonality calculated after subtraction of clonality of unconventional T-cells (γδ T-cells, iNKT, MAIT and GEM T-cells). (b) Frequency of TCRs in blood of healthy control, participants with active TB and lung tissue of active TB positive HIV negative individuals. (c) Frequency of unconventional TCRs in lung tissue pieces.

3.3.8 Frequency of DURT TCRs in the lungs of subjects with active or prior TB and HIV co-infection

Having explored the impact of active TB and HIV co-infection on the overall TCR repertoire, we next examined the impact of these clinical phenotypes on specific DURT cell frequencies in the blood and lung. The invariant TCRα of iNKT was detected in 77/79 lung or blood samples (figure 3.7a), but was not significantly enriched in the lung compared to the peripheral blood of any subgroup, nor did the frequency differ between HIV negative and HIV positive subjects, in either the blood or in the lung (figure 3.7a). Therefore, while nearly all subjects had iNKT-cells present in their blood and lung tissue, there did not appear to be any consistent increase or decrease in iNKT-cells associated with any disease group or compartment.

Although MAIT cells TCRs have been defined, there is growing data to suggest that MAIT TCRs are more heterogonous than initially believed (Greenaway et al 2013). Therefore, for MAIT cells we used three definitions (see appendix III) to identify MAIT TCRs: a) classic MAIT TCR; b) extended MAIT TCR ($MAIT_{Ext}$); and c) MAIT-like TCR, the "total" MAIT repertoire (classic + extended + MAIT-like) were detected in 78/79 samples (figure 3.7b). With this extended definition, the frequency of total MAIT TCRs were not significantly enriched in lung lesions compared to peripheral blood, except for participants with previous TB and HIV co-infection (figure 3.7b). This suggests the marginal increase in MAIT frequency in the all lung samples observed in figure 3.1f is driven by the previous TB HIV positive subgroup. It is hard to speculate what the reasons for this association might be, however, it does indicate that MAIT cells per se are not depleted from the lung tissue in HIV infected individuals. In addition, we see no clear expansion of either MAITs or iNKTs in the lung of subjects with active TB compared to those with prior TB or non-TB controls. For γδ T-cells, as previously observed in figure 3.6b, their frequency was higher in the blood of HIV negative participants with active TB (figure 3.7c), but otherwise, they did not appear to be significantly enriched in the lungs of participants in any of the disease subgroups. Thus, based on these data, there did not appear to be any data supporting the idea that DURTs are specifically or preferentially recruited to the site of active TB lesions.

As for MAITs, we expanded the definition of CD1-rectricted TCR beyond the canonical published GEM TCR sequence. The CD1b-restricted T-cell recognizing GMM is encoded by TRAV01-02 and TRAJ09 (van Rhijn et al 2013). Its CDR3α amino acid sequence is

“CAVRNTGGFKTIF,” which is the result of a rearrangement of amino acids in the motif. By expanding the motif to include ‘classic’ GEM TCRs (i.e., CAVRDTGGFKTIF) and GEM-like TCRs (CAVXXTGGFKTIF), all TCRα rearrangements was searched using the motif CAVXXTGGFKTIF. The most frequently detected clonotypes in lung were CAVRNTGGFKTIF (‘classic’ n=45/45), CAVRDTGGFKTIF (n=45/45), and CAVLNTGGFKTIF (n=38/45). Of the 26 AA clonotypes identified, all used Jα9; but only ~10% used Vα1-02; instead, the most common (47%) was Vα20. Although these TCRs were commonly detected within the cohort, their abundance was generally low (figure 3.7d). One participant with previous TB HIV positive (193) had significant increases in classic GEM – but the frequency was similar in blood and lung.

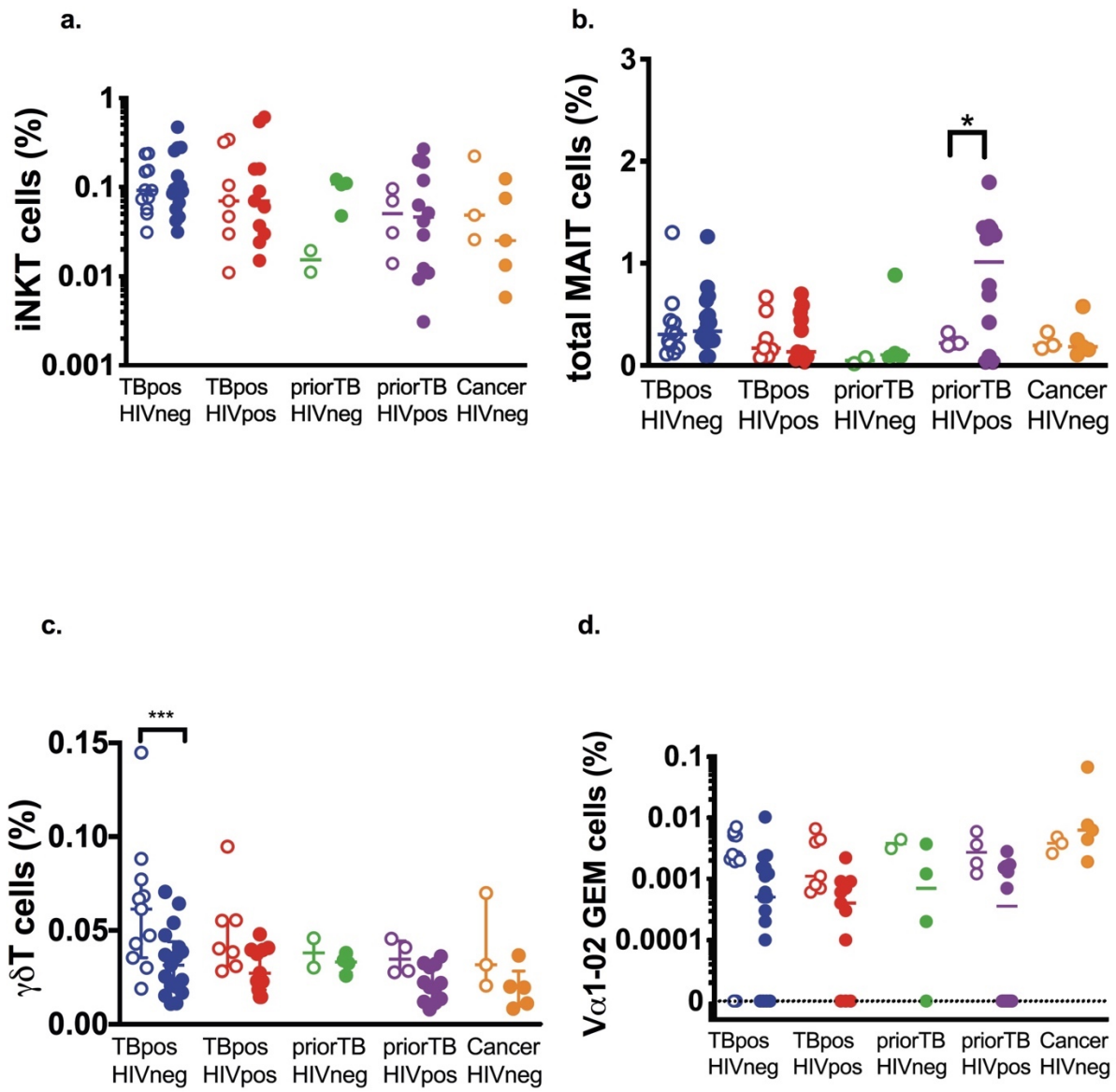


Figure 3.7: Donor unrestricted T-cells in blood (open circles) and lung tissue (solid circles). (a) iNKT-cells (b) total (classic + extended + MAIT-like) MAIT-cells (c) γδ T-cells and (d) GEM T-cell in blood and lung sections (A, B C). Statistics – non parametric Mann-Whitney t-test p = * (< 0.05), p = * (< 0.001)**

3.3.9 TCRδ clonotypes in the lung

Finally, we focused on the TCRδ clonotypes to see if there was any evidence of compartmentalization of γδ T-cells within TB infected lung tissue. Over 30,000 unique TCRδ clonotypes were found among the participants studied. Surprisingly, of the 10,737 found in the lung, only 15.6% were also found in the blood (figure 3.8a). While there were individual variations in which Vδ gene segments were used, the Vδ1 gene was most frequently used by the TCRδ clonotypes in the lung irrespective of disease state (figure 3.8b). This is somewhat different to blood where the distribution of Vδ1 and Vδ2 were roughly even (figure 3.8c). Overall, gene segment use in the lung was skewed towards Vδ1, and a population of Vδ1Jδ3 T-cells was more apparent in the lung than in blood.

Next, the top 10 most abundant TCRδ clonotypes from each sample were examined. The top 10 PBMC TCRδ clonotypes consisted of 270 sequences, of which 269 were unique. For the lung samples, there were 319 unique clonotypes from 430 sequences (figure 3.8d). Of these, 72 were shared with blood. These top 10 TCRδ clonotypes accounted for an average of 56 ± 17.5% (range 13-90%) of all TCRδ clonotypes in the lung sample (figure 3.8e). This fraction was significantly lower among HIV negative participants with active TB compared to HIV positive subjects with active TB or HIV negative participants with previous TB (figure 3.8e). This may suggest that HIV negative participants with active TB have a more diverse γδ T-cell response to *Mtb*.

These "top 10" clonotypes were further evaluated to determine which ones were truly enriched in the lung by determining their frequency in blood relative to the sum of the lung samples (1-3 samples per participant). For the 72 TCRδ clonotypes that were abundant both in blood and in lung, most were present at a relatively low frequency (<0.001) or were more abundant in blood (figure 3.8f - left). Only 6 of the 72 were increased >5-fold in the lung. Then, the remaining 247 TCRδ clonotypes were compared. Of these, 143 were present at a frequency >0.001 and most were >5-fold enriched in the lung (figure 3.8f - middle). Based on these results, all of 30,000 TCRδ clonotypes were evaluated in this manner. With this approach, 106 unique TCRδ clonotypes were identified that were both abundant (productive frequency >0.001) and lung-enriched (lung/blood >5) present at the lung frequency. From then clonotypes that were abundant or were enriched in the lung (lung/blood ratio >5) were defined (figure 3.8f - right).

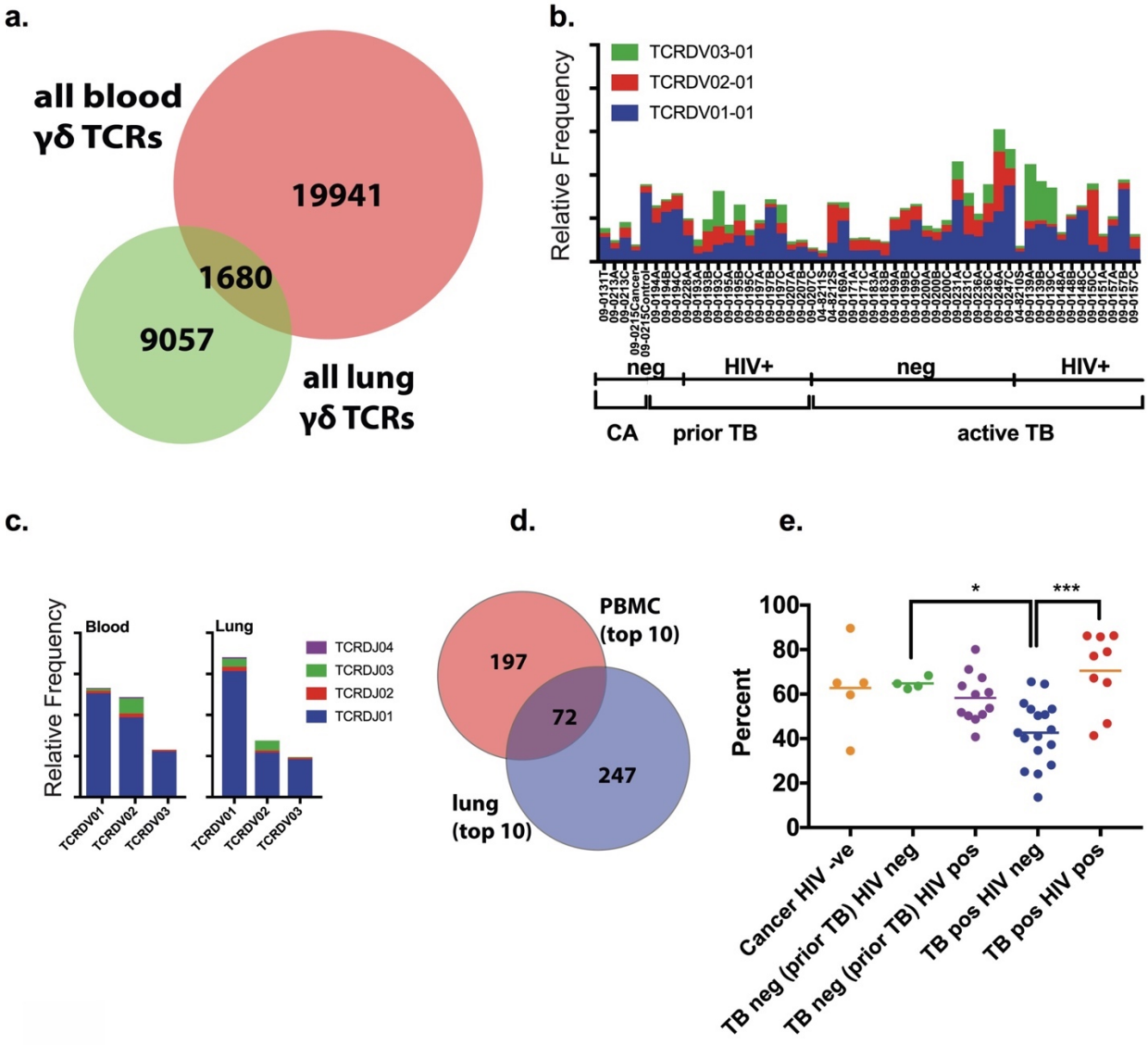
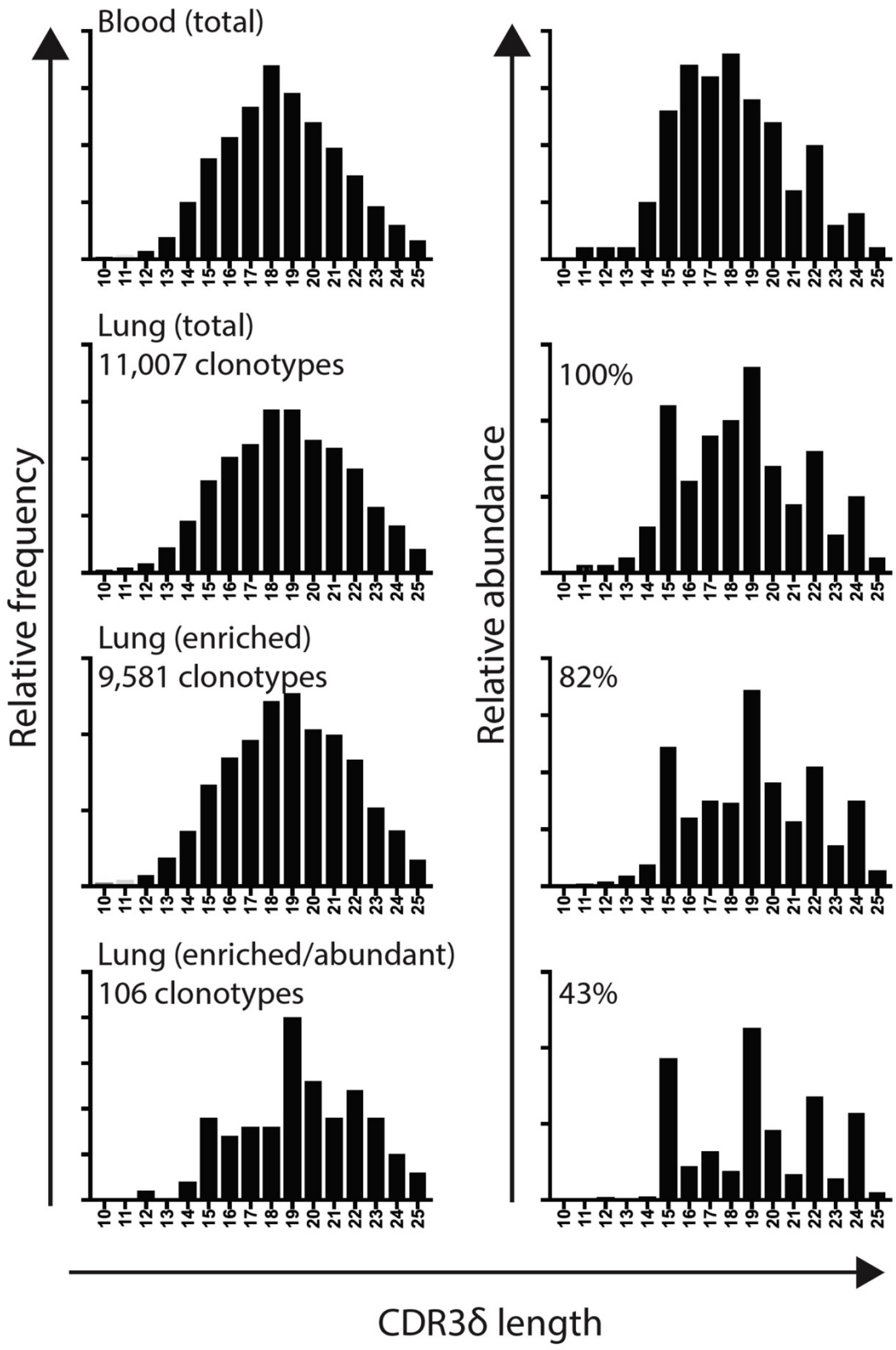

a.
all blood γδ TCRs
19941
1680
9057
all lung γδ TCRs
b.
TCRDV03-01
TCRDV02-01
TCRDV01-01
Relative Frequency
neg
HIV+
neg
HIV+
CA
prior TB
active TB
c.
Blood
Lung
TCRDJ04
TCRDJ03
TCRDJ02
TCRDJ01
Relative Frequency
TCRDV01
TCRDV02
TCRDV03
d.
PBMC (top 10)
197
72
247
lung (top 10)
e.
Percent
*
***
Cancer HIV -ve
TB neg (prior TB) HIV neg
TB neg (prior TB) HIV pos
TB pos HIV neg
TB pos HIV pos
f.
L/B >5
freq > 0.001
combined lung frequency
blood frequency
n=6
n=35
n=31
n=45
n=98
n=50
n=42
n=12
9,057 (30%)
1,676 (5%)
19,940 (65%)

Figure 3.8: Distribution of unique δ-TCR clonotypes. (a) A venn diagram of all unique δ-TCR clonotypes from all participants. (b) The use of Vδ1, Vδ2, or Vδ3 gene segments by δ-TCR clonotypes in the lung. (c) An analysis of the pairing of Vδ1, Vδ2, or Vδ3 with Jδ1, Jδ2, Jδ3, or Jδ4, gene segments. (d) An overlap of 269 unique "top 10" δ-TCR clonotypes from blood vs. the 319 unique δ-TCR clonotypes from lung. (e) The percent of total δ-TCR sequences represented by the "top 10 abundant" clonotypes in each sample, categorized by clinical subgroup. (f) δ-TCR clonotypes preferentially detected in the lung; left, the 72 clonotypes shared with the "top 10" blood clonotypes; middle, the remaining 247 "top 10" lung clonotypes"; right, all 30,673 unique clonotype. Statistics, Mann-Whitney t-test. p = * (< 0.05), p = ** (< 0.001).

3.3.10 Enriched and abundant TCRδ T-cells in the lung.

Although the 106 unique TCRδ clonotypes represent fewer than 1% of the 10,737 unique clonotypes identified in the lung, they make up 43% of the productive TCRδ rearrangements. In considering the CDR3δ lengths of these clonotypes, this population appeared to be skewed, with a bias towards a length of 19 (figure 3.9a, left). To ensure the results were not due to sequencing errors, the distribution of CDR3δ lengths based on their productive frequency (figure 3.9a, right) was also determined. This analysis shows that the abundant TCRs in the lung are biased towards a CDR3δ length of 15 or 19. This skewing is apparent in the total population of lung TCRδ clonotypes and becomes more obvious after selecting for lung-enriched clonotypes, and especially those that are enriched and abundant. Thus, even though total frequency of TCRδ+ T-cells is reduced in the lung compared to the blood during TB infection, many clonotypes found in the lung are not detected in blood and are enriched and abundant in the lung. As an example of the abundance and enrichment of these 106 TCRδ clonotypes, twelve distinct clonotypes from 10 subjects are shown (figure 3.9b). Several are enriched more than 100-fold in the lung and are present with productive frequencies of >1%.

a.


Blood (total)
Lung (total)
11,007 clonotypes
Lung (enriched)
9,581 clonotypes
Lung (enriched/abundant)
106 clonotypes
100%
82%
43%
Relative frequency
Relative abundance
CDR3δ length
10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

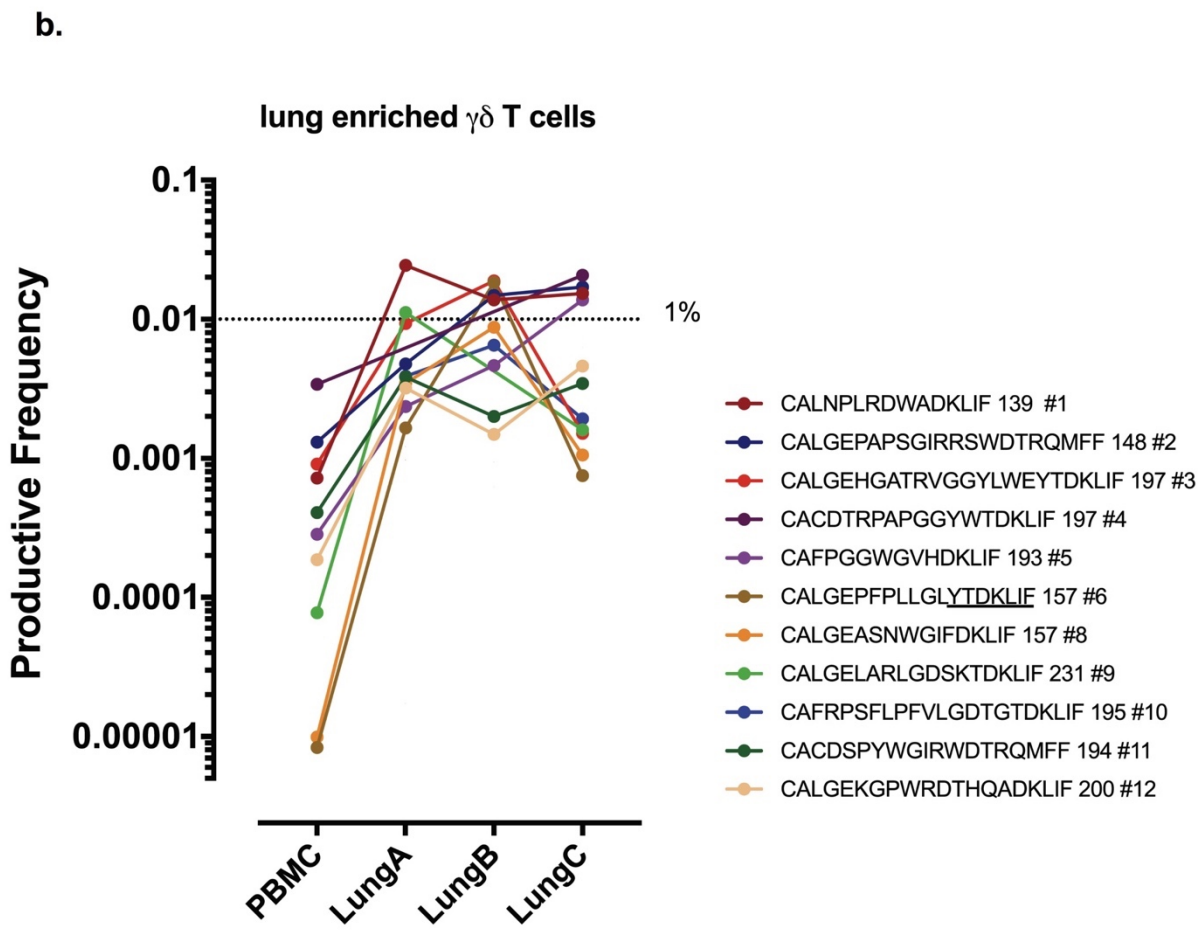


Figure 3.9: Enriched and abundant δ-TCR clonotypes. (a) The distribution of CDR3δ lengths based on the relative frequency (left) or abundance (right) of all unique clonotypes or clonotypes enriched in the lung, or enriched and abundant in lung. (c) Of the 106 enriched and abundant δ-TCR clonotypes detected in lung, productive frequency of the top 12 in blood and lung are shown.

3.4 Discussion

I observed that T-cell immune responses to *Mtb* infection in the human lung were different, in terms of cell types and magnitude of response, from systemic circulation responses. We used a different approach of analysis of TCR in blood and lung tissue in participants with chronic TB cases and lung cancer controls as well as healthy controls (TB negative, HIV negative) to confirm that there were tissue resident T-cells in the lung that do not recirculate in blood. Since the TCR repertoire is a mirror of the human immune response, its characteristics have been widely investigated in infectious and other diseases to study the state of the immune system and the progression of these diseases (Sabet et al. 2007; Wells et al. 2007; Clarêncio et al. 2006; Chaudhry et al. 2013; Di Sante et al. 2015; O'Connell et al. 2014; Schrama, Ritter, and Becker 2017). TCR repertoire can reflect the function, status and composition of T-cell populations (Degauque et al. 2011; Torikai et al. 2012).

T-cells express an antigen-specific TCR, which enables recognition of foreign peptide antigens vastly when bound to MHC molecules (Garcia et al. 1996; DeWitt et al. 2018). Among genetically unrelated individuals that share a dominant MHC allele, a minority of T-cells recognizing a common viral peptide share a common TCR-β sequence (Venturi et al. 2008). Thus, generalizing information derived from TCR sequences is often limited to the dominant MHC type, rarely present in >40% of a given population (Santos et al. 2016). TCR repertoire diversity of both αβ and γδ T-cells can be affected by clonal expansion following the stimulation of *Mtb* antigens and affects a patient's immune response to the pathogen (Luo et al. 2012; Cheng et al. 2018).

TCR repertoire analysis results in this study showed that there was a higher clonal expansion of T-cells and many unique T-cell clones in the lung than peripheral blood. However, clonality was not different between lung pieces. To eliminate the effect of 'contaminating' blood in lung tissue T-cell clonality, cells from lung tissue and matched blood were FACS sorted based on expression of CD69 cell surface marker followed by extraction of DNA for alpha TCR immunosequencing. CD69 is expressed by tissue resident memory T-cells (discussed in detail in chapters1 and 2) and TCR analysis of Lung CD69+ cells showed that not only was clonal expansion higher in lung CD69+ T-cells than PBMC, but there were also more unique T-cell clones compared to Lung CD69- or PBMC. This observation is in agreement with flow cytometry data that showed tissue resident memory T-cells in the lung

that are absent in circulation. Additionally, because in general Lung CD69- T-cells have higher clonality than blood cells but less clonality than lung CD69+ T-cells, this suggests that the lung has another population of cells that does not express CD69, in agreement with flow cytometry data discussed earlier and other studies (Schenkel and Masopust 2014; Hombrink et al. 2016; Oja et al. 2017).

The TCR sequencing approach we employed enabled us to identify DURT TCRs as well as conventional TCRs in the same sample. We thus sought to find out if the DURTs we detected in our samples skewed the overall clonality reported. The frequency of DURTs did not affect the overall clonal expansion of T-cells and could imply that there are shared features between conventional and unconventional TCRs in the TB cases, as has been reported earlier. T-cells specific for a lipid antigen presented by the highly conserved antigen-presenting molecule CD1b shared several features with T-cells specific for peptide antigens restricted by polymorphic MHC (DeWitt et al. 2018). Comparison of percent productive TCRs of unconventional T-cells between lung pieces showed no difference between the pieces, and γδ T-cells were constantly higher than GEM, iNKT and MAIT-cells.

Among the unconventional T-cells, the γδ T-cells are thought to play a critical role in anti-*Mtb* immunity (Casetti and Martino 2008; Holtmeier and Kabelitz 2005; Davey et al. 2011). Results presented here are in agreement with previous studies as the frequency of total γδ T-cells, Vδ2 and Vγ9Vδ2 were depleted in peripheral circulation of TB cases compared to healthy controls. From sequencing results, total γδ T-cells were depleted in the lung tissue compared to the peripheral circulation of TB cases, suggesting that these cells are not recruited to the lung during TB. However, Vδ1 cells were expanded in the peripheral blood of TB cases compared to healthy controls as shown by FACS staining. Since the antibody panel for FACS staining did not contain markers for all the γδ T-cell subsets, the observation that only Vδ1 cells were enriched in the TB cases suggests effect of *Mtb* on the γδ T-cells probably varies according to subset.

γδT-cells in peripheral blood can also be present in increased proportions in some individuals with tuberculosis (Balbi et al. 1993) and some clonotypic γδ T-cells exhibited clonal expansion during BCG infection and re-infection (Chen and Letvin 2003). This observation is different from our own and could be explained by the nature of participants studied by Balbi et al and those studied here. While our study looked at chronic TB cases, Balbi study was

done on early active TB, and our study could not report on the early changes that occur on γδ T-cells at the onset of active TB disease. Although there was an overall high frequency of γδ T-cells in the systemic circulation than lung, there were unique and abundant γδ T-cell clones in the lung that were not shared with γδ T-cells in peripheral circulation, and lung γδ T-cells were skewed towards the use of CDR3δ length of 15 and 19. This suggests the existence of tissue specific non-recirculation γδ T-cell clones in the lung. This is in agreement with a previous study of *Bordetella pertussis* in which aerosol infection of mice established γδ T cells with a Trm cell phenotype (CD69+CD103+) (Misiak et al. 2017). The functional role of these lung tissue-specific γδ T-cell clones in tuberculosis warrant further investigation particularly in newly diagnosed active TB individuals.

MAIT-cells may act as early sensors of *Mtb* infection in airways and provide early cytokines to activate macrophages against infection. By both flow cytometry and TCR sequencing, there was depletion of MAIT-cells in the peripheral blood of TB cases compared to healthy controls. It has also been confirmed by other studies that MAIT-cell frequencies are decreased during active tuberculosis (and non-tuberculosis mycobacterial lung disease) in diverse geographic settings (Sharma et al. 2015; Le Bourhis et al. 2010; Jiang et al. 2014; Kwon et al. 2015). The MAIT TCR motif can be variable and using both classic and extended definition of MAIT sequences based on TRAV01-02 TRAJ33 (Greenaway et al. 2013), MAIT-cells were detected in 78/79 samples analyzed from TB cases. There was an enrichment of MAIT-cells in the lung but not PBMC of participants with previous TB HIV positive suggesting that HIV probably does not deplete MAIT cells in the lung tissue or the possibility of non-recirculating MAIT-cells in the lung.

In this study, frequency of GEM and iNKT-cells was not measured by FACS due to unavailability of tetramers or a suitable cell surface marker. From TCR sequencing, there was a significant loss of GEM T-cells in peripheral blood and lung of TB cases compared to healthy control peripheral blood. No compartment specific differences within in TB cases was observed for GEM T-cells. Surprisingly, the frequency of iNKT-cells was similar between healthy controls and TB cases and between compartments of TB cases. This is in contrast to most clinical studies that have been done on early *Mtb* infection (Sutherland et al. 2009; Im et al. 2008; Montoya et al. 2008), however, iNKTs are dispensable in chronic TB in mice studies (Behar et al. 1999; Sugawara et al. 2002; Sousa et al. 2000). Compared to other DURTs, GEM T-cells and iNKT-cells were, in general, present in very low frequencies.

The CDR3 length has a profound effect on the shape of TCR, and the analysis of CDR3 length variation can reveal the structure-function relationships of different TCRs recognizing specific antigens (Rock et al. 1994; Cheng et al. 2018). The unique CDR3 length distribution of the δ chain exhibited a single/multi-peak-shaped pattern (Gaussian distribution) in total blood and lung clonotypes, similar to a previous study looking at TCR beta variable gene family in PBMCs from healthy donors (Yang et al. 2013). However, most of the unique and enriched clonotypes in the lung were skewed towards a length of 19 amino acids confirming that the clonotypes found in the lung are not detected in blood, consistent with a tissue resident T-cell clone.

Studies between peripheral blood and pleural fluid from patients with tuberculous pleuritis observed significant changes in the TCRB repertoire of T lymphocytes between peripheral blood and the pleural fluid (Gambón-Deza et al. 1995). Expanded T-cell clones were found in the pleural effusion (Zhou et al. 2013; Luo et al. 2012; Gambón-Deza et al. 1995) compared to PBMC. Oligoclonal expansions in the - pleural effusion mononuclear cells - PEMCs was shown by Li and colleagues (Li et al. 2014). In contrast, the TCR repertoire in peripheral blood was polyclonal and there was just a fraction of TCR repertoire overlapping between pleural effusion and peripheral blood. We observed a higher clonality of T-cell clones in lung tissue than peripheral circulation in this study. While it is likely that in comparison there are more TB antigens in the lung tissue than systemic circulation which is driving the clonal expansion of T-cells, it is also possible that clonality in the lung is driven by more effector memory T-cells in the lung tissue compared to blood cells that are mainly naive as described in chapter 2 of this thesis. Indeed, it was shown that TB specific memory T-cells were readily expanded in the mouse lung following *Mtb* challenge (Carpenter et al. 2016b).

The epidemic of tuberculosis has been exacerbated by the HIV pandemic which affects the ability of T-cells, particularly CD4 T-cells, to mount a robust and appropriate response to *Mtb*. In our hands, although HIV coinfected individuals were on anti-retroviral therapy, HIV had a significant effect on T-cell clonality especially in systemic circulation compared to lung tissue. The impact of HIV coinfection on *Mtb*-specific T-cell repertoires in the lung warrant extensive investigation.

We have provided a comprehensive analysis of the TCR of both conventional and unconventional T-cells in peripheral blood and lung tissue in a cohort of chronic TB

participants, providing insights into the dynamics of the TCR repertoire at the site of TB disease and in circulation. For comparison, peripheral blood from healthy controls was studied to measure changes in T-cells in TB disease. Since TCR repertoire diversity can be influenced by many factors including other coinfections, the extent of the likely contribution of TCRs specific to non-TB antigens is not clear in these findings. However, the study has shown that HIV infection has significant contribution to the clonal expansion of T-cells, and this was tested formally in a subset of HIV negative participants. Given that participants with active TB were on anti-TB treatment, the effect of treatment has also not been measured and it was reported that chemotherapy can substantially change the TCR repertoire (Luo et al. 2012). It was shown that TCR repertoire diversity inversely correlated with disease severity as patients with mild disease had the highest relative complexity while severely infected patients had the lowest complexity (Luo et al 2012).

In summary, we have shown that DURTs (except Vδ1 T-cells – enriched – and iNKT – unchanged) are depleted in peripheral circulation of chronic TB cases and also shown that there is high clonal expansion of T-cell clones at the site of TB disease than in systemic circulation. Some of the expanded clones are unique to the lung and are not shared with T-cell clones in circulation. In agreement with flow cytometry data discussed in chapter 2 of this thesis, there are tissue specific T-cells that either do not recirculate or that recirculate but very infrequently between lung and blood as evidenced by more unique T-cell clones in lung CD69+ T-cells compared to blood T-cells from the same individual. HIV coinfection remains a key driver of TB pathology and has been shown here to increase the clonality of T-cells, particularly in circulation. Although no differences were observed between lung tissue pieces, even from the same individual, it is possible this observation could be different in initial TB infection and was missed by the fact that this cohort was made of chronic TB cases. Indeed, heterogeneity of immune responses at the granuloma level has been observed in animal model studies where infection course is easy to track, and coinfections are limited.

CHAPTER 4: CHARACTERIZATION OF B-CELLS IN TUBERCULOSIS: PHENOTYPE AND FUNCTIONAL ROLE

4.1 Introduction

Whereas this thesis was initially set out to study T-cells in TB infected human lung and understand how they differed from T-cells in peripheral blood in terms of phenotype, function and specificity, I constantly observed that there was an enrichment of B-cells in TB infected human lung compared to peripheral blood. Thus, I set out to also characterize the B-cell phenotypes in blood and lung tissue in the context of TB disease and also measured the specificity of B cells in the two compartments together with lung associated lymph nodes. These findings are extensively explained in this chapter.

It is acknowledged that T-cells are important for the control of TB disease. By contrast, the significance of B-cells and humoral immunity in shaping the immune response to *Mtb* is less clear (Maglione and Chan 2009; Casadevall and Pirofski 2011; Glatman-Freedman and Casadevall 1998). Detailed studies of how B-cells affect tuberculosis immunity are somewhat limited and have mostly been done in mice. However, these studies do provide evidence that B-cells and humoral immunity are necessary for the development of optimal anti-TB immunity and for restricting excessive inflammation in the acute phase of infection (Maglione, Xu, and Chan 2007; Vordermeier et al. 1996; Bosio, Gardner, and Elkins 2000; Kozakiewicz et al. 2013; Maglione et al. 2008; Torrado et al. 2013; Maglione and Chan 2009). Infection of B-cell deficient mice with *Mtb,* for example, results in enhanced pathology and increased bacterial loads (Vordermeier et al. 1996; Maglione, Xu, and Chan 2007), depending on the route of infection, either the lung (Maglione et al 2007) or systemic (Vordemeier et al 1996). The lungs of *Mtb*-infected B-cell-deficient mice display enhanced neutrophil recruitment (Maglione et al 2007) which is associated with excessive lung pathology and with poor bacillary control (Eruslanov et al. 2005; Keller et al. 2006). Moreover, increased lung pathology and *Mtb* dissemination outside the lung in B-cell deficient mice, could be prevented by adoptive transfer of B-cells, but not immunoglobulins (Bosio, Gardner, and Elkins 2000), suggesting B-cell activity in TB maybe not be limited to antibody production. In support of this, B-cell-depletion with rituximab has been shown to alter the IL-17/Th17 response (Hamel et al. 2008; Hu et al. 2007; Matsushita et al. 2008), although the effect was not consistent. In addition, a subset of B-cells in the blood of humans with tuberculous infection can suppress Th17 response (Zhang et al. 2012) and it has been

shown that B-cells can regulate the pulmonic inflammatory response, in the early acute phase of TB (Kozakiewicz et al. 2013). Differences between antibody functional profiles between LTBI and active TB have been linked to differential activation of innate immunity and *Mtb* killing within primary macrophages, suggesting that antibodies may not only mark disease states but also contribute functionally to infection outcome (Lu et al. 2016). Overall, these studies support a role for B-cells in the immune response to TB, generally by limiting lung damage.

Within the TB infected lung, B-cells, plasma cells, antibodies and antibody-responsive innate immune cells bearing Fc receptors (FcR) are all abundant in TB granulomas (Phuah et al. 2012; Tsai et al. 2006). By histology, B-cells are a conspicuous cellular component of the lung granulomatous response in tuberculous mice (Slight et al. 2013; Tsai et al. 2006; Maglione, Xu, and Chan 2007; Gonzalez-Juarrero et al. 2001; J. Turner et al. 2001; Khader et al. 2009), non-human primates (Slight et al. 2013; Phuah et al. 2012), and humans (Slight et al. 2013; Tsai et al. 2006; Ulrichs et al. 2004, 2005). B-cells form prominent aggregates with characteristic features of the germinal center (Slight et al 2013, Maglione et al 2007, Khader et al 2009, Ulrichs et al 2005, Ulrichs et al 2004). A recent detailed study in non-human primates at the granuloma level revealed that B-cells can significantly modulate cytokine production, bacterial burden, and inflammation levels in the TB lesions (Phuah et al. 2016). Here, B-cell cytokine production within granulomas was found highly variable, even among individual granulomas from the same animal, and there was a higher median CFU in granuloma that lacked B-cells. This variability in the immunologic environment among granulomas within the lung has previously been reported for T-cells in the same system (Gideon et al. 2015b), and suggest that B-cells have a role in controlling infection in at least a subset of granulomas or animals (Phuah et al 2016).

The canonical function of B-cells is the production of pathogen specific antibodies. Antibodies may function in TB in several ways. They exert their functions by either directly blocking of host T-cell invasion and neutralization of bacterial products, or indirectly through Fc-mediated complement and cell activation mechanisms through Fc receptors (Ravetch and Clynes 1998). Antibodies may also form immune complexes with soluble bacterial antigens or enter the cell through pinocytosis/endocytosis and recognize bacteria in endosomal compartments (Lu et al. 2016). Furthermore, intracellular FcRs, such as TRIM21 (McEwan et al. 2013), may allow for the recognition of bacterial components within the cytoplasm.

Since the granulomas contain an array of immune cells beyond macrophages (Nunes-Alves et al. 2014; Tsai et al. 2006; Ulrichs et al. 2004), the ultimate impact of functional antibodies is likely to be a product of how they direct bacterial fate within macrophages as well as engage the collaborative activity of the local cellular microenvironment (Lu et al. 2016). Multiple studies suggest mAbs play a role in murine models of TB infection (Achkar and Casadevall 2013; Balu et al. 2011; Buccheri et al. 2009; Hamasur et al. 2004; Pethe et al. 2001; Teitelbaum et al. 1998, 1999). Passive immunization of mice with either human or murine polyclonal sera showed protection against infection (Roy et al. 2005; Guirado et al. 2006). Immune sera from infected mice was also able to reverse the lung neutrophilia and the excessive Th17 response in B-cell-deficient mice with TB (Kozakiewicz et al. 2013). This is biologically plausible as immunoglobulins are known to be effective modulators of inflammation (Casadevall and Pirofski 2011; Brady 2005). In human TB, the transfer of antibodies from healthcare workers but not active TB patients showed moderate protection against *Mtb* in an aerosol mouse challenge model (Li et al. 2017). In this study, three of the seven healthcare workers who made protective antibody responses were IGRA negative, suggesting a protective antibody response could have prevented latent TB infection. In a separate study, antibodies from individuals with LTBI were found to enhance several macrophage responses against intracellular *Mtb*, including phagolysosomal maturation and inflammasome activation independent of pyroptosis, that could lead to increase bacterial control (Lu et al. 2016).

The protective function of anti-MTB antibodies, at least in part, may depend on the target antigen and host cell type, the local environment, and the route of administration (mucosa vs. periphery) (Falero-Diaz et al. 2000; Pethe et al. 2001; López et al. 2009; Chambers, Gavier-Widén, and Hewinson 2004; Hamasur et al. 2004; Williams et al. 2004; Roy et al. 2005). Antibodies from patients with active TB disease have been reported to recognize a different repertoire of *Mtb* antigens than those from individuals with controlled latent TB, and antibody titers correlated with mycobacterial burden (Achkar and Casadevall 2013). Recently, it was demonstrated that antibody levels did not correlate with total B-cell frequencies within the same sample, as patients with very low B-cell frequencies still had average levels of PPD reactive antibodies (Joosten et al. 2016).

In addition to antibody production, B-cells may also have both direct and indirect influence on TB infection by modulating immune responses to *Mtb* infection as antigen-presenting

cells or through the production of cytokines (Glatman-Freedman and Casadevall 1998; Teitelbaum et al. 1998; Bosio, Gardner, and Elkins 2000; Chambers, Gavier-Widén, and Hewinson 2004; Roy et al. 2005; Maglione et al. 2008; Maglione, Xu, and Chan 2007; Achkar, Chan, and Casadevall 2015). Antigen uptake by B-cells typically occurs via the B-cell-receptor; however, live mycobacteria can also infect B-cells through macropinocytosis, resulting in MHC class II antigen presentation (Lombardi et al. 1987; Zhu et al. 2016). B-cells and the humoral immune response modulate T-cell immunity, including the development of memory T-cell responses during infection (Lund and Randall 2010; Maglione and Chan 2009) and vaccine-induced protection against secondary challenge with intracellular pathogens such as Chlamydia (Igietseme et al. 2004) and Francisella (Rawool et al. 2008). Regulatory B-cells may play an important role in modulating inflammation during infection, although this may depend on the *Mtb* strain and phase of infection (Magniole et al 2007, Bosio et al 2000, Kozakiewicz et al 2013).

There is no consensus in the variation of B-cell dynamics during TB disease in humans, as B-cell frequencies in active TB have been reported to be unaltered (Barcelos et al. 2006); increased (Wu et al. 2009); or decreased (Corominas et al. 2004; Hernandez et al. 2010) compared to healthy controls. Compared to healthy donors, LTBI individuals have been reported to have decreased B-cell frequencies (Corominas et al 2004), whereas those successfully treated for TB had increased B-cell frequencies (Barcelos et al 2006). In addition, patients with multi-drug resistant (MDR) TB were reported to have decreased frequencies of unswitched, IgD+CD27+ B-cells and decreased plasma cell frequencies, which are frequently observed during chronic inflammation (Abreu et al. 2014). Plasma cells differentiate from activated memory B-cells, a population that is relatively upregulated in LTBI and patients with active TB, suggesting that *Mtb* does not alter the normal development of antibody producing cells and thus also allow the production of *Mtb* specific antibodies. Acute plasmablast response to *Mtb* originates from reactivated memory B-cells and indicates a mucosal origin (Zimmermann et al. 2016). There were no differences in antibody levels between patients with active TB disease and those successfully treated for TB, supporting that plasma cell differentiation and function of differentiated plasma cells is not affected in a similar manner as total B-cell function in those with active TB disease (Joosten et al. 2007). In this chapter, the phenotype and specificity of B-cells to *Mtb* is investigated in detail. In the first set of experiments, a cohort of HIV negative participants with active TB, LTBI cases and

healthy controls was used. The second study in the lung and lung lymph nodes is done using tissue obtained from the TB lung cohort outlined earlier.

4.2 Methods

4.2.1 PBMC and Single cell suspension

B-cells were isolated from PBMC and lung tissue as indicated in section 2.2.1. The B-cell phenotype panel is detailed in the appendix section.

4.2.1 Immunohistochemistry staining

Formalin fixed paraffin embedded tissue sections were cut into 4uM thick serial sections using Shandon Finesse 325 microtome (Thermo Scientific). After sectioning onto microscope slides, tissue sections were deparaffinized in xylene and rehydrated in a series of ethanol dilutions. Sections were incubated 10 min in 3% hydrogen peroxide to quench endogenous peroxidase. The sections were then stained with the following antibodies: anti-CD3 antibody (SP7) (ab16669), Abcam, MA, USA; anti-CD68 monoclonal antibody (KP1), Abcam, MA, USA; anti-CD20 monoclonal antibody (mAb) clone L26 (Abcam). The slides were acquired using Nanozoomer 2.0RS (Hamamatsu)

4.2.2 Conjugation of biotinylated protein antigens.

Varying amounts of streptavidin – APC or streptavidin - PE was added to 10µg of biotinylated protein five times, each time mixing up and down by pipetting. After every addition of streptavidin, the protein was incubated at room temperature for 20 mins away from direct light. After the fifth addition of streptavidin, every protein – streptavidin conjugate was 125µL in volume. Finally, 5µL Biotin (ThermoFischer) to quench unbound streptavidin to give a final bait volume of 130µL. The conjugated protein was stored at 4^{o}C for no more than 28 days before use.

4.2.3 B-cell baiting with biotinylated protein antigen

Streptavidin antigen baits, 5uL of bait per 5 x 10$^{\wedge 6}$ cells were incubated for 20 mins on ice in the dark followed by washing twice with PBS. The cells were then surface stained with 2uL of surface antibody per test (see antibody appendix) for 20 minutes on ice in the dark followed by washing twice with PBS. Live dead cell marker (near IR, Invitrogen) at a dilution of 1:200 was added together with cell surface antibodies. After final wash, cells were

resuspended in 2% PFA and acquired on FACSARIA Fusion and data analysed using FlowJo Software v.9.9 (Treestar Inc, Ashland, OR).

4.3 Results

4.3.1 Low frequency of B-cells in peripheral circulation in active and latent TB infection

There is no clear consensus on how B-cell frequencies change in humans during TB disease (Joosten et al. 2016). Therefore, we first sought to determine the phenotype of peripheral blood B-cells in our study population, using PBMCs from participants with active TB (GeneXpert positive) treatment naive, latent TB and healthy controls (the last two categories defined based on Quantiferon TB gold interferon gamma release assay test). Phenotyping was carried out using a limited number of markers and the gating strategy in figure 4.1a. Overall, there were significantly fewer total B-cells (CD19+CD45+) in subjects with active or latent TB compared to healthy control, but, surprisingly, no differences between LTBI and active TB groups (figure 4.1b). Total B-cells were further analyzed for the co-expression of the B-cell phenotype makers CD21 and CD10. Although these are not standard B-cell phenotypic markers they were chosen as CD21- B-cells have been described in several conditions of chronic immune activation (Thorarinsdottir et al. 2016), and are comprised of activated memory and atypical B-cells; and atypical B-cells have been reported to be elevated in active TB (Joosten et al 2016). The addition of CD10 allows identification of immature or transitional B-cells. The majority of B-cells in the PBMCs of all subjects are CD21+CD10 negative, but this is significantly higher in subjects with LTBI compared to either healthy controls or individuals with active TB (figure 4.1c). In agreement with published work, the great majority of CD21- B-cells in PBMCs were CD10-, conforming to an activated memory or atypical phenotype rather than transitional B-cells (Thorarinsdottir et al 2016). Consistent with their presence in chronic inflammatory conditions, these CD21-CD10- B-cells were significantly higher in subjects with active TB compared to both healthy controls and those with LTBI (figure 4.1d). Finally, there was a higher frequency of immature CD21+CD10+ B-cells in healthy controls than in either subjects with LTBI or active TB (figure 4.1e). The difference between subjects with active TB and LTBI was not significant, although the frequency of immature B-cells tended to be lower in the active TB group. Overall, these data suggest a distinct transition between healthy controls and disease states in the B-cell compartment within PBMCs, although more precise phenotyping is required. In addition, they clearly confirm other studies demonstrating that TB infection in humans induces a B-cell response that is detectable in peripheral blood.

a.

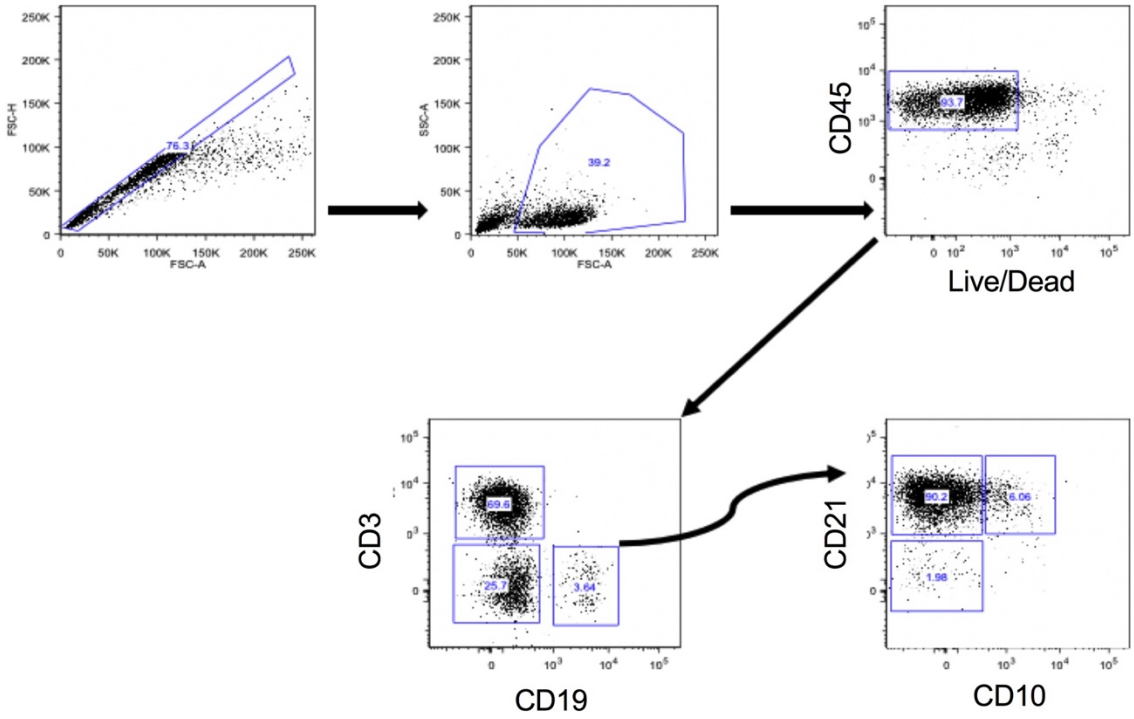


Figure 4.1: B-cells frequencies in peripheral blood. a) B-cell phenotype gating strategy on peripheral blood.

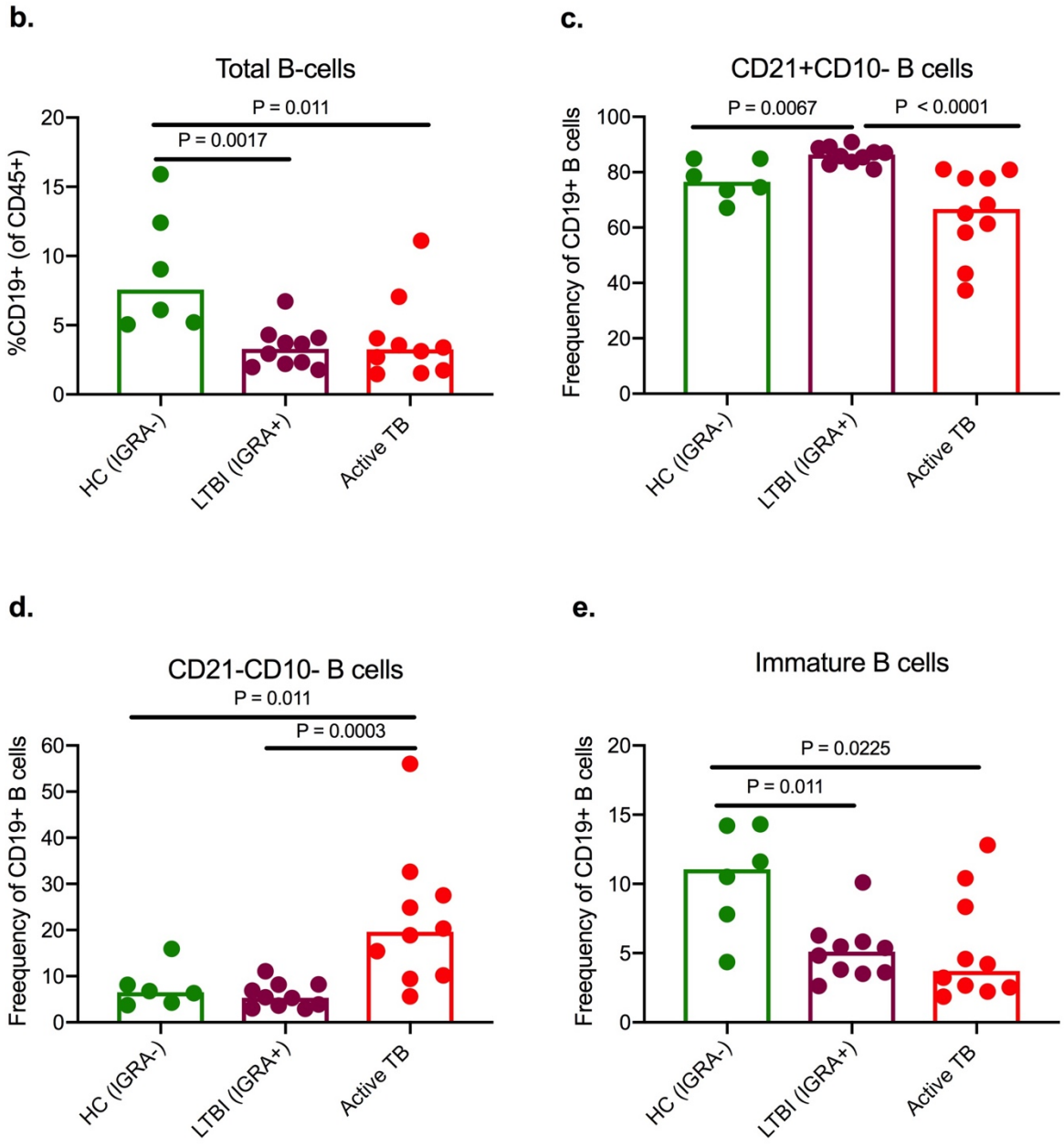


Figure 4.1: B-cells frequencies in peripheral blood. b-e) Frequency of B-cell phenotypes, total B-cells (CD19+) (b); CD21+ CD10- B-cells (c); CD21-CD10- B-cells (d) and immature B-cells (CD21+CD10+ B-cells (e). Healthy controls (HC) and Latent TB (LTBI) defined based on the Quantiferon TB gold test. Statistics; non-parametric Mann-Whitney t-test.

4.3.2 B-cells are enriched in the lung in chronic TB

One possible explanation for the observed difference in B-cell frequencies in peripheral circulation of healthy controls, LTBI and active TB participants (figure 4.1) is that B-cells get recruited to the lung, the primary site of infection during TB disease. To test this hypothesis, B-cells from fresh lung tissue of a cohort of participants with chronic TB who underwent lung resection were analyzed together with blood from the same cohort. As a fraction of total CD45+ve cells we observed a relative enrichment of CD19+ve B-cells in the lung compared to PBMC from the same cohort (figure 4.2 a (matched) and b (all samples). For comparison, we measured the total T-cells frequencies in lung and blood from the same subjects and found no significant difference (figure 4.2c). Presence of B-cells in lung was confirmed by immunohistochemistry staining of lung tissue from the same cohort. Figure 4.2d shows a representative example in which abundant B-cell clusters, staining positive for the B-cell marker CD20, can be observed in comparison to CD3+ve T-cells and CD68+ve macrophages. Additional staining of B-cell marker CD20 in other lung samples are shown in figure 4.2e. These cells are clearly found within the lung parenchyma and are not limited to the vascular system. Moreover, as shown in this image, CD20+ve B-cells are typically found in distinct clusters, suggestive of inducible Bronchio Alveolar Lymphoid Follicles (iBALT) (Moyron-Quiroz et al. 2006; Rangel-Moreno et al. 2006). These have previously been described in close proximity to TB lesions in mouse and NHP models of TB infection (Kaushal et al 2015), and in human TB infected lung tissue (Ulrichs et al. 2004).

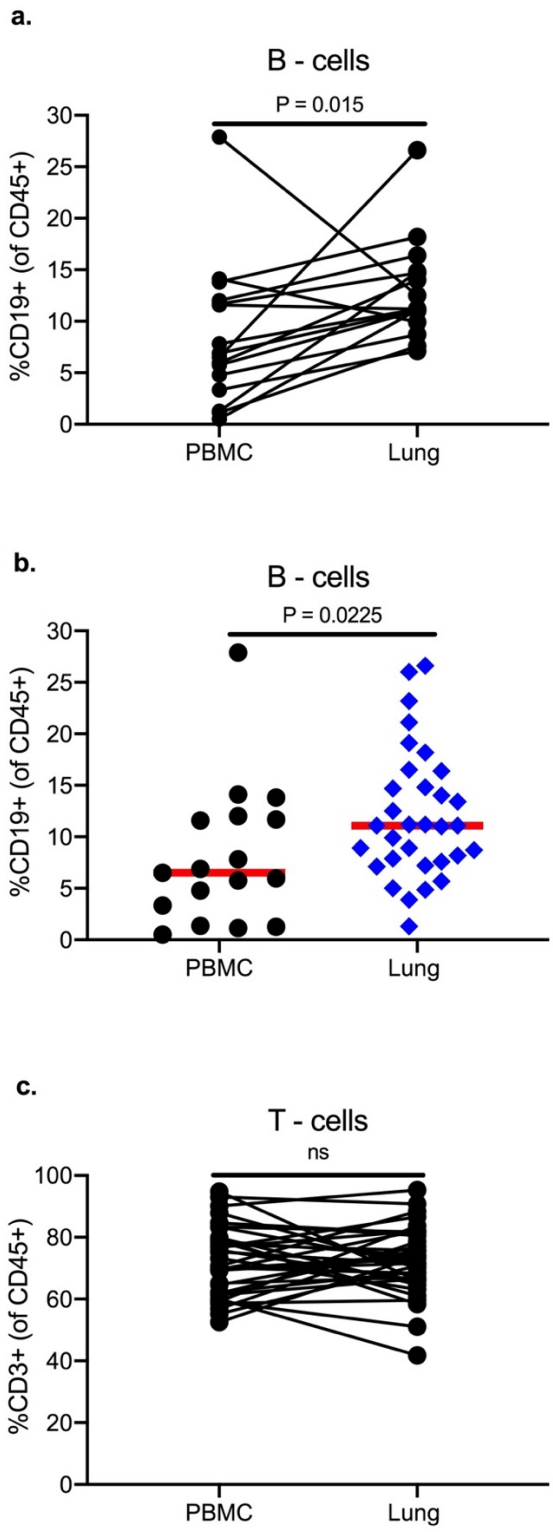


Figure 4.2: Enriched B-cell in lung of TB cases of previous TB episodes or active disease: B-cells frequencies in matched blood and lung tissue samples (a) and all samples (b); T-cell frequencies in PBMC and lung are unaltered (c). Statistics; non-parametric Wilcoxon t-test for paired comparisons and Mann-Whitney for unpaired comparisons.

d.

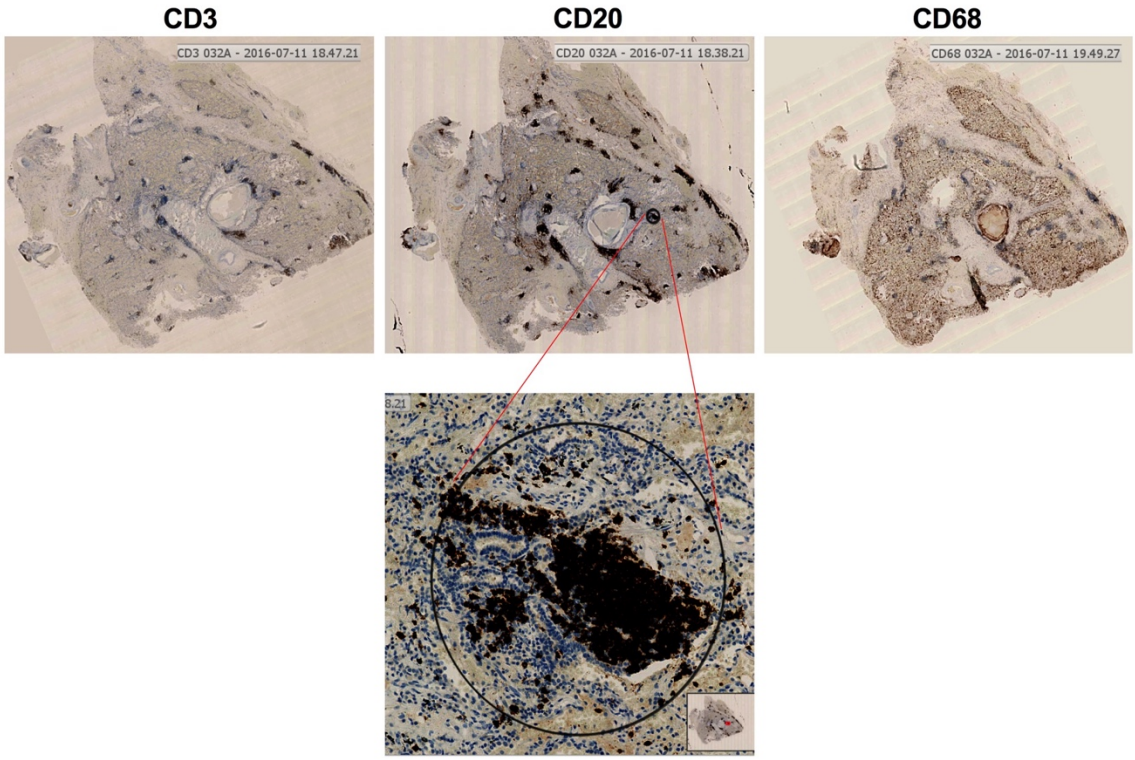


Figure 4.2: Enriched B-cell in lung of TB cases. (d) histology staining of lung tissue from TB infected lung showing numerous B-cell clusters in the lung (middle image), cross reference of the same tissue section with CD3 and CD68 is provided.

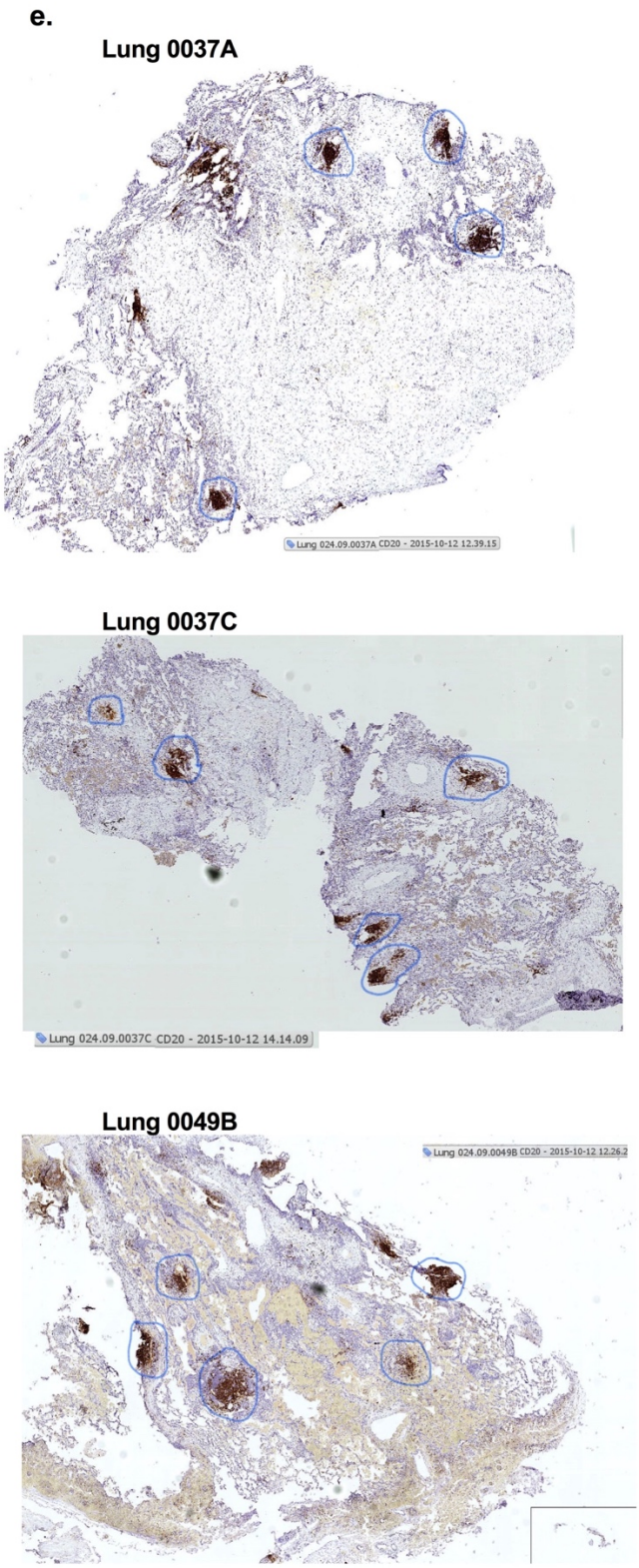


Figure 4.2: Enriched B-cell in lung of TB cases. (e) histology staining of lung tissues (three different samples) from TB infected lung showing numerous B-cell clusters (CD20+ve B cell stain) in the lung (blue circles).

4.3.3 High frequency of memory B-cells in the lung including plasmablasts

To explore the memory and activation status of B-cells enriched in the lung during TB infection we examined co-expression of CD27 and CD38 to distinguish naïve B-cells (CD27CD38-) from total memory B-cells (CD27+); transitional B-cells (CD27-CD38+) and plasma blasts (CD27++CD38++). The representative flow cytometry plot is shown in figure 4.3a. In addition to PBMC and lung tissue, lung lymph nodes from a fraction of the same participants were available and analyzed. There was a significantly greater proportion of memory B-cells in the lung than in PBMC and lymph nodes (figure 4.3b). Memory B-cells were also significantly enriched in lung lymph nodes compared to matched blood (figure 4.3b). Plasmablasts, were significantly enriched in lung tissue compared to PBMC figure 4.3c (matched) and 4.3 d (all samples). Plasmablasts were also higher in lung tissue than lung lymph nodes but the difference did not reach statistical significance. In addition, the difference between plasmablasts in lung lymph nodes and PBMC did not reach statistical significance (figure 4.3d). Finally, there was a significantly lower frequency of both naïve and transitional B-cells in lung compared to both PBMC and lung lymph nodes (figure 4.3e, f). Thus, lung tissue from subjects with active or prior TB infection are enriched for memory B-cells and antibody secreting plasma cells. This enrichment of B-cell memory phenotypes in the lung tissue supports the existence of a distinct population of B-cells with in the lung parenchyma that may not be represented in circulating blood.

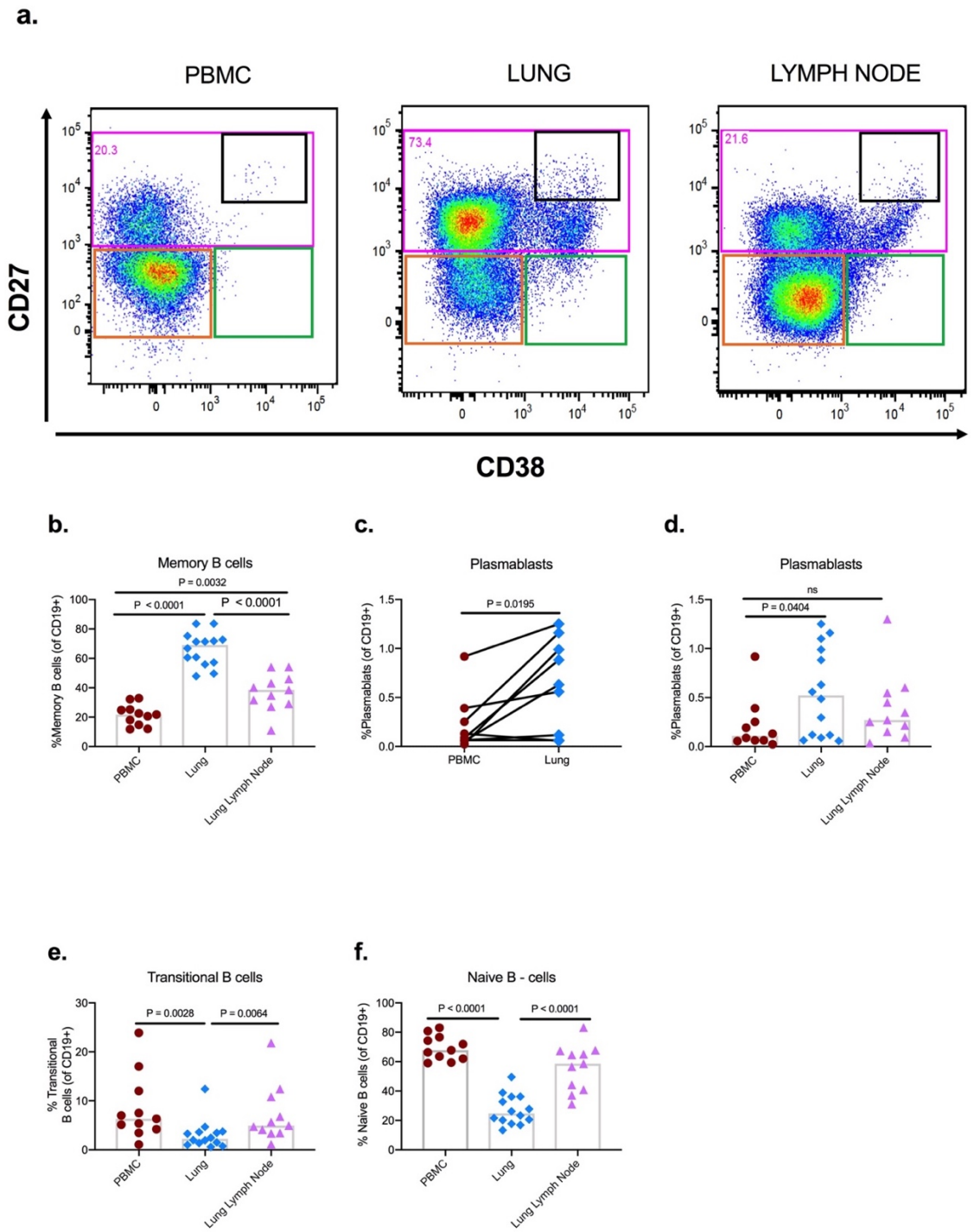


Figure 4.3: B-cell phenotypes in blood, lung and lung lymph nodes of chronic TB participants. (a) Representative FACS plots, samples pre-gated on CD19+CD45+ cells. Total memory B-cells (magenta gate), plasmablast (black gate), transitional B-cells (green gate) and Naïve B-cells (orange gate (b)Memory B-cells (after subtracting plasmablasts). (c -d) frequency of plasmablasts in matched lung – blood and all samples. (e) transitional B-cells and (f) naïve B-cells. Statistics; non-parametric Mann-Whitney t-test for unpaired comparisons and Wilcoxon for paired comparisons.

4.3.4 Evidence of Tissue resident B-cells within TB infected lung

To further explore the B-cells observed within the lung tissue for subjects infected with TB, we next examined expression of markers of tissue residency and immune activation (CD69, HLA-DR), and the pro-apoptotic death receptor Fas or CD95, on B-cell in PBMC, lung and lung lymph nodes. Lung tissue was enriched for CD69+ve B-cells compared to peripheral blood B-cells and lung lymph nodes (figure 4.4 a). Recent data suggest that the lung contains tissue resident B-cells, which, like their T-cell counterparts, express high levels of CD69 (Allie et al 2019). In line with this, CD69 expression was higher on B-cells in lung homogenate than in either PBMC or lung lymph nodes (figure 4.4a). Consistent with a Trm phenotype, CD69+ve B-cells in the lung are predominantly memory cells rather than naïve B-cells or plasmablasts (figure 4.4b). Indeed, in the lung the frequency of CD69+ve B-cells is similar to that of CD69+ve T-cells (figure 4.4c), whilst CD69+ve T-cells are much more abundant in the lung lymph nodes. Overall, these data support the existence a population of Trm B-cells within TB infected lung tissue, which, like Trm T-cells, may have limited recirculation in the blood.

B-cell memory arises from antibody class switching from IgM to other immunoglobulin isotypes (Tangye et al. 2002) and most memory B-cells would be IgM-ve (Fondere et al. 2003; Tuaillon et al. 2006). However, IgM+ve memory B-cells have also been described on the basis of expression of CD27 and these IgM+ve memory B-cells are thought to be generated independently of germinal center (Tangye and Good 2007; Weller et al. 2004; Küppers et al. 1993). Thus, we measured frequency of IgM in peripheral blood, lung tissue and lung lymph nodes (figure 4.4 d-f). Peripheral blood CD19+ B-cells had a significantly higher level of IgM+ve expressing cells than IgM-ve cells (figure 4.4 e). Consistent with a memory phenotype, the majority of B-cells in the lung tissue were IgM-ve, while the lung lymph nodes had equal expression of IgM+ve and IgM-ve cells (figure 4.4e). Within the lung tissue, CD69- B-cells were predominantly IgM-ve while some of CD69+ B-cells were also IgM+ve (figure 4.4f). There was no difference between IgM+ve and IgM-ve in the lung lymph nodes on the basis of CD69 expression (figure 4.4f)

An expanded CD95+ve population of B-cells has been described in individuals with immune thrombocytopenia (Flint et al. 2016). In addition, CD95 is reported to be upregulated on

activated memory B-cells (Wei, Jung, and Sanz 2011) supporting the hypothesis that CD69 expression on these cells relates to tissue residence rather than activation. We therefore measured CD95 levels in lung tissue and lung lymph nodes in a few participants in our cohort (figure 4.4g). In both lung tissue and lung lymph nodes, the mean fluorescent intensity (MFI) of CD95 was higher in CD69+ve B-cells than CD69-ve B-cells. This data is consistent with the previous report of CD95 upregulation on memory B cells (Wei, Jung, and Sanz 2011). As with CD95, HLA-DR expression on B-cells has been associated with activation (Wei, Jung, and Sanz 2011). Furthermore, HLA-DR expression has been reported to be lower on memory B-cells than on naïve B-cells (Harp et al. 2010). Indeed, we observed that in the lung tissue and lung lymph nodes, the MFI of HLA-DR was significantly higher on CD69+ B-cells than on CD69- B-cells (figure 4.4h). Therefore, low of expression of HLA-DR on lung B-cells is consistent with a tissue resident, non-activated B-cell population within the human lung. We observed that HLA-DR expression was much higher on B-cells than T-cells in all compartments (figure 4.4i), reflecting their role as antigen presenting cells. The fact that HLA-DR expression is reduced on B-cells in the lung (figure 4.4j) but remains high in lung associated lymph nodes is consistent with a role for B-cells in antigen presentation in the latter compartment, but not in the lung.

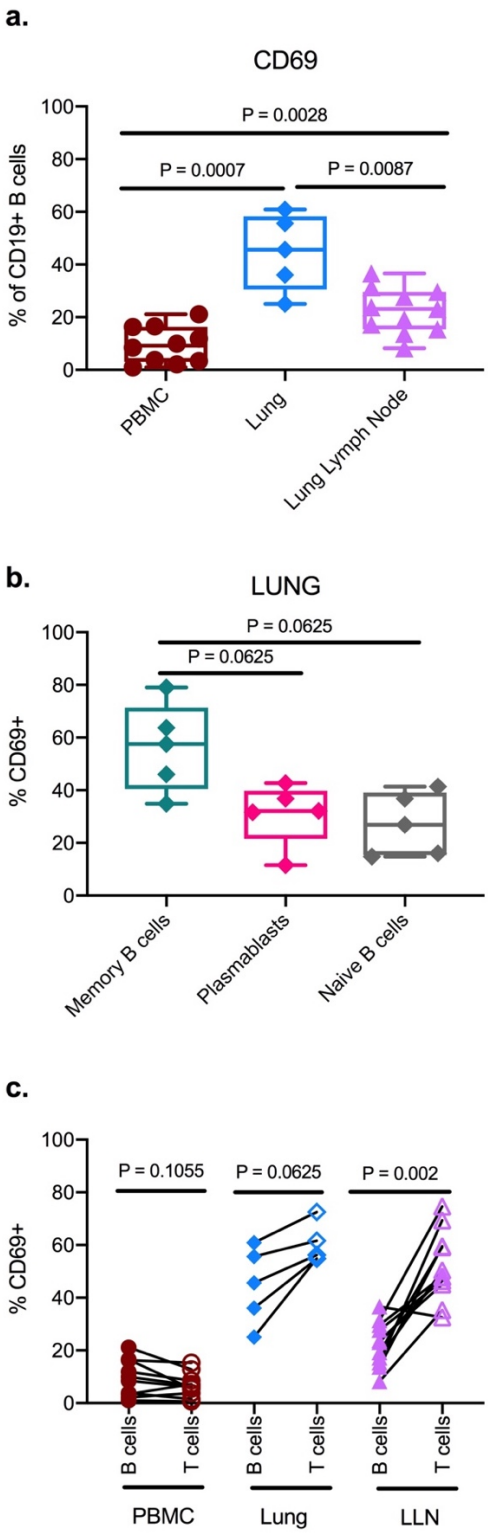


Figure 4.4: Expression of CD69 on B-cells; (a) total CD19+ B-cells in all three sample types sampled, (b) expression of B-cell phenotypes in the lung tissue (c) paired comparison of CD69 expression on B-cells (CD19+) T-cells (CD3+). LLN = lung lymph node. Statistics; non-parametric Mann-Whitney t-test for unpaired comparisons and Wilcoxon for paired comparisons.

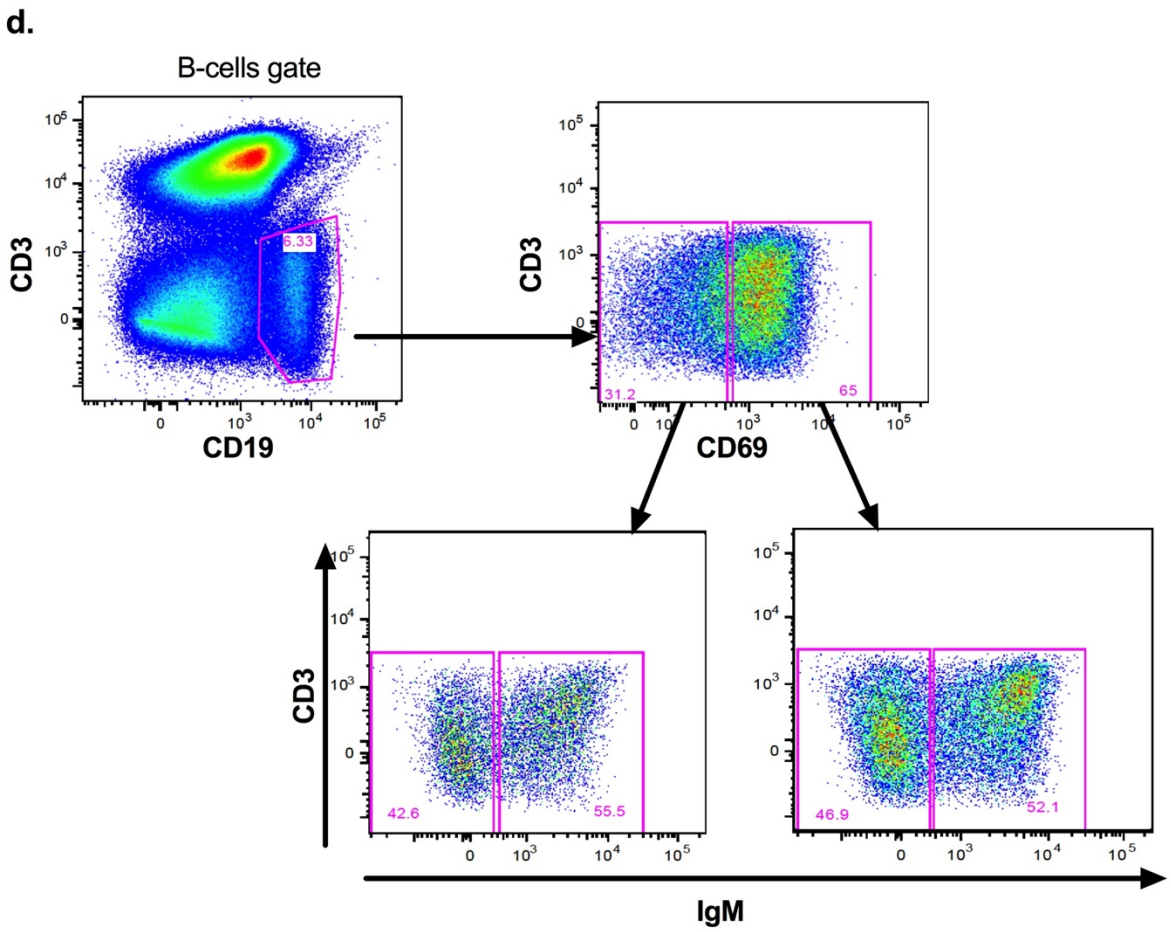


Figure 4.4: Frequency of IgM on B-cells; (d) representative flow cytometry plots of lung tissue.

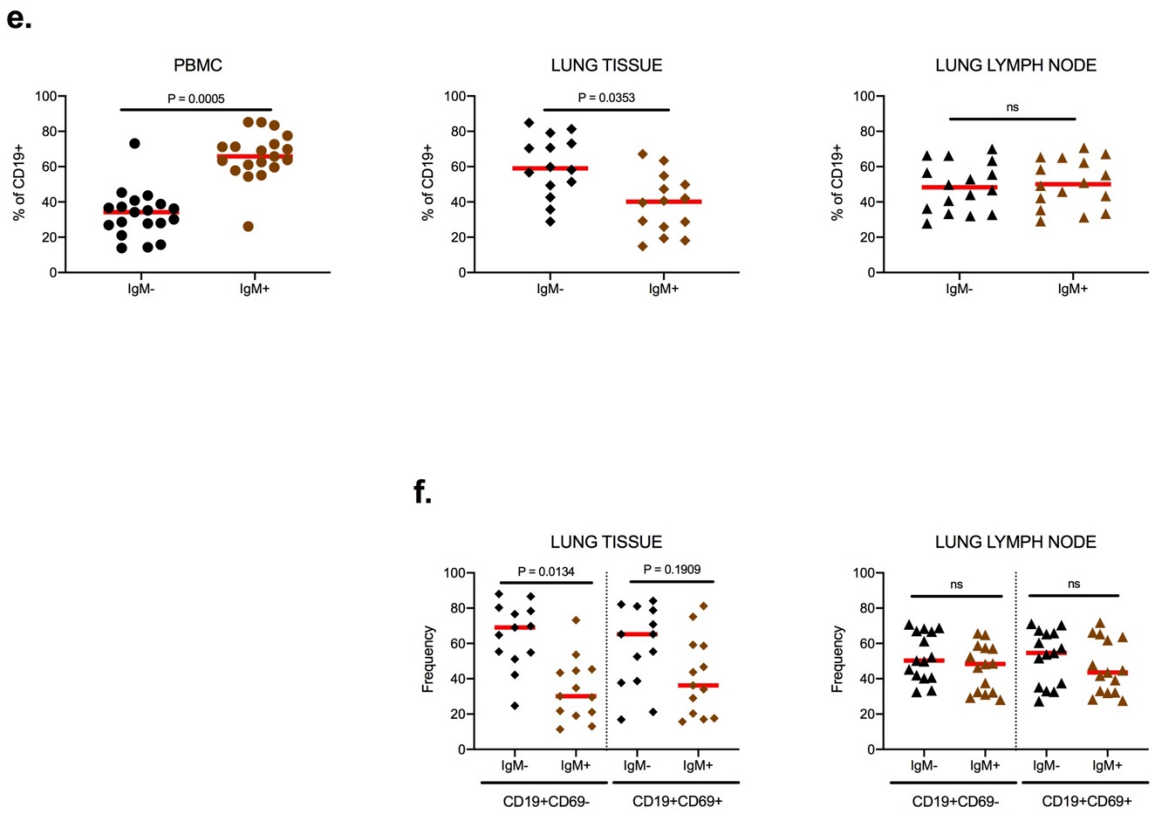


Figure 4.4: Frequency of IgM on B-cells (e) total CD19+ B-cells in blood, lung tissue and lung lymph nodes, (f) IgM expression on CD69+ and CD69- B-cells in lung tissue and lung lymph nodes. Statistics; non-parametric paired Mann-Whitney t-test

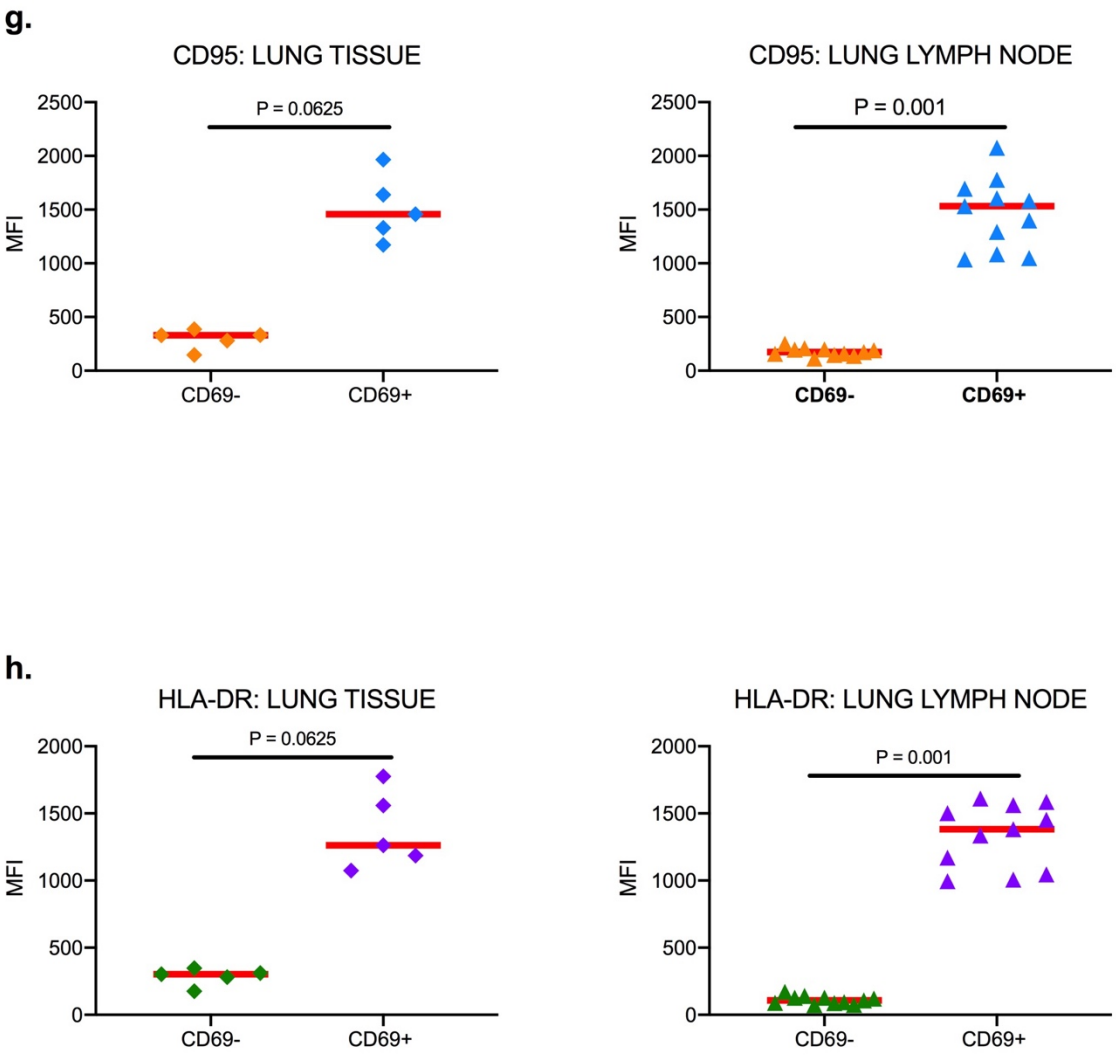


Figure 4.4: Expression of apoptosis marker - CD95 – (g) and activation marker -HLA-DR – (h) on B-cells on CD69-ve and CD69+ve B-cells in lung tissue and lung lymph nodes. Statistics; non-parametric paired Mann-Whitney t-test.

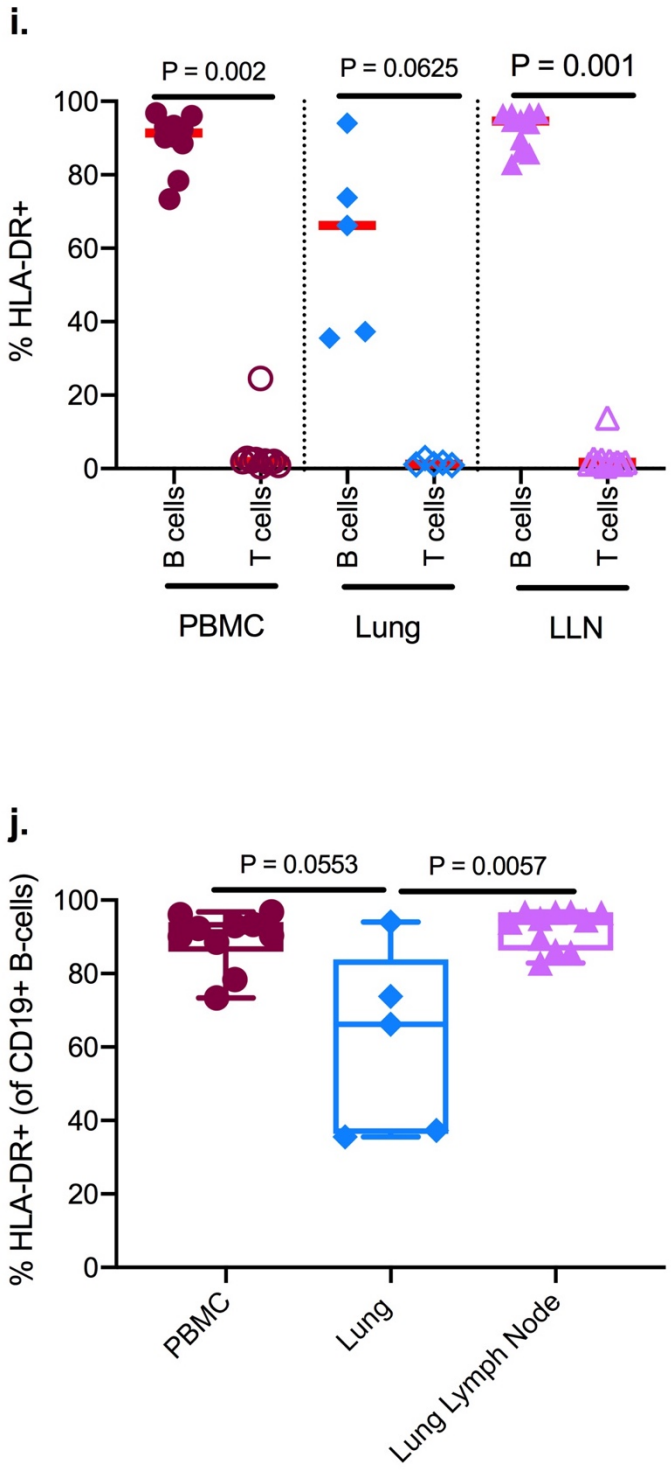


Figure 4.4: Expression of HLA-DR. (i) paired comparison of HLA-DR on B-cells (CD19+) and T-cells (CD3+), (j) HLA-DR on B-cells in lung tissue and lung lymph nodes. Statistics; non parametric Mann-Whitney t-test.

4.3.5 Apparent lack of TB specificity in lung tissue and lung lymph node B-cells

Having found evidence of a potential tissue resident B-cell population within TB infected human lung tissue, we next asked whether, as for T-cells Trm, these B-cells were enriched for TB specificity. To test for specificity of B-cells to TB, we used a B-cell baiting method that uses biotin coated with antigen, which then bind to B-cells recognizing the coating antigen via their surface B-cell receptor (BCR) (Marttila et al. 2000; Lu et al. 2016). A total of four biotinylated antigens were used to generate *Mtb* specific baits – ESAT-6/CP-10, Ag85A/B, LAM and PPD. In our hands these reagents had a high background and to counter this, a negative control sample in which both streptavidin PE and APC fluorochromes were added to PBMC was used to set the gates. Only B-cells binding high levels of both PE and APC baits were considered as bona fide TB specific B-cells (figure 4.5a). For PBMC, bait positive B-cells were pre-gated on activated, IgM- B-cells while for lung tissue (and lymph nodes) bait positive B-cells were pre-gated on total memory B-cells. Surprisingly, *Mtb* specific B-cells, as measured by this method, were less frequent, as a fraction of total B-cells, in lung tissue from TB infected subjects and lung lymph nodes than in matched PBMC samples, reaching statistical significance in lung lymph nodes (figure 4.5b). This data does not rule out the possibility that alternative antigen targets, or epitopes conformations, are more important in the lung and were missed. In addition, plasmablasts B-cells, which are enriched in our lung samples, express low level of BCR, and thus may not bind the *Mtb* baits efficiently. Thus, future work is needed to confirm this finding.

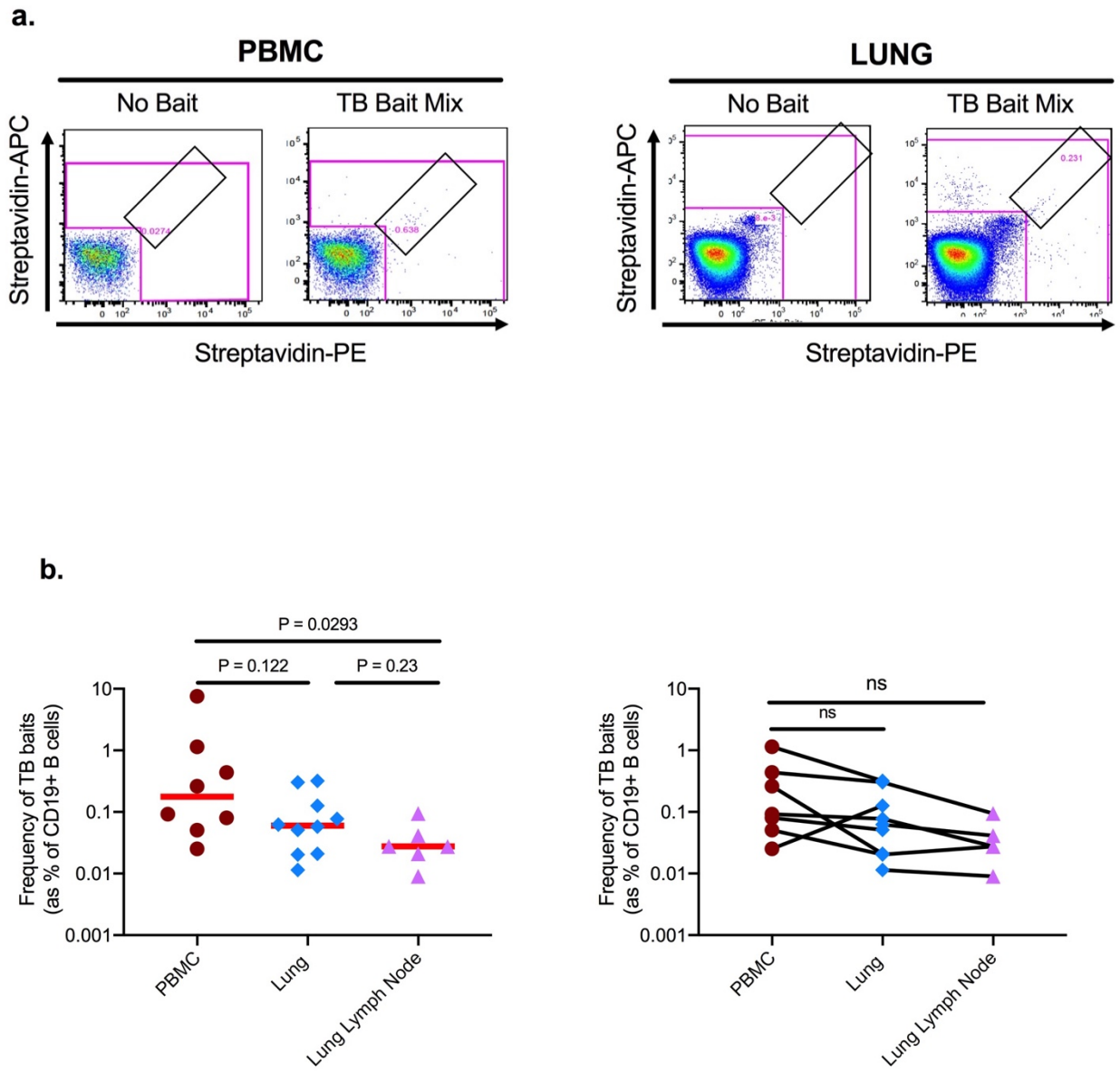


Figure 4.5: Frequency of *Mtb* specific B-cells. (a) Representative flow cytometry plots of PBMC and lung of the same participant. Bona fide TB specific B-cells in black diagonal gate. (b) Cumulative frequency of TB specific B-cells in the PBMC, lung and lymph node captured by TB specific baits (all samples left and matched samples right). Statistics; non-parametric Mann-Whitney t-test for unpaired comparisons and Wilcoxon for paired comparisons.

4.4 Discussion

While cellular immunity is important, the role of humoral immunity in tuberculosis is uncertain (Achkar, Chan, and Casadevall 2015; Kozakiewicz et al. 2013). However, new data from human studies show that individuals with latent and active TB have divergent humoral signatures and antibodies in latent TB infection can drive macrophages to kill intracellular bacteria (Lu et al. 2016). Furthermore, general B-cell function is impaired during active TB and LTBI, and this B-cell dysfunction compromises cellular host immunity during *Mtb* infection (Joosten et al. 2016). To further explore the potential role of B-cells in human tuberculosis, analysis of B-cell phenotype and specificity during infection and TB disease was carried out on peripheral blood of participants with active TB, latent TB infection (LTBI) and healthy controls. In addition, using the AHRI TB lung cohort, B-cells were analyzed at the site of disease in fresh human lung tissue and lung lymph nodes. Consistent with previous work (Joosten et al 2016), we find that total B-cell frequency is diminished in subjects with LTBI and active TB, compared to controls, and that the frequency of certain specific B-cell subsets differs between subjects with active infection and LTBI. In the lung, we find that B-cells are present in the lung tissue homogenate of TB infected individuals and make up a greater fraction of total CD45+ve leukocytes than in matched blood. Histological staining of sections taken from these lung samples confirm the presence of CD20 expressing B-cells within the lung parenchyma, typically clustered together in iBALT like structures. Using a subset of lung samples, with matched blood and lung lymph nodes we find B-cells within the lung homogenate predominantly possess a memory phenotype, with very few naïve cells; whilst both blood and lung lymph nodes are primarily made up of naïve cells. Phenotyping of these cells shows a high fraction express the canonical T-cell Trm marker CD69. Together with strong skewing towards a memory phenotype, and the clustering of B-cells within iBALT like structures, this suggests that human TB infected lung tissue contains a population of tissue resident B-cells that are likely to be distinct from those in circulation. In addition, both lung tissue and lung lymph nodes are enriched for plasma blasts B-cells, compared to matched blood, implying active antibody secretions within the lung and draining lymph nodes of these individuals. However, using fluorescent TB-baits we are unable to identify a significant population of TB specific B-cells with either lung tissue or lung lymph nodes. Indeed, the frequency of B-cells binding to TB baits was significantly lower in both tissues than in matched blood samples.

Considering the blood data, it is important to note that B-cell phenotyping is highly complex and definitions between studies are not always consistent. Moreover, for practical reasons, many of the classical markers of B-cell differentiation, such and CD27 and IgD were not included together in one staining antibody cocktail. However, despite these limitations, the frequency of total B-cells and immature B-cells in subjects with LTBI and active TB, and an enrichment of CD21- B-cell in active TB alone, are in agreement with a previous study by Joosten et al. (Joosten et al. 2016). In this study, they went on to show that atypical B-cells from subjects with active TB, defined as CD21-CD27-, displayed impaired proliferation, immunoglobulin- and cytokine- production (Joosten et al 2016). Similar, exhausted B-cells have also been reported in other conditions like HIV (Moir and Fauci 2014). However, using our markers we are unable to distinguish atypical and activated memory B-cells, both of which lack CD21 and CD10. The apparent enrichment of CD21+CD10- B-cells specifically within subjects with LTBI is intriguing. However, as above, conclusions from this observation are limited, as several different B-cell subsets fall within this definition, including naïve and conventional memory B-cells. Thus, more in-depth phenotyping is clearly required to explore this observation further.

The decrease of B-cells in systemic circulation of participants with LTBI and active TB could result from a recruitment of B-cells to the site of TB disease in the lung. Although, for this lung project healthy control lung samples were not available and so it is not possible to compare our finding with healthy non-TB infected controls. It is notable, however that the relative enrichment of B-cells in the lung compared to PBMC is greater than that for T-cells in the same subjects. An 8-fold increase in absolute number of B-cells in mouse lungs during *Mtb* infection was reported compared to very early stages of infection and these levels remained elevated during chronic infection (Tsai et al. 2006). Moreover, by histology, these lungs showed a strong clustering of B-cells together with dendritic cells (DCs), surrounded by T-cells (Tsai 2006), similar to the B-cell clusters observed in histological sections from our TB infected lung tissue. These B-cell follicles or iBALT, have been reported in human lungs affected by TB disease previously (Maglione, Xu, and Chan 2007; Turner et al. 2001; Ulrichs et al. 2004). Several studies suggest that the organization of B-cells into defined structures in the lung during TB is particularly important as B-cells interact with macrophages and T-cells (Maglione et al. 2008; Monson et al. 2011; Parker 1993; Martinez, Helming, and Gordon 2009; Nimmerjahn and Ravetch 2008) and can affect differential macrophage activation states and T helper cell polarization depending on antigen presentation

or cytokine secretion. In addition, B-cell and macrophages can interact by Fc receptor engagement, which can further modulate pro- or anti-inflammatory reactions (Gallo, Gonçalves, and Mosser 2010; Willcocks, Smith, and Clatworthy 2009). Consequently, the B-cells in the lung can possibly modulate the local environment of tuberculous granulomas via a range of pathways (Phuah et al. 2016).

The observation that memory B-cells and plasmablasts are enriched in the lung is of interest as these are the B-cell subsets that would produce and secrete antibodies during reactivation of TB disease in individuals with LTBI who fail to contain *Mtb* within solid granulomas (Small et al. 1994). Unfortunately, this study did not test antibody profiles in lung supernatants or peripheral circulation and is an area that needs to be explored further. It is worth noting that the interpretation of such antibody profile results needs to be put into context and the phase of TB disease of the cohort studied must be clearly defined as factors such as treatment, disease state and infection can affect antibody profiles (Lu et al. 2016). There were similar levels of naïve and transitional B-cells in lung lymph nodes and peripheral circulation reflecting that B-cells from lymph nodes seed into the general circulation before they home into the lung in response to infection.

The expression of CD69 on B-cells in lung homogenate has been interpreted as evidence of the existence of a population of lung tissue resident B-cells in our TB infected lung. The fact that we also observe an enrichment of memory B-cells in lung compared to matched PBMCs would seem to support this hypothesis, and the existence of iBALT suggests one potential physical location for such a population. Although researchers have primarily focused on tissue resident T-cells, a recent study demonstrated the existence of antigen specific lung resident B-cells that did not recirculate in blood and were important for the early immune response to influenza (Allie et al. 2019). These tissue resident B-cells expressed high levels of CD69, supporting our hypothesis that at least a fraction of the B-cells within TB infected human lung tissue might be non-recirculating resident memory B-cells. Given the diseased state of the lung tissue studied, however, it is possible that upregulation of CD69 related to immune activation rather than upregulation of a tissue resident program. The fact that expression of HLA-DR is low on the lung B-cells argues against this, as this marker is reported to be upregulated on activated B-cells (Wei, Jung, and Sanz 2011), but we cannot exclude this possibility. In addition, CD95 and HLA-DR themselves have distinct functional roles in B-cells that may be of relevance to the activity of B-cells within the lung. Indeed, we

observed a higher MFI of both CD95 and HLA-DR on CD69+ve B-cells, but not CD69-ve B-cells, in the lung tissue and lung lymph nodes. CD95 or Fas is a member of the tumor necrosis factor receptor family that can mediate apoptosis following binding it's ligand CD95L or FasL, via recruitment of a number of proapoptotic factors including caspase-8 to form the death-inducing signaling complex (Peter et al. 2015; Jalali et al. 2018). It is likely therefore, that lung B-cells are more resistant to apoptotic cell death, at least through this pathway. HLA-DR is an MHC class II molecule and speaks to the role of B-cells as professional antigen presenting cells. Thus, the high expression of HLA-DR on B-cells from lung lymph nodes is expected. In addition, as lung tissue and lung lymph nodes were acquired from the same subjects as a part of the lung resection procedure and processed in the same way, the fact that HLA-DR expression is significantly different between lung and lymph node B-cells argues against the differences observed between tissue and blood B-cells being an artefact of sample processing. Lower HLA-DR expression in the lung could also relate to skewed memory subsets as naïve B-cells tend to express higher levels of HLA-DR than memory cells (Harp et al. 2010).

To further characterize the B-cells and the observation that peripheral blood cells were mainly of a naïve phenotype while lung tissue B-cells were of a memory phenotype (based on expression of CD27 and CD38), we measured the expression of IgM on B-cells, as immunoglobulin class switching by B-cells is one of the mechanisms through which memory B-cells are generated. Our observation that lung tissue B-cells are mainly IgM-ve while peripheral blood B-cells are IgM+ve points to the fact the B-cells in the lung are more affinity matured and thus consist of a memory phenotype. However, IgM+ memory B-cells have also been observed as some CD27+ B-cells that have undergone somatic hypermutation do not undergo isotype class switching (Klein, Rajewsky, and Küppers 1998; Maurer et al. 1990; Agematsu et al. 1997). Together with their CD69 expression, it is possible that some of the IgM-ve B-cells in the lung tissue do not recirculate in blood, consistent with the idea of tissue resident memory B-cells.

Although our phenotyping data have shown that B-cells in lung tissue are mainly memory B-cells and likely some of them are tissue specific (on the basis of CD69 expression), there were surprisingly, fewer antigen specific B-cells in the lung tissue and lung lymph nodes compared to systemic circulation. This result could be explained by the observation that plasmablasts were enriched in lung tissue than peripheral circulation. Plasmablasts are known

to be efficient antibody producing B-cells and probably could not bind our antigen baits well via the B-cell receptor. Additionally, it is possible that there are alternative antigen targets, or epitope conformations that are more important in the lung and were missed by our assay.

The functional role of B-cells and humoral immunity during TB disease is less clear, with human studies providing conflicting results often depending on the phase of TB disease of the cohort studied. This study has provided additional key insights on B-cells dynamics that occur during human TB disease especially at the site of infection. In addition to providing evidence that that B-cells are indeed recruited to the lung during TB disease (in line with observation that B-cells are depleted in circulation in LTBI and active TB individuals), the data shows that B-cells in the lung are mainly memory B-cells and plasmablasts that are probably not recirculating in blood based on CD69 expression. However, by the method employed in this study, there were fewer TB specific B-cells in the lung. Since there is a higher frequency of plasmablasts in the lung, additional functional assays like ELISA and ELISPOT are recommended on lung tissue and systemic circulation.

CHAPTER 5: SYNOPSIS OF RESEARCH FINDINGS

5.1 Introduction

This thesis reports on the various experiments used to address the main objective of learning the phenotype, function and TB specificity of adaptive immune responses at the site of TB disease and understanding how they correlate with the responses detectable in circulation. It was hypothesised in this study that adaptive immune responses to *Mtb* infection at the site of disease are different from the immune responses in circulation and these responses at the site of infection determine the outcome of TB disease. A cohort of study participants undergoing medical lung resection for tuberculosis related lung pathologies from which fresh lung tissue and matched peripheral blood were obtained was used to investigate the adaptive immune responses to TB in the lung and systemic circulation. An array of immunological techniques including flow cytometry and cell sorting, mass cytometry, T-cell receptor sequencing, immunohistochemistry and histology were used to address the specific objectives set out in this thesis of which findings are summarised below.

5.2 Phenotype of T-cells in human lung tissue and peripheral blood during TB disease

The initial step in studying fresh lung tissue T-cells and peripheral blood T-cells involved identification of non-recirculating tissue specific T-cells in order to distinguish them from T-cells in systemic circulation. These non-recirculating tissue specific T-cells are referred to as tissue resident memory (Trm) T-cells. To this end, cell surface markers CD103, CD69 and CD62L that have been well characterised as tissue resident memory T-cell markers (Sathaliyawala et al. 2013; Masopust and Picker 2012; Mackay et al. 2013; Kløverpris et al. 2016c) were used to stain CD4+ and CD8+ T-cells from lung tissue and peripheral blood mononuclear cells (PBMCs). In conformity with the previous studies, this thesis has shown that by flow cytometry staining, lung T-cells are enriched for CD69 and CD103 but downregulate the expression of CD62L in comparison to blood T-cells. These markers were validated in a more comprehensive staining of lung tissue by mass cytometry (CyTOF). Thus, these markers reliably identified T-cells in the lung tissue that are not in circulation reliably distinguishing them from T-cells in systemic circulation.

In addition to identification of lung Trm cells by cell surface staining, other T-cell phenotype markers were analysed between lung tissue and PBMCs including T-cell memory, T-cell exhaustion markers and expression of T-cell regulation transcription factor, FoxP3. By further characterizing T-cells based on co-expression of CD45RA and CCR7 (Rufer et al.

2003; Sallusto, Geginat, and Lanzavecchia 2004), we have shown that lung tissue T-cells are mainly effector memory phenotype (CD45RA-CCR7-) while blood T-cells are mainly of a naïve phenotype (CD45RA+CCR7+). Within the lung tissue, CD69+ T-cells are also predorminantly effector memory phenotype than CD69- T-cells. This effector memory phenotype of lung tissue T-cells suggests that lung cells are better poised for immunological function than peripheral T-cells. We have shown that the expression of PD-1 on CD4+ and CD8+ T cells was varied in both lung and PBMC, and PD-1 expression was enriched on CD69+ than CD69- T-cells in both lung tissue and PBMC. On the contrary, there was generally a higher frequency of CTLA-4 and TIM-3 in circulation than in the lung tissue.

Contrary to our expectation, there was a higher frequency of FoxP3+ CD4+ T-cells in circulation than in the lung tissue. Within the lung tissue, FoxP3 was least expressed on CD103+ CD4+ T-cells. However, there was an enrichment of FoxP3 on lung tissue TB-specific T-cells than on peripheral blood TB-specific T-cells. Taken together, these observations confirm that lung tissue T-cells are phenotypically different from peripheral blood T-cells and this difference has an impact on how T-cells in both compartments respond to *Mtb* infection.

T-cell receptor sequencing as an alternative approach of studying the difference between T-cells in the lung tissue and peripheral blood T-cells is also reported in this thesis. The size of the T-cell response, in general, correlates with the abundance of antigen-specific T-cells in the naïve repertoire (Obar, Khanna, and Lefrançois 2008; Kotturi et al. 2008; Jenkins and Moon 2012). Following antigen recognition, activated T-cells proliferate by clonal expansion and some become part of the memory compartment, where they can reside for many years as clonal populations of cells with identical TCR rearrangements by virtue of their descent from a common naive T-cell (Arstila et al. 1999; Neller et al. 2013; Robins et al. 2009). We postulated that there is a difference in clonal T-cell expansion between T-cells resident in the lung and recirculating T-cells in systemic circulation. Our TCR sequencing data shown in this thesis have revealed two main differences between lung tissue and peripheral blood T-cells. Firstly, there is a higher clonal T-cell expansion in TB infected human lung than matched blood, an observation that agrees with the earlier finding that lung T-cells are mainly effector memory. This clonal expansion in the lung is driven by CD4+ T-cells in the lung and not CD8+ lung T-cells as shown from sequencing pre-sorted T-cells. Secondly, and most important, is the existence of high frequency (up to 20% of total T-cells in the lung) unique

T-cell clones in the lung that do not recirculate. This finding agrees with the other results in this thesis of the existence of CD103+CD69+ T-cells only in the lung but not in circulation. It is likely that these non-recirculating unique T-cell clones in the lung are the ones that would be highly enriched for *Mtb* specificity. In line with this, we have shown the existence of unique and expanded γδ T-cells in TB infected lung tissue that are not in circulation. However, this thesis did not conduct definitive experiments to confirm the *Mtb* specificity of these expanded unique T-cell clones.

5.3 Functionality of lung tissue resident memory T-cells during TB disease

Functional recall within hours of stimulation is a signature of memory T-cells (Sathaliyawala et al 2013; Gideon et al 2015) and functionality of T-cells is often measured by their ability to produce cytokines upon *in vitro* stimulation. Comparison of cytokine production between blood and lung tissue T-cells showed that there was a higher production of TNF-α, IL-2, IFN-γ and IL-17 cytokines by lung tissue T-cells than in systemic circulation after non-specific stimulation. Interestingly, Th17 (IL-17 cytokine producing) T-cells are particularly enriched in TB infected human lung. Within the lung tissue, CD103+CD69+ and CD103-CD69+ subsets produced the most cytokines. This superior functionality of lung tissue T-cells could be mediated, in part, by the fact that they express mostly effector memory phenotype.

5.4 Specificity and magnitude of TB specific T-cells in lung tissue and systemic circulation

T-cells that produce multiple cytokines such as IFN-γ, IL-2, TNF-α, IL-17 and IL-10 to TB have been shown in animal model studies and a subset of these cytokines demonstrated to be critical in humans as well (Flynn and Bloom 1996; O'Garra et al. 2013; Algood, Lin, and Flynn 2005; Lee and Kornfeld 2010; Lin et al. 2010; Green, Difazio, and Flynn 2013). Using an MHC class II -restricted mega *Mtb* peptide pool to measure cytokine production by intracellular cytokine staining, we observed that lung tissue bulk CD4+ T-cells produced TNF-α, IL-2, IFN-γ and IL-17 cytokines but the main cytokine produced was TNF-α. Since TB is primarily a disease of the lung tissue and the observation that lung T-cells are mainly effector memory phenotype, we postulated that T-cell responses to *Mtb* are enriched in the lung tissue than peripheral blood. Indeed, comparison of cytokine production between site of disease and systemic circulation revealed that TB specific responses are highly enriched in

the lung for TNF-α, IL-2 and IL-17. Of note, IL-17 cytokine responses are more frequent in lung tissue samples than blood samples suggesting that these responses are often missed when measured in blood alone. Interestingly, IFN-γ level was similar between PBMC and lung tissue T-cells. Analysis of lung Trm T-cells for TB-specific cytokine production showed that CD103+ CD4+ T-cells produced the least cytokines across all the four cytokines. TB-specific cytokine positive CD4+ lung T-cells were also mainly effector memory phenotype. Although the majority of the responses in the lung was TNF-α mono-functional, when compared to cancer control participants, bifunctional lung T-cells producing TNF-α+IL-17+ or TNF-α+IL-2+ was significant in TB cases. Finally, the frequency of TB-specific T-reg cells was enriched in the lung tissue than matched blood particularly on TNF-α and IL-17 producing cells. The higher expression of FoxP3 on TB-specific lung tissue T-cells than on matched blood TB-specific T-cells suggests that these effector T-cells, though enriched in the lung are sub-optimal in controlling *Mtb*.

5.5 Phenotype and specificity of B-cells in TB infected human lung tissue

From literature, the significance of B-cells and humoral immunity in shaping immune response to the *Mtb* is inexhaustive (Maglione and Chan 2009; Casadevall and Pirofski 2011; Glatman-Freedman and Casadevall 1998). We hypothesised that B-cell phenotypes change during the course of TB disease and that there is a difference in the phenotype of B-cells in the lung tissue and peripheral blood. An initial phenotyping of B-cells in peripheral blood of healthy control (IGRA negative), latent TB (IGRA positive) and active TB participants showed that total B-cell frequency is depleted in blood of active TB participants but there exists an expansion of atypical B-cells in the blood of active TB participants. The observation that B-cells are depleted in systemic circulation of active TB participants is in agreement with a previous study (Joosten et al. 2016) and suggests that B-cells are possibly recruited to the lung during active TB. To test this hypothesis, B-cell frequencies between fresh lung tissue and matched blood were compared. Indeed, there was an enrichment of B-cells in the lung compared to peripheral circulation, and the B-cells in the lung are organised in follicular structures as described by other reports (Kaushal et al 2015; Ulrichs et al 2014, Tsai et al 2006). Like lung T-cells, the B-cells in the lung express high levels of CD69 suggesting that they probably do not recirculate. Furthermore, lung tissue B-cells are mainly memory B-cells while PBMC are mainly naïve B-cells. Although there is an enrichment of B-cells in TB

infected human lung, there are surprisingly fewer Mtb-specific B-cells in the lung compared to TB-specific B-cells in circulation.

5.6 Impact of HIV coinfection on the features of lymphocytes within the lung

There is a substantial increased risk of TB and its extra-pulmonary dissemination is strongly correlated with CD4+ T-cell depletion in individuals infected with HIV-1 (Bell and Noursadeghi 2018). T-cell depletion is evident in peripheral blood, in the respiratory tract and at the site of tuberculin skin test (TST) challenge (Bell et al. 2016; Kalsdorf et al. 2009; Geldmacher et al. 2010), although absolute cell counts can be preserved in well controlled HIV infection (Bunjun et al. 2017). We observed a significantly lower CD4:CD8 ratio in the lung of HIV co-infected participants when compared to patients without HIV, regardless of whether they had active TB or a previous TB infection. Although a similar trend was observed in the PBMC, the difference did not reach statistical significance suggesting that HIV dysregulates recruitment of T-cells to the lung during TB disease. In general, there was a trend of a lower lung tissue CD69+ T-cell frequency in participants without HIV compared to those coinfected with HIV. HIV coinfection did not affect the frequency of CD103 in lung tissue T-cells while the frequency of both markers in peripheral blood was not altered by HIV. PD-1 expression on lung tissue T-cells was lower in HIV coinfected individuals while the levels of PD-1 on peripheral blood T-cells was not affected by HIV coinfection. On the other hand, while HIV coinfection had no effect on CTLA-4 and TIM-3 frequency in lung tissue T-cells, the frequency of CTLA-4 and TIM-3 was higher on peripheral blood of HIV coinfected individuals, particularly on CD8+ T-cells.

T-cell receptor sequencing revealed that HIV coinfection had a bigger impact on T-cell clonality of blood T-cells with HIV negative participants having a lower blood T-cell clonality. This high clonal expansion of T-cells in the periphery of HIV coinfected participants masked tissue clonal expansion of T-cell clones in lung tissue in this subgroup of participants.

HIV coinfection had an impact on cytokine production by lung T-cells following nonspecific *ex vivo* stimulation with PMA + ionomycin. There was a lower production of TNF-α, IL-2 and IL-17 by lung tissue CD4+ T-cells in HIV coinfected participants than those without HIV while IFN-γ production by CD4+ lung T-cells was not affected by HIV coinfection. The level

of cytokine production by lung CD8+ lung tissue T-cells was similar regardless of HIV status, except for TNF-α that was significantly higher in HIV negative participants. Within the lung tissue, HIV coinfection had major impact on CD103+CD69+ and CD103-CD69+ T-cell subset where there was significantly lower cytokine production in coinfected participants. Unlike nonspecific stimulation, TB-specific cytokine production by lung tissue T-cells was not affected by HIV coinfection suggesting that there is no preferential depletion of TB-specific T-cells at the site of TB disease.

5.7 Conclusion and recommendations

This thesis has provided a detailed investigation of adaptive immune responses to *Mtb* in the human lung by comparing fresh lung tissue T and B-cell phenotype, function and specificity with matched peripheral blood in individuals with TB disease. Results reported here have clearly demonstrated that there are compartmentalized responses by T and B-cells to *Mtb* in the human lung and this compartmentalization is driven by cells with different phenotypes between blood and lung tissue, including lung tissue specific non-recirculating T-cells (Trms). These results provide new insights of immune events that occur only in the lung and may be important when interpreting data from human PBMC for correlates of protection or vaccine response. The most significant findings in this thesis are, firstly, the enrichment of Th17 cells in the lung that are less frequent in circulation and these cells are the most depleted in HIV coinfection; in addition, the loss of these cells correlates with elevated plasma IL-1β levels which is a marker of disease severity. Secondly, we have shown the existence of unique and expanded γδ-T cells in TB infected human lung that are not in circulation. Finally, our data suggests the existence of tissue resident B-cells in TB infected human lung that are probably absent in circulation.

There are potential limitations of this thesis, first being the nature of lung tissue samples that were available. The participants enrolled in this study are individuals with advanced chronic TB disease who have failed therapy in most cases, while others have pathological sequalae associated with previous TB episode like bronchiectasis. The implication of this is that we did not address the early immune events that occur in the lung in acute TB disease. The thesis also made use of lung tissue from participants with lung cancer as negative controls because of the experimental, logistical and ethical challenges of getting fresh lung samples from healthy individuals. Secondly, TB-specificity was measured using MHC class II restricted

peptides that is not suitable for measuring CD8+ T-cell responses which have been reported to be important in mouse models and in viral vectored vaccines against TB. Thirdly, our antibody panel for identification of regulatory T-cells lacked CD25 antibody because were limited by the number of fluorochrome channels in the cytometer and many parameters we measured simultaneously. We also did not conduct inhibition assays to test the regulatory nature of the cells we identified as T-regs on the basis of FoxP3 expression.

From the results presented in this thesis, it is recommended that:

a) The functional roles of B-cells in TB infected human lung be studied further to provide further evidence to explain the observation that despite high enrichment of B-cells in TB infected lung tissue than peripheral blood, there are fewer TB-specific B-cells in the lung compared to blood.
b) The specificity of TB-specific T-cells in the lung tissue be investigated further using a broad array of TB-antigens that are not MHC restricted to identify other cell types apart from CD4+ T-cells that are functional in the lung.
c) Additional experiments to formally test the specificity of unique, expanded non-recirculating T-cell clones in the lung be done.
d) The results of this thesis should be considered in designing novel TB vaccines to come up with formulations that generate and establish Trm cells in the lung which can make rapid responses to *Mtb* upon exposure.

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APPENDICES

APPENDIX I: ANTIBODIES USED IN FLOW CYTOMETRY

anti-CD45 V500 Horizon clone HI30 (BD Biosciences)

anti-CD3 Brilliant Violet 785 clone OKT3 (BioLegend)

anti-CD4 Brilliant Ultra Violet 496 clone SK3 (BD Bioscience)

anti-CD8 Brilliant Violet 605 clone RPA-T8 (BioLegend)

anti-CD19 FITC clone HIB19 (BD Bioscience)

anti-CD62L PE-Cy5 (BD Pharmingen)

anti-CD103-APC clone Ber-ACT8 (BD Biosciences)

anti-CD45RA Brilliant Violet 650 clone HI100 (BD Biosciences)

anti-CCR7 PerCP-Cy5.5 clone G043H7 (BioLegend)

anti-CD25 Brilliant Violet 711clone BC96 (BioLegend)

anti-CD56 Brilliant Violet 711 clone HCD56(BioLegend)

anti-CD16 Brilliant Violet 650 clone 3G8 (BioLegend)

anti-CD69 Brilliant Ultra Violet 395 clone FN50 (Brilliant Horizon)

anti-TNFα Alexa700 clone Mab11 (BD Biosciences)

anti-IL-2 PE-CF594 clone 5344.111 (BD Biosciennces)

anti-IFN-g PE-Cy7 clone 4S.B3 (BD Biosciences) or Brilliant Violet 421 clone 4S.B3 (BioLegend)

anti-IL-17 PE clone BL168 (Biolegend)

anti-PD-1 Brilliant Violet 421 clone EH12.1 (BD Biosciences)

anti-CTLA-4 PE L3D10 (Biolegend)

anti-TIM-3 Alexa 700 (R&D Systems)

anti-FoxP3 eFlour 450 clone PCH101(eBioscience)

anti-CD19 Brilliant Violet 650 clone HIB19 (BioLegend)

anti-CD27 Brilliant Violet 510 clone O323 (BioLegend)

anti-CD3 Brilliant Violet 711 clone OKT3 (BioLegend)

anti-CD38 Alexa Flour 700 clone HIT2 (BioLegend)

anti-IgM PerCP- Cy5.5 clone MHM-88 (BioLegend)

anti-CD21 Brilliant Violet 450 clone B-ly4 (BD Biosciences)

anti-CD14 Brilliant Violet 711 clone M5E2 (BioLegend)

anti-CD10 PECy5 (BD Pharmingen)

anti-γδ TCR PE clone B1 (BioLegend)

anti-Vδ1 TCR FITC clone TS8.2 (Thermo Scientific)

anti-Vδ2 TCR PerCp Cy5.5 clone B6 (BioLegend)

anti-Vγ9 TCR APC clone B3 (BioLegend)

anti-Vα7.2 Brilliant Violet 711 clone 3C10 (BioLegend)

anti-CD26 PE-Cy5 clone BA5b (BioLegend)

anti-CD161 Brilliant Violet 605 clone HP-3G10 (BioLegend).

APPENDIX II: CyTOF T-CELL MARKERS

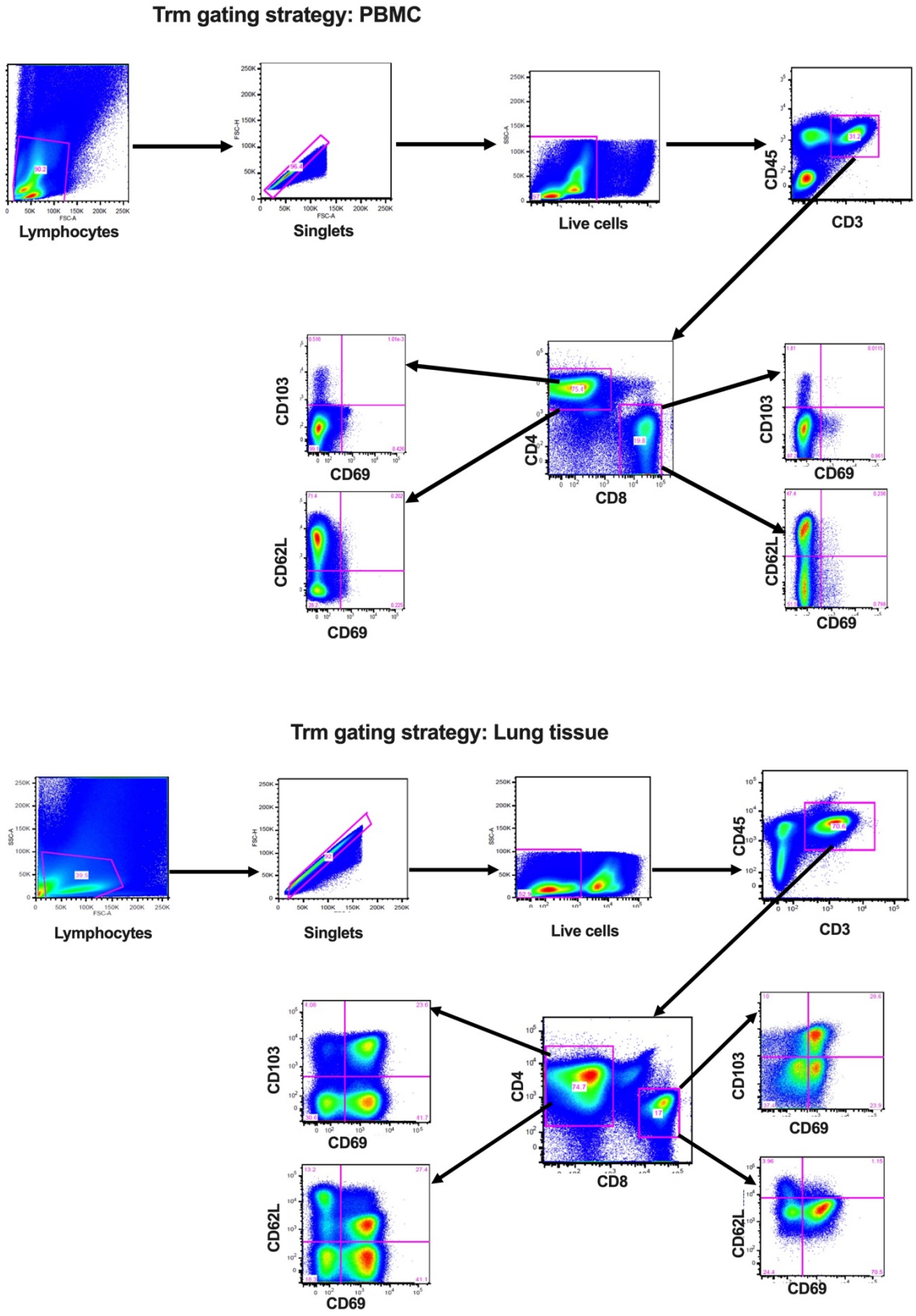
Marker No.	T cell Panel
1	CD45
2	CD14
3	CD57
4	CD16
5	CD19
6	TCR-γδ
7	Vδ2
8	Vα7.2
9	CD56
10	HLA-DR
11	CD8a
12	CD4
13	CD3
14	CD69
15	CD45RO
16	CCR7
17	KLRG-1
18	CD27
19	ICOS
20	CD103
21	OX40
22	TIM-3
23	PD-1
24	CD161
25	CD127
26	CD25
27	CD28
28	CD39
29	CD49A
30	TIGIT
31	CRTH2
32	CXCR5
33	Integrin β7
34	CD62L
35	CCR4
36	CCR6
37	CD107a
38	CXCR3
39	CXCR1
40	CCR5

NOTE: All the markers were designed and validated by ImmunoSCAPE (https://www.immunoscape.com) who collaborated with us on CyToF immune phenotyping before shipping over to AHRI, Durban for staining of samples.

APPENDIX III: DONOR UNRESTRICTED T-CELLS

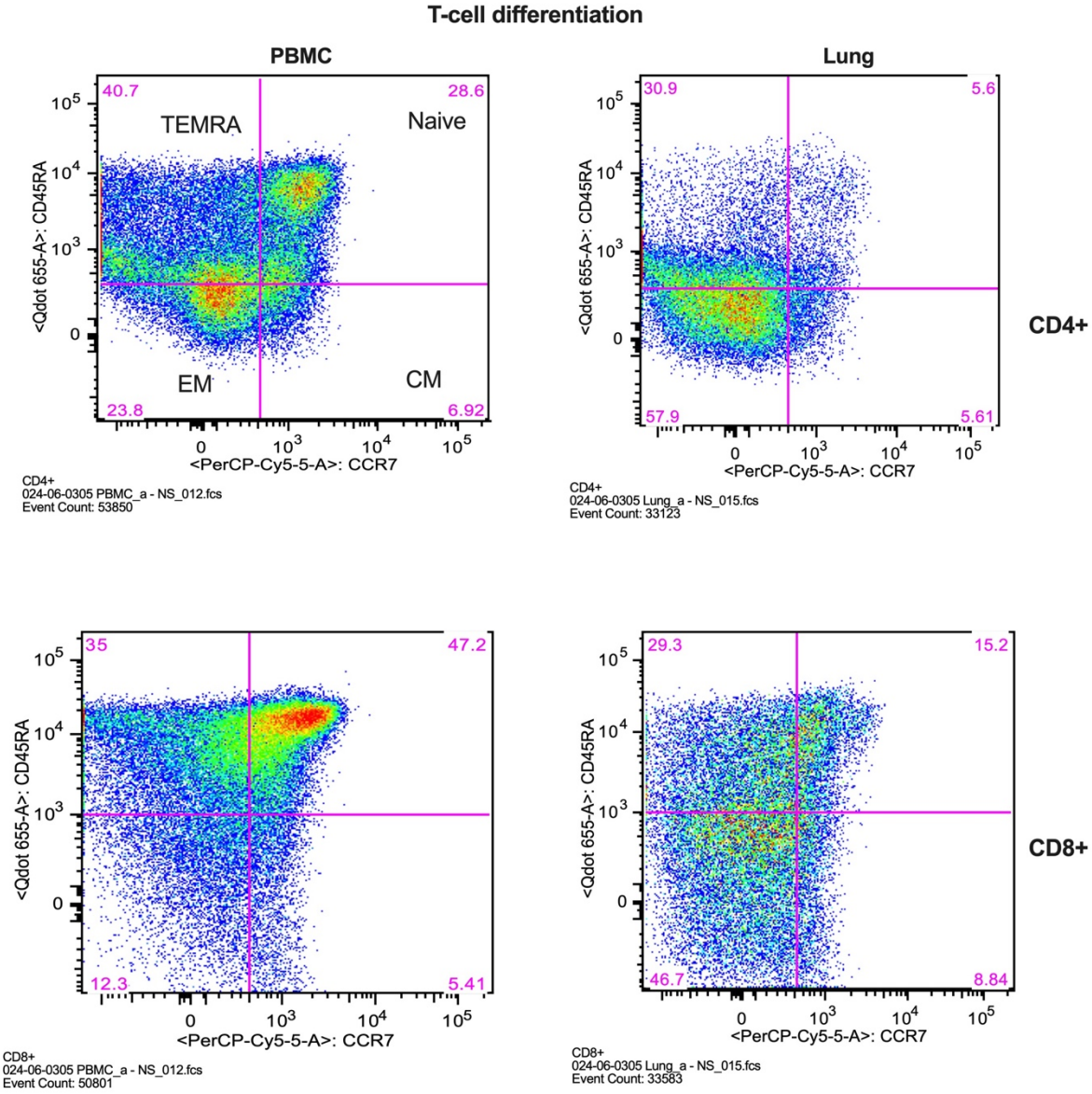
	GEM extended	iNKT	MAIT extended	MAIT-like	γδ-TCR
Va	TRAV01-02	TRAV10	TRAV01-02	TRAV01-02	Vδ1,2,3, 4(TRAV14), 5(TRAV23), 6(TRAV29), 7(TRAV36), 8(TRAV38)
Ja	TRAJ9	TRAJ18	TRAJ33	TRAJ33	Jδ1,2,3,4
CDR3a length	13	15	12	11 - 15	Variable
Number of Unique CDR3a sequences	40	1	8	39	Multiple
Consensus sequence	CAVRxTGGFKTIF CAVLxTGGFKTIF x=any amino acid	N/A	CAVxDSNYQLIW x=R,A,K,L,M,S,T,V	MAIT score = 1	N/A
Reference sequences	CAVRATGGFKTIF CAVRRTGGFKTIF CAVRDTGGFKTIF CAVRCTGGFKTIF CAVRETGGFKTIF CAVRQTGGFKTIF CAVRGTGGFKTIF CAVRHTGGFKTIF CAVRITGGFKTIF CAVRLTGGFKTIF CAVRKTGGFKTIF CAVRMTGGFKTIF CAVRNTGGFKTIF CAVRFTGGFKTIF CAVRPTGGFKTIF CAVRSTGGFKTIF CAVRTTGGFKTIF CAVRWTGGFKTIF CAVRYTGGFKTIF CAVRVTGGFKTIF CAVLATGGFKTIF CAVLRTGGFKTIF CAVLNTGGFKTIF CAVLDTGGFKTIF CAVLCTGGFKTIF CAVLETGGFKTIF CAVLQTGGFKTIF CAVLGTGGFKTIF CAVLHTGGFKTIF CAVLITGGFKTIF CAVLLTGGFKTIF CAVLKTGGFKTIF CAVLMTGGFKTIF CAVLFTGGFKTIF CAVLPTGGFKTIF CAVLSTGGFKTIF CAVLTTGGFKTIF CAVLWTGGFKTIF CAVLYTGGFKTIF CAVLVTGGFKTIF	CVVSDRGSTLGRLYF	CAVRDSNYQLIW CAVADSNYQLIW CAVKDSNYQLIW CAVLDSNYQLIW CAVMDSNYQLIW CAVSDSNYQLIW CAVTDSNYQLIW CAVVDSNYQLIW	CAALDSNYQLIW CAAMDSNYQLIW CAASDSNYQLIW CAASKAAGNKLTF CAAVDSNYQLIW CAFMDSNYQLIW CAGLDSNYQLIW CAGMDSNYQLIW CAIMDSNYQLIW CAPLDSNYQLIW CAPMDSNYQLIW CAPSGSARQLTF CASMDSNYQLIW CASMDSSYKLIF CATMDSNYQLIW CAVESNSGYALNF CAVGDSNYQLIW CAVGYSSASKIIF CAVIDSNYQLIW CAVLDSSYKLIF CAVLNTGGFKTIF CAVLSNDYKLSF CAVMDSSYKLIF CAVNNNNDMRF CAVPNSGGSNYKLTF CAVPSGGSYIPTF CAVQDSNYQLIW CAVRDGDYKLSF CAVRDGNYQLIW CAVRDNDYKLSF CAVRDNFNKFYF CAVRDRDYKLSF CAVRDRDYQLIW CAVRDSDYKLSF CAVRDSSYKLIF CAVRDTGFQKLVF CAVRRDDKIIF CAVSDSSYKLIF CVPMDSNYQLIW	N/A
Reference	Gras et al 2016, van Rhijn and Moody 2015, DeWitt et al 2018		Greenaway et al 2013	Website	

APPENDIX IV: GATING STRATEGY FOR TRM CELLS



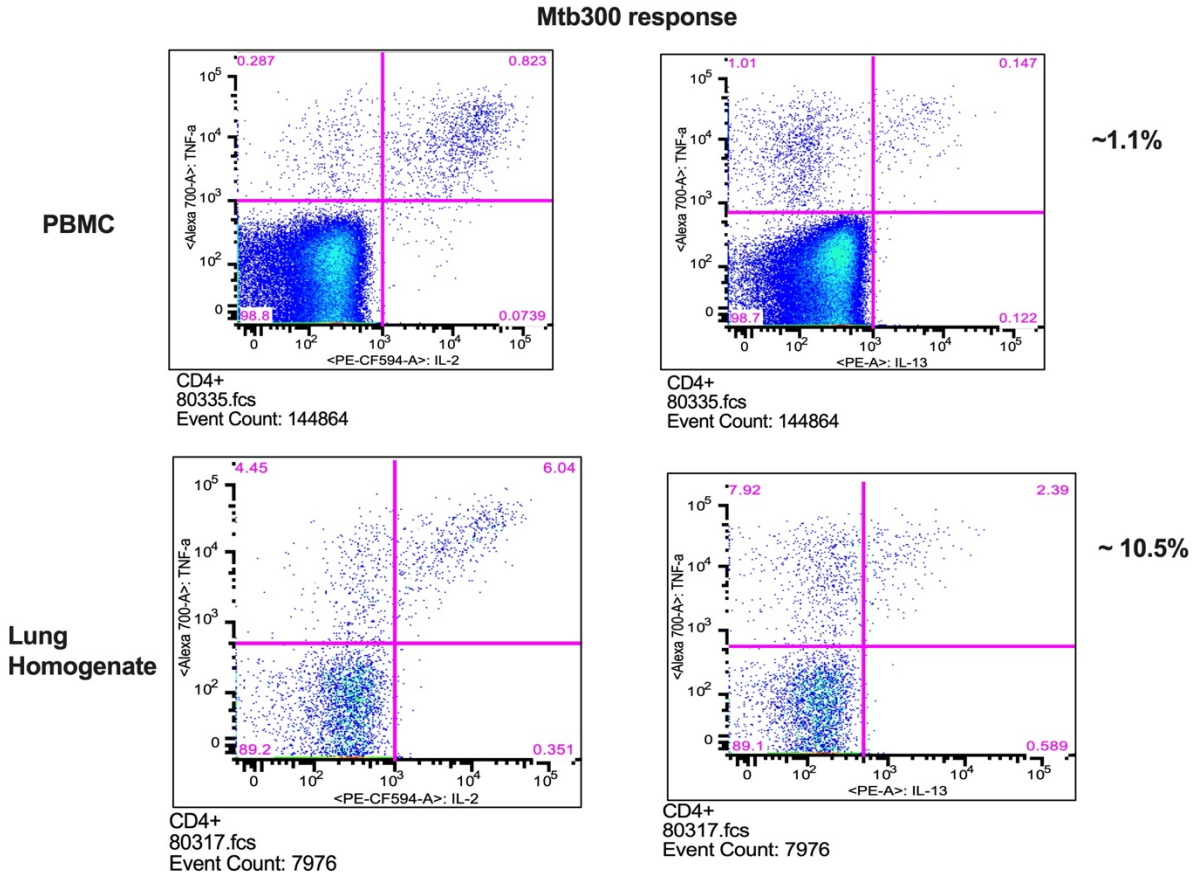
Gating strategy for the identification of Trm cells based on the expression of CD103, CD69 and CD62L. Trm cells are gated from CD4+ and CD8+ live CD45+CD3+ T-cells.

APPENDIX V: T-CELL DIFFERENTIATION IN PBMC AND LUNG



Representative plots of CD4+ and CD8+ T-cells in PBMC and lung tissue on the basis of co-expression of CD45RA and CCR7 markers. CD4+ and CD8+ T-cells identified using the gating strategy in appendix IV. Naïve T-cells (CD45RA+CCR7+), Central memory (CM) T-cells (CD45RA-CCR7+), Effector memory (EM) T-cells (CD45RA-CCR7-) and terminally differentiated effector memory T-cells (TEMRA) (CD45RA+CCR7-).

APPENDIX VI: TB SPECIFIC T-CELLS IN CIRCULATION AND SITE OF DISEASE



Representative flow cytometry plots showing enrichment of Mtb-specific responses in lung homogenate compared to matched PBMC (~ 10.5% vs 1.1% for TNF-α). CD4+ T-cells identified using the gating strategy shown in appendix IV.