UNIVERSITY OF KWAZULU NATAL

AN INVESTIGATION INTO THE TB/HIV MANIPULATION OF THE T-CELL IMMUNE RESPONSE

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DECLARATION

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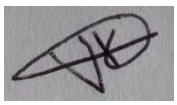

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It always seems impossible until it's done.

- **Nelson Mandela**

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2. TB/HIV Pleurisy Reduces Th17 Lymphocyte Proportion Independent of the Cytokine Microenvironment. VC Korb, A Phulukdaree, UG Lalloo, AA Chuturgoon and D Moodley. *Tuberculosis*. **In Review**. (Manuscript number TUBE_2015_418).

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Review Articles

1. *Mycobacterium tuberculosis*; Manipulator of Protective Immunity. VC Korb, AA Chuturgoon and D Moodley. *International Journal of Molecular Sciences*. Special Issue "Infectious Diseases: Pathophysiology, Diagnostics, Therapies, and Prevention". **In Review**. (Manuscript number 108889).

PRESENTATIONS

The work titled: The Effect of TB/HIV Pleurisy on miR-155 Expression, Stability and IL-35 Production of T-regulatory Lymphocytes. Korb, V., Phulukdaree A., Lalloo, U., Chuturgoon A., and Moodley, D. was presented at the following conference:

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AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
ARV	Antiretroviral
ATP	Adenosine triphosphate
Bak	Bcl-2 homologous antagonist/killer
BAL	Bronchoalveolar lavage
Bax	Bcl-2 associated X protein
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CA	Capsid protein
CCL	C-C motif ligand
CCM	Complete culture medium
CCR	C-C motif receptor
CD	Cluster differentiation
cDNA	Copy DNA
CNS	Conserved non-coding sequence
CTLA4	Cytotoxic T-lymphocyte antigen 4
CXCL	C-X-C motif ligand

CXCR	C-X-C motif receptor
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EBI3	Epstein Barr virus induced gene 3
EEA1	Early endosomal antigen 1
ELISA	Enzyme linked immunosorbent assay
Env	Envelope
ESAT-6	6-kDa early secretory antigen target
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FM	Foamy macrophage
Foxp3	Fork head box p3
gp	Glycoprotein
h	hours
HAART	Highly active antiretroviral therapy
HC	Healthy control
HIVDR	HIV drug resistance
HIV	Human immunodeficiency syndrome
HRP	Horseradish peroxidase

IFN	Interferon
IL	Interleukin
IN	Integrase
iNOS	Inducible nitric oxide synthase
IRF4	Interferon regulatory factor 4
Jak	Janus activated kinase
LAG3	Lymphocyte activation gene 3
LAM	Lipoarabinomannan
LM	Lipomannan
LTR	Long terminal repeat
LXA4	Lipoxin A4
MA	Matrix protein
mAGP	Mycolylarabinogalactan-peptidoglycan
ManLAM	Mannose capped lipoarabinomannan
MAPK	Mitogen activated protein kinase
MDR-TB	Multi drug resistant tuberculosis
MHC	Major histocompatibility complex
min	Minutes
miR	Micro RNA

MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MTB	*Mycobacterium tuberculosis*
NC	Nucleocapsid
NF-κB	Nuclear factor κ B
NK	Natural killer
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside-analog reverse transcriptase inhibitor
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PD-1	Programmed death-1
PDIM	Phthiocerol dimycocerosate
PE	Pleural effusion
PE	Phycoerythrin
PEF	Pleural effusion fluid
PEMC	Pleural effusion mononuclear cell
PerCP-Cy5.5	Peridin-chlorophyll-cyanin 5.5
PGE2	Prostaglandin E2
PI3K	Phosphatidylinositol-3-kinase

PI3P	Phosphatidylinositol-3-phosphate
PIAS	Protein inhibitor of activated STAT
PIM	Phosphatidylinositol mannoside
PR	Protease
PRR	Pattern recognition receptor
pTregs	Periphery derived T-regulatory lymphocytes
qPCR	Quantitative polymerase chain reaction
R	Receptor
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RORC	RAR-related orphan receptor C
RT	Room temperature
RT	Reverse transcriptase
s	Seconds
SA	South Africa
SDS	Sodium dodecyl sulphate
SOCS1	Suppressor of cytokine signalling 1
SP	Spacer peptides
STAT	Signal transducer and activator of transcription

T/XXDR-TB	Totally drug resistant tuberculosis
TTBS	Tris buffered saline and Tween 20
TAR	Trans-activation response element
TB	Tuberculosis
Tconvs	Conventional T-cells
TCR	T-cell receptor
TDM	Trehalose dimycolates
TGF	Transforming growth factor
Th	T-helper
TLR	Toll-like receptor
TMM	Trehalose monomycolates
TNF	Tumor necrosis factor
TPE	Tuberculosis pleural effusion
TRAF6	TNF receptor-associated factor 6
TRAIL-DR5	TNF-related apoptosis inducing ligand death receptor 5
Tregs	T-regulatory lymphocytes
tTregs	Thymus derived T-regulatory lymphocytes
WHO	World Health Organisation
XDR-TB	Extremely drug resistant tuberculosis

ABSTRACT

The human immunodeficiency virus (HIV) is the most powerful risk factor predisposing patients to active tuberculosis (TB) infection, the number one HIV-related cause of death. In South Africa (SA), 62% of TB patients are HIV^+. The pathogenesis of both of these diseases is predominantly orchestrated by a pathogen driven cluster differentiation (CD) 4^+ T-cell response, the nature of which is largely uncharacterised in co-infection. This knowledge gap is especially notable in TB pleurisy, the most common form of extrapulmonary HIV-related TB.

T-helper (Th) 17 and T-regulatory (Treg) lymphocytes are $CD4^+$ T-cell subsets with opposing functions. Th17 cells are characterised by the production of pro-inflammatory interleukin (IL)-17A/F, IL-21 and IL-22, critical in mucosal immunity. Tregs and the lineage specific transcription factor forkhead box p3 (Foxp3) suppress effector lymphocyte function preventing immunopathology. Monocytes play a pivotal role in $CD4^+$ T-cell polarisation by contributing to the cytokine microenvironment. Pathogen-driven alterations in Th17/Treg stability and function, as well as monocyte cytokine production, have been indicated in both TB and HIV mono-infections but have not been assessed in co-infection.

We investigated Treg stability and Th17 proportion, functional characteristics and the associated cytokine milieu in TB/HIV pleurisy *in vitro* and *ex vivo*. We also determined the effect of a TB^+HIV^+ microenvironment on monocyte (THP-1) cytokine production and downstream Th17/Treg polarisation *in vitro* using a supernatant transfer model. Th17, Treg proportion was measured by flow cytometry. Micro (miR) and messenger (m) ribonucleic acid (RNA) expression was assessed by quantitative polymerase chain reaction. Protein levels were determined by western blotting and flow cytometry. The cytokine microenvironment was quantified using enzyme-linked immunosorbent assay and Bio-Plex.

TB^+HIV^+ pleural effusion mononuclear cells (PEMCs) showed increased Foxp3 transcript expression (p=0.0022) but decreased protein levels (p=0.0008). MiR-155 is a direct target of Foxp3 and positively regulates Treg stability by repressing translation of suppressor of cytokine signalling 1 (SOCS1), conferring Treg IL-2 competitive fitness. TB^+HIV^+ pleurisy demonstrated reduced miR-155 levels (p=0.0022) and increased SOCS1 expression (p<0.0001). This was associated with a lower proportion of Tregs *ex vivo* (p=0.0084) and confirmed *in vitro* (p=0.0467). Furthermore, diminished levels of Treg characteristic cytokine IL-35 were observed in co-infected pleural effusion fluid (PEF) (p=0.0258), suggesting a Treg functional deficit. This indicates that the TB/HIV microenvironment decreases the stability and functional capacity of Tregs due to altered miR-155/SOCS1 dynamics.

TB^+HIV^+ PEMCs showed a reduced frequency of Th17 cells *ex vivo* (p=0.0054) and *in vitro* (p=0.0001). This was not associated with alterations in polarising cytokines IL-6, IL-21 and IL-23 or signature cytokines IL-17A and F in PEF. Notably; however, there was upregulation of Th17 signaling molecules; IL-6

(p=0.0022), IL-6 receptor (p=0.0247), signal transducer and activator of transcription (STAT) 3 (p=0.0022) mRNA, and IL-1β mRNA (p=0.0022) and protein (p=0.0008). In this study the TB/HIV microenvironment reduced Th17 proportion by mechanisms independent of Th17 polarising cytokines and signaling molecules and without alteration in the levels of IL-17A/F.

TB/HIV co-infection significantly increased monocyte production of IL-1β (p=0.0217), a Th17 generating cytokine. Following supernatant transfer, the $TB^{+}HIV^{+}$ treated monocyte milieu stimulated significant polarisation of healthy $CD4^{+}$ conventional T-cells into Th17 lymphocytes and Tregs (p<0.0001). However, a significantly greater proportion of Th17 cells (p=0.0001) was observed associated with elevated IL-17A intracellular protein expression (p<0.0001) and 2.278 and 8.191 fold increases in the production of IL-17A and IL-21 respectively. This indicates that the TB/HIV microenvironment modifies monocyte T-cell polarisation towards a pro-inflammatory Th17 phenotype, possibly due to increased IL-1β production.

INTRODUCTION

Tuberculosis ranks as the second leading killer globally from a single infectious agent, the first being HIV. South Africa has the second highest rate of new TB cases [1] and the greatest number of people living with human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) globally [2]. Consequently, 62% of TB patients are HIV positive [3, 4]. Both HIV and TB result in immune dysfunction and synergise to accelerate individual disease pathology [5]. The immunological consequences of simultaneous co-exposure to TB and HIV are largely uncharacterised.

Mycobacterium tuberculosis (MTB) is the causative pathogen of TB, with an estimated 7.3-14.0% annual risk of infection in SA [6]. Tuberculosis is classified as a granulomatous inflammatory condition where effector cells accumulate at the site of mycobacterial infection to form a characteristic tubercle. This physically contains the infection suppressing bacterial replication and preventing dissemination resulting in subclinical disease. However, the granuloma also shields the bacteria from the immune system providing a niche of bacterial survival. Latency is considered the hallmark of protective immunity, classically attributed to the co-ordinated activity of the host $CD4^+$ T-cell responses and successful in approximately 90% of infected individuals [7].

Primary active disease or reactivation is generally considered a failure of $CD4^+$ T-cell immunity. However, rather than the absence of immune activity to explain the re-emergence of a pathogen, active TB infection displays areas of intense immune infiltrate [8, 9]. A contrasting school of thought ascribes active disease progression to MTB mediated dysregulation of the protective $CD4^+$ T-cell response into a pathological productive infection. This results in necrotic liquidation of granulomas and facilitates the extracellular dissemination of bacilli which are expelled out of the lungs on coughing [8].

Typically, HIV causes gradual immunosuppression culminating in AIDS at a $CD4^+$ T-cell count below 200cells/µl. The virus primarily targets and replicates within C-C motif receptor (CCR) 5^+/C-X-C motif receptor (CXCR) 4^+CD4^+ T-cells. Infection is characterised by progressive depletion of $CD4^+$ T-cells and chronic immune activation resulting in exhaustion and failure of the immune system and onset of opportunistic infections, predominantly TB, and AIDS-related malignancies [10]. Both the innate and adaptive immune branches contribute to anti-HIV defence but are insufficient to eradicate the pathogen or maintain long term control of viral replication. Evidence indicates that T-cell responses are significant determinants in elite control of HIV replication, the mechanisms of which are poorly understood [11].

$CD4^+$ T-lymphocytes form the basis of protective immunity and immunopathology in both diseases. The immunological implications of TB/HIV co-infection compared to mono-infections are beginning to be elucidated. Observations include rapid depletion and preferential HIV targeting of MTB specific $CD4^+$ T-cells [12], loss of HIV-specific T-cell functionality [13], increased $CD4^+CCR5^+$ expression and consequently

increased HIV susceptibility and enhanced viral replication [14, 15]. Taken together these findings indicate a conjoined MTB-HIV pathogenic manipulation of $CD4^+$ T-cells which has not been explored in individual subsets.

The $CD4^+$ T-lymphocytes are a heterogonous population that can be broadly classified according to their distinct cytokine and transcription factor profiles. Th17 cells, characterised by the production of IL-17A/F, IL-21 and IL-22 together with the expression of CCR6 and RAR-related orphan receptor C, are a pro-inflammatory subset of effector cells critical in mucosal immunity to bacterial and fungal infections [16]. Regulatory T-cells, categorised by the transcription factor Foxp3, are a suppressive subset of immune cells which maintain peripheral tolerance and limit pro-inflammatory responses preventing immunopathology [17]. An imbalance in Th17 and Treg frequency has been implicated in the pathogenesis of several diseases. Evidence shows pathogen driven alterations in Th17 and Treg proportion and function in both TB and HIV mono-infections that have not been investigated in co-infection.

STUDY RATIONALE AND AIMS

In order to reduce the incidence and mortality of TB/HIV, improvements are mandatory in diagnostics, disease monitoring and treatment [1]. This requires greater understanding of pathogen driven immune dysfunction characteristic of both infections. The $CD4^+$ T-cell response is a convergent point in the host immune response to MTB and HIV. An investigation into the MTB/HIV manipulation of $CD4^+$ T-cells provides clues into the underlying mechanisms mediating pathogenesis of dual infection and gradually lays the foundation for novel therapeutic and prophylactic interventions, ultimately benefiting clinical disease management. With this aim it is imperative to measure the biological responses in TB/HIV endemic areas such as SA.

Although TB most commonly presents as pulmonary disease, with concurrent HIV infection 40-80% of active TB cases are extrapulmonary [18, 19], the most common form of which is TB pleurisy [20]. Clinical and experimental evidence indicates that TB pleurisy provides an optimum model to elucidate immunological mechanisms involved in the defence against MTB *in vivo*. Immunity is compartmentalised in the pleural space [21] and is generally self-limiting, indicating effective anti-MTB activity [21-23]. Furthermore, it has been established that TB infection outcome is determined by localised immune responses which are not accurately reflected systemically [24].

This study was designed to investigate the implications of TB/HIV co-infection on Th17 and Treg lymphocyte responses in PEMCs of a South African population.

Our research objectives were:

1. To determine the effect of TB/HIV pleurisy on Treg stability and functional characteristics *in vitro* and *ex vivo*.
2. To determine the effect of TB/HIV pleurisy on Th17 lymphocyte proportion, functional characteristics and the associated cytokine microenvironment *in vitro* and *ex vivo*.
3. To determine the effect of a TB/HIV co-infected micro-environment on monocyte mediated Th17 and Treg polarisation and the associated cytokine profile *in vitro*.

Pleural effusion fluid samples were obtained by diagnostic pleural tap from TB pleurisy patients with concurrent HIV infection and compared to that from confirmed HIV seronegative patients with non-infectious pleural effusions. The results of *ex vivo* assays were confirmed *in vitro* by treating healthy $CD4^+$ conventional T-cells with PEF and assessment of the equivalent biological parameters.

CHAPTER 1

LITERATURE REVIEW

1.1 Tuberculosis

1.1.1 *Overview of tuberculosis*

Tuberculosis (TB) was declared a global health emergency in 1994 by the World Health Organisation (WHO) and has remained a major public health challenge since [25].

Tuberculosis is spread by the airborne *Mycobacterium tuberculosis* (MTB) and acquired by inhalation of infected droplets. MTB passes to the lungs where the co-ordinated activity of macrophages, dendritic cells (DC) and cluster differentiation (CD) 4^+ T-cells results in the formation of the granuloma and clinically silent infection. Due to host and/or MTB factors, excessive bacterial replication within the granuloma results in its break down with dissemination and transmission of the bacilli via the respiratory route, characteristic of active infection [7]. Although MTB is capable of causing primary disease, the most common manifestation in adults is reactivation of a pre-existing latent infection, termed post primary TB [26], with a life time risk of 10% [27].

Most organs can be infected with MTB; however, the predominant clinical manifestation is pulmonary TB. When MTB has disseminated to other areas of the body, via the blood or lymphatic system, it is referred to as extrapulmonary TB [7].

1.1.2 *Epidemiology of tuberculosis*

According to WHO, in 2013 there were approximately 9 million new cases of active TB infection and 1.5 million deaths globally. Over 95% of these deaths occurred in low- and middle- income developing countries such as South Africa (SA) [1]. South Africa is considered a high burden TB country with an incidence of 450,000 in 2013, the third highest incidence rate globally and the highest rate of drug susceptible and resistant TB in Africa [1, 3] (Figure 1.1). It is estimated that in SA 1 person out every 100 develops active TB each year [1].

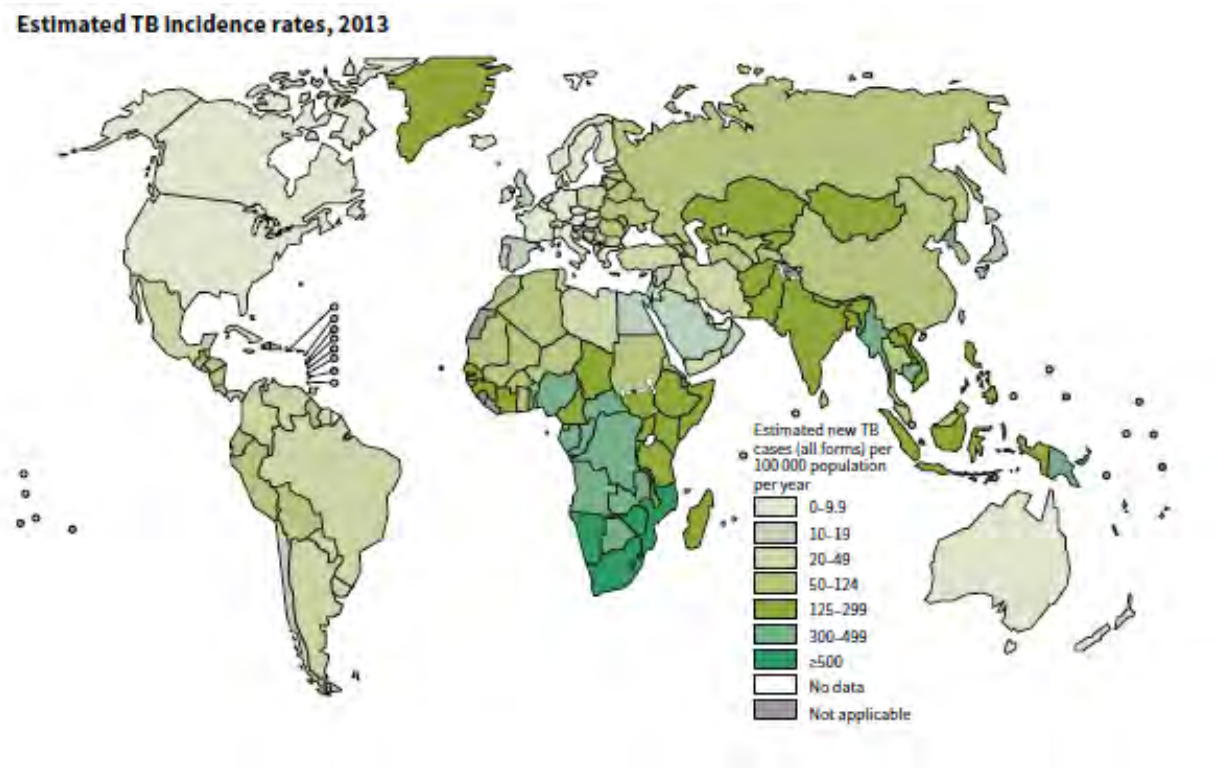

Figure 1.1: Estimated tuberculosis incidence rates (2013) depicting the high burden in South Africa [1].

1.1.3 *Morphology of Mycobacterium tuberculosis*

Mycobacterium tuberculosis is a large, non-motile rod shaped bacterium (Figure 1.2). It is a facultative, obligate aerobe with a slow replication rate of 16-20 hours. The bacterium ranges in length from 2-4 microns and 0.2 - 0.5 microns in width [28].

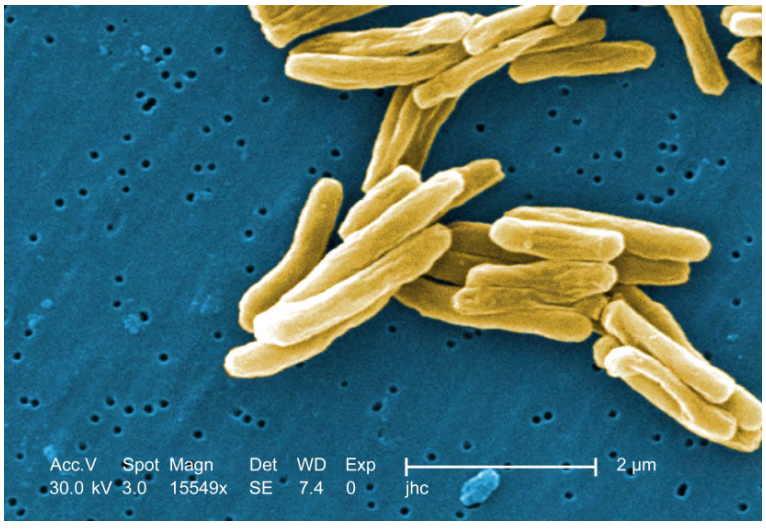

Figure 1.2: Scanning electron micrograph of *Mycobacterium tuberculosis* (15549×) [28].

The cell envelope; the cell wall together with the mycobacterial inner cell membrane, is the major determinant of MTB virulence. The cell wall is made up of two layers - the lower layer adjacent to the cell membrane is composed of peptidoglycan covalently attached to arabinogalactan which in turn is esterified to mycolic acids, forming the mycolylarabinogalactan-peptidoglycan (mAGP) complex. The insoluble mAGP complex is essential for structural integrity and forms a hydrophobic barrier responsible for the viability of MTB and resistance to chemotherapy [29, 30] (Figure 1.3).

In the upper layer, also termed the mycobacterial outer membrane, and intercalated within the mycolic acids of the mAGP complex are interspersed cell wall proteins and the major pathogenic free lipids. Most significantly these include; phthiocerol dimycocerosates (PDIM), cord factor/trehalose dimycolates or monomycolates (TDM/TMM), sulfolipids, phthiocerol-containing lipids, phosphatidylinositol mannosides (PIM), lipomannan (LM) and lipoarabinomannan (LAM). A defining feature of mycobacterium species is this presence of a wide diversity of these complex lipids constructing approximately 60% of the cell envelope [31]. Simplistically, the lipids are the signalling and effector molecules of the bacterium-host interaction and contribute significantly to the success of the pathogen [29, 30] (Figure 1.3).

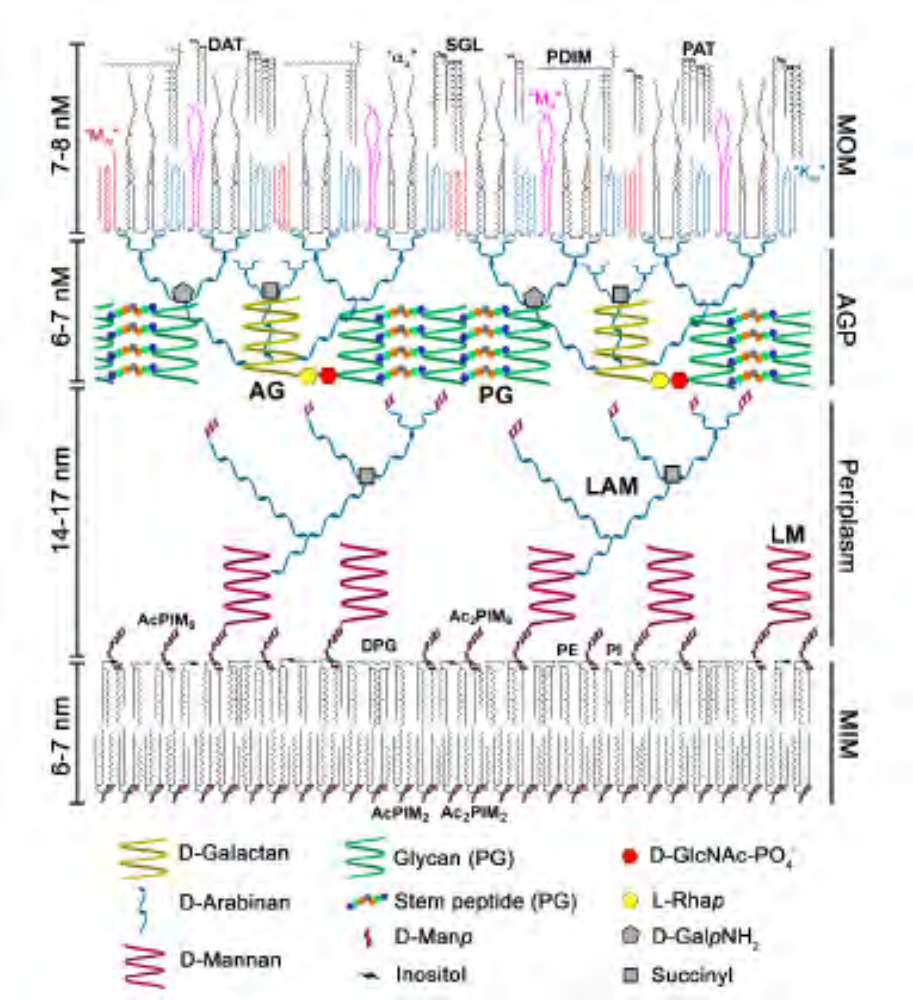

Figure 1.3: Two-dimensional representation of the *Mycobacterium tuberculosis* cell wall. Mycobacterial inner membrane (MIM), mycobacterial outer membrane (MOM) [30].

1.1.4 *The pathogenesis of tuberculosis*

Mycobacterium tuberculosis infection follows a well described set of events. Briefly, bacilli are inhaled as droplets from the atmosphere with the infectious dose estimated at a single bacterium. The innate immune response is initiated when MTB in the alveolar space is recognised by pattern recognition receptors (PRRs), predominantly Toll-like receptors (TLRs) on alveolar and interstitial monocytes/macrophages as well as local DCs, and subsequently engulfed. These antigen presenting cells (APCs) present antigen in association with major histocompatibility complex (MHC) class II molecules to $CD4^+$ T-helper (Th) lymphocytes, and to a lesser extent in association with MHC-I and CD1 to $CD8^+$ T-cells [7, 32].

Up to the initiation of acquired immunity, macrophages remain relatively permissive to intracellular MTB. Experimental host studies identify this as a period of exponential bacterial replication [33]. Primed T-cells then recognise and activate macrophages to institute anti-mycobacterial killing mechanisms through the secretion of interferon (IFN)-γ and tumor necrosis factor (TNF)-α bringing bacterial replication under control, but not eradication. These pro-inflammatory cascades culminate in the remodelling of the infection site into the characteristic tissue nodule, the granuloma. The result is a chronic infection associated with slow or non-replicating bacilli and potentially progressive pathology [7, 34].

1.1.4.1 Macrophages

Macrophages play a dual role in TB infection. *Mycobacterium tuberculosis* targets and replicates within modified phagosomes of macrophages, employing a multitude of strategies to evade clearance by both the innate and adaptive immune systems, yet macrophages are the predominant cell responsible for MTB killing.

Infected macrophages initially respond with a vigorous pro-inflammatory and anti-microbial response mediated by TLR signalling [35]. TLRs are key recognition molecules in the host's innate immune system, TLR-2 being one of the major PRRs for detecting MTB [36, 37]. MTB has a diverse range of TLR-2 ligands including MTB heat shock protein 65 and 70, several types of lipoproteins (LpqH, LprA, LprG, PhoS1), LAM, LM, PIMs and TDM [38-40].

Following MTB engulfment, alveolar macrophages enter the lung interstitium establishing a site of infection [41, 42]. This consequently leads to a localised pro-inflammatory cascade with production of TNF-α, interleukin (IL)-1, IL-6, and IL-12, together with inflammatory chemokines C-C motif ligand (CCL) 2 and C-X-C motif ligand (CXCL) 10 by the infected macrophage. The chemokine gradient recruits waves of neutrophils, natural killer (NK) cells, $CD4^+$, $CD8^+$ and γö T- and B-lymphocytes each producing their own complement of chemokines and cytokines which amplify cellular recruitment and remodelling of the infection site into the granuloma [26, 41-44]. Tumor necrosis factor-α, produced by macrophages and lymphocytes, acts in a positive feedback manner to accentuate macrophage chemokine production thereby emphasising immune cell accumulation and granuloma formation. Persistent TNF-α production is required to

sustain the chemokine gradient and therefore maintain the structure of the granuloma. Once the bacilli have been successfully contained and latency established, the chemokine cascade is down regulated by IFN-γ, produced by lymphocytes at the site of infection, through a negative feedback loop [26].

1.1.4.2 Dendritic cells

Dendritic cells play a central role in the switch from an innate immune response to acquired specific immunity. Bacilli are phagocytosed by DCs surveying the airways resulting in DC production of IL-12, upregulation of MHC class I, II and CD1 associated antigens as well as co-stimulatory molecules CD40/54/80/86. In conjunction, the acquisition of a motile phenotype results in the migration of DCs to the proximal lymph nodes where they prime naïve T-cells [45].

Antigen specific T-cell responses are noted in the mediastinal lymph nodes approximately 10 days following aerogenic murine infection at the earliest [46, 47]. This is the rate limiting step of T-cell priming, the reasons for which are still unclear [48, 49]. This lag permits exponential bacterial replication producing a high pulmonary bacterial load at the onslaught of T-cell immunity [50]. Recent evidence shows that MTB infection of DCs inhibits their migration through the CCL19- C-C motif receptor (CCR) 7 gradient [51]. Furthermore, MTB proteins, Hip1 [52] and mannose capped LAM (ManLAM) [53, 54], have been shown to inhibit DC maturation, cytokine secretion and antigen presentation resulting in impaired T-cell priming and sub optimal inflammatory responses.

1.1.4.3 $CD4^+$ T-lymphocytes

The specialisation of T-lymphocyte responses to produce characteristic cytokine profiles plays a central role in co-ordinating host immunity against MTB infection. $CD4^+$ T-cells are essential for the control of primary infection and the ongoing immune surveillance of the reservoir of persistent bacilli within the granuloma from which reactivation originates [40]. This is illustrated by the fact that selective depletion of $CD4^+$ T-cells by the human immunodeficiency virus (HIV) significantly increases TB reactivation rates to 5-10% per life year [27] and incidence to 25.7 per 100 persons per year, in patients with $CD4^+$ T-cell counts below 50 cells/μl [55].

Classically, innate immunity to MTB is superseded by an antigen specific Th1/IFN-γ response, the nature of which is well described. IFN-γ is a critical mediator of macrophage activation and bactericidal mechanisms. The induction of inducible nitric oxide synthase (iNOS) in macrophages and downstream production of reactive nitrogen intermediates, together with reactive oxygen species, are toxic to MTB [56-58]. Additionally, IFN-γ stimulates autophagic pathways in infected macrophages resulting in maturation of the MTB phagosome to a phagolysome and mycobacterial killing [59].

More recently studies have shown that the newly identified $CD4^+$ T-cell subtypes; pro-inflammatory Th17 and anti-inflammatory T-regulatory lymphocytes (Tregs) are associated with immunity against MTB in addition to Th1. These subsets will be discussed in detail below.

1.1.4.4 $CD8^+$ T-lymphocytes

The role of cytotoxic T-cells has been overshadowed by that of Th cells. It has been demonstrated that $CD8^+$ T-cells partake in MTB infection in several ways [60, 61]: (1) Cytokine production; $CD8^+$ T-lymphocytes have been identified as a source of IFN-γ and therefore mediate macrophage activation and bactericidal mechanisms [58, 62-64]. (2) Granule dependent cytotoxicity; both granulysin and perforin mediated cytotoxic activities have been implicated in the lysis of infected cells and extracellular MTB [62, 65-67]. The bacteria are released into the extracellular environment to be taken up by activated macrophages capable of killing the bacilli. However, $CD8^+$ T-cell dysfunction has been frequently associated with active TB disease [68, 69].

1.1.4.5 B-lymphocytes

Classically, B lymphocytes and associated antibodies are considered to be insignificant role players in TB. However, humoral immunity is involved in shaping the cellular immune response to MTB including direct antigen presentation to T-cells, direct effects of antibody on mycobacteria, cytokine production and granuloma organisation [70-73], binding of antibody complex to APC Fcγ receptors influencing subsequent Th1/IFN-γ responses [8], macrophage activation and bactericidal mechanisms [8, 73] in an organ specific manner [73].

Follicle like B-cell aggregates have been demonstrated in the lungs of human and murine pulmonary TB infections associated with granulomatous inflammation [74-76]. The role of these B-cell centres have not been fully elucidated but are hypothesised to perpetuate local immune responses through cellular proliferation [77] as well as involvement in both B-cell maturation and antigen presentation to T-cells, augmenting the immune response [8, 75].

1.1.5 *The granuloma*

The granuloma serves to physically contain the bacilli preventing dissemination and provide a microenvironment of optimum and localised immune communication. This leads to inhibition of bacterial growth by activating macrophage bactericidal mechanisms and creating an oxygen and nutrient deprived environment [44].

According to the central dogma, the initial granuloma consists of a core of infected macrophages surrounded

by additional macrophages displaying distinct morphology; multi-nucleated giant cells, epitheloid cells and foamy macrophages [78, 79] as well as granulocytes and other mononuclear phagocytes [9, 26, 33, 42]. In the early stages of granuloma development, the nodule undergoes marked neovascularisation due to the production of pro-angiogenic vascular epithelial growth factor by activated macrophages [80]. The blood vessels denote extensive lymphocyte cuffing indicating a recruitment of lymphocytes as well as dendritic cells and macrophages to the site of infection [80]. As the structure matures it develops a significant fibrous sheath of collagen and other extracellular matrix components around the macrophage rich centre. The lymphocytic infiltrate is excluded and aggregates around the fibrous cuff defining the periphery of the nodule [9, 26, 33, 42] (Figure 1.4).

The granuloma has several morphologically distinct forms: solid (composed of dense aggregates of infected and uninfected macrophages and lymphocytes without central necrosis), neutrophilic (extensive granulocytic infiltrates and/or central core of suppuration) and caseous (enlarged necrosis and liquefaction of dead cells in the core of the granuloma which can progress to cavities, surrounded by a cuff of macrophages and lymphocytes) with or without fibrosis or calcification [26, 33, 81].

This solid granuloma characterises the containment or clinically silent stage of infection; a period of stalemate when bacillary load remains constant and the infection enters latency [26, 33, 81]. As the granuloma progresses from solid non-progressive to active cavitary lesions, there is a decrease in the number of blood vessels penetrating the nodule, facilitating caseation in the hypoxic environment [26, 75, 82]. In addition, the killing of MTB within macrophages is suggested to be severely limited as superoxide and nitric oxide production by macrophages is inhibited by hypoxia resulting in uncontrolled bacterial replication anticipating granuloma break down and dissemination [83].

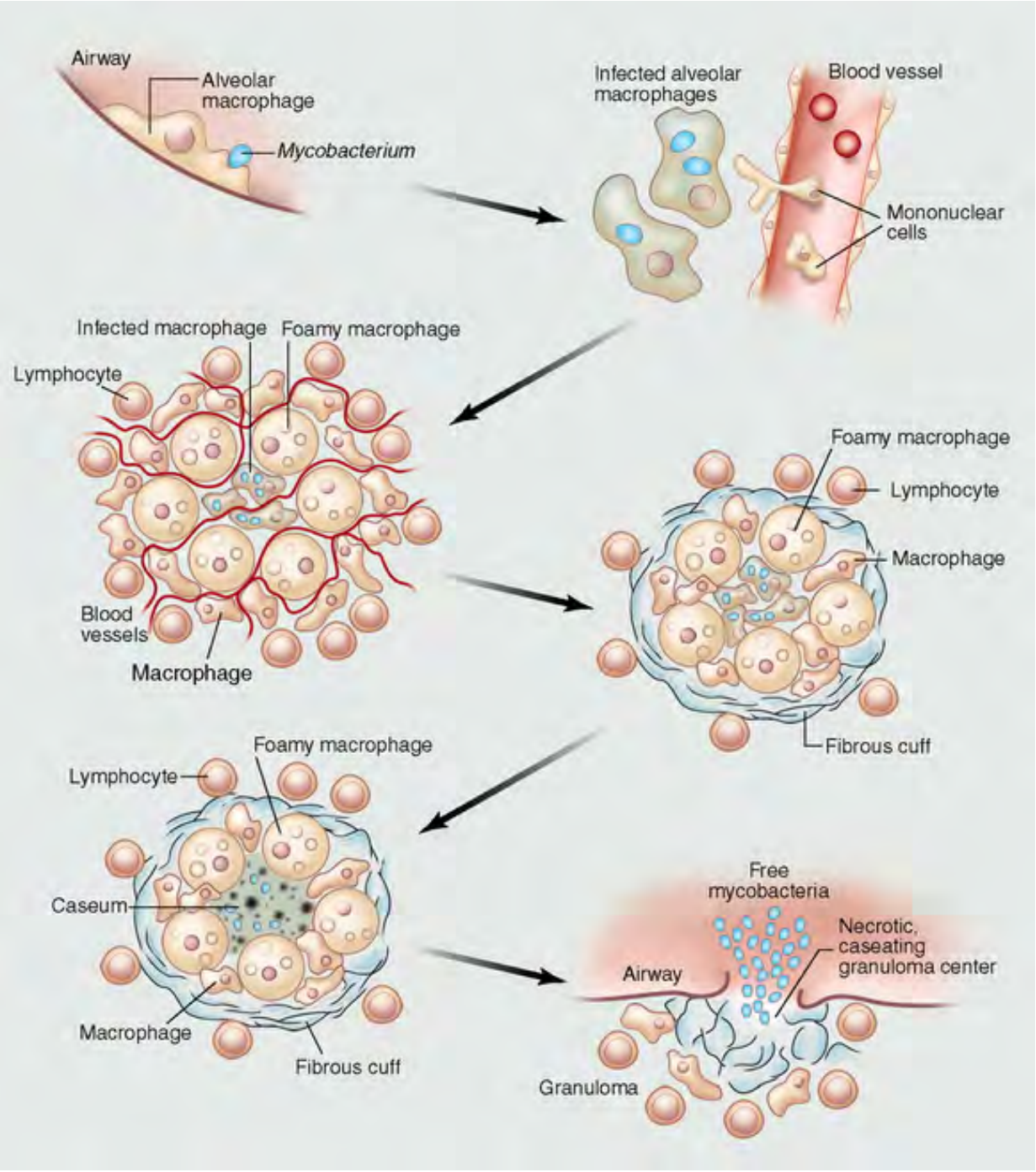

Figure 1.4: The life cycle of *Mycobacterium tuberculosis* [84].

Retrospective histological studies of patients with active disease show marked heterogeneity in granuloma progression seen in a single host. This indicates differential immunological processes occurring separately at each site [26, 85-87] and suggesting that TB progression from latent to active infection is a dynamic evolution determined locally at the granuloma [33].

An alternate school of thought suggests that MTB drives the development of the granuloma in order to physically separate its self within the infected macrophages at the centre from the activated lymphocytes on

the outskirts of the fibrous capsule. In this way the isolated central core serves as a site of bacterial expansion [76, 88-92]. The core of the granuloma is not considered to be the dominant site of immune action but the peripheral cellular infiltrates composed of APCs and lymphocytes, resembling secondary lymphoid organs, potentially orchestrate the immune response [33, 77] (Figure 1.4).

The mycobacterial proteins and lipids have an established granulomatous effect; MTB 6-kDa early secretory antigenic target (ESAT-6) [88] and PIM2 [93], induce matrix metalloproteinase (MMP)-9 expression in pulmonary epithelial cells neighbouring infected macrophages promoting recruitment of macrophages [94]. In addition LM, LAM, TDM/TMM and PIMs are known to induce chemokine and pro-inflammatory cytokine production from mononuclear cells through pattern recognition receptors such as TLR-2 [90, 95-100]. These granulomatous effects provide an accumulation of uninfected host macrophages with suboptimal activation, allowing for continued mycobacterial persistence in the core while physically separated from the bactericidal activity of lymphocytes [89, 90].

1.1.6 *Immune evasion strategies*

Mycobacterium tuberculosis survives and replicates within macrophages establishing chronic persistent infection. This is achieved by arresting phagolysosome biogenesis thereby restricting the unfavourable intracellular environment, preventing host effector mechanisms and shielding MTB from antigen processing pathways.

A feature of phagosome maturation is acidification (pH5.0 and lower) of the phagosome lumen. The acidic environment inhibits bacterial activity, optimises the activity of hydrolytic proteases, ensures correct vesicular trafficking and phagosome-lysosome fusion events and degradation of the pathogen into components for antigen processing and presentation, ultimately resulting in activation of the cell mediated immune response. Acidification is achieved by the recruitment of V-ATPases to the phagosome which actively pump hydrogen ions across the phagosome membrane. *In vitro* and *in vivo* studies observed that MTB containing phagosomes acidify to a minimum of pH6.4 and subsequently fail to fuse with lysosomes [101-104]; MTB has been shown to exclude V-ATPases from the phagosome membrane preventing acidification [105]. This is mediated by a MTB protein tyrosine phosphatase, PtpA, which binds to subunit H of the macrophage V-ATPase inhibiting the trafficking of vesicles containing the V-ATPase complex to the phagosome [106].

Arrest of phagosome-lysosome fusion has been shown to occur due to a failure in early endosomal Rab5 to late endosomal Rab7 conversion, preventing completion of endosomal sorting and membrane trafficking. Rab conversion occurs through a calcium/calmodulin/calmodulin kinase II dependent phosphatidylinositol-3-kinase (PI3K)/phosphatidylinositol-3-phosphate (PI3P) pathway. This cascade mediates the recruitment of the Rab5 effector early endosomal antigen 1 (EEA1) to the phagosome triggering the fusion of phagosomes

with late endosomes. Several bacterial products are observed to be involved; LAM has been shown to interfere in the calcium fluxes required for this pathway consequently inhibiting it [107]. ManLAM has been shown to block the calcium/calmodulin recruitment of PI3K to the phagosome preventing PIP3 generation and EEA1 acquisition [102, 108-110]. A bacterial PI3P phosphatase, SapM, degrades any PI3P which evades the ManLAM block [102, 111, 112]. The combined effect is inhibition of phagolysosome biogenesis.

Macrophage lysosomes are the convergent step between phagocytic and biosynthetic pathways where antigens are processed and complexed with MHC molecules for presentation to T-cells [113]. Accumulation of MTB components in acidic late endosomal compartments optimises processing. The MTB phagosome has limited acidification and hydrolytic activity and therefore a suboptimal capacity to process antigens [114]. The inhibition of phagolysosome biogenesis not only avoids MTB destruction by hydrolytic proteases but also presentation and recognition by primed T-cells [115, 116]. Despite this, cytokine mediated activation of the macrophage is sufficient to overcome MTB mediated phagosome modulation; the phagosome acidifies to pH5.2 with subsequent bactericidal actions and fusion with the lysosome mediated by members of the IFN-inducible p47 GTPase family [117, 118].

1.1.7 *Transmission*

Several factors affecting T-cell and/or macrophage function have been associated with an increased risk of developing active TB [25] including HIV co-infection [119], diabetes mellitus [120], renal impairment [121], TNF-α antagonist therapy [122], cancer [123], alcohol abuse [124] and tobacco use [125].

1.1.7.1 Breakdown of the granuloma

Preceding research dictates that active infection advances from failure to contain infection within the granuloma due to impairment of the host immune response, specifically $CD4^+$ T-cell responses. In accordance, HIV co-infection is the strongest known risk factor for immediate (primary) and delayed (post primary) progression from latent to active disease [119]. By this model, excessive bacterial replication results in progressive necrosis followed by caseation, liquidation of the granuloma and cavitation into the bronchi releasing bacilli. This necessitates development of a productive cough resulting in aerosol transmission of infectious MTB [7, 26].

Mycobacterium tuberculosis evades the immune response to allow persistent infection but simultaneously promotes sufficient immunopathology to ensure its transmission [126]. A more recent school of thought suggests that MTB dysregulation of host lipid synthesis and lipid accumulation is pivotal in transition from latent to active infection, magnified by dysregulation of $CD4^+$ T-cell responses. Histological analysis of biopsies from patients with untreated TB demonstrated that reactivation originated from areas of lipoid pneumonia. Within these areas there was sequestration of abundant lipid rich macrophages, termed foamy

macrophages (FMs) [79, 127, 128]. Furthermore, infection was restricted to the FM and bacilli were predominantly found within the lipid droplets. Cavities originated from tuberculosis pneumonia in individuals who had no histological evidence of caseating granulomas [79, 127, 128]. Whereas previously, tuberculosis pneumonia was regarded as a consequence of cavitation, it is now considered as the origin [127].

Mycolic acids form a significant and characteristic component of MTB, constituting 40-60% of its dry weight [129]. Recently it has been demonstrated that FM generation is specifically induced by oxygenated mycolic acids (oxygenated ketomycolic) and hydroxyl mycolic acids, of virulent mycobacterial strains [79].

The FM is in its self a pro-inflammatory cell [130]. It is indicated as a key player in sustaining persistent bacterial infection and dysregulating T-cell responses driving pathology leading to necrotic cavitation and transmission [33]. FMs show reduced ability to mediate phagocytosis, accompanied by diminished anti-mycobacterial mechanisms [79], lowered antigen processing capacity through down regulation of CD40 and MHC II expression [131], increased Fas/Fas ligand mediated Th1 apoptosis [132], as well as production of high levels of iNOS resulting in T-cell hypo-responsiveness [133].

Mycobacterium tuberculosis induced FMs displayed an elevated continual secretion of TNF-α, a strong inducer of necrosis [79]. In a series of human biopsy samples FMs were only observed in necrotic lesions and their presence correlated to the onset of necrosis, systematically located at the interface between the histiocytes and the necrotic core [33, 79, 127, 128]. The spatial and temporal relationship between FMs and the necrotic centre of the granuloma suggests a causal association.

Together these findings indicate a bacterial driven role of FMs preventing macrophage intracellular killing with simultaneous suppression of T-cell responses resulting in sheltering of MTB and facilitating persistence. This MTB mediated suppression of cell mediated immunity accentuates necrotic loss of granuloma structure leading to excessive bacterial replication and transmission [79, 128].

1.1.7.2 Host cell death

Virulent MTB survive within macrophages by preventing apoptosis, phagosome maturation and antigen processing creating a niche where bacteria remain metabolically active and capable of replication [134]. However, MTB would benefit from cell death once a high intracellular bacterial load has been reached allowing for dissemination of the bacilli. Necrosis is the preferred MTB exit strategy, promoting inflammation and disease progression [135-137].

MTB has been shown to cause plasma membrane microdisruptions in the infected macrophage. Repair of these lesions by exocytosis of endomembranes is required for preventing necrosis and promoting apoptosis. This is achieved by the fusion of golgi and lysosome derived vesicles with the plasma membrane, the process

being dependent on prostaglandin E2 (PGE2) [138]. In the late stages of infection, macrophages infected with virulent MTB strains preferentially synthesise lipoxin A4 (LXA4) which inhibits apoptosis and promotes necrosis shielding the MTB from the innate immune system. The production of LXA4, which blocks PGE2 biosynthesis by down regulation of cyclooxygenase 2, is a natural anti-inflammatory immune mechanism. This is exploited by pathogenic MTB at the infection site, inhibiting plasma membrane repair, driving inflammation and necrosis [138, 139]. Necrotic death of FMs within the granuloma leads to the accumulation of lipid debris at the core of the liquefying granuloma forming the caseum [33].

1.1.8 *Tuberculosis pleurisy*

Although TB most commonly presents as pulmonary disease, with concurrent HIV infection, 40-80% of TB is extrapulmonary [18, 19]. TB pleurisy is considered an early manifestation of TB [18], the initial presenting factor in approximately 25% of cases [140] and the most common form of HIV-related extrapulmonary TB [20]. Furthermore, TB pleurisy is the most common cause of pleural effusions in TB endemic areas [20, 141, 142] such as SA.

A TB pleural effusion (TPE) is a consequence of MTB infection of the pleura characterised by a chronic accumulation of inflammatory cells in the pleural cavity [143]. This can be the result of either primary infection or reactivation and is thought to be due to the breakdown of small subpleural caseous granulomas that release their contents into the pleural space. This is followed by acute inflammation resulting from a delayed hypersensitivity reaction to MTB antigens mediated by $CD4^+$ T-lymphocytes. This compartmentalised inflammatory reaction increases the permeability of pleural capillaries which, together with reduced lymphatic drainage, results in the formation and accumulation of pleural fluid in the pleural cavity. In the initial stages of this process neutrophils dominate the pleural space; however, soon after T-lymphocytes become the chief cell type [143, 144]. TB pleurisy may cause the localised deposition of inelastic fibrotic tissue on the visceral pleura hindering lung expansion and resulting in a negative pleural space pressure permitting the development of a chronic pleural effusion [143].

The TPE most commonly manifests as an acute or sub-acute condition with pyrexia, cough (productive or non-productive potentially with the expectoration of blood), shortness of breath and pleuritic chest pain in over 70% of patients [145]. Other constitutional symptoms include night sweats, weight loss, generalised weakness, malaise and dyspnoea varying in grade according to the magnitude of the effusion [143]. Upon examination of chest radiographs, TPEs are typically unilateral, small to moderate in size, loculated and serous in appearance with or without pulmonary parenchymal changes [18, 146]. This may be associated with tracheal and mediastinal shift away from the TPE, decreased chest movement and dullness on percussion on the side of the TPE [20].

Definitive diagnosis of TPE is based on the demonstration of bacilli in the sputum, pleural fluid or pleural biopsy specimens [147, 148] by acid fast staining (Ziehl-Neelsen, Auramine) and mycobacterial culture regardless of parenchymal or pulmonary involvement [143, 148]. Within the appropriate clinical context a reasonably certain diagnosis can also be based on the demonstration of granulomas in the parietal pleura [143] and a raised pleural fluid adenosine deaminase level above 30-40U/L [20, 143, 149], even among HIV positive individuals with low $CD4^+$ T-cell counts [150]. Frequently pleural fluid analysis allows for the presumptive diagnosis of TPE, this invariably demonstrates a straw coloured exudative effusion with a lymphocytic predominance [148, 151], a protein concentration greater than 3-5g/dl (more than 70% of cases) [20, 140], glucose level below 60mg/dl (greater than 25% of cases) and a pH of less than 7.2 (greater than 10% of cases) [140].

TB/HIV co-infected patients may present with typical TB symptoms; however, more often these patients are minimally symptomatic, with less specific symptoms or asymptomatic [152].

1.1.9 *Treatment of tuberculosis*

According to the South African National Tuberculosis Treatment Guidelines 2014 and the WHO guidelines 2010, uncomplicated extrapulmonary TB is generally treated in the same manner as pulmonary TB: For newly and previously treated adults and children older than 8 years of age and above 30kg in weight, TB treatment is started with four first line drugs – isoniazid, rifampicin, pyrazinamide and ethambutol for a two month intensive phase. This reduces the number of viable bacilli and renders the patient non-infectious within 10-14 days. Approximately 90% of mycobacteria are killed within the first week of treatment. With fewer bacilli the risk of acquired drug resistance decreases and so a 4 month continuation phase is instituted with two drugs – isoniazid and rifampicin. The sterilising activities of these drugs eliminate remaining bacilli preventing relapse (Figure 1.5). This regime is also instituted in TB/HIV co-infection in antiretroviral (ARV) naïve patients [20, 153].

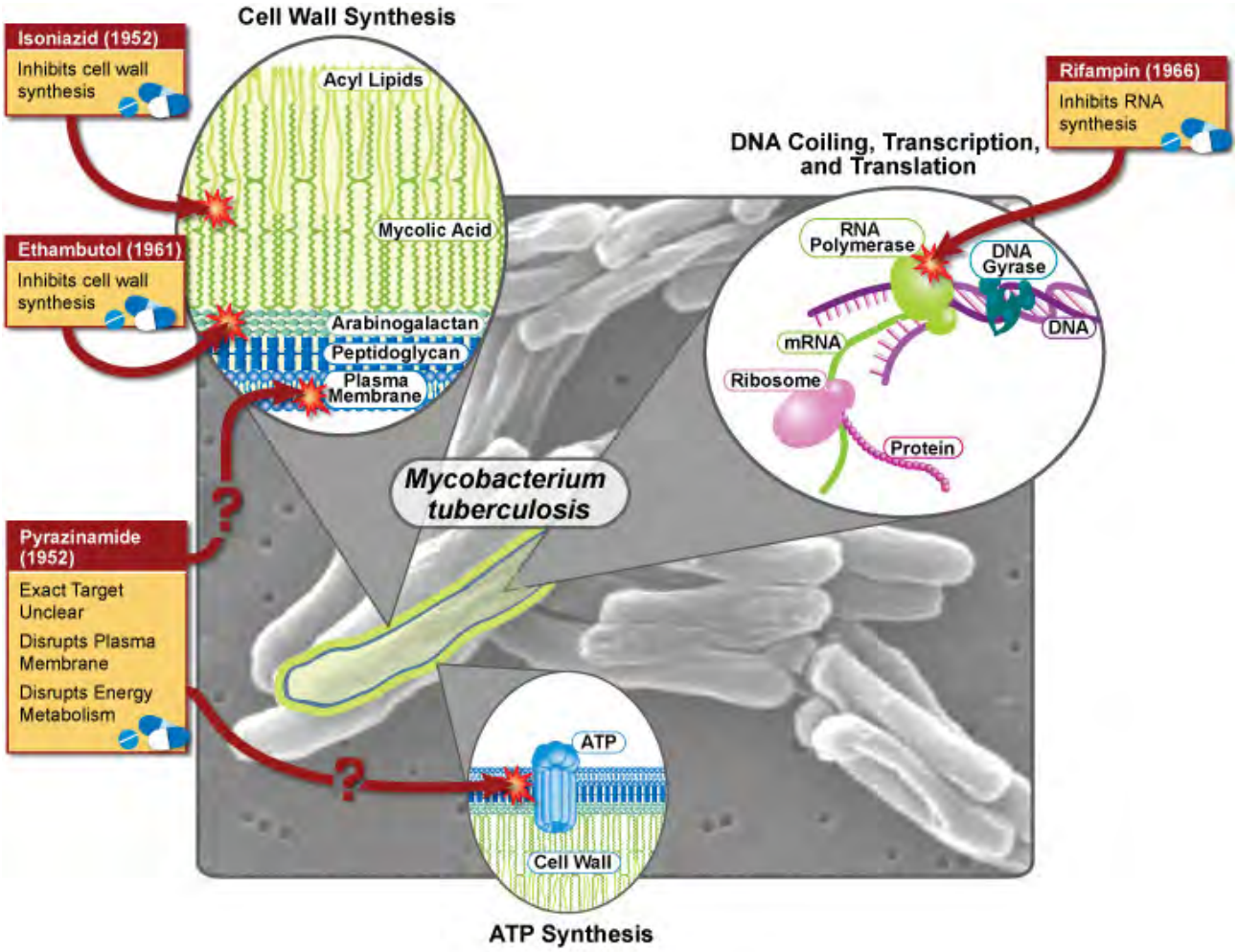

Figure 1.5: First line drugs in the treatment of drug susceptible tuberculosis [154].

1.1.10 *Drug Resistance*

Mycobacterium tuberculosis acquires drug resistant mutations in a sequential manner due to cellular mechanisms and external factors. Most significantly, suboptimal therapy predominantly due to monotherapy, inadequate dosage, treatment interruptions and drug interactions [25].

Multi-drug resistant TB (MDR-TB) is defined as resistance to two of the four first line drugs, isoniazid and rifampicin. Extensively drug resistant TB (XDR-TB) is defined as resistance to any fluoroquinolone and at least one of the 3 of the second line drugs (capreomycin, kanamycin and amikacin) in addition to isoniazid and rifampicin [155, 156]. Totally drug resistant TB (T/XX DR-TB) is a strain of MTB showing resistance to all first and second line TB drugs used, although the terminology is still to be fully defined [25, 157, 158]. Treatment and clinical management is complicated by lengthier, prohibitively expensive, more toxic and less effective treatment regimens with inadequate success rates [159].

According to WHO, drug resistant TB is rising with approximately 210,000 deaths and 480,000 new MDR-

TB cases in 2013. An estimated 9.6% of these are XDR-TB with a mortality rate of 44-49% [1]. However, these figures are thought to be significantly underestimated across sub-Saharan Africa, Asia and Eastern Europe due to the lack of capacity and availability of drug resistance testing facilities, inconsistent testing methods and inadequate treatment [25, 159-161]. During 2013, in SA 1.8% of new cases and 6.7% of retreatment cases were reported to have MDR-TB, with the second highest number of reported XDR-TB cases globally [1]. Furthermore, in 2013 SA became one of four countries reporting cases of T/XXDR-TB [162].

Drug resistant TB is exacerbated by convergence with HIV infection. In a South African study by Gandhi *et al*, of 53 patients diagnosed with XDR-TB 44 were tested for HIV and all were co-infected, 52 of which died with a median survival of 16 days from diagnosis [155]. Further studies have shown a 70-90% HIV co-infection in MDR-TB patients [163-165] and 98-100% in XDR-TB [165] with 80% [166] and 98% [155] mortality in XDR-TB reported.

1.2 Human immunodeficiency virus infection

1.2.1 *Overview of human immunodeficiency virus infection*

HIV is a retrovirus of the lentivirus genus and *Retroviridae* family, transmitted in certain bodily fluids. The virion was identified in 1983 as the causative agent of acquired immunodeficiency syndrome (AIDS) [167, 168]. Infection is characterised by progressive $CD4^+$ T-cell depletion and chronic immune activation resulting in immunodeficiency, immune exhaustion and the onset of opportunistic infections and malignancies [10].

1.2.2 *Epidemiology of human immunodeficiency virus/acquired immunodeficiency syndrome*

As of 2014 an estimated 36.9 million individuals were living with HIV infection worldwide, with approximately 2 million new infections (5,480 daily) and a mortality of 1.2 million during this period. Sub-Saharan Africa is the most affected accounting for 70% of infections globally [169]. In 2013, SA had the highest number of individuals living with HIV infection (6.8 million), new infections (340,000), and the second highest number of AIDS-related deaths (140,000) [2] (Figure 1.6).

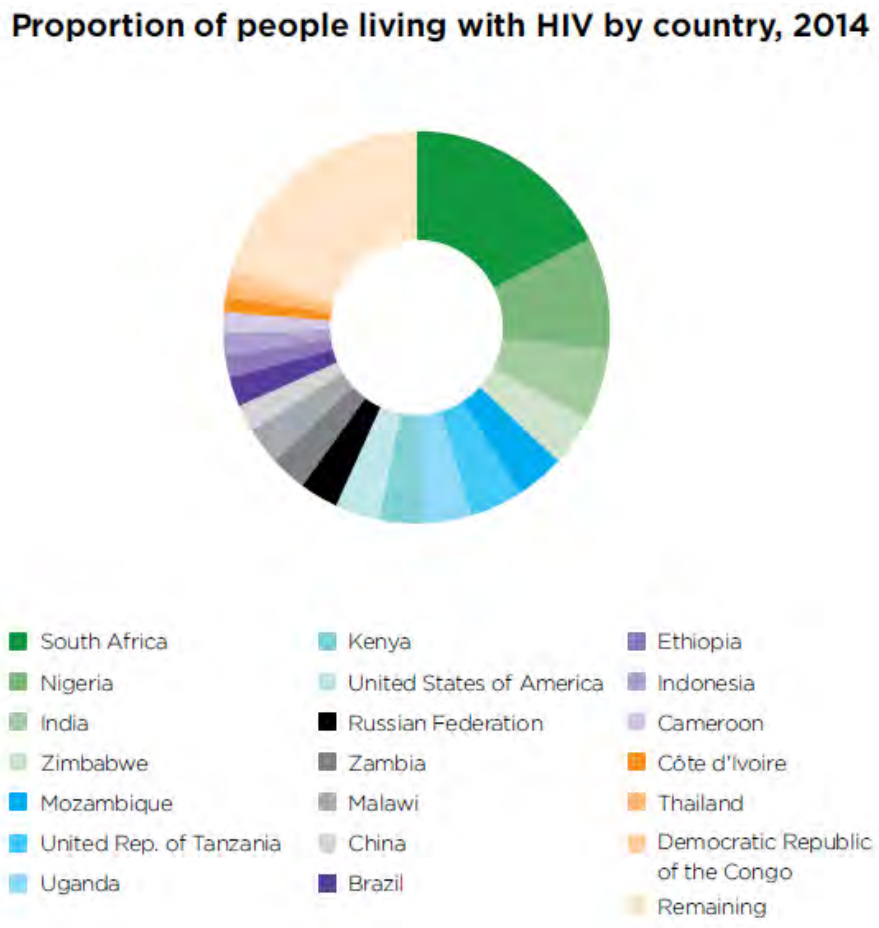

Figure 1.6: Prevalence of human immunodeficiency virus infection (2014) depicting the high burden in South Africa [169].

1.2.3 *Human immunodeficiency virus strains and subtypes*

HIV is a zoonotic virus originating in West and Central Africa from multiple transmissions of the simian immunodeficiency virus from non-human primates [170]. HIV-1 and HIV-2 are two distinct viruses; HIV-1 predominates globally while HIV-2 is relatively uncommon, mainly restricted to West Africa, is less infectious and presents with slower disease progression [10, 171]. In this thesis, HIV refers to HIV-1.

There are four strains of HIV-1 originating from three individual transmission events from chimpanzees (M, N and O) and one event from gorillas (P). Strains N, O and P are restricted to West Africa. Strain M is responsible for the global HIV pandemic and is composed of nine genetically distinct subtypes: A-D, F-H, J and K. In addition, the subtypes can combine genetic material to form a hybrid virus termed a circulating recombinant form, the frequency of which is increasing worldwide. Subtype C is prevalent in Africa and India accounting for approximately 48% of infections globally during 2004-2007. Subtype B is predominately found in Western Europe, North and South America and Australia accounting for 11% of infections in the same period [10, 171].

HIV-1 displays marked genetic diversity and rapid evolution, contributing in a large part to the success of the virus. This is a result of a high rate of replication and mutations caused by the error prone viral reverse transcriptase [172], transcriptional inaccuracy by host ribonucleic acid (RNA) polymerase II and ABOBEC3G/F mediated G-to-A hyper mutation [173]. An infected and untreated individual has a plasma viral load of approximately 10^4-10^5copies/ml with a turnover rate of ~10^{10}/day [174]. This results in 10 mutations with every replication cycle of the viral genome repeated in thousands of generations over the course of infection in a large quasi-species population. Consequently, the virus can evolve rapidly in response to selective pressures with the rise of a genetically distinct HIV-1 population every few years [173, 175, 176].

1.2.4 *Morphology, morphogenesis and replication of human immunodeficiency virus-1*

Mature HIV-1 is spherical virion with a diameter of approximately 120nm. It consists of a lipid bilayer membrane, with envelope (Env) glycoprotein (gp) spikes and an inner layer of matrix proteins (MA). This surrounds a characteristic conical fullerene core housing the rinonucleoprotein (RNP) complex of nucleocapsid (NC), viral RNA and viral enzymes (protease (PR), reverse transcriptase (RT), integrase (IN), Vpu, Vif, Vpr, Nef, Tat and Rev) [175, 177]. The viral genome consists of two identical single-stranded RNA molecules (9.2kb) within the viral particle and a pro-viral double stranded deoxyribonucleic acid (DNA) within the infected host cell [177] (Figure 1.7).

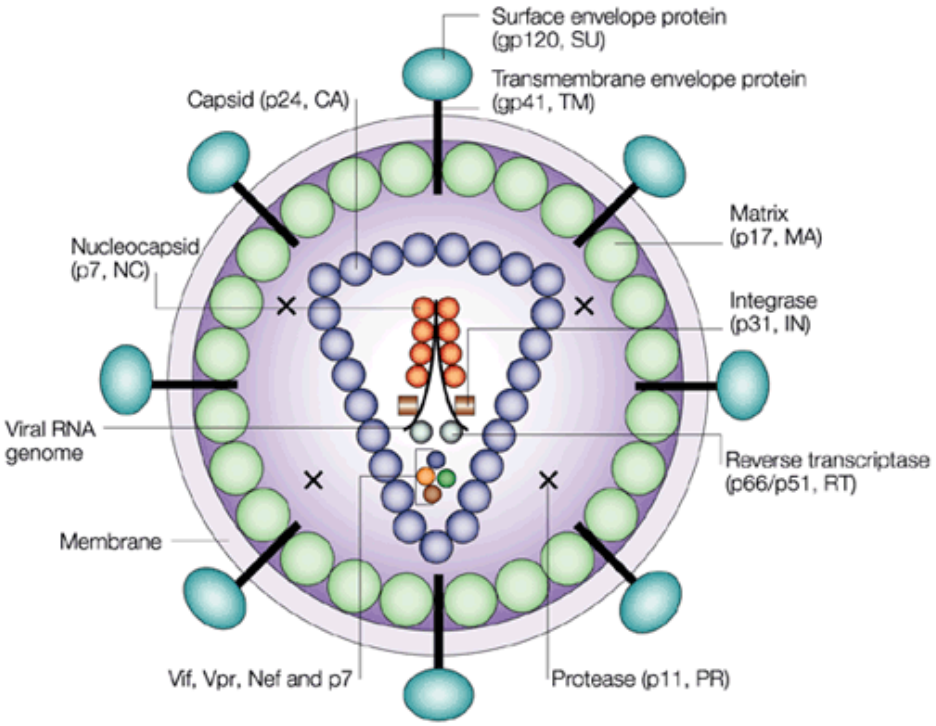

Figure 1.7: The structure of the mature human immunodeficiency virus-1 virion [178].

The initial step in the HIV-1 replication cycle (Figure 1.8) is host cell attachment and viral entry, for which HIV-1 uses a two-receptor model. The Env glycoprotein is a heterodimer of external gp120 covalently linked to the transmembrane domain, gp41, organised in triangular symmetry [179]. Env has an affinity for CD4 receptors and traffics HIV-1 to $CD4^+$ T-cells. The binding of the extracellular domain of gp120 to CD4 initiates a conformational change in gp120 allowing subsequent binding to a chemokine co-receptor; CCR5 or C-X-C motif receptor (CXCR) 4 [180]. The expression of these receptors on the surface of lymphocytes, monocytes, macrophages and DCs are the major determinants of tropism and target cell populations. CD4/co-receptor ligation triggers additional conformational changes in Env permitting gp41 to fuse with the host cell membrane Following fusion, the viral core is released into the cytoplasm of the newly infected cell to initiate replication [176, 177].

Uncoating of the viral capsid is facilitated by MA, Nef and Vif together with host-derived cellular factors. The single stranded viral RNA is then reverse transcribed by HIV-1 RT, a RNA-dependent DNA polymerase, into full length double stranded pro-viral DNA. The pro-viral DNA is transported in the pre-integration complex to the nuclear envelope and is actively transported through the nuclear pore under the guidance of Vpr [181]. Before integration, the pro-viral DNA is present in the host cell nucleus in three forms – linear, 1-long terminal repeat (LTR) or 2-LTR circles. Nef, Tat and Rev are produced in basal amounts by the activation of the LTR promoter region by host cellular factors, including nuclear factor –κB (NF-κB) [182]. In the host cell nucleus HIV-1 IN catalyses the integration of the viral genome into the host chromosomes [176].

Following integration, cellular RNA polymerase II transcribes pro-viral DNA. However, elongation of the viral transcript requires binding of HIV-1 Tat to the HIV-1 trans-activation response element (TAR) [183]. In the early stages of replication only multiply-spliced messenger ribonucleic acids (mRNAs) are generated together with the expression of Tat, Nef and Rev. Once sufficient levels of Rev are achieved, non- or singly-spliced mRNAs are produced. HIV-1 mRNAs are then transported to and translated by cytoplasmic polysomes to produce viral proteins and RNA [184].

Genomic *env* is translated into the precursor protein gp160, glycosylated within the endoplasmic reticulum, transported to and inserted into the host cell membrane. *Gag-pol* is translated to produce Gag polyprotein, and a ribosomal -1 frameshift during translation produces the Gag-Pol polyprotein. Gag is the source of structural proteins for the assembly of the mature virion while Gag-Pol contains the viral enzymes PR, RT and IN. At this point Gag and Gag-Pol polyproteins traffic to the plasma membrane for assembly and budding of new virions [177].

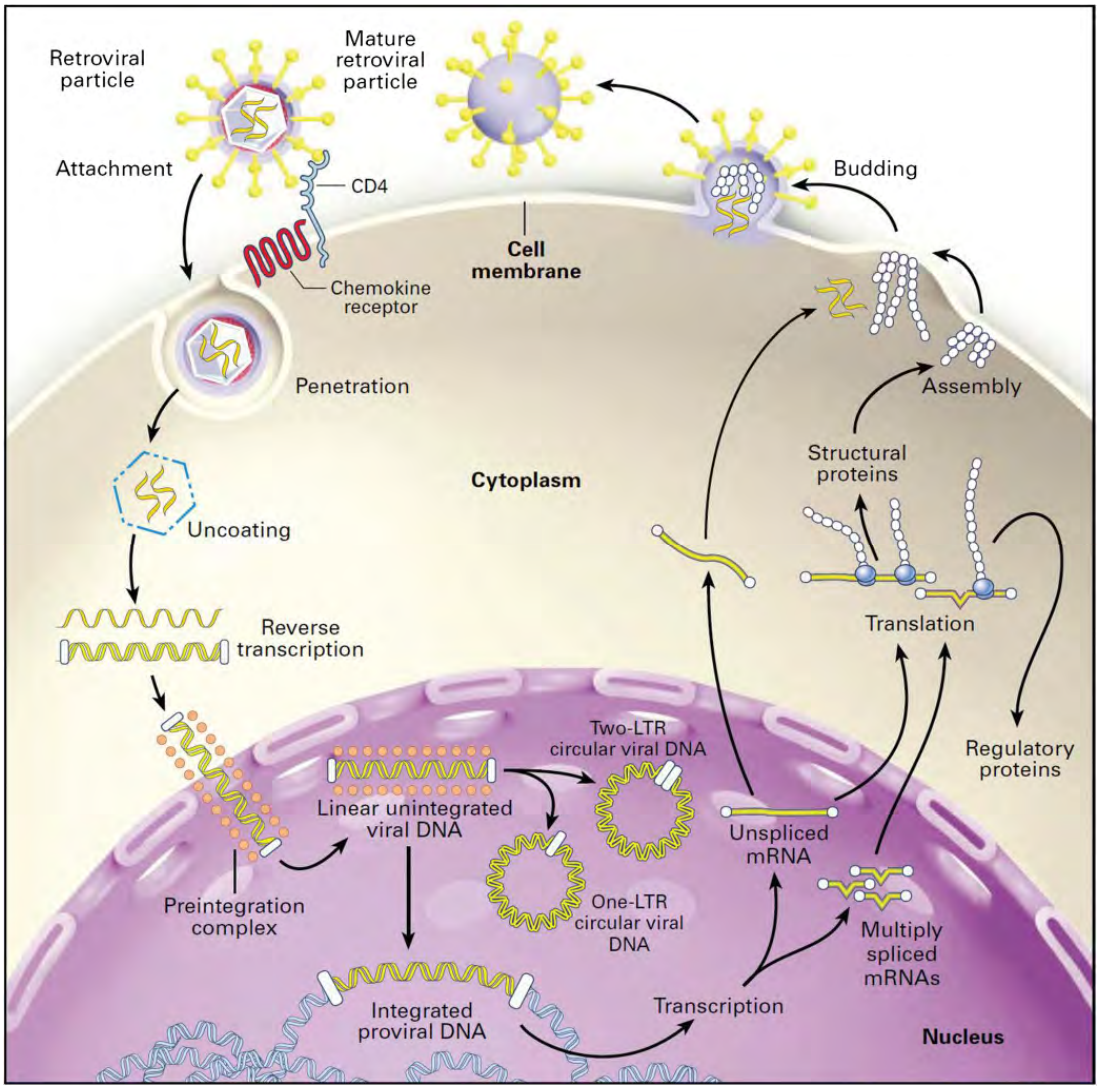

Figure 1.8: The life cycle of human immunodeficiency virus-1 [185].

In the process of replication HIV-1 assembles into two morphologically distinct immature and mature forms (Figure 1.9). HIV-1 is initially budded and released from the infected host cell as a non-infectious donut shaped immature virion. The immature virion is composed of a cellular derived bilayer lipid envelope, and an inner layer of radially extended Gag poly-proteins. Gag is the construction machine of HIV-1 which performs and integrates all of the activities required for HIV-1 assembly [173]. Following translation, the N-terminal MA domain targets Gag to the host cell plasma membrane and binds directly to the inner leaflet by insertion of myristoyl group into the lipid bilayer and by the basic patch which binds acidic phospholipids, notably phosphatidylinositol-(4,5)-biphosphate [186]. During viral assembly and budding Nef, Env and Vpu are involved in the downregulation of CD4 on the host cell membrane preventing interaction with newly formed gp120 [177].

During budding or immediately after release, HIV-1 reorganises the inner morphology of the immature virion through a process of maturation, RNA dimer stabilisation and acquisition of infectivity [173, 175, 187]. Mature HIV-1 structural proteins are derived from the Gag poly-protein which is composed of four domains (MA (matrix protein or p17), CA (capsid protein or p24), NC (nucleocapsid or p7) and p6) and two spacer peptides (SP1, SP2)) thus containing 5 proteolytic cleavage sites (Figure 1.9). Budding results in activation of viral PR which cleaves Gag at these sites to produce a new set of viral proteins. These proteins then reassemble to form the characteristic morphology a mature HIV-1 virion. The MA remains associated with the inner viral membrane to form the matrix layer, NC coats the stabilises the dimeric viral RNA and condenses at the centre of the core to form the nucleocapsid, and approximately 1500 copies of CA assemble by their N-terminals into hexameric rings to form the core that surrounds the RNP complex [175, 177].

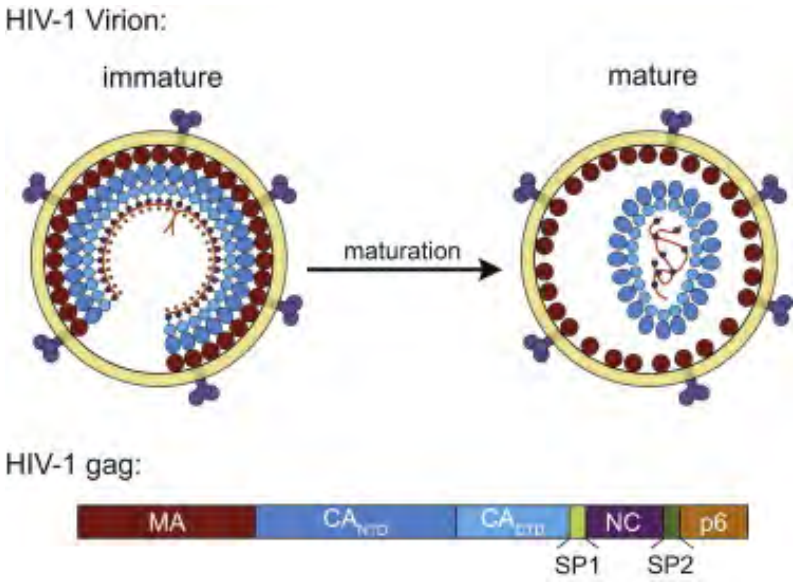

Figure 1.9: The immature and mature human immunodeficiency virus-1 virions and domains of the Gag polyprotein [188].

1.2.5 *Transmission*

HIV-1 is transmitted in certain bodily fluids (blood, pre-seminal fluid and semen, rectal fluids, vaginal fluids and breast milk) when the source comes into contact with a mucous membrane, damaged tissue or direct contact with the blood stream. The routes of exposure include first and foremost sexual, vertical mother-to-child (during pregnancy, delivery and breastfeeding) and intravenous transmission (shared needles such as in intravenous drug use, needle stick injuries, infected blood transfusion). Generally HIV-1 transmission rates are low ranging from 0.001 to 10% per exposure [189, 190]. Viral load is the most significant factor increasing the risk of transmission, with a 2.4 fold greater risk for every $\log_{10}$ increase [191].

1.2.6 *The course of human immunodeficiency virus-1 infection*

The pathogenesis of HIV-1 can be divided into four stages; an eclipse phase, acute infection, asymptomatic chronic infection and AIDS (Figure 1.10).

Infection begins with the transmission of a single founder virus. During the eclipse phase (~1-2 weeks), HIV-1 initiates focal replication in proximal $CD4^+$ T-cells moving to proximal lymphoid organs and then establishing systemic infection through extracellular diffusion, DC transport or T-cell migration. Viremia is undetectable without presentation of an immune response or clinical symptoms [190, 192].

This is followed by acute infection (~2-4 weeks) during which there is a spike in viral replication with plasma viral loads reaching up to 10^8 copies/ml, and consequently the highest risk of transmission during the course of infection [193]. This period is characterised by high numbers of infected cells in the blood and lymph nodes, a transient decrease in peripheral $CD4^+$ T-cells, a significant increase in markers of immune activation as well as marked induction of inflammatory cytokines and chemokines. Killing of productively infected cells is mediated by HIV-specific $CD8^+$ T-cells (cytolysis and Fas mediated apoptosis) in response to HIV-1 antigens expressed on infected cells in association with MHC-I [10, 192]. The advantage of the two step replication model employed by Tat and Rev is the postponement of viral protein synthesis to minimize exposure to $CD8^+$ T-cells and maximise viral production per cell [192]. At approximately three months there is production of HIV neutralising antibodies mediating phagocytosis of infected cells. The innate immune response is also essential for viral control during this time and is largely meditated by NK cells. Both the adaptive and innate immune responses select for the emergence of mutations in key viral epitopes leading to immune escape [10, 192].

The control of replication and exhaustion of activated target cells results in a temporal sharp decrease in plasma viral load by 100-fold or more to a set-point [10, 11, 192]. This value varies from below detectable limits of <40 copies/ml in elite controllers (patients who naturally control viral replication similar to the levels of patients on ARVs) to >10,0000 copies/ml [11, 194]. Clinically, in approximately half of infected individuals, this period can be characterised by flu-like symptoms termed acute retroviral syndrome [10, 192].

This is followed by asymptomatic chronic infection or clinical latency (~1-20 years) where HIV-1 exists in a quasi-steady state with the host. This period is represented by a constant or gradually rising level of viremia with concurrent progressive exhaustion of HIV-specific T-cells; characterised by increased expression of programmed death 1 (PD-1) and loss of effector function [192]. Without treatment, HIV generally progresses to AIDS within 7-10 years. As the final stage, AIDS is diagnosed when a patient has a $CD4^+$ T-cell count below 200 cells/μl and is characterised by opportunistic infections (predominately TB) and viral-related malignancies (non-Hodgkin's lymphoma and Kaposi's sarcoma). There is loss of control over viral replication and viremia increases with a mortality of over 95% if untreated [10, 11, 192].

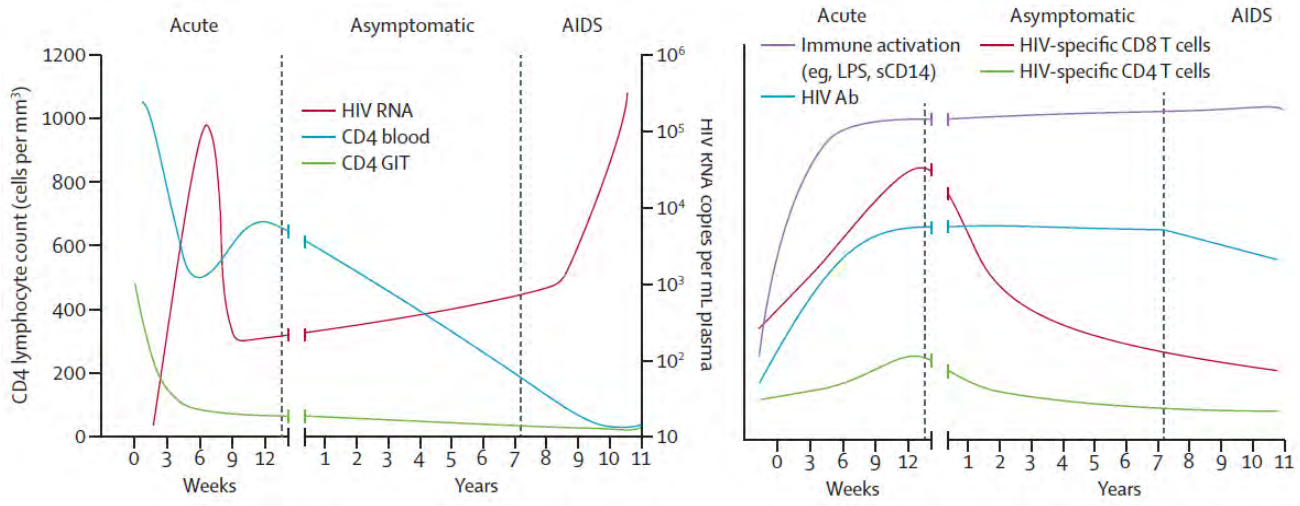

Figure 1.10 The natural history of untreated human immunodeficiency virus-1 infection [10].

1.2.7 *$CD4^+$ T-cell depletion and immune dysfunction*

The initiating HIV-1 founder virus has distinct phenotypic characteristics including CCR5 tropism and the requirement of high surface expression levels of CD4. This lends $CD4^+CCR5^+$ central and effector memory T-cells as the preferred target population. As the infection progresses the HIV-1 Env protein undergoes mutational and conformational evolution allowing HIV-1 to enter cells using CXCR4 and lower expression levels of CD4 representing the extension of infection into new cell types [195].

A characteristic hallmark of HIV infection is the progressive reduction in circulating $CD4^+$ T-cells by a combinatorial effect of reduced capacity to generate and support T-cell function and increased destruction [11, 190].

$CD4^+$ T-cells are eradicated by direct cytopathic infection, HIV-specific $CD8^+$ T-cell and antibody mediated killing of infected cells as well as bystander effects including syncytia formation, chronic immune activation and subsequent senescence [10, 190, 192]. Several viral proteins have been attributed apoptotic effects; host cell surface expression of Env mediates syncytium formation [190] and a proteolytic fragment of caspase 8, Casp8p41 produced by viral proteases induces apoptosis through the Bcl-2 associated X protein (Bax)/Bcl-2 homologous antagonist/killer (Bak) and caspase 9 mitochondrial pathway [196].

Concurrently, HIV-1 replication causes the activation of APCs through ligation of innate immune receptors and the consequent production of pro-inflammatory cytokines and the recruitment and accumulation of HIV-specific effector cells. This pro-inflammatory environment promotes central memory T-cell proliferation increasing the HIV target cell population [11]. Finally, chronic inflammation results in collagen deposition

and progressive fibrotic damage to the fibroblastic reticular cell network, the predominant source of T-cell survival factor IL-7 further accentuating $CD4^+$ T-cell destruction [197].

In contrast to the progressive loss of circulating $CD4^+$ T-cells, the most notable effect on T-cell homeostasis occurs in the gut associated lymphoid tissue, the largest lymphoid organ in the body containing approximately 40% of all lymphocytes. Here, HIV results in the rapid and massive elimination of resting and activated $CCR5^+CD4^+$ T-cells early after infection that is not restored following ARV therapy [11, 198] (Figure 1.10). Further to the loss of total $CD4^+$ T-cells, there is preferential depletion of Th17 cells essential in the immune defence to bacteria and critical in maintenance of the mucosal barrier [199-201]. In the gastrointestinal tract (GIT), T-cell depletion is accompanied by enterocyte apoptosis, breakdown of the intestinal epithelial barrier and enhanced barrier permeability resulting in the increased translocation of microbial products from the GIT into the systemic circulation contributing to systemic immune activation [202].

1.2.8 *Chronic immune activation*

A key correlate of HIV-1 progression is the chronic activation of the innate and adaptive immune systems with the expression of activation (CD38/HLA-DR) [203], senescence (CD57) [204] and exhaustion (PD-1) markers [205], hyperglobulinemia, increased circulating pro-inflammatory cytokines and activation of IFN responsive genes [206]. Several mechanisms have been suggested to drive this biological phenomenon including: (1) Direct actions of HIV as a ligand for TLR-7 and TLR-8 on plasmacytoid DCs and consequent IFN-α production [207]. (2) Activation of other innate receptors through capsid protein [208] or viral DNA [209]. (3) Microbial translocation across the GIT into the systemic circulation – lipopolysaccharide is a potent TLR-4 stimulator resulting in the production of pro-inflammatory cytokines IL-6 and TNF-α [202]. (4) Co-infection with viruses that expand activated T-cells [210]. (5) A reduced ratio of pro-inflammatory Th17 to anti-inflammatory Tregs [200], as well as (6) homeostatic mechanisms driving proliferation to restore depleted lymphocyte populations [11].

1.2.9 *Antiretroviral therapy*

Antiretroviral treatment suppresses viral replication and facilitates reconstitution of the immune system. Expanding access to ARVs has significantly changed the epidemiology of HIV/AIDS; raising prevalence due to the increase in life span but simultaneously decreasing incidence due to the reduced risk of transmission [169]. These drugs are grouped into 6 classes based on the mechanism of action and resistance profiles – nucleoside-analog reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors, fusion inhibitors, integrase inhibitors and chemokine co-receptor agonists (Figure 1.11). Triple drug therapy, termed highly active antiretroviral treatment (HAART) is the most effective treatment approach [10, 176].

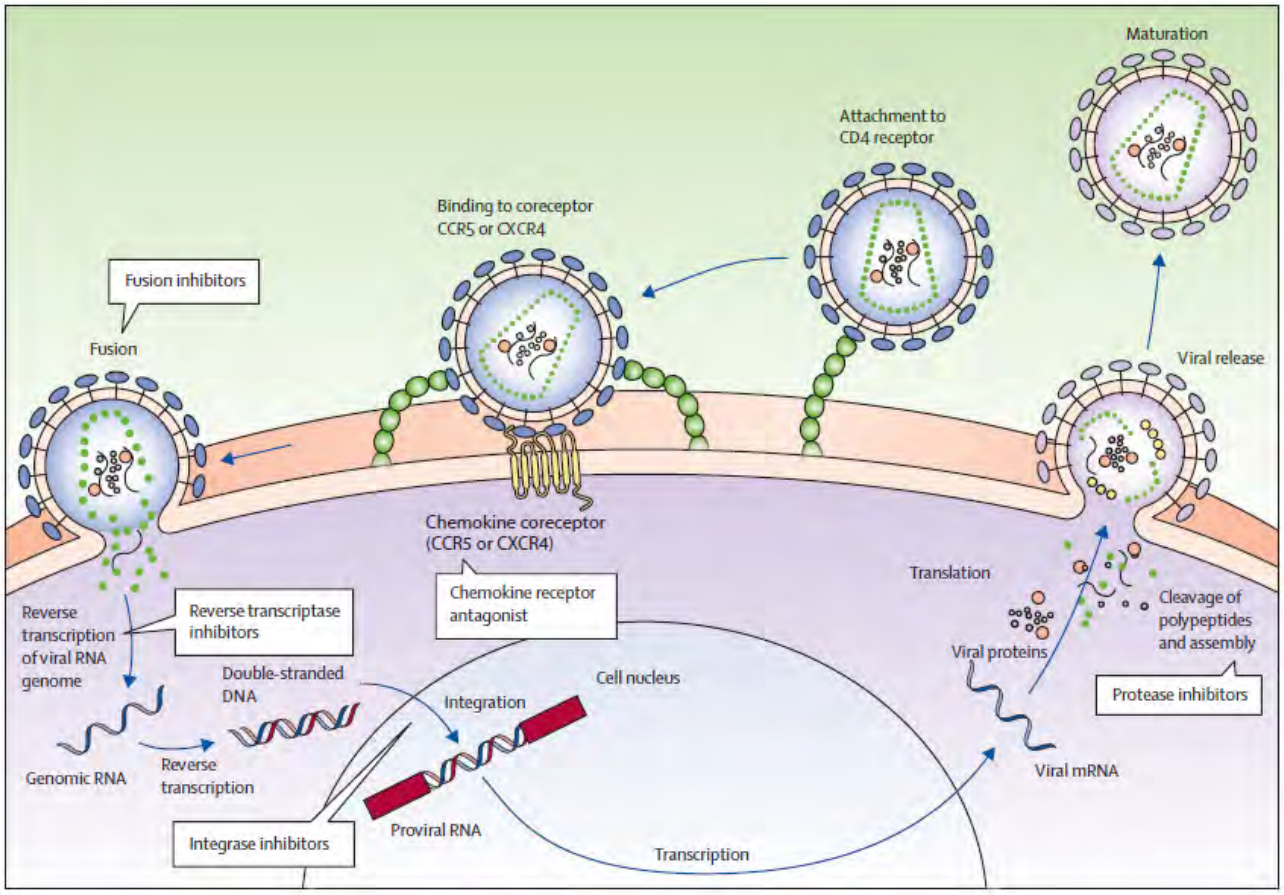

Figure 1.11: The human immunodeficiency virus-1 life cycle illustrating the mechanisms of action of different classes of antiretroviral drugs [10].

The South African National Consolidated Guidelines recommend HAART initiation when the patient's $CD4^+$ T-cell count is below 500cells/µl, WHO clinical stage 3 or 4, pregnant, breastfeeding, or the co-presence of Hepatitis B or active TB. A standard first line combined ARV regimen for adults is composed of two NRTIs (TDF/Tenofovir and 3TC/Lamivudine or FTC/Emtricitabine), as well as a NNRTI (EFV/Efavirenz) provided in a fixed dose combination. This regime is also recommended in HIV/TB co-infection [211]. Following the initiation of ARVs, plasma viral load typically decreases to concentrations below the minimum detection limit of many commercial assays within three months, with a variable restoration of $CD4^+$ T-cell counts [10].

The ability of HIV to mutate and replicate in the presence of ARVs is termed HIV drug resistance (HIVDR). The there is a steady increase in the prevalence of HIVDR in low- and middle-income countries such as SA. The consumption of ARVs despite continued viral replication results in the selection of HIV sub-populations with HIVDR mutations. This is largely a consequence of sub-optimal treatment regimens, poor adherence

and the ability of the drug to select for resistant mutations. This results in the transmission of HIVDR strains, treatment failure and requirement of more expensive second and third line HAART regimes [10, 212].

Despite their success in controlling HIV replication, HAART is unable to eradicate HIV pre-empting lifelong treatment. HIV can survive HAART due to latent infection of long lived memory T-cells, integration of HIV DNA into the host genome without viral replication [192, 213], and anatomical reservoirs poorly accessible to HAART such as the GIT, lymphoid tissue and central nervous system [214, 215].

1.3 Tuberculosis and human immunodeficiency virus co-infection

1.3.1 *Overview of tuberculosis and human immunodeficiency virus co-infection*

Infection with HIV is the most significant risk factor in the progression of TB disease; an HIV positive individual has a 26-31 times greater risk of developing active TB infection. In turn, TB expedites the pathogenesis of HIV, with TB being the most common presenting illness and the leading cause of mortality in this group [1, 169].

1.3.2 *Epidemiology of tuberculosis and human immunodeficiency virus co-infection*

According to WHO, in 2013 at least one third of HIV infected individuals were co-infected with MTB globally. During this time, 1.1 million new TB cases were reported in HIV positive individuals and 1 in 4 HIV-related deaths were attributed to TB. In this same period, 62% of South African TB patients were noted to be HIV positive [1]. Furthermore, SA had the second highest number of TB-related deaths among HIV individuals worldwide; approximately 175 deaths per day [2] (Figure 1.12).

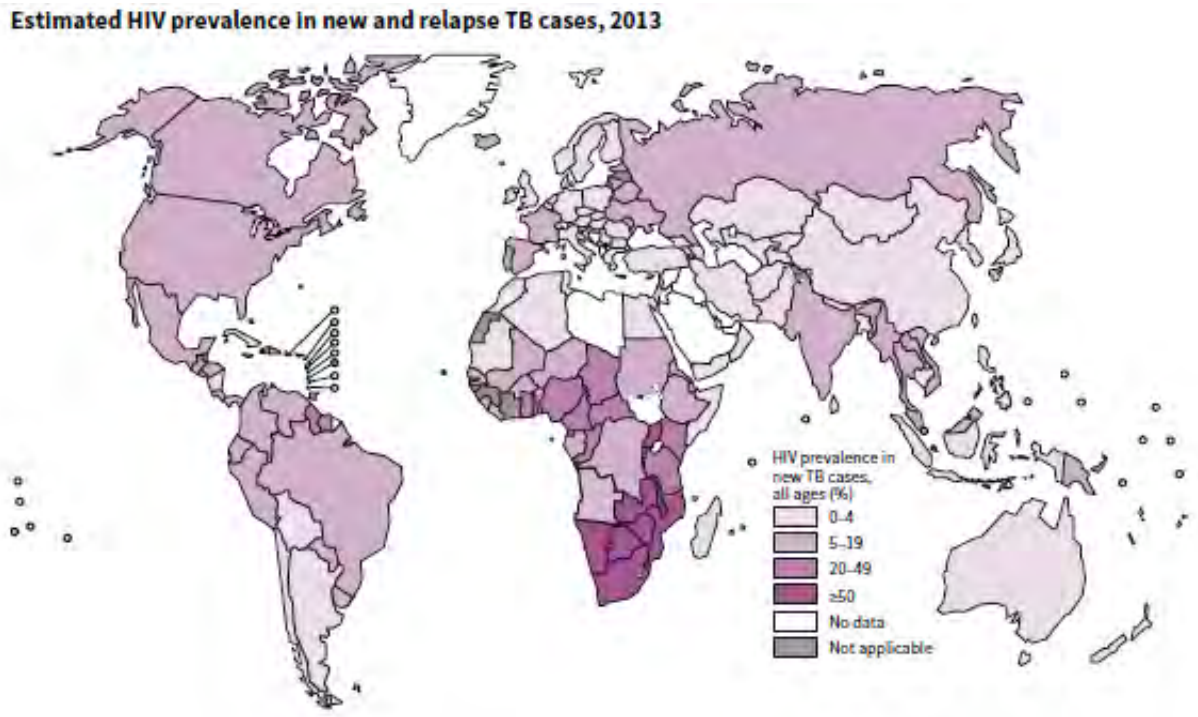

Figure 1.12: Estimated tuberculosis and human immunodeficiency virus co-infection prevalence (2013) depicting the high burden of co-infection in South Africa [1].

1.3.3 *$CD4^+$ T-cell responses in tuberculosis and human immunodeficiency virus co-infection*

Recent studies have begun to elucidate the effect of TB/HIV co-infection on T-cell responses. MTB specific $CD4^+$ T-cells are rapidly depleted from the periphery following HIV infection, partly due to preferential HIV targeting of these cells [12]. In addition, HIV infected patients with latent TB infection show a loss of peripheral and broncho-alveolar lavage (BAL) fluid mono-, bi- [216, 217] and polyfunctional MTB specific $CD4^+$ T-cell responses [216-219]. IL-2 producing MTB-specific $CD4^+$ T-cells correlate inversely to viral load [218], and this is not corrected with ARV treatment [220]. In a macaque model, the peripheral depletion of $CD4^+$ T-cells has been linked to TB reactivation [221].

Both active and latent TB have been associated with lower HIV-specific T-cell functionality in co-infected compared to mono-infected patients. A stronger effect is noted in active infection indicating that MTB load may amplify HIV mediated T-cell dysregulation accounting for expedited HIV progression [13, 222]. Several studies have observed that active TB enhances CCR5 expression and/or the frequency of $CCR5^+CD4^+$ T-cells during HIV co-infection [14, 15], this suggests that TB mediated chronic immune activation leads to a greater pool of $CCR5^+CD4^+$ T-cells that are highly susceptible to HIV infection [223].

Furthermore, it has been shown that a strong $CD4^+$ T-cell response to MTB may simultaneously result in enhanced HIV replication [224], dependent on the MTB strain: Less virulent strains drive potent pro-inflammatory cytokine production and heightened viral replication. More virulent strains, which produce a less immunogenic response, promote lower levels of HIV replication [225]. These findings indicate that MTB antigen specific responses enhance HIV viral replication in line with the higher viral loads observed in co-infected patients compared to HIV non-infected patients who express similar levels of immunosuppression [14, 226].

1.4 T-regulatory lymphocytes

1.4.1 *Overview of T-regulatory lymphocytes*

T-regulatory lymphocytes are a suppressive subset of $CD4^+$ T-lymphocytes (5-10%) characterised by the expression of lineage specific transcription factor forkhead box P3 (Foxp3) [17]. Tregs regulate effector lymphocyte function to maintain immunological tolerance and suppress pro-inflammatory pathways [227, 228]. In the context of TB/HIV co-infection, an imbalance in Treg proportion can either lead to uncontrolled inflammation and consequent immunopathology or down regulation of antigen specific $CD4^+$ T-cells, ultimately resulting in pathogen persistence and establishment of chronic infection [229].

1.4.2 *T-regulatory lymphocyte polarisation*

Under physiological conditions the vast majority of Tregs are generated in the thymus (tTregs), responsible for the maintenance of immune homeostasis and peripheral tolerance. Upon pathological challenge the tTreg T-cell receptor (TCR) repertoire is complemented by peripheral (p)Tregs derived from naïve $CD4^+$ conventional T-cells (Tconv) [229-231]. Together with epigenetic changes, Tregs are polarised by the combination of several transcriptional signals including TCR/CD28 co-stimulation, transforming growth factor-β (TGF-β)/IL-2 cytokine signaling and an autocrine Foxp3 dependent feedback loop. These signals culminate in the activation of a host of transcription factors binding to the *Foxp3* promoter as well as three highly conserved non-coding sequence regions (CNS1-3) in the genomic Foxp3 region [230] (Figure 1.13).

With regard to cytokine signalling, Treg polarisation is mediated by exposure to low-dose antigen in the presence of TGF-β and IL-2 [230, 232]. TGF-β receptor ligation results in SMAD3 binding to CNS1, a prerequisite for Foxp3 induction in $CD4^+$ Tconvs [233]. This is reinforced by IL-2; Tregs constitutively express CD25 (IL-2 receptor (R) α) [228], ligation of which results in activation of Janus activated kinase (Jak)1/3 and subsequent phosphorylation and activation of signal transducer and activator of transcription (STAT) 5 [234]. In turn, STAT5 binds to the promoter region and CNS2 in the *Foxp3* gene inducing Foxp3 expression and Treg lineage commitment [230, 235] (Figure 1.13).

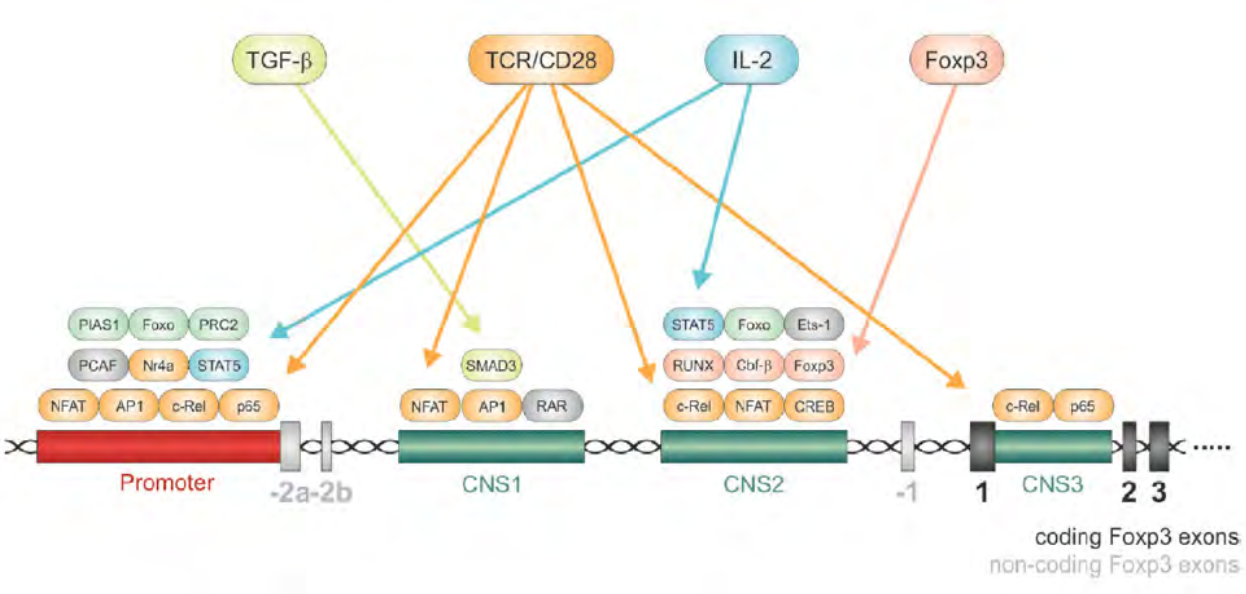

Figure 1.13: Transcriptional regulation of forkhead box P3 expression [230].

Forkhead box P3, a transcription factor of the winged helix family, is indispensable for Treg lineage specification and suppressive function by acting at transcriptional, epigenetic and post transcriptional levels [17, 236]. It maintains the expression of CD25 at the cell surface by binding the *CD25* promoter both directly [237] and indirectly [238, 239]. In this way, IL-2 creates a positive feedback loop for CD25 expression through STAT5 and Foxp3 [240]. Regulatory T-cells do not produce IL-2 [241] but are reliant on IL-2 produced by $CD4^+$ Tconvs [242]. This dependence sets up a negative feedback loop whereby the proliferation of Tregs is directly controlled by the degree of Tconv activation [243].

1.4.3 *Mechanisms of T-regulatory lymphocyte function*

T-regulatory lymphocytes can be divided into subpopulations with disparate suppressive functions and heterogeneous chemokine receptors that promote their differential tissue homing and localisation [244, 245]. Through a broad array of contact dependent and independent mechanisms, Tregs can inhibit the generation and function of effector cells at any point in the inflammatory process [243] including initial activation [246], migration [247] or at the target site [248].

These mechanisms include: (1) The production of inhibitory cytokines (TGF-β, IL-10, IL-35) which suppress T-cell activation and proliferation. (2) The deprivation of cytokines of the common receptor γ-chain family from effector T-cells (particularly IL-2 via expression of CD25) which induces pro-apoptotic protein expression and cell death. (3) The direct killing of DCs and effector cells by granzyme A/B and perforin cytolysis or the TNF-related apoptosis inducing ligand death receptor 5 (TRAIL-DR5) pathway. (4)

Suppression of DC maturation via lymphocyte activation gene 3 (LAG3) and MHC II interactions. (5) Blocking of co-stimulatory molecule expression (CD80/CD86) on DCs via cytotoxic T-lymphocyte antigen 4 (CTLA4), thereby weakening the APC-T-cell interaction. (6) The expression of ectonucleosidases CD39/CD73 which mediate enzymatic hydrolysis of extracellular adenosine triphosphate to inhibitory adenosine thereby disrupting the metabolic state of effector cells via 3', 5'- cyclic adenosine monophosphate. (7) The deprivation of tryptophan from effector T-cells through indoleamine 2,3-dioxygenase induction in DCs, inhibiting T-cell proliferation [243, 249-251] (Figure 1.14). The mechanism of action employed is most likely linked to the cellular target which includes DCs, NK cells, T- and B-lymphocytes [243].

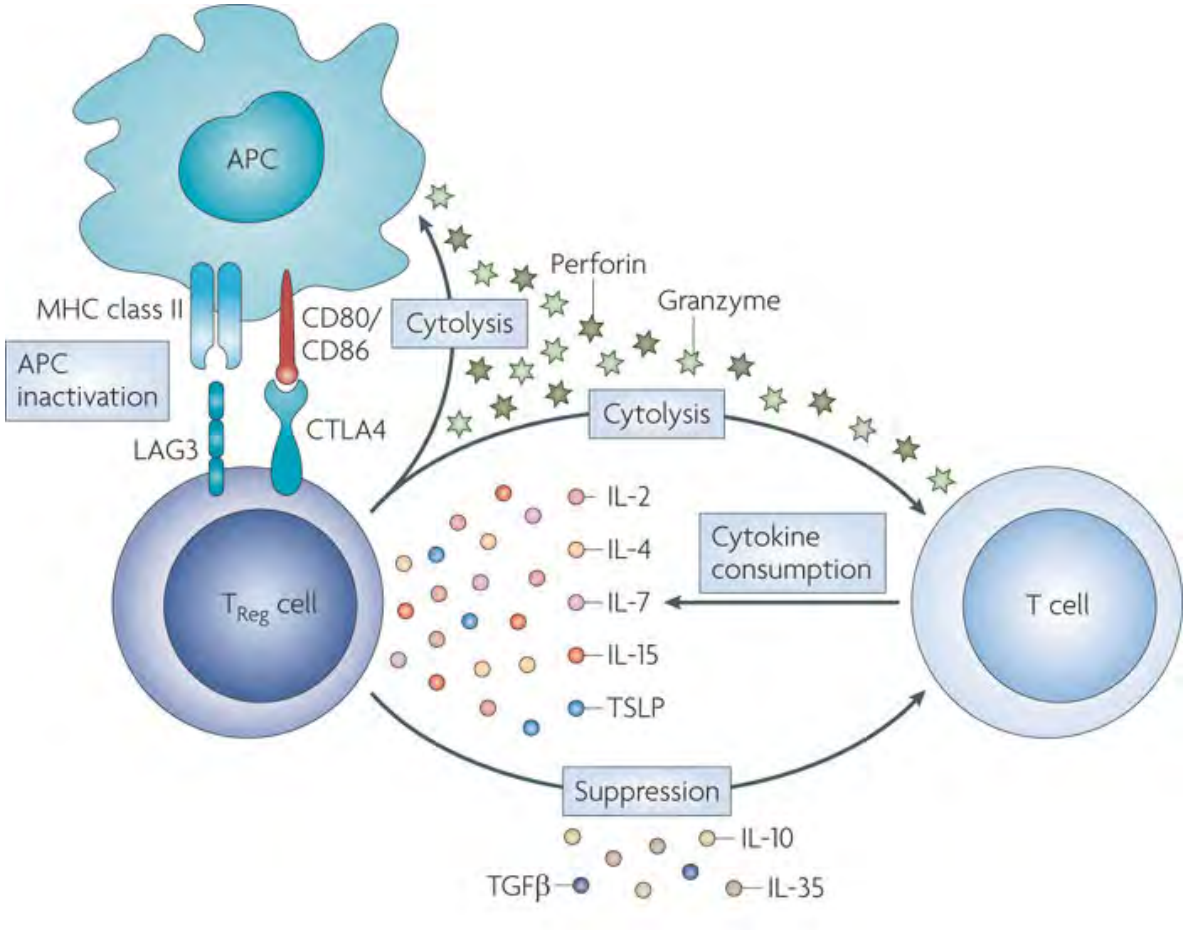

Figure 1.14: Immunosuppressive mechanisms used by T-regulatory lymphocytes [252].

1.4.4 *T-regulatory lymphocytes in tuberculosis*

The reported proportion of Tregs in active TB infection is conflicting. Previous studies have noted a reduced [253] or unchanged [254, 255] frequency of Tregs in the peripheral blood of untreated active TB patients compared to healthy controls. Others have identified Treg expansion at both the disease site and in the peripheral blood in an MTB antigen-specific manner, with a correlation to disease severity and an inverse

correlation to MTB specific immune responses [256-261]. Notably, there is no evidence on the frequency of Tregs in TB/HIV co-infection.

T-regulatory lymphocytes have been shown to down regulate the immune response to MTB preventing tissue injury by resolving granulomatous inflammation. However, impairment of antigen specific T-cell responses simultaneously prevents bacterial clearance and reduces inflammation at the infection site paving the way for chronic infection. More specifically, Treg mediated suppression results in impaired macrophage activation and killing of intracellular bacteria, reduced antigen presenting capacity and pro-inflammatory cytokine production, including MTB specific IFN-γ production by Th1 cells, as well as reduced MTB specific $CD8^+$ and $CD4^+$ T-cell activation, expansion and recruitment to the infection site [223, 255, 258-260, 262-264].

In addition to limiting inflammation, Tregs have also been implicated in the reactivation of latent infection and dissemination of bacilli. During active TB infection Tregs have a higher capacity to inhibit Th1 than Th17 responses. It has been suggested that this differential inhibition of Th1/IFN-γ mediated suppression of bacterial replication while permitting Th17/IL-17 mediated recruitment of pro-inflammatory cells to the site of infection results in chronic inflammatory caseating tubercles and the transmission of bacilli. This indicates that Tregs exert a selective suppression on specific components of the adaptive immune response to MTB [258].

1.4.5 *T-regulatory lymphocytes in human immunodeficiency virus infection*

The majority of HIV studies have reported lower absolute cell counts but increased relative frequency of lymph node [265], mucosa [266] and peripheral [267-271] Tregs compared to healthy controls, resulting in a greater suppressor to effector ratio [272]. Interestingly, HIV controllers and long term non-progressors display tissue and peripheral Treg frequencies similar to or lower than healthy controls [266, 270, 271], indicating that the selective expansion of Tregs is non-protective [273].

Regulatory T-cell functionality is maintained during untreated chronic HIV infection with anti-proliferative capacity similar to that of HIV controllers [274] and significant down regulation of HIV specific $CD4^+$ T-cell immune responses, particularly the inhibition of cytokine production [229, 275-277]. Furthermore, HIV-1 binding to the Treg CD4 enhances Treg survival and suppressive activity particularly the ability to inhibit $CD4^+$ T-cell proliferation and IL-2 production [277]. This is potentially beneficial; suppressing chronic T-cell activation reducing the pool of susceptible $CD4^+$ T-cells [278]. However, this would also inhibit HIV-specific T-cell responses contributing to immune deficiency, heightened virulence and an overall poor prognosis [273, 279].

1.5 T-helper 17 lymphocytes

1.5.1 *Overview of T-helper 17 lymphocytes*

T-helper 17 cells are a pro-inflammatory subset of $CD4^{+}$ effector T-cells [280] characterised by the production of IL-17A/F, IL-21 and IL-22, surface expression of CCR6 and lineage specific transcription factor RAR-related orphan receptor C (RORC) [16]. Under immune homeostasis, Th17 cells predominantly reside at the mucosal surfaces where they play a pivotal role in inflammatory reactions and protective immunity against intra-[281, 282] and extracellular [283, 284] bacterial and fungal infections.

1.5.2 *T-helper 17 lymphocyte polarisation*

Classically, Th17 cells are generated from naïve $CD4^{+}$ Tconvs under TGF-β/IL-21 [285] or TGF-β/IL-6 and IL-21 or IL-23 [286] conditions (Figure 1.15). This leads to Th17 lineage commitment via the activation of STAT3 and downstream transcription factor RORC, which in turn binds to the promoter region of *IL17A/F* [287-290].

Further to this, IL-1β and IL-6 are essential in the expansion and amplification of differentiated and memory Th17 cells and IL-23 is essential for the stabilisation and maintenance of the Th17 phenotype and effector functions [291]. Naive $CD4^{+}$ Tconvs do not express IL-1R and IL-23R; these receptors are induced after exposure to TGF-β and IL-6/IL-21 [285, 292, 293]. Subsequently, IL-1β acts synergistically with IL-6 and IL-23 towards Th17 polarisation by regulating the expression of interferon regulatory factor 4 which then promotes the expression of RORC [289, 294, 295] (Figure 1.15).

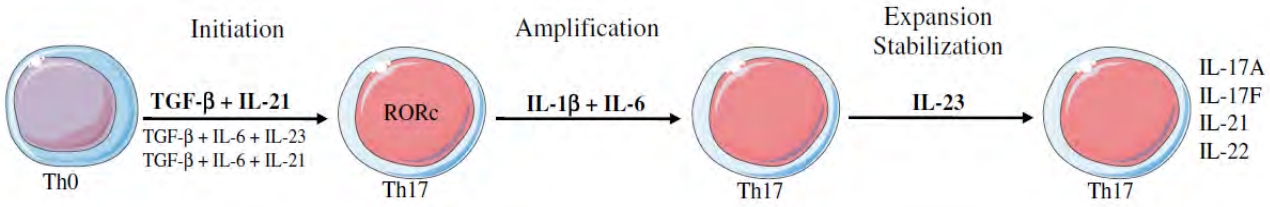

Figure 1.15: Representation of T-helper 17 lymphocyte differentiation [296].

In addition to the key regulators discussed above, other transcription factors such as Baft [297], IκBζ [298] and Runx1 [299] are essential for enhancing Th17 differentiation and production of effector molecules [300].

1.5.3 *Functions of T-helper 17 effector cytokines*

T-helper 17 cells characteristically produce IL-17A/F, IL21 and IL-22. Th17 cells also produce CCL20, granulocyte-macrophage-colony stimulating factor (GM-CSF), IL-8, IL-26 and IL-10, although these are not Th17 specific [300].

1.5.3.1 Interleukin-17A and F

IL-17A is the prototypic member of the IL-17 family consisting of 6 cytokines; IL-17A-F. IL-17A and IL-17F share 44% homology and can form homodimers or heterodimers. They are presumably expressed by induction of the same gene locus and are commonly co-expressed at the level of the single cell [301]. Both IL-17A and F bind to the receptor heterodimer; IL-17RA/IL-17RC [302, 303]. Ligation of this receptor recruits adaptor protein ACT1 which in turn stimulates TNF receptor-associated factor 6 (TRAF6) to activate the canonical NF-κB, and to a lesser extent the mitogen activated protein kinase (MAPK) pathways in target cells [301, 304, 305]. This results in pro-inflammatory gene expression by transcriptional activation [306] and target mRNA stabilisation [307].

IL-17A and F are pivotal in the recruitment, activation and migration of neutrophils and $CCR5^+$ lymphocytes to the site of inflammation [308]. In addition, these cytokines target non-immune cells including epithelial and endothelial cells, fibroblasts and airway smooth muscle cells to produce pro-inflammatory mediators such as antimicrobial molecules (CCL20 and defensin-β4), adhesion molecules, cytokines (IL-6, IL-8, IL-26, TNF-α, IL-1β, granulocyte-CSF, GM-CSF, chemokines (CXCL1, CXCL2, CXCL8, CCL2, CCL7, CCL20, CCL28) as well as PGE2, nitric oxide and MMPs [280, 300, 301, 309, 310] (Figure 1.16).

CCL20 mediates Th17 tissue homing to the site of inflammation via its cognate receptor CCR6, highly expressed on Th17 cells. The autocrine production of CCL20 allows Th17 cells to autonomously regulate their recruitment to inflamed tissues and sustain the Th7 mediated inflammatory cascade [295, 311].

1.5.3.2 Interleukin-21

IL-21, of the IL-2 cytokine family, mediates its actions through a heterodimer of IL-21R and the common cytokine receptor γ chain found on B- and T-lymphocytes as well as other cells of the myeloid lineage. Ligation of the IL-21R complex activates the Jak3/STAT3 pathway resulting in Th17 differentiation [301]. In addition, IL-21 causes proliferation and differentiation of $CD8^+$ T-cells [312] and B-lymphocytes [313], immunoglobulin class switching [314], differentiation and cytotoxic activities of NK cells [315] and IL-8 production by DCs and macrophages [300] (Figure 1.16).

1.5.3.3 Interleukin-22

IL-22, of the IL-10 cytokine family, is produced in response to IL-23/STAT3 signalling [316]. The IL-22R is composed of IL-22R1 and IL-10R2 chains expressed on epithelial cells, keratinocytes and fibroblasts, but not immune cells. Ligation of this receptor activates STAT3 and MAPK pathways resulting in the expression of genes and proteins involved in tissue remodelling and wound healing, epidermal hyperplasia and epithelial barrier function, inflammation, chemotaxis, acute phase reactants and antimicrobial substances, which in turn enhance the inflammatory properties of TNF-α, IL-1β and IL-17A/F [301, 316, 317]. In chronic inflammation, IL-22 exerts either cytoprotective or pro-inflammatory effects depending on the tissue location and cytokine microenvironment [318, 319] (Figure 1.16).

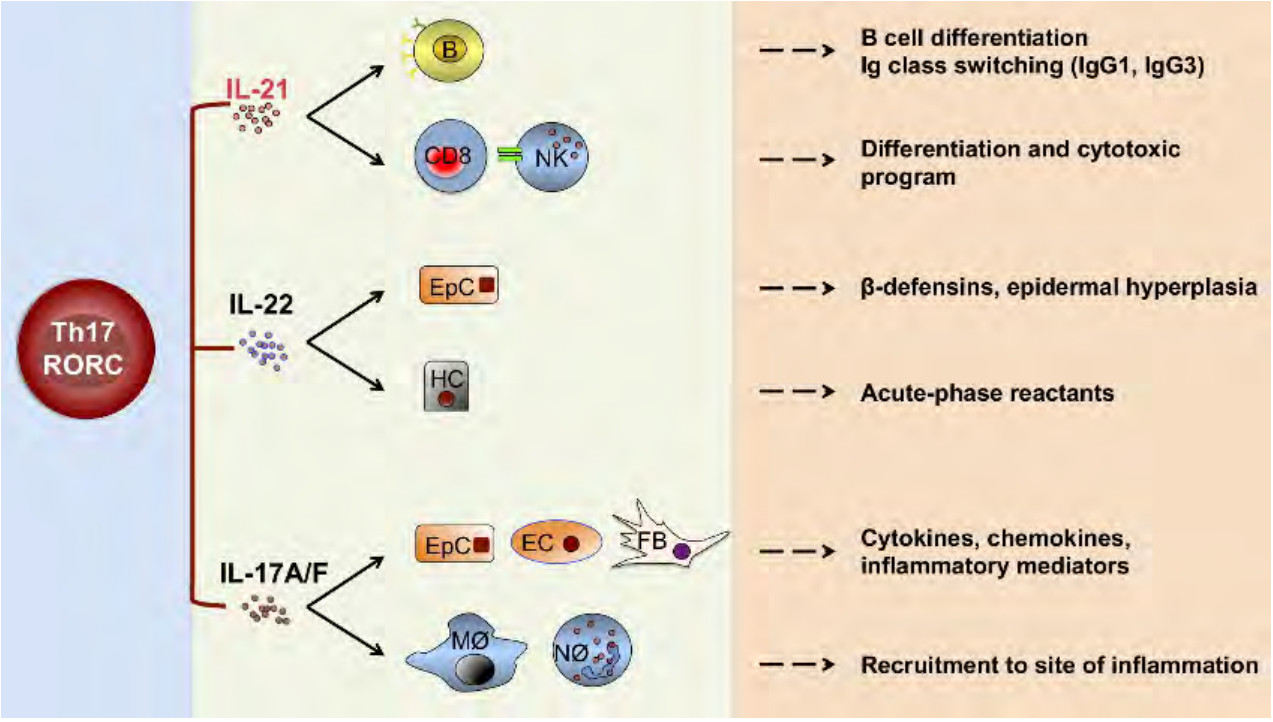

Figure 1.16: The functions of T-helper 17 effector cytokines. Adapted from Maddur *et al* [300].

1.5.4 *T-helper 17 lymphocytes in tuberculosis*

A distinct subset of MTB specific pro-inflammatory Th17 memory T-cells have been identified [320]; however, their frequency and contribution to the immune response is conflicting. Several studies have noted an expansion of Th17 cells and elevated levels of IL-17A in the peripheral blood [253, 321, 322] and pleural fluid [253, 322, 323] during MTB infection with correlation to disease severity [322]. Others have noted a higher frequency of Th17 cells in peripheral blood [320, 324-329] and BAL fluid [320, 328] of latently infected or healthy donors. This suggests that the Th17/IL-17A axis may be associated with either pathology

and/or protection [308]. Furthermore, other studies have found no significant difference in the systemic Th17 count between active, latent and non-infected patients [330, 331]. Notably, there is no evidence on the frequency and function of Th17 cells in TB/HIV co-infection.

Th17/IL-17 axis is suggested to provide protective immunity against MTB by contributing to granuloma formation and maintenance [332-335]. Early expression of pulmonary IL-17 in TB infection is pivotal in granulomatous cellular recruitment by inducing chemokine secretion from non-haematopoietic cells. This predominantly results in neutrophil and $CCR5^+$ lymphocyte recruitment, through the induction of IL-8 and CXCL13 respectively, to form lung lymphoid follicles for optimal macrophage activation and bacterial clearance [308, 336-340]. However, there is a disadvantage to this protective granulomatous strategy. During the chronic stages of infection, Th17 hyperactivity can lead to immunopathology via IL-17-CXCL mediated influx of immune cells and extensive tissue destruction [332, 339, 341-343].

In this way, Th17 cells can take part in both protective and pathological mechanisms in TB infection, not only contributing to the development and organisation of the granuloma but also its breakdown and progression to cavitation and eventual transmission [333, 344]. Th17 immunity has also been shown to contribute to mucosal TB vaccine immunity where CXCL13 induction by IL-17A was critical in protection [308].

1.5.5 *T-helper 17 lymphocytes in human immunodeficiency virus infection*

During HIV infection there is preferential loss of Th17 cells from the mucosa and peripheral blood with reduced levels of IL-17A that is not completely restored by ARV therapy [345-350]. This suggests that HIV infection interferes with the generation of Th17 cells [349, 351]. Interestingly, this phenomenon is absent in HIV-1 infected long-term non-progressors [346] and elite controllers [352]. The loss of Th17 cells has been noted to contribute to HIV pathogenesis [353], correlate highly with immune hyper-activation [354] and negatively correlate with raised viral loads.

Th17 cells show greater HIV susceptibility than other T-cell subsets [354-356]. In addition, this subset is a highly permissive environment for HIV replication *in vitro* and *in vivo*, in both CCR5 and CXCR4 tropic HIV [354, 356, 357]. This has been associated with a significant impairment of Th17 generation from $CD4^+$ Tconvs in the peripheral blood [349]. Th17 targeting correlates with a higher expression of HIV envelope receptors CD4, CXCR4, CCR5 and α4β7 and low levels of autocrine production of CCR5 ligands CCL3 and CCL4, potent mediators of HIV self-protection, resulting in higher gp120 binding [356] and providing a potential reason for their preferential depletion [349, 354, 356]. Furthermore, the over expression of Th17 negative regulators (phosphatase SHP2, suppressor of cytokine signalling 3, and protein inhibitor of activated STAT3) have been observed during HIV infection [358].

1.5.6 *T-regulatory and T-helper 17 lymphocyte antagonism*

There is a cell intrinsic reciprocal relationship between Th17 and Treg developmental pathways. This influences the nature of the immune response with a significant effect on the development and outcome of inflammatory diseases [359].

Th17 cells and Tregs arise from common precursors and carry out opposing functions driven by the cytokine milieu and its effect on specific transcription factors. TGF-β is a common element in Treg and Th17 development by inducing the expression of Foxp3 and RORC in naïve $CD4^+$ Tconv through SMAD2 and 3 respectively. TGF-β alone advances Foxp3 expression and Treg differentiation to maintain peripheral tolerance; the N-terminal domain of Foxp3 interacts with RORC inhibiting *IL-17A* promoter activation preventing Th17 differentiation [359]. However, TGF-β in combination with IL-21, IL-6/IL-21 or IL-6/IL-23 reduces TGF-β induced Foxp3 expression through the activation of STAT3, this in turn overcomes Foxp3 mediated RORC inhibition [296, 360]. In addition, IL-1 inhibits TGF-β induced Foxp3 expression resulting in the enhancement of Th17 differentiation under Th17 polarising conditions [361].

In counterpart to IL-1, IL-6, IL-21 and IL-23; IL-2, secreted by $CD4^+$ Tconv cells, further enforces this reciprocal relationship. IL-2 mediated STAT5 activation opposes Th17 differentiation by STAT5 binding of the *IL17* gene thereby acting as a repressor [362]. IL-2 can also inhibit Th17 polarisation through the transcription factor E26-transformation specific 1 [363] and down regulating IL-6R expression in $CD4^+$ T-cells [364].

References

1. World Health Organisation. 2014. Global tuberculosis report. Geneva, Switzerland.

2. UNAIDS. 2014. AIDSinfo online database. Accessed on 2015-10-14 [http://aidsinfo.unaids.org/].

3. Chatterjee D, Pramanik AK. 2015. Tuberculosis in the African continent: A comprehensive review. Pathophysiology. 22(1):73-83.

4. World Health Organisation. 2014. Tuberculosis country profiles online database. Accessed on 2015-10-14 [http://www.who.int/tb/country/data/profiles/en/].

5. Pawlowski A, Jansson M, Skold M, Rottenberg ME, Kallenius G. 2012. Tuberculosis and HIV co-infection. PLoS Pathogens. 8(2):e1002464.

6. Andrews JR, Hatherill M, Mahomed H, Hanekom WA, Campo M, Hawn TR, Wood R, Scriba TJ. 2015. The dynamics of QuantiFERON-TB Gold in-tube conversion and reversion in a cohort of South African adolescents. American Journal of Respiratory and Critical Care Medicine. 191(5):584-591.

7. Philips JA, Ernst JD. 2012. Tuberculosis pathogenesis and immunity. Annual Review of Pathology: Mechanisms of Disease. 7:353-384.

8. Maglione PJ, Chan J. 2009. How B cells shape the immune response against *Mycobacterium tuberculosis*. European Journal of Immunology. 39(3):676-686.

9. Dheda K, Booth H, Huggett JF, Johnson MA, Zumla A, Rook GA. 2005. Lung remodeling in pulmonary tuberculosis. Journal of Infectious Diseases. 192(7):1201-1210.

10. Maartens G, Celum C, Lewin SR. 2014. HIV infection: Epidemiology, pathogenesis, treatment and prevention. The Lancet. 384(9939):258-271.

11. Lackner A, Lederman MM, Rodriguez B. 2012. HIV pathogenesis: The host. Cold Spring Harbor Perspectives in Medicine. 2(9):a007005.

12. Geldmacher C, Ngwenyama N, Schuetz A, Petrovas C, Reither K, Heeregrave EJ, Casazza JP, Ambrozak DR, Louder M, Ampofo W. 2010. Preferential infection and depletion of *Mycobacterium tuberculosis*–specific CD4 T-cells after HIV-1 infection. Journal of Experimental Medicine. 207(13):2869-2881.

13. Chetty S, Govender P, Zupkosky J, Pillay M, Ghebremichael M, Moosa M-YS, Ndung'u T, Porichis F, Kasprowicz VO. 2015. Co-Infection with *Mycobacterium tuberculosis* impairs HIV-specific $CD8^+$ and $CD4^+$ T-cell functionality. PloS One. 10(3):e0118654.

14. Haridas V, Pean P, Jasenosky LD, Madec Y, Laureillard D, Sok T, Sath S, Borand L, Marcy O, Chan S. 2015. TB-IRIS, T-cell activation, and remodeling of the T-cell compartment in highly immunosuppressed HIV-infected patients with TB. AIDS. 29(3):263-273.

15. Kabue JP, de Swardt D, de Beer C, Glashoff RH. 2013. Short-term antiretroviral therapy fails to reduce the expanded activated CCR5-expressing $CD4^+$ T-lymphocyte population or to restore the depleted naive population in chronically HIV-infected individuals with active pulmonary tuberculosis. AIDS Research and Human Retroviruses. 29(5):769-777.

16. Huber S, Gagliani N, Flavell RA. 2012. Life, death, and miracles: Th17 cells in the intestine. European Journal of Immunology. 42(9):2238-2245.

17. Hori S, Nomura T, Sakaguchi S. 2003. Control of regulatory T-cell development by the transcription factor Foxp3. Science. 299(5609):1057-1061.

18. Barker RD. 2012. Clinical tuberculosis. Medicine. 40(6):340-345.

19. Chaisson RE, Schecter GF, Theuer CP, Rutherford GW, Echenberg DF, Hopewell PC. 1987. Tuberculosis in patients with the acquired immunodeficiency syndrome: Clinical features, response to therapy and survival. American Review of Respiratory Disease. 136(3):570-574.

20. TB DOTS Strategy Co-ordination Directorate. 2014. National Tuberculosis Management Guidelines. National Department of Health, Republic of South Africa.

21. Barnes PF, Mistry SD, Cooper CL, Pirmez C, Rea TH, Modlin RL. 1989. Compartmentalization of a $CD4^+$ T-lymphocyte subpopulation in tuberculous pleuritis. Journal of Immunology. 142(4):1114-1119.

22. Roper W, Waring J. 1955. Primary serofibrinous pleural effusion in military personnel. American Review of Tuberculosis. 71(5):616-634.

23. Balboa L, Romero MM, Basile JI, y García CAS, Schierloh P, Yokobori N, Geffner L, Musella RM, Castagnino J, Abbate E. 2011. Paradoxical role of $CD16^+CCR2^+CCR5^+$ monocytes in tuberculosis: Efficient APC in pleural effusion but also mark disease severity in blood. Journal of Leukocyte Biology. 90(1):69-75.

24. Gideon HP, Phuah J, Myers AJ, Bryson BD, Rodgers MA, Coleman MT, Maiello P, Rutledge T, Marino S, Fortune SM. 2015. Variability in tuberculosis granuloma T-cell responses exists, but a balance of pro-and anti-inflammatory cytokines is associated with sterilization. PLoS Pathogens. 11(1):e1004603.

25. Parida S, Axelsson-Robertson R, Rao M, Singh N, Master I, Lutckii A, Keshavjee S, Andersson J, Zumla A, Maeurer M. 2015. Totally drug-resistant tuberculosis and adjunct therapies. Journal of Internal Medicine. 277(4):388-405.

26. Russell DG. 2007. Who puts the tubercle in tuberculosis? Nature Reviews Microbiology. 5(1):39-47.

27. Corbett EL, Watt CJ, Walker N, Maher D, Williams BG, Raviglione MC, Dye C. 2003. The growing burden of tuberculosis: Global trends and interactions with the HIV epidemic. Archives of Internal Medicine. 163(9):1009-1021.

28. Carr JH, Butler R. 2006. Scanning Electron Micrograph of *Mycobacterium tuberculosis*. Centres for Disease Control and Prevention. Accessed on 2015-10-15 [http://phil.cdc.gov/phil/details.asp?pid=9997#modalIdString_CDCImage_0]

29. Brennan P. 2003. Structure, function, and biogenesis of the cell wall of *Mycobacterium tuberculosis*. Tuberculosis. 83(1):91-97.

30. Minnikin DE, Lee OY, Wu HH, Nataraj V, Donoghue HD, Ridell M, Watanabe M, Alderwick L, Bhatt A, Besra GS. 2015. Pathophysiological implications of cell envelope structure in *Mycobacterium tuberculosis* and related taxa. In: Tuberculosis - Expanding Knowledge. 7:145-175. Edited by Ribón DW. InTech. ISBN 978-953-51-2139-8.

31. Kolattukudy P, Fernandes ND, Azad A, Fitzmaurice AM, Sirakova TD. 1997. Biochemistry and molecular genetics of cell-wall lipid biosynthesis in mycobacteria. Molecular Microbiology. 24(2):263-270.

32. Jo EK, Yang CS, Choi CH, Harding CV. 2007. Intracellular signalling cascades regulating innate immune responses to Mycobacteria: Branching out from Toll-like receptors. Cellular Microbiology. 9(5):1087-1098.

33. Russell DG, Cardona P-J, Kim M-J, Allain S, Altare F. 2009. Foamy macrophages and the progression of the human tuberculosis granuloma. Nature Immunology. 10(9):943-948.

34. Pandey AK, Sassetti CM. 2008. Mycobacterial persistence requires the utilization of host cholesterol. Proceedings of the National Academy of Sciences USA. 105(11):4376-4380.

35. Thoma-Uszynski S, Stenger S, Takeuchi O, Ochoa MT, Engele M, Sieling PA, Barnes PF, Röllinghoff M, Bölcskei PL, Wagner M. 2001. Induction of direct antimicrobial activity through mammalian Toll-like receptors. Science. 291(5508):1544-1547.

36. Underhill DM, Ozinsky A, Smith KD, Aderem A. 1999. Toll-like receptor-2 mediates mycobacteria-induced proinflammatory signaling in macrophages. Proceedings of the National Academy of Sciences USA. 96(25):14459-14463.

37. Means TK, Wang S, Lien E, Yoshimura A, Golenbock DT, Fenton MJ. 1999. Human Toll-like receptors mediate cellular activation by *Mycobacterium tuberculosis*. Journal of Immunology. 163(7):3920-3927.

38. Pecora ND, Gehring AJ, Canaday DH, Boom WH, Harding CV. 2006. *Mycobacterium tuberculosis* LprA is a lipoprotein agonist of TLR-2 that regulates innate immunity and APC function. Journal of Immunology. 177(1):422-429.

39. Sutcliffe IC, Harrington DJ. 2004. Lipoproteins of *Mycobacterium tuberculosis*: An abundant and functionally diverse class of cell envelope components. FEMS Microbiology Reviews. 28(5):645-659.

40. Harding CV, Boom WH. 2010. Regulation of antigen presentation by *Mycobacterium tuberculosis*: A role for Toll-like receptors. Nature Reviews Microbiology. 8(4):296-307.

41. Flynn JL, Chan J. 2005. What's good for the host is good for the bug. Trends in Microbiology. 13(3):98-102.

42. Ulrichs T, Kaufmann SH. 2006. New insights into the function of granulomas in human tuberculosis. Journal of Pathology. 208(2):261-269.

43. Slight SR, Khader SA. 2013. Chemokines shape the immune responses to tuberculosis. Cytokine and Growth Factor Reviews. 24(2):105-113.

44. Algood HMS, Chan J, Flynn JL. 2003. Chemokines and tuberculosis. Cytokine and Growth Factor Reviews. 14(6):467-477.

45. Gonzalez-Juarrero M, Orme IM. 2001. Characterization of murine lung dendritic cells infected with *Mycobacterium tuberculosis*. Infection and Immunity. 69(2):1127-1133.

46. Dorhoi A, Reece ST, Kaufmann SH. 2011. For better or for worse: The immune response against *Mycobacterium tuberculosis* balances pathology and protection. Immunological Reviews. 240(1):235-251.

47. Reiley WW, Calayag MD, Wittmer ST, Huntington JL, Pearl JE, Fountain JJ, Martino CA, Roberts AD, Cooper AM, Winslow GM. 2008. ESAT-6-specific CD4 T-cell responses to aerosol *Mycobacterium tuberculosis* infection are initiated in the mediastinal lymph nodes. Proceedings of the National Academy of Sciences USA. 105(31):10961-10966.

48. Cooper AM. 2009. Cell-mediated immune responses in tuberculosis. Annual Review of Immunology. 27:393-422.

49. Divangahi M, Desjardins D, Nunes-Alves C, Remold HG, Behar SM. 2010. Eicosanoid pathways regulate adaptive immunity to *Mycobacterium tuberculosis*. Nature Immunology. 11(8):751-758.

50. Reece ST, Kaufmann SH. 2012. Floating between the poles of pathology and protection: Can we pin down the granuloma in tuberculosis? Current Opinion in Microbiology. 15(1):63-70.

51. Blomgran R, Ernst JD. 2011. Lung neutrophils facilitate activation of naive antigen-specific $CD4^+$ T-cells during *Mycobacterium tuberculosis* Infection. Journal of Immunology. 186(12):7110-7119.

52. Madan-Lala R, Sia JK, King R, Adekambi T, Monin L, Khader SA, Pulendran B, Rengarajan J. 2014. *Mycobacterium tuberculosis* impairs dendritic cell functions through the serine hydrolase Hip1. Journal of Immunology. 192(9):4263-4272.

53. Srivastava V, Manchanda M, Gupta S, Singla R, Behera D, Das G, Natarajan K. 2009. Toll-like receptor 2 and DC-SIGNR1 differentially regulate suppressors of cytokine signaling 1 in dendritic cells during *Mycobacterium tuberculosis* infection. Journal of Biological Chemistry. 284(38):25532-25541.

54. Geijtenbeek TB, van Vliet SJ, Koppel EA, Sanchez-Hernandez M, Vandenbroucke-Grauls CM, Appelmelk B, van Kooyk Y. 2003. Mycobacteria target DC-SIGN to suppress dendritic cell function. Journal of Experimental Medicine. 197(1):7-17.

55. Holmes CB, Wood R, Badri M, Zilber S, Wang B, Maartens G, Zheng H, Lu Z, Freedberg KA, Losina E. 2006. CD4 decline and incidence of opportunistic infections in Cape Town, South Africa: Implications for prophylaxis and treatment. Journal of Acquired Immune Deficiency Syndromes. 42(4):464-469.

56. Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, Bloom BR. 1993. An essential role for interferon-γ in resistance to *Mycobacterium tuberculosis* infection. Journal of Experimental Medicine. 178(6):2249-2254.

57. Cooper AM, Dalton DK, Stewart TA, Griffin J, Russell D, Orme I. 1993. Disseminated tuberculosis in interferon-γ gene-disrupted mice. Journal of Experimental Medicine. 178(6):2243-2247.

58. Green AM, Difazio R, Flynn JL. 2013. IFN-γ from CD4 T-cells is essential for host survival and enhances CD8 T-cell function during *Mycobacterium tuberculosis* infection. Journal of Immunology. 190(1):270-277.

59. Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V. 2004. Autophagy is a defense mechanism inhibiting BCG and *Mycobacterium tuberculosis* survival in infected macrophages. Cell. 119(6):753-766.

60. Lazarevic V, Flynn J. 2002. $CD8^+$ T-cells in tuberculosis. American Journal of Respiratory and Critical Care Medicine. 166(8):1116-1121.

61. Stenger S. 2001. Cytolytic T-cells in the immune response to *Mycobacterium tuberculosis*. Scandinavian Journal of Infectious Diseases. 33(7):483-487.

62. Serbina NV, Lazarevic V, Flynn JL. 2001. $CD4^+$ T-Cells are required for the development of cytotoxic $CD8^+$ T-Cells during *Mycobacterium tuberculosis* infection. Journal of Immunology. 167(12):6991-7000.

63. Qiao D, Li L, Guo J, Lao S, Zhang X, Zhang J, Wu C. 2011. *Mycobacterium tuberculosis* culture filtrate protein 10-specific effector/memory $CD4^+$ and $CD8^+$ T-cells in tubercular pleural fluid, with biased usage of T-cell receptor Vβ chains. Infection and Immunity. 79(8):3358-3365.

64. Li L, Yang B, Zhang X, Lao S, Wu C. 2014. *Mycobacterium tuberculosis*-specific polyfunctional cytotoxic $CD8^+$ T-cells express CD69. Tuberculosis. 94(3):219-225.

65. Stenger S, Hanson DA, Teitelbaum R, Dewan P, Niazi KR, Froelich CJ, Ganz T, Thoma-Uszynski S, Melián An, Bogdan C. 1998. An antimicrobial activity of cytolytic T-cells mediated by granulysin. Science. 282(5386):121-125.

66. Pitabut N, Sakurada S, Tanaka T, Ridruechai C, Tanuma J, Aoki T, Kantipong P, Piyaworawong S, Kobayashi N, Dhepakson P *et al*. 2013. Potential function of granulysin, other related effector molecules and lymphocyte subsets in patients with TB and HIV/TB co-infection. International Journal of Medical Sciences. 10(8):1003-1014.

67. Serbina NV, Liu C-C, Scanga CA, Flynn JL. 2000. $CD8^+$ CTL from lungs of *Mycobacterium tuberculosis*-infected mice express perforin *in vivo* and lyse infected macrophages. Journal of Immunology. 165(1):353-363.

68. Axelsson-Robertson R, Loxton AG, Walzl G, Ehlers MM, Kock MM, Zumla A, Maeurer M. 2013. A broad profile of co-dominant epitopes shapes the peripheral *Mycobacterium tuberculosis* specific $CD8^+$ T-cell immune response in South African patients with active tuberculosis. PloS One. 8(3):e58309.

69. Day CL, Moshi ND, Abrahams DA, van Rooyen M, O'rie T, de Kock M, Hanekom WA. 2014. Patients with tuberculosis disease have *Mycobacterium tuberculosis*-specific CD8 T-cells with a pro-

apoptotic phenotype and impaired proliferative capacity, which is not restored following treatment. PloS One. 9(4):e94949.

70. Maglione PJ, Xu J, Chan J. 2007. B-cells moderate inflammatory progression and enhance bacterial containment upon pulmonary challenge with *Mycobacterium tuberculosis*. Journal of Immunology. 178(11):7222-7234.

71. Maglione PJ, Xu J, Casadevall A, Chan J. 2008. Fcγ receptors regulate immune activation and susceptibility during *Mycobacterium tuberculosis* infection. Journal of Immunology. 180(5):3329-3338.

72. Junqueira-Kipnis AP, Kipnis A, Henao Tamayo M, Harton M, Gonzalez Juarrero M, Basaraba RJ, Orme IM. 2005. Interleukin-10 production by lung macrophages in CBA xid mutant mice infected with *Mycobacterium tuberculosis*. Immunology. 115(2):246-252.

73. Torrado E, Fountain JJ, Robinson RT, Martino CA, Pearl JE, Rangel-Moreno J, Tighe M, Dunn R, Cooper AM. 2013. Differential and site specific impact of B-cells in the protective immune response to *Mycobacterium tuberculosis* in the mouse. PLoS One. 8(4):e61681.

74. Bosio CM, Gardner D, Elkins KL. 2000. Infection of B-cell deficient mice with CDC 1551, a clinical isolate of *Mycobacterium tuberculosis*: Delay in dissemination and development of lung pathology. Journal of Immunology. 164(12):6417-6425.

75. Tsai MC, Chakravarty S, Zhu G, Xu J, Tanaka K, Koch C, Tufariello J, Flynn J, Chan J. 2006. Characterization of the tuberculous granuloma in murine and human lungs: Cellular composition and relative tissue oxygen tension. Cellular Microbiology. 8(2):218-232.

76. Gonzalez-Juarrero M, Turner OC, Turner J, Marietta P, Brooks JV, Orme IM. 2001. Temporal and spatial arrangement of lymphocytes within lung granulomas induced by aerosol infection with *Mycobacterium tuberculosis*. Infection and Immunity. 69(3):1722-1728.

77. Ulrichs T, Kosmiadi GA, Trusov V, Jörg S, Pradl L, Titukhina M, Mishenko V, Gushina N, Kaufmann SH. 2004. Human tuberculous granulomas induce peripheral lymphoid follicle-like structures to orchestrate local host defence in the lung. Journal of Pathology. 204(2):217-228.

78. Cáceres N, Tapia G, Ojanguren I, Altare F, Gil O, Pinto S, Vilaplana C, Cardona P-J. 2009. Evolution of foamy macrophages in the pulmonary granulomas of experimental tuberculosis models. Tuberculosis. 89(2):175-182.

79. Peyron P, Vaubourgeix J, Poquet Y, Levillain F, Botanch C, Bardou F, Daffé M, Emile J-F, Marchou B, Cardona P-J. 2008. Foamy macrophages from tuberculous patients' granulomas constitute a nutrient-rich reservoir for *M. tuberculosis* persistence. PLoS Pathogens. 4(11):e1000204.

80. Alatas F, Alatas O, Metintas M, Ozarslan A, Erginel S, Yildirim H. 2004. Vascular endothelial growth factor levels in active pulmonary tuberculosis. CHEST Journal. 125(6):2156-2159.

81. Flynn JL, Chan J. 2001. Immunology of tuberculosis. Annual Review of Immunology. 19(1):93-129.

82. Ulrichs T, Kosmiadi GA, Jörg S, Pradl L, Titukhina M, Mishenko V, Gushina N, Kaufmann SH. 2005. Differential organization of the local immune response in patients with active cavitary tuberculosis or with non-progressive tuberculoma. Journal of Infectious Diseases. 192(1):89-97.

83. Robinson MA, Baumgardner JE, Good VP, Otto CM. 2008. Physiological and hypoxic O_2 tensions rapidly regulate NO production by stimulated macrophages. American Journal of Physiology-Cell Physiology. 294(4):1079-1087.

84. Russell DG, Barry CE, Flynn JL. 2010. Tuberculosis: What we don't know can, and does, hurt us. Science. 328(5980):852-856.

85. Lin PL, Pawar S, Myers A, Pegu A, Fuhrman C, Reinhart TA, Capuano SV, Klein E, Flynn JL. 2006. Early events in *Mycobacterium tuberculosis* infection in cynomolgus macaques. Infection and Immunity. 74(7):3790-3803.

86. Capuano SV, Croix DA, Pawar S, Zinovik A, Myers A, Lin PL, Bissel S, Fuhrman C, Klein E, Flynn JL. 2003. Experimental *Mycobacterium tuberculosis* infection of cynomolgus macaques closely resembles the various manifestations of human *M. tuberculosis* infection. Infection and Immunity. 71(10):5831-5844.

87. Kaplan G, Post FA, Moreira AL, Wainwright H, Kreiswirth BN, Tanverdi M, Mathema B, Ramaswamy SV, Walther G, Steyn LM. 2003. *Mycobacterium tuberculosis* growth at the cavity surface: A microenvironment with failed immunity. Infection and Immunity. 71(12):7099-7108.

88. Volkman HE, Pozos TC, Zheng J, Davis JM, Rawls JF, Ramakrishnan L. 2010. Tuberculous granuloma induction via interaction of a bacterial secreted protein with host epithelium. Science. 327(5964):466-469.

89. Agarwal N, Bishai WR. 2010. Subversion from the sidelines. Science. 327(5964):417-418.

90. Rhoades E, Hsu F, Torrelles J, Turk J, Chatterjee D, Russell D. 2003. Identification and macrophage-activating activity of glycolipids released from intracellular *Mycobacterium bovis* BCG. Molecular Microbiology. 48(4):875-888.

91. Russell DG. 2001. *Mycobacterium tuberculosis*: Here today, and here tomorrow. Nature Reviews Molecular Cell Biology. 2(8):569-586.

92. Russell DG, Mwandumba HC, Rhoades EE. 2002. Mycobacterium and the coat of many lipids. Journal of Cell Biology. 158(3):421-426.

93. Bansal K, Kapoor N, Narayana Y, Puzo G, Gilleron M, Balaji KN. 2009. PIM2 induced COX-2 and MMP-9 expression in macrophages requires PI3K and Notch1 signaling. PLoS One. 4(3):e4911.

94. Taylor JL, Hattle JM, Dreitz SA, Troudt JM, Izzo LS, Basaraba RJ, Orme IM, Matrisian LM, Izzo AA. 2006. Role for matrix metalloproteinase 9 in granuloma formation during pulmonary *Mycobacterium tuberculosis* infection. Infection and Immunity. 74(11):6135-6144.

95. Dao D, Kremer L, Guérardel Y, Molano A, Jacobs W, Porcelli S, Briken V. 2004. *Mycobacterium tuberculosis* lipomannan induces apoptosis and interleukin-12 production in macrophages. Infection and Immunity. 72(4):2067-2074.

96. Dahl KE, Shiratsuchi H, Hamilton BD, Ellner JJ, Toossi Z. 1996. Selective induction of transforming growth factor-β in human monocytes by lipoarabinomannan of *Mycobacterium tuberculosis*. Infection and Immunity. 64(2):399-405.

97. Jones BW, Means TK, Heldwein KA, Keen MA, Hill PJ, Belisle JT, Fenton MJ. 2001. Different Toll-like receptor agonists induce distinct macrophage responses. Journal of Leukocyte Biology. 69(6):1036-1044.

98. Gilleron M, Ronet C, Mempel M, Monsarrat B, Gachelin G, Puzo G. 2001. Acylation state of the phosphatidylinositol mannosides from *Mycobacterium bovis* bacillus Calmette Guerin and ability to induce granuloma and recruit natural killer T-cells. Journal of Biological Chemistry. 276(37):34896-34904.

99. Perez RL, Roman J, Roser S, Little C, Olsen M, Indrigo J, Hunter RL, Actor JK. 2000. Cytokine message and protein expression during lung granuloma formation and resolution induced by the mycobacterial cord factor trehalose-6, 6'-dimycolate. Journal of Interferon and Cytokine Research. 20(9):795-804.

100. Yamagami H, Matsumoto T, Fujiwara N, Arakawa T, Kaneda K, Yano I, Kobayashi K. 2001. Trehalose 6, 6′-dimycolate (cord factor) of *Mycobacterium tuberculosis* induces foreign body and hypersensitivity type granulomas in mice. Infection and Immunity. 69(2):810-815.

101. Russell D, Purdy G, Owens R, Rohde K, Yates R. 2005. *Mycobacterium tuberculosis* and the four-minute phagosome: By arresting the maturation of phagosomes, *M-tuberculosis* avoids being delivered to lysosomes. ASM News. 71(10):459-463.

102. Deretic V, Singh S, Master S, Harris J, Roberts E, Kyei G, Davis A, De Haro S, Naylor J, Lee HH. 2006. *Mycobacterium tuberculosis* inhibition of phagolysosome biogenesis and autophagy as a host defence mechanism. Cellular Microbiology. 8(5):719-727.

103. Vergne I, Chua J, Singh SB, Deretic V. 2004. Cell biology of *Mycobacterium tuberculosis* phagosome. Annual Reviews of Cell and Developmental Biology. 20:367-394.

104. Mwandumba HC, Russell DG, Nyirenda MH, Anderson J, White SA, Molyneux ME, Squire SB. 2004. *Mycobacterium tuberculosis* resides in non-acidified vacuoles in endocytically competent alveolar macrophages from patients with tuberculosis and HIV infection. Journal of Immunology. 172(7):4592-4598.

105. Sturgill-Koszycki S, Schlesinger PH, Chakraborty P, Haddix PL, Collins HL, Fok AK, Allen RD, Gluck SL, Heuser J, Russell DG. 1994. Lack of acidification in mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. Science. 263(5147):678-681.

106. Wong D, Bach H, Sun J, Hmama Z, Av-Gay Y. 2011. *Mycobacterium tuberculosis* protein tyrosine phosphatase (PtpA) excludes host vacuolar H^+–ATPase to inhibit phagosome acidification. Proceedings of the National Academy of Sciences USA. 108(48):19371-19376.

107. Vergne I, Chua J, Deretic V. 2003. Tuberculosis toxin blocking phagosome maturation inhibits a novel Ca^{2+}/calmodulin-PI3K hVPS34 cascade. Journal of Experimental Medicine. 198(4):653-659.

108. Fratti RA, Backer JM, Gruenberg J, Corvera S, Deretic V. 2001. Role of phosphatidylinositol 3-kinase and Rab5 effectors in phagosomal biogenesis and mycobacterial phagosome maturation arrest. Journal of Cell Biology. 154(3):631-644.

109. Purdy GE, Owens RM, Bennett L, Russell DG, Butcher BA. 2005. Kinetics of phosphatidylinositol-3-phosphate acquisition differ between IgG bead-containing phagosomes and *Mycobacterium tuberculosis*-containing phagosomes. Cellular Microbiology. 7(11):1627-1634.

110. Shukla S, Richardson E, Athman J, Shi L, Wearsch P, McDonald D, Banaei N, Boom W, Jackson M, Harding C. 2014. *Mycobacterium tuberculosis* lipoprotein LprG binds lipoarabinomannan and determines its localization in the cell wall envelope and affects phagolysosomal fusion. PloS Pathogens. 10(12):e1004596.

111. Saleh MT, Belisle JT. 2000. Secretion of an acid phosphatase (SapM) by *Mycobacterium tuberculosis* that is similar to eukaryotic acid phosphatases. Journal of Bacteriology. 182(23):6850-6853.

112. Vergne I, Chua J, Lee H-H, Lucas M, Belisle J, Deretic V. 2005. Mechanism of phagolysosome biogenesis block by viable *Mycobacterium tuberculosis*. Proceedings of the National Academy of Sciences USA. 102(11):4033-4038.

113. Harding CV, Geuze HJ. 1992. Class II MHC molecules are present in macrophage lysosomes and phagolysosomes that function in the phagocytic processing of *Listeria monocytogenes* for presentation to T-cells. Journal of Cell Biology. 119(3):531-542.

114. Ramachandra L, Noss E, Boom WH, Harding CV. 2001. Processing of *Mycobacterium tuberculosis* antigen 85B involves intraphagosomal formation of peptide–major histocompatibility complex II complexes and is inhibited by live bacilli that decrease phagosome maturation. Journal of Experimental Medicine. 194(10):1421-1432.

115. Beatty WL, Ullrich H-J, Russell DG. 2001. Mycobacterial surface moieties are released from infected macrophages by a constitutive exocytic event. European Journal of Cell Biology. 80(1):31-40.

116. Ullrich H-J, Beatty WL, Russell DG. 2000. Interaction of *Mycobacterium avium*-containing phagosomes with the antigen presentation pathway. Journal of Immunology. 165(11):6073-6080.

117. MacMicking JD, Taylor GA, McKinney JD. 2003. Immune control of tuberculosis by IFN-γ-inducible LRG-47. Science. 302(5645):654-659.

118. Via L, Fratti R, McFalone M, Pagan-Ramos E, Deretic D, Deretic V. 1998. Effects of cytokines on mycobacterial phagosome maturation. Journal of Cell Science. 111(7):897-905.

119. Kwan CK, Ernst JD. 2011. HIV and tuberculosis: A deadly human syndemic. Clinical Microbiology Reviews. 24(2):351-376.

120. Jeon CY, Murray MB. 2008. Diabetes mellitus increases the risk of active tuberculosis: A systematic review of 13 observational studies. PLoS Medicine. 5(7):e152.

121. Wu V-C, Wang C-Y, Shiao C-C, Chang C-H, Huang H-Y, Huang T-M, Lai C-F, Lin M-C, Ko W-J, Wu K-D. 2013. Increased risk of active tuberculosis following acute kidney injury: A nationwide, population-based study. PloS One. 8(7):e69556.

122. Dixon W, Watson K, Lunt M, Hyrich K, Silman A, Symmons D. 2006. Rates of serious infection, including site-specific and bacterial intracellular infection, in rheumatoid arthritis patients receiving anti–tumor necrosis factor therapy: Results from the British society for rheumatology biologics register. Arthritis and Rheumatism. 54(8):2368-2376.

123. Vento S, Lanzafame M. 2011. Tuberculosis and cancer: A complex and dangerous liaison. The Lancet Oncology. 12(6):520-522.

124. Stoffels K, Allix-Béguec C, Groenen G, Wanlin M, Berkvens D, Mathys V, Supply P, Fauville-Dufaux M. 2013. From multidrug-to extensively drug-resistant tuberculosis: Upward trends as seen from a 15-year nationwide study. PLoS One. 8(5):e63128.

125. Maurya V, Vijayan V, Shah A. 2002. Smoking and tuberculosis: An association overlooked. International Journal of Tuberculosis and Lung Disease. 6(11):942-951.

126. Rath P, Huang C, Wang T, Wang T, Li H, Prados-Rosales R, Elemento O, Casadevall A, Nathan CF. 2013. Genetic regulation of vesiculogenesis and immunomodulation in *Mycobacterium tuberculosis.* Proceedings of the National Academy of Sciences USA. 110(49):4790-4797.

127. Hunter RL, Jagannath C, Actor JK. 2007. Pathology of postprimary tuberculosis in humans and mice: Contradiction of long-held beliefs. Tuberculosis. 87(4):267-278.

128. Welsh KJ, Risin SA, Actor JK, Hunter RL. 2011. Immunopathology of postprimary tuberculosis: Increased T-regulatory cells and DEC-205-positive foamy macrophages in cavitary lesions. Clinical and Developmental Immunology. 2011:e307631.

129. Brennan PJ, Nikaido H. 1995. The envelope of mycobacteria. Annual Review of Biochemistry. 64(1):29-63.

130. Baldán Á, Gomes AV, Ping P, Edwards PA. 2008. Loss of ABCG1 results in chronic pulmonary inflammation. Journal of Immunology. 180(5):3560-3568.

131. Ordway D, Henao-Tamayo M, Orme IM, Gonzalez-Juarrero M. 2005. Foamy macrophages within lung granulomas of mice infected with *Mycobacterium tuberculosis* express molecules characteristic of dendritic cells and anti-apoptotic markers of the TNF receptor-associated factor family. Journal of Immunology. 175(6):3873-3881.

132. Ríos-Barrera VA, Campos-Peña V, Aguilar-León D, Lascurain LR, Meraz-Ríos MA, Moreno J, Figueroa-Granados V, Hernández-Pando R. 2006. Macrophage and T-lymphocyte apoptosis during experimental pulmonary tuberculosis: Their relationship to mycobacterial virulence. European Journal of Immunology. 36(2):345-353.

133. Nabeshima S, Nomoto M, Matsuzaki G, Kishihara K, Taniguchi H, Yoshida S-i, Nomoto K. 1999. T-cell hyporesponsiveness induced by activated macrophages through nitric oxide production in mice infected with *Mycobacterium tuberculosis*. Infection and Immunity. 67(7):3221-3226.

134. Gan H, Lee J, Ren F, Chen M, Kornfeld H, Remold HG. 2008. *Mycobacterium tuberculosis* blocks crosslinking of annexin-1 and apoptotic envelope formation on infected macrophages to maintain virulence. Nature Immunology. 9(10):1189-1197.

135. Gil O, Guirado E, Gordillo S, Díaz J, Tapia G, Vilaplana C, Ariza A, Ausina V, Cardona P-J. 2006. Intragranulomatous necrosis in lungs of mice infected by aerosol with *Mycobacterium tuberculosis* is related to bacterial load rather than to any one cytokine or T-cell type. Microbes and Infection. 8(3):628-636.

136. Lee J, Hartman M, Kornfeld H. 2009. Macrophage apoptosis in tuberculosis. Yonsei Medical Journal. 50(1):1-11.

137. Behar SM, Divangahi M, Remold HG. 2010. Evasion of innate immunity by *Mycobacterium tuberculosis*: Is death an exit strategy? Nature Reviews Microbiology. 8(9):668-674.

138. Divangahi M, Chen M, Gan H, Desjardins D, Hickman TT, Lee DM, Fortune S, Behar SM, Remold HG. 2009. *Mycobacterium tuberculosis* evades macrophage defenses by inhibiting plasma membrane repair. Nature Immunology. 10(8):899-906.

139. Chen M, Divangahi M, Gan H, Shin DS, Hong S, Lee DM, Serhan CN, Behar SM, Remold HG. 2008. Lipid mediators in innate immunity against tuberculosis: Opposing roles of PGE2 and LXA4 in the induction of macrophage death. Journal of Experimental Medicine. 205(12):2791-2801.

140. Porcel-Perez J, Vives SM, Esquerda SA, Jover SA. 2004. Cuttoff values of biochemical tests on pleural fluid: Their usefulness in differential diagnosis of 1,040 patients with pleural effusion. Anales de Medicina Interna. 21(3):113-117.

141. Liam CK, Lim KH, Wong CMM. 2000. Causes of pleural exudates in a region with a high incidence of tuberculosis. Respirology. 5(1):33-38.

142. Valdes L, Alvarez D, Valle JM, Pose A, San Jose E. 1996. The etiology of pleural effusions in an area with high incidence of tuberculosis. CHEST Journal. 109(1):158-162.

143. Porcel JM. 2009. Tuberculous pleural effusion. Lung. 187(5):263-270.

144. Light RW. 2007. Tuberculosis pleural effusions. In: Pleural diseases. 5th edn. 13:211-224. Lippincott Williams and Wilkins. Philadelphia, Pennsylvania. ISBN 9780781769570.

145. Perez-Rodriguez E, Light RW. 2008. Effusions from infections: Tuberculosis. In: Textbook of pleural diseases. 2nd edn. 27:367-378. Edited by Light R, Lee Y. CRC Press. Boca Raton, Florida. ISBN 9780340940174.

146. Viedma EC, Dus MJL, González-Molina A, Aldás JLS. 2006. A study of loculated tuberculous pleural effusions treated with intrapleural urokinase. Respiratory Medicine. 100(11):2037-2042.

147. Gopi A, Madhavan SM, Sharma SK, Sahn SA. 2007. Diagnosis and treatment of tuberculous pleural effusion in 2006. CHEST Journal. 131(3):880-889.

148. Ruan S-Y, Chuang Y-C, Wang J-Y, Lin J-W, Chien J-Y, Huang C-T, Kuo Y-W, Lee L-N, Chong-Jen JY. 2012. Revisiting tuberculous pleurisy: Pleural fluid characteristics and diagnostic yield of mycobacterial culture in an endemic area. Thorax. 67(9):822-827.

149. Liang Q-L, Shi H-Z, Wang K, Qin S-M, Qin X-J. 2008. Diagnostic accuracy of adenosine deaminase in tuberculous pleurisy: A meta-analysis. Respiratory Medicine. 102(5):744-754.

150. Baba K, Hoosen AA, Langeland N, Dyrhol-Riise AM. 2008. Adenosine deaminase activity is a sensitive marker for the diagnosis of tuberculous pleuritis in patients with very low CD4 counts. PLoS One. 3(7):e2788.

151. Lin M-T, Wang J-Y, Yu C-J, Lee L-N, Yang P-C, Group T. 2009. *Mycobacterium tuberculosis* and polymorphonuclear pleural effusion: Incidence and clinical pointers. Respiratory Medicine. 103(6):820-826.

152. Bassett IV, Wang B, Chetty S, Giddy J, Losina E, Mazibuko M, Bearnot B, Allen J, Walensky RP, Freedberg KA. 2010. Intensive tuberculosis screening for HIV-infected patients starting antiretroviral therapy in Durban, South Africa. Clinical Infectious Diseases. 51(7):823-829.

153. World Heath Organisation. 2010. Guidelines for the treatment of tuberculosis 4th edition. Geneva, Switzerland.

154. Butler R, Carr J, Townsend K. 2012. First-line treatment of tuberculosis (TB) for drug-Sensitive TB. National Institute of Allergy and Infectious Diseases (NIAID). Accessed on 2015-10-20 [https://www.niaid.nih.gov/topics/tuberculosis/understanding/whatistb/visualtour/pages/firstline.aspx]

155. Gandhi NR, Moll A, Sturm AW, Pawinski R, Govender T, Lalloo U, Zeller K, Andrews J, Friedland G. 2006. Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. The Lancet. 368(9547):1575-1580.

156. Migliori GB, Besozzi G, Girardi E, Kliiman K, Lange C, Toungoussova O, Ferrara G, Cirillo D, Gori A, Matteelli A. 2007. Clinical and operational value of the extensively drug-resistant tuberculosis definition. European Respiratory Journal. 30(4):623-626.

157. Velayati AA, Masjedi MR, Farnia P, Tabarsi P, Ghanavi J, ZiaZarifi AH, Hoffner SE. 2009. Emergence of new forms of totally drug-resistant tuberculosis bacilli: Super extensively drug-resistant tuberculosis or totally drug-resistant strains in Iran. CHEST Journal. 136(2):420-425.

158. Velayati AA, Farnia P, Masjedi MR. 2013. The totally drug resistant tuberculosis (TDR-TB). International Journal of Clinical and Experimental Medicine. 6(4):307-309.

159. Günther G. 2014. Multidrug-resistant and extensively drug-resistant tuberculosis: A review of current concepts and future challenges. Clinical Medicine. 14(3):279-285.

160. Zumla A, George A, Sharma V, Herbert RHN, Oxley A, Oliver M. 2015. The WHO 2014 Global tuberculosis report—further to go. The Lancet Global Health. 3(1):10-12.

161. Zumla A, Abubakar I, Raviglione M, Hoelscher M, Ditiu L, McHugh TD, Squire SB, Cox H, Ford N, McNerney R *et al*. 2012. Drug-resistant tuberculosis: Current dilemmas, unanswered questions, challenges, and priority needs. Journal of Infectious Diseases. 205(suppl 2):228-240.

162. Klopper M, Warren RM, Hayes C, van Pittius NCG, Streicher EM, Müller B, Sirgel FA, Chabula-Nxiweni M, Hoosain E, Coetzee G. 2013. Emergence and spread of extensively and totally drug-resistant tuberculosis, South Africa. Emerging Infectious Diseases. 19(3):449-455.

163. Buthelezi S. Situational analysis of TB drug resistance in KwaZulu-Natal province, Republic of South Africa. In: 2nd Meeting of the WHO Global XDR-TB Task Force. 2008. World Health Organisation. Geneva, Switzerland.

164. Brust J, Lygizos M, Chaiyachati K, Scott M, van der Merwe TL, Moll AP, Li X, Loveday M, Bamber SA, Lalloo UG. 2011. Culture conversion among HIV co-infected multidrug-resistant tuberculosis patients in Tugela Ferry, South Africa. PloS One. 6(1):e15841.

165. Gandhi NR, Shah NS, Andrews JR, Vella V, Moll AP, Scott M, Weissman D, Marra C, Lalloo UG, Friedland GH. 2010. HIV coinfection in multidrug- and extensively drug-resistant tuberculosis results in high early mortality. American Journal of Respiratory and Critcal Care Medicine. 181(1):80-86.

166. Frieden TR, Sterling T, Pablos-Mendez A, Kilburn JO, Cauthen GM, Dooley SW. 1993. The emergence of drug-resistant tuberculosis in New York City. New England Journal of Medicine. 328(8):521-526.

167. Gottlieb MS, Schroff R, Schanker HM, Weisman JD, Fan PT, Wolf RA, Saxon A. 1981. *Pneumocystis carinii* pneumonia and mucosal candidiasis in previously healthy homosexual men: Evidence of a new acquired cellular immunodeficiency. New England Journal of Medicine. 305(24):1425-1431.

168. Barré-Sinoussi F, Chermann J-C, Rey F, Nugeyre MT, Chamaret S, Gruest J, Dauguet C, Axler-Blin C, Vézinet-Brun F, Rouzioux C. 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science. 220(4599):868-871.

169. UNAIDS. 2015. How AIDS Changed Everything - MDG 6: 15 Years, 15 Lessons of Hope from the AIDS Response. Geneva, Switzerland.

170. Hahn BH, Shaw GM, De KM, Sharp PM. 2000. AIDS as a zoonosis: Scientific and public health implications. Science. 287(5453):607-614.

171. Hemelaar J. 2012. The origin and diversity of the HIV-1 pandemic. Trends in Molecular Medicine. 18(3):182-192.

172. Roberts JD, Bebenek K, Kunkel TA. 1988. The accuracy of reverse transcriptase from HIV-1. Science. 242(4882):1171-1173.

173. Ganser-Pornillos BK, Yeager M, Sundquist WI. 2008. The structural biology of HIV assembly. Current Opinion in Structural Biology. 18(2):203-217.

174. Perelson AS, Neumann AU, Markowitz M, Leonard JM, Ho DD. 1996. HIV-1 dynamics *in vivo*: Virion clearance rate, infected cell life-span and viral generation time. Science. 271(5255):1582-1586.

175. Sakuragi J-i. 2011. Morphogenesis of the infectious HIV-1 virion. Frontiers in Microbiology. 2:242-246.

176. Arts EJ, Hazuda DJ. 2012. HIV-1 antiretroviral drug therapy. Cold Spring Harbor Perspectives in Medicine. 2(4):a007161.

177. Sierra S, Kupfer B, Kaiser R. 2005. Basics of the virology of HIV-1 and its replication. Journal of Clinical Virology. 34(4):233-244.

178. Robinson HL. 2002. New hope for an AIDS vaccine. Nature Reviews Immunology. 2(4):239-250.

179. Gelderblom HR. 1991. Assembly and morphology of HIV: Potential effect of structure on viral function. AIDS. 5(6):617-638.

180. Clapham PR, McKnight Á. 2002. Cell surface receptors, virus entry and tropism of primate lentiviruses. Journal of General Virology. 83(8):1809-1829.

181. Le Rouzic E, Benichou S. 2005. The Vpr protein from HIV-1: Distinct roles along the viral life cycle. Retrovirology. 2(1):11-24.

182. Wu Y. 2004. HIV-1 gene expression: Lessons from provirus and non-integrated DNA. Retrovirology. 1(1):13-22.

183. Karn J, Stoltzfus CM. 2012. Transcriptional and post transcriptional regulation of HIV-1 gene expression. Cold Spring Harbor Perspectives in Medicine. 2(2):a006916.

184. Pollard VW, Malim MH. 1998. The HIV-1 rev protein. Annual Reviews in Microbiology. 52(1):491-532.

185. Pasternak AO, Lukashov VV, Berkhout B. 2013. Cell-associated HIV RNA: A dynamic biomarker of viral persistence. Retrovirology. 10(1):41-55.

186. Chukkapalli V, Hogue IB, Boyko V, Hu W-S, Ono A. 2007. Interaction between HIV-1 Gag matrix domain and phosphatidylinositol-(4, 5)-bisphosphate is essential for efficient Gag-membrane binding. Journal of Virology. 82:2405–2417.

187. Frankel AD, Young JA. 1998. HIV-1: Fifteen proteins and an RNA. Annual Review of Biochemistry. 67(1):1-25.

188. Monroe EB, Kang S, Kyere SK, Li R, Prevelige PE, Jr. 2010. Hydrogen/deuterium exchange analysis of HIV-1 capsid assembly and maturation. Structure. 18(11):1483-1491.

189. Boily M-C, Baggaley RF, Wang L, Masse B, White RG, Hayes RJ, Alary M. 2009. Heterosexual risk of HIV-1 infection per sexual act: Systematic review and meta-analysis of observational studies. The Lancet Infectious Diseases. 9(2):118-129.

190. Swanstrom R, Coffin J. 2012. HIV-1 pathogenesis: The virus. Cold Spring Harbor Perspectives in Medicine. 2(12):a007443.

191. Quinn TC, Wawer MJ, Sewankambo N, Serwadda D, Li C, Wabwire-Mangen F, Meehan MO, Lutalo T, Gray RH. 2000. Viral load and heterosexual transmission of human immunodeficiency virus type 1. New England Journal of Medicine. 342(13):921-929.

192. Coffin J, Swanstrom R. 2013. HIV pathogenesis: Dynamics and genetics of viral populations and infected cells. Cold Spring Harbor Perspectives in Medicine. 3(1):a012526.

193. Cohen MS, Shaw GM, McMichael AJ, Haynes BF. 2011. Acute HIV-1 infection. New England Journal of Medicine. 364(20):1943-1954.

194. Phillips RE, Rowland-Jones S, Nixon DF, Gotch FM, Edwards JP, Ogunlesi AO, Elvin JG, Rothbard JA, Bangham C, Rizza CR. 1991. Human immunodeficiency virus genetic variation that can escape cytotoxic T-cell recognition. Nature. 354(6353):453-459.

195. Wilen CB, Tilton JC, Doms RW. 2012. HIV: Cell binding and entry. Cold Spring Harbor Perspectives in Medicine. 2(8):a006866.

196. Sainski AM, Natesampillai S, Cummins NW, Bren GD, Taylor J, Saenz DT, Poeschla EM, Badley AD. 2011. The HIV-1-specific protein Casp8p41 induces death of infected cells through Bax/Bak. Journal of Virology. 85(16):7965-7975.

197. Zeng M, Southern PJ, Reilly CS, Beilman GJ, Chipman JG, Schacker TW, Haase AT. 2012. Lymphoid tissue damage in HIV-1 infection depletes naive T-cells and limits T-cell reconstitution after antiretroviral therapy. PLoS Pathogens. 8(1):e1002437.

198. Mehandru S, Poles MA, Tenner-Racz K, Manuelli V, Jean-Pierre P, Lopez P, Shet A, Low A, Mohri H, Boden D. 2007. Mechanisms of gastrointestinal $CD4^+$ T-cell depletion during acute and early human immunodeficiency virus type 1 infection. Journal of Virology. 81(2):599-612.

199. Liang SC, Tan XY, Luxenberg DP, Karim R, Dunussi-Joannopoulos K, Collins M, Fouser LA. 2006. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. Journal of Experimental Medicine. 203(10):2271-2279.

200. Prendergast A, Prado JG, Kang Y-H, Chen F, Riddell LA, Luzzi G, Goulder P, Klenerman P. 2010. HIV-1 infection is characterized by profound depletion of $CD161^+$ Th17 cells and gradual decline in regulatory T-cells. AIDS. 24(4):491-502.

201. Brenchley JM, Paiardini M, Knox KS, Asher AI, Cervasi B, Asher TE, Scheinberg P, Price DA, Hage CA, Kholi LM. 2008. Differential Th17 CD4 T-cell depletion in pathogenic and nonpathogenic lentiviral infections. Blood. 112(7):2826-2835.

202. Brenchley JM, Price DA, Schacker TW, Asher TE, Silvestri G, Rao S, Kazzaz Z, Bornstein E, Lambotte O, Altmann D. 2006. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. Nature Medicine. 12(12):1365-1371.

203. Giorgi JV, Liu Z, Hultin LE, Cumberland WG, Hennessey K, Detels R. 1993. Elevated levels of $CD38^+CD8^+$ T-cells in HIV infection add to the prognostic value of low $CD4^+$ T-cell levels: Results of 6 years of follow-up. Journal of Acquired Immune Deficiency Syndromes. 6(8):904-912.

204. Brenchley JM, Karandikar NJ, Betts MR, Ambrozak DR, Hill BJ, Crotty LE, Casazza JP, Kuruppu J, Migueles SA, Connors M. 2003. Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of $CD8^+$ T cells. Blood. 101(7):2711-2720.

205. Day CL, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, Mackey EW, Miller JD, Leslie AJ, DePierres C. 2006. PD-1 expression on HIV-specific T-cells is associated with T-cell exhaustion and disease progression. Nature. 443(7109):350-354.

206. Hyrcza MD, Kovacs C, Loutfy M, Halpenny R, Heisler L, Yang S, Wilkins O, Ostrowski M, Der SD. 2007. Distinct transcriptional profiles in *ex vivo* $CD4^+$ and $CD8^+$ T-cells are established early in human immunodeficiency virus type 1 infection and are characterized by a chronic interferon response as well as extensive transcriptional changes in $CD8^+$ T-cells. Journal of Virology. 81(7):3477-3486.

207. Meier A, Chang JJ, Chan ES, Pollard RB, Sidhu HK, Kulkarni S, Wen TF, Lindsay RJ, Orellana L, Mildvan D. 2009. Sex differences in the Toll-like receptor–mediated response of plasmacytoid dendritic cells to HIV-1. Nature Medicine. 15(8):955-959.

208. Manel N, Hogstad B, Wang Y, Levy DE, Unutmaz D, Littman DR. 2010. A cryptic sensor for HIV-1 activates antiviral innate immunity in dendritic cells. Nature. 467(7312):214-217.

209. Yan N, Regalado-Magdos AD, Stiggelbout B, Lee-Kirsch MA, Lieberman J. 2010. The cytosolic exonuclease TREX1 inhibits the innate immune response to human immunodeficiency virus type 1. Nature Immunology. 11(11):1005-1013.

210. Hsue PY, Hunt PW, Sinclair E, Bredt B, Franklin A, Killian M, Hoh R, Martin JN, McCune JM, Waters DD. 2006. Increased carotid intima-media thickness in HIV patients is associated with increased cytomegalovirus-specific T-cell responses. AIDS. 20(18):2275-2283.

211. South African Department of Health. 2014. National Consolidated Guidelines: For the prevention of mother-to-child transmission of HIV (PMTCT) and the management of HIV in children, adolescents and adults. Department of Health. Pretoria, South Africa.

212. World Health Organisation. 2012. HIV drug resistance report. Geneva, Switzerland.

213. Finzi D, Blankson J, Siliciano JD, Margolick JB, Chadwick K, Pierson T, Smith K, Lisziewicz J, Lori F, Flexner C. 1999. Latent infection of $CD4^+$ T-cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. Nature Medicine. 5(5):512-517.

214. Chun T-W, Nickle DC, Justement JS, Meyers JH, Roby G, Hallahan CW, Kottilil S, Moir S, Mican JM, Mullins JI. 2008. Persistence of HIV in gut-associated lymphoid tissue despite long-term antiretroviral therapy. Journal of Infectious Diseases. 197(5):714-720.

215. Cusini A, Vernazza PL, Yerly S, Decosterd LA, Ledergerber B, Fux CA, Rohrbach J, Widmer N, Hirschel B, Gaudenz R. 2013. Higher CNS penetration-effectiveness of long-term combination antiretroviral therapy is associated with better HIV-1 viral suppression in cerebrospinal fluid. Journal of Acquired Immune Deficiency Syndromes. 62(1):28-35.

216. Kalsdorf B, Scriba TJ, Wood K, Day CL, Dheda K, Dawson R, Hanekom WA, Lange C, Wilkinson RJ. 2009. HIV-1 infection impairs the bronchoalveolar T-cell response to mycobacteria. American Journal of Respiratory and Critical Care Medicine. 180(12):1262-1270.

217. Jambo KC, Sepako E, Fullerton DG, Mzinza D, Glennie S, Wright AK, Heyderman RS, Gordon SB. 2011. Bronchoalveolar $CD4^+$ T-cell responses to respiratory antigens are impaired in HIV-infected adults. Thorax. 66(5):375-382.

218. Day CL, Mkhwanazi N, Reddy S, Mncube Z, van der Stok M, Klenerman P, Walker BD. 2008. Detection of polyfunctional *Mycobacterium tuberculosis*–specific T-cells and association with viral load in HIV-1–infected persons. Journal of Infectious Diseases. 197(7):990-999.

219. Sutherland JS, Young JM, Peterson KL, Sanneh B, Whittle HC, Rowland-Jones SL, Adegbola RA, Jaye A, Ota MO. 2010. Polyfunctional $CD4^+$ and $CD8^+$ T-cell responses to tuberculosis antigens in HIV-1 infected patients before and after antiretroviral treatment. Journal of Immunology. 184(11):6537-6544.

220. Jambo KC, Banda DH, Afran L, Kankwatira AM, Malamba RD, Allain TJ, Gordon SB, Heyderman RS, Russell DG, Mwandumba HC. 2014. Asymptomatic HIV-infected individuals on antiretroviral therapy exhibit impaired lung $CD4^+$ T-cell responses to mycobacteria. American Journal of Respiratory and Critical Care Medicine. 190(8):938-947.

221. Diedrich CR, Mattila JT, Klein E, Janssen C, Phuah J, Sturgeon TJ, Montelaro RC, Lin PL, Flynn JL. 2010. Reactivation of latent tuberculosis in cynomolgus macaques infected with SIV is associated with early peripheral T-cell depletion and not virus load. PloS One. 5(3):e9611.

222. Day CL, Abrahams DA, Lerumo L, Janse van Rensburg E, Stone L, O'rie T, Pienaar B, de Kock M, Kaplan G, Mahomed H *et al*. 2011. Functional capacity of *Mycobacterium tuberculosis*-specific T-cell responses in humans is associated with mycobacterial load. Journal of Immunology. 187(5):2222-2232.

223. Jasenosky LD, Scriba TJ, Hanekom WA, Goldfeld AE. 2015. T-cells and adaptive immunity to *Mycobacterium tuberculosis* in humans. Immunological Reviews. 264(1):74-87.

224. Ranjbar S, Ly N, Thim S, Reynes J-M, Goldfeld AE. 2004. *Mycobacterium tuberculosis* recall antigens suppress HIV-1 replication in anergic donor cells via $CD8^+$ T-cell expansion and increased IL-10 levels. Journal of Immunology. 172(3):1953-1959.

225. Ranjbar S, Boshoff HI, Mulder A, Siddiqi N, Rubin EJ, Goldfeld AE. 2009. HIV-1 replication is differentially regulated by distinct clinical strains of *Mycobacterium tuberculosis*. PLoS One. 4(7):e6116.

226. Barnabas RV, Webb EL, Weiss HA, Wasserheit JN. 2011. The role of co-infections in HIV epidemic trajectory and positive prevention: A systematic review and meta-analysis. AIDS. 25(13):1559-1573.

227. Josefowicz SZ, Lu L-F, Rudensky AY. 2012. Regulatory T-cells: Mechanisms of differentiation and function. Annual Review of Immunology. 30:531-564.

228. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. 1995. Immunologic self-tolerance maintained by activated T-cells expressing IL-2 receptor alpha-chains (CD25): Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. Journal of Immunology. 155(3):1151-1564.

229. Holmes D, Jiang Q, Zhang L, Su L. 2008. Foxp3 and Treg cells in HIV-1 infection and immuno-pathogenesis. Immunological Research. 41(3):248-266.

230. Huehn J, Beyer M. 2015. Epigenetic and transcriptional control of Foxp3^{+} regulatory T-cells. Seminars in Immunology. 27(1):10-18.

231. Whiteside TL. 2014. Regulatory T-cell subsets in human cancer: Are they regulating for or against tumor progression? Cancer Immunology, Immunotherapy. 63(1):67-72.

232. Nelson BH. 2004. IL-2, regulatory T-cells and tolerance. Journal of Immunology. 172(7):3983-3988.

233. Tone Y, Furuuchi K, Kojima Y, Tykocinski ML, Greene MI, Tone M. 2008. Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer. Nature Immunology. 9(2):194-202.

234. Bensinger SJ, Walsh PT, Zhang J, Carroll M, Parsons R, Rathmell JC, Thompson CB, Burchill MA, Farrar MA, Turka LA. 2004. Distinct IL-2 receptor signaling pattern in CD4^{+}CD25^{+} regulatory T-cells. Journal of Immunology. 172(9):5287-5296.

235. Zorn E, Nelson EA, Mohseni M, Porcheray F, Kim H, Litsa D, Bellucci R, Raderschall E, Canning C, Soiffer RJ. 2006. IL-2 regulates Foxp3 expression in human CD4^{+}CD25^{+} regulatory T-cells through a STAT-dependent mechanism and induces the expansion of these cells *in vivo*. Blood. 108(5):1571-1579.

236. Fontenot JD, Gavin MA, Rudensky AY. 2003. Foxp3 programs the development and function of CD4^{+}CD25^{+} regulatory T-cells. Nature Immunology. 4(4):330-336.

237. Camperio C, Caristi S, Fanelli G, Soligo M, Del Porto P, Piccolella E. 2012. Forkhead transcription factor Foxp3 upregulates CD25 expression through cooperation with RelA/NF-κB. PLoS One. 7(10):e48303.

238. Wu Y, Borde M, Heissmeyer V, Feuerer M, Lapan AD, Stroud JC, Bates DL, Guo L, Han A, Ziegler SF *et al*. 2006. Foxp3 controls regulatory T-cell function through cooperation with NFAT. Cell. 126(2):375-387.

239. Ono M, Yaguchi H, Ohkura N, Kitabayashi I, Nagamura Y, Nomura T, Miyachi Y, Tsukada T, Sakaguchi S. 2007. Foxp3 controls regulatory T-cell function by interacting with AML1/Runx1. Nature. 446(7136):685-689.

240. Lin JX, Li P, Liu D, Jin HT, He J, Ata Ur Rasheed M, Rochman Y, Wang L, Cui K, Liu C *et al*. 2012. Critical role of STAT5 transcription factor tetramerization for cytokine responses and normal immune function. Immunity. 36(4):586-599.

241. Papiernik M, de Moraes ML, Pontoux C, Vasseur F, Penit C. 1998. Regulatory CD4 T-cells: Expression of IL-2R alpha chain, resistance to clonal deletion and IL-2 dependency. International Immunology. 10(4):371-378.

242. Amado IF, Berges J, Luther RJ, Mailhe MP, Garcia S, Bandeira A, Weaver C, Liston A, Freitas AA. 2013. IL-2 coordinates IL-2-producing and regulatory T-cell interplay. Journal of Experimental Medicine. 210(12):2707-2720.

243. Benoist C, Mathis D. 2012. Treg cells, life history and diversity. Cold Spring Harbor Perspectives in Biology. 4(9):a007021.

244. Feuerer M, Hill JA, Kretschmer K, von Boehmer H, Mathis D, Benoist C. 2010. Genomic definition of multiple *ex vivo* regulatory T-cell subphenotypes. Proceedings of the National Academy of Sciences USA. 107(13):5919-5924.

245. Feuerer M, Hill JA, Mathis D, Benoist C. 2009. Foxp3$^+$ regulatory T-cells: Differentiation, specification, subphenotypes. Nature Immunology. 10(7):689-695.

246. Tang Q, Adams JY, Tooley AJ, Bi M, Fife BT, Serra P, Santamaria P, Locksley RM, Krummel MF, Bluestone JA. 2006. Visualizing regulatory T-cell control of autoimmune responses in non-obese diabetic mice. Nature Immunology. 7(1):83-92.

247. Davidson TS, Shevach EM. 2011. Polyclonal Treg cells modulate T-effector cell trafficking. European Journal of Immunology. 41(10):2862-2870.

248. Feuerer M, Shen Y, Littman DR, Benoist C, Mathis D. 2009. How punctual ablation of regulatory T-cells unleashes an autoimmune lesion within the pancreatic islets. Immunity. 31(4):654-664.

249. Vignali DA, Collison LW, Workman CJ. 2008. How regulatory T-cells work. Nature Reviews Immunology. 8(7):523-532.

250. Huang C-T, Dutta A, Miaw S-C, Drake C, Powell J, Pardoll D. 2015. Infectious tolerance of LAG-3$^+$ regulatory T-cells with attenuated T-bet induction Journal of Immunology. 194(suppl 1):197-215.

251. Shevach EM. 2009. Mechanisms of Foxp3$^+$ T-regulatory cell mediated suppression. Immunity. 30(5):636-645.

252. Rochman Y, Spolski R, Leonard WJ. 2009. New insights into the regulation of T-cells by γc family cytokines. Nature Reviews Immunology. 9(7):480-490.

253. Wang GQ, Yang CL, Yue DF, Pei LH, Zhong H, Niu JX. 2014. The changes and its significance of Th17 and Treg cells and related cytokines in patients with tuberculosis pleurisy. Allergy, Asthma and Clinical Immunology. 10(1):28-33.

254. Nemeth J, Winkler H-M, Boeck L, Adegnika AA, Clement E, Mve TM, Kremsner PG, Winkler S. 2011. Specific cytokine patterns of pulmonary tuberculosis in central Africa. Clinical Immunology. 138(1):50-59.

255. Chiacchio T, Casetti R, Butera O, Vanini V, Carrara S, Girardi E, Di Mitri D, Battistini L, Martini F, Borsellino G. 2009. Characterization of regulatory T-cells identified as $CD4^{+}CD25^{high}CD39^{+}$ in patients with active tuberculosis. Clinical and Experimental Immunology. 156(3):463-470.

256. Pang H, Yu Q, Guo B, Jiang Y, Wan L, Li J, Wu Y, Wan K. 2013. Frequency of regulatory T-cells in the peripheral blood of patients with pulmonary tuberculosis from Shanxi Province, China. PLoS One. 8(6):e65496.

257. Guyot-Revol V, Innes JA, Hackforth S, Hinks T, Lalvani A. 2006. Regulatory T-cells are expanded in blood and disease sites in patients with tuberculosis. American Journal of Respiratory and Critical Care Medicine. 173(7):803-810.

258. Marin ND, Paris SC, Velez VM, Rojas CA, Rojas M, Garcia LF. 2010. Regulatory T-cell frequency and modulation of IFN-γ and IL-17 in active and latent tuberculosis. Tuberculosis. 90(4):252-261.

259. Ribeiro-Rodrigues R, Resende Co T, Rojas R, Toossi Z, Dietze R, Boom WH, Maciel E, Hirsch CS. 2006. A role for $CD4^{+}CD25^{+}$ T-cells in regulation of the immune response during human tuberculosis. Clinical and Experimental Immunology. 144(1):25-34.

260. Chen X, Zhou B, Li M, Deng Q, Wu X, Le X, Wu C, Larmonier N, Zhang W, Zhang H. 2007. $CD4^{+}CD25^{+}FoxP3^{+}$ regulatory T-cells suppress *Mycobacterium tuberculosis* immunity in patients with active disease. Clinical Immunology. 123(1):50-59.

261. He X-Y, Xiao L, Chen H-B, Hao J, Li J, Wang Y-J, He K, Gao Y, Shi B-Y. 2010. T-regulatory cells and Th1/Th2 cytokines in peripheral blood from tuberculosis patients. European Journal of Clinical Microbiology and Infectious Diseases. 29(6):643-650.

262. de Waal Malefyt R, Haanen J, Spits H, Roncarolo M-G, Te Velde A, Figdor C, Johnson K, Kastelein R, Yssel H, De Vries JE. 1991. Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T-cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. Journal of Experimental Medicine. 174(4):915-924.

263. Ding L, Shevach E. 1992. IL-10 inhibits mitogen-induced T-cell proliferation by selectively inhibiting macrophage costimulatory function. Journal of Immunology. 148(10):3133-3139.

264. Fiorentino DF, Zlotnik A, Mosmann T, Howard M, O'garra A. 1991. IL-10 inhibits cytokine production by activated macrophages. Journal of Immunology. 147(11):3815-3822.

265. Bandera A, Ferrario G, Saresella M, Marventano I, Soria A, Zanini F, Sabbatini F, Airoldi M, Marchetti G, Franzetti F *et al*. 2010. $CD4^{+}$ T-cell depletion, immune activation and increased production of regulatory T-cells in the thymus of HIV-infected individuals. PLoS One. 5(5):e10788.

266. Shaw JM, Hunt PW, Critchfield JW, McConnell DH, Garcia JC, Pollard RB, Somsouk M, Deeks SG, Shacklett BL. 2011. Increased frequency of regulatory T-cells accompanies increased immune activation in rectal mucosa of HIV-positive non-controllers. Journal of Virology. 85(21):11422-11434.

267. Montes M, Sanchez C, Lewis DE, Graviss EA, Seas C, Gotuzzo E, White AC, Jr. 2011. Normalization of FoxP3^{+} regulatory T-cells in response to effective antiretroviral therapy. Journal of Infectious Diseases. 203(4):496-499.

268. Bi X, Suzuki Y, Gatanaga H, Oka S. 2009. High frequency and proliferation of CD4^{+}Foxp3^{+} Treg in HIV-1-infected patients with low CD4 counts. European Journal of Immunology. 39(1):301-309.

269. Suchard MS, Mayne E, Green VA, Shalekoff S, Donninger SL, Stevens WS, Gray CM, Tiemessen CT. 2010. Foxp3 expression is upregulated in CD4^{+} T-cells in progressive HIV-1 infection and is a marker of disease severity. PLoS One. 5(7):e11762.

270. Hunt PW, Landay AL, Sinclair E, Martinson JA, Hatano H, Emu B, Norris PJ, Busch MP, Martin JN, Brooks C *et al*. 2011. A low T-regulatory cell response may contribute to both viral control and generalized immune activation in HIV controllers. PLoS One. 6(1):e15924.

271. Schulze zur Wiesch J, Thomssen A, Hartjen P, Tóth I, Lehmann C, Meyer-Olson D, Colberg K, Frerk S, Babikir D, Schmiedel S *et al*. 2011. Comprehensive analysis of frequency and phenotype of T-regulatory cells in HIV Infection: CD39 expression of Foxp3^{+} T-regulatory cells correlates with progressive disease. Journal of Virology. 85(3):1287-1297.

272. Presicce P, Orsborn K, King E, Pratt J, Fichtenbaum CJ, Chougnet CA. 2011. Frequency of circulating regulatory T-cells increases during chronic HIV infection and is largely controlled by highly active antiretroviral therapy. PLoS One. 6(12):e28118.

273. Chevalier MF, Weiss L. 2013. The split personality of regulatory T-cells in HIV infection. Blood. 121(1):29-37.

274. Angin M, Kwon DS, Streeck H, Wen F, King M, Rezai A, Law K, Hongo TC, Pyo A, Piechocka-Trocha A. 2012. Preserved function of regulatory T-cells in chronic HIV-1 infection despite decreased numbers in blood and tissue. Journal of Infectious Diseases. 205(10):1495-1500.

275. Weiss L, Donkova-Petrini V, Caccavelli L, Balbo M, Carbonneil C, Levy Y. 2004. Human immunodeficiency virus–driven expansion of CD4^{+}CD25^{+} regulatory T-cells, which suppress HIV-specific CD4 T-cell responses in HIV-infected patients. Blood. 104(10):3249-3256.

276. Kinter A, McNally J, Riggin L, Jackson R, Roby G, Fauci AS. 2007. Suppression of HIV-specific T-cell activity by lymph node CD25^{+} regulatory T-cells from HIV-infected individuals. Proceedings of the National Academy of Sciences USA. 104(9):3390-3395.

277. Ji J, Cloyd MW. 2009. HIV-1 binding to CD4 on $CD4^{+}CD25^{+}$ regulatory T-cells enhances their suppressive function and induces them to home to, and accumulate in, peripheral and mucosal lymphoid tissues: An additional mechanism of immunosuppression. International Immunology. 21:283–294.

278. Card CM, McLaren PJ, Wachihi C, Kimani J, Plummer FA, Fowke KR. 2009. Decreased immune activation in resistance to HIV-1 infection is associated with an elevated frequency of $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T-cells. Journal of Infectious Diseases. 199(9):1318-1322.

279. Munier CM, Kelleher AD, Kent SJ, De Rose R. 2013. The role of T-cell immunity in HIV-1 infection. Current Opinion in Virology. 3(4):438-446.

280. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang Y-H, Wang Y, Hood L, Zhu Z, Tian Q *et al*. 2005. A distinct lineage of CD4 T-cells regulates tissue inflammation by producing interleukin 17. Nature Immunology. 6(11):1133-1141.

281. Zhou X, Chen Q, Moore J, Kolls JK, Halperin S, Wang J. 2009. Critical role of the interleukin-17/interleukin-17 receptor axis in regulating host susceptibility to respiratory infection with chlamydia species. Infection and Immunity. 77(11):5059-5070.

282. Zhang X, Gao L, Lei L, Zhong Y, Dube P, Berton MT, Arulanandam B, Zhang J, Zhong G. 2009. A MyD88-dependent early IL-17 production protects mice against airway infection with the obligate intracellular pathogen *Chlamydia muridarum*. Journal of Immunology. 183(2):1291-1300.

283. Kudva A, Scheller EV, Robinson KM, Crowe CR, Choi SM, Slight SR, Khader SA, Dubin PJ, Enelow RI, Kolls JK. 2011. Influenza A inhibits Th17-mediated host defense against bacterial pneumonia in mice. Journal of Immunology. 186(3):1666-1674.

284. Ye P, Garvey PB, Zhang P, Nelson S, Bagby G, Summer WR, Schwarzenberger P, Shellito JE, Kolls JK. 2001. Interleukin-17 and lung host defense against *Klebsiella pneumoniae* infection. American Journal of Respiratory Cell and Molecular Biology. 25(3):335-340.

285. Yang L, Anderson DE, Baecher-Allan C, Hastings WD, Bettelli E, Oukka M, Kuchroo VK, Hafler DA. 2008. IL-21 and TGF-β are required for differentiation of human Th17 cells. Nature. 454(7202):350-352.

286. Manel N, Unutmaz D, Littman DR. 2008. The differentiation of human Th17 cells requires transforming growth factor-β and induction of the nuclear receptor ROR-γt. Nature Immunology. 9(6):641-649.

287. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, Cua DJ, Littman DR. 2006. The orphan nuclear receptor ROR-γt directs the differentiation program of proinflammatory IL-17^{+} T-helper cells. Cell. 126(6):1121-1133.

288. Mudter J, Yu J, Zufferey C, Brüstle A, Wirtz S, Weigmann B, Hoffman A, Schenk M, Galle PR, Lehr HA. 2011. IRF4 regulates IL-17A promoter activity and controls ROR-γt-dependent Th17 colitis *in vivo*. Inflammatory Bowel Diseases. 17(6):1343-1358.

289. Brüstle A, Heink S, Huber M, Rosenplänter C, Stadelmann C, Yu P, Arpaia E, Mak TW, Kamradt T, Lohoff M. 2007. The development of inflammatory Th17 cells requires interferon-regulatory factor 4. Nature Immunology. 8(9):958-966.

290. Hirahara K, Ghoreschi K, Laurence A, Yang X-P, Kanno Y, O'Shea JJ. 2010. Signal transduction pathways and transcriptional regulation in Th17 cell differentiation. Cytokine and Growth Factor Reviews. 21(6):425-434.

291. Yang XO, Pappu BP, Nurieva R, Akimzhanov A, Kang HS, Chung Y, Ma L, Shah B, Panopoulos AD, Schluns KS *et al*. 2008. T-helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR-α and ROR-γ. Immunity. 28(1):29-39.

292. Lee W-W, Kang SW, Choi J, Lee S-H, Shah K, Eynon EE, Flavell RA, Kang I. 2010. Regulating human Th17 cells via differential expression of IL-1 receptor. Blood. 115(3):530-540.

293. Volpe E, Servant N, Zollinger R, Bogiatzi SI, Hupe P, Barillot E, Soumelis V. 2008. A critical function for transforming growth factor-β, interleukin 23 and proinflammatory cytokines in driving and modulating human Th17 responses. Nature Immunology. 9(6):650-657.

294. Chung Y, Chang SH, Martinez GJ, Yang XO, Nurieva R, Kang HS, Ma L, Watowich SS, Jetten AM, Tian Q. 2009. Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. Immunity. 30(4):576-587.

295. Sundrud MS, Trivigno C. 2013. Identity crisis of Th17 cells: Many forms, many functions, many questions. Seminars in Immunology. 25(4):263-272.

296. Noack M, Miossec P. 2014. Th17 and regulatory T-cell balance in autoimmune and inflammatory diseases. Autoimmunity Reviews. 13(6):668-677.

297. Schraml BU, Hildner K, Ise W, Lee W-L, Smith WA-E, Solomon B, Sahota G, Sim J, Mukasa R, Cemerski S. 2009. The AP-1 transcription factor Batf controls Th17 differentiation. Nature. 460(7253):405-409.

298. Okamoto K, Iwai Y, Oh-Hora M, Yamamoto M, Morio T, Aoki K, Ohya K, Jetten AM, Akira S, Muta T. 2010. IκBζ regulates Th17 development by cooperating with ROR nuclear receptors. Nature. 464(7293):1381-1385.

299. Zhang F, Meng G, Strober W. 2008. Interactions among the transcription factors Runx1, ROR-γt and Foxp3 regulate the differentiation of interleukin 17–producing T-cells. Nature Immunology. 9(11):1297-1306.

300. Maddur MS, Miossec P, Kaveri SV, Bayry J. 2012. Th17 cells: Biology, pathogenesis of autoimmune and inflammatory diseases, and therapeutic strategies. The American Journal of Pathology. 181(1):8-18.

301. Korn T, Bettelli E, Oukka M, Kuchroo VK. 2009. IL-17 and Th17 Cells. Annual Review of Immunology. 27:485-517.

302. Toy D, Kugler D, Wolfson M, Bos TV, Gurgel J, Derry J, Tocker J, Peschon J. 2006. Cutting edge: Interleukin 17 signals through a heteromeric receptor complex. Journal of Immunology. 177(1):36-39.

303. Shen F, Gaffen SL. 2008. Structure-function relationships in the IL-17 receptor: Implications for signal transduction and therapy. Cytokine. 41(2):92-104.

304. Hot A, Zrioual S, Toh M-L, Lenief V, Miossec P. 2011. IL-17A- versus IL-17F-induced intracellular signal transduction pathways and modulation by IL-17RA and IL-17RC RNA interference in rheumatoid synoviocytes. Annals of the Rheumatic Diseases. 70(2):341-348.

305. Sønder SU, Saret S, Tang W, Sturdevant DE, Porcella SF, Siebenlist U. 2011. IL-17-induced NF-κB activation via CIKS/Act1 physiologic significance and signaling mechanisms. Journal of Biological Chemistry. 286(15):12881-12890.

306. Kao C-Y, Huang F, Chen Y, Thai P, Wachi S, Kim C, Tam L, Wu R. 2005. Up-regulation of CC chemokine ligand 20 expression in human airway epithelium by IL-17 through a JAK-independent but MEK/NF-κB-dependent signaling pathway. Journal of Immunology. 175(10):6676-6685.

307. Datta S, Novotny M, Pavicic PG, Zhao C, Herjan T, Hartupee J, Hamilton T. 2010. IL-17 regulates CXCL1 mRNA stability via an AUUUA/tristetraprolin-independent sequence. Journal of Immunology. 184(3):1484-1491.

308. Gopal R, Rangel-Moreno J, Slight S, Lin Y, Nawar HF, Junecko BF, Reinhart TA, Kolls J, Randall TD, Connell TD. 2013. Interleukin-17-dependent CXCL13 mediates mucosal vaccine–induced immunity against tuberculosis. Mucosal Immunology. 6(5):972-984.

309. Linden A, Laan M, Anderson G. 2005. Neutrophils, interleukin-17A and lung disease. European Respiratory Journal. 25(1):159-172.

310. Pelletier M, Maggi L, Micheletti A, Lazzeri E, Tamassia N, Costantini C, Cosmi L, Lunardi C, Annunziato F, Romagnani S. 2010. Evidence for a cross-talk between human neutrophils and Th17 cells. Blood. 115(2):335-343.

311. Yu Q, Lou X-m, He Y. 2015. Preferential recruitment of Th17 cells to cervical cancer via CCR6-CCL20 pathway. PLoS One. 10(3):e0120855.

312. Tian Y, Cox M, Kahan S, Ingram J, Bakshi R, Zajac A. 2015. IL-21 signaling modulates the differentiation, distribution, and abundance of CD8 T-cell subsets. Journal of Immunology. 194(suppl 1):200-208.

313. Diehl SA, Schmidlin H, Nagasawa M, Blom B, Spits H. 2012. IL-6 Triggers IL-21 production by human $CD4^+$ T-cells to drive STAT3-dependent plasma cell differentiation in B-cells. Immunology and Cell Biology. 90(8):802-811.

314. Avery DT, Bryant VL, Ma CS, de Waal Malefyt R, Tangye SG. 2008. IL-21 induced isotype switching to IgG and IgA by human naive B-cells is differentially regulated by IL-4. Journal of Immunology. 181(3):1767-1779.

315. Denman CJ, Senyukov VV, Somanchi SS, Phatarpekar PV, Kopp LM, Johnson JL, Singh H, Hurton L, Maiti SN, Huls MH *et al*. 2012. Membrane-bound IL-21 promotes sustained *ex vivo* proliferation of human natural killer cells. PLoS One. 7(1):e30264.

316. Wolk K, Sabat R. 2006. Interleukin-22: A novel T- and NK-cell derived cytokine that regulates the biology of tissue cells. Cytokine and Growth Factor Reviews. 17(5):367-380.

317. Wolk K, Haugen HS, Xu W, Witte E, Waggie K, Anderson M, Vom Baur E, Witte K, Warszawska K, Philipp S. 2009. IL-22 and IL-20 are key mediators of the epidermal alterations in psoriasis while IL-17 and IFN-γ are not. Journal of Molecular Medicine. 87(5):523-536.

318. Wolk K, Witte E, Witte K, Warszawska K, Sabat R. 2010. Biology of interleukin-22. Seminars in Immunopathology. 32(1):17-31.

319. Sonnenberg GF, Nair MG, Kirn TJ, Zaph C, Fouser LA, Artis D. 2010. Pathological versus protective functions of IL-22 in airway inflammation are regulated by IL-17A. Journal of Experimental Medicine. 207(6):1293-1305.

320. Scriba TJ, Kalsdorf B, Abrahams D-A, Isaacs F, Hofmeister J, Black G, Hassan HY, Wilkinson RJ, Walzl G, Gelderbloem SJ. 2008. Distinct, specific IL-17-and IL-22-producing $CD4^+$ T-cell subsets

contribute to the human anti-mycobacterial immune response. Journal of Immunology. 180(3):1962-1970.

321. Marín ND, París SC, Rojas M, García LF. 2012. Reduced frequency of memory T-cells and increased Th17 responses in patients with active tuberculosis. Clinical and Vaccine Immunology. 19(10):1667-1676.

322. Jurado JO, Pasquinelli V, Alvarez IB, Peña D, Rovetta AI, Tateosian NL, Romeo HE, Musella RM, Palmero D, Chuluyán HE. 2012. IL-17 and IFN-γ expression in lymphocytes from patients with active tuberculosis correlates with the severity of the disease. Journal of Leukocyte Biology. 91(6):991-1002.

323. Wang T, Lv M, Qian Q, Nie Y, Yu L, Hou Y. 2011. Increased frequencies of T-helper type 17 cells in tuberculous pleural effusion. Tuberculosis. 91(3):231-237.

324. Bandaru A, Devalraju KP, Paidipally P, Dhiman R, Venkatasubramanian S, Barnes PF, Vankayalapati R, Valluri V. 2014. Phosphorylated STAT3 and PD-1 regulate IL-17 production and IL-23 receptor expression in *Mycobacterium tuberculosis* infection. European Journal of Immunology. 44(7):2013-2024.

325. Cowan J, Pandey S, Filion L, Angel J, Kumar A, Cameron D. 2012. Comparison of interferon-γ, interleukin (IL)-17-and IL-22-expressing CD4 T-cells, IL-22 expressing granulocytes and proinflammatory cytokines during latent and active tuberculosis infection. Clinical and Experimental Immunology. 167(2):317-329.

326. Kumar NP, Anuradha R, Suresh R, Ganesh R, Shankar J, Kumaraswami V, Nutman TB, Babu S. 2011. Suppressed type 1, type 2, and type 17 cytokine responses in active tuberculosis in children. Clinical and Vaccine Immunology. 18(11):1856-1864.

327. Chen X, Zhang M, Liao M, Graner MW, Wu C, Yang Q, Liu H, Zhou B. 2010. Reduced Th17 response in patients with tuberculosis correlates with IL-6R expression on $CD4^+$ T-cells. American Journal of Respiratory and Critical Care Medicine. 181(7):734-742.

328. Perreau M, Rozot V, Welles HC, Belluti-Enders F, Vigano S, Maillard M, Dorta G, Mazza-Stalder J, Bart PA, Roger T. 2013. Lack of *Mycobacterium tuberculosis*–specific interleukin 17A producing $CD4^+$ T-cells in active disease. European Journal of Immunology. 43(4):939-948.

329. Hur Y-G, Gorak-Stolinska P, Ben-Smith A, Lalor MK, Chaguluka S, Dacombe R, Doherty TM, Ottenhoff TH, Dockrell HM, Crampin AC. 2013. Combination of cytokine responses indicative of latent TB and active TB in Malawian adults. PLoS One. 8(11):e79742.

330. Marín ND, París SC, Rojas M, García LF. 2013. Functional profile of $CD4^+$ and $CD8^+$ T-cells in latently infected individuals and patients with active TB. Tuberculosis. 93(2):155-166.

331. Peng M, Wang Z, Yao C, Jiang L, Jin Q, Wang J, Li B. 2008. Interleukin 17-producing γδ T-cells increased in patients with active pulmonary tuberculosis. Cellular and Molecular Immunology. 5(3):203-208.

332. Torrado E, Cooper AM. 2010. IL-17 and Th17 cells in tuberculosis. Cytokine and Growth Factor Reviews. 21(6):455-462.

333. Umemura M, Yahagi A, Hamada S, Begum MD, Watanabe H, Kawakami K, Suda T, Sudo K, Nakae S, Iwakura Y. 2007. IL-17 mediated regulation of innate and acquired immune response against pulmonary *Mycobacterium bovis* bacille Calmette-Guerin infection. Journal of Immunology. 178(6):3786-3796.

334. Redford PS, Boonstra A, Read S, Pitt J, Graham C, Stavropoulos E, Bancroft GJ, O'Garra A. 2010. Enhanced protection to *Mycobacterium tuberculosis* infection in IL-10 deficient mice is accompanied by early and enhanced Th1 responses in the lung. European Journal of Immunology. 40(8):2200-2210.

335. Yoshida YO, Umemura M, Yahagi A, O'Brien RL, Ikuta K, Kishihara K, Hara H, Nakae S, Iwakura Y, Matsuzaki G. 2010. Essential role of IL-17A in the formation of a mycobacterial infection-induced granuloma in the lung. Journal of Immunology. 184(8):4414-4422.

336. Seiler P, Aichele P, Bandermann S, Hauser AE, Lu B, Gerard NP, Gerard C, Ehlers S, Mollenkopf HJ, Kaufmann SH. 2003. Early granuloma formation after aerosol *Mycobacterium tuberculosis* infection is regulated by neutrophils via CXCR3-signaling chemokines. European Journal of Immunology. 33(10):2676-2686.

337. Silva MT. 2010. When two is better than one: Macrophages and neutrophils work in concert in innate immunity as complementary and cooperative partners of a myeloid phagocyte system. Journal of Leukocyte Biology. 87(1):93-106.

338. Miossec P, Kolls JK. 2012. Targeting IL-17 and Th17 cells in chronic inflammation. Nature Reviews Drug Discovery. 11(10):763-776.

339. Freches D, Korf H, Denis O, Havaux X, Huygen K, Romano M. 2013. Mice genetically inactivated in interleukin-17A receptor are defective in long-term control of *Mycobacterium tuberculosis* infection. Immunology. 140(2):220-231.

340. Gopal R, Monin L, Slight S, Uche U, Blanchard E, Junecko BAF, Ramos-Payan R, Stallings CL, Reinhart TA, Kolls JK. 2014. Unexpected role for IL-17 in protective immunity against hypervirulent *Mycobacterium tuberculosis* HN878 infection. PLoS Pathogens. 10(5):e1004099.

341. Torrado E, Robinson RT, Cooper AM. 2011. Cellular response to mycobacteria: Balancing protection and pathology. Trends in Immunology. 32(2):66-72.

342. Etna MP, Giacomini E, Severa M, Coccia EM. 2014. Pro-and anti-inflammatory cytokines in tuberculosis: A two-edged sword in TB pathogenesis. Seminars in Immunology. 26(6):543-551.

343. Cruz A, Fraga AG, Fountain JJ, Rangel-Moreno J, Torrado E, Saraiva M, Pereira DR, Randall TD, Pedrosa J, Cooper AM. 2010. Pathological role of interleukin 17 in mice subjected to repeated BCG vaccination after infection with *Mycobacterium tuberculosis*. Journal of Experimental Medicine. 207(8):1609-1616.

344. Wozniak TM, Saunders BM, Ryan AA, Britton WJ. 2010. *Mycobacterium bovis* BCG-specific Th17 cells confer partial protection against *Mycobacterium tuberculosis* infection in the absence of interferon-γ. Infection and Immunity. 78(10):4187-4194.

345. Mehandru S, Poles MA, Tenner-Racz K, Horowitz A, Hurley A, Hogan C, Boden D, Racz P, Markowitz M. 2004. Primary HIV-1 infection is associated with preferential depletion of $CD4^+$ T-lymphocytes from effector sites in the gastrointestinal tract. Journal of Experimental Medicine. 200(6):761-770.

346. Ciccone EJ, Greenwald JH, Lee PI, Biancotto A, Read SW, Yao MA, Hodge JN, Thompson WL, Kovacs SB, Chairez CL. 2011. $CD4^+$ T-cells, including Th17 and cycling subsets, are intact in the gut mucosa of HIV-1-infected long-term nonprogressors. Journal of Virology. 85(12):5880-5888.

347. Gaardbo JC, Hartling HJ, Gerstoft J, Nielsen SD. 2012. Incomplete immune recovery in HIV infection: Mechanisms, relevance for clinical care and possible solutions. Clinical and Developmental Immunology. 2012:e670957.

348. He Y, Li J, Zheng Y, Luo Y, Zhou H, Yao Y, Chen X, Chen Z, He M. 2012. A randomized case–control study of dynamic changes in peripheral blood Th17/Treg cell balance and interleukin-17 levels in highly active antiretroviral-treated HIV type 1/AIDS patients. AIDS Research and Human Retroviruses. 28(4):339-345.

349. DaFonseca S, Niessl J, Pouvreau S, Wacleche VS, Gosselin A, Cleret-Buhot A, Bernard N, Tremblay C, Jenabian M-A, Routy J-P. 2015. Impaired Th17 polarization of phenotypically naive $CD4^+$ T-cells during chronic HIV-1 infection and potential restoration with early ART. Retrovirology. 12(1):38-60.

350. Singh A, Vajpayee M, Ali SA, Chauhan NK. 2014. Cellular interplay among Th17, Th1, and Treg cells in HIV-1 subtype “C” infection. Journal of Medical Virology. 86(3):372-384.

351. Bixler SL, Mattapallil JJ. 2013. Loss and dysregulation of Th17 cells during HIV infection. Clinical and Developmental Immunology. 2013:e852418.

352. Brandt L, Benfield T, Mens H, Clausen LN, Katzenstein TL, Fomsgaard A, Karlsson I. 2011. Low level of regulatory T-cells and maintenance of balance between regulatory T-cells and Th17 cells in HIV-1 infected elite controllers. Journal of Acquired Immune Deficiency Syndromes. 57(2):101-108.

353. Schuetz A, Deleage C, Sereti I, Rerknimitr R, Phanuphak N, Phuang-Ngern Y, Estes JD, Sandler NG, Sukhumvittaya S, Marovich M. 2014. Initiation of ART during early acute HIV infection preserves mucosal Th17 function and reverses HIV-related immune activation. PLoS Pathogens. 10(12):e1004543.

354. El Hed A, Khaitan A, Kozhaya L, Manel N, Daskalakis D, Borkowsky W, Valentine F, Littman DR, Unutmaz D. 2010. Susceptibility of human Th17 cells to human immunodeficiency virus and their perturbation during infection. Journal of Infectious Diseases. 201(6):843-854.

355. McKinnon LR, Nyanga B, Chege D, Izulla P, Kimani M, Huibner S, Gelmon L, Block KE, Cicala C, Anzala AO. 2011. Characterization of a human cervical $CD4^+$ T-cell subset coexpressing multiple markers of HIV susceptibility. Journal of Immunology. 187(11):6032-6042.

356. Alvarez Y, Tuen M, Shen G, Nawaz F, Arthos J, Wolff MJ, Poles MA, Hioe CE. 2013. Preferential HIV infection of $CCR6^+$ Th17 cells is associated with higher levels of virus receptor expression and lack of CCR5 ligands. Journal of Virology. 87(19):10843-10854.

357. Rodriguez-Garcia M, Barr FD, Crist SG, Fahey JV, Wira CR. 2014. Phenotype and susceptibility to HIV infection of $CD4^+$ Th17 cells in the human female reproductive tract. Mucosal Immunology. 2014(7):1375–1385.

358. Bixler SL, Sandler NG, Douek DC, Mattapallil JJ. 2013. Suppressed Th17 levels correlate with elevated PIAS3, SHP2, and SOCS3 expression in CD4 T-cells during acute simian immunodeficiency virus infection. Journal of Virology. 87(12):7093-7101.

359. Zhou L, Lopes JE, Chong MM, Ivanov, II, Min R, Victora GD, Shen Y, Du J, Rubtsov YP, Rudensky AY *et al*. 2008. TGF-β induced Foxp3 inhibits Th17 cell differentiation by antagonizing ROR-γt function. Nature. 453(7192):236-240.

360. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, Weiner HL, Kuchroo VK. 2006. Reciprocal developmental pathways for the generation of pathogenic effector Th17 and regulatory T-cells. Nature. 441(7090):235-238.

361. Ikeda S, Saijo S, Murayama MA, Shimizu K, Akitsu A, Iwakura Y. 2014. Excess IL-1 signaling enhances the development of Th17 cells by downregulating TGF-β induced Foxp3 expression. Journal of Immunology. 192(4):1449-1458.

362. Laurence A, Tato CM, Davidson TS, Kanno Y, Chen Z, Yao Z, Blank RB, Meylan F, Siegel R, Hennighausen L. 2007. Interleukin-2 signaling via STAT5 constrains T-helper 17 cell generation. Immunity. 26(3):371-381.

363. Moisan J, Grenningloh R, Bettelli E, Oukka M, Ho I-C. 2007. Ets-1 is a negative regulator of Th17 differentiation. Journal of Experimental Medicine. 204(12):2825-2835.

364. Liao W, Lin J-X, Wang L, Li P, Leonard WJ. 2011. Modulation of cytokine receptors by IL-2 broadly regulates differentiation into helper T-cell lineages. Nature Immunology. 12(6):551-559.

CHAPTER 2

The Effect of TB/HIV Pleurisy on miR-155 Expression, Stability and IL-35 Production of T-regulatory Lymphocytes

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Abstract

T-regulatory cells (Tregs), characterised by forkhead box P3 (Foxp3), suppress effector lymphocyte function through the production of interleukin (IL)-35. MicroRNA (miR)-155 is a direct target of Foxp3 and positively regulates Treg stability by repressing translation of suppressor of cytokine signalling 1 (SOCS1), conferring Treg IL-2 competitive fitness. There is conflicting data on the frequency of Tregs in both tuberculosis (TB) and human immunodeficiency virus (HIV) infection indicating a pathogen-driven effect on Treg stability which has not been assessed in co-infection.

Objectives: We investigated Foxp3, miR-155 and SOCS1 expression, Treg stability and IL-35 production in TB/HIV pleurisy *in vitro* and *ex vivo.*

Methods: Foxp3 mRNA and miR-155 expression by qPCR, SOCS1 protein by western blotting, Foxp3 protein and Treg frequency by flow cytometry, and IL-35 concentration by ELISA.

Results: $TB^{+}HIV^{+}$ pleural effusion mononuclear cells showed increased Foxp3 mRNA (p=0.0022 but decreased Foxp3 protein expression (p=0.0008), together with reduced miR-155 (p=0.0022) and increased SOCS1 expression (p<0.0001). This was associated with a diminished proportion of $CD4^{+}CD25^{+}Foxp3^{+}STAT5^{+}$ Tregs *ex vivo* (p=0.0084) and confirmed *in vitro* (p=0.0467). Furthermore, a reduced pleural fluid IL-35 concentration (p=0.0258) was observed suggesting a Treg functional deficit.

Conclusions: The TB/HIV microenvironment decreases the stability and functional capacity of Tregs due to altered miRNA-155/SOCS1 dynamics.

Keywords

TB/HIV co-infection, Tregs, Foxp3, miR-155, IL-35, SOCS1

2.1 Introduction

TB and HIV are the leading causes of death due to infectious disease globally. HIV infected individuals have a 26-31 times greater risk of developing active TB infection with TB being the leading cause of HIV-related mortality (1). South Africa has the second highest TB incidence and the largest number of individuals living with HIV/AIDS worldwide (1-3), consequently 62% of TB patients are HIV positive (2). This highlights the importance of co-infection research where the immunological consequences of *Mycobacterium tuberculosis*-HIV interactions are largely uncharacterised.

Regulatory T-cells, specified by the transcription factor Foxp3, are a suppressive subset of $CD4^+CD25^+$ T-cells (5-10%). Tregs regulate effector lymphocyte function through contact dependent and independent mechanisms, such as the production of anti-inflammatory IL-35 (4). Under physiological conditions thymus derived (t)Tregs are responsible for the maintenance of immune homeostasis and peripheral tolerance. Upon pathological challenge tTregs are predominately replaced by periphery derived (p)Tregs from naïve $CD4^+$ T-cells to limit immunopathology during inflammation (5-7). Studies have shown that Treg homeostasis is regulated by miRNAs (8-10), a class of single stranded non-coding RNAs that post transcriptionally regulate gene expression (11, 12). MiR-155 is a direct target of Foxp3 which stabilises Tregs and confers IL-2 competitive fitness by targeting and repressing SOCS1 (8-10, 13, 14).

An imbalance in Treg stability has been associated with various pathologies. In TB/HIV co-infection Treg hyperactivity and down regulation of antigen specific $CD4^+$ T-cells may ultimately result in pathogen persistence and establishment of chronic infection. In contrast, loss of a stable Treg pool results in an excessive pro-inflammatory response which amplifies chronic immune activation and heightened virulence in HIV and drives granulomatous immunopathology in TB (6). There is conflicting data on the frequency and function of Tregs in both TB (15-20) and HIV (21-29) indicating a pathogen-driven effect on Treg stability which has not been assessed in co-infection.

Studies show that 40-80% of HIV-related TB is extrapulmonary (30, 31), the most common form of which is TB pleurisy (32). This is a consequence of *M. tuberculosis* infection of the pleura characterised by a chronic accumulation of inflammatory cells in the pleural space and resultant pleural effusion (PE) (33). TB infection outcome is determined by localised immune responses which are not accurately reflected systemically (34). Moreover, Tregs are noted to expand and accumulate at the pleural infection site exerting potent localised immune suppression (19, 35). Therefore we investigated Foxp3, miR-155 and SOCS1 expression, Treg stability and IL-35 production in TB/HIV pleurisy *in vitro* and *ex vivo*.

2.2 Methods

2.2.1 Study population

This study protocol was approved by the institutional Biomedical Research Ethics Committee (BF170/11). Participants were recruited at the Department of Pulmonary and Critical Care, Inkosi Albert Luthuli Central Hospital and the Department of Internal Medicine, Prince Mshiyeni Memorial Hospital (Durban, South Africa). Newly diagnosed TB patients presenting with a TB pleural effusion and concurrent HIV-infection (n=9) were enrolled. Active TB infection was confirmed by clinical diagnosis with bacterial or radiographic evidence; AFB culture positive sputum or pleural fluid microscopy (Gram, Zhiel-Neelsen, Auramine), *M. tuberculosis* DNA (Gene Xpert), pleural histology and pleural fluid cytology. The matched control group was composed of patients presenting with a non-infectious pleural effusion (malignancy, cardiac failure, end stage renal disease) and HIV seronegative (n=9). Exclusion criteria included anti-tuberculosis or anti-retroviral therapy, immunomodulatory treatment and conditions. Blood and pleural fluid was sampled by routine venesection and diagnostic pleural tap respectively, after informed consent was obtained from each participant. A summary of the demographics and clinical parameters of the study population are presented in Table 2.1.

Table 2.1: Summary of demographic and clinical parameters in the study population

Parameter	$TB^{-}HIV^{-}$ *n*=9	$TB^{+}HIV^{+}$ *n*=9
Age, years, median (range)	58 (32-76)	37 (18-64)
Gender		
Male	5 (56)	4 (44)
Female	4 (44)	5 (56)
Race		
African	5 (56)	9 (100)
Indian	4 (44)	0
TB diagnostic criteria		
Culture positive		8 (89)
Smear positive		2 (22)
PCR-MTBDR		9 (100)
Resistance to any one first line TB drug		1 (11)
MDR-TB		1 (11)
XDR-TB		0
CD4 count, mm3, mean (range)		59 (9-153)
Past history of TB infection	0	1 (11)
Current smoker	2 (22)	1 (11)
Ex-smoker	2 (22)	0
Alcohol use	2 (22)	3 (33)
Definitive cause of PE		
Tuberculosis	0	9 (100)
Malignancy	4 (44)	0
End stage renal disease	2 (22)	0
Nephrotic syndrome	1 (11)	0
Heart failure	2 (22)	0
Co-Morbidities		
Diabetes mellitus	3 (33)	0
Hypertension	5 (56)	1 (11)
Hepatitis B	0	1 (11)

Values expressed as n (%) unless otherwise stated

2.2.2 Pleural effusion fluid and mononuclear cell isolation

Cell free pleural effusion fluid (PEF), pleural effusion mononuclear cells (PEMCs) and peripheral blood mononuclear cells (PBMCs) were extracted from heparinised pleural effusion fluid and blood by differential gradient centrifugation within 2 hours (h) of pleural tap/venipuncture. Briefly, 5 ml of sample was layered onto equivolume Histopaque-1077 (catalogue number 10771-500, Sigma, USA) and centrifuged (1,491rpm, 30 minutes (min), room temperature (RT)). PEF, represented as the uppermost layer, was aspirated and centrifuged (10,000rpm, 10min, 4°C) to remove any cellular constituents. Harvested PEF was pooled from 5 patients in each group equally and stored at -80°C until further use. Buffy coats containing mononuclear cells were aspirated and rinsed in 0.1M phosphate buffered saline (PBS; 1,491rpm, 20min, RT)

2.2.3 $CD4^+$ T-cell isolation

For the *in vitro* component of the study, $CD4^+$ conventional T-lymphocytes (Tconvs) were purified by negative magnetic selection from PBMCs of a healthy BCG vaccinated volunteer (10ml, EDTA, one blood draw), using the Human CD4 T-Lymphocyte Enrichment Set-DM (catalogue number 557939, BD Biosciences, USA). PBMCs were labelled with biotinylated monoclonal antibodies (5µl/10×10^6 cells, 15min, RT) against antigens on erythrocytes, platelets and non-$CD4^+$ peripheral leukocytes. The cells were washed (iMag Buffer, 1,491rpm, 20min) and incubated with streptavidin conjugated magnetic nanoparticles (5µl/10×10^6 cells, 30min, RT). The cell suspension was placed in a magnetic field (8min, iMagnet; BD Biosciences, USA) and the enriched $CD4^+$ T-cell supernatant aspirated. Negative selection of the positive fraction was performed three times to increase the yield of the enriched fraction. The purity of the enriched fraction was increased by further negative selection and assessed by flow cytometry.

2.2.4 Cell culture and treatment

Healthy $CD4^+$ Tconvs were seeded in triplicate (8×10^4cells/well, U-bottomed microplate) in PEF conditioned complete culture medium (CCM; RPMI 1640, 10% foetal calf serum (FCS), 1% Penstrep-Fungizone, 1% L-glutamine, final concentrations) in a 1:1 ratio (24h, 37°C, 5% CO_2). Treatments included TB^-HIV^- PEF and TB^+HIV^+ PEF. Concurrently, cells were activated with Dynabeads Human T-activator CD3/CD28 bead solution (catalogue number 11131D, Gibco by Life Technologies, Norway) (2µl/well). Following incubation, cells were harvested, washed once (0.1M PBS; 1,491rpm, 20min, RT) and the activation beads removed by magnetic selection (1min, iMagnet). The cells were immediately prepared for flow cytometric staining

2.2.5 Flow cytometric analysis

For surface staining, $2.5x10^5$ cells were re-suspended in staining buffer (25µl, PBS containing 1% heat inactivated FCS, 0.09% w/v sodium azide) and labelled with the respective antibodies; fluorescein isothiocyanate (FitC) anti-CD4 (1:5, catalogue number 555346) peridin-chlorophyll-cyanin 5.5 (PerCP-Cy5.5) anti-CD25 (1:20, catalogue number 560503) (20min, 4°c, in the dark). For intracellular staining, cells were fixed in 4% paraformaldehyde (500µl, 30min, RT), washed (staining buffer; 1,491rpm, 20min, RT), permeabilised in 75% cold methanol (500µl, 10min, -20°C) and washed again. Cells were stained with the appropriate intracellular antibody; phycoerythrin (PE) anti-Foxp3 (1:5, catalogue number 560046), Alex Flour® 647 anti-STAT5 (1:5, catalogue number 612599) (60min, RT, in the dark). Antibodies were purchased from BD Biosciences, USA. Analysis was performed using the Accuri C6 flow cytometer (BD Biosciences, USA) and data analysed using FlowJo vX (10.0.7r2). Samples were run in triplicate and 30,000 events acquired per sample.

2.2.6 Molecular profiling

Total RNA was extracted from PEMCs using Qiazol Lysis Reagent (Qiagen, USA) following an in-house protocol. Briefly, cells were added to Qiazol Lysis Reagent (1:1) and incubated at RT (5min) and subsequently frozen (-80°C, overnight). Chloroform (200µl) was added, incubated at RT (3min) and centrifuged (8,200rpm, 15min, 4°C). Isopropanol (500µl) was added to the aqueous phase and frozen (-80°C, overnight). The samples were centrifuged (8,200rpm, 20min, 4°C) and the RNA pellet washed in cold ethanol (75%, 1ml). The samples were then centrifuged (6,400rpm, 15min, 4°C), the ethanol aspirated and the RNA pellet re-suspended in 15µl of nuclease free water. Total RNA was quantified on a Nanodrop™ 2000 UV-Vis Spectrophotometer (Thermoscientific, USA) and purity assessed with the A_{260}/A_{280} ratio.

Copy DNA (cDNA) was synthesised using the RT^2 First Strand Kit (catalogue number 330401, Qiagen, USA) according to the manufacturer's instructions. Briefly, 1µg of RNA pooled equally from 5 patients per group was added to a genomic DNA elimination mix (5 × gDNA elimination buffer, H_20) to a total volume of 10µl and incubated at 42°C (5min) followed by 4°C (1min). Subsequently, reverse transcription mix (10µl, 5 × RT buffer 3, primer and external control mix, RT enzyme mix, H_20) was added and incubated at 42°C (15min) followed by 95°C (5min), on the GeneAmp® PCR System 9700 (Applied Biosystems, USA), to produce cDNA.

Real time PCR was used to quantify Foxp3 mRNA expression. A 25µl reaction consisted of 12.5µl RT^2 SYBR Green Mastermix (catalogue number 330500, Qiagen, United States), 8.5µl nuclease free water, 2µl cDNA and 1µl sense and anti-sense primers. Foxp3 primer sequence: sense 5'-ACACGGACAGGATTGACAGA-3' anti-sense 5'-CAAATCGCTCCACCAACTAA-3' (450nM). The mRNA expression was compared and normalised to a housekeeping gene, 18S sense 5'-

ACACGGACAGGATTGACAGA-3' antisense 5'-CAAATCGCTCCACCAACTAA-3'. Cycling conditions were as follows: initial denaturation (95°C, 10min) followed by 40 cycles of denaturation (95°C, 30 seconds (s)), annealing (64°C, 30s) and extension (72°C, 1min) on a CFX Real-Time PCR Detector (Bio-Rad, USA). The quantification and melt curves were analysed using CFX Manager™ Software v3.0 (Bio-Rad, USA). The mRNA levels were calculated using the method described by Livak and Schmittgen (2001) (36) and represented as fold changes and relative expression to the control. Samples were run in triplicate and the experiments were repeated twice.

2.2.7 miRNA expression

cDNA was synthesised using the miScript II RT Kit (catalogue number 218160, Qiagen, USA) according to the manufacturer's guidelines. Briefly, 1µg of RNA pooled equally from 5 patients per group was added to a reverse transcription mastermix (5x miScript HiSpec Buffer, 10x miScript Nucleics Mix, miScript Reverse Transcriptase Mix, H_20) to a total volume of 20µl and incubated at 37°C (60min) followed by 95°C (5min) on the T100™ Thermocycler (Bio-Rad, USA), to produce cDNA.

Quantification of mature miR-155 was performed using miScript Primer Assays (catalogue number 218300, Qiagen, USA) in combination with miScript SYBR Green PCR kit (Qiagen, USA) according to the product instructions. A 25µl reaction consisted of 12.5µl 2 x QuantiTect SYBR Green PCR Master Mix, 8.5µl nuclease free water, 2µl cDNA, 2.5µl 10x miScript Universal Primer and 2.5µl 10 x miScript Primer Assay. Human RNU6B (catalogue number 331001, Qiagen, USA) was run as an internal control. The reaction underwent an initial activation step at 95°C (15min) followed by 40 cycles of denaturation (94°C, 15s), annealing (55°C, 30s) and extension (70°C, 30s) on the CFX96 Touch Real-Time PCR Detection System. The quantification and melt curves were analysed using CFX Manager™ Software v3.0. MiR-155 levels were calculated using the method described by Livak and Schmittgen (2001) (36) and represented as fold changes and relative expression to the control. Samples were run in triplicate and the experiments were repeated twice.

2.2.8 Western blotting

Protein was isolated from fresh PEMCs using Cytobuster™ Protein Extraction Reagent (catalogue number 71009, Novagen™ Merck Millipore, Germany), supplemented with protease and phosphatase inhibitors (catalogue numbers 05892791001 and 04906837001 respectively, Roche, USA). Cytobuster™ (500ul) was added to approximately $1x10^5$ cells, vortexed (1min), incubated on ice (10min) and centrifuged (1,491rpm, 10min, 4°C). The supernatant containing the crude protein extract was aspirated, pooled equally from 5 patients in each group, and stored at -80°C until further use. Protein concentration was quantified using the bicinchoninic acid (BCA) assay (Sigma, Germany) and samples standardised to 0.4mg/ml. Protein extracts

were denatured by boiling (5mins) in a 1:1 Laemli buffer (0.5M Tris-HCl (pH 6.8), 3% glycerol, 10% sodium dodecyl sulphate (SDS), 1% bromophenol blue, 12% β-mercaptoethanol in dH_20).

Protein samples (25µl) were electrophoresed on a 7.5% SDS-polyacrylamide gel (1.5h, 150 volts) and transferred to a nitrocellulose membrane using the Trans-Blot® Turbo™ Transfer System (1h, 350mA, Bio-Rad, USA). Membranes were blocked (1h) with 5% bovine serum albumin (BSA) in Tris-buffered saline and Tween 20 (TTBS; 137mM NaCl, 2.7mM KCl, 24mM Tris, 0.5% Tween 20, dH_20, pH 7.6).

The membrane was probed overnight (4°C) with rabbit anti-SOCS1 (catalogue number sc-9021, Santa Cruz Biotechnology, USA) diluted 1:1,000 in 5% BSA/TTBS. The membrane was then washed (TTBS, 4 x 10mins) and probed (1h, RT) with horse radish peroxidase (HRP)-conjugated anti-rabbit IgG (catalogue number sc-2004, Santa Cruz Biotechnology) diluted 1:5,000 in 5% BSA/TTBS. The membrane was washed again (TTBS, 4 x 10mins). A HRP-conjugated goat polyclonal antibody specific for β-actin diluted 1:5,000 in 5% BSA/TTBS was used as a loading control and for normalisation of protein expression. The membrane was developed using LumiGLO® Chemiluminescent Substrate System (catalogue number 51-61-00, KPL, USA) and images were captured on an Alliance 2.7 Gel Documentation System (Uvitec, UK). The density of the protein bands was quantified using UviBand Advanced Image Analysis Software v12.14 (Uvitec, UK). The experiment was performed in triplicate. Results are presented as relative band intensity (RBI) normalised against β-actin.

2.2.9 IL-35 ELISA

Quantification of PEF IL-35 was measured by enzyme-linked immunosorbent assay (ELISA) using the Legend Max™ Human IL-35 Heterodimer Kit (catalogue number 439507, Biolegend, USA) according to the manufacturer's protocol. Briefly, cell free PEF (50µl, diluted 1:10 in assay buffer) was added to wells of the pre-coated plate and incubated (2h, 350rpm, RT). The plate was washed 4 times (300µl wash buffer), detection antibody (100µl/well) added and incubated (1h, 350rpm, RT). The plate was washed 4 times after which Avidin-HRP solution (100µl/well) was added and incubated (30min, 350rpm, RT). The plate was washed 5 times, substrate solution F (100µl/well) added and the plate incubated (10min, RT, in the dark). The reaction was stopped by the addition of stop solution (100µl/well) and the absorbance read at 450nm with a reference at 570nm. Standards and samples were run in duplicate. The minimum detection limit was 0.13 ± 0.01ng/ml. Results were analysed using an 8-point standard curve constructed from recombinant protein standards.

2.2.10 Statistical analysis

Statistical analysis was performed using GraphPad Prism software (v5.0). The unpaired t-test with Welch correction was used in *in vitro* analyses and the non-parametric Mann-Whitney test in *ex vivo* analyses to

avoid the influence of potential outlier values in the small sample size. Outlier values were calculated and excluded using the interquartile range. Data is expressed as mean ± standard error of the mean (SEM), p values of less than 0.05 were considered statistically significant.

2.3 Results

2.3.1 TB/HIV mediated increase in Foxp3 mRNA but decrease in Foxp3 protein expression

Foxp3 is indispensable for Treg lineage specification and immunosuppressive activities. We performed qPCR to evaluate the expression of Foxp3 mRNA. $TB^{+}HIV^{+}$ co-infection caused a significant 2.345 fold increase in the Foxp3 mRNA expression in PEMCs compared to a $TB^{-}HIV^{-}$ conditions (19.140 ± 0.161 vs. 17.952 ± 0.164 relative expression (relative cycle threshold, ΔCt), p=0.0022) (Figure 2.1). In contrast $TB^{+}HIV^{+}$ PEMCs showed a significant 1.661 fold decrease in Foxp3 protein (1594.222 ± 86.189 vs. 959.556 ± 127.654 PE-MFI, p=0.0008) (Figure 2.2) compared to $TB^{-}HIV^{-}$ PEMCs.

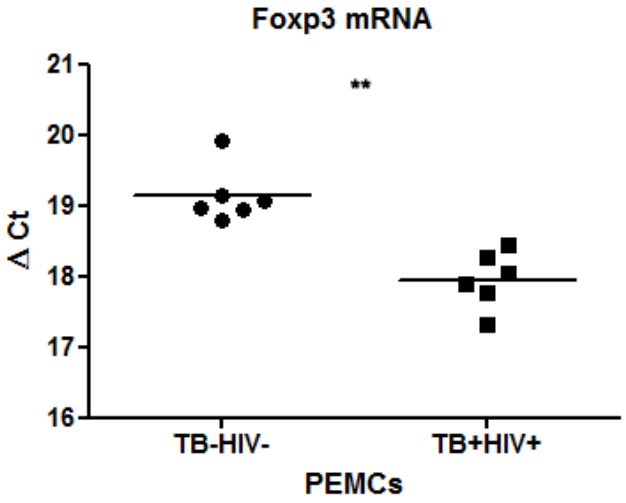

Figure 2.1: Relative expression of forkhead box P3 mRNA. Pooled $TB^{+}HIV^{+}$ pleural effusion mononuclear cells (PEMCs) showed a significant increase in forkhead box P3 (Foxp3) relative mRNA expression represented as a lower relative cycle threshold (ΔCt) (n=6 replicates in each group, p=0.0022, Mann-Whiney test). Data represents the mean.

A B

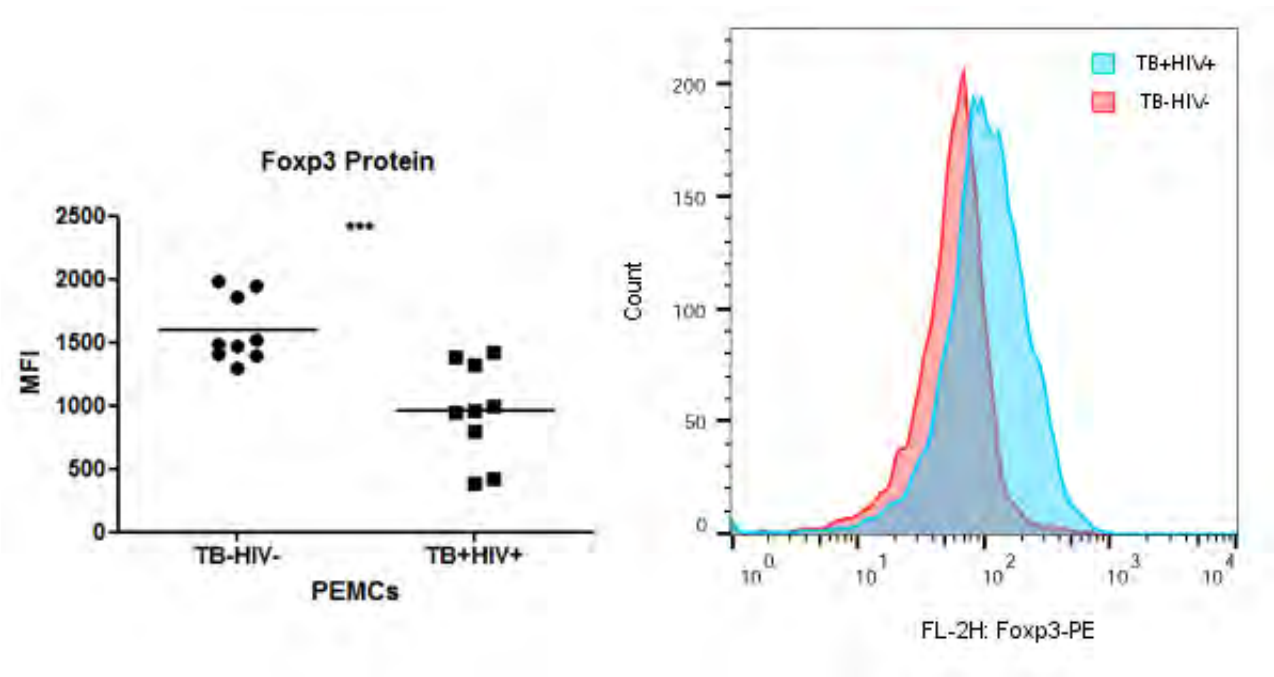

Figure 2.2: Change in forkhead box P3 relative protein expression. (A) $TB^{+}HIV^{+}$ pleural effusion mononuclear cells (PEMCs) showed a significant decrease in forkhead box P3 (Foxp3)-phycoerythrin (PE) mean fluorescence intensity (MFI) ($TB^{-}HIV^{-}$ n=5, $TB^{+}HIV^{+}$ n=5, p=0.0008, Mann Whitney test). Data represents the mean. (B) Histogram showing the shift in Foxp3-PE fluorescence in PEMCs.

2.3.2 Reduced miR-155 expression in $TB^{+}HIV^{+}$ pleurisy

MiR-155 is a direct target of Foxp3, because we observed reduced Foxp3 protein levels in co-infection we questioned whether miR-155 expression (using qPCR) was also decreased. Pooled $TB^{+}HIV^{+}$ PEMCs showed a 2.143 fold decrease in miR-155 expression (3.855 ± 0.073 vs. 4.843 ± 0.044 relative expression (ΔCt), p=0.0022) compared to $TB^{-}HIV^{-}$ PEMCs (Figure 2.3).

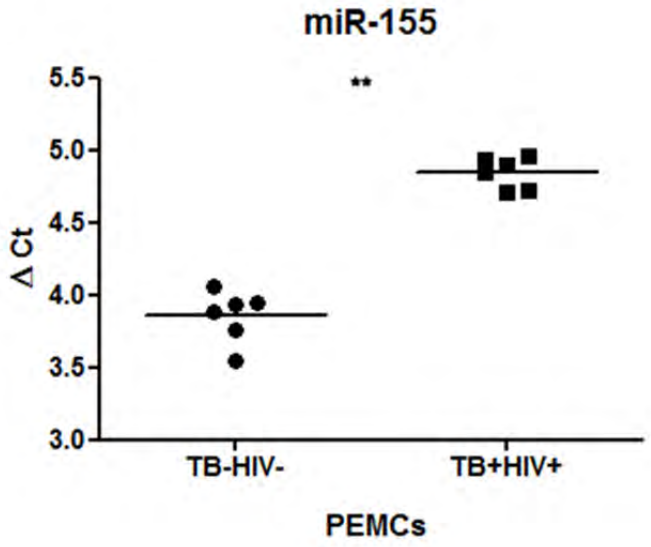

Figure 2.3: Relative expression of microRNA-155. Pooled $TB^{+}HIV^{+}$ pleural effusion mononuclear cells (PEMCs) showed a significant decrease in relative microRNA (miR)-155 expression represented as a higher relative cycle threshold (ΔCt) (n=6 replicates in each group, p=0.0022, Mann-Whiney test). Data represents the mean.

2.3.3 Increased SOCS1 expression in $TB^{+}HIV^{+}$ pleurisy

MiR-155 suppresses the translation of SOCS1, a negative regulator of IL-2 receptor signalling and inhibitor of Foxp3 expression. As we found reduced expression of Foxp3 protein and miR-155 mRNA we interrogated if this was linked by increased SOCS1 expression using western blotting. $TB^{+}HIV^{+}$ PEMCs showed a significant 3.751 fold increase in SOCS1 relative protein expression (1.481 ± 0.231 RBI vs. 5.555 ± 0.440 RBI, $p<0.0001$) compared to $TB^{-}HIV^{-}$ PEMCs (Figure 2.4).

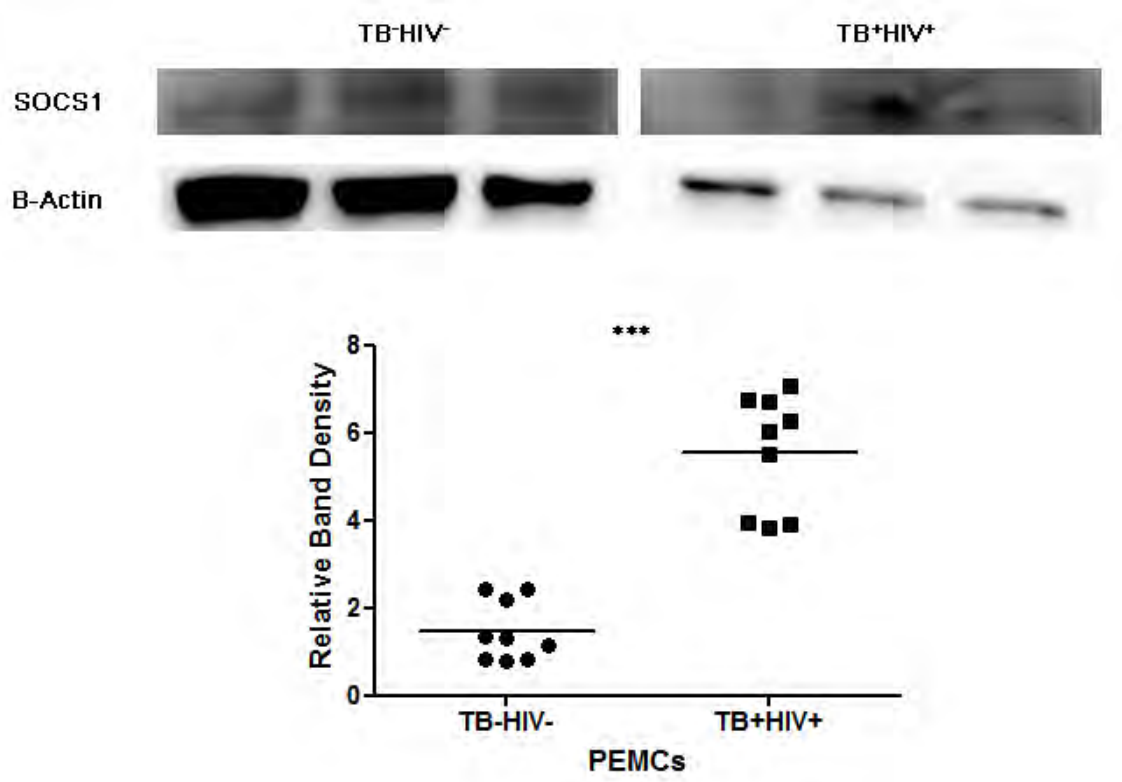

Figure 2.4: Relative protein expression of suppressor of cytokine signalling 1. Pooled $TB^{+}HIV^{+}$ pleural effusion mononuclear cells (PEMCs) showed a significantly greater relative protein expression of the 24kDa suppressor of cytokine signalling 1 (SOCS1), represented as western blot relative band density (RBI) ($n=9$ replicates in each group, $p<0.0001$, Mann-Whiney test). Data represents the mean.

2.3.4 Reduced Treg proportion in TB^+HIV^+ pleurisy

MiR-155 positively regulates Treg stability by suppressing SOCS1 translation and conferring IL-2 competitive fitness. We questioned whether a decreased expression of miR-155 and increased expression of SOCS1 resulted in loss of Treg stability. The number of Tregs in PEF was quantified by flow cytometry. The proportion of $CD4^+CD25^+Foxp3^+STAT5^+$ Tregs in TB^+HIV^+ pleurisy was significantly 21.049% (1.664 fold) lower than TB^-HIV^- controls (52.769 ± 5.413% vs. 31.720 ± 3.821%, p=0.0084) (Figure 2.5).

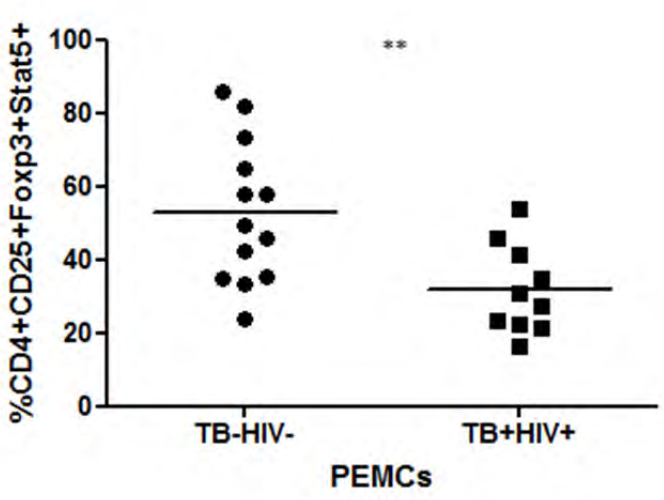

Figure 2.5: Frequency of T-regulatory lymphocytes *ex vivo*. The proportion of $CD4^+CD25^+Foxp3^+STAT5^+$ T-regulatory cells (Tregs) was significantly lower in TB^+HIV^+ pleural effusion mononuclear cells (PEMCs) (TB^-HIV^- n=7, TB^+HIV^+ n=5, p=0.0084 Mann Whitney test). Data represents the mean.

2.3.5 Lower IL-35 concentration in $TB^{+}HIV^{+}$ pleurisy

IL-35 is a suppressive Treg characteristic cytokine. We investigated whether a loss of Treg stability was associated with a loss of suppressive activity by quantifying IL-35 in PEF by ELISA. There was a significant 3.442 fold decrease in IL-35 concentration in the $TB^{+}HIV^{+}$ cohort compared to the control group (18.784 ± 4.911ng/ml vs. 5.457 ± 0.905ng/ml, p=0.0258) (Figure 2.6).

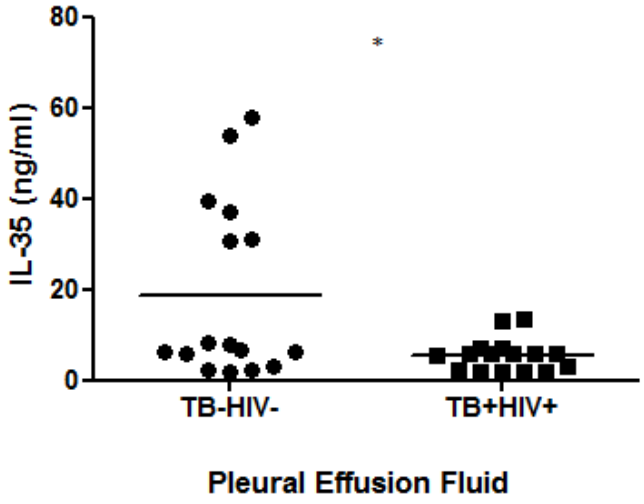

Figure 2.6: Concentration (ng/ml) of interleukin-35 in pleural effusion fluid. The $TB^{+}HIV^{+}$ cohort showed significantly lower pleural effusion fluid interleukin (IL)-35 levels than the controls ($TB^{-}HIV^{-}$ n=8, $TB^{+}HIV^{+}$ n=8, p=0.0258, Mann Whitney test). Data represents the mean.

2.3.6 Loss of Treg stability confirmed *in vitro*

To confirm our *ex vivo* results, we mimicked *in vivo* conditions by treating $CD4^+$ Tconvs with PEF conditioned CCM. In line with the *ex vivo* results, TB^+HIV^+ PEF treatment resulted in a significant 5.756% (1.206 fold) lower count of $CD4^+CD25^+Foxp3^+STAT5^+$ Tregs compared to TB^-HIV^- PEF (33.656 ± 1.719% vs. 27.900 ± 2.024%, p=0.0467) (Figure 2.7).

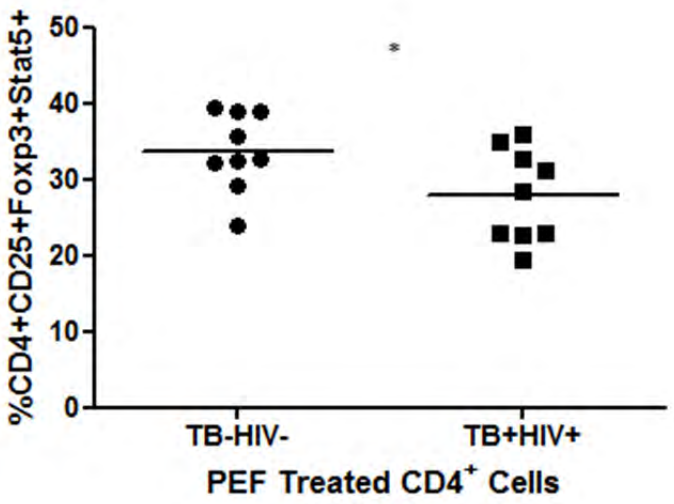

Figure 2.7: Frequency of T-regulatory lymphocytes *in vitro*. The proportion of $CD4^+CD25^+Foxp3^+STAT5^+$ T-regulatory cells (Tregs) was significantly lower in $CD4^+$ T-cells treated with TB^+HIV^+ pleural effusion fluid (PEF) conditioned CCM *in vitro* (n=9 replicates in each group, p=0.0467, unpaired t-test with Welch correction). Data represents the mean.

2.4 Discussion

Regulatory T-cells suppress the activation and function of effector T-cells to maintain immune homeostasis and limit immunopathology. Previous studies have shown a loss of Treg stability in TB and HIV independently; however, the maintenance of a stable and functional Treg pool has not been assessed in co-infection. Failure to maintain lineage identity can result in a compromised immune response; this is significant given the role of Tregs in mitigating dysregulated inflammation characteristic in TB and HIV.

MiRNAs are predominantly negative post transcriptional regulators which destabilise and degrade target mRNA or repress translation, fine tuning gene expression in pleiotropic biological pathways, including immune cell lineage commitment and homeostasis (12, 37). Computational analysis indicates that miRNAs may regulate up to 60% of all human protein coding genes (38). Altered miRNA patterns have been identified in latent and active TB (39-51) as well as HIV infection (52-57). However, a literature search shows that there has been no adequate assessment of miRNA expression in TB/HIV co-infection.

Foxp3, a transcription factor of the winged helix family, is indispensable for Treg lineage specification and immunosuppressive activities by acting at transcriptional, epigenetic and post transcriptional levels (58, 59). Our study shows a 1.661 fold decrease in Foxp3 protein expression in TB/HIV pleurisy. This is in keeping with previous studies that observed reduced Foxp3 protein in an inflammatory and infectious micro-environment (60, 61).

Foxp3 target genes that are critical in Treg homeostasis have been identified, particularly the MIR155 host gene (*MIR155HG*) (62, 63). miR-155 has been implicated in the pathogenesis of several bacterial and viral infections (11). *MIR155HG* has a Foxp3 binding site in intron 2, approximately 500 base pairs upstream of the pre-mir-155 sequence (62, 63). Foxp3-driven miR-155 expression drives the size and stability of the Treg subset through modulation of the IL-2 receptor (IL-2R) pathway (8, 10, 13). MiR-155 knockout mice exhibit a reduced frequency of Tregs in both the thymus and peripheral lymphoid tissues (8).

IL-2 is critical in Treg stabilisation, survival and maintenance in the periphery (7, 58, 64). Signalling is mediated by CD25 (IL-2Rα), constitutively expressed on Tregs, activation of Janus activated kinase (Jak)1/3 and subsequent phosphorylation of signal transducer and activator of transcription (STAT) 5 (65). In turn pSTAT5 binds to the promoter region and conserved non-coding sequence 2 (CNS2) element in the *Foxp3* locus (66), directly controlling Foxp3 heritable expression and transcriptional initiation (7). MiR-155 stabilises Tregs by binding to the 3'UTR of SOCS1, a negative regulator of IL-2R signalling, and suppressing its translation. This in turn confers competitive fitness to the Treg population allowing heightened sensitivity to IL-2 (8, 10, 13).

In support of previous TB (67) and HIV (55, 57) studies, our work showed a significant 2.143 fold reduction in miR-155 expression and 3.751 fold increase in SOCS1 protein expression during TB^+HIV^+ pleurisy, a novel observation in TB/HIV co-infection. Continuous Foxp3 expression has been shown to be indispensable for miR-155 expression (10) therefore reduced miR-155 levels are likely a result of diminished Foxp3 protein and *MIR155HG* targeting. Based on this, we propose that decreased miR-155 expression facilitates SOCS1 mediated loss of Treg stability by increasing the threshold of IL-2 signalling resulting in a reduced proportion of Tregs. A significant 21.049% lower proportion of Tregs was observed in TB^+HIV^+ pleurisy *ex vivo* indicating that the TB^+HIV^+ microenvironment is not conducive to Treg stability. This was validated by mimicking *in vivo* conditions resulting in a significant 5.756% lower frequency of Tregs *in vitro* confirming that the TB^+HIV^+ micro-environment does not maintain a stable Treg pool. These observations provide mechanistic insight into the loss of Treg stability during co-infection.

We next questioned whether a loss of Treg stability was associated with diminished suppressive activity as Tregs from miR-155 knock out models retained suppressive capacity *in vivo* and *in vitro* (8, 13, 37, 68, 69); however, the amount of Foxp3 protein has been shown to directly correlate to Treg suppressive function (70, 71). IL-35, a heterodimer of Epstein-Barr-virus-induced-gene-3 (EBI3) and IL-12p35/α, is a suppressive cytokine of the IL-12 family that is primarily produced by Tregs (4). It is not constitutively expressed (72-74) but its production is potentiated during Treg mediated active suppression of effector T-cells and is required for maximal Treg suppressive activity in mice *in vivo* and *in vitro* (4). The IL-35 receptor shares the gp130 subunit with IL-27 receptor and the IL-12Rβ2 with the IL-12 receptor (75, 76), ligation of which results in auto-phosphorylation of Jak1/2 with subsequent phosphorylation of STAT1 and 4 (75). IL-35 potently inhibits effector T- and B-cells, particularly Th1 and Th17 lineages, by the expansion of Tregs and production of IL-10 (77). Furthermore, IL-35 has the ability to convert naïve $CD4^+$ T-cells into suppressive IL-35 producing T-cells termed $iT_{reg}35$, that lack expression of Foxp3, TGF-β and IL-10, by the process of infectious tolerance (73).

To the best of our knowledge this is the first study to assess IL-35 in TB, HIV or co-infection. We showed a 3.442 fold decrease in IL-35 concentration in TB^+HIV^+ pleurisy thus implying a Treg functional deficit. EBI3 is a downstream target of Foxp3 explaining the preferential IL-35 production by Tregs (4) and therefore reduced Foxp3 protein would likely result in reduced EBI3 transcription and subsequently IL-35 concentrations.

A loss of Treg stability is of benefit to both pathogens. Tregs have been shown to down regulate antigen specific MTB responses preventing tissue injury by resolving granulomatous inflammation; however, hyperactive inflammation results in caseous necrosis, cavitation and transmission of bacilli, hallmarks of active infection and the ultimate goal of the bacterial life cycle. In terms of HIV, loss of Treg immune suppression inhibits HIV-specific T-cell responses contributing to immune deficiency, heightened chronic immune activation and viral replication (78, 79).

Notably, despite a significant decrease in Foxp3 protein expression, a 2.345 fold increase in Foxp3 mRNA was observed in TB/HIV pleurisy. These disparate transcript and protein results indicate a potent pro-Treg signal at the molecular level which is circumvented by post transcriptional and/or translational modifications (80). Research into the post translational negative regulation of Foxp3 protein expression has identified inflammatory signals (IL-6, TNF-α, CCL3) that mediate Foxp3 polyubiquitination and subsequent proteasome-mediated degradation (61, 81-84). Our previous work showed a significantly increased concentration of TNF-α in $TB^{+}HIV^{+}$ pleural fluid compared to a $TB^{-}HIV^{-}$ cohort prompting further investigation (manuscript in preparation). In this way the extracellular environment may regulate cellular intrinsic transcriptional programs and modulate Treg stability and functionality through modulation of Foxp3 polyubiquitination (80). Furthermore, in pathological settings Foxp3 mRNA is negatively regulated by miR-31 (8), miR-15a (85), miR-16 (85), miR-24 (86) and miR-210 (86, 87). The role of poly ubiquitination and miRNA regulation in Foxp3 expression needs to be further investigated in TB/HIV co-infection.

2.5 Concluding Remarks

$TB^{+}HIV^{+}$ pleurisy induces reduced Foxp3 protein expression and loss of Treg stability *in vitro* and *ex vivo* due to altered miR-155/SOCS1 dynamics. This was associated with diminished IL-35 levels implying a Treg functional deficit. A decreased functional Treg pool may contribute to the pathogenesis of TB and HIV by an excessive and dysregulated immune response.

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References

1. WHO. Global tuberculosis report. Geneva, Switzerland: World Health Organisation, 2014.

2. Chatterjee D, Pramanik AK. Tuberculosis in the African continent: A comprehensive review. Pathophysiol 2015; 22(1):73-83.

3. AIDSinfo online database [Internet]. UNAIDS. 2014 [cited 2015-10-14]. Available from: http://aidsinfo.unaids.org/.

4. Collison LW, Workman CJ, Kuo TT, Boyd K, Wang Y, Vignali KM, et al. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. Nature 2007; 450(7169):566-569.

5. Whiteside TL. Regulatory T-cell subsets in human cancer: Are they regulating for or against tumor progression? Cancer Immunol Immunother 2014; 63(1):67-72.

6. Holmes D, Jiang Q, Zhang L, Su L. Foxp3 and Treg cells in HIV-1 infection and immuno-pathogenesis. Immunol Res 2008; 41(3):248-266.

7. Luo CT, Li MO. Transcriptional control of regulatory T-cell development and function. Trends Immunol 2013; 34(11):531-539.

8. Kohlhaas S, Garden OA, Scudamore C, Turner M, Okkenhaug K, Vigorito E. Cutting edge: The Foxp3 target miR-155 contributes to the development of regulatory T-cells. J Immunol 2009; 182(5):2578-2582.

9. Chen L, Ma H, Hu H, Gao L, Wang X, Ma J, et al. Special role of Foxp3 for the specifically altered microRNAs in Regulatory T-cells of HCC patients. BMC Cancer 2014; 14:489-498.

10. Lu LF, Thai TH, Calado DP, Chaudhry A, Kubo M, Tanaka K, et al. Foxp3-dependent microRNA155 confers competitive fitness to regulatory T-cells by targeting SOCS1 protein. Immunity 2009; 30(1):80-91.

11. Eulalio A, Schulte L, Vogel J. The mammalian microRNA response to bacterial infections. RNA Biol 2012; 9(6):742-750.

12. Jeker LT, Marone R. Targeting microRNAs for immunomodulation. Curr Opin Pharmacol 2015; 23:25-31.

13. Yao R, Ma Y-L, Liang W, Li H-H, Ma Z-J, Yu X, et al. MicroRNA-155 modulates Treg and Th17 cells differentiation and Th17 cell function by targeting SOCS1. PLoS One 2012; 7(10):e46082.

14. Vigorito E, Kohlhaas S, Lu D, Leyland R. miR-155: An ancient regulator of the immune system. Immunol Rev 2013; 253(1):146-157.

15. Wang GQ, Yang CL, Yue DF, Pei LH, Zhong H, Niu JX. The changes and its significance of Th17 and Treg cells and related cytokines in patients with tuberculosis pleurisy. Allergy Asthma Clin Immunol 2014; 10(1):28-33.

16. Nemeth J, Winkler H-M, Boeck L, Adegnika AA, Clement E, Mve TM, et al. Specific cytokine patterns of pulmonary tuberculosis in central Africa. Clin Immunol 2011; 138(1):50-59.

17. Chiacchio T, Casetti R, Butera O, Vanini V, Carrara S, Girardi E, et al. Characterization of regulatory T-cells identified as $CD4^{+}CD25^{high}CD39^{+}$ in patients with active tuberculosis. Clin Exp Immunol 2009; 156(3):463-470.

18. Pang H, Yu Q, Guo B, Jiang Y, Wan L, Li J, et al. Frequency of regulatory T-cells in the peripheral blood of patients with pulmonary tuberculosis from Shanxi Province, China. PLoS One 2013; 8(6):e65496.

19. Guyot-Revol V, Innes JA, Hackforth S, Hinks T, Lalvani A. Regulatory T-cells are expanded in blood and disease sites in patients with tuberculosis. Am J Respir Crit Care Med 2006; 173(7):803-810.

20. Marin ND, Paris SC, Velez VM, Rojas CA, Rojas M, Garcia LF. Regulatory T-cell frequency and modulation of IFN-γ and IL-17 in active and latent tuberculosis. Tuberculosis 2010; 90(4):252-261.

21. Ndhlovu LC, Loo CP, Spotts G, Nixon DF, Hecht FM. Foxp3 expressing $CD127^{lo}$ $CD4^{+}$ T-cells inversely correlate with $CD38^{+}CD8^{+}$ T-cell activation levels in primary HIV-1 infection. J Leukoc Biol 2008; 83(2):254-262.

22. Bandera A, Ferrario G, Saresella M, Marventano I, Soria A, Zanini F, et al. $CD4^{+}$ T-cell depletion, immune activation and increased production of regulatory T-cells in the thymus of HIV-infected individuals. PLoS One 2010; 5(5):e10788.

23. Shaw JM, Hunt PW, Critchfield JW, McConnell DH, Garcia JC, Pollard RB, et al. Increased frequency of regulatory T-cells accompanies increased immune activation in rectal mucosa of HIV-positive non-controllers. J Virol 2011; 85(21):11422-11434.

24. Montes M, Sanchez C, Lewis DE, Graviss EA, Seas C, Gotuzzo E, et al. Normalization of $FoxP3^{+}$ regulatory T-cells in response to effective antiretroviral therapy. J Infect Dis 2011; 203(4):496-499.

25. Bi X, Suzuki Y, Gatanaga H, Oka S. High frequency and proliferation of $CD4^{+}Foxp3^{+}$ Treg in HIV-1-infected patients with low CD4 counts. Eur J Immunol 2009; 39(1):301-309.

26. Suchard MS, Mayne E, Green VA, Shalekoff S, Donninger SL, Stevens WS, et al. Foxp3 expression is upregulated in $CD4^+$ T-cells in progressive HIV-1 infection and is a marker of disease severity. PLoS One 2010; 5(7):e11762.

27. Hunt PW, Landay AL, Sinclair E, Martinson JA, Hatano H, Emu B, et al. A low T-regulatory cell response may contribute to both viral control and generalized immune activation in HIV controllers. PLoS One 2011; 6(1):e15924.

28. Schulze zur Wiesch J, Thomssen A, Hartjen P, Tóth I, Lehmann C, Meyer-Olson D, et al. Comprehensive analysis of frequency and phenotype of T-regulatory cells in HIV Infection: CD39 expression of $Foxp3^+$ T-regulatory cells correlates with progressive disease. J Virol 2011; 85(3):1287-1297.

29. Presicce P, Orsborn K, King E, Pratt J, Fichtenbaum CJ, Chougnet CA. Frequency of circulating regulatory T-cells increases during chronic HIV infection and is largely controlled by highly active antiretroviral therapy. PLoS One 2011; 6(12):e28118.

30. Barker RD. Clinical tuberculosis. Medicine 2012; 40(6):340-345.

31. Chaisson RE, Schecter GF, Theuer CP, Rutherford GW, Echenberg DF, Hopewell PC. Tuberculosis in patients with the acquired immunodeficiency syndrome: Clinical features, response to therapy and survival. Am Rev Respir Dis 1987; 136(3):570-574.

32. TB DOTS Strategy Co-ordination Directorate. National Tuberculosis Management Guidelines. Republic of South Africa: National Department of Health 2014.

33. Porcel JM. Tuberculous pleural effusion. Lung 2009; 187(5):263-270.

34. Gideon HP, Phuah J, Myers AJ, Bryson BD, Rodgers MA, Coleman MT, et al. Variability in tuberculosis granuloma T-cell responses exists, but a balance of pro-and anti-inflammatory cytokines is associated with sterilization. PLoS Pathog 2015; 11(1):e1004603.

35. Toossi Z, Hirsch C, Wu M, Mayanja-Kizza H, Baseke J, Thiel B. Distinct cytokine and regulatory T-cell profile at pleural sites of dual HIV/tuberculosis infection compared to that in the systemic circulation. Clin Exp Immunol 2011; 163(3):333-338.

36. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25(4):402-408.

37. Dai R, Ahmed SA. MicroRNA, a new paradigm for understanding immunoregulation, inflammation, and autoimmune diseases. Transl Res 2011; 157(4):163-179.

38. Friedman RC, Farh KK-H, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 2009; 19(1):92-105.

39. Spinelli SV, Diaz A, D'Attilio L, Marchesini MM, Bogue C, Bay ML, et al. Altered microRNA expression levels in mononuclear cells of patients with pulmonary and pleural tuberculosis and their relation with components of the immune response. Mol Immunol 2013; 53(3):265-269.

40. Yi Z, Fu Y, Ji R, Li R, Guan Z. Altered microRNA signatures in sputum of patients with active pulmonary tuberculosis. PLoS One 2012; 7(8):e43184.

41. Yi Z, Fu Y, Wu X, Li J, Xu F. Circulating microRNAs in patients with active pulmonary tuberculosis. J Clin Microbiol 2011; 49:e545911.

42. Berry MP, Graham CM, McNab FW, Xu Z, Bloch SA, Oni T, et al. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. Nature 2010; 466(7309):973-977.

43. Maertzdorf J, Repsilber D, Parida SK, Stanley K, Roberts T, Black G, et al. Human gene expression profiles of susceptibility and resistance in tuberculosis. Genes Immun 2011; 12(1):15-22.

44. Wang C, Yang S, Sun G, Tang X, Lu S, Neyrolles O, et al. Comparative miRNA expression profiles in individuals with latent and active tuberculosis. PloS One 2011; 6(10):e25832.

45. Liu Y, Wang X, Jiang J, Cao Z, Yang B, Cheng X. Modulation of T cell cytokine production by miR-144* with elevated expression in patients with pulmonary tuberculosis. Mol Immunol 2011; 48(9–10):1084-1090.

46. Maertzdorf J, Weiner J, Mollenkopf H-J, Network T, Bauer T, Prasse A, et al. Common patterns and disease-related signatures in tuberculosis and sarcoidosis. Proc Natl Acad Sci U S A 2012; 109(20):7853-7858.

47. Wu J, Lu C, Diao N, Zhang S, Wang S, Wang F, et al. Analysis of microRNA expression profiling identifies miR-155 and miR-155* as potential diagnostic markers for active tuberculosis: A preliminary study. Hum Immunol 2012; 73(1):31-37.

48. Marín ND, París SC, Rojas M, García LF. Functional profile of $CD4^+$ and $CD8^+$ T-cells in latently infected individuals and patients with active TB. Tuberculosis 2013; 93(2):155-166.

49. Zhang H, Sun Z, Wei W, Liu Z, Fleming J, Zhang S, et al. Identification of serum microRNA biomarkers for tuberculosis using RNA-seq. PLoS One 2014; 9(2):e88909.

50. Miotto P, Mwangoka G, Valente IC, Norbis L, Sotgiu G, Bosu R, et al. miRNA signatures in sera of patients with active pulmonary tuberculosis. PLoS One 2013; 8(11):e80149.

51. Xu Z, Zhou A, Ni J, Zhang Q, Wang Y, Lu J, et al. Differential expression of miRNAs and their relation to active tuberculosis. Tuberculosis 2015; 95(4):395-403.

52. Adoro S, Cubillos-Ruiz JR, Chen X, Deruaz M, Vrbanac VD, Song M, et al. IL-21 induces antiviral microRNA-29 in CD4 T-cells to limit HIV-1 infection. Nat Commun 2015; 6:e7562.

53. Narla AV, Bhakta N, Freedman J, Tanriverdi K, Maka K, Deeks S, et al. Unique circulating microRNA profiles and endothelial function in HIV infection. J Am Coll Cardiol 2015; 65(10S):eA1478.

54. Yang Z, Yang J, Wang J, Lu X, Jin C, Xie T, et al. Identify potential regulators in HIV-1 latency by joint microRNA and mRNA analysis. Cell Physiol Biochem 2015; 36(2):569-584.

55. Sun G, Li H, Wu X, Covarrubias M, Scherer L, Meinking K, et al. Interplay between HIV-1 infection and host microRNAs. Nucleic Acids Res 2012; 40(5):2181-2196.

56. Witwer KW, Watson AK, Blankson JN, Clements JE. Relationships of PBMC microRNA expression, plasma viral load, and $CD4^+$ T-cell count in HIV-1-infected elite suppressors and viremic patients. Retrovirology 2012; 9(5):1-15.

57. Bignami F, Pilotti E, Bertoncelli L, Ronzi P, Gulli M, Marmiroli N, et al. Stable changes in $CD4^+$ T-lymphocyte miRNA expression after exposure to HIV-1. Blood 2012; 119(26):6259-6267.

58. Huehn J, Beyer M. Epigenetic and transcriptional control of $Foxp3^+$ regulatory T-cells. Semin Immunol 2015; 27(1):10-18.

59. Arvey A, van der Veeken J, Samstein RM, Feng Y, Stamatoyannopoulos JA, Rudensky AY. Inflammation-induced repression of Foxp3-bound chromatin in regulatory T-cells. Nat Immunol 2014; 15(6):580-587.

60. Miyao T, Floess S, Setoguchi R, Luche H, Fehling Hans J, Waldmann H, et al. Plasticity of $Foxp3^+$ T-cells reflects promiscuous Foxp3 expression in conventional T-cells but not reprogramming of regulatory T-cells. Immunity 2012; 36(2):262-275.

61. Chen L, Wu J, Pier E, Zhao Y, Shen Z. mTORC2-PKBα/Akt1 serine 473 phosphorylation axis is essential for regulation of Foxp3 stability by chemokine CCL3 in psoriasis. J Invest Dermatol 2013; 133(2):418-428.

62. Marson A, Kretschmer K, Frampton GM, Jacobsen ES, Polansky JK, MacIsaac KD, et al. Foxp3 occupancy and regulation of key target genes during T-cell stimulation. Nature 2007; 445(7130):931-935.

63. Zheng Y, Josefowicz SZ, Kas A, Chu T-T, Gavin MA, Rudensky AY. Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T-cells. Nature 2007; 445(7130):936-940.

64. Zheng Y, Josefowicz S, Chaudhry A, Peng XP, Forbush K, Rudensky AY. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. Nature 2010; 463(7282):808-812.

65. Bensinger SJ, Walsh PT, Zhang J, Carroll M, Parsons R, Rathmell JC, et al. Distinct IL-2 receptor signaling pattern in $CD4^{+}CD25^{+}$ regulatory T-cells. J Immunol 2004; 172(9):5287-5296.

66. Zorn E, Nelson EA, Mohseni M, Porcheray F, Kim H, Litsa D, et al. IL-2 regulates Foxp3 expression in human $CD4^{+}CD25^{+}$ regulatory T-cells through a STAT-dependent mechanism and induces the expansion of these cells *in vivo*. Blood 2006; 108(5):1571-1579.

67. Rajaram MV, Ni B, Morris JD, Brooks MN, Carlson TK, Bakthavachalu B, et al. *Mycobacterium tuberculosis* lipomannan blocks TNF biosynthesis by regulating macrophage MAPK-activated protein kinase 2 (MK2) and microRNA miR-125b. Proc Natl Acad Sci U S A 2011; 108(42):17408-17413.

68. O'Connell RM, Kahn D, Gibson WSJ, Round JL, Scholz RL, Chaudhuri AA, et al. MicroRNA-155 promotes autoimmune inflammation by enhancing inflammatory T-cell development. Immunity 2010; 33(4):607-619.

69. Murugaiyan G, Beynon V, Mittal A, Joller N, Weiner HL. Silencing microRNA-155 ameliorates experimental autoimmune encephalomyelitis. J Immunol 2011; 187(5):2213-2221.

70. Wan YY, Flavell RA. Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. Nature 2007; 445(7129):766-770.

71. Williams LM, Rudensky AY. Maintenance of the Foxp3-dependent developmental program in mature regulatory T-cells requires continued expression of Foxp3. Nat Immunol 2007; 8(3):277-284.

72. Li X, Mai J, Virtue A, Yin Y, Gong R, Sha X, et al. IL-35 is a novel responsive anti-inflammatory cytokine: A new system of categorizing anti-inflammatory cytokines. PloS One 2012; 7(3):e33628.

73. Collison LW, Chaturvedi V, Henderson AL, Giacomin PR, Guy C, Bankoti J, et al. IL-35-mediated induction of a potent regulatory T-cell population. Nature Immunol 2010; 11(12):1093-1101.

74. Dixon KO, van der Kooij SW, Vignali DAA, van Kooten C. Human tolerogenic dendritic cells produce IL-35 in the absence of other IL-12 family members. Eur J Immunol 2015; 45(6):1736-1747.

75. Collison LW, Delgoffe GM, Guy CS, Vignali KM, Chaturvedi V, Fairweather D, et al. The composition and signaling of the IL-35 receptor are unconventional. Nat Immunol 2012; 13(3):290-299.

76. Collison LW, Vignali DA. Interleukin-35: Odd one out or part of the family? Immunol Rev 2008; 226(1):248-262.

77. Choi J, Leung PC, Bowlus C, Gershwin ME. IL-35 and autoimmunity: A comprehensive perspective. Clin Rev Allergy Immunol 2015; 49(3):327-332.

78. Chevalier MF, Weiss L. The split personality of regulatory T-cells in HIV infection. Blood 2013; 121(1):29-37.

79. Munier CM, Kelleher AD, Kent SJ, De Rose R. The role of T-cell immunity in HIV-1 infection. Curr Opin Virol 2013; 3(4):438-446.

80. van Loosdregt J, Coffer PJ. Post-translational modification networks regulating Foxp3 function. Trends Immunol 2014; 35(8):368-378.

81. Gao Y, Tang J, Chen W, Li Q, Nie J, Lin F, et al. Inflammation negatively regulates Foxp3 and regulatory T-cell function via DBC1. Proc Natl Acad Sci U S A 2015; 112(25):3246-3254.

82. van Loosdregt J, Fleskens V, Fu J, Brenkman AB, Bekker CP, Pals CE, et al. Stabilization of the transcription factor Foxp3 by the deubiquitinase USP7 increases Treg-cell-suppressive capacity. Immunity 2013; 39(2):259-271.

83. Chen Z, Barbi J, Bu S, Yang H-Y, Li Z, Gao Y, et al. The ubiquitin ligase Stub1 negatively modulates regulatory T-cell suppressive activity by promoting degradation of the transcription factor Foxp3. Immunity 2013; 39(2):272-285.

84. Nie H, Zheng Y, Li R, Guo TB, He D, Fang L, et al. Phosphorylation of Foxp3 controls regulatory T-cell function and is inhibited by TNF-α in rheumatoid arthritis. Nat Med 2013; 19(3):322-328.

85. Liu X, Robinson SN, Setoyama T, Tung SS, D'Abundo L, Shah MY, et al. Foxp3 is a direct target of miR15a/16 in umbilical cord blood regulatory T-cells. Bone Marrow Transplant 2014; 49:793-799.

86. Badran B, Kazan H, Burny A. Human $CD4^{+}CD25^{+}CD127^{low}$ regulatory T-cells: Transcriptional profile, microRNA signature and regulation of Foxp3 and CTLA-4 expression by differentially expressed microRNAs. J Acquir Immun Defic Syndr 2013; 62:70-71.

87. Zhao M, Wang L-t, Liang G-p, Zhang P, Deng X-j, Tang Q, et al. Up-regulation of microRNA-210 induces immune dysfunction via targeting Foxp3 in $CD4^{+}$ T-cells of psoriasis vulgaris. Clin Immunol 2014; 150(1):22-30.

CHAPTER 3

TB/HIV Pleurisy Reduces Th17 Lymphocyte Proportion Independent of the Cytokine Microenvironment

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Abstract

T-helper (Th) 17 cells are a pro-inflammatory subset of $CD4^+$ effector T-cells critical in mucosal immunity. Imbalances in Th17 cell proportion have been implicated in the pathogenesis of several diseases; however, this has not been adequately explored in tuberculosis (TB) and human immunodeficiency virus (HIV) co-infection. Since Th17 cells are predominantly mucosally associated, we assessed Th17 proportion and associated microenvironment in pleural effusions from patients co-infected with TB/HIV. Our results show that TB^+HIV^+ pleurisy results in significantly reduced frequency of $CD4^+IL\text{-}17^+RORC^+STAT3^+$ Th17 cells compared to TB^-HIV^- *ex vivo* (p=0.0054) and was confirmed in conditioned media studies *in vitro* (p=0.0001). This was not associated with alterations in Th17 polarising cytokines interleukin (IL)-6, IL-21 and IL-23 or changes in Th17 signature cytokines IL-17A and F. However, the mRNA expression of Th17 signaling molecules, IL-6 (p=0.0022), IL-6R (p=0.0247), IL-1β (p=0.0022) and signal transducer and activator (STAT) 3 (p=0.0022) were significantly upregulated. Notably, TB^+HIV^+ pleural fluid contained significantly higher concentrations of IL-1β (p=0.0008), IL-22 (p=0.0115), IL-31 (p=0.0210), TNF-α (p=0.0251) and IFN-γ (p=0.0026) than TB^-HIV^- pleural fluid *ex vivo*. Taken together, this suggests a reduced portion of Th17 lymphocytes in TB/HIV pleurisy is independent of locally mediated cytokine polarisation.

Keywords

Tuberculosis, HIV, co-infection, Th17, IL-1β, IL-17

3.1 Introduction

Th17 cells are a pro-inflammatory subset of $CD4^+$effector T-cells [1] characterised by the production of IL-17A/F, surface expression of C-C chemokine receptor (CCR)6 and lineage specific transcription factor RAR-related orphan rector (ROR)C [2]. Th17 cells predominantly reside at mucosal surfaces where they play a pivotal role in inflammatory reactions and protective immunity against intra-[3, 4] and extracellular [5, 6] bacterial and fungal infections. An imbalance in Th17 proportion has been implicated in the pathogenesis of TB.

Approximately one third of the global population suffers from TB; a granulomatous disease spread by the airborne pathogen *Mycobacterium tuberculosis* (MTB). MTB cell wall component, trehalose-6-6'-dimycolate (TDM/cord factor) has been shown to induce the production of Th17 differentiating cytokines from antigen presenting cells through the C-type lectin Mincle pathway [7]. Conflicting studies have noted an expansion of the Th17/IL-17 axis in the peripheral blood [8-10] and pleural fluid [9, 10], and a reduced frequency of Th17 cells in peripheral blood [11-15] and broncho-alveolar lavage (BAL) fluid [11] during active MTB infection, with correlation to disease severity [9].

HIV is the most powerful risk factor predisposing for active TB infection which remains the number one communicable HIV-related cause of death [16]. In South Africa, approximately 62% of TB patients are HIV positive [17]. HIV infection is characterised by chronic immune activation and systemic depletion of $CD4^+$ T-cells. The preferential loss of Th17 cells from the mucosa and peripheral blood with reduced levels of IL-17 during HIV infection is well documented. This loss is not completely restored by antiretroviral therapy [18-23] suggesting that HIV infection interferes in the generation of Th17 cells [24].

Th17 lymphocytes are significant in the pathogenesis of TB and a potential predictor of disease severity; however, data on the proportion and role of Th17 cells in TB are conflicting. Furthermore, the confounding effect of HIV co-infection on Th17 frequency has not been investigated. Notably, 40-80% of HIV-related TB is extrapulmonary [25, 26] the most common form being TB pleurisy [27] and the immunological mechanisms of which are not accurately reflected systemically [28]. Taken together with the mucosal predominance of Th17 cells, this study characterised the proportion of Th17 lymphocytes and the cytokine microenvironment in TB/HIV pleurisy *in vitro* and *ex vivo*.

3.2 Methods

3.2.1 Study population

This study protocol was approved by the institutional Biomedical Research Ethics Committee (BF170/11). Participants were recruited at the Department of Pulmonary and Critical Care, Inkosi Albert Luthuli Central Hospital and the Department of Internal Medicine, Prince Mshiyeni Memorial Hospital (Durban, South Africa). Newly diagnosed TB patients presenting with a TB pleural effusion and concurrent HIV-infection (n=13) were enrolled. Active TB infection was confirmed by clinical diagnosis with bacterial or radiographic evidence; AFB culture positive sputum or pleural fluid microscopy (Gram, Zhiel-Neelsen, Auramine), MTB DNA (Gene Xpert), pleural histology and pleural fluid cytology. The matched control group was composed of patients presenting with a non-infectious pleural effusion (malignancy, cardiac failure, end stage renal disease) and confirmed HIV seronegative (n=8). Exclusion criteria included anti-tuberculosis or anti-retroviral therapy, immunomodulatory treatment and conditions. Blood and pleural fluid were sampled by routine venesection and diagnostic pleural tap respectively, after informed consent was obtained from each participant. A summary of the demographics and clinical parameters of the study population are represented in Table 3.1.

Table 3.1: Summary of demographic and clinical parameters of the study population

Parameter	TB^-HIV^- *n*=8	TB^+HIV^+ *n*=13
Age, years, median (range)	58 (46-78)	36 (18-64)
Gender		
Male	6 (75)	6 (46)
Female	2 (25)	7 (54)
Race		
African	5 (63)	13 (100)
Indian	3 (37)	0
TB diagnostic criteria		
Histopathology		1 (8)
Culture positive		8 (62)
Smear positive		3 (23)
PCR-MTBDR		8 (62)
Clinical with radiography		3 (23)
Resistance to any one first line TB drug		1 (8)
MDR-TB		1 (8)
XDR-TB		0
CD4 count, mm3, mean (range)		169 (9-463)
Past history of TB infection	0	1 (8)
Current smoker	3 (37)	2 (15)
Ex-smoker	1 (13)	0
Alcohol use	2 (25)	3 (23)
Definitive cause of PE		
Tuberculosis	0	13 (100)
Malignancy	5 (63)	0
End stage renal disease	2 (25)	0
Heart failure	1 (13)	0
Co-Morbidities		
Diabetes mellitus	3 (37)	0
Hypertension	5 (63)	1 (8)
Hepatitis B	0	2 (15)

Values expressed as n (%) unless otherwise stated

3.2.2 Pleural effusion fluid and mononuclear cell isolation

Cell free pleural effusion fluid (PEF), pleural effusion mononuclear cells (PEMCs) and peripheral blood mononuclear cells (PBMCs) were extracted from heparinised pleural effusion fluid and blood by differential gradient centrifugation within 2 hours (h) of pleural tap/venipuncture. Briefly, 5 ml of sample was layered onto equivolume Histopaque-1077 (catalogue number 10771-500, Sigma, USA) and centrifuged (1,491rpm, 30 minutes (min), room temperature (RT)). PEF, represented as the uppermost layer, was aspirated and centrifuged (10,000rpm, 10min, 4°C) to remove any cellular constituents. Harvested PEF was pooled from 5 patients in each group equally and stored at -80°C until further use. Buffy coats containing mononuclear cells were aspirated and rinsed in 0.1M phosphate buffered saline (PBS; 1,491rpm, 20min, RT).

3.2.3 $CD4^+$ T-cell isolation

For the *in vitro* component of the study, $CD4^+$ conventional T-lymphocytes (Tconvs) were purified by negative magnetic selection from PBMCs of a healthy BCG vaccinated volunteer (10ml, EDTA, one blood draw), using the Human CD4 T-Lymphocyte Enrichment Set-DM (catalogue number 557939, BD Biosciences, USA), according to the manufacturer's instructions. PBMCs were labelled with biotinylated monoclonal antibodies (5µl/10×10^6 cells, 15min, RT) against antigens on erythrocytes, platelets and non-$CD4^+$ peripheral leukocytes. The cells were washed (iMag Buffer, 1,491rpm, 20min) and incubated with streptavidin conjugated magnetic nanoparticles (5µl/10×10^6 cells, 30min, RT). The cell suspension was placed in a magnetic field (8min, iMagnet; BD Biosciences, USA) and the enriched $CD4^+$ T-cell supernatant aspirated. Negative selection of the positive fraction was performed three times to increase the yield of the enriched fraction. The purity of the enriched fraction was increased by further negative selection and assessed by flow cytometry.

3.2.4 Cell culture and treatment

Healthy $CD4^+$ T-cells were seeded in triplicate (8×10^4cells/well, U-bottomed microplate) in PEF conditioned complete culture medium (CCM; RPMI 1640, 10% foetal calf serum (FCS), 1% Penstrep-Fungizone, 1% L-glutamine, final concentrations) in a 1:1 ratio (24h, 37°C, 5% CO_2). Treatments included TB^-HIV^- PEF and TB^+HIV^+ PEF. Concurrently, cells were activated with Dynabeads Human T-activator CD3/CD28 bead solution (catalogue number 11131D, Gibco, Norway) (2µl/well). Following incubation, cells were harvested, washed once (0.1M PBS, 1,491rpm, 20min, RT) and the activation beads removed by magnetic selection (1min, iMagnet). The supernatants were stored at -80°C for downstream cytokine profiling and the cells immediately prepared for flow cytometric staining.

3.2.5 Flow cytometric analysis

For surface staining, $2.5x10^5$ cells were re-suspended in staining buffer (25µl, 0.1M PBS containing 1% heat inactivated FCS, 0.09% w/v sodium azide) and labelled with fluorescein isothiocyanate (FitC) anti-CD4 (1:5, catalogue number 555346) (20mins, 4°c, in the dark). For intracellular staining, cells were fixed in 4% paraformaldehyde (500µl, 30min, RT), washed (staining buffer; 1,491rpm, 20min, RT), permeabilized in 75% cold methanol (500µl, 10min, -20°C) and washed again. Cells were stained with the appropriate intracellular antibodies; peridin-chlorophyll-cyanin 5.5 (PerCP-Cy5.5) anti-IL17A (1:20, catalogue number 560799), phycoerythrin (PE) anti-RORC2 (1:40 catalogue number IC6006P) and Alex Fluor® 647 anti-pSTAT3 (1:5 catalogue number 557815) (60min, RT, in the dark). Analysis was performed using the Accuri C6 flow cytometer (BD Biosciences, USA) and data analysed using FlowJo vX (10.0.7r2). Samples were run in triplicate and 30,000 events acquired. Antibodies were purchased from BD Biosciences, USA, with the exception of anti-RORC2 which was purchased from R&D Systems.

3.2.6 Molecular profiling

Total RNA was extracted from PEMCs using Qiazol Lysis Reagent (Qiagen, USA) following an in-house protocol. Briefly, cells were added to Qiazol Lysis Reagent (1:1) and incubated at RT (5min) and subsequently frozen (-80°C, overnight). Chloroform (200µl) was added, incubated at RT (3min) and centrifuged (8,200rpm, 15min, 4°C). Isopropanol (500µl) was added to the aqueous phase and frozen (-80°C, overnight). The samples were centrifuged (8,200rpm, 20min, 4°C) and the RNA pellet washed in cold ethanol (75%, 1ml). The samples were then centrifuged (6,400rpm, 15min, 4°C), the ethanol aspirated and the RNA pellet re-suspended in 15µl of nuclease free water. Total RNA was quantified on a Nanodrop™ 2000 UV-Vis Spectrophotometer (Thermoscientific, USA) and purity assessed using the A_{260}/A_{280} ratio.

Copy DNA (cDNA) was synthesised using the RT^2 First Strand Kit (catalogue number 330401, Qiagen, USA) according to the manufacturer's instructions. Briefly, 1µg of RNA pooled equally from 5 patients per group was added to a genomic DNA elimination mix (5 × gDNA elimination buffer, H_20) to a total volume of 10µl and incubated at 42°C (5min) followed by 4°C (1min). Subsequently, reverse transcription mix (10µl, 5 × RT buffer 3, primer and external control mix, RT enzyme mix, H_20) was added and incubated at 42°C (15min) followed by 95°C (5min), on the GeneAmp® PCR System 9700 (Applied Biosystems, USA), to produce cDNA.

Real-time PCR was used to quantify STAT3, IL-6, IL-6 receptor (R) and IL-1β mRNA levels. A 25µl reaction consisted of 12.5µl RT^2 SYBRGreen Masterrmix (catalogue number 330500, Qiagen, United States), 8.5µl nuclease free water, 2µl cDNA and 1µl sense and anti-sense primers. Primer sequences: STAT3 sense 5'- TCTCAACTTCAGACCCGTCAACA-3' anti-sense 5'-ACAGCTCCACGATTCTCTCCTCC-3' (450nM), IL-6 sense 5′-AAATTCGGTACATCCTCGACGG-3′ antisense 5′- GGAAGGTTCAGGTTGTTTTCTGC-3' (500nM), IL-6R sense 5′-

TGAGCTCAGATATCGGGCTGAAC-3′ antisense 5′-CGTCGTGGATGACACAGTGATG-3’ (450nM), IL-1β sense 5′-CAGCTACGAATCTCCGACCAC-3′ antisense 5′-GGCAGGGAACCAGCATCTTC-3’ (600nM). The mRNA expression was compared and normalised to a housekeeping gene, 18S sense 5’-ACACGGACAGGATTGACAGA-3’ antisense 5’-CAAATCGCTCCACCAACTAA-3’. Cycling conditions were as follows: initial denaturation (95°C, 10min) followed by 40 cycles of denaturation (95°C, 30 seconds (s)), annealing (STAT3: 64°C, IL-6: 60°C, IL-6R: 60°C, IL-1β: 60°C for 30s) and extension (72°C, 1min) on a CFX Real-Time PCR Detector (Bio-Rad, USA).The quantification and melt curves were analysed using CFX Manager™ Software v3.0 (Bio-Rad, USA). The mRNA levels were calculated using the method described by Livak and Schmittgen (2001) [29] and represented as fold changes and relative expression to the control. Samples were run in triplicate and the experiments were repeated twice.

3.2.7 Cytokine profiling

Cytokine profiling was performed using the Bio-Plex Pro Human Th17 Cytokine Panel (IL-1β, IL-4, IL-6, IL-10, IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IL-31, IL-33, IFN-γ, sCD40L, TNF-α) (catalogue number 171AA001M, Bio-Rad, USA) according to the manufacturer’s instructions. Briefly, standards and samples (PEF; 1:6 and supernatant; 1:3), were diluted in standard and sample diluent HB respectively. The beads (50µl) were added to the assay plate and washed twice (100µl wash buffer, Bio-Plex Pro™ Wash Station). Standards, samples and controls were added and incubated (50µl, 1h in the dark, RT, 350rpm). The plate was washed three times and incubated with detection antibody (25µl, 30min in the dark, RT, 350rpm). The plate was washed three times and incubated with streptavidin-PE (50µl, 10min in the dark, RT, 350rpm). The plate was washed three times and the beads re-suspended in assay buffer (125µl, 30s, in the dark, RT, 350rpm). Data acquisition was performed on a Bio-Plex 200 and analysis was performed using Bio-Plex Manager™ software v6.0. Standards and samples were run in duplicate. Results were analysed using a 5-paramter logistic regression constructed from recombinant protein standards. The results were normalised to the treatment and then compared between groups.

3.2.8 Statistical analysis

Statistical analysis was performed using GraphPad Prism software (v5.0). The unpaired t-test with Welch correction was used in *in vitro* analyses and the non-parametric Mann-Whitney test in *ex vivo* analyses to avoid the influence of potential outlier values in the small study size. Outlier values were calculated and excluded using the interquartile range. Data is expressed as mean ± standard error of the mean (SEM), p values of less than 0.05 were considered statistically significant.

3.3 Results

3.3.1 Reduced proportion of Th17 lymphocytes in TB^+HIV^+ pleurisy

To investigate the propensity of TB/HIV co-infected pleural effusion microenvironment to promote Th17 generation we treated healthy $CD4^+$ Tconvs with PEF conditioned CCM. Interestingly, we observed that the proportion of $CD4^+$IL-$17^+$$RORC^+$$STAT3^+$cells was significantly 1.168 fold lower in $CD4^+$ Tconvs treated with TB^+HIV^+ PEF compared to TB^-HIV^- PEF *in vitro* (64.400 ± 1.256% vs. 55.156 ± 1.299%, p=0.0001). This trend was corroborated when Th17 proportion was directly investigated in patients. PEMC samples from TB^+HIV^+ patients showed a significant 1.769 fold lower number of $CD4^+$IL-$17^+$$RORC^+$$STAT3^+$cells compared to TB^-HIV^- controls (64.100 ± 5.045% vs. 36.233 ± 6.968% p=0.0054), this was associated with a significant 1.575 fold decrease in IL-17A protein expression (1454.000 ± 117.167 vs. 923.445 ± 64.418 PerCP-Cy5.5 mean fluorescence intensity (MFI), p=0.0040) (Figure 3.1).

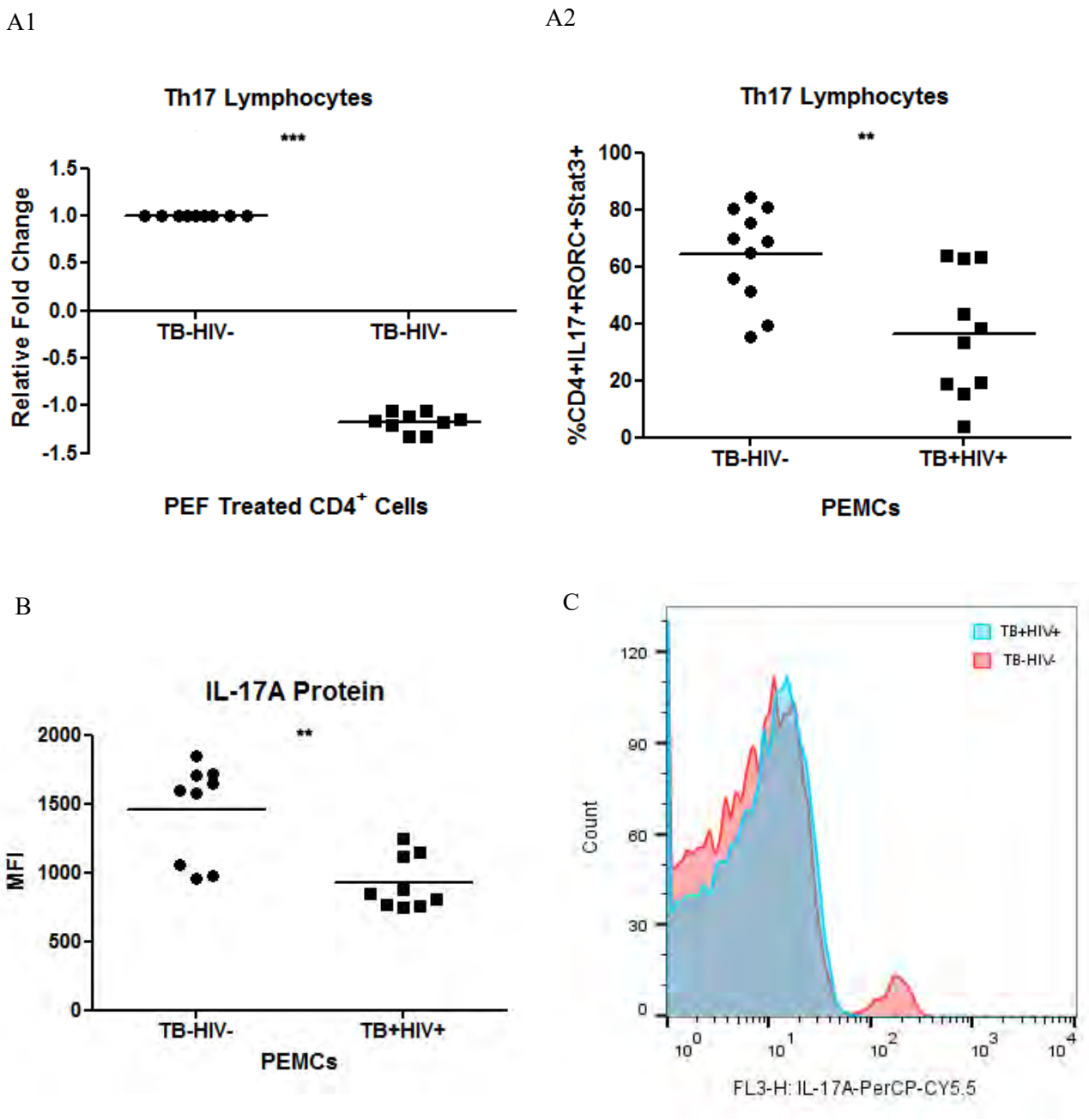

Figure 3.1: T-helper 17 lymphocyte frequency and interleukin-17A protein expression. (A1) Relative fold change in the proportion of $CD4^{+}IL\text{-}17^{+}RORC^{+}STAT3^{+}$ T-helper (Th) 17 lymphocytes in pleural effusion fluid (PEF) treated $CD4^{+}$ conventional T-cells *in vitro* ($n=9$ replicates in each group, $p=0.0001$, unpaired t-test with Welch correction). (A2) Reduced percentage of $CD4^{+}IL\text{-}17^{+}RORC^{+}STAT3^{+}$ Th17 lymphocytes in pleural effusion mononuclear cells (PEMCs) *ex vivo* ($TB^{-}HIV^{-}$ $n=5$, $TB^{+}HIV^{+}$ $n=5$, $p=0.0054$, Mann Whitney test). (B) Interleukin (IL)-17A protein expression was reduced in $TB^{+}HIV^{+}$ PEMCs represented by peridin-chlorophyll-cyanin5.5 (PerCP-Cy5.5) mean fluorescence intensity (MFI) ($TB^{-}HIV^{-}$ $n=5$, $TB^{+}HIV^{+}$ $n=5$, $p=0.0040$, Mann Whitney test). (C) Histogram showing the shift in IL-7A-PerCP-Cy5.5 fluorescence in PEMCs. Data represents the mean.

3.3.2 $TB^{+}HIV^{+}$ mediated in mRNA levels

We then assessed the molecular response of PEMCs with respect to Th17 polarisation. PEMCs from $TB^{+}HIV^{+}$ patients showed a significant upregulation in the transcript expression of molecules which promote Th17 differentiation; a notable 30.647 fold increase in STAT3 (17.037 ± 0.089 vs 12.140 ± 0.103 relative expression (ΔCt), p=0.0022), 11.662 fold increase in IL-1β (14.638 ± 0.043 vs. 11.102 ± 0.035 relative expression (ΔCt), p=0.0022), 3.076 fold increase in IL-6 (19.727 ± 0.143 vs. 18.380 ± 0.284 relative expression (ΔCt), p=0.0022) and a 1.657 fold increase in IL-6R (18.752 ± 0.099 vs. 18.092 ± 0.230 relative expression (ΔCt), p=0.0247) (Figure 3.2).

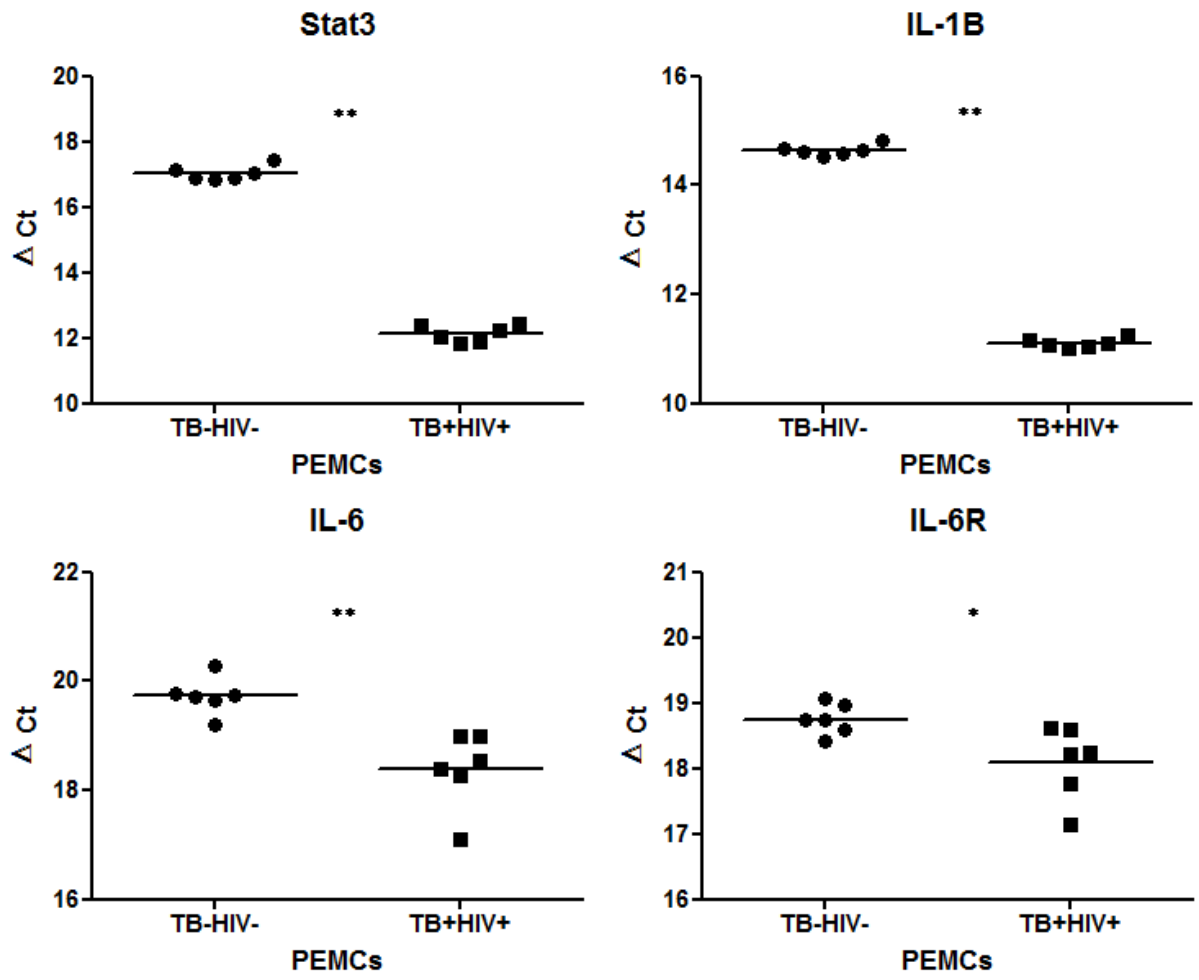

Figure 3.2: Relative messenger RNA expression represented as relative cycle threshold (ΔCt) in pooled pleural effusion mononuclear cells (PEMCs) (n=6 replicates in each group, Mann-Whiney test). Signal transducer and activator of transcription 3 (STAT3) (p=0.0022), interleukin (IL)-1β (p=0.0022), IL-6 (p=0.0022) and IL-6 receptor (IL-6R) (p=0.0247). Data represents the mean.

3.3.3 The TB^+HIV^+ cytokine microenvironment

Since we found increased expression of mRNA transcripts related to Th17 polarisation in TB^+HIV^+ PEMCs, we questioned whether in fact these were translated into secreted cytokine using a Bioplex multi-analyte cytokine screen.

Despite the reduced frequency of Th17 cells in TB^+HIV^+ PEF treatments and pleurisy, there was no significant change in the Th17 effector cytokines IL-17A and IL-17F concentrations in both the *in vitro* and *ex vivo* assays (Figure 3.3 and 3.4). Interestingly, in the TB^+HIV^+ and TB^-HIV^- patient cohorts, IL-17F was elevated by 6.460 fold (18.281 ± 2.383pg/ml vs. 118.090 ± 8.077pg/ml, $p<0.0001$) and 7.844 fold (11.938 ± 2.449pg/ml vs. 93.637 ± 15.357pg/ml, p=0.0009) compared to IL-17A respectively (Figure 3.4).

With respect to Th17 generating cytokines, there were no significant alteration between the TB^+HIV^+ and TB^-HIV^- PEF treatments *in vitro* indicating that the reduced proportion of Th17 lymphocytes observed in $CD4^+$ Tconvs treated with TB^+HIV^+ PEF was not a result of the cytokine milieu (Figure 3.3). In the patient samples, we observed a 3.708 fold increase in IL-1β (4.918 ± 1.421pg/ml vs. 18.238 ± 3.064pg/ml, p=0.0008) but without a significant change in the levels of IL-6, IL-21 or IL-23. This substantiates the *in vitro* result and infers that a reduced proportion of Th17 lymphocytes found in TB^+HIV^+ pleurisy is independent of the cytokine microenvironment *ex vivo* (Figure 3.4).

Th1 cytokines have established roles in TB and HIV pathogenesis, accordingly a 3.626 fold increase in TNF-α (18.233 ± 2.615pg/ml vs. 66.112 ± 13.264pg/ml, p=0.0251) and a 13.559 fold increase in IFN-γ (87.792 ± 27.282pg/ml vs. 1190.386 ± 361.906pg/ml, p=0.0026) concentration was found in TB^+HIV^+ PEF (Figure 3.4).

Notably, a 2.643 fold increase in IL-22 (26.379 ± 8.903pg/ml vs. 69.729 ± 10.318pg/ml, p=0.0115) and a 3.055 fold increase in IL-31 (158.430 ± 35.615pg/ml vs. 483.957 ± 115.651pg/ml, p=0.0210) was observed in TB/HIV pleurisy, both of which are largely uncharacterised in TB and HIV infection (Figure 3.4).

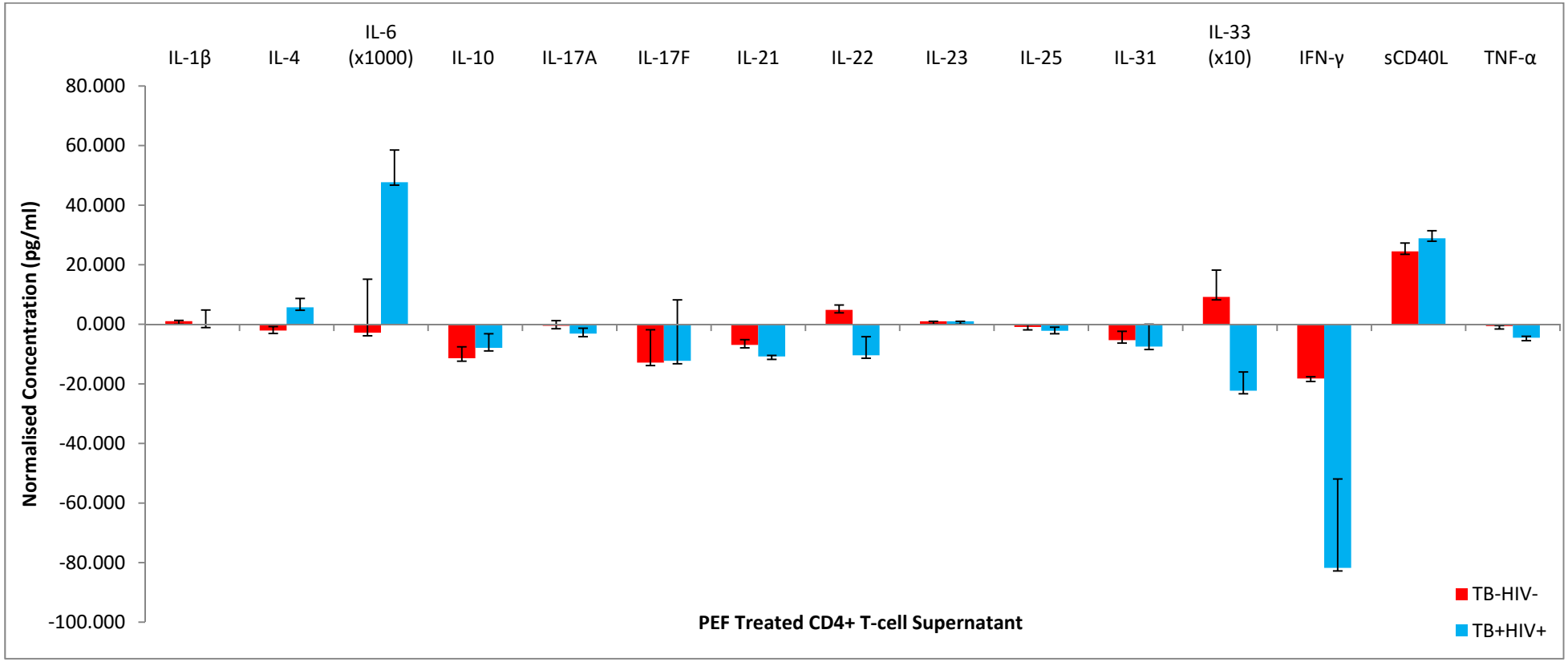

Figure 3.3: Cytokine profiling of supernatants from $CD4^+$ T-cells treated with pleural effusion fluid conditioned media. Results were normalised to the treating pleural effusion fluid (PEF) (*n*=2 replicates in each group, Unpaired t-test with Welch correction). IL; interleukin, IFN; interferon, sCD40L; soluble CD40 ligand, TNF; tumor necrosis factor. Data represents the mean ± SEM.

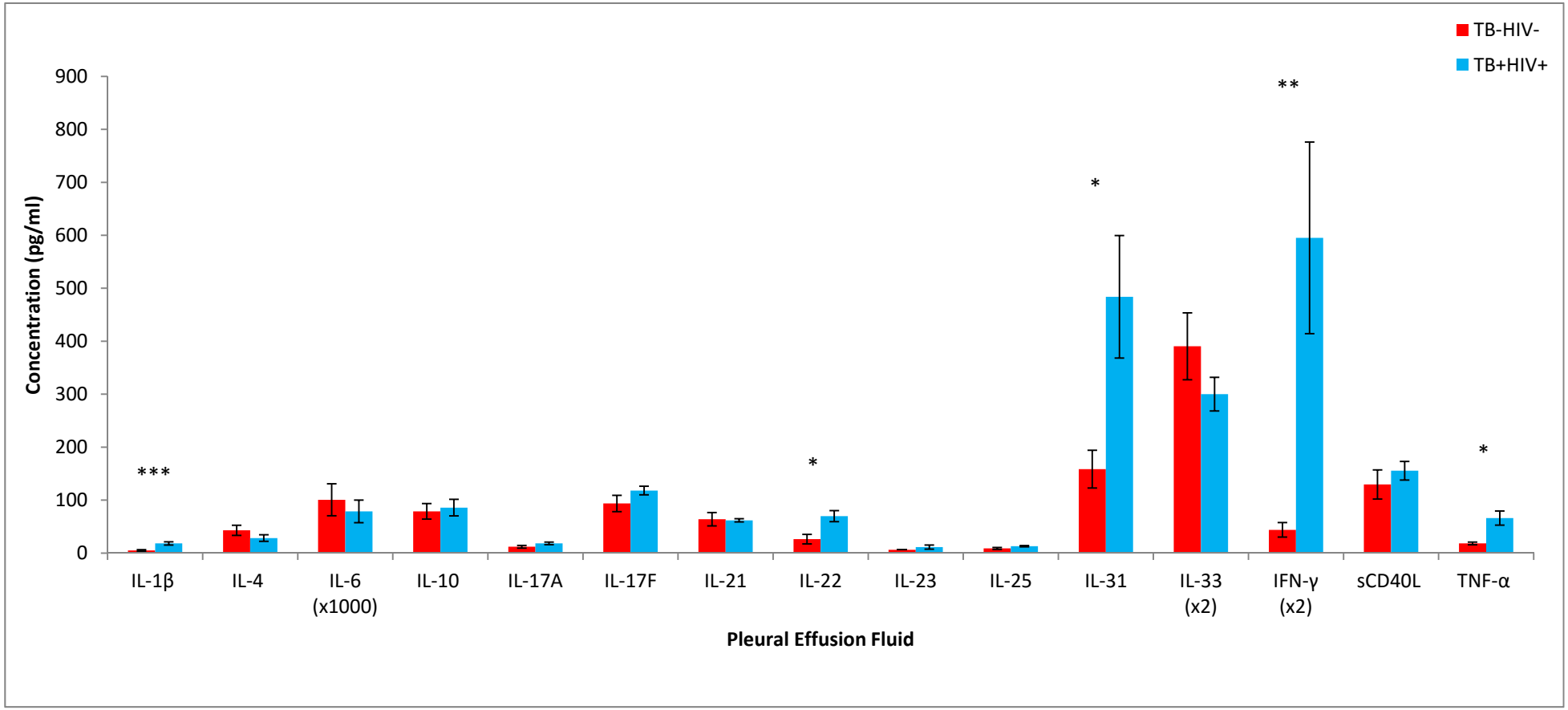

Figure 3.4: Cytokine profiling of pleural effusion fluid. $TB^{+}HIV^{+}$ pleural effusion fluid (PEF) showed increased concentrations of interleukin (IL)-1β (*p*=0.0008), IL-22 (*p*=0.0115), IL-31 (*p*=0.0210), interferon (IFN)-γ (*p*=0.0026) and tumor necrosis factor (TNF)-α (*p*=0.0251). (IL-1β, IL-4, IL-6, IL-10, IFN-γ, TNF-α; $TB^{-}HIV^{-}$ *n*=8, $TB^{+}HIV^{+}$ *n*=12. IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IL-31, IL-33, sCD40L; $TB^{-}HIV^{-}$ *n*=5, $TB^{+}HIV^{+}$ *n*=5, Mann-Whitney test). Data represents the mean ± SEM. sCD40L; soluble CD40 ligand.

3.4 Discussion

The Th17/IL17 axis is suggested to provide protective immunity against MTB by contributing to development and organisation of the granuloma [30-33]. Early expression of pulmonary IL-17 is pivotal in granulomatous cellular recruitment by inducing chemokine secretion from non-haematopoietic cells. This predominantly results in neutrophil and $CCR5^+$ lymphocyte recruitment, through the induction of IL-8 and chemokine C-X-C motif ligand (CXCL) 13 respectively, to form lung lymphoid follicles for optimal macrophage activation and bacterial clearance [34-37]. However, during the chronic stages of infection, Th17 hyperactivity can lead to immunopathology via IL-17-CXCL mediated influx of immune cells [30, 38-40], breakdown of the granuloma, cavitation and transmission [31, 41]. Th17 immunity has also been shown to contribute to mucosal TB vaccine immunity by IL-17A induction of CXCL13 [34]. However, there is no consensus on the proportion of Th17 cells in TB infection and the significance of this contribution to disease pathogenesis.

This is further confounded by HIV co-infection. Th17 cells show greater HIV susceptibility than other T-cell subsets [42-44]. Th17 lymphocyte targeting has been correlated with a higher expression of HIV envelope receptors CD4, CXCR4, CCR5 and α4β7 and low levels of autocrine production of CCR5 ligands CCL3 and CCL4, resulting in higher gp120 binding [43]. This is associated with a significant impairment in the generation of Th17 cells from peripheral naïve $CD4^+$ T-cells [22]. In addition, the over expression of negative regulators in Th17 differentiation (phosphatase SHP2, suppressor of cytokine signalling (SOCS) 3 and protein inhibitor of activated STAT (PIAS) 3) have been to linked to Th17 deficiency in a simian immunodeficiency virus model [45]. Notably, HIV-1 infected long-term non-progressors [19] and elite controllers [46] preserve the Th17 subset in peripheral blood and mucosa.

In our study, a significantly lower proportion of $CD4^+IL\text{-}17^+RORC^+STAT3^+$ lymphocytes was found in TB^+HIV^+ pleurisy compared to TB^-HIV^- pleurisy both *in vitro* and *ex vivo*. Classically, Th17 cells differentiate under TGF-β/IL-21 [47] or TGF-β/IL-6 and IL-21/IL-23 [48] conditions. This leads to Th17 lineage commitment via the activation of STAT3 and downstream transcription factor RORC, as well as interferon regulating factor 4 (IRF4), which in turn bind to the promoter region of *IL17A/F* [49-52]. Further to this IL-1β and IL-6 are significant in the expansion of differentiated and memory Th17 cells and IL-23 for the stabilisation and maintenance of the Th17 phenotype and effector functions [53]. Despite the reduction in Th17 frequency, there was no significant difference in the concentrations of IL-6, IL-21, and IL-23 between the two groups both *in vitro* and *ex vivo* indicating that the reduced proportion of Th17 cells is not a result of decreased polarising cytokines. In support of this, previous studies have shown that HIV infection is characterised by high levels of TGF-β [54], IL-6 [55] and IL-1 suggesting that failure to generate Th17 cells is not due to a lack of promoting cytokines [24]

Notably, TB^+HIV^+ pleurisy was associated with a significant 3.708 fold increase in IL-1β pleural fluid concentration and a 11.662 fold increase in IL-1β mRNA levels in PEMCs. Th17 cells express higher IL-1

receptor levels than other CD4 subsets [56, 57], in turn IL-1β upregulates the expression of transcription factors IRF4 and RORC and acts synergistically with IL-6, IL-23 and TGF-β to stimulate Th17 differentiation and maintain IL-17A, IL-17F, IL-21 and IL-22 cytokine production in effector cells [51, 56-60]. Very recently, a clinical isolate of the hypervirulent W-Beijing strain of MTB has been shown to induce a potent IL-17 protective response in a murine model through the induction of dendritic cell IL-1β and the subsequent formation of lung lymphoid follicles [34]. Furthermore, it was shown that blocking of IL-1/IL-1R signalling led to almost complete inhibition of IL-17A production during MTB stimulation in PBMCs [61]. Our results indicate that Th17 lymphocytes in TB/HIV pleurisy may be defective in their ability to respond to IL-1β

In the current study we found a 1.657 and 3.076 fold increase in IL-6R and IL-6 mRNA levels in co-infection, respectively but no increase in IL-6 cytokine levels in pleural fluid. This was interesting as recently the upregulation of IL-6 has been identified as a potential biomarker of mycobacterial infection *in vitro* [62]. During active TB infection IL-6 levels are increased in plasma [63, 64] and broncho-alveolar lavage (BAL) fluid [64, 65] with positive correlation to disease severity [63]. Furthermore, IL-6 is noted to be elevated in all phases of HIV chronic infection [66] and a clinical trial has shown that increased IL-6 levels are associated with increased all-cause mortality in HIV [67]. Our findings suggest that post transcriptional regulation of key Th17 polarising cytokines may influence the cytokine microenvironment.

Despite a significant decrease in the frequency of Th17 cells in TB^+HIV^+ pleurisy, there was no significant difference in the concentration of effector cytokines IL-17A and IL-17F between the two groups *in vitro* and *ex vivo*. In line with our findings, it has been observed that IL-17 mRNA levels in BAL cells of active TB patients and healthy controls do not differ significantly [68]. This may be a result of IL-17A production by $CD8^+$ T-cells [69], γöT-cells [70], natural killer cells [71], neutrophils [72] innate lymphoid cells [73] and mast cells [74]. The contribution of these different cell types to IL-17 associated diseases is still unknown [37]. γöT-cells, enriched at mucosal surfaces, such as the lung, have been indicated as a major source of early IL-17A production in response to MTB [75, 76] and found to be at significantly higher frequency in the peripheral blood of active TB patients compared to healthy donors [76]. γöT-cells can respond to IL-23 and IL-1β amplifying Th17 responses [75, 77] and therefore it is possible that the IL-17 observed in TB^+HIV^+ pleurisy may be a result of a IL-1β - γöT-cell pathway.

Notably, IL-17F was present at a significantly higher concentration than IL-17A in both TB^+HIV^+ and TB^-HIV^- groups, 6.460 and 7.844 fold respectively. IL-17F alone is generally less inflammatory than IL-17A; however, its effects are augmented when combined with TNF-α [78], also significantly increased in TB^+HIV^+ PEF. In support of our results, a previous study noted a decreased frequency of IL-17A producing $CD4^+$ T-cells but increased frequency of IL-17F producing $CD4^+$ T-cells in the peripheral blood of HIV infected patients [79], implying a HIV mediated selective defect in IL-17A production.

TNF-α and IFN-γ have established roles in the pathogenesis of TB [80, 81] and HIV [82-84], their significantly increased expression observed in TB^+HIV^+ pleurisy is not unexpected. IFN-γ is the preeminent cytokine in MTB immune defence, its primary function is the activation of macrophages instituting mycobacterial killing mechanisms [85-87] which is enhanced by TNF-α [88-90]. HIV-1 Tat protein induces production of TNF-α and IFN-γ in human monocytes/macrophages [83], in turn these cytokines synergistically stimulate HIV replication *in vitro* [91]. This suggests that in co-infection TNF-α and IFN-γ provide protective immunity against MTB but promote replication of HIV [92], indicating that defence against one pathogen allows injury by another. Furthermore, IFN-γ has been shown to limit the size of the Th17 population during MTB infection [93]. In our TB^+HIV^+ cohort we observed significantly a higher concentration of IFN-γ over IL-17A (65.116 fold increase, p=0.0006) or IL-17F (10.080 fold increase, p=0.0092) possibly contributing to the reduction in Th17 lymphocytes in co-infection.

Our investigations showed a 2.643 fold increase in IL-22 levels in TB^+HIV^+ pleurisy. IL-22 has been implicated in pathogenesis of TB [11, 94, 95] and HIV [96-98]. However, it remains to be determined if IL-22 is involved in protective or pathogenic immunity in co-infection or the reparative process following the immuno-pathological consequences of these diseases. IL-22 significantly exceeded the concentration of IL-17A (3.814 fold, p<0.0001) but not IL-17F in TB^+HIV^+ pleurisy. This is in line with previous work where IL-22 was found to surpass IL-17A in TB^+ pleural fluid [95]. The raised level of IL-22 in the face of decreased frequency in Th17 cells could represent contribution by other cell types. In this regard, during experimental MTB infection, IL-22 was found to be predominantly produced by Th1 and Th22 cells rather than Th17 cells [99]. IFN-γ suppresses PBMC IL-17A production and Th17 activity [1, 11, 99-102] but not IL-22 production [11, 99] allowing for effective IL-22 production in the absence of Th17 cells, in line with our results.

The 3.055 fold increase in IL-31 concentration in TB^+HIV^+ pleurisy is novel. IL-31 is generally associated with Th2 mediated skin inflammation or allergic responses in the airways and GIT and has not been characterised in TB/HIV co-infection or mono-infection. IL-31 mRNA is upregulated in the lungs after antigen challenge [103], the targets of which are primarily bronchial epithelial cells, pulmonary fibroblasts and macrophages [104] where it upregulates pro-inflammatory cytokine and chemokine gene expression involved in cellular recruitment [103, 105-107], in the context of TB this may contribute to granuloma organisation. IL-31 can also directly inhibit IL-17A/F mRNA synthesis in Th17 cells through the regulation of PIAS molecules [108] possibly augmenting the observed decrease in Th17 frequency.

In HIV infection, the depletion of Th17 cells at the mucosal surface is associated with increased translocation of microbial into the systemic circulation rendering a patient more prone to opportunistic infections and suggested to be a predominant cause of chronic immune activation and HIV progression [24, 109-112]. At the respiratory mucosa, a loss of Th17 cells decreases mucosal defence to inhaled MTB and

together with reduced IL-17-mediated cellular recruitment and granuloma organisation may result in the progression of active TB infection.

3.5 Concluding remarks

A reduced proportion of Th17 cells were observed in $TB^{+}HIV^{+}$ pleurisy independent of alteration in the levels of Th17 polarising cytokines and IL-17A/F effector cytokines in the localised microenvironment.

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References

1. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang Y-H, et al. A distinct lineage of CD4 T-cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 2005; 6:1133-41.

2. Huber S, Gagliani N, Flavell RA. Life, death, and miracles: Th17 cells in the intestine. *Eur J Immunol* 2012; 42:2238-45.

3. Zhou X, Chen Q, Moore J, Kolls JK, Halperin S, Wang J. Critical role of the interleukin-17/interleukin-17 receptor axis in regulating host susceptibility to respiratory infection with chlamydia species. *Infect Immun* 2009; 77:5059-70.

4. Zhang X, Gao L, Lei L, Zhong Y, Dube P, Berton MT, et al. A MyD88-dependent early IL-17 production protects mice against airway infection with the obligate intracellular pathogen *Chlamydia muridarum*. *J Immunol* 2009; 183:1291-300.

5. Kudva A, Scheller EV, Robinson KM, Crowe CR, Choi SM, Slight SR, et al. Influenza A inhibits Th17-mediated host defense against bacterial pneumonia in mice. *J Immunol* 2011; 186:1666-74.

6. Ye P, Garvey PB, Zhang P, Nelson S, Bagby G, Summer WR, et al. Interleukin-17 and lung host defense against *Klebsiella pneumoniae* infection. *Am J Respir Cell Mol Biol* 2001; 25:335-40.

7. Schoenen H, Bodendorfer B, Hitchens K, Manzanero S, Werninghaus K, Nimmerjahn F, et al. Cutting edge: Mincle is essential for recognition and adjuvanticity of the mycobacterial cord factor and its synthetic analog trehalose-dibehenate. *J Immunol* 2010; 184:2756-60.

8. Marín ND, París SC, Rojas M, García LF. Reduced frequency of memory T-cells and increased Th17 responses in patients with active tuberculosis. *Clin Vaccine Immunol* 2012; 19:1667-76.

9. Jurado JO, Pasquinelli V, Alvarez IB, Peña D, Rovetta AI, Tateosian NL, et al. IL-17 and IFN-γ expression in lymphocytes from patients with active tuberculosis correlates with the severity of the disease. *J Leukoc Biol* 2012; 91:991-1002.

10. Wang GQ, Yang CL, Yue DF, Pei LH, Zhong H, Niu JX. The changes and its significance of Th17 and Treg cells and related cytokines in patients with tuberculosis pleurisy. *Allergy Asthma Clin Immunol* 2014; 10:28-33.

11. Scriba TJ, Kalsdorf B, Abrahams D-A, Isaacs F, Hofmeister J, Black G, et al. Distinct, specific IL-17-and IL-22-producing $CD4^+$ T-cell subsets contribute to the human anti-mycobacterial immune response. *J Immunol* 2008; 180:1962-70.

12. Bandaru A, Devalraju KP, Paidipally P, Dhiman R, Venkatasubramanian S, Barnes PF, et al. Phosphorylated STAT3 and PD-1 regulate IL-17 production and IL-23 receptor expression in *Mycobacterium tuberculosis* infection. *Eur J Immunol* 2014; 44:2013-24.

13. Cowan J, Pandey S, Filion L, Angel J, Kumar A, Cameron D. Comparison of interferon-γ, interleukin (IL)-17-and IL-22-expressing CD4 T-cells, IL-22 expressing granulocytes and proinflammatory cytokines during latent and active tuberculosis infection. *Clin Exp Immunol* 2012; 167:317-29.

14. Kumar NP, Anuradha R, Suresh R, Ganesh R, Shankar J, Kumaraswami V, et al. Suppressed type 1, type 2, and type 17 cytokine responses in active tuberculosis in children. *Clin Vaccine Immunol* 2011; 18:1856-64.

15. Chen X, Zhang M, Liao M, Graner MW, Wu C, Yang Q, et al. Reduced Th17 response in patients with tuberculosis correlates with IL-6R expression on $CD4^+$ T-cells. *Am J Respir Crit Care Med* 2010; 181:734-42.

16. World Health Organisation. Global tuberculosis report 2014. Geneva, Switzerland: World Health Organisation; 2014.

17. Chatterjee D, Pramanik AK. Tuberculosis in the African continent: A comprehensive review. *Pathophysiol* 2015; 22:73-83.

18. Mehandru S, Poles MA, Tenner-Racz K, Horowitz A, Hurley A, Hogan C, et al. Primary HIV-1 infection is associated with preferential depletion of $CD4^+$ T-lymphocytes from effector sites in the gastrointestinal tract. *J Exp Med* 2004; 200:761-70.

19. Ciccone EJ, Greenwald JH, Lee PI, Biancotto A, Read SW, Yao MA, et al. $CD4^+$ T-cells, including Th17 and cycling subsets, are intact in the gut mucosa of HIV-1-infected long-term nonprogressors. *J Virol* 2011; 85:5880-8.

20. Gaardbo JC, Hartling HJ, Gerstoft J, Nielsen SD. Incomplete immune recovery in HIV infection: Mechanisms, relevance for clinical care and possible solutions. *Clin Dev Immunol* 2012; 2012:e670957.

21. He Y, Li J, Zheng Y, Luo Y, Zhou H, Yao Y, et al. A randomized case–control study of dynamic changes in peripheral blood Th17/Treg cell balance and interleukin-17 levels in highly active antiretroviral-treated HIV type 1/AIDS patients. *AIDS Res Hum Retroviruses* 2012; 28:339-45.

22. DaFonseca S, Niessl J, Pouvreau S, Wacleche VS, Gosselin A, Cleret-Buhot A, et al. Impaired Th17 polarisation of phenotypically naive $CD4^+$ T-cells during chronic HIV-1 infection and potential restoration with early ART. *Retrovirology* 2015; 12:38-60.

23. Singh A, Vajpayee M, Ali SA, Chauhan NK. Cellular interplay among Th17, Th1, and Treg cells in HIV-1 subtype “C” infection. *J Med Virol* 2014; 86:372-84.

24. Bixler SL, Mattapallil JJ. Loss and dysregulation of Th17 cells during HIV infection. *Clin Dev Immunol* 2013; 2013:e852418.

25. Barker RD. Clinical tuberculosis. *Medicine* 2012; 40:340-5.

26. Chaisson RE, Schecter GF, Theuer CP, Rutherford GW, Echenberg DF, Hopewell PC. Tuberculosis in patients with the acquired immunodeficiency syndrome: Clinical features, response to therapy and survival. *Am Rev Respir Dis* 1987; 136:570-4.

27. TB DOTS Strategy Co-ordination Directorate. National Tuberculosis Management Guidelines. Republic of South Africa: National Department of Health 2014.

28. Gideon HP, Phuah J, Myers AJ, Bryson BD, Rodgers MA, Coleman MT, et al. Variability in tuberculosis granuloma T-cell responses exists, but a balance of pro-and anti-inflammatory cytokines is associated with sterilization. *PLoS Pathog* 2015; 11: e1004603.

29. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; 25:402-8.

30. Torrado E, Cooper AM. IL-17 and Th17 cells in tuberculosis. *Cytokine Growth Factor Rev* 2010; 21:455-62.

31. Umemura M, Yahagi A, Hamada S, Begum MD, Watanabe H, Kawakami K, et al. IL-17 mediated regulation of innate and acquired immune response against pulmonary *Mycobacterium bovis* bacille Calmette-Guerin infection. *J Immunol* 2007; 178:3786-96.

32. Redford PS, Boonstra A, Read S, Pitt J, Graham C, Stavropoulos E, et al. Enhanced protection to *Mycobacterium tuberculosis* infection in IL-10 deficient mice is accompanied by early and enhanced Th1 responses in the lung. *Eur J Immunol* 2010; 40:2200-10.

33. Yoshida YO, Umemura M, Yahagi A, O’Brien RL, Ikuta K, Kishihara K, et al. Essential role of IL-17A in the formation of a mycobacterial infection-induced granuloma in the lung. *J Immunol* 2010; 184:4414-22.

34. Gopal R, Rangel-Moreno J, Slight S, Lin Y, Nawar HF, Junecko BF, et al. Interleukin-17-dependent CXCL13 mediates mucosal vaccine–induced immunity against tuberculosis. *Mucosal Immunol* 2013; 6:972-84.

35. Seiler P, Aichele P, Bandermann S, Hauser AE, Lu B, Gerard NP, et al. Early granuloma formation after aerosol *Mycobacterium tuberculosis* infection is regulated by neutrophils via CXCR3-signaling chemokines. *Eur J Immunol* 2003; 33:2676-86.

36. Silva MT. When two is better than one: Macrophages and neutrophils work in concert in innate immunity as complementary and cooperative partners of a myeloid phagocyte system. *J Leukoc Biol* 2010; 87:93-106.

37. Miossec P, Kolls JK. Targeting IL-17 and Th17 cells in chronic inflammation. *Nat Rev Drug Discov* 2012; 11:763-76.

38. Torrado E, Robinson RT, Cooper AM. Cellular response to mycobacteria: Balancing protection and pathology. *Trends Immunol* 2011; 32:66-72.

39. Etna MP, Giacomini E, Severa M, Coccia EM. Pro-and anti-inflammatory cytokines in tuberculosis: A two-edged sword in TB pathogenesis. *Semin Immunol* 2014; 26:543-51.

40. Cruz A, Fraga AG, Fountain JJ, Rangel-Moreno J, Torrado E, Saraiva M, et al. Pathological role of interleukin 17 in mice subjected to repeated BCG vaccination after infection with *Mycobacterium tuberculosis*. *J Exp Med* 2010; 207:1609-16.

41. Wozniak TM, Saunders BM, Ryan AA, Britton WJ. *Mycobacterium bovis* BCG-specific Th17 cells confer partial protection against *Mycobacterium tuberculosis* infection in the absence of interferon-γ. *Infect Immun* 2010; 78:4187-94.

42. McKinnon LR, Nyanga B, Chege D, Izulla P, Kimani M, Huibner S, et al. Characterisation of a human cervical $CD4^+$ T-cell subset coexpressing multiple markers of HIV susceptibility. *J Immunol* 2011; 187:6032-42.

43. Alvarez Y, Tuen M, Shen G, Nawaz F, Arthos J, Wolff MJ, et al. Preferential HIV infection of $CCR6^+$ Th17 cells is associated with higher levels of virus receptor expression and lack of CCR5 ligands. *J Virol* 2013; 87:10843-54.

44. El Hed A, Khaitan A, Kozhaya L, Manel N, Daskalakis D, Borkowsky W, et al. Susceptibility of human Th17 cells to human immunodeficiency virus and their perturbation during infection. *J Infect Dis* 2010; 201:843-54.

45. Bixler SL, Sandler NG, Douek DC, Mattapallil JJ. Suppressed Th17 levels correlate with elevated PIAS3, SHP2, and SOCS3 expression in CD4 T-cells during acute simian immunodeficiency virus infection. *J Virol* 2013; 87:7093-101.

46. Brandt L, Benfield T, Mens H, Clausen LN, Katzenstein TL, Fomsgaard A, et al. Low level of regulatory T-cells and maintenance of balance between regulatory T-cells and Th17 cells in HIV-1 infected elite controllers. *J Acquir Immune Defic Syndr* 2011; 57:101-8.

47. Yang L, Anderson DE, Baecher-Allan C, Hastings WD, Bettelli E, Oukka M, et al. IL-21 and TGF-β are required for differentiation of human Th17 cells. *Nature* 2008; 454:350-2.

48. Manel N, Unutmaz D, Littman DR. The differentiation of human Th17 cells requires transforming growth factor-β and induction of the nuclear receptor ROR-γt. *Nat Immunol* 2008; 9:641-9.

49. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, et al. The orphan nuclear receptor ROR-γt directs the differentiation program of proinflammatory IL-17$^+$ T-helper cells. *Cell* 2006; 126:1121-33.

50. Mudter J, Yu J, Zufferey C, Brüstle A, Wirtz S, Weigmann B, et al. IRF4 regulates IL-17A promoter activity and controls ROR-γt-dependent Th17 colitis *in vivo*. *Inflamm Bowel Dis* 2011; 17:1343-58.

51. Brüstle A, Heink S, Huber M, Rosenplänter C, Stadelmann C, Yu P, et al. The development of inflammatory Th17 cells requires interferon-regulatory factor 4. *Nat Immunol* 2007; 8:958-66.

52. Hirahara K, Ghoreschi K, Laurence A, Yang X-P, Kanno Y, O'Shea JJ. Signal transduction pathways and transcriptional regulation in Th17 cell differentiation. *Cytokine Growth Factor Rev* 2010; 21:425-34.

53. Yang XO, Pappu BP, Nurieva R, Akimzhanov A, Kang HS, Chung Y, et al. T-helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR-α and ROR-γ. *Immunity* 2008; 28:29-39.

54. Lotz M, Seth P. TGF-β and HIV infection. *Ann N Y Acad Sci* 1993; 685:501-11.

55. Nixon DE, Landay AL. Biomarkers of immune dysfunction in HIV. *Curr Opin HIV AIDS* 2010; 5:498-503.

56. Lee W-W, Kang SW, Choi J, Lee S-H, Shah K, Eynon EE, et al. Regulating human Th17 cells via differential expression of IL-1 receptor. *Blood* 2010; 115:530-40.

57. Chung Y, Chang SH, Martinez GJ, Yang XO, Nurieva R, Kang HS, et al. Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. *Immunity* 2009; 30:576-87.

58. Sundrud MS, Trivigno C. Identity crisis of Th17 cells: Many forms, many functions, many questions. *Semin Immunol* 2013; 25:263-72.

59. Zielinski CE, Mele F, Aschenbrenner D, Jarrossay D, Ronchi F, Gattorno M, et al. Pathogen-induced human Th17 cells produce IFN-γ or IL-10 and are regulated by IL-1β. *Nature* 2012; 484:514-8.

60. Ikeda S, Saijo S, Murayama MA, Shimizu K, Akitsu A, Iwakura Y. Excess IL-1 signaling enhances the development of Th17 cells by downregulating TGF-β induced Foxp3 expression. *J Immunol* 2014; 192:1449-58.

61. van de Veerdonk FL, Teirlinck AC, Kleinnijenhuis J, Kullberg BJ, van Crevel R, van der Meer JW, et al. *Mycobacterium tuberculosis* induces IL-17A responses through TLR4 and dectin-1 and is critically dependent on endogenous IL-1. *J Leukoc Biol* 2010; 88:227-32.

62. Singh PP, Goyal A. Interleukin-6: A potent biomarker of mycobacterial infection. *Springerplus* 2013; 2:686-93.

63. Verbon A, Juffermans N, Van Deventer S, Speelman P, Van Deutekom H, Van Der Poll T. Serum concentrations of cytokines in patients with active tuberculosis (TB) and after treatment. *Clin Exp Immunol* 1999; 115:110-3.

64. Tsao TCY, Hong J, Huang C, Yang P, Liao SK, Chang KSS. Increased TNF-α, IL-1β and IL-6 levels in the bronchoalveolar lavage fluid with the upregulation of their mRNA in macrophages lavaged from patients with active pulmonary tuberculosis. *Tuber Lung Dis* 1999; 79:279-85.

65. Law K, Weiden M, Harkin T, Tchou-Wong K, Chi C, Rom WN. Increased release of interleukin-1β, interleukin-6, and tumor necrosis factor-α by bronchoalveolar cells lavaged from involved sites in pulmonary tuberculosis. *Am J Respir Crit Care Med* 1996; 153:799-804.

66. Breen E, Rezai A, Nakajima K, Beall G, Mitsuyasu R, Hirano T, et al. Infection with HIV is associated with elevated IL-6 levels and production. *J Immunol* 1990; 144:480-4.

67. Kuller LH, Tracy R, Belloso W, De Wit S, Drummond F, Lane HC, et al. Inflammatory and coagulation biomarkers and mortality in patients with HIV infection. *PLoS Med* 2008; 5:e203.

68. Dheda K, Chang J, Lala S, Huggett J, Zumla A, Rook G. Gene expression of IL-17 and IL-23 in the lungs of patients with active tuberculosis. *Thorax* 2008; 63:566-8.

69. Happel KI, Zheng M, Young E, Quinton LJ, Lockhart E, Ramsay AJ, et al. Cutting edge: Roles of Toll-like receptor 4 and IL-23 in IL-17 expression in response to *Klebsiella pneumoniae* infection. *J Immunol* 2003; 170:4432-6.

70. Stark MA, Huo Y, Burcin TL, Morris MA, Olson TS, Ley K. Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17. *Immunity* 2005; 22:285-94.

71. Michel M-L, Keller AC, Paget C, Fujio M, Trottein F, Savage PB, et al. Identification of an IL-17–producing NK1. 1^{-} iNKT cell population involved in airway neutrophilia. *J Exp Med* 2007; 204:995-1001.

72. Ferretti S, Bonneau O, Dubois GR, Jones CE, Trifilieff A. IL-17, produced by lymphocytes and neutrophils, is necessary for lipopolysaccharide-induced airway neutrophilia: IL-15 as a possible trigger. *J Immunol* 2003; 170:2106-12.

73. Takatori H, Kanno Y, Watford WT, Tato CM, Weiss G, Ivanov II, et al. Lymphoid tissue inducer–like cells are an innate source of IL-17 and IL-22. *J Exp Med* 2009; 206:35-41.

74. Mrabet-Dahbi S, Metz M, Dudeck A, Zuberbier T, Maurer M. Murine mast cells secrete a unique profile of cytokines and prostaglandins in response to distinct TLR2 ligands. *Exp Dermatol* 2009; 18:437-44.

75. Lockhart E, Green AM, Flynn JL. IL-17 production is dominated by γδ T cells rather than CD4 T-cells during *Mycobacterium tuberculosis* infection. *J Immunol* 2006; 177:4662-9.

76. Peng M, Wang Z, Yao C, Jiang L, Jin Q, Wang J, et al. Interleukin 17-producing γδ T-cells increased in patients with active pulmonary tuberculosis. *Cell Mol Immunol* 2008; 5:203-8.

77. Sutton CE, Lalor SJ, Sweeney CM, Brereton CF, Lavelle EC, Mills KH. Interleukin-1 and IL-23 induce innate IL-17 production from γδ T cells, amplifying Th17 responses and autoimmunity. *Immunity* 2009; 31:331-41.

78. Zrioual S, Ecochard R, Tournadre A, Lenief V, Cazalis M-A, Miossec P. Genome-wide comparison between IL-17A-and IL-17F-induced effects in human rheumatoid arthritis synoviocytes. *J Immunol* 2009; 182:3112-20.

79. Salem M, Ronit A, Gaardbo JC, Lund TT, Ullum H, Gerstoft J, et al. Altered balance between IL-17A- and IL-17F-producing Th17 cells in HIV-infected patients. *J Acquir Immune Defic Syndr* 2013; 63:28-30.

80. Dlugovitzky D, Bay ML, Rateni L, Fiorenza G, Vietti L, Farroni MA, et al. Influence of disease severity on nitrite and cytokine production by peripheral blood mononuclear cells (PBMC) from patients with pulmonary tuberculosis (TB). *Clin Exp Immunol* 2000; 122:343-9.

81. Morosini M, Meloni F, Marone Bianco A, Paschetto E, Uccelli M, Pozzi E, et al. The assessment of IFN-γ and its regulatory cytokines in the plasma and bronchoalveolar lavage fluid of patients with active pulmonary tuberculosis. *Int J Tuber Lung Dis* 2003; 7:994-1000.

82. Vaidya SA, Korner C, Sirignano MN, Amero M, Bazner S, Rychert J, et al. Tumor necrosis factor-α is associated with viral control and early disease progression in patients with HIV type 1 infection. *J Infect Dis* 2014; 210:1042-6.

83. Haij NB, Leghmari K, Planès R, Thieblemont N, Bahraoui E. HIV-1 Tat protein binds to TLR4-MD2 and signals to induce TNF-α and IL-10. *Retrovirology* 2013; 10:123-34.

84. Norris PJ, Pappalardo BL, Custer B, Spotts G, Hecht FM, Busch MP. Elevations in IL-10, TNF-α, and IFN-γ from the earliest point of HIV type 1 infection. *AIDS Res Hum Retroviruses* 2006; 22:757-62.

85. Cavalcanti YVN, Brelaz MCA, Neves JKdAL, Ferraz JC, Pereira VRA. Role of TNF-α, IFN-γ, and IL-10 in the development of pulmonary tuberculosis. *Pulm Med* 2012; 2012:e745483.

86. Fenton MJ, Vermeulen MW, Kim S, Burdick M, Strieter RM, Kornfeld H. Induction of gamma interferon production in human alveolar macrophages by *Mycobacterium tuberculosis*. *Infect Immun* 1997; 65:5149-56.

87. Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, Bloom BR. An essential role for interferon-γ in resistance to *Mycobacterium tuberculosis* infection. *J Exp Med* 1993; 178:2249-54.

88. Mohan VP, Scanga CA, Yu K, Scott HM, Tanaka KE, Tsang E, et al. Effects of tumor necrosis factor-α on host immune response in chronic persistent tuberculosis: Possible role for limiting pathology. *Infect Immun* 2001; 69:1847-55.

89. Stenger S. Immunological control of tuberculosis: Role of tumour necrosis factor and more. *Ann Rheum Dis* 2005; 64:24-8.

90. Flynn JL, Goldstein MM, Chan J, Triebold KJ, Pfeffer K, Lowenstein CJ, et al. Tumor necrosis factor-α is required in the protective immune response against *Mycobacterium tuberculosis* in mice. *Immunity* 1995; 2:561-72.

91. Han X, Becker K, Degen H, Jablonowski H, Strohmeyer G. Synergistic stimulatory effects of tumour necrosis factor-α and interferon-γ on replication of human immunodeficiency virus type 1 and on apoptosis of HIV-1-infected host cells. *Eur J Clin Invest* 1996; 26:286-92.

92. Pawlowski A, Jansson M, Skold M, Rottenberg ME, Kallenius G. Tuberculosis and HIV co-infection. *PLoS Pathog* 2012; 8:e1002464.

93. Cruz A, Khader SA, Torrado E, Fraga A, Pearl JE, Pedrosa J, et al. Cutting edge: IFN-γ regulates the induction and expansion of IL-17-producing CD4 T-cells during mycobacterial infection. *J Immunol* 2006; 177:1416-20.

94. Zhang M, Zeng G, Yang Q, Zhang J, Zhu X, Chen Q, et al. Anti-tuberculosis treatment enhances the production of IL-22 through reducing the frequencies of regulatory B-cell. *Tuberculosis* 2014; 94:238-44.

95. Matthews K, Wilkinson KA, Kalsdorf B, Roberts T, Diacon A, Walzl G, et al. Predominance of interleukin-22 over interleukin-17 at the site of disease in human tuberculosis. *Tuberculosis* 2011; 91:587-93.

96. Missé D, Yssel H, Trabattoni D, Oblet C, Lo Caputo S, Mazzotta F, et al. IL-22 participates in an innate anti-HIV-1 host resistance network through acute-phase protein induction. *J Immunol* 2007; 178:407-15.

97. Chege D, Chai Y, Huibner S, Kain T, Wachihi C, Kimani M, et al. Blunted IL17/IL22 and pro-inflammatory cytokine responses in the genital tract and blood of HIV-exposed, seronegative female sex workers in Kenya. *PLoS One* 2012; 7:e43670.

98. Hu J, Li Y, Chen L, Yang Z, Zhao G, Wang Y, et al. Impact of IL-22 gene polymorphism on human immunodeficiency virus infection in Han Chinese patients. *J Microbiol Immunol Infect* 2014; 11:10-7.

99. Behrends J, Renauld J-C, Ehlers S, Hölscher C. IL-22 is mainly produced by IFN-γ secreting cells but is dispensable for host protection against *Mycobacterium tuberculosis* infection. *PLoS One* 2013; 8:e57379.

100. Stumhofer JS, Laurence A, Wilson EH, Huang E, Tato CM, Johnson LM, et al. Interleukin-27 negatively regulates the development of interleukin-17 producing T-helper cells during chronic inflammation of the central nervous system. *Nat Immunol* 2006; 7:937-45.

101. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, et al. Interleukin 17–producing $CD4^+$ effector T-cells develop via a lineage distinct from the T-helper type 1 and 2 lineages. *Nat Immunol* 2005; 6:1123-32.

102. Lazarevic V, Chen X, Shim J-H, Hwang E-S, Jang E, Bolm AN, et al. T-bet represses Th17 differentiation by preventing Runx1-mediated activation of the gene encoding ROR-γt. *Nat Immunol* 2011; 12:96-104.

103. Dillon SR, Sprecher C, Hammond A, Bilsborough J, Presnell SR, Haugen HS, et al. Interleukin 31, a cytokine produced by activated T cells, induces dermatitis in mice. *Nat Immunol* 2004; 5:752-60.

104. Jawa RS, Chattopadhyay S, Tracy E, Wang Y, Huntoon K, Dayton MT, et al. Regulated expression of the IL-31 receptor in bronchial and alveolar epithelial cells, pulmonary fibroblasts, and pulmonary macrophages. *J Interferon Cytokine Res* 2008; 28:207-19.

105. Ip WK, Wong CK, Li ML, Li PW, Cheung PF, Lam CW. Interleukin-31 induces cytokine and chemokine production from human bronchial epithelial cells through activation of mitogen-activated protein kinase signalling pathways: implications for the allergic response. *Immunology* 2007; 122:532-41.

106. Cheung PF-Y, Wong C-K, Ho AW-Y, Hu S, Chen D-P, Lam CW-K. Activation of human eosinophils and epidermal keratinocytes by Th2 cytokine IL-31: Implication for the immunopathogenesis of atopic dermatitis. *Int Immunol* 2010; 22:453-67.

107. Horejs-Hoeck J, Schwarz H, Lamprecht S, Maier E, Hainzl S, Schmittner M, et al. Dendritic cells activated by IFN-γ/STAT1 express IL-31 receptor and release proinflammatory mediators upon IL-31 treatment. *J Immunol* 2012; 188:5319-26.

108. Zhang Q, Putheti P, Zhou Q, Liu Q, Gao W. Structures and biological functions of IL-31 and IL-31 receptors. *Cytokine Growth Factor Rev* 2008; 19:347-56.

109. Favre D, Lederer S, Kanwar B, Ma Z-M, Proll S, Kasakow Z, et al. Critical loss of the balance between Th17 and T regulatory cell populations in pathogenic SIV infection. *PLoS Pathog* 2009; 5:e1000295.

110. Brenchley JM, Paiardini M, Knox KS, Asher AI, Cervasi B, Asher TE, et al. Differential Th17 CD4 T-cell depletion in pathogenic and nonpathogenic lentiviral infections. *Blood* 2008; 112:2826-35.

111. Brenchley JM, Price DA, Schacker TW, Asher TE, Silvestri G, Rao S, et al. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat Med* 2006; 12:1365-71.

112. Douek DC, Roederer M, Koup RA. Emerging concepts in the immunopathogenesis of AIDS. *Annu Rev Med* 2009; 60:471.

CHAPTER 4

TB/HIV Pleurisy Promotes Monocyte Mediated Th17 Polarisation

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Abstract

Monocytes produce cytokines that direct T-cell polarisation. Tuberculosis (TB) and human immunodeficiency virus (HIV) infection alter monocyte function and the pathogenesis of both of these diseases is largely orchestrated by sub-optimal T-cell responses.

Objectives: We constructed an *in vitro* supernatant transfer model to investigate the effect of TB/HIV pleural exudate on monocyte (THP-1) cytokine production and subsequent cytokine expression and polarisation of $CD4^+$ conventional T-cells (Tconvs) into T-helper (Th) 17 and T-regulatory (Treg) lymphocyte subsets, compared to a non-infectious pleural effusion.

Design: HIV-related TB most commonly presents as TB pleurisy and monocyte dysfunction is induced by secreted factors from MTB, HIV or infected cells. The effect of TB/HIV co-infection on monocyte behavior is largely uncharacterised therefore a unicellular model needs to firstly be investigated to elucidate more complex findings in multicellular systems.

Methods: Th17 and Treg frequency was assessed by flow cytometry, monocyte and $CD4^+$ T-cell cytokine production was measured with ELISA and Bio-Plex platforms respectively.

Results: TB^+HIV^+ pleurisy altered monocyte cytokine production resulting in increased production of interleukin (IL)-1β (p=0.0217), a Th17 generating cytokine. Following supernatant transfer, the TB^+HIV^+ treated monocyte milieu caused significant polarisation of $CD4^+$ Tconvs into Th17 cells and Tregs (p<0.0001). However, a significantly greater proportion of Th17 lymphocytes (p=0.0001) was observed, associated with elevated intracellular IL-17A protein expression (p<0.0001) and 2.278 and 8.191 fold increases in the production of Th17 characteristic cytokines, IL-17A and IL-21 respectively.

Conclusion: The $TB^/$HIV microenvironment modifies monocyte mediated T-cell polarisation towards a pro-inflammatory Th17 phenotype, possibly due to increased IL-1β production.

Keywords

Tuberculosis, HIV, TB/HIV co-infection, Th17, IL-1β, monocytes

4.1 Introduction

Monocytes are innate effectors mediating the inflammatory response to pathogens as a source of macrophage and dendritic cell precursors, as well as antigen presentation, phagocytosis, and the production of antimicrobial substances [1, 2]. In addition, monocytes produce cytokines mediating T-cell polarisation at the site of inflammation [2-4].

Tuberculosis, is the second leading killer from a single infectious agent, *Mycobacterium tuberculosis* (MTB), globally, the first being the HIV [5]. Following inhalation of MTB, infected macrophages produce chemokines recruiting monocytes to the infection site [6]. These monocytes show increased peripheral and localised activation and frequency [7-9], killing MTB by Toll-like receptor (TLR) [10] and inducible nitric oxide synthase (iNOS) [11] mediated mechanisms. However, during MTB infection studies have observed differentiation of phenotypically and functionally immature monocyte derived macrophages [8], attenuated IFN-γ mediated antimycobacterial mechanisms [12], aberrant cytokine production [7, 8, 12] and increased monocyte apoptosis and necrosis [13, 14] which has been associated with tissue destruction and cavitation [9]. This indicates that MTB causes significant monocyte dysfunction contributing to disease pathogenesis. This is further complicated by HIV co-infection.

In South Africa 62% of TB patients are HIV co-infected. HIV increases the risk of active TB by 26-31 times with TB being the leading cause of HIV-related mortality [5]. Monocytes are less permissive to HIV-infection than other cell types. However, monocytes from HIV infected patients show impaired maturation and response to TLR ligands [15], alterations in gene expression related to TLR signalling and cytokine responses, cytokine-cytokine receptor interaction, activation, apoptosis and proteasome function [16-18], associated with disease pathogenesis [16].

The pathogenesis of TB and HIV is primarily mediated by dysregulated $CD4^+$ T-cell responses. During pathogen defence, monocytes mediate T-cell polarisation. Both TB and HIV cause monocyte dysfunction, but the effect of co-infection on monocyte mediated $CD4^+$ T-cell polarisation has not been explored.

The majority of HIV-related TB is extrapulmonary [19, 20], most commonly TB pleurisy [21]. Classically, direct MTB/HIV infection is thought to cause monocyte dysfunction; however, more recently secreted factors from MTB, HIV or infected cells are considered to have a greater effect [12, 16]. The consequences of TB/HIV co-infection on monocyte behavior is largely uncharacterised therefore a unicellular model needs to be initially probed to lay the foundation for observations in a multicellular system. We investigated an *in vitro* supernatant transfer model to question whether a TB/HIV pleural exudate altered monocyte (THP-1) cytokine production sufficiently to affect subsequent cytokine expression and polarisation of $CD4^+$ Tconvs into Th17/Treg subsets, differentially from a non-infectious inflammatory microenvironment.

4.2 Methods

4.2.1 Study population

This study protocol was approved by the institutional Biomedical Research Ethics Committee (BF170/11). Participants were recruited at Inkosi Albert Luthuli Central Hospital and Prince Mshiyeni Memorial Hospital (Durban, South Africa). Newly diagnosed TB patients presenting with a TB pleural effusion and concurrent HIV-infection (n=5) were enrolled. Active TB was confirmed by clinical diagnosis with bacterial or radiographic evidence; AFB culture positive sputum or pleural fluid, microscopy (Gram, Zhiel-Neelsen, Auramine), MTB DNA (Gene Xpert), pleural histology and pleural fluid cytology. The matched control group presented with a non-infectious pleural effusion (malignancy, cardiac failure, renal disease) and confirmed HIV seronegative (n=5) (Table 4.1). Exclusion criteria included anti-tuberculosis or anti-retroviral therapy, immunomodulatory treatment and conditions. Blood and pleural fluid were sampled by routine venesection and diagnostic pleural tap respectively, after informed consent was obtained.

Table 4.1: Summary of the demographic and clinical parameters of the study population

Parameter	TB^-HIV^- *n*=5	TB^+HIV^+ *n*=5
Age, years, median (range)	55 (46-61)	36 (18-64)
Gender		
Male	5 (100)	2 (40)
Female	0	3 (60)
Race		
African	3 (60)	5 (100)
Indian	2 (40)	0
TB diagnostic criteria		
Culture positive		4 (80)
Smear positive		1 (20)
PCR-MTBDR		5 (100)
Resistance to any one first line TB drug		1 (20)
MDR-TB		1 (20)
XDR-TB		0
CD4 count, mm^3, mean (range)		51 (12-123)
Past history of TB infection	0	1 (20)
Current smoker	2 (40)	1 (20)
Ex-smoker	1 (20)	0
Alcohol use	1 (20)	2 (40)
Definitive cause of PE		
Tuberculosis	0	5 (100)
Malignancy	2 (40)	0
End stage renal disease	2 (40)	0
Heart failure	1 (20)	0
Co-Morbidities		
Diabetes mellitus	2 (40)	0
Hypertension	3 (60)	1 (20)
Hepatitis B	0	1 (20)

Values expressed as n (%) unless otherwise stated

4.2.2 Pleural effusion fluid isolation

Cell free pleural effusion fluid (PEF) was extracted from heparinised pleural effusion samples by differential gradient centrifugation within 2 hours (h) of pleural tap. Briefly, 5 ml of sample was layered onto equivolume Histopaque-1077 (catalogue number (CN) 10771-500, Sigma, USA) and centrifuged (1,491rpm, 30 minutes (min), room temperature (RT)). PEF was aspirated and centrifuged (10,000rpm, 10min, 4°C) to remove any cellular constituents. Harvested PEF was pooled from 5 patients in each group equally and stored at -80°C.

4.2.3 THP-1 cell culture and treatment

Human acute monocytic leukaemic (THP-1) cells were obtained from ATCC. Cells were maintained in complete culture medium (CCM; RMPI 1640, 10% foetal calf serum (FCS), 1% L-glutamine, 1% Penstrep-Fungizone, 0.05mM 2-mercaptoethanol, 1mM sodium pyruvate) at 37°C, 5% CO_2. Prior to treatment, 7×10^6 cells were stimulated with 10µg/ml lipopolysaccharide in CCM (4h, 37°C, 5% CO_2). Stimulated THP-1 cells were harvested, washed (0.1M phosphate buffered saline (PBS), 1,000rpm, 10min, RT) and seeded in triplicate (3×10^5 cells/ml) in PEF conditioned CCM in a 1:1 ratio for 24h (37°C, 5% CO_2). Treatments included TB^-HIV^- and TB^+HIV^+ PEF. Supernatants were harvested by centrifugation (1,000rpm, 10min, RT) and stored at -80°C for cytokine profiling and downstream supernatant transfer.

4.2.4 ELISA

Quantitation of IL-1β, IL-6, IL-10, IL-12p70, TNF-α and IFN-γ in TB^+HIV^+ PEF, TB^-HIV^-PEF and PEF treated THP-1 supernatants was measured by enzyme-linked immunosorbent assay (ELISA) using OptEIA™ Sets (CN IL-1β 557953, IL-6 555220, IL-10 555157, IL-12p70 555183, TNF-α 555212, IFN-γ 555142, BD Biosciences, Canada). Microwells were coated with capture antibody (100µl/well, 4°C, overnight). The plates were washed three times (wash buffer; 300µl/well) and blocked with assay diluent (10% FCS in 0.1M PBS, 200µl/well, 1h, RT, 350rpm). The plates were washed 3 times; standards and samples (100µl) added and incubated (2h, RT, 350rpm). The plates were washed five times. IL-1β: detection antibody was added (100µl/well, 1h, RT, 350rpm), the plate washed five times and streptavidin-horseradish peroxidase (HRP) added (100µl/well, 30min, RT, 350rpm). IL-6, IL-10, IL-12p70, TNF-α, IFN-γ: working detector was added (detection antibody and Streptavidin-HRP reagent; 100µl/well, 1h, RT, 350rpm). All plates were washed 7 times and substrate solution added (100µl/well, 30min, RT). Stop solution (50µl/well) was added and the absorbance immediately read at 450nm with a reference of 570nm using a µQuant (Bio-Tek, USA) spectrophotometer. Standards and samples were run in triplicate. The results were normalised to the PEF and then compared between groups.

4.2.5 Peripheral blood mononuclear cell isolation

For use in $CD4^+$ T-cell isolation peripheral blood mononuclear cells (PBMCs) were extracted from whole blood (10ml, EDTA, one blood draw) of a healthy BCG vaccinated volunteer by differential gradient centrifugation within 2h of venipuncture. Briefly, 5 ml of sample was layered onto equivolume Histopaque-1077 and centrifuged (1,491rpm, 30min, RT). Buffy coats were aspirated and rinsed (0.1M PBS; 1,491rpm, 20min, RT).

4.2.6 $CD4^+$ T-cell isolation

$CD4^+$ T-lymphocytes were purified by negative magnetic selection using the Human CD4 T-Lymphocyte Enrichment Set-DM (CN 557939, BD Biosciences, USA). PBMCs were labelled with biotinylated monoclonal antibodies (5µl/10×10^6 cells, 15min, RT) against antigens on erythrocytes, platelets and non-$CD4^+$ leukocytes. The cells were washed (iMag Buffer, 1,491rpm, 20min) and incubated with streptavidin conjugated magnetic nanoparticles (5µl/10×10^6 cells, 30min, RT). The cell suspension was placed in a magnetic field (8min, iMagnet; BD Biosciences, USA) and the enriched $CD4^+$ T-cell supernatant aspirated. Negative selection of the positive fraction was performed three times to increase the yield.

4.2.7 $CD4^+$ T-cell culture and treatment

$CD4^+$ T-cells were seeded in triplicate (8×10^4cells/well, U-bottomed microplate) in THP-1 supernatant conditioned CCM (RPMI 1640, 10% FCS, 1% Penstrep-Fungizone, 1% L-glutamine, final concentrations) in a 1:1 ratio (24h, 37°C, 5% CO_2). Treatments included THP-1 supernatant: TB^-HIV^- PEF and THP-1 supernatant: TB^+HIV^+ PEF. Concurrently, cells were activated with Dynabeads Human T-activator CD3/CD28 bead solution (CN 11131D, Gibco, Norway) (2µl/well). Cells were harvested, washed (0.1M PBS, 1,491rpm, 20min, RT) and the activation beads removed by magnetic selection (1min, iMagnet). The supernatants were stored at -80°C for cytokine profiling and the cells immediately prepared for flow cytometric staining. This experiment was repeated three times.

4.2.8 Flow cytometric analysis

For surface staining, $2.5x10^5$ cells were re-suspended in staining buffer (25µl, 0.1M PBS, 1% FCS, 0.09% sodium azide) and labelled with surface antibodies (20min, 4°C); fluorescein isothiocyanate (FitC) anti-CD4 (1:5, CN 555346), peridin-chlorophyll-cyanin 5.5 (PerCP-Cy5.5) anti-CD25 (1:20, CN 560503). For intracellular staining, cells were fixed in 4% paraformaldehyde (500µl, 30min, RT), washed (staining buffer; 1,491rpm, 20min, RT), permeabilized (75% methanol, 500µl, 10min, -20°C) and washed again. Cells were stained with intracellular antibodies (60min, RT); phycoerythrin (PE) anti-Foxp3 (1:5, CN 560046), PerCP-Cy5.5 anti-IL17A (1:20, CN 560799), PE anti-RORC2 (1:40, CN IC6006P). Analysis was performed on an

Accuri C6 flow cytometer (BD Biosciences, USA) and data analysed using FlowJo vX (10.0.7r2). Samples were run in triplicate and 30,000 events acquired. Antibodies were purchased from BD Biosciences, USA, with the exception of anti-RORC2 which was purchased from R&D Systems, USA.

4.2.9 Cytokine profiling

Cytokines were measured using the Bio-Plex Pro Human Th17 Cytokine Panel (IL-1β, IL-4, IL-6, IL-10, IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IL-31, IL-33, IFN-γ, sCD40L, TNF-α) (CN 171AA001M, Bio-Rad, USA). Standards and samples (THP-1 supernatants and CD4 T-cell supernatants, 1:3) were diluted in standard and sample diluent HB respectively. The beads (50µl) were added to the assay plate and washed twice (100µl wash buffer, Bio-Plex Pro™ Wash Station). Standards, samples and controls were added (50µl, 1h, RT, 350rpm). The plate was washed three times and incubated with detection antibody (25µl, 30min, RT, 350rpm). The plate was washed three times and incubated with streptavidin-PE (50µl, 10min, RT, 350rpm). The plate was washed three times and the beads re-suspended in assay buffer (125µl, 30 seconds, RT, 350rpm). Data acquisition was performed on a Bio-Plex 200 (Bio-Rad, USA) and analysed using Bio-Plex Manager™ software v6.0. Samples, standards and controls were run in triplicate. Results were analysed using a 5-paramter logistic regression constructed from recombinant protein standards. The results were normalised to the treatment and then compared between groups.

4.2.10 Statistical analysis

The unpaired t-test with Welch correction was performed using GraphPad Prism software (v5.0). Outlier values were calculated using the interquartile range. Data is expressed as mean ± standard error of the mean (SEM), $p<0.05$ was considered statistically significant.

4.3 Results

4.3.1 TB/HIV pleurisy increases monocyte production of IL-1β

We first investigated whether $TB^{-}HIV^{-}$ and $TB^{+}HIV^{+}$ pleural exudates differentially affected monocyte cytokine production by measuring IL-1β, IL-6, IL-10, IL-12p70, TNF-α and IFN-γ levels in supernatants of THP-1 cells cultured in PEF conditioned media. A significantly 219.737 fold increase in THP-1 production of IL-1β, a Th17 generating cytokine was observed (0.076 ± 0.201pg/ml vs. 16.700 ± 2.335pg/ml, p=0.0217) following $TB^{+}HIV^{+}$ PEF treatment (Figure 4.1).

Interestingly, both monocyte activating cytokines IL-6 (-0.769 ± 1.731pg/ml vs. -20.577 ± 1.855pg/ml, p=0.0160) and TNF-α (2.500 ± 0.120pg/ml vs -2.847 ± 0.139pg/ml, p=0.0002), were reduced to a significantly greater extent in the supernatants of THP-1 cells cultured in $TB^{+}HIV^{+}$ media compared to $TB^{-}HIV^{-}$ conditions. No significant change in IL-10, IL-12p70 and IFN-γ production was observed (Figure 4.1).

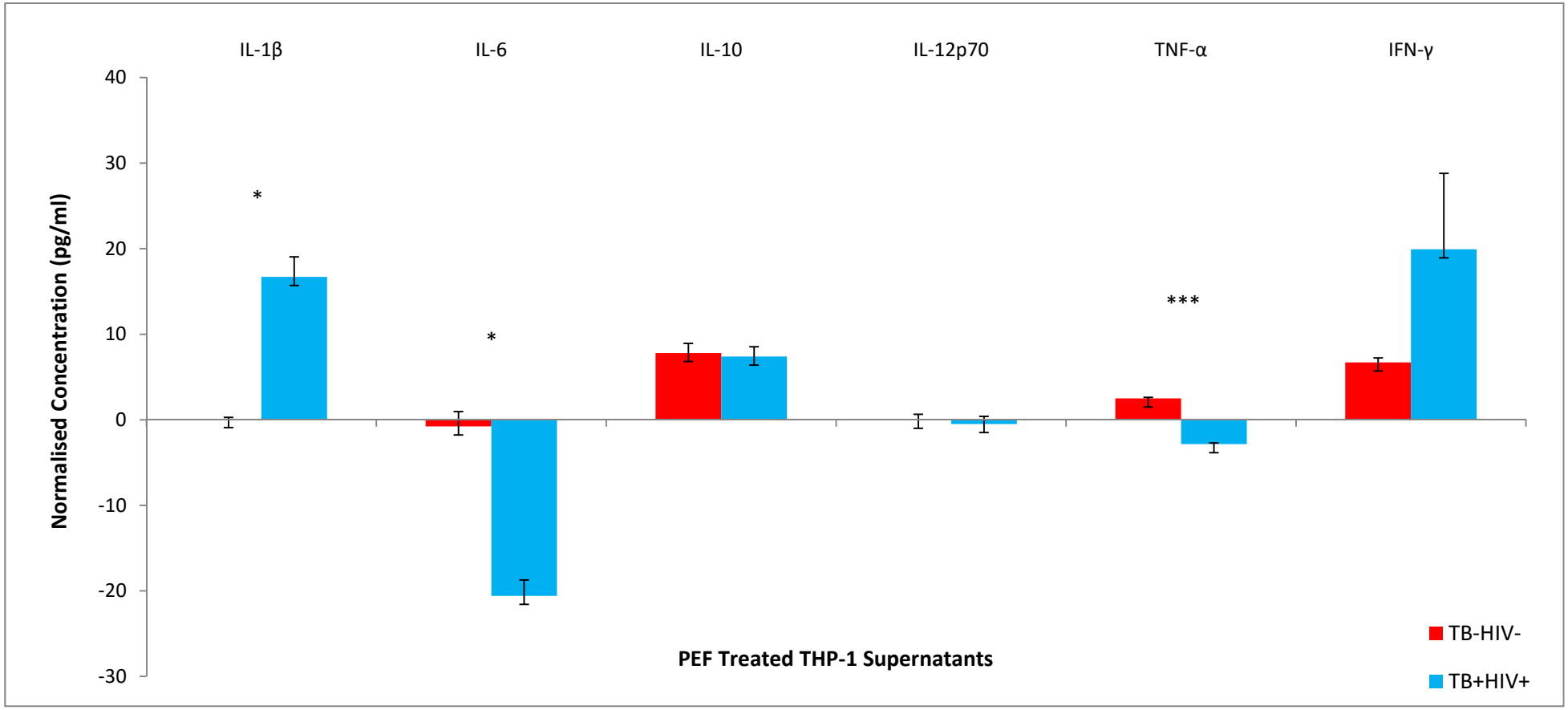

Figure 4.1: Normalised cytokine concentrations (pg/ml) of supernatants of THP-1 monocytes cultured in $TB^{-}HIV^{-}$ and $TB^{+}HIV^{+}$ PEF conditioned media. The $TB^{+}HIV^{+}$ pleural exudate resulted in significantly increased monocyte production of interleukin (IL)-1β (p=0.0217) but significantly decreased IL-6 (p=0.0160) and tumour necrosis factor (TNF)-α (p=0.0002) ($TB^{-}HIV^{-}$ n=3, $TB^{+}HIV^{+}$ n=3, Unpaired t-test with Welch correction). IFN; interferon.

4.3.2 TB/HIV co-infection increases monocyte mediated Th17 polarisation

We then assessed whether differentially conditioned monocytes and their resulting cytokine milieu were sufficient to effect downstream polarisation of $CD4^+$ Tconvs into Treg and Th17 subsets. Supernatants of THP-1 cells cultured in TB^+HIV^+ PEF caused a 1.671 fold increase in the frequency of $CD4^+CD25^+Foxp3^+$ Tregs (33.329 ± 2.470% vs. 55.700 ± 2.249%, $p<0.0001$), this was associated with a 1.429 fold increase in the lineage specific transcription factor, forkhead box P3 (Foxp3) protein expression (1759.000 ± 135.100 vs. 2514.333 ± 67.804 PE mean fluorescence intensity (MFI), $p=0.0004$) (Figure 4.2).

More notably, TB^+HIV^+ THP-1 supernatants resulted in a 2.174 fold increase in the proportion of $CD4^+$IL-$17A^+RORC^+$ Th17 lymphocytes (32.211 ± 1.036% vs. 70.022 ± 1.235%, $p<0.0001$) with a corresponding 1.287 fold increase in Th17 characteristic cytokine, IL-17A intracellular protein expression (1387.444 ± 19.194 vs 1785.111 ± 23.668 PerCP-Cy5.5 MFI, $p<0.0001$) (Figure 4.3).

Furthermore, while TB^-HIV^- THP-1 supernatant conditions resulted in similar proportions of Tregs and Th17 cells (33.329 ± 2.470% vs. 32.211 ± 1.036%, $p=0.6875$), the TB^+HIV^+ monocyte milieu resulted in a significant 14.322% (1.257 fold) greater proportion of Th17 lymphocytes than Tregs (55.700 ± 2.249% vs. 70.022 ± 1.235%, $p=0.0001$). This strongly indicates that TB^+HIV^+ PEF treatment resulted in a monocyte cytokine milieu that predominantly promotes Th17 differentiation, and further suggests that co-infection is associated with a pro-inflammatory microenvironment (Figure 4.4).

2A 2B 2C

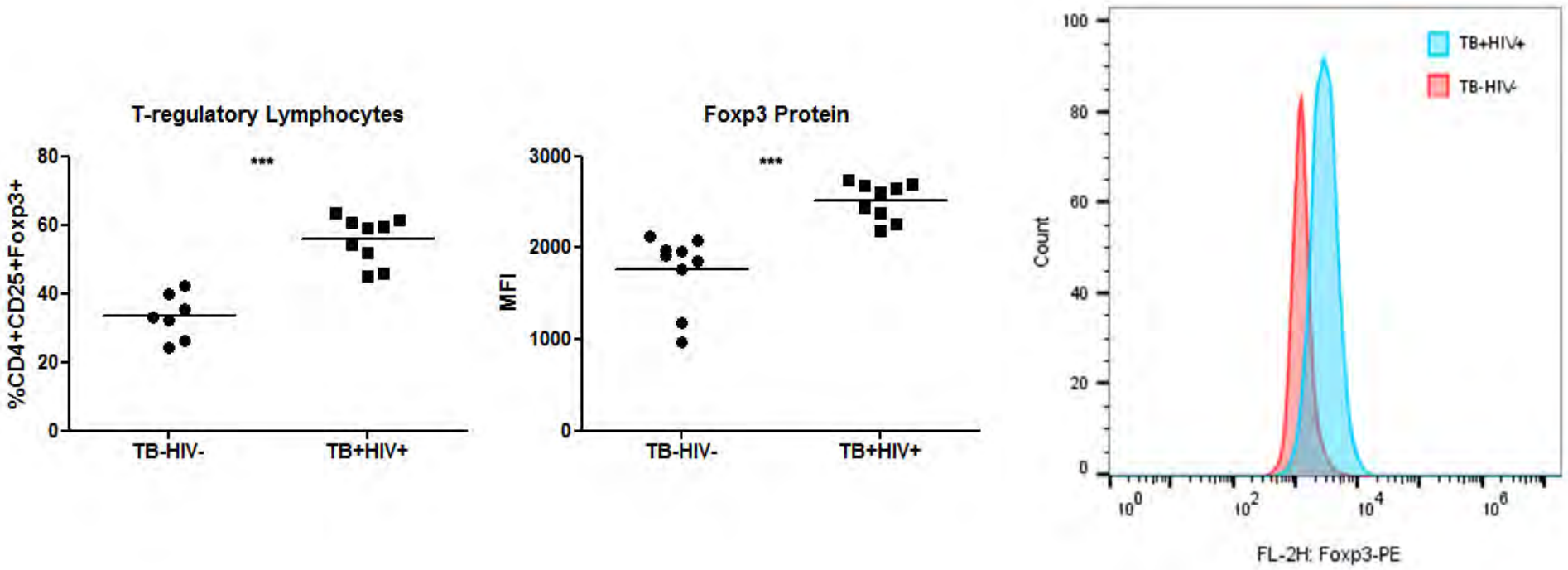

Figure 4.2: Change in T-regulatory lymphocyte frequency and forkhead box P3 protein expression. Supernatants of THP-1 cells treated with TB^+HIV^+ conditioned media resulted in a significantly elevated (A) proportion of $CD4^+CD25^+Foxp3^+$ T-regulatory lymphocytes ($p<0.0001$) and (B) Forkhead box P3 (Foxp3)-phycoerythrin (PE) mean florescence intensity (MFI) ($p=0.0004$) (TB^-HIV^- $n=9$, TB^+HIV^+ $n=9$, Unpaired t-test with Welch correction). Data represents the mean. (C) Histogram showing the shift in Foxp3-PE fluorescence in $CD4^+$ T-cells.

3A 3B 3C

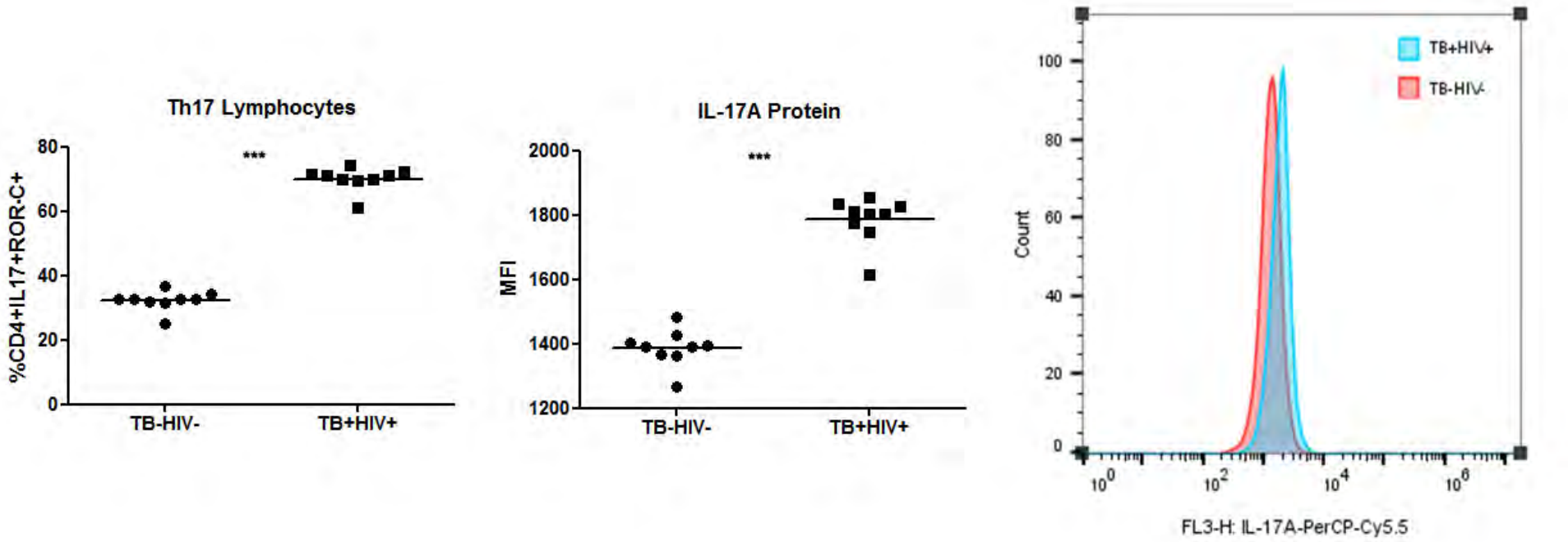

Figure 4.3: Change in T-helper 17 lymphocyte frequency and interleukin-17A protein expression. Supernatants of THP-1 cells treated with TB^+HIV^+ conditioned media resulted in a significantly elevated (A) proportion of $CD4^+IL\text{-}17A^+RORC^+$ T-helper (Th) 17 lymphocytes ($p<0.0001$) and (B) interleukin (IL)-17A-peridin-chlorophyll-cyanin 5.5 (PerCP-Cy5.5) mean fluorescence intensity (MFI) ($p<0.0001$) (TB^-HIV^- $n=9$, TB^+HIV^+ $n=9$, Unpaired t-test with Welch correction). Data represents the mean. (C) Histogram showing the shift in IL-17A- PerCP-Cy5.5 fluorescence in $CD4^+$ T-cells

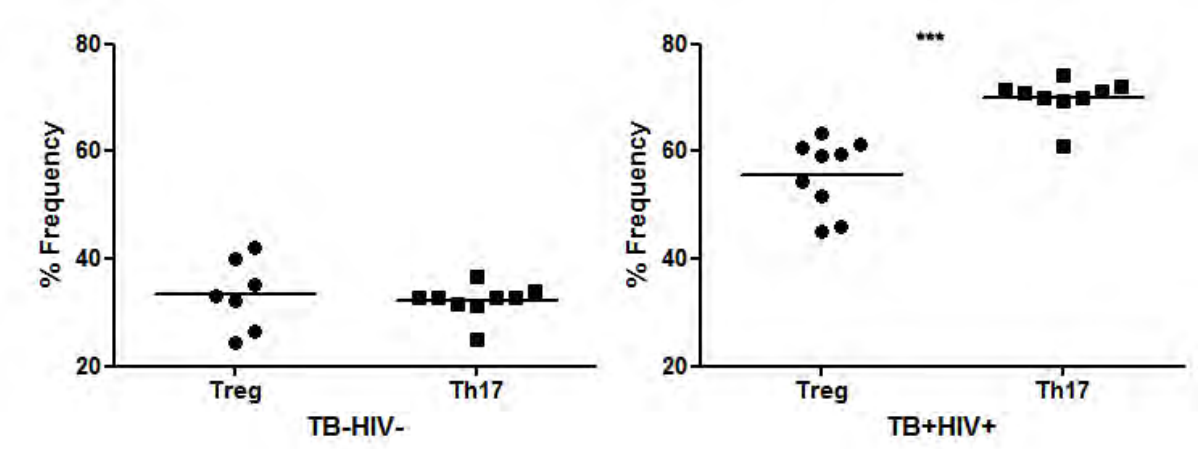

Figure 4.4: Comparison of T-regulatory and T-helper 17 lymphocyte proportion in $CD4^+$ conventional T-cells treated with supernatants of THP-1 monocytes cultured in PEF conditioned media. TB^+HIV^+ pleurisy resulted in a significantly increased proportion of T-helper (Th) 17 cells in comparison to T-regulatory (Treg) lymphocytes (p=0.0001) (TB^-HIV^- n=9, TB^+HIV^+ n=9, Unpaired t-test with Welch correction).

4.3.3 Increased $CD4^+$ T-cell production of IL-17A and IL-21

Next we questioned if there was a differential cytokine microenvironment associated with $CD4^+$ T-cell polarisation by performing a Bio-Plex cytokine screen of the extracellular milieu of $CD4^+$ Tconvs treated with supernatants of THP-1 monocytes cultured in PEF conditioned media.

TB^+HIV^+ supernatant transfer resulted in increased $CD4^+$ Tconv production of Th17 characteristic cytokines IL-17A (2.278 fold; 6.340 ± 0.855pg/ml vs. 14.440 ± 1.738pg/ml, p=0.0527) and IL-21 (8.191 fold; 3.850 ± 0.012pg/ml vs. 31.535 ± 12.710pg/ml, p=0.0527) compared to TB^-HIV^- conditions. Although not significant these were the only two cytokines produced by $CD4^+$ Tconvs (Figure 4.5).

Notably, $CD4^+$ Tconvs treated with supernatants of monocytes cultured in TB^+HIV^+ conditioned media were associated with a significantly greater 148.360 fold decrease in the concentration of IL-1β compared to TB^-HIV^- pleural exudate (-0.805 ± 0.078pg/ml vs. -119.430 ± 5.646pg/ml, p=0.0023). Furthermore, IFN-γ, a cytokine produced by and acting on effector $CD4^+$ T-cells in TB and HIV infection, showed a significantly larger 6.133 fold decrease in levels following TB^+HIV^+ treatment compared to TB^-HIV^- conditions (-82.290 ± 19.312 vs. -504.700 ± 21.171, p=0.0007) (Figure 4.5).

The extracellular milieu of $CD4^+$ Tconvs following TB^+HIV^+ supernatant transfer also showed significantly greater decreases in Th2 related cytokines IL-4 (-3.185 ± 0.240pg/ml vs. -14.325 ± 2.070pg/ml, p=0.0332),

IL-22 (-6.515 ± 0.707pg/ml vs. -35.405 ± 1.671pg/ml, p=0.0039), IL-25 (-1.120 ± 0.554pg/ml vs. -4.105 ± 0.488pg/ml, p=0.0272) and IL-31 (-32.555 ± 5.395pg/ml vs. -109.120 ± 1.882pg/ml, p=0.0055) compared to $TB^{-}HIV^{-}$ treatment (Figure 4.5).

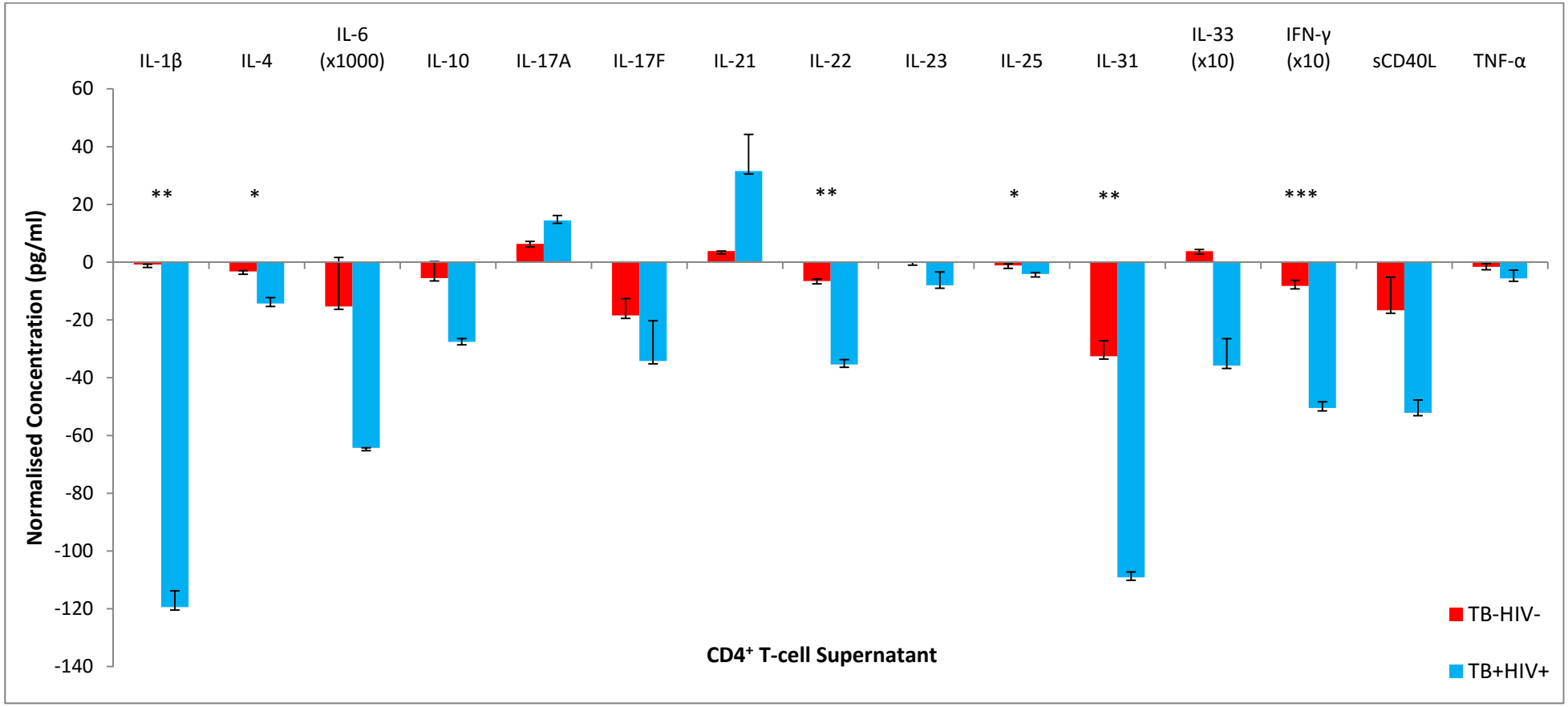

Figure 4.5: Normalised cytokine concentrations (pg/ml) of $CD4^+$ conventional T-cell supernatants treated with supernatants of monocytes cultured in PEF conditioned media. The TB^+HIV^+ conditions resulted in significantly reduced concentrations of interleukin (IL)-1β (p=0.0023), IL-4, (p=0.0332), IL-22 (p=0.0039), IL-25 (p=0.0272), IL-31 (p=0.0055) and interferon (IFN)-γ (p=0.0007) (TB^-HIV^- n=3, TB^+HIV^+ n=3, Unpaired t-test with Welch correction). sCD40L; soluble CD40 ligand, TNF; tumour necrosis factor.

4.4 Discussion

Through differential cytokine production, monocytes play an essential role in directing T-cell responses by influencing their polarisation and expansion [22]. Previous studies have shown conflicting alterations in monocyte cytokine production during MTB (TNF-α, IL-1β, IL-6, IL-10, IL-12p40 and IL-12p70 [8, 12, 13]), and HIV (IL-1β, IL-6, IL8, IL-10 and TNF-α [18, 23]) infection suggesting a modification in the nature of the $CD4^+$ T-cell response induced and maintained by monocytes. This has not been assessed in co-infection.

In this study a TB^+HIV^+ microenvironment resulted in a 219.737 fold greater increase in monocyte IL-1β production compared to a non-infectious pleural exudate, this is in line with previous research in both MTB [12, 24] and HIV infection [25]. In contrast, a significantly lower level of monocyte stimulating cytokines IL-6 and TNF-α was observed in the extracellular milieu, possibly representing cytokine levels following THP-1 receptor ligation.

IL-1β/IL-1 receptor (R) signalling has been highlighted as critically required for the early differentiation of Th17 cells at the site of inflammation and production of IL-17A during MTB infection [26-30]. Th17 cells are a pro-inflammatory subset of $CD4^+$ effector T-cells [31] that contribute to pathogen clearance by the production of pro-inflammatory IL-17A/F, IL-21 and IL-22 [32]. During homeostasis, Th17 cells play a pivotal role in mucosal immunity against intra-[33, 34] and extracellular [35, 36] bacterial and fungal infections and have been implicated in the pathogenesis of TB and HIV.

Th17 cells express higher IL-1R levels than other CD4 subsets [37, 38]. IL-1β ligation upregulates the expression of transcription factor interferon regulatory factor 4 (IRF4) and in turn, RAR-related orphan receptor C (RORC) which bind to the promoter region of *IL17A/F* stimulating Th17 differentiation and maintaining IL-17A, IL-17F, IL-21 and IL-22 cytokine production [37-42]. IL-21, in combination with TGF-β promotes Th17 lineage commitment via the activation of STAT3 and RORC [43]. The autocrine production of IL-21 by Th17 cells creates a feed forward cycle of Th17 polarisation [44].

$CD4^+$ Tconvs treated with supernatants of monocytes cultured in TB^+HIV^+ conditioned media were associated with a 2.174 and 1.287 fold increase in Th17 lymphocyte proportions and intracellular IL-17A protein expression respectively. Furthermore, following TB^+HIV^+ supernatant transfer, $CD4^+$ Tconvs produced 2.278 and 8.191 fold greater amounts of IL-17A and IL-21 respectively with significantly less IL-1β in the extracellular milieu, likely representing the use of IL-1β in monocyte IL-1R binding.

This suggest that under TB^+HIV^+ conditions increased monocyte production of IL-1β drives Th17 polarisation resulting in the predominant Th17 phenotype. In support of this, $CD14^+$ monocytes have been observed to spontaneously and specifically promote Th17 responses in blood derived $CD4^+$ T-cells in an IL-1β dependent manner [3]. Moreover, a clinical isolate of the hypervirulent W-Beijing strain of MTB has been shown to induce a potent IL-17 response through the induction of IL-1β [45].

Tregs, specified by the transcription factor Foxp3, are a suppressive subset of $CD4^+CD25^+$ T-cells which regulate effector lymphocyte function through contact dependent and independent mechanisms [46]. The cytokine milieu of TB^+HIV^+ PEF treated monocytes also resulted in a 1.671 fold increase in Tregs with a corresponding 1.429 fold increase in Foxp3 protein expression compared to TB^-HIV^- conditions. This; however, was not associated with an increased production of Treg cytokine IL-10 implying that Tregs do not contribute significantly to the inflammatory microenvironment in TB/HIV pleurisy. Moreover, $CD4^+$ Tconvs treated with supernatants of monocytes cultured in TB^+HIV^+ conditioned media showed a significant 14.322% greater proportion of Th17 cells compared to Tregs indicting a skewing towards a Th17 polarisation and a predominant pro-inflammatory environment during TB/HIV pleurisy.

There is a reciprocal relationship between Th17 and Treg developmental pathways exhibiting antagonism as well as plasticity [47-49]. Th17 cells and Tregs arise from common precursors and carry out opposing functions driven by the cytokine milieu and its effect on specific transcription factors. Foxp3 inhibits Th17 differentiation by antagonising the effects of RORC, inhibiting *IL-17A* promoter activation. IL-1β inhibits TGF-β induced Foxp3 expression enhancing Th17 differentiation under Th17 polarising conditions [55]. Taken together, IL-1β actively promotes Th17 differentiation and survival by upregulating lineage specific transcription factors while suppressing Foxp3 expression and Treg polarisation. We suggest that IL-1β mediated Foxp3 down regulation may be a potential mechanism facilitating a predominant Th17 lymphocyte generation in a TB^+HIV^+ microenvironment.

The balance of Th17 and Tregs at the infection site influences the outcome of the immune response. The Th17/IL-17 axis is suggested to provide protective immunity against MTB by contributing to granuloma formation and maintenance [50-53]. Early expression of pulmonary IL-17 is pivotal in cellular recruitment by inducing chemokine secretion from non-haematopoietic cells. This results in neutrophil and $CCR5^+$ lymphocyte recruitment, through the induction of IL-8 and chemokine C-X-C motif ligand (CXCL) 13 respectively, to form lung lymphoid follicles for optimal macrophage activation and bacterial clearance [45, 54-56]. However, during the chronic stages of infection, IL-17-CXCL mediated influx of immune cells results in extensive tissue destruction and cavitation [50, 51, 57-60]. Th17 responses also contribute to mucosal TB vaccine immunity by IL-17A induction of CXCL13 [45]. In co-infection, the TB^+HIV^+ monocyte mediated promotion of a Th17 pro-inflammatory phenotype may aid in granuloma organisation providing a niche of MTB survival and at later stages facilitating transmission, as well as accentuating hyper-immune activation contributing to viral replication.

We observed a significantly reduced concentration of IFN-γ, IL-22 and Th2 related cytokines; IL-4, IL-25 and IL-31 in the extracellular milieu of $CD4^+$ Tconvs treated with supernatants of monocytes cultured in TB^+HIV^+ PEF conditioned media. IFN-γ is the preeminent cytokine in MTB immune defence and acts on $CD4^+$ T-cells stimulating apoptosis to limit the T-cell immune response [61, 62]. This suggests that a decline in IFN-γ levels is possibly due to ligation of IFN-γ receptors on $CD4^+$ Tconvs. T-cells do not express the IL-

22 receptor complex and IL-22 has no effect on resting or activated immune cells *in vitro* or *in vivo* [63]. Together with a short *in vivo* half-life of IL-22 in animal models (1.3-2 hours) [64] this indicates that a reduced concentration of IL-22 is likely due to cytokine breakdown in the extracellular environment. Likewise, MTB has been shown to drive Th2 polarisation by altering the balance of polarising cytokines in an IL-1β dependent manner [65]; however, the fact that $CD4^+$ Tconvs did not produce Th2 cytokines under TB^+HIV^+ conditions leads us to conclude that the decreased amounts of IL-4, IL-25 and IL-31 in the microenvironment is likely the result of cytokine degradation.

4.5 Concluding Remarks

The microenvironment in TB/HIV pleurisy alters monocyte cytokine production sufficiently to skew $CD4^+$ Tconv polarisation towards a pro-inflammatory Th17 phenotype, potentially due to increased monocyte IL-1β production. In this way TB/HIV co-infection modifies the adaptive immune response from the initial interactions with the innate immune system.

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References

1. Italiani P, Boraschi D. From monocytes to M1/M2 macrophages: Phenotypical vs. functional differentiation. *Front Immunol* 2014; **5**:514-535.

2. Serbina NV, Jia T, Hohl TM, Pamer EG. Monocyte-mediated defense against microbial pathogens. *Annu Rev Immunol* 2008; **26**:421-452.

3. Evans HG, Gullick NJ, Kelly S, Pitzalis C, Lord GM, Kirkham BW*, et al. In vivo* activated monocytes from the site of inflammation in humans specifically promote Th17 responses. *Proc Natl Acad Sci U S A* 2009; **106**:6232-6237.

4. Jakubzick C, Gautier EL, Gibbings SL, Sojka DK, Schlitzer A, Johnson TE*, et al.* Minimal differentiation of classical monocytes as they survey steady-state tissues and transport antigen to lymph nodes. *Immunity* 2013; **39**:599-610.

5. World Heath Organisation. *Global tuberculosis report 2014.* Geneva, Switzerland: World Heath Organisation 2014.

6. Gonzalez-Juarrero M, Shim TS, Kipnis A, Junqueira-Kipnis AP, Orme IM. Dynamics of macrophage cell populations during murine pulmonary tuberculosis. *J Immunol* 2003; **171**:3128-3135.

7. Castaño D, García LF, Rojas M. Increased frequency and cell death of $CD16^+$ monocytes with *Mycobacterium tuberculosis* infection. *Tuberculosis* 2011; **91**:348-360.

8. Chávez-Galán L, Ocaña-Guzmán R, Torre-Bouscoulet L, García-de-Alba C, Sada-Ovalle I. Exposure of monocytes to lipoarabinomannan promotes their differentiation into functionally and phenotypically immature macrophages. *J Immunol Res* 2015; **2015**: e984973

9. Balboa L, Romero MM, Basile JI, y García CAS, Schierloh P, Yokobori N*, et al.* Paradoxical role of $CD16^+CCR2^+CCR5^+$ monocytes in tuberculosis: Efficient APC in pleural effusion but also mark disease severity in blood. *J Leukoc Biol* 2011; **90**:69-75.

10. Thoma-Uszynski S, Stenger S, Takeuchi O, Ochoa MT, Engele M, Sieling PA*, et al.* Induction of direct antimicrobial activity through mammalian Toll-like receptors. *Science* 2001; **291**:1544-1547.

11. Nicholson S, Bonecini-Almeida Mda G, Lapa e Silva JR, Nathan C, Xie QW, Mumford R*, et al.* Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis. *J Exp Med* 1996; **183**:2293-2302.

12. Castaño D, Barrera LF, Rojas M. *Mycobacterium tuberculosis* alters the differentiation of monocytes into macrophages *in vitro*. *Cell Immunol* 2011; **268**:60-67.

13. Sánchez MD, García Y, Montes C, París SC, Rojas M, Barrera LF*, et al.* Functional and phenotypic changes in monocytes from patients with tuberculosis are reversed with treatment. *Microbes Infect* 2006; **8**:2492-2500.

14. Chávez-Galán L, Sada-Ovalle I, Baez-Saldaña R, Chávez R, Lascurain R. Monocytes from tuberculosis patients that exhibit cleaved caspase 9 and denaturalized cytochrome C are more susceptible to death mediated by Toll-like receptor 2. *Immunology* 2012; **135**:299-311.

15. Jiang W, Lederman MM, Salkowitz JR, Rodriguez B, Harding CV, Sieg SF. Impaired monocyte maturation in response to CpG oligodeoxynucleotide is related to viral RNA levels in human immunodeficiency virus disease and is at least partially mediated by deficiencies in α/β interferon responsiveness and production. *J Virol* 2005; **79**:4109-4119.

16. Van den Bergh R, Florence E, Vlieghe E, Boonefaes T, Grooten J, Houthuys E*, et al.* Transcriptome analysis of monocyte-HIV interactions. *Retrovirology* 2010; **7**:53-68.

17. Pulliam L, Sun B, Rempel H. Invasive chronic inflammatory monocyte phenotype in subjects with high HIV-1 viral load. *J Neuroimmunol* 2004; **157**:93-98.

18. Wu JQ, Sassé TR, Wolkenstein G, Conceicao V, Saksena MM, Soedjono M*, et al.* Transcriptome analysis of primary monocytes shows global down-regulation of genetic networks in HIV viremic patients versus long-term non-progressors. *Virology* 2013; **435**:308-319.

19. Barker RD. Clinical tuberculosis. *Medicine* 2012; **40**:340-345.

20. Chaisson RE, Schecter GF, Theuer CP, Rutherford GW, Echenberg DF, Hopewell PC. Tuberculosis in patients with the acquired immunodeficiency syndrome: Clinical features, response to therapy and survival. *Am Rev Respir Dis* 1987; **136**:570-574.

21. TB DOTS Strategy Co-ordiantion Directorate. *National Tuberculosis Management Guidelines.* Republic of South Africa: National Department of Health 2014.

22. Geissmann F, Auffray C, Palframan R, Wirrig C, Ciocca A, Campisi L*, et al.* Blood monocytes: Distinct subsets, how they relate to dendritic cells, and their possible roles in the regulation of T-cell responses. *Immunol Cell Biol* 2008; **86**:398-408.

23. Collini P, Noursadeghi M, Sabroe I, F Miller R, H Dockrell D. Monocyte and macrophage dysfunction as a cause of HIV-1 induced dysfunction of innate immunity. *Curr Mol Med* 2010; **10**:727-740.

24. Wallis R, Amir-Tahmasseb M, Ellner J. Induction of interleukin 1 and tumor necrosis factor by mycobacterial proteins: The monocyte western blot. *Proc Natl Acad Sci U S A* 1990; **87**:3348-3352.

25. Jalbert E, Crawford TQ, D'Antoni ML, Keating SM, Norris PJ, Nakamoto BK, *et al.* IL-1β enriched monocytes mount massive IL-6 responses to common inflammatory triggers among chronically HIV-1 infected adults on stable anti-retroviral therapy at risk for cardiovascular disease. *PloS One* 2013; **8**:e75500.

26. van de Veerdonk FL, Teirlinck AC, Kleinnijenhuis J, Kullberg BJ, van Crevel R, van der Meer JW, *et al. Mycobacterium tuberculosis* induces IL-17A responses through TLR4 and dectin-1 and is critically dependent on endogenous IL-1. *J Leukoc Biol* 2010; **88**:227-232.

27. Fremond CM, Togbe D, Doz E, Rose S, Vasseur V, Maillet I, *et al.* IL-1 receptor-mediated signal is an essential component of MyD88-dependent innate response to *Mycobacterium tuberculosis* infection. *J Immunol* 2007; **179**:1178-1189.

28. Mayer-Barber KD, Barber DL, Shenderov K, White SD, Wilson MS, Cheever A, *et al.* Cutting edge: Caspase-1 independent IL-1β production is critical for host resistance to *Mycobacterium tuberculosis* and does not require TLR signaling *in vivo*. *J Immunol* 2010; **184**:3326-3330.

29. Gomez L, Camargo J, Castiblanco J, Ruiz-Narváez E, Cadena J, Anaya J. Analysis of IL-1β, TAP1, TAP2 and IKBL polymorphisms on susceptibility to tuberculosis. *Tissue Antigens* 2006; **67**:290-296.

30. Wilkinson RJ, Patel P, Llewelyn M, Hirsch CS, Pasvol G, Snounou G, *et al.* Influence of polymorphism in the genes for the interleukin (IL)-1 receptor antagonist and IL-1β on tuberculosis. *J Exp Med* 1999; **189**:1863-1874.

31. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang Y-H, *et al.* A distinct lineage of CD4 T-cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 2005; **6**:1133-1141.

32. Huber S, Gagliani N, Flavell RA. Life, death, and miracles: Th17 cells in the intestine. *Eur J Immunol* 2012; **42**:2238-2245.

33. Zhou X, Chen Q, Moore J, Kolls JK, Halperin S, Wang J. Critical role of the interleukin-17/interleukin-17 receptor axis in regulating host susceptibility to respiratory infection with chlamydia species. *Infect Immun* 2009; **77**:5059-5070.

34. Zhang X, Gao L, Lei L, Zhong Y, Dube P, Berton MT, *et al.* A MyD88-dependent early IL-17 production protects mice against airway infection with the obligate intracellular pathogen *Chlamydia muridarum*. *J Immunol* 2009; **183**:1291-1300.

35. Kudva A, Scheller EV, Robinson KM, Crowe CR, Choi SM, Slight SR, *et al.* Influenza A inhibits Th17-mediated host defense against bacterial pneumonia in mice. *J Immunol* 2011; **186**:1666-1674.

36. Ye P, Garvey PB, Zhang P, Nelson S, Bagby G, Summer WR, *et al.* Interleukin-17 and lung host defense against *Klebsiella pneumoniae* infection. *Am J Respir Cell Mol Biol* 2001; **25**:335-340.

37. Lee W-W, Kang SW, Choi J, Lee S-H, Shah K, Eynon EE, *et al.* Regulating human Th17 cells via differential expression of IL-1 receptor. *Blood* 2010; **115**:530-540.

38. Chung Y, Chang SH, Martinez GJ, Yang XO, Nurieva R, Kang HS, *et al.* Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. *Immunity* 2009; **30**:576-587.

39. Brüstle A, Heink S, Huber M, Rosenplänter C, Stadelmann C, Yu P, *et al.* The development of inflammatory Th17 cells requires interferon-regulatory factor 4. *Nat Immunol* 2007; **8**:958-966.

40. Ikeda S, Saijo S, Murayama MA, Shimizu K, Akitsu A, Iwakura Y. Excess IL-1 signaling enhances the development of Th17 cells by downregulating TGF-β induced Foxp3 expression. *J Immunol* 2014; **192**:1449-1458.

41. Sundrud MS, Trivigno C. Identity crisis of Th17 cells: Many forms, many functions, many questions. *Semin Immunol* 2013; **25**:263-272.

42. Zielinski CE, Mele F, Aschenbrenner D, Jarrossay D, Ronchi F, Gattorno M, *et al.* Pathogen-induced human Th17 cells produce IFN-γ or IL-10 and are regulated by IL-1β. *Nature* 2012; **484**:514-518.

43. Yang L, Anderson DE, Baecher-Allan C, Hastings WD, Bettelli E, Oukka M, *et al.* IL-21 and TGF-β are required for differentiation of human Th17 cells. *Nature* 2008; **454**:350-352.

44. Wei L, Laurence A, Elias KM, O'Shea JJ. IL-21 is produced by Th17 cells and drives IL-17 production in a STAT3-dependent manner. *J Biol Chem* 2007; **282**:34605-34610.

45. Gopal R, Rangel-Moreno J, Slight S, Lin Y, Nawar HF, Junecko BF, *et al.* Interleukin-17-dependent CXCL13 mediates mucosal vaccine–induced immunity against tuberculosis. *Mucosal Immunol* 2013; **6**:972-984.

46. Collison LW, Workman CJ, Kuo TT, Boyd K, Wang Y, Vignali KM, *et al.* The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* 2007; **450**:566-569.

47. Ichiyama K, Yoshida H, Wakabayashi Y, Chinen T, Saeki K, Nakaya M, *et al.* Foxp3 Inhibits RORγt-mediated IL-17A mRNA transcription through direct interaction with RORγt. *J Biol Chem* 2008; **283**:17003-17008.

48. Zhou L, Lopes JE, Chong MM, Ivanov, II, Min R, Victora GD, *et al.* TGF-β induced Foxp3 inhibits Th17 cell differentiation by antagonizing ROR-γt function. *Nature* 2008; **453**:236-240.

49. Noack M, Miossec P. Th17 and regulatory T-cell balance in autoimmune and inflammatory diseases. *Autoimmun Rev* 2014; **13**:668-677.

50. Torrado E, Cooper AM. IL-17 and Th17 cells in tuberculosis. *Cytokine Growth Factor Rev* 2010; **21**:455-462.

51. Umemura M, Yahagi A, Hamada S, Begum MD, Watanabe H, Kawakami K, *et al.* IL-17 mediated regulation of innate and acquired immune response against pulmonary *Mycobacterium bovis* bacille Calmette-Guerin infection. *J Immunol* 2007; **178**:3786-3796.

52. Redford PS, Boonstra A, Read S, Pitt J, Graham C, Stavropoulos E, *et al.* Enhanced protection to *Mycobacterium tuberculosis* infection in IL-10 deficient mice is accompanied by early and enhanced Th1 responses in the lung. *Eur J Immunol* 2010; **40**:2200-2210.

53. Yoshida YO, Umemura M, Yahagi A, O'Brien RL, Ikuta K, Kishihara K, *et al.* Essential role of IL-17A in the formation of a mycobacterial infection-induced granuloma in the lung. *J Immunol* 2010; **184**:4414-4422.

54. Seiler P, Aichele P, Bandermann S, Hauser AE, Lu B, Gerard NP, *et al.* Early granuloma formation after aerosol *Mycobacterium tuberculosis* infection is regulated by neutrophils via CXCR3-signaling chemokines. *Eur J Immunol* 2003; **33**:2676-2686.

55. Silva MT. When two is better than one: Macrophages and neutrophils work in concert in innate immunity as complementary and cooperative partners of a myeloid phagocyte system. *J Leukoc Biol* 2010; **87**:93-106.

56. Miossec P, Kolls JK. Targeting IL-17 and Th17 cells in chronic inflammation. *Nat Rev Drug Discov* 2012; **11**:763-776.

57. Torrado E, Robinson RT, Cooper AM. Cellular response to mycobacteria: Balancing protection and pathology. *Trends Immunol* 2011; **32**:66-72.

58. Etna MP, Giacomini E, Severa M, Coccia EM. Pro-and anti-inflammatory cytokines in tuberculosis: A two-edged sword in TB pathogenesis. *Semin Immunol* 2014; **26**:543-551.

59. Cruz A, Fraga AG, Fountain JJ, Rangel-Moreno J, Torrado E, Saraiva M, *et al.* Pathological role of interleukin 17 in mice subjected to repeated BCG vaccination after infection with *Mycobacterium tuberculosis*. *J Exp Med* 2010; **207**:1609-1616.

60. Wozniak TM, Saunders BM, Ryan AA, Britton WJ. *Mycobacterium bovis* BCG-specific Th17 cells confer partial protection against *Mycobacterium tuberculosis* infection in the absence of interferon-γ. *Infect Immun* 2010; **78**:4187-4194.

61. Cooper AM, Adams LB, Dalton DK, Appelberg R, Ehlers S. IFN-γ and NO in mycobacterial disease: New jobs for old hands. *Trends Microbiol* 2002; **10**:221-226.

62. Dalton DK, Haynes L, Chu C-Q, Swain SL, Wittmer S. Interferon-γ eliminates responding CD4 T-cells during mycobacterial infection by inducing apoptosis of activated CD4 T-cells. *J Exp Med* 2000; **192**:117-122.

63. Wolk K, Kunz S, Witte E, Friedrich M, Asadullah K, Sabat R. IL-22 increases the innate immunity of tissues. *Immunity* 2004; **21**:241-254.

64. Wu D, Huang Z, Liu H, Huang Y, Yan X. Uses of interleukin-22 (IL-22) in treating and preventing nerve damage diseases or neurodegenerative diseases. US Patent 20, 147, 293; 2015.

65. Dwivedi VP, Bhattacharya D, Chatterjee S, Prasad DV, Chattopadhyay D, Van Kaer L*, et al.* *Mycobacterium tuberculosis* directs T-helper 2 cell differentiation by inducing interleukin-1β production in dendritic cells. *J Bioll Chem* 2012; **287**:33656-33663.

CHAPTER 5

CONCLUSION

TB/HIV co-infection is a global pandemic, of which SA is one of the worst affected countries. The pathogenesis of the individual diseases is largely driven by pathogenic dysregulation of $CD4^+$ T-cell responses, the subset specific alterations of which have not been assessed in co-infection. TB pleurisy is the most common form of extrapulmonary HIV-related TB and provides an optimal localised immune model to study host responses to co-infection, which is often neglected.

This study characterised the nature of the Th17 and Treg lymphocyte responses in TB/HIV pleurisy. The comparison of biological parameters in a TB^+HIV^+ and non-infectious pleural effusion indicates that the reduced Treg stability (p=0.0084), Th17 proportion (p=0.0054) and monocyte mediated skewing towards Th17 polarisation (p<0.0001) is specific to co-infection and precludes the interpretation of a generalised pleuritic response. This pilot study is based on a small sample size and this may have limited the extent of our findings, these results should be validated in a larger cohort.

The TB/HIV microenvironment was not conducive to Treg stability (52.769 ± 5.413% vs. 31.720 ± 3.821%, p=0.0084) or Th17 polarisation (64.100 ± 5.045% vs. 36.233 ± 6.968% p=0.0054) *ex vivo* and confirmed *in vitro* (p=0.0467 and p=0.0001 respectively), as evidenced by a significantly reduced frequency of these subsets. Furthermore an impairment of both pro- and anti-inflammatory $CD4^+$ T-cell immune responses indicates a suppression of localised immunity as a whole. In contrast, the TB^+HIV^+ microenvironment altered monocyte cytokine production to promote both Treg (33.329 ± 2.470% vs. 55.700 ± 2.249%, p<0.0001) and Th17 (32.211 ± 1.036% vs. 70.022 ± 1.235%, p<0.0001) polarisation *in vitro*. This suggests that the loss of Th17 and Treg localised responses at the infection site is not initiated by a monocytic innate immune response or may reflect the differences in a multicellular compared to unicellular model.

A loss of Treg stability *ex vivo* was associated with an IL-35 functional deficit (p=0.0258) and is likely due to altered miR-155/SOCS1 dynamics and loss of IL-2 competitive fitness. We were unable to identify a potential mechanism of diminished Th17 proportion but determined that it was not a result of alterations in the measured polarising signals at the molecular or cytokine level. This prompts further investigation into other inhibitory cytokines and epigenetic modifications involved in Th17 lymphocyte generation. Interestingly, there was no impairment in the levels of Th17 effector cytokines (IL-17A/F) observed.

Notably, despite Th17/Treg suppression, a higher proportion of Th17 cells than Tregs was found in PEMCs *ex vivo* (31.720 ± 3.821% vs. 36.233 ± 6.968%, p=0.5490) and *in vitro* (27.900 ± 2.024% vs. 55.156 ±

1.299%, p=0.0004) indicating a predominantly pro-inflammatory microenvironment. This trend was mirrored in the THP-1 monocyte model where the supernatants of monocytes cultured in $TB^{+}HIV^{+}$ conditioned media resulted in $CD4^{+}$ Tconv polarisation towards a chiefly pro-inflammatory Th17/IL-17A phenotype (55.700 ± 2.249% vs. 70.022 ± 1.235%, p=0.0001). This was potentially the result of MTB/HIV mediated increase in monocyte IL-1β production (219.737 fold, p=0.0217). IL-1 β was also significantly upregulated at the transcript and protein level (p=0.0022 and p=0.0008 respectively) in $TB^{+}HIV^{+}$ pleurisy, likely providing a mechanism of Th17 preponderance. Simplistically, despite a suppressed immunity as a whole, a prevailing pro-inflammatory environment advances both TB and HIV pathogenesis potentially by promoting granulomatous pathology and bacterial transmission, as well as immune hyperactivation and increased viral replication respectively.

Appendix 1: Ethical approval

UNIVERSITY OF
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08 March 2012

Ms. Vanessa Korb
Department of Medical Biochemistry
Room219, second floor
DDMRI Building
Nelson R Mandela School of Medicine
University of KwaZulu-Natal

Dear Ms Korb

FINAL APPROVAL

PROTOCOL: The Immunoregulatory Mechanisms in Active TB infection. REF: BF170/11

The Biomedical Research Ethics Committee (BREC) has considered the abovementioned application.

The study was provisionally approved by a sub-committee of BREC on 17 February 2012 pending appropriate responses to queries raised. Your responses have been noted by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given full ethics approval and may begin as from 08 March 2012.

This approval is valid for one year from **08 March 2012.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2004), South African National Good Clinical Practice Guidelines

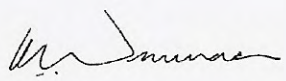
(2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/ResearchEthics11415.aspx.
BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The following Committee members were present at the meeting that took place on 11 October 2011:

Professor D Wassenaar	Chair
Professor V Rambiritch	Pharmacology
Prof D Pudifin	Medicine
Prof C Rout	Anaesthetics
Dr S Singh	Dentistry
Dr U Govind	Private Pract. - Gen. Practitioner
Professor S Collings	Psychology
Prof R Bhimma	Paediatrics and Child Health
Mrs T Makhanya	Community Representative
Dr D Singh	Anaesthetics
Dr Z Khumalo	KZN Health (External)
Professor J Tsoka-Gwegweni	Medicine
Dr K Naidoo	Family Medicine
Prof J Singh	Legal Representative
Dr T Hardcastle	Surgery - Trauma

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely



PROFESSOR D R WASSENAAR
Chair: Biomedical Research Ethics Committee

Appendix 2: Corresponding peripheral blood results for Chapter 2 as well as healthy control and human immunodeficiency virus mono-infected cohorts

2.1 Foxp3 gene expression

Foxp3 is indispensable for Treg lineage specification and immunosuppressive activities. We performed qPCR to evaluate the expression of Foxp3 transcript. Compared to the healthy control (HC) cohort, the TB^-HIV^- PBMCs showed as significant 1.552 ± 0.240 fold increase (p=0.0077) and the TB^+HIV^+ PBMCs showed a significant -4.118 ± 0.240 fold decrease (*p*=0.0077) in Foxp3 mRNA expression suggesting increased and deceased immunosuppressive capacity respectively, at the molecular level (Table A2.1).

Relative to TB^-HIV^- PBMCs, TB^+HIV^+ PBMCs showed a remarkable -6.085 ± 0.707 (*p*=0.0048) fold decrease in Foxp3 mRNA expression, this is a reversal of the trend observed in PEMCs indicating a disparate localised and systemic Treg immune response between cohorts (Table A2.1).

Furthermore, in both the TB^-HIV^- and TB^+HIV^+ cohorts, Foxp3 mRNA was expressed at significantly higher levels at the localised pleural effusion site than the peripheral blood (2.457 ± 0.167, p=0.0028 and 33.935 ± 4.298, *p*=0.0050 fold increases respectively) implying a greater immunosuppressive capacity at the site of infection (Table A2.1). Foxp3 mRNA was undetectable in PBMCs from the HIV mono-infected cohort.

Table A2.1: Forkhead box P3 mRNA expression in pooled peripheral blood and pleural effusion mononuclear cells

Population	Relative Fold Change	p-value
HC PBMC* : TB^-HIV^- PBMC	1.552 ± 0.240	0.0077
HC PBMC* : TB^+HIV^+ PBMC	-4.118 ± 0.886	0.0077
TB^-HIV^- PBMC* : TB^+HIV^+ PBMC	-6.085 ± 0.707	0.0048
TB^-HIV^- PBMC* : TB^-HIV^- PEMC	2.457 ± 0.167	0.0028
TB^+HIV^+ PBMC* : TB^+HIV^+ PEMC	33.935 ± 4.298	0.0050

Results expressed as mean ± SEM, *n*=6 replicates in each group, Mann-Whitney test. * Indicates control group used in analyses. Healthy control (HC).

2.2 Foxp3 protein expression

The change in relative Foxp3 protein expression, represented as mean fluorescence intensity (MFI), was measured by flow cytometry. PBMC Foxp3 protein expression did not significantly differ between HC PBMCs and $TB^{-}HIV^{-}$ or $TB^{+}HIV^{+}$ PBMCs. However, HIV mono-infection showed a significant -1.726 fold decrease in PBMC Foxp3 protein (1107.333 ± 143.329 vs. 641.533 ± 7.613 phycoerythrin (PE)-MFI, p=0.0002) indicating a loss of immunoregulatory capability (Table A2.2).

In comparison to the HIV mono-infected population, the co-infected PBMCs showed a significant 1.269 fold increase in Foxp3 protein (641.533 ± 7.613 vs. 814.333 ± 36.032 PE-MFI, p=0008) suggesting increased immunosuppressive activity in co-infection (Table A2.2).

In line with the PEMC results, there was significant -1.193 fold decrease in $TB^{+}HIV^{+}$ PBMC Foxp3 protein expression compared to the $TB^{-}HIV^{-}$ population (971. 222 ± 45.938 vs. 814.333 ± 36.032 PE-MFI, p=0.0360), highlighting a similar trend in Foxp3 protein levels across the localised and systemic compartments (Table A2.2).

Furthermore there was no significant difference in Foxp3 protein expression between $TB^{+}HIV^{+}$ PBMCs and PEMCs implying an even distribution of immunosuppressive function. However, there was a notable 1.641 fold increase in the level of Foxp3 protein in the $TB^{-}HIV^{-}$ PEMCs compared to PBMCs (971. 222 ± 45.938 vs. 1594.222 ± 86.189 PE-MFI, p<0.0001) implying an expansion of immunosuppressive activity at the infection site in non-infectious pleural effusions (Table A2.2).

Table A2.2: Forkhead box P3 relative protein expression in peripheral blood and pleural effusion mononuclear cells

Population	Foxp3-PE (MFI)	p-value
HC PBMC	1107.333 ± 143.329	
TB^-HIV^- PBMC	971. 222 ± 45.938	
TB^+HIV^+ PBMC	814.333 ± 36.032	
HIV^+ PBMC	641.533 ± 7.613	
TB^-HIV^- PEMC	1594.222 ± 86.189	
TB^+HIV^+ PEMC	959.556 ± 127.654	
HC PBMC : TB^-HIV^- PBMC		0.6958
HC PBMC : TB^+HIV^+ PBMC		0.3485
HC PBMC : HIV^+ PBMC		0.0002
HIV^+ PBMC : TB^+HIV^+ PBMC		0.0008
TB^-HIV^- PBMC : TB^+HIV^+ PBMC		0.0360
TB^-HIV^- PBMC : TB^-HIV^- PEMC		<0.0001
TB^+HIV^+ PBMC : TB^+HIV^+ PEMC		0.2238

Results expressed as mean ± SEM, HC PBMC *n*=4, TB^-HIV^- PBMC *n*=3, TB^+HIV^+ PBMC *n*=3, HIV^+ PBMC *n*=5, TB^-HIV^- PEMC *n*=5, TB^+HIV^+ PEMC *n*=5, Mann-Whitney test. Healthy control (HC), HIV mono-infected (HIV^+), forkhead box P3 (Foxp3), phycoerythrin (PE), mean fluorescence intensity (MFI)

2.3 MicroRNA-155 expression

We measured miR-155 expression, a direct target of Foxp3, by qPCR. Relative to healthy controls, both $TB^{-}HIV^{-}$ and $TB^{+}HIV^{+}$ PBMCs showed a significant reduction in the expression of miR-155 (-2.336 ± 0.212, p=0.0004 and -5.747 ± 0.635, p=0.0004 fold change respectively). In contrast, PBMCs from HIV mono-infected patients showed a 2.371 ± 0.258 (p=0.0004) fold increase. Taken together, this implies miR-155 dysregulation in all patient groups (Table A2.3).

In comparison to the HIV mono-infected population, the co-infected PBMCs showed a remarkable -12.607 ± 0.955 fold decline in the expression of miR-155 (p=0.0004) (Table A2.3).

In line with the PEMC results, $TB^{+}HIV^{+}$ PBMCs showed a -2.458 ± 0.160 fold reduced expression (p=0.0004) of miR-155 compared to $TB^{-}HIV^{-}$ PBMCs indicating a similar immunological trend across compartments (Table A2.3).

In both the $TB^{-}HIV^{-}$ and $TB^{+}HIV^{+}$ cohorts, miR-155 expression was significantly raised in PEMCs as compared to PBMCs (1.468 ± 0.090, p=0.0004 and 2.102 ± 0.207, p=0.0004 respectively) (Table A2.3).

Table A2.3: MicroRNA-155 expression in pooled peripheral blood and pleural effusion mononuclear cells

Population	Relative Fold Change	p-value
HC PBMC* : TB^-HIV^- PBMC	-2.336 ± 0.212	0.0004
HC PBMC* : TB^+HIV^+ PBMC	-5.747 ± 0.635	0.0004
HC PBMC* : HIV^+ PBMC	2.371 ± 0.258	0.0004
HIV^+ PBMC* : TB^+HIV^+ PBMC	-12.607 ± 0.955	0.0004
TB^-HIV^- PBMC* : TB^+HIV^+ PBMC	-2.458 ± 0.160	0.0004
TB^-HIV^- PBMC* : TB^-HIV^- PEMC	1.468 ± 0.090	0.0004
TB^+HIV^+ PBMC* : TB^+HIV^+ PEMC	2.102 ± 0.207	0.0004

Results expressed as mean ± SEM, *n*=6 replicates in each group, Mann-Whitney test. * Indicates control group used in analyses. Healthy control (HC), HIV mono-infected (HIV^+).

2.4 T-regulatory lymphocyte frequency

MicroRNA-155 positively regulates Treg stability by suppressing SOCS1 translation and conferring IL-2 competitive fitness. We measured Treg frequency using flow cytometry. The proportion of $CD4^{+}CD25^{+}Foxp3^{+}STAT5^{+}$ Tregs did not significantly differ between the HC PBMCs and $TB^{-}HIV^{-}$ or $TB^{+}HIV^{+}$ PBMCs. However, HIV mono-infection showed a significant -3.269 fold decrease in PBMC Tregs compared to HC (39.592 ± 4.757% vs. 12.111 ± 1.115%, $p<0.0001$) highlighting a loss of Treg stability (Table A2.4).

In comparison to the HIV mono-infected population, the co-infected PBMCs showed a significant 3.004 fold increase in Treg number (12.111 ± 1.115% vs. 36.384 ± 6.820%, $p=0.0280$) indicating a more stable Treg population (Table A2.4).

In contrast to the PEMC trend, there was no significant difference in the number of Tregs between $TB^{-}HIV^{-}$ and $TB^{+}HIV^{+}$ PBMC populations, highlighting a differential Treg response across the localised and systemic compartments (Table A2.4).

Furthermore there was no significant difference in Treg frequency between $TB^{+}HIV^{+}$ PBMCs and PEMCs suggesting both compartments are equally conducive to Treg stability and submitting that Tregs do not accumulate at the infection site. However, there was a notable 1.975 fold increase in the number of Tregs in the $TB^{-}HIV^{-}$ PEMCs compared to PBMCs (26.723 ± 2.799% vs. 52.769 ± 5.413%, $p=0.0010$) suggesting a microenvironment more favourable towards Treg stability and expansion at the infection site in non-infectious pleural effusions (Table A2.4).

Table A2.4: T-regulatory lymphocyte frequency in peripheral blood and pleural effusion mononuclear cells

Population	$CD4^+CD25^+Foxp3^+STAT5^+$ (%)	p-value
HC PBMC	39.592 ± 4.757	
TB^-HIV^- PBMC	26.723 ± 2.799	
TB^+HIV^+ PBMC	36.384 ± 6.820	
HIV^+ PBMC	12.111 ± 1.115	
TB^-HIV^- PEMC	52.769 ± 5.413	
TB^+HIV^+ PEMC	31.720 ± 3.821	
HC PBMC : TB^-HIV^- PBMC		0.0644
HC PBMC : TB^+HIV^+ PBMC		0.9326
HC PBMC : HIV^+ PBMC		<0.0001
HIV^+ PBMC : TB^+HIV^+ PBMC		0.0280
TB^-HIV^- PBMC : TB^+HIV^+ PBMC		0.1425
TB^-HIV^- PBMC : TB^-HIV^- PEMC		0.0010
TB^+HIV^+ PBMC : TB^+HIV^+ PEMC		0.5579

Results expressed as mean ± SEM, HC PBMC *n*=6, TB^-HIV^- PBMC *n*=7, TB^+HIV^+ PBMC *n*=6, HIV^+ PBMC *n*=5, TB^-HIV^- PEMC *n*=7, TB^+HIV^+ PEMC *n*=5, Mann-Whitney test. Healthy control (HC), HIV mono-infected (HIV^+).

2.5 IL-35 concentration

We quantified IL-35, a suppressive Treg characteristic cytokine, by ELISA. There was no significant difference between IL-35 in HC plasma and that of the $TB^{-}HIV^{-}$ cohort. However, compared to HC plasma there was a notable -5.122 fold decrease in co-infected plasma (25.106 ± 7.101 vs. 4.902 ± 0.656, p=0.0015) and -12.178 fold decrease HIV mono-infected plasma (25.106 ± 7.101 vs. 2.062 ± 0.142, p<0.0001) implying a Treg functional deficit (Table A2.5).

In comparison to the HIV mono-infected plasma, the co-infected plasma showed a significant 2.377 fold increase in IL-35 levels (2.062 ± 0.142 vs. 4.902 ± 0.656, p=0.0154) indicating greater Treg immunosuppressive activity (Table A2.5).

In line with the PEF results, there was a remarkable -6.599 fold decrease in IL-35 concentration in $TB^{+}HIV^{+}$ plasma relative to $TB^{-}HIV^{-}$ (32.348 ± 9.431 vs. 4.902 ± 0.656, p= 0.0579), suggesting a Treg functional insufficiency in the $TB^{+}HIV^{+}$ cohort and a similar IL-35 trend across the localised and systemic compartments between cohorts (Table A2.5).

Furthermore there was no significant difference in IL-35 levels between $TB^{-}HIV^{-}$ or $TB^{+}HIV^{+}$ plasma and PEF compartments suggesting even distribution of Treg functional responses (Table A2.5).

Table A2.5: Interleukin-35 concentration in plasma and pleural effusion fluid

Population	IL-35 (ng/ml)	p-value
HC Plasma	25.106 ± 7.101	
TB^-HIV^- Plasma	32.348 ± 9.431	
TB^+HIV^+ Plasma	4.902 ± 0.656	
HIV^+ Plasma	2.062 ± 0.142	
TB^-HIV^- PEF	18.784 ± 4.911	
TB^+HIV^+ PEF	5.457 ± 0.905	
HC Plasma : TB^-HIV^- Plasma		0.5431
HC Plasma : TB^+HIV^+ Plasma		0.0015
HC Plasma : HIV^+ Plasma		<0.0001
HIV^+ Plasma : TB^+HIV^+ Plasma		0.0154
TB^-HIV^- Plasma : TB^+HIV^+ Plasma		0.0579
TB^-HIV^- Plasma : TB^-HIV^- PEF		0.9075
TB^+HIV^+ Plasma : TB^+HIV^+ PEF		0.6427

Results expressed as mean ± SEM, HC plasma *n*=9, TB^-HIV^- plasma *n*=9, TB^+HIV^+ plasma *n*=11, HIV^+ plasma *n*=8, TB^-HIV^- PEF *n*=8, TB^+HIV^+ PEF *n*=8, Mann-Whitney test. Healthy control (HC), HIV mono-infected (HIV^+), interleukin (IL).

Appendix 3: MicroRNA-21 expression

MicroRNA-21 has been shown to positively regulate peripheral Treg development by indirectly regulating the expression of Foxp3. Relative to HC, both $TB^{-}HIV^{-}$ and $TB^{+}HIV^{+}$ PBMCs showed a significant reduction in the expression of miR-21 (-1.522 ± 0.054, p=0.0004 and -1.565 ± 0.047, p=0.0004 fold change respectively). In contrast, PBMCs from HIV mono-infected patients showed a 2.258 ± 0.298 (p<0.0001) fold increase. Taken together, this implies miR-21 dysregulation in all patient groups (Table A3.1).

In comparison to the HIV mono-infected population, the co-infected PBMCs showed a significant -3.254 ± 0.029 fold decline in the expression of miR-21 (p=0.0004) (Table A3.1).

Both $TB^{+}HIV^{+}$ PBMCs (-1.008 ± 0.061, p=0.7304) and PEMCs (-1.120 ± 0.041, p=0.0062) showed a decrease in miR-21 expression compared to $TB^{-}HIV^{-}$ counterparts indicating a similar immunological trend across compartments, although this was only significant in the pleural fluid compartment (Table A3.1). These results were not included in Chapter 2 as despite the significance the fold change was not considered strong enough.

In both the $TB^{-}HIV^{-}$ and $TB^{+}HIV^{+}$ cohorts, miR-21 expression was significantly raised in PEMCs as compared to PBMCs (1.387 ± 0.091, p=0.0014 and 1.271 ± 0.101, p=0.0062 fold change respectively) (Table A3.1).

Table A3.1: MicroRNA-21 expression in pooled peripheral blood and pleural effusion mononuclear cells

Population	Relative Fold Change	p-value
HC PBMC* : TB^-HIV^- PBMC	-1.522 ± 0.054	0.0004
HC PBMC* : TB^+HIV^+ PBMC	-1.565 ± 0.047	0.0004
HC PBMC* : HIV^+ PBMC	2.258 ± 0.298	<0.0001
HIV^+ PBMC* : TB^+HIV^+ PBMC	-3.254 ± 0.029	0.0004
TB^-HIV^- PBMC* : TB^+HIV^+ PBMC	-1.008 ± 0.061	0.7304
TB^-HIV^- PEMC* : TB^+HIV^+ PEMC	-1.120 ± 0.041	0.0062
TB^-HIV^- PBMC* : TB^-HIV^- PEMC	1.387 ± 0.091	0.0014
TB^+HIV^+ PBMC* : TB^+HIV^+ PEMC	1.271 ± 0.101	0.0062

Results expressed as mean ± SEM, *n*=6 replicates in each group, Mann-Whitney test. * Indicates control group used in analyses. Healthy control (HC), HIV mono-infected (HIV^+).

Appendix 4: The $2^{-\Delta\Delta Ct}$ method for the analysis of quantitative PCR

The $2^{-\Delta\Delta Ct}$ method refers to the method of analysis used to obtain a relative fold change of a treated sample compared to an appropriate control group as described by Livak and Schmittgen (2001) [1]. The table below extracted directly from the original article highlights an example of relative quantification.

Table A4.1: An example of relative mRNA quantification using the $2^{-\Delta\Delta Ct}$ method [1].

TABLE 1

Treatment of Replicate Data Where Target and Reference Are Amplified in Separate Wells[a]

Tissue	c-*myc* C_T	GAPDH C_T	ΔC_T (Avg. c-*myc* C_T − Avg. GAPDH C_T)	$\Delta\Delta C_T$ (Avg. ΔC_T − Avg. $\Delta C_{T,Brain}$)	Normalized c-*myc* amount relative to brain $2^{-\Delta\Delta C_T}$
Brain	30.72	23.70			
	30.34	23.56			
	30.58	23.47			
	30.34	23.65			
	30.50	23.69			
	30.43	23.68			
Average	30.49 ± 0.15	23.63 ± 0.09	6.86 ± 0.17	0.00 ± 0.17	1.0 (0.9–1.1)
Kidney	27.06	22.76			
	27.03	22.61			
	27.03	22.62			
	27.10	22.60			
	26.99	22.61			
	26.94	22.76			
Average	27.03 ± 0.06	22.66 ± 0.08	4.37 ± 0.10	−2.50 ± 0.10	5.6 (5.3–6.0)

[a] Total RNA from human brain and kidney were purchased from Clontech. Using reverse transcriptase, cDNA was synthesized from 1 μg total RNA. Aliquots of cDNA were used as template for real-time PCR reactions containing either primers and probe for c-*myc* or primers and probe for GAPDH. Each reaction contained cDNA derived from 10 ng total RNA. Six replicates of each reaction were performed.

References

1. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25(4):402-408.

Appendix 5: Corresponding peripheral blood results for Chapter 3 as well as healthy control and human immunodeficiency virus mono-infected cohorts

5.1 Th17 lymphocyte frequency

The frequency of Th17 lymphocytes was measured by flow cytometry. Compared to the HC cohort (52.925 ± 5.113%), $TB^{-}HIV^{-}$ (-1.983 fold, 26.692 ± 2.135%, p=0.0006), $TB^{+}HIV^{+}$ (-1.969 fold, 26.877 ± 7.907%, p=0.0179) and HIV mono-infected (-4.137 fold, 12.792 ± 1.543%, p<0.0001) groups all showed a significantly lower proportion of peripheral $CD4^{+}IL\text{-}17^{+}RORC^{+}STAT3^{+}$ Th17 cells indicating a suppression of the Th17 pro-inflammatory immune response (Table A5.1).

There was no significant difference in Th17 frequency between HIV mono-infected and TB/HIV co-infected groups (Table A5.1).

In contrast to PEMC results, there was no significant difference in Th17 proportion between $TB^{-}HIV^{-}$ and $TB^{+}HIV^{+}$ PBMCs indicating a similar peripheral Th17 pro-inflammatory response between cohorts (Table A5.1).

There was also no significant difference in Th17 frequency between $TB^{+}HIV^{+}$ PBMCs and PEMCs suggesting an even distribution of this subset between localised and systemic compartments, with the microenvironment in both compartments being equally conducive to Th17 generation. In contrast, $TB^{-}HIV^{-}$ PEMCs showed a 2.327 fold increase (26.692 ± 2.135% vs 62.100 ± 5.045%, p=0.0001) in the proportion of Th17 lymphocytes compared to PBMCs implying a potent localised Th17 response in non-infectious pleural effusions (Table A5.1).

Table A5.1: T-helper 17 lymphocyte frequency in peripheral blood and pleural effusion mononuclear cells

Population	$CD4^+IL\text{-}17^+RORC^+STAT3^+$ (%)	p-value
HC PBMC	52.925 ± 5.113	
TB^-HIV^- PBMC	26.692 ± 2.135	
TB^+HIV^+ PBMC	26.877 ± 7.907	
HIV^+ PBMC	12.792 ± 1.543	
TB^-HIV^- PEMC	62.100 ± 5.045	
TB^+HIV^+ PEMC	36.233 ± 6.968	
HC PBMC : TB^-HIV^- PBMC		0.0006
HC PBMC : TB^+HIV^+ PBMC		0.0179
HC PBMC : HIV^+ PBMC		<0.0001
HIV^+ PBMC : TB^+HIV^+ PBMC		0.2904
TB^-HIV^- PBMC : TB^+HIV^+ PBMC		0.9663
TB^-HIV^- PBMC : TB^-HIV^- PEMC		0.0001
TB^+HIV^+ PBMC : TB^+HIV^+ PEMC		0.5362

Results expressed as mean ± SEM, HC PBMC *n*=6, TB^-HIV^- PBMC *n*=7, TB^+HIV^+ PBMC *n*=6, HIV^+ PBMC *n*=5, TB^-HIV^- PEMC *n*=5, TB^+HIV^+ PEMC *n*=5, Mann-Whitney test. Healthy control (HC), HIV mono-infected (HIV^+).

5.2 IL-17A protein expression

Relative protein expression of the Th17 characteristic cytokine, IL-17A, was measured by intracellular flow cytometry and represented as peridin-chlorophyll-cyanin 5.5 (PerCP-Cy5.5) MFI. Compared to the HC cohort (1097. 667 ± 70.949 PerCP-Cy5.5 MFI), $TB^{+}HIV^{+}$ PBMCs did not show a significant change in IL-17A protein expression, however both $TB^{-}HIV^{-}$ (-1.253 fold, 876.333 ± 35.230 PerCP-Cy5.5 MFI, p=0.0491), and HIV mono-infected (-1.347 fold, 815.067 ± 25.429 PerCP-Cy5.5 MFI, p=0.0012) groups showed a significantly lower expression of IL-17A indicating a reduced pro-inflammatory immune response in these groups (Table A5.2).

There was no significant difference in PBMC IL-17A protein expression between HIV mono-infected and TB/HIV co-infected groups (Table A5.2).

In contrast to PEMC results, there was no significant difference in IL-17A protein expression between $TB^{-}HIV^{-}$ and $TB^{+}HIV^{+}$ PBMCs indicating a similar peripheral Th17 pro-inflammatory response between cohorts (Table A5.2).

There was also no significant difference in IL-17A protein expression between $TB^{+}HIV^{+}$ PBMCs and PEMCs implying a similar IL-17A mediated pro-inflammatory response between localised and systemic compartments. In contrast, $TB^{-}HIV^{-}$ PEMCs showed a 1.659 fold increase in IL-17A protein expression compared to PBMCs (876.333 ± 35.230 vs. 1454.000 ± 117.167 PerCP-Cy5.5 MFI, p=0.0026) implying a potent and differential localised IL-17A pro-inflammatory response in non-infectious pleural effusions (Table A5.2).

Table A5.2: Interleukin-17A relative protein expression in peripheral blood and pleural effusion mononuclear cells

Population	IL-17A-PerCP-Cy5.5 (MFI)	p-value
HC PBMC	1097. 667 ± 70.949	
TB^-HIV^- PBMC	876.333 ± 35.230	
TB^+HIV^+ PBMC	895.500 ± 16.280	
HIV^+ PBMC	815.067 ± 25.429	
TB^-HIV^- PEMC	1454.000 ± 117.167	
TB^+HIV^+ PEMC	923.445 ± 64.418	
HC PBMC : TB^-HIV^- PBMC		0.0491
HC PBMC : TB^+HIV^+ PBMC		0.1012
HC PBMC : HIV^+ PBMC		0.0012
HIV^+ PBMC : TB^+HIV^+ PBMC		0.1498
TB^-HIV^- PBMC : TB^+HIV^+ PBMC		0.8099
TB^-HIV^- PBMC : TB^-HIV^- PEMC		0.0026
TB^+HIV^+ PBMC : TB^+HIV^+ PEMC		0.5287

Results expressed as mean ± SEM, HC PBMC *n*=4, TB^-HIV^- PBMC *n*=3, TB^+HIV^+ PBMC *n*=3, HIV^+ PBMC *n*=5, TB^-HIV^- PEMC *n*=5, TB^+HIV^+ PEMC *n*=5, Mann-Whitney test. Healthy control (HC), HIV mono-infected (HIV^+),interleukin (IL), peridin-chlorophyll-cyanin5.5 (PerCP-CY5.5), mean fluorescence intensity (MFI).

5.3 Cytokine profiling

Compared to HC plasma:

TB^-HIV^- plasma showed significantly increased concentrations of IL-1β (105.133 fold, 0.015 ± 0.001pg/ml vs. 1.577 ± 0.663pg/ml, *p*=0.0050), IL-6 (32.263 fold, 1.786 ± 1.087pg/ml vs. 57.622 ± 11.328pg/ml, *p*=0.0050), IL-10 (27.521 fold, 0.530 ± 0.001pg/ml vs. 14.586 ± 4.123pg/ml, *p*=0.0134), IL-31 (47.475 fold, 0.668 ± 0.158pg/ml vs. 31.713 ± 3.296pg/ml, *p*=0.0080), IL-33 (7.670 fold, 121.568 ± 29.205pg/ml vs. 932.409 ± 253.009pg/ml, *p*=0.0283), IFN-γ (12.695 fold, 0.210 ± 0.001pg/ml vs. 2.666 ± 2.278pg/ml, *p*=0.0106), sCD40L (10.374 fold, 24.493 ± 9.868pg/ml vs. 254.083 ± 53.920pg/ml, *p*=0.0040) and TNF-α (8.578 fold, 0.683 ± 0.514pg/ml vs. 5.859 ± 0.751pg/ml, *p*=0.0050). There was no significant change in the levels of IL-4, IL-17A, IL-17F, IL-21, IL-22, IL-23 and IL-25 (Figure A5.1).

TB^+HIV^+ plasma showed significantly elevated levels of IL-1β (284.00 fold, 0.015 ± 0.001pg/ml vs. 4.272 ± 0.401pg/ml, *p*=0.0039), IL-4 (4.757 fold, 5.749 ± 3.179pg/ml vs. 27.335 ±7.571pg/ml, *p*=0.0356), IL-6 (647.542 fold, 1.786 ± 1.087pg/ml vs. 2108.583 ± 1156.510pg/ml, p=0.0032), IL-10 (30.219 fold, 0.530 ± 0.001pg/ml vs. 16.016 ± 3.870pg/ml, *p*=0.0035), IL-25 (23.825 fold, 0.080 ± 0.001pg/ml vs. 1.906 ± 0.465pg/ml, *p*=0.0084), IL-31 (121.708 fold, 0.668 ± 0.158pg/ml vs. 81.301 ± 13.738pg/ml, *p*=0.0080), IFN-γ (357.610 fold, 0.210 ± 0.001pg/ml vs. 75.098 ± 21.629pg/ml, *p*=0.0032), sCD40L (10.229 fold, 24.493 ± 9.868pg/ml vs. 240.541 ± 78.887pg/ml, *p*=0.0081), TNF-α (26.432 fold, 0.683 ± 0.514pg/ml vs. 18.053 ± 4.089pg/ml, *p*=0.0044) and no significant alterations in IL-17A, IL-17F, IL-21, IL-22, IL-23 and IL-33 (Figure A5.1)

Plasma from HIV mono-infected individuals showed significantly raised levels of IL-1β (29.867 fold, 0.015 ± 0.001pg/ml vs. 0.448 ± 0.030pg/ml, *p*=0.0286), IL-31 (60.012 fold, 0.668 ± 0.158pg/ml vs. 40.088 ± 18.623pg/ml, *p*=0.0265), sCD40L (11.745 fold, 24.493 ± 9.868pg/ml vs. 287.665 ± 68.737pg/ml, *p*=0.0286) and TNF-α (12.750 fold, 0.683 ± 0.514pg/ml vs. 8.708 ± 1.315pg/ml, *p*=0.0286). Concentrations of IL-4, IL-6, IL-10, IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IL-33 and IFN-γ were not significantly changed (Figure A5.1).

HIV mono-infected compared to TB^+HIV^+ co-infected plasma:

Relative to plasma from HIV mono-infected patients, there was a significant 9.536 fold increase in IL-1β (0.448 ± 0.300pg/ml vs. 4.272 ± 0.401pg/ml, *p*=0.0036) in TB/HIV co-infected patients. No significant difference in the levels of IL-4, IL-6, IL-10, IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IL-31, IL-33, IFN-γ, sCD40L, TNF-α was observed (Figure A5.2).

TB^-HIV^- compared to TB^+HIV^+ plasma:

Compared to TB^-HIV^-, the plasma of patients with TB^+HIV^+ co-infection showed significantly elevated concentrations of IL-1β (2.709 fold, 1.577 ± 0.663pg/ml vs. 4.272 ± 0.401pg/ml, p=0.0058), IL-17F (84.750 fold, 0.120 ± 0.001pg/ml vs. 10.170 ± 3.529pg/ml, p=0.0376), IL-31 (2.564 fold, 31.713 ± 3.296pg/ml vs 81.301 ± 13.738pg/ml, p=0.0180), IFN-γ (11.266 fold, 6.666 ± 2.278pg/ml vs. 75.098 ± 21.629pg/ml, p=0.0169) and a significant reduction in IL-33 (-3.154 fold, 932.409 ± 253.009pg/ml vs. 295.644 ± 105.002pg/ml, p=0.0379). There was no significant alteration in IL-4, IL-6, IL-10, IL-17A, IL-21, IL-22, IL-23, IL-25, sCD40L and TNF-α levels (Figure A5.3).

When comparing results between TB^-HIV^-/TB^+HIV^+ PEF and plasma we observed a similar trend for IL-1β, IL-31 and IFN-γ, all significantly raised in plasma and PEF of TB^+HIV^+ patients. In contrast, IL-22 showed an inverse trend; significantly increased in TB^+HIV^+ PEF compared to TB^-HIV^- PEF but decreased in TB^+HIV^+ plasma, however this was not significant in plasma. TNF-α was increased in TB^+HIV^+ plasma and PEF but it was only a significant result in PEF. IL-17F was increased in TB^+HIV^+ plasma and PEF but it was only a significant result in plasma. IL-33 was decreased in TB^+HIV^+ plasma and PEF but it was only significant in plasma (Figure A5.3 and Figure 2B).

TB^-HIV^- PEF compared to plasma:

TB^-HIV^- PEF relative to TB^-HIV^- plasma showed significantly elevated concentrations of IL-4 (2.278 fold, 18.895 ± 7.147pg/ml vs. 43.042 ± 9.590pg/ml, p=0.0200), IL-6 (1745.528 fold, 57.622 ± 11.328pg/ml vs. 100580.806 ± 30145.472pg/ml, p<0.0001), IL-10 (5.404 fold, 14.586 ± 4.123pg/ml vs. 78.820 ± 14.729pg/ml, p=0.0033), IL-17F (780.308 fold, 0.120 ± 0.001pg/ml vs. 93.637 ± 15.357pg/ml, p=0.0006), IL-21 (4.656 fold, 13.737 ± 8.650pg/ml vs. 63.959 ± 12.567pg/ml, p=0.0047), IL-25 (7.502 fold, 1.170 ± 0.650pg/ml vs. 8.777 ± 1.872pg/ml, p=0.0021), IL-31 (4.996 fold, 31.713 ± 3.296pg/ml vs. 158.430 ± 35.615pg/ml, p=0.0367), IFN-γ (13.170 fold, 6.666 ± 2.278pg/ml vs. 87.793 ± 27.282pg/ml, p=0.0064) and TNF-α (3.112 fold, 5.859 ± 0.753pg/ml vs. 18.233 ± 2.615pg/ml, p=0.0004). No change in IL-1β, IL-17A, IL-22, IL-23, IL-33, sCD40L levels were observed (Figure A5.4) indicating a differential systemic and localised cytokine response.

TB^+HIV^+ PEF compared to plasma:

In comparison to TB^+HIV^+ plasma, TB^+HIV^+ PEF showed significantly raised levels of IL-1β (4.272 ± 0.401pg/ml vs. 18.239 ± 3.064pg/ml, p=0.0004), IL-6 (2108.583 ± 1156.513pg/ml vs. 78778.906 ± 21300.368pg/ml, p<0.0001), IL-10 (16.016 ± 3.870pg/ml vs. 85.835 ± 15.680pg/ml, p=0.0005), IL-17A (0.035 ± 0.001pg/ml vs 18.281 ± 2.383pg/ml, p=0.0003), IL-17F (10.170 ± 3.529pg/ml vs. 118.090 ± 8.077pg/ml, p=0.0004), IL-21 (1.866 ± 0.673pg/ml vs. 61.792 ± 2.995pg/ml, p=0.0004), IL-22 (1.230 ±

0.001pg/ml vs. 69.729 ± 10.318pg/ml, p=0.0003), IL-25 (1.906 ± 0.465pg/ml vs. 12.945 ± 1.240pg/ml, p=0.0004), IL-31 (81.301 ± 13.738pg/ml vs 483.957 ± 115.651pg/ml, p=0.0002), IL-33 (295.644 ± 105.002pg/ml vs. 600.367 ± 63.523pg/ml, p=0.0205), IFN-γ (75.098 ± 21.629pg/ml vs. 1190.386 ± 361.906pg/ml, p=0.0003), TNF-α (18.053 ± 4.089pg/ml vs. 66.112 ± 13.264pg/ml, p=0.0058). There was no significant difference in IL-4, IL-23 or sCD40L concentrations (Figure A5.5) indicating a differential systemic and localised cytokine response.

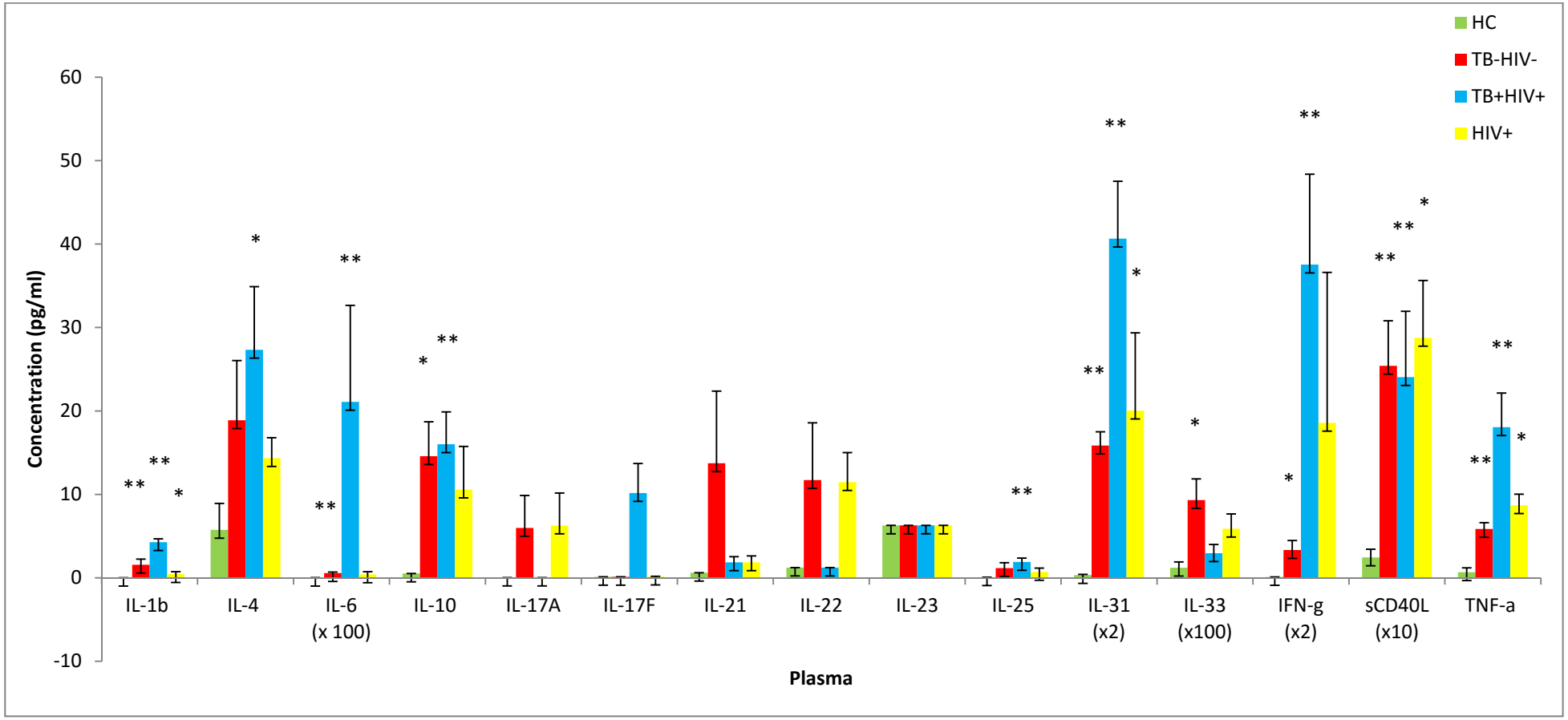


Figure A5.1: Cytokine profiling of plasma. Significance determined relative to the healthy control (HC) cohort. Interleukin (IL)-1β, IL-4, IL-6, IL-10, interferon (IFN)-γ, tumour necrosis factor (TNF)-α; HC *n*=4, $TB^{-}HIV^{-}$ *n*=8, $TB^{+}HIV^{+}$ *n*=12, HIV^{+} *n*=4. IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IL-31, IL-33, soluble CD40 ligand (sCD40L); HC *n*=4, $TB^{-}HIV^{-}$ *n*=5, $TB^{+}HIV^{+}$ *n*=5, HIV^{+} *n*=4, Mann-Whitney test. Data represents the mean ± SEM.

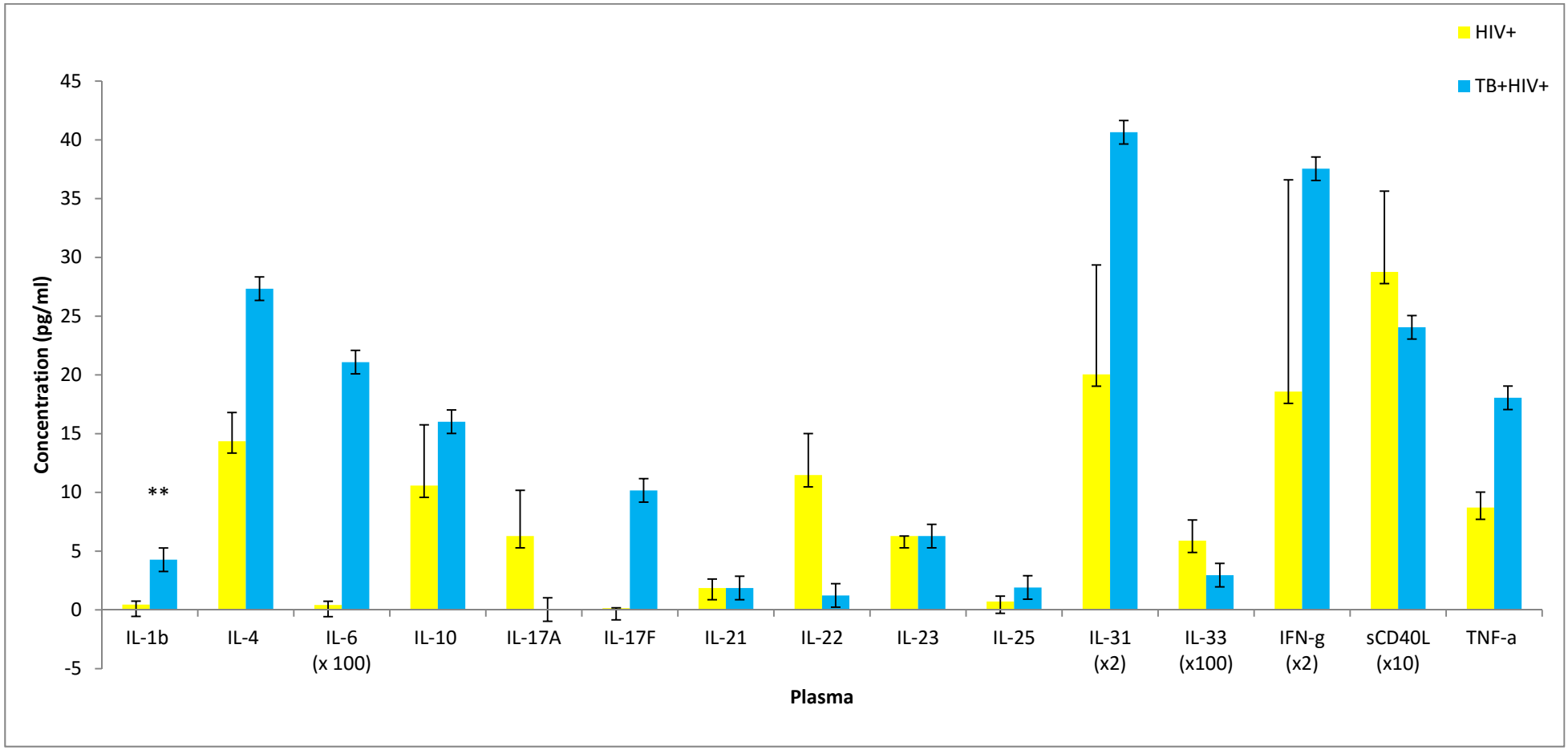


Figure A5.2: Cytokine profiling HIV mono-infected and $TB^{+}HIV^{+}$ co-infected plasma. Interleukin (IL)-1β, IL-4, IL-6, IL-10, interferon (IFN)-γ, tumour necrosis factor (TNF)-α; $TB^{+}HIV^{+}$ *n*=12, HIV^{+} *n*=4. IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IL-31, IL-33, soluble CD40 ligand (sCD40L); $TB^{+}HIV^{+}$ *n*=5, HIV^{+} *n*=4, Mann-Whitney test. Data represents the mean ± SEM.

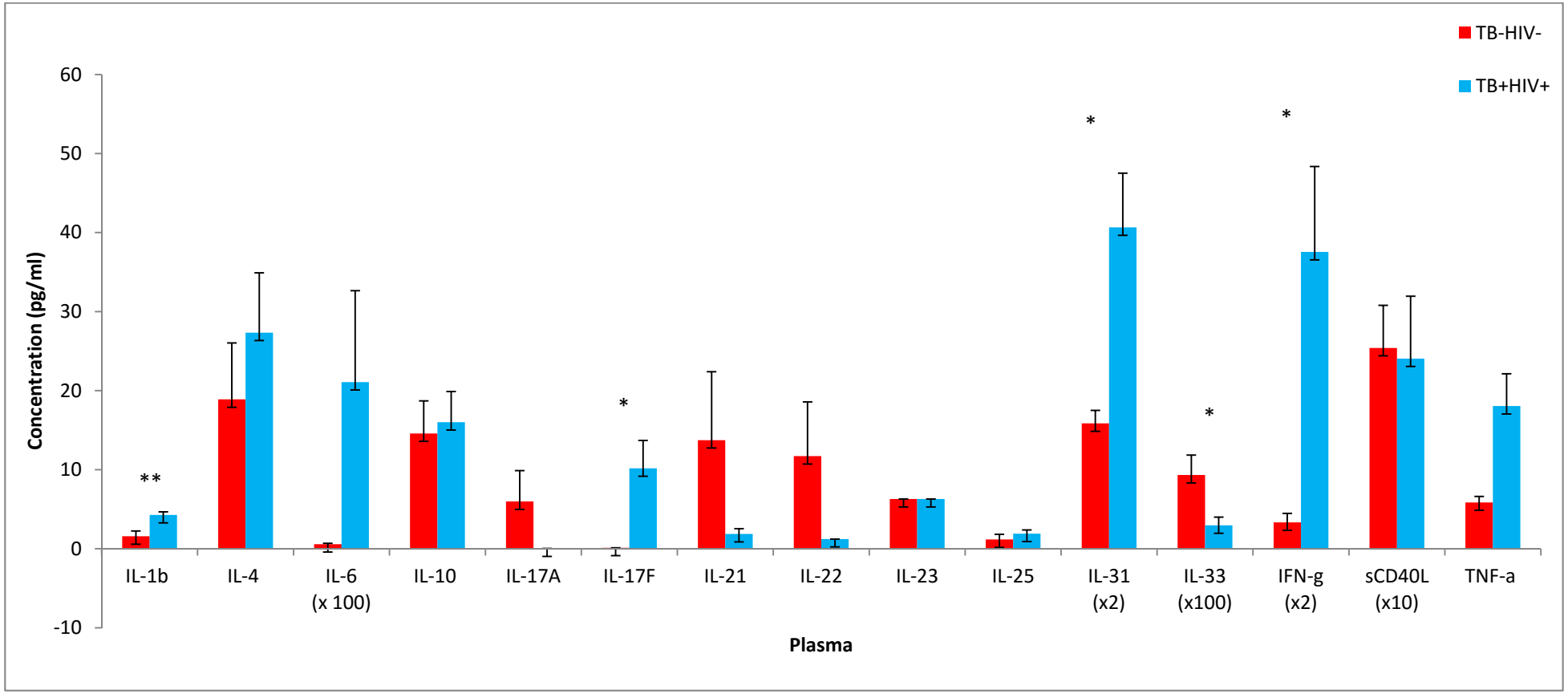


Figure A5.3: Cytokine profiling of $TB^{-}HIV^{-}$ and $TB^{+}HIV^{+}$ plasma. Interleukin (IL)-1β, IL-4, IL-6, IL-10, interferon (IFN)-γ, tumor necrosis factor (TNF)-α; $TB^{-}HIV^{-}$ *n*=8, $TB^{+}HIV^{+}$ *n*=12. IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IL-31, IL-33, soluble CD40 ligand (sCD40L); $TB^{-}HIV^{-}$ *n*=5, $TB^{+}HIV^{+}$ *n*=5, Mann-Whitney test. Data represents the mean ± SEM.

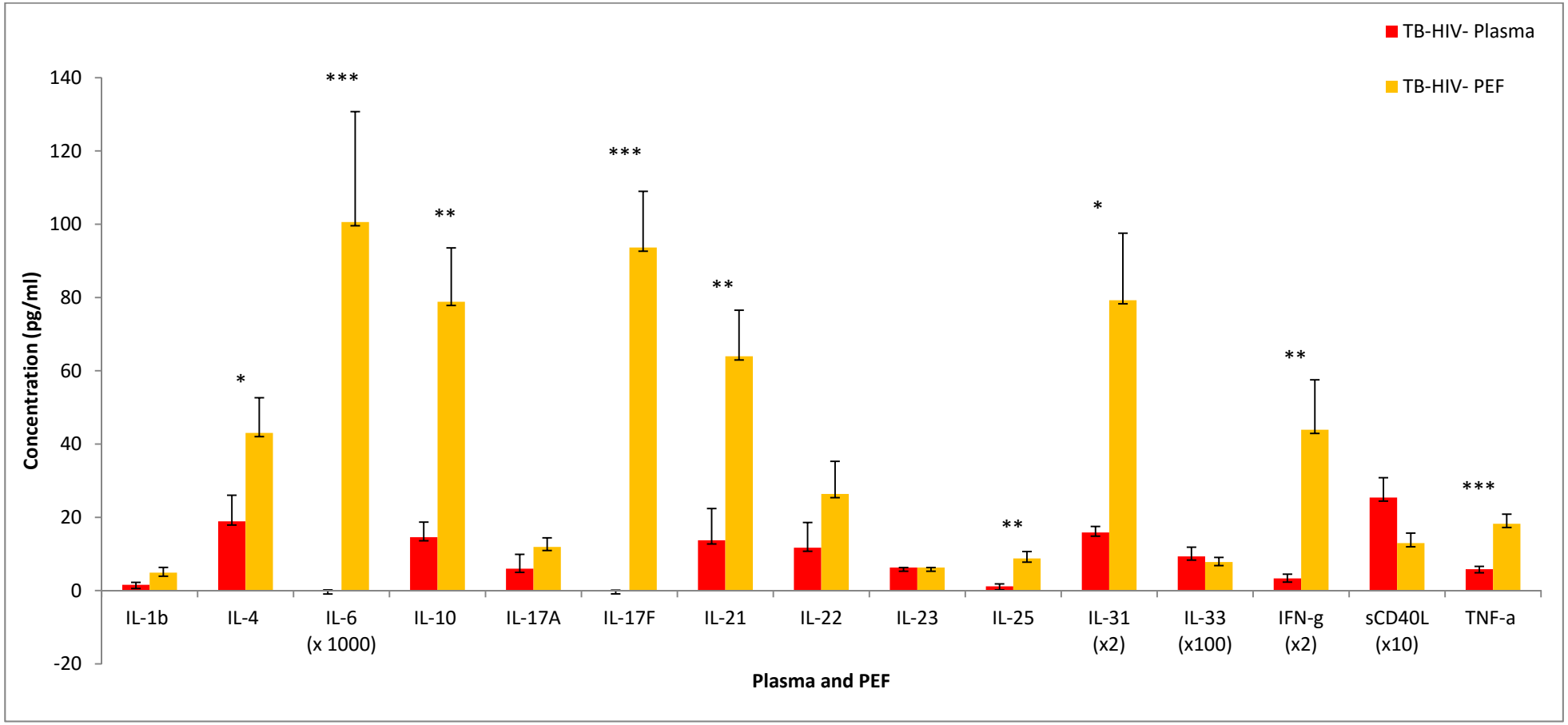


Figure A5.4: Cytokine profiling of TB$^-$HIV$^-$ plasma and pleural effusion fluid (PEF). Interleukin (IL)-1β, IL-4, IL-6, IL-10, interferon (IFN)-γ, tumour necrosis factor (TNF)-α; TB$^-$HIV$^-$ plasma and PEF *n*=8. IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IL-31, IL-33, soluble CD40 ligand (sCD40L); TB$^-$HIV$^-$ plasma and PEF *n*=5, Mann-Whitney test. Data represents the mean ± SEM.

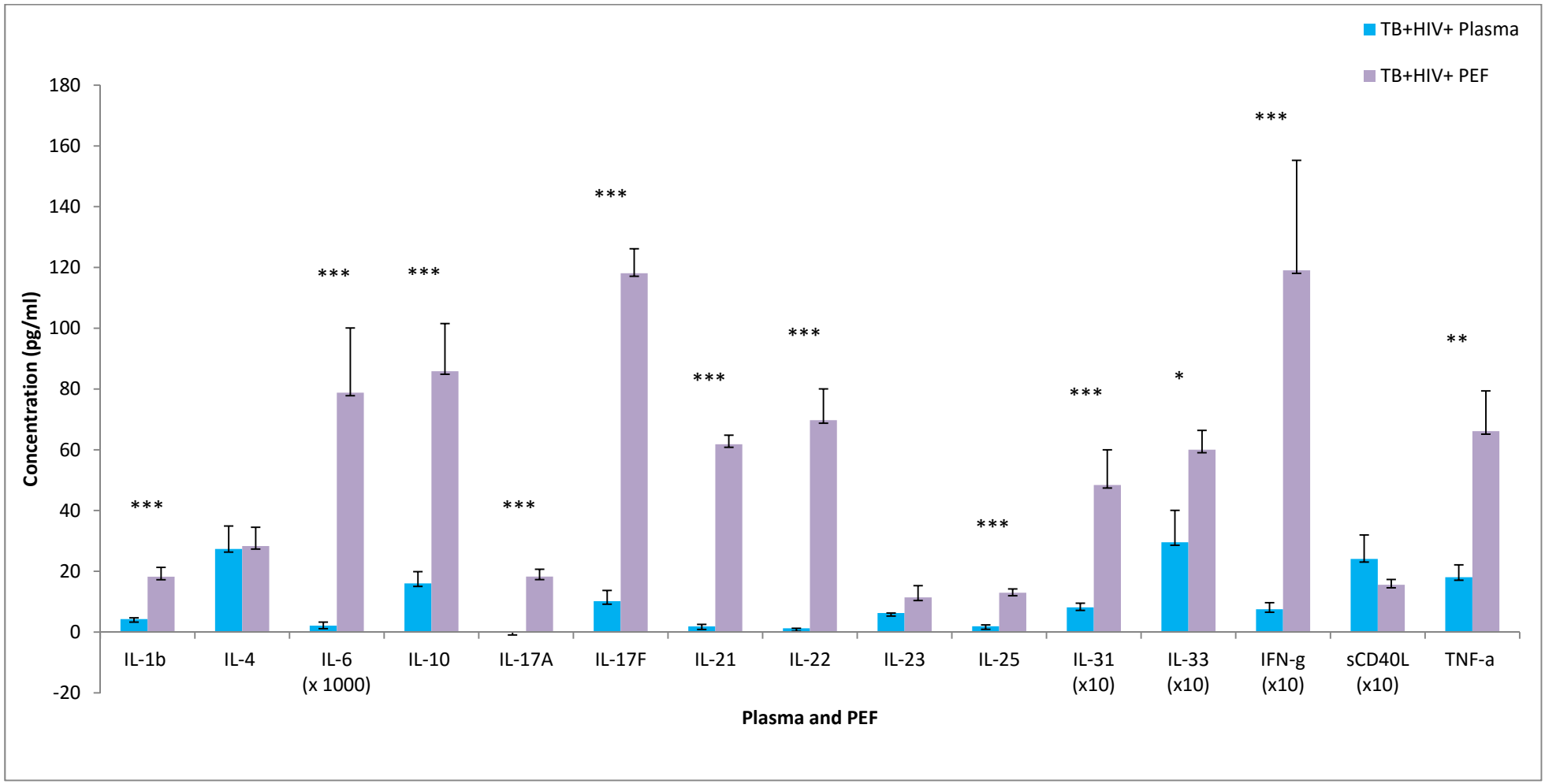


Figure A5.5: Cytokine profiling of $TB^{+}HIV^{+}$ plasma and pleural effusion fluid (PEF). Interleukin (IL)-1β, IL-4, IL-6, IL-10, interferon (IFN)-γ, tumour necrosis factor (TNF)-α; $TB^{+}HIV^{+}$ plasma and PEF *n*=12. IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IL-31, IL-33, soluble CD40 ligand (sCD40L); $TB^{+}HIV^{+}$ plasma and PEF *n*=5, Mann-Whitney test. Data represents the mean ± SEM.

5.4 mRNA expression of Th17 signalling molecules

Messenger RNA expression of Th17 signalling molecules, STAT3, IL-1β, IL-6 and IL-6R, was measured by qPCR. Relative to the HC PBMCs, $TB^{-}HIV^{-}$ PBMCs showed significantly increased fold expression of STAT3 (2.347 ± 0.247, p=0.0048) and decreased fold expression of IL-6R (-1.178 ± 0.082, p=0.0048) without significant changes in IL-1β (1.068 ± 0.047) and IL-6 (1.017 ± 0.099) (Table A5.3).

$TB^{+}HIV^{+}$ PBMCs showed significantly elevated expression of STAT3 (6.303 ± 0.670, p=0.0048), IL-6 (3.603 ± 0.583, p=0.0048) and IL-6R (1.695 ± 0.119, p=0.0048) and significant reduction on IL-1β (-2.657 ± 0.140, p=0.0048) fold expression compared to HC PBMCs (Table A5.3).

Taken together, this suggests that infectious and non-infectious microenvironments have a differential and selective effect on peripheral mechanisms of the pro-inflammatory response.

HIV mono-infected PBMCs showed a remarkable fold rise in STAT3 (161.827 ± 15.714, p=0.0048), IL-1β (20.233 ± 1.040, p=0.0048), IL-6 (231.298 ± 61.258, p=0.0131) and IL-6R (19.496 ± 1.476, p=0.0075) indicating up-regulation at all stages of Th17 signalling measured (Table A5.3).

Compared to HIV mono-infected PBMCs, TB/HIV co-infected PBMCs showed a notable decline in the expression of STAT3 (-26.127 ± 2.098, p=0.0048), IL-1β (-53.462 ± 3.249, p=0.0048), IL-6 (-51.420 ± 5.166, p=0.0048) and IL-6R (-11.193 ± 0.130, p=0.0048) implying a suppression of Th17 signalling networks peripherally (Table A5.3).

In line with the trends seen in the PEMCs, $TB^{+}HIV^{+}$ PBMCs showed significant fold elevations in the mRNA expression of STAT3 (2.693 ± 0.154, p=0.0048), IL-6 (3.473 ± 0.338, p=0.0048) and IL-6R (1.953 ± 0.056, p=0.0048) relative to $TB^{-}HIV^{-}$ PBMCs indicating a more active peripheral Th17 signalling during co-infection. However, in contrast to the PEMCs, $TB^{+}HIV^{+}$ PBMCs showed marked fold decrease in IL-1β (-2.807 ± 0.075, p=0.0048) mRNA highlighting a differential peripheral IL-1β response between groups (Table A5.3).

In non-infectious pleural effusions, there was a significant down regulation of STAT3 (-1.262 ± 0.071, p=0.0048), IL-1β (-5.742 ± 0.282, p=0.0048) and IL-6R (-1.117 ± 0.076, p=0.0048) mRNA expression together with a significant up-regulation of IL-6R (2.257 ± 0.227, p=0.0048) mRNA between systemic and localised compartments (PBMC vs. PEMC) (Table A5.3).

In co-infected individuals, STAT3 (9.018 ± 0.701, p=0.0048), IL-1β (5.705 ± 0.095, p=0.0048) and IL-6 (1.906 ± 0.592, p=0.0075) mRNA was significantly elevated and IL-6R (-1.447 ± 0.209, p=0.0048) significantly down regulated in the localised PEMCs compared to the systemic samples (Table A5.3).

Taken together this suggests that in both infectious and non-infectious pleural effusions there is a differential Th17 signalling network between localised and systemic compartments.

Table A5.3: Signal transducer and activator of transcription 3, interleukin -1β, -6, and 6 receptor messenger RNA expression

Population	Relative Fold Change (p-value)			
	STAT3	**IL-1β**	**IL-6**	**IL-6R**
HC PBMC* : TB^-HIV^- PBMC	2.347 ± 0.247 (0.0048)	1.068 ± 0.047 (0.0637)	1.017 ± 0.099 (0.9357)	-1.178 ± 0.082 (0.048)
HC PBMC* : TB^+HIV^+ PBMC	6.303 ± 0.670 (0.0048)	-2.657 ± 0.140 (0.0048)	3.603 ± 0.583 (0.0048)	1.695 ± 0.119 (0.0048)
HC PBMC* : HIV^+ PBMC	161.827 ± 15.714 (0.0048)	20.233 ± 1.040 (0.0048)	231.298 ± 61.258 (0.0131)	19.496 ± 1.476 (0.0075)
HIV^+ PBMC* : TB^+HIV^+ PBMC	-26.127 ± 2.098 (0.0048)	-53.462 ± 3.249 (0.0048)	-51.420 ± 5.166 (0.0048)	-11.193 ± 0.130 (0.0048)
TB^-HIV^- PBMC* : TB^+HIV^+ PBMC	2.693 ± 0.154 (0.0048)	-2.807 ± 0.075 (0.0048)	3.473 ± 0.338 (0.0048)	1.953 ± 0.056 (0.0048)
TB^-HIV^- PBMC* : TB^-HIV^- PEMC	-1.262 ± 0.071 (0.0048)	-5.742 ± 0.282 (0.0048)	2.257 ± 0.227 (0.0048)	-1.117 ± 0.076 (0.0048)
TB^+HIV^+ PBMC* : TB^+HIV^+ PEMC	9.018 ± 0.701 (0.0048)	5.705 ± 0.095 (0.0048)	1.906 ± 0.592 (0.0075)	-1.447 ± 0.209 (0.0048)

Results expressed as mean ± SEM, *n*=6 replicates in each group, Mann-Whitney test. * Indicates control group used in analyses. Healthy control (HC), HIV mono-infected (HIV^+), signal transducer and activator of transcription 3 (STAT3), interleukin (IL); receptor (R).