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Immunological Analysis of Pericardial Tuberculosis

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Thesis Presented for the Degree of

DOCTOR OF PHILOSOPHY

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DECLARATION

I, Kerry Matthews, hereby declare that the work on which this dissertation is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university. I empower the university to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

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ABSTRACT

Kerryn Matthews

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Immunological Analysis of Pericardial Tuberculosis

Pericardial tuberculosis (TB) offers a relevant human model to study TB at the site of disease and to determine the effect of HIV-1 infection. 96 Patients with pericardial TB were recruited into this study, 68 of whom were HIV-1 infected. Where clinically indicated, pericardiocentesis was performed to drain pericardial fluid and blood was drawn.

A whole blood and pericardial fluid overnight restimulation assay with *M.tb*-specific antigens showed high levels of antigen-specific IFN- γ production, irrespective of the HIV status of patients. However, there was decreased cellular production of IFN- γ in pericardial fluid in response to stimulation with the *M.tb*-specific antigen ESAT-6 in HIV-1 infected compared to HIV-1 uninfected patients as shown by ELISpot assay.

FACS analysis showed antigen experienced T cells present at the site of disease in HIV-1 uninfected and a more intermediate, less differentiated phenotype in the pericardial fluid of HIV-1 infected patients. HIV-1 uninfected pericardial fluid CD4⁺ cells secreted IFN- γ and TNF cytokines by themselves, whereas HIV-1 infected pericardial fluid showed more IL-2 secreting and polyfunctional IL-2, GM-CSF in combination with IFN- γ and TNF secreting CD4⁺ cells, pointing to a less differentiated cell type present at the site of disease.

The pericardial space had high levels of pro-inflammatory transcript as determined by RT-PCR, but also pro-fibrotic, with compartmentalization of pro-collagen genes to the site of disease, irrespective of the HIV status of the patients. Matrix metalloproteinases and their endogenous inhibitors, involved in destruction

and repair of the epithelial surface of the pericardium during *M.tb* infection were also prominently expressed.

The inflammatory cytokine IL-17 was not abundant in pericardial fluid or serum of TB pericarditis patients, while the opposite was true for IL-22.

These data provide novel insight into the immune response in the pericardium to TB infection. Furthermore, HIV-1 infection caused a dysregulation of the immune response.

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LIST OF ABBREVIATIONS

°C	degrees Celsius
+	positive
ABI	Applied Biosystems
AChE	acetylcholinesterase
Acr	Alpha-crystallin
AFB	Acid-fast bacilli
Alexa700	Alexa Fluor 700
APC	allophycocyanin
ARG1	Arginase 1
ART	anti-retroviral treatment
AVP	Avidin Peroxidase
B	Blank
BCG	Bacille Calmette-Guérin
BD	Beckton Dickenson
Bo	Maximum binding
CAMP	Cathelicidin Anti-Microbial Peptide
CD	Cluster of Differentiation
cDNA	cyclic Deoxyribonucleic acid
CFF	Cell Free Pericardial Fluid
CFP	Culture Filtrate Protein
CO ₂	Carbon dioxide
CPGR	Centre for Proteomic and Genomic Research
CSF	Cerebrospinal Fluid

CT	Cycle Threshold
CTGF	Connective Tissue Growth Factor
CXCL	Chemokine (C-X-C) ligand
DEFB4	Defensin B4
dH ₂ O	distilled water
dNTPs	deoxynucleotide triphosphate
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EIA	Enzyme Immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay
ELISpot	Enzyme-Linked Immunospot
ESAT	Early Secretory Antigenic Target
F	Female
FACS	Fluorescence Activated Cell Sorting
FHS	Faculty of Health Sciences
FITC	fluorescein isothiocyanate
FoxP3	Forkhead Box Protein 3
g	G-Force
gDNA	genomic Deoxyribonucleic acid
GM-CSF	Granulocyte Macrophage – Colony Stimulating Factor
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
H ₂ O ₂	Hydrogen Peroxide
H ₂ SO ₄	Hydrogen Sulphide, ELISA stop solution
H37Rv	Laboratory strain of <i>Mycobacterium tuberculosis</i> , virulent
HI-FCS	Heat Inactivated - Fetal Calf Serum

HIV	Human Immunodeficiency Virus
hk	heat killed
HLA-DR	human leukocyte antigen complex on MHC class II
HNE	Human Neutrophil Elastase
HRP	Horse-radish Peroxidase
ICS	Intracellular Cytokine Staining
IFN- γ	Interferon-gamma
Ig	Immunoglobulin
IGRA	Interferon Gamma Release Assay
IL	Interleukin
IMPI Africa	Investigation of the Management of Pericarditis in Africa
IP-10	Interferon inducible protein-10
IQR	Inter Quartile Range
kDa	kiloDalton
Ki67	Proliferation marker
LCN2	Lipocalin 2
LJ	Lowenstein-Jensen
LTBI	Latent tuberculosis infection
M	Male
M	Molar
<i>M.tb</i>	<i>Mycobacterium tuberculosis</i>
<i>M.tb</i>	<i>Mycobacterium tuberculosis</i>
MAP	MultiAnalyte Profiling
mg	milligram
Mg ²⁺	Magnesium

MHC	major histocompatibility complex
ml	millilitre
mm	millimetre
MMP	Matrix Metalloproteinase
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
MW	molecular weight
ng	nanograms
NHLS	National Health Laboratory Services
NIL	Background or Unstimulated
nm	nanometers
NSB	non-specific binding
OPD	o-Phenylenediamine dihydrochloride
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDAR	Pre-Designed Assay Reagents
PE	phycoerythrin
PE-Cy7	Phycoerythrin conjugated to cychrome 7
PerCP	Peridinin chlorophyll protein
PerCP-Cy5.5	Peridinin chlorophyll protein conjugated to cychronme 5.5
PFC	Pericardial Fluid Cells
pg	picogram
PHA	Phytohemagglutinin
PMA	phorbal 12-myristate 13-acetate

PPD	Purified Protein Derivative
Pst S-1	Phosphate specific Substrate binding protein - 1
QC	Quality Control
QDot 605	Quantum Dots 605
QFT-G	QuantiFERON-TB Gold
QFT-GIT	QuantiFERON-TB Gold In Tube
r	recombinant
RD	Region of Difference
REC	Research Ethics Committee
RFU	Relative Fluorescence Units
RIN	RNA Integrity Number
RNA	Ribonucleic acid
ROX	Reference dye
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute Medium
RT	reverse transcription
RT-PCR	Real-time – Polymerase Chain Reaction
SATVI	South African Tuberculosis Vaccine Initiative
SEB	staphylococcal enterotoxin B
SFC	Spot Forming Cells
SPARC	Secreted protein and rich in cysteine
TB	Tuberculosis
TGF	Transforming Growth Factor
Th	T-helper
TIMP	Tissue Inhibitor of Matrix Metalloproteinase

TMB	Tetramethyl-benzidine
TNF	Tumor Necrosis Factor
TST	Tuberculin Skin Test
U	units
UK	United Kingdom
ZN	Ziehl-Neelson
β	beta
μg	microgram
μl	microlitre

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CHAPTER 1

Introduction

The Immunology of tuberculosis at the site of disease

The aim of this chapter is to summarise what is known about the immunology of tuberculosis (TB) at the site of disease. Much is known about TB in the lung, but the immunology and pathogenesis at extrapulmonary sites of disease may be vastly different. This chapter introduces tuberculosis, the role of HIV-1 co-infection and extrapulmonary TB disease. The main aim of this chapter is to describe the extrapulmonary form of TB disease – pericardial TB, and the immunological background as known so far.

1. Tuberculosis (TB)

Tuberculosis (TB) is estimated to have caused death before 8000BC (1) and it now claims more lives than any other microbial pathogen each year. TB is a chronic necrotizing granulomatous disease that is spread through the inhalation of infectious nuclei droplets containing the causative organism, *Mycobacterium tuberculosis* (*M.tb*) (2). *M.tb* was first discovered by Robert Koch in 1882 and has continued to be an object of dedicated study since. More than 2 billion people worldwide are infected with *M.tb*, but less than 5% of people infected with this organism develop primary progressive TB disease and 5-10% of people develop reactivation TB disease (Fig1.1). The remaining 90% contain the infection as Latent TB infection (LTBI), showing no signs or symptoms of TB disease (3).

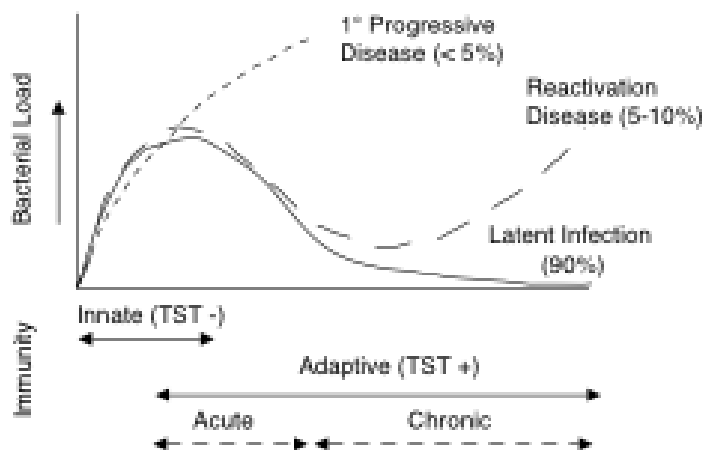


Figure 1.1. Natural history of *M.tb* infection (4)

In 2009 it was estimated that there were approximately 9.4 million incident cases and 1.3 million deaths due to tuberculosis and this continues to rise, especially in Sub-Saharan Africa. South Africa has an overall TB case notification rate of approximately 984 per 100,000 people, currently one of the highest rates in the world (3).

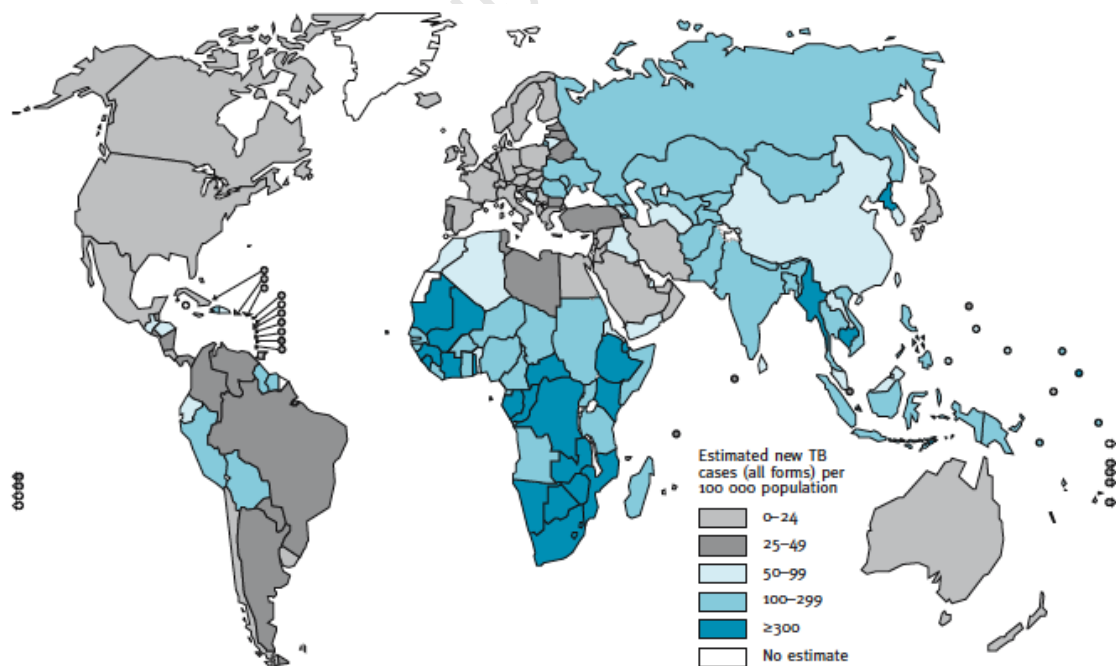


Figure 1.2. Estimated worldwide incidence of TB, 2009 (3)

Possible reasons for this mammoth incidence rate is the combination of poverty, poor nutrition, lack of education, time to diagnosis and HIV-1 co-infection.

1.1. Diagnosis of Tuberculosis in patients

Diagnosis of tuberculosis in patients remains a challenge, with clinicians having to rely on clinical examination along with the tuberculin skin test (TST) and/or direct microscopic analysis of bacterial culture or sputum smear. Culture of *M.tb* from sputum, blood or other bodily fluid remains the current gold standard, however, culture can take up to 8 weeks and the *M.tb* bacillus is not always cultured from a high proportion of samples.

1.1.1. Tuberculin skin test (TST)

The TST has been the only tool for diagnosing TB infection in symptomatic and non-symptomatic patients in the last century. A small amount of tuberculin injected under the epidermis causes the accumulation of antigen-specific lymphocytes in the area; and thereby an inflammatory reaction (5). The inflammatory reaction that occurs is measured by the red, swollen, indurated area at the site of injection. In many parts of the world, TST is of little value because the tuberculin or purified protein derivative (PPD) that is used in the test contains a crude extract of more than 200 antigens that are shared among *M.tb*, *Mycobacterium bovis*, the Bacille Calmette-Guérin (BCG) vaccine strain and most environmental mycobacteria (6). The specificity of the test is low in diagnosing active TB and the test cannot discriminate between latent and active TB (2), especially in an HIV-1 infected person, where sensitivity of the TST is further compromised (7) and (8).

1.1.2. Sputum smear staining for *M.tb*

M.tb has a waxy mycolic acid cell wall that is resistant to Gram-staining and thus acid-fast staining or Ziehl-Neelson (ZN) staining was developed. Sputum expressed from a symptomatic patient can be stained using this technique to identify acid-fast bacilli (AFB+), indicative of *M.tb* (Figure 1.3) (3).

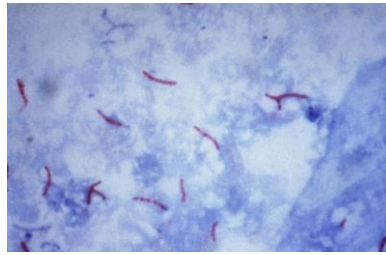


Figure 1.3. Ziehl-Neelson staining showing *Mycobacterium tuberculosis* in red (Courtesy of George Kubica, CDC) (9).

“The revised definition of a new sputum smear-positive pulmonary TB case is based on the presence of at least one AFB+ in at least one sputum...” (3).

1.1.3. Sputum, blood or fluid culture for *M.tb*

Sputum is first digested and decontaminated before sedimentation to isolate any organisms. The sediment is then inoculated onto egg-based, complete or Ogawa media, depending on availability, cost and preference of the testing laboratory. Blood and bodily fluids are also decontaminated and small volumes are inoculated onto either of these media. Blood agar can also be used and can be grown at different temperatures to culture mycobacteria, however this is not very specific for *M.tb*.

The conventional method for culturing *M.tb* uses Lowenstein-Jensen (LJ) media slopes to grow the mycobacteria. The slopes are inspected weekly for growth and ZN staining for AFB+ and further biochemical tests are performed to distinguish *M.tb* from other mycobacteria. These cultures can take between 6-12 weeks to positively identify *M.tb* from them, but drug susceptibility testing can be done at the same time from inoculated broth (10).

The rapid radiometric culture system or BACTEC (Becton-Dickinson) is now accepted for the culture isolation of *M.tb* using an enriched Middlebrook 7H12 containing ^{14}C labeled palmitic acid. The multiplying mycobacteria use ^{14}C and release $^{14}\text{CO}_2$ as they grow. The $^{14}\text{CO}_2$ is detected in an ionic chamber with an electronic detector in the instrument. This method is quicker than the conventional LJ slope culture methods and can differentiate *M.tb* from mycobacteria other than *M.tb*.

Nucleic acid amplification of *M.tb* (NAAT) is another method of detecting *M.tb* in blood or other bodily fluids, but sensitivity differs between laboratories (11). A fully automated NAAT, the Xpert MTB/Rif test for TB has recently been endorsed by the World Health Organization (WHO), allowing rapid molecular detection of TB and drug resistance (12). However, the effectiveness of this method is yet to be proven for site of disease samples.

1.2. Immunodiagnosis of Tuberculosis

As culture for *M.tb* may take up to 6 weeks and is not always possible, other *in vitro* immunologic tests have been developed to assist in the diagnosis of TB in patients.

1.2.1. Diagnosis based on RD1 encoded antigens

Due to the low specificity and sensitivity of the tuberculin skin test, the need to identify antigens of higher specificity has arisen. There are more than 100 open reading frames (ORFs) absent from BCG substrains compared to *M.tb*. These genes are spread over 14-16 chromosomal regions known as region of difference (RD) 1-16 (13). Before 1921, when BCG was internationally disseminated for use as a vaccine, RD1 was deleted and is absent from all BCG substrains. RD1 is a 9.5kb genomic section consisting of 9 ORFs (14).

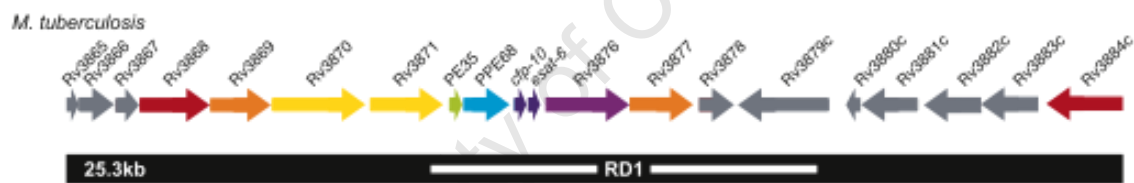


Figure 1.4. Linear alignment of the RD1 regions in *M.tb* (15)

RD1 consists of genes that encode for the attenuated recombinant Early Secreted Antigenic Target, 6kDa (ESAT-6) and the 10kDa Culture Filtrate Protein (CFP-10) (Figure 1.4). CFP-10 has about 40% similarity with ESAT-6 and both represent potent T and B cell antigens secreted by *M.tb* (16). CFP-10 (100 amino acids) and ESAT-6 (95 amino acids) induce high levels of Interferon-gamma (IFN- γ) from T cells when they re-encounter these antigens *in vitro*, and it is this function that can be used as a diagnostic measure of tuberculosis infection.

ESAT-6 and CFP-10, used either as recombinant antigens or as mixtures of overlapping peptides, have been shown to be more sensitive than the TST (17). These TB specific antigens can be used as stimulants in *in vitro* assays such as the Enzyme-Linked Immunosorbent Assay (ELISA) based QuantiFERON-TB Gold, or the Enzyme-Linked Immunosorbent Assay (ELISA) based TSPOT.TB to measure IFN- γ concentrations. These two blood tests may offer an improvement over the skin test. They are based on the detection of IFN- γ released by T cells in response to the *M.tb*-specific antigens; ESAT-6 and CFP-10, and may offer more accurate tools for TB control and elimination (18).

1.2.2. Diagnosis based on non-RD1 encoded antigens

Other antigens of interest, that fall outside of the RD1 region, such as Alpha-Crystallin 1 (Acr1) and Alpha-Crystallin 2 (Acr2) are also of significance in studying TB-immune biology. Acr1 is encoded by the Alpha-Crystallin (*acr*) gene. The *acr* gene is required for the log phase growth as well as assists in intracellular replication of *M.tb* (19).

The expression of Acr1 is induced after cellular exposure to hypoxia and/or nitric oxide. It is associated with the non-replicating state of *M.tb* during latent infection. Acr2 is a protein that is upregulated by unfavourable conditions such as heat shock and oxidative stress (20). It is expressed very early after the mycobacterium has been taken up by monocytes or macrophages, and is thought to be infection stage specific. Another antigen that can be used as a serological agent in the diagnosis of tuberculosis is the Phosphate specific Substrate binding protein-1 (Pst S-1), also known as 38kDa. It is a glycoprotein that is associated with the cell wall of *M.tb* and is associated with current active disease (21). These antigens, along with the live and heat killed laboratory *M.tb* strain, H37Rv, can also be used for stimulating T cells in laboratory assays.

1.3. Normal host response to *M.tb*

After inhalation, the *M.tb* bacillus is ingested and transported to regional lymph nodes by macrophages and other cells (22) (Figure 1.5). The bacilli may be killed and eliminated, but they may also replicate within the macrophages, and subsequently present *M.tb* antigen to CD4 lymphocytes. This leads to the cell-mediated immune response (23). IL-12 is responsible for clonal expansion of Th1 cells, which results in IFN- γ production and subsequently further release of chemokines, pro-inflammatory cytokines and TNF.

As a result, a granuloma forms to contain the *M.tb* bacilli and thus prevent bacterial replication and assist in intracellular killing (24). The granuloma consists of the infected macrophages, surrounded and contained by foamy macrophages, multinucleate Langerhans giant cells (25) and in the periphery CD4 and CD8 lymphocytes and B cells. The final layer of an established granuloma is fibroblasts and the subsequent development of collagen fibres. If the granuloma fails to contain the *M.tb* bacilli, it breaks open and releases the bacteria, that infect surrounding macrophages, eventually forming cavities elsewhere in the lung and spread to the rest of the body causing disseminated disease (23).

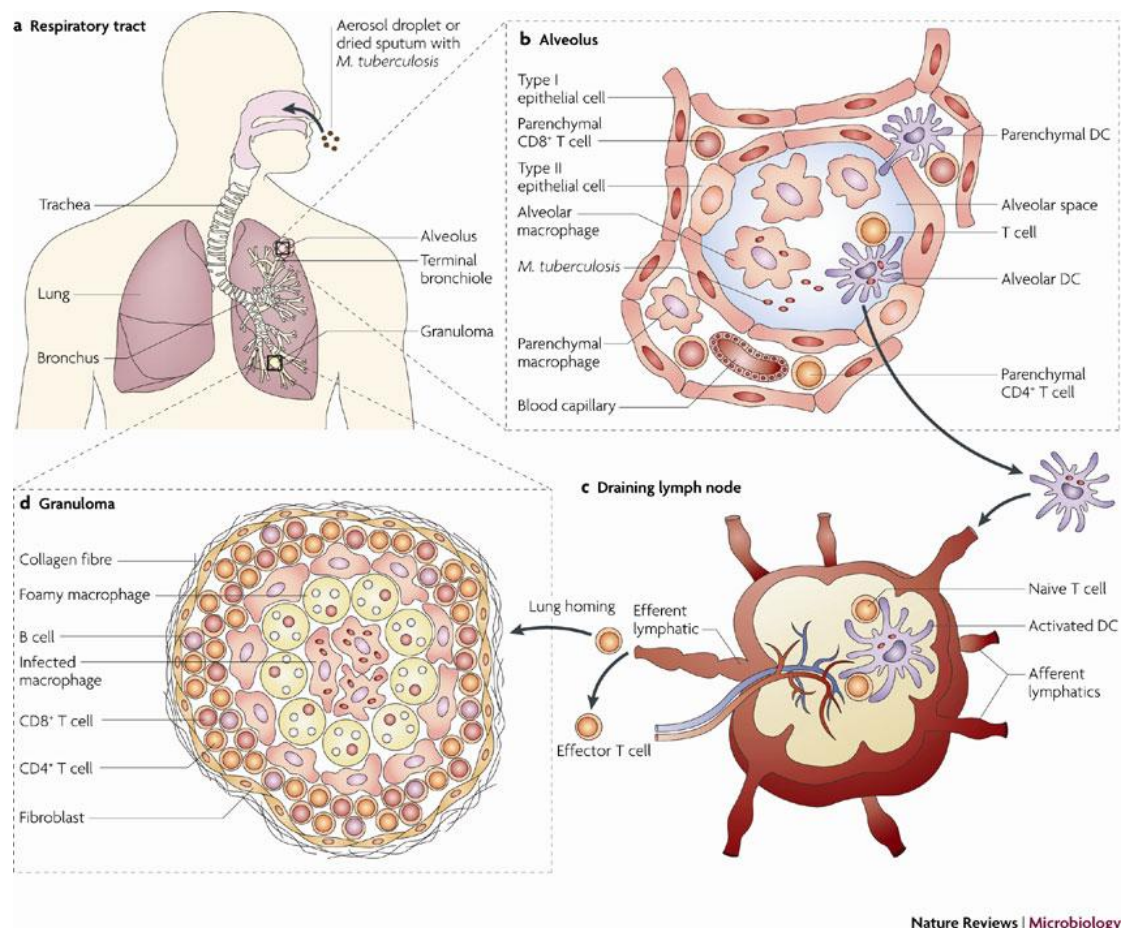


Figure 1.5. The normal host response to eliminate and contain *M.tb* (26)

- inhalation of the *M.tb* bacilli
- ingestion by alveolar macrophages
- transport to the draining lymph node and *M.tb* antigen presentation and cellular activation and expansion
- granuloma formation to contain the *M.tb* bacilli

2. Human Immunodeficiency Virus (HIV)

The human immunodeficiency virus is a Retrovirus, which is part of the Lentivirus genus (27). HIV-1 is made up of two copies of single stranded RNA, each with its own complete copy of the viral genes, surrounded by a conical capsid made up of the p24 protein (Figure 1.6). This capsid is surrounded by host-cell derived plasma membrane. When the capsid buds from the host cells, it takes some of the host-cell plasma membrane with it and develops the envelope (28). The envelope includes the glycoproteins gp120 for adherence to new host cells and gp41, which aids in fusion to the new cell (29). Nucleocapsid proteins, p6, p7 and reverse transcriptase and integrase enzymes are tightly bound to the single stranded RNA, along with other viral genes (regulatory genes such as *tat*, *rev*, *nef*, *vif*, *vpr* and *vpu* and structural genes such as *env*, *gag* and *pol*) and viral protease (30).

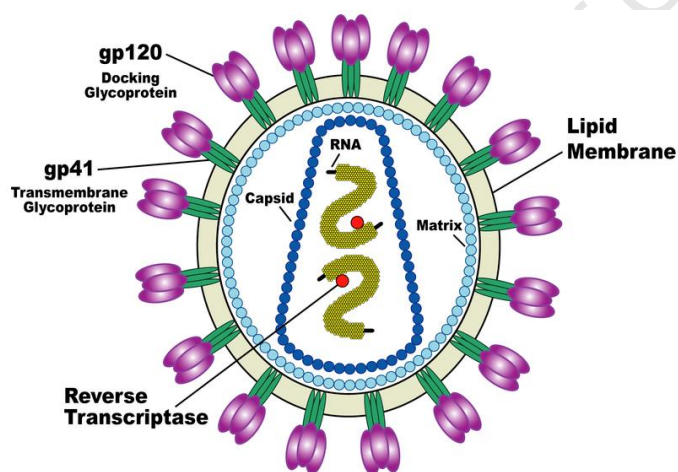


Figure 1.6. Diagrammatic representation of the structure of HIV-1 (31)

HIV-1 is transmitted via bodily fluids from an infected person to an uninfected person.

During acute infection, viral replication occurs rapidly, with decline in circulating CD4⁺ T cells and increase and activation of CD8⁺ T cells. CD8⁺ T cells suppress HIV during the acute phase of infection before they fall in numbers as CD4⁺ T cells rebound, suppressing

HIV replication to some extent (Figure 1.7) during the chronic phase of HIV infection in which the virus is active in the lymph nodes and other tissues such as the brain.

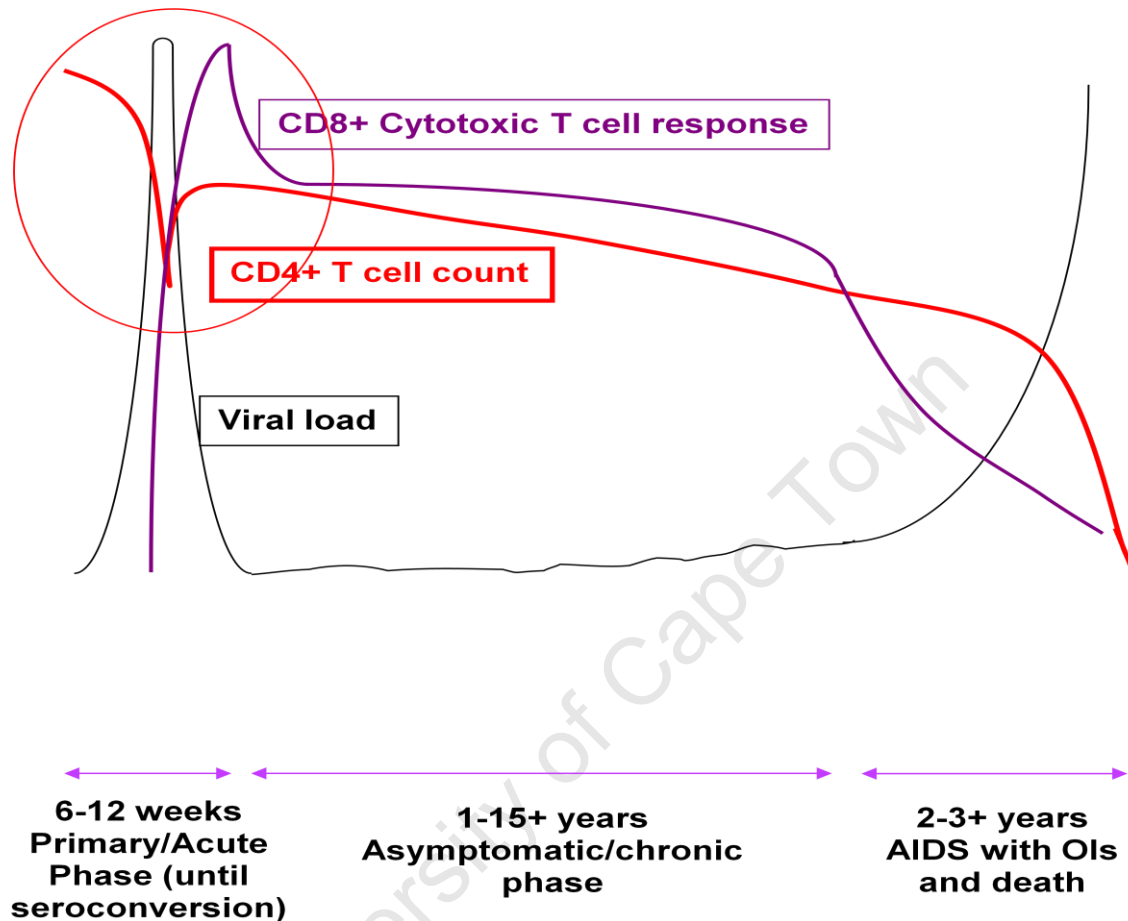


Figure 1.7. Diagrammatic representation of the stages of HIV infection and the immune response that is elicited (Courtesy of Katalin A. Wilkinson, Adapted from (32))

2.1. Host response to HIV-1

HIV-1 specific cell mediated immunity is generally unable to effectively suppress HIV-1 replication except in the few cases of “elite controllers”. HIV-1 causes the loss of proliferating CD4⁺ T cells, but IFN- γ secreting CD4⁺ T cells remain plentiful (33), resulting in skewing toward one functional population of CD4⁺ T cell. Memory T cell responses to Epstein-Barr Virus (EBV) and Cytomegalovirus (CMV) infections have been studied in detail. These infections are effectively controlled by cell-mediated immunity, and are examples of an effective immune response, while HIV-1-specific T cell responses are incompletely effective. The presence of three functional populations of cells distinguishes EBV and CMV-specific CD4⁺ T cell responses: cells that secrete IL-2 alone, cells that secrete both IL-2 and IFN- γ , and cells that secrete IFN- γ alone. The IL-2 response shows antigen clearance, the IFN- γ response is typical of antigen persistence and high antigen load, while the polyfunctional IL-2 and IFN- γ response is typical of continued low level antigen expression (34). In HIV-1 infection, there is also skewing of memory CD8⁺ T cell responses toward those that secrete IFN- γ and no evidence of CD8⁺ T cells capable of proliferation (35).

2.2. The effect of HIV-1 on the TB specific immune response

TB is a key opportunistic infection in HIV-1 infected individuals and is the leading cause of death amongst HIV-1 infected patients. In South Africa, the prevalence of HIV-1 in new TB cases is estimated at more than 50% (Figure 1.8) (3). HIV-1 infects CD4 lymphocytes and integrates itself into the host DNA by reverse transcription. The virus also attacks and kills helper CD4 T cells causing CD4 T cell depletion. Due to this immunosuppression, once the TB organism is inhaled, the HIV-1 infected patients immune system do not contain the infection in the form of a granuloma as efficiently (36). HIV-1 infection can cause TB infection to rapidly progress to symptomatic disease (37). HIV-1 infection can also cause latent TB infection to re-activate. In individuals without HIV-1 infection, the lifetime risk of developing active tuberculosis disease is 10%, but for infected patients, the risk of developing TB can be as high as 7-10% per year (38) and an incidence of up to 36% has been reported (39).

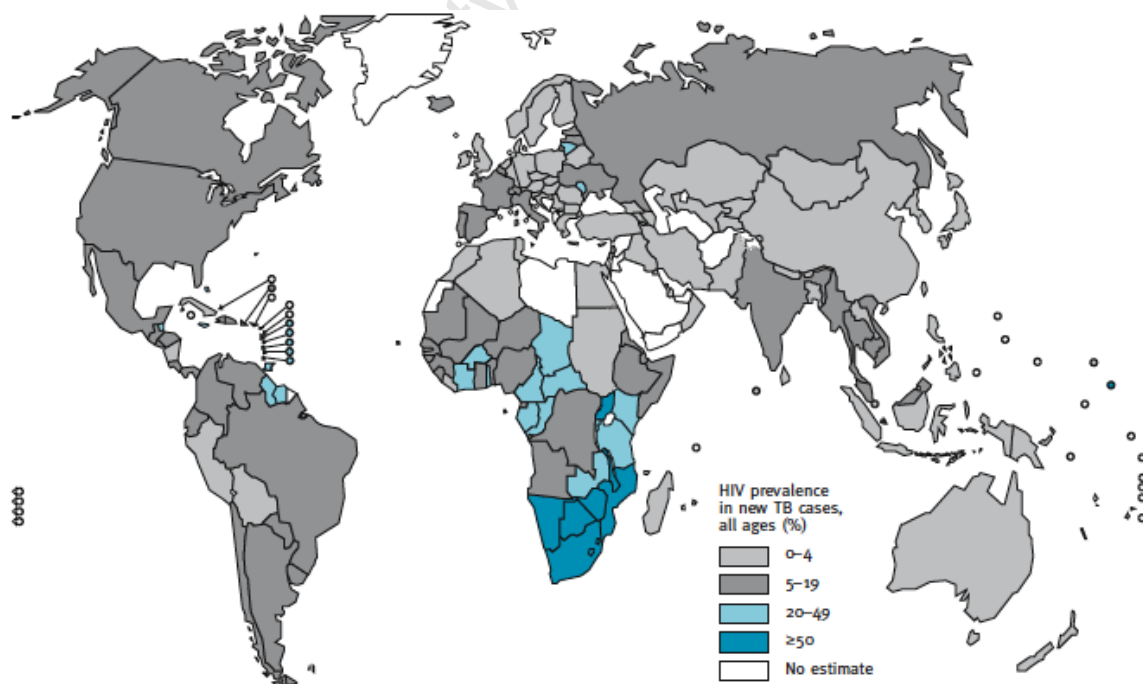


Figure 1.8. Estimated HIV-1 prevalence in new TB cases, 2009 (3)

Latent infection is considered a strong risk factor for TB in HIV-1 infected individuals. TB in HIV-1 infected patients may occur at all stages of HIV-1-related immunosuppression. The clinical features of TB disease with early HIV-1 disease resembles TB infection in HIV-1 uninfected patients. Advanced immunosuppression caused by HIV-1 results in atypical chest X-ray patterns and extrapulmonary disease. The most common manifestations of extrapulmonary TB in HIV-1 infected patients are lymphadenitis, pleurisy, disseminated TB and pericarditis (36).

TB, together with HIV-1 exerts a cofactor effect leading to an accelerated decline in the immune function (Figure 1.9). HIV-1 causes loss of CD4 lymphocytes (40), impairs macrophage recruitment and function (41). HIV-1 impairs the functional capacity of CD4 lymphocytes by impairing T cell proliferation (42) and prevents these cells from secreting Th1 cytokines (IL-2, IFN- γ and IL-12) and instead produce more Th2 cytokines (IL-4 and IL-10) (43).

Increased HIV-1 replication at extrapulmonary sites of disease can cause a decreased immune containment at that site (44), further hindering the host response to TB or other opportunistic infections (45). This immunological response is not well characterised in the extrapulmonary forms of disease, especially in pericardial TB, due to the difficulties associated with access to the disease site.

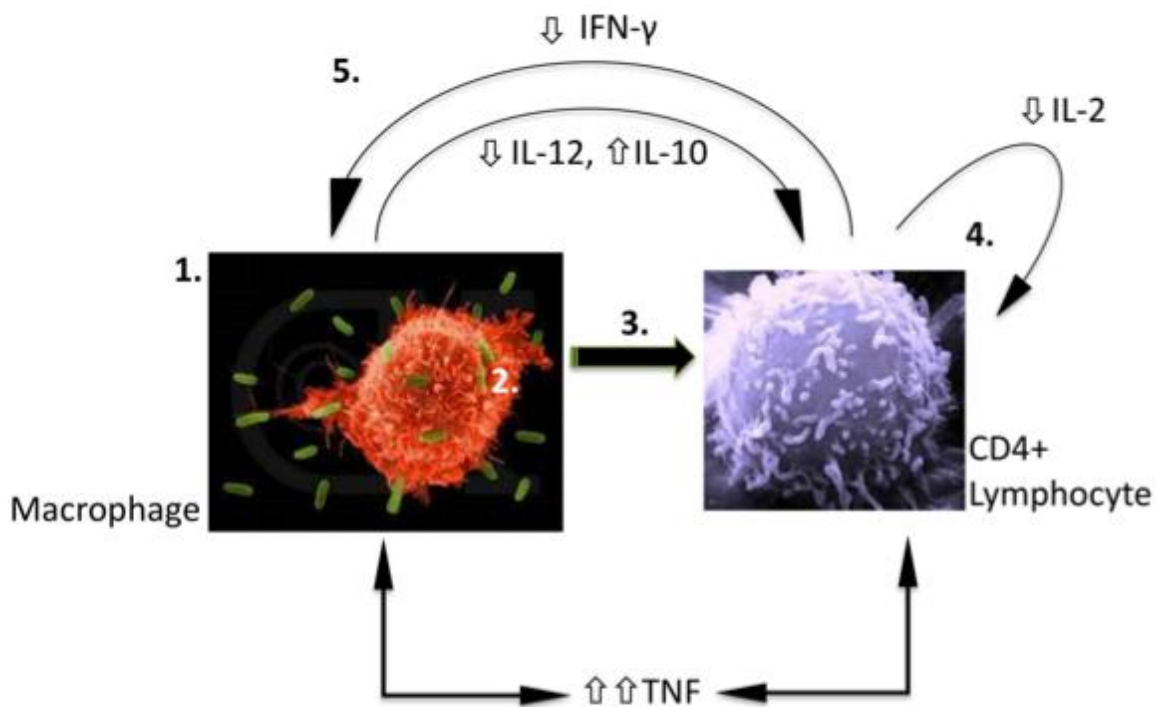


Figure 1.9. Potential mechanisms of the impact of HIV-1 on immunity to *M.tb* infection (adapted from (46))

HIV-1 may affect :

1. the binding and phagocytosis of *M.tb* by macrophages;
2. *M.tb* intracellular killing and antigen processing;
3. *M.tb* antigen presentation;
4. CD4 T-cell activation and proliferation
5. effector cytokine production.

3. Pericarditis

The pericardium is a double-layered enclosed sac that surrounds the heart. The parietal pericardium is the outer fibrinous layer constituted by multiple collagen layers interspersed with elastin fibrils, and the inner visceral serous layer is made up of microvilli for the secretion of pericardial fluid (47). The pericardial cavity (between the two layers) is filled with a thin layer (15-20ml) of serous pericardial fluid that helps reduce friction as the heart moves within the pericardial sac (48). Pericarditis is an inflammation of this delicate tissue wrapper.

3.1. *Different forms of Pericarditis*

Pericarditis can be the result of many infectious and non-infectious causes. The non-infectious causes can be immunoreactive, neoplastic, metabolic and traumatic (cardiac injury, pericardial perforation, and even injury caused by toothpicks (49)), but is mostly idiopathic (undefined) (50-52). Infectious causes are mostly viral infections, followed by bacterial (50, 52). Pericardial abnormalities are reported in approximately 20% of all HIV-1 infections, however this frequency is decreased with the use of anti retroviral treatment (53).

Normal pericardial fluid was studied in the attempt to better diagnose pericardial infections and the use of fluid:serum lactate dehydrogenase (LDH) and protein ratios are thought to be important (54). Protein levels of IL-6, IL-8 and IFN- γ are found to be elevated in pericardial fluids from malignant, bacterial infections and other idiopathic causes of pericarditis, where lymphocytic effusions exist. These cytokines are consistently undetectable in matching serum samples. The most common infiltrating cells are CD3+CD4+ lymphocytes, as well as some neutrophils in other pericardial effusions caused by infections other than TB (55).

Differential diagnosis of pericarditis such as malignant, bacterial and rheumatoid arthritis is also done using the LDH and protein composition of pericardial fluid as well as PCR, but culture is still the gold standard for infectious causes of pericarditis (56).

3.2. *Pericardial tuberculosis*

Tuberculosis is responsible for approximately 70% of all pericarditis cases in the developing world (associated with large pericardial effusions and mostly constrictive pericarditis), compared to the low 4% in the industrialized countries. In the Western Cape, more than half of the patients that present with pericardial tuberculosis are also HIV-1 infected (57).

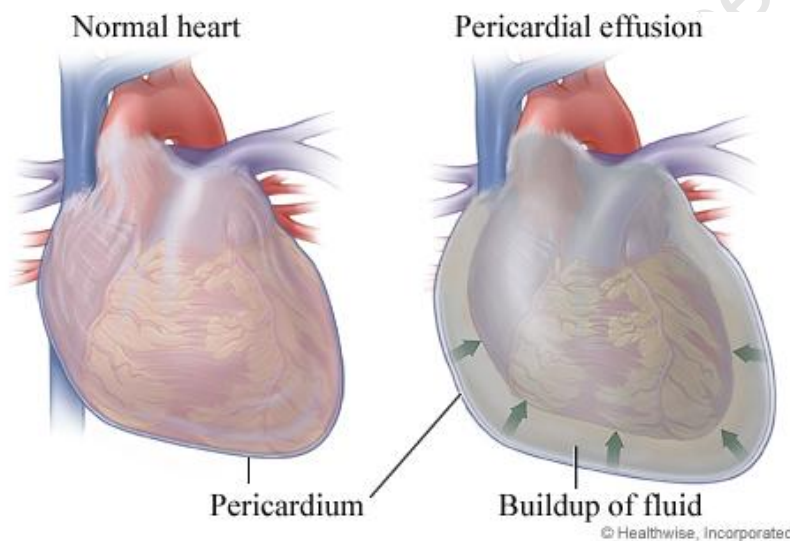


Figure 1.10. Diagrammatic representation of pericardial effusion (58)

Symptoms of tuberculous pericarditis can include fever, night sweats, shortness of breath, cough, malaise and fatigue (59). Diagnosis currently depends on the histopathological study of the pericardium or other tissue, demonstration of acid-fast bacilli in the pericardial tissue or fluid, culture of *M.tb* from pericardial tissue or fluid, the presence of tuberculosis elsewhere in the body or response to a specific treatment (60). Non-invasive methods of

diagnosis include echocardiography, CT and MRI imaging, but these are expensive, require specialized personnel and are seldom available in the rural areas of the developing world (57).

Tuberculous pericardial effusions usually occur as a result of spread of mycobacteria from the mediastinal lymph nodes (61). Mayosi, *et al.*(57), recognized four pathological stages of tuberculous pericarditis:

- (1) The relative abundance of mycobacteria, along with early granuloma formation with loose organization of macrophages and T cells and fibrinous exudation with initial polymorphonuclear leukocytosis.
- (2) Serosanguineous effusion with predominantly lymphocytic exudates with monocytes and foam cells.
- (3) Pericardial thickening caused by fibrin, collagenosis and fibrosis after the effusion has been absorbed.
- (4) Constrictive scarring may occur in which the heart becomes enclosed in a fibrotic calcified sac that causes constrictive pericarditis.

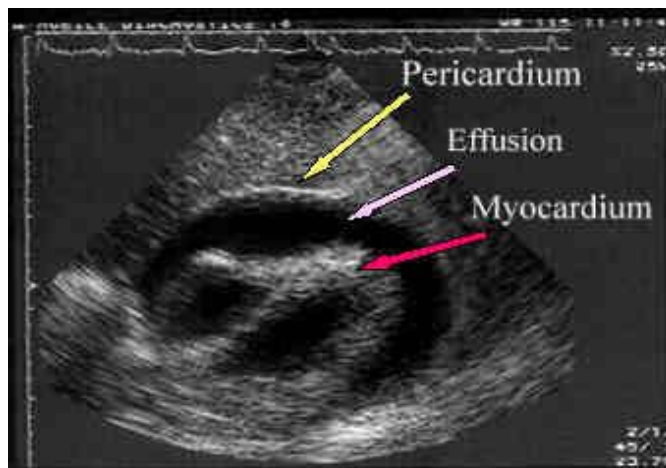


Figure 1.11. Transthoracic echocardiogram with doppler demonstrating massive pericardial effusion (62)

Pericardial tuberculosis can present in three clinical forms – pericardial effusion (Figure 1.10 and 1.11), constrictive pericarditis and a combination of effusion and constriction (Figure 1.12) (63). Effusive pericarditis develops if fluid accumulates slowly in the pericardium. If fluid accumulates rapidly, the heart can become compressed and cardiac tamponade may occur. Cardiac tamponade is the hemodynamic result of fluid accumulation. Constrictive pericarditis occurs due to fibrotic thickening of the pericardium that restricts diastolic filling and cardiac output. Fibrin deposition and scarring also occurs and prevents the movement of fluid in the pericardial cavity. Effusive-constrictive pericarditis is a mixture of effusive and constrictive pericarditis, where there is the presence of a large pericardial effusion, but the right atrial pressure remains elevated after pericardiocentesis, showing evidence of constriction of the visceral pericardium (57). If the fluid resolves and fibrosis occurs, the disease is thought to progress from an effusive-constrictive to a constrictive stage (64).

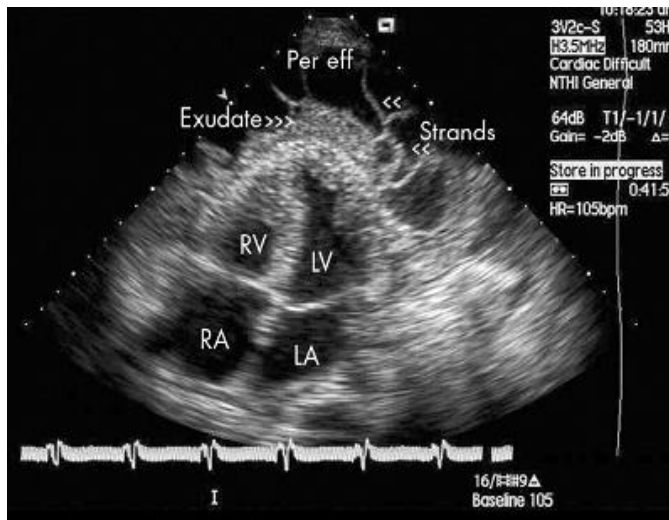


Figure 1.12. Echocardiograph showing a large pericardial exudate, as well as fibrotic strands, indicative of TB pericarditis (65)

When clinically indicated, the pericardial fluid that accumulates is removed by pericardiocentesis, an invasive procedure done under the guidance of fluoroscopic echocardiography. A pigtail catheter is used to drain the fluid from the pericardial cavity. The fluid can be sent to the laboratory for testing and diagnosis (66), and can provide an invaluable source of immunological analysis (67).

4. Immunology of TB pericarditis

4.1. Immunology of TB at the site of disease

In HIV-1 uninfected patients with pleural TB, CD4 lymphocytes are concentrated to the disease site and mostly produce IFN- γ (68). One of the earliest studies done at a TB site of disease was on pleural TB. Barnes, et al (1993), show that mRNA for IFN- γ and IL-2 were increased in pleural fluid compared to blood in pleural TB patients without HIV-1 infection, while IL-4 mRNA was downregulated in the pleural fluid compared to blood. When looking at the protein levels, they also found that IFN- γ was increased in the pleural fluid compared to matching blood samples, while IL-2 and IL-4 proteins were not detectable. The mRNA transcript for the regulatory IL-10 and pro-inflammatory IL-12, as well as the protein was also upregulated in the pleural fluid compared to blood in these patients, showing compartmentalization of Th1 cytokines and IL-10 to the site of disease in active TB (69). TNF protein levels were different in serum and pleural fluid in patients without TB infection, and significantly elevated in the pleural fluid of patients with TB (70).

Pleural TB has been described as a hypersensitivity reaction rather than direct TB infection (71). Compartmentalization of bronchoalveolar immune responses during TB disease are not as a result of sequestration of cells to the site of disease and an increase of local T cell numbers, but increased responsiveness of the resident T cells during active TB (68). It was also shown that there are activated T cells in the bronchoalveolar lavage of TB patients (68). These cells are responsible for the local release of IFN- γ by CD4 and CD8 T cells, but this response alone, is insufficient to bestow protective immunity.

Other inflammatory cytokines, such as IL-6 was found to be compartmentalized to the site of active disease, while IL-18 and TNF was also found in pleural effusions of patients with TB.

IL-17 was only found in a few patients with pleural TB and its role in TB is controversial (72). These are discussed further in Chapter 5 and 6.

In HIV-1 infected patients, IFN- γ is increased in both serum and pleural fluid compared to TB patients without HIV-1 (73), while IL-10 is increased in the serum of HIV-1 infected patients, the pleural fluid levels are similar between the two groups. IFN- γ plays a major role in *M.tb* immunity, both in pathogenesis and protection, and is one of the most studied cytokines. It regulates expression of a host of genes involved in antimycobacterial effector functions. By binding to the IFN- γ receptor, IFN- γ activates macrophages to produce TNF, IL-12, IL-1 and IL-6 (74).

In HIV-1 infected patients with TB meningitis, there is a presence of CD4 T cells in the cerebrospinal fluid (CSF) with a naïve (CD45RA+) and activated (CCR5+CD45RO-) phenotype, while the CD8 T cells are also activated (CD38+CD95+) (75) and these are the same phenotype as found in the lungs of pulmonary TB patients (76). CSF CD4+ T cells mostly express CCR5 and not CCR7, indicative of less differentiated cells (77), while CD8 effector cells are compartmentalized to the site of TB meningitis (78). CSF from TB meningitis patients also display increased levels of IFN- γ , IL-1 β , IL-6, IL-8, IL-10 and TNF (75, 79).

There are only a very small number of studies that have looked at the immune response at the site of disease in pericardial TB. One of these, a study in Cape Town, South Africa on TB pericarditis showed a decrease in IL-4 production and an increase in IFN- γ and TNF in pericardial effusions (80), which is consistently seen in pleural effusions (69, 81-85). IL-6 is increased in pericardial TB effusions compared to bacterial effusions, however decreased

compared to malignant pericarditis (86), which is also consistent in other sites of effusions studied (87). IL-10 is also increased in pericardial TB effusions (86), but to low levels (88).

Cell specific studies have shown that the repertoire of CD4 T cells accumulating at the site of TB disease (including pericardial TB) show broad clonal heterogeneity and there is compartmentalization of antigen specific CD4 T cells to the site of disease (89). Tuberculous pericardial effusions are characterised by a Th1-dominant immune response (86). IFN- γ producing CD4⁺ lymphocytes dominate in HIV-1 negative patients who have TB, whereas CD8⁺ lymphocytes seem to play a more important role in HIV-1 positive patients. There is also an absence of NK cells in the pericardial fluid compared to blood (80).

4.2. The fibrotic response in TB pericarditis

Constrictive pericarditis occurs within six months in up to 25% of patients with tuberculous pericardial effusion despite treatment with anti-tuberculosis medication and corticosteroids. Preliminary data suggests that HIV-1 infected patients tend to develop constriction less often than HIV-1 uninfected patients (90). However, little is known about the immune characteristics and modulations occurring in the pericardium during tuberculosis infection and even less is known about pericardial fibrosis, a long-term complication that arises in constrictive pericarditis patients.

Fibrosis during pericardial TB has not been studied, and most literature is on hepatic, pulmonary and pleural fibrosis. TGF- β has been implicated in the development of fibrosis because it is a potent fibrogenic cytokine which stimulates the synthesis and deposition of extracellular matrix (ECM) components, which include type I and IV collagens (91), whereas IL-1 β and TNF inhibit collagen production (92). Connective Tissue Growth Factor (CTGF) is

also associated with fibrosis by stimulating collagen and fibronectin production, it is upregulated by TGF- β , and its effect is blocked by IL-4 (93).

IFN- γ inhibits fibroblast proliferation and production in vitro (94), whereas in contrast IL-4 stimulates fibroblast proliferation and the production of collagen (95, 96). A shift in this balance towards the IL-4 side is associated with an increase in fibrosis (97). IL-13 shares part of the IL-4 receptor component for signal transduction (98) and shares most of the biological activities of IL-4, including pro-fibrotic action (99). Matrix Metalloproteinases (MMPs) are responsible for the breakdown of collagen and other components of the extracellular matrix (ECM). The functions of MMPs are regulated by their endogenous inhibitors, the Tissue Inhibitors of Matrix Metalloproteinases (TIMPs) (100). These proteins play a role in the regulation of ECM remodeling (Figure 1.13).

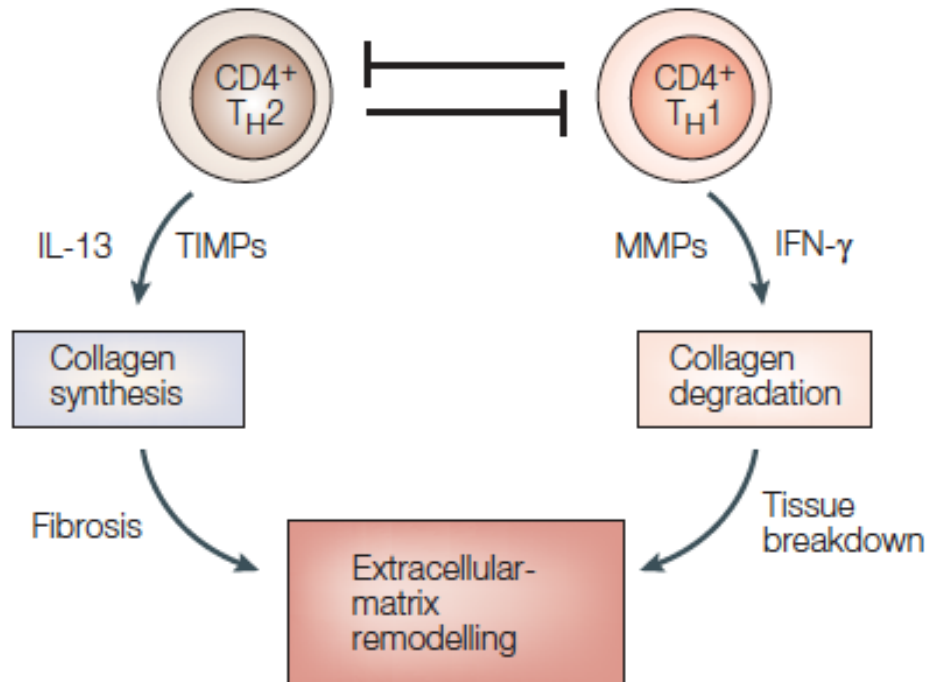


Figure 1.13. Diagrammatic representation of the role of Th1 and Th2 cells in extracellular remodeling (101)

Marquis, et al. (2008) have shown that certain genes are upregulated during pulmonary fibrosis in mice. These genes *COL1A1*, *COL1A2*, *COL4A1*, *COL4A2*, *MMP2*, *TIMP1*, *ARG1* and *SPARC* are associated with the fibrotic response (102). *COL1A1* and *COL1A2* encode a major component of Type I collagen (fibrillar collagen), and *COL4A1* and *COL4A2* are precursors for Type IV collagen (basal lamina). *SPARC* (Secreted Protein and Rich in Cysteine) has a fibrotic response regulatory function and is increased when fibrosis is low. *ARG1* accelerates the development of fibrosis and is associated with a Th2 response, IL-13 and TGF- β secretion (101).

IL-1 β , IL-6 and TNF can directly decrease collagen synthesis and procollagen mRNA expression in cardiac fibroblasts (103). IL-1 β and TNF increase the breakdown of collagen by increasing MMP2, MMP3 and MMP9 activity (103, 104) at transcriptional and post-transcriptional level. TGF- β is a potent stimulant of collagen synthesis via increased transcription and decreased degradation of collagen via increasing TIMP and decreasing MMP (Figure 1.14) (105).

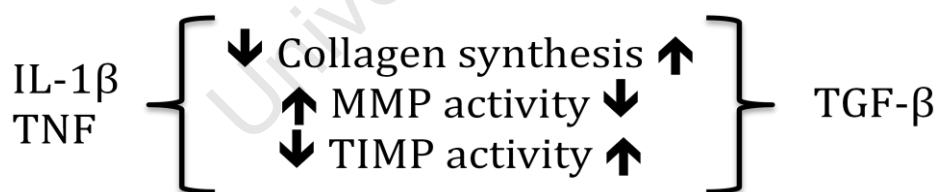


Figure 1.14. The role of cytokines in Fibrosis (adapted from (105))

The exact role of these proteins in the fibrotic response during TB pericarditis have not been studied, and will be addressed in Chapter 5 of this thesis.

5. Hypotheses

1. There is an increase of *M.tb*-specific cytokine release at the disease site compared to blood.
2. Memory T cells are recruited to the site of disease in response to *M.tb*-specific antigens and elicit an inflammatory response.
3. There is a relationship between the clinical presentation of TB pericarditis and the phenotype of immune cells present at the site of disease and in blood.
4. Specific genes are upregulated during TB pericarditis, that determine whether a pure inflammatory or pro-fibrotic response occurs and these genes are responsible for the transcription of proteins at the site of disease.
5. The inflammatory cytokines IL-17 and IL-22 play a role in the inflammatory response against *M.tb*.

6. Aims

Pericardial TB offers a relevant human model to study TB at the site of disease. There are 5 main aims that will be described in this thesis.

1. Determination of the *Mycobacterium tuberculosis* antigen-specific cytokine production at the site of disease and in the blood. CHAPTER 3
2. Determination of the phenotype and function of T cells at the site of disease and in the blood. CHAPTER 4
3. Determination of the mRNA transcript profile of cells in pericardial fluid and blood. CHAPTER 5
4. ELISA and Luminex determination of proteins arising from transcripts found to be upregulated during mRNA transcript profiling. CHAPTER 5
5. Investigation of the regulation of IL-17 and IL-22 in the context of human tuberculosis. CHAPTER 6

CHAPTER 2

Materials and Methods

1. Patients and recruitment

1.1. *Pericardial TB patients*

Ethical clearance was obtained for this study from the University of Cape Town (FHS REC 402/2005). The patients presented to Somerset, Karl Bremer or GF Jooste Hospitals in Cape Town and were subsequently referred to the Cardiac Clinic at Groote Schuur Hospital in Observatory to be recruited into the Investigation of the Management of Pericarditis in Africa (IMPI Africa) registry. All patients were over the age of 18. When clinically indicated, pericardiocentesis was performed under fluoroscopic guidance through a pig-tailed catheter. The case definition of pericardial tuberculosis has previously been defined (106), with either definite TB pericarditis (tubercle bacilli are found in stained smear or culture of pericardial fluid) or probable TB pericarditis (evidence of pericardial effusion in a patient with tuberculosis demonstrated elsewhere in the body and/or empirical diagnosis by the clinical team, based on clinical signs, symptoms and response to TB treatment). Counseling and testing for HIV-1 were inclusion criteria for the study. Patients were followed up for 1 year after inclusion into the study.

1.2. *Open-heart Surgery Control patients*

Patients undergoing open-heart surgery for heart bypass or valve replacement surgery were recruited from the Cardiac Unit in Groote Schuur Hospital. Ethical clearance (FHS REC 289/2007) was obtained from the University of Cape Town. All patients tested HIV-1 negative and had no evidence or past history of treatment for TB disease.

2. Sample types and preparations

2.1. Collection of Whole Blood and Pericardial Fluid

2.1.1. Pericardial TB patients

Blood was obtained before the pericardiocentesis procedure and collected in two BD Vacutainer (Preanalytical Solutions, UK) tubes. At the time of pericardiocentesis, pericardial fluid was collected from all patients and decanted into four 10ml BD Vacutainer vials (Preanalytical Solutions, UK). Blood and pericardial fluid for RNA extraction was also collected in a PAXgene Blood RNA stabilization tube (Preanalytix). Blood for serum separation was collected in a 5ml BD Vacutainer SSTTM Advance vial (BD Diagnostics, UK).

2.1.2. Open-heart Surgery Control patients

Blood was obtained before surgery and collected in a BD Vacutainer (Preanalytical Solutions, UK) tube. Blood for serum separation was collected in a 5ml BD Vacutainer SSTTM Advance vial (BD Diagnostics, UK). At the time of open-heart surgery, pericardial fluid was collected from all patients and decanted into a 10ml BD Vacutainer vial (Preanalytical Solutions, UK).

2.2. Separation of Peripheral Blood Mononuclear Cells (PBMC)

Whole blood was diluted 1:1 with 1 X Dulbecco's Phosphate Buffered Saline (PBS) (Sigma[®], UK) and slowly pipetted drop-wise over a 10ml layer of Ficoll-Paque PLUS (GE Healthcare Bio-Sciences AB) in a 50ml universal Falcon tube (Sterilin). The tube was centrifuged in a Sigma 4K15 laboratory centrifuge with a swing-out rotor at 700g for 20 minutes with the brakes off. The whole blood separated into layers and the clear buffy layer was removed using a plastic pipette. The buffy layer consisting of the PBMC was washed in RPMI-1640 Medium (Sigma[®]) without additives and centrifuged at 600g for 10 minutes with

the brakes on. The RPMI was poured off leaving a pellet of PBMC behind. This pellet was resuspended in 10ml RPMI with 10% heat inactivated Fetal-Calf Serum (RPMI/10%HI-FCS) (Sigma), after which the number of PBMC was counted. A 1:1 dilution of PBMC in RPMI/10%HI-FCS was added to a 0.4% trypan blue solution (Sigma®) and counted in a 10-chamber plastic counting slide (Immune Systems LTD, UK). Assays using the PBMC were set-up and the remaining PBMC were frozen in a 1:1 dilution of RPMI/10%HI-FCS and Cell freeze medium (20%DMSO in HI-FCS).

2.3. Separation of Pericardial Fluid Cells (PFC)

Pericardial Fluid was transferred into 50ml Falcon universal tubes (Sterilin) and centrifuged at 700g for 20 minutes with the brakes off. A 1.8ml sample of cell free fluid was removed and stored in a cryotube vial (NUNC) at -80°C until needed. The remainder of the cell free fluid was poured off leaving a pellet consisting of Pericardial Fluid Cells (PFC). These cells were washed and counted in the same manner as the PBMC. Assays using the PFC were set-up and the remaining PFC were frozen in a 1:1 dilution of RPMI/10%HI-FCS and Cell freeze medium.

2.4. Separation of Serum from Blood

Blood in the BD Vacutainer serum vial, which contained Heparin, was centrifuged at 3000 g for 10 minutes. The separated serum was removed and stored with 0.1% of Sodium Azide in a cryotube vial (NUNC) at -80°C for future analysis.

3. Chosen antigen types and selection

3.1. Region of Difference (RD) -1 encoded antigens

The endotoxin free *M.tb*-specific RD-1 encoded antigens, deleted from the vaccine strain BCG, Early Secretory Antigenic Target (ESAT) -6 (molecular weight (MW) = 11800) and Culture Filtrate Protein (CFP) -10 (MW = 12600) (both from Proteix, Prague, Czech Republic) were used in all assays. They were used at a final concentration of 10µg/ml unless otherwise stated.

3.2. Non-RD-1 encoded *M.tb* specific antigens

The endotoxin free non-RD1 encoded *M.tb*-specific Alpha-crystallin (Acr) -1 (MW = 14000) and -2 (MW = 19300) antigens, associated with heat-shock and oxidative stress of the *M.tb* bacterium were used along with the *M.tb* cell wall associated 38kDa (MW = 38000) antigen (all at 10µg/ml) (all from Proteix, Prague, Czech Republic) for the IFN-γ ELISpot assay.

3.3. Positive controls

Positive controls in the form of phytohemagglutinin (PHA, 5µg/ml) and Purified Protein Derivative (PPD, Statens Serum Institute, Denmark, 10000U and 10µg/ml) were used, along with staphylococcal enterotoxin B (SEB, Sigma-Aldrich, 5 and 10µg/ml) to stimulate cells. Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, 10ng/ml) and Ionomycin (Sigma-Aldrich, 10ng/ml) were used to restimulate 4-day cultures.

3.4. Whole *M.tb* stimulation

The heat killed (hk) laboratory strain H37Rv was used to stimulate cells for flow cytometry at a multiplicity of infection of 1 mycobacterium: 1 cell (MOI 1:1). Live H37Rv was used to stimulate whole blood to determine the role of IL-17 in *M.tb* infection also at a MOI 1:1.

4. Determination of the HIV status of the patients

4.1. Determine HIV-1/2 Ag/Ab Combo test

The Determine HIV-1/2 is an *in vitro*, qualitative immunochromatographic test for the detection of antibodies to HIV-1 and HIV-2 in human serum, plasma or whole blood. In our assay, where serum was not available, we used cell free pericardial fluid. All patients were tested for HIV status using this assay as permitted by Ethical clearance (REC 402/2005 – re-issued 2009). The Determine HIV-1/2 test card was coated with HIV-1/2 recombinant antigen and synthetic peptides. As the sample (50µl) was added to the test pad, it migrated through the pad, reconstituted and mixed with the selenium colloid-antigen conjugate. The mixture continued to migrate through the solid phase to the immobilized recombinant antigens and synthetic peptides at the patient window site. If antibodies to HIV-1 and/or HIV-2 were present in the sample, the antibodies in the sample would bind to the antigen-selenium colloid and to the antigen, forming a red line at the window site. If there were no antibodies, no red line was formed. A control line was included in the assay after the patient window site to ensure the validity of the assay. The assay was read after a minimum of 15 minutes.

5. Determination of the HIV viral load of HIV-1 infected patients

5.1. Nucleic Acid Sequence-based amplification

Measurement of HIV viral load was performed at the National Health Laboratory Services (NHLS) in Groote Schuur Hospital, using nucleic acid sequence-based amplification (NASBA) on stored serum and cell free pericardial fluid samples from ten patients included in the FACS analysis experiments described in Chapter 4. The limit of detection by this assay was 300 copies of HIV RNA / ml sample.

6. Determination of *M.tb* antigen-specific cytokine production at the site of disease and in blood

6.1. Whole blood and pericardial fluid overnight re-stimulation assay

A 1ml volume of whole blood and pericardial fluid was each pipetted into 4 separate wells of a 24 well plate (NUNC). These were stimulated with the following antigens: ESAT-6, CFP-10 and the positive control PHA, all at a final concentration of 10µg/ml. The 4th well was left unstimulated as a negative control. The plate was incubated in a 37°C-humidified incubator with 5% CO₂ overnight. The following morning, the supernatants were removed and filtered by a syringe driven filter unit with a 0.22mm Millex GP filter (Millipore, Ireland). The IFN-γ produced in the assay was measured by an in-house IFN-γ sandwich ELISA (described later).

6.2. IFN-γ-specific ELISpot

A 96 well pre-coated IFN-γ ELISpot plate (PRe coated One-step, MABTECH, code 3420-2APT-10) was washed 5 times with 200µl of 1 X PBS (Sigma®). The plate was blocked for 30 minutes at room temperature with 200µl per well of RPMI/10% HI-FCS. The blocking medium was removed and the PBMC and PFC were added at 200 000 cells in 100µl per well to 8 wells. Cells were stimulated with *M.tb* specific antigens as follows: 5µg/ml ESAT-6, 2.5µg/ml CFP-10, 4µg/ml Acr1, 20µg/ml Acr2, 2.5µg/ml 38kDa, 200 U/ml PPD and 5µg/ml Phytohemagglutinin (PHA) as a positive control. The 8th well was unstimulated to act as a negative control.

The cells were incubated overnight in a 37°C humidified incubator with 5% CO₂. The following morning, the wells were washed 5 times with 200µl of 1 X PBS. A secondary antibody (alkaline phosphatase-conjugated detection monoclonal antibody 7-B6-1,

MABTECH) which is a biotinylated catcher antibody was added at a final concentration of 0.5mg/ml in 100µl of filtered 1 X PBS containing 0.5% HI-FCS per well. The plate was incubated for 2 hours at room temperature before it was washed 5 times with PBS. Filtered substrate solution, BCIP/NBT-plus, was added at 100µl per well, until spots started to develop. The reaction was stopped with tap water and left to dry. The plate was read in an automated Immunospot reader (CTL, Cellular Technology Ltd), which recorded the number of spots per well. Background spots from the unstimulated well were subtracted from the antigen stimulated wells to give the corrected Spot Forming Cells (SFC) per well. The results were expressed as SFC/ 10^6 cells by multiplying the number of spots per well by the dilution factor of 5.

6.3. Depletion of CD4 or CD8 T cells from PBMC and PFC

6.3.1. Preparation of the PBMC and PFC

PBMC and PFC from 4 HIV-1 infected TB pericarditis patients with sufficient cell numbers were used for the following depletion assays. The cells were thawed and added to warm RPMI/10%HI-FCS, washed by centrifugation for 10 minutes at 600g, and set up in 10ml of RPMI/10%HI-FCS. The cells were counted and washed again. The cells were resuspended at 10^7 /ml of wash buffer (PBS/0.1% HI-FCS) and added to 3 separate eppendorf tubes at 2.0×10^6 cells / tube, labeled anti-CD4, anti-CD8 and anti-CD4+ anti-CD8+ and left on ice until needed.

6.3.2. Preparation of the magnetic beads

The anti-CD4 (Dyna/Invitrogen, 111.45) and anti-CD8 (Dyna/Invitrogen, 111.47) bead vials were thoroughly vortexed and 50µl of beads per 10^7 /ml PBMC for depletion were removed and dispensed into clean eppendorf tubes. These were placed in 1ml wash buffer, mixed, and

placed in the magnet (Dyna/Invitrogen) for 1 minute. The magnet binds the beads and the liquid can be removed. The beads were resuspended in the required wash buffer until use.

6.3.3. Depletion assay

The required volume of beads was added to each of the designated tubes, the tubes were flicked and kept on ice for a further 30 minutes, with flicking every 5-10 minutes to ensure adequate binding of the beads to the cells. After the 30 minutes, the tubes were inserted into the magnet and the contents were aspirated and dispensed into new eppendorf tubes. The tube containing the bound beads was discarded from the magnet. The depleted fractions were spun down and resuspended in RPMI/10%HI-FCS to make up the original cell count to 2.0×10^6 /ml. These cells were plated onto an IFN- γ specific ELISpot plate, along with undepleted PBMC and PFC as positive controls, and then stimulated with ESAT-6, CFP-10, PHA and an unstimulated control, assayed and counted as described above.

6.3.4. FACS confirmation of depletion efficiencies

Any remaining cells were used for FACS staining to ascertain the efficiency of the depletions. The cells were fixed using BD Cytofix/Cytoperm for 20 minutes at 4°C and washed with 1 X BD Permeabilisation buffer. Before surface staining with markers for CD3, CD4 and CD8 (Table 2.1), the viability of the cells was stained for using a viability marker VIVID (Invitrogen) that binds to amines upregulated during cell death, which is read in the violet channel of a BD LSRII. The viability marker was prepared by adding 2 μ l undiluted VIVID to 2ml PBS. It was mixed and 200 μ l of the diluted mixture was added to each tube. The tubes were left to incubate for 30 minutes, washed in 1 X BD Permeabilisation buffer and then stained for with surface antibodies for 30 minutes at 4°C. The cells were washed

and resuspended in 1 X Permeabilisation buffer for acquisition. Analysis was done using FlowJo v9 (www.treestar.com).

Table 2.1. Antibody and conjugate, supplier and volume of surface antibody used to determine adequate depletion of PBMC and PFC by magnetic bead assay

Antibody and Conjugate	Supplier	Volume (µl)
CD3-FITC	BD Biosciences	5
CD4-APC	BD Biosciences	3
CD8-Cy5.5PerCP	BD Biosciences	3
VIVID (1:1000)	Invitrogen	200

The percentage of the CD4 and CD8 cells gated were used to determine the depletion efficiency for each assay by the following calculation:

$$100 - \left[\frac{\% \text{CD4 measured in CD4 depleted sample}}{\% \text{CD4 measured in CD8 depleted sample}} \times 100 \right] = \% \text{ depleted}$$

7. Phenotype and Function of T cells at the site of disease and in the blood

7.1. Surface phenotype of PFC and PBMC

PBMC and PFC from HIV-1 uninfected and HIV-1 infected patients were set up at 400 000 cells per well in 200µl in 6 wells of a 96-well plate (NUNC) and were incubated overnight at 37°C with 5% CO₂. The first 5 wells were stimulated with hKH37Rv (MOI 1:1). The last well was left unstimulated. Brefeldin A (5µg/ml) was added for the last 5 hours to the 4th well (Table 2.2). Brefeldin A blocks Golgi transport, thus allowing the accumulation of cytokines inside the cell. The cells were harvested into 5ml BD Falcon Polystyrene Round Bottom tube (BD Biosciences), and washed with 1ml of FACS wash buffer (PBS/2% HI-FCS) and centrifuged at 300g for 5 minutes. The supernatant was poured off and the pellet was loosened by vortexing the tubes. Surface staining (all BD Biosciences, except for FoxP3) was performed according to Table 2.2.

Once the antibody was added to the cells, they were left to incubate for 20 minutes at 4°C in the dark after which 500µl of FACS wash buffer was added and the tube was spun at 300g for 5 minutes to wash off the excess staining antibodies. The supernatant was removed and the pellet was loosened by vortex. FACS-FIX buffer (PBS/1.6% Paraformaldehyde) was added to tubes 1-3 and 5-6 and stored at 4°C in the dark until analyzed.

Table 2.2. Different surface markers stained for with antibodies conjugated to a specific colour tag

Tube/well	APC (µl)	PerCP (µl)	FITC (µl)	PE (µl)
1 (proliferating T cells)	CD4 (3)	CD8 (5)	HLA-DR (5)	CD71 (3)
2 (Memory T cells)	CD4 (3)	CD8 (5)	CD28 (5)	CD45RA (3)
3 (Memory T cells)	CD4 (3)	CD8 (5)	CD45RO (5)	CD45RA (3)
4 (T regs)	CD3 (3)	CD4 (5)	-	FOXP3 * (10)
5 (NK cells)	CD3 (3)	-	CD16 (5)	CD56 (3)
6 (unstimulated)	-	-	-	-

*FOXP3 is an intracellular stain that is stained for after surface staining is complete.

To stain for intracellular FoxP3 (eBiosciences), freshly prepared eBioscience Fix/Perm buffer (1 part Fix/Perm concentrate: 3 parts Fix/Perm diluent) was added to tube 4 and left to incubate at 4°C for 45 minutes. The tube was centrifuged at 300g for 5 minutes after which the supernatant was decanted. The pellet was loosened by vortex, before being washed twice with eBioscience Permeabilisation buffer. The supernatant was poured off and the pellet loosened. Normal rat serum was added to block non specific binding sites, in order to prevent background staining, and incubated for 15 minutes at 4°C. Then without washing, intracellular anti-human FoxP3-PE was added straight to the pellet and incubated at 4°C for 30 minutes. The pellet was washed again with permeabilisation buffer and spun at 300g for 5 minutes, the supernatant poured off and the pellet was loosened by vortex. FACS-FIX buffer was added and all the tubes were analyzed together. A four colour BD Bioscience FACS Calibur Flow Cytometer was used to acquire the cells and the program CellQuest Pro was used to record the readings. FlowJo version 8.8.6 (www.treestar.com) was used to analyze the results.

7.2. Intracellular staining of PFC and PBMC

A further sample of cells from HIV-1 uninfected and HIV-1 infected patients were also analysed for T cells producing IFN- γ , IL-2, TNF, IL-17 and GM-CSF using Intracellular Cytokine Staining (ICS). Recombinant ESAT-6 and CFP-10 (5 μ g/ml each), hkB37Rv (MOI 1:1) and PPD (10000U) were used as stimulants, as well as SEB (10 μ g/ml) and no antigen as the negative control.

Two million cells were stimulated with the *M.tb* specific antigens for 2 hours after-which Brefeldin A (5 μ g/ml) was added for the last 16 hours. Cells were washed in 1 X PBS (Sigma[®]) and then fixed with FACS FIX for 20 minutes at 4°C. Cells were spun down at 300g for 10 minutes and frozen in a 1:1 dilution of RPMI/10%HI-FCS and cell freeze medium for future staining.

The frozen cells were thawed, resuspended in 1 X PBS and washed at 300g for 10min. The cells were separated into 2 tubes of 1x10⁶ each and stained according to Table 2.3 and 2.4, for 1 hour at 4°C in the dark.

The South African Tuberculosis Vaccine Initiative (SATVI) determined the optimal volumes of antibody to be used after series of titration tests were run on PBMC from healthy donors.

Table 2.3. Table of marker, conjugate, supplier and volume of antibody used to stain PBMC and PFC in Tube 1 for the ICS assay

Tube 1	Conjugate	Supplier	Volume (µl)
CD3	Pacific Blue	BD Biosciences	1
CD4	QDot 605	Invitrogen	0.5
CD8	Cy5.5PerCP	BD Biosciences	3
IFN- γ	Alexa700	eBiosciences	1
IL-17	Alexa647	eBiosciences	3
IL-2	FITC	BD Biosciences	5
TNF	Cy7PE	eBioscience	0.5
GM-CSF	PE	BD Biosciences	2

The excess stain was washed off using 1 X Perm Wash Buffer (BD Biosciences) and the cells were resuspended in 200µl of Perm Wash Buffer for acquisition. An 8-colour panel was used on the BD LSRII flow cytometer and FACS Diva was used to acquire the data. Analysis of the data was done using FlowJo Software version 8.8.6 (www.treestar.com). Boolean gating was applied in the ICS staining to look at cells bearing multiple fluorescent markers.

Table 2.4. Table of marker, conjugate, supplier and volume of antibody used to stain PBMC and PFC in Tube 2 for the ICS assay

Tube 2	Conjugate	Supplier	Volume (µl)
CD3	Pacific Blue	BD Biosciences	1
CD4	QDot 605	Invitrogen	0.5
CD8	Cy5.5PerCP	BD Biosciences	3
IFN- γ	Alexa700	eBiosciences	1
CD45RA	APC	BD Biosciences	3
CD27	FITC	BD Biosciences	3
CCR5	PE	BD Biosciences	5

7.3. Compensation beads

Compensation beads (BD 552843) were used to adequately compensate each parameter on the BD LSRII during the acquisition of the cells. One drop of the positive anti-mouse Ig, κ beads were added to each of the polystyrene round bottom tubes with 50µl of 1 X Perm Wash Buffer (BD Biosciences). Beads were stained with each of the single antibody-conjugates (Table 2.5.) for 1hour at 4°C. The beads were washed with 1 X Perm Wash before 1 drop of the negative control beads in FBS were added to each tube. The beads were resuspended in 150µl of 1 X Perm Wash and acquired separately on the BD LSRII.

Table 2.5. Table of conjugate, supplier and volume of antibody used to stain the compensation beads for the ICS assay

Conjugate	Supplier	Volume
Pacific Blue	BD Biosciences	1
QDot 605	Invitrogen	0.5
Cy5.5PerCP	BD Biosciences	4.5
Alexa700	eBiosciences	2
Alexa647 / APC	eBiosciences	3
FITC	BD Biosciences	5
Cy7PE	eBioscience	0.5
PE	BD Biosciences	3

Compensation beads were stained for each assay that was performed to ensure comparability between samples run on different days. Compensation of each sample was done using the compensation wizard on FlowJo software and the compensation matrix was applied to each tube of the samples run. The compensation matrix was manually checked for each tube to ensure that tubes were not under or over compensated for any reason.

8. mRNA Transcript profiling of pericardial fluid and blood

8.1. RNA extraction from pericardial fluid and blood

Whole blood and pericardial fluid were stored in RNA PAXgene tubes (PreAnalytics, 762165) at -20°C until extracted in batches, using the PreAnalytics kit (762174). The tubes were left to thaw at room temperature for a minimum of 2 hours and centrifuged at 5000g for 10 minutes. The supernatant was poured off and the pelleted cells were loosened by vortex. The cells were resuspended in 5ml of RNA-free water and the tubes were centrifuged again at 5000g for 10 minutes. The supernatant was poured off and 350µl of Buffer BR1 was added.

The cells were thoroughly resuspended and then transferred to a 1.5ml Eppendorf tube to which 300µl of Binding Buffer BR2 was added, along with 40µl of proteinase K. These were mixed by vortexing for 5 seconds and incubated for 10 minutes at 55°C. The lysate was pipetted directly onto a PAXgene Shredder spin column placed in a 2ml processing tube and centrifuged for 3 minutes at 13000g. The entire flow-through fraction was transferred to a 1.5ml microcentrifuge tube containing 350µl of 100% pure-grade ethanol (Sigma®). These were mixed by vortex and centrifuged briefly to remove any drops from the inside of the tube lid.

Approximately 700µl of the sample was added into the PAXgene RNA spin column and centrifuged for 1 minute at 13000g. The spin column was placed in a new processing tube and the remaining sample was added. The column was centrifuged for 1 minute at 13000g before 350µl wash buffer 1 was added to the spin column in a new 2ml processing tube. The column was centrifuged for 1 minute at 13000g and then placed in a new processing tube, to which 80µl of DNase I incubation mix was added and incubated at room temperature for 15 min.

The spin column was washed with 350µl of wash buffer 1 and centrifuged for 1 minute at 13000g. The spin column was placed in a new processing tube before 500µl of wash buffer 2 was added. The column was centrifuged for 1 minute at 13000g before it was washed again with another 500µl of wash buffer 2.

The PAXgene spin column was placed in a 1.5ml microcentrifuge tube to which 40µl of elution buffer BR5 was added directly to the membrane. The column was centrifuged for 1 minute at 13000g. Another 40µl of BR5 was added and the column was once again centrifuged in the same microcentrifuge tube. The elute was incubated at 65°C for 5 minutes and then chilled immediately on ice. The RNA samples were stored at -80°C for future RT-PCR assays to be performed.

8.2. Determination of the quality and quantity of RNA in pericardial fluid and blood

RNA concentration was determined using the Nanodrop ND 1000 (Thermo Scientific, USA). With the sampling arm opened, 1.2µl of RNase-free water was pipetted onto the lower measurement pedestal and the sampling arm was closed to initiate spectral measurement using the operating software on the computer. After blanking with 1.2µl of water, 1.2µl of sample was pipetted onto the lower measurement pedestal and the sampling arm closed again to initiate spectral measurement. The sample column was automatically drawn between the upper and lower measurement pedestals and the spectral measurement was made. After the first measurement was completed, both pedestals were wiped with a soft laboratory wipe and the next sample was placed and measured in the same manner. The spectral measurement determines overall concentration as well as purity of the RNA using different wavelengths of light at 230, 260 and 280 nanometers (nm) which passes through the sample and is measured as absorbance. For RNA samples to be considered free of contaminants and salts the 260/280

ratio should be between 1.8 and 2.0 and 260/230 ratios should be > 1.5. The software then determines the concentration of the sample. Concentration of the RNA samples were given as nanograms/ μl (ng/ μl). The concentration was used to determine the amount of RNA to reverse transcribe to obtain optimal amounts of cDNA for each sample.

8.3. Reverse Transcription of RNA to cDNA

RNA was reverse transcribed to cDNA using the Quantitect Reverse Transcription kit (Qiagen, 205313). The template RNA, which was stored at -80°C , was thawed on ice. The gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix and RNase-free water were thawed at room temperature ($15\text{-}25^{\circ}\text{C}$). When the solutions were thawed, tubes were gently flicked and the residual liquid was collected by centrifugation. Elimination of genomic DNA contamination of the template RNA was done according to the volumes in Table 2.6, and left to incubate for 2 minutes at 42°C and then placed immediately on ice. Template RNA was used at $1\mu\text{g}$ per reaction.

Table 2.6. Genomic DNA Elimination Reaction Components

Component	Volume/reaction
gDNA Wipeout Buffer	$2\mu\text{l}$
Template RNA	Variable ($1\mu\text{g}$), $x\mu\text{l}$
RNase-free water	$12\mu\text{l} - x\mu\text{l}$
Total Volume	$14\mu\text{l}$

The reverse-transcription master mix was prepared on ice according to Table 2.7. and added to the gDNA elimination mixture containing the template RNA. The mix was incubated for 15 minutes at 42°C followed by an incubation at 95°C for 3 minutes to inactivate the

Quantiscript Reverse Transcriptase. The resulting cDNA was stored at -20°C until real-time RT-PCR was performed using the ABI Prism 7000.

Table 2.7. Reverse-Transcription Reaction Components

Component	Volume/reaction
Reverse-transcription master mix	
Quantiscript Reverse Transcriptase (Contains RNase inhibitor)	1µl
Quantiscript RT Buffer, 5x (contains Mg ²⁺ and dNTPs)	4µl
RT Primer Mix	1µl
Template RNA	
Entire genomic DNA elimination reaction volume	14µl
Total Volume	20µl

8.4. Real Time – Polymerase Chain Reaction (RT-PCR)

RT-PCR using Inventoried TaqMan Gene Expression Assays in the Pre-Designed Assay Reagents (PDAR) format from Applied Biosystems (ABI) were used for specific human genes listed in Table 2.8.

Table 2.8. List of PDAR and catalogue numbers used for RT-PCR

PDAR	Gene description	Catalogue number	Amplicon Length
Genes involved in the Th1 pathway			
IL-1 β	Interleukin-1beta	Hs00174097_m1	94
IL-2	Interleukin-2	Hs00174114_m1	94
IL-6	Interleukin-6	Hs00985639_m1	66
IL-18	Interleukin-18	Hs01038788_m1	115
IL-24	Interleukin-24	Hs01114274_m1	67
TNF	Tumor Necrosis Factor	Hs00174128_m1	80
IFN- γ	Interferon-gamma	Hs00174143_m1	79
IP-10	Interferon inducible factor-10	Hs99999049_m1	73
Genes involved in the Th2 pathway			
IL-4	Interleukin-4	Hs00174122_m1	70
IL-13	Interleukin-13	Hs00174379_m1	82
Genes involved in the Th17 pathway			
IL-17A	Interleukin-17A	Hs00174383_m1	80
IL-22	Interleukin-22	Hs00220924_m1	68
IL-23p19	Interleukin-23p19	Hs00372324_m1	107
Genes involved in regulation of the immune system			
TGF- β	Transforming growth factor-beta	Hs00171257_m1	63
FoxP3	Forkhead Box P3	Hs00203958_m1	64
IL-10	Interleukin-10	Hs00174086_m1	119
Neutrophil associated genes			

IL-8	Interleukin-8	Hs00174103_m1	101
LCN2	Lipocalin 2	Hs00194353_m1	103
CAMP	Cathelicidin Anti-Microbial Peptide	Hs00189038_m1	86
DEFB4	Defensin B4	Hs00175474_m1	81
HNE	Human Neutrophil Elastase	Hs00357734_m1	66
Growth Factor genes			
EGF	Epidermal Growth Factor	Hs01099999_m1	70
CTGF	Connective Tissue Growth Factor	Hs00170014_m1	60
GM-CSF	Granulocyte Macrophage – Colony Stimulating Factor	Hs00171266_m1	113
Genes involved in fibrosis			
IL-9	Interleukin-9	Hs00174125_m1	92
Col1a1	Pro-collagen 1a1	Hs00164004_m1	66
Col1a2	Pro-collagen 1a2	Hs00164099_m1	68
Col4a1	Pro-collagen 4a1	Hs00266237_m1	75
Col4a2	Pro-collagen 4a2	Hs01098873_m1	65
ARG1	Arginase 1	Hs00223403_m1	61
SPARC	Secreted Protein, Acidic, Cysteine- Rich	Hs01552265_m1	69
Matrix Metalloproteinases and their endogenous inhibitors			
MMP1	Matrix Metalloproteinase 1	Hs00899658_m1	64
MMP2	Matrix Metalloproteinase 2	Hs00234422_m1	83
MMP3	Matrix Metalloproteinase 3	Hs00968308_m1	98
MMP7	Matrix Metalloproteinase 7	Hs01042796_m1	64

MMP8	Matrix Metalloproteinase 8	Hs01029057_m1	67
MMP9	Matrix Metalloproteinase 9	Hs00234579_m1	54
MMP10	Matrix Metalloproteinase 10	Hs00233987_m1	82
MMP11	Matrix Metalloproteinase 11	Hs00968295_m1	60
TIMP1	Tissue Inhibitor of Matrix Metalloproteinase 1	Hs00171558_m1	104
TIMP2	Tissue Inhibitor of Matrix Metalloproteinase 2	Hs00234278_m1	73
TIMP3	Tissue Inhibitor of Matrix Metalloproteinase 3	Hs00165949_m1	59

Set-up of the assay was at a final volume of 25µl in a 96-well plate and consisted of 2 X PCR Master Mix (Applied Biosystems), containing a passive ROX dye. ROX controls for light spill over between wells of the plate. The PDAR was diluted 1:200 in the final volume and a 1µl volume of 1µg of cDNA was added. The remaining volume consisted of RNase and DNase free water (Applied Biosystems). The plate was sealed tightly with a plastic optical plate sealer (Applied Biosystems) and centrifuged at 1200rpm for 1 minute to allow the mixture to collect at the bottom of the wells and also to remove any bubbles that may have formed during pipetting.

The plate was covered by a protective mat and inserted into an ABI Prism 7000 RT-PCR machine. ABI Prism 7000 SDS software was used for the plate and instrument set-up.

Universal cycling conditions consisting of a holding stage of 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute were used for the reactions.

The PCR cycle at which the gene of interest starts to be exponentially detected above the threshold line (Figure 2.1) is known as the cycle threshold (CT). This analysis was done using the ABI Prism 7000 SDS software. The CT of each sample was exported to Microsoft Excel after which the CT of the gene of interest was normalized to human beta-actin within the same sample, by subtracting the beta-actin CT from the gene of interest CT to give delta CT (Figure 2.1).

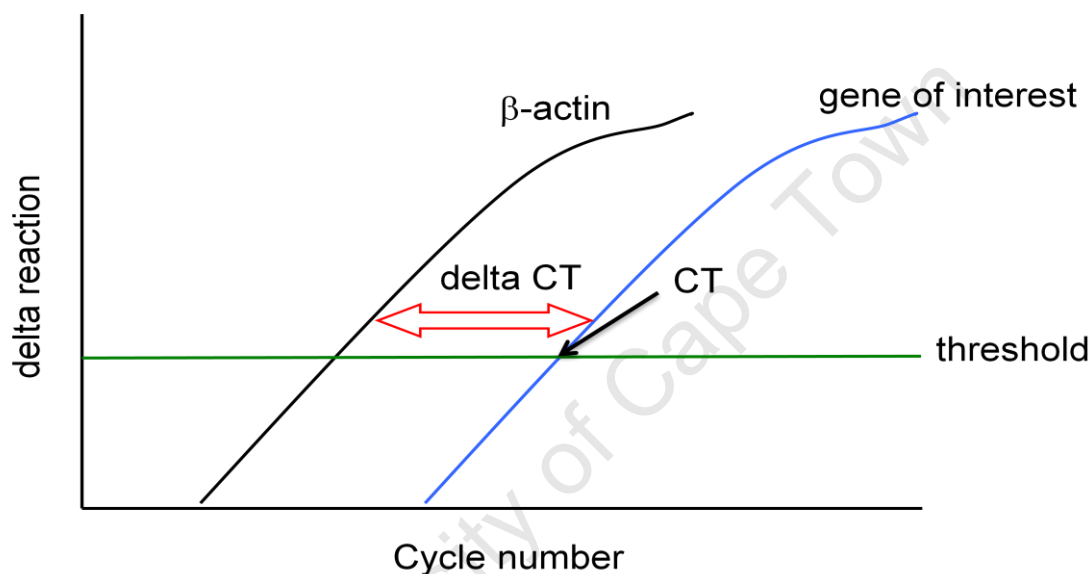


Figure 2.1. Diagrammatic representation of the RT-PCR analysis showing the threshold, Cycle Threshold (CT) of the gene of interest as well as the calculation of the delta CT

Some gene transcript failed to be amplified in the 40 cycles and these were marked as “invalid”. Analysis was done using Graphpad Prism 5 software. Delta CT were compared for HIV-1 uninfected and HIV-1 infected patients. The fold induction or repression of the pericardial fluid gene transcript level over the blood level was also calculated for analysis. This was done by subtracting the delta CT of the blood from the delta CT of the pericardial fluid (delta delta CT) and by adding this number to the mathematical equation $2^{-\text{delta delta CT}}$.

The result was log transformed to normalize the data, thus a positive result indicates upregulation of the gene transcript in the pericardial fluid compared to blood, and a negative result indicates down regulation or repression of the gene transcript in the pericardial fluid compared to blood.

9. Determination of the presence of proteins in serum and pericardial fluid by ELISA

Where ELISA assays were bought from the same manufacturer, the methodology was identical, so as to prevent repetition, the assays were batched accordingly and the reagents were tabulated before a combined general description of the assays was given. For in-house ELISA assays or single assays bought from other manufacturers, the assay, including all of the reagents was described.

9.1. BD Biosciences

In-house Interferon gamma ELISA

An in-house IFN- γ ELISA was carried out on the supernatants that were removed from the whole blood and pericardial fluid overnight restimulation assays. The ELISA plate (Costar 3590, 96 well EIA/RIA plate, high binding) was coated with mouse anti-human IFN- γ monoclonal capture antibody (BD Bioscience 551221) at a final concentration of 2 μ g/ml in 0.1 Molar (M) carbonate coating buffer. The plate was incubated at 4°C overnight. The plate was washed twice with PBS-Tween wash buffer (PBS/0.05% Tween-20) using an automated ELISA washer. The plate was then blocked with PBS/10% HI-FCS for 2 hours at room temperature before it was washed two more times. The samples were added at 100 μ l per well. The standard was recombinant human IFN- γ (BD Bioscience 554617) with an initial concentration of 10 000 pg/ml. This was serially diluted 1:3 with PBS-Tween/10% HI-FCS to give standards with a concentration of 6666, 3333, 1111, 370, 123, 41 and 0 pg/ml. The standards were added at 100 μ l per well in duplicate wells, followed by samples at 100 μ l/well. The plate was incubated overnight at 4°C in a plastic box to contain any spillages. The next morning, the plate was washed 4 times before adding 100 μ l of biotinylated mouse anti-human IFN- γ detection antibody (BD Bioscience 554550) at 1 μ g/ml in PBS-Tween/10% HI-FCS. The plate was incubated at room temperature for 45 minutes before it was washed 6

times. Avidin-Peroxidase (Sigma A3151) at 1µg/ml was diluted 1:1000 in PBS-Tween/10%HI-FCS and 100µl was added to each well. The plate was placed in the dark at room temperature for 30 minutes before it was washed 8 times with PBS-Tween. Freshly made OPD (SigmaFast OPD tablet set) colour developer solution (1 citrate buffer tablet and 1 OPD tablet in dH₂O) was added at 100µl per well and incubated in the dark for approximately 5 minutes. The reaction was stopped by adding 50µl of 2M H₂SO₄ (Sigma 320501) to each well. The plate was read at an absorbance of 490nm on a BioRad plate reader and the results were recorded. The concentrations were calculated from a standard curve that was constructed from the standard concentrations.

9.2. Bender MedSystems

An ELISA kit to measure Interleukin (IL) -18 was purchased from Bender MedSystems (Vienna, Austria).

Table 2.9. Summary of IL-18 ELISA

Cytokine	IL-18
Catalogue number	BMS267/CE
Microwell plate coated with coating antibody (supplied)	Monoclonal antibody to human IL-18
Wash Buffer (supplied)	Concentrate 20 X, use at 1 X, dilute with dH ₂ O (PBS/1%Tween 20)
Blocking solution	PBS/1%BSA
Sample diluent (supplied)	Concentrate 20 X, used at 1 X, diluted with dH ₂ O (PBS/1%Tween 20 and 10%BSA)
Detection Antibody and dilution	1 X Biotin-conjugate anti-human IL-18 monoclonal antibody 1:100
Standard Protein	Human IL-18 standard
Standard Range	78 – 5000pg/ml
Enzyme conjugate and dilution	Streptavidin-HRP 1:400
Developing reagent	Tetramethyl-benzidine (TMB) Substrate
Stop solution	1M Phosphoric acid

The microwells were washed twice with 400µl of 1 X wash buffer with soaking of the wells for 10-15 seconds between each wash. The standards were serially diluted 1:2 to make a 7 point standard curve within the detection ranges of each of the standards and added at 100µl per designated well, with 100µl of sample diluent added as the blanks of the assay. To each of the sample wells, 50µl of sample diluent followed by 50µl of sample were added. The biotin-conjugated detection antibody was prepared and 50µl was added to all the wells. The plates were covered with adhesive film and incubated on a microplate shaker at 200rpm for 2 hours at room temperature. After the incubation, the plate was washed 6 times, with soaking of the wells between each wash and 100µl of the prepared Streptavidin-HRP was added to all of the wells. Adhesive film was used to cover the plate and the plate was left to incubate on a microplate shaker at 200rpm for 1 hour at room temperature. The plate was washed 6 times with soaking in between washes and 100µl of the TMB substrate solution was added to all of the wells. The addition of 100µl of the Stop solution to all wells stopped the reaction and the absorbance was read at 450nm in a plate reader.

9.3. eBioscience

IL-1 β , IL-6, IL-12p70, IL-13, IL-17 and IL-23p19 Ready-SET-Go! kits were purchased from eBioscience (BIOCOM Biotech).

Table 2.10. Summary of IL-1 β , IL-6, IL-12p70, IL-13, IL-17 and IL-23p19 ELISA

Cytokine	IL-1 β	IL-6	IL-12p70	IL-13	IL-17A	IL-23p19
Catalogue number	88-7010	88-7066	88-7126	88-7439	88-7371	88-7237
Coating Antibody and dilution	Purified anti-human IL-1 β (CRM56) 1:250	Purified anti-human IL-6 (MQ2-13A5) 1:250	Purified anti-human IL-12p70 (B-T21) 1:250	Purified anti-human IL-13 (PVM13-1) 1:250	Purified anti-human IL-17A (eBio 64CAP17) 1:250	Purified anti-human IL-23p19 (eBio 473P19) 1:250
Coating Buffer	1 packet ELISA Coating Buffer powder, added to 1L dH ₂ O					
Washing solution	PBS/0.05% Tween					
Blocking solution	1 X Assay Diluent					
Assay Diluent	5 X concentrate, diluted with dH ₂ O and filtered using a 0.22 μ m filter					
Detection Antibody and dilution	Biotin conjugate anti-human IL-1 β (CRM57) 1:250	Biotin conjugated anti-human IL-6 (MQ2-39C3) 1:250	Biotin-conjugate anti-human IL-12p70 (C8.6) 1:250	Biotin-conjugate anti-human IL-13 (polyclonal antibody) 1:250	Biotin-conjugate anti-human IL-17A (eBio64DE C17) 1:250	Biotin-conjugate anti-human IL-23p19 (C8.6) 1:250
Standard Protein and	Recombi-nant human	Recombi-nant human	Recombi-nant human	Recombi-nant human	Recombi-nant human	Recombi-nant human

concentration	IL-1β 1μg/ml	IL-6 1μg/ml	IL-12p70 1μg/ml	IL-13 1μg/ml	IL-17A 1μg/ml	IL-23p19 1μg/ml
Standard Range	7 – 500 pg/ml	3 – 200 pg/ml	7 – 500pg/ml			31 – 2000 pg/ml
Enzyme conjugate and dilution	Avidin-HRP 1:250					
Developing reagent	1 X TMB solution					
Stop solution	2M H ₂ SO ₄					

Costar 3590, 96 well EIA/RIA, high binding plates were coated with 100 μ l of coating antibody in coating buffer, sealed with adhesive film and incubated overnight at 4°C. The plates were washed 5 times with 250 μ l per well wash buffer, with soaking for 1 minute between each wash. The wells were blocked with 200 μ l of 1 X assay diluent and incubated at room temperature for 1 hour and then washed as before. The standards were serially diluted 1:2 to give 7 points of a standard curve and added in duplicate to the designated wells. Assay diluent was added as the blank or 0 pg/ml standard. Serum and cell free pericardial fluid samples were added undiluted at 100 μ l per well to sample wells, the plates were sealed and incubated overnight at 4°C. The next day, the plates were washed as before and 100 μ l per well of detection antibody diluted in assay diluent was added to all wells. The plates were sealed and incubated at room temperature for 1 hour and then washed 5 times as before. Diluted Avidin-HRP in assay diluent was added at 100 μ l per well and incubated for 1 hour at room temperature. The plate was washed 7 times with soaking for 1 minute in between washes before 100 μ l of the substrate solution was added to each well. The reaction was left to develop for 15 minutes before 50 μ l of stop solution was added to each well. The plates were read at 450nm on a BioRad plate reader.

9.4. Koma Biotech

ELISA kits to measure IL-8 and IL-22 were purchased from Koma Biotech (South Korea).

Table 2.11. Summary of IL-8 and IL-22 ELISA

Cytokine	IL-8	IL-22
Catalogue number	KO131216	KO131234
Coating Antibody and concentration	Goat anti-Human IL-8 0.5µg/ml	Rabbit anti-Human IL-22 1µg/ml
Coating Buffer	50mM Carbonate-Bicarbonate Buffer, pH 9.6	
Washing solution	PBS/Tween 0.05%	
Blocking solution	PBS/1% BSA	
Assay Diluent	PBS/Tween 0.05% with 0.1% BSA	
Detection Antibody and concentration	Biotinylated Goat anti-Human IL-8 0.25µg/ml	Biotinylated Rabbit anti-Human IL-22 0.5µg/ml
Standard Protein	Recombinant Human IL-8	Recombinant Human IL-22
Standard Range	31 – 2000pg/ml	
Enzyme conjugate and dilution	Avidin-HRP 1:2000	
Developing reagent	TMB Substrate Reagent A – TMB solution Reagent B – Substrate solution	
Stop solution	2M H ₂ SO ₄	

The ELISA Microtiter 96 well plates (Costar 3590, 96 well EIA/RIA plate, high binding) were coated with 100µl per well of diluted coating antibody in coating buffer and incubated overnight at 4°C. The next day the plate was washed 5 times with wash buffer. The wells were blocked with 200µl blocking solution per well and incubated for 1 hour at room temperature, after which the plates were washed 5 times with wash buffer. The standards were serially diluted 1:2 to obtain a 7-point standard curve, within the range indicated in

Table 2.11. Assay diluent was included as the 0 pg/ml nil standard. The standards were added in duplicate at 100µl per well. The cell free pericardial fluid and serum samples were added undiluted to each of the sample wells at 100µl per well. Standards and samples were incubated for 2 hours at room temperature and then the plate was washed 5 times. Detection antibody was added at 100µl per well to each well of the plate and incubated for 2 hours at room temperature. After washing the plates 5 times with wash buffer, Enzyme conjugate, Avidin-HRP was diluted 1:2000 in assay diluent and 100µl was added to each well. The plates were incubated for 30 minutes at room temperature and washed 5 times. The TMB solution was mixed immediately prior to use and 100µl of the solution was added to each well. After sufficient colour development (5-15min at room temperature), 50µl of Stop solution was added to each well. The plates were then read on a microtiter plate reader at 450nm.

9.5. Mabtech

In-house IL-10 ELISA

The IL-10 specific ELISA was performed using antibodies from MABTECH (3430-1H, developed in-house by Dr Katalin A. Wilkinson). The ELISA plate (Costar 3590, 96 well EIA/RIA plate, high binding) was coated with monoclonal IL-10 capture antibody (9D7) with a final concentration of 2mg/ml in PBS. The plate was incubated at 4°C overnight. The plate was washed twice with PBS using an automated ELISA washer. The plate was then blocked with 0.05% Tween in PBS for 1 hour at room temperature before it was washed twice with PBS/0.05% Tween. The samples were added at 100µl per well. The standard was recombinant human IL-10 (Sigma I9276), with an initial concentration of 2000pg/ml. This was serially diluted 1:2 with PBS/0.05% Tween to give standards with a concentration of 1000, 500, 250, 125, 62, 31 and 0 pg/ml. The standards were added at 100µl per well in duplicate wells. The plate was incubated for 2 hours at room temperature. The plate was washed 3 times with PBS/0.05% Tween before adding 100µl of biotinylated monoclonal antibody (12G8) at 1mg/ml in PBS/0.05% Tween. The plate was then incubated at room temperature for 1 hour before it was washed 3 times. Streptavidin-HRP at 1mg/ml was diluted 1:1000 in PBS/0.05% Tween and 100µl was added to each well. The plate was placed in the dark at room temperature for 1 hour before it was washed 3 times. TMB substrate solution (Sigma T0440) was added at 100µl per well and incubated in the dark for approximately 20 minutes. The reaction was stopped by adding 50µl of 2M H₂SO₄ to each well. The plate was read at an absorbance of 450nm on a plate reader. The concentrations were calculated from a standard curve that was constructed from the standard concentrations.

9.6. R&D Systems

TGF- β , TIMP1, TIMP2, MMP2 / TIMP1 and MMP9 / TIMP2 DuoSet ELISA were purchased from R&D Systems.

Table 2.12. Summary of TGF- β , TIMP1, TIMP2, MMP2 / TIMP1 and MMP9 / TIMP2 DuoSet ELISA

Cytokine	TGF- β 1	TNF	TIMP1	TIMP2	MMP2 / TIMP1	MMP9 / TIMP2
Catalogue number	DY240	DY210	DY970	DY971	DY1496	DY1453
Coating Antibody and concentration	Mouse anti-human TGF- β 1 (Part 840116) 2 μ g/ml	Mouse anti-human TNF (Part 840119) 4 μ g/ml	Mouse anti-human TIMP1 (Part 840294) 2 μ g/ml	Mouse anti-human TIMP2 (Part 840528) 2 μ g/ml	Goat anti-human MMP2 (Part 841249) 2.4 μ g/ml	Mouse anti-human MMP9 (Part 841175) 2 μ g/ml
Coating Buffer	1 X PBS					
Washing solution	PBS/Tween 0.05%					
Blocking solution	PBS/ Tween 5% with 0.05% NaN ₃	PBS/1% BSA			PBS/1% BSA with 0.05% NaN ₃	
Assay Diluent	PBS/ Tween 0.05% with 1.4% BSA	PBS/1% BSA			50mM Tris, 10mM CaCl ₂ , 0.15M NaCl and 0.05% Brij [®] 35	
Detection Antibody and concentration	Biotinylated chicken anti-human TGF- β 1 (Part 840117) 300ng/ml	Biotinylated goat anti-human TNF (Part 840120) 75ng/ml	Biotinylated goat anti-human TIMP1 (Part 840295) 50ng/ml	Biotinylated goat anti-human TIMP2 (Part 840529) 50ng/ml	Biotinylated goat anti-human TIMP1 (Part 841250) 200ng/ml	Biotinylated goat anti-human TIMP2 (Part 841176) 100ng/ml

Standard Protein	Recombi- nant human TGF-β1 145ng/ml	Recombi- nant human TNF 290ng/ml	Recombi- nant human TIMP1 100ng/ml	Recombi- nant human TIMP2 60ng/ml	Recombi- nant human MMP2/ TIMP1 complex 80ng/ml	Recombi- nant human MMP9/ TIMP2 complex 18μg/ml
Standard Range	31 – 2000 pg/ml	15 – 1000 pg/ml	31 – 2000pg/ml		150 –10000 pg/ml	325– 25000 pg/ml
Enzyme conjugate and dilution	Streptavidin-HRP (Part 890803) 1:200					
Developing reagent	TMB 1 part Colour Reagent A – H ₂ O ₂ 1 part Colour Reagent B – TMB (DY994)					
Stop solution	2M H ₂ SO ₄					

The ELISA plates (Costar 3590, 96 well EIA/RIA plate, high binding) were coated with coating antibody diluted in 1 X PBS and incubated at 4°C overnight. The plates were washed 3 times with wash buffer using an automated ELISA washer. The plates were then blocked with the relative blocking solution for 1 hour at room temperature before they were washed 3 more times. The samples were added at 100 μ l per well. The standards were serially diluted 1:2 in assay diluent for a 7-point standard curve and added at 100 μ l per well in duplicate wells. The plates were incubated for 2 hours at RT. The plates were washed 3 times before adding 100 μ l per well of the respective biotinylated detection antibody diluted in assay diluent. The plates were incubated for 2 hours at RT before they were washed as before. Streptavidin-HRP was diluted in reagent diluent and added at 100 μ l per well. The plates were placed in the dark at RT for 20 minutes before they were washed 3 times. Freshly made substrate solution was added at 100 μ l per well

and the plates were incubated in the dark for approximately 20 minutes. The developing reaction was stopped by adding 50 μ l of stop solution to each well. The plate was read at an absorbance of 450nm on a plate reader. The concentrations were calculated from a standard curve that was constructed from the standard concentrations.

9.7. Roche

A Cell Death Detection ELISA (11 544 675 001) was purchased to qualitatively and quantitatively determine the cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) detectable after cell death in cell free pericardial fluid. The anti-histone solution as well as the anti-DNA-POD solution were each reconstituted in 1ml dH₂O for 10 minutes and then mixed thoroughly by vortexing. The coating solution was prediluted by adding 1ml coating buffer concentrate to 9ml dH₂O, and adding 9ml of this solution to the 1ml anti-histone solution already prepared. The coating solution was added at 100µl per well to each of the wells and incubated for 1 hour at RT. The coating solution was aspirated and 200µl of incubation buffer (supplied) was added per well. The plate was left to incubate for 30 minutes at RT, before the plate was washed 3 times with 300µl of wash solution (diluted 40ml in 360ml dH₂O) per well. Cell free pericardial fluid was added to the wells, undiluted, at 100µl per well. Cell free pericardial fluid from an open-heart surgery patient was used as a control for the samples. The background of the assay was determined by adding 100µl of incubation buffer into 2 wells. The plate was covered and left to incubate for 90 minutes at RT. After the incubation, the plate was washed 3 times as before and 100µl of the conjugate solution (1ml anti-DNA-POD in 9ml incubation buffer) was added to each of the wells, except the blank wells. The plate was covered and left to incubate for a further 90 minutes at RT. The plate was washed 3 times and 100µl per well of the substrate solution (3 x 5mg ABTS substrate tables dissolved in 15ml Substrate buffer) was added. The plate was incubated on a plate shaker at 250rpm until the colour developed (10 – 20 minutes). The plate was read at 405nm on an automated ELISA plate reader.

The results were calculated by subtracting the background absorbance from each sample and calculating the specific enrichment of mono- and oligonucleosomes released into the cytoplasm from these values using the following formula;

$$\text{Enrichment factor} = \frac{\text{mU (absorbance) of the sample}}{\text{mU of the corresponding control}}$$

10. Luminex Assays

R&D Systems Fluorokine[®] MultiAnalyte Profiling (MAP) Human MMP Base kits (LMP000) were used to analyse serum and cell free pericardial fluid from TB pericarditis and open-heart surgery control patients for specific MMPs. MMP1 (LMP901), MMP2 (LMP902), MMP3 (LMP513), MMP7 (LMP907) and MMP8 (LMP908) were analysed on 1 plate together at a 1:10 dilution of sample, while MMP9 (LMP911) was analysed separately at a 1:100 dilution of sample on another plate.

10.1. Preparations

10.1.1. Sample Preparation

The samples were prepared before set-up of the assay. For the MMP1, MMP2, MMP3, MMP7 and MMP8 plate, 15µl of samples were diluted 10-fold with Calibrator Diluent RD5-37 (Part 895853) and mixed thoroughly. For the MMP9 plate, the 10-fold dilution of the samples were further diluted 10 times with the same Calibrator Diluent to give 100-fold diluted samples.

10.1.2. Reagent Preparation

All the reagents were brought to RT before use. The wash buffer was made by adding 20ml of Wash Buffer Concentrate to 500ml of dH₂O. The lyophilized Standard MMP Cocktail (Part 895851) were reconstituted with Calibrator Diluent RD5-37 to give top standards as indicated on the standard card (Table 2.13) and allowed to sit for 15 minutes with gentle agitation before making dilutions. Dilutions were carried out in polypropylene tubes and 1:3 serial dilutions were done to give a 7-point standard curve. Calibrator Diluent was used as the blank.

Table 2.13. MMP standard concentrations as per standard card

Analyte	Top concentration (pg/ml)	Microparticle region for aquisition
MMP1	6800	4
MMP2	55000	13
MMP3	9700	26
MMP7	91500	30
MMP8	80000	43
MMP9	47900	47

10.1.3. Diluted Microparticle Preparation

Each microparticle concentrate vial was vortexed to resuspend the beads and then diluted in the mixing bottle provided. For a plate of 96 wells, 50µl of each of the MMP1, MMP2, MMP3, MMP7 and MMP8 microparticle concentrate was added to 5ml of microparticle diluent 3 (Part 895857) and mixed gently. MMP9 (50µl) was added to 5ml diluent for the separate plate.

10.1.4. Diluted Biotin Antibody Preparation

Each biotin antibody vial was vortexed and 50µl was added to the 5.25ml vial of Biotin Antibody Diluent 2 (Part 895832).

10.1.5. Streptavidin-PE Preparation

The Streptavidin-PE vial was vortexed before diluting the 100 X concentrate to a 1 X concentration by adding 55µl of the Streptavidin-PE to 5.5ml of 1 X wash buffer.

10.1.6. Instrument settings

A bead region was set for each of the analytes being measured. The minimum events measured were set at 0, while 50 events per bead were collected. The flow rate of the samples was 60µl per minute, with the sample size set at 50µl.

10.2. Assay Procedure

The reagents, working standards and samples were all prepared as above before starting. The filter-bottomed microplates (Part 640448) were pre-wet with 100µl of wash buffer before removing with a vacuum manifold, designed specifically to accommodate a microplate (BioRad). The microparticle mixture was added to the dried microplate at 50µl per well to each well along with the 50µl of the diluted standards and samples per well to the designated wells. The plates were sealed and incubated for 2 hours at RT on a horizontal orbital microplate shaker set at 500rpm. After incubation the plates were washed by removal of the liquid by vacuum and adding 100µl of wash buffer per well for a total of 3 washes. The diluted Biotin Antibody Cocktail was added at 50µl to all 96 wells, the plates were sealed with a foil cover and incubated for 1 hour at RT on the shaker before the plates were washed 3 times as before. After washing, 50µl diluted Streptavidin-PE was added to all wells, the plates were covered with a foil cover and incubated at RT for 30 minutes on the shaker and then the plates were washed 3 times as before. Wash buffer (100µl) was added to each well after washing and the plate was left for 2 minutes at RT on the shaker before reading on a BioRad BioPlex Analyzer.

10.3. Calculation of results

A standard curve was created for each of the analytes measured. The median Relative Fluorescence Units (RFU) per analyte was read off the standard curve giving the corresponding MMP concentration.

11. Determination of the role of IL-17 in whole blood

11.1. Blocking of IFN- γ by neutralizing antibodies

Whole blood from healthy laboratory donors was diluted 1:5 with RPMI (Sigma) and 1ml was added per well into 16 wells of a 24 well plate. Live H37Rv *M.tb* laboratory strain was plated at a MOI = 1:1 per well into 8 wells. The other 8 wells were left unstimulated. Anti-IFN- γ (eBioscience, 16-7318-85) at 5 μ g/ml was added to 4 of the stimulated wells and to 4 of the unstimulated wells. The blood was left to incubate for 24, 48 and 96 hours before supernatant was removed for IL-17, IL-22 and IFN- γ ELISA analysis (as described above). At the set-up of the experiments, 1ml of diluted whole blood was spun and the supernatant was removed to give a baseline background of cytokine. In 5 healthy donors, the left over blood from the 24 hour incubation, after supernatant was removed, was added to Paxgene RNA stabilization tubes for RNA extraction (described above) and gene transcript for IL-17A and IFN- γ was determined using the PDAR described in Table 2.8.

An assay to determine the functional activity of the IFN- γ neutralizing antibody was not set up, but could have been included by stimulating whole blood with different doses of active IFN- γ and neutralizing antibody and using the IFN- γ ELISA to detect the recovery of the IFN- γ protein. If the neutralizing antibody has activity against IFN- γ , then no IFN- γ protein should be recovered.

12. Determination of a distinct subset of Th17 cells with the ability to produce IL-22 but not IL-17

12.1. PBMC culture conditions

PBMC in RPMI/10% HI-FCS from healthy donors were plated at 0.3×10^6 cells per well of Costar 96 well round bottom tissue culture plate (Corning). The cells were stimulated in duplicate with PPD ($\mu\text{g/ml}$), SEB ($5\mu\text{g/ml}$) and an unstimulated control. Each of the duplicate stimulated wells had one of the following specific culture conditions:

- Neat environment – IgG1, $5\mu\text{g/ml}$ (eBiosciences)
- Th1 environment – recombinant IL-12p70, 10ng/ml (eBiosciences) and anti-human IL-4, $5\mu\text{g/ml}$ (eBiosciences)
- IFN- γ environment – recombinant IFN- γ , 10ng/ml (BD Biosciences)

The cells were left to incubate for 4 days at 37°C , 5% CO_2 . After 4 days, half of the supernatant was removed and stored at -20°C for IL-17, IL-22 and IFN- γ ELISA as described above. PMA (10ng/ml) and Ionomycin (10ng/ml), along with BFA ($10\mu\text{g/ml}$) were added to all the wells for a further 4 hours to restimulate the cells to produce cytokine. After the 4 hours, the cells were washed in PBS (Sigma) for 10 minutes at 1680rpm and fixed in FACS FIX solution for 20 minutes at 4°C . After fixing the cells were washed in FACS wash buffer (PBS/2%HI-FCS), duplicates were pooled and frozen in a 1:1 mixture of RPMI/10% HI-FCS and cell freeze solution until batch staining was done.

12.2. ICS 8 colour assay

Stimulated and fixed PBMC were thawed and washed in PBS. They were incubated in BD cytofix/cytoperm for 20 minutes at 4°C and then washed in 1 X BD Permeabilisation buffer. The Perm buffer was decanted and the cells were stained with $200\mu\text{l}$ per tube of diluted VIVID (as described in Section 6.3.4) for 30 minutes at 4°C and then stained according to Table 2.9. at

4°C for 1 hour. After staining, the cells were washed in 1 X Perm buffer and acquired using a BD Fortessa LSRII flow cytometer and analysed using FlowJo v9 (www.treestar.com).

Table 2.14. Staining panel for IL-22 and IL-17 producing cells

Tube 1	Conjugate	Supplier	Volume (µl)
VIVID	Pacific Blue	Invitrogen	200
CD3	QDot 605	Invitrogen	0.5
CD8	Cy5.5PerCP	BD Biosciences	3
IFN- γ	Alexa700	eBiosciences	1
IL-17	Alexa647	eBiosciences	3
Ki67	FITC	BD Biosciences	0.25
IL-22	PE	BD Biosciences	1

13. Statistical analysis

All data was analysed using Graphpad Prism 5 Software (Graphpad Software, Inc.). The normality of samples was tested using D'Agostino and Pearson omnibus normality test. Normally distributed paired samples were compared using a student t-test, while non-parametric paired samples were analysed using the Wilcoxon-matched pairs test. Unpaired, non-parametric samples were tested using the Mann-Whitney U test and unpaired, normally distributed samples were compared using the unpaired student t-test. Fisher's exact test was used to test for differences in age and gender of all patients included in the study. A p-value <0.05 was considered statistically significant. Data are quoted as median (IQR) unless otherwise stated.

Multiple comparison corrections were not carried out as the data sets compared are complex and difficult to group accordingly. It has also been argued that use of multiple testing corrections is an inefficient way to perform empirical research such as this, since multiple testing adjustments control false positives at the potential expense of many more false negatives.

14. Summary of all patients included in this thesis

Table 2.15. Characteristics of all TB pericarditis patients included in this thesis

Total of 96 TB pericarditis patients included in this thesis			
Median Age (IQR) years	33.0 (28.0 – 44.0)		
Gender (%)	58 Male (60.4)	37 Female (38.5)	1 unknown (0.1)
HIV status (%)	28 HIV-1 uninfected (29.2)	68 HIV-1 infected (70.8)	
TB diagnosis (%)	44 Definite TB (45.8)	52 Probable TB (54.2)	

The characteristics of the patients included in this thesis were broken down by their inclusion into the assays in each chapter

CHAPTER 3

Table 2.16. Characteristics of the patients included in the overnight restimulation and ELISpot assay

	Combined TB pericarditis (n=56)	HIV-1 uninfected (n=15)	HIV-1 infected (n=41)	HIV-1 uninfected vs HIV-1 infected, p- value
Median Age in years (IQR)	(n=55) 33.0 (28.0-44.0)	49.0 (31.0-58.0)	(n=40) 31.5 (28.0-39.0)	0.011
Gender, Male (M)/Female (F)	36M/20F	12M/3F	24M/17F	0.210
TB diagnosis, Definite/Probable	31 Definite/25 Probable	8 Definite/7 Probable	23 Definite/18 Probable	1.000
Median CD4 count (IQR) cells/μl [number available]	172.0 (88.25-415.5) [n=52]	447.0 (381.0-649.0) [n=15]	107.0 (72.5-270.5) [n=37]	<0.0001
Number on Anti Retroviral Treatment	1	/	1	n/a

CHAPTER 4

Table 2.17. Characteristics of the patients included in the 4-colour flow cytometry analysis

	HIV-1 uninfected (n=8)	HIV-1 infected (n=9)	p-value
Median Age in years, (IQR)	53.5 (33.75 - 66.25)	30.0 (20.0 - 35.5)	0.021
Gender, Male/Female	5M/3F	3M/6F	0.347
TB diagnosis, Definite/Probable	2 Definite/6 Probable	4 Definite/5 Probable	0.620
Median CD4 count, cells/μl (IQR)	381.0 (187.0 - 649.0)	211.5 (104.5 - 320.0)	0.094
Number on Anti retroviral treatment	0	1	n/a

Table 2.18. Characteristics of the patients included in the 8-colour flow cytometry analysis

Patient subset	HIV-1 uninfected (n=10)	HIV-1 infected (n=10)	p-value
Median Age in years, (IQR)	57.5 (32.8 – 68.3)	29.5 (27.3 – 30.3)	0.003
Gender, Male/Female	7M/3F	5M/5F	0.650
TB diagnosis, Definite/Probable	4 Definite/6 Probable	4 Definite/6 Probable	1.000
Median CD4 count, cells/μl (IQR), [number available]	381.0 (187.0 - 566.0) [n=7]	169.5 (106.0 – 259.3) [n=4]	0.072
Number on Anti retroviral treatment	0	0	n/a

CHAPTER 5

Table 2.19. Characteristics of the patients included in the mRNA Transcript Profiling of blood and pericardial fluid

	Combined TB pericarditis (n=29)	HIV-1 uninfected (n=12)	HIV-1 infected (n=17)	HIV-1 uninfected vs HIV-1 infected, p-value
Median Age in years, (IQR)	32 (28-47)	47 (26-69)	31 (28-38)	0.199
Gender, Male/Female (M/F)	19M/10F	9M/3F	10M/7F	0.450
TB diagnosis Definite/Probable	11 Definite/18 Probable	3 Definite/9 Probable	8 Definite/9 Probable	0.273
Median CD4 count cells/μl, (IQR)	187.0 (109.5-404.0)	366.0 (204.3- 498.0)	118.0 (77.0- 270.5)	0.002

Table 2.20. Characteristics of TB pericarditis and Open-heart surgery control patients included in the protein profiling of blood and pericardial fluid

	Combined TB pericarditis (n= 89)	Open-heart Surgery controls (n=31)	TB pericarditis vs Open-heart Surgery controls, p-value
Median Age in years, (IQR)	33 (27 – 44)	51 (38 - 64)	<0.0001
TB Diagnosis Definite/Probable	40 Definite/49 Probable	No evidence of TB	/
Gender, Male/Female (M/F)	53M/35F (1 unknown)	10M/21F	0.0273
Median CD4 count cells/μl, (IQR)	167 (83.5 – 343) n=73	Not recorded	/
Number on Anti retroviral treatment	3	All HIV-1 uninfected	/

Table 2.21. Characteristics of HIV-1 uninfected and HIV-1 infected TB pericarditis and included in the protein profiling of blood and pericardial fluid

	HIV-1 uninfected (n=27)	HIV-1 infected (n=61)	HIV-1 uninfected vs HIV-1 infected, p-value
Median Age in years, (IQR)	51 (27.8 – 58.3)	32 (26.5 – 39)	0.012
TB Diagnosis Definite/Probable	9 Definite/18 Probable	31 Definite/30 Probable	0.862
Gender, Male/Female (M/F)	17M/10F	36M/25F	0.999
Median CD4 count cells/μl, (IQR)	381 (184.5 – 651) n=22	105 (65 – 266) n=51	<0.0001
Number on Anti retroviral treatment	0	3	/

CHAPTER 3

Determination of the *M.tb* antigen-specific response in blood and at the site of disease in TB pericarditis patients

1. Introduction

Tuberculous pericardial effusions usually occur as a result of spread of mycobacteria from the mediastinal lymph nodes (61) which causes the accumulation of fluid containing lymphocytes and other cells. Echocardiography is used to assess the presence of this effusion (107), which often has to be drained to prevent tamponade and other heart dysfunctions (108). This fluid contains cytokines involved in inflammation, such as IFN- γ , Tumor Necrosis Factor (TNF) and Interleukin (IL)-6. It has been suggested that pericardial effusions arise due to a hypersensitivity reaction that is coordinated by Th1 lymphocytes (80). Whether the presence of these cytokines is a consequence of the *M.tb* infection or not is yet to be determined as well as the effect that HIV-1 plays on the phenotype of *M.tb* specific cells to the site of disease.

Interferon-Gamma Release Assays (IGRA) have been used in various clinical settings for the detection of TB disease or latent TB infection (LTBI) in conjunction with the tuberculin skin test (TST). Two immunodiagnostic tests are marketed for this reason: QuantiFERON®-TB Gold (QFT-G, Cellestis, Carnegie, Australia) is a whole-blood based assay using ELISA for detection of IFN- γ secreted in response to the RD-1 encoded ESAT-6 and CFP-10 antigens, and the in-tube version of QFT-G (QFT-GIT) containing an additional *M.tb*-specific peptide (TB7.7). ESAT-6 and CFP-10 are strong targets of Th1 cells in *M.tb* infection and their ability to elicit dominant specific T cell responses, result in these tests having relatively high sensitivity (109).

Many studies have evaluated the use of the IFN- γ release as a tool to study immune responses on *M.tb* site of disease samples. A study using pleural site of disease samples showed fold increases of IFN- γ measured in response to ESAT-6 in the pleural fluid compared to the blood (110). My study therefore used this test in an informative manner to determine specific *M.tb* responses at the site of TB disease.

Another immunological method to determine antigen-specific cytokine production by cells is the ELISpot assay. The ELISpot was shown to be more sensitive than the TST for the detection of active TB in HIV-1 infected children, but this sensitivity was not very high (TST 36% versus ELISpot 73%) (111).

A commercial kit, the T-SPOT.TB, has also been marketed, that can potentially replace the TST in diagnosing TB in HIV-1 infected individuals (8), while the IFN- γ ELISpot has been shown to assist in the diagnosis of TB in pleural fluid (110, 112) and bronchoalveolar lavage fluid (113) as well as cerebrospinal fluid in TB meningitis patients (114). The clinical usefulness of the ELISpot assay on pericardial fluid cells of a single suspected TB pericarditis case has been suggested (115), but no study targeted pericardial TB cases with concurrent HIV infection.

In this study I have conducted a detailed analysis of the cells present at the disease site in HIV-1 uninfected and infected patients with TB pericarditis. I used an IFN- γ overnight restimulation assay to assess *M.tb* antigen-specific production by whole blood and pericardial fluid and assessed the influence that HIV infection has on the ability of the T cells to produce IFN- γ in this assay. I also used an IFN- γ specific ELISpot assay to determine the cell specific

IFN- γ production by cells in the blood and in the pericardial fluid when re-stimulated with *M.tb*-specific antigens.

2. Results

2.1. Patient description and characteristics

A total of 56 (15 HIV-1 uninfected and 41 HIV-1 infected) patients with TB pericarditis were included in this chapter and are summarized in Table 3.1. The median age of the patients was 33.0 years (IQR 28.0 - 44.0) and there was a difference in ages when stratifying by HIV status. HIV-1 infected patients were significantly younger than their non-HIV-1 infected counterparts ($p=0.011$). There were no differences in gender or whether the patients had a definite or probable TB diagnosis. The absolute CD4 counts were significantly lower in the HIV-1 infected patients with a median of 107 cells/ μ l (IQR 72.5 - 270.5) compared to 447.0 cells/ μ l (IQR 381.0 - 649.0) in the HIV-1 uninfected patients ($p<0.0001$). Of the 41 HIV-1 infected patients included in this chapter, only 1 was on ARV treatment of less than 2 weeks duration at the time of sampling. The remaining 40 patients were all referred to the HIV clinic for initiation of treatment.

Table 3.1. Patient characteristics of patients included in Chapter 3

	Combined TB pericarditis (n=56)	HIV-1 uninfected (n=15)	HIV-1 infected (n=41)	HIV-1 uninfected vs HIV-1 infected, p-value
Median Age in years (IQR)	(n=55) 33.0 (28.0-44.0)	49.0 (31.0-58.0)	(n=40) 31.5 (28.0-39.0)	0.011
Gender, Male (M)/Female (F)	36M/20F	12M/3F	24M/17F	0.210
TB diagnosis, Definite/Probable	31 Definite/25 Probable	8 Definite/7 Probable	23 Definite/18 Probable	1.000
Median CD4 count (IQR) cells/μl [number available]	172.0 (88.25-415.5) [n=52]	447.0 (381.0-649.0) [n=15]	107.0 (72.5-270.5) [n=37]	<0.0001

Number on Anti Retroviral Treatment	1	/	1	n/a

2.2. Increased *M.tb* antigen-specific IFN- γ production at the site of disease

Figure 3.1. shows the overall IFN- γ response in the blood and pericardial fluid of all patients with pericardial TB (n=56). The median background (NIL) IFN- γ concentration in the blood was 0.0ng/ml (IQR 0.0 - 0.0), with only a few patients having detectable IFN- γ , while the median background concentration of the pericardial fluid was 0.9ng/ml (IQR 0.1 - 3.6), significantly higher ($p>0.0001$) compared to blood.

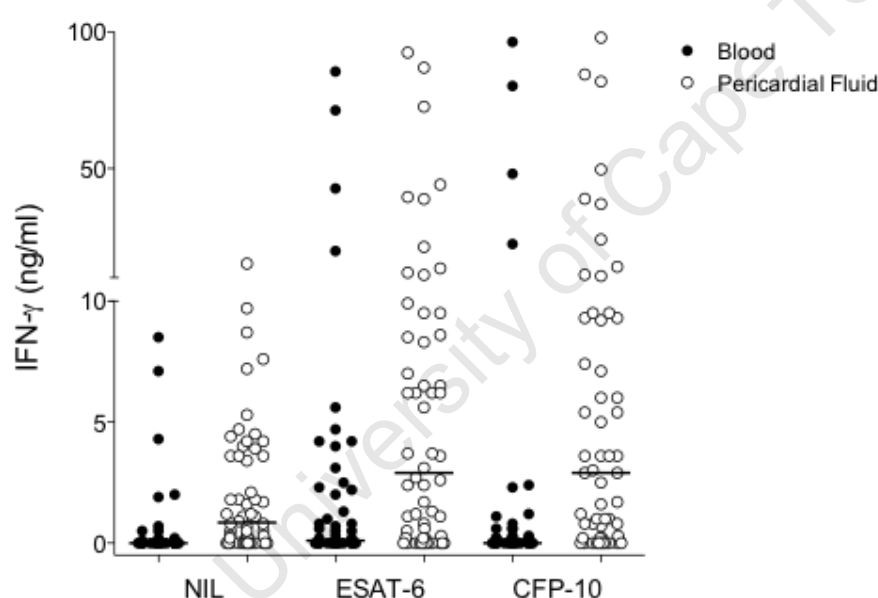


Figure 3.1. Significantly elevated *M.tb* antigen-specific IFN- γ secretion in pericardial fluid compared to blood in all patients with pericardial TB, irrespective of stimulation

IFN- γ (ng/ml) concentration in blood compared to pericardial fluid in unstimulated (NIL), as well as stimulated (ESAT-6 and CFP-10) samples, showing significant increase of IFN- γ production in the pericardial fluid compared to blood for all patients with pericardial TB (n=56).

When stimulating with ESAT-6, IFN- γ secretion in the blood increased to a median of 0.1ng/ml (IQR 0.0 - 1.8, $p<0.0001$), and the pericardial fluid to 2.9ng/ml (IQR 0.2 - 8.6, $p<0.0001$). When stimulating with CFP-10, the median IFN- γ in the blood remained 0.0ng/ml (IQR 0.0 - 1.8, $p=0.0015$), while in the pericardial fluid IFN- γ concentration increased significantly to a median of 2.9ng/ml (IQR 0.2 - 9.3, $p<0.0001$). All stimulations resulted in a statistically significant increase in the amount of IFN- γ measured in the specific compartment compared to the unstimulated control (Table 3.2).

Table 3.2. Statistical evaluation of the comparisons between unstimulated (NIL) and stimulated (ESAT-6 and CFP-10) whole blood and pericardial fluid of all TB pericarditis patients

Compared to NIL	Combined TB pericarditis (n=56)	
	Blood	Pericardial Fluid
ESAT-6	$p<0.0001$	$p<0.0001$
CFP-10	$p=0.0015$	$p<0.0001$

2.3. The Effect of HIV on the *M.tb* antigen-specific IFN- γ production in blood and in pericardial fluid

Figure 3.2 shows IFN- γ production of *M.tb*-specific antigen stimulated whole blood and pericardial fluid from 15 HIV-1 uninfected (Figure 3.2A) and 41 HIV-1 infected (Figure 3.2B) patients. While in general the concentrations of IFN- γ in the pericardial fluid of HIV-1 uninfected patients tended to be higher, similar increase in response to antigenic stimulation was observed in both HIV-1 uninfected and HIV-1 infected groups of patients, with no statistically significant differences between them. All stimulations caused a statistically significant increase in the amount of IFN- γ measured in the specific compartment compared to the unstimulated control (Table 3.3), except for when stimulating the pericardial fluid in the HIV-1 uninfected group with CFP-10 ($p=0.0904$).

In the HIV-1 uninfected patients (Figure 3.2A), the median concentration of IFN- γ in the unstimulated blood was 0.0ng/ml (IQR 0.0 - 0.0), compared with 1.2ng/ml (IQR 0.0 – 3.9) in the pericardial fluid ($p=0.001$). ESAT-6 stimulation resulted in a further increase to median 6.2ng/ml (IQR 0.0 – 11.0), and CFP-10 stimulation to 3.6ng/ml (IQR 0.0 – 10.6) IFN- γ in the pericardial fluid, both differences being statistically significant when compared to the respective blood concentrations ($p=0.001$).

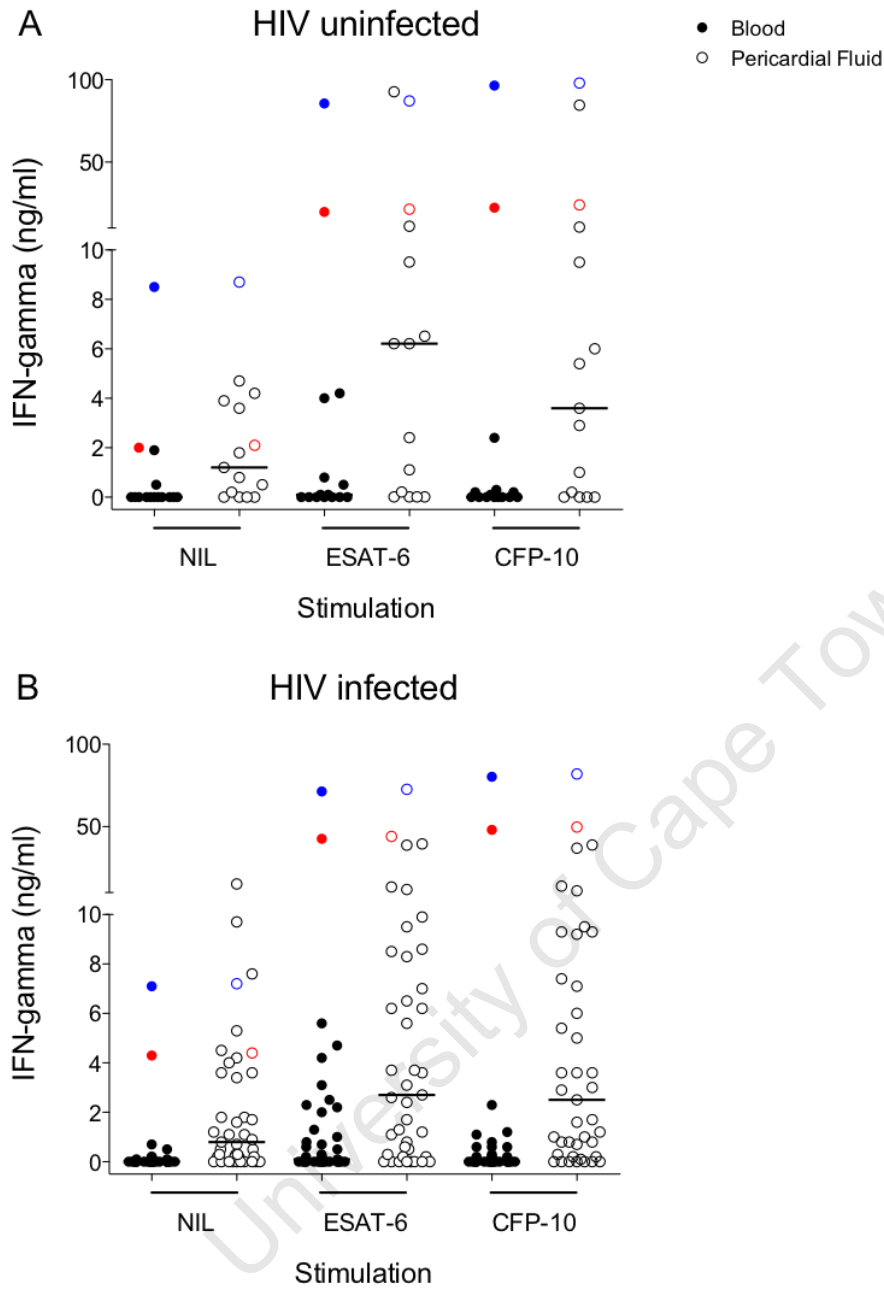


Figure 3.2. Significantly increased *M.tb* antigen specific IFN- γ secretion in pericardial fluid compared to blood irrespective of the HIV status of TB pericarditis patients

IFN- γ (ng/ml) in blood compared to pericardial fluid in unstimulated (NIL), as well as stimulated (ESAT-6 and CFP-10) samples, showing significant increase of IFN- γ production in the pericardial fluid compared to blood for both HIV-1 uninfected (A, n=15) and HIV-1 infected (B, n=41) patients. Blue and red open and closed circles indicate patients with similar levels of IFN- γ in blood and pericardial fluid.

Table 3.3. Statistical evaluation of the comparisons between unstimulated (NIL) and stimulated (ESAT-6 and CFP-10) whole blood and pericardial fluid of HIV-1 uninfected and HIV-1 infected TB pericarditis patients

Compared to NIL	HIV-1 uninfected (n=15)		HIV-1 infected (n=41)	
	Blood	Pericardial Fluid	Blood	Pericardial Fluid
ESAT-6	p=0.0142	p=0.0020	p<0.0001	p<0.0001
CFP-10	p=0.0059	p=0.0904	p=0.0078	p<0.0001

In the HIV-1 infected patients (Figure 3.2B), the median concentration of IFN- γ in unstimulated blood was 0.0ng/ml (IQR 0.0 - 0.0), compared with 0.8ng/ml (IQR 0.1 – 3.6) in the pericardial fluid (p=0.0004). Upon stimulation with ESAT-6 the IFN- γ concentration increased to a median of 2.7ng/ml (IQR 0.25 – 8.4) in the pericardial fluid (p=0.0017) compared to blood of 0.1ng/ml (IQR 0.0 – 1.65). Similarly, using CFP-10 as a stimulant, the concentration of IFN- γ increased to a median of 2.5ng/ml (IQR 0.25 – 8.3) in the pericardial fluid (p=0.0007) compared to blood of 0.0ng/ml (IQR 0.0 – 0.25).

It is interesting to note, that in 2 HIV-1 uninfected and 2 HIV-1 infected patients (Blue and red open and closed circles, Figure 3.2), there was similar IFN- γ levels in their blood as well as in their pericardial fluid, perhaps suggestive of a plateau of IFN- γ able to be produced by the T cells in both the blood and the pericardial fluid regardless of stimulation or not. These patients had no clinical features different from that of the other patients and their ages were similar to the rest of the patients in their separate HIV groups. Of the 4 patients, 3 were male and all were alive at the 6-month follow-up visit. The 2 HIV-1 infected patients were not on anti-retroviral treatment (ART) and there was no difference in CD4 counts.

2.4. IFN- γ specific ELISpot results

In order to determine the frequency of IFN- γ producing cells at the disease site compared to blood, ELISpot analysis using PFC and PBMC was performed. Figure 3.3 and Table 3.3 show the combined ELISpot results for all the patients after background (NIL) was subtracted (n=32).

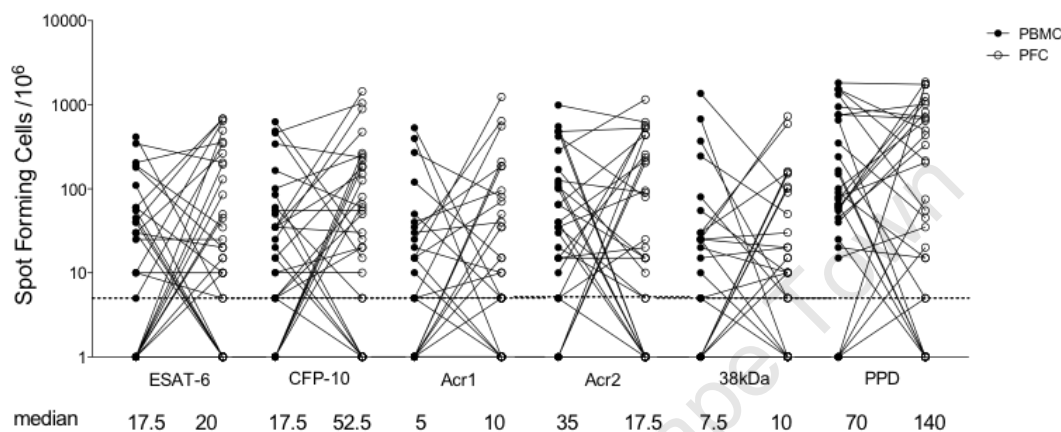


Figure 3.3. Combined IFN- γ specific ELISpot results show heterogeneous spread between PBMC and PFC

IFN- γ Spot Forming Cells/ 10^6 in response to ESAT-6, CFP-10, Acr1, Acr2, 38kDa and PPD per patient. There is no significant difference between PBMC and PFC for any of the antigens.

There was IFN- γ production detectable by both blood and pericardial fluid cells, however there was no clear concentration of IFN- γ producing cell numbers in either the periphery or in the pericardium. The results were further stratified by HIV status to determine if HIV-1 had any effect. Figure 3.4 and Table 3.3 show the results of 9 HIV-1 uninfected (Figure 3.4A) and 23 HIV-1 infected (Figure 3.4B) patients.

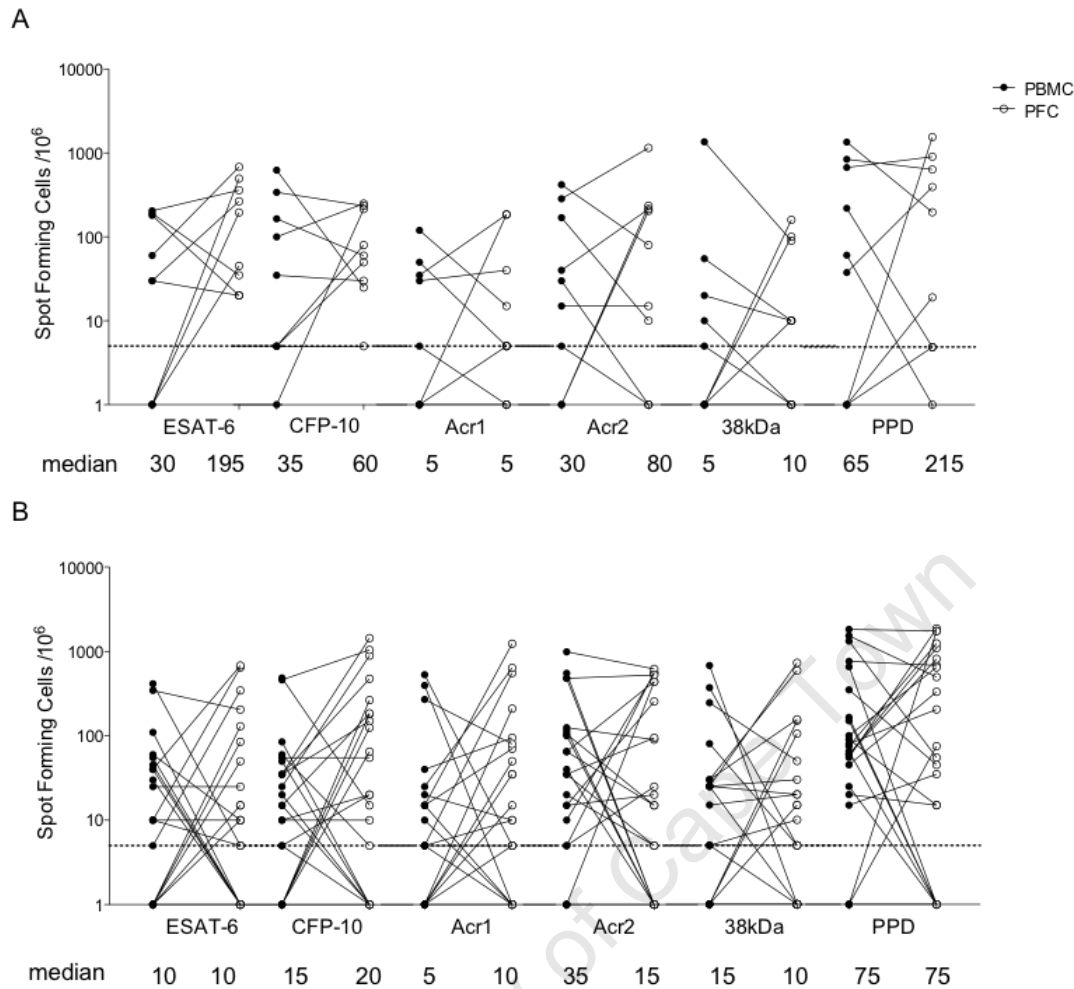


Figure 3.4. Heterogeneous spread of IFN- γ producing cells in blood and pericardial fluid in HIV-1 uninfected and HIV-1 infected TB pericarditis patients

IFN- γ Spot Forming Cells/ 10^6 in response to ESAT-6, CFP-10, Acr1, Acr2, 38kDa and PPD per patient. There is no significant difference between PBMC and PFC for any of the antigens except for significantly higher numbers of SFC/million pericardial fluid cells in response to ESAT-6 ($p=0.009$) in the HIV-1 uninfected patients (A, $n=9$) compared to the HIV-1 infected (B, $n=23$) patients.

The strongest ELISpot response was to PPD in both HIV-1 infected and uninfected patients in both compartments. Again there was significant heterogeneity between patients in response to all antigenic stimuli, with no clear concentration of antigen specific IFN- γ secreting cells at the

disease site compared to blood. The median response to all antigens tended to be higher in the pericardial fluid compared to blood of HIV-1 uninfected patients, but this was not the case for HIV-1 infected patients.

Table 3.4. Summary of the Median IFN- γ -SFC/10⁶ PBMC and PFC for the combined ELISpot results as well as the HIV-1 uninfected and HIV-1 infected patients and comparisons between the groups

		Combined (n=32)		HIV-1 uninfected (n=9)		HIV-1 infected (n=23)		HIV-1 uninfected vs HIV-1 infected
Antigen	Cell type	Median SFC/10 ⁶ (IQR)	p-value	Median SFC/10 ⁶ (IQR)	p-value	Median SFC/10 ⁶ (IQR)	p-value	p-value
ESAT-6	PBMC	17.5 (1.00-58.8)	0.225	30.0 (1.00-185)	0.138	10.0 (1.00-45.0)	0.828	0.365
	PFC	20.0 (2.00-203)		195.0 (27.5-428)		10.0 (1.00-85.0)		0.009
CFP-10	PBMC	17.5 (5.00-58.8)	0.086	35.0 (5.00-253)	1.0	15.0 (1.00-50.0)	0.056	0.364
	PFC	52.5 (5.00-208)		60.0 (27.5-225)		20.0 (1.00-185)		0.388
ACR1	PBMC	5.0 (1.00-28.8)	0.174	5.00 (1.00-42.5)	0.527	5.00 (1.00-20.0)	0.178	0.896
	PFC	10.0 (1.00-77.5)		5.00 (3.00-113)		10.0 (1.00-80.0)		0.832
ACR2	PBMC	35.0 (5.00-123)	0.690	30.0 (3.00-228)	0.547	35.0 (5.00-115)	0.926	0.784
	PFC	17.5 (1.00-250)		80.0 (5.50-225)		15.0 (1.00-435)		0.670
38kDa	PBMC	7.5 (1.00-28.8)	0.919	5.00 (1.00-37.5)	0.779	15.0 (1.00-30.0)	0.744	0.589
	PFC	10.0 (1.00-80.0)		10.0 (1.00-95.0)		10.0 (1.00-50.0)		0.983
PPD	PBMC	70.0 (21.3-579)	0.710	65.0 (1.00-848)	0.813	75.0 (25.0-350)	0.733	0.850
	PFC	140.0 (5.00-789)		215.0 (5.00-863)		75.0 (1.00-815)		0.900
NIL	PBMC	2.0 (0.250-6.00)	0.718	5.00 (1.00-52.5)	1.0	2.0 (0.0-6.00)	0.736	0.188
	PFC	4.50 (1.00-7.50)		10.0 (3.00-25.0)		3.0 (0.0-5.00)		0.022

An interesting separation into two patterns of response was noted: “pericardial dominance” with low antigen specific responses in blood and higher responses in the pericardial fluid (Figure 3.5) and “peripheral dominance” with a high response in blood and a lower response in

the pericardial fluid as shown in Figure 3.6. This pattern was evident in both HIV-1 uninfected and HIV-1 infected patients, with the exception of the response to ESAT-6 in the pericardial fluid cells: the median response was 195 SFC/ 10^6 in the HIV-1 uninfected patients, compared to 10 SFC/ 10^6 in the HIV-1 infected patients ($p=0.009$) as shown in Figure 3.4 and Table 3.4.

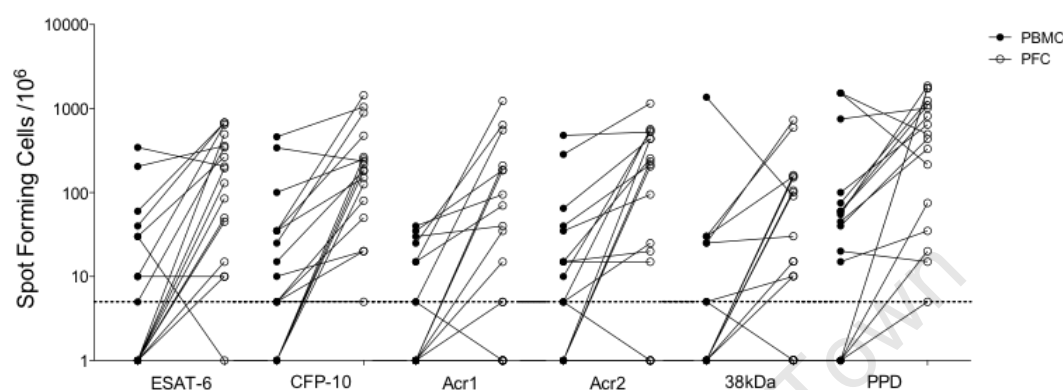


Figure 3.5. Pericardial dominance of some TB pericarditis patients

*“Pericardial dominance” showing low antigenic recognition by the PBMC SFC/ 10^6 cells in the blood and high antigenic recognition by the PFC SFC/ 10^6 in pericardial fluid for all *M.tb*-specific antigens.*

It was also interesting to note that without stimulation, there was increased background IFN- γ SFC/ 10^6 in the PFC of HIV-1 uninfected compared to HIV-1 infected TB pericarditis patients ($p=0.022$). Background (NIL) was always subtracted from each of the results, and negative values were changed to 0 SFC/ 10^6 for analysis.

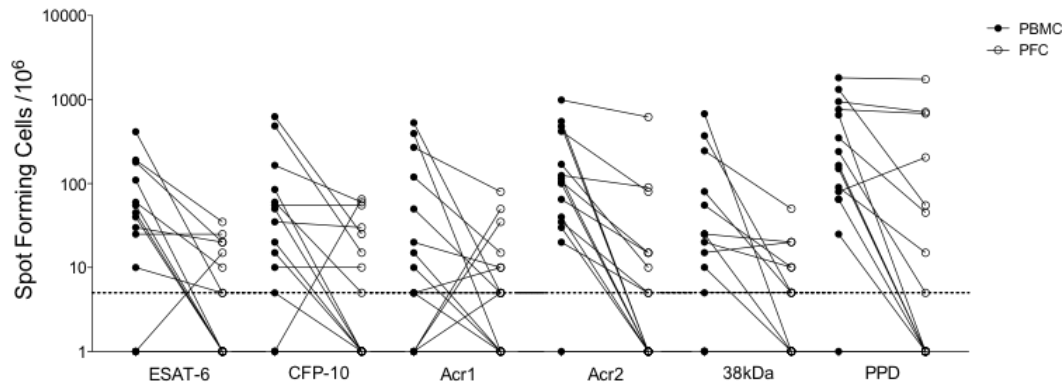


Figure 3.6. Peripheral dominance

“Peripheral dominance” with high antigenic recognition by the PBMC SFC/10⁶ cells in the blood and low antigenic recognition by the PFC SFC/10⁶ in the pericardial fluid for all M.tb-specific antigens.

In order to analyze *M.tb* specific antigenic recognition by cells at the site of disease, the numbers of SFC/10⁶ for ESAT-6 was summarized and divided by the number of PPD specific spots (Figure 3.7). This ratio reflects the number of RD1 specific cells over the number of PPD specific cells. There was significantly more *M.tb*-specific recruitment to the site of disease in HIV-1 uninfected patients compared to HIV-1 infected patients ($p=0.029$), thus implying that HIV may hinder responses to *M.tb* antigens in these patients and in this way interfere with the normal host response to the organism.

2.5. Depletion assays to determine if a CD4 or CD8 T cell response is responsible for the IFN- γ specific cellular response in the blood and site of disease

2.5.1. IFN- γ specific ELISpot

I next determined whether the IFN- γ response seen in the blood and pericardial fluid was produced by CD4 or CD8 T cells in the PBMC and pericardial fluid cells. The hypothesis was that in HIV-1 infected patients; IFN- γ production would be skewed towards CD8 T cells due to the documented CD4 T cells depletion caused by HIV-1 infection (116). Depletion of CD4 and CD8 cells was performed on PBMC and PFC from 4 HIV-1 infected TB pericarditis patients as described in Chapter 2. These were used for an IFN- γ specific ELISpot using ESAT-6, CFP-10, PHA and an unstimulated control. Two of the patients had no response to any antigen when stimulating the undepleted PBMC or PFC and were excluded from the analysis. The remaining 2 patient's results are tabulated in Table 3.5.

Table 3.5. SFC/10⁶ PBMC and PFC for undepleted, CD4 depleted and CD8 depleted samples per antigen for Sample 1 and Sample 2

ELISpot results		Sample 1			Sample 2		
Cell type	Antigen	Undepleted	CD4 depleted	CD8 depleted	Undepleted	CD4 depleted	CD8 depleted
PBMC	ESAT-6	5	5	0	30	10	20
	CFP-10	0	5	0	0	0	0
	PHA	445	350	145	500+	400+	250+
	NIL	0	0	0	10	10	0
PFC	ESAT-6	20	50	10	210	60	120
	CFP-10	10	15	0	0	0	0
	PHA	500+	500+	140	800+	750+	500+
	NIL	5	25	10	70	45	35

Both samples showed large responses in the PHA stimulated wells, indicating the cells were able to respond to mitogen. Sample 1 showed increased SFC/10⁶ PBMC and PFC for the CD4 depleted cells, showing that in this patient, the response is more likely due to the CD8 T cells recognition, whereas sample 2 showed the opposite trend, indicating that CD4 T cells are

eliciting the IFN- γ response in this patient. It is of note that the PFC had large background (NIL) IFN- γ responses in undepleted as well as the depleted cells.

2.5.2. FACS confirmation of CD4 and CD8 depletion

Any remaining cells from the depletion assays were analysed by FACS as this is a more accurate confirmation of the depletion of the CD4 and CD8 cells by the magnetic bead method. A representation of the gating strategy to determine the depletion efficiency is shown in Figure 3.8.

The percentage of the CD4 and CD8 cells gated for in Figure 3.8C and 3.8D were used to determine the depletion efficiency for each assay. The proportions of CD4 and CD8 measured by FACS are tabulated in Table 3.5 and the depletion efficiencies calculated are tabulated in Table 3.6.

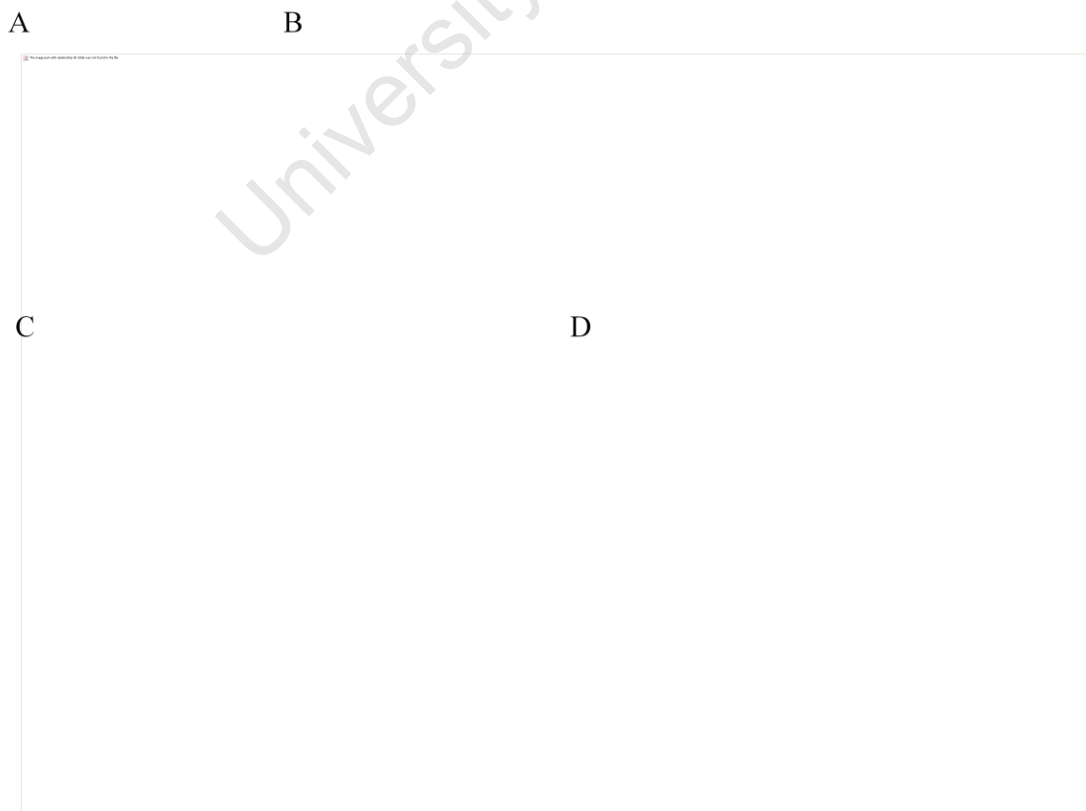


Figure 3.8. Representative dot plot showing the gating strategy for determination of the accuracy of the depletion assays

A. shows the Side Scatter (Ssc) plotted versus CD3-FITC, the purple gate denotes the CD3 population.

B. shows the CD3 population plotted versus VIVID, which is measured in the Pacific Blue channel. VIVID negative cells are viable live cells and these were picked out in the purple gate.

C. shows the viable CD3 cells plotted versus CD4-APC, giving the CD4 population

D. shows the viable CD3 cells plotted versus CD8-Cy5.5PerCP, giving the CD8 population.

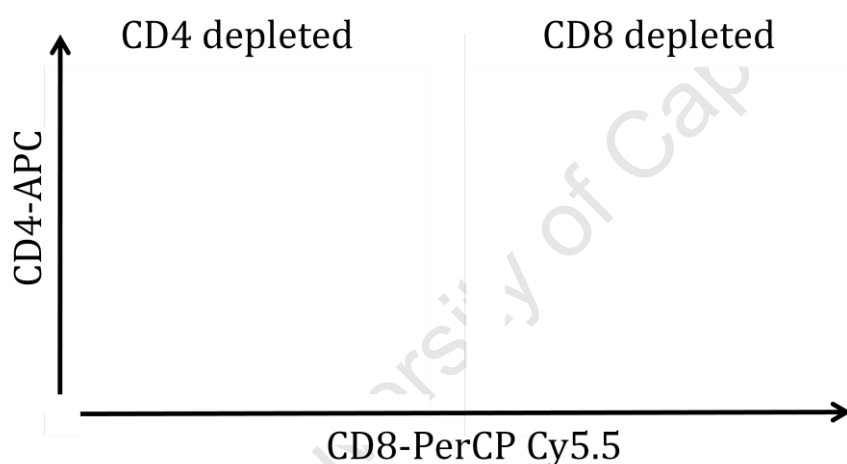


Figure 3.9. Representative dot plot showing the proportions of CD4 and CD8 cells left when depleting CD4 or CD8 cells in the same sample

Table 3.6. Proportions of CD4 and CD8 cells measured in Sample 1 and Sample 2

Sample 1	PBMC		PFC	
Absolute CD4 count = 86				
CD4 depleted	% Viable CD3 cells	No cells available	% Viable CD3 cells	64.6
	% CD4 of CD3		% CD4 of CD3	9.38
	% CD8 of CD3		% CD8 of CD3	73.3
CD8 depleted	% Viable CD3 cells	No cells available	% Viable CD3 cells	61.3
	% CD4 of CD3		% CD4 of CD3	64.2

	% CD8 of CD4		% CD8 of CD4	18.0
Sample 2	PBMC		PFC	
Absolute CD4 count = 427				
CD4 depleted	% Viable CD3 cells	86.2	% Viable CD3 cells	74.9
	% CD4 of CD3	17.2	% CD4 of CD3	2.61
	% CD8 of CD3	73.6	% CD8 of CD3	79
CD8 depleted	% Viable CD3 cells	82.7	% Viable CD3 cells	73.2
	% CD4 of CD3	70.8	% CD4 of CD3	77.4
	% CD8 of CD4	17.6	% CD8 of CD4	16.1

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Table 3.7. Depletion efficiency in Sample 1 and Sample 2 for CD4 and CD8 cells

Sample 1	Population	Depletion efficiency
PFC	CD4 cells	85.34%
	CD8 cells	75.44%
Sample 2	Population	Depletion efficiency
PBMC	CD4 cells	75.71%
	CD8 cells	76.09%
PFC	CD4 cells	96.63%
	CD8 cells	79.62%

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3. Discussion

I found significantly increased concentrations of IFN- γ in the pericardial fluid compared to the blood in the unstimulated as well as *M.tb*-specific antigen stimulated samples, irrespective of HIV status of the patients in the whole blood and pericardial fluid overnight restimulation assay. When comparing the HIV-1 uninfected group with the HIV-1 infected patients there seemed to be higher concentrations of IFN- γ in both blood and pericardial fluid of the HIV-1 uninfected patients, but no statistically significant differences were noted.

Studies in high endemic areas of TB, such as South Africa, where this study took place, report high background levels of IFN- γ in pleural fluid, similar to our pericardial fluid samples (117). IFN- γ concentrations were significantly increased at the site of disease compared to blood, especially when stimulating with *M.tb* antigens, and there were no differences when comparing HIV-1 uninfected to HIV-1 infected TB pericarditis patients.

The IFN- γ specific ELISpot was used to determine cell specific IFN- γ production at the site of disease and in blood to ascertain if the increase of IFN- γ at the site of disease is a result of the increased numbers of IFN- γ producing cells, or the same numbers of T cells in the pericardium as in the blood, but with an enhanced ability to produce IFN- γ in response to *M.tb* antigens.

However, the IFN- γ specific ELISpot showed heterogeneous results for all patients. In TB pericarditis patients uninfected and infected with HIV, there were diverse results for both blood and pericardial fluid for all antigens, with no clear concentration of IFN- γ -specific cells recruited to the site of disease, except for ESAT-6 that was significantly recognized by PFC in the HIV-1 uninfected group when compared to the HIV-1 infected group ($p=0.009$). This is similar to the response of ESAT-6 stimulated pleural fluid cells at the site of disease in pleural

TB disease in HIV-1 uninfected patients (110). Concentration of *M.tb* ESAT-6 specific IFN- γ secreting cells at the pericardial disease site exclusively in the HIV-1 uninfected patients may indicate the contribution of these cells to host defense in containing *M.tb* at sites of replication. ESAT-6 functions in virulence of *M.tb* as its pore forming activity could allow direct access to epithelial cells, which may allow mycobacteria to amplify Matrix metalloproteinase (MMP) 9 (118). MMP9 plays a role in human susceptibility to TB and its increased activity is correlated with worse outcomes (119). Therefore the T cell response against ESAT-6 must play a role in clearance or protection only seen in HIV-1 uninfected patients, while HIV-1 infected patients do not mount an efficient protective response.

A trend that was noticeable for both HIV-1 infected and uninfected groups, is that an increase in the IFN- γ cell-specific response in the pericardial fluid compared to blood for one antigen can predict an increase in the IFN- γ response of the pericardial fluid cells to other *M.tb*-specific antigens, showing “pericardial dominance”. The opposite is also true for an increase in the blood compared to pericardial fluid, showing “peripheral dominance”. This could be as a result of the fluctuations of the different proportions of the distinct subsets of memory T cells present at the site of disease during the time of pericardiocentesis and in the periphery during the time of blood sampling. The ELISpot assay measures IFN- γ produced by effector T cells in the samples. The proportions of these IFN- γ producing T cells may be lower in one patient compared to another, as the time of infection of the pericardium to pericardiocentesis may differ in patients. Since repeat effusions were not clinically indicated in these patients, I was not able to obtain repeat pericardial fluid samples from these patients and I could not investigate this hypothesis any further.

Depletion assays were done on PBMC and PFC from 4 HIV-1 infected patients to determine if the ELISpot response was as a result of CD4 or CD8 T cells producing IFN- γ , as the dominance of CD8 T cells at the disease site of HIV-1 infected patients has been described in pericardial TB patients (80). FACS analysis confirmed efficient depletion of both CD4 and CD8 cells in these samples. In 2 of these patients, no ELISpot response was detected, perhaps indicating the lack of viable activated, effector and antigen specific lymphocytes present after cryopreservation. The other 2 patients showed opposite responses to each other. The first patient showed a CD4 dominant response and the second showed a CD8 dominant response. This could be as a result of the absolute CD4 count measured at the time of pericardiocentesis as the patient showing a CD4 dominant response had a CD4 count of 427 cells/ μ l, much higher than the patient eliciting a CD8 response with a CD4 count of only 86 cells/ μ l. The CD8 T cells in this patient, may be substituting for the role of the CD4 T cells in the immune response to these *M.tb* antigens. With a loss of CD4 T cells during HIV infection, a reliance on CD8 T cells is required. These cells are able to secrete both IFN- γ and TNF and have anti-microbial activity against cells infected with *M.tb*, but protection against the development of active TB occurs only with CD4 restoration due to anti retroviral treatment (120).

4. Conclusion

T cells in whole blood and pericardial fluid in patients with TB pericarditis recognize *M.tb*-specific antigens and elicit strong IFN- γ production in response irrespective of the HIV status of the patients. However, HIV tends to decrease the overall cellular IFN- γ response at the site of disease and in blood due to the depletion of CD4 cells and these patients may elicit a more dominant CD8 response to *M.tb*-specific antigens. Further studies into the exact phenotype and function of CD4 T cells at the site of disease as well as the CD8 response in HIV-1 uninfected and infected TB pericarditis patients will be discussed in Chapter 4.

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CHAPTER 4

Determination of the phenotype and function of T cells present in the blood and at the site of disease in TB pericarditis patients and the effect of HIV

1. Introduction

Pericardial effusions are generally lymphocytic with CD4⁺ T cells dominating in HIV-1 uninfected and CD8⁺ T cells in HIV-1 infected pericardial TB patients (80). However, the exact memory phenotype of the T cells at the disease site has not been fully characterized.

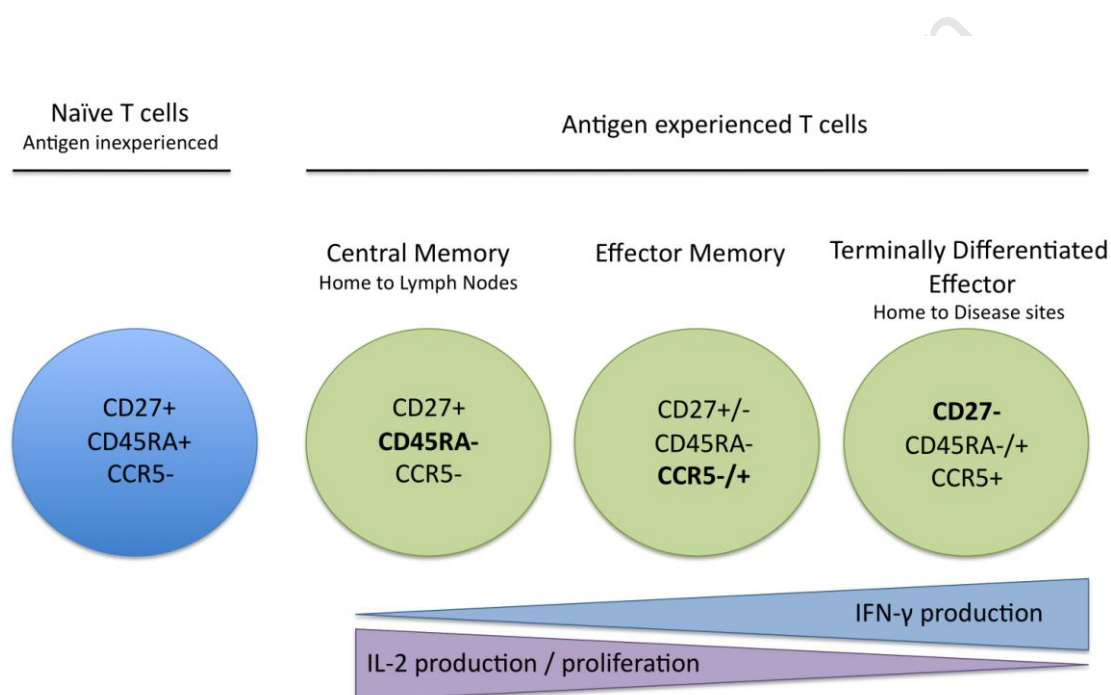


Figure 4.1. CD4 and CD8 T cell differentiation by marker (Adapted from Katalin A. Wilkinson)

Activation of CD4⁺ T cells requires co-stimulation via CD27/CD28 – CD80/CD86 ligation (121), while reactivation of memory CD4⁺ T cells is enhanced by CD28 co-stimulation, it is not imperative. Loss of CD28 rather than CD27 marks the latest stage of CD4⁺ T cell

differentiation, but not CD8⁺ T cell differentiation (122, 123). CD45RA is lost by T cells upon priming by antigens and differentiates naïve and memory T cells (124-126), while CD45RO is a marker of all memory T cells (127). Memory T cells that migrate to inflamed peripheral tissues and display immediate effector function with less need for co-stimulation are phenotypically distinct from central memory T cells that home to secondary lymphoid organs, have no effector function, but are able to proliferate and differentiate to effector cells in response to antigenic stimulation (Figure 4.1)(128, 129).

Studies have shown, using pleural tuberculosis as a model, that the antigen specific T cells at the disease site are predominantly CD4⁺, able to exert effector function rapidly (by releasing IFN- γ within 6 hours after antigen contact), and lack the chemokine receptor CCR7, the absence of which differentiates effector from central memory cells (110). Effector cells also express the chemokine receptor CCR5, which is also a coreceptor of most transmitted HIV-1 (130) and are found at sites of inflammation (131).

In HIV-1 infected patients, the HIV-1 specific response is largely comprised of CD4⁺ and CD8⁺ T cells with an intermediate differentiation state (122, 132). Central memory T cells are depleted in chronic HIV infection (133) and can be restored by anti retroviral treatment (116). It has been shown, that HIV-1 viral load can affect the memory phenotype of CD4⁺ T cells specific to cytomegalovirus (CMV) in the blood of HIV and CMV co-infected individuals, towards an earlier, less differentiated state (132), suggesting that HIV-1 replication affects the phenotype of CD4⁺ T cells of other specificities, resulting in reduced ability of the immune system to control other pathogens. Depletion of CD4⁺ cells results in more than half of all memory cells being destroyed (134), and during early HIV-1 infection, naïve cells comprise large proportions of the T cell population (135).

CD4⁺ T cells secreting IFN- γ play an essential role in protective immunity against TB (136). However, recent evidence suggests that polyfunctional CD4⁺ T cells secreting IFN- γ in combination with other cytokines, such as TNF and IL-2, may also contribute (137). These cells can be found in the blood of HIV-1 infected people, but their ability to secrete more than one cytokine is negatively correlated with the viral load (138). Antiretroviral treatment on the other hand, was shown to restore the polyfunctionality of *M.tb* specific CD4⁺ T cells (120). We know that HIV-1 infection severely impairs the frequency of polyfunctional cells expressing IFN- γ , TNF and IL-2 in bronchoalveolar lavage of people without active TB (139), but whether these T cells are present at other tuberculous disease sites, or the effect that HIV co-infection might present, is not known.

The cytokine GM-CSF is thought to be involved in anti-mycobacterial immunity and is expressed by a variety of cells including T cells and macrophages. In mice where GM-CSF was knocked out, there was decrease in inflammation and in Th1 responses in the lungs (140). *M.tb*-specific GM-CSF expressing CD4⁺ T cells have also been detected in children with TB or with latent TB infection (141) as well as in granulomatous tissues in latently infected individuals (142).

The immune response is also controlled by regulatory T cells (Tregs). The most specific marker for the Treg subpopulation, is the unique forkhead and winged-helix family transcription factor, forkhead box P3 or FoxP3. FoxP3 binds DNA, localizes to the nucleus and can act as a transcriptional repressor (143).

In a study done by Ribeiro-Rodrigues *et al.* (144), it was shown that Tregs might cause the depression of IFN- γ secreting T cells in active TB patients. When these patients were treated for TB, there was a significant decrease in the frequencies of regulatory T cells, as well as

improved IFN- γ secretion compared to the initial samples. Tregs help to control the critical balance between immune-mediated suppression of *M.tb* and the immunopathology in patients with TB infection.

The exact phenotype of cells present at the site of disease in TB pericarditis is unknown. My study, therefore, aimed to identify the memory phenotype of CD4⁺ and CD8⁺ T cells at the site of disease and to determine the functionality of these cells.

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2. Results

2.1. Determination of the phenotype of CD4+ memory cells at the site of disease and in blood by 4-colour flow cytometry

The main aim of this part of the study was to determine the exact phenotype of cells present in the blood and in the pericardial fluid. In order to understand the effect of HIV-1 on the phenotype of T cells at the site of disease, the TB pericarditis patients were stratified by HIV-1 status for analysis.

2.1.1. Patient characteristics

There were 8 HIV-1 uninfected and 9 HIV-1 infected patients included in this section of the study. Only 8 of the HIV-1 infected patients had matching blood and pericardial fluid FACS analysed, the remaining patient had only pericardial fluid cells stained and analysed. The characteristics of all the patients included are summarized in Table 4.1.

Table 4.1. Characteristics of patients included in the 4-colour flow cytometry

Patient subset	HIV-1 uninfected (n=8)	HIV-1 infected (n=9)	p-value
Median Age in years, (IQR)	53.5 (33.75 - 66.25)	30.0 (20.0 - 35.5)	0.021
Gender, Male/Female	5M/3F	3M/6F	0.347
TB diagnosis, Definite/Probable	2 Definite/6 Probable	4 Definite/5 Probable	0.620
Median CD4 count, cells/ μ l (IQR)	381.0 (187.0 - 649.0)	211.5 (104.5 - 320.0)	0.094
Number on Anti retroviral treatment	0	1	n/a

The HIV-1 infected patients with TB pericarditis were significantly younger than their HIV-1 uninfected counterparts ($p=0.021$) and while there was no statistically significant difference in CD4 counts between groups, the HIV-1 infected patients had lower CD4 counts than those

without HIV-1 infection. There were no differences in the genders or in the TB diagnosis of the patients.

2.1.2. Gating Strategy used for 4-colour FACS

Figure 4.2 shows the gating strategy for the determination of the proportions of CD3, CD4 and CD8 cells in blood and pericardial fluid.

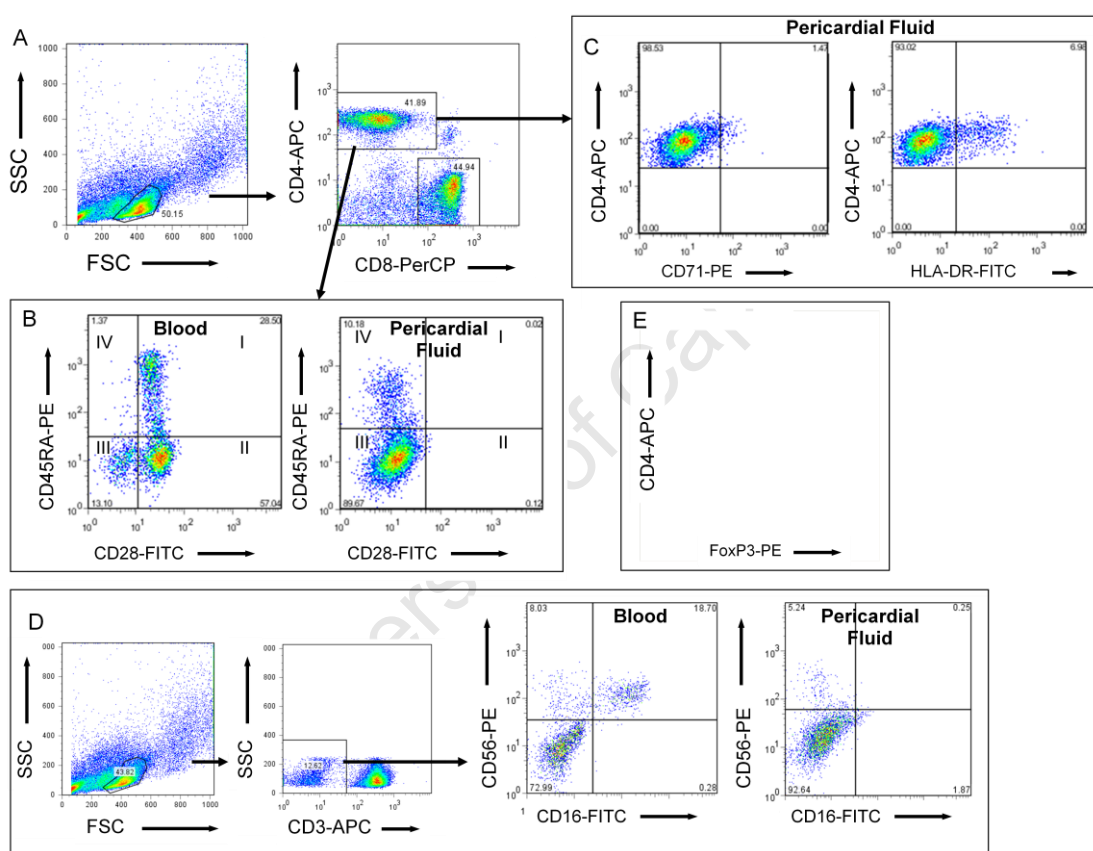


Figure 4.2. Representative scatter dot plots illustrating the gating strategy in 4-colour flow cytometry

A. Selection of CD4 and CD8 positive cells: a scatter plot using Side Scatter (SSC) and Forward Scatter (FSC) was applied to select for lymphocytes. These were further divided into CD4 and CD8 positive cells by gating on CD4-PE and CD8-PerCP fluorochromes

B. CD4 positive cells were further analysed for their surface expression of CD45RA-PE and CD28-FITC as follows (I) $CD4^+CD28^+CD45RA^+$, (II) $CD4^+CD28^+CD45RA^-$, (III) $CD4^+CD28^-CD45RA^-$, (IV) $CD4^+CD28^-CD45RA^+$ cells

C. CD4 positive cells were also analysed for their surface expression of CD71-PE and HLA-DR-FITC

D. Representative example of gating on NK cells: CD3-APC negative cells were selected from the lymphocytes, and analysed as $CD16-FITC^+CD56-PE^+$ NK cells

E. Representative example of gating for $CD4^+FoxP3^+$ cells by gating on surface CD4-APC and intranuclear FoxP3-PE

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2.1.3. Determination of the composition of lymphocytes in blood and pericardial fluid

The analysed results are shown in Figure 4.3 and Table 4.2 for HIV-1 uninfected as well as HIV-1 infected TB pericarditis patients. Overall pericardial fluid had increased proportions of lymphocytes compared to blood, although this did not reach statistical significance. There were increased CD3+ T cells at the site of disease (86.9%) compared to blood (53.3%) in HIV-1 uninfected patients ($p=0.021$), however this trend was not significant in the patients infected with HIV-1 (Blood 58.8% versus pericardial fluid 91.2%).

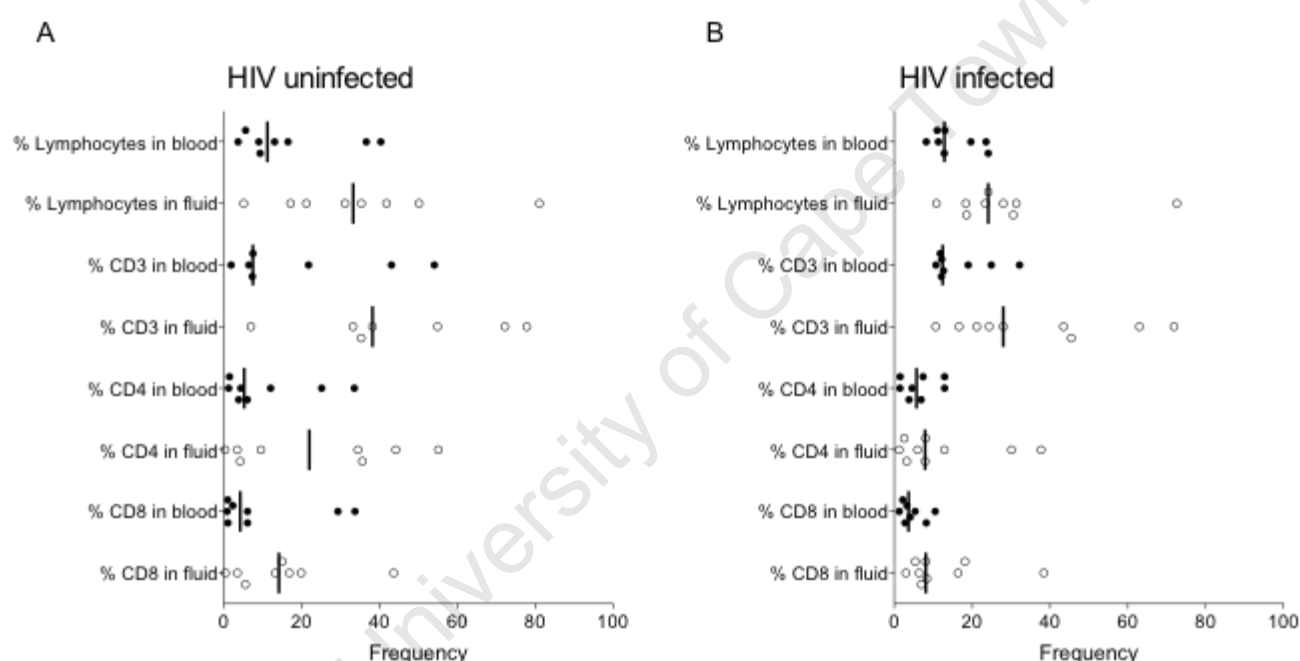


Figure 4.3. Frequencies of the lymphocytic composition of blood and pericardial fluid from TB pericarditis patients

A. HIV-1 uninfected patients (n=8) and

B. HIV-1 infected TB pericarditis patients (blood n=8, pericardial fluid n=9)

There were increased CD8+ T cells at the site of disease (8.1% versus blood 3.7%) in the HIV-1 infected patients ($p=0.031$), and a trend towards decreased CD4+ T cells (7.9% versus blood

5.7%), albeit not significant. There was no significant difference for any cell type when comparing HIV-1 uninfected to HIV-1 infected patients.

Table 4.2 Composition of the blood and pericardial fluid of HIV-1 uninfected and HIV-1 infected TB pericarditis patients

	HIV-1 uninfected (n=8)			HIV-1 infected (n=9)			HIV-1 uninfected vs HIV-1 infected	
	Blood	Pericardial Fluid	p-value	Blood	Pericardial Fluid	p-value	p-value	
							Blood	Pericardial fluid
Lymphocytes (FSC/SSC)	11.2 (6.5-31.6)	33.3 (18.2-48.1)	0.078	12.9 (11.1-22.6)	23.8 (18.4-29.9)	0.437	0.721	0.279
CD3+ of Lymphocytes	53.3 (29.6-76.6)	86.9 (72.6-90.5)	0.021	58.8 (53.6-83.6)	91.2 (79.3-96.3)	0.094	0.382	0.065
CD3+ of all cells	7.5 (6.5-43.1)	38.3 (33.3-72.3)	0.078	12.4 (11.9-23.4)	28.0 (18.9-54.2)	0.078	0.536	0.408
CD4+ of Lymphocytes	36.2 (14.3-44.8)	48.3 (16.9-62.0)	0.195	27.8 (12.2-40.7)	30.9 (19.6-50.5)	1.0	0.442	0.481
CD4+ of all cells	5.3 (2.1-21.9)	22.0 (3.7-42.1)	0.109	5.7 (2.1-11.5)	7.9 (2.9-21.5)	0.687	0.879	0.370
CD8+ of Lymphocytes	19.8 (14.5-41.1)	28.1 (20.0-44.2)	0.641	27.1 (17.9-47.8)	34.7 (22.5-55.2)	0.164	0.505	0.481
CD8+ of all cells	4.2 (1.0-23.6)	14.2 (4.1-19.2)	0.547	3.7 (2.4-7.5)	8.1 (5.9-17.3)	0.031	0.879	0.815

2.1.4. Determination of the surface memory subsets of CD4⁺T cells present in the blood and in the pericardial fluid

A summary of the CD4⁺ T cell memory phenotypes is shown in Figure 4.5 and Table 4.3.

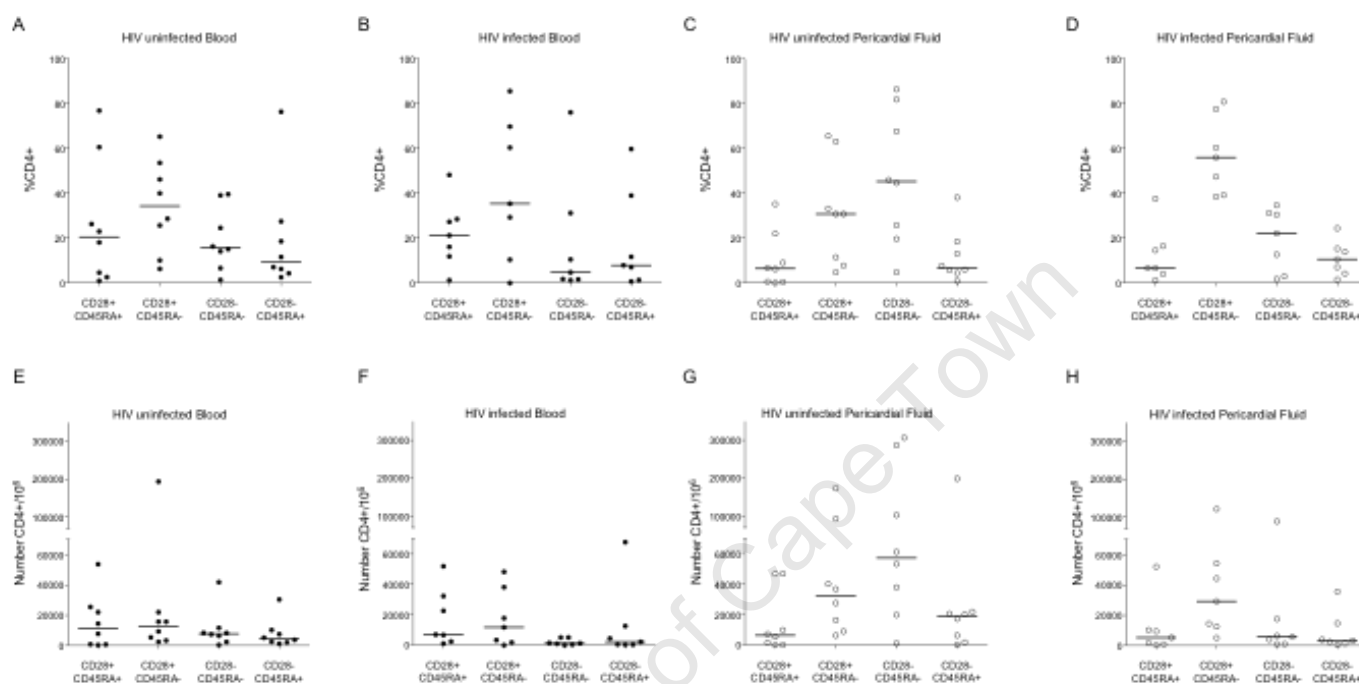


Figure 4.4. Surface phenotype of CD4⁺ T cells in blood and pericardial fluid

A – D. Frequency of CD4⁺ expressing CD28 and CD45RA in HIV-1 uninfected (n=8) and HIV-1 infected (n=7) blood and pericardial fluid

E – H. Number of CD4⁺ expressing CD28 and CD45RA per 10⁶ cells in HIV-1 uninfected (n=8) and HIV-1 infected (n=7) blood and pericardial fluid

Table 4.3. Frequency of CD4 memory phenotypes in blood and pericardial fluid of HIV-1 uninfected and HIV-1 infected TB pericarditis patients

%CD4+	HIV-1 uninfected (n=8)			HIV-1 infected (n=7)			HIV-1 uninfected vs HIV-1 infected	
	Blood	Pericardial Fluid	p-value	Blood	Pericardial Fluid	p-value	p-value	
							Blood	Pericardial fluid
CD28+CD45 RA+	20.6 (3.1 – 52.0)	6.5 (0.5 – 18.8)	0.109	21.2 (11.9 – 28.5)	6.7 (4.0 – 16.5)	0.313	0.955	0.536
CD28+CD45 RA-	34.4 (14.0 – 51.8)	30.8 (8.7 – 55.6)	1.000	35.4 (10.4 – 69.8)	56.0 (39.2 – 77.6)	0.218	0.613	0.040
CD28-CD45RA-	15.7 (8.5 – 35.5)	45.3 (21.2 – 78.4)	0.016	4.7 (1.6 – 31.2)	22.1 (3.0 – 31.2)	0.438	0.336	0.016
CD28-CD45RA+	9.3 (4.8 – 25.3)	6.8 (4.7 – 17.1)	0.844	7.9 (1.3 – 39.0)	10.6 (4.2 – 15.3)	1.000	0.955	0.867
CD45RO+	42.5 (26.2 – 66.3)	57.2 (25.9 – 74.3)	0.297	21.1 (10.1 – 47.8)	57.2 (43.7 – 67.4)	0.008	0.189	0.955

The frequency as well as total number of cells in blood and pericardial fluid were analysed.

The frequency of CD4+CD28-CD45RA- cells were significantly increased in the pericardial fluid compared to blood of HIV-1 uninfected patients (Figure 4.4A and C, p=0.016). The total number of CD4+CD28-CD45RA- cells were significantly increased in both blood (Figure 4.4E and F, p=0.029) and pericardial fluid (Figure 4.4G and H, p=0.014) of HIV-1 uninfected patients compared to the HIV-1 infected patients. HIV-1 infected patients had significantly increased total numbers of CD4+CD28+CD45RA- cells at the site of disease compared to blood (Figure 4.4F and H, p=0.016), as well as compared to the frequencies of these cells in the pericardial fluid from HIV-1 uninfected patients (Figure 4.4C and D, p=0.04), demonstrating that HIV-1 infection results in the occurrence of less differentiated CD4+ T cells to the site of TB disease.

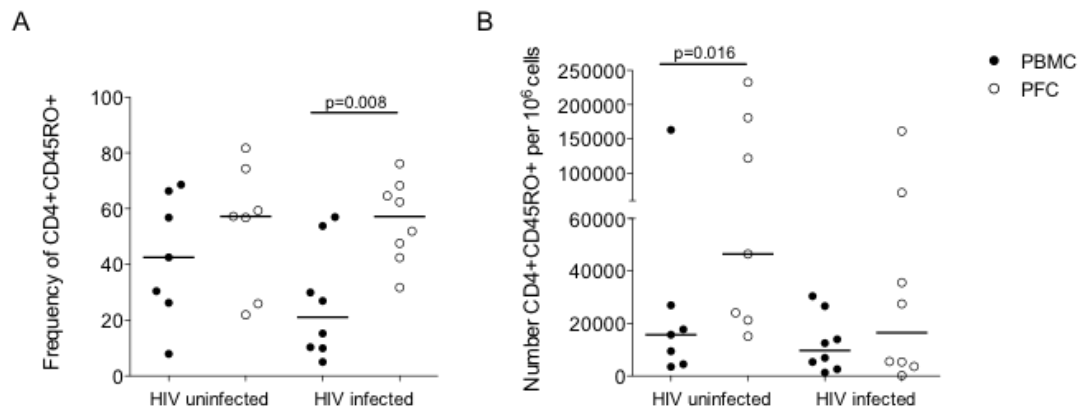


Figure 4.5. Frequency and Number of CD4+ cells expressing CD45RO in blood and pericardial fluid of HIV-1 uninfected (n=7) and HIV-1 infected (n=8) TB pericarditis patients

A. Frequency of CD4+CD45RO+ cells

B. Number of CD4+CD45RO+ per 10⁶ cells

When analyzing the expression of the memory marker CD45RO on CD4+ T cells (Figure 4.5 and Table 4.3), there were significantly increased proportions of CD4+CD45RO+ in the pericardial fluid compared to blood of the HIV-1 infected TB pericarditis patients (Figure 4.5A, p=0.008). However, there were significantly increased total numbers of CD4+CD45RO+ cells in the pericardial fluid compared to blood in the HIV-1 uninfected group (Figure 4.5B, p=0.016). There were no differences when comparing the frequencies or total numbers of CD4+CD45RO+ cells of the HIV-1 uninfected to the HIV-1 infected TB pericarditis patients.

2.1.5. Determination of the proportions of activated and proliferating T cells in the blood and pericardial fluid

PBMC and PFC were surface stained for the transferrin receptor CD71, a surrogate of cycling (proliferating) cells, and the marker for activated cells, HLA-DR. Figure 4.6 shows a trend towards an increase of proliferating CD71+ and activated HLA-DR+ CD4+ T cells in the pericardial fluid compared to blood of the HIV-1 infected patients, significant only for the CD4+CD71+ cells (Figure 4.6B and Table 4.4, $p=0.023$). However, the actual numbers of CD4+HLA-DR+ and CD4+CD71+ cells per 10^6 were much less in the HIV-1 infected pericardial fluid than the frequencies showed (compared to HIV-1 uninfected, not statistically significant). HIV-1 uninfected patients showed significant increased numbers of activated CD4+HLA-DR+ (Figure 4.6C, $p=0.023$) and proliferating CD4+CD71+ (Figure 4.6D, $p=0.039$) cells in the pericardial fluid compared to blood. When comparing both frequencies and numbers of cells of HIV-1 uninfected to HIV-1 infected patients, no differences were noted.

Table 4.4. Summary of CD71 and HLA-DR expression on CD4+ T cells in blood and pericardial fluid

	HIV-1 uninfected (n=8)			HIV-1 infected (n=8)			HIV-1 uninfected vs HIV-1 infected	
	Blood	Pericardial Fluid	p-value	Blood	Pericardial Fluid	p-value	p-value	
							Blood	Pericardial fluid
CD4+ CD71+	12.8 (8.5-16.1)	10.4 (4.3-20.1)	0.844	7.6 (3.0-11.9)	11.2 (7.2-32.0)	0.023	0.130	0.505
CD4+ HLA-DR+	15.1 (8-19.4)	14.9 (12.3-49.7)	0.195	6.7 (4.8-15.3)	15.9 (4.9-34.7)	0.148	0.083	0.798

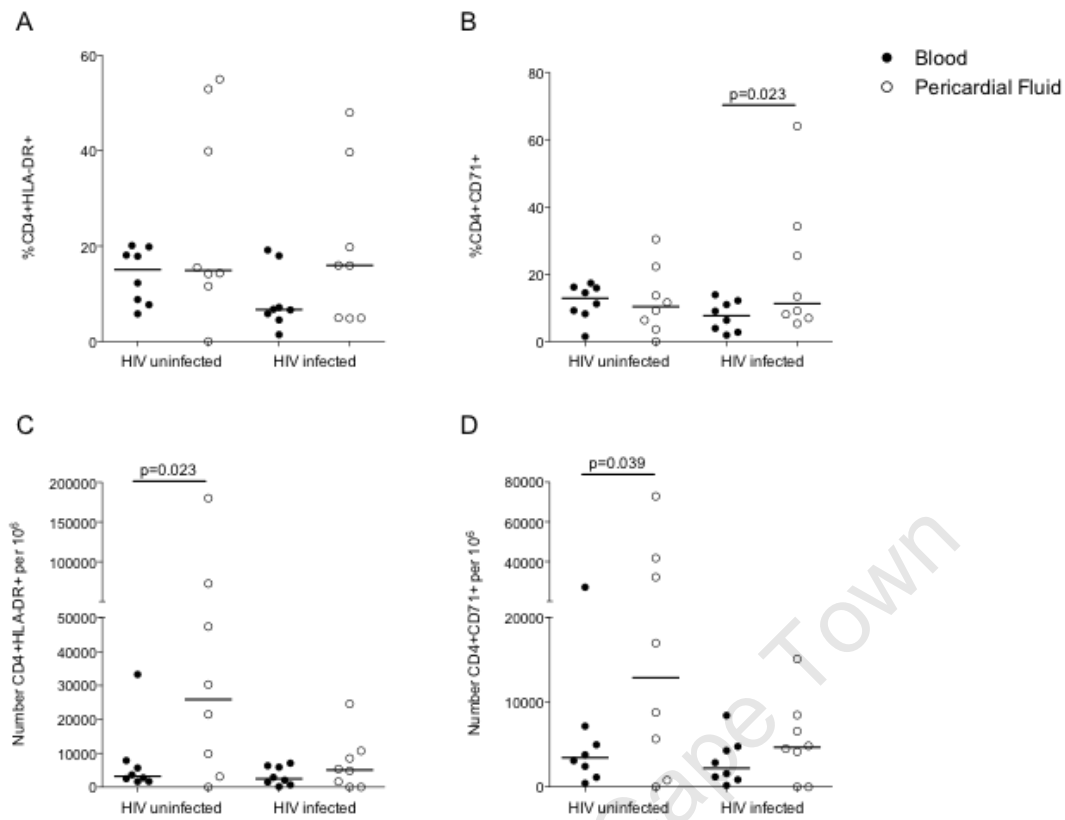


Figure 4.6. Frequency and Number of CD4 cells expressing HLA-DR and CD71 in blood and pericardial fluid from HIV-1 uninfected (n=8) and HIV-1 infected (n=8) TB pericarditis patients

A. Frequency of CD4+HLA-DR+ cells, B. Frequency of CD4+CD71+ cells,

C. Number of CD4+HLA-DR+ cells per 10^6 , D. Number of CD4+CD71+ cells per 10^6

2.1.6. Presence of FoxP3 regulatory T cells in the blood and pericardial fluid of TB pericarditis patients

FoxP3-PE was used to stain for CD3+CD4+ T cells in blood and pericardial fluid, as shown in Figure 4.2. In the HIV-1 uninfected group of patients, the median of CD3+CD4+FoxP3+ cells was very low, with no difference between blood and pericardial fluid. The proportions of CD3+CD4+FoxP3+ cells in the blood and pericardial fluid of the HIV-1 infected patients were similar to each other, and slightly increased compared to the HIV-1 uninfected patients, but not statistically different between the groups. The numbers of CD3+CD4+FoxP3+ cells in blood and pericardial fluid followed the same trend as the frequencies, with low numbers of these cells in the HIV-1 uninfected blood and pericardial fluid and relatively increased numbers in HIV-1 infected blood and pericardial fluid, albeit the differences are not significant.

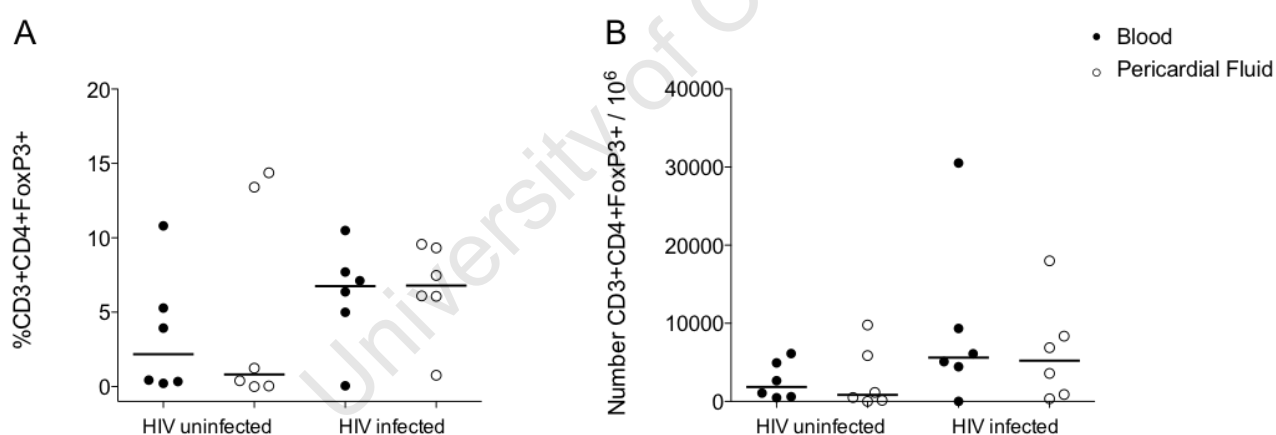


Figure 4.7. Frequency and number of CD3+CD4+FoxP3+ regulatory T cells in blood and pericardial fluid of HIV-1 uninfected (n=6) and HIV-1 infected (n=6) TB pericarditis patients

A. Frequency of CD3+CD4+FoxP3+ regulatory T cells, B. Number of CD3+CD4+FoxP3+ regulatory T cells

Table 4.5. Summary of FoxP3 expression in blood and pericardial fluid

	HIV-1 uninfected (n=6)			HIV-1 infected (n=6)			HIV-1 uninfected vs HIV-1 infected	
	Blood	Pericardial Fluid	p- value	Blood	Pericardial Fluid	p- value	p-value	
							Blood	Pericardial fluid
CD3+CD4+ FOXP3+	2.2 (0.3-6.7)	0.8 (0.03-13.4)	0.688	6.8 (3.8–8.4)	6.8 (4.7-9.4)	0.438	0.394	0.485

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2.1.7. Presence of NK cells in the blood and pericardial fluid of TB pericarditis patients

NK cells are innate cells that also produce the cytokine IFN- γ . They can be identified as CD3-CD56+CD16+ as depicted in the gating strategy in Figure 4.2. There were significantly increased proportions of CD3-CD56+CD16+ NK cells present in the blood compared to pericardial fluid in the HIV-1 uninfected patients with TB pericarditis (Figure 4.8A and Table 4.6, $p=0.035$), although significance was lost when comparing the actual numbers of NK cells (Figure 4.8B, $p=0.055$). In the patients infected with HIV-1, there was no difference between the proportions of NK cells in blood and pericardial fluid. There were no differences when comparing the frequencies or numbers of NK cells in blood and pericardial fluid between HIV-1 uninfected and HIV-1 infected patients.

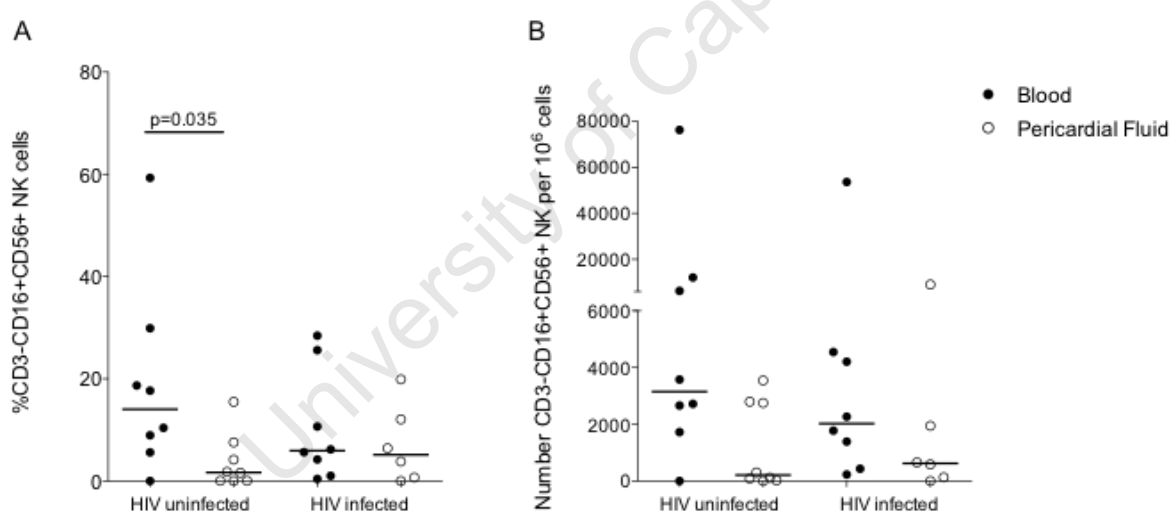


Figure 4.8. Frequency and Number of CD3-CD16+CD56+ NK cells in blood and pericardial fluid of HIV-1 uninfected (n=8) and HIV-1 infected (blood n=8 and pericardial fluid n=6) TB pericarditis patients

A. Frequency of CD3-CD16+CD56+ NK cells

B. Number of CD3-CD16+CD56+ NK per 10⁶ cells

Table 4.6. Summary of the proportions of NK cells present in blood and pericardial fluid

	HIV-1 uninfected (n=8)			HIV-1 infected (n=6)			HIV-1 uninfected vs HIV-1 infected	
	Blood	Pericardial Fluid	p- value	Blood	Pericardial Fluid	p- value	p-value	
							Blood	Pericardial fluid
CD3- CD56+CD16+ NK cells	14.1 (6.4-27.1)	1.7 (0.04-6.7)	0.035	6.2 (2.6-27)	6.4 (0.7-19.9)	0.312	0.673	0.297

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2.2. Determination of the functional phenotype of CD4⁺ cells present at the site of disease and in blood by 8-colour flow cytometry

2.2.1. Patient characteristics

Further groups of HIV-1 uninfected (n=10) and HIV-1 infected (n=10) patients with TB pericarditis were included in this part of the study. Again, the HIV-1 infected TB pericarditis patients were younger compared to the HIV-1 uninfected patients (Table 4.7, p=0.003) and also had a lower CD4 count (not significant due to low number of available CD4 counts). There were no differences in gender or TB diagnosis of the patients in each group and no patients were on antiretroviral treatment at the time of inclusion into the study.

Table 4.7. Characteristics of patients included in the 8-colour flow cytometry

Patient subset	HIV-1 uninfected (n=10)	HIV-1 infected (n=10)	p-value
Median Age in years, (IQR)	57.5 (32.8 – 68.3)	29.5 (27.3 – 30.3)	0.003
Gender, Male/Female	7M/3F	5M/5F	0.650
TB diagnosis, Definite/Probable	4 Definite/6 Probable	4 Definite/6 Probable	1.000
Median CD4 count, cells/μl (IQR), [number available]	381.0 (187.0 - 566.0) [n=7]	169.5 (106.0 – 259.3) [n=4]	0.072
Number on Antiretroviral treatment	0	0	n/a

2.2.2. Gating strategy used in the 8-colour flow cytometry

Figure 4.9 shows the gating strategy used in the 8-colour flow cytometry. Due to the large number of cells needed to stain for all the different stimulations, not all assays were performed on all the patients and the figures will depict this.

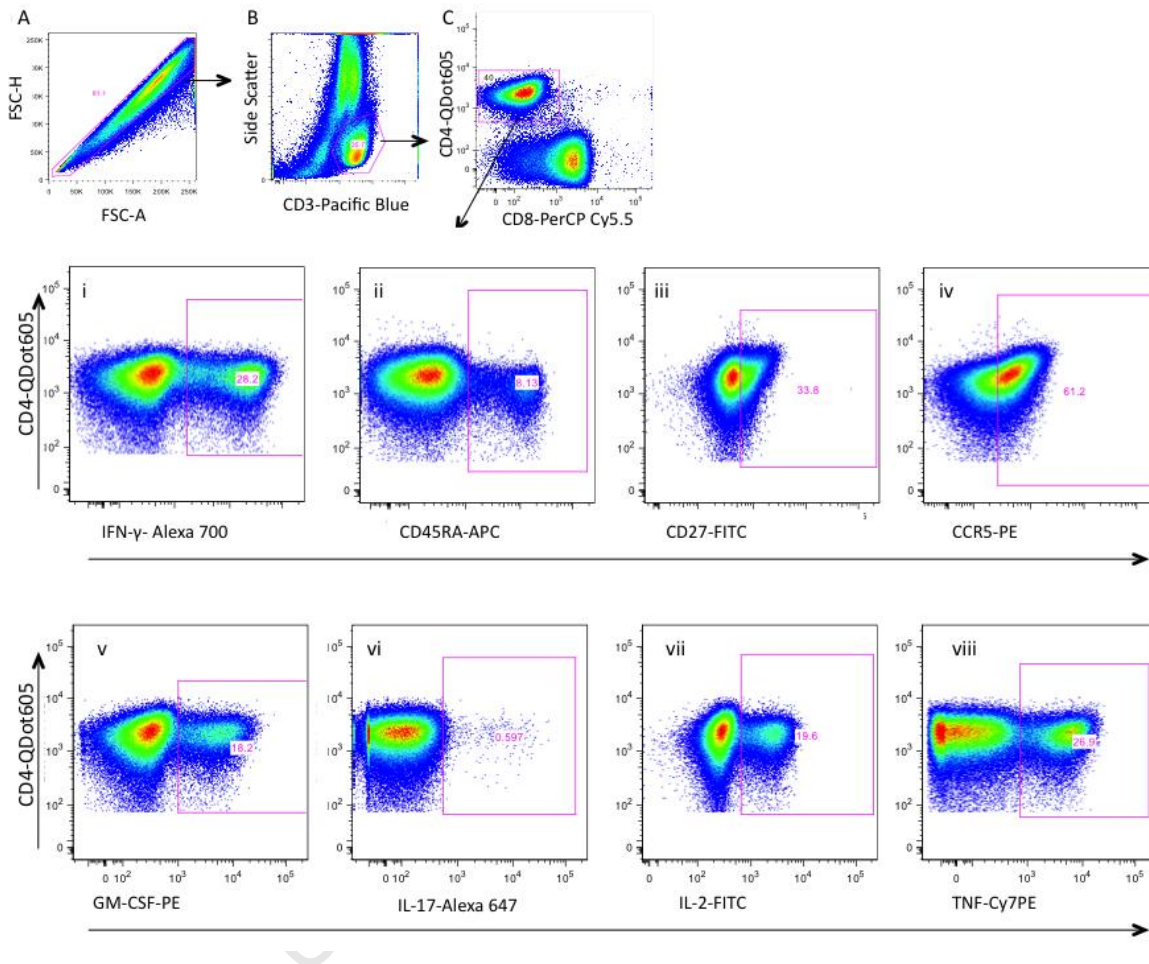


Figure 4.9. Representative scatter dot plots illustrating the gating strategy in 8-colour flow cytometry (SEB stimulation)

- A. Plotting FSC-Area against FSC-Height allowed selection of singlet cells
 - B. CD3⁺ cells were selected using SSC and CD3-PacBlue
 - C. CD4⁺ and CD8⁺ cells were selected for by plotting CD4-Qdot 605 against CD8-PerCP Cy5.5 fluorochromes
- CD4⁺ or CD8⁺ cells on the Y-axes were plotted against:

i. $IFN-\gamma$ -Alexa 700, ii. CD45RA-APC, iii. CD27-FITC, iv. CCR5-PE; and v. GM-CSF-PE, vi. IL-17-Alexa 647, vii. IL-2-FITC and viii. TNF-Cy7PE on the X axes, after which Boolean gating was applied to look at combinations of the markers.

2.2.3. Memory phenotype of CD4⁺ T cells in the blood and pericardial fluid

The exact phenotype of memory cell present in the blood and pericardial fluid of HIV-1 uninfected and HIV-1 infected TB pericarditis patients was determined by staining for CD45RA, the chemokine receptor CCR5 and the co-stimulatory molecule CD27 on CD3⁺CD4⁺ T cells. Figure 4.10 - 4.14 shows these memory cells from the blood and pericardial fluid of HIV-1 uninfected and HIV-1 infected TB pericarditis patients when stimulated with recombinant NIL, SEB, hkH37Rv, PPD and ESAT-6 + CFP-10. Stimulation had no effect on surface markers and the cells showed no differences compared to unstimulated cells (Figure 4.9) for any memory subset in either HIV-1 uninfected or HIV-1 infected TB pericarditis patients.

When stimulating with the *M.tb*-specific antigen ESAT-6+CFP-10 (Figure 4.14) and comparing HIV-1 uninfected and HIV-1 infected patients, the following was noted; there was significantly decreased CCR5⁺CD27⁺CD45RA⁻ CD4⁺ cells in the blood ($p=0.029$), and decreased, but not statistically significant, CCR5⁺CD27⁺CD45RA⁻ CD4⁺ cells in the pericardial fluid ($p=0.055$) of HIV-1 infected patients. There were also increased proportions of CCR5⁻CD27⁻CD45RA⁻ CD4⁺ cells in the pericardial fluid of HIV-1 infected patients ($p=0.031$) compared to HIV-1 uninfected patients. When stimulating with the other antigens, the trend of decreased CCR5⁺CD27⁺CD45RA⁻ CD4⁺ cells (Figure 4.12. hkH37Rv $p=0.012$, Figure 4.13. PPD $p=0.055$) and increased CCR5⁻CD27⁻CD45RA⁻ CD4⁺ cells (Figure 4.12. hkH37Rv $p=0.021$, Figure 4.13. PPD $p=0.043$) in the pericardial fluid of HIV-1 infected patients was also evident.

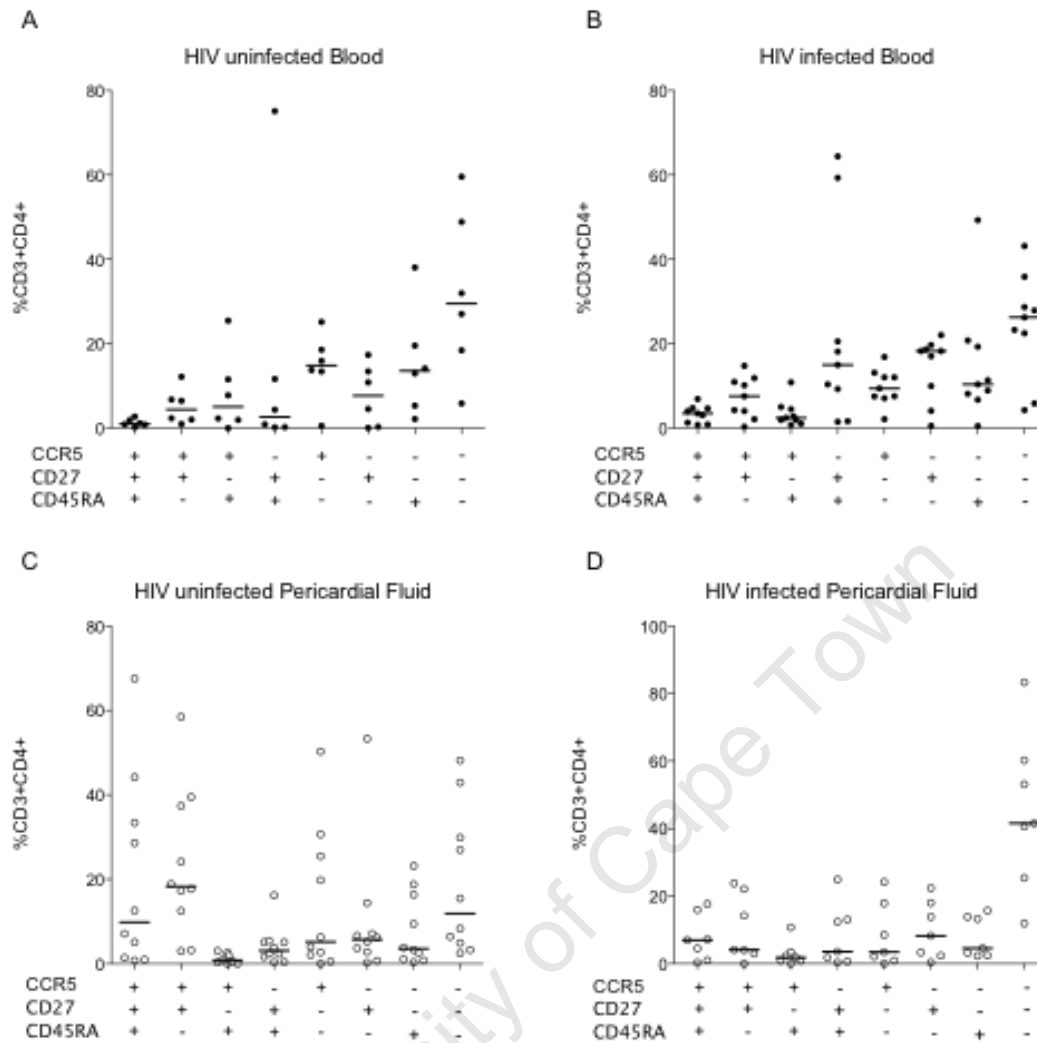


Figure 4.10. Frequency of surface phenotype of unstimulated (NIL) CD3+CD4+ cells in blood and pericardial fluid of HIV-1 infected and HIV-1 uninfected TB pericarditis patients

A. HIV-1 uninfected blood (n=6), B. HIV-1 infected blood (n=9), C. HIV-1 uninfected pericardial fluid (n=10), D. HIV-1 infected pericardial fluid (n=7)

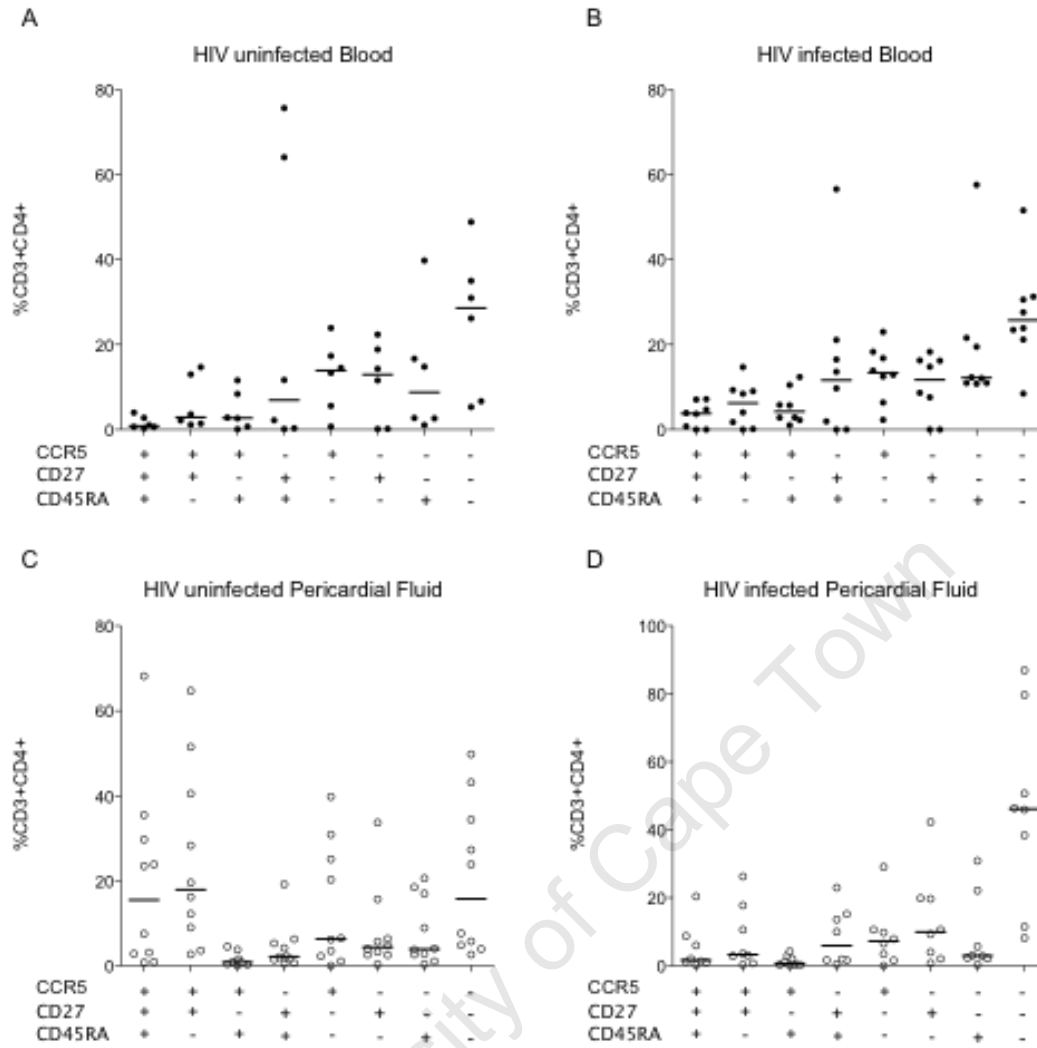


Figure 4.11. Frequency of surface phenotype of CD3+CD4+ cells in blood and pericardial fluid of HIV-1 infected and HIV-1 uninfected TB pericarditis patients when stimulated with the positive control SEB

A. HIV-1 uninfected blood (n=6), B. HIV-1 infected blood (n=8), C. HIV-1 uninfected pericardial fluid (n=10), D. HIV-1 infected pericardial fluid (n=8)

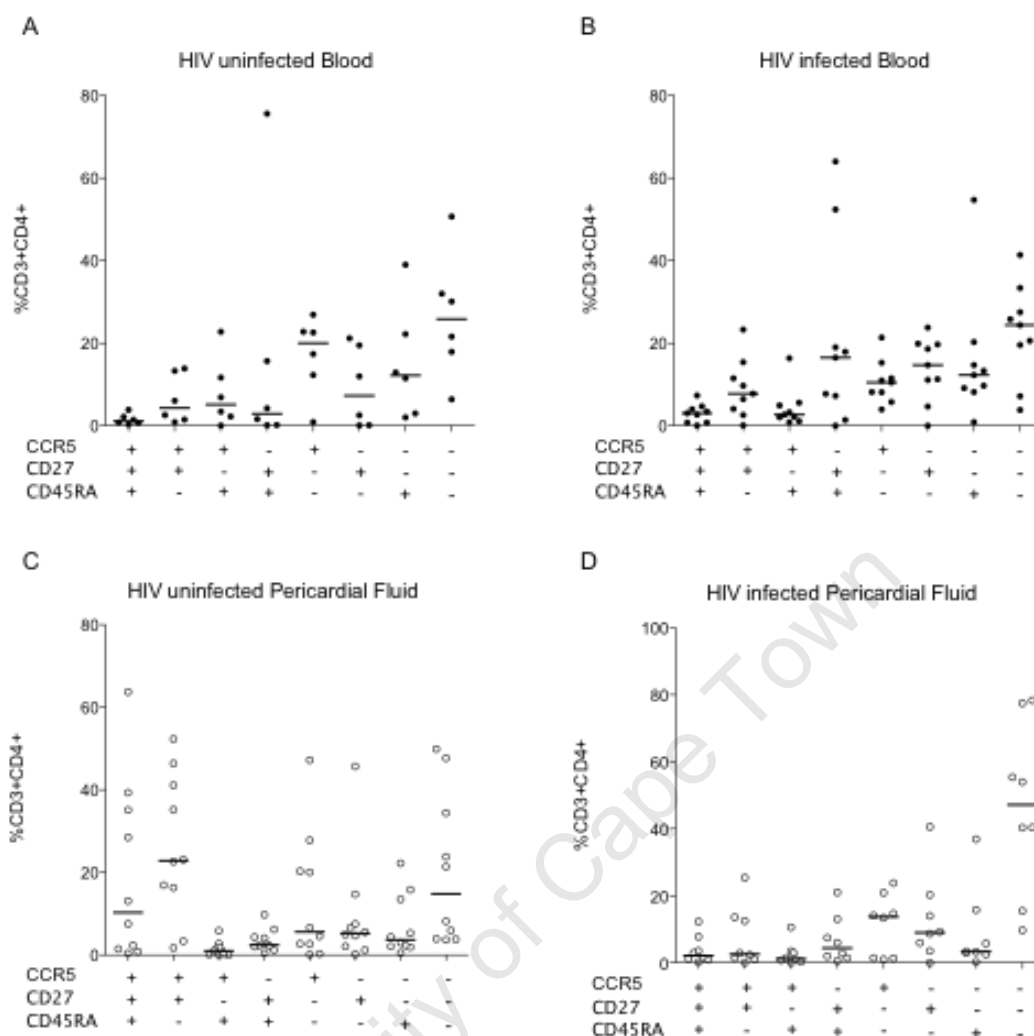


Figure 4.12. Frequency of surface phenotype of CD3+CD4+ cells in blood and pericardial fluid of HIV-1 infected and HIV-1 uninfected TB pericarditis patients when stimulated with hKH37Rv

A. HIV-1 uninfected blood (n=6), B. HIV-1 infected blood (n=9), C. HIV-1 uninfected pericardial fluid (n=10), D. HIV-1 infected pericardial fluid (n=8)

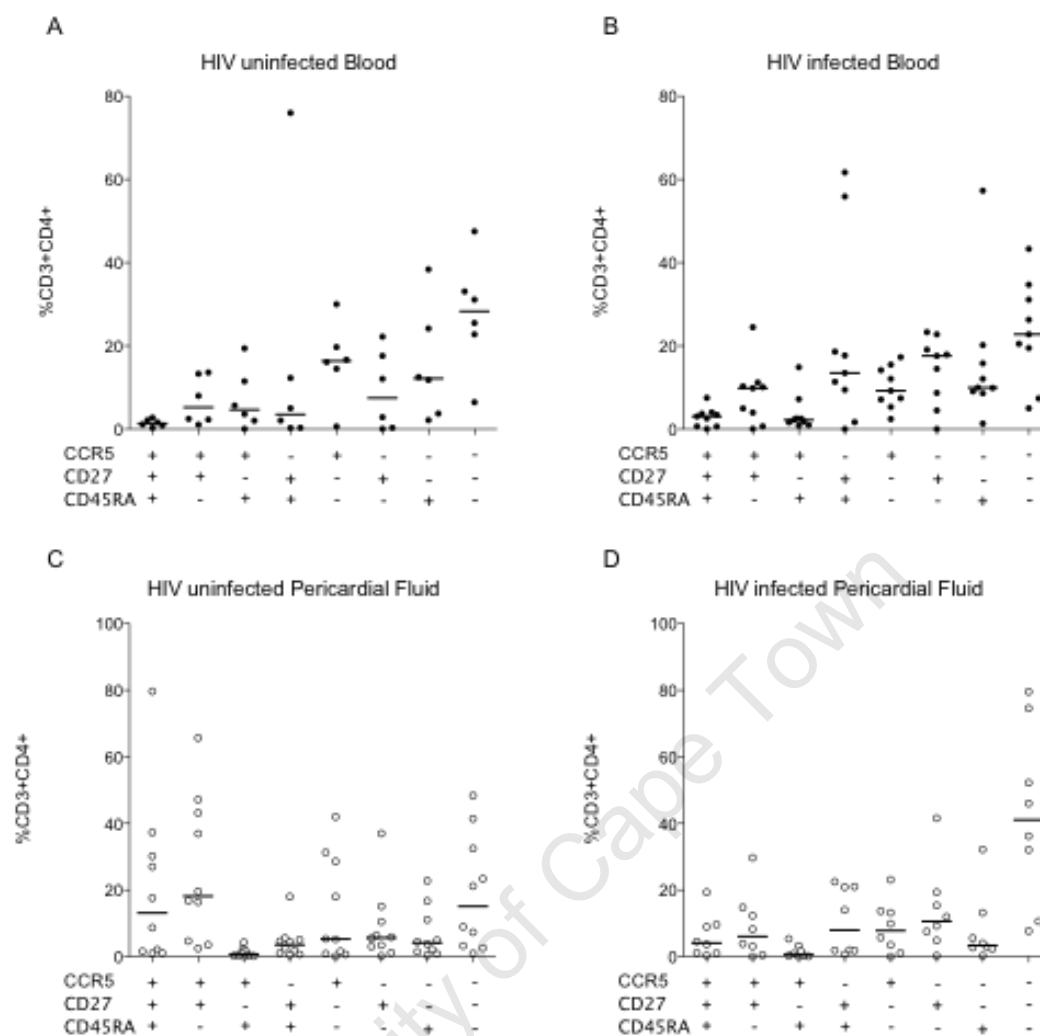


Figure 4.13. Frequency of surface phenotype of CD3+CD4+ cells in blood and pericardial fluid of HIV-1 infected and HIV-1 uninfected TB pericarditis patients when stimulated with PPD

A. HIV-1 uninfected blood (n=6), B. HIV-1 infected blood (n=9), C. HIV-1 uninfected pericardial fluid (n=10), D. HIV-1 infected pericardial fluid (n=8)

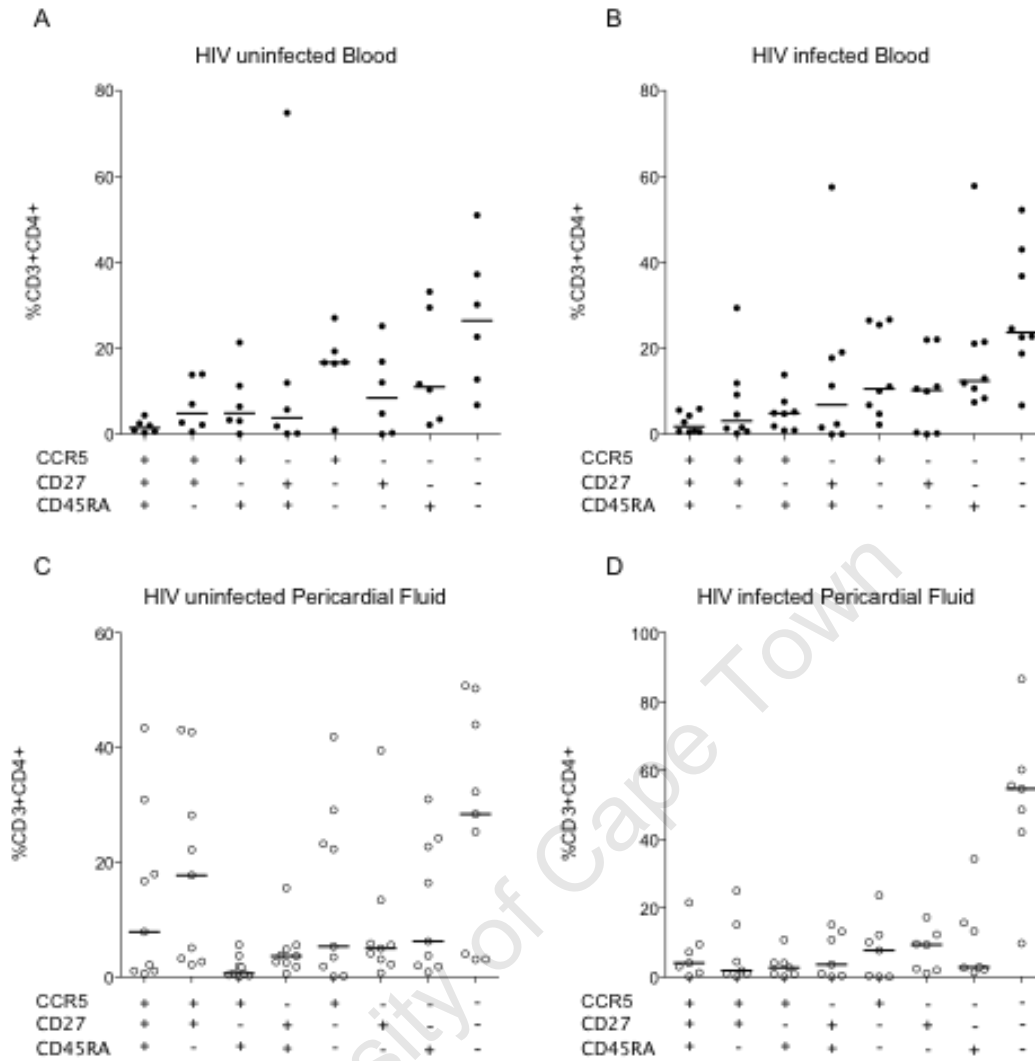


Figure 4.14. Frequency of surface phenotype of CD3+CD4+ cells in blood and pericardial fluid of HIV-1 infected and HIV-1 uninfected TB pericarditis patients when stimulated with the recombinant ESAT-6 + CFP-10

A and B. HIV-1 uninfected (n=6) and HIV-1 infected (n=8) blood

C and D. HIV-1 uninfected (n=9) and HIV-1 infected (n=7) pericardial fluid

2.2.4. Functional phenotype of memory CD4+ T cells in pericardial fluid

I next determined the exact phenotype of cells secreting IFN- γ in the blood and pericardial fluid, as this had not been studied before. The background IFN- γ secretion (NIL) was subtracted from each of the samples before analysis was done. Samples with negative values after background subtraction were changed to 0% for analysis.

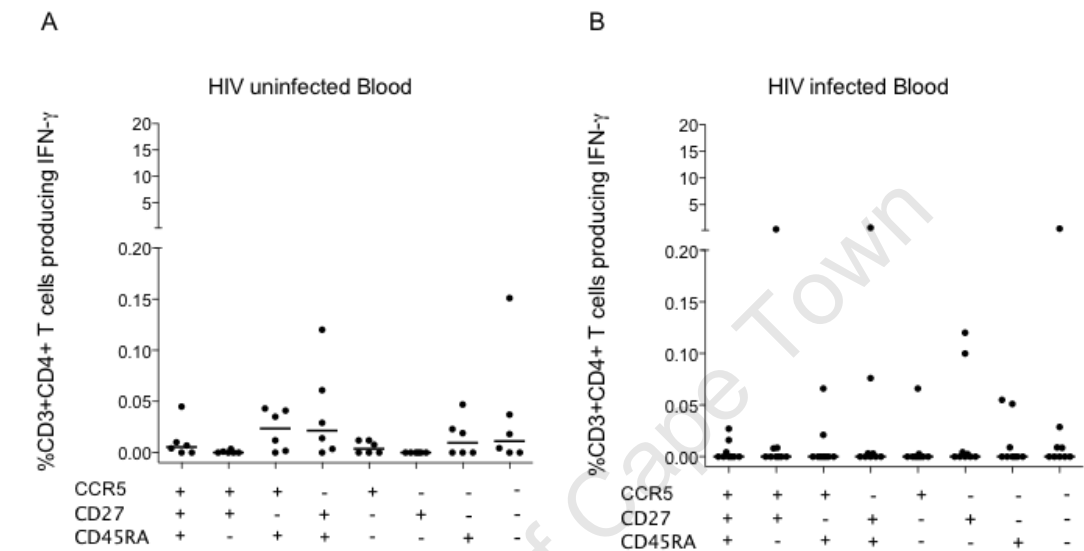


Figure 4.15. Frequency of CD3+CD4+ T cells secreting IFN- γ in response to hKH37Rv in blood

A. HIV-1 uninfected blood (n=6), B. HIV-1 infected blood (n=9)

Overall, the median frequency of memory cells secreting IFN- γ in HIV-1 infected blood was 0% for all combinations. Stimulation with hKH37Rv (Figure 4.15) showed the most prominent CD4+ memory subsets secreting IFN- γ in the blood of HIV-1 uninfected patients were CCR5+CD27-CD45RA+, followed by CCR5-CD27+CD45RA+, CCR5-CD27-CD45RA+ and cells triple negative for all 3 markers.

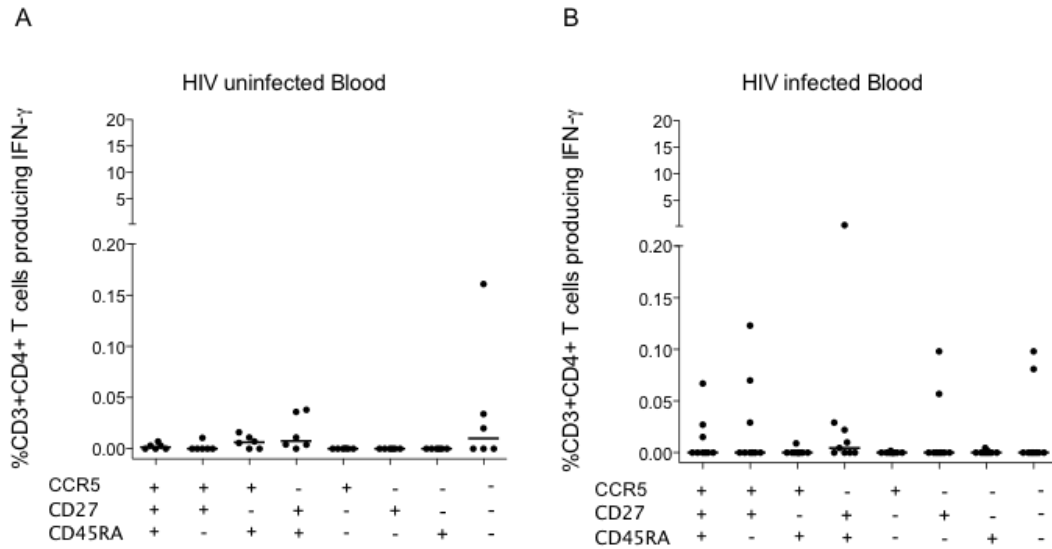


Figure 4.16. Frequency of CD3+CD4+ T cells secreting IFN- γ in response to PPD in blood

A. HIV-1 uninfected blood (n=6), B. HIV-1 infected blood (n=9)

When stimulating blood with PPD (Figure 4.16), a similar pattern emerged. The most prominent CD4⁺ memory subsets secreting IFN- γ in HIV-1 uninfected patients were CCR5-CD27⁺CD45RA⁺, followed by CCR5+CD27-CD45RA⁺ and cells triple negative for all 3 markers. HIV-1 infected blood showed a slight increase in the proportion of CCR5-CD27⁺CD45RA⁺ CD4⁺ cells with the ability to secrete IFN- γ compared to when blood was stimulated with hkH37Rv, whereas ESAT-6 + CFP-10 stimulation showed no memory CD4⁺ cells secreting IFN- γ (Figure 4.17).

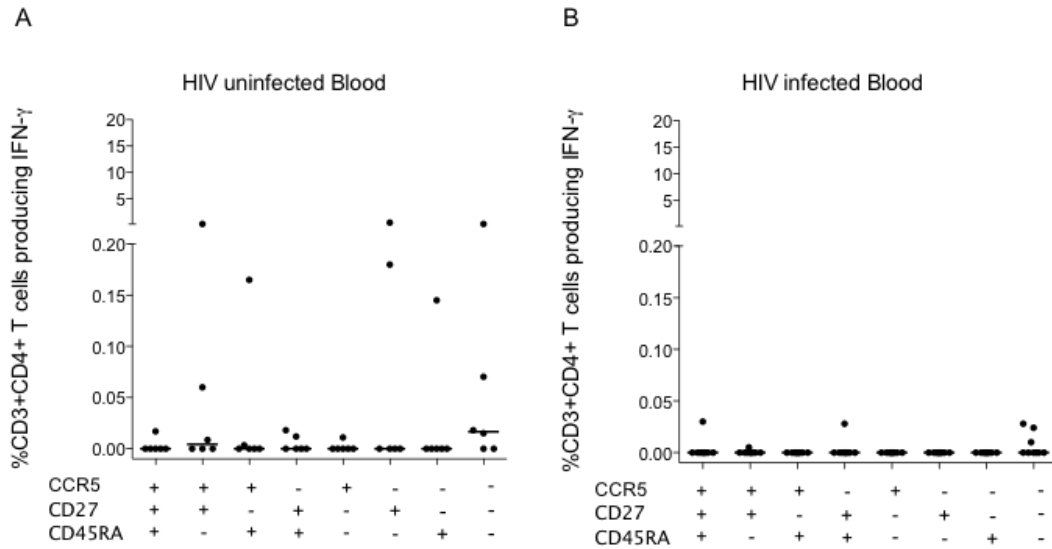


Figure 4.17. Frequency of CD3+CD4+ T cells secreting IFN- γ in response to recombinant ESAT-6 + CFP-10 in blood

A. HIV-1 uninfected blood (n=6), B. HIV-1 infected blood (n=8)

ESAT-6 + CFP-10 stimulation of HIV-1 uninfected PBMC showed that only cells triple negative for CCR5, CD27 and CD45RA were mainly positive for IFN- γ as well as a small proportion of CCR5+CD27+CD45RA- CD4+ cells. There were no statistically significant differences when comparing blood from HIV-1 uninfected to HIV-1 infected TB pericarditis patients for any of the 3 stimulants.

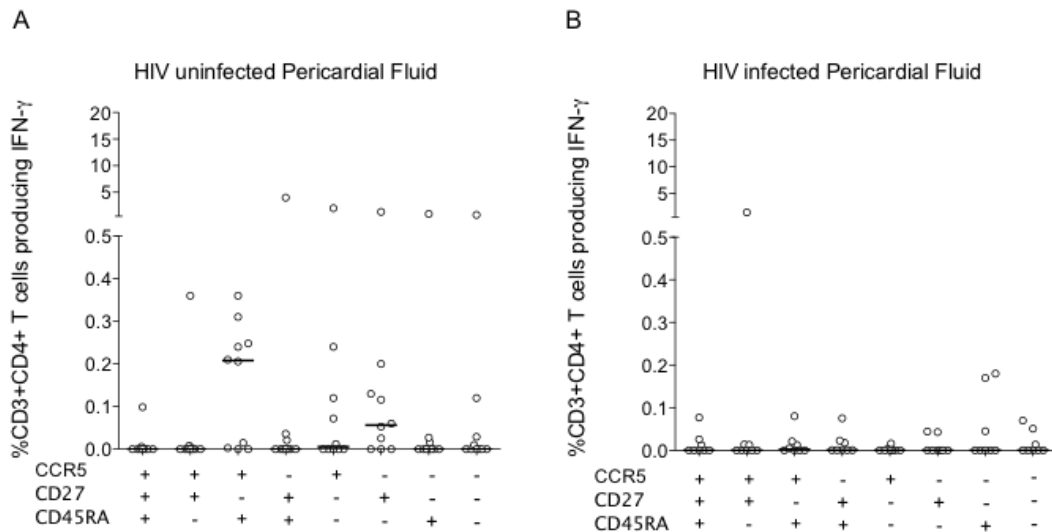


Figure 4.18. Frequency of CD3+CD4+ T cells secreting IFN- γ in response to hKH37Rv in pericardial fluid

A. HIV-1 uninfected pericardial fluid (n=10), B. HIV-1 infected pericardial fluid (n=8)

Stimulation of the PFC with hKH37Rv (Figure 4.18) showed little CD4+ memory subset specific IFN- γ secretion in the pericardial fluid of HIV-1 infected TB pericarditis patients, and there was significantly more CCR5+CD27-CD45RA+ CD4+ T cells secreting IFN- γ in the HIV-1 uninfected patients compared to HIV-1 infected patients ($p=0.033$). There was also a small proportion of CCR5-CD27+CD45RA- CD4+ T cells secreting some IFN- γ in the pericardial fluid of HIV-1 uninfected patients.

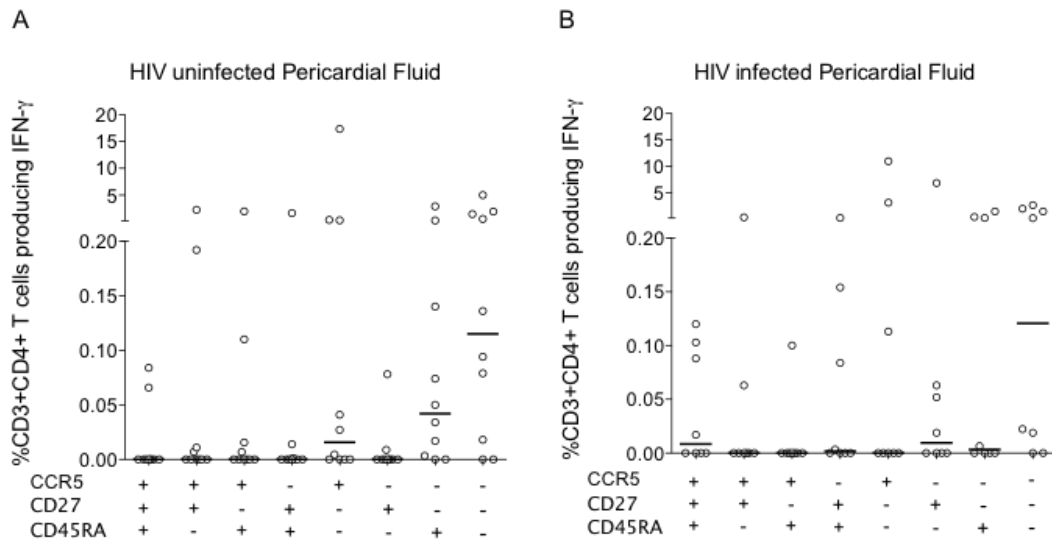


Figure 4.19. Frequency of CD3+CD4+ T cells secreting IFN- γ in response to PPD in pericardial fluid

A. HIV-1 uninfected pericardial fluid (n=10), B. HIV-1 infected pericardial fluid (n=8)

PPD stimulation (Figure 4.19) showed that HIV-1 infected patients had some pericardial fluid cells (CCR5+CD27+CD45RA+, CCR5-CD27+CD45RA- and the triple negative population) with the ability to secrete IFN- γ . The HIV-1 uninfected patients also showed proportions of CCR5+CD27-CD45RA-, CCR5-CD27-CD45RA+ and the triple negative cells secreting IFN- γ . There were no differences when comparing HIV-1 uninfected to HIV-1 infected TB pericarditis patients, although these patients showed different populations of memory cells secreting IFN- γ .

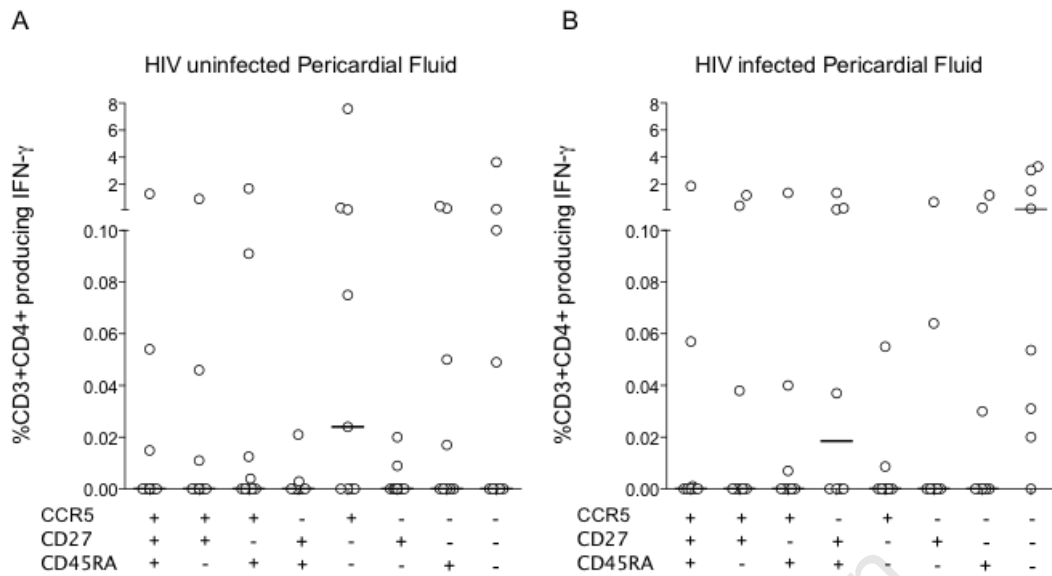


Figure 4.20. Frequency of CD3+CD4+ T cells secreting IFN- γ in response to recombinant ESAT-6 + CFP-10 in pericardial fluid

A. HIV-1 uninfected pericardial fluid (n=8), B. HIV-1 infected pericardial fluid (n=8)

Recombinant ESAT-6 + CFP-10 stimulation (Figure 4.20) showed evidence of CCR5+CD27-CD45RA- CD4+ cells capable of IFN- γ secretion in HIV-1 uninfected patients, while the CCR5-CD27+CD45RA+ and triple negative CD4+ population were the cells secreting IFN- γ in the HIV-1 uninfected patients. There were no differences when comparing any of the memory subsets secreting IFN- γ between HIV-1 uninfected and HIV-1 infected TB pericarditis patients.

2.2.5. Determination of total cytokine secretion by CD4⁺ T cells in blood and pericardial fluid

In addition to staining for intracellular IFN- γ secretion, I evaluated the secretion of other cytokines such as GM-CSF, IL-2, IL-17A and TNF by total CD4⁺ T cells in the blood and pericardial fluid of HIV-1 uninfected and HIV-1 infected TB pericarditis patients. The results are graphically represented in Figures 4.21 – 4.23, after background subtraction (NIL) of cytokines was done.

Stimulation with hkH37Rv (Figure 4.21) showed very different intracellular cytokine profiles in the blood and pericardial fluid of HIV-1 uninfected and HIV-1 infected TB pericarditis patients. HIV-1 uninfected blood cells showed predominantly IFN- γ secreting CD4⁺ cells, followed by IL-2 and TNF, while HIV-1 infected blood showed the same profile, but also with some GM-CSF and IL-17 secreting cells. HIV-1 uninfected pericardial fluid showed CD4⁺ cells with the ability to mostly produce GM-CSF, followed by TNF and then IFN- γ , with small proportions of IL-2 secreting cells, while HIV-1 infected pericardial fluid showed CD4⁺ cells with the ability to produce all 5 cytokines studied. No significant differences were apparent when comparing blood and pericardial fluid or when comparing HIV-1 uninfected to HIV-1 infected patients.

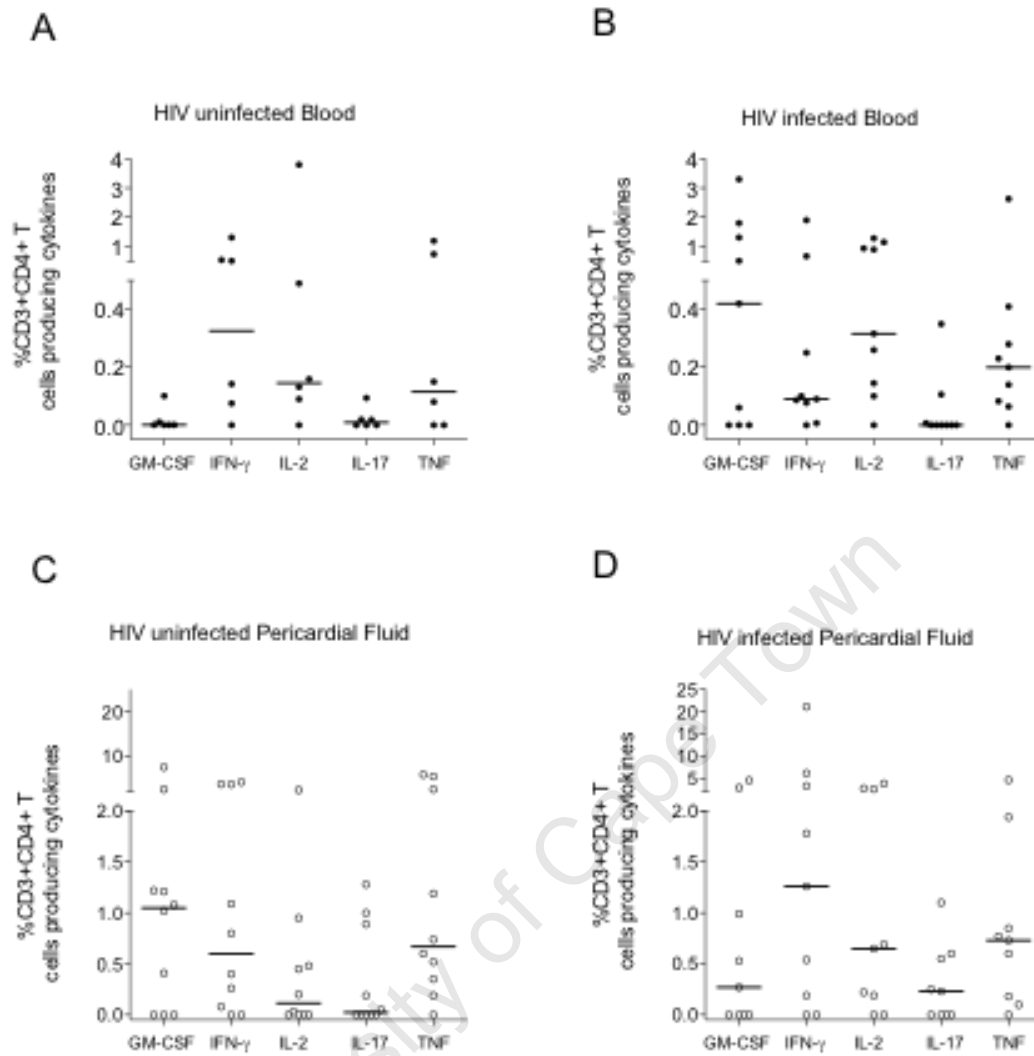


Figure 4.21. Frequency of CD3+CD4+ cells secreting GM-CSF, IFN- γ , IL-2, IL-17 and TNF in response to hKH37Rv stimulation

A. HIV-1 uninfected blood (n=6), B. HIV-1 infected blood (n=10), C. HIV-1 uninfected pericardial fluid (n=10), D. HIV-1 infected pericardial fluid (n=8)

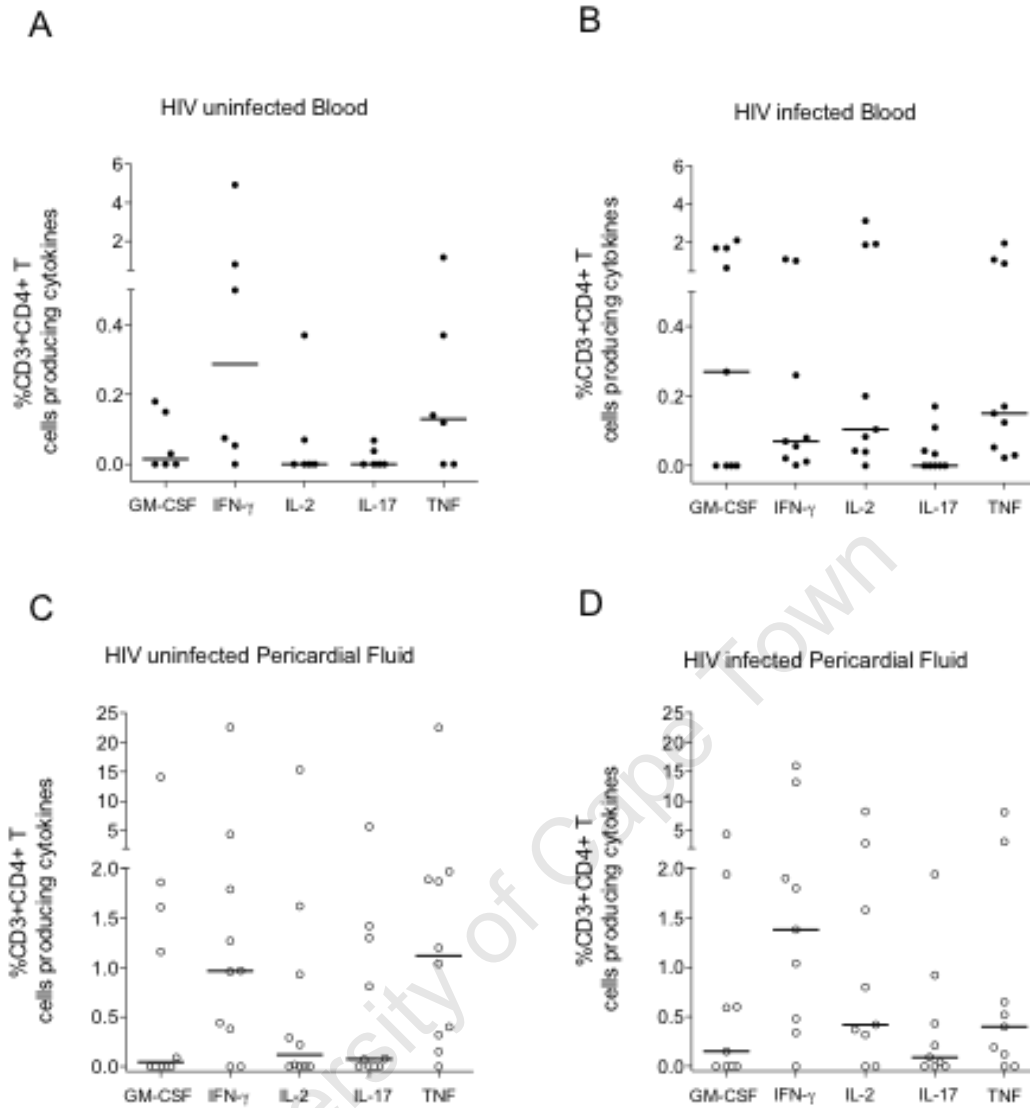


Figure 4.22. Frequency of CD3+CD4+ cells secreting GM-CSF, IFN- γ , IL-2, IL-17 and TNF in response to PPD stimulation

A. HIV-1 uninfected blood (n=6), B. HIV-1 infected blood (n=9), C. HIV-1 uninfected pericardial fluid (n=10), D. HIV-1 infected pericardial fluid (n=9)

PPD stimulation (Figure 4.22) showed a similar trend to the hKH37Rv stimulation, however, both blood and pericardial fluid of the HIV-1 uninfected patients, showed CD4+ cells with the ability to produce mainly IFN- γ and TNF only. The HIV-1

infected blood showed cells mostly secreting GM-CSF, TNF, IL-2 and IFN- γ , while CD4⁺ cells in the pericardial fluid produced IFN- γ , IL-2, TNF and some GM-CSF.

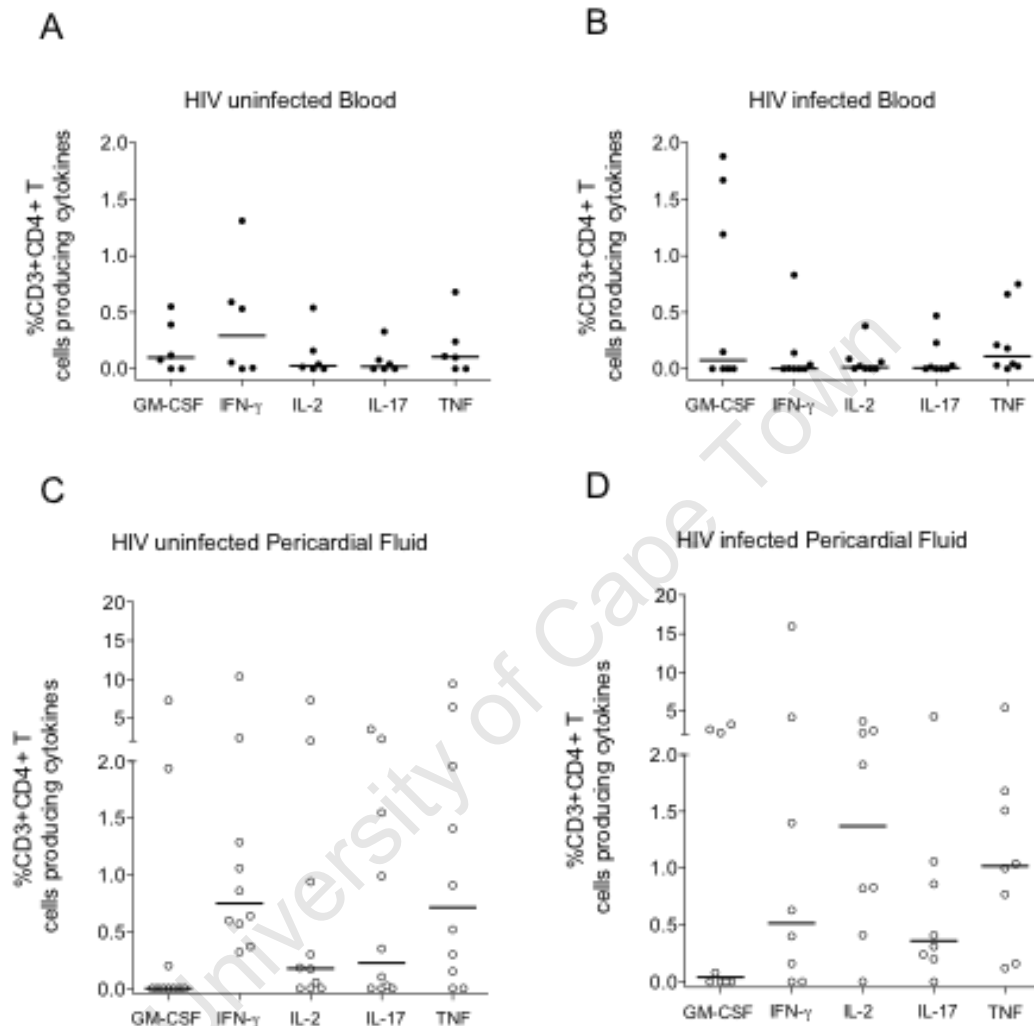


Figure 4.23. Frequency of CD3+CD4⁺ cells secreting GM-CSF, IFN- γ , IL-2, IL-17 and TNF in response to recombinant ESAT-6 + CFP-10

A. HIV-1 uninfected blood (n=6), B. HIV-1 infected blood (n=8), C. HIV-1 uninfected pericardial fluid (n=10), D. HIV-1 infected pericardial fluid (n=8)

ESAT-6 + CFP-10 stimulation (Figure 4.23) of HIV-1 uninfected blood showed low frequencies of detectable intracellular cytokine, with mostly IFN- γ secreting cells,

followed by GM-CSF, TNF and small frequencies of IL-2 and IL-17 secreting CD3+CD4+ T cells, while HIV-1 infected blood cells had mostly TNF secreting cells, followed by GM-CSF, IL-2 and hardly any IFN- γ or IL-17 secreting cells.

When analyzing the pericardial fluid compartment, the HIV-1 uninfected TB pericarditis patients had mostly IFN- γ secreting cells, followed by TNF and small frequencies of IL-2 and IL-17 secreting CD3+CD4+ T cells. The HIV-1 infected patients, on the other hand, had a very different intracellular cytokine profile. Most CD3+CD4+ T cells produced IL-2, followed by TNF, IFN- γ and IL-17, and no GM-CSF.

Blood and pericardial fluid of both HIV-1 uninfected and HIV-1 infected TB pericarditis patients were compared, and while the HIV-1 uninfected patients had similar total cytokine secretion when comparing blood and pericardial fluid, the HIV-1 infected patients, had increased IL-2, TNF and IL-17 secretion by CD3+CD4+ T cells in the pericardial fluid compared to blood (all $p=0.031$). There were no differences when comparing compartments between HIV-1 uninfected and HIV-1 infected TB pericarditis patients.

2.2.6. Determination of the presence of polyfunctional CD4⁺ T cells in the blood and pericardial fluid

Using Boolean gating, the proportions of CD3⁺CD4⁺ T cells with the ability to secrete more than one cytokine were determined. IFN- γ , IL-2 and TNF polyfunctional cells were identified as these are most reported in the literature. The potential role of CD3⁺CD4⁺ T cells expressing various combinations of IFN- γ , IL-2 and TNF has been described in the blood of TB patients and healthy controls vaccinated with novel TB vaccines (137). However, their presence at TB disease sites has not been assessed. I therefore compared frequencies in the blood and pericardial fluid of HIV-1 uninfected and infected patients, and summarized the results for hkB37Rv, PPD and ESAT-6 + CFP-10 stimulations in Figure 4.24 – 4.26.

In the blood and pericardial fluid of the HIV-1 uninfected patients (Figure 4.24), there were low proportions of CD4⁺ T cells expressing all three cytokines in response to hkB37Rv. The majority of antigen specific cells were IFN- γ or TNF single positive, with some cells expressing both IFN- γ and TNF. The overall proportional contribution of CD4⁺ T cells expressing all three, any two, or any one cytokine is represented in the pie charts (Figure 4.27).

In the blood of HIV-1 infected patients, there were mostly IL-2 secreting CD4⁺ cells, followed by some TNF secreting cells. The pericardial fluid showed mostly IFN- γ single secreting cells, followed by cells secreting both TNF and IFN- γ and then IL-2 and TNF single secreting cells. There were no differences when comparing blood and pericardial fluid or when comparing HIV-1 uninfected to HIV-1 infected TB pericarditis patients.

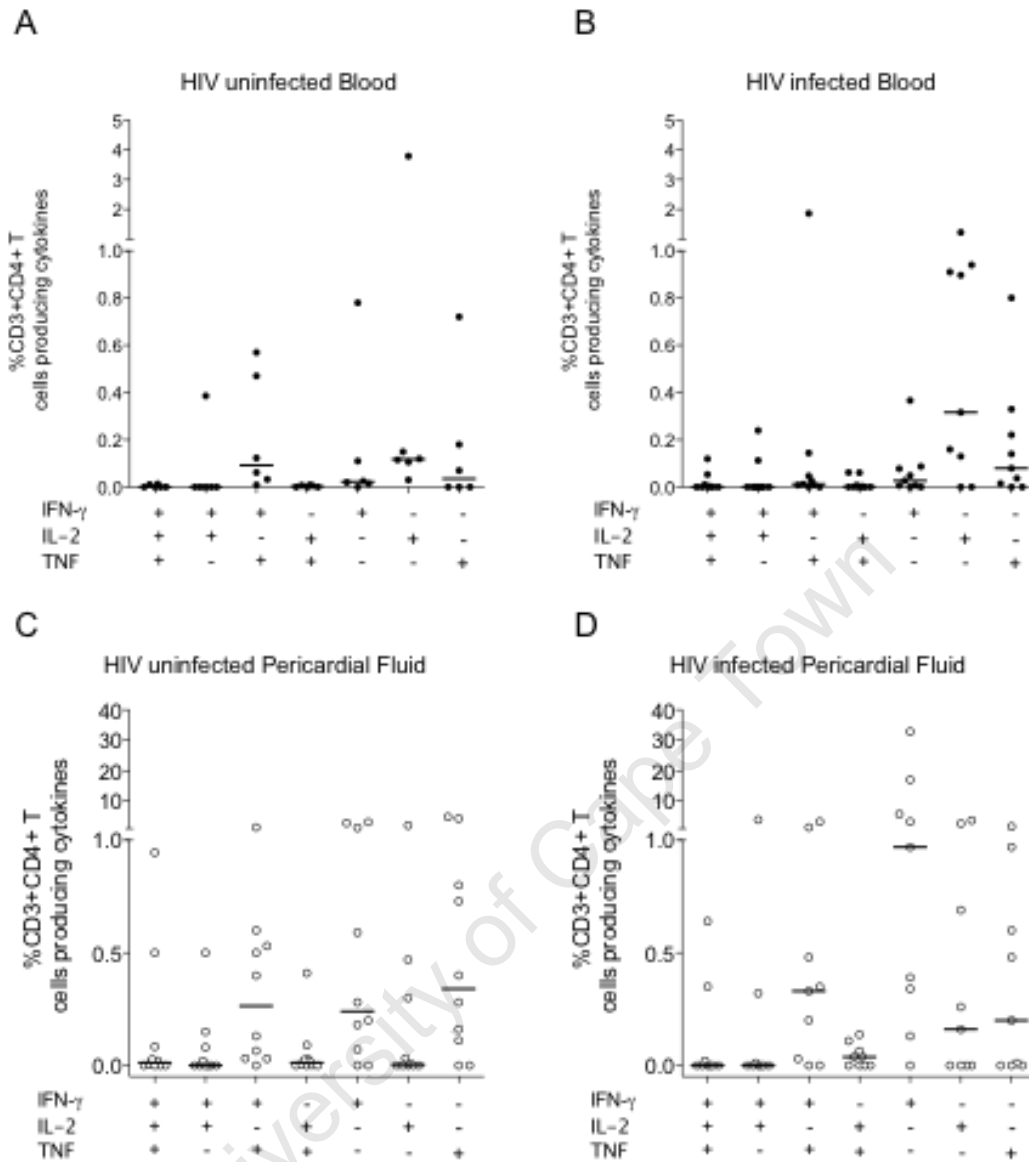


Figure 4.24. Polyfunctional CD3+CD4+ T cells in the blood and pericardium in response to hkh37Rv stimulation

A. HIV-1 uninfected blood (n=6), B. HIV-1 infected blood (n=9), C. HIV-1 uninfected pericardial fluid (n=10), D. HIV-1 infected pericardial fluid (n=9)

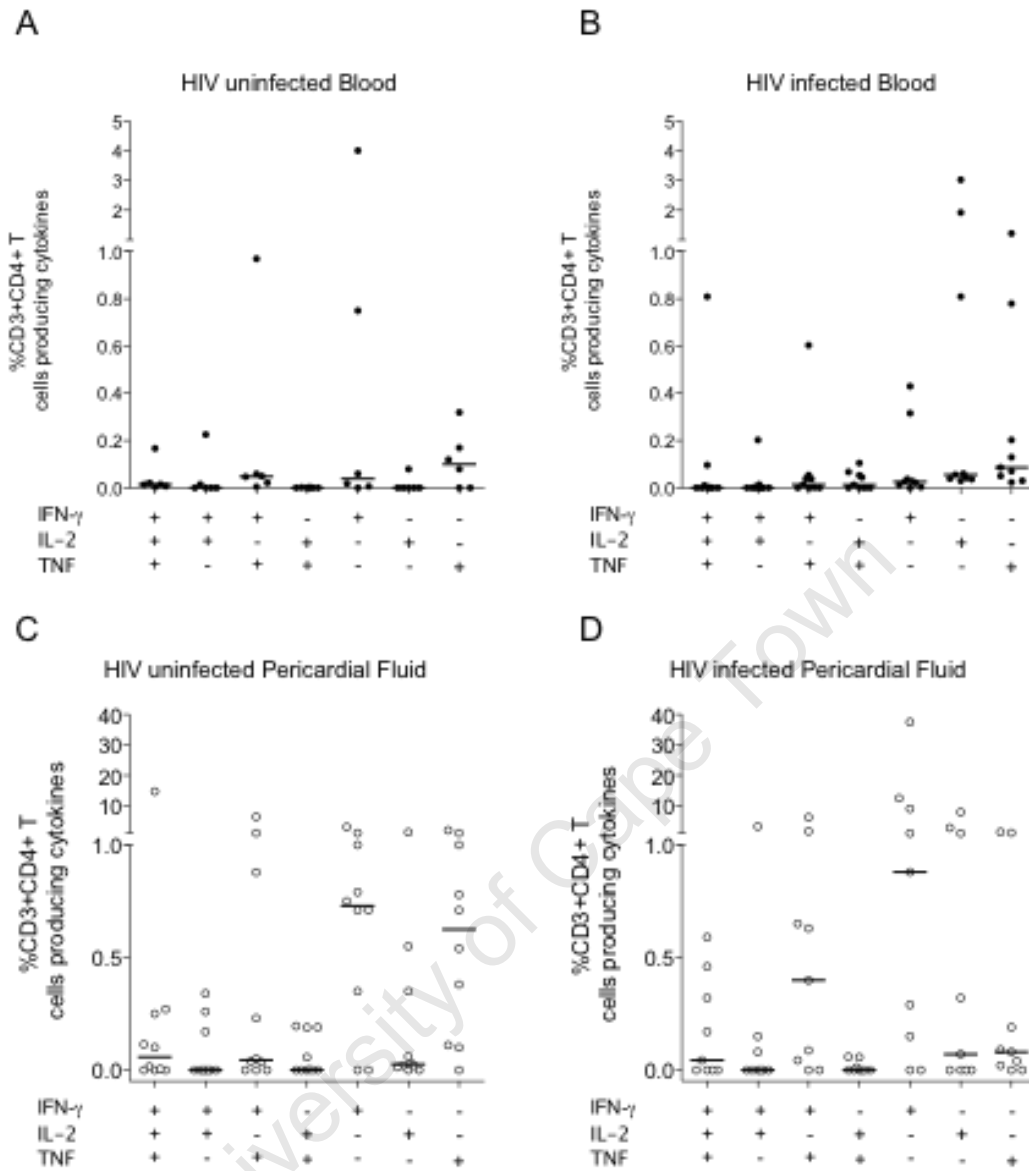


Figure 4.25. Polyfunctional CD3+CD4+ T cells in the blood and pericardium in response to PPD stimulation

A. HIV-1 uninfected blood (n=6), B. HIV-1 infected blood (n=9), C. HIV-1 uninfected pericardial fluid (n=10), D. HIV-1 infected pericardial fluid (n=9)

PPD stimulation (Figure 4.24) of blood from HIV-1 uninfected TB pericarditis patients showed low frequencies of TNF and IFN- γ single secreting cells, followed for cells secreting both these cytokines at the same time. The pericardial fluid also

showed IFN- γ and TNF single secreting cells, some cells expressing TNF and IFN- γ together and a very small proportion of cells secreting all 3 cytokines. When comparing the pie charts of these patients (Figure 4.26), the HIV-1 uninfected blood showed that there was a large proportion of cells expressing all three cytokines. Overall there was very little cytokine secretion by CD4⁺ cells in the blood, such that a small proportion of cytokine secreting cells takes up a disproportionately large piece of the pie.

HIV-1 infected patients showed mostly TNF and IL-2 single secreting cells in the blood, with IFN- γ secreting, followed by IFN- γ and TNF dual secreting cells and then TNF and IL-2 single secreting cells. There was also a small proportion of cells with the ability to produce all three cytokines at the same time.

ESAT-6 + CFP-10 stimulation (Figure 4.25) of blood from HIV-1 uninfected and HIV-1 infected patients showed larger proportions of cytokine secreting cells compared to stimulation with hKH37Rv and PPD. In the HIV-1 uninfected blood, there were mostly single IFN- γ , TNF and IL-2 expressing cells, while the pericardial fluid showed single secreting cells for IFN- γ , TNF and some cells secreting both. HIV-1 infected blood and pericardial fluid showed mostly single cytokine secreting cells, with a small proportion of cells secreting IFN- γ and TNF together and some cells expressing all three cytokines at once.

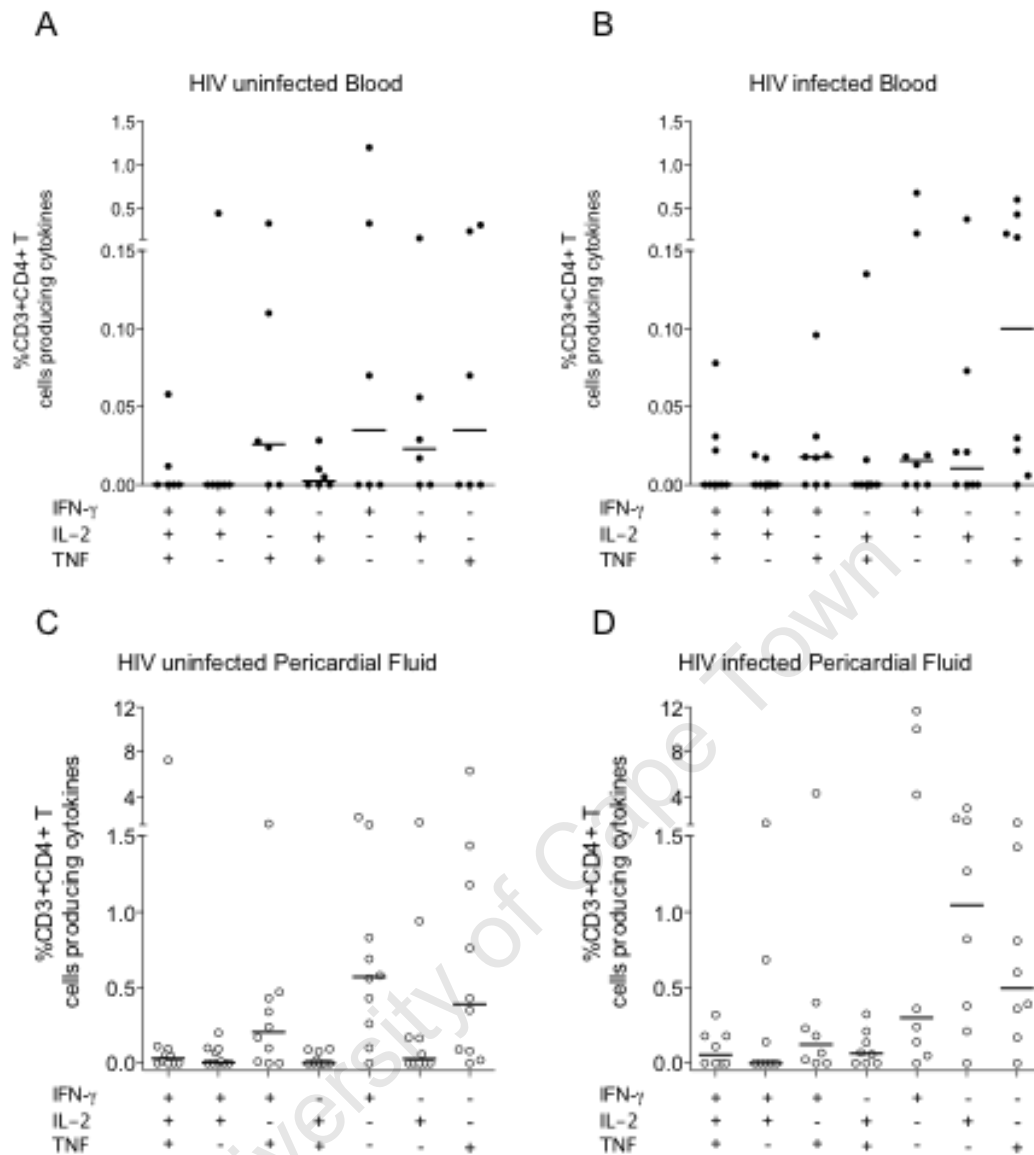


Figure 4.26. Polyfunctional CD3+CD4+ T cells in the blood and pericardium in response to recombinant ESAT-6 + CFP-10 stimulation

A. HIV-1 uninfected blood (n=6), B. HIV-1 infected blood (n=8), C. HIV-1 uninfected pericardial fluid (n=10), D. HIV-1 infected pericardial fluid (n=8)

When comparing HIV-1 uninfected and infected patients (Figure 4.27), the pie charts point towards a greater overall contribution of polyfunctional cells expressing all three and any two cytokines, in the blood of HIV-1 uninfected patients. There was a

shift towards single cytokine expressing cells, with the IL-2 single positive cells proportionally expanded in response to PPD ($p=0.014$) in the blood of HIV-1 infected patients, while the pericardial fluid of HIV-1 infected patients showed a greater tendency towards polyfunctionality.

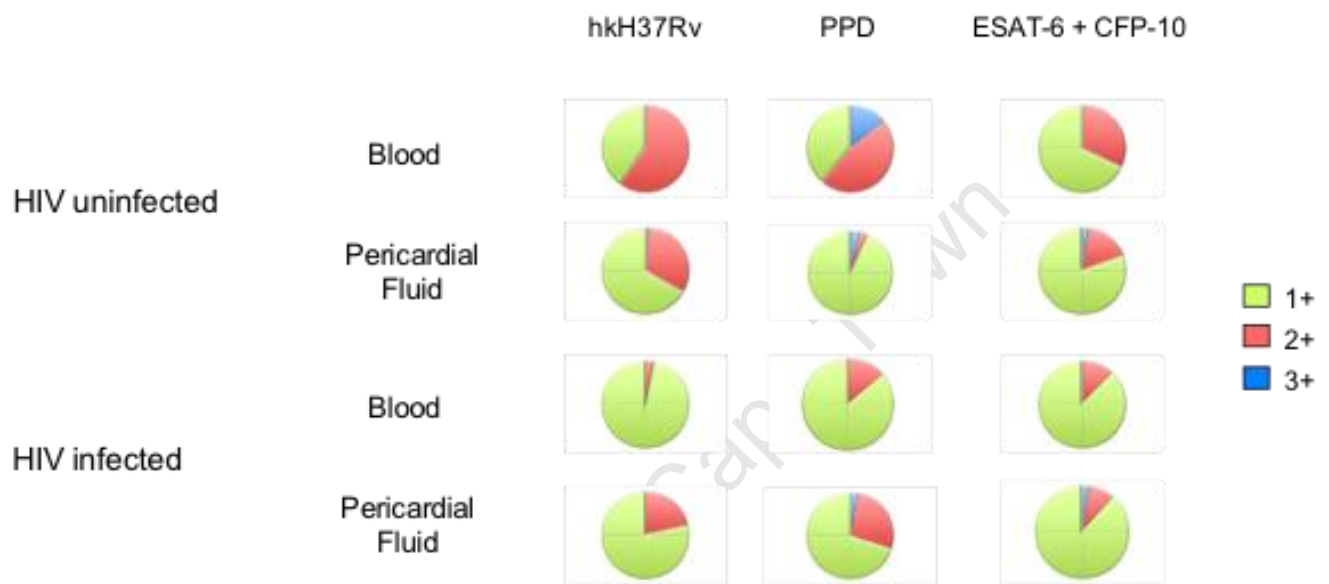


Figure 4.27. Polyfunctional CD3+CD4+ T cells in the blood and pericardium of HIV-1 uninfected and HIV-1 infected TB pericarditis patients

Pie-charts illustrate the overall proportional contribution of cytokine expressing cells in blood and pericardial fluid to the antigen specific response. 1+: any one cytokine, 2+: any two cytokines, 3+: all three cytokines.

As polyfunctional cells include any cell with the ability to secrete more than one cytokine, I analysed the potential of CD4+ T cells to produced GM-CSF, IFN- γ , IL-2, IL-17 and TNF in any combination in blood and pericardial fluid of HIV-1 uninfected and HIV-1 infected TB pericarditis patients.

Stimulation with the positive control SEB (Figure 4.28) showed the capability of the cells to secrete all five cytokines upon stimulation.

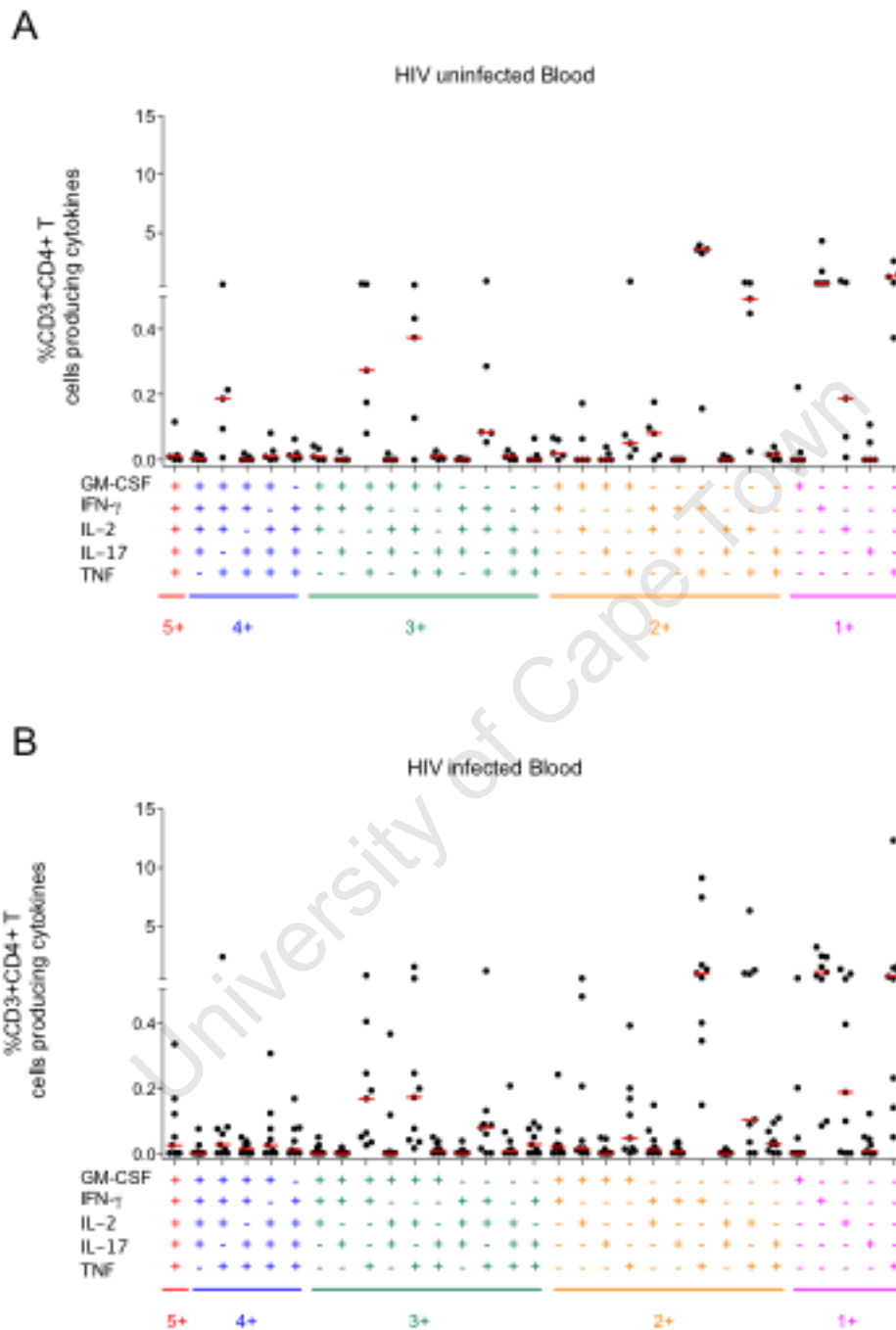


Figure 4.28. Polyfunctional CD4⁺ T cells secreting any combination of GM-CSF, IFN- γ , IL-2, IL-17 and TNF in blood in response to the positive control SEB

A. HIV-1 uninfected Blood, B. HIV-1 infected Blood

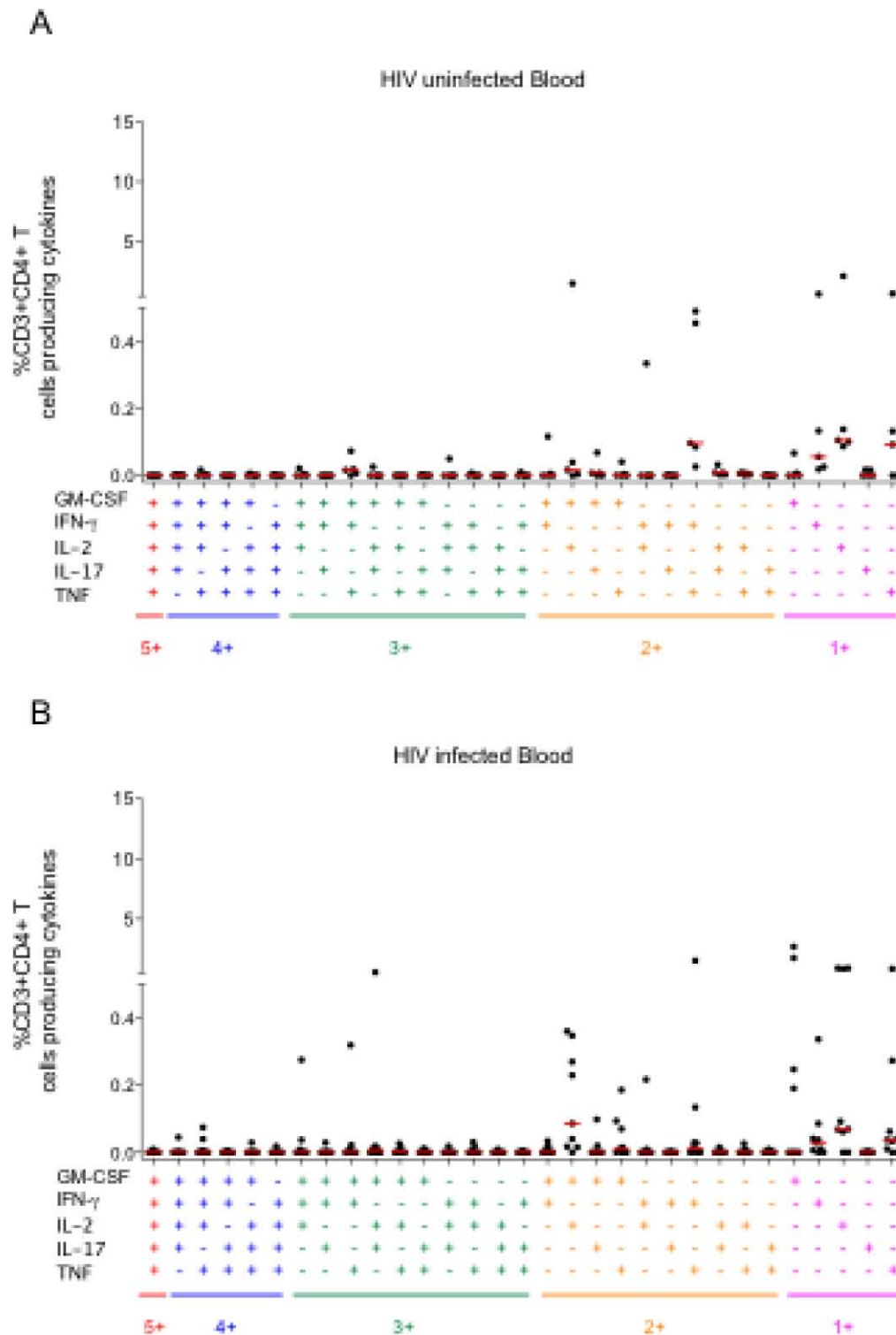


Figure 4.29. Polyfunctional CD4⁺ T cells secreting any combination of GM-CSF, IFN- γ , IL-2, IL-17 and TNF in blood in response to hKH37Rv stimulation

A. HIV-1 uninfected Blood, B. HIV-1 infected Blood

Stimulation of blood with hkH37Rv (Figure 4.29) showed the presence of single positive CD4⁺ T cells mostly secreting IFN- γ , IL-2 and TNF by in both HIV-1 uninfected and infected patients. HIV-1 uninfected patients also showed low frequencies of IFN- γ and TNF dual secreting CD4⁺ T cells in blood, while low frequencies of GM-CSF and IL-2 dual producing CD4⁺ T cells were present in the blood of HIV-1 infected patients.

Stimulation with PPD (Figure 4.30) also showed mainly single cytokine producing CD4⁺ T cells in the blood of both HIV-1 uninfected (IFN- γ and TNF) and HIV-1 infected (GM-CSF, TNF and IL-2) patients. HIV-1 uninfected blood also showed some CD4⁺ T cells with the ability to dually secrete IFN- γ and TNF, while HIV-1 infected patients showed proportions of CD4⁺ T cells able to produce GM-CSF and IL-2, as well as GM-CSF and TNF in combination.

ESAT-6+CFP-10 stimulation (Figure 4.31) showed single cytokine producing CD4⁺ T cells in the blood of HIV-1 uninfected (all cytokines except for TNF) with only GM-CSF single producing CD4⁺ T cells in the blood of HIV-1 infected patients. Both HIV-1 uninfected and HIV-1 infected patients showed CD4⁺ T cells with the ability to secrete GM-CSF and TNF in combination, while HIV-1 uninfected patients also showed some IFN- γ and TNF dual producing CD4⁺ T cells in blood.

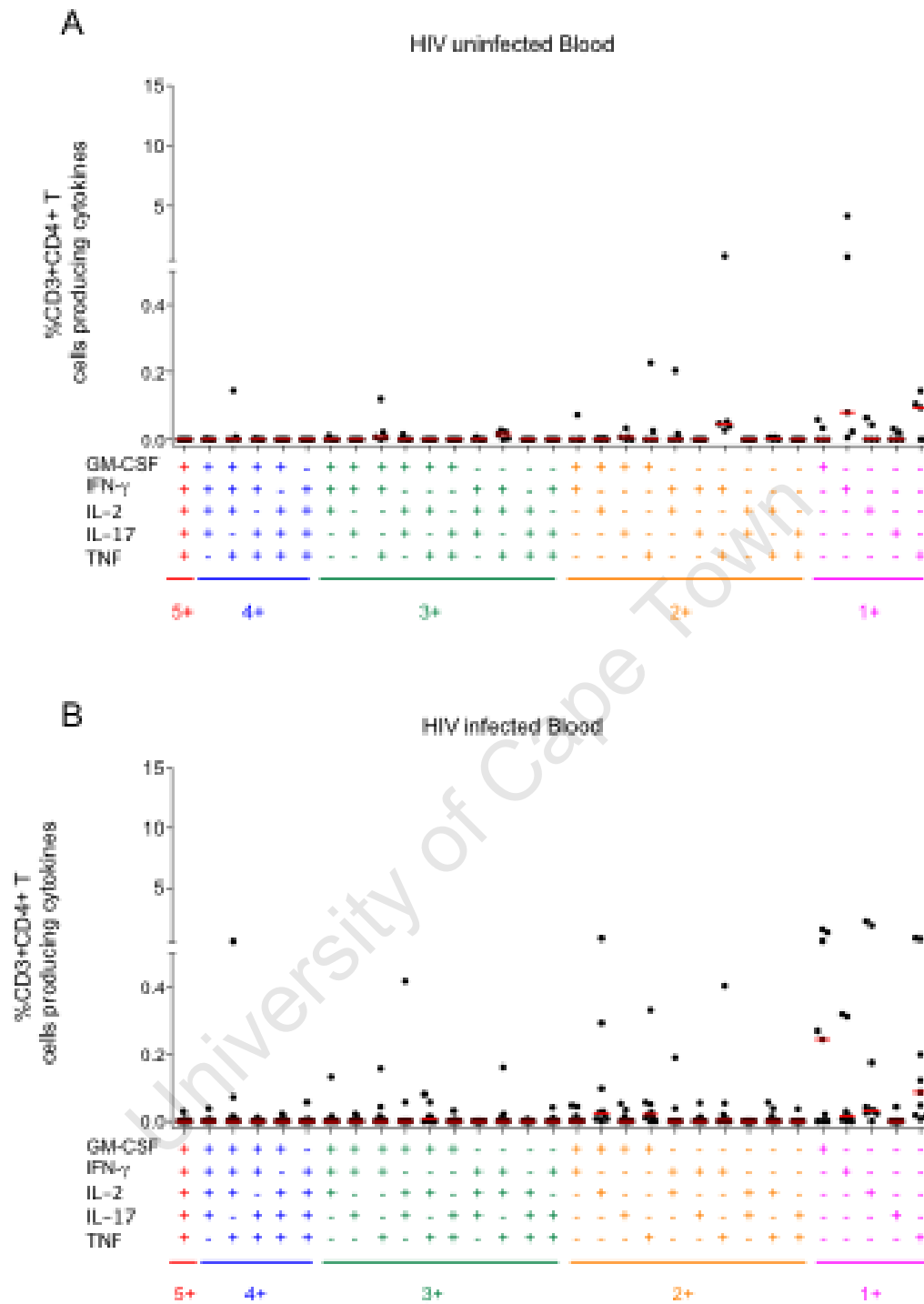


Figure 4.30. Polyfunctional CD4⁺ T cells secreting any combination of GM-CSF, IFN- γ , IL-2, IL-17 and TNF in blood in response to PPD stimulation

A. HIV-1 uninfected Blood, B. HIV-1 infected Blood

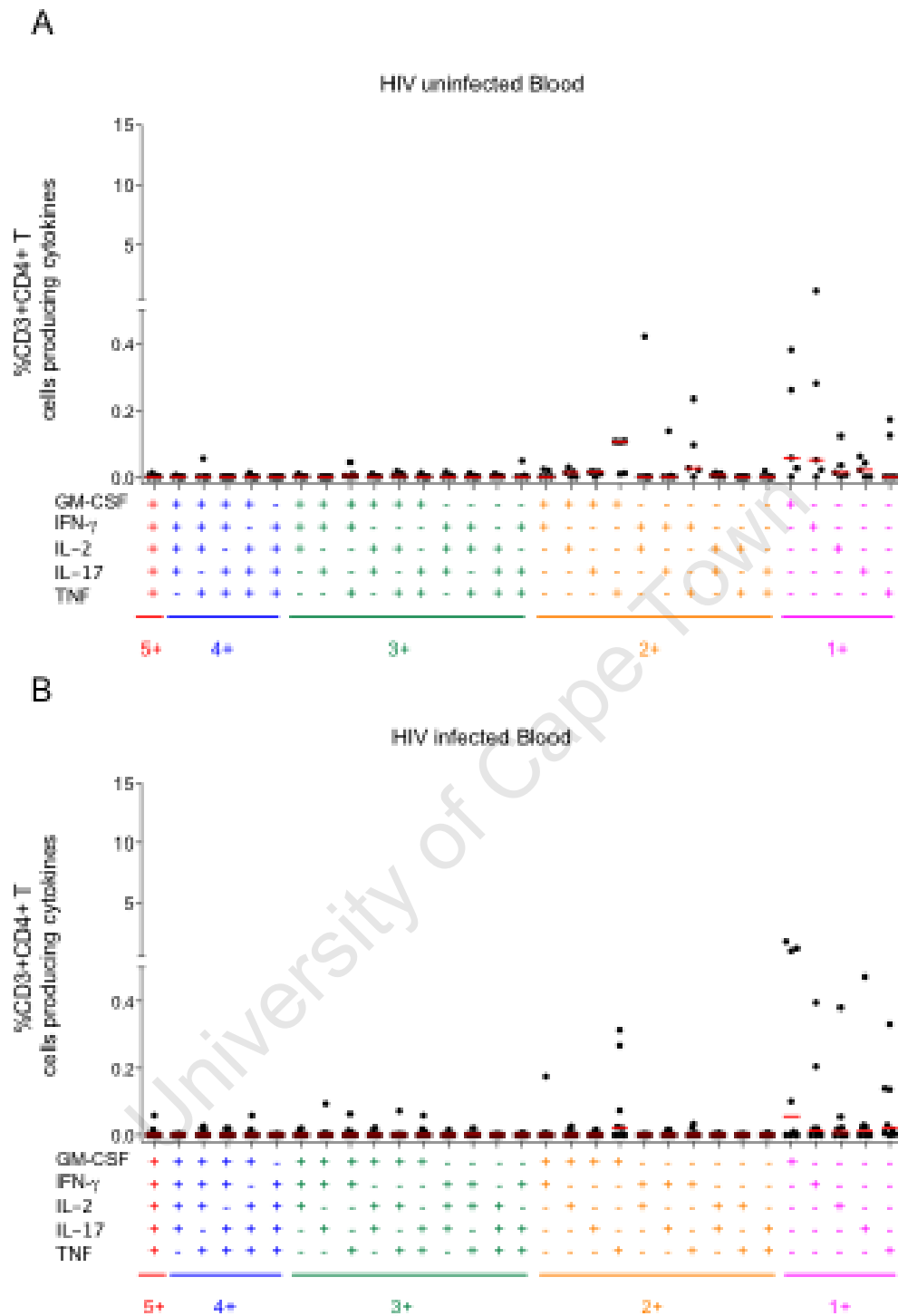


Figure 4.31. Polyfunctional CD4⁺ T cells secreting any combination of GM-CSF, IFN- γ , IL-2, IL-17 and TNF in blood in response to recombinant ESAT-6+CFP-10 stimulation

A. HIV-1 uninfected Blood, B. HIV-1 infected Blood

Pericardial fluid cells are presented in Figures 4.32 – 4.35 and showed some similarities to the blood, but also some different combinations of cytokine producing cells. In response to hkB37Rv stimulation (Figure 4.33), HIV-1 uninfected pericardial fluid showed CD4⁺ T cells producing GM-CSF, IFN- γ , TNF and IL-17 by themselves, with some GM-CSF and IFN- γ , GM-CSF and TNF and some IFN- γ and TNF dual secreting cells. There were also low frequencies of cells able to produce three cytokines in combination; GM-CSF, IFN- γ and TNF as well as GM-CSF, IFN- γ and IL-2 producing CD4⁺ T cells.

Interestingly, stimulation with PPD (Figure 4.34), showed low frequencies of single cytokine producing CD4⁺ T cells in the pericardial fluid of HIV-1 uninfected patients, and higher frequencies of GM-CSF and IFN- γ , IFN- γ and TNF, as well as GM-CSF and TNF dual producing CD4⁺ T cells. There were also low proportions of GM-CSF, IFN- γ and TNF triple positive cells in the pericardial fluid of HIV-1 uninfected TB pericarditis patients. HIV-1 infected patients on the other hand showed mainly IL-2, TNF and IFN- γ single producing CD4⁺ T cells in the pericardial fluid.

Recombinant ESAT-6+CFP-10 stimulation (Figure 4.35) of pericardial fluid showed some single cytokine secreting CD4⁺ T cells (IFN- γ , IL-17 and GM-CSF) in the HIV-1 uninfected patients, but also some dual GM-CSF and IFN- γ as well as GM-CSF and TNF producing cells. Cells with the ability to secrete GM-CSF, IFN- γ , IL-2 and TNF in combination were also detectable in the pericardial fluid of the HIV-1 uninfected TB pericarditis patients. HIV-1 infected patients showed mostly single cytokine secreting cells (IFN- γ , TNF, IL-2 and GM-CSF) but also a small population of GM-CSF, IFN- γ and TNF secreting cells in the pericardial fluid.

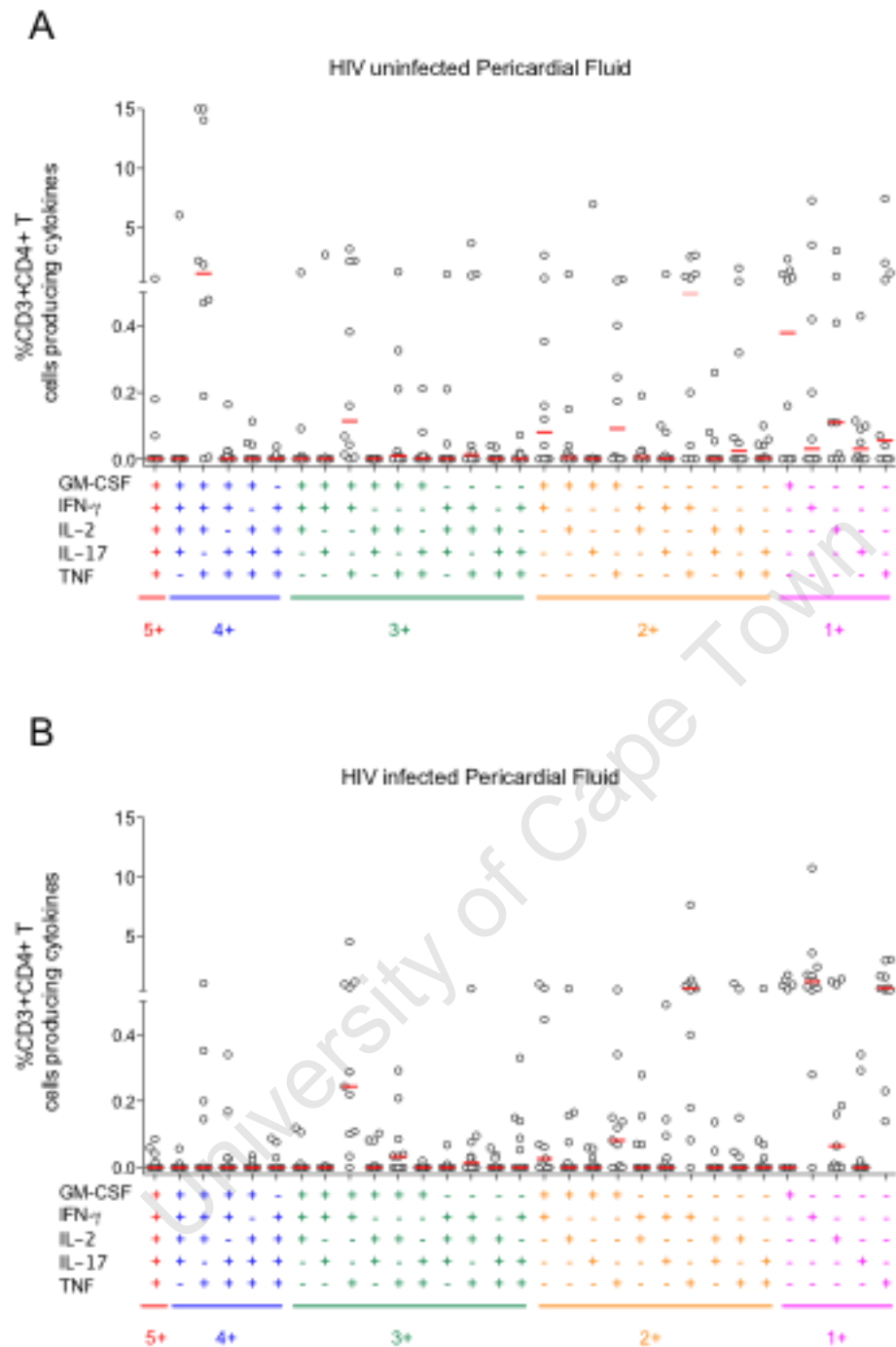


Figure 4.32. Polyfunctional CD4⁺ T cells secreting any combination of GM-CSF, IFN- γ , IL-2, IL-17 and TNF in pericardial fluid in response to the positive control SEB

A. HIV-1 uninfected Pericardial fluid, B. HIV-1 infected Pericardial fluid

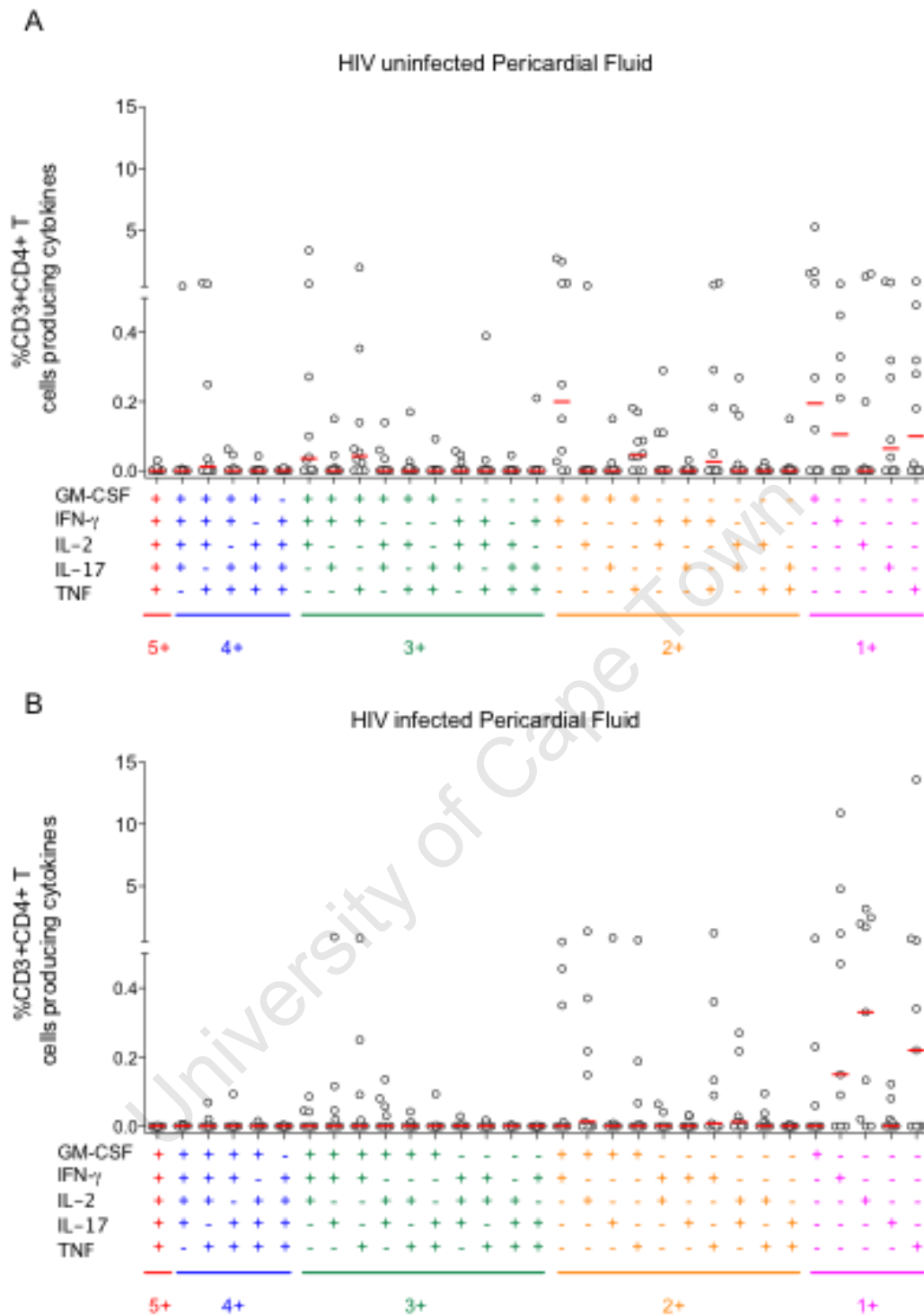


Figure 4.33. Polyfunctional CD4⁺ T cells secreting any combination of GM-CSF, IFN- γ , IL-2, IL-17 and TNF in pericardial fluid in response to hKH37Rv stimulation

A. HIV-1 uninfected Pericardial fluid, B. HIV-1 infected Pericardial fluid

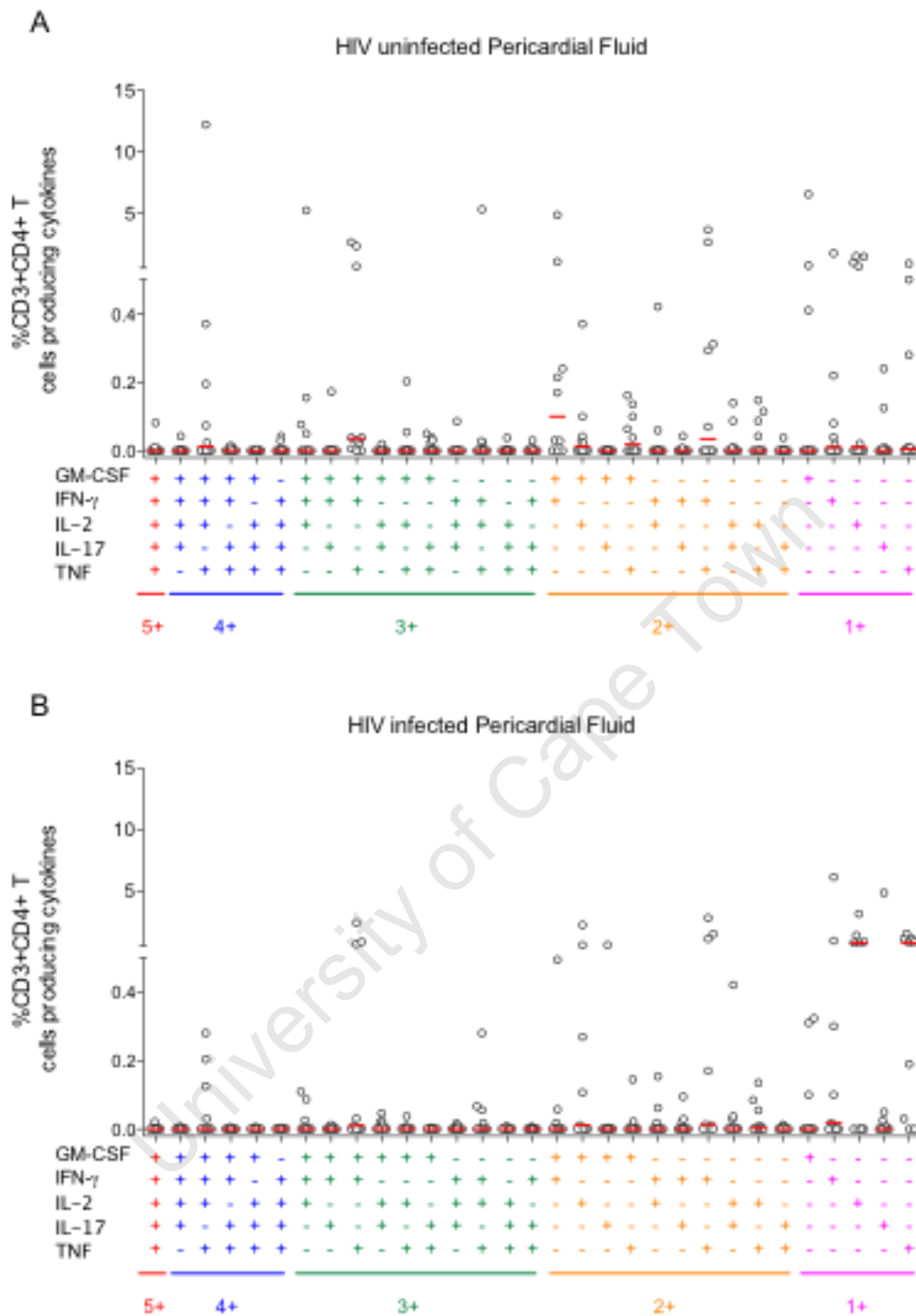


Figure 4.34. Polyfunctional CD4⁺ T cells secreting any combination of GM-CSF, IFN- γ , IL-2, IL-17 and TNF in pericardial fluid in response to PPD stimulation

A. HIV-1 uninfected Pericardial fluid, B. HIV-1 infected Pericardial fluid

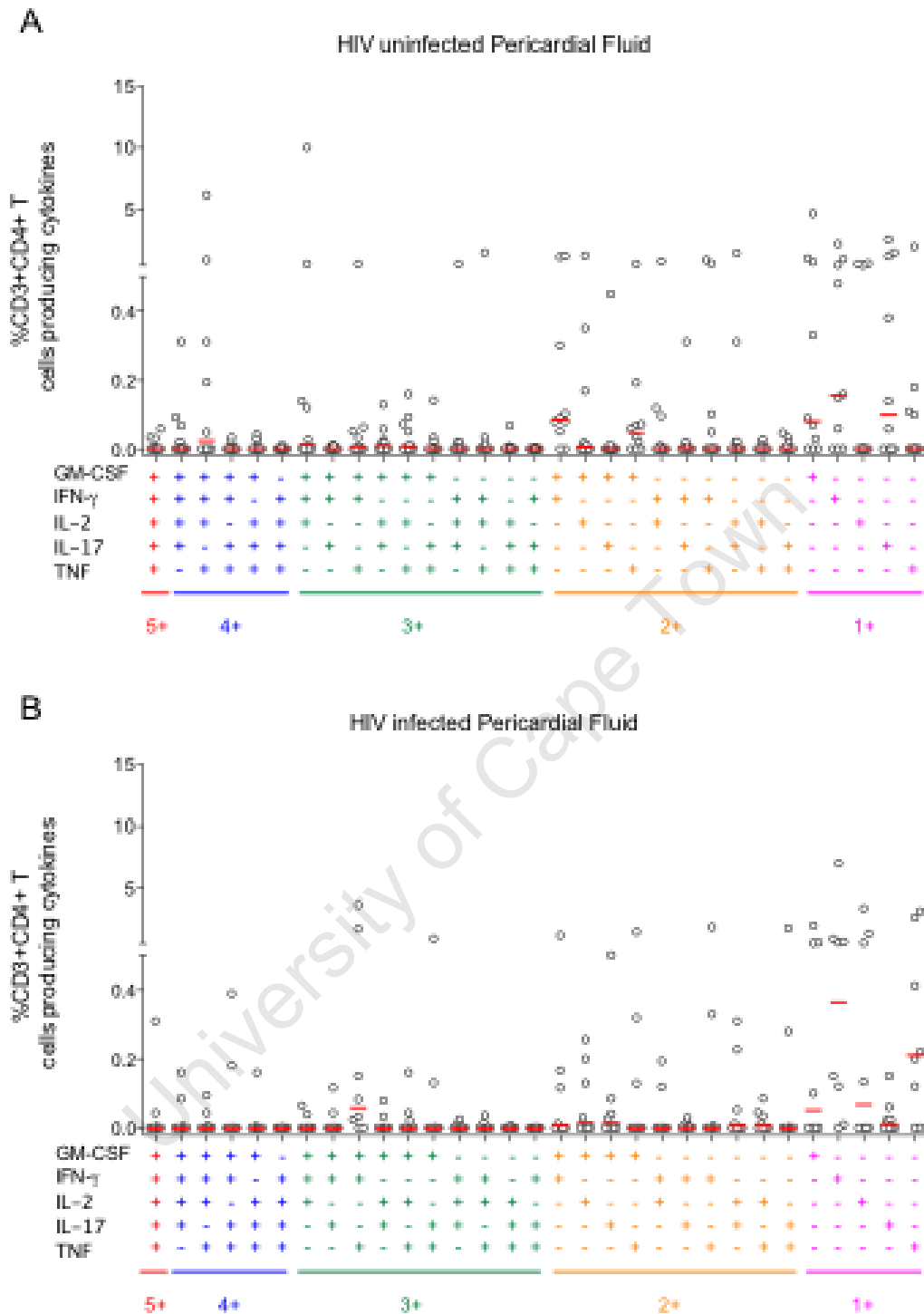


Figure 4.35. Polyfunctional CD4⁺ T cells secreting any combination of GM-CSF, IFN- γ , IL-2, IL-17 and TNF in pericardial fluid in response to recombinant ESAT-6+CFP-10 stimulation

A. HIV-1 uninfected Pericardial fluid, B. HIV-1 infected Pericardial fluid

2.3. CD8+ memory phenotype using 4-colour FACS

Using the same patients and methods as described before, I determined the memory phenotype of the CD8+ T cells present in blood and pericardial fluid. Surface phenotyping (Figure 4.36 and Table 4.8) showed no differences when comparing the frequencies of CD8+ memory cells in blood and pericardial fluid for either HIV-1 uninfected or HIV-1 infected TB pericarditis patients. However, when comparing the numbers of cells in blood and pericardial fluid, in HIV-1 infected TB pericarditis patients, there were significantly increased CD8+CD28+CD45RA- T cells in the pericardial fluid compared to blood (Figure 4.36F and H, Table 4.8, $p=0.023$).

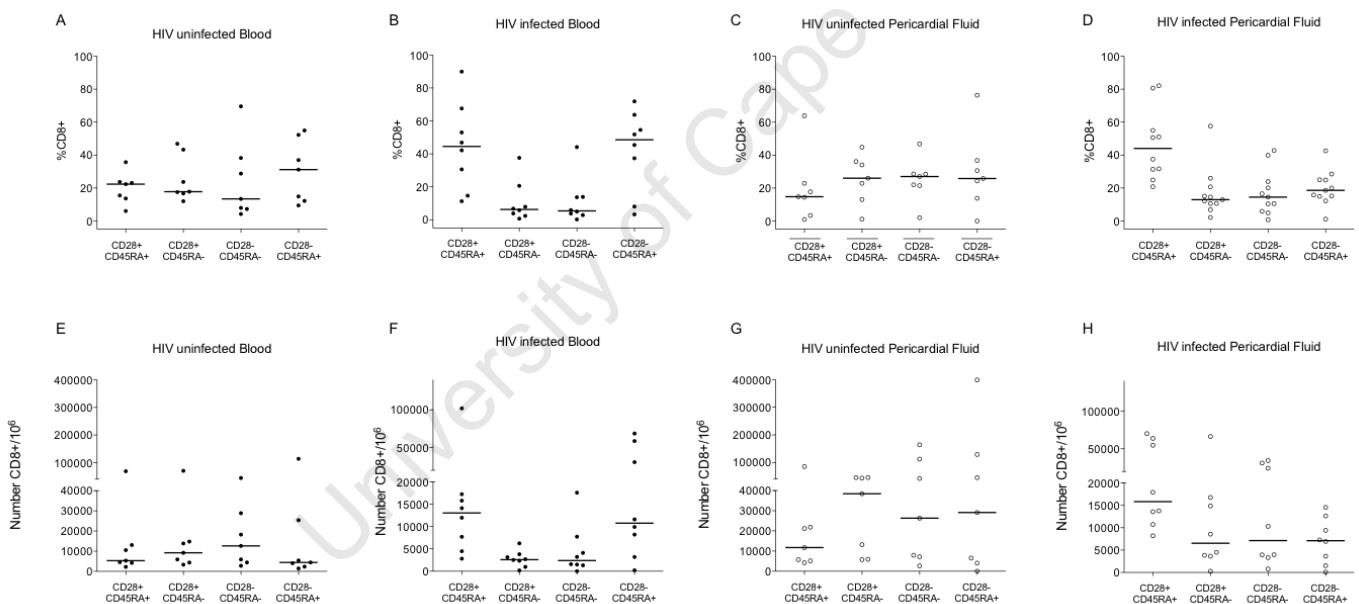


Figure 4.36. Surface phenotype of CD8+ T cells in blood and pericardial fluid

A – D. Frequency of CD8+ expressing CD28 and CD45RA in HIV-1 uninfected ($n=7$) and HIV-1 infected ($n=8$) blood and pericardial fluid

E – H. Number of CD8+ expressing CD28 and CD45RA per 10^6 cells in HIV-1 uninfected ($n=7$) and HIV-1 infected ($n=8$) blood and pericardial fluid

Table 4.8. Frequency of CD8 memory phenotypes in blood and pericardial fluid of HIV-1 uninfected and HIV-1 infected TB pericarditis patients

	HIV-1 uninfected (n=7)			HIV-1 infected (n=8)			HIV-1 uninfected vs HIV-1 infected	
	Blood	Pericardial Fluid	p-value	Blood	Pericardial Fluid	p-value	p-value	
							Blood	Pericardial fluid
CD8+CD28+ CD45RA+	22.4 (13.7 – 23.7)	14.8 (3.4 - 22.9)	0.938	44.5 (18.6-63.90)	44.1 (29.8-61.4)	0.461	0.072	0.010
CD8+CD28+ CD45RA-	17.8 (16.7 – 43.3)	26.0 (13.0 – 36.1)	0.938	6.3 (2.7-17.5)	12.9 (10.5-20.6)	0.219	0.029	0.147
CD8+CD28- CD45RA-	31.2 (12.2 – 52.3)	25.8 (13.8 – 36.8)	0.938	5.4 (3.1-13.9)	14.5 (5.9-23.9)	0.742	0.189	0.147
CD8+CD28- CD45RA+	13.4 (7.3 – 38.3)	27.0 (21.5 – 28.6)	0.578	48.6 (15.3-61.4)	18.6 (15.0-25.0)	0.055	0.463	0.365
CD8+ CD45RO+	12.7 (8.5 - 44.1)	60.0 (12.1 - 65.7)	0.188	16.2 (10.4 - 44.1)	21.3 (15.2 - 44.5)	0.219	0.779	0.432

When comparing HIV-1 uninfected to HIV-1 infected TB pericarditis patients, some differences were noted. There were increased frequencies of CD8+CD28+CR45RA- in the blood (p=0.029) of HIV-1 uninfected compared to HIV-1 infected patients, while there were increased frequencies of CD8+CD28+CD45RA+ in the pericardial fluid (p=0.010) of HIV-1 infected compared to HIV-1 uninfected patients, again showing that HIV-1 infection causes less differentiated cells to be present at the site of disease, even in the CD8 T cell compartment. The total numbers of CD8+CD28+CD45RA- (p=0.004) were decreased, while CD8+CD28-CD45RA-

($p=0.029$) cells were significantly increased in the blood of HIV-1 infected compared to HIV-1 uninfected TB pericarditis patients.

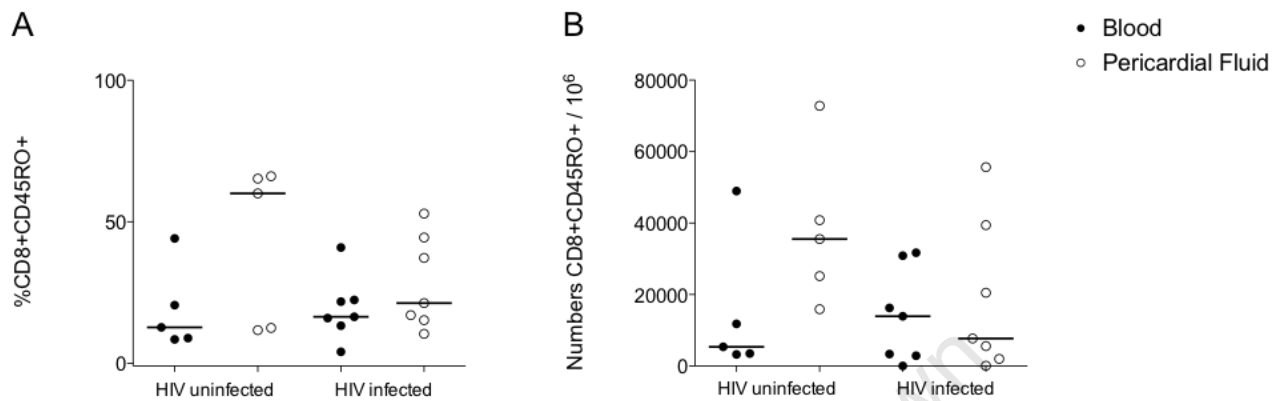


Figure 4.37. CD8+CD45RO+ memory T cells in blood and pericardial fluid of HIV-1 uninfected (n=5) and HIV-1 infected (n=7) TB pericarditis patients

A. Frequency of CD8+CD45RO+ memory T cells, B. Number of CD8+CD45RO+ memory T cells

The total frequency and number of CD8+CD45RO+ memory T cells in blood and pericardial fluid of HIV-1 uninfected and HIV-1 infected TB pericarditis patients was also determined (Figure 4.37 and Table 4.8). There were no differences when comparing blood and pericardial fluid, or when comparing HIV-1 uninfected to HIV-1 infected patients for either the frequencies or number of CD8+CD45RO+ cells. The number of cells did follow the same trend as the frequencies in blood and pericardial fluid for both HIV-1 uninfected and HIV-1 infected patients.

2.4. Phenotype and function of CD8+ T cells by 8-colour FACS

Similarly to the phenotyping of CD4+ memory T cells, there were no differences on surface phenotypes when using different stimuli. NIL, SEB, hkB37Rv, PPD and ESAT-6 + CFP-10 stimulated samples are shown in Figures 4.38 – 4.42.

It was interesting to note that there were no differences when comparing blood and pericardial fluid for the HIV-1 uninfected TB pericarditis patients, thus showing similar frequencies of the memory CD8+ subsets in both compartments. The HIV-1 infected patients however, showed consistent trends irrespective of stimulation. There were increased CD8+CCR5-CD27-CD45RA+ (PPD, hkB37Rv and NIL $p=0.016$, ESAT-6+CFP-10 $p=0.047$), CD8+CCR5-CD27+CD45RA+ (PPD $p=0.047$, ESAT-6+CFP-10, hkB37Rv and NIL $p=0.016$) and CD8+ cells triple negative for all 3 markers (NIL and ESAT-6+CFP-10 $p=0.016$, hkB37Rv $p=0.031$ and PPD $p=0.047$) in the blood compared to pericardial fluid of HIV-1 infected TB pericarditis patients. The pericardial fluid showed increased proportions of CD8+CCR5+CD27-CD45RA- (All stimulations $p=0.016$) and CD8+CCR5+CD27+CD45RA+ (All stimulations $p=0.016$) T cells compared to blood of the HIV-1 infected patients.

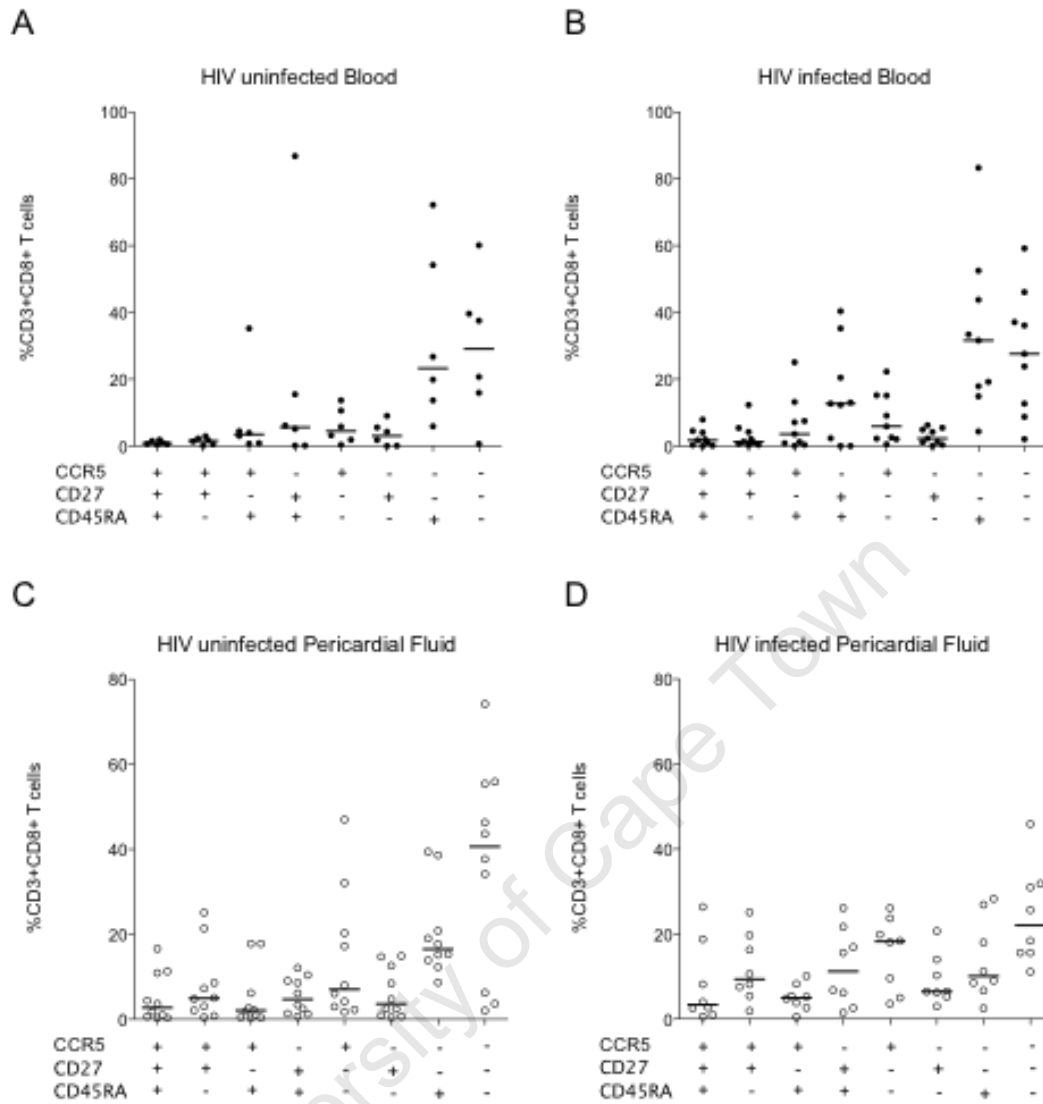


Figure 4.38. Frequency of surface phenotype of unstimulated (NIL) CD3+CD8+ cells in blood and pericardial fluid of HIV-1 infected and HIV-1 uninfected TB pericarditis patients

A. HIV-1 uninfected blood (n=6), B. HIV-1 infected blood (n=9), C. HIV-1 uninfected pericardial fluid (n=10), D. HIV-1 infected pericardial fluid (n=7)

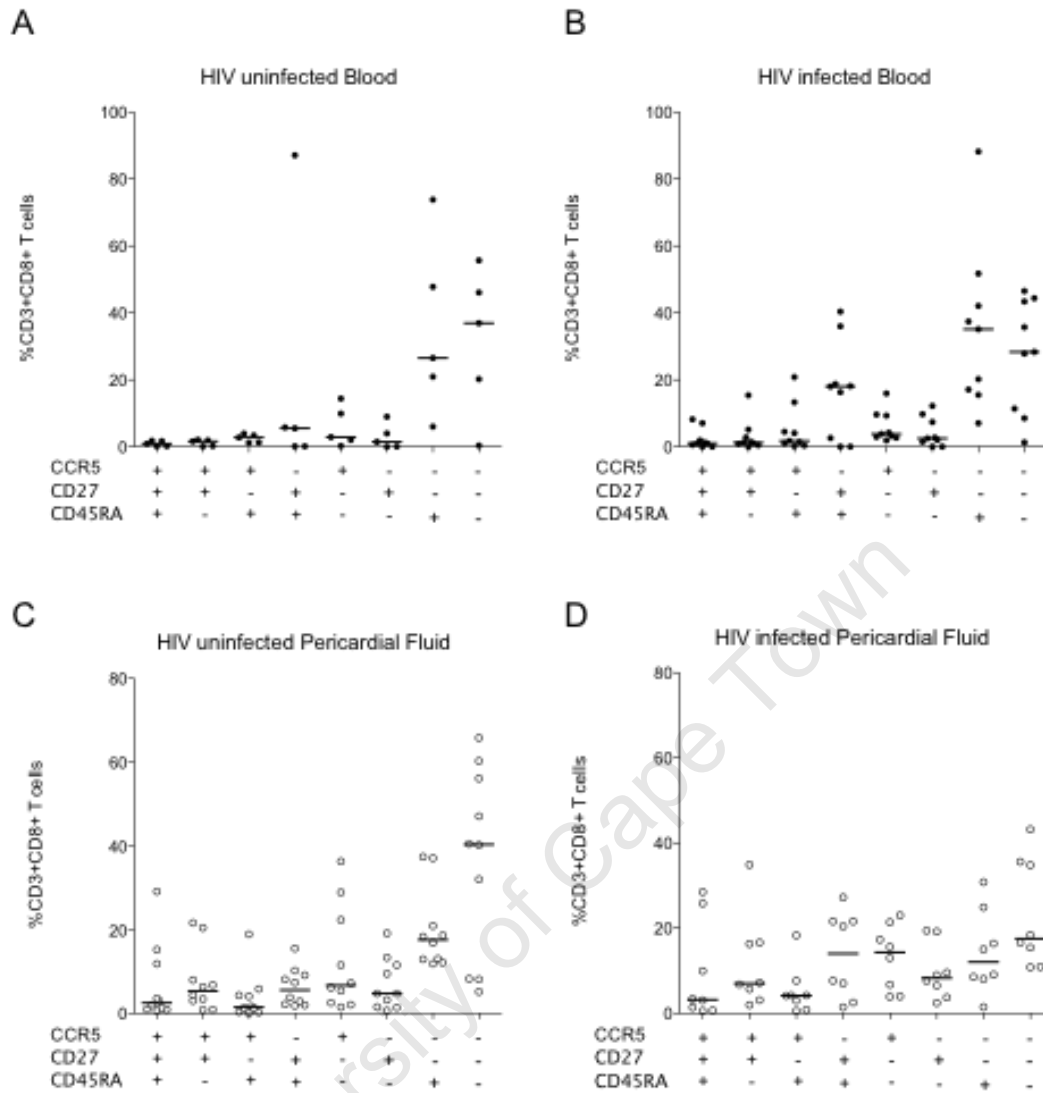


Figure 4.39. Frequency of surface phenotype of CD3+CD8+ cells in blood and pericardial fluid of HIV-1 infected and HIV-1 uninfected TB pericarditis patients when stimulated with the positive control SEB

A. HIV-1 uninfected blood (n=5), B. HIV-1 infected blood (n=8), C. HIV-1 uninfected pericardial fluid (n=10), D. HIV-1 infected pericardial fluid (n=8)

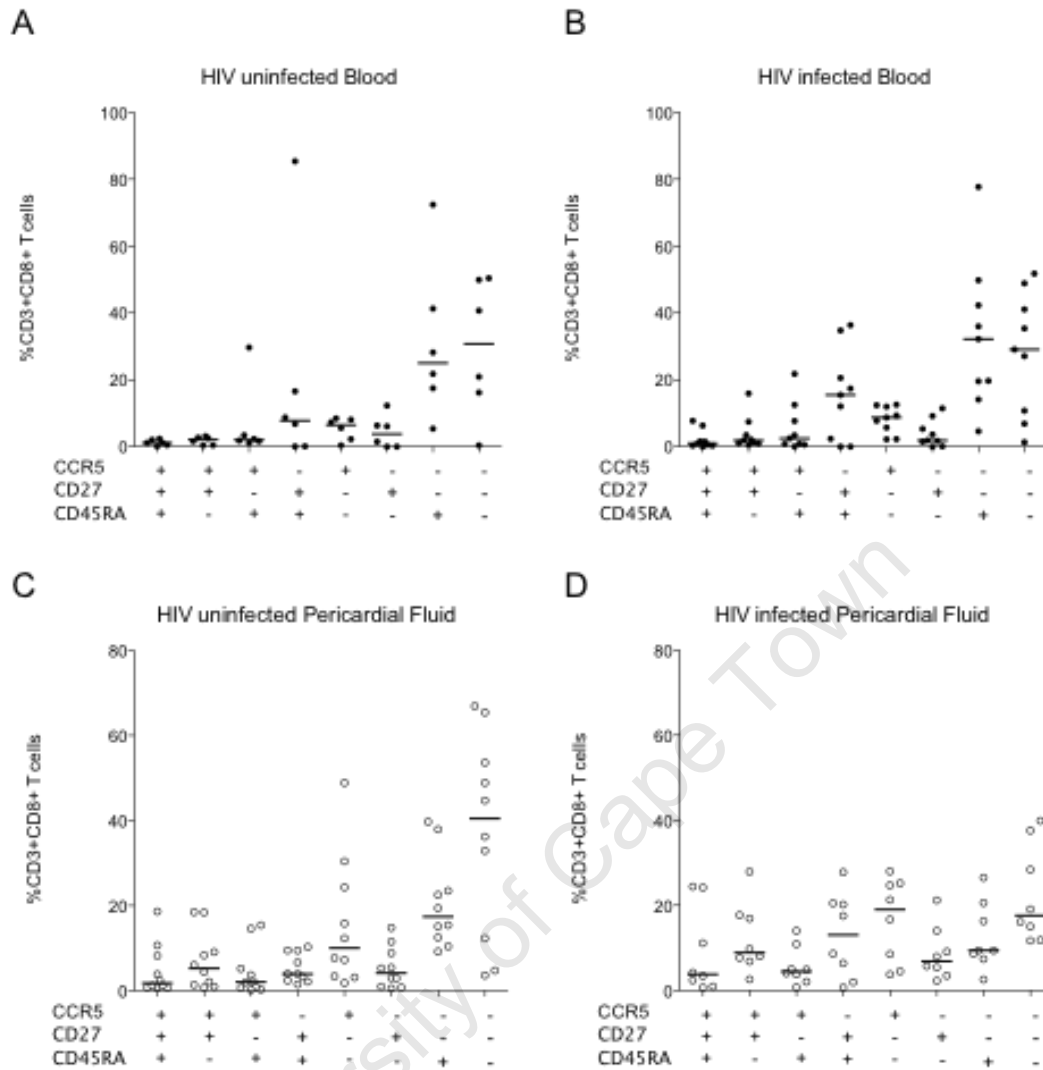


Figure 4.40. Frequency of surface phenotype of CD3+CD8+ cells in blood and pericardial fluid of HIV-1 infected and HIV-1 uninfected TB pericarditis patients when stimulated with hkh37Rv

A. HIV-1 uninfected blood (n=6), B. HIV-1 infected blood (n=9), C. HIV-1 uninfected pericardial fluid (n=10), D. HIV-1 infected pericardial fluid (n=8)

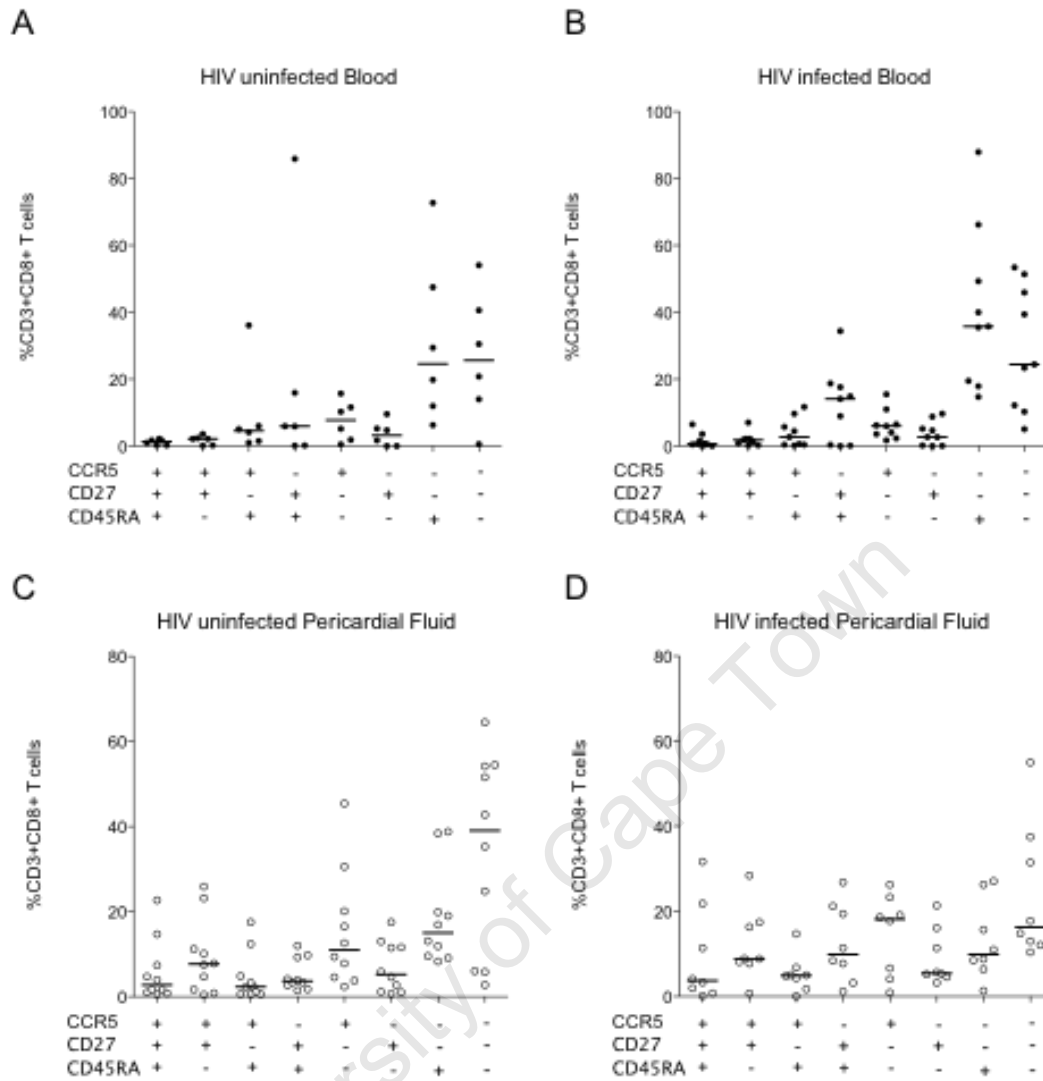


Figure 4.41. Frequency of surface phenotype of CD3+CD8+ cells in blood and pericardial fluid of HIV-1 infected and HIV-1 uninfected TB pericarditis patients when stimulated with PPD

A. HIV-1 uninfected blood (n=6), B. HIV-1 infected blood (n=9), C. HIV-1 uninfected pericardial fluid (n=10), D. HIV-1 infected pericardial fluid (n=8)

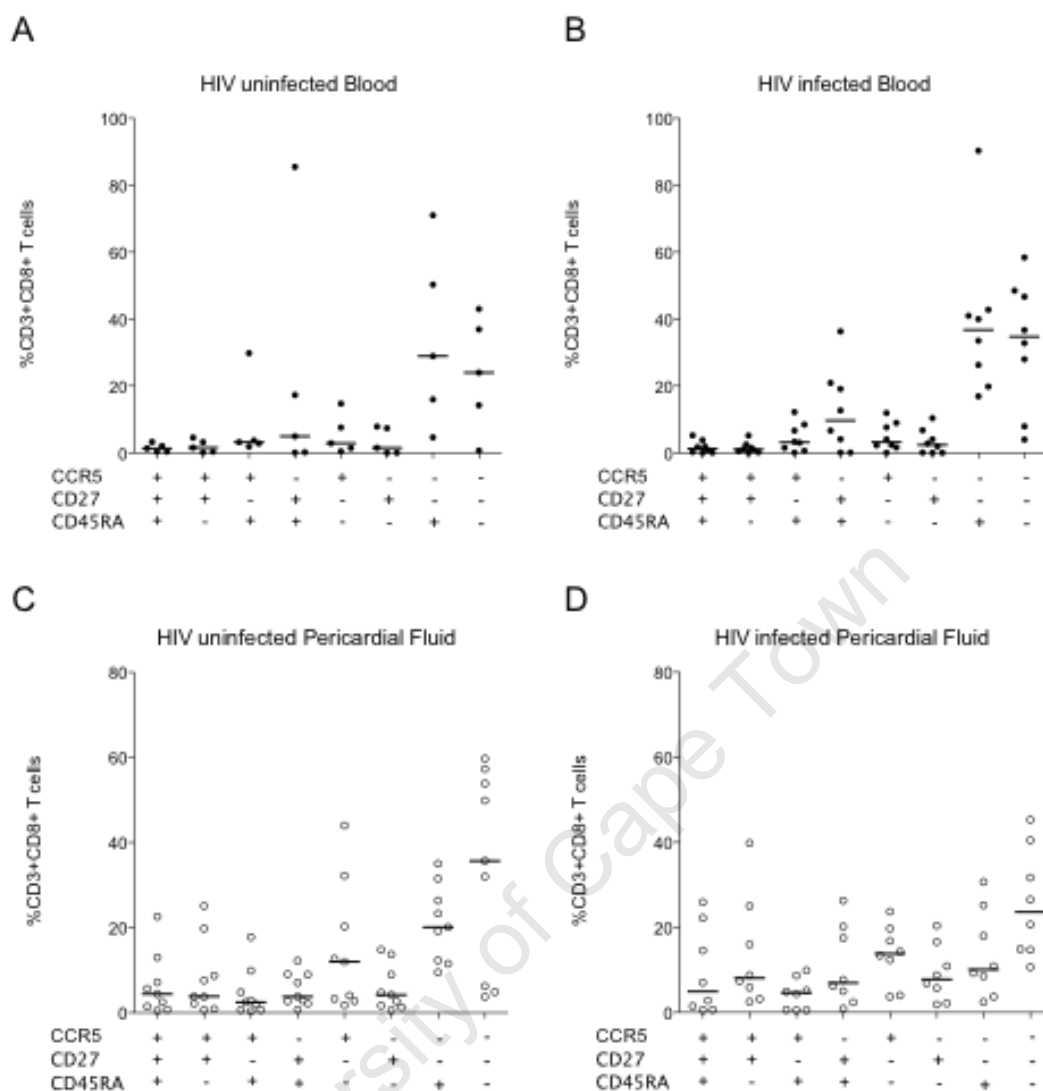


Figure 4.42. Frequency of surface phenotype of CD3+CD8+ cells in blood and pericardial fluid of HIV-1 infected and HIV-1 uninfected TB pericarditis patients when stimulated with the recombinant ESAT-6 + CFP-10

A and B. HIV-1 uninfected (n=5) and HIV-1 infected (n=8) blood

C and D. HIV-1 uninfected (n=9) and HIV-1 infected (n=8) pericardial fluid

There were increased proportions of CD8+ triple negative cells in the pericardial fluid of HIV-1 uninfected compared to HIV-1 infected patients when stimulating with PPD and hkH37Rv ($p=0.0009$) and when not stimulated (NIL $p=0.004$). Blood showed

increased proportions of CD8+CCR5+CD27+CD45RA- (ESAT-6+CFP-10 and PPD p=0.002, hkH37Rv and NIL p=0.0004), CD8+CCR5+CD27-CD45RA- (ESAT-6+CFP-10 p=0.002, PPD, hkH37Rv and NIL p=0.0004), CD8+CCR5-CD27+CD4RA+ (all stimulations p=0.016), CD8+CCR5-CD27-CD45RA+ (PPD p=0.036, NIL and hkH37Rv p=0.0004) and CD8+ triple negative cells (PPD p=0.012, hkH37Rv p=0.002 and NIL p=0.0004) in HIV-1 infected compared to HIV-1 uninfected TB pericarditis patients.

The functional capacity of the memory subsets of CD8+ cells were not analysed as IFN- γ + cells were very few as the stimulating antigens were soluble proteins, which may not be the optimal form to be recognised by CD8+ T cells. The overall cytokine secreting capacity of CD3+CD8+ T cells in response to stimulation with hkH37Rv, PPD and recombinant ESAT-6 + CFP-10 (Table 4.9) after background subtraction were analysed. There was very little cytokine secretion in both blood and pericardial fluid of HIV-1 uninfected and HIV-1 infected TB pericarditis patients and there were no differences when comparing blood and pericardial fluid in each group for any of the stimulations. However, when comparing HIV-1 uninfected to HIV-1 infected TB pericarditis patients, there was significantly increased IFN- γ (p=0.05) and GM-CSF (p=0.005) secreting cells in the blood of HIV-1 infected compared to HIV-1 uninfected patients, while there was increased GM-CSF (p=0.04) secreting cells in the pericardial fluid of the HIV-1 uninfected patients after stimulation by hkH37Rv.

Table 4.9. Proportion of CD3+CD8+ T cells secreting cytokines in blood and pericardial fluid of HIV-1 uninfected and HIV-1 infected patients

CD3+CD8+ T cells secreting cytokines						
hkH37Rv	HIV-1 uninfected			HIV-1 infected		
	Blood (n=6)	Fluid (n=10)	p-value	Blood (n=9)	Fluid (n=9)	p-value
IFN-γ	0.01 (0 - 0.11)*	0.42 (0 - 0.88)	0.188	0.22 (0.04 - 0.3)*	0.11 (0 - 0.44)	0.945
IL-2	0.12 (0 - 0.69)	0.22 (0.03 - 1.68)	0.688	0.43 (0.1 - 0.94)	0.12 (0 - 2.18)	0.238
TNF	0.02 (0 - 0.12)	0.4 (0.04 - 0.61)	0.063	0.05 (0.01 - 0.2)	0.19 (0.04 - 0.39)	0.326
IL-17	0 (0 - 0.14)	0.06 (0 - 0.35)	0.250	0.04 (0 - 0.42)	0.14 (0.01 - 0.28)	0.938
GM-CSF	0.02 (0 - 0.09)§	1.41 (0.2 - 2.7)\$	0.063	0.18 (0.14 - 0.73)§	0 (0 - 0.31)\$	0.461
PPD	HIV-1 uninfected			HIV-1 infected		
	Blood (n=6)	Fluid (n=10)	p-value	Blood (n=9)	Fluid (n=9)	p-value
IFN-γ	0.06 (0.02 - 0.26)	0.03 (0.0 - 1.43)	0.625	0.09 (0.0 - 0.24)	0.0 (0.0 - 0.36)	1.000
IL-2	0.10 (0.03 - 0.24)	1.07 (0.06 - 5.57)	0.094	0.06 (0.01 - 0.17)	0.62 (0.02 - 1.47)	0.195
TNF	0.08 (0.0 - 0.18)	0.38 (0.0 - 1.03)	0.313	0.02 (0.0 - 0.15)	0.18 (0.0 - 0.45)	0.383
IL-17	0.04 (0.01 - 0.11)	0.02 (0.0 - 0.35)	0.438	0.04 (0.0 - 0.17)	0.24 (0.02 - 0.40)	0.469
GM-CSF	0.04 (0.0 - 0.28)	0.50 (0.0 - 1.45)	0.156	0.0 (0.0 - 0.23)	0.04 (0.0 - 0.86)	0.563
ESAT-6+CFP-10	HIV-1 uninfected			HIV-1 infected		
	Blood (n=6)	Fluid (n=10)	p-value	Blood (n=8)	Fluid (n=8)	p-value
IFN-γ	0.02 (0 - 0.13)	0.37 (0 - 1.94)	0.125	0.03 (0 - 0.09)	0.44 (0.12 - 1.86)	0.625
IL-2	0.02 (0 - 0.1)	1.31 (0.24 - 5.51)	0.063	0.01 (0 - 0.08)	0.66 (0.1 - 2.54)	0.875
TNF	0.06 (0 - 0.09)	0.08 (0 - 1.67)	0.313	0.04 (0 - 0.08)	0.33 (0.14 - 0.78)	0.063
IL-17	0 (0 - 0.1)	0.37 (0.02 - 0.65)	0.063	0.01 (0 - 0.5)	0.19 (0 - 0.5)	0.500
GM-CSF	0.04 (0.02 - 0.08)	0.74 (0 - 3.6)	0.125	0.07 (0 - 0.65)	0.5 (0.04 - 2.33)	1.000

* p=0.05

§ p=0.005

\$ p=0.04

2.5. Determination of the viral load in blood and pericardial fluid

Paired serum and cell free pericardial fluid from 10 HIV-1 infected patients with pericardial TB from the above described flow cytometry experiments was sent to the NHLS for measurement of HIV-1 viral load.

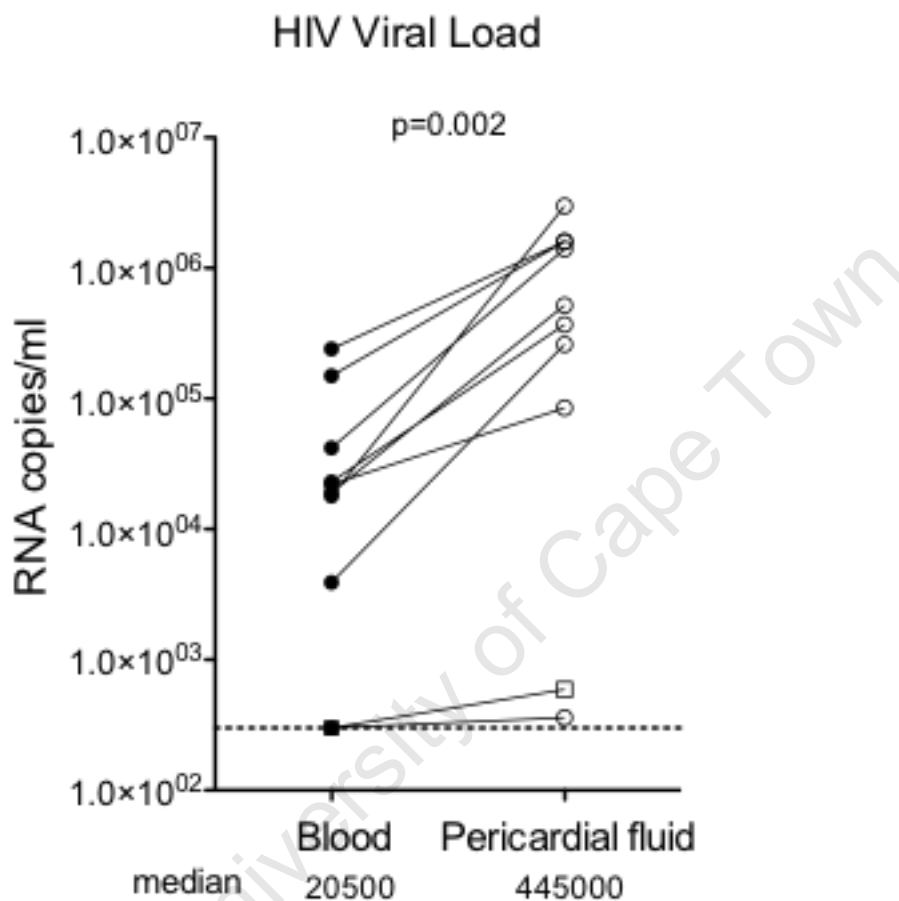


Figure 4.43. HIV-1 viral load is increased in pericardial fluid compared to blood
HIV-1 RNA copies / ml in matched pericardial fluid and serum from HIV-1 infected patients. Squares represent patients on ARV treatment at enrolment into the study.

There were significantly increased HIV-1 RNA copies / ml in the pericardial fluid compared to the serum ($p=0.002$) in these patients, showing that the pericardium is an active site of HIV-1 replication. There was 1 patient on ARV treatment at the time of enrolment into the study and this patient showed suppressed viral load in the blood,

but measurable viral load in the pericardial fluid. The viral load in the pericardial fluid indirectly correlated with the proportion of CD4+ lymphocytes ($r=-0.8095$, $p=0.0218$).

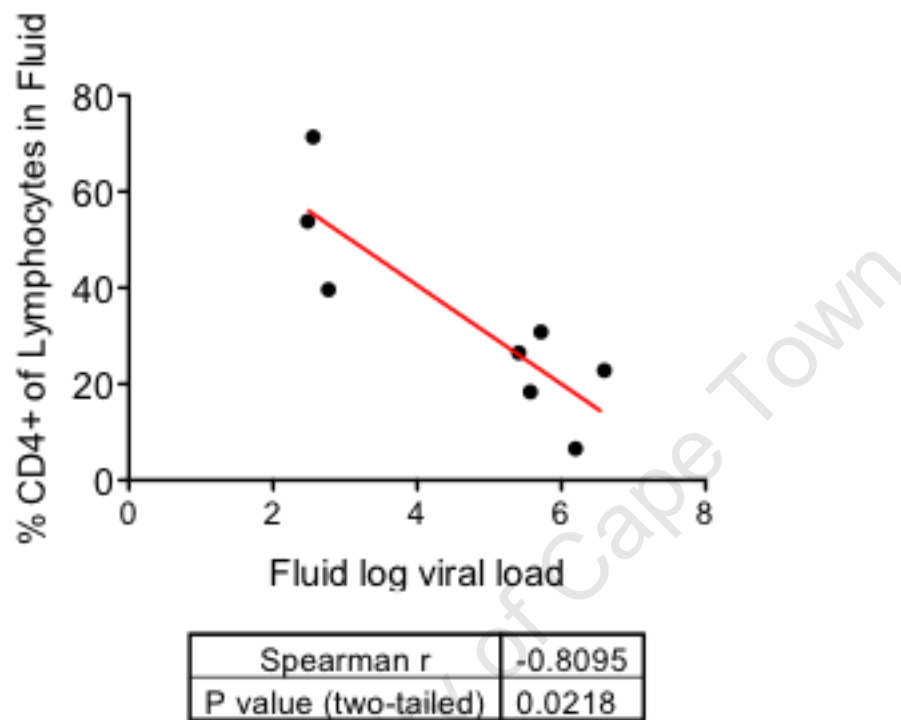


Figure 4.44. Pericardial fluid HIV-1 viral load inversely correlates with the proportions of CD4+ lymphocytes in pericardial fluid.

3. Discussion

I determined the memory phenotype of CD4⁺ and CD8⁺ T cells in blood and pericardial fluid of HIV-1 uninfected and HIV-1 infected TB pericarditis patients using flow cytometry. The overall cytokine secreting capacity of both CD4⁺ and CD8⁺ T cells in both blood and pericardial fluid was also determined, as well as the effect that HIV infection has.

There was a trend towards larger proportions of CD3⁺ lymphocytes in the pericardial fluid compared to blood, significant for HIV-1 uninfected patients only. A previous study comparing radiographically involved lung segment biopsies of pulmonary tuberculosis between HIV-1 infected and uninfected patients also found higher proportions of lymphocytes in the involved segments of HIV-1 uninfected patients (145), together with lower proportions of CD4⁺ T cells, and increased proportions of CD8⁺ T cells in the HIV-1 infected patients. Nevertheless a dominance of CD8⁺ T cells at the disease site of HIV-1 infected patients has been described in pericardial TB patients (80). The detailed surface phenotype of T cells at the disease site in pericardial TB has not been previously described. Using the combination of CD28 and CD45RA, as well as CD27 and CCR5 on CD4⁺ T cells, I found a number of differences between cells present at the site of disease compared to the periphery, and also between HIV-1 uninfected and HIV-1 infected TB pericarditis patients.

The majority of CD4⁺ T cells at the site of disease in the HIV-1 uninfected group were of the more differentiated, antigen experienced phenotype CD45RA-CD28- and CD45RA⁺CD28-, that do not need co-stimulation in order to exert effector function upon antigen recognition. Eight-colour flow cytometry further indicated that the

majority of pericardial fluid cells in HIV-1 uninfected patients expressed the disease site homing CCR5 receptor. By contrast, CD4 T cells at the disease site in the HIV-1 infected patients were skewed towards a less mature phenotype, with larger proportions of CD45RA-CD28⁺ cells, that need co-stimulation, and very small proportions of CD45RA⁺CD28⁻ cells, as well as an almost complete lack of CCR5 positive cells.

The surface phenotype of CD4 T cells responsible for secreting IFN- γ in the pericardium of HIV-1 uninfected patients in response to the *M.tb* laboratory strain H37Rv was terminally differentiated CD27-CD45RA⁺ cells expressing CCR5, while PPD and ESAT-6 + CFP-10 stimulation showed the predominant cells were effector memory CD27⁻ CD45RA⁻ expressing CCR5. In the same time, responses were almost entirely focused on the triple negative CD45RA-CD27-CCR5⁻ cells in the HIV-1 infected patients. It was also interesting to note that there was lack of recognition of hkh37Rv (whole *M.tb*) by the pericardial fluid cells in the HIV-1 infected patients. This may be due to the complex nature of this antigen that needs processing and presentation by antigen presenting cells, which are functionally compromised by HIV-1 infection. The cells were not co-stimulated with anti-CD28 and anti-CD49d as often done in such experiments, as the exact phenotype *ex vivo* was to be determined. Co-stimulation may have resulted in increased proportions of IFN- γ -producing cells, but would not be specific for *M.tb*. The lack of co-stimulation, however, does offer more direct comparison with the ELISpot assay. ESAT-6 and CFP-10 are single recombinant proteins that may be easier for cells to process and present and may result in different and increased antigenic responses.

It is well established that HIV-1 infection is associated with chronic and systemic immune activation (146). Generalized immune activation has also been described in pulmonary TB patients co-infected with HIV-1 (147). The transferrin receptor CD71, expressed on cycling cells, has been shown to represent activated and proliferating T cells (148). CD71 expression on CD4⁺ T cells was assessed and found to be proportionally elevated in HIV-1 infected patients at the disease site, compared to blood. However, when the actual numbers were evaluated, CD4⁺CD71⁺, CD4⁺HLA-DR⁺ and CD4⁺CD45RO⁺ cells were significantly increased in the pericardial fluid compared to blood in the HIV-1 uninfected patients. These cell numbers also tended to be higher than in HIV-1 infected pericardial fluid (not significant), showing that HIV-1 infection may play a role in diminishing the numbers of cells at the site of infection, but not the overall proportions.

Intracellular cytokine staining further indicated the expression of more inflammatory cytokines TNF and IL-17 on CD4⁺ T cells at the site of disease, significantly so in the HIV-1 infected patients. Increased IL-2 secretion was noted at the disease site of the HIV-1 infected patients only. Since IL-2 secretion is characteristic of T cells with a less differentiated phenotype (149), these findings are congruent with the presence of phenotypically less differentiated T cells at the disease site of the HIV-1 infected patients.

It has been described that HIV-1 viral load and HIV-1 replication can affect the maturation of CD4⁺ T cells of other antigenic specificities, such as cytomegalovirus (CMV), towards an earlier, less differentiated state, which might adversely affect their ability to control those pathogens (132). My data indicates, that the same appears true

in case of *M.tb* specific CD4⁺ T cells. The significantly elevated viral load in the pericardial fluid compared to the blood further supports this idea. There was also an inverse correlation of viral load and CD4⁺ cells in the pericardial fluid of HIV-1 infected TB pericarditis patients. The shift of balance towards a predominantly CD8⁺ dominated phenotype at the disease site in the HIV-1 infected patients, without significant *M.tb* antigen specific cytokine secretion by these CD8⁺ T cells indicates that the majority of CD8⁺ T cells at the disease site are not *M.tb* specific, and may rather be HIV-1 specific. The stimulating antigens were proteins, and thus the lack of peptide antigens used to stimulate blood and pericardial fluid cells is another reason for not detecting a more prominent *M.tb*-specific CD8⁺ T cell cytokine response. This further argues that the increase in IL-17 and TNF production, as well as the increased HIV-1 viral load show that the CD8⁺ T cells present at the site of disease are more likely to be HIV-1 specific. My study focused on *M.tb* antigen specific T cell responses, and it was not within its scope to determine HIV-1 antigen specific CD8⁺ T cell responses. However, this aspect warrants further investigation in future studies.

I did however determine the surface phenotype of the CD8⁺ cells present at the disease site. My study has shown skewing of less differentiated CD8 memory cells to the site of disease in HIV-1 infected TB pericarditis patients, compared to blood of HIV-1 infected patients, but also compared to the HIV-1 uninfected TB pericarditis patients. While the expansion of CD8⁺CD28⁻CD45RA⁻ T cells in HIV-1 infected individuals has been reported (150, 151), studies have shown that CD8⁺CD28⁻ T cell accumulation during HIV-1 infection can cause accelerated inflammatory reactions and immune activation (152). It has also been described that HIV-1-specific T cells

preferentially accumulate as preterminally differentiated T cells (CD45RO+CCR7-) (151) and the accumulation of CR45RO+ cells may “block” the terminal differentiation of CD8+ cells during HIV-1 infection (122, 153, 154). Intraindividual diversity of CD45RA+ on CD8+ cells in some HIV-1 infected TB pericarditis patients might represent a population of resting memory cells that may not have come into contact with *M.tb* or HIV-1 antigen for some time, but on antigenic stimulation, might re-express CD45RO (155), however CD8+CD45RO+ cells in blood and pericardial fluid are more prominent in the HIV-1 uninfected TB pericarditis patients. The altered redistribution of memory CD8+ and CD4+ T cells may be caused by high turnover of HIV-1 specific T cells in response to persistent infection, and not by a “block” in the maturation of these cells (151, 156, 157). High viral load could also be an explanation for the lack of terminally differentiated CD8+ T cells (158), cells responsible for cytokine secretion.

Since Natural Killer (NK) cells also produce IFN- γ in response to pathogens (159), CD3 negative cells displaying CD16 and CD56 on their surface were analysed (160). There were very low proportions of NK cells in the pericardium of HIV-1 uninfected patients compared to the blood, indicating that NK cells are less likely to contribute to IFN- γ secretion at the disease site. However, in HIV-1 infected patients, there were similar proportions of NK cells at the disease site and blood, indicating that a proportion of IFN- γ produced at the site of disease could originate from these cells, but total numbers of NK cells were also found to be lower at the disease site of these patients. A reduction of NK cells at the disease site has been previously demonstrated in HIV-1 uninfected pleural tuberculosis patients (161) as well as in HIV-1 infected pericardial TB patients (80).

It has been suggested that regulatory T cells are expanded at the disease sites of tuberculosis patients (162). Intracellular FoxP3 staining marks activated and regulatory CD4⁺ T cells (163). While the proportions of CD3⁺CD4⁺FoxP3⁺ T cells tended to be elevated in both blood and pericardial fluid of HIV-1 infected patients, there were no significant differences between blood and fluid of either patient group. Actual numbers of CD3⁺CD4⁺FoxP3⁺ cells followed the same trend.

Polyfunctional T cells that secrete multiple cytokines, most often studied as various combinations of IFN- γ , IL-2 and TNF, have recently been implicated as both correlates of protection and pathology in tuberculosis (137), highlighting the difficulties of evaluating human immunity to tuberculosis. My data shows that there were low proportions of CD4⁺ T cells secreting all three of the above cytokines in both HIV-1 infected and uninfected patients, and their presence was dependent on the stimulating antigen. It was interesting to note, that while some PPD specific triple positive T cells were present in the blood of HIV-1 uninfected patients, they were essentially absent from the blood of the HIV-1 infected patients. At the same time, low proportions of PPD and ESAT-6 + CFP-10 specific triple positive T cells were detectable at the disease site of both HIV-1 infected and uninfected patients

I have also shown the presence of polyfunctional CD4⁺ T cells secreting combinations of GM-CSF, IFN- γ , IL-2, IL-17 and TNF. Again, there were low frequencies of CD4⁺ T cells secreting the above five cytokines in blood and pericardial fluid of both HIV-1 uninfected and HIV-1 infected TB pericarditis patients. Different combinations of cytokine secreting CD4⁺ cells depended on the

stimulating antigen. The only antigen to elicit secretion of four cytokines in combination by pericardial fluid CD4⁺ T cells was recombinant ESAT-6+CFP-10.

Cytokine responses were infrequent in blood of both HIV-1 uninfected and HIV-1 infected patients, with mostly single and dual cytokine secreting CD4⁺ T cells present. Pericardial fluid also showed mostly single and dual cytokine secreting cells, with some cells producing a combination of three cytokines in response to all three antigens of interest. Of note, HIV-1 uninfected patients tended to produce mostly IFN- γ and TNF, either by themselves, or in combinations with GM-CSF. GM-CSF co-expression with IFN- γ and TNF by *M.tb*-specific CD4⁺ T cells was reported in children (164), but has not to my knowledge been studied at the site of TB disease. GM-CSF was found to be important in maintaining CD4 cell counts above 350 cells/ μ l during acute HIV-1 infection (165). HIV-1 infected patients produced mainly IL-2, with some GM-CSF, IFN- γ and TNF secreting cells, again showing less differentiated CD4⁺ cells in HIV-1 infected patients, consistent with the less mature phenotype of CD4⁺ T cells at the disease site of HIV-1 infected patients. GM-CSF pre-ART levels have shown to be a predictor of future IRIS onset (166) as well as elevated in TB-IRIS PBMC stimulated cultures (167). However, GM-CSF levels were also increased in *M.tb* and HIV-1 co-culture of macrophages *in vitro* and was shown to enhance viral replication (168), but plasma concentrations may be beneficial

Polyfunctional CD4⁺ T cells have been associated with protective immunity (169) but also with TB disease (170) and polyfunctional CD8⁺ T cells were detected in cured TB patients, but not in controls (171). Polyfunctional *M.tb*-specific CD4⁺ and CD8⁺ T cell responses were maintained in the blood of HIV-1 infected patients without

active TB disease, however the functional capacity of these cells were affected by HIV-1 (172). In my study polyfunctional CD4⁺ T cells were more prominent at the site of disease, a site of ongoing HIV-1 replication and chronic inflammation. I have also shown skewing of the CD4⁺ and CD8⁺ memory phenotype towards a less differentiated phenotype possibly resultant from high turnover of T cells at the site of disease. These cells may therefore be a result of pathology of TB pericarditis and may be capable of significant secondary responses at a later stage rather than offering protection during TB pericarditis.

University of Cape Town

4. Conclusion

Until now, little was known about the immune response of the pericardium to tuberculosis infection. This immune response is further complicated by the presence of HIV-1 co-infection. My data shows that HIV-1 replication at the disease site affects the maturation of *M.tb* specific CD4⁺ and CD8⁺ memory cells, resulting in the presence of less differentiated cells at the site of disease. The functional capacity of both CD4⁺ and CD8⁺ T cells were diminished at the site of disease in HIV-1 infected patients. Early antiretroviral treatment may play a role to preserve the capacity of the immune system to control *M.tb* at the site of disease.

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CHAPTER 5

Determination of the mRNA transcript and protein profile of blood and pericardial fluid and the effect of HIV

1. Introduction

The pericardium is a site of active *M.tb* replication, which results in the recruitment of effector T cells (Chapter 4) to the site in order to elicit specific immune responses, such as cytokine release. These cytokines may be transcribed by the cells already at the site of disease and then released to perform their function, or cells newly arriving to the site of disease may release proteins without the need for transcription to occur. There are multiple pathways of genes and their proteins that are needed to efficiently eliminate the *M.tb* bacilli from the site of disease and these have to work coherently together to achieve this.

1.1. *Th1 and Th2 pathway genes and proteins*

IFN- γ is important for activation of macrophages and resistance to *M.tb* infection (173). TNF has an important role in the maintenance of granulomas during latent TB infection. IFN- γ was found to be increased in the lungs of TB patients and is thought to be the hallmark of active TB and has been used in diagnostic settings (174-176). IL-4 on the other hand is a prototypical Th2 cytokine and is usually not detectable (69). The Th1/Th2 balance has nevertheless been postulated as contributing to pulmonary TB (177) and the pattern of cytokines produced at the site of disease may determine the nature of the inflammatory response to TB. Some studies have suggested that mRNA for some cytokine genes such as IFN- γ or IL-4 might be better predictors of the extent of TB disease than IFN- γ protein alone (178). Other cytokines associated with these pathways may also be informative.

IL-2, for example, is responsible for the clonal expansion of antigen specific T cells (179) and is found in the pericardium at lower levels in TB patients compared to those with malignant pericarditis (86). IL-2 is also responsible for the activation of cytotoxic T cells and natural killer cells (69) in response to antigen. IL-6 is a general marker of inflammation at sites of disease and is released by monocytes, fibroblasts and some T cells (180). IL-18 has been described as an IFN- γ inducible factor and is a cytokine with multiple functions that affect both the innate and the adaptive immune responses (181, 182). IL-18, in the presence of IL-12 induces Th1 CD4 T cell polarization, and in the absence of IL-12, Th2 CD4 T cell polarization. IL-24 on the other hand, is a member of the IL-10 family, but is immunomodulatory, opposite to IL-10 (183). IL-24 is decreased in TB patients and mediates pro-inflammatory functions such as enhancing IFN- γ in a manner that is not yet known, and reducing FoxP3 expression (183). IL-24 also activates monocytes to produce TNF and IL-6 (184). CXCL-10, also known as Interferon inducible protein (IP)-10 is expressed in lymphocytes and monocytes and is involved in the recruitment of activated Th1 cells to the site of inflammation (185).

1.2. Th17 pathway genes and proteins

IL-17 was first described as a pro-inflammatory cytokine produced by T cells. Later the T cells specific for the production of IL-17 were classified as distinct from Th1 and Th2 cells, called Th17 cells. IL-17 has a family of 6 members, A-F. IL-17F is 50% homologous to IL-17A, but not all of the effects overlap (186). IL-17A is able to increase IL-6 and other pro-inflammatory cytokine secretion as well as induce neutrophils and their maturation (187, 188). IL-22 is a member of the IL-10 family and is also released by Th17 cells (189), and has recently been described to be

released by a distinct subset of cells, Th22 (190). IL-22 has been associated with many inflammatory diseases and thus may be associated with inflammation during *M.tb* infection (191). IL-23 is made up of a p19 and the p40 subunit of IL-12 and is an essential mediator of inflammation as it induces IL-17 production by Th17 cells (192).

1.3. Regulatory genes and proteins

Regulatory cytokines such as IL-10 are needed to modulate the inflammatory response and thereby prevent damage to the host (193). IL-10 has also been associated with poorer clinical outcome in TB patients and the IFN- γ /IL-10 ratio in pulmonary and extrapulmonary TB patients, including one pericardial TB case, has showed a direct relationship to disease severity (194). TGF- β has a broad spectrum of effects and can be separated into 3 distinct categories; Th17 cell differentiation and proliferation, immunoregulatory effects and enhancement of extracellular matrix (ECM) gene regulation (195). FoxP3, considered to be the most accurate marker of Treg cells, was described in Chapter 4 in detail.

1.4. Neutrophil associated genes and proteins

IL-8 or CXCL-8 is a chemokines that attracts neutrophils, monocytes and T cells, directly and indirectly to sites of inflammation (196). It is essential to normal host responses to *M.tb* and anti-IL-8 treatment has been shown to inhibit granuloma formation (197). IL-8 has been found in BAL in patients infected with TB (198) and was found to be upregulated in patients who had died from TB (199). Neutrophils release antimicrobial peptides called defensins and these target killing by disrupting the organisms microbial membrane (200). Defensins also induce IL-8 expression

from epithelial cells by increasing expression of TNF and IL-1 and decreasing IL-10 by monocytes. Other products released by neutrophils are cathelicidin antimicrobial peptide (CAMP) and lipocalin 2. These neutrophil peptides have been shown to restrict growth of *M.tb* in culture (201). Human neutrophil elastase (HNE) has been implicated in inflammatory damage and is an indicator of neutrophil degranulation (202, 203).

1.5. Growth Factors

Growth factors have been shown to play a role in the ECM remodeling during tissue repair and 3 genes were analysed that may play a role in the repair of the damage caused by *M.tb* infection in the pericardium. Connective tissue growth factor (CTGF) has been implicated in tissue remodeling and affects both the construction of the ECM, by increasing MMP, and destruction of the ECM, by increasing TIMP molecules by activating myofibroblasts (204) and mediating some actions of TGF- β (205-207). Granulocyte macrophage – colony-stimulating factor (GM-CSF) is a white blood cell growth factor that stimulates the production of monocytes and granulocytes (neutrophils) from developing cells. It has a direct effect on fibroblast differentiation to increase wound healing, rather than affecting the proliferation or collagen production by fibroblasts (208-210). Epidermal growth factor (EGF) is another protein that affects cell growth, proliferation and differentiation and is increased in HIV-1 uninfected patients with pleural TB compared to those with pulmonary TB (211).

1.6. Fibrosis related genes and proteins

Shifts in the synthesis versus the breakdown of the ECM regulate the net increase or decrease of collagen. Fibrosis occurs when the synthesis of new collagen by myofibroblasts exceeds the rate at which it is degraded. IL-9 is a growth factor produced by activated Th2 cells (212, 213) and is known to reduce lung fibrosis by reducing collagen production (214). IL-9 is upregulated in PBMC from TB patients (215).

Collagen is produced by fibroblasts and is one of the major components of the extracellular matrix. IL-4 increases collagen production, whereas IFN- γ decreases its production (216), while TGF- β induces collagen production by fibroblasts through the action of Smad 3 (217). CTGF also promotes collagen matrix contraction in fibroblasts (218). Marquis, et al. 2008, identified that Col1a1, Col1a2, Col4a1 and Col4a2 were differentially regulated in mice in response to *M.tb* infection (102). Over production of collagen, by any means, is responsible for fibrotic strand formation and thus fibrosis.

Arginase-1 (ARG1) eliminates nitrogen in the body by catalyzing the hydrolysis of arginine to ornithine and urea (101) and its gene expression is regulated by IL-4 and IL-13 (219, 220). Arginase downregulates chronic inflammation and type 1 responses, protects mice from lethal Th2 pathologies, inhibits CD4 T cell proliferation and cytokine production and suppresses fibrosis (221).

Secreted Protein Acidic and Rich in Cysteine (SPARC) is also known as osteonectin as it was first discovered in bone marrow. SPARC mediates cell-matrix interactions,

acts as a counter adhesive protein and modulates growth factor activity and is a cell-cycle inhibitor (222). It is also known to enhance cell migration and proliferation. SPARC has not been investigated in the context of TB infection before and so direct functions are not known, but in its absence, collagen is decreased in mice (223). SPARC is known to increase the activity of MMP2 (224) and so may regulate the fibrotic response.

1.7. Matrix Metalloproteinases and their endogenous inhibitors

Matrix Metalloproteinases (MMPs) are endopeptidases that use zinc or calcium in their active sites (225). There are 25 MMP molecules that have been identified, and 24 of these are found in mammals (100). MMPs are classified into groups based on their functions (Table 5.1). There are 4 known Tissue Inhibitor of Matrix Metalloproteinases (TIMPs) and these inhibit at a 1:1 ratio of TIMP:MMP through interaction of the TIMP molecule N-terminal domain to the active site of the MMP (226).

MMPs and TIMPs are regulators of tissue repair and remodelling. Dysregulation of either of these molecules can result in fibrosis via various mechanisms. MMP8 and MMP9 can process human IL-8, thereby increasing IL-8 activity and thus neutrophils to sites of disease (100, 227). MMP7 is also a regulator of neutrophil migration through the transepithelial membrane following lung injury (100) and the absence of MMP7 results in a lack of neutrophils. MMPs also have specific effects on cytokines; MMP7 was shown to activate TNF in isolated macrophages (228), while MMP2, MMP3 and MMP9 can activate IL-1 β (229), but MMP3 can also degrade mature IL-1 β depending on the environment (230). MMP3 and MMP9 can activate TGF- β (231,

232). MMPs cleave many chemokines and so regulate the immune response in this way (100). The differential expression of TIMP molecules can also affect the innate and adaptive immune response. TIMP1 is increased in lung injury, which in turn increases vascular permeability and so neutrophils and other inflammatory cells can infiltrate areas of damage (233, 234).

Table 5.1. Classification and function of MMP molecules measured in this study (adapted from (100))

	Group	Function
MMP1	Collagenase-1	Degrades fibrillar collagen I and II
MMP2	Gelatinase-A	Cleaves chemokines, degrades procollagens and elastin
MMP3	Stromelysin-1	Degrades basal membrane collagen IV, laminin and fibronectin
MMP7	Matrilysin	Degrades elastin
MMP8	Collagenase-2	Recruits neutrophils
MMP9	Gelatinase-B	Degrades fibrin, procollagens and elastin
MMP10	Stromelysin-2	Not known
MMP11	Stromelysin-3	Not known

The aim of this part of the study was to determine the mRNA transcript profile of whole blood and pericardial fluid in TB pericarditis patients and to confirm their protein expression by ELISA. The modulatory effect of HIV-1 infection was also determined.

2. Materials and Methods

Matched whole blood and pericardial fluid from TB pericarditis patients, stored at -20°C in PaxGene RNA stabilization tubes until the time of RNA extraction and reverse transcription, as described in Chapter 2, were used for real time-PCR in this part of the study. Patients were firstly selected based on HIV status and TB diagnosis and then on available RNA in each sample. The concentration of RNA to be used for RT-PCR was determined using the nanodrop method. RNA with little or no protein contamination was selected. RNA was used at a total concentration of 1µg per reaction for reverse transcription to cDNA.

The housekeeping gene, human β -actin was used to normalize the measured gene transcript levels for both blood and pericardial fluid using the delta CT method (as described in Chapter 2). Human β -actin gene transcript was measured in duplicate for each blood and pericardial fluid sample, while other genes were assayed only once. In samples where the gene did not reach the cycle threshold in the 40 cycles for either the blood or pericardial fluid, the CT was allocated a 40. If this occurred in both blood and pericardial fluid from a sample, that sample was excluded from analysis and figures depict this.

The genes assayed were categorized according to their function or pathway that they are involved in. The pericardial fluid was also normalized to the blood using the log fold induction method as described in Chapter 2 to allow for easier comparisons between the two compartments.

The proteins of gene transcript measured, that were found to be differentially regulated or involved in the immune response against *M.tb*, were determined using ELISA on matching serum and cell free pericardial fluid (CFF) as described in Chapter 2. Luminex assays were used to determine the MMP protein levels in serum and CFF of TB pericarditis and open-heart surgery control patients. These will also be discussed in this chapter.

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3. Results

3.1. mRNA Transcript profiling

3.1.1. Patient characteristics

A total of 29 patients with TB pericarditis were included in this part of the study and are described in Table 5.1. Of the 29 patients, 12 were HIV-1 uninfected and 17 were HIV-1 infected. There were 11 definite and 18 probable TB pericarditis patients, 19 of whom were male and 10 female. The median age was 32 (28-47) years old with a median CD4 count of 187 (109.5-404.0) cells/ μ l. There were no differences in proportions of definite or probable TB diagnosis, gender or age between HIV status groups. The CD4 count was significantly decreased in the HIV-1 infected patients ($p=0.002$).

Table 5.2. Characteristics of Combined, HIV-1 uninfected and HIV-1 infected TB pericarditis patients included in mRNA transcript profiling

	Combined TB pericarditis (n=29)	HIV-1 uninfected (n=12)	HIV-1 infected (n=17)	HIV-1 uninfected vs HIV-1 infected, p-value
Median Age in years, (IQR)	32 (28-47)	47 (26-69)	31 (28-38)	0.199
Gender, Male/Female (M/F)	19M/10F	9M/3F	10M/7F	0.450
TB diagnosis Definite/Probable	11 Definite/18 Probable	3 Definite/9 Probable	8 Definite/9 Probable	0.273
Median CD4 count cells/μl, (IQR)	187.0 (109.5-404.0)	366.0 (204.3-498.0)	118.0 (77.0-270.5)	0.002
Number on Anti Retroviral treatment	0	0	0	/

3.1.2. Pro-inflammatory Gene Transcripts

Genes were split according to their pathways or roles played in the immune response. This section summarises the genes associated with inflammation. Figure 5.1 and Table 5.3 shows increased gene transcript of IL-1 β ($p=0.006$) and IFN- γ ($p=0.002$) in the blood compared to pericardial fluid of TB pericarditis patients (the lower the delta CT, the higher the gene expression), while there was an abundance of gene transcript for IL-6 ($p<0.0001$) in the pericardial fluid compared to blood when using the delta CT method. TNF was also increased in the pericardial fluid ($p=0.018$) compared to blood. There were no differences between blood and pericardial fluid for IL-2, IL-18, IL-24 or IP-10.

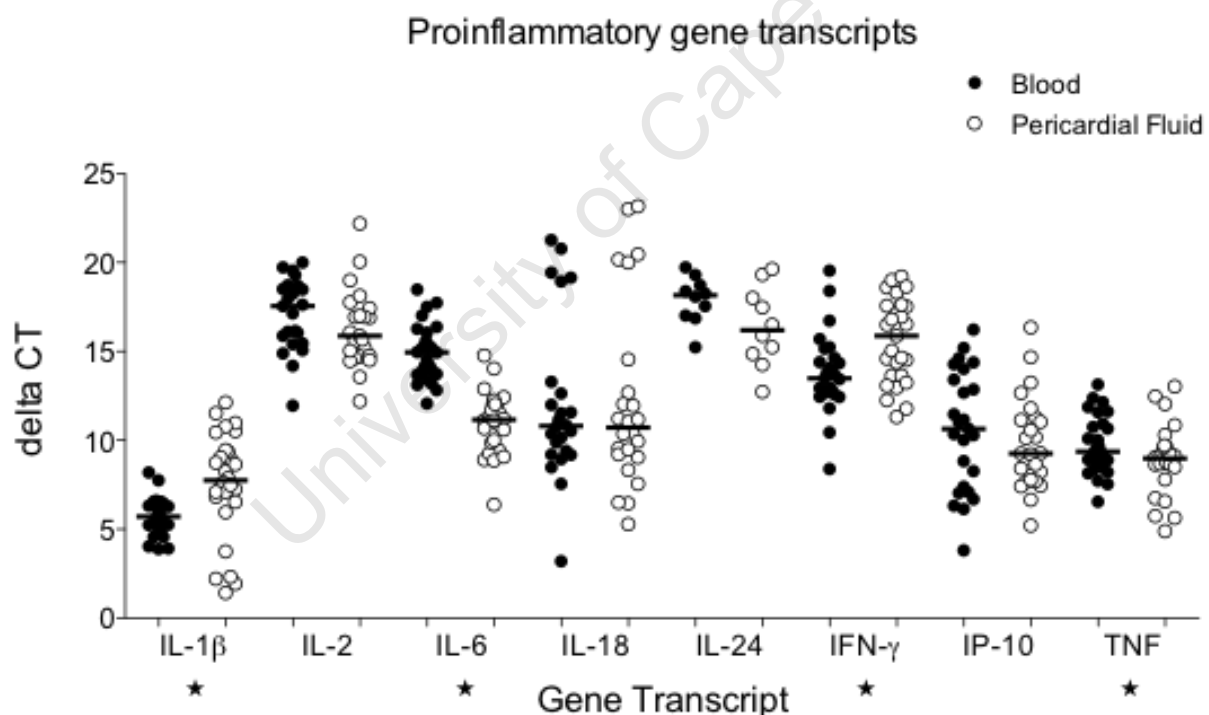


Figure 5.1. Median delta CT of Pro-inflammatory gene transcript levels in whole blood and pericardial fluid of TB pericarditis patients

Stars below the x-axis show significant differences. Patients with undetectable gene transcript in both blood and pericardial fluid were excluded from analysis

Table 5.3. Median delta CT (IQR) of Pro-inflammatory gene transcripts for blood and pericardial fluid for all TB pericarditis patients

Gene	Sample	n=	Median delta CT (IQR)	Blood vs Fluid, p-value
IL-1 β	Blood	28	5.72 (5.1 - 6.31)	0.006
	Fluid	28	7.76 (6.60 - 9.43)	
IL-2	Blood	23	17.6 (15.5 - 18.7)	0.110
	Fluid	23	15.9 (14.7 - 17.4)	
IL-6	Blood	28	15.0 (13.8 - 15.9)	<0.0001
	Fluid	28	11.2 (9.90 - 12.1)	
IL-18	Blood	24	10.8 (9.25 - 13.1)	0.700
	Fluid	24	10.7 (9.09 - 14.1)	
IL-24	Blood	10	18.2 (17.0 - 18.9)	0.131
	Fluid	10	16.2 (14.7 - 18.3)	
IFN- γ	Blood	27	13.5 (12.7 - 14.6)	0.002
	Fluid	27	15.9 (13.6 - 17.6)	
IP-10	Blood	25	10.6 (7.24 - 13.7)	0.275
	Fluid	25	9.26 (7.75 - 11.2)	
TNF	Blood	28	9.36 (8.46 - 10.9)	0.018
	Fluid	28	8.98 (8.53 - 9.62)	

When determining the mean log fold induction of pericardial fluid over blood (Figure 5.2), there was increased IL-2, IL-6, IL-24, IP-10 and TNF in the pericardial fluid of those with measurable gene expression, while, IL-1 β , IL-18 and IFN- γ were decreased in the pericardial fluid compared to blood.

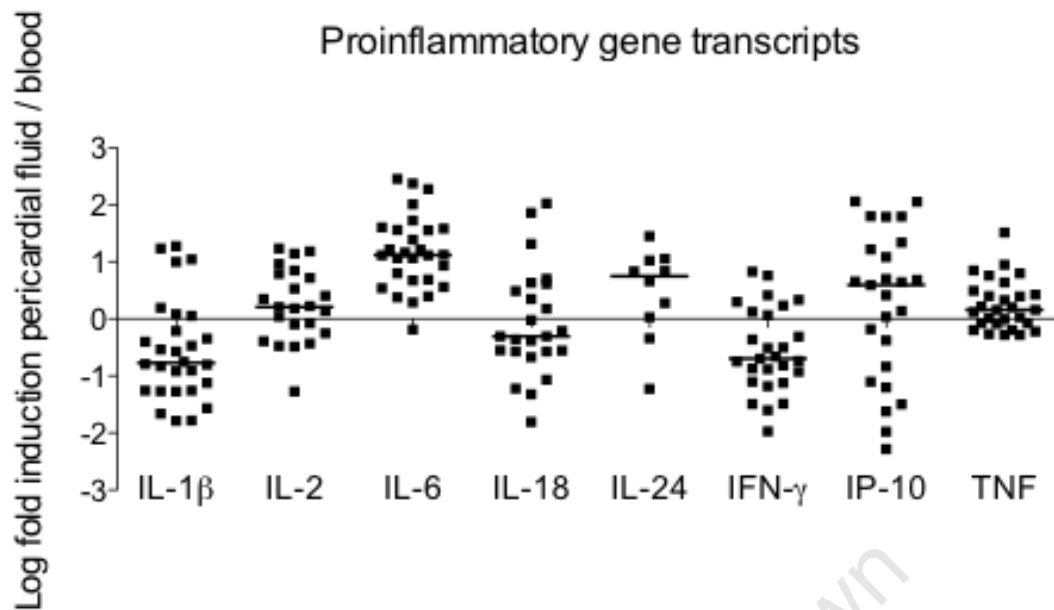


Figure 5.2. Mean log fold induction of pericardial fluid over blood for Pro-inflammatory gene transcripts of all TB pericarditis patients

Patients with undetectable gene transcript in both blood and pericardial fluid were excluded from analysis

The effect of HIV on the pro-inflammatory transcript profile of pericardial fluid and blood of TB pericarditis patients is summarized in Table 5.4. In the HIV-1 uninfected group, there was increased gene transcript of IL-6 ($p=0.001$) and IP-10 ($p=0.042$) in the pericardial fluid compared to blood. In the HIV-1 infected group, there was decreased gene transcript of IL-1 β ($p=0.016$) and IFN- γ ($p=0.011$), but increased IL-6 ($p=0.0004$) and TNF ($p=0.029$) in the pericardial fluid compared to blood.

When comparing the pro-inflammatory gene transcript profile of HIV-1 uninfected and HIV-1 infected patients for blood and pericardial fluid a number of differences are noted. TNF was decreased in the blood of HIV-1 infected patients ($p=0.008$). IL-

2 (p=0.009) and IL-18 (p=0.017) were significantly less abundant in the presence of HIV-1 in the pericardial fluid of TB pericarditis patients.

Table 5.4. Median (IQR) delta CT of Pro-inflammatory gene transcripts in blood and pericardial fluid of HIV-1 uninfected and HIV-1 infected TB pericarditis patients

	HIV-1 uninfected			HIV-1 infected			HIV-1 uninfected vs HIV-1 infected	
							Blood	Pericardial fluid
Gene	Blood	Pericardial fluid	p-value	Blood	Pericardial fluid	p-value	p-value	p-value
IL-1β	5.7 (4.6 - 6.0)	7.5 (3.8 - 9.1)	0.206	5.8 (5.1-6.5)	7.9 (7.0-10.0)	0.016	0.397	0.495
IL-2	16.1 (14.9 - 18.5)	14.8 (14.5 - 16.9)	0.083	17.8 (16.1-19.2)	17.2 (15.7-18.8)	0.677	0.103	0.009
IL-6	14.1 (13.6 - 16.4)	11.7 (10.0 - 12.3)	0.001	14.6 (13.6-15.4)	11.1 (9.7-12.0)	0.0004	0.259	0.510
IL-18	10.4 (8.5 - 12.0)	9.5 (6.5 - 12.0)	0.898	11.2 (10.2-19.2)	13.0 (10.4-20.1)	0.296	0.120	0.017
IL-24	17.6 (16.9-18.2)	15.4 (14.4-17.5)	0.250	18.6 (17.0-19.4)	17.0 (14.6-19.4)	0.313	0.257	0.476
IFN-γ	13.7 (12.7 - 15.2)	14.7 (13.1 - 17.6)	0.083	13.2 (12.6-14.6)	16.2 (13.9-17.6)	0.011	0.824	0.675
IP-10	11.2 (8.8 - 14.4)	8.7 (7.7 - 11.2)	0.042	10.4 (7.0-12.7)	9.7 (7.8-12.8)	0.761	0.341	0.255
TNF	8.5 (8.2 - 9.5)	8.5 (6.6 - 9.6)	0.365	10.0 (9.2-11.7)	9.1 (8.7-10.0)	0.029	0.008	0.204

Next, I normalized the fold induction of pericardial fluid over blood for HIV-1 uninfected and HIV-1 infected TB pericarditis patients (Figure 5.3) for the pro-inflammatory gene transcripts. While there were no differences when comparing the log fold induction of HIV-1 uninfected with HIV-1 infected patients, the normalization of the pericardial fluid to the blood reiterates the differences between blood and pericardial fluid when comparing HIV-1 status groups.

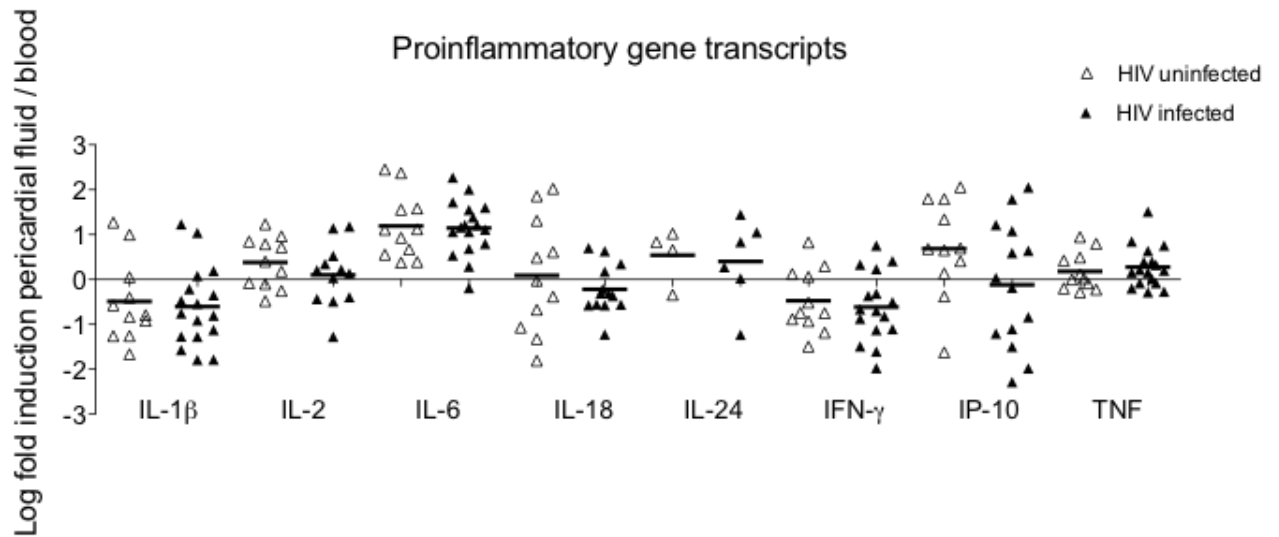


Figure 5.3. Mean log fold induction of pericardial fluid over blood for Pro-inflammatory gene transcripts of HIV-1 uninfected and HIV-1 infected TB pericarditis patients

Patients with undetectable gene transcript in both blood and pericardial fluid were excluded from analysis

3.1.3. Th2 Gene Transcripts

IL-4 and IL-13 are cytokines produced by Th2 cells and these downregulate Th1 cytokine production. It is evident that gene transcript for both these cytokines are relatively low, and even absent in some patients (Figure 5.4 and Table 5.5).

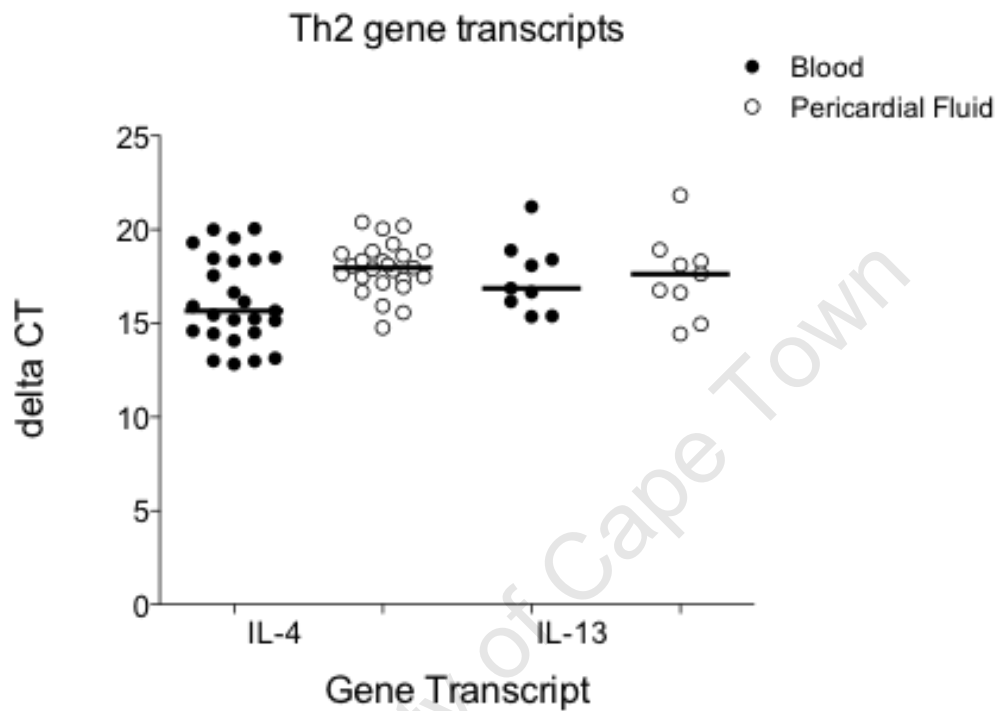


Figure 5.4. Median delta CT of Th2 gene transcript levels in whole blood and matching pericardial fluid of TB pericarditis patients

Patients with undetectable gene transcript in both blood and pericardial fluid were excluded from analysis

Table 5.5. Median delta CT (IQR) of Th2 related gene transcripts for blood and pericardial fluid from all TB pericarditis patients

Gene	Sample	n=	Median delta CT (IQR)	Blood vs Fluid, p-value
IL-4	Blood	25	15.7 (14.5 - 18.4)	0.0005
	Fluid	25	18.0 (17.2 - 18.6)	
IL-13	Blood	9	16.9 (15.8 - 18.6)	0.953
	Fluid	9	17.6 (15.8 - 18.6)	

IL-4 was only detectable in 25/29 patients, while IL-13 in 9/29 patients. However, there was decreased IL-4 gene transcript in the pericardial fluid compared to the blood (p=0.0005) of TB pericarditis patients, with no difference between blood and pericardial fluid for IL-13. The reduction in transcript for both genes in pericardial fluid compared to blood is illustrated in Figure 5.5, showing the mean log fold induction.

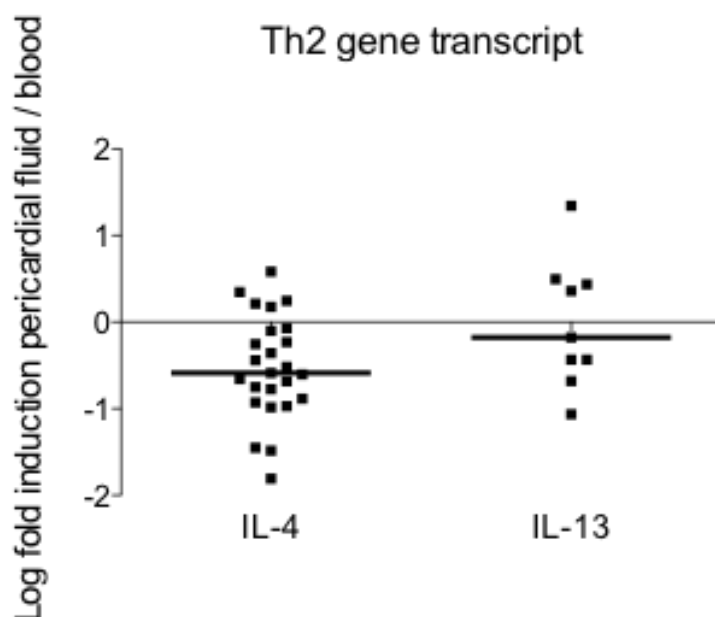


Figure 5.5. Mean log fold induction of pericardial fluid over blood for Th2 gene transcripts of all TB pericarditis patients

Patients with undetectable gene transcript in both blood and pericardial fluid were excluded from analysis

When stratifying the patients by HIV-1 status (Table 5.4), IL-4 gene transcript was significantly decreased in the pericardial fluid compared to blood in HIV-1 uninfected ($p=0.006$) and HIV-1 infected patients ($p=0.035$).

	HIV-1 uninfected			HIV-1 infected			HIV-1 uninfected vs HIV-1 infected	
							Blood	Pericardial fluid
Gene	Blood	Pericardial fluid	p-value	Blood	Pericardial fluid	p-value	p-value	p-value
IL-4	14.3 (13.0 - 15.4)	17.3 (15.8 - 18.2)	0.006	17.6 (15.5-18.5)	18.3 (17.6-18.9)	0.035	0.003	0.067
IL-13	16.9	17.6	0.750	17.5	17.4	0.844	0.548	1.000

	(15.4-18.1)	(16.6-18.3)		(16.0-19.5)	(14.8-19.6)			

Table 5.6. Median (IQR) delta CT of Th2 related gene transcripts in blood and pericardial fluid of HIV-1 uninfected and HIV-1 infected TB pericarditis patients

There was more abundant IL-4 gene transcript in the blood ($p=0.003$) of HIV-1 uninfected patients. There were no differences in IL-13 gene transcript levels between HIV-1 uninfected and HIV-1 infected blood and pericardial fluid, as only 3/9 patients with detectable IL-13 were HIV-1 uninfected.

Calculating the mean log fold induction (Figure 5.6) showed decreased transcription for IL-4 at the site of TB disease in both HIV-1 uninfected and HIV-1 infected patients and significantly more so for the HIV-1 uninfected ($p=0.025$), while IL-13 tended to be decreased in HIV-1 uninfected pericardial fluid (not significant).

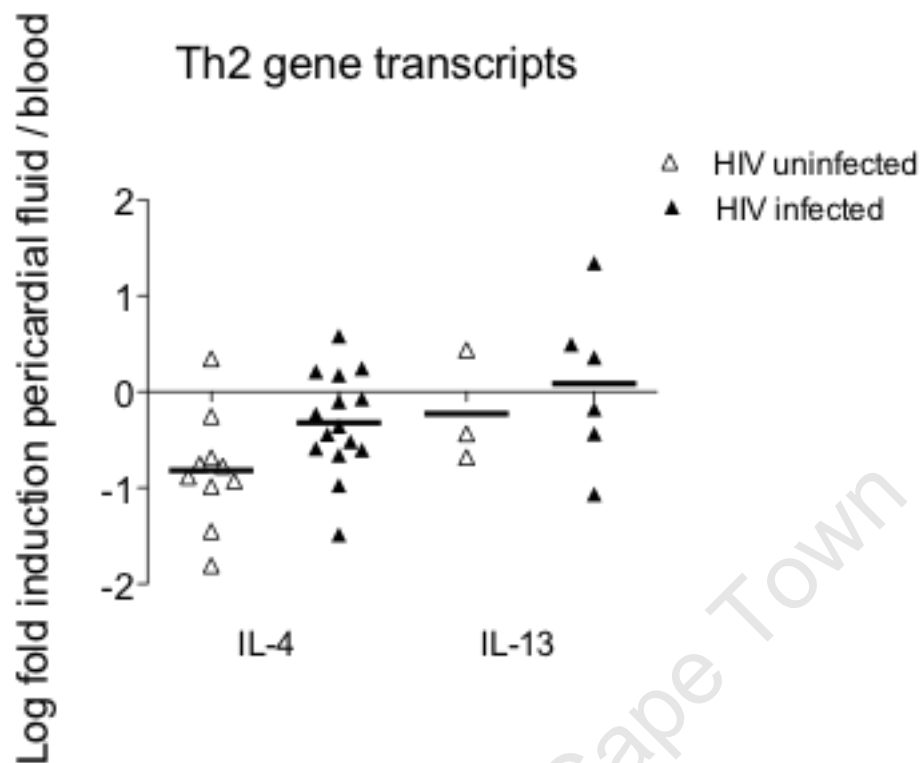


Figure 5.6. Mean log fold induction of pericardial fluid over blood for Th2 gene transcripts of HIV-1 uninfected and HIV-1 infected TB pericarditis patients

Patients with undetectable gene transcript in both blood and pericardial fluid were excluded from analysis

3.1.4. *Th17 Related Gene Transcripts*

The Th17 related gene transcripts analysed were IL-17, IL-22 and IL-23p19 (Figure 5.7 and Table 5.7). While IL-23p19 was detectable in all blood and pericardial fluid samples from all 29 patients and at relatively high levels, only 8/20 and 4/29 patients had detectable IL-22 and IL-17 gene transcript respectively, and these were at low levels.

There were no differences in median delta CT between blood and pericardial fluid for any of the transcripts (Figure 5.7 and Table 5.7).

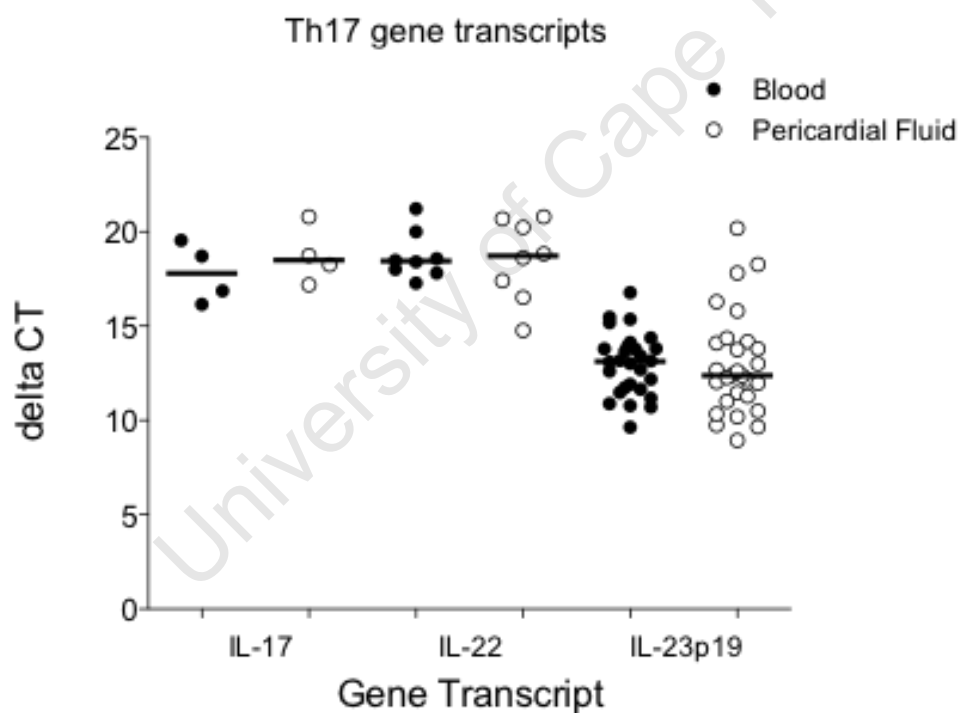


Figure 5.7. Median delta CT of Th17 gene transcript levels in whole blood and matching pericardial fluid of TB pericarditis patients

Patients with undetectable gene transcript in both blood and pericardial fluid were excluded from analysis

Table 5.7. Median delta CT (IQR) of Th17 related gene transcripts for blood and pericardial fluid from all TB pericarditis patients

Gene	Sample	n=	Median delta CT (IQR)	Blood vs Fluid, p-value
IL-17	Blood	4	17.8 (16.3 - 19.3)	0.625
	Fluid	4	18.5 (17.5 - 20.3)	
IL-22	Blood	8	18.4 (17.9 - 19.6)	0.844
	Fluid	8	18.7 (16.9 - 20.6)	
IL-23p19	Blood	28	13.1 (11.7 - 13.9)	0.406
	Fluid	28	12.4 (11.1 - 14.2)	

When comparing HIV-1 uninfected and HIV-1 infected TB pericarditis patients (Table 5.8), it was noted that the 4 patients with detectable gene transcript for IL-17 were all HIV-1 uninfected. There were no differences between blood and pericardial fluid for any of the gene transcripts for HIV-1 uninfected or HIV-1 infected patients. However, when comparing HIV-1 uninfected to HIV-1 infected TB pericarditis patients, IL-23p19 was increased in both blood ($p=0.024$) and pericardial fluid ($p=0.021$) in the HIV-1 uninfected patients compared to HIV-1 infected.

Table 5.8. Median (IQR) delta CT of Th17 related gene transcripts in blood and pericardial fluid of HIV-1 uninfected and HIV-1 infected TB pericarditis patients

	HIV-1 uninfected			HIV-1 infected			HIV-1 uninfected vs HIV-1 infected	
							Blood	Pericardial fluid
Gene	Blood	Pericardial fluid	p-value	Blood	Pericardial fluid	p-value	p-value	p-value
IL-17	17.8 (16.3-19.3)	18.5 (17.5-20.3)	0.625	none	none	/	/	/
IL-22	18.5 (17.3 - 18.6)	18.8 (14.8 - 20.8)	0.625	18.4 (17.9-20.6)	18.6 (17.0-20.5)	0.813	0.786	1.000
IL-23p19	11.8 (10.9 - 13.4)	11.3 (10.2 - 12.6)	0.008	13.8 (12.4-14.8)	13.0 (12.1-16.0)	0.925	0.024	0.021

Figure 5.8 shows the log fold induction of pericardial fluid over blood for all patients.

The trend noted for all the TB pericarditis patients was evident for both HIV-1 uninfected and HIV-1 infected patients (Figure 5.9), with decreased IL-17 and increased IL-22 and IL-23p19 transcript levels in the pericardial fluid compared to blood, but no statistically significant differences between them.

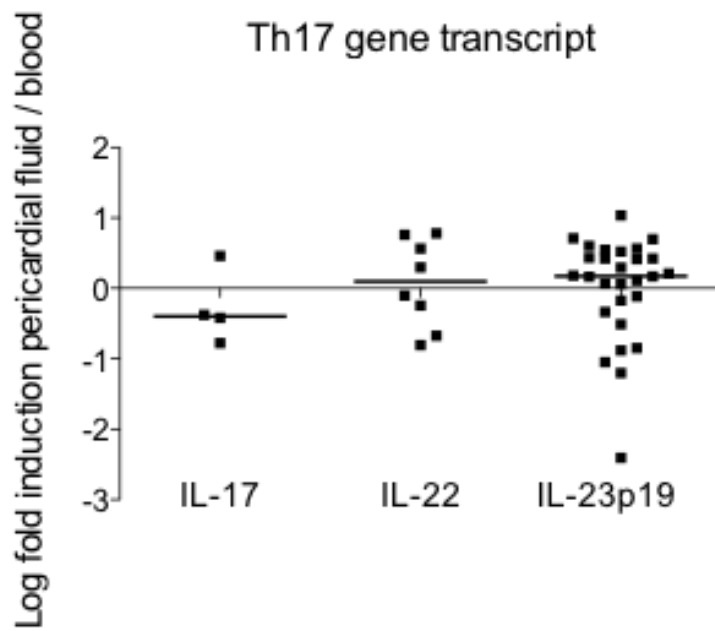


Figure 5.8. Mean log fold induction of pericardial fluid over blood for Th17 gene transcripts of all TB pericarditis patients

Patients with undetectable gene transcript in both blood and pericardial fluid were excluded from analysis

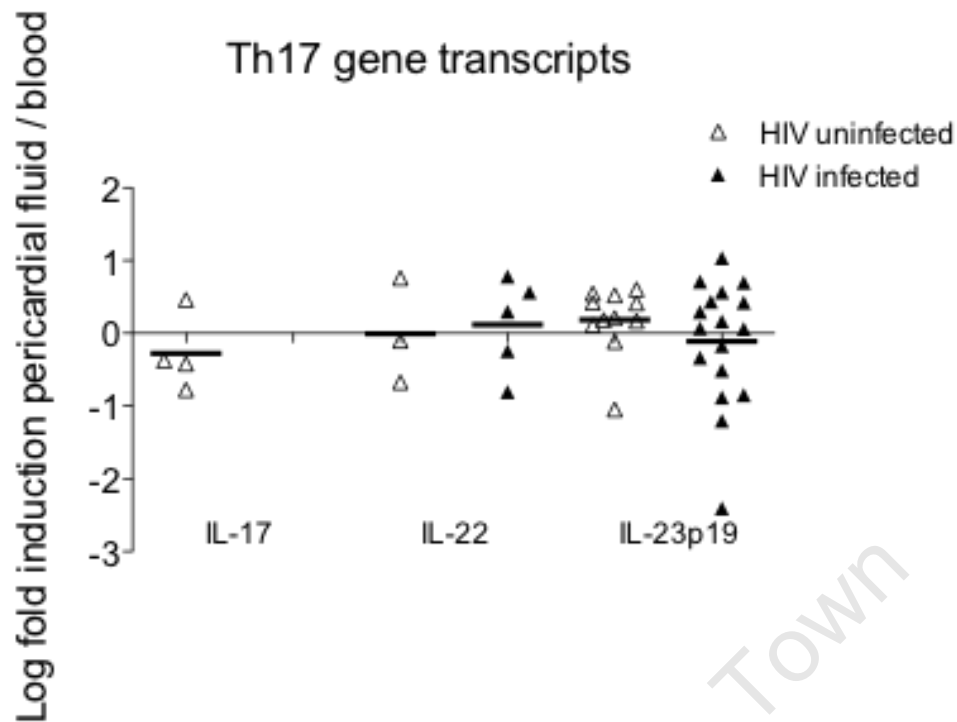


Figure 5.9. Mean log fold induction of pericardial fluid over blood for Th17 gene transcripts of HIV-1 uninfected and HIV-1 infected TB pericarditis patients

Patients with undetectable gene transcript in both blood and pericardial fluid were excluded from analysis

3.1.5. Regulatory Gene Transcripts

IL-10, TGF- β and FoxP3 are regulatory genes that were analysed in blood and pericardial fluid of TB pericarditis patients (Figure 5.10 and Table 5.9). TGF- β transcript was very abundant in both blood and pericardial fluid, but significantly higher in the blood compared to pericardial fluid ($p < 0.0001$). Transcript for IL-10 ($p = 0.004$) and FoxP3 ($p = 0.0001$) were significantly higher in the pericardial fluid compared to blood and this is evident when considering the log fold induction of pericardial fluid over blood (Figure 5.11.).

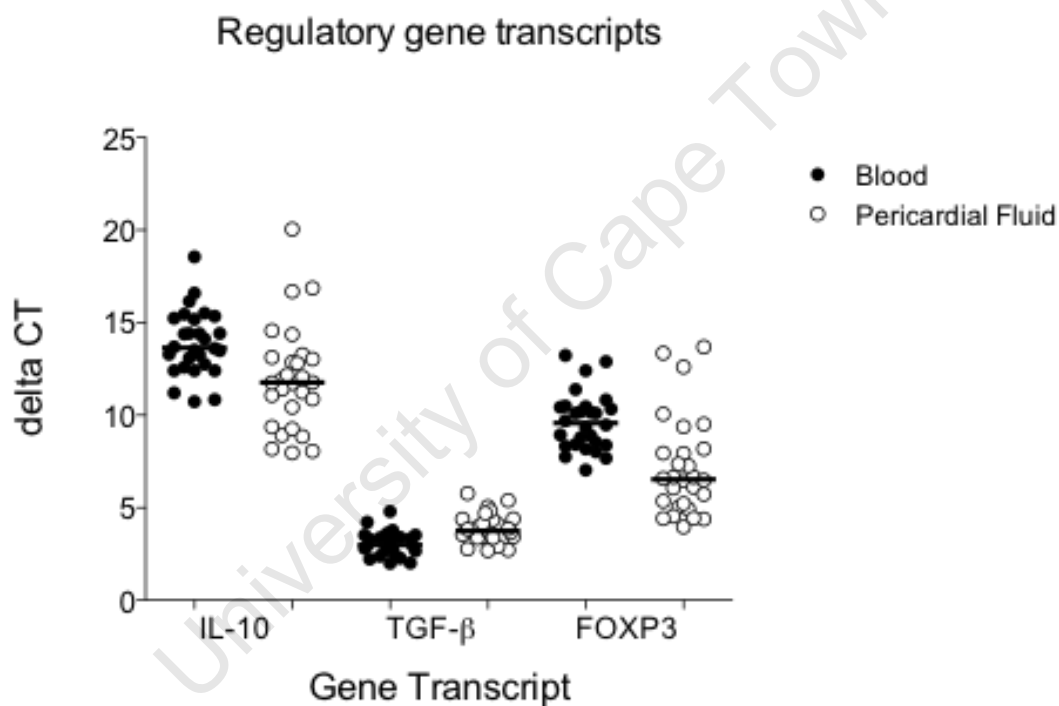


Figure 5.10. Median delta CT of Regulatory gene transcript levels in whole blood and matching pericardial fluid of TB pericarditis patients

Patients with undetectable gene transcript in both blood and pericardial fluid were excluded from analysis

Table 5.9. Median delta CT (IQR) of Regulatory gene transcripts for blood and pericardial fluid from all TB pericarditis patients

Gene	Sample	n=	Median delta CT (IQR)	Blood vs Fluid, p-value
IL-10	Blood	28	13.6 (12.6 - 15.2)	0.004
	Fluid	28	11.8 (9.6 - 13.1)	
TGF-β	Blood	28	3.0 (2.7 - 3.5)	<0.0001
	Fluid	28	3.8 (3.4 - 4.4)	
FoxP3	Blood	28	9.6 (8.4 - 10.5)	0.0001
	Fluid	28	6.5 (5.1 - 8.1)	

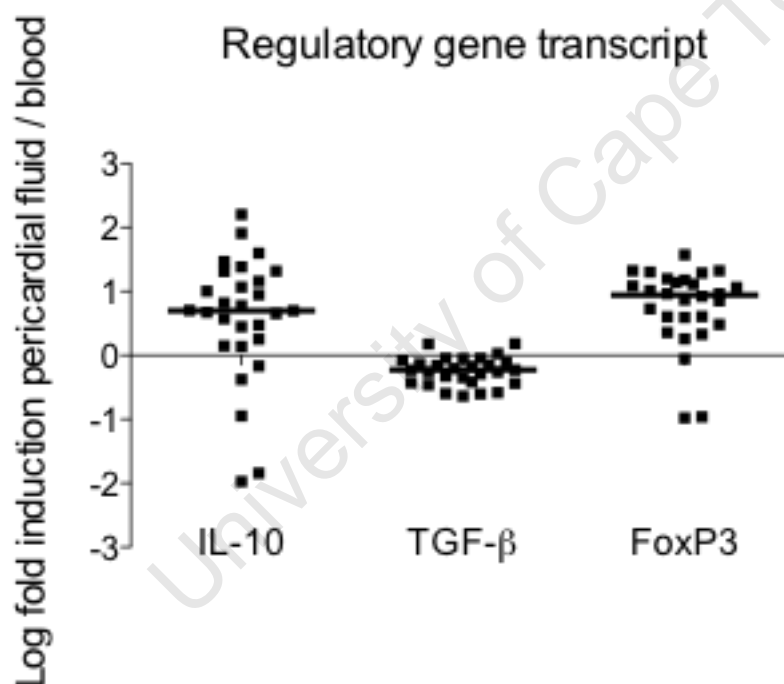


Figure 5.11. Mean log fold induction of pericardial fluid over blood for Regulatory gene transcripts of all TB pericarditis patients

Patients with undetectable gene transcript in both blood and pericardial fluid were excluded from analysis

When taking HIV infection into consideration (Table 5.10), it was noted that in HIV-1 uninfected patients, there was increased gene transcript for IL-10 ($p=0.002$) in the pericardial fluid compared to blood, but no differences in the HIV-1 infected group.

Table 5.10. Median (IQR) delta CT of Regulatory gene transcripts in blood and pericardial fluid of HIV-1 uninfected and HIV-1 infected TB pericarditis patients

	HIV-1 uninfected			HIV-1 infected			HIV-1 uninfected vs HIV-1 infected	
							Blood	Pericardial fluid
Gene	Blood	Pericardial fluid	p-value	Blood	Pericardial fluid	p-value	p-value	p-value
IL-10	13.7 (12.4 - 14.4)	11.5 (9.3-12.1)	0.005	13.6 (12.7-15.3)	11.9 (9.7-13.9)	0.088	0.707	0.452
TGF-β	3.0 (2.4 - 3.5)	3.7 (2.9 - 4.1)	0.001	3.0 (2.7-3.5)	3.8 (3.5-4.4)	0.001	0.851	0.312
FoxP3	8.4 (8.0 - 9.3)	6.5 (4.4 - 7.4)	0.001	10.2 (9.2-10.9)	6.7 (5.5-9.8)	0.005	0.007	0.110

TGF- β gene transcript abundance was less in the pericardial fluid compared to blood in HIV-1 uninfected ($p=0.001$) and HIV-1 infected patients ($p=0.001$). FoxP3 gene transcript was more abundant in the pericardial fluid compared to blood, irrespective of the HIV-1 status of the patients (HIV-1 uninfected $p=0.001$, HIV-1 infected $p=0.005$).

The comparison between HIV-1 uninfected and HIV-1 infected blood and pericardial fluid revealed no differences for any of the regulatory gene transcripts measured.

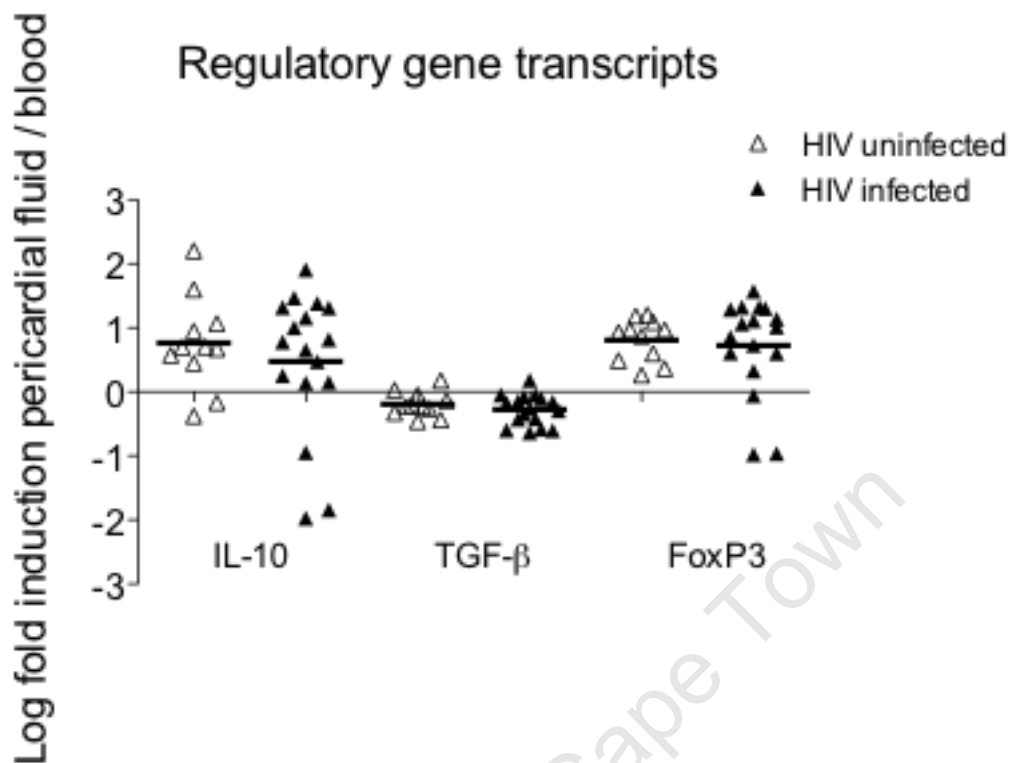


Figure 5.12. Mean log fold induction of pericardial fluid over blood for Regulatory gene transcripts of HIV-1 uninfected and HIV-1 infected TB pericarditis patients

Patients with undetectable gene transcript in both blood and pericardial fluid were excluded from analysis

Normalization of the pericardial fluid to the blood for HIV-1 uninfected and HIV-1 infected patients (Figure 5.12.) illustrated the increase of IL-10 and FoxP3 and decrease of TGF- β in the pericardial fluid of these patients more noticeably.

3.1.6. Neutrophil Related Gene Transcripts

Neutrophil related gene transcripts were differentially expressed in the blood and pericardial fluid from TB pericarditis patients. (Figure 5.13 and Table 5.11).

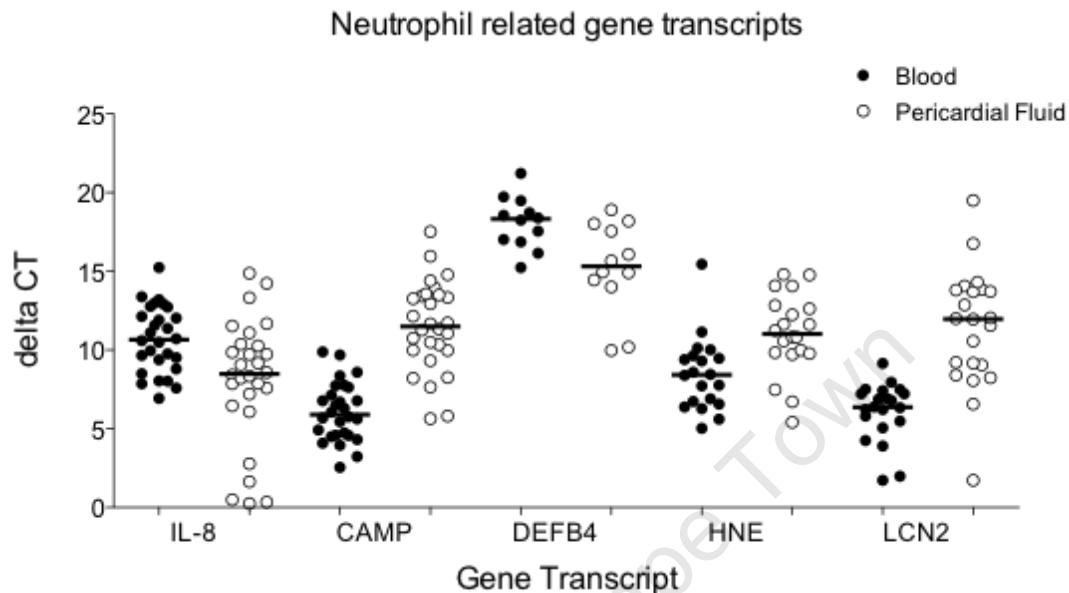


Figure 5.13. Median delta CT of Neutrophil related gene transcript levels in whole blood and matching pericardial fluid of TB pericarditis patients

Patients with undetectable gene transcript in both blood and pericardial fluid were excluded from analysis

The gene transcript for the neutrophil chemoattractant chemokine IL-8 (CXCL8) was increased in the pericardial fluid compared to the blood ($p=0.003$), along with DEFB4 ($p=0.002$), although only 12/29 patients had detectable DEFB4. CAMP ($p<0.0001$), LCN2 ($p=0.0002$) and HNE ($p=0.003$) were all significantly lower in the pericardial fluid compared to the blood and this is represented by the log fold inductions shown in Figure 5.14.

Table 5.11. Median delta CT (IQR) of Neutrophil gene transcripts for blood and pericardial fluid from all TB pericarditis patients

Gene	Sample	n=	Median delta CT (IQR)	Blood vs Fluid, p-value
IL-8	Blood	28	10.7 (8.9 - 12.6)	0.003
	Fluid	28	8.9 (6.6 - 10.3)	
CAMP	Blood	28	5.9 (4.7 - 7.5)	<0.0001
	Fluid	28	11.5 (10.0 - 13.5)	
LCN2	Blood	22	6.4 (4.9 - 7.3)	0.0002
	Fluid	22	12.0 (8.9 - 13.8)	
DEFB4	Blood	12	18.3 (16.9 - 19.3)	0.002
	Fluid	12	15.3 (14.1 - 17.9)	
HNE	Blood	20	8.42 (6.6 - 9.6)	0.003
	Fluid	20	11.0 (9.8 - 12.8)	

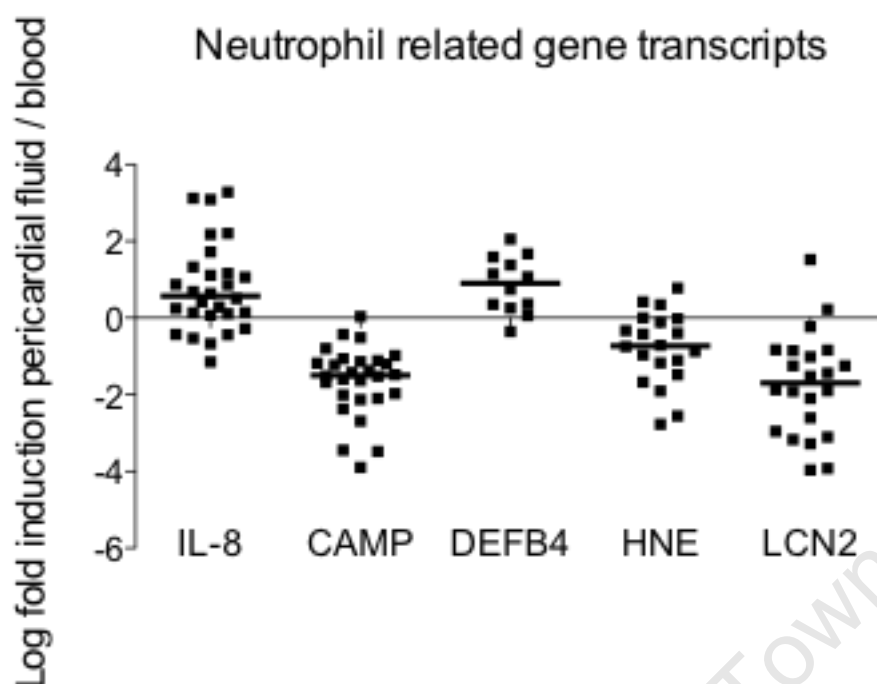


Figure 5.14. Mean log fold induction of pericardial fluid over blood for Neutrophil related gene transcripts of all TB pericarditis patients

Patients with undetectable gene transcript in both blood and pericardial fluid were excluded from analysis

When analysed by HIV status, CAMP ($p=0.002$) and LCN2 ($p=0.014$) gene transcript were significantly lower in the pericardial fluid of HIV-1 uninfected patients (Table 5.12). In the HIV-1 infected TB pericarditis patient group, IL-8 ($p=0.001$) and DEFB4 ($p=0.008$) were higher in the pericardial fluid compared to blood, while CAMP ($p=0.0003$), LCN2 ($p=0.001$) and HNE ($p=0.012$) were significantly higher in the blood compared to pericardial fluid.

When comparing HIV-1 uninfected and HIV-1 infected patients, only two differences were observed. IL-8 ($p=0.005$) was more abundant in the blood of HIV-1 uninfected

patients. The mean log fold induction of pericardial fluid over blood showed no differences between HIV-1 uninfected and infected patients (Figure 5.15.).

Table 5.12. Median (IQR) delta CT of Neutrophil related gene transcripts in blood and pericardial fluid of HIV-1 uninfected and HIV-1 infected TB pericarditis patients

	HIV-1 uninfected			HIV-1 infected			HIV-1 uninfected vs HIV-1 infected	
							Blood	Pericardial fluid
Gene	Blood	Pericardial fluid	p-value	Blood	Pericardial fluid	p-value	p-value	p-value
IL-8	8.8 (8.0 - 10.5)	8.3 (2.8 - 10.3)	0.577	11.9 (10.2-13.0)	8.6 (7.0-10.7)	0.001	0.005	0.814
CAMP	4.6 (4.1 – 7.2)	11.7 (9.3 - 13.6)	0.002	6.5 (5.19-8.07)	11.3 (10.0-13.5)	0.0003	0.074	0.814
LCN2	6.8 (5.0 - 7.2)	12.0 (9.2 - 13.8)	0.014	6.2 (4.3-7.4)	11.6 (8.2-13.8)	0.001	0.694	0.599
DEFB4	17.6 (16.9 - 18.5)	15.3 (11.2 - 17.5)	0.250	18.6 (16.5-19.7)	15.3 (14.2-18.0)	0.008	0.461	0.808
HNE	7.3 (6.3 - 9.1)	10.8 (8.1 - 14.1)	0.055	8.9 (7.0-10.0)	11.0 (10.0-12.8)	0.012	0.177	0.908

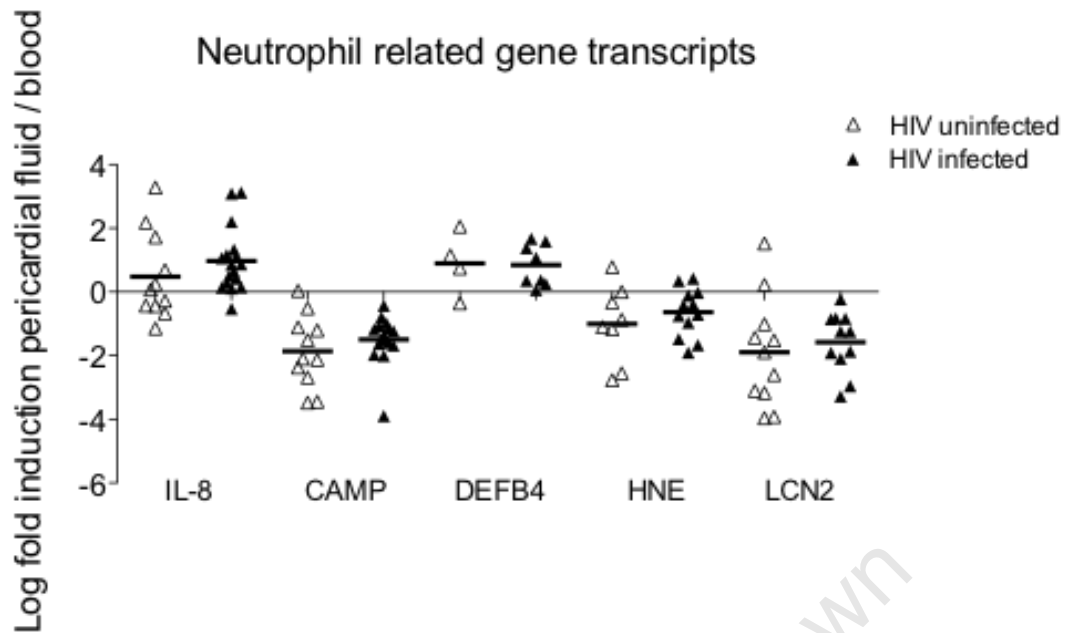


Figure 5.15. Mean log fold induction of pericardial fluid over blood for Neutrophil related gene transcripts of HIV-1 uninfected and HIV-1 infected TB pericarditis patients

Patients with undetectable gene transcript in both blood and pericardial fluid were excluded from analysis

3.1.7. Growth Factor Gene Transcripts

Three gene transcripts related to Growth Factors were analysed in this study (Figure 5.16 and Table 5.13). CTGF ($p<0.0001$) and GM-CSF ($p=0.014$) were significantly higher in pericardial fluid compared to blood in TB pericarditis patients. EGF ($p<0.0001$), however, was significantly lower at the site of disease compared to blood and this is illustrated in Figure 5.17 showing the mean log fold induction of pericardial fluid over blood.

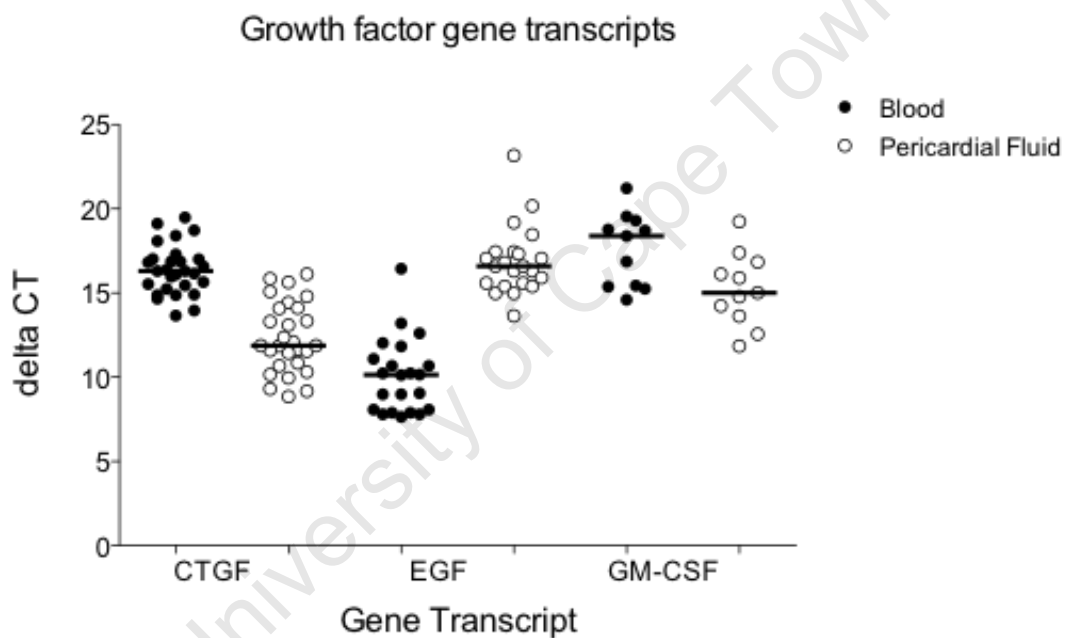


Figure 5.16. Median delta CT of Growth Factor gene transcript levels in whole blood and matching pericardial fluid of TB pericarditis patients

Patients with undetectable gene transcript in both blood and pericardial fluid were excluded from analysis

Table 5.13. Median delta CT (IQR) of Growth factor related gene transcripts for blood and pericardial fluid from all TB pericarditis patients

Gene	Sample	n=	Median delta CT (IQR)	Blood vs Fluid, p-value
CTGF	Blood	28	16.3 (15.3 - 17.0)	<0.0001
	Fluid	28	11.9 (10.7 - 14.1)	
EGF	Blood	22	10.1 (8.0 - 11.3)	<0.0001
	Fluid	22	16.6 (15.5 - 17.5)	
GM-CSF	Blood	11	18.4 (15.4 - 19.3)	0.014
	Fluid	11	15.0 (13.6 - 16.8)	

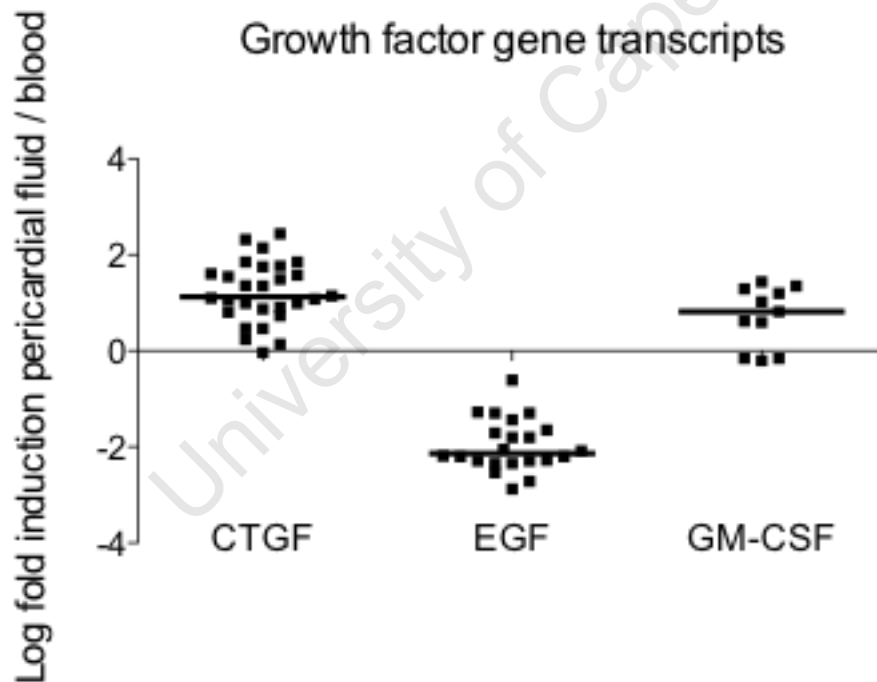


Figure 5.17. Mean log fold induction of pericardial fluid over blood for Growth factor gene transcripts of all TB pericarditis patients

Patients with undetectable gene transcript in both blood and pericardial fluid were excluded from analysis

Table 5.14 summarizes the comparison between blood and pericardial fluid for HIV-1 uninfected and HIV-1 infected TB pericarditis patients.

Table 5.14. Median (IQR) delta CT of Growth factor related gene transcripts in blood and pericardial fluid of HIV-1 uninfected and HIV-1 infected TB pericarditis patients

	HIV-1 uninfected			HIV-1 infected			HIV-1 uninfected vs HIV-1 infected	
	Blood	Pericardial fluid	p-value	Blood	Pericardial fluid	p-value	Blood	Pericardial fluid
Gene	Blood	Pericardial fluid	p-value	Blood	Pericardial fluid	p-value	p-value	p-value
CTGF	16.2 (15.5 - 17.6)	13.1 (11.3 – 14.3)	0.001	16.2 (15.5-17.6)	13.1 (11.2-14.3)	0.0004	0.906	0.090
EGF	9.0 (7.8 - 10.5)	15.9 (15.2 - 16.8)	0.004	10.2 (8.02-11.9)	16.9 (15.5-18.6)	0.0001	0.344	0.138
GM-CSF	18.8 (16.9 – 20.3)	15.0 (14.2 - 17.7)	0.156	18.8 (16.9-20.3)	15.0 (14.2-17.7)	0.125	0.178	0.662

CTGF was significantly higher in pericardial fluid for both HIV-1 uninfected ($p=0.001$) and HIV-1 infected ($p=0.0004$) patients, while EGF was significantly elevated in the blood compared to pericardial fluid irrespective of HIV-1 status (HIV-1 uninfected, $p=0.004$ and HIV-1 infected, $p=0.0001$). Gene transcript abundance of GM-CSF were no different between blood and pericardial fluid in either HIV-1 uninfected or infected TB pericarditis patients. When comparing the gene transcript levels of these genes between HIV-1 uninfected and HIV-1 infected blood and pericardial fluid no differences were evident.

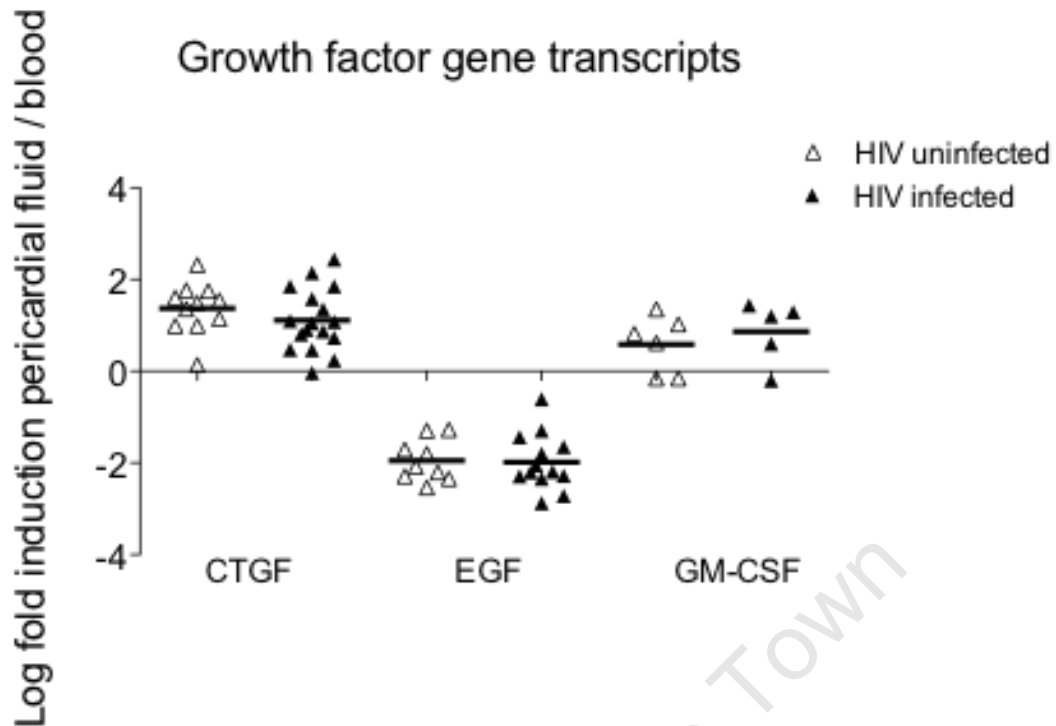


Figure 5.18. Mean log fold induction of pericardial fluid over blood for Growth factor gene transcripts of HIV-1 uninfected and HIV-1 infected TB pericarditis patients

Patients with undetectable gene transcript in both blood and pericardial fluid were excluded from analysis

Normalization of pericardial fluid to blood in HIV-1 uninfected and infected patients (Figure 5.18.) demonstrated the trend of increased CTGF and GM-CSF, as well as decreased EGF transcript in the pericardial fluid for both HIV-1 uninfected and infected TB pericarditis patients, however no statistical significance between groups existed.

3.1.8. Fibrosis Related Gene Transcripts

Genes related to fibrosis, including initiation (*IL-9*), establishment (*Col1a1*, *Col1a2*, *Col4a1*, *Col4a2*) and the control and regulation of the fibrotic response; *ARG1* and *SPARC* were measured in TB pericarditis patients (Figure 5.19 and Table 5.15).

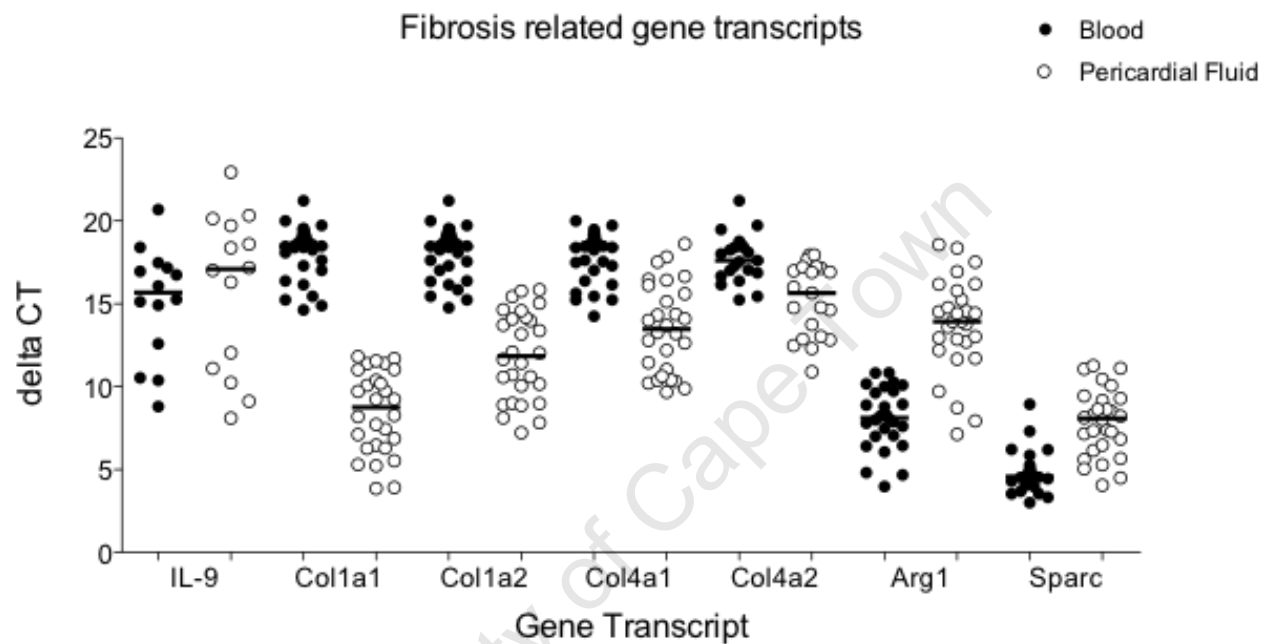


Figure 5.19. Median delta CT of Fibrosis related gene transcript levels in whole blood and matching pericardial fluid of TB pericarditis patients

Patients with undetectable gene transcript in both blood and pericardial fluid were excluded from analysis

IL-9 was not different between blood and pericardial fluid. The procollagen genes; Col1a1, Col1a2, Col4a1 (all $p < 0.0001$) and Col4a2 ($p = 0.0004$) were more abundant in the pericardial fluid and very low in the blood of all TB pericarditis patients. ARG1 and SPARC were significantly lower in the pericardial fluid compared to blood (both $p < 0.0001$).

Table 5.15. Median delta CT (IQR) of Fibrosis related gene transcripts for blood and pericardial fluid from all TB pericarditis patients

Gene	Sample	n=	Median delta CT (IQR)	Blood vs Fluid, p-value
IL-9	Blood	14	15.7 (12.1 - 17.2)	0.855
	Fluid	14	17.1 (10.9 - 19.8)	
Col1a1	Blood	28	18.5 (17.1 - 19.1)	<0.0001
	Fluid	28	8.76 (6.34 - 10.9)	
Col1a2	Blood	28	18.4 (16.5 - 19.1)	<0.0001
	Fluid	28	11.8 (9.26 - 14.1)	
Col4a1	Blood	28	18.4 (16.5 - 18.8)	<0.0001
	Fluid	28	13.5 (10.7 – 16.0)	
Col4a2	Blood	21	17.6 (16.8 - 18.5)	0.0006
	Fluid	21	15.7 (13.0 - 17.2)	
ARG1	Blood	28	8.1 (7.0 - 9.7)	<0.0001
	Fluid	28	13.9 (12.3 - 15.7)	
SPARC	Blood	28	4.6 (4.1 - 5.0)	<0.0001
	Fluid	28	8.4 (6.2 - 9.3)	

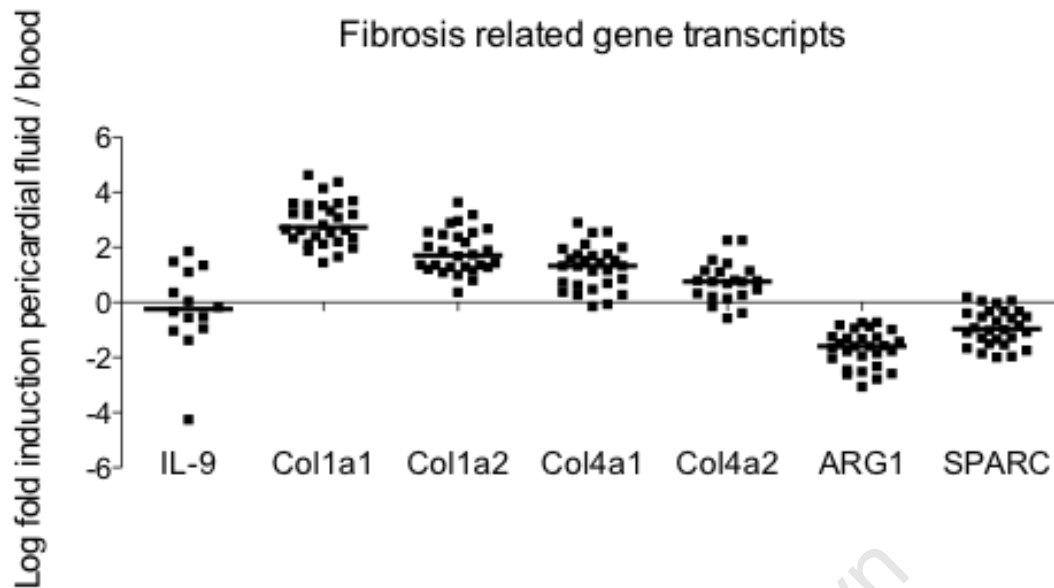


Figure 5.20. Mean log fold induction of pericardial fluid over blood for Pro-inflammatory gene transcripts of all TB pericarditis patients

Patients with undetectable gene transcript in both blood and pericardial fluid were excluded from analysis

The determination of the mean log fold induction of pericardial fluid over blood showed an increase of the procollagen genes in the pericardial fluid (Figure 5.20), with IL-9, ARG1 and SPARC lower in pericardial fluid.

HIV infection had no effect on the regulation of these genes at all (Table 5.16). The significant trend of increased procollagen genes and decreased ARG1 and SPARC in the pericardial fluid compared to blood was apparent in both HIV-1 uninfected and infected TB pericarditis patients.

Mean log fold induction of pericardial fluid over blood showed that HIV-1 tended to decrease the transcription of all these genes, albeit not significantly (Figure 5.21).

Table 5.16. Median (IQR) delta CT of Fibrosis gene transcripts in blood and pericardial fluid of HIV-1 uninfected and HIV-1 infected TB pericarditis patients

	HIV-1 uninfected			HIV-1 infected			HIV-1 uninfected vs HIV-1 infected	
							Blood	Pericardial fluid
Gene	Blood	Pericardial fluid	p-value	Blood	Pericardial fluid	p-value	p-value	p-value
IL-9	16.5 (15.1 - 18.0)	17.8 (15.8 - 19.9)	1.000	13.9 (10.4-17.3)	13.7 (9.4-19.8)	0.945	0.282	0.345
Col1a1	18.3 (17.0 - 18.7)	6.9 (6.3 - 10.2)	0.001	18.5 (16.9-19.4)	9.72 (7.6-11.2)	0.0003	0.347	0.158
Col1a2	18.1 (16.4 - 18.6)	10.2 (8.98 - 12.1)	0.001	18.5 (16.9-19.4)	13.4 (11.1-14.6)	0.0003	0.279	0.067
Col4a1	17.3 (15.6 - 18.6)	12.7 (10.7 -14.0)	0.002	18.5 (17.5-19.2)	14.4 (10.7-16.6)	0.0004	0.100	0.158
Col4a2	17.2 (16.8 - 18.1)	15.2 (14.2 – 17.0)	0.020	18.4 (16.2-19.5)	16.0 (12.8-17.2)	0.007	0.275	0.972
ARG1	8.0 (7.1 – 10.0)	14.5 (13.0 - 15.8)	0.001	8.3 (6.7-9.7)	13.6 (10.7-15.4)	0.0003	0.925	0.188
SPARC	4.6 (4.0 - 4.9)	7.2 (5.7 - 8.6)	0.010	4.6 (4.3-5.7)	8.30 (7.1-9.4)	0.0004	0.525	0.204

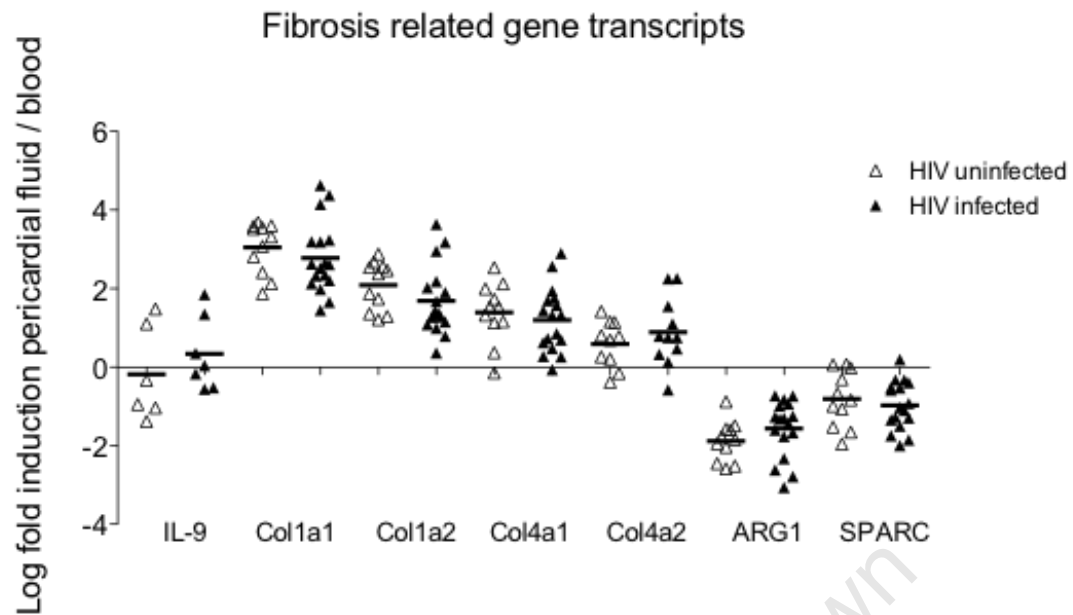


Figure 5.21. Mean log fold induction of pericardial fluid over blood for Fibrosis related gene transcripts of HIV-1 uninfected and HIV-1 infected TB pericarditis patients

Patients with undetectable gene transcript in both blood and pericardial fluid were excluded from analysis

3.1.9. MMP and TIMP Gene Transcripts

Matrix Metalloproteinases are responsible for the repair and rebuilding of the extracellular matrix and their endogenous inhibitors, TIMPs are responsible for keeping the MMPs under control. I measured MMP1, 2, 3, 7, 8, 9, 10 and 11 as well as TIMP1, 2 and 3 (Figure 5.22 and Table 5.17).

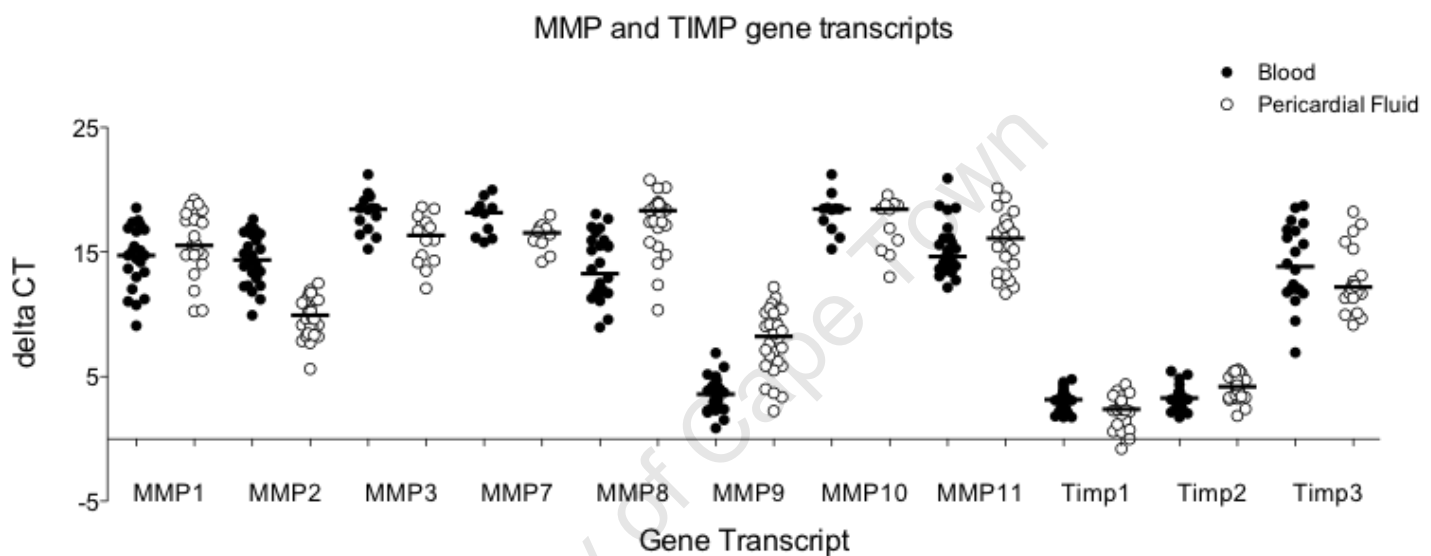


Figure 5.22. Median delta CT of MMP and TIMP gene transcript levels in whole blood and matching pericardial fluid of TB pericarditis patients

Patients with undetectable gene transcript in both blood and pericardial fluid were excluded from analysis

Of all the MMPs evaluated, MMP9 transcript was the most abundant overall. While there were no differences between blood and pericardial fluid for MMP1, 10 and 11, there were differential levels for the rest of the transcripts. Transcript levels of MMP2 ($p<0.0001$), 3 ($p=0.017$) and 7 ($p=0.027$) were more abundant in the pericardial fluid compared to blood, whereas the opposite was true for MMP8 ($p=0.0002$) and MMP9 ($p<0.0001$). TIMP1 and 2 were highly abundant in both blood

and pericardial fluid, however, a statistically significant difference was found between blood and fluid in as much as TIMP1 was significantly elevated in the pericardial fluid ($p=0.001$), while TIMP2 was significantly elevated in blood ($p=0.002$). TIMP3 was less abundant and detected equally in both blood and pericardial fluid of TB pericarditis patients. Figure 5.23 shows the mean log fold induction of pericardial fluid over blood which depict the trend for up and down regulation of MMP and TIMP genes in the pericardial fluid as noted by the delta CT analysis.

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Table 5.17. Median delta CT (IQR) of MMP and TIMP gene transcripts for blood and pericardial fluid from all TB pericarditis patients

Gene	Sample	n=	Median delta CT (IQR)	Blood vs Fluid, p-value
MMP1	Blood	22	14.7 (12.8 - 16.8)	0.023
	Fluid	22	15.5 (14.6 - 17.7)	
MMP2	Blood	28	14.4 (13.0 - 16.4)	<0.0001
	Fluid	28	9.9 (8.6 - 11.2)	
MMP3	Blood	14	18.4 (16.7 - 19.2)	0.017
	Fluid	14	16.3 (14.3 - 17.5)	
MMP7	Blood	10	18.2 (16.1 - 18.9)	0.027
	Fluid	10	16.5 (15.5 - 17.1)	
MMP8	Blood	26	13.3 (11.8 - 15.9)	0.0002
	Fluid	26	18.3 (16.7 - 18.8)	
MMP9	Blood	28	3.6 (2.4 - 4.3)	<0.0001
	Fluid	28	8.2 (6.0 - 10.1)	
MMP10	Blood	11	18.5 (16.9 - 18.6)	0.278
	Fluid	11	18.4 (15.1 - 18.8)	
MMP11	Blood	23	14.6 (13.6 - 16.2)	0.438
	Fluid	23	16.1 (13.3 - 17.2)	
TIMP1	Blood	28	3.2 (2.8 - 3.7)	0.001
	Fluid	28	2.4 (1.6 - 2.9)	
TIMP2	Blood	28	3.3 (2.8 - 3.7)	0.002
	Fluid	28	4.2 (3.4 - 4.7)	
TIMP3	Blood	20	13.8 (11.8 - 14.7)	0.162
	Fluid	20	12.2 (11.3 - 14.7)	

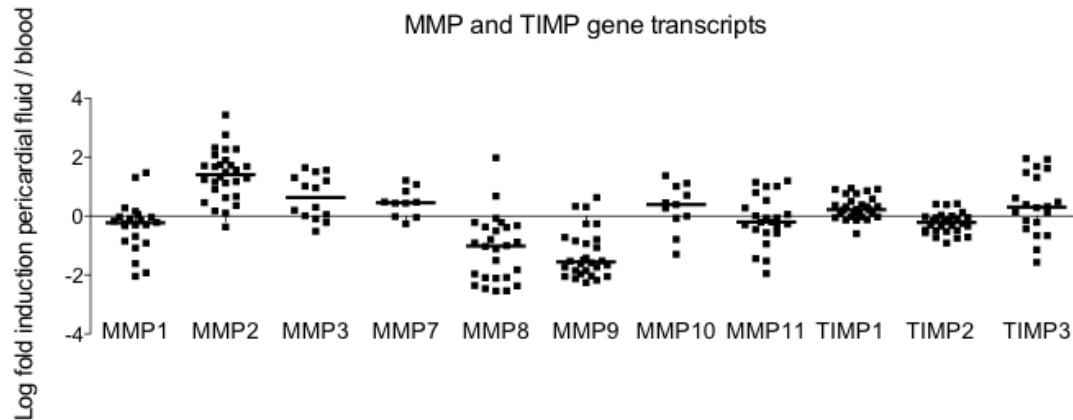


Figure 5.23. Mean log fold induction of pericardial fluid over blood for MMP and TIMP gene transcripts of all TB pericarditis patients

Patients with undetectable gene transcript in both blood and pericardial fluid were excluded from analysis

HIV infection did not seem to influence the levels of either MMP or TIMP transcripts (Table 5.18). MMP2 was more abundant in pericardial fluid compared to blood in both HIV-1 uninfected and HIV-1 infected patients (both $p=0.0005$), and MMP9 was more abundant in the blood of both sets of patients (HIV-1 uninfected $p=0.027$, HIV-1 infected $p=0.0003$). MMP3 ($p=0.049$) and TIMP1 ($p=0.003$) were higher in the pericardial fluid of HIV-1 infected patients compared to blood, while the opposite was true for MMP8 ($p=0.004$) and TIMP3 ($p=0.008$). There were no differences between HIV-1 uninfected and HIV-1 infected blood and pericardial fluid or mean log fold induction of pericardial fluid over blood from TB pericarditis patients (Figure 5.24 and Table 5.18).

Table 5.18. Median (IQR) delta CT of MMP and TIMP gene transcripts in blood and pericardial fluid of HIV-1 uninfected and HIV-1 infected TB pericarditis patients

	HIV-1 uninfected			HIV-1 infected			HIV-1 uninfected vs HIV-1 infected	
							Blood	Pericardial fluid
Gene	Blood	Pericardial fluid	p-value	Blood	Pericardial fluid	p-value	p-value	p-value
MMP1	15.1 (13.7 - 16.8)	16.3 (14.8 - 18.8)	0.240	14.7 (11.0 - 17.2)	15.1 (14.0 - 17.6)	0.054	0.393	0.555
MMP2	14.0 (13.5 - 16.5)	9.6 (8.2 - 11.7)	0.001	14.4 (12.3 - 16.3)	10.1 (9.10 - 11.2)	0.0005	0.742	0.655
MMP3	17.7 (16.5 - 18.5)	15.4 (13.7 - 17.6)	0.250	18.5 (17.2 - 19.5)	16.5 (14.6 - 17.7)	0.049	0.540	0.540
MMP7	18.3 (16.9 - 18.7)	16.7 (14.6 - 17.2)	0.031	16.2 (15.8 - 20.0)	16.4 (15.9 - 17.0)	1.000	0.667	1.000
MMP8	13.3 (11.8 - 15.7)	17.5 (16.7 - 19.2)	0.102	13.1 (11.7 - 16.4)	18.4 (15.9 - 18.8)	0.004	0.980	0.980
MMP9	4.3 (3.1 - 5.0)	8.4 (5.9 - 10.1)	0.014	3.6 (2.3 - 3.9)	8.06 (6.0 - 10.3)	0.0003	0.054	0.888
MMP10	18.5 (17.3 - 18.5)	15.5 (14.9 - 18.1)	0.250	18.4 (16.2 - 19.7)	18.5 (16.9 - 18.9)	0.813	0.788	0.315
MMP11	14.6 (13.4 - 17.7)	15.8 (14.3 - 17.0)	0.301	15.1 (13.6 - 16.2)	15.4 (12.7 - 17.6)	0.952	0.766	0.560
TIMP1	3.0 (2.3 - 3.5)	2.6 (2.3 - 3.1)	0.206	3.4 (2.9 - 3.7)	2.35 (1.1 - 2.7)	0.003	0.230	0.279
TIMP2	3.3 (2.4 - 3.7)	4.3 (3.4 - 4.7)	0.067	3.3 (2.9 - 4.1)	4.08 (3.3 - 4.9)	0.008	0.672	1.000
TIMP3	13. (11.9 - 17.1)	11.8 (10.7 - 14.8)	0.203	14.1 (11.8 - 16.7)	12.4 (11.3 - 15.3)	0.465	0.820	0.704

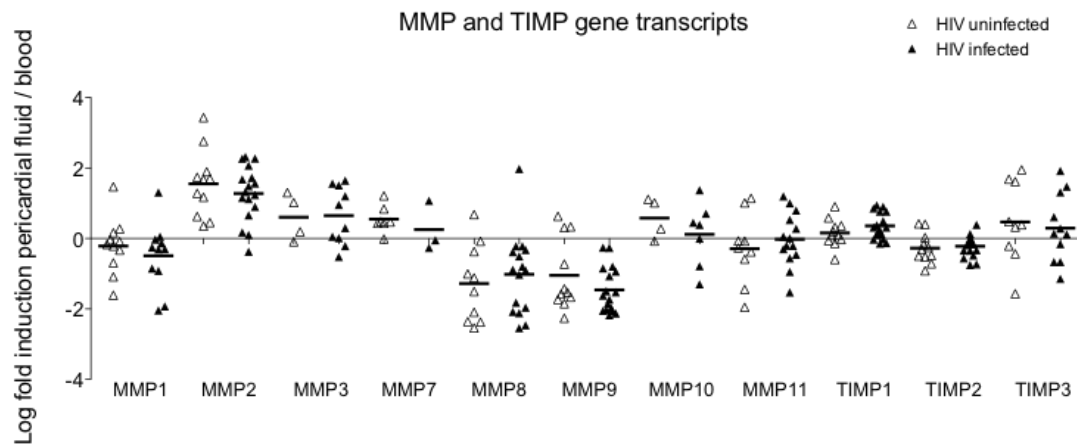


Figure 5.24. Mean log fold induction of pericardial fluid over blood for MMP and TIMP gene transcripts of HIV-1 uninfected and HIV-1 infected TB pericarditis patients

Patients with undetectable gene transcript in both blood and pericardial fluid were excluded from analysis

3.2. Measurement of Protein levels by ELISA

3.2.1. Patient Characteristics

The baseline characteristics of TB pericarditis patients and open-heart surgery control patients included in this part of the study are compared in Table 5.19. TB pericarditis patients were much younger than the open-heart surgery control patients (<0.0001), perhaps biased by the type of surgery the patients were undergoing, as these were surgeries normally needed in the older population, such as bypass and valve replacement. There were 10 male and 21 female open-heart surgery participants, statistically different from the 53 male and 35 female TB pericarditis participants. All open-heart surgery patients were HIV-1 uninfected and had no evidence of TB or previous TB treatment. Their CD4 counts were not recorded. The TB pericarditis patients all had a diagnosis of definite or probable TB and they had a median CD4 count of 167cells/ μ l.

Table 5.19. Characteristics of Combined TB pericarditis and Open-heart Surgery control patients included in the protein analysis by ELISA

	Combined TB pericarditis (n= 89)	Open-heart Surgery controls (n=31)	TB pericarditis vs Open- heart Surgery controls, p-value
Median Age in years, (IQR)	33 (27 – 44)	51 (38 - 64)	<0.0001
TB Diagnosis Definite/Probable	40 Definite/49 Probable	No evidence of TB	/
Gender, Male/Female (M/F)	53M/35F (1 unknown)	10M/21F	0.0273
Median CD4 count cells/μl, (IQR)	167 (83.5 – 343) n=73	Not recorded	/
Number on Anti retroviral treatment	3	All HIV-1 uninfected	/

TB pericarditis patients stratified by HIV status are compared in Table 5.20. The HIV-1 infected patients were significantly younger than their HIV-1 uninfected counterparts ($p=0.012$). There were no differences between the groups for TB diagnosis or gender; however, the CD4 count of the HIV-1 infected patients was much lower than those uninfected with HIV-1 ($p<0.0001$). Three of the HIV-1 infected patients were on ARV treatment for less than 2 weeks duration at the time of recruitment. The remaining patients were sent for HIV-1 counseling and referred to the ARV clinic.

Table 5.20. Characteristics of HIV-1 uninfected and HIV-1 infected TB pericarditis patients included in the protein analysis by ELISA

	HIV-1 uninfected (n=27)	HIV-1 infected (n=61)	HIV-1 uninfected vs HIV-1 infected, p-value
Median Age in years, (IQR)	51 (27.8 – 58.3)	32 (26.5 – 39)	0.012
TB Diagnosis Definite/Probable	9 Definite/18 Probable	31 Definite/30 Probable	0.862
Gender, Male/Female (M/F)	17M/10F	36M/25F	0.999
Median CD4 count cells/μl, (IQR)	381 (184.5 – 651) n=22	105 (65 – 266) n=51	<0.0001
Number on Anti retroviral treatment	0	3	/

3.2.2. Pro-inflammatory cytokines

TB pericarditis patients had increased IL-1 β ($p=0.001$), IL-6 ($p<0.0001$) and IFN- γ ($p<0.0001$) in pericardial fluid compared to serum. Open-heart surgery control patients had elevated IL-6 protein in their pericardial fluid compared to serum ($p=0.0002$), although levels were fairly low. IL-6 along with IL-18, were the only pro-inflammatory cytokines at detectable levels in the pericardial fluid of the control patients (Figure 5.25)

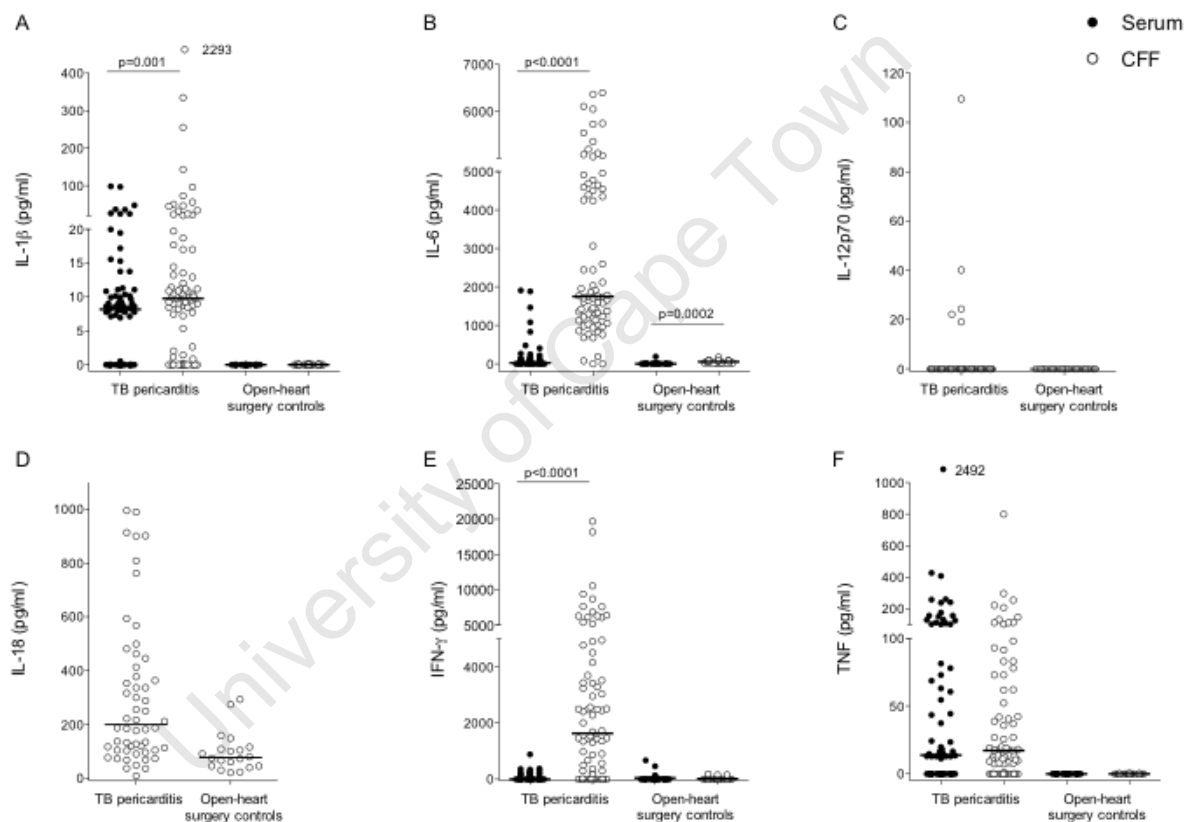


Figure 5.25. Pro-inflammatory cytokine levels measured in serum and cell free pericardial fluid (CFF) of TB pericarditis patients and open-heart surgery control patients

A. IL-1 β , B. IL-6, C. IL12p70, D. IL-18, E. IFN- γ and F. TNF

When stratifying the TB pericarditis patients by HIV-1 status, IL-1 β , IL-6 and IFN- γ were significantly higher in pericardial fluid compared to blood in both HIV-1 uninfected and infected TB pericarditis patients (Figure 5.26). However, when comparing HIV-1 uninfected and HIV-1 infected TB pericarditis patients, only TNF was found to be significantly lower in pericardial fluid of HIV-1 infected patients ($p=0.048$).

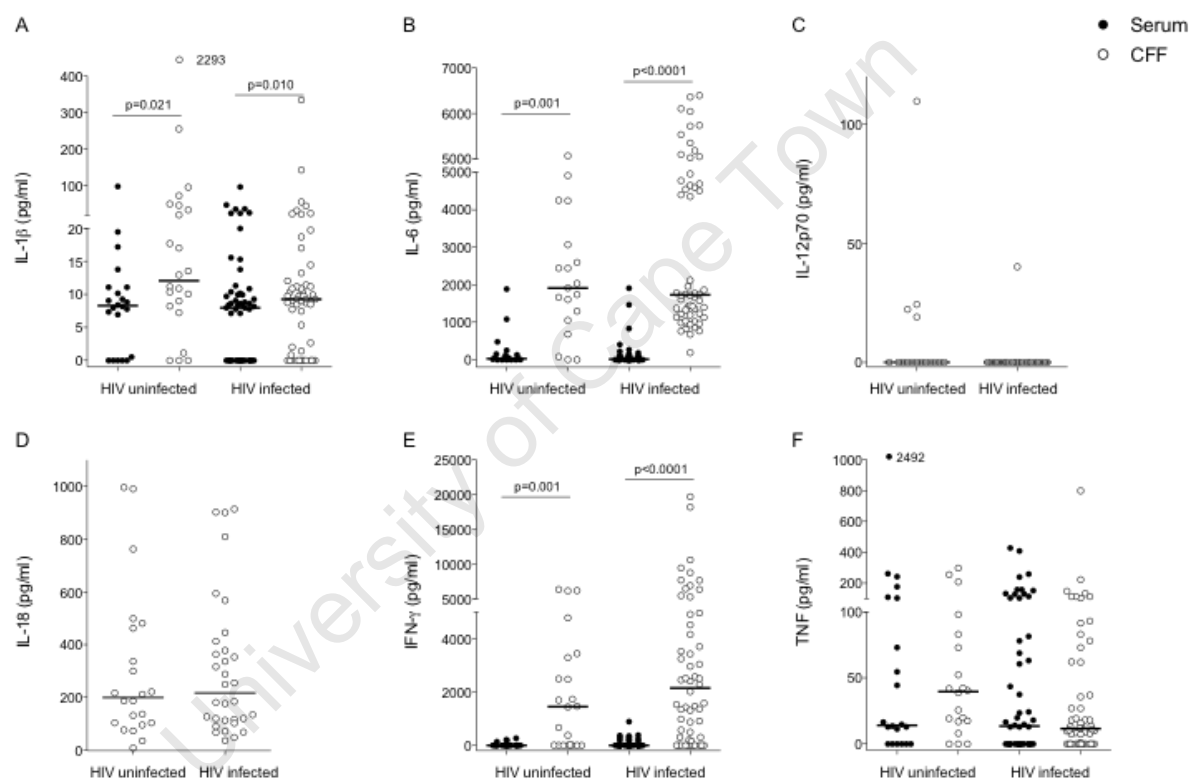


Figure 5.26. Pro-inflammatory cytokine levels measured in serum and cell free pericardial fluid (CFF) of HIV-1 uninfected and HIV-1 infected TB pericarditis patients

A. IL-1 β , B. IL-6, C. IL12p70 (only measured in CFF), D. IL-18 (only measured in CFF), E. IFN- γ and F. TNF

3.2.3. Th2 related proteins

IL-13 was the only Th2 protein assayed by ELISA. IL-13 was detectable at very low concentrations in the CFF of both TB pericarditis patients and controls (median 9.2pg/ml for TB pericarditis patients and 6.4pg/ml for controls), with no significant difference between them. It is interesting to note that IL-13 was significantly higher in the CFF of pericardial TB patients compared to their serum (serum median 0pg/ml, $p<0.0001$) (Figure 5.27).

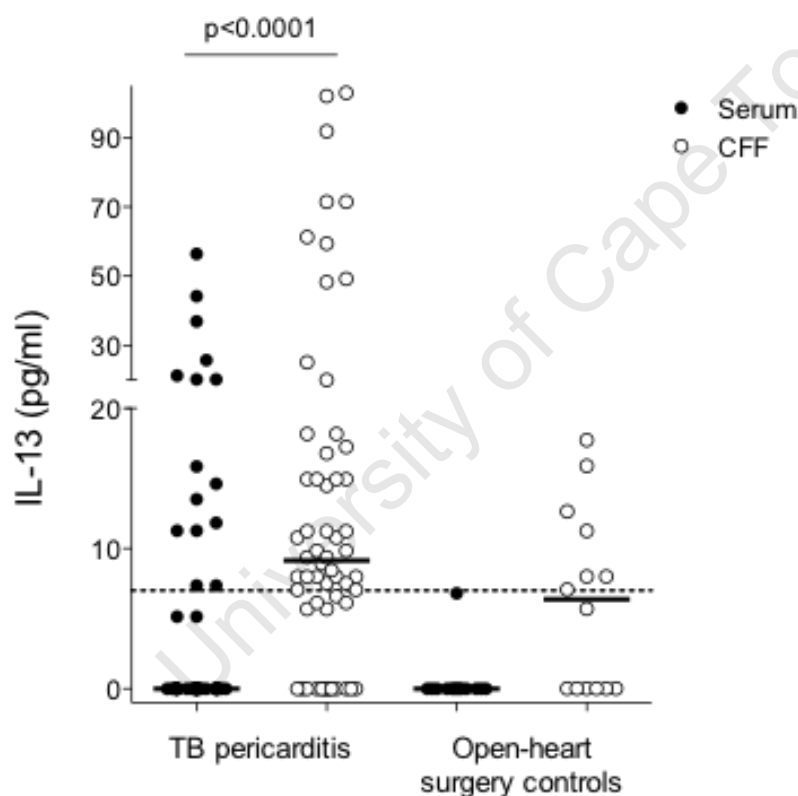


Figure 5.27. IL-13 protein levels in serum and cell free pericardial fluid of TB pericarditis and open-heart surgery control patients

The dotted line shows the lower limit of detection of 7pg/ml for this ELISA

When stratifying the TB pericarditis patients by their HIV status, IL-13 concentrations were higher in the pericardial fluid of both HIV-1 uninfected and HIV-

The scatter plot displays IL-13 protein levels (pg/ml) for two groups: HIV uninfected and HIV infected TB pericarditis patients. The y-axis represents the concentration of IL-13, with a dashed horizontal line indicating the lower limit of detection at 7 pg/ml. The x-axis labels the two groups. For the HIV uninfected group, data points are clustered at low levels, mostly below 10 pg/ml. For the HIV infected group, data points are more widely distributed, with many points above the 7 pg/ml detection limit. A horizontal line with a vertical endcap is drawn across the plot at approximately 10 pg/ml, representing the mean for the HIV infected group. The plot shows a significant overlap between the two groups, particularly in the range of 7 to 20 pg/ml.

The dotted line shows the lower limit of detection of 7pg/ml for this ELISA

3.2.4. Th17 pathway cytokines

Of the cytokines measured in this group, only IL-22 was elevated in the pericardial fluid of TB pericarditis patients ($p < 0.0001$) irrespective of HIV status (HIV-1 uninfected $p = 0.004$ and HIV-1 infected $p < 0.0001$). All Th17 related cytokine levels were absent in the serum or CFF of open-heart surgery control patients (Figure 5.29).

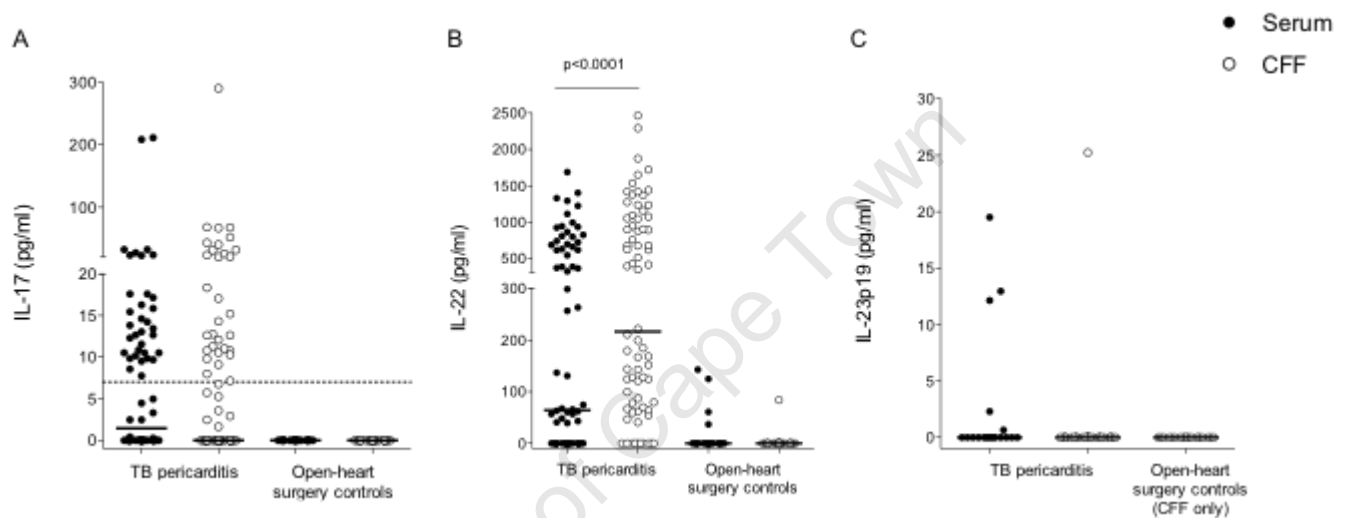


Figure 5.29. Th17 related cytokine levels measured in serum and cell free pericardial fluid (CFF) of TB pericarditis patients and open-heart surgery control patients

A. IL-17A, B. IL-22 and C. IL-23p19

The dotted line shows the lower limit of detection of 7pg/ml for the IL-17A ELISA

Of note, IL-22 measured in the serum of a TB pericarditis patient, had measureable IL-22 in the corresponding pericardial fluid, and a positive correlation of IL-22 between the two sites was identified ($p < 0.0001$, $r = 1.000$, Figure 5.31).

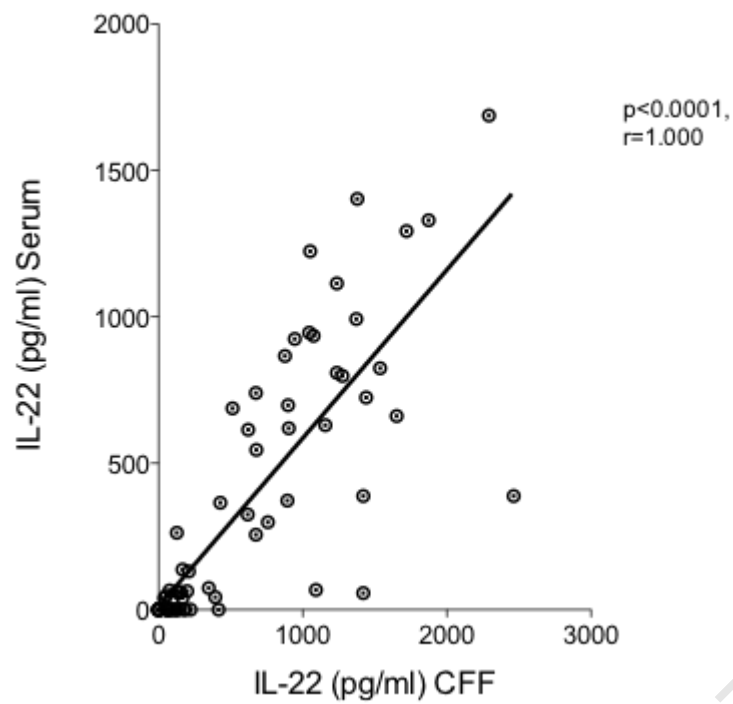


Figure 5.30. Direct correlation of IL-22 in serum and CFF of pericardial TB patients

3.2.5. Regulatory cytokines

While IL-10 was not different in serum or CFF of TB pericarditis patients, TGF- β was increased in the serum of TB pericarditis patients ($p<0.0001$). IL-10 and TGF- β (Figure 5.31) were significantly raised in the serum of the open-heart surgery control patients (IL-10 $p=0.005$ and TGF- β $p=0.02$), but levels were fairly small and infrequent. IL-10 was significantly increased in both serum ($p=0.004$) and pericardial fluid ($p<0.0001$) of TB pericarditis patients, and TGF- β was significantly elevated in the serum of TB pericarditis patients ($p<0.0001$) compared to the control patients.

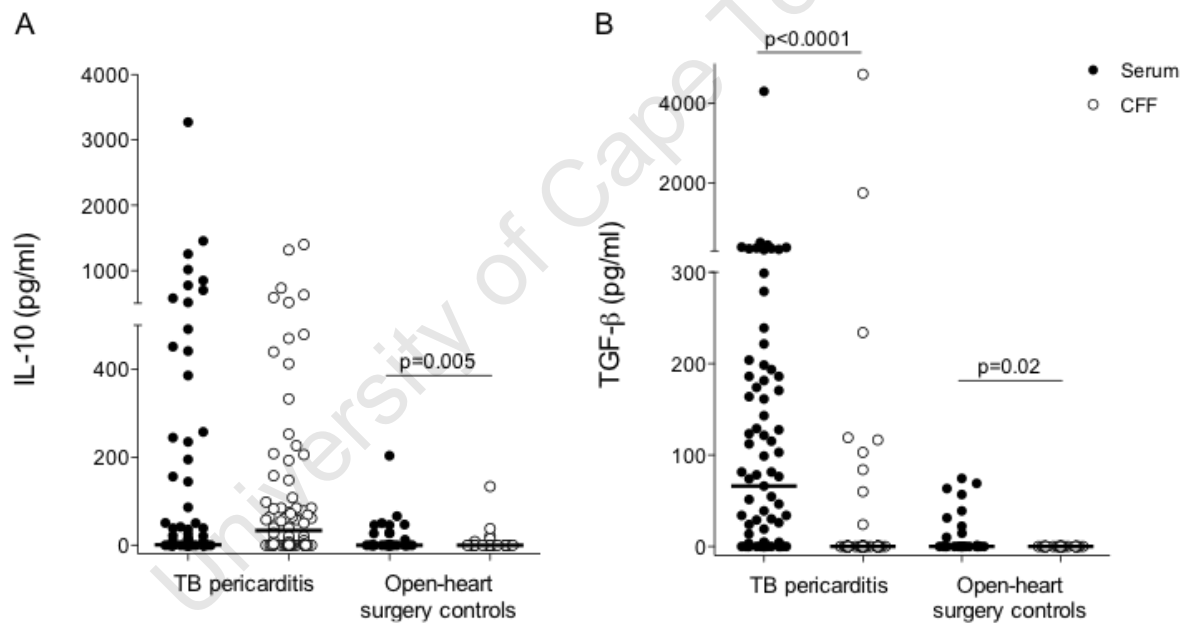


Figure 5.31. Regulatory cytokine levels measured in serum and CFF of TB pericarditis and open-heart surgery control patients

A. IL-10 and B. TGF- β

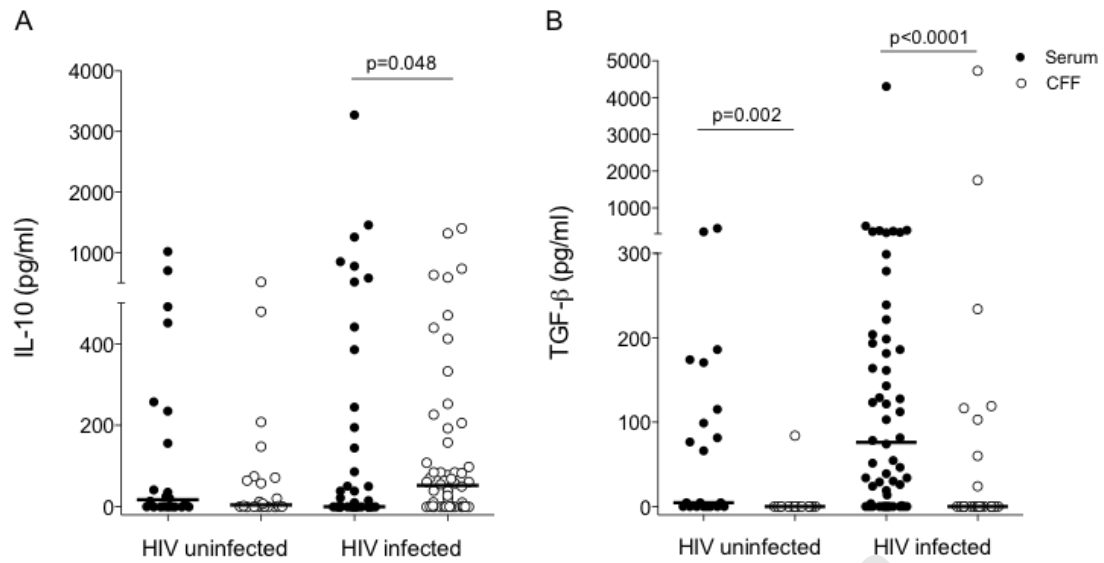


Figure 5.32. Regulatory cytokine levels measured in serum and CFF of HIV-1 uninfected and HIV-1 infected TB pericarditis patients

A. *IL-10* and B. *TGF-β*

TGF-β was significantly elevated in the serum of TB pericarditis patients, irrespective of HIV status (HIV-1 uninfected $p=0.002$, HIV-1 infected $p<0.0001$, Figure 5.32), while IL-10 was raised in the pericardial fluid of HIV-1 infected patients only ($p=0.048$). However, there were no differences in either serum or CFF when comparing HIV-1 uninfected and HIV-1 infected patients.

3.2.6. Neutrophil related proteins

IL-8 was the only neutrophil related protein measured in the serum and CFF of TB pericarditis patients only. IL-8 was significantly elevated in the pericardial fluid compared to serum of TB pericarditis patients (Figure 5.33, $p < 0.0001$).

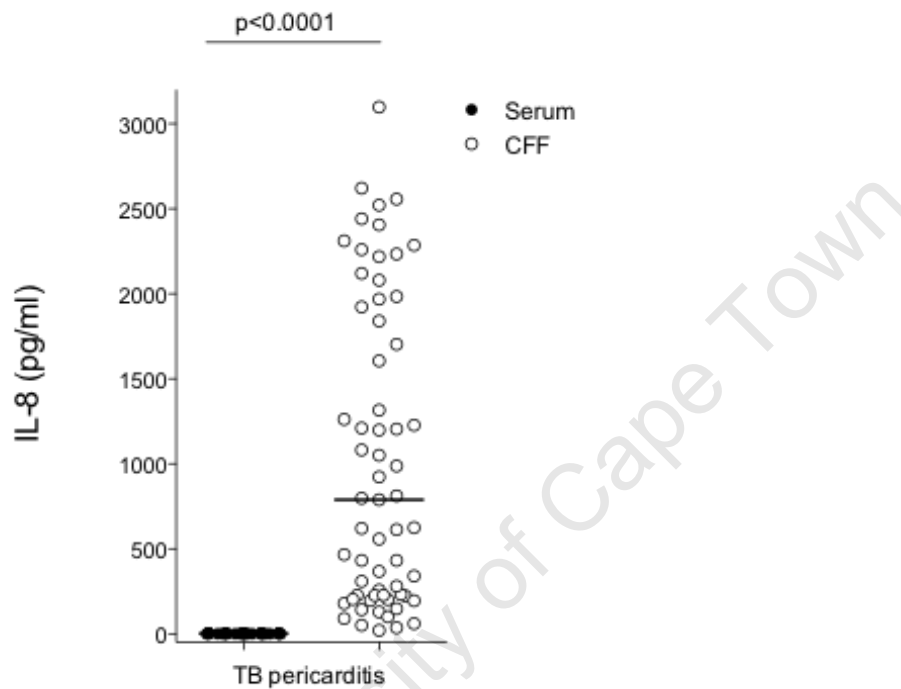


Figure 5.33. IL-8 protein levels measured in serum and CFF of TB pericarditis patients

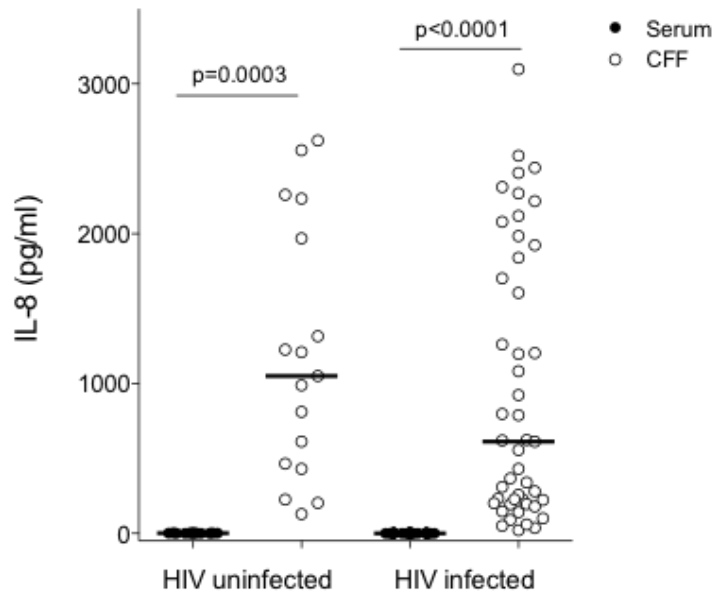


Figure 5.34. IL-8 protein levels measured in serum and CFF of HIV-1 uninfected and HIV-1 infected TB pericarditis patients

When comparing by HIV status (Figure 5.34 and Supplementary Table 5q), there was increased IL-8 measured in the pericardial fluid of both HIV-1 uninfected ($p=0.0003$) and HIV-1 infected ($p<0.0001$) TB pericarditis patients. There tended to be more IL-8 in the pericardial fluid of HIV-1 uninfected patients compared to those infected with HIV-1, however this was not significant ($p=0.162$).

3.2.8. *MMP and TIMP proteins*

Due to the limited volumes of serum and cell free pericardial fluid remaining towards the end of the study, MMP molecules were analysed using Luminex technology.

MMP1, MMP2, MMP3, MMP7, MMP8 and MMP9 were assayed in TB pericarditis patients as well as open-heart surgery controls. The MMP2 and MMP9 protein levels measured confirmed the presence and trend of the mRNA transcript levels of MMP2 and MMP9 genes in blood and pericardial fluid of the TB pericarditis patients.

MMP2 was significantly higher in pericardial fluid compared to serum ($p=0.0001$) and MMP9 was elevated in the serum ($p<0.0001$). MMP7 protein was also significantly raised in the serum compared to pericardial fluid ($p=0.0003$), as opposed to its gene.

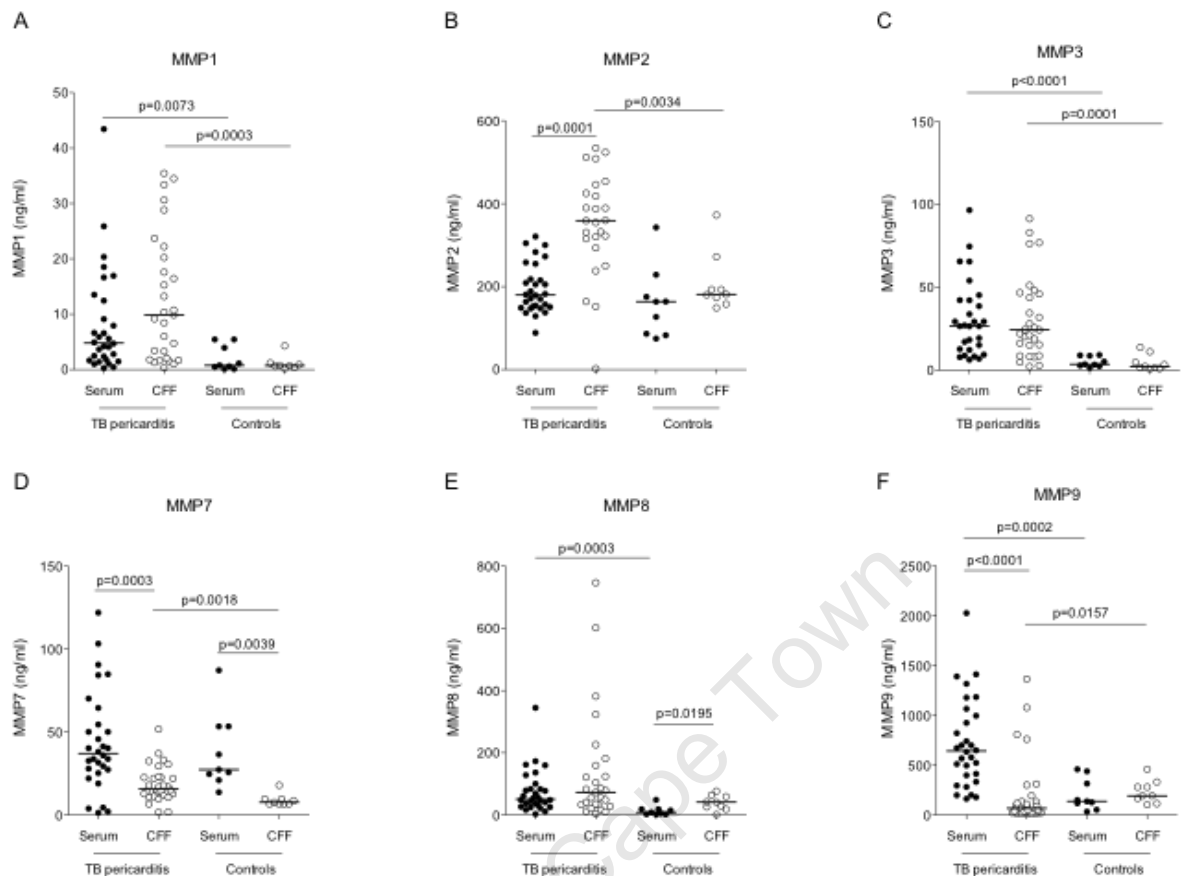


Figure 5.35. MMP protein levels measured in serum and cell free pericardial fluid of TB pericarditis and open-heart surgery controls

A. MMP1, B. MMP2, C. MMP3, D. MMP7, E. MMP8 and F. MMP9

When comparing TB pericarditis patients and open-heart surgery control patients, MMP1 and MMP3 were significantly elevated in both the serum and pericardial fluid of TB pericarditis patients compared to the control patients (Figure 5.35), while MMP2 and MMP7 were elevated in the pericardial fluid only of TB pericarditis versus control patients (all $p<0.005$). MMP9, however, was significantly increased in the serum of TB pericarditis patients compared to the open-heart surgery control patients ($p=0.0002$), while the control patients had slightly elevated protein present in

their pericardial fluid compared to the pericardial fluid of the TB pericarditis patients ($p=0.0157$).

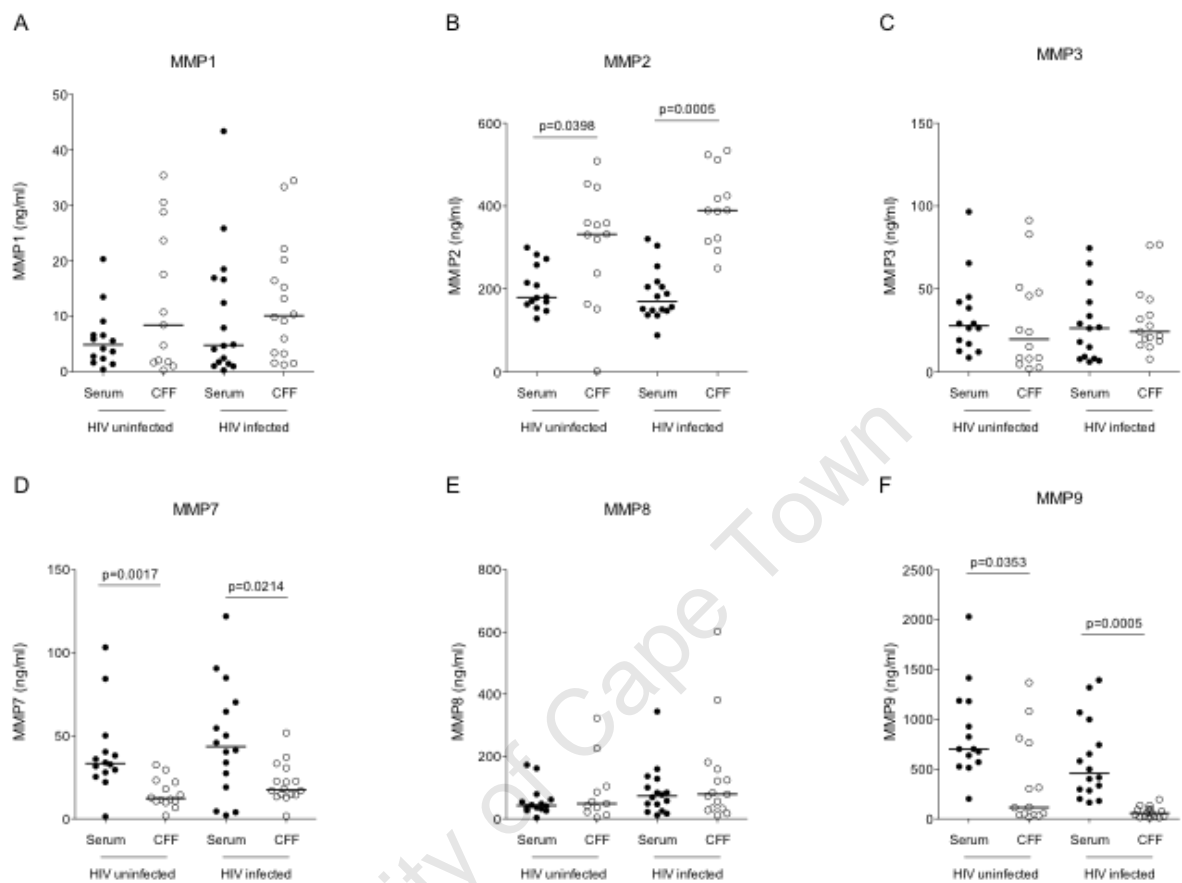


Figure 5.36. MMP protein levels measured in serum and cell free pericardial fluid of HIV-1 uninfected and HIV-1 infected TB pericarditis patients

A. MMP1, B. MMP2, C. MMP3, D. MMP7, E. MMP8 and F. MMP9

When stratifying the TB pericarditis patients by HIV status, MMP2 was significantly elevated in pericardial fluid compared to serum, while MMP7 and MMP9 were significantly increased in serum compared to pericardial fluid, for both HIV-1 uninfected and HIV-1 infected patients (all $p<0.05$, Figure 5.36).

As TIMP molecules bind to MMP molecules in an inhibitory fashion, the presence of MMP2/TIMP1 and MMP9/TIMP2 complexes was analysed in both blood and pericardial fluid from TB pericarditis and open-heart surgery control patients, using complex specific ELISA kits.

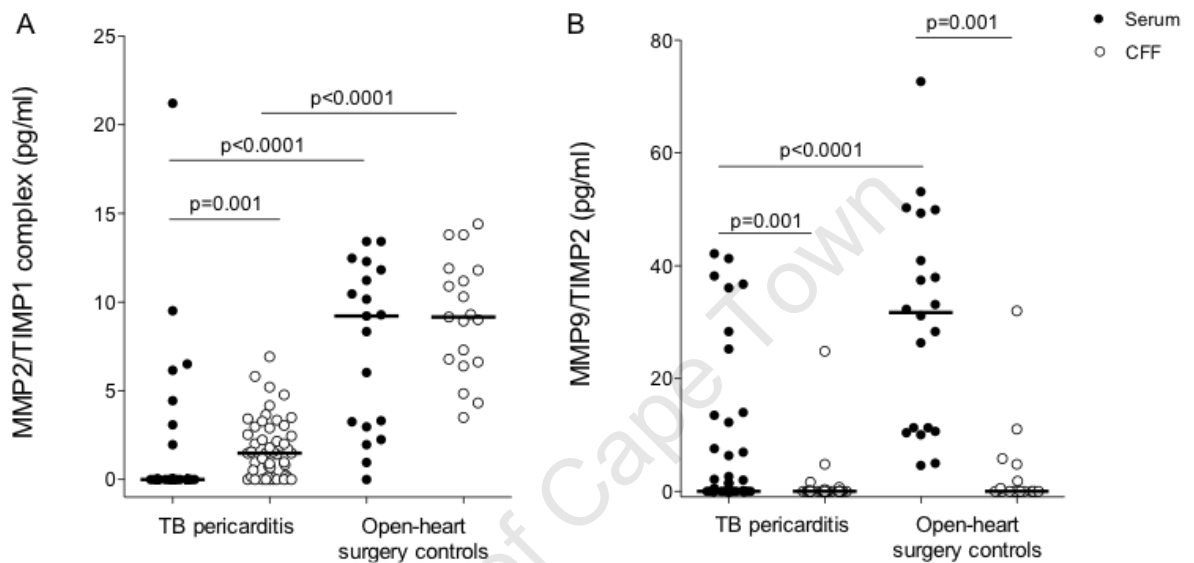


Figure 5.37. MMP2/TIMP1 and MMP9/TIMP2 complex levels in serum and cell free pericardial fluid of TB pericarditis and open-heart surgery control patients

A. MMP2/TIMP1 and B. MMP9/TIMP2

When comparing TB pericarditis and open-heart surgery controls (Figure 5.37), the MMP2/TIMP1 complex was found to be significantly elevated in both the serum and CFF of the open-heart surgery control patients (both $p<0.0001$), while MMP9/TIMP2 was significantly increased in serum of control patients compared to TB pericarditis patients ($p<0.0001$). When comparing serum and pericardial fluid in the TB pericarditis patients, there was elevated MMP2/TIMP1 complex in the CFF compared

to serum ($p=0.001$), and slightly elevated, although significant, MMP9/TIMP2 complex measured in the serum compared to CFF ($p=0.001$).

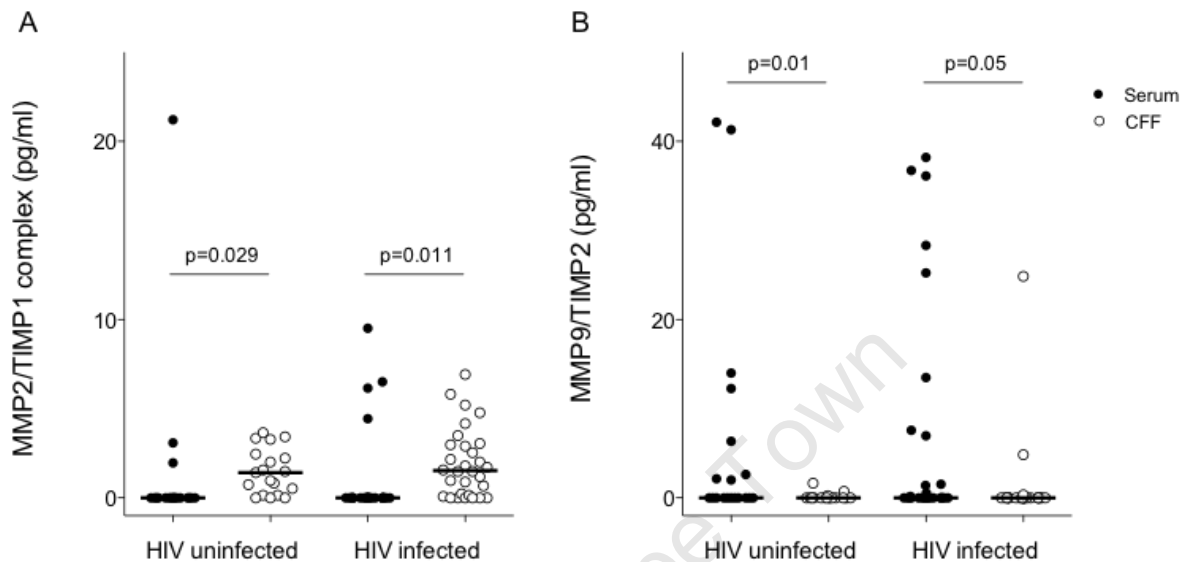


Figure 5.38. MMP2/TIMP1 and MMP9/TIMP2 complex levels in serum and cell free pericardial fluid of HIV-1 uninfected and HIV-1 infected TB pericarditis patients

A. MMP2/TIMP1 and B. MMP9/TIMP2

When stratified by HIV status (Figure 5.38) MMP2/TIMP1 was consistently increased in the CFF compared to serum in both HIV-1 uninfected ($p=0.029$) and HIV-1 infected ($p=0.011$) patients, and MMP9/TIMP2 was elevated in the serum compared to CFF in both HIV-1 uninfected ($p=0.01$) and HIV-1 infected ($p=0.05$) TB pericarditis patients. There were no differences when comparing HIV-1 uninfected to HIV-1 infected TB pericarditis patients.

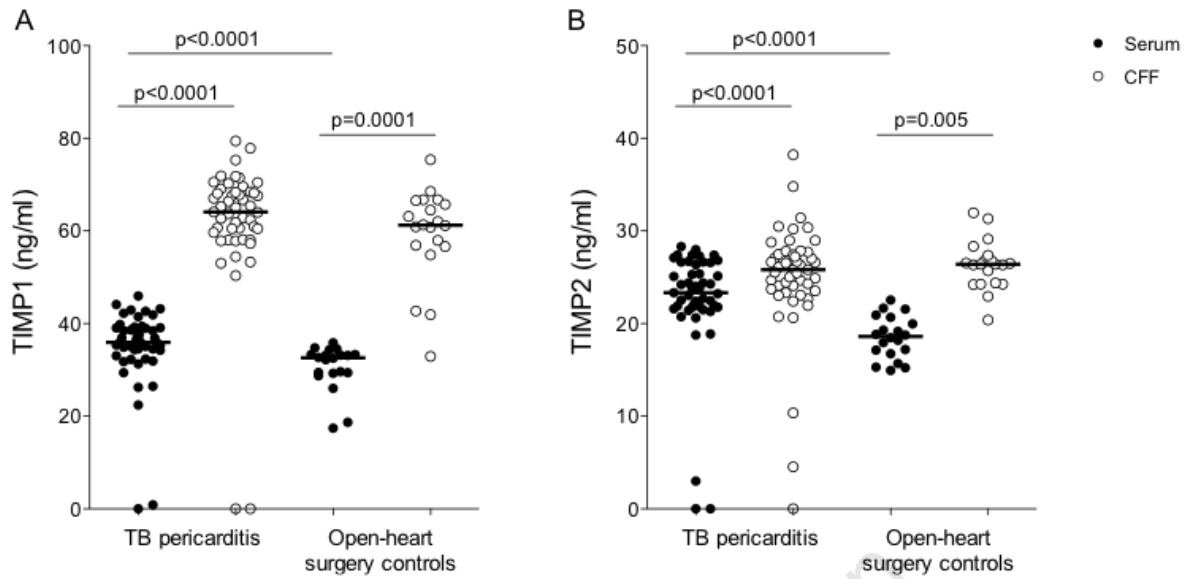


Figure 5.39. TIMP1 and TIMP2 levels in serum and cell free pericardial fluid of TB pericarditis and open-heart surgery control patients

A. TIMP1 and B. TIMP2

TIMP1 and TIMP2 proteins were analysed separately by ELISA in serum and CFF from TB pericarditis and open-heart surgery control patients (Figure 5.39). Both these proteins were significantly increased in CFF compared to serum (all $p < 0.005$), however TB pericarditis patients had more TIMP1 and TIMP2 in serum compared to the control patients (both $p < 0.0001$).

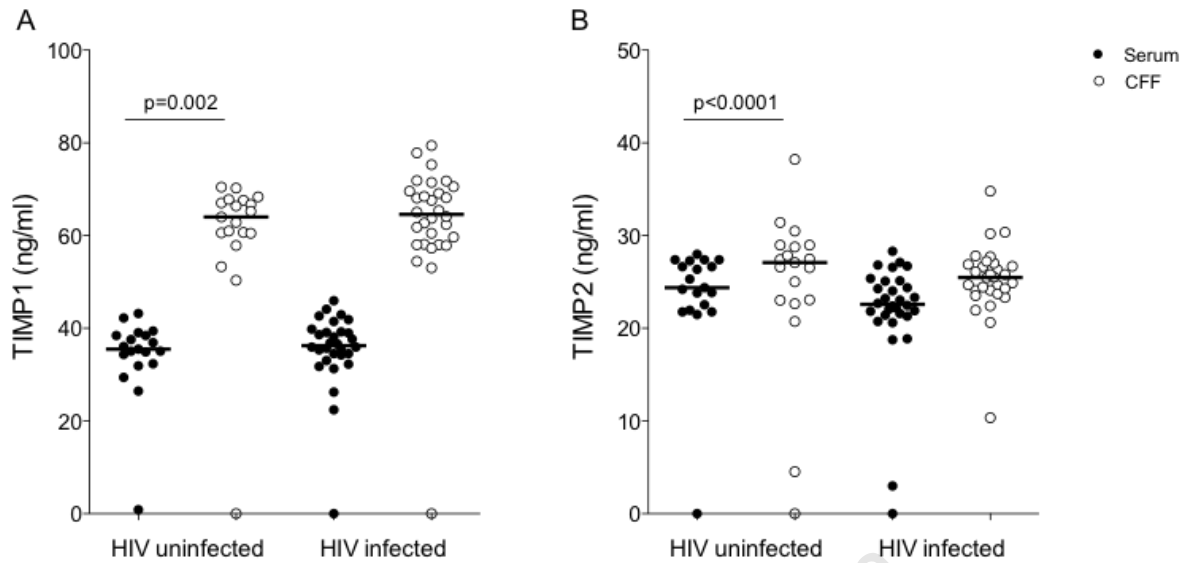


Figure 5.40. TIMP1 and TIMP2 levels in serum and cell free pericardial fluid of HIV-1 uninfected and HIV-1 infected TB pericarditis patients

A. TIMP1 and B. TIMP2

When stratifying the TB pericarditis patients by HIV status (Figure 5.40), TIMP1 showed consistent elevation in the CFF compared to serum for both HIV-1 uninfected ($p=0.002$) and HIV-1 infected ($p<0.0001$) TB pericarditis patients, while TIMP2 remained elevated only in the CFF of HIV-1 infected patients compared to serum ($p=0.004$). There were however, no differences when comparing HIV-1 uninfected to HIV-1 infected TB pericarditis patients.

3.2.9. Cell Death Detection ELISA

To determine the level of DNA degradation from apoptotic or necrotic cells, Cell Death Detection ELISA on the CFF from TB pericarditis and open-heart surgery control patients was performed. There was significantly more cell death occurring in the pericardium of patients infected with TB than in the open-heart surgery control patients ($p < 0.0001$) (Figure 5.41). The HIV status of the TB pericarditis patients had no influence on the amount of cell death detected by the ELISA in the pericardial fluid (data not shown).

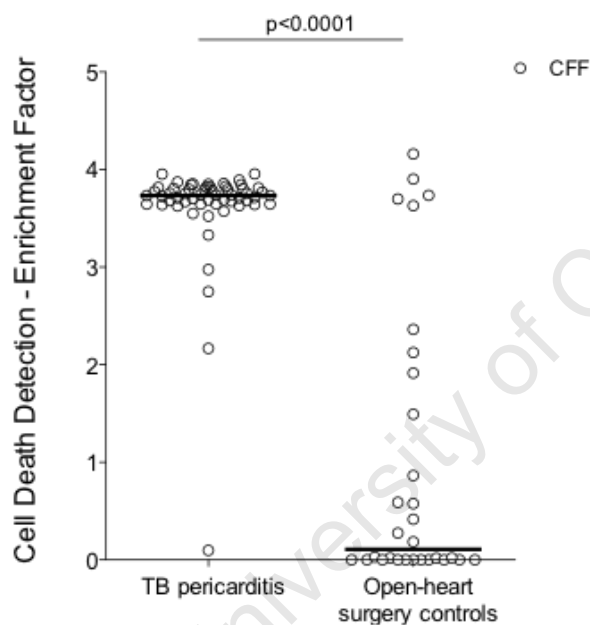


Figure 5.41. Cell Death Detection ELISA shows more cell death in TB pericarditis patients

3.3. Correlations and manipulations of data

Genes identified to play a role in the activation or stimulation of other genes and proteins were tested to see if there were any correlations in either the blood or pericardial fluid of TB pericarditis patients. It was determined that MMP9 ($r=0.672$, $p<0.0001$) and MMP10 ($r=0.748$, $p=0.007$) mRNA measured in the pericardial fluid was positively correlated with IL-1 β in the pericardial fluid (Figure 5.42). MMP9 transcripts measured in the pericardial fluid also correlated directly with IL-8 transcript in the pericardial fluid (Figure 5.43). The procollagen genes measured in the pericardial fluid correlated positively with each other (Figure 5.44). In the HIV-1 uninfected patients, MMP9 measured by Luminex in CFF correlated positively with IL-22 in both serum ($r=0.665$, $p=0.018$) and CFF ($r=0.658$, $p=0.02$) measured by ELISA (Figure 4.45).

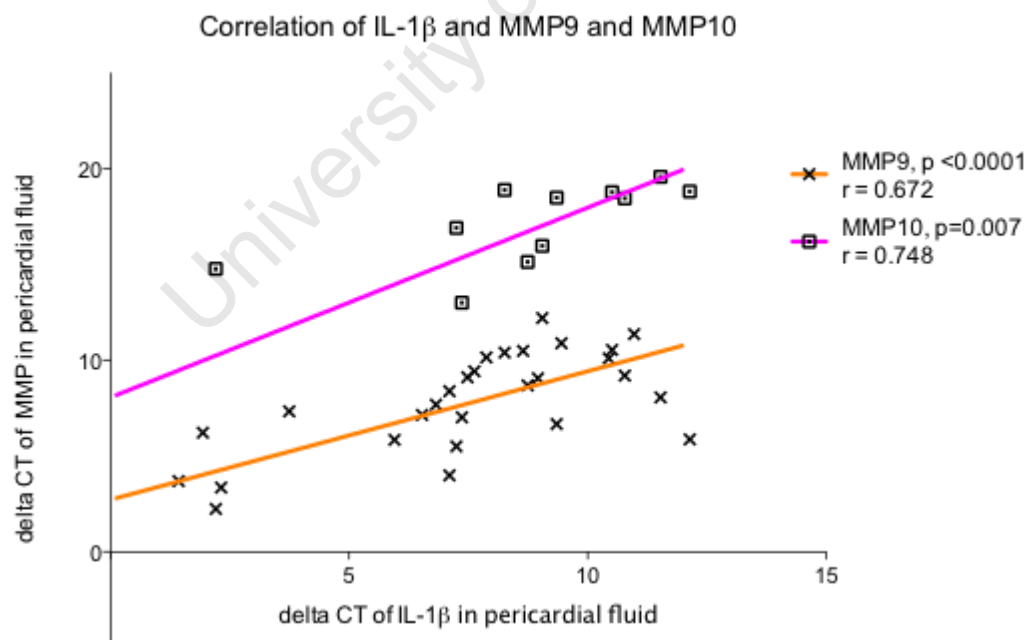


Figure 5.42. Positive correlation of IL-1 β and MMP9 and MMP10 in pericardial fluid of TB pericarditis patients

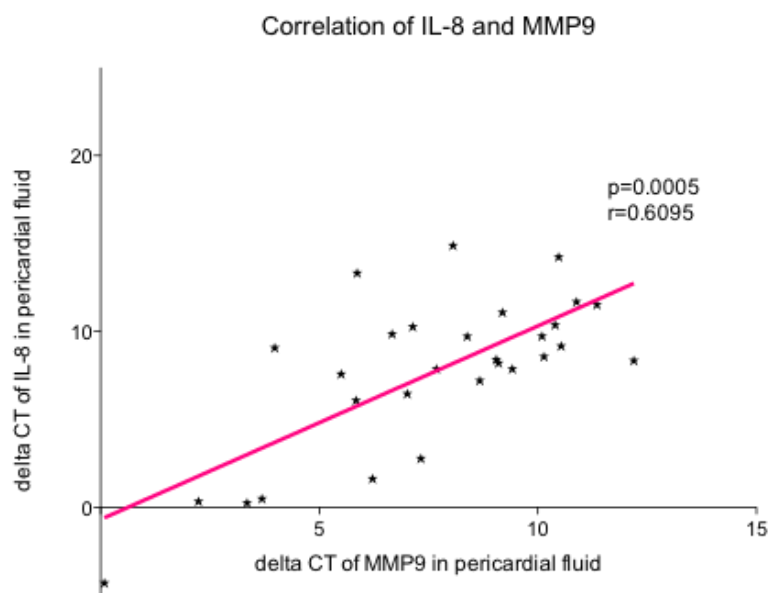


Figure 5.43. Positive correlation of IL-8 and MMP9 in pericardial fluid of TB pericarditis patients

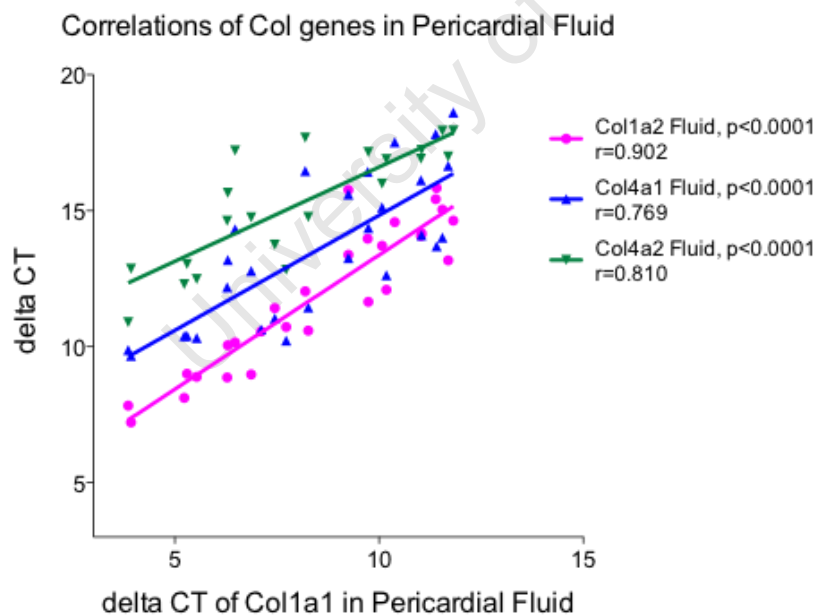


Figure 5.44. Positive correlation of procollagen genes in pericardial fluid of TB pericarditis patients

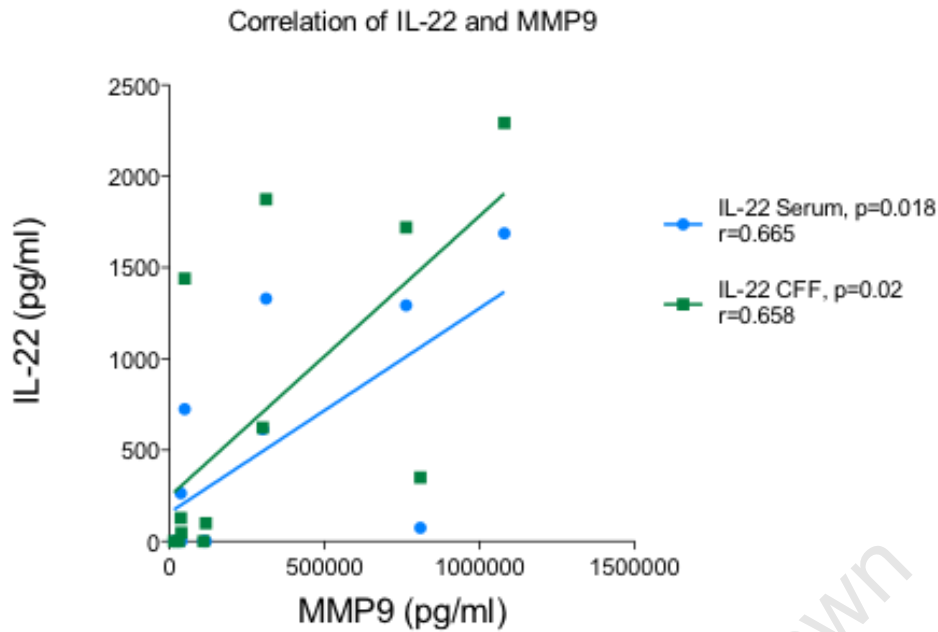


Figure 5.45. Positive correlation of IL-22 in serum and CFF and MMP9 in CFF of HIV-1 uninfected patients

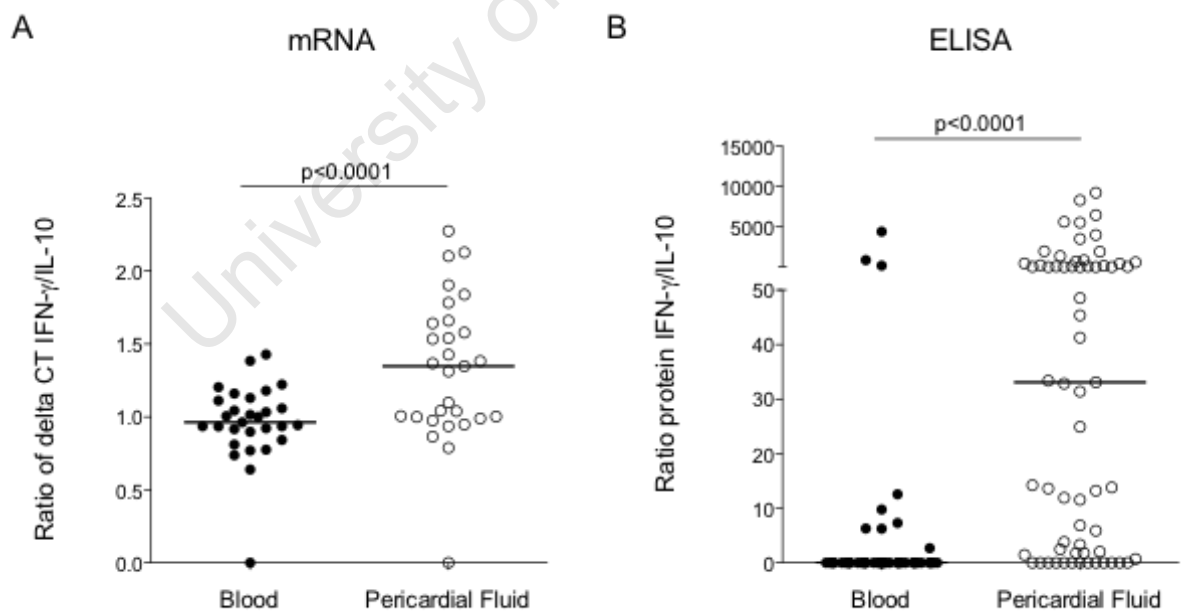


Figure 5.46. Ratio of IFN- γ /IL-10 is significantly increased in Pericardial fluid

A. The ratio of delta CT of IFN- γ /IL-10 mRNA in blood and pericardial fluid

B. The ratio of protein levels of IFN- γ /IL-10 in blood and pericardial fluid

The ratio of IFN- γ /IL-10 was analysed using both the RT-PCR and ELISA results for both blood and pericardial fluid as an increased ratio was shown to associate with disease severity in extrapulmonary TB (194). Both mRNA and protein levels of IFN- γ /IL-10 were significantly increased in the pericardial fluid compared to blood (both $p < 0.0001$, Figure 5.46), and this was irrespective of the HIV status of the TB pericarditis patients (data not shown). This analysis was not carried out on the open-heart surgery control patients as these patients had no IFN- γ or IL-10 detectable in their serum or CFF, thus the ratio would be 0.

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4. Discussion

I have evaluated the mRNA transcript profile of pericardial TB by measuring 42 genes, and the protein profile by measuring 24 secreted products. Low IFN- γ gene transcript was detected in the pericardial fluid cells compared to blood, as well as in the HIV-1 infected patients. One study looked at IFN- γ mRNA in lungs of TB patients and showed that the lower levels of IFN- γ mRNA were found in the lungs of patients with the highest levels of lung damage (235). The pericardium, during *M.tb* infection, is undergoing constant inflammation and degradation of the extracellular matrix, causing immense damage and scarring, and this could account for the low levels of IFN- γ mRNA measured in the pericardial fluid. Another reason for low levels of IFN- γ mRNA, but increased protein, is cells recruited to the site of disease are activated, short-lived T cells primed for cytokine release. They immediately release cytokines, such as IFN- γ , just before they die or apoptose. The same may be true for cells producing IL-1 β and MMP1. A Cell Death Detection ELISA was performed to determine the amount of cell death in normal (open-heart surgery controls) pericardial fluid as well as in pericardial fluid infected with *M.tb* and/or HIV. TB infection causes significant cell death ($p < 0.0001$, Figure 5.42) with little or no cell death occurring in normal pericardial fluid, due to the absence of infiltrate containing cells, irrespective of HIV status.

Regulatory IL-10 mRNA was significantly elevated in pericardial fluid compared to blood of all TB pericarditis patients, especially in the HIV-1 uninfected patients. IL-10 protein however, was significantly elevated in the pericardial fluid compared to blood of HIV-1 infected patients ($p = 0.048$), but was not different for HIV-1 uninfected patients. *M.tb* has been shown to stimulate IL-10 production by human

macrophages (236) which delays the activation of macrophages and T cells to allow the organism time to target and enter the macrophages. Once inside the macrophage, IL-10 production prevents apoptosis, thereby enhancing the survival of *M.tb* inside the cell (237). IL-10 decreases chronic inflammation through many mechanisms (238) and is also shown to directly inhibit fibrosis (239). IL-10 has been shown to decrease mRNA for TNF (240), IFN- γ and IL-12 (241), the cytokines needed to kill *M.tb* organisms.

As the ratio of IFN- γ /IL-10 was linked to disease severity (194), I analysed both mRNA and protein levels of these proteins. It was determined that the IFN- γ /IL-10 ratio was significantly elevated in the pericardial fluid compared to blood for both mRNA and protein levels in TB pericarditis patients (Figure 5.46) and this was not influenced by HIV-1 (data not shown). The IFN- γ /IL-10 ratio was not determined in the open-heart surgery control patients as they had little or no IFN- γ or IL-10 present in either serum or CFF samples. This points to an out of control inflammatory response occurring in the pericardium during *M.tb* infection, with over production of IFN- γ and a lack of regulatory IL-10.

FoxP3 transcript was significantly upregulated in the pericardial fluid compared to blood ($p=0.0001$) irrespective of HIV status, however HIV-1 uninfected patients had higher FoxP3 mRNA in their blood compared to HIV-1 infected TB pericarditis patients ($p=0.007$). FoxP3 is the best marker for regulatory T cells, and it is known that FoxP3 mRNA is increased in PBMC from patients with TB compared to healthy controls and significantly increased in TB patients who developed extrapulmonary TB compared to those with pulmonary TB (162).

IL-6 and TNF mRNA were significantly elevated in the pericardial fluid compared to the blood of TB pericarditis patients. IL-6 was not affected by the HIV status of the patients, whereas HIV-1 infected patients tended to have lower mRNA for TNF in the pericardial fluid compared to blood. TNF mRNA was also detected in pleural fluid of patients with TB (242). IL-6 is a pro-inflammatory cytokine that is elevated during infections, myocardial infarctions and even surgery (243). Other studies have shown that TB patients spontaneously produce IL-6 and TNF pointing to unspecified systemic inflammation, and not necessarily confined to sites of disease and these proteins have been identified as contributors to heart failure (244) and progressive left ventricular dysfunction (245).

IP-10 mRNA was found to be increased in pleural TB effusions (246). IP-10 was significantly elevated in the pericardial fluid compared to blood of HIV-1 uninfected patients ($p=0.042$), but was similar in blood and pericardial fluid of the HIV-1 infected group. IP-10 is induced by IFN- γ secretion, which was more plentiful in the HIV-1 uninfected patients.

TGF- β gene transcript was measured in high abundance in both the pericardial fluid (median delta CT = 3.8) and blood (median delta CT = 3.0), and significantly increased in the blood of TB pericarditis patients. High levels of TGF- β can inhibit IL-2 production and may partially account for the low IFN- γ mRNA (241, 247), and in turn result in poor recruitment of *M.tb* specific antigen experienced T cells to the site of infection (248), especially in the HIV-1 infected patients (as shown in Chapter 4). TGF- β gene transcript is increased in children with TB, and correlates with the

extent of TB disease, playing a pivotal role in the balance between wound healing, excessive fibrosis and tissue damage (249) and while TGF- β has beneficial anti-inflammatory effects (250) it also plays a role in the stimulation of collagen synthesis. Femtomolar concentrations of TGF- β is known to elicit direct chemotactic responses from neutrophils (251) and from monocytes (252), while picomolar concentrations activate monocytes to synthesize IL-1 β and TNF (252, 253) and has a deactivating effect on macrophages by inhibiting pro-inflammatory cytokine and chemokine synthesis (254, 255). The abundance of gene transcript for TGF- β in both blood and pericardial fluid may regulate pro-inflammatory cytokines downstream, influencing the outcome of the immune response.

There was little IL-4 mRNA present in either blood or pericardial fluid of TB pericarditis patients, although transcript was increased in HIV-1 uninfected patients blood ($p=0.002$) and pericardial fluid ($p=0.035$). Pericardial TB has been shown to elicit a Th1 dominant immune response and low IL-4 mRNA adds to this imbalance.

IL-17A mRNA or protein was not different between blood and pericardial fluid, and there was no mRNA detectable in the HIV-1 infected patients and only small levels of protein, perhaps indicating downregulation of Th17 cells by HIV in the pericardial environment due to the increased levels of IFN- γ protein which is known to suppress Th17 function. IL-23p19 was not differentially expressed in blood or pericardial fluid of all TB pericarditis patients combined, however when stratified by HIV, there was increased gene transcript in both blood ($p=0.024$) and pericardial fluid ($p=0.021$) of HIV-1 uninfected patients, again showing impaired Th17 immune response in the pericardial environment in HIV-1 infected patients (256, 257). The corresponding

protein was not detected in either blood or pericardial fluid (both medians = 0pg/ml). IL-23p19 protein is needed for Th17 cell function (258).

IL-22 is another cytokine released by Th17 cells and although there was no difference in transcript levels between blood or pericardial fluid, the protein was significantly raised in both blood and pericardial fluid of TB pericarditis patients compared to the open-heart surgery control patients (both $p < 0.0001$), and significantly increased in the pericardial fluid compared to blood of TB pericarditis patients ($p < 0.0001$), in both HIV-1 uninfected ($p = 0.004$) and HIV-1 infected ($p < 0.0001$) patients. IL-22 is a pro-inflammatory cytokine that may contribute to the inflammatory response at the site of disease (191) and may be produced by a distinct population of cells unable to produce IL-17, now termed Th22 (190, 191). This hypothesis will be investigated in Chapter 6 of this thesis.

Of interest was the significant direct correlation between IL-22 (Figure 5.31) measured in the serum and pericardial fluid of TB pericarditis patients. This is of great interest for using this protein as a biomarker of disease or for diagnosis purposes using serum only. This may imply that IL-22 is made by specific cells at the disease site, which then filters into the blood. IL-22 was also shown to be elevated in pleural fluid (190) and correlates with plasma levels (personal communication Thomas Scriba), however, it would also need to be studied at other disease sites.

Despite the reported increase of IL-22 protein and transcripts or IL-22-expressing cells in *M.tb* infection of macaques (259, 260) or in disease sites of humans with TB, it remains unknown what role IL-22 plays in host defence against *M.tb*. One

possibility may be the well-described function of IL-22 as a regulator of keratinocyte mobility, epidermal differentiation and wound healing (261-264). The success of *M.tb* as pathogen is dependent on its destruction of the pulmonary extracellular matrix. This process which is, at least partly, driven by MMPs brings about cavitation and thereby creates an immunoprivileged niche (265). Increased IL-22 at the TB disease site may be involved in the healing response, rather than anti-mycobacterial immune mechanisms. The correlations of IL-22 in pericardial fluid with MMP9 levels in pericardial fluid and blood of HIV-1 uninfected patients support this hypothesis (Figure 4.45). Notably, peripheral blood MMP9 levels were previously associated with severity of TB disease (266) and higher levels of MMP9 were detected in pleural fluid from patients with TB pleural effusions, compared with patients with congestive heart failure (267).

The neutrophil chemoattractant IL-8 was investigated to determine the role of neutrophils and their antimicrobial secretions during *M.tb* infection of the pericardium. IL-8 mRNA and protein was significantly elevated in the pericardial fluid compared to blood in all TB pericarditis patients, regardless of the HIV-1 status of the patients. IL-8 can be increased via many mechanisms including activation by certain MMP molecules. MMP8 and MMP9 increase IL-8 activity (100) and in fact the delta CT of the MMP9 and IL-8 genes measured in pericardial fluid directly correlate with each other ($r=0.6095$, $p=0.0005$, Figure 5.44), showing this may hold true in TB pericarditis. Further mechanistic studies could shed more light on this matter. Neutrophil counts were not performed, and this is a limitation of this study, but cytology of the pericardial fluid revealed the cellular composition of the fluid to

be inflammatory lymphocytes with the presence of some neutrophils (personal communication Mpiko Ntsekhe).

The gene transcripts for CAMP, LCN2 and HNE are all significantly elevated in the blood compared to pericardial fluid of TB pericarditis patients. Defensin B4 is the only product of neutrophils to be raised in the pericardial fluid compared to blood of TB pericarditis patients, especially so in HIV-1 infected patients ($p=0.008$), however the delta CT levels of DEFB4 were very high, indicating low levels of the transcript. These molecules are important for killing the *M.tb* organism, and their low level in the pericardium could indicate absence of neutrophils or that these cells have already degranulated.

Cytokines play a role in the inflammatory and regulatory response in the pericardium during TB, but some also regulate the fibrotic response at sites of fibrosis. Collagen is a major component of the extracellular matrix (ECM) and dysregulation of its transcription can lead to overproduction and fibrosis (268). There was evidence of compartmentalization of collagen to the site of disease, as all the procollagen transcripts (*Col1a1*, *Col1A2*, *Col4A1* and *Col4a2*) measured were significantly elevated in the pericardial fluid compared to blood of all the TB pericarditis patients, and HIV-1 infection did not affect this expression. The procollagen transcript levels positively correlated with each other (Figure 5.44) showing that the pericardium is a collagen rich environment, which is in constant need of regulation to repair damage caused by *M.tb* and/or HIV-1, but also to prevent fibrosis.

The fibrotic regulators *ARG1* and *SPARC* (102) were significantly decreased in the pericardial fluid compared to blood in all TB pericarditis patients, even when stratified by HIV-1 status (all $p < 0.005$), indicating evidence of an dysregulated pro-fibrotic environment in the pericardium.

IL-1 β , TNF and IL-6 can directly decrease collagen synthesis and procollagen mRNA expression in myofibroblasts during cardiac injury (103). They function by increasing the breakdown of collagen by increasing collagenase MMP activity, both at transcriptional and post-transcriptional levels (103, 269). However, none of these cytokines correlated with procollagen expression in either blood or pericardial fluid from TB pericarditis patients (data not shown).

IL-1 β is known to increase MMP1, MMP2, MMP3 and MMP9 (270-273) expression, while MMP2, MMP3 and MMP9 can activate IL-1 β (229). I have shown that IL-1 β mRNA positively correlates with MMP9 and MMP10 mRNA in the pericardial fluid of TB pericarditis patients (Figure 5.42).

Some MMPs, such as MMP1, are collagenases and cleave fibrillar collagen. MMP1 is specifically expressed in macrophages found around granulomas in the lung. MMP1 was found to be *M.tb* specific (274) and not regulated by BCG (275). However, in my study, MMP1 protein was increased in TB pericarditis patients compared to controls, and was similar in blood and pericardial fluid, irrespective of HIV status. It does not seem to be dysregulated, merely increased, during *M.tb* infection of the pericardium. MMP3 protein was elevated in both blood and pericardial fluid of TB pericarditis patients compared to the open-heart surgery

control patients, consistent with MMP3 mRNA, and while HIV-1 infection did not alter the expression of the protein, HIV affected the mRNA abundance, thus suggesting again, dysregulated collagenase activity in the pericardium during concurrent *M.tb* and HIV-1 infection.

TGF- β is a potent stimulator of collagen synthesis by increasing transcription and decreasing degradation of collagen by increasing TIMP activity or decreasing MMP (collagenase) activity (105) and this may be mediated through CTGF by activating myofibroblasts to stimulate ECM production (204, 276). CTGF mRNA is significantly elevated in the pericardial fluid compared to blood in both HIV-1 uninfected ($p=0.001$) and HIV-1 infected ($p=0.0004$) TB pericarditis patients. CTGF did not positively correlate with TGF- β mRNA (data not shown), and may not mediate myofibroblast function in this way.

I have shown that TGF- β mRNA is largely abundant in both the blood and pericardial fluid of TB pericarditis patients (delta CT 3.0 in blood and 3.8 in pericardial fluid), significantly more so in the blood compared to the pericardial fluid ($p<0.0001$), irrespective of HIV status (HIV-1 uninfected and HIV-1 infected, $p=0.001$). TGF- β protein was only detected in serum from TB pericarditis patients ($p<0.0001$ compared to pericardial fluid) and more so in serum from HIV-1 infected patients and not detected in the open-heart surgery control patients. The gene transcript level of TGF- β was abundant in the pericardial fluid and there was an absence of protein, suggesting that the protein was used relatively quickly in either the fibrotic or regulatory response to *M.tb* and/or HIV-1.

MMP2 and MMP9 are capable of activating TGF- β (277). Both MMP2 and MMP9 form part of the gelatinase group of MMP molecules that break down collagens, fibrin and gelatin. However, these 2 molecules are differentially regulated. MMP2 mRNA and protein was significantly higher in pericardial fluid compared to blood (mRNA $p < 0.0001$, protein $p = 0.0001$) while MMP9 mRNA and protein was significantly higher in blood compared to pericardial fluid (mRNA $p < 0.0001$, protein $p < 0.0001$) of all TB pericarditis patients, irrespective of the HIV-1 status of the patients. Although there was no correlations of either with TGF- β (data not shown), MMP9 mRNA was expressed similarly to TGF- β in both blood and pericardial fluid. A study limitation is that serum and not plasma was collected from patients in this study. Serum is not the best sample to measure MMPs and the clotting agents may alter some of the levels measured (278, 279)

Sime, *et al.* showed that a one dose delivery of TGF- β to the bronchial epithelium of mice initiated a local fibrotic response in BAL for up to 10 days, and pulmonary tissue fibrosis progressed for 60 days after, despite the absence of continued TGF- β expression. The fibrosis was extensive and produced scarring of the pleura and the persistence may be independent of continued TGF- β production (280).

A TGF- β independent mechanism of inducing fibrosis (281), is increasing collagen production via IL-13. IL-13 is produced by Th2 cells (282) and shares the IL-4R α receptor with IL-4. IL-13 has many functions when it comes to collagen synthesis; it increases total collagen and type I collagen, inhibits the degradation of collagen by inhibiting the collagenases MMP1 and MMP3 and thereby enhancing collagen deposition, and it specifically induces procollagen gene expression (283). Blocking

of IL-13 in mice with schistosomiasis decreased collagen deposition by more than 85% (284, 285) and the absence of fibrosis is associated with low IL-4 and IL-13 (286). IL-13 mRNA, however, was not differentially expressed in blood or pericardial fluid in TB pericarditis patients, although the protein was elevated in pericardial fluid in all TB pericarditis patients ($p<0.0001$) as well as those infected with HIV-1 ($p=0.0001$), though protein levels were small. IL-13 did not correlate with any MMP genes or cytokine that I measured (data not shown).

MMP2/TIMP1 and MMP9/TIMP2 complexes were determined in serum and cell free pericardial fluid from open-heart surgery control and TB pericarditis patients by ELISA. This ELISA measures bound MMP and TIMP molecules, by using a MMP capture antibody and a TIMP secondary antibody. MMP2/TIMP1 protein was significantly elevated in the pericardial fluid compared to serum of TB pericarditis patients ($p=0.001$). In the open-heart surgery patients, while there were no differences in MMP2/TIMP1 protein concentrations in serum and CFF, there was significantly more protein in both serum and CFF compared to the TB pericarditis patients (both $p<0.0001$). This could indicate a dysregulation in the binding of TIMP1 with MMP2 in the milieu of TB pericarditis, especially as there is more unbound TIMP1 protein in both serum ($p<0.0001$) and CFF (not significant) of TB pericarditis patients compared to the control patients (Figure 5.41). Open-heart surgery control patients had increased levels of MMP9/TIMP2 complex in their serum ($p<0.0001$) compared to the TB pericarditis patients. Unbound TIMP2 was significantly elevated in the serum of TB pericarditis patients, suggestive of a dysregulation in the binding of TIMP2 to MMP9. HIV infection did not seem to modulate this dysregulation.

There needs to be a balance between MMP and TIMP molecules to prevent fibrosis, as over production of either TIMP molecules or MMP molecules could cause over production of ECM components by preventing the MMP action of degradation and thus leading to development of fibrosis. This does not seem to be the case, whilst there does seem to be some dysregulation of the binding of these specific complexes in the pericardial fluid, over production of MMP molecules could lead to more ECM breakdown, over-activation of some inflammatory cytokines and chemokines, causing ongoing inflammation and further damage to the pericardium, thus causing more production of collagen and other ECM components to further develop fibrosis, as a long term complication of the disease.

Overall, the data show that the pericardium is a pro-fibrotic environment and that any dysregulation caused by an infection, i.e. *M.tb* or HIV-1, could stimulate an inflammatory response and thus, via this response, a fibrotic response. It would depend on the amount and duration of inflammation that occurs as to when the fibrotic response takes over.

5. Conclusions

I have shown, using RT-PCR and ELISA, that the pericardial space during *M.tb* infection is largely pro-inflammatory, with increased Th1 genes and cytokines and decreased Th2 IL-4 and regulatory IL-10 genes and cytokines. There is a large amount of cell death at the site of disease during *M.tb* infection. The pericardial space is highly pro-fibrotic, with compartmentalization of procollagen genes and a lack of fibrotic regulators as well as over production of pro-fibrotic cytokines. There is dysregulation between MMP and TIMP molecule production and binding in the pericardium that may over-stimulate the synthesis of inflammatory and pro-fibrotic cytokines. The response in the pericardium can best be described by the following figure:

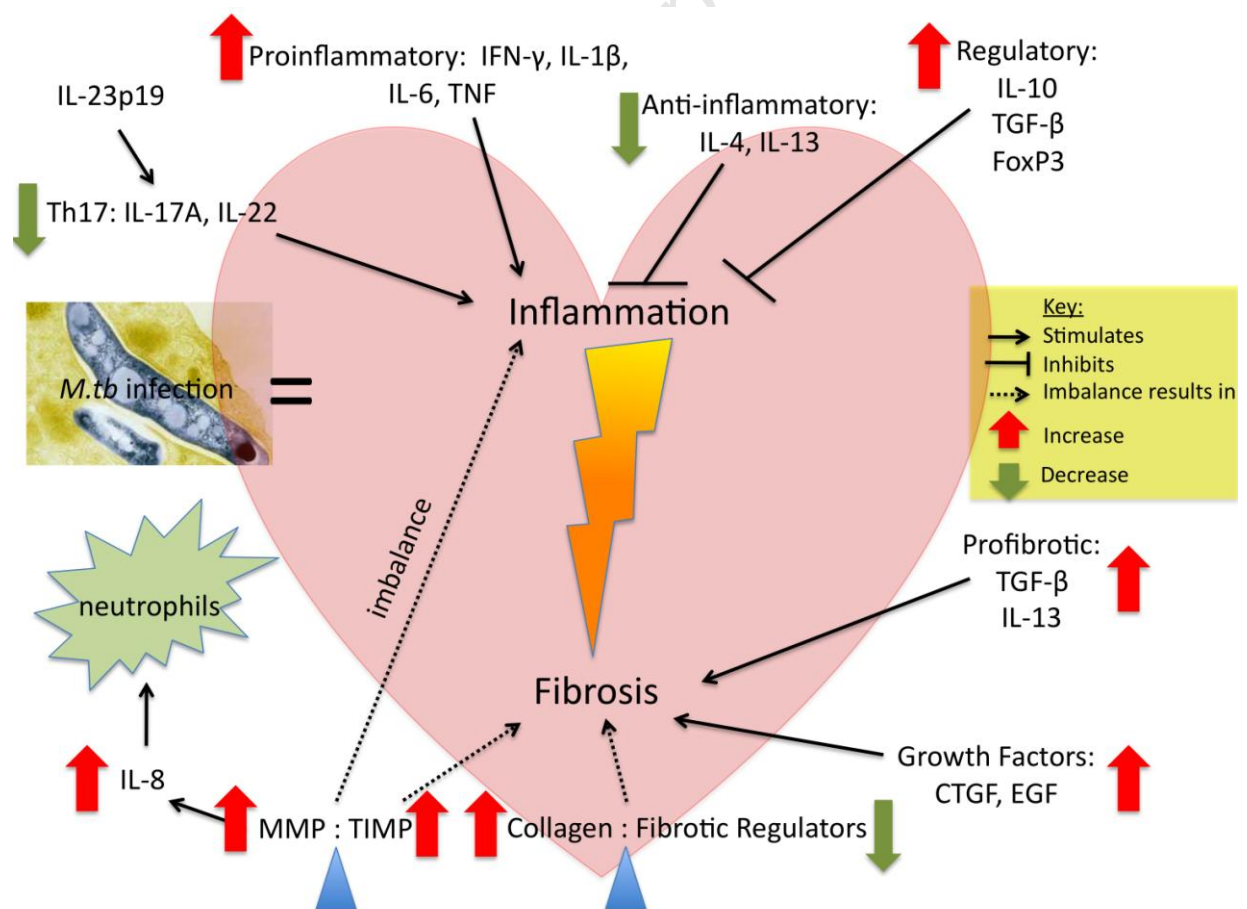


Figure 5.47. Illustration depicting the balance between inflammation and fibrosis in the pericardium during *M.tb* infection

HIV-1 infection plays a role by decreasing the mRNA response for the pro-inflammatory genes IL-2, IL-18 and TNF, Th2 released IL-4, Th17 released IL-17, regulatory FoxP3, neutrophil related IL-8 and CAMP and the growth factor CTGF in either blood, pericardial fluid or both. HIV-1 infected patients had increased IL-10 and IL-13 proteins in pericardial fluid compared to blood, while there was no differences for the HIV-1 uninfected patients. HIV-1 causes a dysregulated immune response at the site of TB disease, causing an environment in which *M.tb* can flourish and stimulate a continuous inflammatory response to further damage the pericardium.

University of Cape Town

CHAPTER 6

Investigation of the regulation of IL-17 and IL-22 in the context of human tuberculosis

1. Introduction

Human infection with *Mycobacterium tuberculosis* (*M.tb*) is asymptomatic in most people and controlled, at least in part, by a T cell response comprising CD4 and CD8 cells, and expression of the Th1 cytokines, IFN- γ and TNF (287). However, this control fails in approximately 9.3 million people worldwide who develop clinical tuberculosis (TB) each year (3). Sites of established disease in humans represent compartments of failed immune control, offering the opportunity to study immune mediators and understand mechanisms of immune control in humans.

The inflammatory response is compartmentalized to the TB disease site, and is strongly Th1 driven. Significantly higher levels of IFN- γ , TNF and IL-12 are found in pleural fluid of TB pleuritis patients, compared with peripheral blood, or non-tuberculous effusions (69) and antigen-specific Th1 cells are recruited to the pleural fluid (110). IFN- γ and TNF- α levels were also increased in another disease site, tuberculous pericardial fluid, compared with malignant and other infectious causes of pericarditis (86). Similarly, recruitment of IFN- γ expressing Th1 cells to the lungs of *M.tb*-infected individuals was observed after bronchoscopic instillation of PPD (288).

IL-17A is an inflammatory cytokine that was first described as a mediator of inflammatory diseases (289). This cytokine has since been implicated in protective immunity against extracellular bacterial and fungal pathogens (289). IL-17 also plays

a role in the control of murine *M.tb* infection as Th17 memory cells, induced by TB vaccination, mediated recruitment of protective Th1 cells to the lung by upregulating chemokines (290). Upon infection, IL-17 may also trigger recruitment of neutrophils to the inflamed lung and facilitate granuloma formation (291). It was recently showed that peripheral blood mycobacteria-specific IL-17-producing T cells are induced in humans with *M.tb* infection or TB disease (191). However, IL-17 was undetectable by ELISA in BAL fluid from patients with pulmonary TB. Since BAL involves instillation of considerable volumes of saline, cytokines present at low levels may have been diluted to undetectable levels (191).

IL-22 is a novel inflammatory cytokine produced by Th17 cells and is linked to many inflammatory diseases such as Crohn's disease and rheumatoid arthritis and it may contribute to the inflammation during *M.tb* infection. IL-22 production was found to increase after BCG vaccination of macaques (292) and mRNA and protein levels were increased after cardiac surgery with cardiopulmonary bypass (293). Scriba, *et al.*, showed that Th1 cells inhibit IL-17, but not IL-22 cytokine production and that IL-22 may mediate pulmonary inflammation in active TB (191).

These studies suggest that IL-17 and/or IL-22-producing CD4 T cells may play a role in the human immune response to mycobacteria. Here, I hypothesized that IL-17 contributes to the inflammatory response at the disease site of human TB and this response is inhibited by the IFN- γ response, while IL-22 is not affected. I also hypothesized that IL-22 is produced by a distinct subset of cells (Th22)(294) that do not produce IL-17 in a Th1 environment.

2. Results

2.1. Neutralization of IFN- γ in whole blood does not affect IL-17 and IL-22 secretion

Neutralization of IFN- γ protein, by neutralizing antibody, was done on whole blood from 10 healthy PPD sensitised laboratory volunteers. After 0, 24, 48 and 96 hours, supernatant was removed and analysed for IFN- γ , IL-17A and IL-22 protein secretion. IFN- γ ELISA was done to determine the functionality of the neutralizing antibody with live *M.tb* H37Rv stimulation and without.

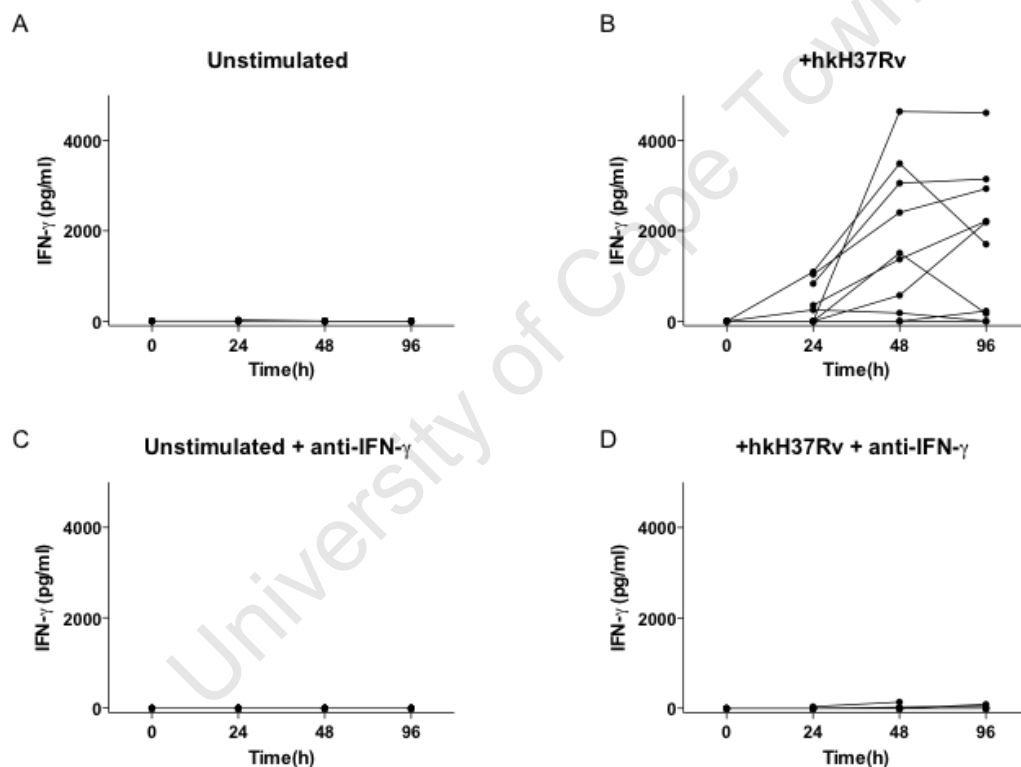


Figure 6.1. The effect of H37Rv stimulation and blocking with anti-IFN- γ antibody on IFN- γ protein in whole blood

Before-and-after plots of whole blood neutralizing assays for all 10 donors showing:

A. IFN- γ in unstimulated whole blood, B. IFN- γ in whole blood over 96 hours when stimulated with H37Rv, C. the effect of no stimulation but with the addition of anti-

IFN- γ antibody on IFN- γ , D. IFN- γ levels when stimulated with H37Rv and with the addition of anti-IFN- γ antibody

Figure 6.1B shows that live H37Rv stimulated IFN- γ production that was detectable from 24 hours and for up to 96 hours in the supernatant from stimulated whole blood. The unstimulated control (Figure 6.1A) shows that this is a specific response and cannot be reproduced by incubation alone. Blocking with IFN- γ neutralizing antibody alone, i.e. without H37Rv stimulation (Figure 6.1C), showed no IFN- γ detectable up to 96 hours indicating that the antibody had no background producing effect. Stimulation with H37Rv and blocking with anti-IFN- γ (Figure 6.1D) showed almost complete absence of IFN- γ protein detectable, which lasted for up to 96 hours, showing the presence of the IFN- γ neutralizing antibody.

The effect of neutralizing IFN- γ protein on the levels of IL-17 and IL-22 detectable in whole blood was determined next. Figure 6.2 shows the effect on IL-17 protein. Although H37Rv stimulation caused a slight, but not significant, increase in IL-17 production in whole blood of some donors, neutralization of IFN- γ did not cause significant increase of IL-17 production. Mostly the levels of IL-17 measured in the samples with and without anti-IFN- γ remained the same and this was also true when determining the effect on IL-22 production (Figure 6.3).

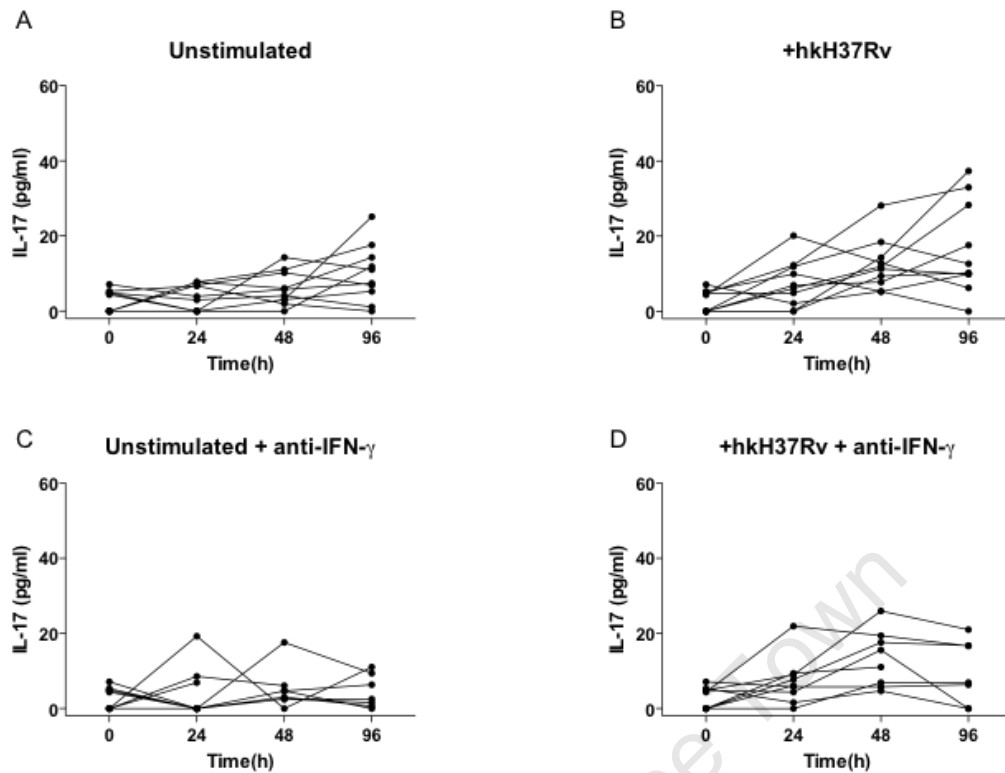


Figure 6.2. The effect of H37Rv and blocking of IFN- γ antibody on IL-17A protein in whole blood

Before-and-after plots of whole blood neutralizing assays for all 10 donors showing:

A. IL-17 when whole blood is not stimulated, B. IL-17 in whole blood over 96 hours when stimulated with H37Rv, C. the effect of no stimulation but with the addition of anti-IFN- γ antibody on IL-17, D. IL-17 levels when stimulated with H37Rv and with the addition of anti-IFN- γ antibody

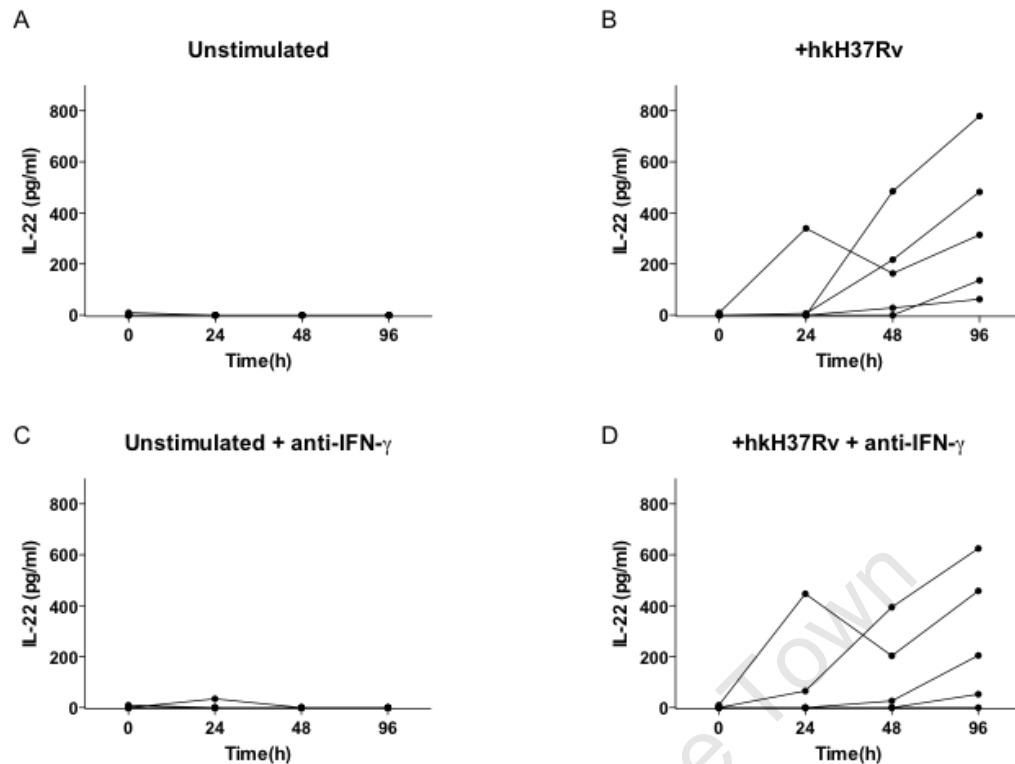


Figure 6.3. The effect of H37Rv and blocking of IFN- γ antibody on the production of IL-22 protein in whole blood (n=5)

Before-and-after plots of whole blood neutralizing assays for 5 donors showing:

A. IL-22 when whole blood is not stimulated, B. IL-22 in whole blood over 96 hours when stimulated with H37Rv, C. the effect of no stimulation but with the addition of anti-IFN- γ antibody on IL-22, D. IL-22 levels when stimulated with H37Rv and with the addition of anti-IFN- γ antibody

The effect of IFN- γ protein neutralization on the gene transcript for IFN- γ or IL-17 (Figure 6.4) was next determined.

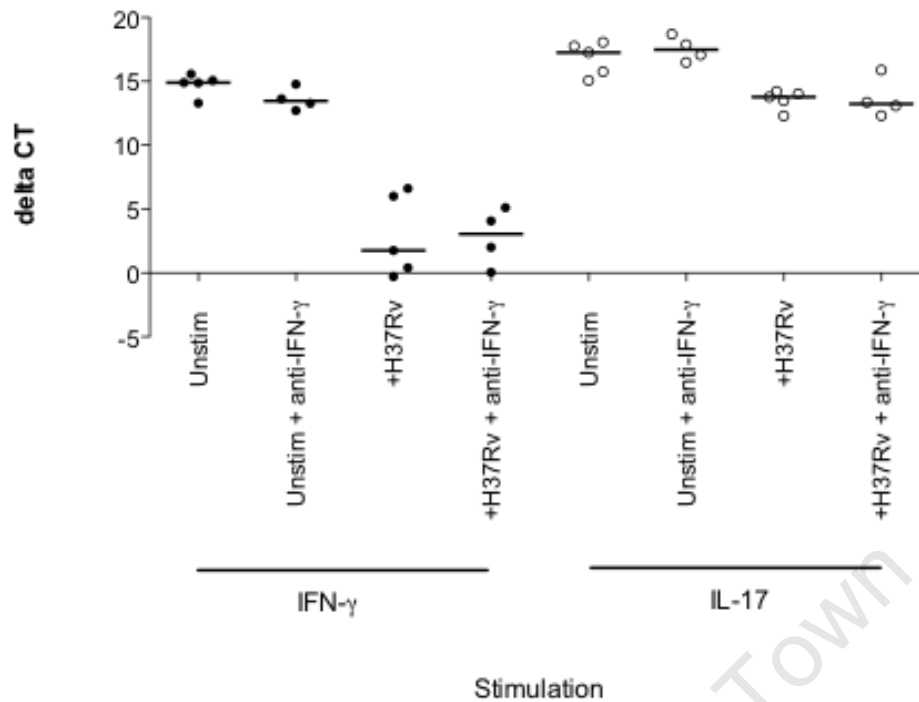


Figure 6.4. The effect of stimulation with H37Rv and the addition of anti-IFN- γ antibody on the mRNA gene transcript levels of IFN- γ and IL-17

H37Rv stimulation increased IFN- γ gene transcription (decreased delta CT, not significant, $p=0.063$) and blocking IFN- γ in stimulated samples tended to decrease IFN- γ gene transcription slightly (median delta CT 1.76 vs. median delta CT 3.05). Stimulation with H37Rv also increased IL-17 gene transcription in blood (not significant, $p=0.063$). The neutralizing antibody did not affect transcript levels.

IL-22-PE was stained for using a panel consisting of CD3-Pacific Blue, CD4-QDot 605, CD8-PerCPCy5.5 and IL-17-Alexa 647, using predetermined volumes for these antibodies. The volumes for IL-22-PE were altered each time to determine the best volume of IL-22 to use in this panel. I used 0, 0.1, 0.3, 1 and 3 μ l per tube of IL-22-PE in the panel (Figure 6.5).

The percent IL-22 positive cells were determined by gating on CD3+CD4+IL-22+ cells and these values were plotted against the volume of IL-22-PE used (Figure 6.6A). The ratio of the Mean Fluorescence Index (MFI) for the IL-22 positive cells over the MFI of the IL-22 negative cells was also calculated to determine the best ratio per volume of IL-22 antibody used (Figure 6.6B). Using these analysis it was determined that the optimal volume of IL-22-PE to use was 1 μ l per tube.

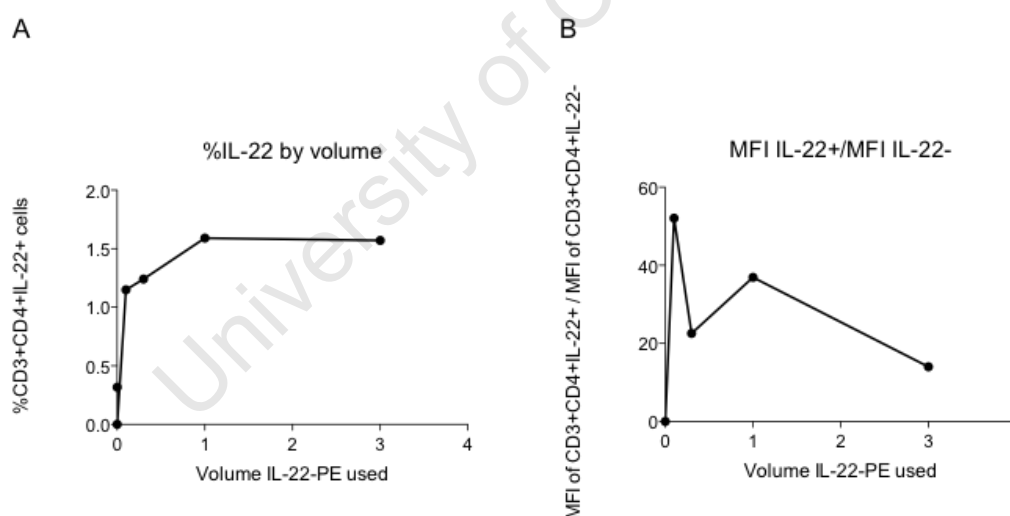


Figure 6.6. Determination of the volume of IL-22-PE to be used in the panel

A. shows the %CD3+CD4+IL-22+ producing cells for each volume of IL-22-PE used, and B. shows the Mean Fluorescence Index (MFI) of the CD3+CD4+IL-22+ cells over the MFI of the CD3+CD4+IL-22- cells

2.2.2. Four-day proliferation assay to determine the proportions of IL-17, IL-22 and IFN- γ expressing CD4⁺ cells

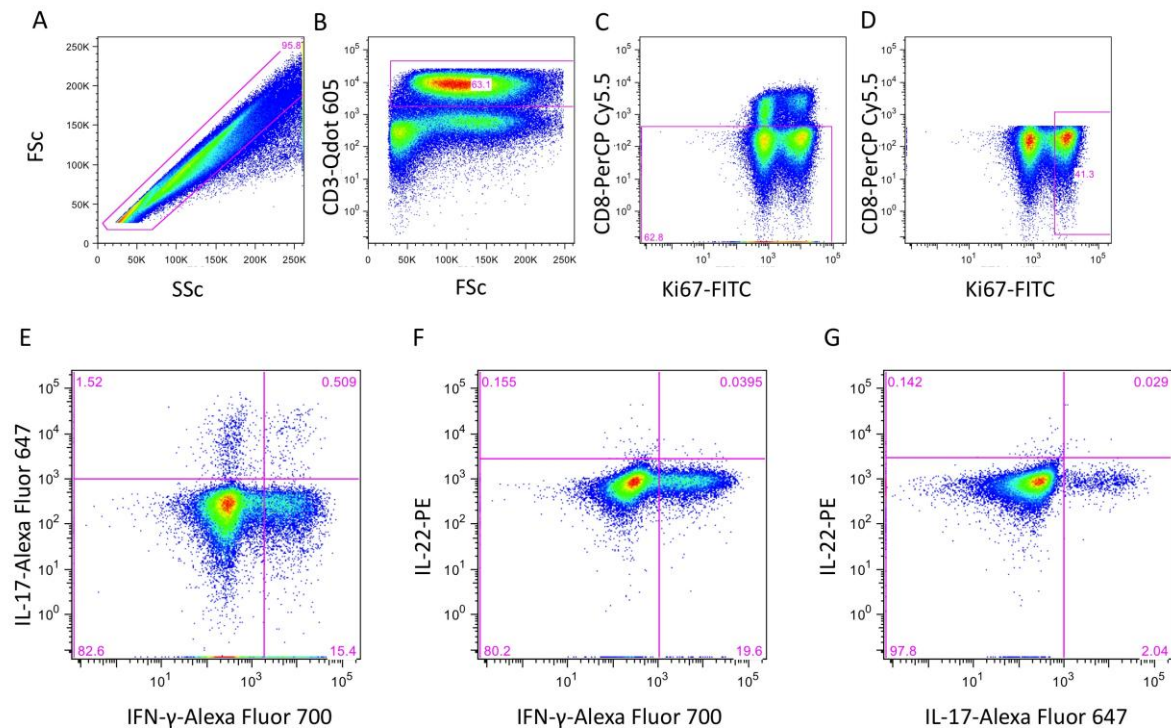


Figure 6.7. Representative gating strategy to determine proliferating CD4⁺ cells expressing cytokines

A. Exclusion of doublet cells by plotting Forward Scatter (FSc) versus Side Scatter (SSc), B. Selection of CD3⁺ cells by plotting CD3-QDot 605 versus FSc, C. Selection of all CD4⁺ cells by selecting CD8-PerCP Cy5.5 negative cells when plotted versus Ki67-FITC, D. Selection of proliferating CD4⁺ cells by selecting the Ki67⁺ cells, E. Representative plot to determine IL-17⁺ and IFN- γ ⁺ expressing cells in CD4⁺Ki67⁺ cells, F. Representative gating plot to determine IL-22⁺ and IFN- γ ⁺ expressing cells in CD4⁺Ki67⁺ cells, G. Representative gating plot to determine IL-22⁺ and IL-17⁺ expressing cells in CD4⁺Ki67⁺ cells

A total of 10 PPD TST positive healthy laboratory donors were recruited for blood sampling. After the 4-day proliferation assay, PBMC were harvested, stained and acquired on the BD LSRII Fortessa. Figure 6.7 shows a representative sample of the gating strategy employed to firstly determine proliferating Ki67+ CD4+ cells and then to determine the capacity for cytokine secretion by these cells.

Cytokine secretion by cells from the unstimulated background (NIL) culture condition was subtracted from the PPD stimulated cells. Figure 6.8 shows the proportion of proliferating CD4+ cells expressing IFN- γ , IL-17 and IL-22 during neat (IgG1), recombinant (r)-IFN- γ and Th1 culture conditions under the stimulation of PPD. The neat culture conditions showed relatively high proportions of IFN- γ expressing cells, which significantly increased under the r-IFN- γ conditions ($p=0.016$), but decreased under Th1 conditions compared to neat ($p=0.008$) and compared to r-IFN- γ ($p=0.008$) conditions.

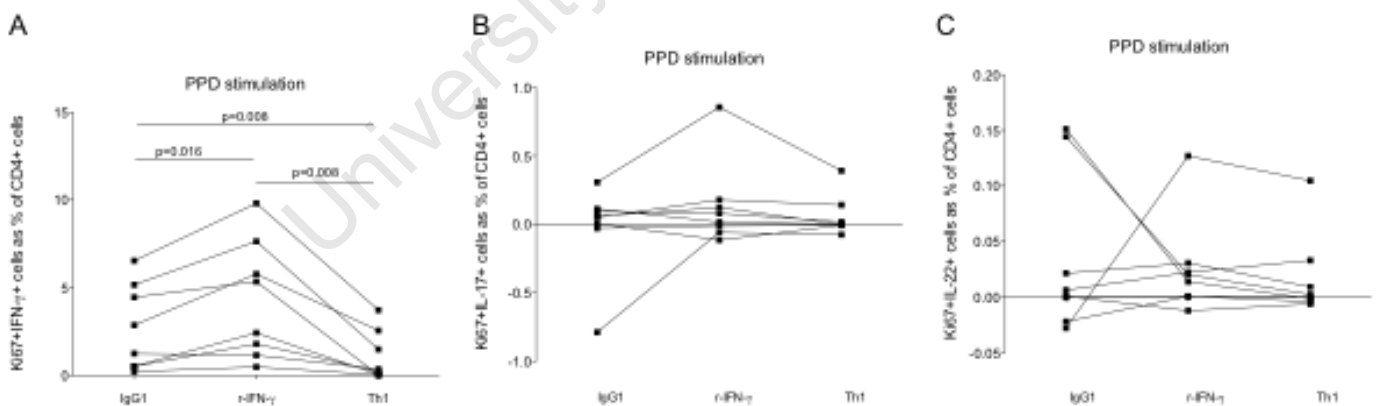


Figure 6.8. Ki67+cytokine expressing cells as a percentage of CD4+ cells in response to PPD stimulation under neat (IgG1), r-IFN- γ and Th1 cytokine conditions after background subtraction

A. Ki67+IFN- γ + cells as a proportion of CD4+ cells, B. Ki67+IL-17+ cells as a proportion of CD4+ cells and C. Ki67+IL-22+ cells as a proportion of CD4+ cells

When determining proliferating CD4⁺ cells expressing IL-17 in response to PPD stimulation under the 3 culture conditions, some donors showed a slight decrease in the percentage of IL-17 expressing cells during Th1 culture conditions, but the majority showed no differences between culture conditions. The same was true for IL-22 expressing CD4⁺ cells, although the proportion of IL-22⁺ cells was very small and not very reliable (Figure 6.8).

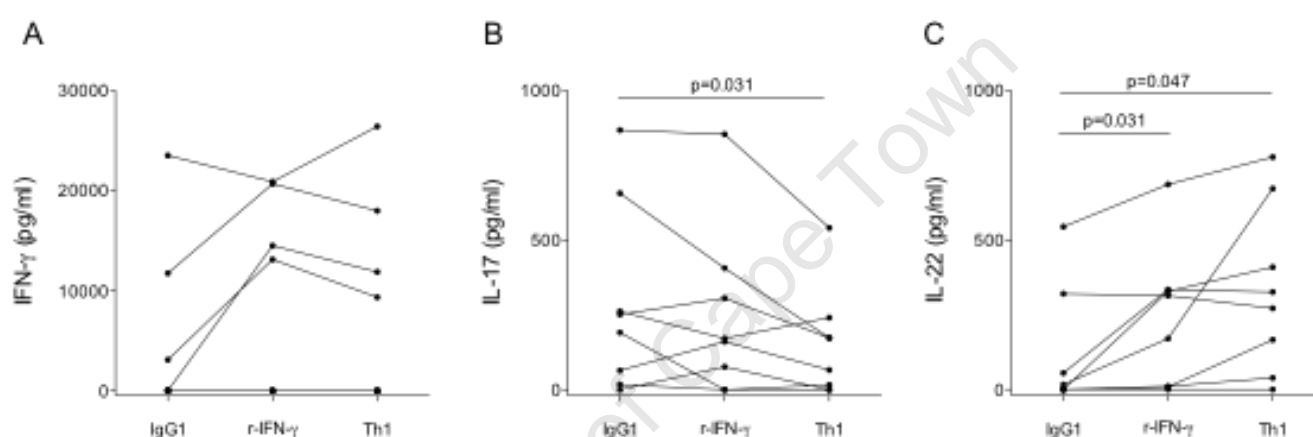


Figure 6.9. ELISA determination of cytokine secretion in response to PPD stimulation under neat (IgG1), r-IFN-γ and Th1 culture conditions

A. IFN-γ (pg/ml), B. IL-17 (pg/ml) and C. IL-22 (pg/ml)

Supernatant from the assays were stored to allow for protein detection for these cytokines by ELISA. IFN-γ protein concentrations were different for each donor studied. Three donors had increased IFN-γ concentrations under r-IFN-γ culture conditions, which decreased under Th1 conditions, while for one donor the opposite trend was true. There were no statistically significant differences when comparing IFN-γ secretion in response to PPD stimulation for any of the culture conditions (Figure 6. 9).

IL-17 production was significantly decreased under Th1 conditions compared to the neat culture condition ($p=0.031$), while IL-22 was significantly increased under r-IFN- γ ($p=0.031$) and Th1 ($p=0.047$) compared to neat culture conditions, showing that a Th1 environment suppresses IL-17 cytokine secretion but favours IL-22.

3. Discussion

The role of IL-17 and IL-22 in the inflammatory response during *M.tb* infection has not been fully characterised. From Chapter 5, I have shown that there is compartmentalization of IFN- γ to the pericardial space during TB infection, characteristic of the TB-associated inflammatory response (86, 110, 288). IL-17 protein and mRNA was not abundant in pericardial fluid of TB pericarditis patients, in contrast to murine studies (290, 291). Large IFN- γ concentrations are known to suppress IL-17 expression by Th17 cells as shown in PBMC (191) and I hypothesised that this may be taking place in the pericardium. IL-22 was readily detected in the pericardial fluid and serum of TB pericarditis patients along with IL-22 mRNA. These were consistent with findings in pleural fluid (190).

I went on to determine the effects of live *M.tb* stimulation and IFN- γ neutralization on IL-17 secretion in whole blood. IL-17 is also produced by neutrophils (295), which are present in whole blood and these cells may produce more IL-17 in the absence of IFN- γ . Limitations of this part of this study were that a negative isotype control was not included. There was also no biological positive control included to prove that the anti-IFN- γ antibody worked, such as TNF, which is upregulated by IFN- γ . However, neutralization of IFN- γ in whole blood showed no increase of IL-17 cytokine production or IL-17 gene transcription. There was also no effect on IL-22 cytokine production.

I used a 4-day proliferation assay to determine cell specific IL-17 and IL-22 secretion under various cytokine environments, as Th1 conditions and not just IFN- γ , may play a role in suppressing IL-17 cytokine production. The role of these cytokine culture

conditions on IL-22 secretion was also studied. IL-22, but not IL-17, was shown to be produced by human TH17 skin homing memory cells (294) and IFN- γ was shown to suppress IL-17 but not IL-22 secretion (191).

While no CD4⁺ cell specific changes in IL-17 or IL-22 expression were found, Th1 culture conditions were shown to decrease IL-17 and increase IL-22 protein expression during PPD stimulation, consistent with other studies (191). IL-22 expressing proliferating CD4⁺ cells were not readily detected in the PBMC of the healthy PPD positive donors after 4 days culture. IFN- γ ⁺ secreting proliferating CD4⁺ cells also decreased in Th1 cytokine culture conditions compared to the neat conditions. This could indicate the culture conditions were too long and the IL-22 and IFN- γ specific cells were missed, due to their short life-span. However, IL-22 and IFN- γ protein in the culture supernatant was readily detectable. A shorter term assay may add more to this study.

Hitherto, the exact role of IL-17 during human *M.tb* infection is unconfirmed, although it is thought to mediate host protective responses against infection, while IL-22 may aid in the repair process during TB infection of the pericardium (from Chapter 5).

4. Conclusion

I have confirmed that live *M.tb* infection causes IL-17 and IL-22 production in whole blood *in vitro* and that a Th1 environment suppressed IL-17, but increased IL-22 protein secretion *in vitro*. In the pericardial space, the robust Th1 environment may be responsible for the low IL-17 and increased IL-22 in the pericardial fluid of TB pericarditis patients.

University of Cape Town

CHAPTER 7

General Discussion and Overall Conclusions

1. General Discussion

TB is a disease that elicits a pro-inflammatory cytokine response, the hallmark of which is IFN- γ release (287). In my study, IFN- γ was measured in whole blood and pericardial fluid in an overnight restimulation assay using the *M.tb*-specific antigens ESAT-6 and CFP-10. IFN- γ was produced in the pericardial fluid and this was significantly increased in the stimulated compared to unstimulated samples, as well as compared to blood samples. There were no differences, however, when comparing HIV-1 uninfected to HIV-1 infected samples, although the HIV-1 infected patients tended to have slightly decreased IFN- γ concentrations in pericardial fluid.

IFN- γ production by PBMC and pericardial fluid cells was also measured using ELISpot. ELISpot showed increased cellular production of IFN- γ at the site of disease in HIV-1 uninfected patients when stimulated with ESAT-6 ($p=0.009$), similar to pleural TB (110), implicating that HIV-1 uninfected TB pericarditis patients mount a protective T cell response in an attempt to clear the organism. ESAT-6, which is implicated in virulence of *M.tb* (296), induces MMP9 in epithelial cells neighbouring *Mycobacterium marinum* infected macrophages (118) and while MMP9 enhances the recruitment of macrophages and is critical for granuloma maturation (119), it is also involved in extracellular matrix repair (100). Peripheral blood MMP-9 levels were previously associated with severity of TB disease (266) and higher levels of MMP-9 were detected in pleural fluid from patients with TB pleural effusions, compared with patients with congestive heart failure (267). I found a direct correlation of MMP9

measured at the site of disease and IL-22 measured in serum and cell free pericardial fluid in HIV-1 uninfected patients only. IL-22 is well described as a regulator of keratinocyte mobility, epidermal differentiation and wound healing (261-264). The success of *M.tb* as pathogen is dependent on its destruction of the pulmonary extracellular matrix. This process which is, at least partly, driven by MMPs brings about cavitation and thereby creates an immunoprivileged niche (265). Increased IL-22 at the TB disease site may be involved in the healing/regeneration response as well as an anti-mycobacterial immune mechanism and therefore may be induced by the *M.tb* specific antigen ESAT-6. As this is not seen in the HIV-1 infected patients, HIV infection may hinder an efficient protective response against *M.tb* in the pericardium.

The other *M.tb* specific antigens tested in this study showed no clear concentration of IFN- γ producing cells at the site of disease in either HIV-1 uninfected or HIV-1 infected TB pericarditis patients. An interesting phenomenon of “pericardial dominance” or “peripheral dominance” for the recognition of all *M.tb* antigens was evident for each patient, irrespective of their HIV status, indicating that time from infection and development of symptoms to pericardiocentesis may play an important role in the types and numbers of cells present at the site of disease and highlights the difficulties of studying the human immune response to tuberculosis, especially at sites of disease.

FACS analysis showed antigen experienced memory T cells present at the site of disease in HIV-1 uninfected TB pericarditis patients and a more intermediate, less differentiated phenotype in pericardial fluid of HIV-1 infected TB pericarditis patients. CD4⁺ cells from HIV-1 uninfected pericardial fluid had a tendency to

secrete IFN- γ and TNF cytokines by themselves, whereas HIV-1 infected pericardial fluid CD4⁺ cells were more prone to express IL-2 by itself, and IFN- γ , IL-2 and TNF in combination, again pointing to a less differentiated, and more polyfunctional, memory cell type present at the site of disease.

GM-CSF and IL-17, along with IFN- γ , TNF and IL-2 secreting CD4⁺ T cells were identified in blood and pericardial fluid. There were no CD4⁺ T cells secreting all five cytokines in either compartment of either HIV-1 uninfected or HIV-1 infected patients and very low frequencies of IL-17 secreting cells in any combination. GM-CSF production by itself, however, was more evident in HIV-1 infected blood cells and HIV-1 uninfected pericardial fluid cells. GM-CSF has been shown to be a correlate of protection in HIV -1 infected patients with high CD4 counts (165), but has also been associated with the onset of TB-IRIS (166, 167). IL-17-producing T cells are induced in humans with *M.tb* infection or TB disease (191), but I have shown low frequencies at the site of disease and suppression of protein in an *in vitro* Th1 cytokine environment. This offers an explanation as to the absence of IFN- γ and IL-17 co-producing CD4⁺ T cells, as well as the low IL-17 cytokine secretion in the pericardial fluid of all TB pericarditis patients.

The role of polyfunctional CD4⁺ T cells in *M.tb* infection has been described as protective (169), but also as a correlate of TB disease (170). Polyfunctional CD4⁺ T cells were more abundant during peak plasma viremia in SIV and *M.tb* co-infected macaques (297). I propose that in the context of TB pericarditis, polyfunctional CD4⁺ cells may arise due to pathology and high antigen loads, rather than offer protection during *M.tb* infection of the pericardium, especially in HIV co-infected

individuals as the phenotype of CD4⁺ T cells is skewed towards less differentiated cells.

CD8⁺ T cells were also shown to contribute to the immune response at the site of infection, but responses may be HIV-1 rather than *M.tb*-specific. CD8⁺ T cells may compensate for the loss of CD4⁺ T cells at the site of disease, especially as I have shown the pericardium to be a site of ongoing HIV-1 replication, which correlates with loss of CD4⁺ T cells. I have also shown skewing of the memory phenotype of CD8⁺ T cells at the site of disease in HIV-1 infected TB pericarditis patients. The altered redistribution of memory CD8⁺ and CD4⁺ T cells may be caused by high turnover of HIV specific T cells in response to persistent infection, and not by a block in the maturation of these cells (151, 156, 157).

The role of cells other than T cells in the pericardium was not evaluated in this study, but some involvement by neutrophils and macrophages may be evident from the data generated. IL-8 mRNA correlates directly with MMP9 mRNA in pericardial fluid. ESAT-6 has been shown to increase MMP9 during *M.avium* infection (118), and in TB pericarditis, may induce IL-8, which in turn attracts neutrophils to the site of disease. However, most TB pericardial effusions have shown lymphocytic infiltrates and only small numbers of neutrophils present (80). The neutrophils that are present, may not be sufficient to eliminate the infection and thus macrophages and T cells are recruited. Increased IL-12p70 and TNF in the pericardial fluid may indicate the presence of macrophages at the site of disease and the direct correlation of IL-1 β mRNA and MMP9 transcript levels in the pericardial fluid shows an infection

associated inflammatory response, resulting in more Th1 cytokines released at the site of disease.

Transcript for pro-inflammatory cytokine related genes IL-2 ($p=0.005$) and IL-18 ($p=0.009$) were significantly decreased in pericardial fluid of HIV-1 infected patients compared to HIV-1 uninfected patients. IL-2 is needed for clonal expansion of antigen experienced T cells (179) and IL-18 induces Th1 CD4⁺ T cell polarization (181), and their absence may reflect lack of functionality of the less differentiated cells recruited at the site of disease in HIV-1 infected patients.

Other genes downregulated during HIV co-infection were IL-4 and IL-23p19 in both blood and pericardial fluid. IL-4 is an important Th2 cytokine that can balance and downregulate an overwhelming Th1 response, and its absence is evidence of the dysregulated immune response to *M.tb* and/or HIV during TB pericarditis. IL-4 was not detected in previous pericardial (86) or pleural (69) TB studies, consistent with my data.

IL-23p19 protein was not readily detected in serum or CFF of either HIV-1 uninfected or HIV-1 infected TB pericarditis patients. *M.tb* growth was controlled by IL-23p19 deficient mice that showed an almost complete loss of Th17 cells and IL-17 mRNA, but no effect on IFN- γ (298). HIV-1 infected TB pericarditis patients showed an absence of IL-17 mRNA and little or no protein in the pericardial fluid, indicating a possible defect in the IL-23-IL-17 axis and thereby Th17 cell differentiation. IL-17, but not IL-22 cytokine production is suppressed by Th1 conditions, another

mechanism with which to explain the absence of IL-17 at the site of disease during *M.tb* infection.

CD4+FoxP3+ T cells in both blood and pericardial fluid were identified and no differences between the compartments were found. There were smaller proportions of these cells in both blood and pericardial fluid of HIV-1 uninfected patients, albeit not significantly. However, when FoxP3 mRNA was measured, it was significantly elevated in the pericardial fluid compared to blood of both HIV-1 uninfected ($p=0.0005$) and HIV-1 infected ($p=0.005$) patients, and significantly lower in the blood of HIV-1 infected patients ($p=0.004$) compared to HIV-1 uninfected patients. This data points to a dysregulation in the transcription of FoxP3 by some mechanism. IFN- γ is known to decrease FoxP3 transcription in T lymphocytes (299) and CD28 co-stimulation is required to signal developing thymocytes to express FoxP3 and to initiate Treg cell differentiation (300). Activated NK cells have also been shown to downregulate FoxP3 expression by T lymphocytes (301) and TGF- β is a known Treg suppressor (253, 302). There is a lack of NK cells at the site of disease in TB pericarditis, thus activated NK cells may not result in downregulation of FoxP3 in this context. I have, however shown elevated IFN- γ levels at the site of disease in all TB pericarditis patients, as well as elevated TGF- β protein in the blood and elevated mRNA in both blood and pericardial fluid. These may suppress FoxP3 transcription, which could explain the absence of FoxP3+ T cells in blood and pericardial fluid.

I have shown that the pericardial space is highly pro-fibrotic during *M.tb* infection, with compartmentalized pro-collagen gene expression in the pericardium. There were less, but not significantly so, pro-collagen genes measured in the HIV-1 infected TB

pericarditis patients compared to HIV-1 uninfected. There was also a lack of fibrotic regulators (*ARG1* and *SPARC*) at the site of disease. CTGF, which is implicated in the development of fibrosis, was also significantly increased in pericardial fluid of HIV-1 uninfected TB pericarditis patients. Both may support the observation that HIV-1 infected patients tend to develop constrictive pericarditis less often than HIV-1 uninfected patients (90).

TGF- β protein, a suppressor of T cell responses to *M.tb* (303) and inducer of the fibrotic response (268), was present at only low levels in the pericardium of all TB pericarditis patients, irrespective of the HIV status. However, mRNA was readily detected at high levels in both blood and pericardial fluid. TGF- β is synthesized as an inactive protein bound to a latency-associated peptide (LAP) that needs to be cleaved by proteolytic cleavage, MMP2 or MMP9, acidic environments and other mechanisms to become active (304, 305). *M.tb* infection of the pericardium may impair the translation and activation of TGF- β and may be a reason as to why the protein is reduced.

TB pericarditis patients also have increased MMP2 and decreased MMP9 protein and mRNA in the pericardium compared to blood. MMP2 and MMP9 are responsible for breakdown of some components of the ECM to prevent fibrosis (100) in the pro-fibrotic pericardial space and may be used up during this response. I have shown that MMP2 mRNA and protein were significantly elevated in the pericardium compared to blood, irrespective of the HIV status of the patients, whereas MMP9 protein and mRNA were significantly elevated in the blood compared to pericardial fluid. In open-heart surgery control patients, there was significantly more MMP2/TIMP1

complex protein concentrations in serum and cell free pericardial fluid compared to TB pericarditis patients (both $p < 0.0001$). This could indicate a dysregulation in the binding of TIMP1 with MMP2 in the milieu of TB pericarditis, especially as there is more unbound TIMP1 protein in both serum and CFF of TB pericarditis compared to control patients. The same was true for MMP9/TIMP2 complex binding. Over production of either TIMP or MMP molecules could cause over production of ECM components by preventing MMP action of degradation, leading to development of fibrosis in these patients (100).

This study has shown that *M.tb* infection of the pericardium elicits a strong Th1 cytokine response, with impairment or down regulation of the regulatory response. HIV infection somewhat diminishes this Th1 response, by depleting and skewing CD4+ T cells at the site of disease towards a less differentiated memory phenotype, with decreased IFN- γ and TNF and increased IL-2 production.

The pericardium was also shown to be a pro-fibrotic environment, under regulation of fibrotic mediators and matrix metalloproteinases. Any disturbance of the normal balance may set the fibrotic response out of control and TB pericarditis patients may go on to constrict at a later stage after infection.

2. Overall Conclusions

The overall aim of the study presented in this thesis was to immunologically characterise TB pericarditis and to determine the effect that HIV has on the immune response.

TB pericarditis offers a relevant human model to study TB at the site of disease. TB is responsible for more than 70% of pericarditis cases in developing countries, compared to 4% in the rest of the world, which resulted in the Investigation of the Management of Pericarditis In Africa (IMPI Africa) trial commencing in 2005. The Cardiac Clinic in Groote Schuur Hospital is one of the 16 study sites in South Africa, and one of 50 in Africa. Immunology studies form part of Phase II of recruitment to this trial, and form the foundation of this thesis. Where clinically indicated, pericardiocentesis was performed on patients recruited into this study and blood and pericardial fluid samples were analysed in the laboratory.

The hypotheses tested included: (1) There is an increase of *M.tb*-specific cytokine release at the site of disease compared to blood, (2) Memory T cells are recruited to the site of disease in response to *M.tb*-specific antigens and elicit an inflammatory response, (3) There is a relationship between the clinical presentation of TB pericarditis and the phenotype of immune cells present at the site of disease and in blood, (4) Specific genes are upregulated during TB pericarditis, that determine whether a pure inflammatory or pro-fibrotic response occurs and these genes are responsible for the transcription of proteins at the site of disease, and (5) The novel inflammatory cytokine IL-17 plays a role in the inflammatory response against *M.tb*.

I used an overnight *M.tb*-specific restimulation assay to show that T cells preferentially recognise *M.tb*-specific antigens in pericardial fluid compared to blood, and more so in the HIV-1 uninfected TB pericarditis patients compared to those infected with HIV. ELISpot assays showed no clear concentration of IFN- γ -specific cells to the site of disease, except for ESAT-6-specific pericardial fluid cells in the HIV-1 uninfected patients compared to HIV-1 infected TB pericarditis patients ($p=0.009$). There was, however, evidence of “peripheral” or “pericardial dominance” in recognition of *M.tb*-specific antigens, which could reflect the sampling time difference between patients from infection to pericardiocentesis.

Flow cytometry of PBMC and pericardial fluid cells was performed to determine the exact phenotype of CD4 and CD8 T cells in blood and pericardial fluid. The phenotype of cells in pericardial fluid has not been analysed in such detail before, and this novel information may give insight in the dysregulation of the immune response at the site of TB disease. There were increased lymphocyte proportions recruited to the site of disease compared to blood of the HIV-1 uninfected patients. Ongoing HIV replication at the disease site, as shown by the increased viral loads, may result in CD4⁺ T cell depletion in HIV-1 infected TB pericarditis patients, and may be responsible for the increased proportions of CD8⁺ T cells in pericardial fluid compared to blood in these patients.

Pericardial fluid cells showed a more differentiated effector memory CD4⁺ and CD8⁺ T cell phenotype in the HIV-1 uninfected patients, while HIV was shown to skew the phenotype towards the presence of less differentiated T cells in the pericardial fluid. HIV-1 infected patients also showed decreased actual numbers of activated

CD4+HLA-DR+, proliferating CD4+CD71+ and memory CD4+CD45RO+ cells, which may result from overall CD4+ T cell depletion, however the proportions of these cells were relatively high. I also determined the functional capacity of the cells present in blood and pericardial fluid. HIV-1 uninfected patients showed single cytokine producing CD4+ T cells, secreting TNF and IFN- γ at the site of disease, while HIV-1 infected patients showed slightly more CD4+ T cells with polyfunctional profiles secreting GM-CSF in combination with IFN- γ and TNF. There were also increased CD4+ T cells secreting IL-2, a cytokine secreted by less differentiated memory cells to induce proliferation of antigen experienced cells, consistent with the less mature phenotype of CD4+ T cells at the disease site of HIV-1 infected patients. These polyfunctional cells may be as a result of the pathology of TB pericarditis, rather than a correlate of protection, due to the ongoing inflammation occurring at the site of disease.

A cell death detection ELISA showed high levels of cell death of pericardial fluid cells at the site of disease, irrespective of the HIV status of the patients. This result may imply that short-lived cells are recruited to the site of infection to release cytokines and die, reflecting the low frequencies of cytokine secreting cells at the site of disease, while IFN- γ protein levels are significantly raised.

The mRNA transcript and protein profile of blood and pericardial fluid of TB pericarditis patients has never been described in literature. Using these techniques, I have shown that the pericardial space is largely pro-inflammatory, with increased Th1 (IL-2, IL-6, IL-24, IP-10 and TNF) genes and (IL-1 β , IL-6, IFN- γ and TNF) cytokines, decreased Th2 released IL-4 as well as regulatory IL-10 genes and

cytokines. The pericardial space is also largely pro-fibrotic, showing compartmentalization of pro-collagen genes as well as a lack of fibrotic regulators (*ARG1* and *SPARC*) and over production of pro-fibrotic genes (TGF- β and CTGF). Dysregulation between MMP and TIMP molecule production and binding may also stimulate the synthesis of inflammatory and pro-fibrotic cytokines.

HIV infection plays a role by decreasing the mRNA response for the pro-inflammatory genes IL-2, IL-18 and TNF, Th2 released IL-4, Th17 released IL-17, regulatory FoxP3, neutrophil related IL-18 and CAMP and the growth factor CTGF in either blood, pericardial fluid or both. HIV-1 infected patients had increased IL-10 and IL-13 proteins in pericardial fluid compared to blood, while there was no differences for the HIV-1 uninfected patients. HIV-1 causes a dysregulated immune response at the site of TB disease.

I have also shown that *M.tb* does in fact cause the production of novel pro-inflammatory cytokines, IL-17 and IL-22 in whole blood. I have also determined that Th1 cytokine environments suppress IL-17 but not IL-22 protein production and that there may be a defect in the IL-17-IL-23 axis during pericardial TB which adversely effects Th17 differentiation and IL-17 production.

In summary, I have shown that the *M.tb*-specific antigen ESAT-6 is recognised by T cells in the pericardial fluid of HIV-1 uninfected patients, which in turn secrete IFN- γ to combat the pathogen. ESAT-6 interacts directly with epithelial cells to induce gelatinase MMP9 transcription and protein production, which in turn increases IL-22 and IL-8 release in the pericardium. IL-22 is a pro-inflammatory cytokine that is also

involved in wound healing and may also induce MMP9 in this way. IL-8 causes neutrophils to migrate to the site of infection to engulf the bacilli, but cause extensive tissue damage and thus induce the repair process by collagens. MMP9 also induces pro-inflammatory IL-1 β release, which in turn increases other Th1 cytokine secretion to efficiently combat the pathogen.

However, there is no efficient immune response mounted against *M.tb* in the pericardium of HIV-1 infected patients. This may be due to the high turnover of antigen inexperienced T cells at the site of disease as well as the skewing of the memory phenotype of cells towards the presence of less differentiated CD4⁺ and CD8⁺ T cells, unable to elicit a robust protective immune response, allowing ongoing HIV replication and depletion of cells, which in turn allows *M.tb* organisms to flourish in an environment of ongoing inflammation.

The novel findings of this work include:

- There is increased recognition of *M.tb*-specific antigens by T cells in the pericardial fluid
- There is a phenomenon of “Pericardial” and “Peripheral dominance” in the recognition of *M.tb*-specific antigens
- There are more differentiated antigen experienced CD4⁺ and CD8⁺ T cells in HIV-1 uninfected TB pericarditis patients, while HIV skews the maturational phenotype of memory T cells in the pericardium towards less differentiated CD4⁺ and CD8⁺ T cells

- HIV skews the cell-specific cytokine response towards a more polyfunctional T cell profile, probably as a result of pathology of TB pericarditis, rather than protection.
- The pericardial space has been shown to be largely pro-inflammatory, but it is also pro-fibrotic, with compartmentalization of pro-collagen genes and lack of fibrotic regulators, irrespective of the HIV status of the patients.
- There is dysregulation between MMP and TIMP molecule production and binding, which may over stimulate the synthesis of inflammatory and pro-fibrotic cytokines
- The novel pro-inflammatory cytokine IL-22, but not IL-17, may be involved in TB-induced pathology as well as the resultant repair of the epithelial surface of the pericardium during TB infection.

CHAPTER 8

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