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Analysis of gamma-delta T cells in black South African patients with active tuberculosis

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2014
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UNIVERSITY OF THE WITWATERSRAND

ANALYSIS OF GAMMA-DELTA T CELLS IN BLACK SOUTH AFRICAN PATIENTS WITH ACTIVE TUBERCULOSIS

A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree, Master of Medicine in Haematopathology.

Dr Qanita Sedick

Johannesburg, 2014

DECLARATION

I declare that this research report is my own and unaided work. It is being submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Medicine in Haematopathology. It has not been submitted before for any degree or examination, nor has it been prepared with the assistance of any organisation, body or person outside the University of the Witwatersrand, Johannesburg.

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ABSTRACT

Mycobacterium Tuberculosis is the leading cause of morbidity and mortality due to infectious diseases worldwide. South Africa has ~20% of the world's HIV associated Tuberculosis and has the second largest reported numbers of multidrug resistant (MDR) Tuberculosis in the world.

Given the complexity of the mycobacterium and its ability to evade the immune system, there is a need for dissecting the immunological response to Tuberculosis including innate like lymphocytes such as gamma-delta T cells. Gamma-delta T cells are of particular relevance as they react to phospho-proteins of mycobacteria. Gamma-delta T cells can be divided into two subsets. Gamma-delta T cells using the Vdelta2 (VD2) segment as the variable segment in their T cell receptor and gamma-delta T cells using an alternative variable segment (non VD2 T cells).

We aimed to enumerate both subsets of gamma-delta T cells in the immunological response to Tuberculosis. We collected samples from three patient populations at the Charlotte Maxeke Johannesburg Academic Hospital for comparison: HIV positive patients with no evidence of Tuberculosis disease, HIV positive patients with active pulmonary Tuberculosis and a healthy control group. We used a nine colour flow cytometric panel to enumerate the frequency of gamma-delta T cells in these participant groups.

We found that the VD2 T cell subset was reduced in the HIV positive group and the dual HIV positive TB positive group compared with healthy controls, which mirrored the loss of CD4 T cells in these patients. Conversely, the non VD2 subset of gamma-delta T cells showed a statistically significant increased frequency in HIV positive patients and dual HIV positive TB positive patients compared to healthy controls. The frequency of gamma-delta T cells, expressed as a percentage of total T cells, was significantly increased in HIV positive patients and not non- significantly increased in the HIV positive TB positive groups compared to healthy controls.

This skewing of the gamma-delta T cell repertoire in HIV positive patients and HIV positive patients with active Tuberculosis may have specific immune implications. The mechanism of the loss of VD2 T cells in HIV and HIV associated Tuberculosis has not been elucidated. The loss of VD2 gamma-delta T cells in HIV and HIV associated Tuberculosis may underlie susceptibility to Tuberculosis disease.

ACKNOWLEDGMENTS

Dr. Melinda Suchard, my supervisor for this project who has helped me tremendously in gaining a better understanding of immunological concepts as well as the art of critical thinking and writing;

Dr. Dave Murdoch, co-supervisor who supplied the critical monoclonal antibody for the panel – gamma-delta T cell antibody;

Ntombeni Mashabane for her extensive help with sample preparation in the flow laboratory;

University of the Witwatersrand for providing the relevant funding;

National Health Laboratory Service staff

DEDICATION

All praise is due to my Creator of the Universe for blessing me with the ability to complete this research.

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ABBREVIATIONS

AFB: Acid Fast Bacilli

ADCC: Antibody dependent cytotoxicity

AZT: Azidothymidine

BCG: Bacillus Calmette-Guerin

CD: Cluster of differentiation

CFP-10: Culture filtrate protein-10

D/O: Disorder

ESAT: Early secreted target-6

Efavirenz: EVF

EDTA: Ethylenediaminetetra-acetic acid

XDR: Extensive drug resistant Tuberculosis

FMO: Fluorescence minus one

FSC: Forward scatter

H-gates: Histogram gates

HX: History

HIV: Human Immunodeficiency virus

HAART: Highly active anti-retroviral treatment

HMBPP: Hydroxy-3-methyl-but-2-enyl pyrophosphate

IFN-gamma: Interferon gamma

IGRA: Interferon-gamma release assays

ICD: Intra-costal drain

INSUFF: Insufficient

LTBI: Latent Tuberculosis Infection

3TC: Lamivudine

LPD: Lipodystrophy

LAD: Lymphadenopathy

MHC: Major histocompatibility complex

MFI: Mean Fluorescence Intensity

MAIT: Mucosal associated Invariant T cells

MDR: Multidrug resistance

MDR TB: Multidrug resistant Tuberculosis

MTB: Mycobacterium Tuberculosis

NK: Natural killer

NHLS: National Health Laboratory Service

NO: Nitric oxide

N/A: Not applicable

NOD: Nucleotide binding oligomerization domain-like receptor

NF-KB: Nuclear factor- kappa B

PRR's: Pathogen recognition receptors

PMT: Photomultiplier tube voltages

PBMC: Peripheral blood mononuclear cells

PBS: Phosphate buffered saline

PNP: Peripheral neuropathy

D4T: Stavudine

SAH: Subarachnoid haemorrhage

SSC: Side scatter

TDF: Tenofovir

TLR: Toll like receptors

TB: Tuberculosis

TNF: Tumor necrosis factor

TCR: T cell receptor

TX: Treatment

U + E: Urea and electrolytes

VDJ: Variability, Diversity, Junctional

WHO: World Health Organization

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1. INTRODUCTION

1.1. Tuberculosis in the third world

Tuberculosis (TB) remains a global health problem. According to the Global Tuberculosis Report, 2013, an estimated 8.6 million people developed Tuberculosis and 1.3 million of these patients died from the disease in 2012. An estimated 1.1 million (13%) of the 8.6 million people who developed TB in 2012 were HIV positive. About 75% of these cases were in the African Region. In 2012, an estimated 450 000 people developed Multi Drug Resistant Tuberculosis (MDR-TB) and there were an estimated 170 000 deaths from MDR-TB. The majority of cases worldwide in 2012 were in the South-East Asia (29%), Africa (19%) and Western Pacific (19%) regions. The TB incidence rate in South Africa is currently around 1000 or more cases per 100 000 people (1, 2).

Most Tuberculosis patients are in their 20s to 40s resulting in a devastating socio-economic loss. Population expansion, poor case detection and cure rates in developing countries, ongoing transmission, overcrowding in hospitals and prisons, migration due to wars and famine, drug abuse and social decay has all impacted on the TB disease burden (3).

In addition, co-infection with HIV also worsens the disease burden with ~50% of HIV positive patients at risk of TB. The WHO estimates that 80% of HIV infected TB patients reside in Africa (4).

The emergence of multi-drug resistant (MDR) TB (strains of M.TB resistant to two first line drugs) and extensive drug resistant (XDR) TB (strains of M.TB resistant to fluoroquinolone, kanamycin and amikacin) has also been reported in 55 countries around the world. MDR-TB is difficult and expensive to treat while XDR-TB is virtually untreatable in most developing countries (5, 6).

1.2. History of Tuberculosis disease

Mycobacterium Tuberculosis is an ancient organism that has inhabited the earth for centuries. Egyptian mummies which existed several thousand years BC have shown evidence of spinal Tuberculosis. Tuberculosis references can be found in the ancient Babylonian and Chinese scriptures. In more recent times, molecular genetic studies have revealed that Tuberculosis is ~3 million years old (3).

1.3. Natural history of Mycobacterium Tuberculosis

Tuberculosis is usually spread by individuals with active pulmonary TB. These Tuberculosis patients usually reside in close proximity to other family and community members, thus facilitating the spread of the disease (3).

Mycobacterium Tuberculosis is usually acquired by inhaling aerosolized mycobacteria from the environment. These bacterial droplets are ~1-5µm and remain suspended in the air for several hours. The risk of an individual contracting the infection depends on the infectiousness and proximity of the contact, the bacillary load and the immune status of the host (7).

1.3.1. Primary infection

The epithelium of the lung is the primary site of infection. The inhaled droplet nuclei are able to penetrate the terminal alveoli where they are engulfed by phagocytes. Phagocytes are cells of the innate immune system and include macrophages and dendritic cells. Once internalized by these phagocytes, the mycobacterium is able to replicate intra-cellularly. Some of these infected cells may be able to cross the alveolar barrier in the lung and cause systemic dissemination. Dissemination occurs to the lymph nodes and to various other extra pulmonary sites such as the liver, kidney and bone marrow. The initial focus of infection in the lung epithelium and lymph nodes is called the ghon complex and represents primary TB infection (8, 9).

1.3.2. Latent Tuberculosis infection (LTBI)

In most individuals who are not immune compromised, an effective immune response develops soon after infection which halts further replication of the mycobacterium (3). Granulomas

represent a part of this immune response. Granulomas are confined areas of necrotic tissue that wall off the infection and prevent dissemination. The tubercle bacilli are also destroyed within these caseating granulomas if an effective immune response in the host is established (7, 10, 11).

Despite the development of an adaptive immune response to the mycobacterium, the pathogen is often able to survive in this protected niche in the lung and avoid complete elimination for many years, resulting in latent TB infection (10, 11).

1.3.3. Reactivation of latent Tuberculosis

Latent Tuberculosis is defined as infection with M.TB in foci within granulomas which remain in a non-replicating state but retains the ability to emerge and cause active TB when immune deregulation occurs (12).

When the host immune response is compromised, as occurs with old age, diabetes mellitus, malnutrition, renal failure and immunosuppressive treatments, reactivation of latent Tuberculosis can occur (7, 13-15). HIV is the leading cause of reactivation due to depletion of $CD4^+$ T cells (4, 15). With reactivation, the dormant bacilli, found in the macrophages within the granulomas migrate towards a new area of the lung epithelium to establish a new focus of infection (16-19). Reactivation often occurs in the upper lobes of the lung where severe necrosis and cavitation occurs (17, 20).

1.3.4. Symptoms of disease from Tuberculosis

Patients may develop influenza like symptoms that resolve within a few days. Progressive primary infection or reactivation of pulmonary Tuberculosis manifests with numerous classical symptoms including a cough which is productive of sputum or blood, drenching night sweats, fatigue, fever and loss of weight. Patients may also experience difficulties in breathing, chest pains and wheezing. If systemic dissemination has occurred, patients will present with organ symptoms depending on the site of the Tuberculosis reactivation.

1.4. The immunology of Tuberculosis

1.4.1. The innate immune response to Tuberculosis

Mycobacterium Tuberculosis has a predilection for lung tissue and is able to penetrate the terminal alveoli where it is engulfed by phagocytic cells of the innate immune system to form a phagolysosome. Recognition of the bacillus occurs when pathogen recognition receptors (PRRs) on the phagocytes identify certain pathogen-associated molecular patterns (PAMP) of the mycobacterium (21).This process is followed by recognition of the mycobacterium by Toll-like receptors (TLR), nucleotide binding oligomerization domain-like receptors (NOD) and C-type lectins (CD201, DC-SIGN and Dectin-1) which are all receptors of the innate immune response (22, 23). Interaction of the mycobacterium with these receptors initiates an intra-cellular signaling cascade through nuclear factor–K B (NF-KB) resulting in a pro-inflammatory and antimicrobial response. The pro-inflammatory response to the mycobacterium allows the migration of monocytes, neutrophils and lymphocytes to the focal site of infection in the lung with the consequent formation of a phagolysosome (22, 23).

Innate immunity is the first line of defense against infections present in all multicellular organisms. Components of the innate immune system in humans include epithelia, phagocytic cells, natural killer (NK) cells, cytokines, complement components and plasma proteins (24).

Natural killer (NK) cells are lymphocytes capable of killing microbe infected cells and activating phagocytes by secreting interferon-gamma (IFN-gamma). NK cells do not express immunoglobulin or T cell receptors. They are regulated by cell surface stimulatory and inhibitory receptors which recognize major histocompatibility complex molecules. NK cells also express CD56 and the receptor CD16 that binds to IgG antibodies on cells. These receptor mediated signals stimulate the NK cells to release their cytotoxic granules which are able to kill opsonized targets in a process which is known as antibody dependent cytotoxic killing (ADCC) (24). During Tuberculosis infection, natural killer cells are able to kill microbial pathogens via this cytotoxic response (25).

1.4.2. The Adaptive Immune response to Tuberculosis

Adaptive immunity also plays a crucial role in the host response to the mycobacterium. Dendritic cells engulf bacilli and migrate to regional lymph nodes to prime $CD4^+$ T and $CD8^+$ T cells. The activated T cells are stimulated by chemokine signaling and migrate back to the focus of infection in the lung to begin manufacturing of the granuloma (26).

The granuloma is an enclosed area of inflammation which is able to contain the tubercle bacilli from the rest of the lung epithelium thus attempting to limit the spread of bacteria. Within the granuloma, numerous cells are actively involved in forming an effective host immune response to the mycobacterium (27). Macrophages carrying Tuberculosis antigens present peptide antigens to $CD4^+$ T cells in an immunological synapse resulting in the production of tumor necrosis factor (TNF) and IFN-gamma (28). These cytokines activate macrophages which generate nitric oxide (NO). This NO along with other substances is toxic to the bacilli and is thought to eliminate it (29-31). Cytotoxic T cells can directly kill the mycobacterium via the granulysin effect (29-31). This process represents a protective immune response which is manifested clinically by a positive tuberculin skin test and resistance to re-infection (7, 26).

1.4.3. Tuberculosis evades the immune response

Despite this protective immune response to Mycobacterium Tuberculosis, the pathogen has developed numerous strategies to modulate and evade the immune response and persist in the host. The mycobacterium is able to avoid destruction by lysosomes in the phagolysosome and is also able to block maturation of the phagolysosome (32). The mycobacterium is able to modulate calcium signaling cascades which inhibits phagosome maturation and leads to enhanced intracellular survival (33-36). Mycobacterium tuberculosis can also inhibit apoptosis of macrophages which escapes host defenses (37).

1.4.4. VDJ recombination and diversity of T cells

There are two classes of T cells which are involved in adaptive immunity. The majority of the T cells in the body have alpha-beta T cell receptors. Another subset of T cells have gamma-delta T cell receptors and comprise <5% of all circulating T cells in healthy adults.

The T cell receptor (TCR) recognizes antigen but is unable to transmit signals to the T cell. Therefore, associated with the TCR is a complex of proteins called the CD3 molecules (three protein structures) and the zeta chain that make up the TCR complex. The CD3 and the zeta chains transmit some of the signals that are initiated when the TCR recognizes antigen. Signal transduction occurs through tyrosine phosphorylation (24).

The diversity of these antigen receptors on lymphocytes is produced as a result of VDJ recombination. This process involves the use of different combinations of V, D and J segments of the TCR gene in different clones of lymphocytes which introduces variability in T cell receptors and results in a diverse repertoire of lymphocytes with different receptors which are capable of recognizing different antigens (24, 38).

Most T cells recognize only protein antigens in the context of MHC and have a wide range of specificities of antigen due to the diversity of their TCR repertoire (39). Gamma-delta T cells have a limited diversity of their TCR repertoire. The first crystallographic structure of the human gamma delta TCR was described in 2001 as a result of their unique surface expression of novel molecules (38, 40, 41). Gamma- delta T cells are referred to as invariant T cells. Gamma-delta T cells comprise <5% of all circulating T cells in healthy adults (38, 42). Gamma-delta T cells are the predominant cell type found on mucosal surfaces including the epithelial layers of the gut, tongue, lung and female reproductive tract (38, 43).

1.4.5. Invariant T cells in Tuberculosis

Mycobacterium Tuberculosis is structurally composed of a cell wall which is surrounded by a thick waxy mixture of lipids and polysaccharides and a high content of mycolic acids (44-47). $CD4^+$ and $CD8^+$ T cells are largely responsible for immune responses to peptides. Cells which respond to lipid antigens are CD1-resticted T cells (natural killer T cells) and cells which respond to phospholipids are the gamma-delta T cells. CD1-restricted T cells and gamma-delta T cells are unique T cell types which are specifically adapted to recognize these lipid and phospho-antigens on mucosal and epithelial surfaces (44, 45). Recent evidence suggests that gamma-delta T cells are important in the host immunity to mycobacteria (48). Gamma-delta T cells have been shown to recognize non-peptide metabolites of isoprenolol biosynthesis and lipid extracts of mycobacteria

(38, 49). Gamma-delta T cells do not require uptake, processing or intracellular loading on the MHC molecule for antigen presentation and are dependent on cell to cell contact (38, 49). These gamma-delta T cells express pro-inflammatory cytokines such as IFN-gamma which are cytotoxic to the mycobacterium (47, 50).

1.4.6. Sputum testing for Tuberculosis

TB diagnosis currently includes a detailed medical history, clinical examination as well as radiological, microbiological, immunological, molecular and histological investigations. The tuberculin skin test is an in vivo test which becomes positive six to eight weeks after exposure to the bacilli. The skin test is based on a delayed type hypersensitivity response. A skin reaction of >20mm is due to active tuberculosis disease. This test, however, lacks sensitivity and specificity (7, 51).Culture based detection of the mycobacterium in sputa has remained the gold standard of diagnosis until recently. Sputum acid fast bacilli (AFB) by microscopy is the most common method of screening (52). The culture growth of Mycobacterium Tuberculosis takes on average two or more weeks. In addition, only 44% of all new cases (15-20% of children) are actually identified by the presence of (AFB) on sputum smears. The sensitivity of sputum smear microscopy is as low as ~35% in settings with TB and HIV co-infection. This results in difficulty in initiating anti-Tuberculosis treatment where AFBs are not detected by sputum smear microscopy (52). Tuberculosis drugs have potential side effects which can be debilitating and thus empiric Tuberculosis treatment is not ideal. Tuberculosis is treated for a period of six to nine months. Tuberculosis symptoms resolve after a few weeks of treatment and for these reasons, compliance is a major problem facing the health sector. As a result of noncompliance and failure to complete the full six months of anti-tuberculosis treatment, multidrug resistant and extensive drug resistant strains of Mycobacteria Tuberculosis are emerging with increasing frequency (5).

1.4.7. Newer methods of testing for Tuberculosis

More sensitive and specific tests based on the cell-mediated immune response to Tuberculosis include the IFN-gamma release assays (IGRA) which are able to detect T cell responses after stimulation by two mycobacterial antigens, the early secreted antigen target-6 (ESAT-6) and the culture filtrate protein-10 (CFP-10) (53-58). IGRA includes the Quantiferon Gold assay and the

ELISPOT test both of which have been FDA approved (59-62). While these assays can differentiate individuals who have been previously exposed to Tuberculosis from those who have never been exposed, they cannot differentiate latent from active disease and therefore lack utility in areas of high background Tuberculosis like South Africa (57, 60-63).

This scenario is currently evolving with the roll out of the Xpert MTB/RIF (Cepheid) in South Africa which is a molecular assay with an increased sensitivity, reduced turn-around time and easy detection of rifampicin resistance (64). The Xpert MTB/RIF testing has shown superior performance for the rapid diagnoses of Mycobacterium Tuberculosis compared to current assays in an HIV and TB endemic areas such as South Africa (64).

Molecular testing, however, still requires the presence of a suitable sample and may have limitations in groups who struggle to produce sputum as well as in non-sputum samples such as cavitatory fluids (65).

1.4.8. Need to understand the immunological response to TB

Due to this inadequacy of diagnostic tools and weak laboratory–based diagnoses of active TB, many cases remain under-diagnosed, further contributing to the morbidity, mortality and the continued transmission of the disease especially in those patients co-infected with HIV.

As a result there is a clear need for the development of cost-effective and simple new tools for the reliable detection of Tuberculosis in HIV-infected and uninfected patients.

Due to the pressure for strengthening earlier diagnosis in the paucibacillary stage, including sputum-negative pulmonary Tuberculosis, extra pulmonary Tuberculosis, childhood and neonatal Tuberculosis and Tuberculosis with HIV co-infection, there is an urgent need to understand the immune response to Tuberculosis in order to develop new systems based on the immunological response to Mycobacterium Tuberculosis (52). A deeper understanding of the immunology of Tuberculosis may also lead to improved therapeutics and adjunctive treatments as well as monitoring tools for treatment outcome (66, 67).

1.5. Gamma-delta T cells in Tuberculosis and HIV

It has long been known that it is the immune control of the mycobacterium that determines active Tuberculosis. A person can be latently infected with Tuberculosis, yet appear completely healthy (68). When his CD4 count falls beyond a certain point, however, he manifests with disease.

Numerous studies have recently shown that gamma-delta T cells have an important role in defending the host against a very wide range of infections. Gamma-delta T cells are unique in the immune system as they have been shown to combine conventional adaptive functions with rapid, innate like responses in the initiation of immune responses (69). Six mechanisms have been attributed to gamma-delta T cells and recently reviewed (70).

These mechanisms are listed below:

1. Gamma-delta T cells lyse as well as eliminate infected cells via the production of granzymes
2. Gamma-delta T cells produce a diverse range of cytokines and chemokines which regulates other immune cells
3. Gamma-delta T cells assist B cell function. The B cells are then able to produce IgE
4. Gamma-delta T cells present antigens for alpha-beta T cell priming
5. Gamma-delta T cells can trigger dendritic cell maturation
6. Gamma-delta T cells can regulate stromal cell function via growth factor production

Given this diverse function in the immunological system, it is not surprising that gamma-delta T cells have also been shown to have an important role in HIV and Tuberculosis in several studies.

There are conflicting studies regarding the distribution of gamma delta T cells during Tuberculosis infection. Studies show that gamma-delta T cells are increased in the peripheral blood and lungs of Tuberculosis patients (71, 72). It has been shown that gamma-delta T cells express an activation phenotype even after 5 weeks of Tuberculosis treatment (40). Gamma-delta T cells have also been shown to be involved in the formation of the Tuberculosis granuloma in humans, playing a role in the innate immune response to Tuberculosis (73). IL-17 producing gamma-delta T cells were increased in the peripheral blood of TB patients compared to healthy controls (74). The frequency

of gamma delta T cells in patients with mild Tuberculosis was increased compared to patients with advanced and pulmonary or miliary Tuberculosis (75).

In contrast, some studies have suggested that gamma-delta T cells are reduced in the peripheral blood of TB and HIV infected patients compared to healthy controls (48). It was hypothesized that the reduction in the gamma-delta T cells in the peripheral blood of patients with active Tuberculosis may be a result of sequestration of reactive gamma-delta T cells to the site of infection in the lung epithelia (40). Another study, however, showed that gamma delta T cell subsets from broncho-alveolar lavage samples of patients with active Tuberculosis were functionally impaired (76).

Several animal models have supported the role of gamma-delta T cells as modulators of Tuberculosis infection.

Macaque Vgamma2Vdelta2 T cells have the capability to recognize mycobacterium phospho-antigen (E)-4-hydroxy-3-methyl-but-2-enylpyrophosphate (HMBPP) (77). In a macaque model of infection with Mycobacterium Bovis Bacillus Calmette-Guerin (BCG), the gamma-delta T cells demonstrated clonal expansion during primary exposure. The expansion of gamma-delta T cells in the pulmonary compartment of these monkeys was associated with a decline of bacterial burden, resolution of active infection and immunity against fatal Tuberculosis. These findings demonstrated the importance of gamma-delta T cells in the innate immune response to Mycobacterium Tuberculosis in macaque monkeys (78). Recent studies have also shown that HIV infected macaque monkeys with high viral loads inhibit gamma-delta T cell responses in the blood and lungs. Tenofavir and indinavir anti-retroviral treatment of these monkeys were able to restore the capacity of gamma-delta T cells to proliferate and undergo pulmonary migration in active Tuberculosis re-infection, suggesting the vital and protective role of gamma-delta T cells in dual HIV+ TB+ infection (79).

Recently, it was found that Vdelta2gamma-delta T cells were the essential populations producing IFN-gamma in response to BCG immunization in infants and children suggesting that these unconventional T cells are significant in the mycobacterial immune response (80).

1.6. Subsets of gamma-delta T cells

Gamma-delta T cells are comprised of two subsets: Gamma-delta T cells which use the Vdelta2 segment (VD2) as their variable segment and those that do not(41). These subsets belong to the same class of T cell types but have slightly different antigen specificities (24).Studies have found that these gamma-delta T cell subsets behave differently in HIV patients with opportunistic infections (81).

1.7. Detection of gamma-delta T cells

All lymphocytes arise from stem cells in the bone marrow. B lymphocytes undergo VDJ recombination in the bone marrow and T lymphocytes undergo VDJ recombination in the thymus. All lymphocytes are morphologically similar in appearance but differ with regard to function, lineage and phenotype (82). Different subtypes of B and T lymphocytes can be distinguished by surface proteins on the cell membrane. The standard nomenclature for these proteins is the CD or cluster of differentiation (24).

Multicolour flow cytometry is a means of representing a very large amount of data involving cell surface receptors and cellular activation from a single sample. Flow cytometry can enable characterization of complex populations and immunological mechanisms within the human body (83-87).

Given the importance of gamma-delta T cells in the immune system and their role in innate and adaptive immune responses, we aimed to assess the frequency of gamma-delta T cells in HIV and Tuberculosis infected patients in the South African setting where these infections are most prevalent. In order to delineate all the cell subsets for specific assessment of gamma-delta T cell functions in HIV and TB populations, a series of T and B cell markers were chosen for our nine colour (multiparameter) panel. Included in our panel but not analyzed as part of this dissertation are markers of NK T cells. The chosen markers are indicated in table 1.

Table 1: Panel of CD markers selected

CD number	Main cellular expression	Function
CD19	Most B cells	B cell activation; forms a co receptor complex with CD21 and CD81 which delivers signals that synergize with signals from B cell antigen receptor complex (24)
CD3	T cells	A lineage specific marker for T cells; for cell surface expression of and signal transduction by the T cell antigen receptor (24)
CD4	T helper cells(also found on monocytes and macrophages)	Signaling and adhesion co-receptor in class 11 MHC-restricted antigen-induced T cell activation (24)
CD8 Alpha	Cytotoxic T lymphocytes	Signaling and adhesion co-receptor in class 1 MHC-restricted antigen-induced T cell activation (binds to class 1 MHC molecules (24)
Gamma delta TCR	Mucosal associated invariant T cells; T cells with gamma delta TCR	Recognize lipid and non-protein antigens on epithelial surfaces (24)
VD2 TCR	A subset of gamma-delta T cells using the Vdelta2 variable segment in their TCR	Recognize lipid and non-protein antigens on epithelial surfaces (24)
CD16	NK cells	Fc receptor (24)
CD56	NK cells	Responsible for adhesion (24)
CD6B11	NK T cells	Express the alpha beta TCR but recognize glycolipid and other non-peptide antigens displayed by non-polymorphic MHC-like molecules, e.g. CD1d (24)

Table 2: Our lineage description was as follows

CELL MARKER:	DESCRIPTION:
CD3+	T cell
CD3+ CD4+	CD4 T helper cell
CD3+ CD8 ALPHA +	CD8 cytotoxic T cell
CD3+ GAMMA DELTA +	Gamma-delta T cell
CD3+ VD2 TCR+	VD2 TCR subset of gamma-delta T cell
CD3+ GAMMA DELTA+ CD8+	Gamma-delta T cell expressing CD8+
CD3+ GAMMA DELTA+ VD2 TCR-	Gamma-delta T cell that is not in the VD2 TCR subset (likely VD1 TCR subset)

1.8. Overall aims of the study

The primary aim of this study was to enumerate the frequency of gamma-delta T cells by flow cytometry in HIV+ patients and HIV+ TB+ infected patients in comparison with healthy controls in a South African setting.

The secondary aim of the study was to assess whether:

1. Gamma-delta T cell frequency was correlated with CD4 count, used as a marker of HIV disease progression.

2. The frequency of the VD2 TCR subset of gamma-delta T cells differed in healthy controls compared with HIV or HIV/TB co-infected patients.

2. MATERIALS AND METHODS

2.1. Study samples

Ethics approval was obtained from the University of Witwatersrand human ethics committee and the following ethics number received: M10228.

The study was a prospective study which was performed over a period of 2 months. A total number of 49 samples were collected. Samples from patients were collected at the Charlotte Maxeke Johannesburg Academic Hospital from 1 July 2011 until 31 August 2011. Samples were analyzed at the National Health Laboratory Services, department of Molecular Medicine and Haematology based at the Charlotte Maxeke Johannesburg Academic Hospital.

Seventeen control samples were collected in ethylene-diamine-tetra-acetic acid (EDTA) anti-coagulated tubes from health care workers at the Charlotte Maxeke Johannesburg Academic Hospital. Participants signed informed consent for blood to be used for the study. Controls were given the option of free HIV testing with appropriate counseling by independent counselors not involved with the study. Those that consented were taken to an HIV testing centre where a rapid HIV test was performed**.** Seventeen control patients were enrolled of which four (A6, A8, A12, A13) refused consent for the HIV testing but were included in the study. One control patient tested HIV positive. This patient was subsequently referred to the HIV clinic and was excluded from the study analysis. During processing of samples, further exclusion of samples was required. This resulted in samples A1, A2, A3 and A4 being excluded. These samples were excluded because the antibody we used to mark gamma-delta T cells for this batch was found to stain sub-optimally. Samples A14, A15 were also excluded from our analysis because there were too few events noted on these sample plots. Therefore, 10 control samples in total were analyzed in total.

Patient samples were collected included two groups. The first group included patients that were HIV positive and had no symptoms or signs of current Tuberculosis. TB microscopy was not collected routinely on this patient group and exclusion of Tuberculosis was based on absence of

symptoms and signs of Tuberculosis. Symptoms for which patients were screened included productive cough, night sweats and loss of weight. Signs included pleural effusions and signs of immunosuppression such as cachexia. Data was collected on a patient data sheet (Appendix 4). These samples were obtained from the Charlotte Maxeke Johannesburg Academic hospital HIV outpatient clinic. A total number of seventeen patients were collected in this group. During sample processing, it was found that B5 had too few cells for analysis and was thus excluded from the analysis. A total of 16 samples in this group were thus used in the final analysis.

The second group included patients that were HIV positive with recent onset (previous two weeks) of smear positive Tuberculosis and that had only been on anti-Tuberculosis treatment for less than 4 days or had not started anti Tuberculosis treatment. These samples were obtained from the medical and infectious disease wards at the Charlotte Maxeke Johannesburg Academic Hospital. The sputum results for smear positivity of each patient were obtained from the microbiology laboratory. All patients who had been on anti-Tuberculosis therapy for longer than 4 days and who did not have smear-positive sputum result were excluded. There were no pregnant women in the cohort. The age range for all patients and health care workers was between 20-60 years old. A total number of fifteen patients in this group were collected. There were no exclusions in this group. Therefore, all the samples in this group were used in the analysis.

Table 3: Enrollment and exclusion numbers

	GROUP A (CONTROLS)	**GROUP B(HIV+TB-)**	**GROUP C (HIV+TB+)**
TOTAL NUMBER SAMPLES COLLECTED	17 samples	17	15
EXCLUSIONS	A1, A2, A3, A4 excluded due to poor staining A7 excluded due to HIV positivity A14, A15 excluded due to too few cells for analysis	B5 excluded due to too few cells for analysis	No exclusions in this group
FINAL SAMPLES ANALYSED	10 samples	16 samples	15 samples

2.2. CD4 testing

Blood for CD4 testing was taken from the patient and sent to the National Health Laboratory Service (NHLS) laboratory for testing according to their standard procedure. The CD4 testing was part of routine investigations performed on the patient.

2.3. Peripheral blood mononuclear cell (PBMC) isolation

Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation over Ficoll Hypaque gradients. The PBMC's were spun at a speed of 3000 rcf (relative centrifugal force) and washed three times in phosphate buffered saline (PBS). An antibody cocktail was prepared which consisted of each appropriate volume of the nine antibodies multiplied by the number of samples for testing. The staining volume of each antibody was obtained by titration (see below). Each

sample was then stained using the antibody cocktail for each day, to minimize pipetting inaccuracies.

2.4. Flow cytometric acquisition and analysis

All the data was recorded on the LSRII flow cytometer (BD Biosciences) using FACSDIVA software (BD Biosciences) for acquisition. FlowJo software (Treestar, USA) was used for analysis. Approximately 200 000 events were recorded for each sample.

2.4.1. Quality control:

Daily quality control of the instrument was performed as per routine laboratory procedure by use of 1 X beads (midlevel fluorescence beads) (BD Biosciences). Any shift of fluorescence compared to the beads previous position is recorded and only minor shifts which remain within set gates are considered acceptable.

2.4.2. Compensation

BD Comp Beads are polystyrene particles that have been coupled to an antibody specific for the IGH Kappa light chain of a mouse. Each set contains a negative control which has no binding capacity. When these are mixed with a mouse antibody conjugated to a fluorochrome, BD Comp Beads are able to provide distinct positive and negative stained populations. BD Comp Beads were thus used in the experiment to set compensation levels and to standardize experiments.

For compensation, nine tubes were prepared which each contained one antibody in the previously titrated volume. A positive and negative compensation bead was included in each tube (IgG capture beads, BD Biosciences). These tubes were then run on the LSR and photomultiplier tube voltages (PMTs) were adjusted according to expected brightness of the specific fluorochrome using histogram (H) gates. For each flourochrome, a gate was applied around the population where the fluorochrome was originally expressed on the first run. H-gates were applied to each individual fluorochrome for each sample tested and PMT’s adjusted to bring the population within the gate.

2.4.3. Titration of antibodies

When preparing a sample for flow cytometric analysis, titration of antibodies is essential to find the optimal concentration of antibody that approaches the saturation level of binding sites. This will ensure that a fluorescent signal emission is proportional to the antigen in the sample. CD4 Alexa700 and CD8 PerCP CY5.5 had been titrated for the initial antibody panel. As illustrated in Appendix 9, the titration of CD8 Beta was performed. A series of dilutions of the antibody was prepared as follows:

Table 4: Titration of CD8 Beta

TUBE	1	2	3	4	5	6	7	8
VOLUME OF CD8 BETA(µL)	0.625	1.25	2.5	5	7.5	10	20	UNSTAINED TUBE
TOTAL STAINING VOLUME(µL)	50	50	50	50	50	50	50	50

The mean fluorescence intensity for the antibody was plotted against the antibody staining volume. The optimal mean fluorescence intensity for the antibody was chosen according to the point of saturation for the specific antibody.

Due to fluorescence spillover of antibodies, the initial antibody panel was altered which required further titration of another set of antibodies. These antibodies were serially titrated. The mean and median fluorescence intensity was determined and the final optimal volume for each antibody was chosen .The final volume chosen for $CD4^{+}$Alexa700 was 2,5ul and for $CD8^{+}$ Alpha was 5ul. The other antibodies had been previously titrated by our laboratory.

2.4.4. Fluorescence-minus-one (FMO) experiments

Fluorescence-minus-one (FMO) experiments involve omitting one fluorochrome at a time from each tube and looking for spillover fluorescence from the remaining fluorochromes into the channel of the omitted colour. FMO's were prepared for each antibody. Results of the FMO experiment required redesign of the panel as shown below.

Table 5: Initial and new antibody panel

ANTIBODY	INITIAL FLUOROCHROME	FINAL FLUOROCHROME	CLONE NUMBER	MANUFACTURER
CD4	PercP	Alexa 700	RPA-T4	Bio Legend, SanDiego, California
CD3	AmCyan	AmCyan	SK7	BD Biosciences, SanJose, California
CD8 Alpha	Alexa700	PerCP-CY5.5	RPZ-T8	Bio Legend, SanDiego, California
CD8 Beta	APC	APC	R22-33	BD Biosciences, SanJose, California
CD16/CD56	Pacific blue	Pacific blue	CD16:3G8 CD56:B159	BD Biosciences, SanJose, California
VD2 TCR	FITC	FITC	δG9	BD Biosciences, SanJose, California
CD19	PE-Cy7	PE-Cy7	REF:IM3628	Beckman Coulter, Brea, California
GAMMA DELTA TCR	PE-Cy5/PerCP	PE-Cy5/PerCP	REF:IM2662	Beckman Coulter, Brea, California
INKT 6B 11	PE	PE	6B11	Bio Legend, SanDiego, California

The initial FMO experiments indicated spillover. Spillover is the phenomenon that occurs when fluorescence emission of one fluorochrome is detected in a second detector designed to measure signals from a second fluorochrome. In the CD3 FMO experiments, omission of CD3 AmCyan while running a panel comprising the other eight fluorochromes showed spillover into the AmCyan channel (figure 1). Omission of CD4 PerCP-CY5.5 while running a panel comprising the other eight fluorochromes also showed spillover into the PerCP-CY5.5 channel (figure 2). Spillover into the CD8 Beta APC channel also occurred (figure 3).

These spillover effects necessitated a substitution in the panel. We used CD4 Alexa 700 and CD8 Alpha PerCP-Cy5.5 instead for the new antibody panel and reanalyzed FMOs. No spillover effects occurred with our new panel.

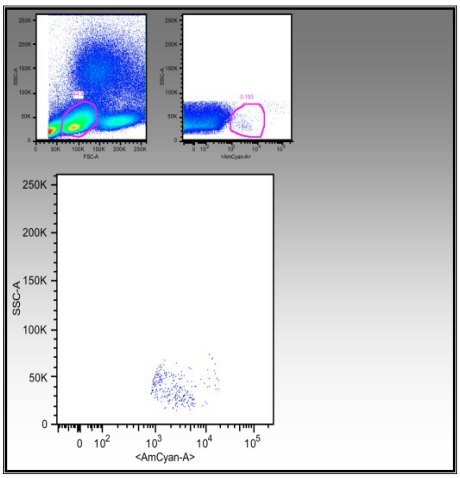


Figure 1: Spillover into the AmCyan channel. The above plot was from a sample in which the fluorochrome AmCyan was omitted. There should be no events in the AmCyan channel. This plot shows events on the x axis (AmCyan) which indicates that another fluorochrome is spilling over into the AmCyan channel.

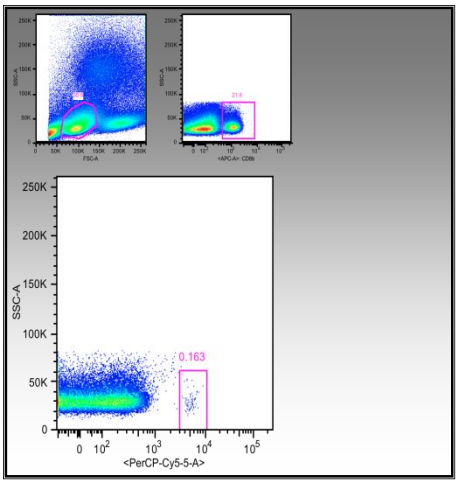


Figure 2: Spillover into PerCP-CY5.5. The above plot was obtained in an experiment which omitted PerCP-Cy5.5 from the tube. All other fluorochromes were present in the tube. In view of the omission of PerCP-Cy5.5 from the tube, no events are expected to occur in the PerCP-Cy5.5 channel. However, events in this channel are noted (see boxed events), indicating spillover of another fluorochrome into the PerCP-Cy5.5 channel

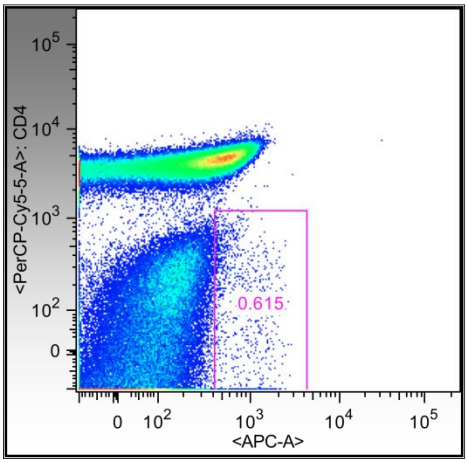


Figure 3: Spillover of other fluorochromes into CD8 Beta APC. This plot similarly shows events in the APC channel when APC is omitted which indicates spillover of another fluorochrome into APC channel.

2.5. Manual adjustment of compensation matrix

Digital compensation was originally performed using FlowJo software. Digital compensation did not fully compensate each fluorochrome against each other as is common in multicolour panels.Therefore, manual compensation was performed by iteratively plotting each fluorochrome against every other fluorochrome and adjusting the compensation matrix until the single–stained and unstained compensation beads showed equal fluorescence in all channels other than the stained fluorochrome channel.

2.6. Exclusions

Although manual compensation was performed for each sample,it was not possible to fully eliminate compensation issues from all the samples.Samples A1,A2,A3 and A4 belonged to the same batch of samples which were collected and analysed on the same day.These samples were excluded because the antibody the used to mark gamma-delta T cells for this batch was found to stain suboptimally.Sample numbers A14, A15 and B5 were excluded from our data because their were too few events noted in these plots.

2.7. Gating strategy

Lymphocytes were selected on the basis of forward scatter and low side scatter followed by the selection of T cells on the basis of CD3 positivity, which is lineage specific for T cells. B cells were identified using CD19. From the total CD3 population, the other T cell subsets were selected. These included CD4, CD8 Alpha, CD8 Beta, NK- T cells and gamma-delta T cells. There were two gamma- delta T cell subsets included in the study: gamma-delta TCR and VDelta2TCR (VD2 TCR).

As depicted in figure 5, gamma delta TCR was plotted on the y-axis and VD2 TCR was plotted on the x–axis. The frequency of total gamma-delta T cells was calculated by adding the top two quadrants of each plot. The VD2 TCR subset was evaluated by adding the top and bottom right hand quadrants of each plot.

2.8. Statistical analysis

Descriptive statistics using medians, minimum and maximum values were used to characterize age, gender CD4 count and number of patient on HAART. Age differences between groups was analyzed using a Chi Square test. CD4 count between group B and C was analyzed using a Mann Whitney test. The proportion of patients on HAART were compared between groups B and C using a two tailed Fishers Exact test. These tests were performed using Graph-Pad Prism software, version 6 (La Jolla, California).

Gamma delta T cells, VD2 T cells, CD4 and CD8 frequencies in HIV, HIV+ TB+ and control groups were compared using non-parametric one way analysis of variation (ANOVA) testing with Kruskal-Wallis. Graph Pad Prism software was used. Post-hoc comparisons were done using Dunns analysis. The levels of significance was chosen as $p<0.05$. Correlations were performed using the Spearman non-parametric method.

3. RESULTS:

The study population included black South African patients from the HIV clinic and respiratory wards at the Charlotte Maxeke Johannesburg Academic Hospital. Appendix 8 shows the patient demographic profiles. A comparison with respect to age, gender, HAART therapy and CD4 counts was done using a uni-variate analysis on Graph-Pad Prism software version 6 (La Jolla, California).

Gender differences were evaluated in the cohort. There was a total number of 18 males and 31 females in the cohort. There were 4 males and 13 females in group A. There were 8 males and 9 females in group B. There were 6 males and 9 females in group C. Differences in gender was analyzed using a Chi Square test. No difference with respect to gender was found between the groups ($p=0.3458$) (table 6).

Table 6: Gender differences in the cohort

	GROUP A	GROUP B	GROUP C	TOTAL NUMBER
MALES	4 (23% of group A)	4 (30% of group B)	6 (40% of group C)	18 (36% of total)
FEMALES	13	9	9	31
TOTAL	17	13	15	49

All three groups were assessed for age differences. The median age in group A was 40 years, in group B was 38 years and in group C was 34 years. The youngest person was 20 years old in group A and group C. The oldest person was 84 years old in group A. Age differences were analyzed using a Chi-square test. There was no difference found between the groups with respect to age (p=0.2916) (table 7).

Table 7: Age differences in the cohort

AGE IN YEARS	GROUP A	GROUP B	GROUP C
MINIMUM AGE	20	31	20
MEDIAN AGE	40	38	34
MAXIMUM AGE	84	71	52

The minimum CD4 count was 10 x 10^6/l in group C and the maximum CD4 count was 739 x 10^6/l in group B. Using a Mann Whitney test, the median CD4 count (median=90 µl) was statistically lower than the median CD4 count in group B (median=465 µl) (p=0.002) (table 8).

Table 8: CD4 counts in the cohort

	GROUP B	**GROUP C**
MINIMUM (µl)	45	10
MEDIAN (µl)	465	90
MAXIMUM (µl)	739	477

In group B, 15 patients were on HAART and 2 patients were not on HAART. In group C, 7 patients were on HAART and 8 patients were not on HAART. Using a 2 tailed Fishers exact test, there was a significant difference in the proportion of patients receiving HAART therapy and those not on HAART therapy (p=0.021). Table 9 shows the number of patients on HAART therapy for each group.

Table 9: Number of patients on HAART therapy

	HAART THERAPY
GROUP B	15 patients on HAART, 2 patients not on HAART
GROUP C	7 patients on HAART, 8 patients not on HAART

3.1. Frequencies of Gamma-delta T cell subsets differ between HIV infected patients and healthy controls

It was hypothesized that there may be differences in the frequency of gamma-delta T cells in HIV infected patients compared to healthy controls. The total gamma-delta T cells as well as the VD2 subset ($CD3^+$, Gamma-delta TCR+,VD2 TCR+) and the non-VD2 subset ($CD3^+$, Gamma-delta TCR+, VD2 TCR-) was analyzed.

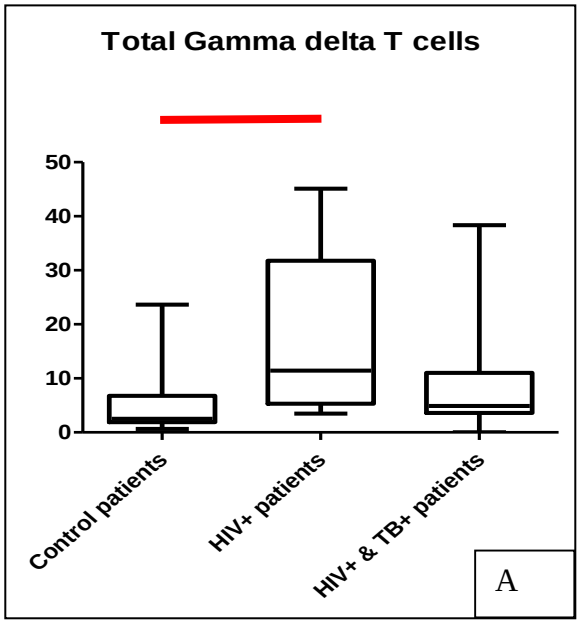
The frequency of total gamma-delta T cells as a percentage of T cells was higher in HIV infected patients compared to healthy controls (p=0.0052, median 11.4% vs.2.53%). HIV infected patients also had higher values compared to HIV+TB+ patients (median 4.92%) although this was not statistically significant.

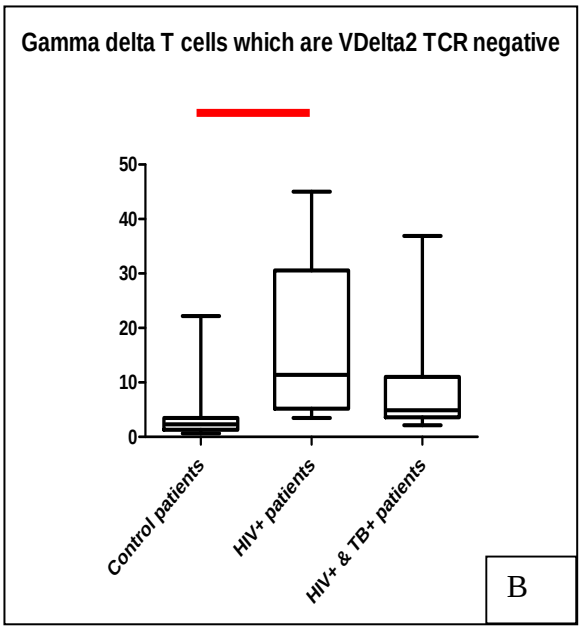
Similarly, the non-VD2subset, as a frequency of total T cells, was also higher in HIV infected patients compared to healthy controls (p=0.0005, median 11.4% vs.2.3%)

These results differed to those noted for the VD2 subset of gamma-delta T cells. The frequency of the VD2 subset as a percentage of total CD3 T cells was higher in healthy controls compared to HIV+TB+ patients (p=0.0318 , median 5.88% vs.0.87%). The VD2 subset was also higher in the healthy controls than the HIV+ group (median 0.87%) although this did not reach statistical significance.

There was no difference between controls and HIV+ and HIV+TB+ groups in the frequency of gamma-delta T cells expressing the cytotoxic T cell marker CD8 Alpha ($p=0.1252$).

There was no significant correlation between the CD4 count and the frequency of gamma-delta T cells, or the VD2 subset, as a percentage of CD3 T cells, in HIV infected or HIV+TB+ patients (data not shown).





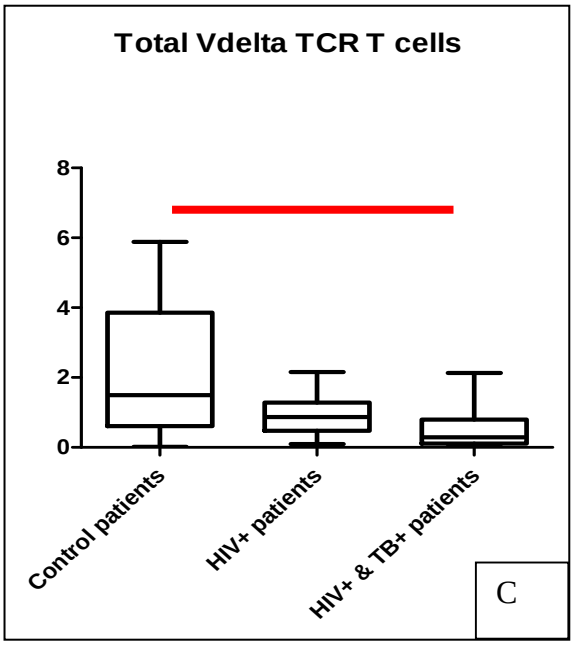


Figure 4:

A: The frequency of total gamma-delta T cells, as a percentage of the CD3 population, was higher in HIV infected patients than healthy controls (p=0.0052).

B: The non VD2 subset also had a higher frequency in HIV infected patients than controls (p=0.0005).

C: In contrast, the VD2 subset had a lower frequency in HIV+TB+ patients compared to healthy controls (p=0.0318).

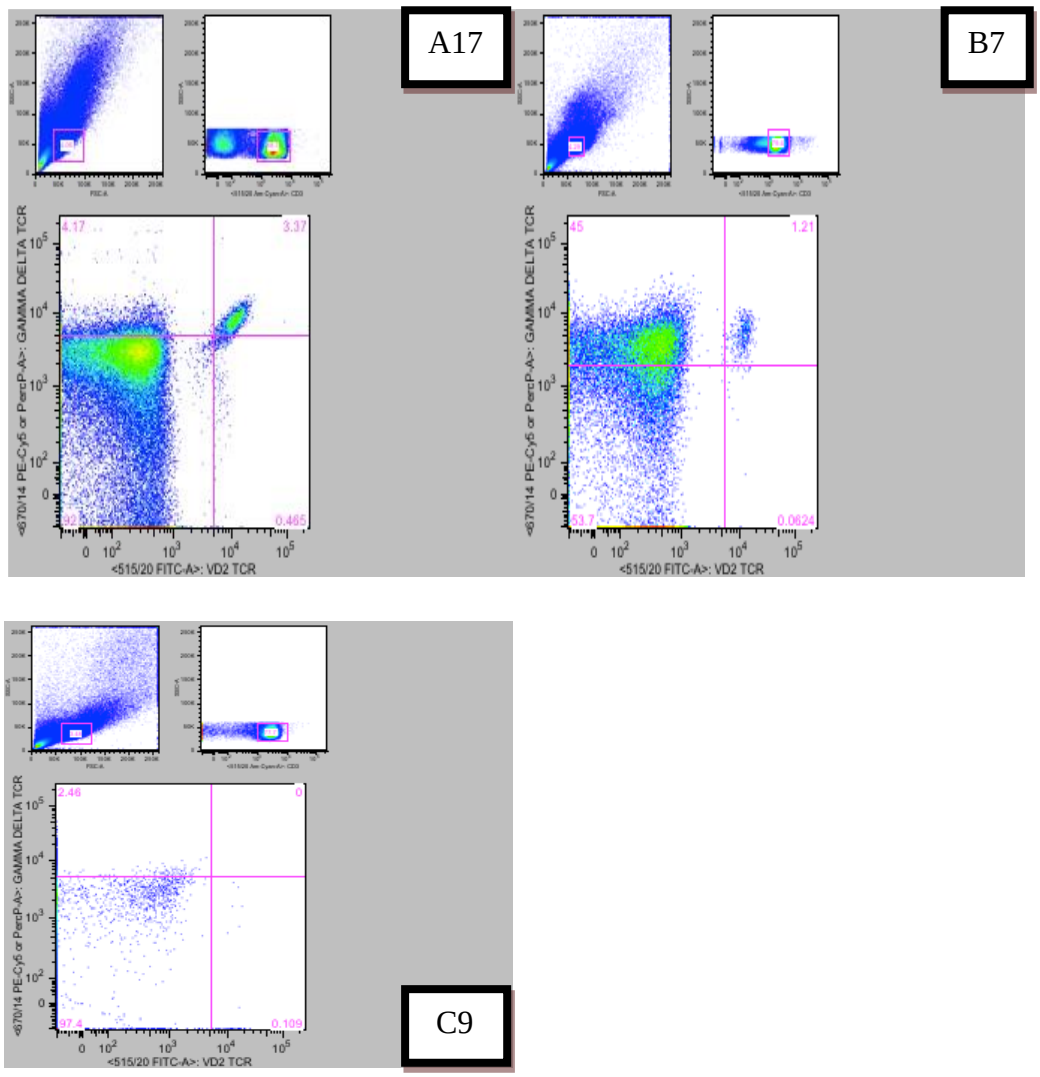


Figure 5: Flow cytometric plots of gamma-delta T cells. The first lymphocytes were gated according to forward and side scatter. Then CD3 positive cells were gated. Then the gamma delta TCR was plotted on the y-axis and VD2 TCR is plotted on the x–axis. The frequency of total gamma-delta T cells was calculated by adding the top two quadrants. The VD2 TCR subset was evaluated by adding the top and bottom right hand quadrants.

A17=healthy control patient. B7=HIV+ patient. C9=HIV+TB+ patient. The VD2 subset appears absent in this HIV+TB+ patient.

3.2. Cytotoxic T cells and CD4 T helper cells

The frequency of cytotoxic T cells ($CD3^+$, CD8 $Alpha^+$) as a percentage of $CD3^+$ T cells was significantly increased in HIV+TB+ patients compared to healthy controls (p=0.0009, median =73.4% compared to 40%) (Figure 6). The HIV+ group (median 59.3%) also had higher cytotoxic T cell frequency than the control group (median 40%), not reaching statistical significance. This is likely due to the lower CD4 frequency, as a percentage of $CD3^+$ T lymphocytes, known to occur in HIV.

T helper cell ($CD3^+CD4^+$) frequency was lower in HIV infected and HIV+TB+ patients compared to healthy controls (p=0.0409, medians=35% for HIV+ patients, 30% for HIV+TB+ patients and 53,8% for healthy controls).

The absolute CD4 count was negatively correlated with the $CD8^+$T cell frequency for both HIV infected (p=0.009 and Spearman rho=-0.062) and HIV+TB+ patients (p<0.001 and Spearman rho=-0.814) (figure 8).

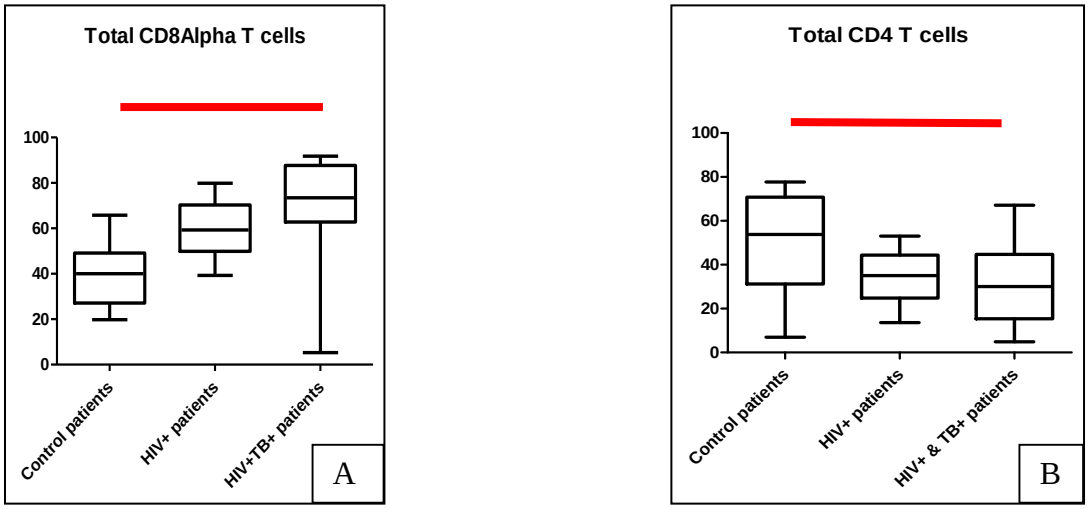


Figure 6: Cytotoxic T cells ($CD3^+$, CD8 $Alpha^+$), as a percentage of $CD3^+$ T cells, had a higher frequency in HIV+TB+ patients compared to controls (p=0.0009). Helper T cells ($CD3^+$, $CD4^+$), as a percentage of $CD3^+$ T cells, were lower in HIV+TB+ patients compared to healthy controls (p=0.0409).

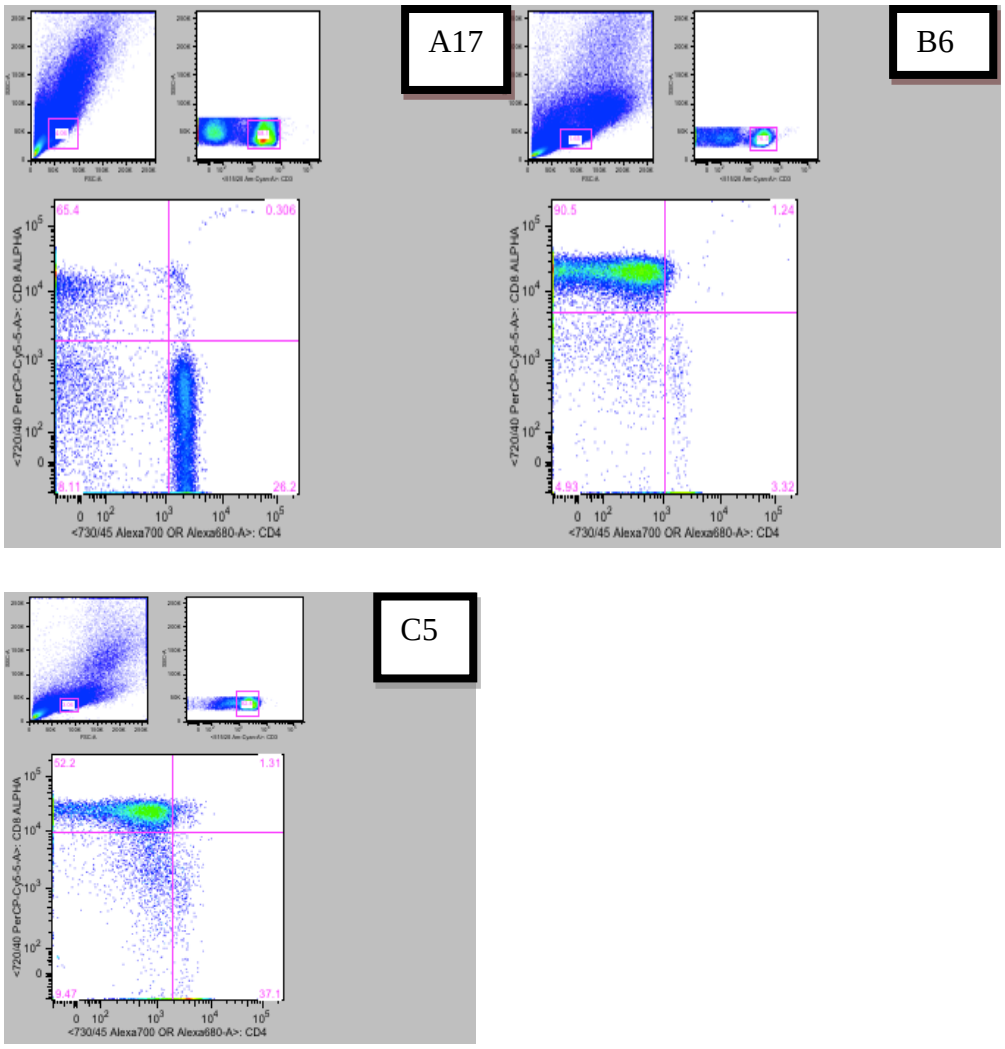
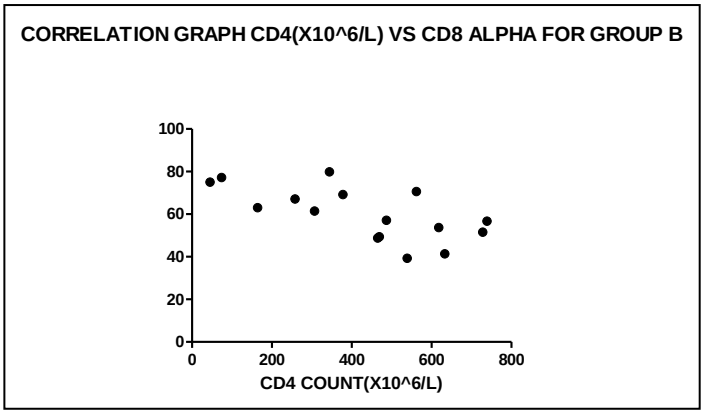


Figure 7: Flow cytometric plots of helper ($CD3^+CD4^+$) and cytotoxic ($CD3^+$ $CD8^+$) T cells. CD4 is plotted on the X axis and the CD8 Alpha is plotted on the Y axis of the largest plots. Total cytotoxic T cell frequency was calculated by adding the top right and left quadrants. Total CD4 T cell frequency was calculated by adding the top and bottom right quadrants.

A17=healthy control, B6=HIV+ patient, C5=HIV+TB+ patient.

Cytotoxic T cells ($CD3^+$ $CD8^+$) as a percentage of CD3 are increased in HIV+ and HIV+TB+ patients compared to healthy controls. T helper cells ($CD3^+$, $CD4^+$) are markedly reduced in HIV+ and HIV+TB+ patients compared to healthy controls.



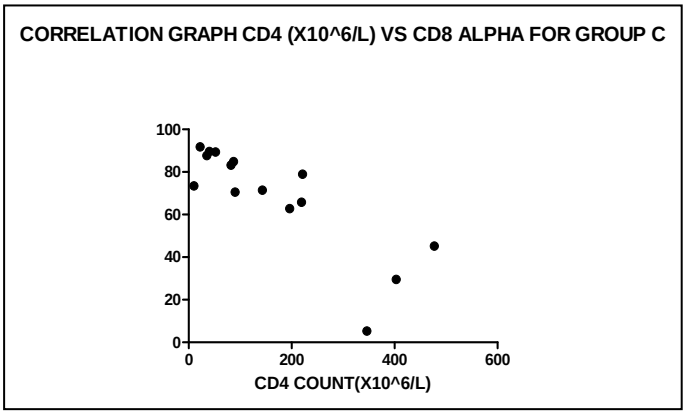


Figure 8: These graphs show inverse correlations between CD4 count on the X axis and CD8 Alpha, as a percentage of CD3 T cells, on the Y axis, for group B (rho=-0.063, p=0.009) and group C (rho=-0.814, p<0.001).

4. DISCUSSION

Gamma-delta T cells are a unique population of immune cells which have been a subject of recent interest due to their distinct property of combining conventional adaptive functions with rapid innate immune responses (70). Their diverse scope of antigen recognition has placed them at the forefront of research into prevalent viral and bacterial infections worldwide (70). In addition, gamma-delta T cells have the potential to become reagents in immunotherapies and vaccines for cancers, autoimmune disease and infections such as HIV and Tuberculosis (88-91). There is, however, a paucity of data on gamma-delta T cell frequency in HIV infections and human Tuberculosis in Africa, and no data from the South African population.

Gamma-delta T cells in HIV infected Tuberculosis patients was enumerated. A multi-colour flow cytometric panel which allows the inclusion of many markers simultaneously was used. This enabled multiplexing the markers CD3, gamma-delta TCR (expressed by all gamma-delta T cells) and VD2 TCR (a subset of gamma-delta T cells expressing the VD2 variable gene in their T cell receptor). Markers of conventional T helper cells (CD4), cytotoxic T cells (CD8 Alpha) and B cells (CD19) were included. Additionally markers of other innate-like lymphocytes (NKT cells)

were included (CD16, CD56, CD8Beta and CD6B11). Analysis of the NKT cell subset will not form part of this dissertation.

A healthy control group and an HIV infected patient group with and without active Tuberculosis was enrolled. The Tuberculosis group comprised HIV positive patients that were smear positive for acid fast bacilli in sputum. The HIV group was screened for Tuberculosis symptoms by a questionnaire and directed investigations if necessary. The control group comprised health care workers from the Charlotte Maxeke Johannesburg Hospital. Testing for latent Tuberculosis was not performed due to ethical, logistical and financial constraints in offering isoniazid treatment to participants found to be harboring latent Tuberculosis. Most health care workers and HIV infected patients in our setting are expected to have been previously exposed to Tuberculosis (92, 93). There was no statistical difference in age or gender between groups in our cohort.

The frequency of total gamma-delta T cells, expressed as a percentage of total T cells, were significantly increased in HIV infected patients compared to health care workers. There was a trend to higher gamma-delta T cells in the HIV+ TB+ group compared to healthy controls; however this did not reach statistical significance.

Increased gamma-delta T cells in HIV and dual HIV+ TB+ patients may not reflect an increase in absolute numbers, rather an increase in proportion of CD3+ T cells as a result of the known loss of CD4+ T cells that occurs with HIV infection.

When divided into VD2 and non-VD2 subsets of gamma-delta T cells, the non-VD2 subset showed a similar pattern of increased frequency in HIV patients, and a trend to higher frequencies in HIV+ TB+ patients, in comparison with healthy controls.

Paradoxically, however, the VD2 T cell subset was significantly reduced in the HIV+ TB+ group compared with healthy controls, and showed a non-significant trend to lower levels in the HIV group. The reduced VD2 cells in HIV and in HIV+ TB+ patients, as a frequency of CD3+ T cells, mirrors the loss of CD4+ T cells in these patients and likely reflects a loss in absolute numbers of these cells.

There is thus a skewing in the gamma-delta T cell repertoire in HIV infected patients with a decrease in the VD2 subset in proportion to the non-VD2 subset. This decrease in the VD2 subset was particularly marked in HIV infected patients with active Tuberculosis. The non-VD2 subset appears elevated in frequency but this may be a reflection of lower CD4+ and VD2 T cells in the total CD3+ population.

Gamma-delta T cells are of interest in Tuberculosis as they react to phospho-antigens found in the lipid wall of the mycobacterium (44-47). Gamma-delta T cells are non-MHC restricted, unlike the more common alpha-beta T cells. They express the T cell receptor in a limited repertoire (45, 46, 94, 95).

Recent evidence suggests that gamma-delta T cells are essential immune regulators of several viruses. Vaccinia virus infected mice have increased numbers of IFN-gamma secreting splenic gamma-delta T cells after 2 days of infection corresponding with an innate immune response (96). Mice infected with West Nile Virus show reduced numbers of gamma-delta T cells to primary and secondary infection and it is suggested that gamma-delta T cells form memory T cells in this setting (97). Gamma-delta T cells are also expanded in CMV infections in humans (98, 99).

Regarding the patient groups in our cohort, there was a statistically significant difference noted in the proportion of patients receiving HAART therapy. More HIV+TB+ patients (group C) were not on HAART therapy compared to group B patients. Several studies have found that HAART therapy increases the VD2 T cell subset in HIV+ patients (95, 100).It is difficult to assess whether the differences in the VD2 T cell subset noted in our group is associated with active Tuberculosis or with longer HAART duration and higher CD4 counts.

The high proportion of patients with previous TB in group B (16 patients of 17 with known previous TB) illustrates high burden of disease in our setting and highlights the logistical difficulty in obtaining cohorts with only HIV infection and no history of Tuberculosis disease. It was logistically difficult to find a Tuberculosis only (HIV negative) group at our tertiary care centre. Thus, the relative contribution of HIV disease and TB disease to the skewed gamma-delta T cell repertoire is difficult to determine and would require further studies. Decreased frequency of VD2

T cells compared with healthy controls may be associated either with HIV or previous TB infection in group B

The loss of VD2 TCR that occurs with HIV infection is now well established (91). The findings of a reduction in the VD2 TCR subset in HIV+ patients with active Tuberculosis is supported by a similar study which recruited HIV infected patients with opportunistic infections including Tuberculosis, candidiasis, hepatitis C, herpes zoster and salmonella (81).The VD2 subset was reduced in HIV+ patients with opportunistic infections compared to asymptomatic HIV infected patients. In addition, this study performed stimulation testing and found a reduction in the production of IFN-gamma from VD2 TCR T cells in HIV+ patients. This data suggests that a decreased frequency and function of VD2 T cells may be associated with the presence of opportunistic infections (81).

Most of the patients in our cohort had lower than detectable viral loads. The VD2 TCR subset of gamma delta T cells correlated directly with CD4 T cell count and inversely with viral loads in a Chinese cohort (101).The VD2 TCR response is preferentially depleted during HIV infection (102).The depletion of the VD2 TCR subset occurs early during the disease with an ongoing depletion noted during progression of HIV (103). Potential immunotherapeutic strategies for increasing the VD2 TCR response in HIV disease are being explored (104).

With regards to CD4+ T cell and CD8+ T cell frequencies, it was found that healthy controls had the highest frequency of CD4+ T cells and the HIV+TB+ co-infected group had the lowest frequency. HIV+TB+ patients had more CD8+ T cells as a percentage of CD3+ T cells compared to HIV+ patients (not significant) and to healthy controls (significant). The high CD8 frequency, when expressed as a percentage of CD3+ T cells, is likely due to the HIV-associated loss in absolute numbers of CD4 T cells. Supporting this interpretation, the CD4 frequency of both HIV+ and HIV+TB+ patients was inversely proportional to CD8+ T cells. These findings are in keeping with the known decline in CD4+ T cells during HIV progression. Multiple mechanisms for CD4 T cell decline have been described. These include cytopathic effect of the virus and death of uninfected cells (24, 105).

Similarly, the immune responses of peptide-specific CD4+ and CD8+ T cells and non-peptide specific Vgamma2Vdelta2 T cells during clinical quiescence of latent mycobacterium tuberculosis infection in HIV infected humans has been analysed (106). The study was performed in China using 4 groups of patients as follows: HIV+ with active Tuberculosis, HIV+ with latent Tuberculosis, HIV+ patients with no Tuberculosis and a healthy control group with no HIV infection. Active Tuberculosis patients were identified by clinical symptoms, abnormal chest X-rays, tuberculosis culture positive and smear-positive acid fast bacilli in sputum cultures. The patients with latent Tuberculosis were identified using a T-SPOTTB assay. Absolute cell numbers were calculated based on flow cytometric data and complete blood counts. The study found that the frequency and absolute numbers of CD4+ T cells in all 3 HIV infected groups were significantly lower than the healthy controls, as expected. The HIV+ patients with active Tuberculosis had lower CD4+T cell counts than the HIV+ patients with latent Tuberculosis. Mean absolute numbers of CD8+ T cells in the HIV+ patients with latent Tuberculosis were significantly higher than those in the HIV+ patients with active Tuberculosis. Both percentage and absolute numbers of gamma-delta T cells and VD2 T cells in the HIV+ patients with active Tuberculosis were also significantly lower than those in HIV+ patients with latent Tuberculosis (106).

We did not include analysis of cell function in our study. However, in studies of antigen specific T cell responses was found that potent immune responses of HMBPP-specific gamma-delta T cells and VD2 T cells occurred. PPD-specific CD8+ T cells were associated with the latent stage of Tuberculosis. It was concluded that gamma-delta and CD8+T cells are anti-Tuberculosis effector T cells in HIV infected patients with low CD4 T cell counts (106). Another study found that the IFN-gamma VD2 T cells in HIV+ Latent Tuberculosis patients were much greater than those in the HIV+ active Tuberculosis group (106).

HIV+ patients with no concomitant Tuberculosis have been studied using flow cytometry to analyze the function of VD2 T cells. Samples were stimulated with mycobacterium tuberculosis. VD2 T cells were found to be reduced after stimulation (106). It has been suggested that the continuous antigenic stimulation that occurs with HIV infection may be the reason for the reduction of VD2 T cell frequency in HIV progression (94).

Another activation study showed similar results. PBMC's were stimulated for 7 days with microbial antigens which included candida albicans, salmonella typhimurium as well as mycobacterium tuberculosis. The VD1 and VD2 T cells were quantified as a percentage of CD3+ T cells. The VD2 T cells were increased in response to stimulation with microbial antigens *ex vivo* (107).

Bordon et al used spectratyping to determine the size distribution of VD2 T cell transcripts in peripheral blood mononuclear cells in HIV positive subjects and showed that the decreased VD2 T cell frequency in HIV+ patients seems to recover with the initiation and duration of HAART (100). Other studies have supported this concept showing that interruption of HAART, leads to a reduction in total gamma-delta T cells (95). Poccia et al showed that HIV positive patients with opportunistic infections including candidiasis, Tuberculosis, herpes zoster and salmonella had markedly increased gamma-delta T cell subsets as a percentage of CD3 after only 3 months of HAART (95).

Other studies have shown a reduction of gamma-delta T cells in the peripheral blood of patients with active Tuberculosis and have hypothesized that this may be a reflection of a migration of these gamma-delta T cells to the site of infection in the lung epithelia (44, 49, 108). Increased numbers of gamma-delta intraepithelial lymphocytes (IEL) were noted in most HIV infected patients suggesting that they may contribute to the first line of defense against infectious organisms at the site of infection (109). Pleural effusion fluid contained gamma-delta T cells (49). Gamma-delta T cell subsets taken from broncho-alveolar lavages from active Tuberculosis patients displayed a functional anergy (not able to produce cytokines) compared to peripheral gamma delta T cells (76).

A major strength of this study was that it is one of the first studies to analyze gamma-delta T cells in black South African patients. A prospective study design which allowed us to screen and enroll candidates for the study using appropriate exclusion and inclusion criteria was chosen. All patients and controls were screened and enrolled and samples were sent for CD4 counting on the same day as enrollment. Flow cytometry was performed on fresh blood rather than cryo-preserved material and therefore is more likely representative of *in vivo* cell frequencies. All samples were processed

within 4 hours of blood drawn. The controls consisted of a healthy health care worker group as well as an HIV infected control group.

Multi-colour flow cytometry also has the advantage of being able to analyze a lot of information about different cell types in the body in a single sample using a small amount of blood. A stringent daily quality control of the flow cytometer was ensured by daily running of 1 x beads (87).

Limitations of this study included the absence of a Tuberculosis positive but HIV negative control group which was difficult to obtain in our setting where HIV infection is prevalent. Not all of the healthy controls gave consent for HIV testing and counseling. Only thirteen out of the seventeen healthy controls agreed to HIV testing. One out of these thirteen that consented was found to be HIV positive and was consequently excluded from the study. The others were all found to be HIV negative.

Gamma-delta T cell frequency alone was analyzed. The functional properties such as cell proliferation and cytokine production were not analyzed. This would have entailed additional stimulation time per sample and further funding which was not available.

A disadvantage of multi-colour flow cytometry is the complex compensation issues that can occur when using multiple fluorochromes. The compensation difficulties experienced was complex and required panel adjustments following the FMO experiments. Even with the new panel, the compensation was not perfect in the groups after manual adjustment of the compensation matrix. The compensation issues in the HIV+TB+ group were particularly evident. This may have also been due to increased incidence of non-specific binding of monoclonal antibodies (personal communication Prof Debbie Glencross) which occurs in dual infected HIV+ TB+ patients. The best compensation set attempted was used. During the flow cytometric analysis, height and width for forward and side scatter was not acquired. This would have been beneficial to exclude debris to allow easier manual compensation. The cells were spun at 3000 RCF which is routine in the laboratory. This speed may have been too high for HIV and TB co-infected samples and contributory to suboptimal cell yields. Centrifugation speeds were not optimized specifically for HIV/TB co-infected samples.

5. CONCLUSION:

Gamma-delta T cells in HIV infected Tuberculosis patients were enumerated. The VD2 subset expressed as a frequency of total T cells was significantly reduced in the HIV+ TB+ group compared with healthy controls, and showed a non-significant trend to lower levels in the HIV group. Therefore, a skewing of the gamma-delta T cell repertoire in the dual HIV and TB infected patients with a decrease in the VD2 subset occurred. This mechanism of alteration in gamma-delta T cell frequency, in particular the loss of VD2 T cells, deserves attention in elucidating susceptibility to Tuberculosis disease in HIV infected patients.

The frequency of total gamma-delta T cells, expressed as a percentage of total T cells, was significantly increased in HIV infected patients compared to health care workers. This may, however, be a reflection of lower CD4 T cells and VD2 gamma delta T cells in the total CD3 positive population.

6. RECOMMENDATIONS:

Further studies which would be of value are the assessment of the VD2 T cell subset of gamma-delta T cells in the pleural fluid of active Tuberculosis patients for comparison with peripheral blood frequencies in a South African setting. This would enable a better understanding of the function of these cells at the site of infection. Studies including activation markers for gamma-delta T cells such as IFN-gamma and interleukins should also be included in future studies for assessment of gamma-delta T cell function.

Due to the complex nature of multicolour flow cytometric compensation and poor sample quality of HIV positive samples, including paucity of lymphocytes and non-specific binding of monoclonal antibodies which occurs with HIV infection, it is recommended that confirmatory studies using 5-7colour flow cytometric panels with carefully selected markers and fluorophores performed to potentially avoid compensation issues.

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APPENDIX

1. Plagiarism declaration
2. Statement of principles for postgraduate supervision
3. Control and patient consent forms
4. Patient data sheet
5. Control data sheet
6. Ethics forms:

    6.1. Clearance certificate

    6.2. Letter to human research ethics committee requesting amendment

    6.3. Approval for amendment from ethics committee

    6.4. Approval by Charlotte Maxeke Johannesburg Academic Hospital for conduction of research

7. Master of medicine grant award
8. Patient demographic spreadsheet
9. Example of titrations-CD8 Beta titration analysis including flow cytometric plots and graphs.