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DIAGNOSIS OF ACTIVE TUBERCULOSIS USING FLOW CYTOMETRY IN HIV POSITIVE INDIVIDUALS IN A TUBERCULOSIS ENDEMIC SETTING

Suvarna Buldeo

A research report submitted to the Faculty of Health Sciences, University of Witwatersrand, Johannesburg, in partial requirements for the degree of Master of Medicine in the branch of Clinical Pathology

Johannesburg, 2012

I, Dr Suvarna Buldeo declare that this research report is my own work. I enrolled the patients, collected the samples and performed the assay as well as the flow cytometric and statistical analysis. It is being submitted for the degree of Master in Medicine in the branch of Clinical Pathology in the University of Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.



DR S BULDEO

3rd day of April, 2012.

This research protocol was approved by the Post Graduate Committee, University of the Witwatersrand

To my sister-in-law

Dr Nerissa Pather, who

due to the absence of the 'ideal' TB diagnostic assay,

capable of diagnosing smear negative pulmonary and extra-pulmonary TB,

endured its most severe complications.

Thank you for revealing that the Human Spirit

Can triumph over All,

Including the "Great Killer"

Tuberculosis.

To my husband, Alvin

and my parents, Anita and Satish.

Thank you for your ongoing support and encouragement.

PUBLICATIONS AND PRESENTATIONS

- Optimisation of a flow cytometry assay for the diagnosis of active tuberculosis (TB) in HIV infected individuals in an endemic setting

  Buldeo SB, Murdoch DM, Suchard MS and Stevens, WS
  Presented as a poster at PathVine Conference, Stellenbosch, September 2010

- Pulmonary immune-compartment specific interferon gamma responses in HIV infected individuals with active tuberculosis (TB) in an area of high TB prevalence

  Buldeo S, Murdoch DM and Suchard MS

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ABSTRACT

The diagnosis of tuberculosis (TB) in Human Immunodeficiency Virus (HIV) infected individuals is a challenge due to atypical presentations including negative sputum smear and culture results, which renders traditional microbiological tests inadequate in this population. Immune based assays are an attractive alternative, but current commercial blood interferon gamma (IFNγ) release assays have limited utility in areas of high *Mycobacterium tuberculosis (M. tuberculosis)* prevalence. Immune responses in the lung differ from those in peripheral blood, hence a better understanding of lung compartment-specific immune response to TB may assist in developing new diagnostic assays.We evaluated the IFNγ production from induced sputum (ISp) and blood samples of 31 HIV positive, smear-negative TB suspects who were being investigated for active TB. We analysed the IFNγ responses of total lymphocytes in ISp samples; and total, CD4+ T lymphocytes and memory CD27- CD4+ T lymphocytes in blood. IFNγ was quantitated after stimulation with two *M. tuberculosis* antigens, PPD (purified peptide derivative) and ESAT6 (early secretory antigen 6). Eleven sputum samples were excluded due to poor sample quality.We observed that IFNγ secretion from total lymphocytes in sputum was, significantly higher than blood after stimulation with either ESAT6 (0.64% vs 0.10%, p = 0.03) or PPD (2.04% vs 0.21%, p = 006), and the PPD specific IFNγ secreting lymphocytes in sputum could be used to differentiate between the *M. tuberculosis* negative and positive groups. None of the lymphocyte populations analysed in blood differentiated these two *M. tuberculosis* groups. The counterintuitive finding is that, median PPD specific IFNγ secreting lymphocytes in sputum were significantly *lower* (p = 0.04) in the *M. tuberculosis* positive (0.23%) cohort when compared to the *M. tuberculosis* negative group (2.04%). The sensitivity and specificity of this finding to discriminate active and latent TB at a diagnostic threshold of 1.25% PPD-specific IFNγ secreting total sputum

lymphocytes is 78% and 70% respectively. The routine diagnostic utility of measuring T lymphocyte immune responses in sputum by flow cytometry however is not practical due to limitations imposed by the required cellular content and viability, as well as the need to process these samples within two hours of collection. These results however, are valuable and add insight into the lung compartment specific immune response to *M. tuberculosis* antigens.

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ABBREVIATIONS

AFB	-	Acid Fast Bacilli
AIDS	-	Acquired Immune Deficiency syndrome
APC	-	Allophycocyanin
BAL	-	Broncho-alveolar lavage
BCG	-	Bacille-Calmette Guerin
CMJAH	-	Charlotte Maxeke Johannesburg Academic Hospital
CR	-	Complement Receptor
CXR	-	Chest X-ray
EDTA	-	Ethylenediaminetetraacetic acid
ESAT6	-	Early Secretory Antigen 6
FCS	-	Foetal calf serum
FITC	-	Fluorescein isothiocyanate
FMO	-	Fluorescence Minus One
FSC	-	Forward scatter
HJH	-	Helen Joseph Hospital
HIV	-	Human Immunodeficiency Virus
IFNγ	-	Interferon Gamma
IGRA	-	Interferon Gamma Release Assays
IRIS	-	Immune reconstitution inflammatory syndrome
ISp	-	Induced Sputum
LTBI	-	Latent tuberculosis infection
LPA	-	Line probe assay
M. tuberculosis	-	*Mycobacterium tuberculosis*
MFI	-	Mean Fluorescence Intensity
MOTTs	-	Mycobacteria other than tuberculosis
MR	-	Mannose Receptor
NKT	-	Natural Killer T
PBMC	-	Peripheral Blood Mononuclear cells
PBS	-	Phosphate Buffered Saline
PCR	-	Polymerase Chain Reaction

PE	-	Phycoerythrin
PerCP	-	Peridinin chlorophyll protein
PPD	-	Purified Peptide Derivative
RD1	-	Region of Difference
R10	-	Tissue culture medium plus antibiotics and FCS
SEB	-	Staphylococcal Enterotoxin B
TB	-	Tuberculosis
Th	-	T helper
TLR	-	Toll Like Receptor
TNFα	-	Tumour Necrosis Factor alpha
TST	-	Tuberculin Skin Test

CHAPTER ONE

1.0 Background

1.1 Introduction

M. tuberculosis causes about two to three million deaths annually and globally has the highest mortality for any single infectious agent (1). South Africa is classified as a high TB and Human Immunodeficiency virus (HIV) burden country. According to the World Health Organisation TB database (54) the South African TB prevalence in 2010 was 400 000, with a rate of 795 per 100 000 population. The prevalence of TB has doubled over the last decade. The increased burden of the disease in South Africa has been attributed to overcrowding, poor ventilation and malnutrition but currently, as shown in Table 1.1, is fuelled by the prevailing HIV epidemic.This increase in prevalence is likely due to an increased risk of infection, reactivation of latent TB or the rapid progression to active TB in *M. tuberculosis* and HIV co-infected individuals (2).

Table 1.1 WHO 2010 TB data for South Africa (54) shows the high percentage of HIV infection in patients with TB.

TB patients with known HIV status	213 006
% of TB patients with known HIV status	54
TB patients that are HIV-positiv	128 457
% of tested TB patients that are HIV-positive	60

TB remains one of the great killers despite the availability of adequate chemotherapeutic agents and a vaccine. The lack of diagnostic assays with high sensitivity and specificity is partly responsible for the inadequate control of the disease (3). Additionally, culture of the organism which is the gold standard for diagnosing TB requires 7- 42 days to detect bacterial growth (4), resulting in delayed diagnosis and further spread of TB. Furthermore, the diagnosis of *M. tuberculosis* infection in HIV co-infected individuals is challenging as patients present atypically, often having less cavitatory disease and more extra-pulmonary disease. Routine diagnostic approaches such as sputum smears for acid fast bacilli (AFB) and typical clinical and radiological features are therefore not appropriate in this group (5). Importantly these undiagnosed patients who often are AFB sputum negative (6) are capable of transmitting mycobacteria despite its lack of detection in the sputum (7) and therefore serve as a point source for further spread of the disease (8).

In 2005, the World Health Organisation declared the increasing global epidemic of TB an emergency requiring that urgent and extraordinary actions be undertaken. This increased the awareness that new prevention, diagnostic and treatment strategies were needed to control this epidemic in the era of HIV. A quick and accurate diagnostic tool for *M. tuberculosis* could save a quarter of the approximately two million lives lost to TB each year (3). For the reasons stated above, a diagnostic test for active TB that can be applicable in a high TB burden area and that does not rely on the direct detection of AFB in sputa is urgently required in HIV and *M. tuberculosis* co-infected individuals. Measurement of *M. tuberculosis* specific immune responses is a good candidate for this diagnostic approach. Elucidation of the immune response to *M. tubeculosis* lead to the discovery that *M. tuberculosis* specific IFNγ response could be used as a biomarker of the disease. The

immune response to *M. tuberculosis,* as well as the principle and limitations of commercially available IFNγ based assays are discussed in detail below.

Flow cytometry offers a novel approach to measure *M. tuberculosis* specific IFNγ secretion. A previous publication (9) suggested that the use of a simple flow cytometric assay performed on ISp may differentiate active from latent TB. This promising finding was, however, from an area of low TB prevalence. We chose to apply this methodology to a group of HIV infected patients in a high TB prevalence setting. The aims of this study were to: elucidate the pulmonary-specific T lymphocyte immune responses to *M. tuberculosis* by comparing the IFNγ responses in blood and sputum; and to evaluate the potential application of this assay to diagnose active TB in this group of patients who are most in need of improved diagnostics, that is, individuals with two negative smears for acid fast bacilli but in whom clinical suspicion of active TB persists.

1.2.0 TB diagnostic assays

1.2.1 Historical background

The high burden of TB in South Africa can be likened to that of England a century ago. England's triumph in controlling the disease can be attributed to an improvement in socio-economic factors but also to the active TB research that occurred during that period. At the end of the nineteenth century, a microbiologist named Robert Koch identified *M. tuberculosis* as the causative agent in TB. A few years later, he attempted to develop a therapy using a sterile filtrate from *in vitro* cultures, but this induced a very strong inflammatory response that caused a severe necrotic reaction, called Koch's phenomenon, in patients with active TB. This lead to the discovery of the tuberculin skin test (TST) which is in use to date, almost 100 years later (10), and despite its numerous limitations. This highlights the lack of research interest in this field over the last century. The recent establishment of the global Stop TB Strategy however, has created renewed awareness and interest in the development of new *M. tuberculosis* diagnostic assays (11).

1.2.2 Current active TB diagnostic assays and their limitations in HIV and TB co-infection

The diagnosis of active TB is currently based on clinical signs and symptoms, sputum smear microscopy results, x-ray changes and culture. More recently molecular amplification techniques such as the Polymerase Chain Reaction (PCR) with improved extraction methodologies to improve sensitivity have become available. Despite further recent advances to these technologies, their sensitivity and specificity largely remains suboptimal in the setting of HIV, (Table 1.2) (5) (4). The recent introduction of the Xpert MTB/RIF (GeneXpert, Cepheid), which employs a molecular amplification method, has created excitement in South Africa, as even with an HIV prevalence of 60-80% in TB

suspects, the sensitivity of GeneXpert for smear positive patients has been reported as greater than 90% (correctly detected 551 of 561 patients) and for sputum smear negative patients as 72% (correctly detected 124 of 171 patients) (12). However, while this represents an improvement over existing methods, this does not solve all the diagnostic dilemmas in culture negative or even GeneXpert negative, HIV infected patients.

Table 1.2 Impact of HIV on TB Diagnosis.

TECHNIQUE and its LIMITATIONS	ADVANCES	IMPACT of HIV
Clinical presentation and radiology: not specific as bacterial pneumonia and non-infective diseases can produce similar features.	-	Atypical presentation : Lack of a persistent cough or night sweats; more extra-pulmonary disease; less cavitatory disease on chest x-ray (CXR)
Smear Microscopy: requires skill, labour intensive and direct detection of organism needed, hence sputum cannot be used to diagnose extra-pulmonary disease.	Use of fluorescent microscopy to increase sensitivity	Paucibacillary and extra-pulmonary disease more common.
Culture: despite being the gold standard, often leads to delay in diagnosis owing to prolonged time required for growth detection.	Liquid culture systems that are more sensitive and decrease growth detection time to 7 days.	Paucibacillary disease and higher contamination rates, which lead to further delays in diagnosis and invalid results.
PCR : *M.tuberculosis* needs to be present in sample and extra-pulmonary infection cannot be detected from sputum samples.	Line probe (LPA) and real time assays that simultaneously detect infection with *M. tuberculosis* and regions of drug resistance. Examples are: Genotype MTBDR, Hain Lifesciences, Germany and Xpert MTB/RIF (GeneXpert, Cepheid), respectively.	The GeneXpert has a sensitivity of almost 100% in smear positive samples but is decreased (66-72%) in smear negative cases, which are more prevalent in HIV and TB co-infection.

Adapted from references (3) and (27).

1.2.3 Diagnosis of latent TB infection and the IGRA

Latent tuberculosis infection (LTBI) is defined as the absence of clinical signs or symptoms of active TB in an individual who has a history of exposure to *M. tuberculosis* and a corresponding positive TST (13). It remains critical to differentiate active from latent TB, since a diagnosis of TB commits an individual to a 6 month multi-drug anti-tuberculosis regimen. Furthermore this places the patient at risk of unwarranted toxicity and drug resistance (14). The commercial assays available to diagnose latent TB include the TST and IGRAs.

The TST, which is an *in vivo* test, involves the injection of a Tuberculin antigen intradermally and the presence of a wheal and its diameter is read 48 – 72 hours after injection. The TST has been the standard diagnostic test for LTBI, despite having a lower sensitivity in HIV positive patients and cross-reacting with components of the Bacille Calmette-Guerin (BCG) vaccine, as well as with Mycobacteria other than tuberculosis (MOTTs) (25). Blood based immune-mediated assays that detect IFNγ production have recently been developed to detect *M. tuberculosis* infection. These make use of the Region of difference (RD1) antigens found almost exclusively in *M. tuberculosis* but not in other mycobacteria or the BCG vaccine (15). This eliminates the cross-reactivity from BCG vaccination and most MOTTs, and is an improvement over the TST assay. The other advantages of IGRA assays over the TST is that a single patient visit is required; the turnaround time is halved; there are no boosting effects on the patients immune system; the results are more reproducible and objective, and this assay is run in the presence of controls (16).

The blood based immune ex-vivo assays include the enzyme-linked immunosorbent assay known as Quantiferon-TB Gold in-Tube (Pro-Gen Diagnostics, South Africa), which is a whole blood tube method and the T.Spot TB test (Oxford Immunotech, Calicom trading, South Africa), a histochemical blot method. The RD1 antigens used include early secretory antigen 6 (ESAT6) and culture filtrate protein 10 (CFP 10). Despite their increased specificity, their value in advanced HIV infection remains controversial, requiring further evaluation studies. Studies have demonstrated that the immunological response produced by these antigens are weak in HIV positive individuals who progress to the Acquired Immunodeficiency Syndrome (AIDS) (17) .

Most studies show that although the commercial IGRAs are unable to differentiate between active and latent TB, they are capable of differentiating individuals exposed to TB from those that have never been exposed. Hence, they have gained favour in settings of low TB prevalence for detecting patients with latent TB who can be treated with anti-tuberculosis eradication therapy in these areas. However, use of these assays in routine TB diagnosis in highly TB endemic areas remains unlikely (18). In South Africa, Isoniazid (INH), an anti-tuberculosis drug is recommended as monotherapy to prevent reactivation of latent *M. tuberculosis* infection in some immunosuppressed groups, including those that are HIV infected. Multi-drug eradication therapy is not recommended for latent TB infection in high burden countries. Hence we require diagnostic tools that can differentiate active from latent TB, not merely exposure to *M. tuberculosis* from non-exposure. Thus, a better understanding of the immune responses to *M. tuberculosis* is required to develop an immune based test to distinguish active from latent infection.

1.3 Transmission and clinical spectrum of TB in HIVuninfected and infected individuals

The discovery of new immunodiagnostic tools such as the interferon gamma release assays (IGRA) have improved the understanding of the immuno-pathogenesis of human TB. New evidence suggests that fifty percent of HIV uninfected individuals exposed to *M. tuberculosis* do not develop positive immuno-diagnostic tests, meaning that the adaptive immune system was not activated as these tests measure antigen specific memory responses (19). This implies that the innate immune system of these individuals is capable of sterilizing immunity, that is the complete eradication of the bacteria (20)**.** Of the remaining fifty percent, that is patients with latent TB as evidenced by positive immunodiagnostic tests, only five percent progress to active disease (19).

The transmission of TB in HIV infected individuals is similar to that in HIV negative cases but the progression of the disease is altered, which results in an increase in morbidity and mortality in co-infected individuals (2). HIV increases susceptibility to *M. tuberculosis* infection, reactivation and re-infection as well as accelerates the progression from latent to active disease (2). The spread of *M. tuberculosis* occurs when mycobacteria are aerosolised during coughing and then inhaled by close contacts. The mycobacteria may be contained and therefore remain dormant or latent within macrophages. HIV destroys the cells integral to the control of *M. tuberculosis*, namely the CD4+ T lymphocytes. When the immune control is disrupted, as is in the case of HIV, reactivation of TB occurs (21). *M. tuberculosis* and HIV also act synergistically to compound the insult on the host's immune system (22) as evidenced by the presence of poorly formed granulomas in co-infected individuals. This immune insult results in an increase in mortality. HIV positive patients who commence anti-retroviral therapy are also at risk of developing TB associated immune

reconstitution inflammatory syndrome (IRIS), which is an over-exuberant immunological response to the mycobacterium with deleterious consequences (4) (23).

1.4 Innate and adaptive immunological responses to *M.tuberculosis*

M. tuberculosis is an aerobic obligatory intracellular pathogen which enters the body via the respiratory route and has a predilection for the lung due to its rich oxygen supply (19).

The innate mechanisms initiated to control the bacilli include plasma lysozymes, lysis by natural killer cells (lysis of pathogen directly or infected phagocytes) and phagocytosis. The CD1d restricted Natural Killer T (NKT) cells that are capable of recognizing lipid antigen also contribute to the protection against *M. Tuberculosis* (24). The *M. tuberculosis* bacteria bind to complement receptors (CR1, CR2, CR3 and CR4), mannose receptors (MR), Toll-like receptors (TLR2 and TLR4) and other cell surface receptors (CD14 receptor, surfactant protein receptors) on monocytes, macrophages and dendritic cells (25). The bacilli may be phagocytosed by these cells resulting in phagosome-lysosome fusion, the release of acidic hydrolases and degradation of the bacilli. Alternate mechanisms of phagocyte degradation of the engulfed bacteria include the generation of reactive oxygen intermediates (hydrogen peroxide), reactive nitrogen intermediates (nitric oxide), production of cytokines including Tumour Necrosis Factor alpha (TNFα) and IFNγ, and programmed cell death (FAS-ligand mediated or TNFα induced apoptosis) (25).

The role of antibodies in protection against mycobacterial infections remains controversial. While antibodies do form against *M. tuberculosis,* they do not lead to the clearance of the organism or immunity to re-infection. Previous explanations for the failure of antibodies to protect against *M. tuberculosis* have been based on the T helper 1/T helper 2 (Th)

paradigm that Th1 cells, orchestrating cellular immunity are important in protecting the body against intracellular pathogens while Th2 responses, with resultant antibody responses, protect against extra-cellular pathogens. The modern view however is that a mixed response rather than a specific Th1 or Th2 response is required to protect from either an intracellular or extracellular pathogen (26). More recent studies have therefore further explored the role of antibodies and one such study has shown that mice that are unable to produce antibodies have a three to eight-fold increase in viable bacilli compared to normal controls (27). B-cell deficient mice also show decreased production of the cytokines, IFNγ and TNFα, which are central to TB control (28). Human vaccine studies also support the role of antibodies in protecting against severe mycobacterial infection, and the mechanism of action of these antibodies is most likely opsonisation which leads to increased phagocytosis of the organism and antigen presentation (26).

The cell-mediated immune response, involving T lymphocytes is recognised as the classical protective response in *M. tuberculosis* infection. Both CD4+ and CD8+ T lymphocytes are important in mediating this response (29). HIV co-infection provides compelling evidence for the role of CD4+ T lymphocytes in protection against *M. tuberculosis* disease. The primary effector function of CD4+ T lymphocytes is IFNγ production (30). The role of CD8+ T lymphocytes has been controversial, but recent work shows that these cells protect the host by virtue of their cytotoxic activity and production of IFNγ; but that the cytotoxic activity requires the help of CD4+ T lymphocytes (31). The CD4+ T lymphocytes however are the predominant producers of IFNγ (32). IFNγ is the major activator of macrophages, enhances antigen presentation and recruitment of CD4+ and CD8+ T lymphocytes to the site of mycobacterial infection (25).

1.5 Measurement of immune responses as diagnostic tools for TB

The advantage of measuring various immune responses is that the organism does not have to be present in the sample, that is, it is an indirect measure of infection. Hence, it can be used to diagnose smear or culture negative disease as well as aid in the diagnosis of extra-pulmonary infection (9). Researchers have reviewed the possibility of using absolute numbers of lymphocytes, granulocytes and natural killer T cells as tools to diagnose active TB (33). Quantitation of antibody responses is a simple immunodiagnostic method, but as discussed earlier, antibodies have largely been disregarded as diagnostic tools for active TB. Current research is focused on identifying antibodies to immunodominant antigens that could be used to diagnose individuals with active TB (34). The measurement of cell-mediated immune responses is widely used, and two assay platforms are currently available to measure *M. tuberculosis* specific IFNγ responses.

1.6.0 IFNγ response in the diagnosis of active TB using flow cytometry and ISp

1.6.1 The advantage of using pulmonary samples

It has recently become apparent that immune responses are not uniform throughout the body, but may differ at different sites, such as blood, gastrointestinal tract, genitourinary tract, lung etc (35). The lung immune response is the major line of defense against *M. tuberculosis* even in extra-pulmonary or disseminated infection, as in most cases the lung is the portal of entry of the bacilli (9). For respiratory infections therefore, assessment of lung immune responses may be more relevant for response to infection than blood immune responses.

Broncho-alveolar lavage (BAL) fluid has been shown to be more sensitive for cytokine based assays than blood since cytokine production in response to *M. tuberculosis* antigens is ten times greater in BAL fluid than blood (36). This may relate to the fact that the lung is the primary site of infection or that two percent of the body's lymphocytes reside in the blood and approximately ten percent are located in the lung (36).

BCG vaccinated individuals show cross reactivity in the blood but not BAL, when these samples are stimulated with PPD, which is known to share epitopes with BCG. Pulmonary samples may therefore have an additional advantage over peripheral blood in areas where BCG vaccination is practiced (9, 36). The other advantage of using respiratory-based specimens, is that, these samples may simultaneously be utilised for microbiological detection (9).

BAL is the sample retrieved after bronchoscopic infusion of saline into the distal broncho-alveolar tree. Obtaining lung fluid this way is invasive, requires a skilled pulmonologist,

expensive equipment and the patient requires anaesthesia (37). ISp, in contrast to BAL, is less invasive and does not require anaesthesia or expensive equipment. ISp refers to the lung fluid expectorated after a patient is nebulised with hypertonic saline for approximately twenty minutes. Sputum is mainly expectorated without assistance, hence requires less skill to perform (8). Many studies have revealed that ISp produces a similar *M. tuberculosis* microbiological yield to BAL (38). A recent paper (39) comparing ISp and BAL for the diagnosis of sarcoidosis found good T-lymphocyte correlation between the two sampling procedures.

1.6.2 The use of IFNγ measurement to detect active disease

Respiratory based samples produce superior cytokine results when compared to blood, function as a distinct immunological compartment, and additionally can be used to differentiate between active and latent TB. Recent work investigating the use of the ELISpot on broncho-alveolar lavage fluid was found to be capable of differentiating active and latent TB infection by comparing the lung specific immune responses to blood in an HIV negative population (36). This group of investigators also used this method in an immunosuppressed female and were able to reproduce this finding (40).

The Breen et al study (9) evaluated the role of ISp in TB diagnosis in a population comprising both HIV positive and negative individuals. This group used PPD as the stimulating mycobacterial antigen and analysed cytokine production by flow cytometry. This study concluded that the cytokine response in ISp was capable of diagnosing active TB infection in HIV positive and negative individuals and this was irrespective of the site of the infection. This method was capable of diagnosing pulmonary as well as extra-pulmonary TB. Problems identified with this study were the false positive results obtained

in three individuals with latent infection. The false positives may be explained by their choice of stimulating antigen, namely PPD which is a very potent antigen (41), or may represent "true positives in waiting", that is, may reflect individuals more likely to reactivate, who therefore require follow up (8) (42). Nonetheless, the suggested ability of ISp to differentiate between active and latent TB warrants further exploration, as flow cytometry is now available in 62 laboratories throughout South Africa for the purpose of CD4 T lymphocyte counting.

1.6.3 Flow cytometry versus the commercially available IGRA

The IGRAs are the first real immunological advance specific to *M. tuberculosis* diagnostics (43). These tests are superior to the TST in detecting patients who have been infected with *M. tuberculosis* (44) and show a better correlation with progression to active disease in those that are latently infected (42). The limitation of this technique is that it can only evaluate a single cytokine, the source of the cytokine production cannot be determined for either the test or the control, and it has a high indeterminate rate, especially in the setting of HIV (15).

Subsequently, the evaluation of the cytokine response to *M. tuberculosis* antigens has been found to be superior if analysed by flow cytometry, rather than using the histoblot or whole blood IGRAs, as flow cytometry has a higher sensitivity and specificity (14). Flow cytometry also allows for the simultaneous detection of the cells responsible for the cytokine production, in addition to the quantitation of cytokine production. It provides a more flexible platform in which the number of stimulants, the number of cytokines measured and the composition of the secreting cells can be user defined (43). The flow cytometry based assay is also less influenced by CD4+ T lymphocyte count as IFNγ

results are reported as a percentage of CD4+ T lymphocyte, and would therefore be the method of choice in HIV and *M. tuberculosis* co-infected individuals with low CD4 counts (15).

In addition, the use of blood samples instead of compartment specific immune responses, suggests that active TB may be differentiated from latent/non- TB by studying the flow cytometric detection of IFNγ production from memory CD4+ T lymphocytes, which are characterized as being CD27 negative (45). Classical markers of memory phenotype such as CD45RO do not reproduce this finding (45) . CD27 is a member of the TNF receptor family where it acts as a receptor for co-stimulation. It is down- regulated when a naïve T cell encounters an antigen and progresses to a terminal memory cell resulting in reduced expression of CD27. The importance of this phenotype was elucidated in a murine-based study (46) which showed that the lung IFNγ producing CD4+ T lymphocytes were CD27 negative. The hypothesis is that these cells traffic through the blood en-route to the lung at the time of active *M. tuberculosis* infection (45).

1.7.0 Study objective

The hypothesis to be tested is that lung and blood specific T lymphocyte immune responses, particularly *M. tuberculosis* specific IFNγ secretion, differs in HIV infected patients with active TB, compared with HIV infected patients without active TB, and that this may form the basis of a flow cytometric diagnostic assay for active TB.

1.7.1 The aims of this study were to:

- Optimise the flow cytometric panel for detection of IFNγ from sputum and blood.
- Quantitate the *M. tuberculosis* specific IFNγ secreting total lymphocytes, CD4+ T lymphocytes and memory CD27- CD4+ T lymphocytes in sputum and blood.
- Compare the percentage of *M. tuberculosis* specific IFNγ secreting lymphocytes from blood with that from sputum
- Compare the two TB antigens, ESAT6 and PPD, in their ability to stimulate IFNγ secretion
- Compare the IFNγ responses in sputum and blood with the final microbiological diagnosis on each patient.

CHAPTER TWO

2.0 Materials and Methods

2.1.0 Optimisation of laboratory techniques

2.1.1 Immunofluorescence panel

A four colour fluorescence panel was used to label the respective cells. CD3, a marker of the T cell receptor, was labelled with fluorescein isothiocyanate (FITC), CD4 was labelled with a tandem peridinin chlorophyll protein (PerCP Cy3.5), CD27 was labelled with allophycocyanin (APC) and IFNγ with phycoerythrin (PE). All fluorescently labelled monoclonal antibodies were manufactured by Becton Dickinson, USA, including those that were generously provided by Dr David Murdoch, Duke University, USA.

2.1.2 Titration of monoclonal antibodies

This procedure was used to determine the minimum amount of monoclonal antibody needed to achieve good discrimination between a negative and positive cell population while mimimising background staining. This was determined by comparing the mean fluorescence intensity achieved with varying concentrations of antibody. In addition, the signal to noise ratio (S:N) was calculated, by dividing the mean fluorescence intensity (MFI) of the negative and positive populations. The titration volume with the highest MFI and S:N ratio was selected.

2.1.3 Fluorescence minus one (FMO) controls

This procedure was used to detect spill over from one fluorochrome in the flow cytometric panel into an incorrect channel. Fluorescence minus one (FMO) controls are carried out by eliminating a single fluorophore from each experiment and verifying that it was not detected.

2.1.4 Optimisation of the flow cytometric assay using cells from ISp

Due to the lack of established processing methods, ISp was collected from eight AFB negative patients being worked up for other chronic lung disorders. The aim was to identify an appropriate mucolytic agent, as well as the volume and time required for mucolysis, by determining cellular viability. The second aim was to identify the optimal filtration method by comparing the cellular yield obtained when either a 40um or a 100um filter was used. Cellular viability was assessed by counting the number of all cells, followed by viable cells only. Total cell counts were performed after staining with trypan blue (Sigma Aldrich, USA), and cells were counted using, a Neubauer Haemocytometer and the calculation described in Appendix 6. The erythrosin stain (Sigma Aldrich, USA) was used to stain the viable cells, after which the percent viability was calculated. Finally, the optimal gating strategy to identify lymphocytes in ISp was determined (Figure 3.2).

2.1.5 Optimisation of the concentration of ESAT6 and PPD

Based on a recent review (6), two concentrations of ESAT6 and PPD (5ug/ml and 10ug/ml) were selected to stimulate peripheral blood mononuclear cells (PBMC) from healthy controls. PBMC were isolated according to the procedure described in section 2.3.2.2. The concentration that yielded the greater production of percentage IFNγ specific total lymphocytes was selected and IFNγ production was quantified using MFI.

2.2 Patient recruitment

This was a prospective study. Adult, HIV positive patients undergoing diagnostic work-up for suspected *M. tuberculosis* disease despite producing two negative AFB sputum smear samples were randomly enrolled from the TB Focal Point, Helen Joseph Hospital (HJH) or the Infectious Diseases ward at Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) between June 2010 and March 2011. Patients on anti-tuberculosis chemotherapy for more than one week, with a history of asthma, chronic obstructive lung disease, on an immunosuppressant regimen or with a recent life-threatening event were excluded from the study. Enrollment was terminated when a similar number of patients were represented in the *M.tuberculosis* negative and positive groups.

Ethics approval was obtained from the University of Witwatersrand, Human Research Ethics Committee/M090517 (Appendix 1). The study details were discussed and a written information sheet (Appendix 2) handed out to all participants prior to obtaining written informed consent (Appendix 3). A clinical data sheet (Appendix 4) was used to record all relevant clinical details. Study participation did not disrupt the participants' routine diagnostic work-up.

2.3.0 Sample acquisition and processing

Sputum induction was performed within the sputum induction facility at HJH and in the Infectious Diseases ward at CMJAH. These areas are equipped with ultraviolet lamps and an N95 mask was worn during the induction procedure. Participants were instructed to clear the mouth and nostrils of residual secretions prior to induction by rinsing with water. Twenty millilitres of 5% hypertonic saline (Sabax, Adcock Ingram, South Africa) was added to a nebuliser mask and delivered in six litres of oxygen per minute. The induction continued for fifteen to twenty minutes. Sputum was collected into a sterile container. Five to six millilitres of venous blood was collected into an Acid Citrate Dextrose tube.

The intracellular cytokine staining protocol (Appendix 5) used was adapted from the standard operating procedure employed by the HIV Immunology laboratory, National Institute of Communicable Diseases, Johannesburg. The details of this protocol are described below. A reagent template (Appendix 6) was used to ensure that exact reagents and staining master-mixes were made for each experiment. Both blood and sputum samples were processed simultaneously and within two hours of collection.

2.3.1 Isolation of cells from sputum

An equivalent volume of Sputolysin (Caldon biotech, USA) was added the ISp sample, agitated for twenty minutes at room temperature then, filtered by adding a 100um filter into a 50ml conical tube. The filtrate was washed and centrifuged at a centrifugation rate of 400g for 10 minutes, in phosphate buffered saline (PBS, Philemon Diagnostics) to which foetal calf serum (FCS, Sigma Aldrich, USA) was added. Five milliliters of FCS was added to each 500ml aliquot of PBS. The supernatant was decanted after washing and 1500ul of culture medium was added as described below.

2.3.2 Isolation of peripheral blood mononuclear cells (PBMC)

The anticoagulated blood sample was inverted three times, layered on 4.5 ml histopaque (Sigma Aldrich, USA) and centrifuged at 800g for twenty minutes,with the brake off to facilitate identification of the buffy coat. The buffy coat was removed with a plastic pipette and transferred into a sterile tube. The cells were washed twice, in the PBS plus FCS cocktail, by centrifugation at a rate of 400g for 10 minutes with the break on. The cells were then stimulated at outlined below.

2.3.3 In vitro antigen stimulation

The PBMCs and sputum cell preparations were diluted in two millilitres of tissue culture medium, RPMI 1640 supplemented with FCS, 2mM L-glutamine GibcoBRL, 100U/ml penicillin G and 100ug/ml streptomycin sulphate. All tissue culture reagents were purchased from Sigma Aldrich, USA.

During the washing and incubation steps described above, the stimulatory and unstimulatory tubes were prepared as follows. Five milliliter polypropylene tubes with caps (BD Biosciences, USA) were used for culture. Each blood or ISp sample required four culture tubes (PPD, ESAT6, Staphylococcal Enterotoxin B (SEB) and one unstimulated). Ten ug/ml of ESAT6 and PPD, both purchased from the Statens Seruminstitut, Denmark were added to their respective tubes. We stimulated PBMCs with both ESAT6 and PPD to determine whether ESAT6 as a RD1 antigen was more predictive of active TB than PPD in a high TB burden country, and to determine whether PPD induced significantly more IFNγ than ESAT6. One ug/ml of SEB (Sigma Aldrich, USA) was added to the positive control tube. The PPD, ESAT6 and SEB were added to culture

media to ensure a final volume of 250ul in each tube. Antigen was omitted from the negative control, hence 250ul of culture media was added to the tube marked as 'negative control'. The co-stimulatory cocktail comprised of one microlitre each of co-stimulatory antibodies, CD28 and CD49d (Becton Dickinson, USA) as well as 0.5ul Brefeldin A (Becton Dickinson, USA), plus culture media to obtain a final volume of 250ul.The co-stimulatory cocktail was immediately added to all tubes. Brefeldin A is a cytokine secretion inhibitor, hence maintaining IFNγ within the cell so that it could be detected by flow cytometry.

Five hundred microlitres of the cells and tissue culture medium solution was then added to each of the four sample tubes. The final sample volume was one milliltre (250ul stimulatory mix + 250 co-stimulatory mix + 500ul of the cell solution). The samples were gently shaken, then incubated in 5% CO2 for 16 to 18 hours at 37 ˚C with the cap loosened. After stimulation, 100ul ethylenediamine tetra-acetic acid (EDTA) (Becton Dickinson, USA) was added, the samples gently shaken and incubated in the dark, at room temperature for 15 minutes. This process was performed to detach any adherent cells. The samples were then washed, in PBS supplemented with FCS, by centrifugation at a rate of 300g for five minutes. The supernatant was decanted.

2.3.4 Fluorescence labelling

During the incubation and wash steps above, a monoclonal antibody master-mix was prepared for surface (CD4Percp Cy3.5 and CD27 APC) and intra-cellular staining (IFNγ PE and CD3 FITC). PBS was added to each of the staining mixtures to ensure that 100ul could be added to each tube.

One hundred microlitres of the surface markers mixture was added to each of the tubes containing the washed cells, and incubated in the dark, at room temperature for 20 minutes. Thereafter the cells were washed in PBS supplemented with FCS, by centrifugation at a rate of 300g for five minutes. The cells were then permeabilised by adding 250ul of Cytofix/Cytoperm (BD Biosciences, USA) to each tube and incubated in the dark, at room temperature for 20 minutes. The samples were then washed twice with BD wash buffer (included in the cytofix/cytoperm kit, BD Biosciences) by centrifugation at a rate of 500g for eight minutes. The supernatant was decanted.

One hundred microlitres of the intra-cellular stain mixture was added to each tube and incubated in the dark, at 4 ˚C for 45 minutes. Following which, the samples were washed with BD wash buffer as described above, and Cellfix solution (BD Biosciences) was added. The samples were now ready for flow cytometric analysis. CD3, is normally used as a surface marker to stain for the T cell receptor (TCR), since is down regulated during stimulation, a cytoplasmic CD3 label was used.

2.4 Flow cytometry analysis

2.4.1 Acquisition and quality control

Four colour flow cytometry was performed on the LSRII flow cytometer (BD Biosciences). BD Cytometer set up and tracking beads were run daily which allows the BD Facs Diva software to automatically characterise, track and report fluorescent intensities. 1 X Rainbow beads (Spherotech Inc.), which are mid level fluorescence beads were run prior to each experiment in order to standardise the mean fluorescence intensity of each flourochrome throughout the duration of the study. Colour spill-over was minimised using digital compensation. Compensation refers to the subtraction of light contamination from secondary detectors, which occur as a result of spectral overlap. Compensation was performed prior to each experiment, using mouse anti-IgG capture compensation beads (Becton Dickinson, USA) and calculated digitally using the FacsDiva software.

Each sample was allowed to run for five to eight minutes. Sample acquisition was stopped when the cellular events rate dropped to less than one hundred events so that the full volume of the sample was analysed.

2.4.2 Analysis

Samples were analysed using FloJo software (TreeStar, USA). A FloJo template was created to enable the measurement of percentage IFNγ specific lymphocytes from total lymphocytes, CD4+ T lymphocytes and memory CD27- CD4+ T lymphocytes.
T lymphocytes were identified by gating on CD3, as CD3+ is a marker for the T cell receptor. The gate for each sample set was drawn using the negative control, which is the unstimulated blood sample.

The percentage IFNγ secreting lymphocytes for each target population outlined above was determined utilising a sequential gating strategy, Figures 3.2 to 3.3. Forward Scatter (FSC) Area and FSC Height Plot was used to exclude doublets or clumps of cells. Lymphocytes were then identified from their characteristic cluster on side scatter (SSC) and FSC area. After gating on the lymphocyte population, the CD3+ and CD4+ co-expressing lymphocytes were used to identify the CD4+ T lymphocytes. Finally gating on the CD3+ CD4+ lymphocytes with dim to absent expression of CD27 identified the memory, CD27- CD4+ T lymphocytes.

2.5 Diagnostic classification

TB culture is the current 'gold standard' used to diagnose active disease. A microbiological classification was therefore used to categorise the study population. All participants were smear negative at enrolment but were being investigated for active TB, hence repeat sputum microscopy as well as TB culture were requested on sputum or other tissue from all study participants. These results were used to assign final diagnosis as follows:

- *M. tuberculosis* Positive/Active TB: patients who have positive microscopy or culture results subsequent to enrolment.
- *M. tuberculosis* Negative/Latent or non-TB: patients who remain microscopy and culture negative.

Flow cytometric detection of IFNγ responses from blood or ISp lymphocytes were statistically analysed to determine whether these responses could be used to discriminate between the *M.tuberculosis* negative and positive groups

2.6 Data analysis

The percentage of IFNγ producing cells was determined for each cellular population. Tests for normality were performed on all data to be analysed, leading to use of non-parametric statistics for all data that was not normally distributed. Data was summarized using descriptive non-parametric statistics such as median values. The Mann-Whitney test was used to compare IFNγ production between groups. A value of $p < 0.05$ was considered significant. A receiver operating curve was used to establish sensitivity and specificity. Correlations were calculated by non-parametric Spearman's test. GraphPad Prism version 5 was used for all statistical analyses and to draw graphs.

Data was analysed as raw values, and with the unstimulated background IFNγ secretion subtracted from the stimulated values. Only the raw data has been reported. The subtracted data showed similar trends.

CHAPTER THREE

3.0 Results

3.1.0 Optimisation of laboratory techniques

3.1.1 Titration of monoclonal antibodies

Each monoclonal antibody in the panel was titrated to identify the minimum volume that could be used to provide good quality results while limiting the expense of these antibodies. Table 3.1 represents the results of the titration experiment for CD27 APC where a final volume of 10ul was selected as this volume produced the best S:N ratio.The titration volumes obtained for CD27 APC, CD4 PerCP Cy3.5, CD3 FITC and IFNγ PE were 10ul, 15ul, 10ul and 15ul respectively.

Table 3.1 Analysis of CD27 APC titration. For CD27 APC, this table shows the increase in the mean fluorescence intensities of both the positive population (signal) and the negative population (noise) with increasing volumes of antibody used, as well as the calculated signal to noise ratio.

CD27 APC			
	NegMed	PosMed	S:N
CD27 APC 2.5uL	31.7	1979	62.43
CD27 APC 5uL	50	4039	80.78
CD27 APC 10uL	54.6	5438	99.60
CD27 APC 20uL	72.5	6452	88.99
CD27 APC 40uL	83.2	6607	77.55
CD27 APC 80uL	99.7	6945	69.66

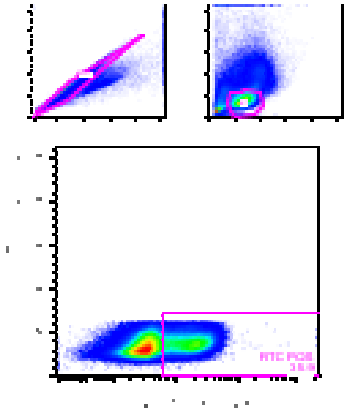
The optimal signal to noise ratio is highlighted in red. The first column shows the phenotypic marker, the fluorescent label followed by the clone of the monoclonal antibody then titration volume.

NegMed, negative median. PosMed, positive median. S:N, signal to noise ratio

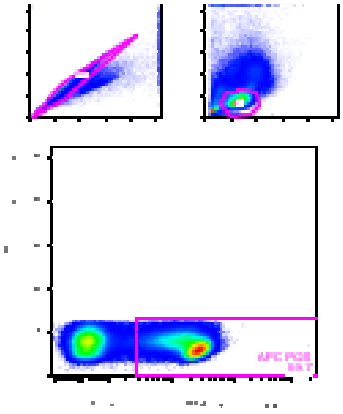
3.1.2 FMO experiment

Flow cytometric analysis of the FMO experiment did not detect any spill-over amongst the four fluorochromes used in the panel. Figure 3.1 Panel A below shows the flow cytometric scatterplots for the FMO experiment in which there was no staining in the PE (IFNγ) channel when PE was omitted but the other flourochromes were correctly identified. The presence of PE is, however, noted in the control sample, Figure 3.1 Panel B.

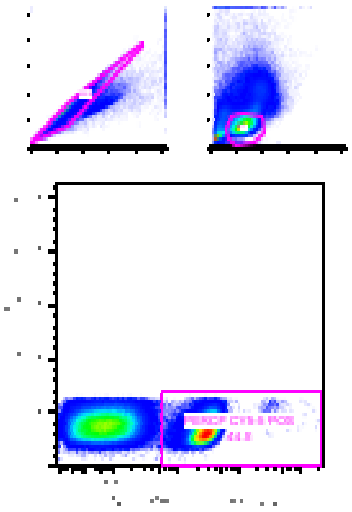
Panel A



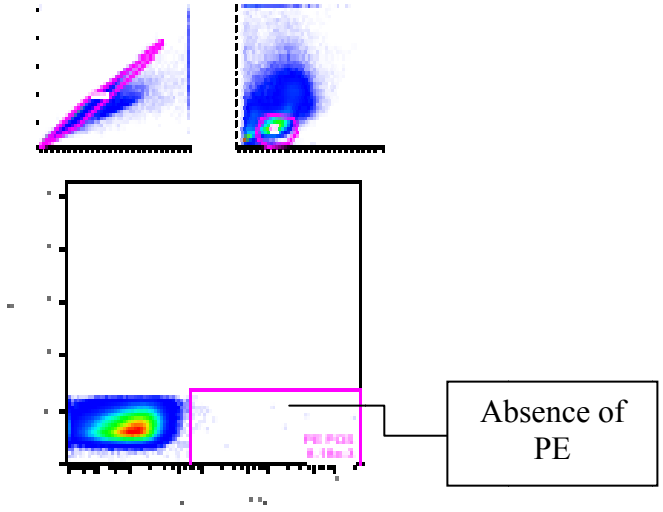
FITC



APC



PerCP Cy3.5



PE

Panel B

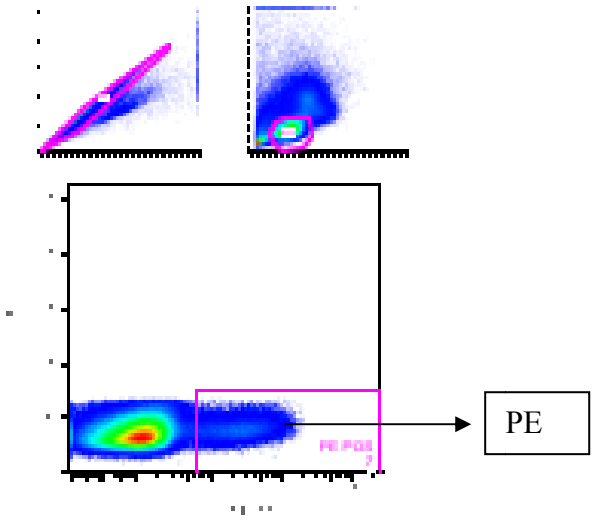


Figure 3.1 Flow cytometry dot-plots of the FMO experiment

PE is not detected when it is omitted (**Panel A)** but is detected in the control (**Panel B**). Panel A from left to right represents FITC, APC, PerCP Cy3.5 and PE respectively on the X-axis. SSC is represented on the Y-axis of all plots

3.1.3 Optimisation of sputum samples

Upon optimisation of the flow cytometry analysis for sputum samples, the lymphocyte population in sputum was found to occupy the same area as lymphocytes in blood on the forward and side scatter flow cytometry dot- plot, Figures 3.2 and 3.3.

3.1.4 Concentration of ESAT6 and PPD used to stimulate the samples

PPD and ESAT6 were each evaluated at two concentrations, five ug/ml and ten ug/ml, for maximal IFNγ secretion by a healthy control individual. Table 3.2 shows the PE (IFNγ) MFI produced after stimulation with either five ug/ml or ten ug/ml of PPD and ESAT6. The MFI values from the 10ug/ml concentration produced marginally higher MFI values than the five ug/ml concentration. Ten ug/ml each of PPD and ESAT6 was selected as the concentration with which to stimulate further samples.

Table 3.2 IFNγ Mean Fluorescence Intensity after stimulation with ESAT6 and PPD

Concentration of Antigen (ug/ml)	*IFNγ MFI*
ESAT6 5	911
ESAT6 10	948
PPD 5	1051
PPD 10	1158

Mean fluorescence intensity (MFI) for both PPD and ESAT6 was marginally higher using 10ug/ml than 5ug/ml

3.1.5 Quantitation of *M. tuberculosis* specific IFNγ secreting lymphocytes in blood and sputum

All participants produced IFNγ in response to SEB, which was used as the positive control. For blood samples, IFNγ secreting lymphocytes were quantified as a frequency of total lymphocytes, CD4 + T lymphocytes or memory, CD27- CD4+ T lymphocytes, Figures 3.2 to 3.5 respectively. For sputum, samples only IFNγ secretion as a frequency of total lymphocytes was included in the statistical analysis, as numbers of IFNγ events in the CD4+ or CD27- CD4+ T lymphocyte subsets became too small for reliable interpretation, Figure 3.2. Gates for IFNγ positivity were set according to the unstimulated sample and copied into the stimulated tubes.

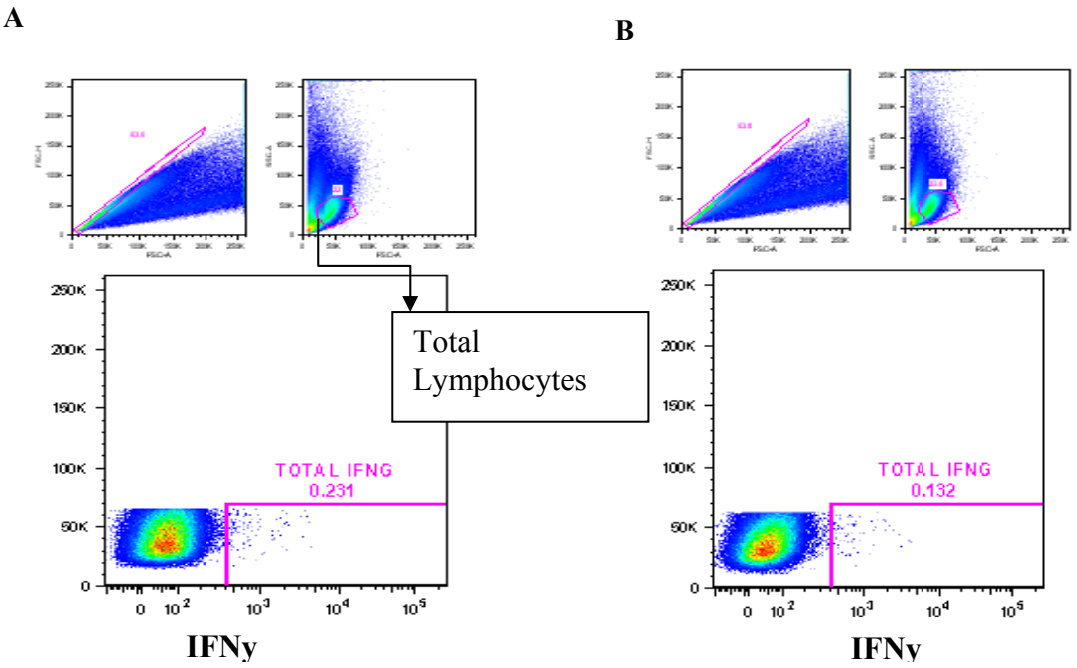


Figure 3.2 Flow cytometry dot plots showing gating strategy for IFNγ production from the total sputum lymphocyte population

(A) The top left plot shows exclusion of doublets by gating cells with proportional forward scatter area (x-axis) against forward scatter height (y-axis). The top right plot shows gating of total lymphocytes according to forward scatter (x-axis) and side scatter (y-axis). The large lower plot shows the IFNγ produced from total lymphocytes in response to ESAT6. The position of the gate was determined in the unstimulated control tube **(B)** and copied into the stimulated tubes.

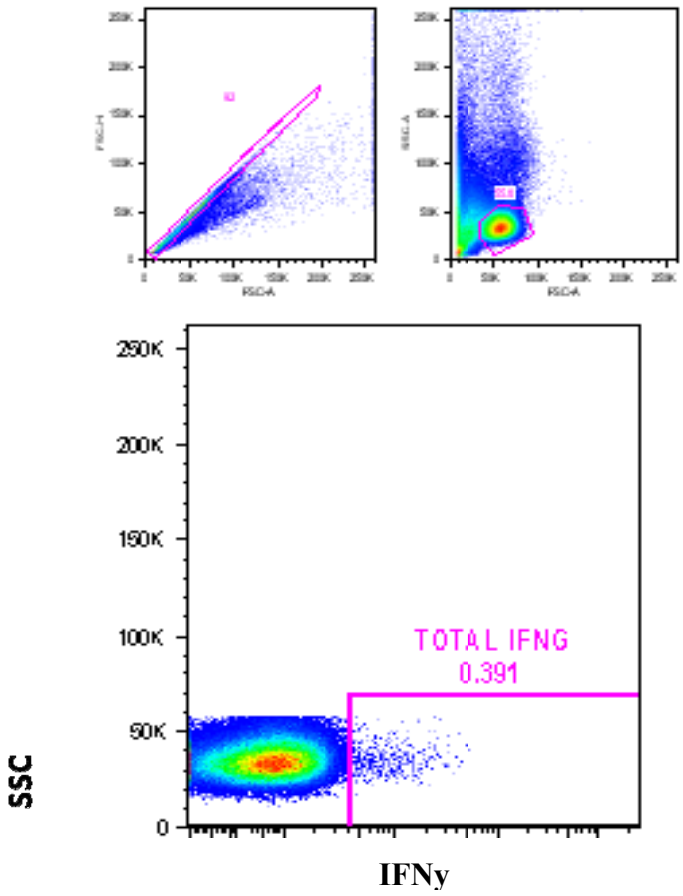


Figure 3.3 Flow cytometry dot plots showing gating strategy for IFNγ production from the total blood lymphocyte population.

The top left plot shows exclusion of doublets by gating cells with proportional forward scatter area (x-axis) against forward scatter height (y-axis). The top right plot shows gating total lymphocytes according to forward scatter (x-axis) and side scatter (y-axis). The large lower plot shows the IFNγ produced from total blood lymphocytes in response to ESAT6. The position of the gate was determined in the unstimulated control tube and copied into the stimulated tubes.

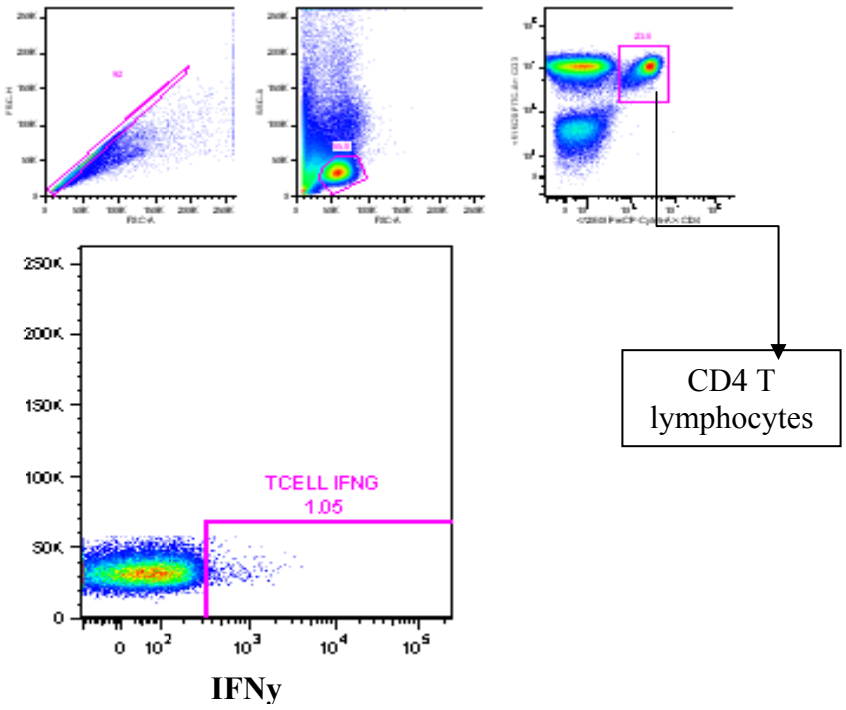


Figure 3.4 Flow cytometry dot plot showing gating strategy for IFNγ production as a frequency of CD4+ T lymphocytes

The first two top left plots are described in Figure 3.3. The top right plot shows dual positive CD3+ (y-axis) and CD4+ (x-axis) lymphocytes. The large lower plot shows the percentage of IFNγ secreting CD3+ CD4+ lymphocytes in response to ESAT6. The position of the gate was determined in the unstimulated control tube and copied into the stimulated tubes.

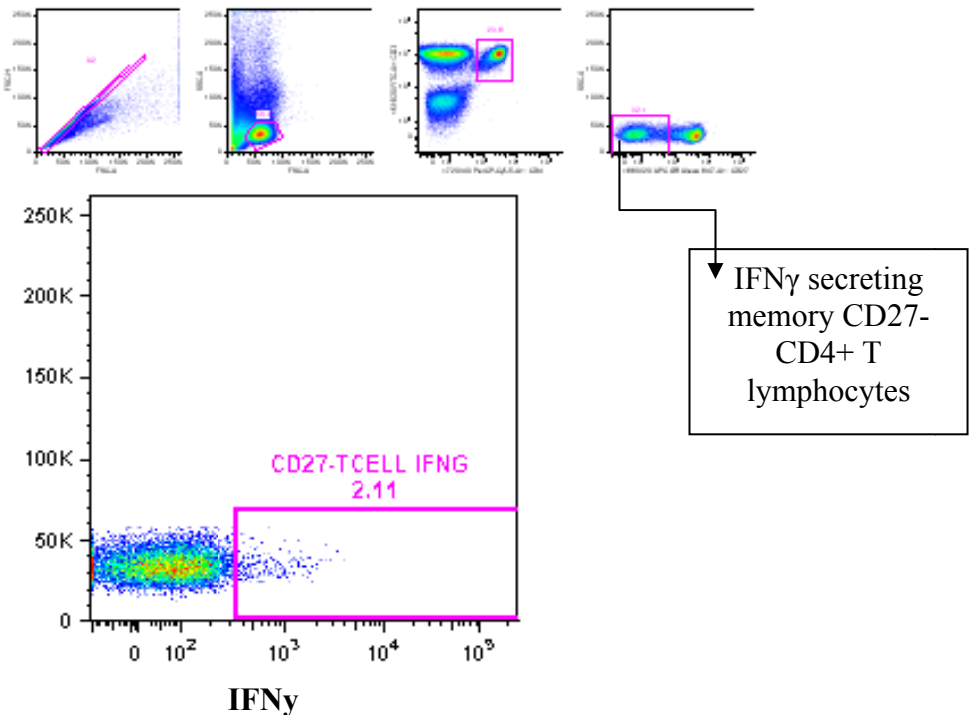


Figure 3.5 Flow cytometry dot plots showing the gating strategy for IFNγ production as a frequency of memory CD27- CD4+ T lymphocytes

The first three top left plots are as described in Figures 3.3 and 3.4. The top right plot comprises only CD3+CD4+ lymphocytes and shows the CD27- (CD27 on x-axis and SSC on y-axis) CD3+CD4+ lymphocytes. The large lower plot shows the percentage of IFNγ secreting memory CD27- CD4+ T lymphocytes. The position of the gate was determined in the unstimulated control tube and copied into the stimulated tubes.

3.2 Study population

Thirty-one HIV infected, smear negative TB suspects who met the inclusion and exclusion criteria were recruited, including five from HJH and twenty-six from CMJAH. Blood samples were analysed on all 31 patients. One patient was unable to expectorate following sputum induction. Despite optimisation of sputum processing techniques, eleven sputum samples were excluded due to assay related factors including poor lymphocyte viability and the presence of non-specific monoclonal binding, leaving nineteen samples suitable for analysis. According to the diagnostic classification outlined in section 2.5, seventeen patients were classified as *M. tuberculosis* positive and fourteen were classified as *M. tuberculosis* negative from the whole group. Of the 19 patients with available sputum samples, 9 were *M. tuberculosis* positive and 10 *M. tuberculosis* negative. The diagnosis of positivity was made either on AFB smears or culture of *M. tuberculosis* from the ISp sample taken during this study or subsequent samples from any site. PCR analysis (GenoType MTBDR, Hain Lifesciences, Germany) of all positive results confirmed the isolates as *M. tuberculosis.* All patients were suspected of having pulmonary TB, while four patients were subsequently found to have additional extra pulmonary sites of *M. tuberculosis.*

The relevant patient information is summarised in Table 3.3. The study population comprised 48% females and 35% were on anti-retroviral therapy. The median age of the study population was 37 years old (range, 17 to 61 years) and the median CD4 count was 117 cells/ul (range, 15 to 503 cells/ul). IFNγ secretion from blood lymphocytes showed no correlation with CD4 count or age (data not shown).

Table 3.3 Summary of patient characteristics

STUDY NO.	HOSPITAL	AGE	SEX	DATE of PREVIOUS TB (treatment duration)	HAART	? EXTRA PULMONARY *M. tuberculosis*	CD4	AFB/CULTURE RESULTS
1	HJH	61	female	no	no	no	-	Negative
2	HJH	39	female	yes- 2008 (6/12)	yes	no	229	Negative
3	HJH	53	female	no	no	no	276	Negative
4*	HJH	50	female	no	no	yes - shoulder abscess AFB positive	-	Positive - shoulder abscess
5*	HJH	54	female	no	no	cervical lymph nodes	-	Positive - cervical lymph node
6	CMJAH	49	female	unknown (1/52 in Feb 2010)	no	no	43	Negative
7*	CMJAH	27	male	no	no	no	88	Negative
8*	CMJAH	28	male	no	yes	no	-	Negative
9	CMJAH	25	male	yes - 2006 (6/12)	yes	no	112	Negative
10*	CMJAH	36	male	no	yes	no	57	Positive – sputum
11*	CMJAH	35	male	no	yes	no	178	Positive - cervical lymph node
12	CMJAH	36	female	no	no	no	185	Negative
13	CMJAH	52	male	no	no	?TB Meningitis	45	Positive - pleural fluid and BMA
14	CMJAH	33	male	no	yes	no	49	Negative (but high fluid ADA)
15	CMJAH	39	male	yes - 2007 (6/12)	no	? Miliary	-	Positive – sputum
16*	CMJAH	54	female	no	no	no	252.5	Positive – sputum
17	CMJAH	35	female	yes - 2007 (6/12)	no	no	15	Positive – sputum
18	CMJAH	17	male	no	no	no	-	Positive – sputum
19	CMJAH	34	female	no	no	no	193	Positive – sputum
20*	CMJAH	29	male	no	no	no	-	Positive – sputum
21	CMJAH	39	female	yes - 2008 (6/12)	yes	no	-	Negative
22*	CMJAH	35	male		no	? TB Meningitis	173	Negative
23*	CMJAH	34	female	yes-2008 (6/12)	yes	no	111	Negative
24	CMJAH	28	female	no	yes	no	122	Positive – sputum
25	CMJAH	34	male	yes- 2008 (6/12)	no	no	56	Negative (but marked inflammatory infiltrate)
26	CMJAH	41	male	yes - 2007 (6/12)	yes	no	152	Positive – sputum
27*	CMJAH	51	male	no	no	no	51	Positive – sputum
28	CMJAH	29	female	no	yes	no	500	Negative
29	CMJAH	40	male	no	no	no	25	Positive – sputum
30	CMJAH	30	female	no	no	yes - ?TB Meningitis	503	Positive – sputum
31*	CMJAH	30	male	no	no	no	150	Positive – sputum

HAART, Highly Active Antiretroviral therapy. ADA, Adenosine Deaminase. BMA, bone marrow aspirate.* indicates patients in whom only blood was available for analysis, as sputum was not available or suitable for meaningful interpretation. ? indicates unknown or possible

3.3 Cell yield in blood and sputum samples

The mean number of lymphocyte acquired varied amongst samples but sputum samples produced a lower mean than blood samples. The mean number of lymphocytes acquired for each of the lymphocyte populations in the unstimulated sample for blood and sputum are tabulated in Table 3.4.

Table 3.4 Mean lymphocyte counts acquired from the unstimulated tubes for blood and sputum

LYMPHOCYTE POPULATION	*M. TUBERCULOSIS* POSITIVE (MEAN CELL NUMBER)	*M. TUBERCULOSIS* NEGATIVE (MEAN CELL NUMBER)
Blood (n=31)		
Total lymphocytes (range)	218252 (18452 - 468361)	269108 (45428 – 683946)
CD4+ T lymphocytes (range)	58539 (319 - 291471)	48951 (3084 – 237097)
CD27- CD4+ T Lymphocytes (range)	7363 (113 – 34516)	15190 (298 – 10077)
Sputum (n=31)		
Total lymphocytes (range)	41970 (37 – 156939)	30261 (539 – 184662)
CD4+ T lymphocytes	Insufficient to gate	Insufficient to gate
CD27- CD4+ T lymphocytes	Insufficient to gate	Insufficient to gate

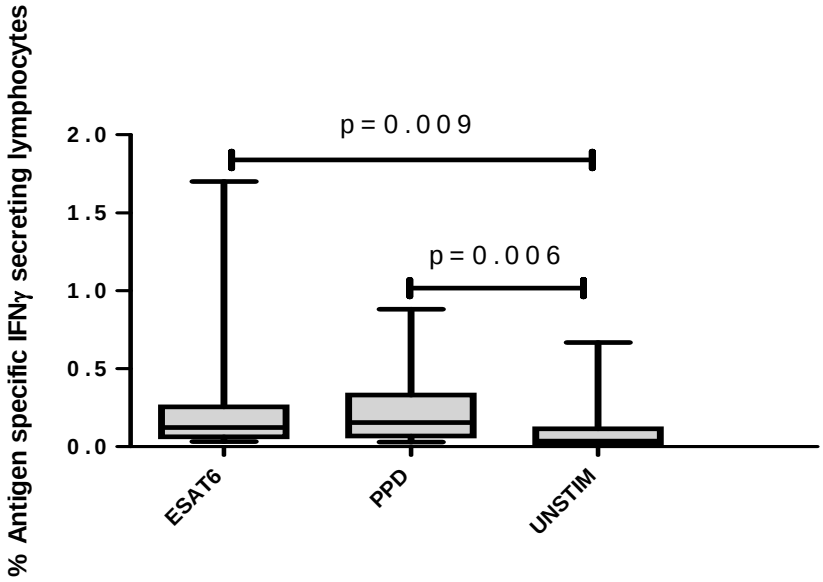
3.4 The effect of *M. tuberculosis* antigen stimulation in blood and sputum

The percentage of IFNγ secretion was quantified as described in 3.1.3. The percentage of *M. tuberculosis* specific IFNγ secreting lymphocytes in blood and sputum of the *M. tuberculosis* positive and negative groups were compared to that from the respective unstimulated samples to determine whether IFNγ secretion was largely antigen specific. Figures 3.6 and 3.7 show the comparison between the percentage of *M. tuberculosis* specific (stimulation with either PPD or ESAT6) and non-specific (unstimulated samples) IFNγ secreting total lymphocytes in blood and sputum samples.

In the blood samples from the *M. tuberculosis* positive group Figure 3.6.A, the median PPD (0.15%) specific or ESAT6 (0.12%) specific IFNγ secreting lymphocytes were significantly higher than the unstimulated sample (0.03%. $p = 0.006$ and $p = 0.009$ respectively). In the blood samples from the *M. tuberculosis* negative group, Figure 3.6.B, the median PPD (0.20%) specific IFNγ secreting lymphocytes remained significantly higher than the median from the unstimulated sample (0.08%. $p = 0.03$) but this significance (p,0.35) was not noted when ESAT6 (median,0.09%) was used as the stimulant.

In sputum, Figure 3.7, median PPD specific and ESAT6 specific responses were not significantly higher than the unstimulated samples for either the *M. tuberculosis* positive or negative groups. The median percentage PPD, ESAT6 and unstimulated IFNγ secreting lymphocytes in the *M. tuberculosis* positive were 0.23%, 0.64% and 0.27% respectively, and in the *M. tuberculosis* negative group were 2.04%, 3.18% and 2.08% respectively.

A



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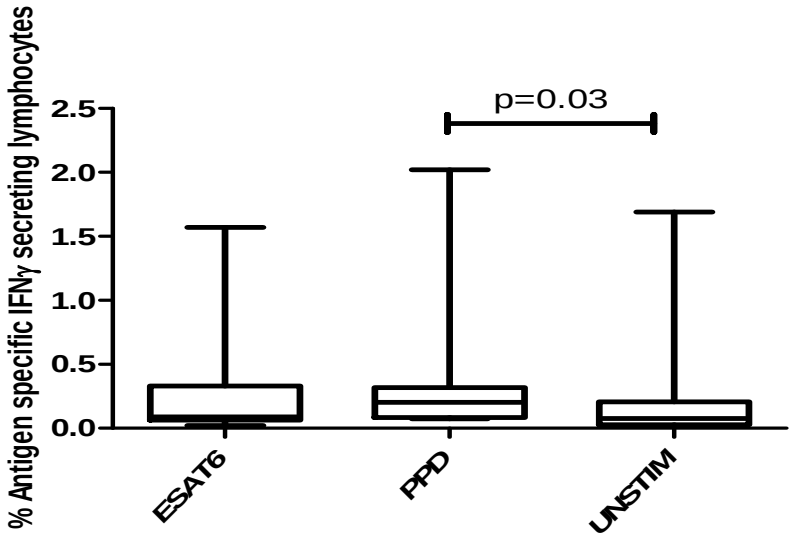


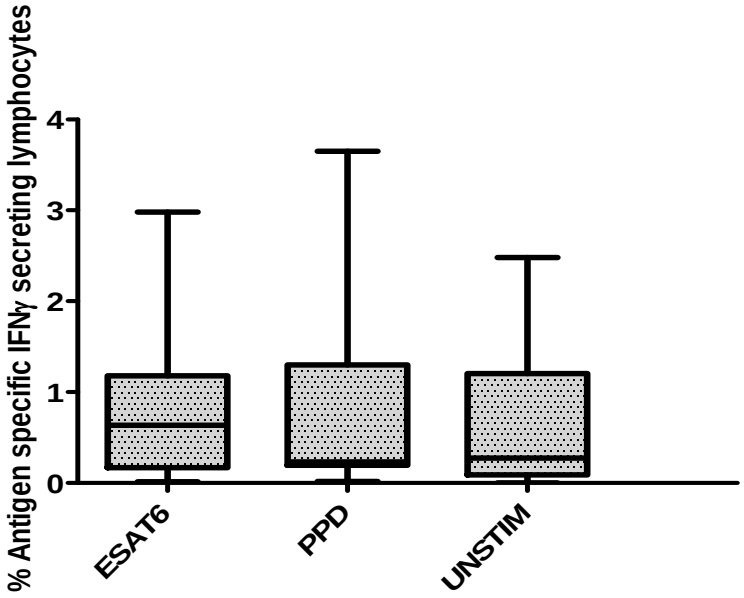
Figure 3.6 IFNγ secretion as a percentage of total lymphocytes in unstimulated samples and following stimulation with either ESAT6 or PPD in blood

In the *M. tuberculosis* positive **(A)** group, PPD and ESAT6 specific IFNγ secretion is significantly higher than the unstimulated samples. In the *M. tuberculosis* negative **(B)** group, PPD but not ESAT6 specific IFNγ secretion was significantly higher than the unstimulated samples. No significant differences were noted between ESAT6 and PPD in their ability to induce IFNγ secretion. Median % PPD and ESAT6 specific and unstimulated IFNγ responses in *M. tuberculosis* positive are 0.15%, 0.12% and 0.03% respectively and median responses in *M. tuberculosis* negative are 0.20%, 0.09% and 0.08% . Blood, n = 31.

3.5 Comparison of PPD and ESAT6 as optimal stimuli for IFNγ induction

We compared the magnitude of the IFNγ response by lymphocytes in blood and sputum to assess which antigen was the more powerful stimulant. The two stimuli were compared in the *M. tuberculosis* positive and negative groups. Data representing the frequency of *M. tuberculosis* specific IFNγ secreting lymphocyte as a percentage of total lymphocytes in both blood and sputum is shown in Figure 3.6 and 3.7 respectively. This analysis shows that IFNγ induction by total blood and sputum lymphocytes was not significantly different when stimulated with either PPD or ESAT6. Similarly, in blood, there was no significant difference in the two stimuli for IFNγ production by CD4+ T lymphocytes or memory CD27- CD4+ T lymphocytes in any patient group analysed (data not shown).

A



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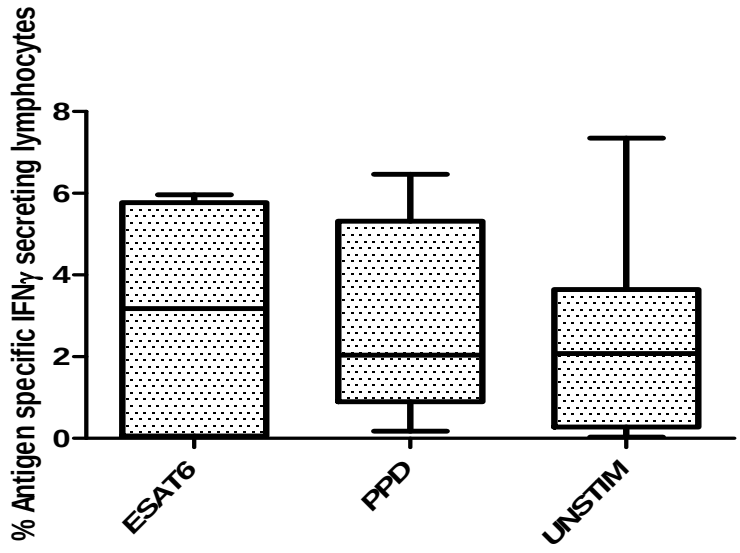


Figure 3.7 IFNγ secretion as a percentage of total lymphocytes in the unstimulated samples and following stimulation with either ESAT6 or PPD in sputum.

IFNγ secretion does not show statistical differences between the unstimulated and stimulated samples. No significant differences were noted between ESAT6 and PPD in their ability to induce IFNγ secretion. Median % PPD and ESAT6 specific and unstimulated IFNγ responses in *M. tuberculosis* positive **(A)** is 0.23%, 0.64% and 0.27% respectively; and 2.04%, 3.13% and 2.08% respectively in the *M. tuberculosis* negative **(B)** population. Sputum, n=19.

3.6 IFNγ secretion from sputum is higher than blood

The percentage of IFNγ secreting lymphocytes as a frequency of total blood lymphocytes was compared to the frequency of the total sputum lymphocytes to determine whether the lung produced significantly more IFNγ. Only blood samples with corresponding sputum samples were used in this analysis (n=19). This data is represented in Figure 3.8. The median percentage of ESAT6 specific IFNγ secreting lymphocytes in blood and sputum were 0.10% and 0.64% respectively in the *M. tuberculosis* positive group and, 0.10% and 3.18% respectively in the *M. tuberculosis* negative group. The median PPD specific IFNγ secreting lymphocytes in blood and sputum were 0.15% and 0.23% respectively in the *M. tuberculosis* positive group and, 0.21% and 2.04% in the *M. tuberculosis* negative group respectively. Thus, the percentage of *M. tuberculosis* specific IFNγ secreting lymphocytes was higher in sputum than blood. The difference between blood and sputum was significantly different in the *M. tuberculosis* positive group when stimulated with ESAT6 ($p = 0.03$) but in the *M. tuberculosis* negative group the difference was significant after PPD stimulation ($p = 0.006$). Comparison of SEB specific IFNγ secreting lymphocytes in blood and sputum was not significantly different (data not represented, $p= 0.48$).

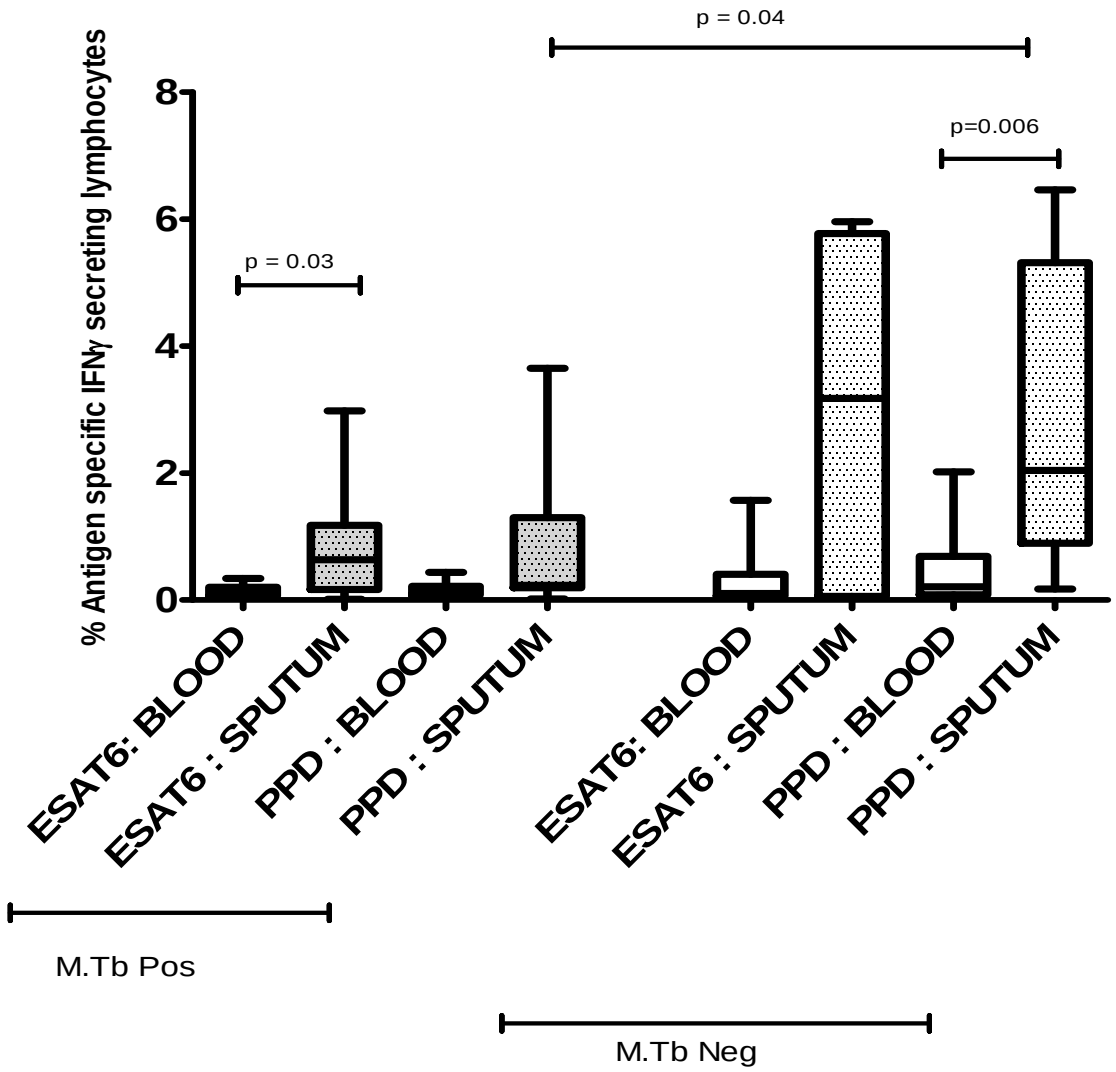


Figure 3.8 ESAT6 and PPD specific IFNγ secreting lymphocytes in blood and sputum of both the *M. tuberculosis* positive and negative groups

M. tuberculosis specific lymphocytes were more frequent in sputum compared to blood, reaching statistical significance in the *M. tuberculosis* positive group with ESAT6, and with PPD stimulation in the *M. tuberculosis* negative group. Sputum PPD specific IFNγ secreting lymphocytes were significantly higher in the *M. tuberculosis* negative group when compared to the *M. tuberculosis* positive group. Only blood samples with corresponding sputum samples were analysed (n=19).

3.7.0 Comparison of IFNγ secretion in sputum and blood to final microbiological diagnosis

In order to assess whether IFNγ responses could be used diagnostically, we compared the results to the microbiological tests and used the diagnostic classification outlined above. The percentage of IFNγ secreting lymphocytes in sputum and blood of the *M. tuberculosis* positive group was compared to that of the *M. tuberculosis* negative group to determine whether this approach could be used to differentiate active TB from background mycobacterial exposure in a TB endemic setting. Lung based samples were shown to be capable of differentiating active and latent TB in low prevalance settings. In addition blood memory CD4+ T lymphocytes which are characterised as being CD27- have been shown to differentiate activate TB from latently infected or unexposed individuals.

3.7.1 PPD but not ESAT6 specific IFNγ responses in sputum differentiates *M. tuberculosis* positive and negative patients

In the ISp, PPD stimulation resulted in significantly more IFNγ secretion in the *M. tuberculosis* negative group than the *M. tuberculosis* positive group, Figure 3.8. The median percentage of PPD specific IFNγ secreting lymphocytes were 2.04% in the *M. tuberculosis* negative group and 0.23% in the *M. tuberculosis* positive group.

Stimulation with ESAT6 resulted in higher specific IFNγ secretion in the in the *M. tuberculosis* negative (median, 3.18%) compared with the *M. tuberculosis* positive group (median, 0.64 %). However this did not reach significance (Figure 3.8).

3.7.2 Sensitivity and specificity of PPD specific lymphocytes in sputum to differentiate *M. tuberculosis* positive and negative patients

A receiver operating curve (ROC), Figure 3.9, was generated to calculate the cut-off value for sputum PPD stimulated IFNγ production with the best sensitivity and specificity for diagnosis of active TB. A frequency of PPD specific IFNγ secreting lymphocytes at a diagnostic threshold of 1.20 % had a sensitivity of 78% (95% CI, 40% to 97) and specificity of 70% (95% CI, 35% to 93%) to discriminate smear or culture positive *M. tuberculosis* from smear and culture negative *M. tuberculosis*. The area under the curve was 0.78. The positive and negative predictive values are 70% and 78%, respectively, Table 3.9.

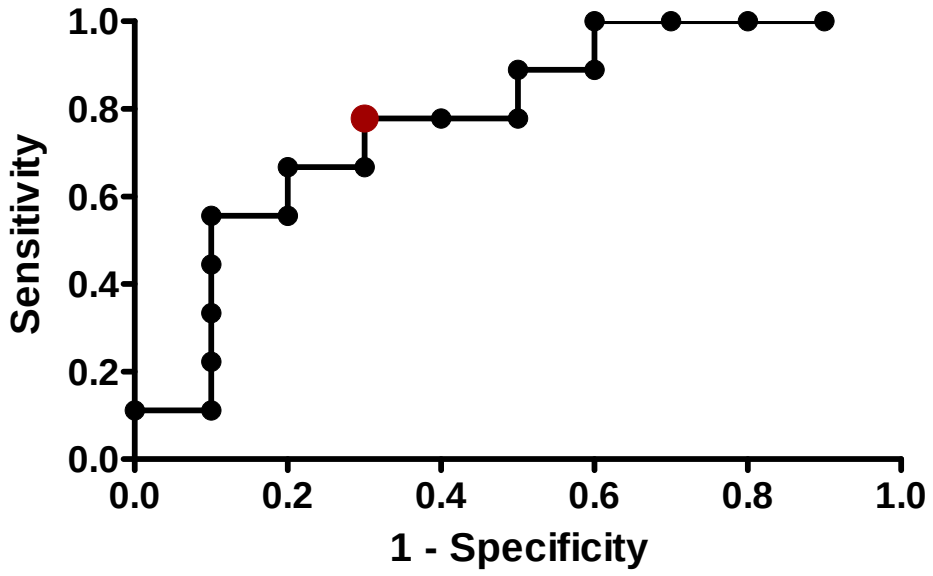


Figure 3.9 ROC curve analysis of sputum PPD induced IFNγ production

The sensitivity and specificity for discriminating smear or culture positive *M. tuberculosis* from smear and culture negative *M. tuberculosis* by percentage PPD specific IFNγ secretion from sputum lymphocytes, at a diagnostic threshold of 1.20, larger red point, are 78% and 70%, respectively.

Table 3.5 Two by Two table to calculate sensitivity, specificity and predictive values

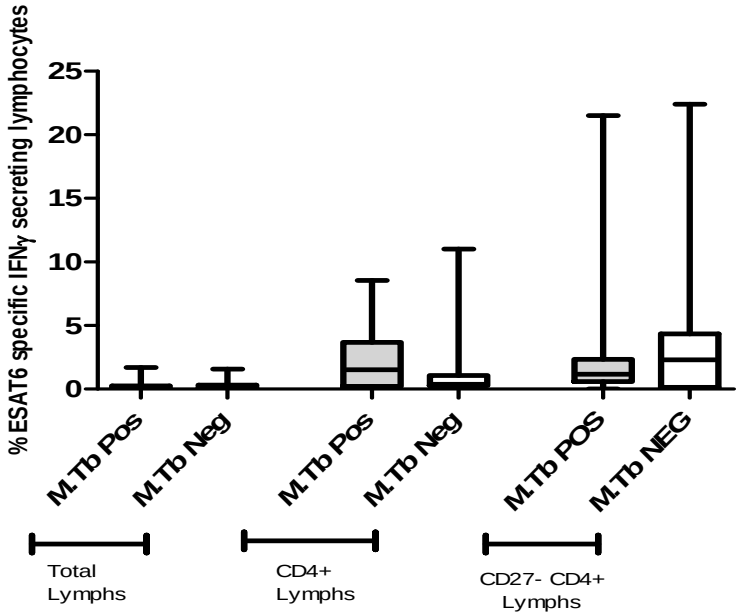
Sputum PPD specific IFNγ	*M. tuberculosis* Positive	*M. tuberculosis* Negative
Positive	**7 (TP)**	**3 (FP)**
Negative	**2(FN)**	**7(TN)**

Using a diagnostic threshold of 1.20 % PPD specific IFNγ secreting lymphocytes in sputum, the assay correctly diagnosed 7 patients with active TB (true positive, TP), incorrectly diagnosed 3 patients without active TB as having TB (false positive FP); incorrectly missed the diagnosis of active TB in 2 patients (false negative, FN) and was correctly negative in 7 patients without active TB (true negative, TN). The sensitivity is 78%, specificity is 70% and positive and negative predictive values are 70% and 78% respectively.

3.7.3 *M. tuberculosis* specific IFNγ responses in blood failed to differentiate the *M. tuberculosis* positive and negative patients

In blood, the percentage of ESAT6 or PPD specific IFNγ secreting lymphocytes (total, CD4+ T lymphocytes or memory CD27- CD4+ T lymphocytes) were not statistically different when the *M. tuberculosis* positive or negative groups were compared, Figure 3.10. The median percentage ESAT6 specific IFNγ secreting total lymphocytes was 0.12 % versus 0.09%, 1.54 % versus 1.07% for CD4+ T lymphocytes and 1.17% versus 2.3% for CD27- CD4+ T lymphocytes for the *M. tuberculosis* positive and negative groups respectively. The median PPD specific IFNγ secretion was 0.15% versus 0.20% for total lymphocytes, 1.20% versus 0.58% for CD4+ T lymphocytes and 2.50% versus 2.96% CD27- CD4+ T lymphocytes for the *M. tuberculosis* positive and negative groups respectively. All these comparisons between the *M. tuberculosis* positive and negative groups produced $p > 0.30$.

A



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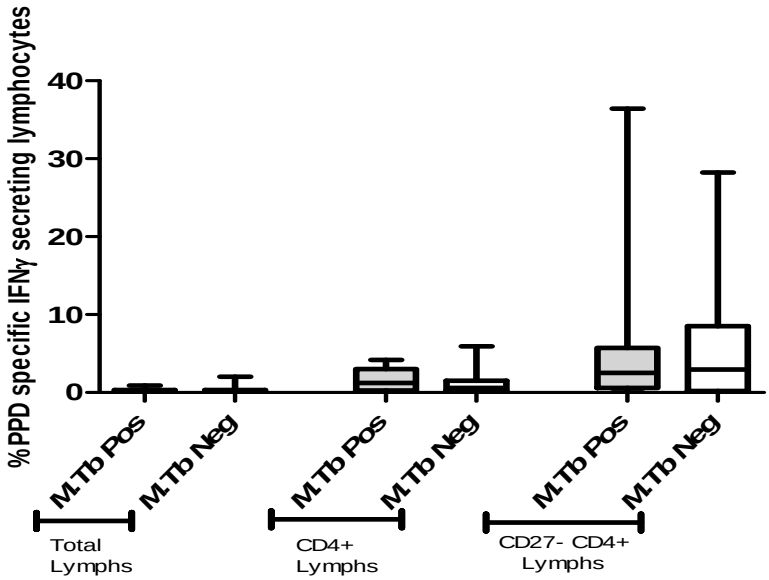


Figure 3.10 ESAT6 (A) and PPD (B) specific IFNγ secreting total, CD4+ and CD27- CD4+ blood T lymphocytes in *M. tuberculosis* positive and negative patients

No statistical differences were noted between the two TB groups for any of the lymphocyte populations analysed. Blood, n = 31.

3.8 IFNγ secretion from CD27- CD4+ T lymphocytes compared to CD27+ CD4+ T lymphocytes

CD27- CD4+ T lymphocytes in this study did not differentiate the active from the latent group. This lead us to investigate the secretion of IFNγ from the two CD27 subsets and to determine whether IFNγ secretion from CD27+ CD4+ T lymphocytes, rather than the CD27- subset, was more informative in our cohort. No difference was noted in ESAT6 stimulated IFNγ secretion by the CD27+ CD4+ T lymphocytes between the *M. tuberculosis* positive and negative groups (data not shown). Interestingly, as shown in Figure 3.11, a higher percentage of IFNγ was secreted by the CD27+ CD4+ T lymphocytes than the the CD27- CD4+ T lymphocytes in both the *M. tuberculosis* positive (median 0.37%) and *M. tuberculosis* negative groups (median = 0.2%) groups (p= 0.01).However, when samples were stimulated with PPD, no difference was observed between the percentage for both the *M. tuberculosis* positive (median CD27+ is 0.12% and CD27- is 0.44%) and negative populations (median CD27+ is 0.12% and CD27- is 0.26%).

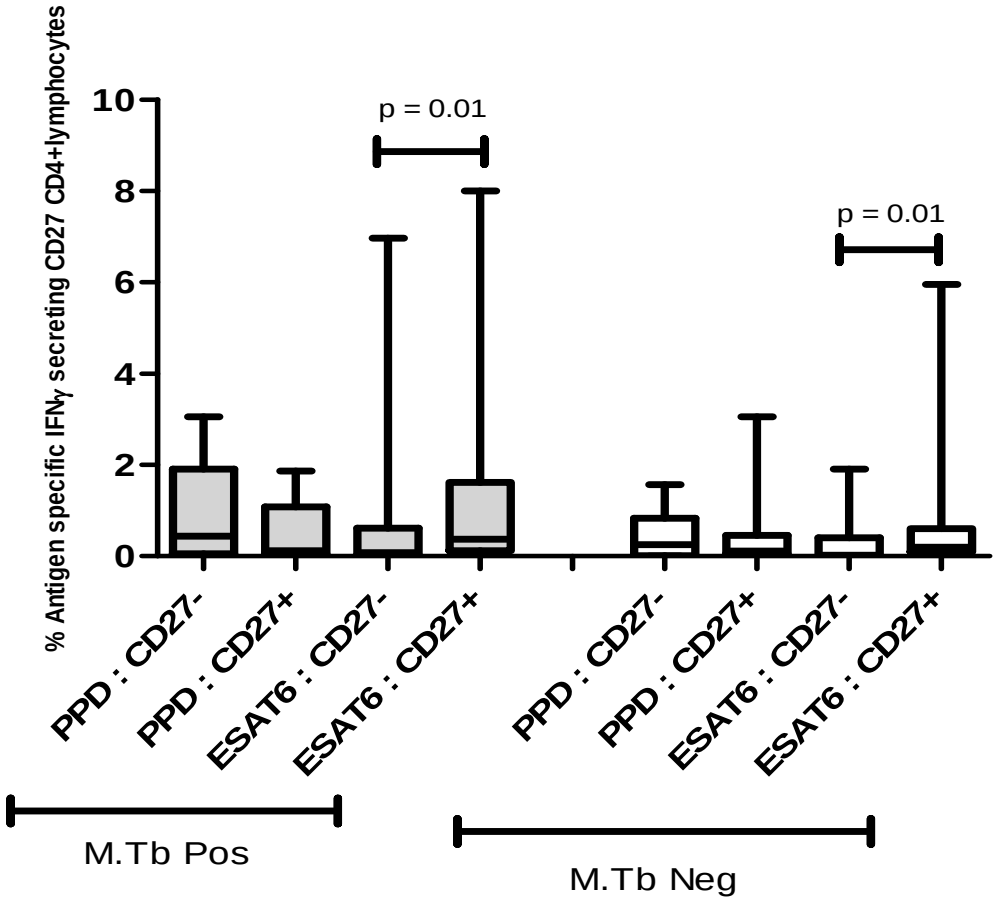


Figure 3.11 IFNγ secretion from CD27 CD4+ T lymphocytes after stimulation with ESAT6 and PPD

ESAT6 specific IFNγ CD4+ T lymphocytes are predominantly CD27+ but PPD specific IFNγ CD4+ T cells can be either CD27+ or CD27- .
Blood, n=31.

CHAPTER FOUR

4.0 Discussion and Conclusion

4.1 Discussion

The HIV epidemic in South Africa has not only lead to a resurgence of TB but has also impacted on the TB diagnostic approach, by emphasizing the need for assays that do not rely on the detection or culture of acid fast bacilli (5). The present study was undertaken to improve the understanding of *M. tuberculosis* lung specific T cell immune responses and to explore the role of flow cytometry in the immuno-diagnosis of active TB disease in a setting with a high prevalence of HIV and *M. tuberculosis* co-infection.

The study population represented the most diagnostically challenging group who gravely require new diagnostic approaches, that is HIV positive individuals admitted with a clinical suspicion of active TB but who are initial sputum smear AFB negative. A microbiological classification was used to categorise patients as *M. tuberculosis* positive (n = 17 for blood, n = 9 for sputum) and *M. tuberculosis* negative (n = 14 for blood, n = 10 for sputum) on the basis of their subsequent smear microscopy and culture results. The *M. tuberculosis* negative group served as the control group, which is clinically more relevant for evaluation of novel diagnostic approaches than using a healthy control group, in a high TB burden setting such as this.

We optimised a simple four colour flow cytometry panel to identify IFNγ secreting lymphocytes in the blood and sputum of HIV and *M. tuberculosis* co-infected individuals. This study confirmed that *M. tuberculosis* specific IFNγ secreting lymphocytes are concentrated in the lung. The frequency of *M. tuberculosis* specific IFNγ secreting lymphocytes in sputum was higher than that of blood. This finding most likely reflects the

potency of the immune response at the primary site of infection, the lung (9). The most significant finding however, is that the percentage of PPD specific IFNγ secreting sputum lymphocytes is significantly higher in the *M. tuberculosis* negative group than the *M. tuberculosis* positive group. The sensitivity and specificity of this method at a diagnostic threshold of 1.20% to diagnose microbiological proven TB in a high prevalence setting is 78% and 70% respectively, yielding a positive and negative predictive value of 70% and 78% respectively.

The higher percentage of IFNγ secretion in the *M. tuberculosis* negative group was counterintuitive at first, and is contradictory to the findings by Breen et al (9). This contradictory result can be attributed to the differences in the study population, with the Breen study population being drawn from an area of low *M. tuberculosis* prevalence with only three patients having latent infection. Two of the three latently infected individuals, and who were recently infected produced IFNγ responses higher than fifty percent of the active TB group (9) . Of interest, latent TB infection has been shown in other studies (47) (48) to produce higher percentages of *M. tuberculosis* specific IFNγ secreting lymphocytes than active disease. A recent publication (48) analysed the immune responses from T lymphocytes in individuals with active and latent *M. tuberculosis* infection and concluded that patients with latent TB had polyfunctional T lymphocyte immune responses, including IFNγ production but T lymphocytes from patients with active TB had single TNFα positivity. This shows that patients with latent TB infection produced IFNγ but patients with active disease did not, they only produced TNFα. The same conclusion was drawn by a similar study (47) who found reduced PPD specific T lymphocytes in active TB when compared to latent infection.

Accurate data on the prevalence of latent TB in South Africa is not available but ranges from 55 to 88% according to data published by local investigators (13) (49) (50). This equates to a possible large proportion of latently infected individuals in the *M. tuberculosis* negative group of this study. Hence, the Breen study (9) compared active disease largely to unexposed individuals, and the current study compared active *M. tuberculosis* to a mixed group, which is likely to comprise largely of latently infected individuals. We believe that these latently infected individuals in the *M. tuberculosis* negative group, of this current study, are responsible for the high IFNγ secretion noted after stimulation with PPD.

We showed that PPD and ESAT6 were of similar ability to induce IFNγ secretion in blood and though both antigens induced significantly more IFNγ in the *M. tuberculosis* positive group, only PPD reached this significance in the *M. tuberculosis* negative group. Sputum IFNγ responses to either antigen were higher in sputum than in blood, this difference reached significance for PPD in the *M. tuberculosis* negative and ESAT6 in the *M. tuberculosis* positive group. The implications of these findings are not clear and may simply reflect small sample size or these differences highlight the complexity of measuring the immune responses in settings where there is a high prevalence of both *M. tuberculosis* and MOTTs as well as the routine practise of BCG vaccination. The choice of antigen to be used in future studies therefore requires careful consideration and should be based on influencing factors such as the use of PPD when analysing blood based samples in areas where administration of the BCG vaccine is routine practise.

M. tuberculosis specific CD27- CD4+ T lymphocytes, as a marker of memory CD4+ T lymphocytes, has gained recognition as quick blood based tool to diagnose active TB (8). Sreitz et al (45) showed that PPD specific IFNγ production from blood CD27- CD4+ T lymphocytes served as a marker for active *M. tuberculosis* disease (sensitivity 100% and specificity of 85%). The current study however, did not detect any difference in antigen specific IFNγ production from blood CD27- CD4+ T lymphocytes between the *M. tuberculosis* positive and *M. tuberculosis* negative populations that has been previously described (45) (51). The possible explanation for this discrepancy is that the population in previous studies differed from the current study. All participants in the current study are HIV positive, and HIV infection can alter CD27 expression and subsequent proliferation of antigen specific CD4+ T lymphocytes (52). In addition, HIV and *M. tuberculosis* co-infection is associated with paucibacillary disease (25) and the loss of CD27 expression on CD4+ T lymphocytes may be linked to the bacterial burden of the disease in the lung (45). Furthermore, a recent study showed that the reduced expression of CD27 on *M. tuberculosis* specific CD4+ T lymphocytes correlates better with persistent active TB rather than newly diagnosed active TB (51). All participants in this study were individuals with newly diagnosed active TB, rather than persistent active disease. Our patient cohort represents the type of patient commonly posing diagnostic difficulties in our setting, and is a group of individuals at risk of increased morbidity and mortality as well as at risk of spreading the infection to other susceptible individuals (4) (5) (6).

An interesting finding is that in the *M. tuberculosis* positive group, ESAT6 specific CD27+ CD4+ T lymphocytes produced significantly more IFNγ than the corresponding CD27- population. This finding is similar to other studies performed in humans but contrary to the murine model (51). IFNγ secretion from CD27 subsets after stimulation with PPD did not

show similar significant differences, which probably highlights the cross reactivity between the BCG vaccine and PPD antigens. A similar study (45) showed that the BCG vaccine alone, in unexposed individuals resulted in 35% CD27- PPD specific CD4+ T lymphocytes. These findings show that the inclusion of CD27, as a marker of memory T cells, into a flow cytometric panel for IFNγ production does not aid TB diagnosis of newly acquired active *M. tuberculosis* infection in a high prevalence setting.

The limitations of this study need to be addressed. The effect of LTBI in the *M. tuberculosis* negative group cannot be quantified, as latency was not diagnosed. Due to ethical and financial constraints, in this cross-sectional study, the author would have been unable to provide Isoniazid prophylaxis to latently infected subjects with appropriate monitoring, and it was therefore decided not to include tests for latency. BCG vaccination status was not documented despite enquiry, as patient self–reporting for a vaccine administered at birth was unreliable. However, BCG vaccination at birth forms part of the South African immunisation schedule implying that most if not all participants were vaccinated. The sample number (n=31 blood; n=19 sputum) was limited due to financial constraints imposed by the high cost of monoclonal antibodies and other reagents. Secondly, all tasks, from obtaining informed consent and collecting samples to preparing and analysing these samples were performed by the author. Sputum sample numbers were further reduced since samples had to be excluded (n = 11) due to quality and technical processing issues. The exclusion of sputum samples was performed independently of the microbiological results. The need to perform a Bartlett score to assess the quality of the sputum samples was overlooked. This would have enabled the exclusion of the unsuitable sputum samples prior to processing. In addition, frequent unstimulated IFNγ secretion was noted in sputum samples and a viability marker was not added to sputum samples to

exclude the effect of dead cells binding non-specifically to the fluorescent labelled monoclonal antibodies. However, most debris was excluded using the FSC-A: FSC-H plot and gating strategy described above. Finally, the sensitivity of smear microscopy and culture, even on ISp, is not 100% hence some of the patients in the *M. tuberculosis* negative group could have been misclassified. The Xpert MTB/RIF (GeneXpert, Cepheid) was not routinely performed at the time of this study, and inclusion of the Xpert MTB/RIF (GeneXpert, Cepheid) would have improved reliability of the sputum results.

The strengths of this study include the study population and sample choice. The study population reflects the group of patients in which new diagnostic approaches are needed, which is HIV positive individuals from a high TB endemic setting who are AFB sputum negative. Furthermore, as explained above the culture negative control group comprised a clinically more relevant group than using healthy individuals. The use of both blood and sputum samples enabled the comparison of the immune response at these two immunologically distinct compartments.

4.2 Conclusion

We have shown that in HIV *M. tuberculosis* co-infected individuals, immune responses in ISp samples can be quantified using flow cytometry techniques. Lung responses showed differences between the *M. tuberculosis* positive group and the *M. tuberculosis* negative group, while these findings were not reproduced in blood even with inclusion of the memory marker, CD27.

Sputum samples offer the following advantages over blood samples, that is, the proportion of *M. tuberculosis* specific IFNγ secreting lymphocytes is higher, the sample is less affected by BCG vaccination and the sample can be split for microbiological assessment. Sputum induction is a simple non-invasive procedure that can easily be performed in an out-patient department. However similar to the recommendations made by a study that used the immunospot system to evaluate IFNγ responses in induced sputum (53), we believe that measuring immune responses in sputum based assays is unlikely to be useful in the routine diagnostic setting. The impracticalities of this method include the logistical difficulties involved in processing sputum for flow cytometry analysis, as samples need to be processed within two hours and despite meeting this requirement often display a paucity of cellular content. Furthermore, the newly introduced molecular testing methods offer a more robust, less labour intensive system with the added advantage of drug susceptibility testing with comparable sensitivity and specificity for sputum smear negative samples.

Nonetheless, these results will assist in increasing the understanding of the lung compartment-specific immune response to TB in high prevalence settings and our understanding of TB pathogenesis and the spectrum of TB specific T lymphocyte immune responses as one progresses from latent to active disease. The finding of lower IFNγ secretion in the lung compartment in the *M. tuberculosis* positive group after stimulation

with PPD in comparison with the *M. tuberculosis* negative group was counter-intuitive, as most blood based assays and a handful of studies analyzing lung specific immune responses have reported increased IFNγ secretion in the TB group in comparison with the control group. This is likely due to a difference in the control group used, with our control group likely having a high prevalence of latent TB and prior studies largely being conducted in areas of low latent TB prevalence. The finding of lower *M. tuberculosis* specific IFNγ secretion in active TB is supported by the findings of Harari (47) and Streitz (48) et al, both of whom also compared the T lymphocyte immune responses in active TB to that of latent infection. These studies however used blood rather than lung based samples. It has been proposed (48) that the reduced IFNγ secreting lymphocytes in the blood of patients with active TB represents either a diminished response or trafficking of these IFNγ secreting cells from the blood to the lung. Our finding supports the theory that as an individual progresses from latent infection to active TB the ability to produce IFNγ is diminished.

We hypothesise from these findings that the percentage of *M. tuberculosis* specific IFNγ secreting lymphocytes is a spectrum ranging from low in unexposed individuals to intermediate in active disease to high in latent TB infection, and that the reduced IFNγ secretion in active disease when compared to latent infection reflects the loss of protection as one progresses from latent to active TB. Furthermore, our findings highlight the importance of studying compartment specific immune responses rather than blood alone, and we recommend more studies in high TB prevalent settings adopt this approach to elucidate the spectrum of IFNγ secretion from latency to active TB.

REFERENCES

1. Doherty TM and Andersen P. Vaccines for tuberculosis: novel concepts and recent progress. Clin Microbiol Rev. 2005 Oct;18(4):687-702.

2. Corbett EL, Watt CJ, Walker N, Maher D, Williams BG, Raviglione MC, et al. The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. Arch Intern Med. 2003 May 12;163(9):1009-21.

3. Keeler E, Perkins MD, Small P, Hanson C, Reed S, Cunningham J, et al. Reducing the global burden of tuberculosis: the contribution of improved diagnostics. Nature. 2006 Nov 23;444 Suppl 1:49-57.

4. Nyendak MR, Lewinsohn DA and Lewinsohn DM. New diagnostic methods for tuberculosis. Curr Opin Infect Dis. 2009 Apr;22(2):174-82.

5. El-Sadr WM and Tsiouris SJ. HIV-associated tuberculosis: diagnostic and treatment challenges. Semin Respir Crit Care Med. 2008 Oct;29(5):525-31.

6. Naidoo K, Padayatchi N and Abdool Karim Q. HIV-Associated Tuberculosis. Clin Dev Immunol. 2011;2011.

7. Anandaiah A, Dheda K, Keane J, Koziel H, Moore DA and Patel NR. Novel Developments in the Epidemic of HIV and TB Co-Infection. Am J Respir Crit Care Med. 2010 Dec 22.

8. Janossy G, Barry SM, Breen RA, Hardy GA, Lipman M and Kern F. The role of flow cytometry in the interferon-gamma-based diagnosis of active tuberculosis and its coinfection with HIV-1--A technically oriented review. Cytometry B Clin Cytom. 2008;74 Suppl 1:S141-51.

9. Breen RA, Hardy GA, Perrin FM, Lear S, Kinloch S, Smith CJ, et al. Rapid diagnosis of smear-negative tuberculosis using immunology and microbiology with induced sputum in HIV-infected and uninfected individuals. PLoS One. 2007;2(12):e1335.

10. Kaufmann SH and Parida SK. Tuberculosis in Africa: learning from pathogenesis for biomarker identification. Cell Host Microbe. 2008 Sep 11;4(3):219-28.

11. Pai M and O'Brien R. New diagnostics for latent and active tuberculosis: state of the art and future prospects. Semin Respir Crit Care Med. 2008 Oct;29(5):560-8.

12. Boehme CC, Nabeta P, Hillemann D, Nicol MP, Shenai S, Krapp F, et al. Rapid molecular detection of tuberculosis and rifampin resistance. N Engl J Med. 2010 Sep 9;363(11):1005-15.

13. Wood R, Liang H, Wu H, Middelkoop K, Oni T, Rangaka MX, et al. Changing prevalence of tuberculosis infection with increasing age in high-burden townships in South Africa. Int J Tuberc Lung Dis. 2010 Apr;14(4):406-12.

14. Fuhrmann S, Streitz M and Kern F. How flow cytometry is changing the study of TB immunology and clinical diagnosis. Cytometry A. 2008 Nov;73(11):1100-6.

15. Leung WL, Law KL, Leung VS, Yip CW, Leung CC, Tam CM, et al. Comparison of intracellular cytokine flow cytometry and an enzyme immunoassay for evaluation of cellular immune response to active tuberculosis. Clin Vaccine Immunol. 2009 Mar;16(3):344-51.

16. van Rooyen CaB, AJ. Gamma Interferon assays as a diagnostic tool in tuberculosis infections. The South African Journal of Epidemiology and Infection. 2007;22(4):107-8

17. Karam F, Mbow F, Fletcher H, Senghor CS, Coulibaly KD, LeFevre AM, et al. Sensitivity of IFN-gamma release assay to detect latent tuberculosis infection is retained in HIV-infected patients but dependent on HIV/AIDS progression. PLoS One. 2008;3(1):e1441.

18. Barth RE, Mudrikova T and Hoepelman AI. Interferon-gamma release assays (IGRAs) in high-endemic settings: could they play a role in optimizing global TB

diagnostics? Evaluating the possibilities of using IGRAs to diagnose active TB in a rural African setting. Int J Infect Dis. 2008 Nov;12(6):e1-6.

19. Dheda K, Schwander SK, Zhu B, van Zyl-Smit RN and Zhang Y. The immunology of tuberculosis: from bench to bedside. Respirology. 2010 Apr;15(3):433-50.

20. Morrison J, Pai M and Hopewell PC. Tuberculosis and latent tuberculosis infection in close contacts of people with pulmonary tuberculosis in low-income and middle-income countries: a systematic review and meta-analysis. Lancet Infect Dis. 2008 Jun;8(6):359-68.

21. W L, editor. Review of Medical Microbiology and Immunology. Tenth ed2008

22. Lawn SD, Bangani N, Vogt M, Bekker LG, Badri M, Ntobongwana M, et al. Utility of interferon-gamma ELISPOT assay responses in highly tuberculosis-exposed patients with advanced HIV infection in South Africa. BMC Infect Dis. 2007;7:99.

23. Sester M, Giehl C, McNerney R, Kampmann B, Walzl G, Cuchi P, et al. Challenges and perspectives for improved management of HIV/Mycobacterium tuberculosis co-infection. Eur Respir J. 2010 Dec;36(6):1242-7.

24. Im JS, Kang TJ, Lee SB, Kim CH, Lee SH, Venkataswamy MM, et al. Alteration of the relative levels of iNKT cell subsets is associated with chronic mycobacterial infections. Clin Immunol. 2008 May;127(2):214-24.

25. Raja A. Immunology of tuberculosis. Indian J Med Res. 2004 Oct;120(4):213-32.

26. Abebe F and Bjune G. The protective role of antibody responses during Mycobacterium tuberculosis infection. Clin Exp Immunol. 2009 Aug;157(2):235-43.

27. Voldemeier HM VN, Harris DP, Ivanyi J. Increase in tuberculosis infection in organs of B cell-deficient mice. Clinical and Experimental Immunology. 1996;106:312-6.

28. Maglione PJ, Xu J and Chan J. B cells moderate inflammatory progression and enhance bacterial containment upon pulmonary challenge with Mycobacterium tuberculosis. J Immunol. 2007 Jun 1;178(11):7222-34.

29. Rueda CM, Marin ND, Garcia LF and Rojas M. Characterization of CD4 and CD8 T cells producing IFN-gamma in human latent and active tuberculosis. Tuberculosis (Edinb). 2010 Nov;90(6):346-53.

30. Chen ZW. Immunology of AIDS virus and mycobacterial co-infection. Curr HIV Res. 2004 Oct;2(4):351-5.

31. Sud D, Bigbee C, Flynn JL and Kirschner DE. Contribution of CD8+ T cells to control of Mycobacterium tuberculosis infection. J Immunol. 2006 Apr 1;176(7):4296-314.

32. Ngai P, McCormick S, Small C, Zhang X, Zganiacz A, Aoki N, et al. Gamma interferon responses of CD4 and CD8 T-cell subsets are quantitatively different and independent of each other during pulmonary Mycobacterium bovis BCG infection. Infect Immun. 2007 May;75(5):2244-52.

33. Sutherland JS, Jeffries DJ, Donkor S, Walther B, Hill PC, Adetifa IM, et al. High granulocyte/lymphocyte ratio and paucity of NKT cells defines TB disease in a TB-endemic setting. Tuberculosis (Edinb). 2009 Nov;89(6):398-404.

34. Wanchu A, Dong Y, Sethi S, Myneedu VP, Nadas A, Liu Z, et al. Biomarkers for clinical and incipient tuberculosis: performance in a TB-endemic country. PLoS One. 2008;3(4):e2071.

35. Sester M, Sotgiu G, Lange C, Giehl C, Girardi E, Migliori GB, et al. Interferon-gamma release assays for the diagnosis of active tuberculosis: a systematic review and meta-analysis. Eur Respir J. 2011 Jan;37(1):100-11.

36. Jafari C, Ernst M, Strassburg A, Greinert U, Kalsdorf B, Kirsten D, et al. Local immunodiagnosis of pulmonary tuberculosis by enzyme-linked immunospot. Eur Respir J. 2008 Feb;31(2):261-5.

37. Dheda K, van Zyl Smit R, Badri M and Pai M. T-cell interferon-gamma release assays for the rapid immunodiagnosis of tuberculosis: clinical utility in high-burden vs. low-burden settings. Curr Opin Pulm Med. 2009 May;15(3):188-200.

38. Conde MB, Soares SL, Mello FC, Rezende VM, Almeida LL, Reingold AL, et al. Comparison of sputum induction with fiberoptic bronchoscopy in the diagnosis of tuberculosis: experience at an acquired immune deficiency syndrome reference center in Rio de Janeiro, Brazil. Am J Respir Crit Care Med. 2000 Dec;162(6):2238-40.

39. Mroz RM, Korniluk M, Stasiak-Barmuta A and Chyczewska E. Comparison of induced sputum and bronchoalveolar lavage fluid cell profile during the treatment of pulmonary sarcoidosis. J Physiol Pharmacol. 2007 Nov;58 Suppl 5(Pt 2):445-52.

40. Strassburg A, Jafari C, Ernst M, Lotz W and Lange C. Rapid diagnosis of pulmonary TB by BAL enzyme-linked immunospot assay in an immunocompromised host. Eur Respir J. 2008 May;31(5):1132-5.

41. Barry S, Lipman M, Breen R and Janossy G. Local immunodiagnosis of pulmonary TB: ELISPOT or flow cytometry, PPD or ESAT-6? Eur Respir J. 2008 Aug;32(2):531-2; author reply 2-3.

42. Diel R, Loddenkemper R, Niemann S, Meywald-Walter K and Nienhaus A. Negative and Positive Predictive Value of a Whole-Blood Interferon-{gamma} Release Assay for Developing Active Tuberculosis: An Update. Am J Respir Crit Care Med. 2011 Jan 1;183(1):88-95.

43. Won DI and Park JR. Flow cytometric measurements of TB-specific T cells comparing with QuantiFERON-TB gold. Cytometry B Clin Cytom. 2010 Mar;78(2):71-80.

44. Kobashi Y, Shimizu H, Ohue Y, Mouri K, Obase Y, Miyashita N, et al. Comparison of T-cell interferon-gamma release assays for Mycobacterium tuberculosis-

specific antigens in patients with active and latent tuberculosis. Lung. 2010 Aug;188(4):283-7.

45. Streitz M, Tesfa L, Yildirim V, Yahyazadeh A, Ulrichs T, Lenkei R, et al. Loss of receptor on tuberculin-reactive T-cells marks active pulmonary tuberculosis. PLoS One. 2007;2(1):e735.

46. Lyadova IV, Oberdorf S, Kapina MA, Apt AS, Swain SL and Sayles PC. CD4 T cells producing IFN-gamma in the lungs of mice challenged with mycobacteria express a CD27-negative phenotype. Clin Exp Immunol. 2004 Oct;138(1):21-9.

47. Streitz M, Fuhrmann S, Powell F, Quassem A, Nomura L, Maecker H, et al. Tuberculin-specific T cells are reduced in active pulmonary tuberculosis compared to LTBI or status post BCG vaccination. J Infect Dis. 2011 Feb 1;203(3):378-82.

48. Harari A, Rozot V, Enders FB, Perreau M, Stalder JM, Nicod LP, et al. Dominant TNF-alpha+ Mycobacterium tuberculosis-specific CD4+ T cell responses discriminate between latent infection and active disease. Nat Med. 2011 Mar;17(3):372-6.

49. Mahomed H, Hawkridge T, Verver S, Geiter L, Hatherill M, Abrahams DA, et al. Predictive factors for latent tuberculosis infection among adolescents in a high-burden area in South Africa. Int J Tuberc Lung Dis. 2011 Mar;15(3):331-6.

50. Shanaube K, Hargreaves J, Fielding K, Schaap A, Lawrence KA, Hensen B, et al. Risk factors associated with positive QuantiFERON-TB Gold In-Tube and tuberculin skin tests results in Zambia and South Africa. PLoS One. 2011;6(4):e18206.

51. Jiang J, Wang X, Cao Z, Liu Y, Dong M, Tong A, et al. Reduced CD27 expression on antigen-specific CD4+ T cells correlates with persistent active tuberculosis. J Clin Immunol. 2010 Jul;30(4):566-73.

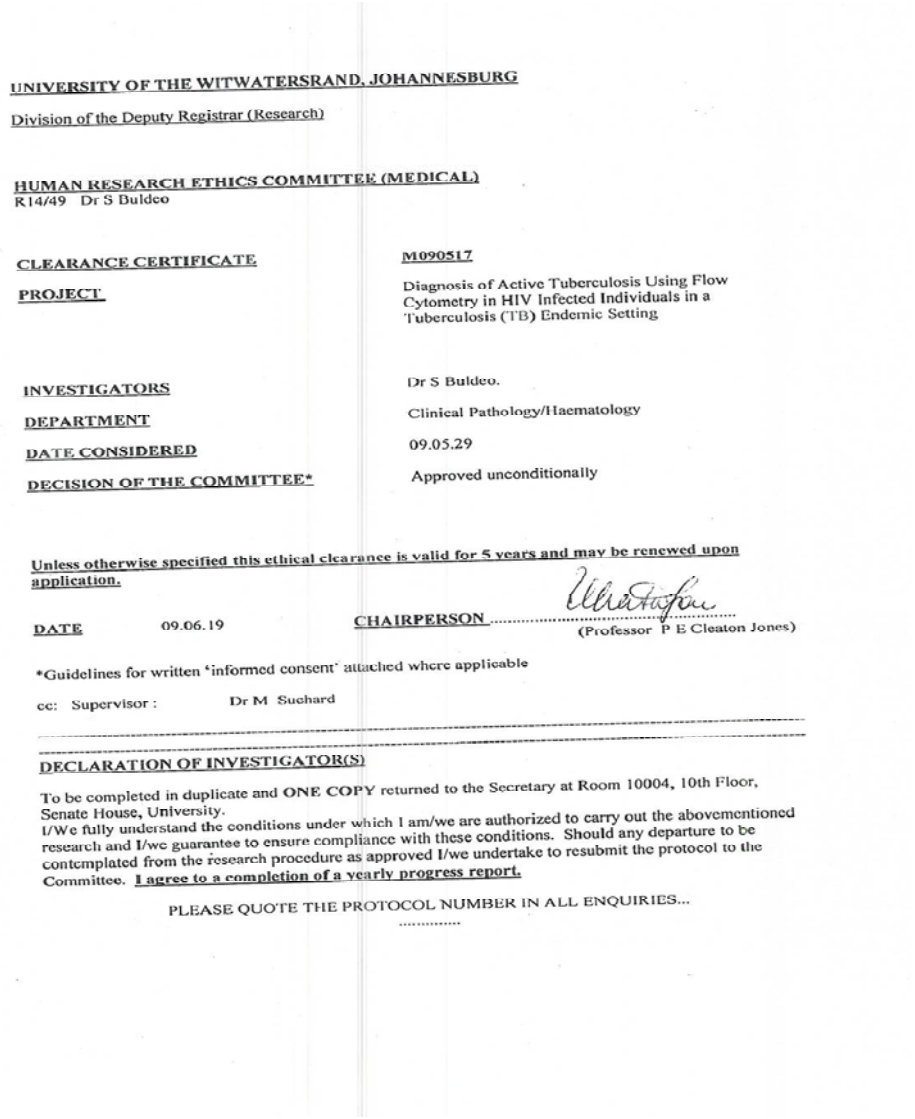
52. Luciano AA, Lederman MM, Valentin-Torres A, Bazdar DA and Sieg SF. Impaired induction of CD27 and CD28 predicts naive CD4 T cell proliferation defects in HIV disease. J Immunol. 2007 Sep 15;179(6):3543-9.

53. Cashmore TJ, Peter JG, van Zyl-Smit RN, Semple PL, Maredza A, Meldau R, et al. Feasibility and diagnostic utility of antigen-specific interferon-gamma responses for rapid immunodiagnosis of tuberculosis using induced sputum. PLoS One. 2010;5(4):e10389.

Web references:

54. World Health Organization. www.who.int/tb/data. Accessed on 23 March 2012.

APPENDIX 1: Ethics clearance certificate



UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Dr S Buldeo

CLEARANCE CERTIFICATE	M090517
PROJECT	Diagnosis of Active Tuberculosis Using Flow Cytometry in HIV Infected Individuals in a Tuberculosis (TB) Endemic Setting
INVESTIGATORS	Dr S Buldeo.
DEPARTMENT	Clinical Pathology/Haematology
DATE CONSIDERED	09.05.29
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 09.06.19 CHAIRPERSON .....
(Professor P E Cleaton Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Dr M Suchard

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and ONE COPY returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. I agree to a completion of a yearly progress report.

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

APPENDIX 2: Patient Information Sheet

STUDY NUMBER:

STUDY TITLE:
Diagnosis of active tuberculosis using flow cytometry in HIV infected individuals in a tuberculosis (TB) endemic setting

INVESTIGATOR: Dr S Buldeo

INSTITUTION: NHLS

DAYTIME AND AFTER HOURS TELEPHONE NUMBER(S):
Dr Suvarna Buldeo 011 4898432/ 0833053665

INTRODUCTION:
Good day, my name is Dr Suvarna Buldeo, I am a doctor currently specialising in Clinical Pathology and am currently working in the Haematology department at Johannesburg General Hospital. I would like to invite you to consider participating in a research study, entitled "Immunodiagnosis of active tuberculosis (TB)using Induced sputum and blood in HIV infected individuals in a TB endemic setting".

- You have presented with a cough, fever and/or night sweats. This could possibly be TB.
- TB is treatable and curable but patients need to take anti-TB drugs for six months or longer. Many complications can occur if the diagnosis is not made promptly and treatment initiated timeously.
- Diagnosis of TB is difficult and takes a long time. This is especially true in HIV positive people as they do not present with the classical signs and symptoms. The purpose of this study is to determine if TB diagnosis can be improved.
- This study will use induced sputum and a blood sample to aid in the rapid diagnosis of TB. I will shortly explain the procedure for induced sputum to you. We are investigating a new assay using these samples. It is an assay using that will use the techniques of flow cytometry to evaluate the immune response to TB. This may assist in determining whether a person has TB or not, sooner than the tests that are currently available. These samples will also be sent for TB culture, which is the gold standard for TB diagnosis. The culture results will therefore be used to assess if a person definitively has TB.

LENGTH OF THE STUDY AND NUMBER OF PARTICIPANTS:

- The study will be performed from November this year to the end of next year.
- Approximately 50 participants will be recruited.
- The participants will be 18 years and older.
- The total amount of time required for your participation in this study will be a maximum of six hours today and another short visit in eight weeks.
- You will be asked to re-visit me in eight weeks for the results of your tests (microscopy and culture of induced sputum).

PROCEDURES:

- If you agree to take part in this study, you will first be asked questions and examined to see if you qualify for this study.
- Please note that one tube of blood and an induced sputum sample are required. This may result in minor discomfort but should not cause any pain.
- Venipunctures (i.e. drawing blood) are normally done as part of routine medical care and present a slight risk of discomfort. Drawing blood may result in faintness, inflammation of the vein, pain, bruising or bleeding at the puncture site. There is also a slight possibility of infection. Your protection is that experienced personnel perform the procedures under sterile conditions. A total of 5 ml of blood (i.e. 2 teaspoons) will be collected over the course of the entire study.
- Induced sputum is a procedure that will be carried out over 20 minutes. It is similar to a nebulisation. You will be asked to breathe in salt water (saline) and then to cough into a bottle. This method will provide a better quality sample than merely coughing into the bottle. Some patients experience discomfort during the procedure. I will assess your lung function before and during the procedure. If there is a 15% or greater loss from baseline the procedure will be stopped. The procedure will also be stopped if you experience severe chest discomfort. A few cases of shortness of breath have occurred during the procedure. These complications occurred in people with underlying lung pathology and that is why these patients have to be excluded from the study (refer to exclusion criteria). However we will be equipped with emergency medication (oxygen masks and bronchodilators) to assist you immediately.
- Your blood and sputum will be sent for **TB** microscopy and culture as well as the new test under investigation in this study that is flow cytometry. At the end of the study left-over blood and/or sputum may be stored for future testing. This may include genetic testing related to HIV and/or TB and ethics approval will be sought prior to use.

BENEFITS:

- The potential benefit from your participation in this study is that the diagnosis of TB (which may have been missed by conventional methods) may be confirmed, as a higher quality sample (i.e., induced sputum) will be used. This may also assist in the diagnosis being made sooner than current diagnostic tests. Hence, treatment will be initiated sooner than with the conventional methods.
- However, you may not benefit from this study.
- Your participation in this study will also contribute to medical knowledge that may help other patients.

RISKS

- Except for the risks associated with the procedure (as mentioned above), no other risks are involved.

RIGHTS AS A PARTICIPANT IN THIS STUDY:

Voluntary:

- Your participation in this study is entirely voluntary and you can decline to participate, or stop at any time, without stating any reason.

Withdrawal:

- Your withdrawal will not affect your access to other medical care.
- I retain the right to withdraw you from the study if it is considered to be in your best interest.

REIMBURSEMENT FOR STUDY PARTICIPATION:

- You will not be paid to participate in this study but you will be reimbursed with R100, 00 for the transport costs incurred. This will be paid to you at your follow up visit.

ETHICAL APPROVAL:

- This clinical study protocol has been submitted to the University of the Witwatersrand, **Human Research Ethics Committee (HREC)** and that committee has granted written approval.
- The study has been structured in accordance with the **Declaration of Helsinki** (last updated: October 2008), which deals with the recommendations guiding doctors in biomedical research involving human participants. A copy may be obtained from me should you wish to review it. I do not have any financial or personal interests that may bias my actions. If you want any information regarding your **rights as a research participant,**

or complaints regarding this research study, you may contact Prof. Cleaton-Jones, Chairperson of the University of the Witwatersrand, Human Research Ethics Committee (HREC), which is an independent committee established to help protect the rights of research participants at (011) 717 2301.

CONFIDENTIALITY:

- All information obtained during the course of this study, including hospital records, personal data and research data will be kept strictly confidential. Data that may be reported in scientific journals will not include any information that identifies you as a participant in this study.
- The information might also be inspected by the University of the Witwatersrand, Human Research Ethics Committee (HREC), as well as your personal doctor.
- These records will be utilised by them only in connection with carrying out their obligations relating to this clinical study.
- Any information uncovered regarding your test results or state of health as a result of your participation in this study will be held in strict confidence. You or your doctor will be informed of any finding of importance to your health but confidential information will not be disclosed to any third party without your written permission. The only exception to this rule will be cases of communicable diseases where a legal duty of notification of the Department of Health exists. In this case, you will be informed of my intent to disclose such information to the authorised state agency.

PARTICIPANT QUESTIONS *?:*

If you have any questions please ask and I will do my best to answer them. If you have additional questions in the future you can reach me, Dr Suvarna Buldeo on 0833053665/011 4898432.

APPENDIX 3: Informed Consent

I hereby confirm that I have been informed by the study doctor, Dr S Buldeo, about the nature, conduct, benefits and risks of the clinical study entitled "Diagnosis of active TB using flow cytometry in HIV infected individuals in a TB endemic setting".

- I have also received, read and understood the above written information (Participant Information Sheet) regarding the clinical study.
- I am aware that the results of the study, including personal details regarding my sex, age, date of birth, initials and diagnosis will be anonymously processed into a study report.
- I may, at any stage, without prejudice, withdraw my consent and participation in the study.
- I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study.

PARTICIPANT:

Printed Name	Signature / Mark or Thumbprint	Date and Time

I, Dr Suvarna Buldeo herewith confirm that the above participant has been fully informed about the nature, conduct and risks of the above study.

STUDY DOCTOR:

Printed Name	**Signature**	**Date and Time**

TRANSLATOR / OTHER PERSON EXPLAINING INFORMED CONSENT……(DESIGNATION):

Printed Name	**Signature**	**Date and Time**

WITNESS (If applicable)**:**

Printed Name	*Signature*	*Date and Time*

I hereby confirm that I have been informed by the study doctor, Dr S Buldeo that at the end of the study left-over blood and/or sputum may be stored for future testing. This may include genetic testing related to HIV and/or TB and ethics approval will be sought prior to use.

I _____

_____

Give permission for my blood and /or induced sputum to be stored for future testing relating to TB and HIV research including genetic research. I understand that this permission for future research on my stored sample will be sought from the Wits Human Research Ethics Committee.

PARTICIPANT:

Printed Name	Signature / Mark or Thumbprint	Date and Time

STUDY DOCTOR: _____

TRANSLATOR: _____

APPENDIX 4: Clinical Data Sheet

PATIENT NAME: **Date:**

DOB: **AGE:**

MEDICAL ILLNESSES:

RESPIRATORY SYMPTOMS:

RESPIRATORY SIGNS:

IS EXTRA-PULMONARY TB SUSPECTED:
Site:
Reason for suspicion:

BCG VACCINATION:

MEDICATION:

CD4 count:

PREVIOUS MICROSCOPY FOR TB:
Date: _____ Result:
Date: _____ Result:
Date: _____ Result:

PREVIOUS TB:
Duration of treatment:

Productive/ non: _____

PLEASE NOTE WHETHER YOUR PATIENT HAS ANY OF THE FOLLOWING EXCLUSION CRITERIA:

General:	**Yes**	**No**
Smear positive TB		
Current TB therapy		
Current immunosuppressive use		

Suitability for Induced Sputum:		
Asthma		
COPD		
Recent life-threatening illness		

APPENDIX 5: Intracellular Staining Protocol for Blood and ISp

Blood and sputum processing

Blood	Induced Sputum
• Add 4.5 ml warm Histopaque to 15ml conical tube. Invert ACD tube 3x and add blood.	
• Centrifuge @ 800g for 20min with the brake off.	• Split sample for microbiology if necessary. Add immunological aliquot to 15ml conical tube.
	• To immunological aliquot add equal volumes of Sputolysin
	• Rock for 20min at RT.
• Transfer buffy coat to 15ml conical tube. Combine both blood tubes. Store serum.	• Filter the suspension with the 100um strainer into 50ml conical tube. Empty 50ml back into 15ml conical tube.
• Wash with PBS and 0.1% FBS. Centrifuge at 400g for 10min – brake on.	• Wash with PBS and 0.1%FBS. Centrifuge at 400g for 10mins.
• Decant and resuspend.	• Decant supernatant and resuspend.
• Repeat wash as above.	• Add 1500ul of R10
	• Remove 20ul and perform cell count and viability
• Add 2ml R10. Perform cell count and viability.	• Dilute if possible to obtain 1 million cells per tube.

Day 1 – PBMC Stimulation

1. Add 0.5ml cells to labelled polypropylene tubes (NB: ensure that tubes have lids & that proper "cloudy" tubes are used for stimulation). *Keep any cells that may be left over – to set FSC and SSC.*
2. Add 0.25ml co-stimulation mix per tube (Reagent 1). Make up co-stim master mix according to number of tubes in experiment. This contains Brefeldin A.
3. Add 0.25ml peptide stimulation mix to appropriate tubes (Reagent 2).
4. Add 0.25ml SEB mix to SEB stimulated tubes (Reagent 3). Always add SEB last as prone to contaminating other tubes. Use a designated pipette.
5. Add 0.25ml R10 to co-stimulated only tubes.
6. Gently shake tubes to mix and incubate at 5%CO_2 at 37^0C with cap loosened and tubes at an angle for 16 hrs. If not acquiring immediately after incubation the samples must be refrigerated at 4^0C.

Day 2 – Surface & Intracellular Staining

1. Remove cells from incubator and record total stimulation time.
2. Add 50µL of FastImmune EDTA to each tube and vortex for 3 sec.
   Prepare surface stain mix and ICS mix.
3. Incubate at room temperature (18-25^{0}C) for 15min in the dark.
4. Add 2.5ml 0.5% Wash Buffer (Reagent 4) (or BD CellWash). (NB: amount added does not have to be accurate).
5. Centrifuge tubes at 300g for 5min.
6. Decant supernatant and gently blot on paper towel for 1 second.
7. Shake tubes to mix cells. Approximately 50µl of fluid remains.
8. Add 150µL (or 100µL depending on surface stain mix protocol – **see Experiment-specific Worksheet**) of prepared surface stain mix to each tube and mix gently (Reagent 5). Always work from a master-mix to maintain consistency.
9. Cover with foil and incubate at room temperature in the dark for 20min.
   Prepare wash buffer.
10. Add 2.5ml 0.5% Wash Buffer (or BD Cell Wash) per tube.
11. Centrifuge at 300g for 5min.
12. Decant supernatant without blotting. Resuspend.
13. Add 250µL BD Cytofix/Cytoperm to each tube & gently mix. Use neat as it is pre-diluted.
14. Cover with foil and incubate at room temperature in the dark for 20min.
    Permeabilisation is toxic to cells, hence the time can be shortened but not prolonged.
    Prepare comp tubes.
15. Add 2.5ml 1X BD Perm/Wash buffer to each tube (Appendix 6). *Make up wash buffer according to number of tubes and number of total washes - i.e. three).*
16. Centrifuge at 500g for 8min (NB: increased centrifugation following permeabilization).
17. Decant supernatant without blotting & resuspend.
18. Repeat wash steps (22 and 23). Two washes are essential to remove the Cytofix/Cytoperm.
19. Decant supernatant and blot as in 13. Resuspend.

From this point forward, take special care to keep samples cold and protect from light.

20. Add 150ul (or 100µL depending on surface stain mix protocol – see Experiment-specific Worksheet & Reagent 7) of prepared ICS mix and gently mix.
21. Cover tubes with foil and incubate at 4°C for 45min.
    Run CST, 1X and comp tubes.
22. Add 2.5ml 1X BD Perm/Wash buffer to each tube.
23. Centrifuge at 500g for 8min.
24. Decant supernatant and gently blot on paper towel for 1 second. Resuspend.

25. Add 300µl 1% paraformaldehyde "Fix" (Reagent 8) (or BD CellFix) to each tube.
26. Store samples at 4^0C until acquisition. Acquire within 6hrs of sample preparation.

COMPENSATION:

1. Label tubes appropriately.
2. Vortex comp beads.
3. Add 1 drop Positive beads (Mouse Ig kappa) to each tube.
4. Add 1 drop Negative beads (Mouse Ig kappa) to negative tube.
5. Add corresponding Ab-PBS mix with a final volume of 50ul.
6. Incubate in dark for 20min.
7. Wash with PBS: 300g for 10min.
8. Decant.
9. Add 200ul 1% Cell Fix.

Reagents

1. Co-stimulation Mix 1

CD28 (1mg/ml) – 1µl
CD49d (1mg/ml) – 1µl
} Make up volume to 125ul with R10.

2. Co stimulation mix 2

Brefeldin A (10mg/ml) – 0.5µl Make up volume to 125µl per reaction with R10

3. Peptide stimulation Mix (PPD, ESAT6 mix concentration pending)

Dilute peptides in R10 to give a volume of 250µl with a 10µg/ml final concentration.

4. SEB Mix

SEB (1mg/ml): Add 1µl to 249ul R10

5. 0.5% Wash Buffer (Gibco – cat: 481819)

Add 5ml Fetal Bovine Serum to 495ml Phosphate buffer saline

6. Surface Stain Mix

Add antibodies for surface staining (see reagent template worksheet) to wash buffer to make up to final volume of 100µl per test. CD3 is downregulated and should be added at ICS step.

7. 1X BD Cytofix/Cyoperm Buffer: Catalogue 554715

Add 1ml 10X Perm/Wash concentrate buffer to 9ml distilled water.

Cytofix/Cytoperm (Fix/Perm) is used neat.
N*ote:* It is important that the BD Perm/Wash™ Buffer be used for dilution of anti cytokine antibodies, rather than a standard staining buffer, in order to maintain cells in a permeabilized state for ICS.

8. Intracellular Stain (ICS) Mix

Add antibodies for intracellular staining (volumes determined by antibody titration) to wash buffer (BD wash buffer as per cytofix/cytoperm) to make up a final volume of 150µl per test.

9. 1% Paraformaldehyde "Fix"

Dilute 37% formalin 1:37 with PBS to give a 1% solution.

10. R10

Add 50ml FBS to 440ml RPMI plus 5ml antibiotics (penicillin/streptomycin/glutathione).

APPENDIX 6: Reagent template

- Each patient will have eight tubes: Blood (PPD/ ESAT6/SEB and unstim). ISp (PPD/ ESAT6/SEB and unstim)
- One positive control will be performed per run

DATE:_____

NAME	HOSPITAL NUMBER	OTHER DETAILS
1.		
2.		
3.		
4.		
3.		
6.		

SURFACE STAIN MIX

FLOUROPHORE	SURFACE BIOMARKER	TITRATION VOLUME	NO OF TUBES	TOTAL
PerCP Cy3.5	CD4	15 ul		
APC	CD27	10 ul		
WASH BUFFER		75ul		
TOTAL		**100UL**		

ICS MIX

FLOUROPHORE	SURFACE BIOMARKER	TITRATION VOLUME	NO OF TUBES	TOTAL
PE	IFNγ	15 ul		
FITC	CD3	15 ul		
WASH BUFFER		70 ul		
TOTAL		**100ul**		

BD WASH BUFFER

WASH BUFFER	DH2O
1ml	9ml

- 2.5ml of diluted wash buffer required per tube per wash.
- Therefore total wash buffer required = 2.5ml x 3 washes x no of tubes.
- Eg. 2.5 x 3 x 6 tubes = 45 ml wash buffer required in total
- Using ratio of 1:9 … 5ml WB : 45ml dH20

PPD and ESAT6

- PPD and ESAT6 are aliquoted into concentrations of 100ug/ml.
- 10 ug/ml are required for the assay.
- Using the equation: C1V1 = C2V2

  (100ug/ml) V1 = (10ug/ml)(1000ul)

  V1 = 100ul

- **hence 100ul of the aliquot is required per tube for the assay**.
- **PPD and ESAT6 are not added to the co-stim tube which serves as the negative control.**

CO-STIM MIX (required for all tubes)

Co-stim	Volume per tube	No of Tubes	Total
CD28 (1mg/ml)	1ul		
CD49d (1mg/ml)	1ul		
Brefeldin A (10mg/ml)	0.5ul		
R10	247.5ul		
Total	**250ul**		

CELL COUNT AND VIABILITY

PATIENT	AVERAGE CELLS /SQUARE	VIABILITY (%)	RSULTS
1.			
2.			
3.			
4.			
3.			
6.			

Haemacytometer Counts	**Turk Cell Count**	**Cell Count (Viable)**	**Cell Count (non-viable)**
Square #1			
Square #2			
Square #3			
Square #4			
Average # Cells / Square			
Cell Concentration (cell/ml) = (Av. # cells/square) x (10,000) x 2 (dilution factor) x % viability			
Viable Cell Concentration (cell/ml) = (Av. # viable cells/square) x (10,000) x 2 (dilution factor)			
% Viability = (Av. # viable cells per square / Av. # total cells per square) x (100)			
Total Number of Viable Cells = (viable cell concentration) x (total volume (in ml))			

Comments:

Reviewed By:_____ Signature:_____

Date (ddmmmyy):_____

NOTE: If the cell count is less than 25 or greater than 100 cells per square, the accuracy of the cell count may be compromised. If the total number of cells per square is greater than 100, increase the volume of the original cell suspension to get the number of cells/square to > 25 and < 100 cells/square. Prepare a new cell/dye suspension and repeat the count. If the total number of cells is less than 25, re-pellet the PBMCs and resuspend in a smaller volume to get the number of cells to > 25 and < 100 cells/square. Prepare a new cell/dye suspension and repeat the count. Take note of the new cell volumes.