Optimisation of a whole blood flow cytometry assay to aid in the diagnosis of Tuberculosis by detecting intracellular cytokines released by $CD4^+$ T-cells.

By

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Abstract

Background: South Africa (SA) sees 8 million new Tuberculosis (TB) cases each year and has a significant problem with Human Immunodeficiency Virus (HIV) and TB co-infection. Latent TB infection (LTBI) is described in persons infected with *mycobacterium tuberculosis (M.tb)* but shows no signs and symptoms of active disease. HIV^+ individuals with LTBI can develop active TB infection more readily than that of HIV^- individuals. Gold standard methods for diagnosing active disease have been criticized for, among other things, their lengthy turnaround times. Currently there is no gold standard for the diagnosis of LTBI. Flow cytometry allows one to measure cytokine responses in $CD4^+$ T-cells following overnight stimulation with TB antigens ESAT-6 and CFP-10 (E/C). Studying these cytokine expression patterns will make it possible to classify patients into active disease vs. LTBI.

Methods:A total of 18 TB^+ patients which included 6 HIV^+patients, were recruited from Tygerberg Hospital, Western Cape. A whole blood no-centrifuge intracellular flow cytometry assay was optimised to study the cytokine expression patterns in $CD4^+$ T-cells that have been stimulated with TB antigens and Staphylococcus Enterotoxin B (SEB), following an 18 hour overnight incubation. $CD3^+CD4^+$ T-cells were delineated into the following subsets: naïve (T_N) ($CD45RO^-CD27^+$), central memory (T_{CM}) ($CD45RO^+CD27^+$),effector memory (T_{EM})($CD45RO^+CD27^-$) and terminally differentiated effector memory cells (T_{DEM}) ($CD45RO^-CD27^-$). The expression patterns and effect of stimulation on cytokines IFN-γ and TNF-α as well as T-cell exhaustion marker TIM3, was assessed.

Results: This study has demonstrated higher levels of IFN-γ expression in the control group compared to that of the TB positive patients (median %IFN-γ 2.960 ± 3.51 versus patient 2.370 ± 2.07; p=0.2800). TNF-α had higher expression in the patient group compared to the control subjects (median %TNF-α 2.415 ± 2.60 versus control 1.340 ± 1.86; p=0.1729). Dual expression of cytokines was almost similar in the two groups (control median % $IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ 0.5400 ± 0.36 versus patient 0.8550 ± 0.60; p=0.3961). TIM3 expression was not significantly different between the four T-cell subsets (median T_N 0.0750 ± 1.89, T_{CM} 0.3400 ± 4.28, T_{EM} 0.0850 ± 2.73, T_{DEM} 0.1600 ± 1.93; p= 0.5877). When comparing the subset distribution in the patient group, T_N cells were the most abundant (median 47.48 ± 20.96) followed by T_{EM} cells (median 21.92 ± 13.25), T_{DEM} cells (median 13.02 ± 20.13) and finally T_{CM} cells (median 11.51 ± 8.62). These results showed a significant difference in expression between the four groups (p=<0.0001).

Conclusion: Through careful titration of antibodies and relevant optimisation steps, we established a flow cytometry assay that may be used to study cytokine patterns in TB patients. The increased TNF-α only expression in the patient group is suggestive of active TB and the increased IFN-γ in the control group could indicate BCG vaccination. TIM3 would be a useful marker in a larger HIV^+ cohort of patients as this will allow identification of functionally exhausted T-cells. In SA, HIV

prevalence is rising and this assay proves its suitability by using minimal volumes of whole blood rather than sputum. By generating intracellular cytokine profiles one would be able to distinguish between active and LTBI which would aid in treatment management of patients.

Opsomming

Agtergrond: Suid-Afrika (SA) het 8000000 nuwe Tuberkulose (TB) gevalle elke jaar en het 'n groot probleem met MIV en TB mede-infeksie. Latente TB-infeksie (LTBI) word beskryf in persone wat besmet is met Mycobacterium tuberculosis (M.tb), maar toon geen tekens en simptome van 'n aktiewe siekte. MIV^+ individue met LTBI kan aktiewe TB-infeksie meer geredelik as dié van MIV^-individue ontwikkel. Goud standaard metodes vir die diagnose van aktiewe siekte is gekritiseer vir, onder andere, hul lang omkeertye. Daar is tans geen goue standaard vir die diagnose van LTBI. Vloeisitometrie laat mens toe om sitokien uitdrukking in $CD4^+$ T-selle te meet na oornag stimulasie met TB antigene ESAT-6 en CFP-10 (E / C). Die bestudering van hierdie sitokien uitdrukking patrone sal dit moontlik maak om pasiënte te klassifiseer as aktiewe siekte of LTBI.

Metodes: 'n Totaal van 18 TB pasiënte wat 6 MIV^+ pasiënte insluit, is gewerf uit die Tygerberg-hospitaal, Wes-Kaap. 'N Heel bloedgeen-centrifuge intrasellulêre vloeisitometrie toets is geoptimaliseer om die sitokien uitdrukking patrone in $CD4^+$ T-selle wat reeds gestimuleer word met TB antigeneen Staphylococcus enterotoksien B (SEB), na aanleiding van 'n 18 uur oornag inkubasie te bestudeer. $CD3^+CD4^+$ T-selle is afgebaken in die volgende onderafdelings: naïef (T_N) ($CD45RO^-CD27^+$), sentralegeheue (T_{CM}) ($CD45RO^+CD27^+$), effektorgeheue (T_{EM}) ($CD45RO^+CD27^-$) enterminaal gedifferensieerde effektorgeheueselle (T_{DEM}) ($CD45RO^-CD27^-$). Die uitdrukkings patrone en effek van stimulasie op sitokiene IFN-γ en TNF-α asook T-seluitputtingmerker TIM3, is bestudeer.

Resultate: Hierdie studie het hoër vlakke van IFN-γ uitdrukking getoon in die kontrole groep in vergelyking met dié van die TB-positiewe pasiënte (gemiddelde% IFN-gamma 2,960 ± 3,51 vs pasiënt 2,370 ± 2,07; p = 0,2800). TNF-α het hoëruitdrukking in die pasiëntgroep in vergelyking met die kontrole kandidate (mediaan% TNF-α 2,415 ± 2,60 teen beheer 1,340 ± 1,86; p = 0,1729). Dubbele uitdrukking van sitokiene was amper soortgelyk in die twee groepe (kontrole mediaan% IFN-γ + TNF-α + 0,5400 ± 0,36 teen pasiënt 0,8550 ± 0,60; p = 0,3961). TIM3 uitdrukking was nie beduidend anders in die vier T-sel deel versamelings nie (mediaan T_N 0,0750 ± 1.89, T_{CM} 0,3400 ± 4.28, T_{EM} 0,0850 ± 2,73, T_{DEM} 0,1600 ± 1,93; p = 0,5877). Wanneer die vergelyking van die subset verspreiding in die pasiëntgroep gedoen was, het $T_N$selle die meeste voorgekom (mediaan 47,48 ± 20,96) gevolg deur TEM selle (mediaan 21,92 ± 13,25), TDEM selle (mediaan 13,02

± 20,13) en uiteindelik TCM selle (mediaan 11,51 ± 8,62). Hierdie resultate toon 'n beduidende verskil in die uitdrukking tussen die vier groepe (p = <0,0001).

Gevolgtrekking: Deur versigtige titrasie van teenliggaampies, ens het ons gestig 'n vloeisitometrietoets wat gebruik kan word om sitokien patrone te studeer in TB-pasiënte. Die verhoogde enkele TNF-α uitdrukking in die pasiëntgroep is n aanduiding van aktiewe TB en die verhoogde IFN-γ in die kontrolegroep kan moontlike BCG inenting aan dui. TIM3 sou 'n nuttige merker in 'n groter MIV^+ kohort van pasiënte wees, want dit sal die identifisering van funksioneel uitgepute T-selle identifiseer. In SA, is die voorkoms van MIV stygend en hierdie toets bewys sy geskiktheid deur die gebruik van minimale volumes van volbloed, eerder as sputum. Deur die opwekking van intrasellulêre sitokien profiele sou 'n mens in staat wees om te onderskei tussen aktiewe en LTBI wat sou help met die behandeling van pasiënte

.

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List of Abbreviations

AIDS	Acquired immune deficiency syndrome
ANOVA	Analysis of Variance
APC	Allophycocyanin
APC750	Allophycocyanin 750
ART	Antiretroviral therapy
BCG	BacilleCalmette – Guerin
BL	Baseline (negative control)
BM	Bone marrow
CD	Cluster of differentiation
CFP – 10	Culture filtrate antigen
CMIA	chemiluminescent microparticle immunoassay
DC	Dendritic cells
DOH	Department of Health
DOTS	Directly observed treatment, short – course
E/C	ESAT-6/CFP-10
ECD	PhycoerythrinTexas Red-X
ELISPOT	Enzyme – linked immunospot technique
EMB	Ethambutol
ESAT – 6	Early secretory antigenic target
FITC	Fluorescein isothiocyanate
FMHS	Faculty of Medicine and Health Sciences
FS	Forward scatter
HIV	Human immunodeficiency Virus
HREC	Health Research Ethics Committee
ICCFC	Intracellular cytokine flow cytometry

ICS	Intracellular cytokine staining
IFN-γ	Interferon gamma
IGRA	Interferon gamma release assay
IL-1β	Interleukin 1 beta
IL-2	Interleukin 2
IL-6	Interleukin 6
INH	Isoniazid
LAM	Lipoarabinomannan
LED	Light emitting diode
LTBI	Latent Tuberculosis infection
(LT)-α3	Lymphotoxin
MDM	Monocyte – derived macrophages
MDR-TB	Multi – drug resistant Tuberculosis
MFI	Mean fluorescent Intensity
MODS	Microscopic observation drug susceptibility
M.tb	Mycobacterium Tuberculosis
NAA	Nucleic Acid Amplification testing
NHLS	National Health Laboratory Services
NS	Not significant
PBMC	Peripheral blood mononuclear cells
PC5	Phycoerythrin-Cyanin 5.1
PC7	Phycoerythrin-Cyanin 7
PCR	Polymerase chain reaction
PD-1	Programmed – death 1
PD-1/L1	Programmed – death 1 ligand 1
PE	Phycoerythrin
PMT	Photomultiplier tube

PVL	Plasma viral load
PZA	Pyrazinamide
QFT – GIT	QuantiFERON – TB Gold In-tube test
RIF	Rifampicin
RLU	Relative light unit
RNA	Ribonucleic acid
RT	Room temperature
SA	South Africa
S/CO	Signal to cut-off
SD	Standard deviation
SEB	Staphylococcus Enterotoxin B
S:N	Signal to noise ratio
SS	Side scatter
SS INT	Side scatter integrel
TB	Tuberculosis
TBH	Tygerberg Hospital
T_{CM}	Central memory T-cell
T_{DEM}	Terminally differentiated effector memory T cells
T_{EM}	Effector memory T-cell
Th1	T-helper 1
Th2	T-helper 2
Th22	T-helper 22
TIM3	T-cell Immunoglobulin and Mucin Domain–Containing Molecule-3
T_N	Naïve T-cell
TNF	Tumor necrosis factor
TNF-α	Tumor necrosis factor alpha
TNFR	Tumor necrosis factor receptor family

T – Spot	T-SPOT.TB test
TST	Tuberculin skin test
WB	Whole blood
WHO	World Health Organisation
XDR-TB	Extensively drug – resistant Tuberculosis
ZN	Ziehl – Neelsen

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Chapter 1 - Literature review

1.1 Introduction

The World Health Organization (WHO) declared Tuberculosis (TB) a global health pandemic more than 20 years ago. However despite recent advances in diagnosis and clinical management, TB to date still remains the second deadliest infectious disease after acquired immune deficiency syndrome (AIDS) (Shu et al., 2012; Elkington 2013). *Mycobacterium Tuberculosis (M.tb)* bacilli gets transmitted by the inhalation of infected aerosol droplets that is produced through the coughing of persons with active pulmonary disease. In most cases this transmission is done without detection, since the patient's signs and symptoms are not specific to TB. Factors such as overcrowded areas, poor hygiene and sanitation, poverty and homelessness, alcoholism, diabetes and age, to mention a few, contribute to the development and spread of this disease and play a huge role in the clinical management and finally the disease outcome (Lienhardt, 2001; Betsou et al., 2011; Shu et al., 2012).

In addition, due to a non-specific clinical picture and diagnostic complications, the pathogenesis of TB is further hampered by human immunodeficiency virus (HIV) co-infection (Shankar et al., 2014). In South Africa (SA) alone more than 70% of people living with TB also have HIV (For, 2011). Immune function of the host is weakened by the co-existence of this duo, as HIV depletes the body's $CD4^{+}$T-cells, which in turn is needed to help control TB. HIV also hampers cytokine production and macrophage function, key features necessary in the immune response, which leads to the inability of the host to suppress latent or initial TB infection (Diedrich & Flynn 2011; Pawlowski et al. 2012). In addition, HIV increases reactivation of latent TB infection up to 20 times and catalyses the progression of TB infection to active disease and similarly TB exacerbates HIV infection (Pawlowski, Jansson, Sköld, Rottenberg, & Källenius, 2012). For the purpose of this thesis, this chapter will give a condensed and simplified overview of TB, the effect on the immune response, TB diagnostics, HIV co-infection and flow cytometry in order to provide context for later chapters.

1.2 Background

1.2.1 Epidemiology

The WHO states that; TB is responsible for ill health amongst millions of people each year and remains one of the deadliest communicable diseases in the world. In 2014, an estimated 9.6 million people developed TB and 1.5 million died from the disease, of whom 400 000 were HIV – positive(WHO, 2015). TB incidence globally (Figure 1.1) is declining slowly each year and it's estimated that 37 million lives were saved between 2000 and 2013 through effective diagnosis and treatment.At present, a six month regimen of four first - line drugs: Isoniazid, Rifampicin, Ethambutol and Pyrazinamide is the recommended treatment.The treatment success rate of newly diagnosed TB cases was 86% in 2013 (WHO 2014).

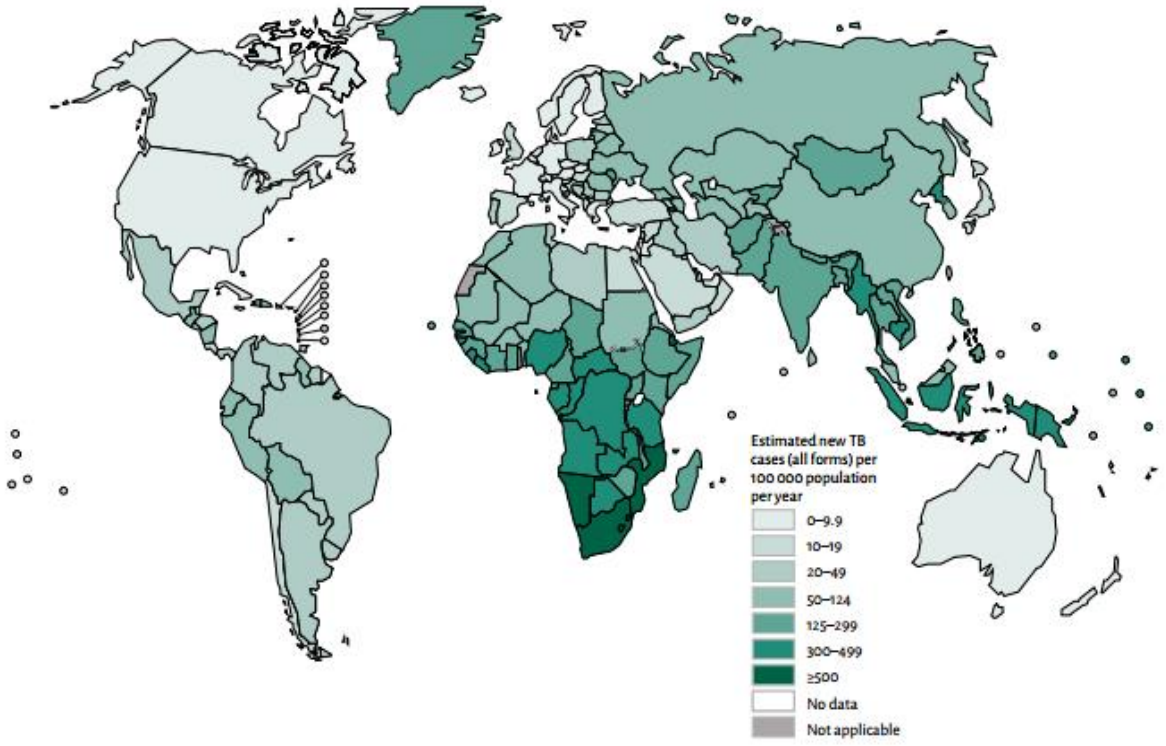


Figure 1.1Global Tuberculosis burden 2014(Image taken from WHO 2015)

South Africa is one of five countries experiencing the highest TB incidence globally with800–1,200 per 100,000 in 2012, which coincided with the onset of the HIV pandemic (Vynnycky et al., 2015). Ninety percent of TB cases in 2013 had co -

infection with HIV while 26 023 cases of multi – drug resistant TB (MDR-TB) was reported in SA. South Africa was also one of three countries with the largest increase in MDR-TB between 2011 and 2012. Treatment for MDR-TB lasts 20 months and treatment success rates are much lower (Tuberculosis & Guidelines, 2014). An estimated 9.7% of people with MDR-TB have extensively drug-resistant TB (XDR-TB) (WHO, 2015). XDR-TB defined as: MDR-TB plus resistance to at least one fluoroquinolone and a second-line injectable, had been reported by 105 countries globally by the end of 2014 (WHO, 2015). Although the WHO 2015 report does not give exact figures for the successful treatment of XDR-TB, it does make mention that the high mortality rate (47%) of XDR-TB patients in SA is most likely due to the rising level of HIV co-infection (WHO, 2015).

1.2.2 Tuberculosis Pathogenesis

TB infection is a marked serious of events, which starts off by a patient with pulmonary TB coughing and subsequently releasing the *M.tb* bacilli into the atmosphere whereby the infectious droplets are then inhaled by others in close proximity (Cooper, 2009). The innate and adaptive immune systems work together to eliminate the invading pathogen from the lungs resulting in the formation of a granuloma. A granuloma is made up of a mass of cells such as lymphocytes, macrophages, dendritic cells, neutrophils and fibroblasts and often has a necrotic centre and multiple granulomas can be seen in patients with active TB (Diedrich & Flynn, 2011). Although these granulomas may heal, it leaves a calcified Ghon focus in the lower zones of the lungs which mark the initial site of infection (Cooper 2009; Elkington 2013). In latent TB infection, the bacteria persist within the granuloma, but the host manages to effectively resist the bacteria. As seen in Figure 1.2, when the granuloma's structure or function becomes compromised it leads to either latent TB infection, disseminated TB or active disease (Diedrich & Flynn, 2011).

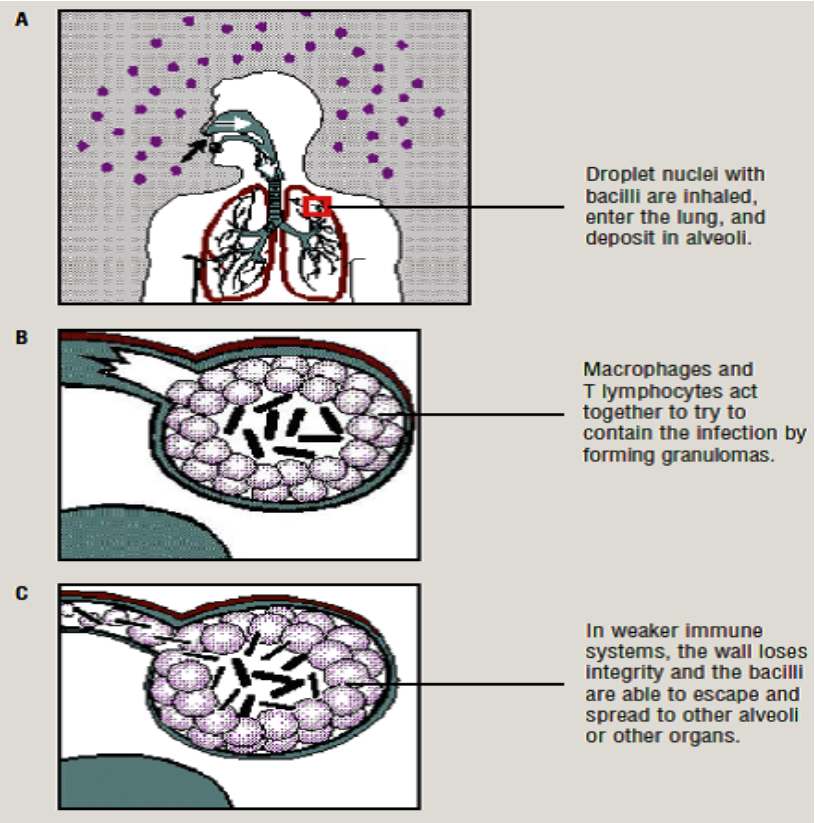


Figure 1.2Pathophysiology of tuberculosis: (A) *M.tb* bacilli inhaled in droplet formation, (B) innate and adaptive immune system working together to contain *M.tb* bacilli in the granuloma, (C) when host becomes immune compromised the granuloma ruptures and is spilled in to the atmosphere through coughing by the host or spread to other organs (Image taken from Knechel 2009).

Alveolar macrophages are the initial target cells for mycobacteria bacilli and are activated by cytokines, enabling them to perform bactericidal effector functions (Pawlowski et al., 2012). Dendritic cells phagocytose the *M.tb* bacilli in the lung tissue and move to the draining lymph nodes whereby the adaptive immune response is initiated and subsequently activates naïve T-cells(Chackerian et al., 2002). The role of these activated T-cells will be discussed in section 1.2.2.1

1.2.2.1 Cell – mediated immunity and TB

T-cells play a major role in containing or spreading TB infection as they are forceful interferon gamma (IFN-γ) producers, which in turn activates crucial anti–mycobacterial activities of macrophages. IFN-γ together with tumor necrosis factor

alpha (TNF-α), that control intracellular infections, and lymphotoxin (LT)-α3, regulate the formation of granulomas and maintain its structural integrity (Kaufmann, 2001). $CD4^+$T helper cells or $CD8^+$cytotoxic T-cells can produce two or more cytokines at the same time and are known to be polyfunctional. Polyfunctional cells have greater effector and proliferative functions as compared to mono- or bi-functional cells and produce IFN-γ, TNF and interleukin 2 (IL-2) (Seder et al. 2008; Jasenosky et al. 2015). T helper 1 – type $CD4^+$ T-cells are the hallmark cells for controlling the pathogenesis of TB (Jeong et al., 2014). Previous studies have shown that $CD4^+$ T-cells that are mono- or bi-functional, i.e. expressingeither TNF-α alone, or both TNF-α and IFN-γ respectivelyare associated with active TB disease and they act synergistically to kill pathogens. On the contrary larger numbers of polyfunctional $CD4^+$T-cellswith the phenotype $TNF^+IFN\text{-}\gamma^+IL\text{-}2^+$,were found predominantly in patients with latent TB infection, as well as patients on TB treatment (Jasenosky et al., 2015).However, Caccamo et al (2010) has found that patients with active TB disease showed a higher frequency of polyfunctional cells (Caccamo et al., 2010). Antigenic stimulation (with cytomegalovirus or Mycobacterium bovis bacillus Calmette–Guérin (BCG) vaccine) of T-cells also leads to activation of the subset cells (central memory and effector memory) into effector $CD4^+$T-cells which lead to their death. However, the extent to which cells differentiate depend on initial antigen exposure or innate – immune factors found in the cells' surrounding environment.

A study done by Seder et al., 2008 has demonstrated as seen in Figure 1.3, that $CD4^+$T-cells mostly express TNF and can co-express IL-2 whether IFN-γ is expressed or not. However, if IL-2 is expressed regardless of TNF, the cell will survive and can be sustained for longer and subsequently differentiate into IFN-γ producing T-cells, because on its own, IL-2 has little effector function (Darrah et al., 2007) Hence, memory $CD4^+$T-cells with effector function secrete IL-2 or TNF or both simultaneously. T-cells can produce IFN-γ and TNF without IL-2. T-cells that secrete IFN-γ only, do not survive for long as memory T-cells and are usually at the final stage of T cell differentiation (Seder et al., 2008).

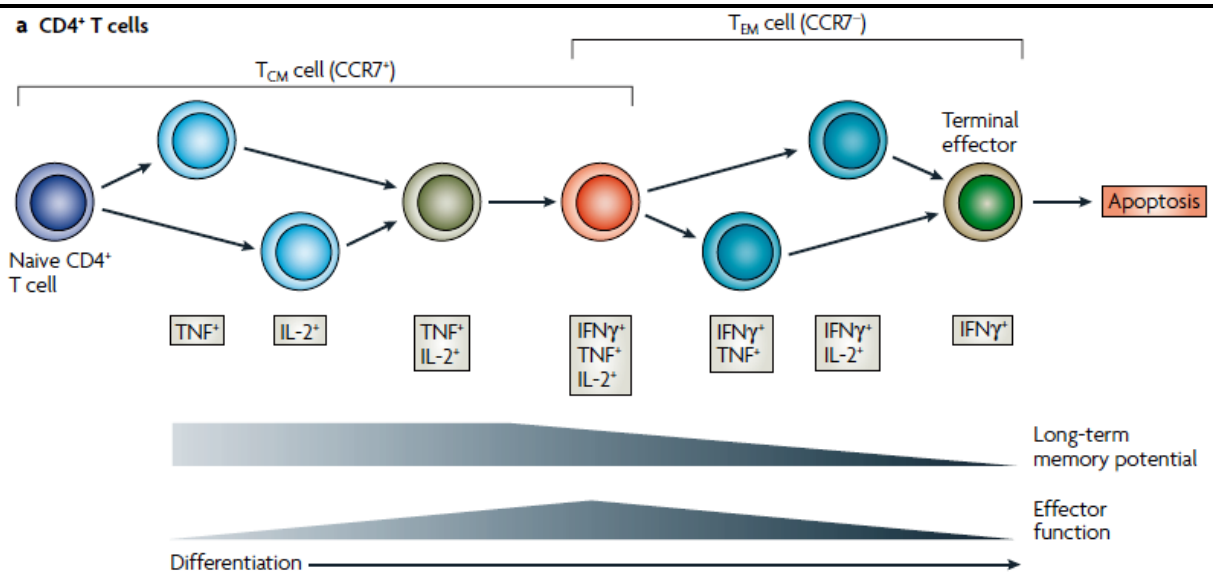


Figure 1.3Linear model for T-cell differentiation (a) $CD4^+$T-cells gain function as they differentiate until they reach the optimal stage of their effector function, which is to produce cytokines. The last stage of differentiation is reached under continued antigenic stimulation whereby T-cells produce only IFN-γ and have a relatively short life span(Image taken from Seder et al. 2008).*T_{CM} – central memory $CD4^+$ T-cell; T_{EM} effector memory $CD4^+$ T-cell.*

In conclusion, $CD4^+$T-cells that differentiate into polyfunctional cells secreting IFN-γ, TNF and IL-2, have three possible outcomes: (i) they can continue as memory or effector cells; (ii) they can differentiate into less functional T-cells or (iii) following activation they can undergo apoptosis and die. An ideal cell population deemed effective in combatting infection should be multifunctional and be able to rapidly mediate effector function and have a reservoir of memory T-cells. (Darrah et al., 2007;Seder et al., 2008).

1.2.2.2 T-cell subsets

$CD4^+$ T-cells differentiate into different types of T-helper (Th) cells which each produce cytokines that assist other cells to perform different functions. Figure 1.4 shows the five lineages of T-helper cells which are Th1, Th2, Th17, T-follicular cells (Tfh) and T-regulatory (Tregs) cells. These groups of cells each perform a different function (Spellberg & Edwards, 2001).

T helper cell subsets

Th1

Th1 cells are the hallmark cells for controlling the pathogenesis of TB and are known to be the leading regulators of type 1 immunity (Jeong et al., 2014)(Appay et al., 2008). Th1 cells produce IFN-γ, TNF-α, IL-2 and lymphotoxin-α. The functions of these cytokines are discussed in Chapter 1, section 1.2.2.1, page 7-9. Another cytokine produced in this group is IL-12 which plays a role in the polarisation of Th1 cells and IFN-γ induction. It also increases cytotoxicity by stimulating the proliferation of antigen-specific cytolytic T cells and NK cells (Appay et al., 2008)

Th2

Th2 cells produce IL-4, IL-5, IL-10 and IL-13. They also produce TNF-α but not IFN-γ. The role of these cytokines are briefly describes as follows :

- IL-4

IL-4 is increased during active TB and is said to play a pathogenic role during the late phase of TB infection. IL-4 down regulates Th1 responses (Sakamoto K, 2012)

- IL-5

Plays a role in the differentiation and activation of eosinophils in the bone marrow (Sanderson 1990)

- IL-10

IL-10's function is to deactivate macrophages and reduce Th1 responses. It limits antigen presentation, decreases reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) and these subsequently have a major effect on the innate and adaptive immune response in TB. IL-10 is an immunosuppresive cytokine that has its effect on macrophages, monocytes, dentritic cells and T-cells. IL-10 plays an important role in TB and has been identified as a biomarker and a correlate of susceptibility to TB (Beamer et al., 2008).

- IL-13

IL-13 is involved in IgE synthesis and subsequently allergic responses and plays a role in airway inflammation (Wynn, 2003).

Th17

Th17 cells develop in the presence of low amounts of TGF-β, IL-1β and TNF and this process is initiated by IL-6 or IL-21. The signature cytokines of this group is IL-17, IL-17F, IL-21 and IL-22. It is suggested that these cells and its produced cytokines have a complex role in different infections caused by bacteria, fungi and viruses and they play a key role in inducing inflammation and tissue damage in animals in autoimmune diseases and infection. There is limited information on human studies done on Th17 cytokines, but mouse studies suggest that a high dose of *M.tb* initiated intratracheally was poorly controlled in the absence of IL-17 (Torrado & Cooper, 2010).

T follicular cells (Tfh)

Tfh cells fall under the $CD4^+$ T-cell subset cells that reside in human secondary lymphoid tissues and are responsible for the activation, expansion and differentiation of B cells into immunoglobulin secreting cells. This process is facilitated by the simultaneous expression of chemokine receptors CXCR5 and the down regulated CCR7. Transcription factor Bcl-6 is required for the differentiation of Tfh cells that

subsequently express IL-21, however; circulating Tfh cells have proven to lack Bcl-6 expression (Ma & Deenick, 2014).

T regulatory cells (Tregs)

Treg cells emerge from the thymus.Upon stimulation they have the phenotype $CD4^{+}CD25^{+}Fox\beta3^{+}$ and has been identified as a role player in immune suppression of chronic diseases and subsequently the suppression of effector T-cell responses during TB infection.Singh et al., (2012) has shown that the Treg cell representation is directly proportional to the TB bacillary load or severity of TB infection as the Treg numbers have declined after successful TB treatment. (Singh et al., 2012;Lastovicka J 2013).

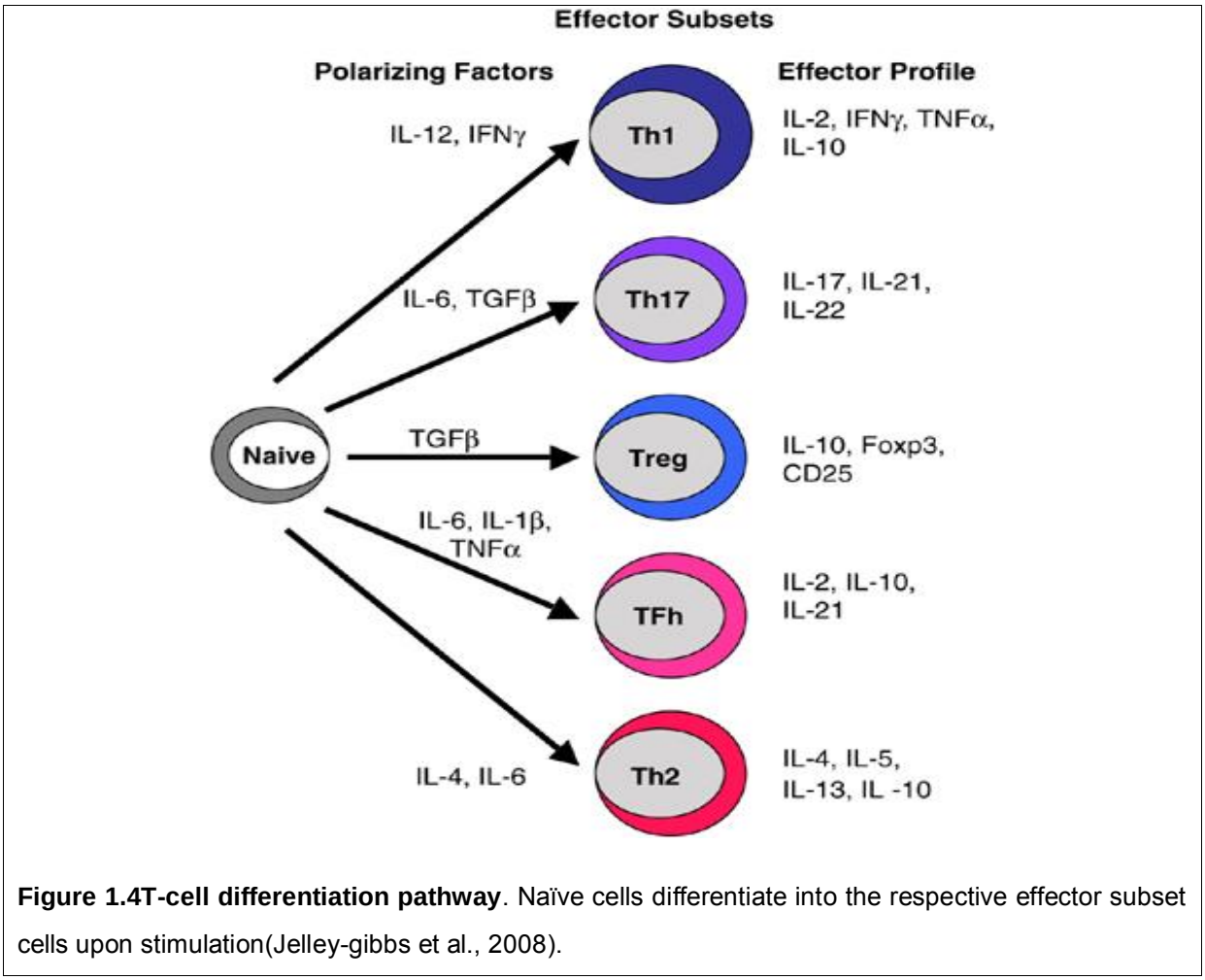


Figure 1.4T-cell differentiation pathway. Naïve cells differentiate into the respective effector subset cells upon stimulation(Jelley-gibbs et al., 2008).

1.2.2.3 Active vs. Latent TB disease

In latent TB infection (LTBI), *M.tb* bacilli are able to persist in the body without causing illness. The host has no clinical signs or symptoms, is not infectious and cannot spread the *M.tb* bacilli. If the hosts' immune system becomes compromised, progression to active disease occurs. Currently a diagnosis of LTBI is made on the bases of a positive tuberculin skin test (TST) and Interferon gamma release assay (IGRA), however, these two tests have been criticized for their accuracy and their short comings will be discussed in detail later in section 1.2.3 (CDC 2015).

In active disease the *M.tb* bacilli overcomes the host's defence mechanisms and multiply in the body. Clinically patients present with symptoms such as fatigue, weight loss and coughing and are able to spread the *M.tb* bacilli to others (CDC 2015).

Treatment between the two disease states diverges with regards to the regimen of drugs and duration of treatment as detailed below in Table 1.1 (Rovina et al., 2013).In addition, the presence of HIV further increases the reactivation of latent TB infection up to 20 times and catalyses the progression of TB infection to active disease, therefore it is imperative to be able to accurately diagnose LTBI (Pawlowski et al., 2012).

Table 1.1 Treatment regimens for LTBI and active disease

Latent TB disease			
Drugs	**Duration**	**Interval**	**Minimum doses**
INH	9 months	Daily Twice weekly*	270 76
INH	6 months	Daily Twice weekly*	180 52
INH + Rifampentine	3 months	Once weekly	12
RIF	4 months	Daily	120
Active disease			
Initial phase INH RIF PZA EMB	8 weeks	Daily	56
Continuation phase INH RIF	18	Daily	126
INH RIF	18	Twice weekly	36

Abbreviations: INH – Isoniazid, RIF – Rifampin, PZA – Pyrazinamide, EMB – Ethambutol
** - Use directly observed therapy (Adapted from CDC 2015).*

1.2.3 Tuberculosis Diagnostics

Despite advances in TB diagnostic techniques, the rate of false negative results remains high (Elkington, 2013). Given the high statistics of TB/HIV-1 co-infection, it is important to efficiently diagnose latent tuberculosis infection (LTBI), as patients co-infected, transplant patients and patients receiving immunosuppressive therapies are more susceptible to develop active disease. Being able to distinguish between the two disease states is imperative as treatment and management of the patient differs between the two (Rovina et al., 2013). In Table 1.2 below, the differences between the different diagnostic tests are detailed.

Table 1.2 Differences between different TB diagnostic tests

Test	Specificity	Sensitivity	Ability to detect LTBI	Detection of *Mtb* resistance to RIF and/or INH	Limitations
TST	High in non-BCG	Low	No	No	Return visit in 48-72 for test results
IGRA	High	High	No	No	Costly
Chest X-ray	Low	Low	No	No	Non confirmatory
ZN microscopy	Low	Low	No	No	Cannot distinguish between *M.tb* and other species
Culture	Low	Low	No	No	Results take up to 6 weeks
NAA	High	Low	No	No	Costly
LAM	High	Low	No	No	Contamination of urine samples with normal flora – may lead to false-positive results
MODS	High	High	No	Yes – both	Training and technical expertise needed. Biosafety level 3 required
LED microscopy	Low	High	No	No	Fluorescent microscope, dark room and expensive light source required
GeneXpert	High	High	No	Yes – RIF only	Costly; Sufficient laboratory infrastructure and staff training required

Abbreviations: TST – Tubercilin skin test, IGRA – Interferon gamma release assay, ZN – Ziehl Nielsen, NAA - Nucleic Acid Amplification testing, LAM – lipoarabinomannan, MODS - microscopic Observation Drug Susceptibility, LED – light emitting diode. (Adapted from Sia & Wieland 2011)

A diagnosis of LTBI is made on the bases of a negative Mantoux TST and IGRA, whereas active disease relies on chest x-rays suggestive of pulmonary TB and a positive Ziehl-Neelsen (ZN) acid fast bacilli (AFB) smear and culture (Sia & Wieland, 2011). The ZN acid fast stain, a microscopic test recommended by the WHO as a diagnostic tool, especially in resource poor settings, has both low sensitivity and specificity. With a detection limit of 5000-10 000 *M.tb* bacilli/ml per sample, a false negative result is more common in HIV patients, children and the elderly due to a low bacillary load and difficulty to produce sputum. Likewise, culture techniques,

regarded as the gold standard for TB diagnosis, has its own draw backs despite the fact that it requires only 10 *M.tb* bacilli/ml as compared to the ZN. It takes 4 – 8 weeks for the bacteria to grow which subsequently delays the onset of treatment (Muwonge et al., 2014).

Similarly the TST, dating back to the early 1930's, is still a current diagnostic tool to identify TB infection despite several limitations on the use of this test (Starke, 1993). This includes return visits from the patient, cross-reactivity with non-tuberculosis mycobacteria and the Bacille Calmette-Guerin (BCG) vaccine (vaccine containing Mycobacterium Bovis) as well as inter-reader variability. In addition, a major limitation of this test is the poor sensitivity in immune compromised patients as well as the inability to distinguish between latent TB and active disease (Frahm et al., 2011).

On the other hand the IGRAs was developed to compensate for some of the TST's short comings as it is more reliable and highly specific (Sester et al., 2011). IGRAs measure the expression of IFN-γ released by T-cells following stimulation of the peripheral blood with 6-kDa early secretory antigenic target (ESAT-6) and 10-kDa culture filtrate antigen (CFP-10) which are encoded in the region of difference 1 of the mycobacterial genome. The specificity of this test is due to the fact that neither ESAT-6 and CFP-10 are found in BCG vaccine strains or in non-tuberculous mycobacteria (Rovina et al., 2013). There are currently two IGRAs namely, the QuantiFERON-TB Gold In –Tube test (QFT-GIT) and the T-SPOT.TB test (T-Spot) (Sester et al., 2011). Since these tests measure IFN-γ as a single parameter of T-cell activation, it will only indicate either the presence or absence of TB infection, and cannot distinguish between active from latent TB, which discredits its specificity but compliments its sensitivity (Rutledge et al., 2010).

In 2010, the WHO endorsed an automated, rapid molecular test that can test for TB and Rifampicin (RIF) resistance simultaneously (WHO, 2013). This real-time PCR assay - GeneXpert MTB/RIF amplifies the MTB specific sequence of the *rpo*B gene that detects mutations within the rifampin-resistance determining region. The instrument which takes 2 hours to generate a result, uses a disposable cartridge containing all needed reagents to simultaneously perform bacterial lysis, nucleic acid extraction, amplification and amplicon detection (Boehme et al., 2010). Although this

assay is more sensitive and replaced the smear microscopy as the primary diagnostic test in SA, it is very expensive making it inaccessible to most endemic regions (WHO, 2013).

Nucleic Acid Amplification testing (NAAT), used to test for pulmonary TB, detects *M.tb* ribosomal RNA directly from both AFB smear positive and negative patients with suspected TB using the Enhanced Amplified Mycobacterium Tuberculosis Direct (MTD) test. The Amplicor*Mtb* test on the other hand is able to detect the *Mtb* DNA in AFB smear – positive respiratory specimens. The two tests – NAAT and AFB smears, always go hand in hand. If both tests are positive, this is indicative of TB. However, if the results are discrepant, doctors make a decision together with the culture results and clinical symptoms (Sia & Wieland, 2011).

Another diagnostic test involves lipoarabinomannan (LAM), a glycoprotein found in the cell wall of *Mtb*, which is released from active or degrading *Mtb* cells during active TB infection. LAM antigens are detected in patients' urine and a positive result indicates active TB disease. This test has the advantage of easier sample collection, especially in children as urine is safer to process and store and infection control procedures are minimal. With regards to specificity, LAM assays is comparable to smear microscopy, however it has a higher and more reliable sensitivity and has the advantage of detecting disseminated TB. However, the greatest diagnostic challenge of differentiating between active and latent disease, is still not addressed by this assay (Minion et al., 2011).

Fluorescent light emitting diode (LED) microscopy uses a fluorochrome stain – auramine that is more sensitive than the ZN stain and less time consuming. However, the need for expensive equipment and a dark room makes this test less popular (Wilson, 2011). The microscopic Observation Drug Susceptibility (MODS) Assay is a broth microtitre method that detects *M.tb* bacilli and Isoniazid (INH) and Rifampin resistance (Moore et al., 2006). *M.tb* grows faster in liquid than on solid media, giving this assay the advantage over the conventional culture method. Anti-TB drugs are also included in the assay which allows for rapid detection of drug resistance. However the use of MODS is not so popular as it requires testing in a biosafety level 3 facility, equipment and supplies are expensive and is therefore not available at smaller laboratories (Wilson, 2011).

Similar to IGRAs, Intracellular cytokine flow cytometry (ICCFC) has a further advantage of being able to measure multiple cytokines released by specific T-cells after stimulation by *M.tb* antigen (Kim et al., 2014). It is a method used for counting and sorting of microscopic particles and examining the characteristics of individual particles that flow in a single file suspended in a stream of fluid (Brown & Wittwer, 2000). The difference in size and internal complexity of the cell can be distinguished from light scattered at different angles when compared to light emitted from fluorescently labelled antibodies, which is able to identify cell surface and cytoplasmic antigens. Due to these unique characteristics, flow cytometry is a powerful tool to use for detailed analysis of complex populations in a short period of time (Orfao et al., 1995). The results obtained upon analysis may be both quantitative and qualitative (Brown & Wittwer, 2000). Flow cytometry can be used to measure the morphological and cell surface antigen characteristics of a single cell. It is able to measure the cell size and inner complexity of the cell such as the shape of the nucleus, the roughness of the membrane and the type of cytoplasmic granules (Brown & Wittwer, 2000).

Flow cytometry is able to enumerate cells and detect which cells specifically release cytokines in response to TB antigen stimulation (Maino & Picker, 1998). It can rapidly and simultaneously determine cytokine production of defined leucocyte subsets in peripheral blood and T-cells at inflammatory sites. The presence of variable concentrations of cellular or soluble receptors also does not compromise the quantitation of cytokine production (Maino & Picker, 1998). With flow cytometry it is possible to directly detect intracellular cytokine expression with fluorochrome–conjugated anti–cytokine antibodies after short periods (4–6h) of activation with different stimuli (Picker et al., 2016). The length of activation may vary depending on the individual need of each laboratory. The sensitivity of cytokine detection is enhanced by disruption of cytokine secretion after stimulation by adding the secretion inhibitor Brefeldin A, followed by a fixation and permeabilization step in order to stain intracellularly (Maino & Picker, 1998). Another fixation step follows and cells may now be acquired on the flow cytometer. The length of time for the entire process to complete is dependent on optimisation of the assay. Optimisation of the assay will be discussed in section 2.8. For the purpose of this thesis, flow cytometry and its potential as a diagnostic tool will be further investigated in this project. As

many flow cytometry studies have investigated measurements on both whole blood and peripheral blood mononuclear cells (PBMC's), we further discuss these options to determine which of the two is best to use during optimization.

1.2.4 TB and HIV co-infection

As mentioned in section 1.1, a large percentage of people within SA also have HIV. Figure 1.5, below demonstrates the coaction of TB and HIV co-infection. Following the phagocytosis of *M.tb,* macrophages become activated and secrete cytokines: TNF-α, interleukin 1 beta (IL-1β) and interleukin 6 (IL-6) which in turn increase HIV-1 replication (Shankar et al., 2014). Effector T-cells however, reach a state of immune exhaustion following continual activation and cytokine secretion. This renders these cells dysfunctional or unresponsive to any downstream antigen stimulation. As HIV infection continues, immune exhaustion become more evident as T-cell effector functions such as its cytokine cytotoxic potential become diminished and progression to AIDS occurs (Shankar et al., 2014). AIDS is characterised by the hallmark loss of $CD4^{+}$T-cells and this contributes to the reactivation of latent TB and makes the host susceptible to new *M.tb* infection.

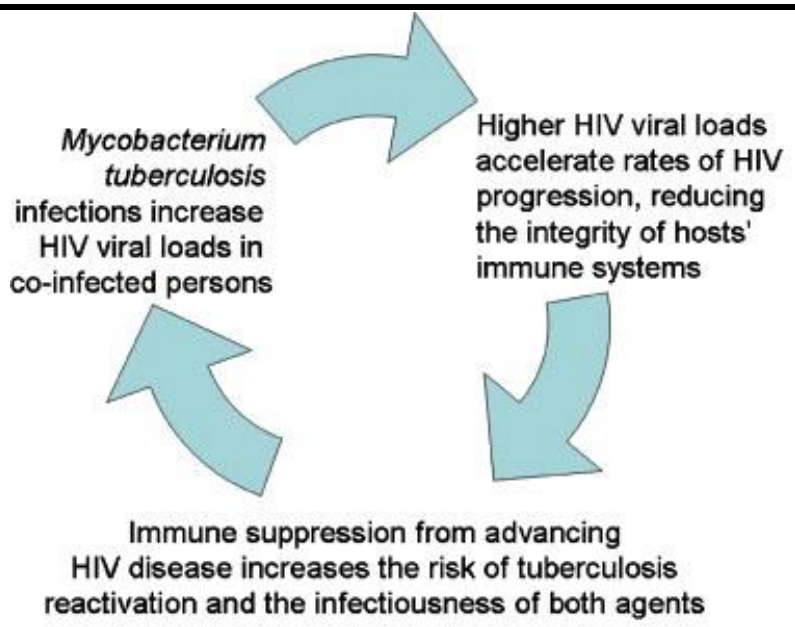


Figure 1.5Epidemiological coaction of Tuberculosis and HIV(Vermund & Yamamoto, 2007)

HIV exacerbates TB by tipping the Th1/Th2 cell balance and impairs the TNF-mediated macrophage response to MTB and this leads to survival of the bacteria (Patel et al., 2007;Pawlowski et al., 2012).

Granuloma formation in TB infection, localizes the infection, prevents the spread of bacilli and offers host protection. HIV compromises granuloma structural integrity. A granuloma typically has a caseous necrotic centre, however in patients with AIDS, a dominant granulocytic infiltrate and necrosis replaces this normal feature (Pawlowski et al., 2012). Diedrich & Flynn, 2011 has divided the granuloma disruption into four steps: (i) viral load increase in the involved tissues, that result in (ii) significant loss of $CD4^{+}$T-cells, alongside (iii) macrophage dysfunction and (iv) disruption of *M.tb* specific T-cell function ultimately causing functional and inimical changes within the granuloma. HIV fuels this defect and results in systemic disease characterized by multiple organs containing defective granulomas that lead to the development of more diffuse lesions (de Noronha et al.,2008). This disease state is known as extra pulmonary TB or disseminated disease. As *M.tb* replication is now increased, mononuclear cells become activated resulting in increased HIV replication and subsequently increased HIV viral load at the site of TB infection (Lawn, 2005).

1.2.5 Markers of T cell exhaustion

One of the fundamental hallmarks of infections particularly in the case of HIV as well as TB is “T cell exhaustion”. This is described as the inability of cells to produce cytokines, to proliferate and to survive in the presence of persistent infections such as HIV as well as TB. As discussed in section 1.2.4, two such markers of exhaustion we will discuss is programmed death – 1 (PD-1) and T-cell immunoglobulin mucin domain - 3 (TIM-3) that plays a role in regulating T cell immunity and cell tolerance (Sakhdari et al., 2012).

1.2.5.1 PD-1 inhibition marker

PD-1 is expressed on activated T-cells such as $CD4^+$ and $CD8^+$, NK cells, B cells and monocytes. When PD-1 binds to its ligands PD-1/L1 and PD-1/L2 which is expressed on dendritic cells, it inhibits T-cell activation and induces T-cell exhaustion (Larsson et al., 2013)(Tousif et al., 2011). This PD-1/PD-L interaction will only occur at the same time as the TCR/MHC II interaction takes place. Studies have shown that in $CD8^+$T-cells, the PD-1 expression is directly proportional to HIV disease severity such as increased viral load and decreased $CD4^+$ T-cell count (Larsson et al., 2013).

Furthermore, Jurado et al. corroborate that the prevention of PD-1 and its ligands is associated with the inhibition of T-cell effector functions in active TB due to increased IFN-γ producing lymphocytes, needed for protective immunity against TB (Jurado et al., 2008)

1.2.5.2 TIM-3 inhibitory molecule

TIM3 is a molecule expressed on IFN-γ Th1 cells, but not on Th2 cells and plays a role in regulating Th1 immunity and tolerance by negatively regulating IFN-γ secretion by inducing cell death when binding to its ligand Galectin–9 (Hastings et al. 2009). This indicates the inhibitory role of TIM-3 on $CD4^+$T-cells(Wang et al., 2011). Furthermore, TIM-3 is also expressed on HIV specific T-cells undergoing exhaustion that produce little to no cytokines (Jones et al., 2008).

TIM-3^{+}CD4^{+}T-cells produce Th1/Th22 cytokines and limit intracellular replication of the *M.tb* bacilli in macrophages. In active TB, TIM-3 expressing CD4^{+}T-cells displayed polarized effector memory phenotypes lacking CD27 (Qiu et al., 2012).

TIM-3 is also a negative regulator of Th1 and Th17 cytokines in T-cells, as IFN-γ production is increased when the TIM-3 pathway is blocked, but does not have an overall effect on cytokine production in the body (Hastings et al., 2009).

1.2.6 CD27 and CD45RO memory markers

Once T-cells are released from the thymus it undergoes different stages of differentiation, and the CD45 antigen isoforms best discriminates between primed and unprimedT-cells. CD45RA is expressed on naïve T-cells, whereas CD45RO is a marker of memory T-cells(Schiött, Lindstedt, Johansson-Lindbom, Roggen, & Borrebaeck, 2004). CD27 is a member of the tumor necrosis factor receptor family (TNFR) which further delineates the different stages of T-cell differentiation. CD27 has a co-stimulatory function, and after initial up-regulation upon T-cell receptor engagement, CD27 expression is gradually irreversibly down regulated following repeated antigenic stimulation (Fritsch et al., 2005). Therefore CD4^{+}CD45RO^{+} memory T-cells, can further be divided into two subsets based on their CD27 expression (Schiött et al., 2004). The T-cell path of differentiation can best be described as follows: naïve T-cells (T_N) - CD45RO^{-}CD27^{+}, that progress into central memory T-cells (T_{CM}) – CD45RO^{+}CD27^{+} which in turn progress into effector memory cells (T_{EM}) – CD45RO^{+}CD27^{+} and with persistent antigenic stimulation become CD45RO^{+}CD27^{-} and then finally to the terminally differentiated effector memory T-cells (T_{DEM}) with the phenotype CD45RO^{-}CD27^{-}(Schiött et al., 2004;Rovina et al., 2013). The sequence of events described above is tabulated in table 1.3 below, and shows the role of the CD4 subsets in the diagnosis of active vs. latent TB disease. Effector memory cells secrete effector cytokines and have a strong antigen recall response, whereas central memory cells require co-stimulation and lack responsiveness toward antigen and T-cell receptor triggering. Rovina et al. 2013 has shown that T_{DEM} cells, lacking CD27 and expressing IFN-γ is an accurate discriminator between active and latent TB disease. Therefore including CD27 and CD45RO in our flow cytometry panel, will assist in distinguishing stages of immune

activation according to T-cell memory phenotype and subsequently assist in determining the disease state.

Table 1.3 Cytokine expression profiles within the different T-cell subsets

T – cell subset	Expression profile	Disease state indicated
Naive T-cells	$CD45RO^{-}$ $CD27^{+}$	Active disease
Central memory	$CD45RO^{+}$ $CD27^{+}$	
Effector memory	$CD45RO^{+}$ $CD27^{-}$	Latent disease/BCG vaccine
Terminally differentiated effector memory	$CD45RO^{-}$ $CD27^{-}$	

1.2.7 IFN-γ and TNF-α cytokines

Protective immunity against TB is crucial and T-cell mediated immune responses play a critical role in controlling *M.tb* infection. IFN-γ released by $CD4^{+}$T-cells activate phagocytes to incorporate the intracellular pathogen and this provides the host with protection against disease (Walzl et al., 2011). Likewise, TNF-α assists in cell apoptosis, cell activation, recruitment and differentiation and these functions make TNF-α pivotal in sustaining protective immune responses in TB (Mootoo et al.,2009). According to Walzl et al. 2011 specific cytokine expression profiles of $CD4^{+}$ T-cells are associated with bacterial loads at the various stages of TB disease (Walzl et al., 2011). Furthermore Pollock et al. 2013 has found that $CD4^{+}$T-cells that are bi-functional secreting IFN-γ/TNF-α or IFN-γ only or TNF-α only were indicative of active TB disease. On the other hand, cells with an effector phenotype secreting TNF-α only was an accurate marker to distinguish between active and latent infection and studies done by Harari et al. 2011 confirmed this. Likewise, a study done by Rovina et al. 2013 corroborate these findings and further demonstrated, along with Rueda et al. 2010 that $CD4^{+}$T-cells expressing IFN-γ and lack CD27, is an accurate indicator of LTBI.

1.3 Whole blood vs. PBMC's

In order to measure immune responses in T-cells, either whole blood or peripheral blood mononuclear cells (PBMC's) can be used to stimulate the T-cells. However depending on the assay requirements, normally a large volume of whole blood is required if PBMC isolations is needed, which in itself is time consuming and laborious. This studying of human T – cell immunity becomes even more challenging even in optimal conditions, especially in children and infants when the volume of blood for assays is limited (Hanekom et al. 2004). Based on this observations, Hanekom et al. (2004) investigated a flow cytometry intracellular cytokine assay utilizing whole blood as the specimen of choice because volumes as little as 200µl of whole blood was sufficient. Similarly, Bourguignon and colleagues agreed with the previous author and further stated that another disadvantage of using PBMC's was the need for liquid nitrogen for the cryopreservation of cells and this may be troublesome in some resource constraint areas (Bourguignon et al., 2014).

The flow cytometry intracellular cytokine assay backgrounds was studied in both whole blood and PBMC's and results indicated that background IFN-y expression was considerably less in whole blood samples compared to PBMC (Figure 1.6) (Hanekom et al., 2004). Despite the low IFN-y expression in whole blood, compared to PBMC's, others continue the investigation of intracellular cytokine expression in whole blood.

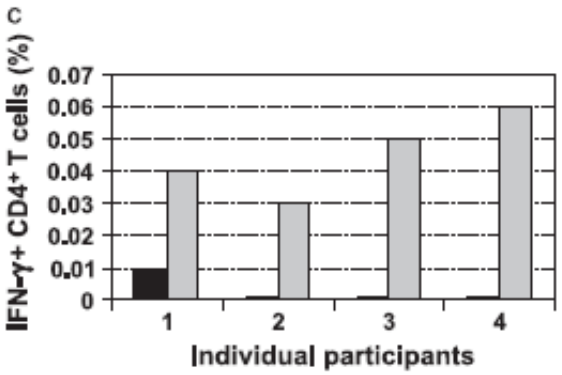


Figure 1.6 IFN-γ background expression. Background expression of IFN-y released by $CD4^+$ T-cells following the incubation of whole blood (black bars) and of freshly isolated PBMC (grey bars) samples of four patients. (Hanekom et al., 2004)

Likewise, Nomura and colleagues also noted that whole blood offers a more physiological environment and this may enhance the T-cell response following antigenic stimulation (Nomura et al., 2000). Whole blood not only preserves natural cell – cell interactions and soluble factors that play a role in cell activation, but also provides more representative *in-vivo* conditions than the alternate PBMC method (Crucian & Sams, 2001).

Thus for the purpose of our study, whole blood is the specimen of choice and is used for optimization purposes as this technique is simple, avoids time-consuming separation steps and is performed in an environment close to *in vivo.*

1.4The present study

From the abovementioned literature review and the growing statistics of TB/HIV-1 co-infection, early and accurate identification of both active and LTBI and effective treatment of active TB is imperative. It is evident that there is a need for new specific TB diagnostic tests.

In this present study we aim to aid in the diagnosis and distinguish between BCG vaccine and TB disease in peripheral blood using flow cytometry by generating TB-specific intracellular cytokine (TNF-α and/or IFN-γ) profiles of $CD4^+$ T-cell populations following exposure to TB specific antigens.

Whole blood and not peripheral blood mononuclear cells (PBMC's) is used for optimization purposes as this technique is simple, avoids time-consuming separation steps and is performed in an environment close to *in vivo.*

Thus the objective of the present study is to optimise the experimental conditions (i.e. antibody volumes and intracellular staining protocols) for this assay prior patient analysis.

For optimisation purposes only 18 TB positive (6 – HIV^+ and 12 HIV^-) patients as well as 10 TB negative and HIV negative along with the appropriate negative (unstimulated) and positive assay controls (SEB) were tested and analysed using flow cytometry to establish a foundation for comparisons.

The samples were tested for the release of TNF-α and IFN-γ (positivity in stimulated $CD4^+$T-cells) in terms of mean fluorescence intensity (MFI). We will also compare the cytokine expression profiles of healthy controls selected to that of newly diagnosed TB cases, before initiation of treatment and compare the results thereof with the gold standard methods to determine assay sensitivity and specificity. If established this technique will potentially enable diagnosticians to distinguish BCG vaccine and TB disease (latent or active).

Chapter 2 – Materials and Methods

2.1 Study cohort/design and Ethical considerations

The present study forms part of a larger study of which ethical approval has been given by the Health Research Ethics Committee (HREC), Faculty of Medicine and Health Sciences (FMHS) at Stellenbosch University - N11/08/246. Further permission to conduct this study was also obtained from the Western Cape Government, Department of Health (DOH) in order to recruit patients from Tygerberg Hospital (TBH). This study forms part of a larger study which focuses on the establishment of a flow cytometry-based TB assay and the validation and implementation of the assay in a clinical set up. However, for the purpose of this study we focus on the optimisation and development of this flow cytometry-based assay.

Eighteen consenting, previously confirmed $HIV^{neg}TB^{pos}$, patients were recruited from TBH while 10 $HIV^{neg}TB^{neg}$ consenting participants which served as the negative control group were recruited from the Division of Haematology, Tygerberg Hospital. HIV diagnostic confirmation of participating patients were screened in the NHLS, Division of Virology Unit, using the HIV p24 Antigen ELISA assay, while a positive TB diagnosis was confirmed in the Division of Medical Microbiology on the Cepheid Gene Xpert® (Sunnyvale, CA, USA). For the control group participation, their TB negative status was confirmed based on the completion of a quarterly TB questionnaire used to screen NHLS staff members. In addition, HIV p24 Antigen ELISA tests were also performed to confirm HIV status.

2.1.1 Inclusion criteria

Patients older than 18 years, who are treatment naïve, HIV negative and TB positive were included.

2.1.2 Exclusion criteria

HIV positive patients with a CD4 count below 350 cells/mm^3 and patients who have previously been confirmed as having MDR/XDR TB have been excluded. In addition, patients who have commenced empiric TB treatment were also excluded from this study.

2.2 Principle of tests

2.2.1 HIV Ag/Ab Combo for HIV screening/confirmation

The Architect HIV Ag/Ab Combo assay (Vironostika, BioMerieux, The Netherlands), is a two-step chemiluminescent microparticle immunoassay (CMIA) which qualitatively detects HIV p24 antigen and antibodies to HIV type 1 and/or type 2 in human serum or plasma. Firstly, the patient's serum, Architect wash buffer, assay diluent and paramagnetic microparticles were all combined. The HIV p24 antigen and HIV-1/HIV-2 antibodies present in the serum bind to the HIV-1/HIV-2 antigen and HIV p24 monoclonal (mouse) antibody coated microparticles. Following a wash step, the HIV p24 antigen and HIV-1/HIV-2 antibodies bind to the acridinium – labelled conjugates consisting of HIV-1/HIV2 antigens (recombinant), synthetic peptides and HIV p24 antibody (mouse monoclonal). After a consecutive wash cycle, pre-trigger (1.32% [w/v] hydrogen peroxide) and trigger (0.35N sodium hydroxide) solutions were added to the reaction mixture and the resulting chemiluminescent reaction was measured as relative light units (RLUs). The amount of HIV antigens and antibodies in the sample is directly proportional to the RLUs detected by the system optics. A positive result was recorded when a sample had a signal to cut off (S/CO) >1.00 and a non-reactive sample had a S/CO <1.00. The cut off signal was determined from the Architect HIV Ag/Ab Combo calibration.

2.3 Sample Collection and processing

Following consent, 6ml of peripheral blood was collected by venepuncture into 6ml Sodium Heparin BD Vacutainer tubes (BD Vacutainer, San Jose, CA). Samples were transported at room temperature from the clinic to Tygerberg Haematology laboratory, within 6 hours of collection.

2.4 Optimization of experimental conditions

Briefly, the following steps will be followed in order optimize the experimental conditions for this assay:

i) Cell stimulation in the presence or absence of antigens such as early secretory antigenic target-6 (ESAT-6), culture filtrate protein-10 (CFP-10) and Staphylococcus Enterotoxin B (SEB)

ii) Cytokine accumulation,

iii) Fixation,

iv) Permeabilization and

v) Flow cytometry.

2.4.1. Baseline intracellular marker protocol

Intracellular and cell surface expression of T-cell markers was determined by staining cells with fluorochrome labelled monoclonal antibodies. Lineage markers anti-CD45 ECD (J33 clone), anti-CD3 PC5 (UCHT 1 clone) and anti-CD4 APC (13B8.2 clone) from Beckman coulter, Inc., USA, were used to characterize T-cells. Antibodies were combined to form a cocktail as shown in Table 2.1 along with supplier details.

Table 2.1 Antibody cocktail for surface markers.

Antibody	Volume	Function	Supplier
CD45-ECD	8µl	Pan-leucocyte marker	Beckman coulter, Inc, USA
CD3-PC5	2µl	T cell receptor	Beckman coulter, Inc, USA
CD4-APC	6µl	Lineage marker	Beckman coulter, Inc, USA
CD27-APC 750	4µl	T cell memory marker	Beckman coulter, Inc, USA
TIM3-PC7	4µl	Marker of T cell exhaustion	BD Biosciences, San Diego, CA, USA
CD45RO – BO	3µl	T cell memory marker	BD Biosciences, San Diego, CA, USA
Total	**27µl** of cocktail to be added to each 50ul aliquot of whole blood.		

Abbreviations: APC- Allophycocyanin, APC 750- Allophycocyanin-Alexa Fluor 750, BO- Brilliant Orange, ECD- Phycoerythrin-Texas Red, PC5- PhycoerythrinCyanin 5, PC7- PhycoerythrinCyanin 7

The sample preparation procedure for flow cytometry analyses is further demonstrated in Figure 2.1. Briefly, whole blood was divided into three aliquots of 200µl each in 5ml FACS Tubes (BD Falcon, BD Biosciences, MA, USA) in order to represent the un-stimulated (negative control tube), one representing and containing ESAT6/CFP10 antigens and another one representing the positive control, containing the SEB antigen. Antigens were added to the respective tubes and a co-stimulant anti – CD28 (Beckman coulter, Inc., USA) needed for T-cell activation was also added to all three representative tubes. After the addition of the antigens to the respective tubes, the three samples were incubated at 37°C for 2 hours, after which 5µl of 5µg/ml Golgi transport inhibitor Brefeldin A was added and samples were further incubated overnight for 18 hours.

After incubation, samples were removed and stained with 1µl Fixable Viability Dye eFluor® 455UV purchased from eBioscience(San Diego, CA, USA) and incubated at room temperature (RT) for 30min.

For each of the three samples a secondary tube was labelled, however the unstimulated sample received an additional tube and this will serve as the unstimulated/unstained control as this tube will receive no monoclonal

antibodies.Following cell stimulation and cytokine accumulation, 50µl from the primary tubes containing the stimulated and un-stimulated whole blood as is the case with the negative control, was pipetted into the four secondary tubes respectively. Twenty seven microliters of the surface marker antibody cocktail as shown in Table 2.1 was added to each of the three representative tubes. The unstimulated/unstained tube received no antibody as this will serve as the unstimulated, unstained control which is used to verify autofluorescence. All samples were incubated for 15 – 20min in the dark at RT.

Following surface marker antibody incubation, the Beckman Coulter PerFix-nc Kit (no centrifuge assay Kit) was used to fix and permeabilize cells. Five microlitres of Buffer 1 (fixative reagent) was added to each representative tube. The tubes were vortexed and incubated in the dark at RT for 15min. After incubation, samples were vortexed again and 300µl of Buffer 2 (permeabilizing reagent) was added and samples were vortexed immediately. Intracellular markers Fluorescein isothiocyanate (FITC) labelled IFN-γ (18µl) and Phycoerythrin (PE) labelled TNF-α (14µl) (volumes determined from optimisation) both from Beckman coulter, Inc., USA, was added, vortexed and placed in the dark to incubate for 15 – 30min at RT. Lastly, 400µl of the Final 1X Reagent (prepared from the 10x concentrated Final Solution) was added to all four tubes and samples were acquired on the NAVIOS.

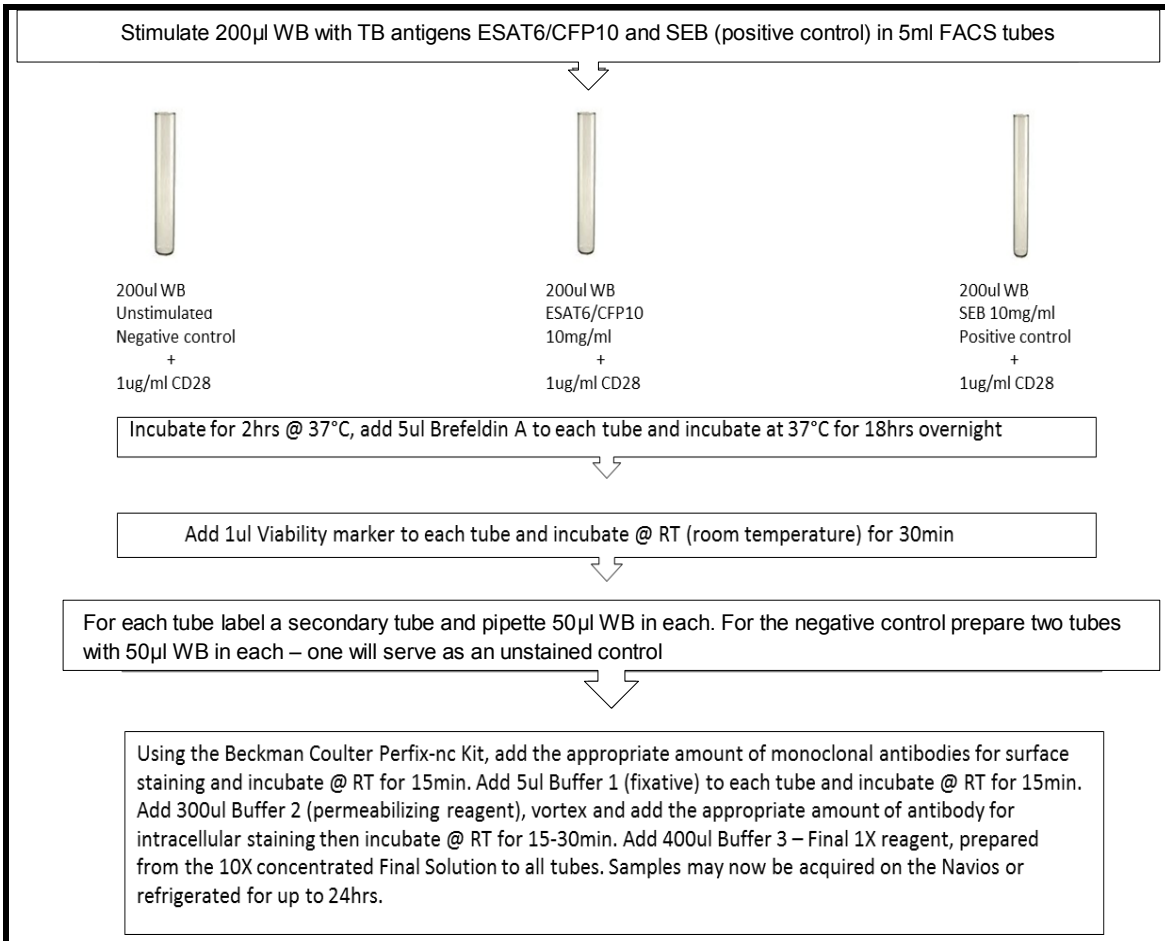


Figure 2.1: A schematic diagram of sample preparation for flow cytometry analysis

2.5 Instrument quality control and compensation

Various steps were taken to ensure that the instrument operates optimally on a daily basis. Before analysing patient samples, the following steps were taken:

2.5.1 Voltage Settings

Voltages were set on an unstained control by adjusting the voltage of each photomultiplier tube (PMT) to bring the population onto scale. Following this, single stained controls were then run on the instrument to further adjust the voltage for each PMT. Furthermore, compensation was performed as explained below and the voltages were verified on the final verification tube.

2.5.2 Compensation

In flow cytometry, spectral overlap occurs when the fluorescence of one fluorochrome e.g. PE, is picked up by another detector e.g. the FITC detector. This happens when the spectral emission of certain fluorochromes overlap. Compensation was therefore performed to adjust for spectral overlap between the fluorochromes and this was done according to the Beckman Coulter specifications. Briefly, 50µl whole blood was added to a 5ml Falcon^{TM} tube, followed by the titrated amount of antibody and the tube is then placed in the Beckman Coulter TQ-prep instrument. Compensation was then performed on the Beckman Coulter Navios instrument using the automated procedure and those settings was then analysed using the Beckman Coulter Kaluza software. Some antibodies in the panel did not have clear separation of their negative and positive populations, and in these instances we used different antibodies for the same fluorochrome. Table 2.2 describes this. Compensation was only re-run after the instrument was serviced or if the daily QC failed repeatedly despite following troubleshooting steps.

Table 2.2 Antibody panel used for project compared to compensation antibody panel

Flow cytometry project antibody panel									
FL1 FITC	**FL2 PE**	**FL3 ECD**	**FL4 PC-5**	**FL5 PC-7**	**FL6 APC**	**FL7 ALEXA 700**	**FL8 APC-750**	**F9 450/50**	**F10 BO**
IFN-γ*	TNF-α*	CD45	CD3	TIM3	CD4	N/A	CD27	Viability	CD45RO
Flow cytometry compensation panel									
FL1 FITC	**FL2 PE**	**FL3 ECD**	**FL4 PC-5**	**FL5 PC-7**	**FL6 APC**	**F7 ALEXA 700**	**F8 APC-750**	**F9 450/50**	**F10 BO**
CD45RA*	CD45RO*	CD45	CD3	CD3	CD4	N/A	CD27	CD45-PB	CD45RO

*Denotes intracellular markers; Abbreviations: PB – Pacific Blue

2.5.3 Daily QC

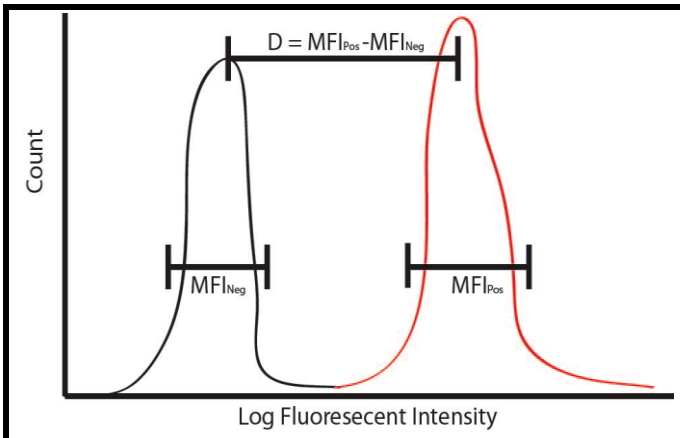
Quality control checks were done every day upon start-up of the instrument. Beckman Coulter Flowcheck Pro fluorospheres were used to assess the stability of the laminar flow (fluidics), and the detector voltages of the instrument. Furthermore, the instrument monitors its performance over time and plots these details on a Levey-Jennings chart. Through this we were able to see if the detector's performance is out of range and perform the necessary corrective action.

2.6 Assay Optimization

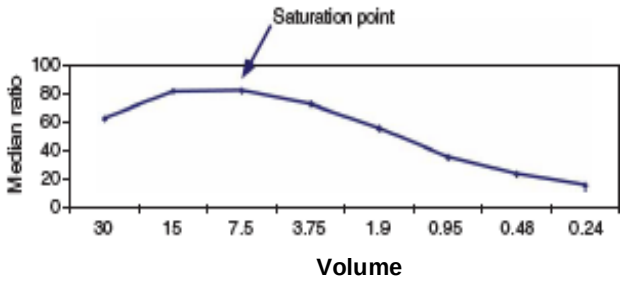
Assay optimisation forms the foundation of the intracellular staining protocol. Optimisation was divided into (i) surface and intracellular monoclonal antibody volume (ii) Overnight Incubation temperature at 37°C or 4°C and (iii) staining with surface markers before permeabilizing cells.

2.6.1 Monoclonal antibody optimisation

In order to use fewer antibody volumes as a way of saving cost, an antibody titration is performed. The optimal antibody volume will result in the highest signal of the positive population and the lowest signal of the negative population. The signal to noise ratio (S:N) = D, is used to quantitatively determine expression of cell separation when the positive and negative populations can clearly be distinguished from each other as seen in figure 2.2 A.



A Signal to noise ratio is calculated by dividing the MFI of the positive population (red) by the MFI of the negative population (black).



B Saturation point on curve indicating the volume of antibody that results in the least amount of background fluorescence

Figure 2.2 Monoclonal Antibody optimisation. Adapted from (Lamoreaux, Roederer, & Koup, 2006) **MFI – Mean fluorescent Intensity*

This is calculated by dividing the mean fluorescence intensity (MFI) of the positive population by the MFI of the negative population. Background noise can be kept to a minimum when small antibody volumes can be used in samples with high cell concentrations (Hulspas, O'Gorman, Wood, Gratama, & Robert Sutherland, 2009). Together with the S:N, the optimal volume was established from the plateau area (saturation point) of the antibody titration curve as demonstrated in figure 2.2B, however, statistical criteria was used as confirmation (Collino, Jaldin-Fincati, & Chiabrando, 2007). Optimizing the antibody volume determines the maximum amount of antibody that results in the least background fluorescence and this is achieved by focusing on the fluorescence intensity of the negative population rather than the difference between the two populations (Hulspas, 2010).

Briefly, 50µl whole blood (WB) was stained with decreasing volumes of surface markers CD4–APC, CD45-ECD, CD3-PC5, TIM3-PC7, CD27-APC 750 and CD45RO-Brilliant Orange as listed in table 2.1. Where the manufacturer's suggested volume was 20µl, antibodies were added as follows: 20µl, 18µl, 16µl, 14µl, 12µl, 10µl, 8µl and 6µl. Where the recommended volume was 10µl, antibodies were added as follows: 10µl, 8µl, 6µl, 4µl, 2µl and 1µl. Tubes were placed in the Beckman Coulter TQ-Prep, where it was incubated and lysed. Each tube was then run in triplicate on the Beckman Coulter, NAVIOS flow cytometer. The optimal volume was determined following visual analysis of density plots, S:N and statistical criteria (data shown in Chapter 3).

For the intracellular markers, the baseline protocol as described above was followed. TNFα-PE and IFNγ-FITC was added in decreasing volumes starting at 20µl down to 6µl. Samples were then fixed and acquired on the NAVIOS in triplicate. The optimal volume of these intracellular markers was determined following visual analysis of density plots, S:N and statistical criteria.

2.6.2 Overnight Incubation with Brefeldin A - 37°C vs. 4°C

In order to determine whether an incubation temperature of 37°C and/or 4°C will have a difference on experimental conditions, both of these temperatures were tested to determine its effects. Two samples of 500µl WB each was stimulated with

1µg/ml SEB and 1µg/ml CD28. Both samples were then incubated at 37°C for 2 hours in an incubator, however; one of the samples was placed in a beaker of water in the incubator. Five micrograms per millilitre of BFA was added to both samples. The sample with the beaker of water was then placed in the refrigerator at 4°C overnight (18 hours) – this was done to maintain a steady decrease in the incubation temperature, and the other sample remained incubated at 37°C. The following day both samples were stained with lineage markers CD45-ECD, CD4-APC and CD3-PC5 as well as a viability marker 7-AAD (Beckman coulter, Inc., USA). Samples were then placed in the TQ-Prep after which it was acquired on the Beckman Coulter NAVIOS flow cytometer.

2.6.3 Staining with surface markers before or after permeabilizing cells

Previous protocols suggest staining of surface markers after fixation and permeabilization of cells, however following this route did not yield any $CD4^+$ T-cells. We experimented with staining stimulated cells before and after fixation, as Lamoreaux et al., 2006 has stated that the fixative can change the epitope and subsequently the avidity of the antibody-antigen reaction. We followed the same stimulation protocol as before, except in this experiment, cells were stained with CD4-APC before fixing and permeabilization. See Chapter 3 for results.

2.7 Flow cytometry gating strategy

Flow cytometric acquisition was performed on a ten colour Beckman Coulter Navios, while data analyses was performed using Kaluza software (Beckman Coulter). A density plot of forward scatter (FS) peak vs. FS integral was drawn and a gate was set around the single cells. These cells were then interrogated on a density plot of side scatter (SS) vs. Viability marker – pacific blue to separate viable cells from dead cells. The viable cells were used in a secondary plot of SS vs. CD45-ECD to put a gate around the lymphocyte population. The lymphocyte population was separated into T-cells and B-cells using a density plot of CD3-PC5 vs. CD45-ECD. The T-cell population of cells was further interrogated and a density plot of CD3-PC5 vs. CD4-APC was drawn and a gate was set around the CD4 population of cells. This CD4

population of cells was further delineated into the respective $CD4^+$ T-cell subsets – data shown in Chapter 3. A detailed gating strategy can be seen in figure 2.3.This gating strategy will be applied to all samples for analyses.

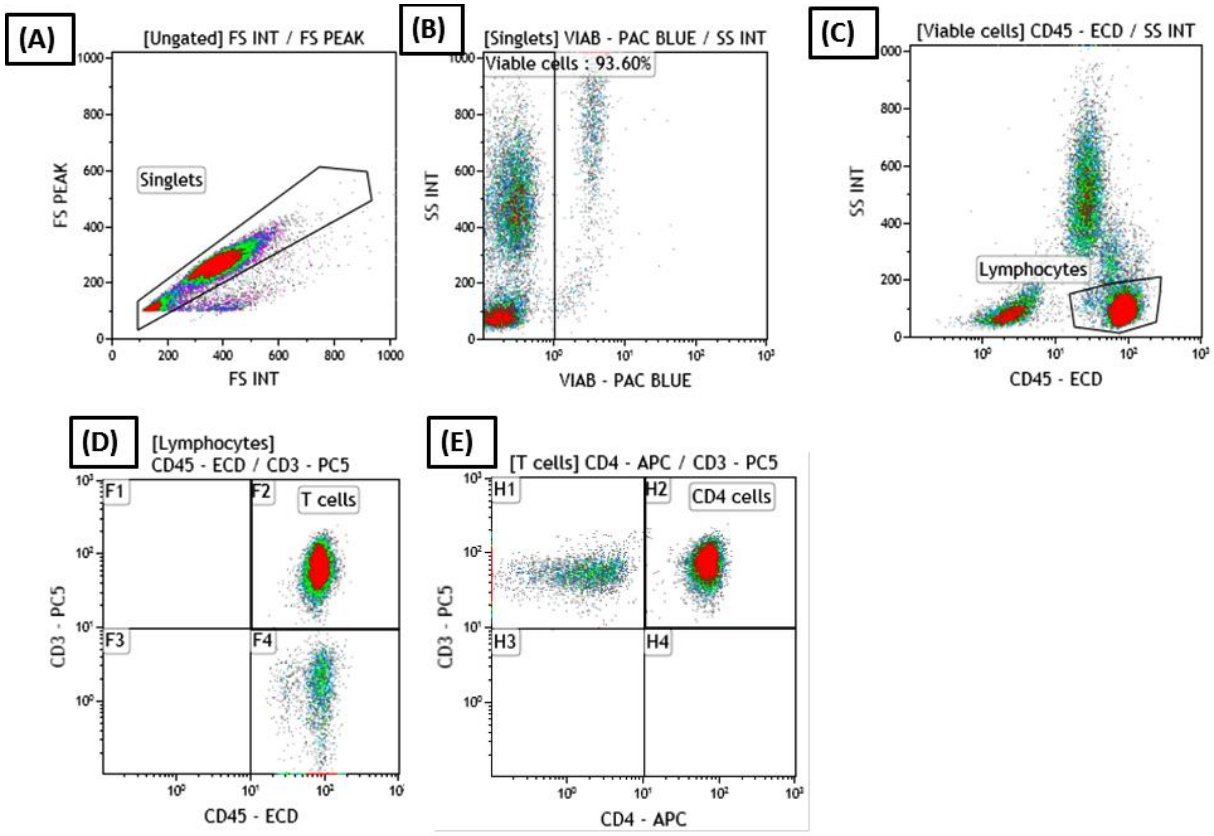


Figure 2.3A detailed sequential gating strategy. (A) Forward Scatter (FS) peak vs. FS integral shows doublet discrimination and a gate is drawn around the single cells. (B) Side scatter (SS) vs. Viability marker, gated on single cells, was used to gate on viable cells. (C) SS vs. CD45-ECD gated on viable cells and a gate was placed around lymphocyte population. (D) A secondary plot of CD3-PC5 vs. CD45-ECD gated on the lymphocyte population was drawn to separate T-cells from B-cells (E) CD4 cells were isolated using CD3-PC5 vs. CD4-APC on density plot gated on the T-cell population.

2.8 Statistics

Box-and-whisker plots were used to present the data and have the following format: the median value is represented by a horizontal line in the box; the box represents the interquartile range (25-75 percentile) of the data, while the whiskers indicate the minimum and maximum values. When comparing two variables, an unpaired T-test was used when the data was normally distributed, while a Mann-Whitney U test was performed for non-parametric data. The repeated measures one-way Analysis of Variance (ANOVA) was used for comparing multiple factors e.g. IFN-γ expression across the $CD4^+$ T-cell subsets within the patient group. This was followed by a Dunn's multiple comparison post-test for non-parametric data. Data is reported as median percentage expression ± standard deviation (SD), with $p<0.05$ indicating significant results and results with a p value >0.05 was considered not-significant (NS). A statistician from the Stellenbosch University, Centre for Evidence Based Health Care, Biostatistics Unit was consulted and it was determined that a minimum of 8 subjects per group was needed for statistical power and to verify that all statistical analyses done were correct for that data set. GraphPad Prism software v.5 (GraphPad Software, San Diego CA, www.graphpad.com) was used for all statistical analyses

2.9 Overview of data analysis

As mentioned above, an overview of the optimization results will be given first in figure 2.4 in order to introduce the different cytokine profile result comparisons within the different subpopulations analysed. Following optimization, patients analysis was performed by comparing statistical analyses of baseline cytokine (IFN-γ, and TNF-α), and TIM-3 expression between TB-positive patients and controls upon stimulation with TB antigens ESAT6 & CFP10 (E/C), SEB (positive assay control) as well as an unstimulated baseline (negative control).

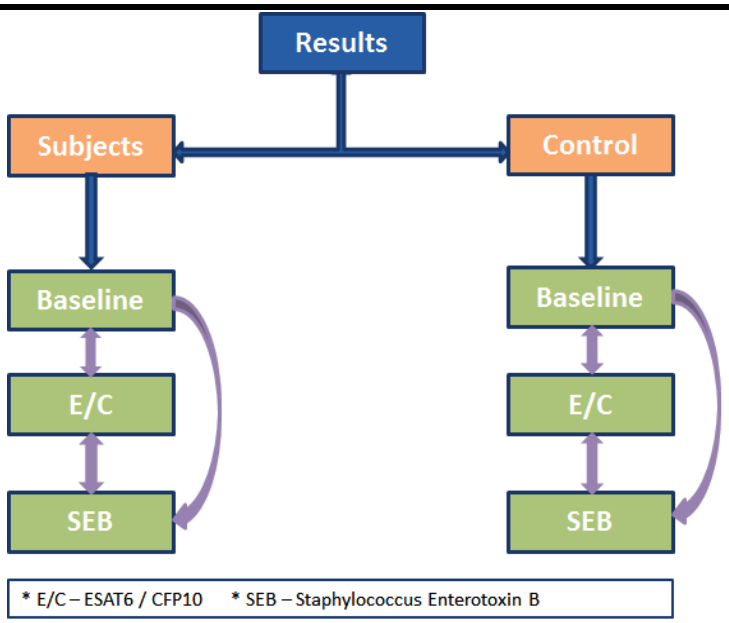


Figure 2.4 Schematic overview of data analysis. Results were divided into study subjects and healthy controls. Each of these groups was further subdivided into baseline (negative control), E/C test condition and SEB (positive control). All results were compared.

Chapter 3 – Results

3.1 Demographics of study participants

A total of 18 patients were recruited for this study, which included either mixed race or black patients. The mean age (years) of this group was 39.11(Range: 18 – 63). The control group consisted of 10 participants aged between 27 and 40 years of age, and had a mean age of 30. The dominant gender was males with 66.67% in the study group and in the control group females were in the majority with 60%. Table 3.1 below summarises the patient demographics. For data analyses, the patient group was combined and analysed as a whole.

Table 3.1 Demographics of study participants

Parameter	TB^+HIV^-	TB^+HIV^+	Control group
No. of Participants	12	6	10
Age range	18 – 63	36 – 58	25– 40
Age (mean years)	37.58	42.16	31.9
% of females	33.33	33.33	60
% of males	66.66	66.66	40
% mixed race	58.33	41.66	70
% black	83.33	16.66	30

3.2 AssayOptimisation

3.2.1 Incubation temperature

In order to determine the effect of incubation temperature on the $CD4^+$ T-cell yield and experimental conditions, two samples of 500µl WB each were incubated overnight at 4°C and 37°C respectively. Results in figure 3.1 below showed no statistical difference between incubation at 37°C vs. 4°C and in conclusion, samples were incubated at 37°C overnight for all other subsequent incubations.

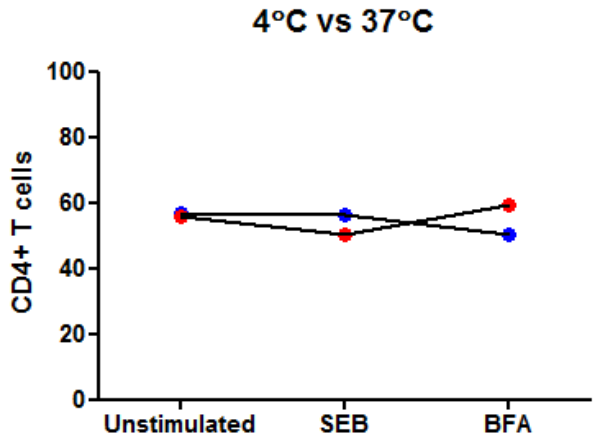


Figure 3.1 Relative percentage (%) CD4$^+$ T-cells per stimulation condition. Incubation of samples at 4°C vs. 37°C. Graph shows no significant difference between the two incubation temperatures. This concludes that samples can be incubated at 37°C. [4°C; 37°C]

3.2.2 Staining with surface markers before or after permeabilizing cells

The intracellular staining kit (PerFixnc, Beckman Coulter,USA) used in this study suggested staining cells with surface markers after permeabilizing cells; however we were unable to detect any CD4$^+$ T-cells, most likely due to antigen loss after fixation. We thus stained cells with surface markers before permeabilizing and fixing cells and this yielded a CD4$^+$ T-cell population, clearly distinguished from the negative cells as seen in figure 3.2 below.As staining of surface marker before permeabilization and fixation was proven to yield the best result, the workflow in the protocol/experimental conditionswas modified for all subsequent staining of other surface markers.

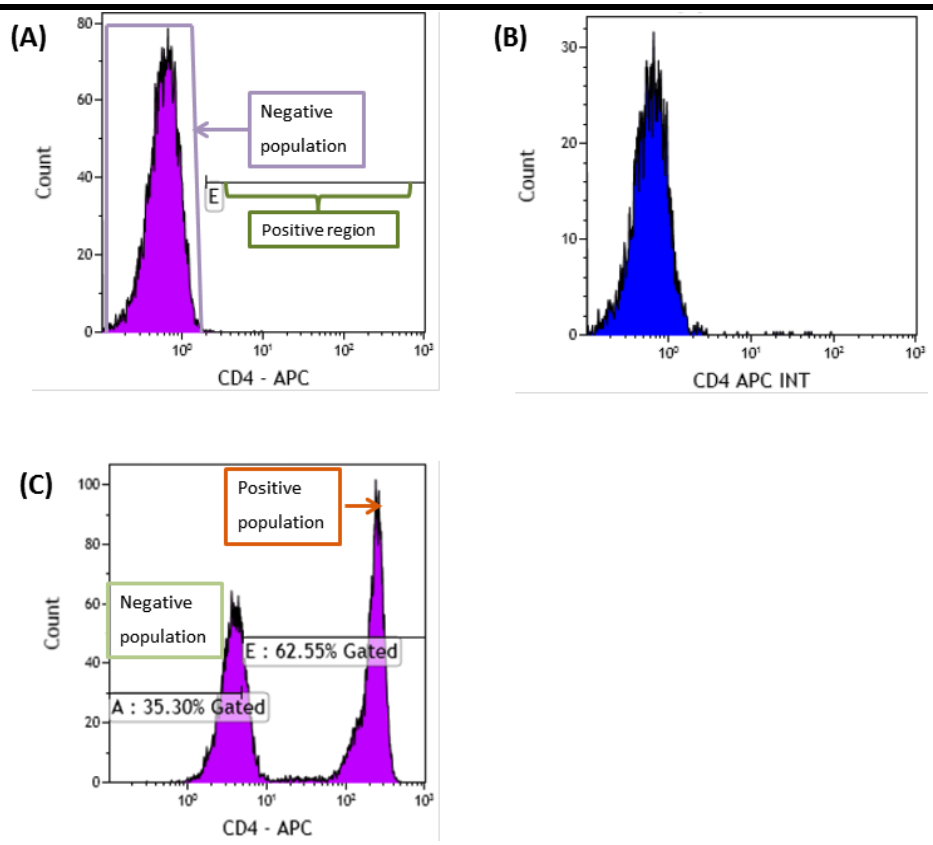


Figure 3.2:The effect of staining with surface markers before and after permeabilizing and fixation. (A) Baseline (unstained) control for surface marker CD4 – APC antibody. **(B)** Cells stained with surface marker, CD4-APC **after** fixing and permeabilizing. Histogram plot shows no significant increase in MFI of a population within the positive region. **(C)** Cells stained with surface marker, CD4-APC **before** fixation, histogram shows an increase in MFI of the positive population and clear separation from the negative population. This concludes that fixative has an effect on the cell surface epitope, and samples should be stained with surface markers before fixing of cells. * *APC - Allophycocyanin*

3.2.3 Antibody titrations

Antibodies were titrated to determine the optimal volume required for the staining protocol to avoid under- or over staining of the cells but also from a cost perspective, The optimal antibody volume was determined using: statistical analyses, visual analyses and signal-to-noise(S:N) ratio, all of which are detailed below. For the purposes of this section, CD3-PC5 is being used as an example. Statistics for the remaining fluorochromes can be found in Addendum 1.

3.2.3.1 Statistical determination of optimal antibody volumes

The unpaired *t*-test was used to determine statistical differences in the MFI of the various volumes used. Table 3.2 shows CD3-PC5 as an example (see Addendum 1 for complete antibody list). Rows 1-3 show a significant difference in MFI (p=0.0253, p=0.0129, and p=0.0006 respectively), suggesting that these volumes cannot be used for optimal staining.

Table 3.2 Statistical determination of optimal antibody volume

#	Antibody	Comparison (µl)	MFI mean difference	P value
1	CD3-PC5	1 vs. 2	-64.22	0.0253 *
2		2 vs. 4	-41.26	0.0129 *
3		4 vs. 6	-26.22	0.0006 ***
4		6 vs. 8	2.01	0.4387 (NS)
5		8 vs. 10	-1.99	0.1022 (NS)

*µl – microlitres, NS – not significant, MFI – mean fluorescence intensity, PC5 - Phycoerythrin-Cyanin5.1, * - p<0.05, ** - p<0.01, *** p<0.001*

The results detailed in Table 3.2 are plotted on a graph as seen in Figure 3.3 and shows the antibody titration curve for CD3-PC5. The saturation point on the graph is reached at 6µl (indicated by red dot) which has a mean percentage positive-staining cells of 99.35% and the MFI = 119.04. This confirms the statistical analyses that showed no significant difference between 6µl and 8µl of antibody. Although we can take 6µl as the optimal volume based on the statistics, we still have to interpret these results together with the visual analyses and S:N ratio.

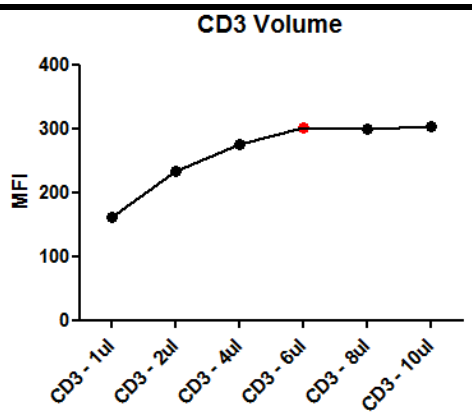


Figure 3.3 Determination of optimal antibody volume using the MFI. The red dot indicates the saturation point at 6µl. Since there is no statistical difference between 6µl and 8µl - 6µl would the volume to use. **MFI – mean fluorescent intensity*

3.2.3.2 Visual analyses of optimal antibody volume

The maximum amount of antibody required, which resulted in the lowest background fluorescence (or “noise”), was determined by comparing the density plots of the different volumes of antibody. Figure 3.4 below shows the dot plots of all volumes for visual analyses. The dot plots show clear separation of the negative and positive populations for each volume of antibody. However, 1µl and 2µl (within the red square) are the only two plots with a negative population closest to that of the unstained control. This indicates that these two volumes will give the least amount of background fluorescence and the S:N ratio will be able to determine which of the two volumes will work best.

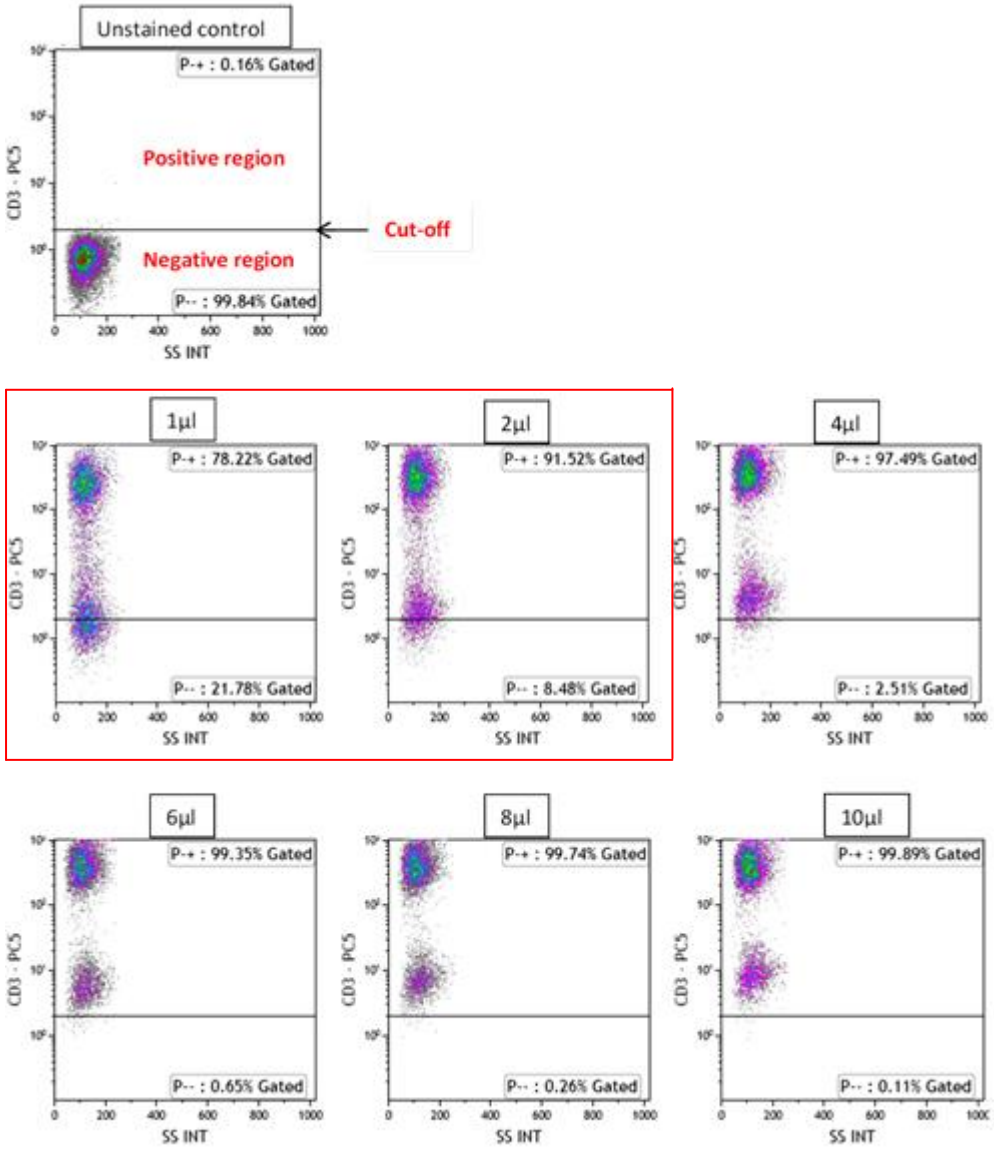


Figure 3.4 Determining the minimum amount of antibody to use. The graphs show antibody on the y-axis vs. Side Scatter on the x-axis. The unstained control is used to set the cut-off for the negative region. All plots show clear separation of the negative and positive populations. The plots within the red square have a portion of their negative populations within the negative region, closest to that of the unstained control and would be the volumes of choice, however these results have to be read together with the S:N ratio. **µl – microliters; PC5 – Phycoerythrin-Cyanin 5.1; SS INT – side scatter integrel*

3.2.3.3 Determination of optimal antibody volume using the S:N ratio

The histogram plots below in figure 3.5 show the separation of the negative and positive populations. The geometric mean is used to determine the MFI for each population and these values were used to calculate the S:N ratio for each volume.

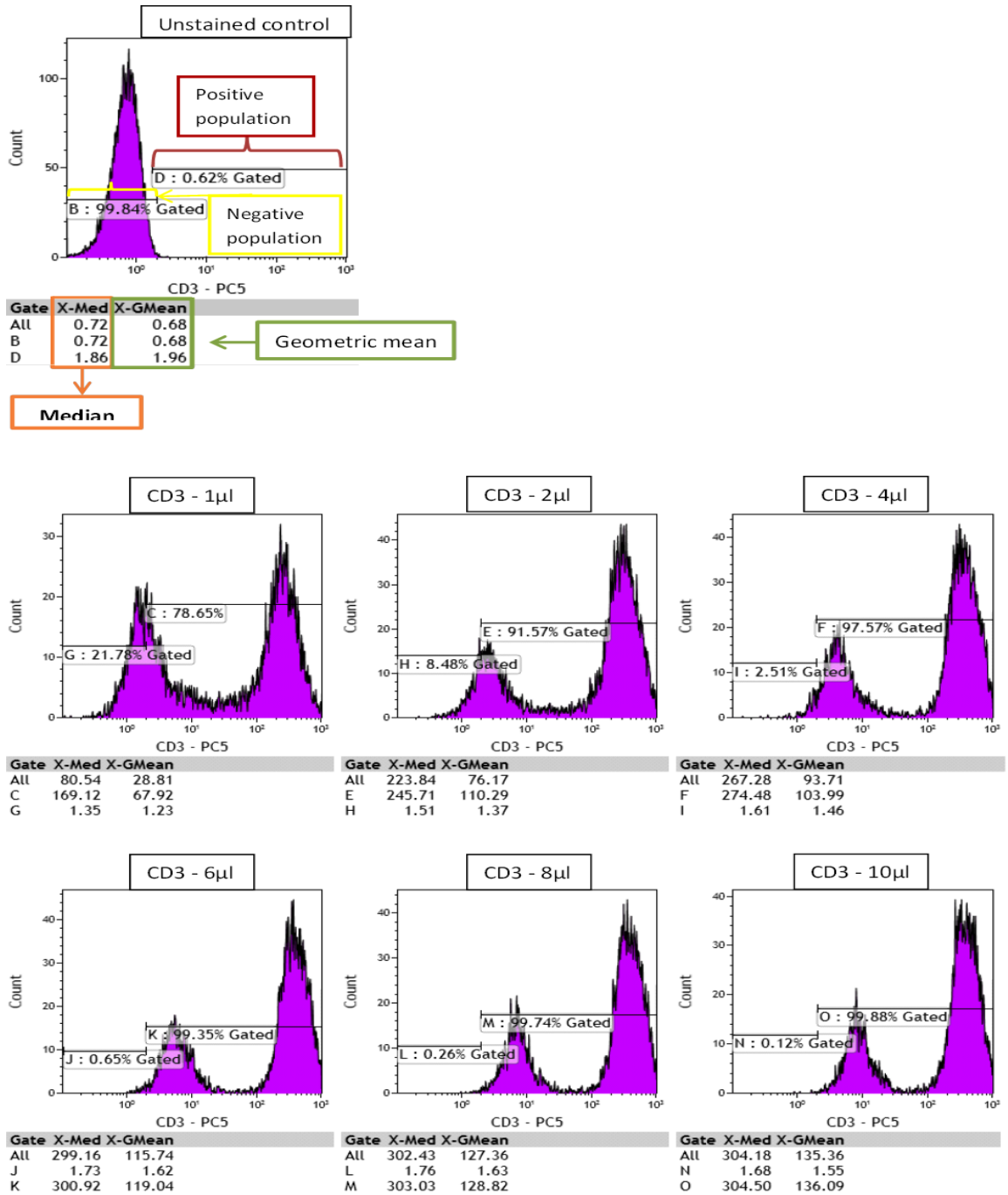


Figure 3.5 Histogram plots for antibody CD3-PC5. The positive MFI is determined from the geometric mean of the positive population and the negative MFI is determined from the geometric mean of the negative population. **PC5 – Phycoerythrin-Cyanin 5.1*

The S:N ratio was calculated by dividing the MFI of the positive population by the MFI of the negative population for each antibody volume. The volume with the highest S:N ratio is the volume that will result in maximum staining of the cells with the least amount of background noise. Table 3.3 below shows the S:N ratio results for the antibody CD3-PC5. Based on the S:N ratio it is determined that 2µl is the optimal volume of CD3-PC5 to be used. See Addendum 2 for complete antibody list.

Table 3.3 Determination of optimal antibody volume using the S:N ratio

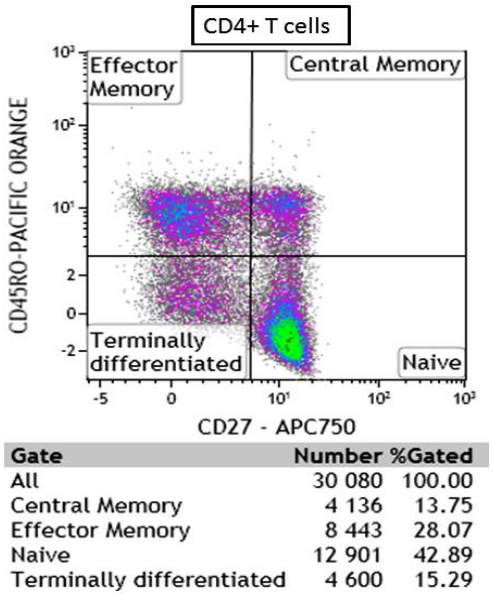
Antibody	Volume (µl)	S:N ratio	MFI of negative population	MFI of positive population
CD3	1	56.31	1.23	69.27
	2	**80.67**	**1.37**	**110.53**
	4	71.45	1.46	104.32
	6	73.48	1.62	119.04
	8	79.03	1.63	128.82
	10	91.89	1.48	136.01

µl – microliter, S:N – signal to noise ratio, MFI – mean fluorescence intensity

Taking all three criteria into consideration i) statistical analyses ii) visual analysis and iii) S:N ratio we have concluded that 2µl is the volume of choice that will give us maximum staining of cells with the least amount of non-specific binding. The statistical analysis indicated 6µl as the volume to be used; however, visually 6µl had both its negative and positive populations within the positive region, indicating a high degree of non-specific binding. Visually, 1µl and 2µl were the two volumes that had some portion of their negative population within the set negative region meaning one of these two volumes could be used. The third criteria determined that 2µl had the highest S:N ratio, hence in conclusion we used 2µl of CD3 – PC5. This same strategy was applied to all surface and intracellular antibodies.

3.3 CD4$^+$ T cell subset distribution

CD4$^+$ T-cell population was further delineated into its respective sub populations based on their CD27 and CD45RO expression. Figure 3.6 below shows the different populations i.e. naïve cells, central memory, effector memory and terminally differentiated T cells. Polarisation towards a specific subset may indicate disease status, e.g. active disease will stimulate expansion of effector memory cells (Jasenosky et al., 2015).



Gate	Number	%Gated
All	30 080	100.00
Central Memory	4 136	13.75
Effector Memory	8 443	28.07
Naive	12 901	42.89
Terminally differentiated	4 600	15.29

Figure 3.6 CD4$^+$ T-cell subsets. CD4$^+$ T cells are further delineated into different subsets based on their CD45RO and CD27 positivity. * *APC750 - Allophycocyanin*

Table 3.4 below shows the percentage expression of each cell type in the control vs. patient groups. Naïve cells ($CD45RO^{-}CD27^{+}$) were the most abundant cell type in both groups and compared to each other they were not significantly different. The differences in expression between the two groups for all cell types were not significant.

Table 3.4 Percentage positivity of $CD4^{+}$ T-cell subset cell expression.

	Cell phenotype	Healthy controls	TB Patients	p-value
Naïve cells (T_N)	$CD45RO^{-}CD27^{+}$	37.11 ± 14.22	47.48 ± 20.96	NS
Central memory(T_{CM})	$CD45RO^{+}CD27^{+}$	19.10 ± 10.79	11.51 ± 8.62	NS
Effector memory(T_{EM})	$CD45RO^{+}CD27^{-}$	23.85 ± 19.96	21.92 ± 13.25	NS
Terminally differentiated effector memory (T_{DEM})	$CD45RO^{-}CD27^{-}$	7.575 ± 9.99	13.02 ± 20.13	NS

p–value> 0.05 = Not significant (NS)

Figure 3.7 below shows the $CD4^+$ subset distribution comparison between healthy controls and patients. In both the patient and control group, naïve cells were the most abundant, followed by the effector memory cells. It was expected that the percentage of naïve cells would be greater in the control group, and the percentage of effector memory cells would be greater in the patient group, however this was not the case. The terminally differentiated cells in the patient group are greater than the central memory cells, and this is expected as there is on-going immune activation leading to more exhausted cells.

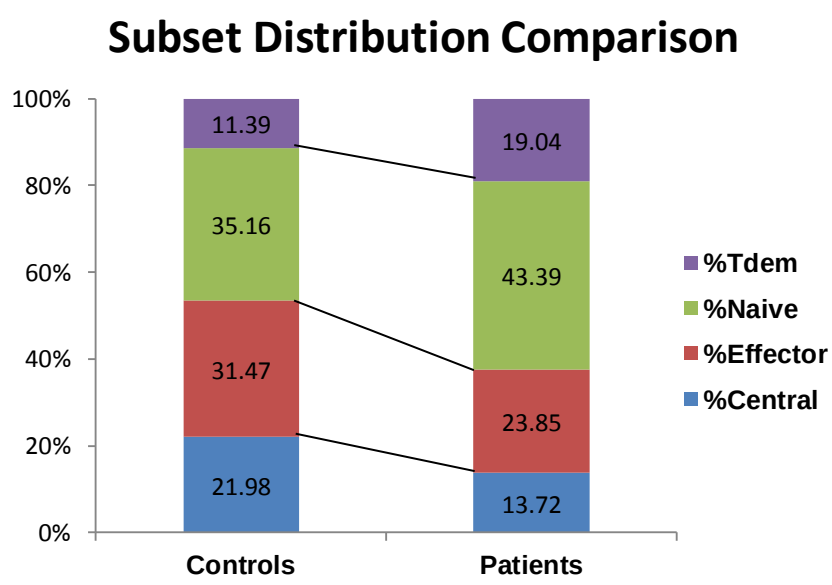


Figure 3.7 Comparison of $CD4^+$ T-cell subset distributions between healthy controls and patient group. Naïve cells were the most abundant cell type in both groups followed by the Effector memory cells. The control group had more central memory cells than terminally differentiated effector memory cells which is expected, whereas the patient group had more terminally differentiated effector memory cell expression than central memory cells.

3.4 Cytokine expression on $CD4^+$ T-cells

In figure 3.8 below cytokine expression of the total $CD4^+$ T-cell population was compared between the healthy control and patient group. In table 3.5 below, single expression of IFN-γ and IFN-γ^+TNF-α^+ dual expression was similar in the two groups and although the TNF-α expression was higher in the patient group, the difference in expression was not significant.

Table 3.5 T-test of cytokine expression

Cytokine	Median %		P - value
	Control	Patient	
IFN-γ	**2.960**	2.370	0.2800 (NS)
TNF-α	1.340	**2.415**	0.1729 (NS)
IFN-γ^+TNF-α^+	0.5400	**0.8550**	0.3961 (NS)

P-value >0.005 = not significant (NS)

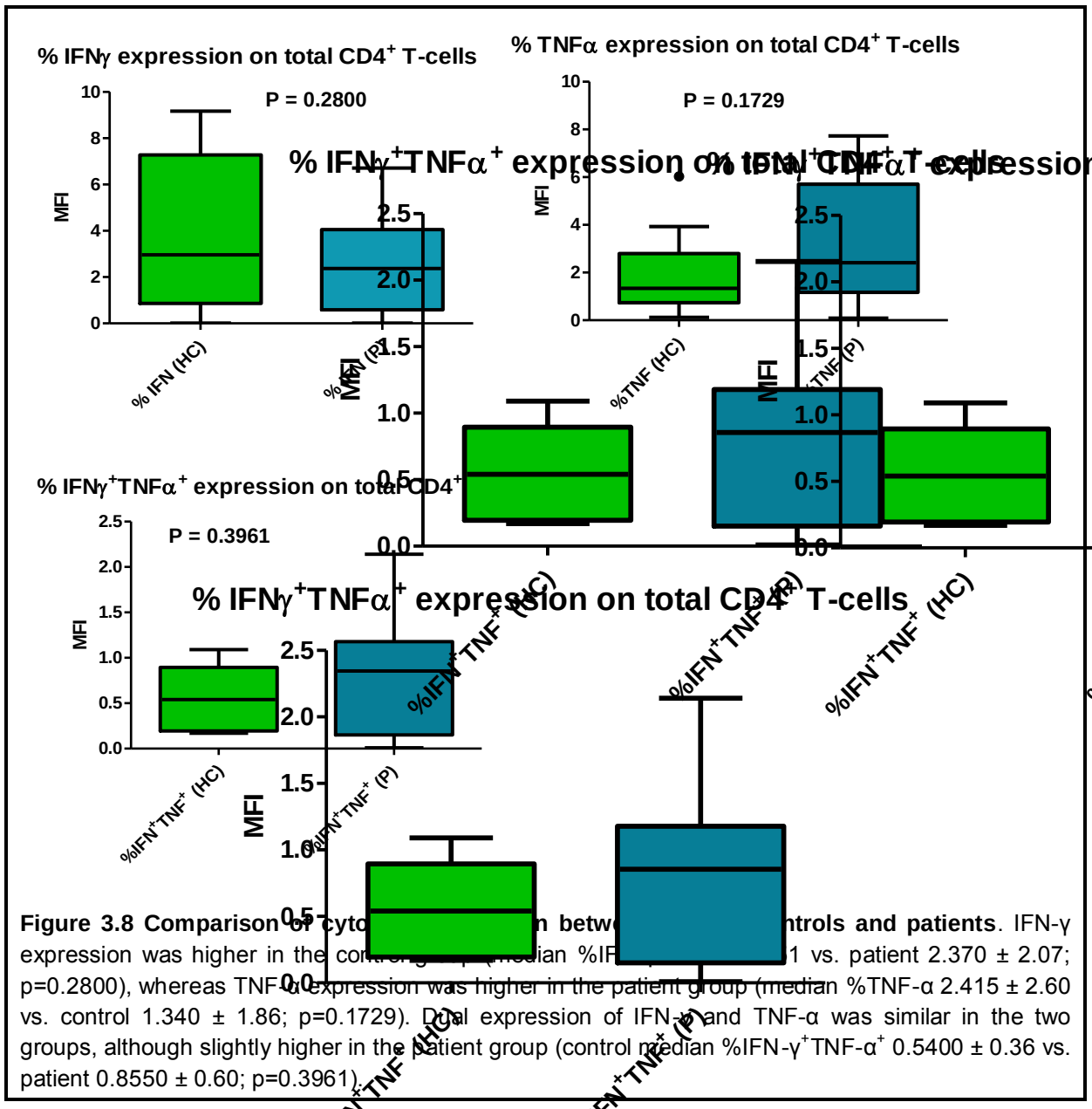


Figure 3.8 Comparison of cyto[illegible]n betw[illegible]ntrols and patients. IFN-γ expression was higher in the con[illegible] (median %IF[illegible]1 vs. patient 2.370 ± 2.07; p=0.2800), whereas TNF-α expression was higher in the patient group (median %TNF-α 2.415 ± 2.60 vs. control 1.340 ± 1.86; p=0.1729). Dual expression of IFN-γ and TNF-α was similar in the two groups, although slightly higher in the patient group (control median %IFN-γ$^+$TNF-α$^+$ 0.5400 ± 0.36 vs. patient 0.8550 ± 0.60; p=0.3961).

3.5 Effect of stimulation on $CD4^+$ T- cells

3.5.1 Effect of stimulation on IFN-γ expression in the patient group

In Table 3.6 IFN-γ expression was compared between the three stimulation conditions. The negative control expressed significantly more IFN-γ than the other two conditions. Figure 3.9 below shows the effect of stimulation on IFN-γ expression.

Table 3.6 Effect of stimulation on IFN-γ on total $CD4^+$ T-cell population within the patient group

	BL%IFN-γ	SEB%IFN-γ	E/C%IFN-γ
Median	**3.475**	2.520	2.370
P-value (<0.05) = 0.0015			
BL %IFN-γ vs. SEB %IFN-γ= No			
BL %IFN-γ vs. E/C %IFN-γ= Yes **			
SEB %IFN-γ vs. E/C %IFN-γ = Yes *			

BL = Baseline (negative control), SEB = Staphylococcus Enterotoxin B (positive control), E/C = ESAT6/CFP10

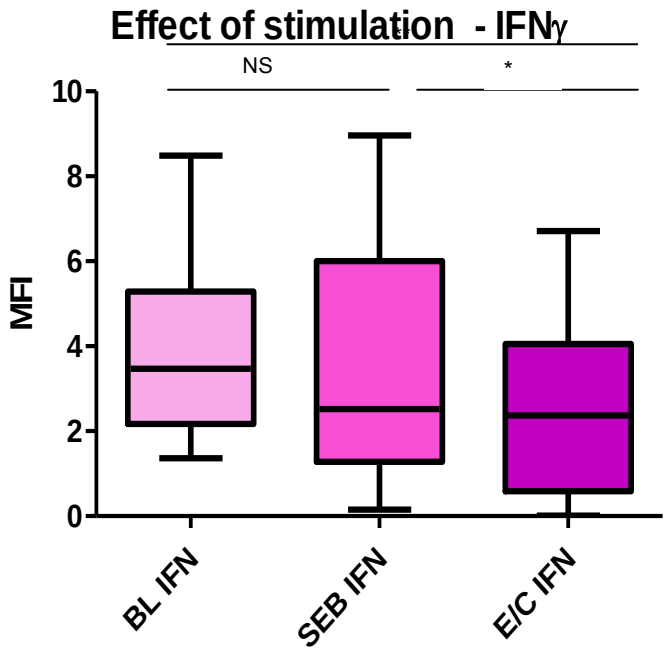


Figure 3.9$CD4^+$ T-cells were stimulated with TB antigens ESAT6 and CFP10 and the effect this had on the IFN-γ cytokine expression was recorded. The results indicate that stimulating cells significantly decreased IFN-γ expression.

3.5.2 Effect of stimulation on TNF-α expression in the patient group

In Table 3.7 TNF-αexpression was compared between the three stimulation conditions. The E/C test condition had the least cytokine expression and was significantly less than the positive control. Figure 3.10 below shows the effect of stimulation on TNF-α expression.

Table 3.7 Effect of stimulation on TNF-α on total $CD4^+$ T-cell population within the patient group

	BL % TNF-α	**SEB % TNF-α**	**E/C% TNF-α**
Median	3.510	**4.500**	2.415
P-value (<0.05) = 0.0064			
BL % TNF-α vs. SEB % TNF-α = No			
BL % TNF-α vs. E/C % TNF-α = No			
SEB % TNF-α vs. E/C % TNF-α = Yes **			

BL = Baseline (negative control), SEB = Staphylococcus Enterotoxin B (positive control), E/C = ESAT6/CFP10

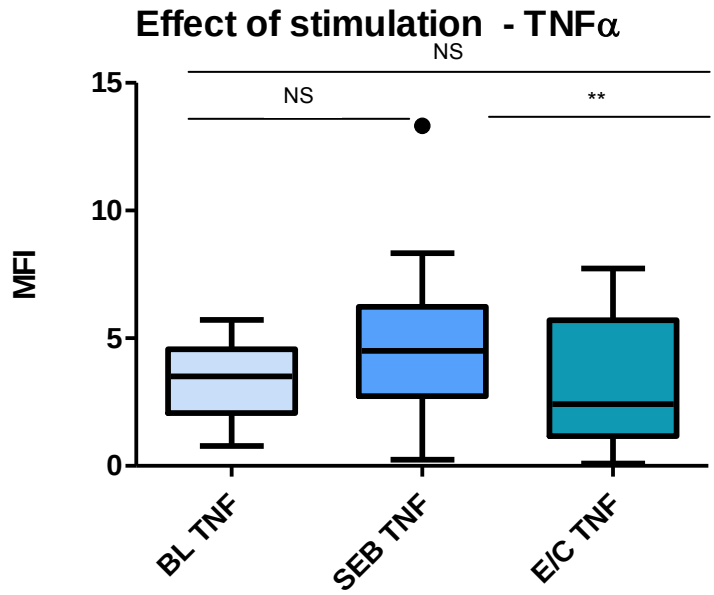


Figure 3.10$CD4^+$ T-cells were stimulated with Tuberculosis antigens ESAT6 and CFP10 and the effect this had on the TNF-α cytokine expression was recorded. The positive control had the most cytokine expression and expressed significantly more TNFα than in the E/C test condition

3.5.3 Effect of stimulation on dual cytokine expression in the patient group

In Table 3.8 IFN-γ^+ TNF-α^+ expression was compared between the three stimulation conditions. There was no statistical difference in cytokine expression between the three conditions. Figure 3.11 below shows the effect of stimulation on IFN-γ^+ TNF-α^+ dual expression.

Table 3.8 Effect of stimulation on % IFN-γ^+ TNF-α^+on total $CD4^+$ T cell population within the patient group

	BL % IFNγ$^+$ TNFα$^+$	**SEB % IFNγ$^+$ TNFα$^+$**	**E/C % IFNγ$^+$ TNFα$^+$**
Median	0.9650	**1.000**	0.8550
P-value (<0.05) = 0.2231			
BL % IFNγ$^+$ TNFα$^+$ vs. SEB % IFNγ$^+$ TNFα$^+$ = No			
BL % IFNγ$^+$ TNFα$^+$ vs. E/C % IFNγ$^+$ TNFα$^+$ = No			
SEB % IFNγ$^+$ TNFα$^+$ vs. E/C % IFNγ$^+$ TNFα$^+$ = No			

BL = Baseline (negative control), SEB = Staphylococcus Enterotoxin B (positive control), E/C = ESAT6/CFP10

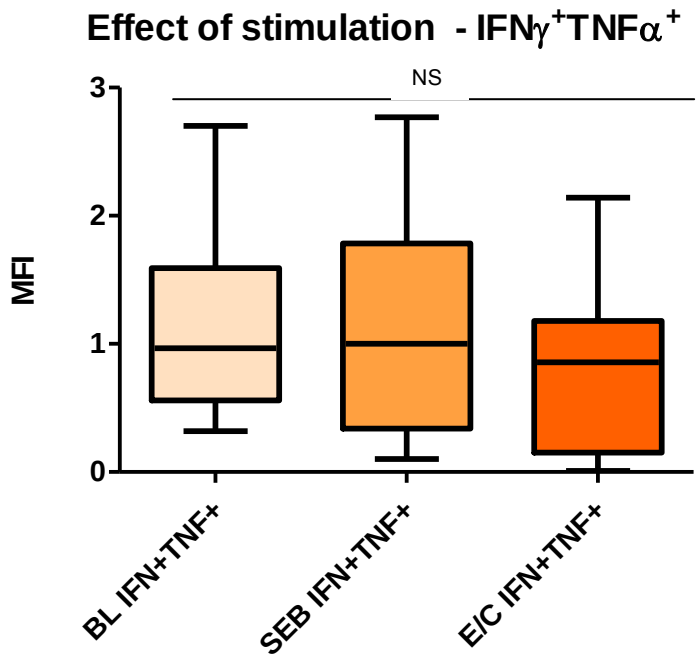


Figure 3.11$CD4^+$ T-cells were stimulated with Tuberculosis antigens ESAT6 and CFP10 and the effect this had on the IFNγ$^+$TNFα$^+$ cytokine expression was recorded. The three conditions displayed similar dual cytokine expression with no statistical difference in the median values.

3.5.4 Effect of stimulation on IFN-γ expression in the control group

In table 3.10 below IFN-γ expression was compared between the three stimulation conditions. The negative control expressed significantly more IFN-γ than the other two conditions. Figure 3.12 below shows the effect of stimulation on IFN-γ expression within the healthy control group.

Table 3.9 Effect of stimulation on IFN-γ on total $CD4^+$ T-cell population within the healthy control group

	BL%IFN-γ	**SEB%IFN-γ**	**E/C%IFN-γ**
Median	**5.110**	3.900	2.960
P-value (<0.05) = <0.0001			
BL %IFN-γ vs. SEB %IFN-γ= No			
BL %IFN-γ vs. E/C %IFN-γ= Yes ***			
SEB %IFN-γ vs. E/C %IFN-γ = Yes *			

BL = Baseline (negative control), SEB = Staphylococcus Enterotoxin B (positive control), E/C = ESAT6/CFP10

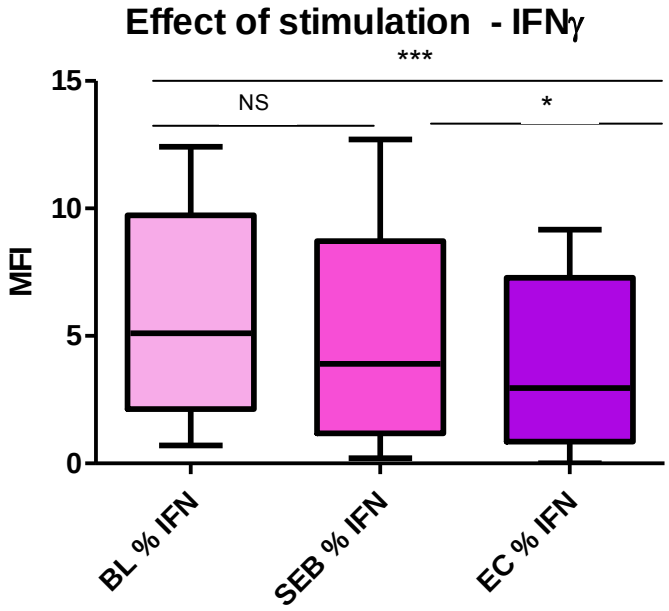


Figure 3.12$CD4^+$ T-cells were stimulated with TB antigens ESAT6 and CFP10 and the effect this had on the IFN-γ cytokine expression was recorded. The box and whisker plots show a significant difference in IFN-γ between BL and EC as well as SEB and EC

3.5.5 Effect of stimulation on TNF-α expression in the control group

In table 3.10 below TNF-α expression was compared between the three stimulation conditions. The E/C test condition had the least cytokine expression and was markedly less than the positive control. Figure 3.13 below shows the effect of stimulation on TNF-α within the healthy control group.

Table 3.10 Effect of stimulation on TNF-α on total $CD4^+$ T cell population within the healthy control group

	BL % TNF-α	**SEB % TNF-α**	**E/C% TNF-α**
Median	**2.270**	2.200	1.340
P-value (<0.05) = 0.3621			
BL % TNF-α vs. SEB % TNF-α = No			
BL % TNF-α vs. E/C % TNF-α = No			
SEB % TNF-α vs. E/C % TNF-α = No			

BL = Baseline (negative control), SEB = Staphylococcus Enterotoxin B (positive control), E/C = ESAT6/CFP10

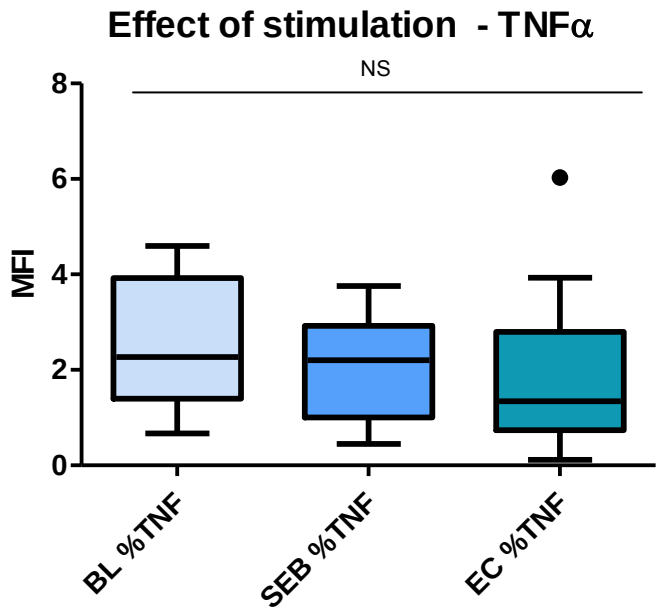


Figure 3.13$CD4^+$ T-cells were stimulated with Tuberculosis antigens ESAT6 and CFP10 and the effect this had on the TNF-α cytokine expression was recorded. Although the E/C condition had the least cytokine expression it was not statistically lower than the two control conditions.

3.5.6 Effect of stimulation on dual cytokine expression in the control group

In table 3.11 below IFN-γ^+ TNF-α^+dual expression was compared between the three stimulation conditions. Although the cytokine expression was similar in the negative and positive control, there was a statistical difference in expression between the negative control and the E/C condition. Figure 3.14 below shows the effect that stimulation had on IFNγ^+TNFα^+ dual expression within the control group.

Table 3.11 Effect of stimulation on IFNγ^+TNFα^+ on total CD4$^+$ T-cell population within the healthy control group

	BL % IFNγ^+ TNFα^+	SEB % IFNγ^+ TNFα^+	E/C % IFNγ^+ TNFα^+
Median	**0.8500**	0.8300	0.5400
P-value (<0.05) = 0.0307			
BL % IFNγ^+ TNFα^+ vs. SEB % IFNγ^+ TNFα^+ = No			
BL % IFNγ^+ TNFα^+ vs. E/C % IFNγ^+ TNFα^+ = Yes *			
SEB % IFNγ^+ TNFα^+ vs. E/C % IFNγ^+ TNFα^+ = No			

BL = Baseline (negative control), SEB = Staphylococcus Enterotoxin B (positive control), E/C = ESAT6/CFP10

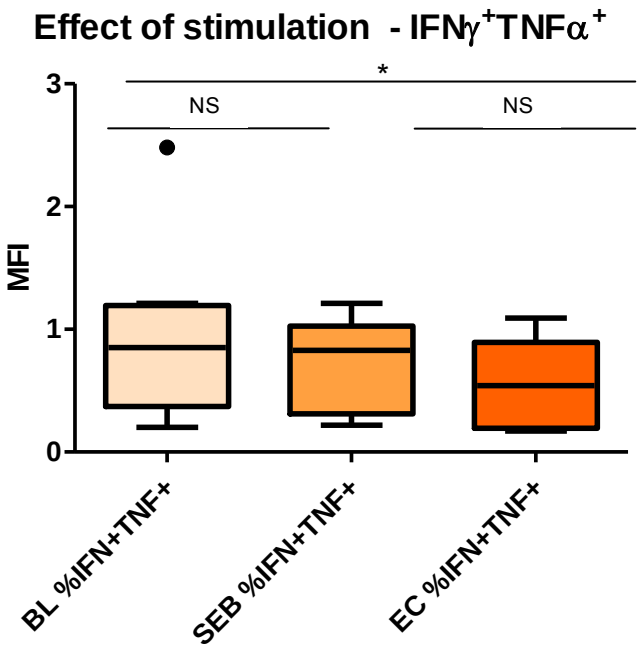


Figure 3.14CD4$^+$ T cells were stimulated with Tuberculosis antigens ESAT6 and CFP10 and the effect this had on the IFNγ^+TNFα^+ cytokine expression was recorded. The cytokine expression in the negative and positive control was almost identical; however the negative control was statistically higher than the EC test condition.

3.6 TIM3 expression on total $CD4^+$ T-cell subsets

TIM3 showed no statistical difference in expression within the $CD4^+$ subsets in both the patient and control group (data not shown). In table 3.12 below central memory cells expressed the most TIM3 in both groups; however it was not significantly higher than the other cell groups. In active TB, it is expected that effector memory cells would express the most TIM3 and patients with HIV would have terminally differentiated $CD4^+$ cells expressing the most TIM3, but this was not the case.

Table 3.12 TIM3 expression on $CD4^+$ T-cell subsets in patient group

	Naïve	**Central memory**	**Effector memory**	**Terminally differentiated**
Median	0.1700	**0.4450**	0.1800	0.2600
P-value (<0.05) = 0.1511				
T_N %TIM3 vsT_{CM} %TIM3 = No				
T_N %TIM3 vsT_{EM} %TIM3 = No				
T_N %TIM3 vs T_{DEM} %TIM3 = No				
T_{CM} %TIM3 vsT_{EM} %TIM3 = No				
T_{CM} %TIM3 vs T_{DEM} %TIM3 = No				
T_{EM} %TIM3 vs T_{DEM} %TIM3 = No				

Chapter 4 – Discussion

In this study we aimed to develop and optimise a blood based flow cytometry assay in order to detect cytokines released from $CD4^+$ T-cells in patients with active TB compared to a control group of healthy individuals. Development and optimisation of such an assay would aid in elucidating optimal reagent volumes and concentrations, while maximising the number of tests that can be performed, thereby lowering the effective cost-per-patient. It would allow us to generate intracellular cytokine (TNF-α and/or IFN-γ) profiles of $CD4^+$ T-cell populations following exposure to TB specific antigens in order to distinguish between active and latent TB in both TB and in some instances HIV and TB co-infected individuals.

4.1 Optimisation of a whole blood flow cytometry assay

From the literature, most flow based TB assays have been performed on PBMC's rather than whole blood. For the purpose of this study, whole blood has been used for analysis in order to allow for a faster turnaround time but also reduce labour and cost and is performed in an environment close to *in vivo.* For the optimization aspects we evaluated incubation temperatures (37^{o}C vs. 4^{o}C) on $CD4^+$ T-cell yield as well as experimental conditions. We also aimed to determine whether staining with surface markers before or after permeabilization has an effect on $CD4^+$ T-cell yield and to determine antibody volumes. From our results, in chapter 3, section 3.3.1 as observed in figure 3.2, page 39, no significant differences were observed between incubation temperatures of 37^{o}C vs. 4^{o}C, therefore all future incubation experiments were conducted at 37^{o}C. Regarding the staining of surface marker prior or after permeabilization, results observed showed that staining prior to permeabilization yielded the best results as observed in chapter 3, section 3.3.2, figure 3.3, page 40. The antibody titration aspect formed the bulk part of the optimization and there was three criteria used to determine antibody volume, namely: 1) statistical analysis, 2) visual analysis and 3) signal to noise ratio. In using these criteria we were able to optimise most of the antibodies in the panel except for TIM3-PC7 and CD45-ECD. These antibodies only had one population of cells that had a high MFI, and because of this the visual analysis and signal to noise ratio

could not be used. The manufacturer suggests using 5µl of TIM3-PC7, but statistically there was no difference between 4µl and 5µl, hence we used 4ul of TIM3-PC7 in the assay. The suggested volume of CD45-ECD is 10µl, however there was no statistical difference between 10µl and 8µl; hence we used 8µl CD45-ECD in the assay. For all the other antibodies in our staining panel, less volume per reaction recommended by the manufacturer was used (see chapter 2, section 2.6.1, Table 2.1, page 28). The titrations performed maintained optimal staining of our cells, which subsequently reduced the effective cost per sample.

4.2 $CD4^+$ T-cell subset distribution

As per the gating strategy in Chapter 2, section 2.8,$CD4^+$ T-cells were further delineated into the following four subsets, namely: naïve cells (T_N), central memory (T_{CM}), effector memory (T_{EM}) and terminally differentiated effector memory cells (T_{DEM}). According to Rovina et al. (2013) polarisation of the $CD4^+$T-cell compartment towards a specific subset may be indicative of a disease state, and certain effector molecules, such as cytokines, are associated with different stages of disease, such as active, or latent disease(Rovina et al., 2013). Furthermore, Chiacchio et al. (2014) has found that HIV uninfected patients have a higher proportion of MTB-specific T_{EM} T-cells, and a lower proportion of T_{CM} $CD4^+$ T-cells in active TB patients, compared to those with latent MTB infection (Chiacchio et al., 2014). Likewise, Petruccioli et al. (2013) also showed that during active TB infection, effector $CD4^+$ T-cells are expanded, and there is a higher proportion of antigen-specific T_{EM} T-cells, while the proportion of T_{CM} $CD4^+$ T-cells is reduced, compared to that of latently-infected individuals (Petruccioli et al., 2013). However; our results showed that naïve cells were the most abundant cell type in both the control and patient group, and their relative proportions were not statistically different from one another. In both patients and controls there was a higher expression of T_{EM} $CD4^+$ T-cells, followed by T_{CM} T-cells. This was consistent with literature, although this was not statistically significant. The population with the lowest relative frequency was that of the terminally differentiated subset, which was expected in the control group and in HIV negative individuals; however one would expect to see an increased frequency of T_{DEM} T-cells in HIV^+ individuals. Due to the fact that the HIV^+ cohort of patients were very few in

number, there was no statistically-significant increase in the T_{DEM} subset , however a larger sample size may confirm this finding.

4.3 IFNγ – single or dual expression)

IFN-γ in conjunction with TNF-α control intracellular infections. The differential expression patterns of these cytokines allows one to classify TB infection as being either active or latent(Kaufmann, 2001). These two cytokines work synergistically to ward off pathogens and the expression of either TNF-α alone, or both TNF-α and IFN-γ is associated with active TB disease. On the contrary, polyfunctional cells expressing $TNF\alpha^{+}IFN\gamma^{+}IL\text{-}2^{+}$ are found in patients with latent TB infection (LTBI) or in patients who are on TB treatment (Jasenosky et al., 2015). As seen in chapter 3, section 3.5, figure 3.9, page 50, we compared the expression of IFN-γ between healthy controls and the patient group. IFN-γ expression was almost similar in the two groups, although slightly higher in the control group, the difference in expression was not statistically significant ($p = 0.2800$).

When individuals are in states of long-term immune control, as in the case of treated TB or latent infection or BCG vaccination, these patients may have an immunological profile that is dominated by dual $IFN\gamma^{+}IL\text{-}2^{+}$ secreting effector memory T-cells (Rovina et al., 2013). As a means to save costs we did not include IL-2 in our staining panel making it difficult to assess whether the IFN-γ expression was indeed single expression or whether it was co-expressed with IL-2. Our control group consisted of National Health Laboratory Services (NHLS) staff members from the Division of Haematology, Tygerberg Hospital, leading to the assumption that these subjects have been exposed to TB and are most likely immunised. We would therefore expect to see the increased dual expression of $IFN\text{-}\gamma^{+}IL\text{-}2^{+}$, however, since we do not have $IL\text{-}2^{+}$ in our staining panel one cannot determine whether the increased IFN-γ expression we see in our control group is increased as a single parameter or whether it is increased together with IL-2, which would then justify our findings.

4.4 TNFa expression is increased in active TB

Observations from literature by (Harari et al., 2011) and Rovina et al (2013), have concluded that TNFα-expressing MTB-specific $CD4^+$ T-cells ("single-positive" cells) were increased in subjects with active TB. This confirms our finding as seen in chapter 3, section 3.5, figure 3.9, page 50, that there was a trend towards increased TNF-α expression in the patient group (median 2.415, SD = 2.606), and although it was not significantly increased (p = 0.1729) we can conclude that based on this result our findings are in line with literature.

4.5 Presence of increased bifunctional $CD4^+$ T-cells is correlated with active disease

In our study we have shown in chapter 3, section 3.5, figure 3.9, page 50, that dual expression of IFN-γand TNF-α on $CD4^+$ T-cells is higher in the patient group than in the control group, although the difference was not statistically significant (p = 0.8550). This is consistent with data reported by Petruccioli et al. (2013) that stated MTB-specific $IFN\gamma^+TNF\alpha^+$ $CD4^+$ T-cells were significantly increased in patients with active TB compared to LTBI patients (p=0.008). Chiacchio et al (2014) in their study showed that $IFN\gamma^+TNF\alpha^+$ $CD4^+$ T-cells associated with HIV-TB; however we had too few HIV^+ patients in our study group to compare to their findings.

4.6 Stimulation with TB antigens has an effect on IFN-γ production

To determine whether cell stimulation had an effect on cytokine production, we compared median expression between the three stimulation conditions i.e. unstimulated baseline ("baseline"), SEB – positive control and TB antigens ESAT6 & CFP10 (E/C). We found that IFN-γ production was significantly decreased in all stimulation conditions in both the control and patient groups. TNF-α production was significantly down regulated in the patient group and although there was decreased production in the control group, it was not of significance. Similarly, there was a significant decrease in $IFN\gamma^+TNF\alpha^+$ dual expression within the control group, however; the decrease in $IFN\gamma^+TNF\alpha^+$ dual expression within the patient group was not significant. From this we can conclude that stimulating $CD4^+$ T-cells does decrease the production of cytokines and whether the effect of stimulation was

significant or not, we could still rely on the cytokine production and expression patterns to classify our results.

4.7 TIM3 expression on $CD4^+$ T-cell subsets

TIM3 is conjugated to fluorochrome PC7, resulting in bright expression of the single TIM3 population. Jayaraman et al (2016) has shown in their study that TIM3 has bimodal expression with clear separation of the negative and the positive population; however this was not the case in our study (Jayaraman et al., 2016).

All four $CD4^+$ T-cell subsets had similar TIM3 expression. Central memory cells had slightly higher expression but it was not significant (p=0.1511). As TB disease progresses you would expect to see T_{DEM} T-cells expressing TIM3 as these cells are functionally exhausted. Also, with our patient cohort being hospital patients with co-infections, we expected to see higher levels of TIM3 within this group, however the TIM3 expression was similar (controls = 3.300, patients = 3.830) indicating no significant difference between the two groups. It may be useful to reinvestigate the TIM3 expression on a larger cohort of patients and also to compare the TIM3 expression between HIV^+ and HIV^-patients.

4.8 Limitations of study

Our healthy control cohort was NHLS staff members within the TBH. All control subjects recruited in this study completed a TB exposure questionnaire which is designed to rule out the chance of the person having active TB, however; it does not rule out latent infection due to the absence of reportable symptoms. Working in a hospital environment exposes you to infections such as TB, and based on this one can assume that it is possible for the control subjects to have been exposed to TB and possibly have LTBI. Rovina et al., (2013) has shown that polyfunctional $TNF\alpha^+IFN\gamma^+IL\text{-}2^+CD4^+$ T-cells are associated with LTBI. Since we did not include IL-2 in our staining panel we cannot confidently say that our healthy controls are not producing IL-2. Our control cohort was not tested for LTBI either as this would've added to the cost of the study significantly. For this reason we cannot distinguish between active and LTBI as we cannot confidently say whether our control group is

healthy or has LTBI to compare to the results of our patient cohort. However, taking into account the area and target population one can assume that the control group would have LTBI.

The patients for our study group were patients from TBH. In order to use patients from local peripheral clinics required a lengthy application process to the Western Cape Department of Health and our timeline could not accommodate such a process. Patients admitted to TBH with active TB however were more than often patients with TB reactivation. Also, these patients were often on empirical TB treatment. Thirty three percent (6/18) of our patient cohort had HIV and this resulted in very low $CD4^+$ T-cell counts for these patients. Since $CD4^+$ T-cells were our cells of interest, we often only managed to acquire 9-12 $CD4^+$ T-cells, making it impossible to further analyse that data as that would lead to our results being skewed and working with such low numbers of $CD4^+$ T-cells can lead to unreliable results. These patients were subsequently excluded from our data analysis. Our patient group consisted mainly of generally very ill patients with underlying diseases or co-infections aggravating the TB and this resulted in a very lengthy recruitment process as newly diagnosed TB, off-treatment patients are very seldom sent to a tertiary institution such as TBH. On 3 occasions where potential patients had newly-diagnosed TB and had not started TB treatment yet, these patients were so ill due to their co-infections, that they were unable to communicate and give informed consent.

A major limitation of this study was our sample size, because with only 18 patients it was impossible to statistically determine cut-off values for our positive and negative results. Although we only needed 8 patients for statistical power, as determined by the statistician, it would've been interesting to set cut-off values to validate our findings in the patient cohort.

4.9 Summary of results and the way forward

In summary, the optimisation of this assay was challenging and lengthy, however it yielded the desired results. In future we will be able to use less antibody per assay and this would decrease the cost of future work. Revision of the staining panel may be necessary so as to include markers such as IL-2. Other studies have heavily

relied on IL-2 expression to confirm whether the IFN-γ and/or TNF-α expression is indeed single expression and not co-expressed with IL-2 as this may change the disease status completely. However; a trend towards increased levels of TNF-α expression in our patient cohort is in line with the findings in other studies.

The increased expression of single IFN-γ in our control cohort can be suggestive of our control subjects being vaccinated with BCG or being latently infected with TB. Both scenarios can be used to describe these subjects, but we cannot confirm this. In future it would be advisable to test the healthy control group for LTBI using the QuantiFERON-TB Gold In –Tube test (QFT-GIT) in order to draw a clear line between healthy persons and those with LTBI. Study subjects should also indicate whether they have been previously vaccinated, although as most people are vaccinated as infants, it may be difficult to get this kind of information. Although we had slightly higher IFN-γ expression (not significant) in our control group as opposed to our patient group, it would be advisable to still include IL-2 in our staining panel to strengthen our findings.

In addition, patients should be recruited from the local community clinic which would ensure that these patients are newly diagnosed TB cases, not on treatment. This would speed up the recruiting process as large numbers of patients visit the clinics on a daily basis. This would also make it easy to recruit newly diagnosed HIV^+ patients in order to differentiate between HIV^+TB^+ and HIV^-TB^+ patients in future studies. Furthermore, these patients could be tested post treatment to compare the cytokine expression patterns and compare it to literature. It would enhance this study greatly if we could recruit more patients in order to determine sample cut-offs, to include IL-2 in our staining panel and to screen our healthy patient cohort to differentiate between healthy and LTBI.

The flow cytometry antibody panel would have to be redesigned to accommodate TIM3 conjugated to a different fluorochrome as TIM3 would indicate those T-cells that are functionally exhausted, and this would assist in classifying our results.

4.10 Conclusion

In our study we went with the whole blood approach to differentiate between active and latent TB within 24 hours. Our assay measured IFN-γ and TNF-α simultaneously at the single cell level which is important as certain cytokine profiles have been shown to be associated with specific disease profiles. Through optimisation we could determine that this assay may be performed from less than 500µl of whole blood and with results being available by the following day, this assay holds the promise for being able to be implemented in a routine setting.

Flow cytometry is a high-throughput technique that can be applied to a number of diagnostic areas. Although it is quite technical, careful controlling of instrument and experimental factors allows for high-quality reproducible results. Furthermore, stimulation assays such as the one described here are routinely used in determining immune responses in vaccine trials, and have been shown to provide reliable results. These types of assay are, however, time-consuming and relatively expensive compared to conventional TB diagnostic tests, although this may be more a reflection of economies-of-scale rather than pure cost analysis. With that said, stimulation-flow cytometry assays are currently the only method that is able to enumerate antigen-specific T-cells while delineating into T-cell subsets. With ongoing development of new instruments and reagents, it is possible that more data per patient can be generated in a single assay, for a decreased price. After careful titration of antibodies, a viability marker and a robust gating strategy, this assay has proven its suitability and usefulness in our set-up.

This study has shown an increase in IFN-γ expression within our patient cohort although we could not distinguish between exposure through immunisation or latent infection in the control group. Future studies would have to determine both the vaccination status of the control group, and whether or not they have latent TB. It was interesting to note the trend towards increased expression levels of TNF-α only within the patient group, as this is suggestive of active TB, however; a larger sample size is needed to confirm this. It is desirable to compare the results of an $HIV^{+}TB^{+}$ cohort to that of an $HIV^{-}TB^{+}$ cohort, as an increase in bifunctional MTB-specific $CD4^{+}$ T-cells would be seen in those patients with HIV. Unfortunately, in a hospital set-up such as in TBH, patients with $HIV^{+}TB^{+}$ have very low $CD4^{+}$ T-cell counts, thereby

making analysis on these patients nearly impossible. A cohort of $HIV^{+}TB^{+}$ patients with $CD4^{+}$ T-cell counts above 350cells/µl would be advisable in future studies.

Furthermore, including markers such as IL-2 in the antibody staining panel may assist in differentiating between active and latent TB which in this setting is necessary due to the high incidence of HIV and TB co-infection. By addressing the limitations in this study, this assay has the potential to significantly impact the current on-going diagnostic challenges in accurately diagnosing latent TB, while ensuring turnaround times of less than 24 hours.

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Addendum 1

Statistical determination of optimal antibody volumes

ROW #	Antibody	Comparison (µl)	MFI mean difference	P value
1	**CD4-APC**	1 vs. 2	-38.04	0.0026 **
2		2 vs. 4	-24.97	0.0036 **
3		4 vs. 6	-18.19	0.0647 (NS)
4		6 vs. 8	-5.500	0.0022 **
5		8 vs. 10	-22.25	0.4394 (NS)

APC –Allophycocyanin

ROW #	Antibody	Comparison (µl)	MFI mean difference	P value
1	**CD45-ECD**	1 vs. 2	-50.55	0.0316 *
2		2 vs. 4	-11.29	0.0475 *
3		4 vs. 6	-60.51	0.0016 **
4		6 vs. 8	3.843	0.0465 *
5		8 vs. 10	3.878	0.0151 *

ECD - PhycoerythrinTexas Red-X

ROW #	Antibody	Comparison (µl)	MFI mean difference	P value
1	**CD27-APC 750**	1 vs. 2	-5.083	0.0031 **
2		2 vs. 4	-2.293	0.0199 *
3		4 vs. 6	-0.3967	0.2776 (NS)
4		6 vs. 8	0.3733	0.2200 (NS)
5		8 vs. 10	0.1000	0.7390 (NS)

APC750 –Allophycocyanin 750

ROW #	Antibody	Comparison (µl)	MFI mean difference	P value
1	**TIM3-PC7**	1 vs. 2	-12.81	0.0017 **
2		2 vs. 3	-6.573	0.0020 **
3		3 vs. 4	-7.303	0.0039 **
4		4 vs. 5	-8.110	0.0820 (NS)

PC7 - Phycoerythrin-Cyanin 7

ROW #	Antibody	Comparison (µl)	MFI mean difference	P value
1	**TNFα-PE**	6 vs. 8	-0.2500	0.0494 *
2		8 vs. 10	-0.8267	0.0116 *
3		10 vs. 12	-0.1000	0.2300 (NS)
4		12 vs. 14	-0.09000	0.5949 (NS)
5		14 vs. 16	-0.4767	0.0508 (NS)
6		16 vs. 18	-0.5067	0.0666 (NS)
7		18 vs. 20	-0.2700	0.0968 (NS)

PE - Phycoerythrin

ROW #	Antibody	Comparison (µl)	MFI mean difference	P value
1	**IFN-γ-FITC**	6 vs. 8	-0.02000	0.7007 (NS)
2		8 vs. 10	-0.05000	0.0377 *
3		10 vs. 12	-0.01000	0.4226 (NS)
4		12 vs. 14	-0.04000	0.0202 *
5		14 vs. 16	-0.03333	0.0099 **
6		16 vs. 18	-0.03333	0.0099 **
7		18 vs. 20	-0.03333	0.0099 **

FITC - Fluorescein isothiocyanate

ROW #	Antibody	Comparison (µl)	MFI mean difference	P value
1	**CD45RO-BO**	0.5 vs. 1	-0.4033	0.3779 (NS)
2		1 vs. 1.5	0.7167	0.0313 *
3		1.5 vs. 2	-0.8067	0.0287 *
4		2 vs. 2.5	-0.4000	0.0970 (NS)
5		2.5 vs. 3	-0.2367	0.0715 (NS)
6		3 vs. 4	-0.2867	0.0459 *
7		4 vs. 5	-0.05667	0.1425 (NS)

BO – Brilliant Orange

Addendum 2

Determination of optimal antibody volume using the S:N ratio.
For antibodies CD45-ECD and TIM3-PC7 the S:N ratio could not be used as both these antibodies had 100% MFI positivity.

Antibody	Volume (µl)	S:N ratio	MFI of negative population	MFI of positive population
CD4	1	48.09	2.06	99.06
	2	45.39	2.52	114.40
	4	26.44	3.27	86.45
	6	**31.21**	**3.21**	**100.18**
	8	19.36	3.54	68.54
	10	15.003	3.64	54.61

Antibody	Volume (µl)	S:N ratio	MFI of negative population	MFI of positive population
CD27	1	10.08	0.73	7.36
	2	9.495	1.09	10.35
	4	**8.33**	**1.32**	**11.00**
	6	7.50	1.42	10.65
	8	6.97	1.50	10.46
	10	6.98	1.51	10.54

Antibody	Volume (µl)	S:N ratio	MFI of negative population	MFI of positive population
CD45RO	0.5	10.16	0.45	4.57
	1	9.89	0.53	5.24
	1.5	9.16	0.62	5.68
	2	9.08	0.74	6.72
	2.5	8.52	0.81	6.90
	3	**7.34**	**0.90**	**6.61**
	4	6.29	1.02	6.42
	5	5.44	1.10	5.98

Antibody	Volume (µl)	S:N ratio	MFI of negative population	MFI of positive population
IFN-γ	6	1.76	0.74	1.30
	8	1.59	0.75	1.19
	10	1.62	0.78	1.27
	12	1.64	0.76	1.25
	14	1.76	0.75	1.32
	16	1.76	0.76	1.34
	18	**1.79**	**0.76**	**1.36**
	20	1.77	0.79	1.40

Antibody	Volume (µl)	S:N ratio	MFI of negative population	MFI of positive population
TNF-α	6	5.76	0.54	3.11
	8	8.80	0.36	3.17
	10	9.80	0.36	3.53
	12	10.05	0.37	3.72
	14	**10.55**	**0.38**	**4.01**
	16	12.34	0.35	4.32
	18	13.26	0.34	4.64
	20	8.14	0.61	4.97