Application of Becton Dickinson FACS^{TM} Combinatorial Antibody Profile (FACS^{TM} CAP) technology to the identification of efficiency of tuberculosis therapy

Bronwyn Kerry Smith

Thesis presented in fulfilment of the requirements for the degree of Master in Medical Science in the Faculty of Medicine and Health Sciences at Stellenbosch University

Supervisor: Professor Gerhard Walzl

March 2015

Declaration

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Abstract

Currently the treatment of individuals with active *Mycobacterium tuberculosis* (*Mtb*) infection involves a standard six-month multi-drug regimen, impacting negatively on treatment adherence, which in turn fuels multi- and extensive drug resistant TB. However, some patients may not require the full six-month regimen due to less extensive disease or rapid early treatment response. The identification of these patients has been problematic but would allow significant cost savings and may impact positively on treatment adherence if treatment duration could be shortened and if this subgroup constituted a significant portion of patients.

The aim of this project was to identify peripheral blood lymphocyte surface markers through a proprietary technology, FACS^{TM} CAP by Becton Dickinson Technologies, to investigate the change in expression during the course of treatment with potential treatment monitoring utility.

Peripheral blood mononuclear cells (PBMCs) were isolated from TB patients (n=33), healthy community controls (n=11) and other lung disease controls (OLD, n=9) at diagnosis of disease, week 4 (after commencement of treatment) and week 24 (end of treatment, EOT). Antibodies to 252 surface markers were used to stain PBMCs, the cells were fixed in 2% paraformaldehyde and data acquired on a FACS Calibur flow cytometer. Post-acquisition compensation and analysis was performed using FlowJo software. The analysis was performed by gating on the lymphocytes and overlaying sample plots on isotype controls.

Statistics analysis included repeated measures ANOVA, paired t-test and independent t-test. Comparisons were made between the expression levels of patient time points (diagnosis, week 4 and week 24) and participant groups (TB, healthy community controls and OLD controls). Sample wells that provided an uncertain demarcation of the positive and negative expression population were flagged and excluded from analysis. After the application of the Bonferroni correction, results revealed five overall treatment response markers (CD120b, CD126, CD62L, CD48 and CD29) that were significantly different (p-value <0.0002) when comparing expression levels at TB diagnosis and EOT (week 24) samples. A comparison of expression between TB at diagnosis and healthy community controls showed a significant difference for four markers (CD48, CD18, CD126 and fMLPr).

Due to the application of the stringent Bonferroni correction, only these few markers were found to be statistically significant therefore all markers with a p-value <0.01 prior to Bonferroni correction, were included for analysis with Ingenuity Pathway Analysis (IPA) and Qlucore Omics Explorer software.

IPA identified 23 biological pathways that were associated with two or more markers with significant changes during treatment. The top nine pathways are discussed and included the inflammatory response, cell migration, differentiation and maturation and crosstalk between cells of the innate and adaptive immune responses.

In conclusion, this project resulted in the identification of three promising biologically significant surface markers that require further validation as candidates for biomarkers of TB treatment response. Future studies will investigate the most promising markers, including those that showed a trend for differences after the Bonferroni correction, in a candidate biomarker project with a new cohort of TB patients undergoing treatment.

Abstrak

Die roetine behandeling van individue met aktiewe *Mikobakterium tuberkulosa* (*Mtb*) infeksie, behels ses maande van multi-middel terapie, 'n tydperk wat gevolglik negatief impakteer op behandelingsgetrouheid en dus bydra tot multi- en ekstensiewe middelweerstandige TB. Dit mag egter wees dat sommige pasiënte, as gevolg van minder verspreide infeksie of 'n versnelde reaksie op vroeë behandeling, nie die volle ses maande-lange behandeling benodig nie. Alhoewel die identifikasie van sulke pasiënte problematies is, kan dit beduidende kostebesparings meebring en moontlik ook 'n positiewe impak op behandelingsgetrouheid hê indien behandelingsduur verkort kan word en indien dié subgroep 'n beduidende deel van die pasiënte uitmaak.

Die doel van die huidige projek was om perifere bloed-limfosiet oppervlaksmerkers te identifiseer met behulp van 'n patente tegnologie, FACS™ CAP van Becton Dickinson, om sodoende die verandering in merker uitdrukking tydens die verloop van behandeling te ondersoek vir moontlike gebruik as behandelings monitering toepassing.

Perifere bloed mononukleêre selle (PBMSe) is geïsoleer van TB pasiënte (n=33), gesonde kontroles (n=11) en kontroles met ander longsiektes (OLD, n=9) tydens diagnose van siekte, week 4 (na begin van behandeling) en week 24 (einde van behandeling, EOT). Teenliggame is gebruik om 252 seloppervlaksmerkers van die PBMSe te merk, die selle is met 2% paraformaldehied gefikseer en die data op 'n FACS Calibur vloeisitometer verkry. FlowJo sagteware is gebruik vir na-verkryging-kompensasie en analise wat gedoen is deur die limfosiete te selekteer, gevolg deur oorlegging van isotipe-kontroles.

Statistiese analises het herhaalde metings-ANOVA, die gepaarde en onafhanklike t-toetse ingesluit. Vergelykings is getref tussen die uitdrukkingsvlakke van verskillende pasiënt-metings (diagnose, 4 weke en 24 weke) en deelnemende groepe (TB, gesonde kontroles en OLD kontroles). Proefdata wat nie tussen die positiewe en negatiewe uitdrukkingspopulasie kon onderskei nie, is van die analise uitgesluit. Na toepassing van die Bonferroni-korreksie het die resultate getoon dat vyf algehele behandelingsrespons-merkers (CD120b, CD126, CD62L, CD48 en CD29) beduidend verskil (p-waarde <0.0002) wanneer die uitdrukkingsvlakke tussen die TB

diagnose en EOT (24 weke) tydstip vergelyk is. Vergelyking van merker uitdrukking tussen TB (by diagnose) en gesonde kontroles het 'n beduidende verskil vir 4 merkers (CD48, CD18, CD126 enfMLPr) aangetoon. Aangesien slegs hierdie merkers statisties beduidend was na toepassing van die streng Bonferroni-korreksie is alle merkers met 'n p-waarde <0.01 voor Bonferroni-korreksie ingesluit vir analise met Ingenuity Pathway Analysis (IPA) en Qlucore Omics Explorer sagteware.

IPA het 23 biologiese paaie geïdentifiseer wat geassosieer is met twee of meer merkers met beduidende veranderinge tydens behandeling. Die belangrikste nege paaie word bespreek en sluit in die inflammatoriese respons, sel-migrasie, -differensiasie, -maturasie en kruiskommunikasie tussen selle van die ingebore en sellulêre immuun sisteme.

Om op te som, hierdie projek het drie belowende biologies beduidende oppervlaksmerkers geïdentifiseer wat verdere ondersoek as kandidaatbiomerkers van TB behandelingsrespons, regverdig. Toekomstige studies sal die mees belowende merkers, insluitende daardie wat 'n tendens in verskille na Bonferroni-korreksie getoon het, navors in 'n kandidaat-biomerkerprojek met 'n nuwe populasie TB pasiënte gedurende TB behandeling.

Acknowledgements

I would like to thank the following people for their support and contribution towards the completion of my degree.

Thank you to my husband, Tarren, for all your emotional, intellectual and financial support and for being so understanding during the stressful times. Thank you for believing in me and helping me carry my load, you are always my pillar of strength and I am privileged to call you my husband.

Thank you to Professor Gerhard Walzl for your support, time, knowledge and guidance during my degree. It has been a privilege to be a member of your team and I am proud to be associated with the SUN Immunology Research Group. Thank you for accepting me as your student, I feel honoured to call you my supervisor and boss.

Thank you to my family for your continued encouragement and support. Thank you to my mom for the love and pride you have in me and for always taking an interest in my work. Thank you to my dad for teaching me that the world is my oyster and that I can do anything I set my mind to. You always knew how to encourage and advise me and show your love and pride in me. I wish you were here to see me graduate but I know you are watching.

Thank you to the research assistants of the SUN Immunology Research Group who provided assistance with samples and especially Belinda Kriel and Reeva Erasmus. Thank you to the ladies in the office who helped to cover my duties when I had too much to do and for the constant encouragement throughout the project.

Thank you to Dr Andre Loxton for proof-reading my thesis and for your encouragement and advice over the years. Thank you to Dr Léanie Kleynhans and Dr Katharina Ronacher for your help and guidance with the Ingenuity Pathway analysis and Qlucore software.

Thank you to Dr Farida Bouzahzah for all the training, guidance, friendship and encouragement that you gave me through the course of the project and during my visits to the USA. Thank you too for your input on my thesis, I appreciate and value everything you have done for me.

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°C	Degrees Celsius
µm	Micrometre
µL	Microliter
AFB	Acid fast bacilli
Alexa488	Alexa Fluor 488
Alexa647	Alexa Fluor 647
ANOVA	Analysis of variance
APC	Allophycocyanin
APCs	Antigen presenting cells
APDF	Welch Approximate Degrees of Freedom
APP	Acute phase proteins
BD	Becton Dickinson
BMGF	Bill and Melinda Gates Foundation
CD	Cluster of differentiation
CD4+	CD4 positive T helper cells
CD8+	CD8 positive cytotoxic T cells
CFP-10	10kDa culture filtrate antigen
CMV	Cytomegalovirus
CO_2	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
CRTH2	Chemo attractant receptor-homologous molecule expressed on Th2 cells
DC	Dendritic cells

dH$_2$0	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DST	Drug susceptibility testing
Dx	Diagnosis
ELISPOT	Enzyme-linked immunosorbent spot assay
EOT	End of treatment
ESAT-6	Early secreted antigen 6
FACS^{TM} CAP	FACS combinatorial antibody profile
FBS	Fetal bovine serum
FDG-PET/CT	[18F]-fluoro-2-deoxy-D-glucose positron emission tomography/computer tomography
FITC	Fluorescein isothiocyanate
FL	Fluorescent
FoxP3	Forkhead box P3
FSC	Forward scatter
GATA3	GATA binding protein 3
HIV	Human immunodeficiency virus
HTS	High throughput system
ICAM3	Intercellular adhesion molecule 3
IFN- γ	Interferon gamma
IFNGR	Interferon gamma receptor
IgE	Immunoglobulin E
IGRA	Interferon gamma release assays
IL-	Interleukin

INH	Isoniazid
IPA	Ingenuity pathway analysis
ITGAL	Integrin alpha L
ITGβ1	Integrin β1
iTregs	Induced T regulatory cells
JAM1	Junctional adhesion molecule 1
kDa	Kilo Dalton
LFA-1	Lymphocyte function-associated antigen 1
LiPAs	Line probe assays
LT-α	Lymphotoxin-α
mBD-2	Mouse beta-defensin 2
MDR	Multi-drug resistant
MGIT	Mycobacterial growth indicator tube
MHC I/II	Major histocompatibility complex
mL	Millilitre
Mtb	*Mycobacterium tuberculosis*
NAAT	Nucleic acid amplification test
NF-κβ	Nuclear factor kappa-light-chain enhancer of activated B cells
NK cell	Natural killer cell
nm	Nanometres
OLD	Other lung disease
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	R-phycoerythrin
PMT	Photomultiplier tube

PPD	Purified protein derivative
PSGL1	P-selectin glycoprotein ligand-1
QFN-GIT	QuantiFERON-TB Gold In-Tube assay
RCF	Relative centrifugal force
RD-	Region of deletion
RIF	Rifampicin
RNA	Ribonucleic acid
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
RORγt	Retinoic acid receptor-related orphan receptor gamma-T
ROS	Reactive oxygen species
SAA	Serum amyloid A
SAP	Serum amyloid P
sIL	Soluble interleukin
SP-A	Surfactant protein A
SSC	Side scatter
STAT4	Signal transducer and activator of transcription protein 4
TAP	Transporter associated with antigen processing
TB	Tuberculosis
TCR	T cell receptor
TFH	T follicular helper cells
TGF-β	Transforming growth factor β
Th	T helper cells
Th1	T helper 1
Th17	T helper 17 cell

Th2	T helper 2 cell
TNF	Tumour necrosis factor
TNFR1	Tumour necrosis factor receptor 1
TNFR2	Tumour necrosis factor receptor 2
Treg	T regulatory cell
W24	Week 24
W4	Week 4
WHO	World Health Organisation
XDR	Extensive-drug resistant

Chapter 1: Introduction

1.1. Epidemiology of tuberculosis

Tuberculosis (TB) is an infectious disease which is caused by the bacterium *Mycobacterium tuberculosis* (*Mtb*) and was responsible for as many as 8.6 million new infections and 1.3 million deaths in 2012 (1). Approximately 13% (1.1 million) of the newly infected patients are HIV-positive with an estimated 75% in the African region. As illustrated in figure 1.1, the incidence rate in South Africa is estimated at about 1000 or more cases per 100 000 people (1).
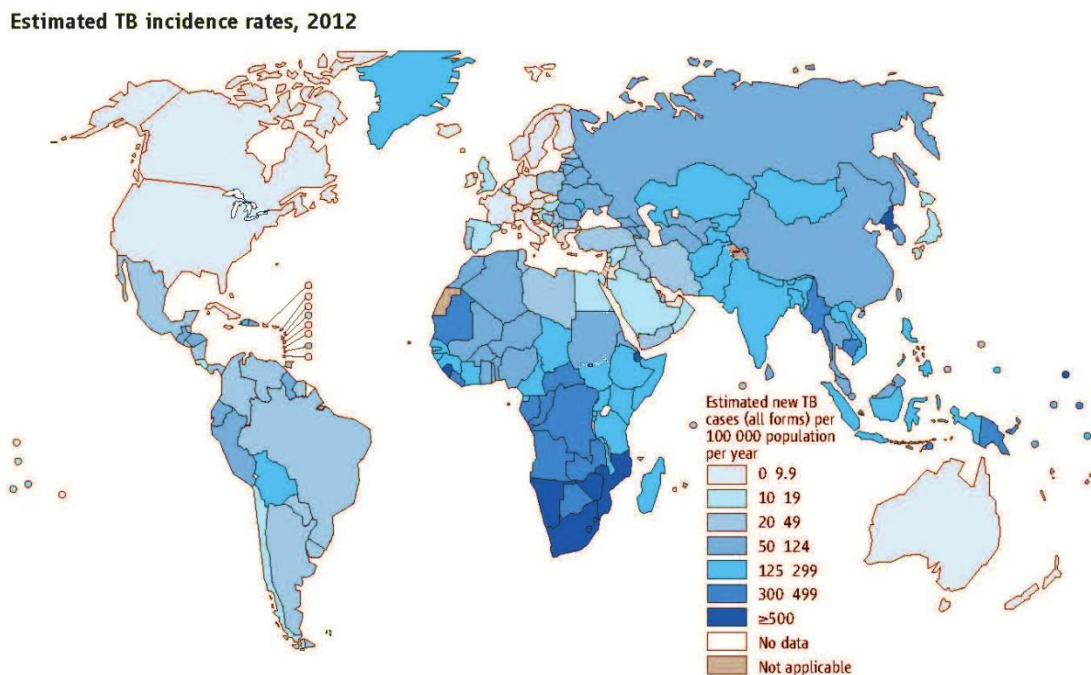
Figure 1.1: WHO incidence rate per 100 000 population per year.

The light blue areas denote a lower incidence rate to the darker blue areas demonstrating the difference in TB incidence globally (1).

The *Mtb* organism is a slow-growing acid-fast bacillus which is transmitted primarily by the respiratory route (2). The disease is spread by infected people who transmit the bacteria by coughing or sneezing and although it can infect other parts of the body, the lungs are the primary site of infection.

Although it is estimated that one third of the world's population is infected with the bacteria, only a small number of people will develop active disease (2). The host immune system is capable of containing the infection even if it cannot eliminate it entirely, which results in the bacteria entering a dormant state and surviving under adverse metabolic conditions. This mechanism enables the bacterium to survive long-term in humans, sometimes for many years, emerging only when there is a disruption in the host immune system. A number of factors may increase the risk of developing active disease such as human immunodeficiency virus (HIV) infection, aging, drug or alcohol abuse, diabetes mellitus and treatment with corticosteroids (2). It has been documented that live bacilli may persist after successful treatment of active TB which could potentially result in a relapse of active disease (3).

1.2. Problems facing tuberculosis

Currently the treatment for TB involves a multi-drug regimen, which must be continued for a minimum of six months until confirmation of cure (4). One of the limitations of a treatment regimen of this duration is that it may lead to poor treatment adherence, which has resulted in the emergence of multi- and extensive drug resistant strains of *Mtb*, requiring an even more aggressive and longer duration of treatment in order to reach cure. Patients often feel better after just one or two months of treatment or conversely the side effects from the drugs may be too severe, which results in discontinuation of treatment. Evidence has been shown that patients who respond early in the treatment regimen may require a shortened course of treatment, which may improve treatment adherence and thus the treatment outcome (4).

The only currently accepted biomarker for treatment response is a conversion to negative sputum culture and smear microscopy at month two of treatment (5).

However, these methods have their limitations. Culture methods are still the gold standard for diagnosis of TB though problems associated with culture include the long delay before a result becomes available (up to 6 weeks before a culture is regarded as negative), contamination of cultures resulting in a false positive result and the high costs of the assay. Smear microscopy provides a simple, inexpensive and fast way of diagnosing TB; however, the sensitivity of the test is poor when there is a bacterial load of less than 10 000 organisms/mL of sputum. The test is labour intensive, requiring a skilled microscopist to examine individual samples, which is problematic in high prevalence areas where large numbers of samples need to be assessed daily (6). Thus it has become necessary to find alternative ways to monitor treatment response to chemotherapy in order to improve clinical management of patients as well as to monitor new anti-TB drugs during clinical trials. The human response to infection and treatment of TB is complex thus it would be likely that a bio signature consisting of multiple markers would have a better prediction outcome than a single marker. The most useful markers would be biomarkers found in readily-available bodily fluids such as blood.

1.3. *Mtb* and Immunity

The immune system, crucial in our defences against *Mtb*, is composed of a number of biological processes that work together to protect the host. The immune system can be categorised into two branches, the innate immune system and the adaptive immune system (7).

The innate immune system is our first line of defence against invading pathogens while the adaptive immune system is a second line of defence, which augments innate effector responses and also protects against infection during re-exposure of the same pathogen. When encountering a pathogen the immune system has to rely on both the innate and the adaptive immune response to ensure clearance of the pathogen (7).

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1.3.1.   Innate immune response

The innate immune response is the branch of the immune system which responds first after exposure of the body to pathogens (7). Once the pathogen penetrates the epithelial surfaces of the body, it is immediately met with phagocytic cells which are able to recognise bacterial surfaces and bind to them. Once this occurs, inflammation takes place, which involves the release of cytokines and chemokine's by activated macrophages into the blood stream, which attract neutrophils, monocytes and adaptive immune cells to the site of infection.

Features of the innate immune system include (8):
- The recognition of pathogens through broad specificity receptors (pattern recognition receptors).
- The response time of the innate immune system is immediate or very fast as it relies on preformed mediators.
- The innate immune response does not improve with repeated exposure as its cells do not generate immunological memory.

The innate immune system consists of two components:
- Chemical factors
- Cellular factors

1.3.1.1.   Chemical factors

Acute inflammation is an innate response which occurs in the event that an infectious agent has penetrated the anatomical barriers (9).Humoral immunity, part of the adaptive immune response, is primed to recognise and protect against extracellular antigens. Chemical factors play a large role in inflammation and these factors include:

- Complement system: Once activated, the complement system results in increased vascular permeability, attraction of phagocytic cells, opsonisation of invading microorganisms and lysis of infected cells. This is the most important

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component of the chemical defence system and a vital process in TB pathogenesis. Phagocytes have an enhanced capacity for engulfing and opsonising *Mtb* in the presence of activated complement (10).

- Interferons: These molecules have an important role in protecting the host against intracellular infections. In TB, Interferon gamma (IFN- γ) plays a critical role in macrophage activation and restriction of bacterial growth. Studies using IFN-γ knockout mice have demonstrated the importance of IFN-γ by showing an inability to control bacterial load and dissemination when infected with *Mtb* thus resulting in decreased survival when compared with wild-type mice (11).

- Defensins: These are present in the lung and gastrointestinal tract and prevent colonisation of microorganisms. In mice β-defensin 2 (mBD-2) may play a role in controlling the growth of the bacilli as well as by creating a link between the innate and adaptive immune response by contributing towards the establishment of a Th1 response (12).

- Surfactants: These are produced by pulmonary epithelial cells and, based on their location within the alveoli, constitute an important first responder of the innate immune response. Surfactants function by regulating opsonisation and phagocytosis of invading microorganisms. It has been reported that mice with a surfactant protein A (SP-A) deficiency are more susceptible to invading microorganisms such as Mtb (13) and SP-A enhances phagocytosis of virulent *Mtb* (14).

- Cytokines: These are proteins which are secreted by cells and are involved in the signalling and interaction between cells. The function of cytokines usually occurs as a cascade where one cytokine stimulates the release of another (15).

- Lysozymes: These break down the cell wall of bacteria and disrupt the cell membrane.

- Interleukin-1: IL-1 signalling is essential for the containment of intracellular pathogens and studies show that it plays a key role in the production and maintenance of granuloma's in *Mtb* infection.
Interleukin-6: IL-6 plays an important role in inflammation and the activation of the acute phase response (16). IL-6 is produced by a variety of cells including T cells, B cells, monocytes and fibroblasts. Previous studies have shown that IL-6 deficient mice develop normally, however they have impaired immune and acute-

phase responses (17) . Unsal *et al.* hypothesised that IL-6 might play a role in reactive thrombocytosis and increased acute-phase reactants seen in patients with pulmonary TB. They showed that patients with reactive thrombocytosis had increased concentrations of IL-6 and those with TB and reactive thrombocytosis had more extensive radiological findings and symptoms (fever, night sweats, weight loss) than patients with normal thrombocyte counts (16).

1.3.1.2. Cellular factors

Haematopoietic stem cells can be categorised into myeloid progenitor and lymphoid progenitor cells (18).   Myeloid progenitor cells give rise to granulocytes, macrophages, megakaryocytes and erythrocytes and lymphoid progenitor cells give rise to T lymphocytes (T cells), B lymphocytes (B cells) and natural killer cells (NK). Dendritic cells (DC) can arise from both myeloid or lymphoid progenitor cells and thus do not fall into either of these categories (18). During the innate immune response granulocytes, macrophages, DC's and NK cells act as the first responders before the adaptive immune response develops.

1.3.1.2.1. Granulocytes

There are three types of granulocytes; neutrophils (CD15+), eosinophils (CCR1+) and basophils (CD22+). These cells are short lived and increase in number during an immune response. Neutrophils are able to internalise and entrap pathogens,in a phagosome and undergo a process of maturation where the phagosome fuses with a lysosome to form a phagolysosome. The phagolysosome is a hostile and acidic environment that encourages the degradation of its contents (19). During active TB infection neutrophils are one of the first responders to arrive at the site of infection in order to eliminate the bacteria. It is suggested that neutrophils perform their function not by direct clearance of the bacteria but rather by targeting the pathogen by degranulation or in assisting the transition from the innate immune response to the adaptive immune response by cytokine and chemokine production (20).

1.3.1.2.2. Macrophages

Monocytes (CD14+ and CD16+) circulate in the peripheral blood and differentiate into macrophages upon migrating into tissue. Macrophages are one of three types of phagocytic cells involved in the immune response and they play an important role in innate immunity and chronic inflammation. Upon inhalation of the *Mtb* bacillus into the lungs, the alveolar macrophages respond by phagocytosing the bacteria. The macrophages stimulate the production of chemokines and cytokines which attract neutrophils and monocytes to the site of infection resulting in the formation of a granuloma which will be discussed in more detail [21].

1.3.1.2.3. Dendritic cells

Dendritic cells are considered to be one of the most important antigen presenting cells (APCs) due to their ability to stimulate the differentiation of naïve T cells. Dendritic cells recognise pathogenic antigens and migrate from the blood into tissue where they mature to perform their phagocytic function. The cells are specialised to take up antigen, migrate to the regional lymph nodes and display it to circulating lymphocytes via the major histocompatibility complex (MHC) molecules in association with CD1. Recognition of the antigen by CD4+ and CD8+ T cells plays an important role in the activation of the adaptive immune response. It has been noted that some virulent strains of *Mtb* are capable of inhibiting the maturation of DC's and therefore their ability to present antigens to lymphocytes through a decreased expression of CD1 [21].

1.3.1.2.4. Natural killer cells (NKs)

Natural killer cells (CD56+) are part of the innate immune system as they do not have antigen specific receptors on their surface. They are able to recognise and destroy abnormal cells.

1.3.2. Adaptive immune response

When an invasion by pathogens cannot be contained by the innate immune response, the adaptive immune system is called into action to help eliminate the harmful pathogens (9). The adaptive response is initiated by dendritic cells that recognise, engulf and present the pathogen to lymphocytes. The activated dendritic cells also secrete cytokines which are imperative to the immune response.

Features of the adaptive immune response include:

- High specificity to pathogens.
- Ability to form memory to specific pathogens which allows a faster response time of the immune system during re-exposure.
- There is a lag time between exposure and response to a pathogen due to the need for clonal expansion of lymphocytes.

The adaptive immune response consists of two classes:

- Antibody response (humoral)
- Cell mediated response

1.3.2.1. Antibody-mediated response (humoral)

This response is initiated by B cells and allows for the production and secretion of antibodies. These antibodies circulate in the blood stream and can bind with specific pathogens that have entered the body. The binding of the antibody and pathogen prevents pathogens such as viruses and toxins from being able to bind to the host cells thus interfering with the infection. B cells and T cells do not work independently of each other but rather complement each other. B cells are professional antigen presenting cells, which through their activation, progression and interaction with CD4+ T helper cells can stimulate T cells to produce cytokines. Reciprocally, these cytokines can aid in regulating the antibody responses of B cells (22). During *Mtb* infection, B cells are able to influence the host immune response and disease outcome by engaging the Fc receptors and influencing the Th1 activation and containment on the mycobacteria (22).

1.3.2.2. Cell mediated response

1.3.2.2.1. T cells

T cells are the primary cells involved in the cell mediated immune response, however, they are not the only cells involved. Two of the important T cell subsets are the T cytotoxic cell (CD8+ T cells) and the T helper cells (CD4+ T cells). The T helper cells secrete mediators such as cytokines which direct the functioning of other T cells, B cells and other phagocytic cells to perform their function. T cytotoxic cells will directly bind to and kill the pathogens involved or the infected cells. APCs ingest and package the pathogens and display them as antigens on their surface. MHC I molecules present antigen from the cytosolic compartment (produced within the cell, i.e. viral particles) to CD8+ cytotoxic T cells, which will destroy the infected cells. MHC II molecules present antigens that have entered the host cell via the endocytic pathway (i.e. through phagocytosis) to T cells, which will subsequently become activated and proliferate. The T helper cells then release cytokines, which activate the antigen presenting cell (i.e. the macrophage to improve its killing function of phagocytosed pathogens) or which will stimulate the B cells to produce antibodies [23].

1.3.2.2.1.1.   CD4+ T helper cells

Once a pathogen has been recognised as such and phagocytosed by APC's, the cells travel to the lymphoid organs where they package and display the pathogens antigens on the surface of the cell via the MHC II molecule. Naïve T helper cells recognise and respond to the antigens by becoming activated, by differentiating and by proliferating (clonal expansion). Some of the CD4+ T cells will differentiate into effector cells and be able to secrete different cytokines that can perform different functions. T cell phenotypes include but are not limited to Th1, Th2, T regulatory (Treg), T follicular (Tfh) and Th17. During the initial activation of the naïve cells, a subset of long-lived memory cells is also formed. These memory cells can remain in the body for a number of years and are specific for the antigen it has just

encountered. These cells have the advantage of being able to respond much faster and more efficiently upon re-exposure to the pathogen (23)

1.3.2.2.1.2.   *CD4+ effector T cells*

These perform their function by differentiating into a type of T helper (Th) cell which results in the secretion of a group of cytokines which can provide assistance to other cells to perform different functions. A big factor in the determination of a T helper cell group and subsequently the secretion of a cytokine group is based on the status of cytokines in the environment at the time of the encounter.   The five dominant lineages of T helper cells which are produced are Th1, Th2 and Th17, T follicular cells (TFH) and T regulatory (Tregs) cells (Figure 1.2). Each type of cell produces a different group of cytokines which results in a different function (24). CD4+ T cells play critical roles in mediating adaptive immunity to a variety of pathogens. They are also involved in autoimmunity, asthma, and allergic responses as well as in tumour immunity. During TCR activation in a particular cytokine milieu, naive CD4 T cells may differentiate into one of several lineages of T helper (Th) cells, including Th1, Th2, Th17, and iTreg, as defined by their pattern of cytokine production and function (13). The T helper cell phenotypes will be discussed under the adaptive immune response.

1.3.2.2.1.3.   *CD8+ cytotoxic T cells*

Pathogen proteins and their peptides that are present in the cytoplasm of a cell will be processed by the proteosome and transported into the endoplasmic reticulum via the transporter associated with antigen processing (TAP) where it will eventually be displayed on the surface of the cell via MHC I molecule, resulting in recognition by naïve CD8+ T cells, followed by activation and clonal expansion of the CD8+ T cells (25). CD8+ cytotoxic cells can directly kill the infected cells by releasing perforin and granulysin, which lyses host cells and induces apoptosis. A subset of memory cells forms in much the same way as in CD4+ T cells and play a role during reinfection (23).

1.3.2.2.2. T helper cell phenotypes (Figure 1.2)

1.3.2.2.2.1.   T helper 1 (Th1)

Th1 cells are known as the principal regulators of type 1 immunity and produce the pro-inflammatory cytokines IFN-γ, IL-2, TNF-a and IL-12. The general consensus is that the CD4+ Th1 phenotype is characterised by the expression of CXCR3 and CCR5 [26].

- IFN-γ: This is the most important cytokine involved in the immune response to *Mtb* infection.   IFN-γ is a pro-inflammatory cytokine and is predominantly secreted by CD4+ helper cells, CD8+ cytotoxic T cells and NK cells although there are other cells that may also contribute to its secretion. The main function of IFN-γ is the activation of macrophages as well as the promotion of Th1 response [27]. Previous studies show that patients with a defect in IFN-γ production are prone to uncontrolled *Mtb* infections, poor granuloma formation and severe progression of disease [28]. Patients with HIV are more susceptible to *Mtb* and this may be due to failure to produce sufficient levels of IFN-γ in the lung [21].

- IL-2: Upon antigen presentation increased expression of the IL-2 receptor permits a rapid and selective expansion of CD4+ and CD8+ T cells. Conversely it has been shown that IL-2 also plays a role in down-regulating the immune response in the prevention of autoimmune diseases [29].

- TNF-α: Plays an important role in recruitment of leukocytes to the site of infection and granuloma formation and maintenance. It has been reported that mice that are deficient in TNF-α are unable to form proper granulomas [30]. TNF-α is also instrumental in the activation of macrophages as well as stimulating apoptosis and reactive oxygen (ROI) and nitrogen intermediates (RNI) [21]. While TNF-α is vital in the containment of *Mtb*, it has also been identified as a main player in the destruction of lung tissue due to the chronic inflammation and necrosis [31]. The characteristic fever and wasting that is commonly seen in patients with *Mtb* can be partially attributed to TNF-α production.

- <u>IL-12</u>: IL-12 is important in the polarisation of Th1 cells and the induction of IFN-γ production. IL-12 also stimulates the proliferation of antigen-specific cytolytic T cells and NK cells thereby enhancing cytotoxicity (21).

- <u>Lymphotoxin-α (LT- α)</u>: LT- α is primarily produced by activated T cells, B cells and NK cells and is known to mediate some important functions of the immune system such as the development of lymph nodes, Peyer's patches and primary B cell follicles (32). Allie *et al* (2010) demonstrated that LT- α deficient mice were highly susceptible to *Mtb* despite their ability to still mount a Th1 response. These mice had uncontrolled bacterial growth with a lack of well demarcated primary granuloma formation similar to that seen in TNF-α deficient mice (32).

1.3.2.2.2.2. T helper 2 (Th2)

The primary cytokine is IL-4 with IL-5, IL-10 and IL-13 making up the rest of the signature cytokine profile. Th2 cells have also been found to produce IL-9 and TNF-α but not IFN-γ. It is accepted that the surface expression of Crth2 (Chemo-attractant receptor-homologous molecule expressed on Th2 cells), CCR3 and CCR4 denote a Th2 subset (26).

- <u>IL-4:</u> IL-4 is elevated in active disease and has been shown to have a pathogenic role during the late phase of *Mtb* infection. IL-4 down-regulates the Th1 responses and a high expression of IL-4 has been associated with cavitation (21).

- <u>IL-5</u>: IL-5 promotes the differentiation and activation of eosinophils in the bone marrow (33).

- <u>IL-9</u>: IL-9 promotes the expansion of mast cells especially in allergic responses and lung inflammation (34).

- <u>IL-10</u>: IL-10 is a powerful immunosuppressive cytokine that affects macrophages, monocytes, DC's and T cells. Its function stretches from deactivation of macrophages and reduced Th1 response to decreasing reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) and also limiting antigen presentation, all which may have big effects on the innate and adaptive immune response in TB. IL-10 is thought to be an important biomarker of disease progression in TB and a correlate of susceptibility to TB (35).

- IL-13: IL-13 is involved in allergic responses and IgE synthesis and contributes to airway inflammation (36).

1.3.2.2.2.3. T helper 17 (Th17)

These cells are derived from CD4+ naïve T cells, in an environment with TGF-β and IL-6, and are characterised by the production of IL-17a, IL-17f, IL-21 and IL-22. The cytokines mediate the host defensive mechanisms in various infections; however, these pro-inflammatory cytokines can cause immunopathology when the response is exaggerated. There are few human studies on cytokines released by Th17 cells but mouse studies have shown that a high dose of *Mtb* delivered intratracheally was poorly controlled in the absence of IL-17 (37). It has been determined that Th17 cells express CCR6 and CCR4 on their surface thus giving them a phenotype of co-expression of CD4, CCR6 and CCR4 (38).

1.3.2.2.2.4.   T follicular cells (Tfh)

Tfh cells are a subset of CD4+ T cells that reside in the secondary lymphoid tissues and function by providing assistance for B cell activation, expansion and differentiation (39). The expression of CXCR5 with a concomitant loss of CCR7 leads to the relocation of Tfh to B cell follicles (40). The differentiation of Tfh relies on the transcription factor Bcl6 as a master regulator (41) and the primary cytokines that are produced during the induction of Tfh production are IL-6 and IL-21.

1.3.2.2.2.5.   T regulatory cells (Tregs)

Tregs are a heterogeneous T cell population that are capable of suppressing the immune system and regulating self-tolerance. Tregs are a critical component of immune cell homeostasis and function by enforcing a dominant negative regulation on other immune cells (42). Singh *et al.* (2012) demonstrated that a high TB bacillary load correlated with increased Tregs which returned to normal levels after treatment indicating that expansion may occur in response to pathogen exposure (43). Natural Tregs emerge from the thymus as single CD4+ cells, which when stimulated by an

antigen, differentiate into Tregs expressing CD25 and FoxP3. Thus it is widely accepted that the phenotype for Tregs is the co-expression of CD4, CD25hi and FoxP3 [44].
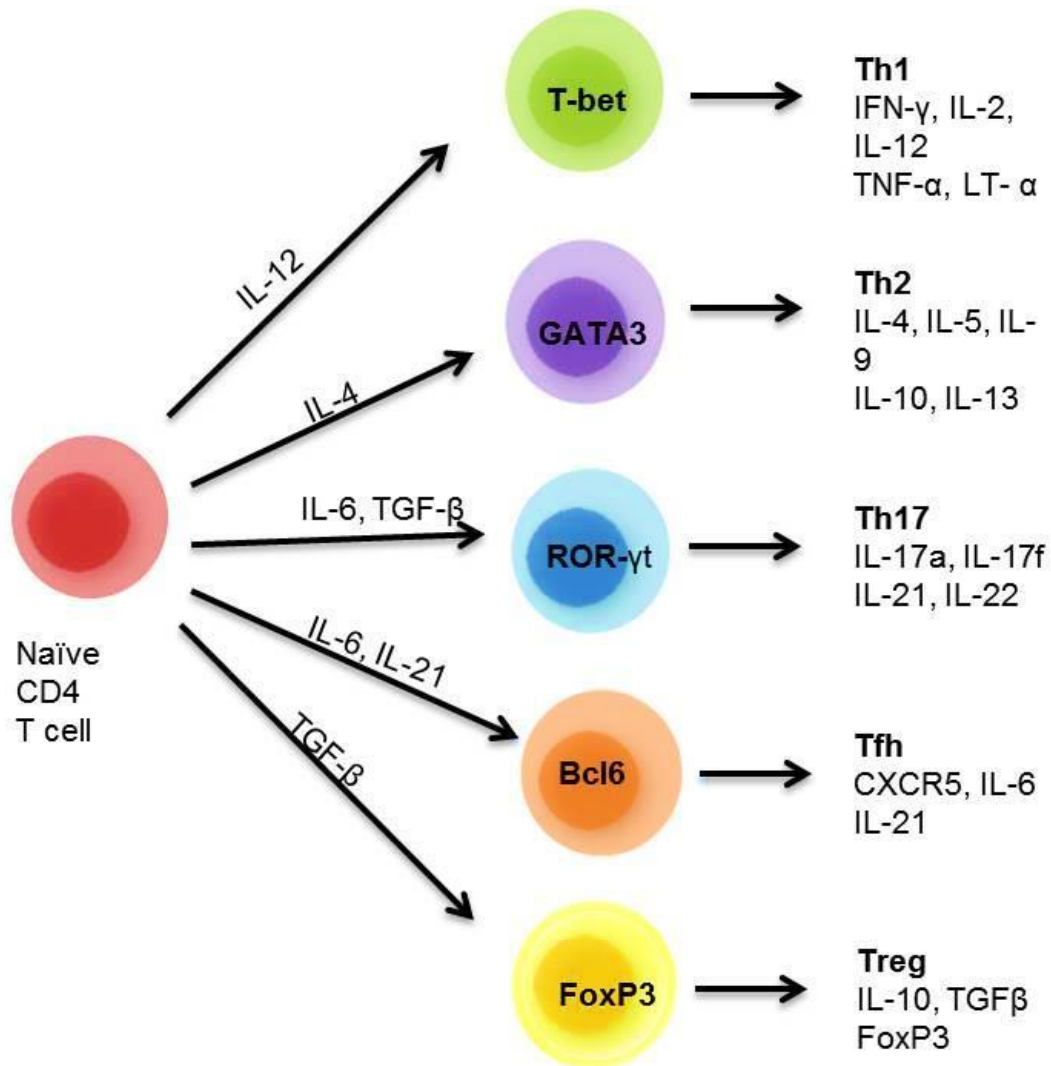
Figure 1.2: Illustration of the T helper cell phenotypes originating from naïve CD4+ T cells.

A naïve CD4 T cell has the ability to give rise to different phenotypes of T cells namely Th1, Th2, Th17, Tfh and Treg cells.

1.4. Cell surface markers

A cell surface marker can be defined as a protein that is present on a cell and distinguishes it from other subsets of cells. Clusters of differentiation (CD) provides a simple and unambiguous nomenclature system to be used in immune phenotyping by identifying subsets of cells based on their surface marker expression (45). Surface markers are able to differentiate between lineages of cells as well as stages in development, activation or proliferation of cells (46). Specific cell surface markers can provide information on whether a cell type will respond to drugs or whether a cell expresses a receptor necessary for specific biological processes (47).

1.5. Granuloma formation

A granuloma is developed by the host as a way to contain or eliminate the pathogen however; it can also provide an environment in which the bacteria can survive for many years after infection. The formation of a granuloma starts shortly after inhalation of the *Mtb* bacillus which, once inhaled, travels to the alveoli and is phagocytosed by alveolar macrophages. The macrophages release cytokines and chemokines, attract additional monocyte-derived macrophages and neutrophils to the site of infection forming the start of the granuloma (48). The centre of the granuloma is predominantly made up of infected macrophages surrounded by epitheloid cells and multinucleated giant cells. Once these cells have arranged themselves in an aggregate around the infected macrophages, lymphocytes create a rim around the granuloma followed by a fibrous capsule (21). Over time the centre becomes necrotic and takes on a caseous appearance, which is thought to be hypoxic and may play a role in the *Mtb* metabolism, altering susceptibility to some anti-tuberculosis treatments (49). The cytokines IFN-γ and TNF-α are both important in the formation and maintenance of the granuloma while IL-10 has been shown to have an opposite effect by acting as a negative regulator of granuloma formation (50).

1.6. Methods of detection

Current methods for diagnosis of TB include the following:

1.6.1. Sputum smear microscopy

Smears stained with carbol fuchsin and fluorochromes such as auramine-rhodamine are widely supported for diagnosis of *Mtb* in clinical samples (51).

1.6.2. Sputum culture

Different media used in culture methods include:

I. Lowenstein-Jensen medium – egg based solid medium which may take between 4-6 weeks for a result.
II. BACTEC liquid culture offers a more sensitive and rapid test to solid media with an available result within 1-3 weeks.
III. Mycobacterial growth indicator tube (MGIT) is a liquid culture medium that is able to provide a result in as little as 8 days while simultaneously allowing for drug susceptibility by the addition of antibiotics.

1.6.3. Nucleic acid amplification test (NAAT)

NAAT's are able to detect TB rapidly and with a high specificity by targeting specific nucleic acid sequences. A range of commercially available tests have been developed and have proved to increase sensitivity and specificity of both smear positive and smear negative samples (51).

1.6.4. Drug resistance tests

The emergence of multi-drug resistant (MDR) and extensive-drug resistant (XDR) strains of *Mtb* have increased the importance of drug susceptibility testing (DST) at diagnosis. Methods have been developed that simultaneously detect infection with *Mtb* and amplify regions of drug resistance.

- Line probe assays (LiPAs): These use a technology where amplified DNA is applied to strips which contain probes specific for Isoniazid (INH) and Rifampin (RIF) resistance.
- Molecular beacons: These are colorimetric based assays that provide an ease of assay interpretation. DNA probes contain an intrinsic fluorophore which, when bound to its complementary sequence, undergo a conformational change and produce a visually detectable signal [51]. One of these tests is the GeneXpert$^{®}$ which can simultaneously give an *Mtb* diagnosis while also testing for rifampicin resistance in approximately 2 hours. The reagents that are used are enclosed in a single cartridge and contain anti-tuberculosis properties which results in a safe and convenient form of diagnosis that can be performed outside of a laboratory [52]

1.6.5. Interferon Gamma release assays (IGRA)

IGRA is a blood-based assay that identifies an immune response to *Mtb* specific antigens (ESAT-6, CFP-10 and TB7.7) by measuring the presence of TB specific effector memory cells after exposure of an individual to TB.

- QuantiFERON-TB Gold In-Tube assay (QFN-GIT): IFN-γ is produced in response to stimulation with ESAT-6, CFP-10 and TB 7.7 and the concentrations are detected using an ELISA test.
- T-SPOT. *TB* assay: T-SPOT. *TB* test measures the number of IFN-γ producing cells in response to ESAT-6 and CFP-10. The principle of the T-SPOT. *TB* assay is based on the enzyme-linked immunosorbent spot assay (ELISPOT).

1.7. Flow cytometry

Flow cytometry refers to the measurement of a single cell as it passes through a region of investigation. It allows a single cell to be studied at a time with a flow rate of over 1000 cells (events) per second. The investigation of each cell is multi-parametric and includes but is not limited to the following properties:

- Size of the cell (forward scatter; FSC)

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- Internal integrity and granularity (side scatter; SSC)
- Cell surface markers or proteins
- Auto-fluorescence

There are three main components of flow cytometry:

- <u>Fluidics system</u>: Through hydrodynamic focusing, cells are separated and allowed to pass single file through one or more lasers.

- <u>Optics system</u>: Once the cells have passed through the lasers, light is either scattered and collected in detectors or it excites fluorochromes and is collected in a photomultiplier tube (PMT). Antibodies are directly conjugated to fluorescent dyes (fluorochromes) which, when passing through a laser, accepts light from a laser and is excited at a specific wavelength which is optimal to that dye. During excitation electrons move from a resting state to an excited state and then back to a resting state. The result of this is an emission of light at a lower energy state which appears as a longer wavelength. It is this difference in the excitation and emission wavelengths that allows us to separate the light using detectors. The laser is scattered when it strikes the cell and the scattered light is collected by a collection lens and focused to a photodiode where it is converted into a current and recorded by the electronic system. Light that is collected in the forward direction is used to determine the size of a cell (forward scatter) and light that is collected at 90 degrees indicates the granularity of the cell (side scatter). Emitted light is collected with a collection lens and directed towards a series of dichroic mirrors and filters until it reaches its designated PMT (53).

- <u>Electronics system</u>: Once emitted light reaches its PMT it is converted into electrons. The electrons are multiplied in order to produce a voltage pulse by either linear or logarithmic amplifiers and analog digital converters. The voltage pulse is sent to the signal processors where it is measured for its height, width and area and is stored in the software for further analysis (53).

1.8. FACS^{TM} CAP Technology (CAP: Combinatorial Antibody Profile)

With the continued and growing identification of cell surface markers using flow cytometry it became necessary to create a platform that allowed the characterisation of a heterogeneous population of cells, using many of the monoclonal antibodies available, simultaneously.   Becton Dickinson (BD) Biosciences developed the FACS™ CAP technology as a multi-dimensional screening tool for rapid identification and characterisation of human cell surface protein expression profiles using high throughput flow cytometry. The antibody array panel includes a selection of 252 antibodies, directly conjugated to the fluorochromes FITC or Alexa488, PE and APC or Alexa647, placed in combinations of three antibodies in each well of a 96 well plate. Isotype controls are included in the plates to assess the amount of background staining and non-specific Fc receptor binding. Staining of the cells occurs using an automated liquid handling system while a semi-automated flow cytometry system and software are used for the acquisition of the cells (54).   A standardised algorithm has been designed to analyse the flow cytometry data. The antibodies present in the FACS^{TM} CAP plates represent intercellular pathways, cell proliferation, cell-cell signalling, chemotaxis, apoptosis, cell adhesion and cell motility while also allowing for the addition of antibodies to monitor specific immune functions and inflammatory responses. BD designed the FACS^{TM} CAP technology to be a valuable tool in the use of stem cell therapy or cell banking by documenting phenotypic variants or changes due to different donors, evaluating isolation protocols, culture or storage media and studying stem cells for differentiation, carcinogenesis and drug targeting (54). This technology can also be applied to a number of experiments involving cell surface proteins such as identifying a set of biomarkers that are present on one set of cells when compared with cells from another patient, time point or different culture conditions all of which would be highly beneficial in the clinical diagnosis or predictive outcome of disease (54).

1.9. Goals and objectives

1.9.1. Context

Evaluating the success of chemotherapy for tuberculosis (TB) currently relies on a battery of inadequate tests and new markers are urgently needed to facilitate the discovery and testing of new TB drugs. The hypothesis underlying this project is that the expression of surface markers on peripheral blood mononuclear cells (PBMCs) of TB patients, from pre-treatment and during standard anti-tuberculosis chemotherapy, will provide clinically valuable markers for diagnosis and early treatment response. This project is a sub-study, in collaboration with Becton Dickinson Technologies (BDT), of an on-going study funded by the Bill and Melinda Gates Foundation (BMGF) and led by the Catalysis Foundation for Health. The goal of the main project was to identify biomarkers for bacterial load during active TB and during therapy. Identification of load biomarkers would assist in the stratification of patients into different treatment arms, depending on their risk for poor outcomes.   A range of sample types (blood, breath condensate, saliva, sputum and urine) were obtained to search for such markers.   Patients also underwent three [18F]-fluoro-2-deoxy-D-glucose positron emission tomography/computer tomography (FDG-PET/CT) imaging studies to further define the extent of inflammation in the lungs and the changes during treatment.

1.9.2. Project goal

The goal of the FACS^{TM} CAP project is to discover host candidate biomarkers for TB treatment response. These markers will be based on PBMC surface molecule expression in particular early treatment response and cure at the end of standard anti-TB therapy. The objective is to use the BDT developed FACS^{TM} CAP technique to discover peripheral blood cell surface markers which can serve as indicators of TB disease status.

1.9.3. Primary objectives

1.9.3.1. Objective 1: Optimization of FACS^{TM} CAP procedures

1.9.3.1.1. Objective 1.1: Assess the effect of cryopreservation on FACS^{TM} CAP performance. Cryopreservation serves as a useful tool for storing live cells for prolonged periods of time allowing retrospective phenotypic and functional analysis. The idea for this study was to use frozen PBMCs from TB patients that were previously prepared and frozen in liquid Nitrogen.

1.9.3.1.2. Objective 1.2: Assess the effect of PBMC culture in the presence of *Mtb* antigen stimulation on marker expression in comparison to unstimulated PBMCs. This experiment would help determine if by inducing TB specific antigen we would have greater chance of finding variation in the expression of these antigens during the course of therapy.

1.9.3.2. Objective 2: To assess the differential PBMC surface marker expression by FACS^{TM} CAP in TB patients during treatment

Chapter 2: Materials and methods

2.1. Recruitment of patients

Patients were recruited from four local communities in Cape Town, South Africa, namely Ravensmead, Uitsig, Adriaanse and Elsiesriver all of which have a high prevalence of TB and a low incidence of HIV.

2.1.1. Inclusion criteria

- All patients had to be between the ages of 16 and 70 years.
- Willing to give informed consent.
- Willing to be tested for HIV and have their results disclosed to the field worker.
- TB patients had to have a newly confirmed diagnosis of pulmonary TB infection or a recurrent infection at least 12 months after completing the previous treatment. TB was confirmed by GeneXpert[®] (Cepheid) testing and MGIT culture on suspected patients. Positive cultures were confirmed to be due to *Mtb* by acid-fast bacilli (AFB) staining, followed by Polymerase chain reaction (PCR, an in-house assay for RD1, RD4, RD9 and RD12) or the Capilia TB assay.
- Healthy community controls had to have a negative sputum GeneXpert[®] test taken at recruitment into the study.
- Other lung disease controls (OLD) had to have a negative sputum GeneXpert[®] test at recruitment as well as a diagnosis of one of the following:

  - febrile illness with chest symptoms
  - radiographic evidence of viral or bacterial pneumonia
  - bronchiectasis with acute exacerbation
  - acute exacerbation of asthma or chronic obstructive pulmonary disease (COPD).

2.1.2.   Exclusion criteria

- People with a haemoglobin level of <10g/l.

- Medical conditions such as Diabetes Mellitus, chronic bronchitis/asthma/emphysema requiring steroid treatment, cancer, pregnancy or steroid treatment within the last 6 months.

- People without a permanent residence or residence in the study area for less than 3 months.

- Participation in a drug or vaccine trial.

- Alcohol abuse or the use of illicit drugs.

- TB patients who had started treatment prior to enrolment were excluded.

- Healthy community controls who had suffered with an acute respiratory infection within the last four weeks.

- Other lung disease controls were excluded if their GeneXpert® test proved to be positive for *Mtb*.

Patients who successfully enrolled in the study made themselves available to have blood drawn at multiple time points. The blood sampling for this study occurred at diagnosis (Dx), 4 weeks after commencement of treatment (Week 4) and at the end of treatment (Week 24, EOT).

In total 48 TB patients were enrolled for this study. Nine patients were dropped from the study for reasons such as pregnancy, conversion to positive HIV status and insufficient cell concentration for plate preparation. Six other patients were dropped for incomplete data due to missed clinic visits or instrument error resulting in poor quality flow cytometry data. In total 33 TB patients, with complete data for three time points, were included for data analysis (S112, S123, S124, S125, S129, S130, S131, S132, S133, S134, S136, S137, S138, S139, S140, S141, S142, S144, S145, S146, S147, S149, S150, S153, S154, S155, S156, S163, S164, S167, S168, S169, S170).

Control groups were represented by 14 healthy community controls and 10 patients with other lung diseases (OLD). Only 11 healthy controls (S148, S157, S158, S179, S180, S181, S182, S194, S195, S197, S198) and 9 patients with OLD (S166, S173,

S175, S177, S184, S186, S187, S188, S189) were analysed due to time constraints. Patient demographics are listed in tables 2.1- tables 2.3 below.

Table 2.1: Patient demographics - TB patients

Patient ID	Gender (M/F)	Age (years)	Chest X-Ray	BCG scar (Y/N)	TTP at Dx (days)	Speciation	Status	Week 8 culture (pos/neg)	EOT outcome
S112	M	52	Active TB and cavities in right lung	Y	4	Positive	Active TB	Negative	Definite cure
S123	M	40	In keeping with Active TB	Y	5	Positive	Active TB	Positive	Definite cure
S124	M	24	Active TB	Y	5	Positive	Active TB	Negative	Definite cure
S125	M	32	Active TB. No clear cavity	Y	8	Positive	Active TB	Positive	Probable cure
S129	M	40	Consistant with active PTB	N	4	Positive	Active TB	Negative	Definite cure
S130	M	29	Compatible with TB	N	6	Positive	Active TB	Negative	Definite cure
S131	F	18	Abnormalities consistent with Active TB	Y	14	Positive	Active TB	Negative	Definite cure
S132	F	17	Abnormalities consistent with Active TB	Y	5	Positive	Active TB	Positive	Definite cure
S133	F	33	Abnormalities consistent with Active TB	Y	6	Positive	Active TB	Inconclusive	Definite cure
S134	F	24	Abnormalities consistent with Active TB	N	3	Positive	Active TB	Negative	Definite cure
S136	M	42	Consistent with Previous TB and possible New Activation.	Y	18	Positive	Active TB	Negative	Probable cure
S137	F	44	Extensive Active TB	Y	7	Positive	Active TB	Negative	Definite cure

Table 2.1: Patient demographics - TB patients continued

S138	M	23	Extensive Active TB	Y	4	Positive	Active TB	Negative	Definite cure
S139	M	38	Abnormalities consistent with Active TB	Y	5	Positive	Active TB	Positive	Definite cure
S140	M	22	Abnormalities consistent with Active TB	Y	6	Positive	Active TB	Positive	Definite cure
S141	M	17	Abnormalities consistent with Active TB	Y	10	Positive	Active TB	Negative	Definite cure
S142	F	43	Abnormalities consistent with Active TB	N	3	Positive	Active TB	Positive	Definite cure
S144	M	31	Abnormalities consistent with Active TB	N	7	Positive	Active TB	Positive	Definite cure
S145	F	19	Abnormalities consistent with Active TB	Y	4	Positive	Active TB	Negative	Definite cure
S146	M	20	Abnormalities consistent with Active TB	N	8	Positive	Active TB	Negative	Definite cure
S147	M	23	Active Bilateral TB	N	5	Positive	Active TB	Inconclusive	Definite cure
S149	F	19	Abnormalities consistent with Active TB	N	3	Positive	Active TB	Positive	Definite cure
S150	F	21	Active TB with slight involvement	Y	6	Positive	Active TB	Negative	Probable cure
S153	M	25	Abnormalities consistent with Active TB	Y	5	Positive	Active TB	Positive	Definite cure
S154	F	42	Active TB bilateral upper zones	Y	4	Positive	Active TB	Positive	Definite cure

Table 2.1: Patient demographics - TB patients continued

S155	F	36	Active TB in Left Upper Lobe	Y	8	Positive	Active TB	Negative	Definite cure
S156	M	64	Active TB in Bilateral Upper Lobes	Y	3	Positive	Active TB	Positive	Definite cure
S163	M	25	Active TB in left lung	N	4	Positive	Active TB	Positive	Not cured
S164	M	45	Abnormalities consistent with Active TB	N	Neg **	not done	Active TB	Negative	Definite cure
S167	M	41	Active TB left lung	N	4	Positive	Active TB	Negative	Definite cure
S168	F	23	Bilateral Active TB	N	4	Positive	Active TB	Negative	Not cured
S169	M	30	Right lung active TB and Pleural effusion	N	4	Positive	Active TB	Negative	Not cured
S170	M	30	Extensive bilateral active TB	Y	6	Positive	Active TB	Negative	Probable Cure
S191	F	50	Not done	N	9	Positive	Active TB	Not applicable	Not applicable
S192	F	47	Not done	N	20	Positive	Active TB	Not applicable	Not applicable
S193	F	52	Not done	N	3	Positive	Active TB	Not applicable	Not applicable
S201	F	41	Not done	Y	5	Positive	Active TB	Not applicable	Not applicable
S203	F	36	Not done	N	9	Positive	Active TB	Not applicable	Not applicable

** Patient S164 was treated as a TB positive patient due to a positive GeneXpert[®] test performed at the National Health Laboratory Service. We were unable to obtain a positive culture result using the research sputum sample.

Table 2.2: Patient demographics - Other lung disease controls

Patient ID	Gender (M/F)	Age (years)	Chest X-Ray	QFN (Pos/Neg)	BCG scar (Y/N)	TTP (days)	Speciation	Status
S166	M	31	Right middle lobe collapse; consolidation	Negative	Y	Negative	Not done	OLD (pneumonia)
S173	F	62	Bilateral disease, not in keeping with TB	Negative	N	Negative	Not done	OLD (pneumonia)
S175	M	49	Mild bilateral bronchiectatic changes	Positive	N	Negative	Not done	OLD (pneumonia secondary to bronchiectasis)
S176	F	40	Normal examination	Negative	N	Negative	Not done	OLD (upper respiratory tract infection)
S177	F	28	Infiltrates in right lower lobe.	Positive	N	Contaminated	Negative	OLD (pneumonia)
S184	M	34	Cardiomegaly and patchy consolidation in RUL and RLL	Positive	Y	Contaminated	Negative	OLD (pneumonia)
S186	F	31	Left lower lobe consolidation	Positive	Y	Negative	Not done	OLD (Pneumonia)
S187	M	23	Left lower lobe pneumonic consolidation and pleural effusion	Positive	Y	Negative	Not done	OLD (pneumonia)
S188	M	39	Large left effusion	Positive	Y	Contaminated	Negative	OLD (parapneumonic effusion)
S190	F	52	Abnormalities in keeping with pneumonia	Positive	Y	Negative	Negative	OLD (pneumonia)

Table 2.3: Patient demographics - Healthy community controls

Patient ID	Gender (M/F)	Age (years)	QFN (Pos/Neg)	BCG scar (Y/N)	TTP (days)	Speciation	Status
S148	M	21	Positive	Y	Negative	Not applicable	Healthy control
S157	M	25	Positive	Y	Contaminated	Negative	Healthy control
S158	F	23	Positive	Y	Negative	Negative	Healthy control
S179	F	34	Positive	Y	Negative	Negative	Healthy control
S180	M	26	Negative	N	Contaminated	Negative	Healthy control
S181	M	27	Negative	Y	Negative	Not applicable	Healthy control
S182	M	27	Negative	Y	Negative	Not applicable	Healthy control
S194	F	30	Negative	N	Negative	Not applicable	Healthy control
S195	M	25	Positive	Y	Negative	Not applicable	Healthy control
S197	F	30	Positive	Y	Negative	Not applicable	Healthy control
S198	M	57	Positive	N	Negative	Not applicable	Healthy control

2.2. Reagents

2.2.1. FACS CAP lyoplates

FACS[TM] CAP lyoplates were manufactured by BD Technologies in North Carolina and BD Biosciences in California. Seventy nine combinations of three antibodies were prepared in tubes at the correct concentrations at BD Technologies, North Carolina (NC) and sent to California. A master plate was created using the combinations from the tubes prepared in NC, from which 500 daughter plates were produced. Due to the high risk for inter-plate variability, the final daughter lyoplates were produced using an automated system and lyophilised to increase stability and ease of transport and storage. For each lot, a number of plates were used for quality control purposes and upon passing the quality control check, were sealed in foil pouches, assigned a lot number and given an expiry date of two years after production. Antibodies used in the FACS[TM] CAP lyoplates were directly conjugated to fluorescent dyes detectable in channels 1, 2 and 4 on the FACSCalibur[TM] (Table 2.4). Although there are a total of 252 antibodies in each plate (Table 2.5), some were used more than once or multiple times to verify accuracy of pipetting and reproducibility between wells.

Table 2.4: List of fluorescent dyes directly conjugated to antibodies in the FACS[TM] CAP plates

Fluorochrome	Abbreviation	Detector channel
Fluorescein isothiocyanate	FITC	FL1
Alexa Fluor 488	Alexa488	FL1
R-phycoerythrin	PE	FL2
Allophycocyanin	APC	FL4
Alexa Fluor 647	Alexa647	FL4

Table 2.5: List of antibodies included in the FACS^{TM} CAP plates 14

CD1a	CD11c	CD27	CD41b	CD49d	CD62P	CD80	CD102	CD119	CD140b	CD172a	CD220	CD294	CXCR2	MET
CD1b	CD13	CD28	CD42a	CD49e	CD63	CD81	CD103	CD120b	CD141	CD172b	CD221	CD318	CXCR5	MIC A/B
CD1d	CD14	CD29	CD42b	CD49f	CD64	CD85	CD104	CD122	CD142	CD178	CD226	CD326	EGFR	NKB1
CD2	CD15	CD30	CD43	CD50	CD66	CD86	CD105	CD123	CD146	CD180	CD227	CDw329	F11 R	NKG2D
CD3	CD16	CD31	CD44	CD51	CD66b	CD87	CD106	CD124	CD147	CD181	CD231	CD336	FGFR3	NKp46
CD4	CD16b	CD32	CD45	CD53	CD69	CD88	CD107a	CD126	CD150	CD183	CD235a	CD337	FMC7	PAC-1
CD4 V4	CD18	CD33	CD45RA	CD54	CD70	CD89	CD107b	CD127	CD151	CD184	CD244	CD349	fMLP receptor	PD-L2
CD5	CD19	CD34	CD45RB	CD55	CD71	CD90	CD108	CD130	CD154	CD195	CD252	CCR1	HER-2/neu	P-glycoprotein
CD6	CD20	CD35	CD45RO	CD56	CD72	CD91	CD109	CD132	CD158a	CD200	CD256	CCR2	HLA-A,B,C	SSEA3
CD7	CD21	CD36	CD46	CD57	CD73	CDw93	CD110	CD133	CD158b	CD201	CD267	CCR3	HLA-DR	SSEA4
CD8	CD22	CD37	CD47	CD58	CD74	CD94	CD112	CD134	CD161	CD205	CD268	CCR4	Integrin b7	TGFBR2
CD9	CD23	CD38	CD48	CD59	CD75	CD95	CD114	CD135	CD162	CD206	CD271	CCR6	KDR	TNF
CD10	CD24	CD39	CD49a	CD61	CD77	CD97	CD116	CD137	CD163	CD208	CD275	CCR7	LAIR1	TRA-1-60 antigen
CD11a	CD25	CD40	CD49b	CD62E	CD79a	CD98	CD117	CD138	CD164	CD210	CD278	CCR8	Le B4 R	TRA-1-81 antigen
CD11b	CD26	CD41a	CD49c	CD62L	CD79b	CD99	CD118	CD140a	CD166	CD212	CD282	CCR9	Lymphotoxin b receptor	TWEAK

Wells A01 – A03 of the FACS^{TM} CAP plates were left empty to allow for the addition of unstained cells for instrument setup. Wells A04 – A08 were also left empty for the addition of unstained cells and single antibody to be used for post-acquisition compensation. Wells B01 – B05 contained isotype controls relevant to each of the classes of antibodies used throughout the plate. Wells B06 – H12 contained the three-colour antibody cocktails

2.2.2.   Additional reagents

Table 2.6 lists the additional reagents necessary for cell preparation, plate preparation and staining of the PBMCs.

Table 2.6: List of additional reagents

Reagent name	Catalogue number	Manufacturer
Phosphate buffered saline	BE17-517Q	Whitehead Scientific
Ficoll-paque	17-1440-03	GE Healthcare Life Sciences
Human FcR Blocking reagent	130-059-901	Miltenyi Biotec
Fetal bovine serum	de14-801fi	Whitehead Scientific
Staining buffer	PBS with 1% FBS	N/A
BD FACS^{TM} CAP lyoplates	N/A	Becton Dickinson
Anti-CD45 FITC	340664	Becton Dickinson
Anti-CD45 PE	555483	Becton Dickinson
Anti-CD45 APC	340943	Becton Dickinson
Anti-CD8 FITC	555634	Becton Dickinson
Anti-CD4 PE	555347	Becton Dickinson
Anti-CD3 Alexa 647	557706	Becton Dickinson
BD calibrate 3 beads	340486	Becton Dickinson
BD APC calibrate beads	340487	Becton Dickinson
16% Paraformaldehyde	19943 LT	Thermo scientific
Tuberculin PPD	2390	Staten Serum Institute
DMSO	D8418	Sigma Aldrich
Trypan Blue	93595	Sigma Aldrich
AIM media	12055091	Life Technologies
Greiner 50mL centrifuge tube	PGRE227261	Lasec
Pasteur pipette 3mL	PLPS135138	Lasec
Cryovials, 2mL	PAXGCRYO-2ML-RB-C	Lasec
P1000 sterile pipette tips	FA111-NXL-1000	WhiteSci
P200 sterile pipette tips	FA108-200	WhiteSci
Disposable 25mL pipette	PLPS162510	Lasec
Disposable 10mL pipette	PGRE607160	Lasec
0,6mL conical tubes	PAXGMCT-060-C	Lasec

2.3. Laboratory Methods

2.3.1. Sample preparation and cell staining

Peripheral blood mononuclear cells (PBMC's) were isolated from the anti-coagulated blood, stained with fluorescent labelled antibodies and data acquired by the FACSCalibur[TM].

2.3.1.1. Sample collection

Each blood draw consisted of approximately 54mL of blood collected into sodium heparin tubes. The tubes were inverted 10 times after venepuncture to ensure the anticoagulant was mixed sufficiently with the blood and preventing the formation of fibrin clots.

2.3.1.2. PBMC preparation

Whole blood was diluted 1:1 with 1 x phosphate buffered saline (PBS) and the diluted blood was layered onto Ficoll in a 50mL disposable tube. The 50mL tubes were centrifuged at 400 relative centrifugal forces (RCF) for 25 minutes with the brake and acceleration on the minimum setting. After centrifugation the PBMC's were easily visualised due to the density gradient layers. Red cells sediment at the bottom of the tube, followed by the layer of Ficoll, a thin band of PBMC's and finally the bulk is made up of plasma and PBS. The PBMC's were easily obtained by discarding the plasma/PBS layer until approximately 1 cm above the PBMC layer. Using a Pasteur pipette, the PBMC's were removed, along with a small amount of Ficoll, and placed in a clean 50mL tube. This was done for each tube and the cells were collected in one 50mL tube. The tube was filled up to 50mL with clean PBS and centrifuged for 10 minutes at 400 RCF (wash step 1). After the wash step, the supernatant was discarded and the pellet re-suspended in 5mL dH_2O to lyse the remaining red blood cells. After swirling the tube 10 times, PBS was added to a total volume of 20mL and the tube was centrifuged at 400 RCF for 10 minutes (wash step 2). The supernatant was discarded and the pellet was re-suspended in 5mL PBS

using a Gilson P1000 pipette. Counting of the PBMC's was performed by mixing 10µL of the cell suspension with 40µL of Trypan Blue and placing 10µL of the solution into a haemocytometer. The cell count was performed and reported as total cell count using the following calculation:

(Cell count X dilution factor in Trypan Blue X 10000) (Volume of cells)
= (Cell count X 50000) (5)

The remainder of the cell suspension was brought to a volume of 15mL by adding PBS and centrifuging at 400 RCF for 10 minutes. The cells were diluted to a concentration of 1 X 10^7 cells/mL in staining buffer composed of 1% fetal bovine serum (FBS) in phosphate buffered saline (PBS). FcR blocking reagent was added at a concentration of 20µL per 1 X 10^7 cells/mL and incubated for 10 minutes, followed by a wash step with PBS. After centrifugation at 400RCF for 8 minutes, the supernatant was discarded and the cells were diluted to a concentration of 1 X 10^6 cells/mL. A minimum of 20mL of cell suspension was needed which in some cases required a cell concentration of less than 1 X 10^6 cells/mL however this was not a frequent occurrence. The cell suspension was passed through a 70µm cell strainer to remove clumps of cells and debris and prevent blockages of the instrument during acquisition.

2.3.1.3. Cryopreservation of PBMCs

Five healthy participants were recruited to compare the effects of cryopreservation on the expression of surface markers on PBMC's. Upon receiving the blood, PBMC isolation was performed as outlined previously. Once the protocol reached the second wash step, the samples were split in half. One half of the cells continued with the protocol as described above (section 2.3.1.2) while the other half was cryopreserved, to be processed at a later stage. Cryopreservation occurred as follows:

The cryomedia of choice was 10% DMSO in 90% FBS and this was achieved by mixing 0.4mL of DMSO in 3.6mL of heat inactivated FBS. The cryomedia was placed on ice until the cells were ready for cryopreservation. After the second wash step of

the cells with PBS, 4mL of cryomedia was added to the cells drop by drop, while mixing, until the entire volume was added. A cell count was performed to determine the number of cells that would be cryopreserved. This was done by using 10μL of cells from the cryomedia suspension and mixing it with 40μL of Trypan blue stain. Counting commenced as detailed previously and the calculation was performed as follows:

(Cell count X dilution factor in Trypan Blue X 10000) (Volume of cells)

= (Cell count X 50000) (4)

The samples were split into two cryovials with 2mL's each and placed into a Nalgene Mr Frosty® to be step-frozen overnight at -80°C. The next morning, the cells were placed into liquid nitrogen to be stored until use. After 4-8 weeks, the cells were thawed quickly in pre-warmed media, washed and the protocol continued as for fresh cells after the second wash with PBS.

2.3.1.4. PPD stimulation of PBMCs

Samples collected from five actively infected TB patients were used to determine if there was a change in expression of surface markers of purified protein derivative (PPD) stimulated PBMC's. Upon receiving the blood, PBMC isolation was performed as outlined previously. Once the protocol reached the second wash step, the samples were split in half. The protocol as described above (section 2.3.1.2) was continued with one half of the cells while the other half was re-suspended in AIM media at a concentration of 1×10^6 cells/mL. The stimulant of choice was PPD at a concentration of 10μg/mL. The cells were incubated overnight at 37°C, 5% CO_2. The following morning the cells were washed twice in PBS and the protocol was continued as described above.

2.3.1.5. Staining of cells in FACS^TM CAP plates

Duplicate lyoplates were prepared for each sample in case of instrument or operator error. A volume of 100μL was added to each well of each lyoplate resulting in a concentration of 1×10^5 cells in each well. Wells A04 – A08 were stained as follows to allow for single stains to be used for post-acquisition compensation (Figure 2.1):

- A04 – Unstained cells
- A05 – CD45 FITC
- A06 – CD45 PE
- A07 – CD45 APC
- A08 - CD4 PE, CD8 FITC, CD3 Alexa Fluor 647

Wells A01 – A03 were left as unlabelled cells and were used to verify the setup of the instrument detectors between the tube and plate acquisition mode. Wells A09 – A12 were left unlabelled as additional setup wells in case of an instrument problem or error (Figure 2.1). At the same time, a tube with 1mL of PBMC's was prepared to be used to set up the instrument prior to loading the plates. The cells were mixed using a multichannel pipette to ensure even and adequate mixing of the cells with the labelled antibodies. The plates were incubated for 30 minutes in the dark. A wash step was performed by adding 100uL of PBS to each well and 1 mL PBS to the tube containing unlabelled cells and centrifuged at 1200rpm for 8 minutes. The supernatant was discarded and the wash step was repeated with 200μL of PBS per well and 1mL per tube. After the second wash, 200μL of a 2% paraformaldehyde solution was added to each well and 1 mL to the tube and the plates and tube were stored in the dark overnight.

	01	02	03	04	05	06	07	08	09	10	11	12
A	Unstained Setup well	Unstained Setup well	Unstained Setup well	Unstained Comp well	CD45 FITC Comp	CD45 PE Comp	CD45 APC Comp	CD4PE, CD8FITC, CD3 Alexa Fluor647				
B												
C		**Lyophilized antibodies B01 to H12**										
D												
E		**B01 – B05 Isotype controls**										
F												
G		**B06 – H12 Three-colour cocktail**										
H												

Figure 2.1: FACS^TM CAP lyoplate layout

Wells A01 – A03 contained unlabelled cells for instrument setup. A04 – A08 were used for single stains for post-acquisition compensation. Wells B01-H12 contained a three-antibody cocktail re-suspended in freshly isolated PBMCs.

2.4. Instrument

A FACSCalibur^TM with the addition of the High Throughput System (HTS) capable of handling 96 well plates was used for sample acquisition. The FACSCalibur^TM is a four colour flow cytometer fitted with an air-cooled argon laser which reads at 488nm and a red diode laser that reads at 633 nm. It also contains the appropriate filters for detection of the fluorochromes FITC, Alexa Fluor 488, PE, APC and Alexa Fluor 647. The software that was used for setup and acquisition of the samples in tube mode was CellQuest™ Pro while the software used for the HTS loader was BD™ Plate Manager software.   The instrument and HTS loader were manufactured and maintained by Becton Dickinson.

2.5. Acquisition of lyoplates on FACSCalibur^{TM}

After an overnight incubation the plates were acquired on the FACSCalibur^{TM} as mentioned above (section 2.4). All samples were acquired uncompensated as post-acquisition compensation was performed using third party software. Prior to the plate being loaded, a tube with unlabelled cells was loaded and acquired and used to set the voltages on each detector. The forward scatter (FSC) and side scatter (SSC) voltages were set such that the lymphocyte population was placed towards the junction of the X and Y axis (Figure. 2.2) to allow space for the granulocyte and monocyte population to fall within the plot. The voltage for each fluorescent detector was adjusted to ensure that the histogram plot fell within the first log decade while still allowing visualisation of the incline of the histogram plot. Once satisfactory settings were obtained, the settings were saved to each patient's folder and identical settings were used for each of the duplicate plates. Each well was mixed prior to acquisition and a volume of 100µL was acquired at a rate of 2µL per second. Acquisition was complete once 30 000 events had been acquired or once the acquired volume was depleted.

Correct operation of the instrument was ensured by using FACS Calibrite beads before each run and maintenance was performed on a regular basis.
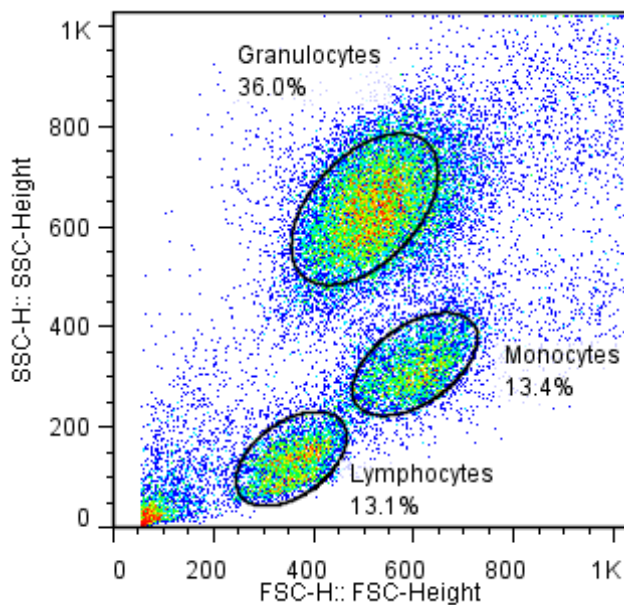
Figure 2.2: Demonstration of FSC and SSC settings during instrument setup.
The instrument settings are set to ensure clear lymphocyte, monocyte and granulocyte populations are evident.

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2.6. Data analysis

The software used for analysis of this project is FlowJo version 7.6.5 (Tree Star Inc., Ashland, OR). As the plates were acquired uncompensated, it was necessary to perform post-acquisition compensation on each plate to be used for analysis. Each sample was prepared in duplicate plates, therefore it was required to first verify the quality of the acquired data for each plate and to make a decision on which of the two plates would be used for analysis. This decision was based on whether or not the required amount of 30 000 events was reached and if there was an instrument error or blockage that resulted in a shift of the population to a point on the graph where it could not be gated accurately (Figure 2.3). At times this required combining wells from plate 1 and plate 2 to have a full set of satisfactory data. Once the best plate was determined, compensation could be performed.
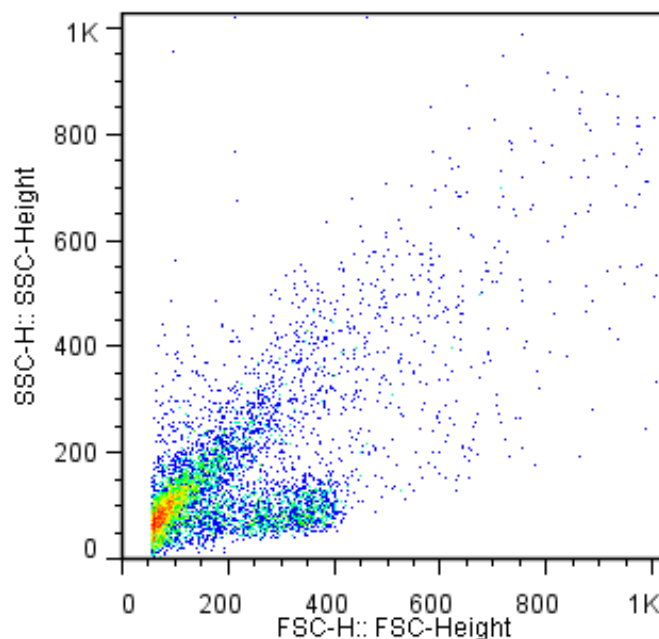
Figure 2.3: Graph demonstrating the effect of an injection probe blockage during acquisition.

There is no clear separation of cell populations with the majority of cells accumulating at the origin of the graph. The lymphocytes cannot to accurately gated therefore this well would be eliminated from analysis.

2.6.1. Post-acquisition compensation of flow cytometry data

A plot of FSC vs. SSC was used to visualise the total cell population and a gate was set around the lymphocyte population (Figure 2.4A). The gated cells were viewed using a plot of histogram vs. FL detector. The negative expression population was visualised (Figure 2.4B) and the positive signals in the FL1 (well A05), FL2 (well A06) and FL4 (well A07) detectors were demarcated (Figure 2.4C). The compensation matrix was created by assigning the negative (unstained) population from well 04 as a universal negative control and the positive population from each channel. The matrix was saved and assigned to the group of samples. Well A08 had a cocktail of three antibodies (CD4 PE, CD8 FITC and CD3 Alexa Fluor-647), one for each detector, and this well was used to verify the compensation was correct (Figure 2.4D).
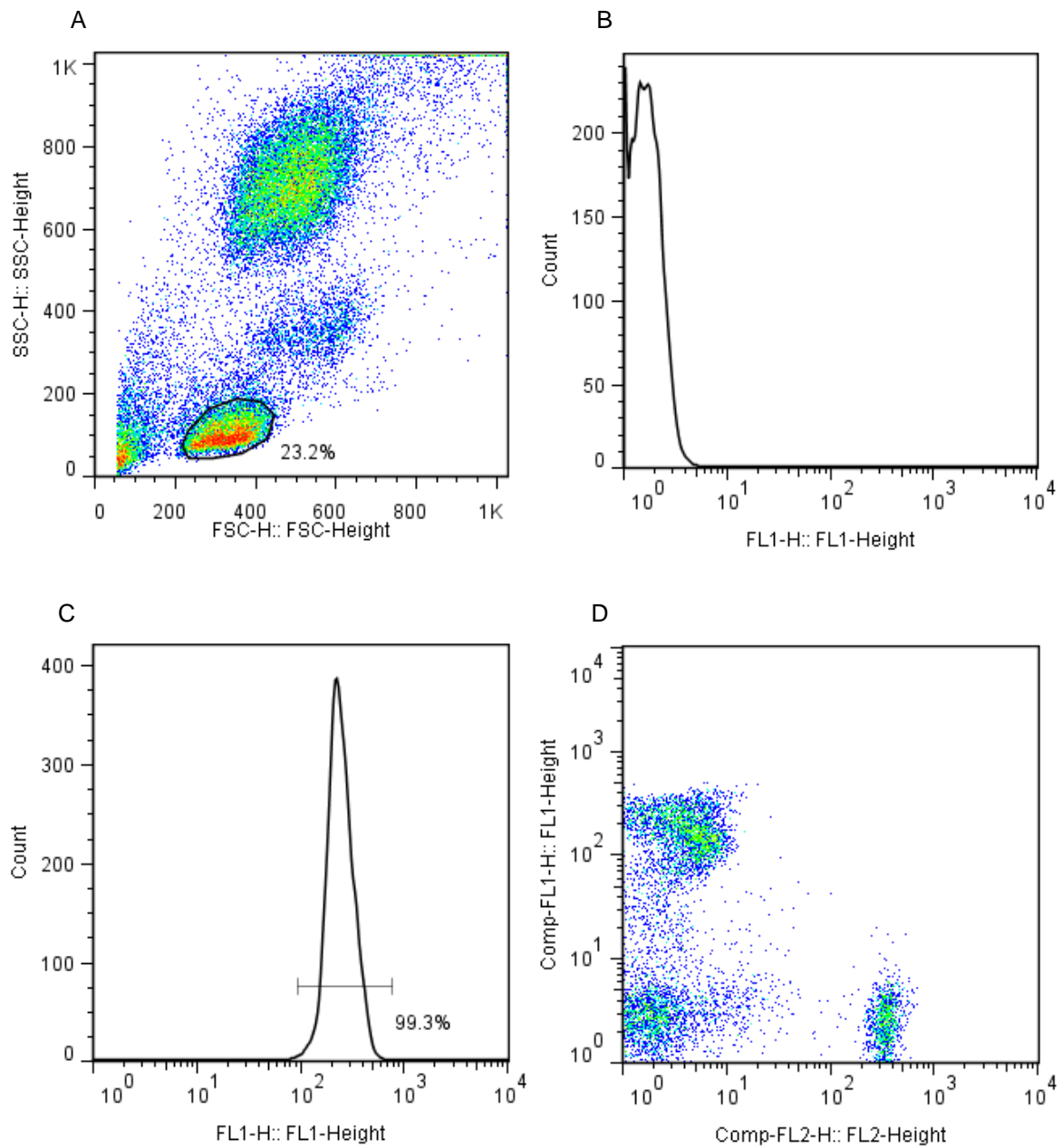
Figure 2.4: Demonstration of the steps involved in post-acquisition compensation.

Graph demonstrating the lymphocyte gate used for post-acquisition compensation (a). Histogram vs FL1 detector of the gated lymphocyte population demonstrating the negative (b) and positive (c) expression populations. Well A08 demonstrating correct post-acquisition compensation using the cocktail consisting of CD4 PE, CD8 FITC and CD3 Alexa Fluor-647 (d).

2.6.2.   Analysis of flow cytometry data

Isotype controls were included as negative controls to be compared against the sample wells to detect a positive signal. As there were two isotype controls for both FL1 and FL4 channels, it was necessary to determine the best control to use going forward. In most cases there was very little difference between them in which case the first control in each channel was used. Once the isotype control was chosen, the positive population gate was set so that when overlayed with the sample wells, anything below this threshold would be considered negative (Figure 2.5).
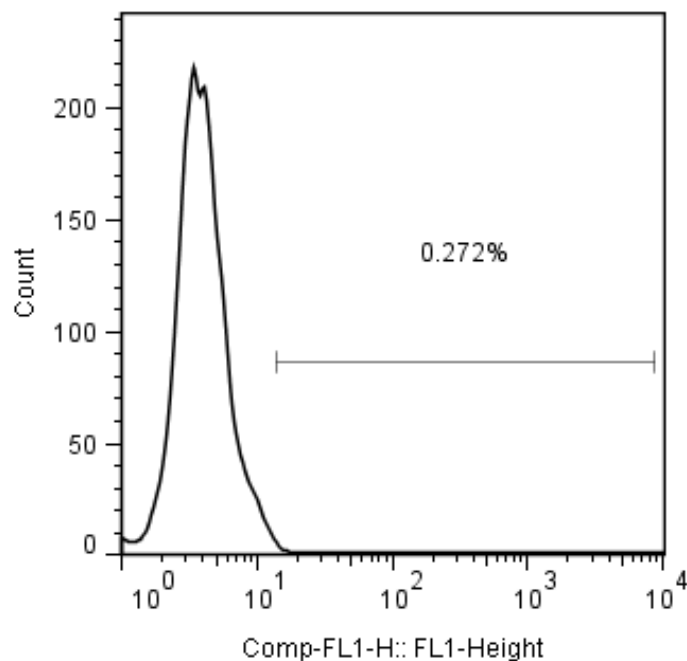
Figure 2.5: Graph demonstrating the positive gate set on the isotype control.

Any expression that falls within the demarcated range is considered as positive. This range was adjusted, based on the dot plot adjustments, if there was a shift in the expression and the demarcation between a positive and negative population was adjusted.

Once the gate was determined for all three channels, it was applied to all sample wells. In the layout editor each channel was assigned a page on which the isotype histogram vs FL detector was placed. This was done 79 times for each channel, once for each well. Starting with the first antibody cocktail well, B06, each sample was overlayed onto the isotype control which resulted in a representation of a positive expression population when compared with the negative expression population defined by the isotype control. The positive expression population was denoted by a percentage of the total events which was automatically saved in the statistics of the software (Figure 2.6).
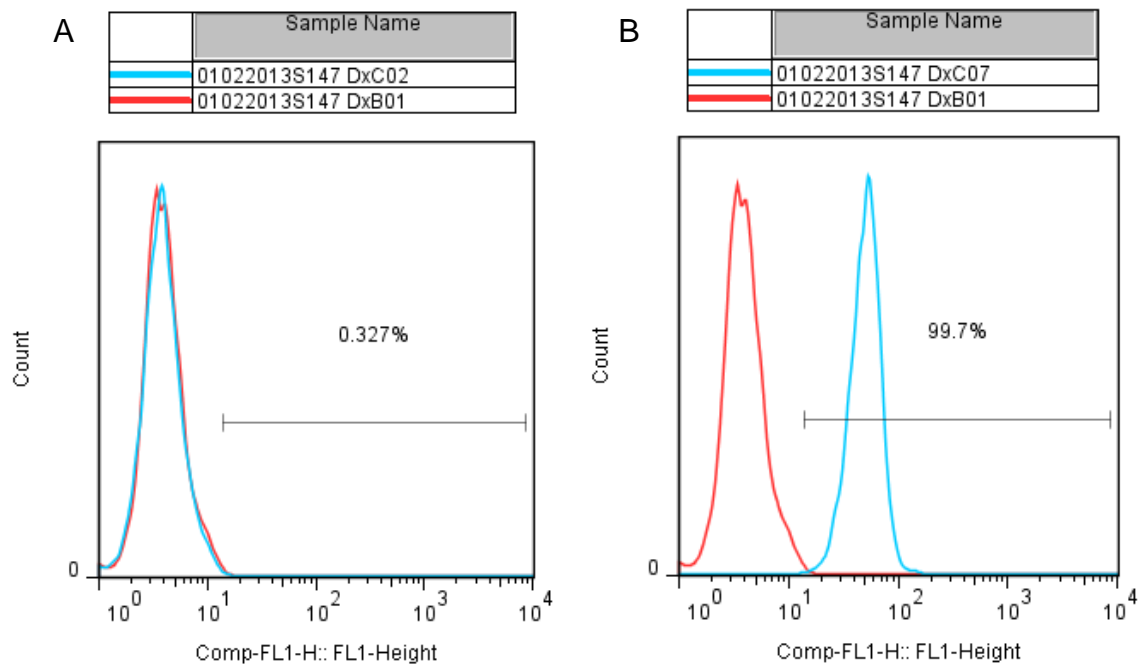
Figure 2.6: Demonstration of a sample well overlayed on the isotype control.

The isotype control (red) denotes a negative expression and is used to differentiate between a sample well (blue) with a negative (a) or a positive (b) expression.

As the demarcation between the positive and negative expression was uncertain in some of the samples, it was necessary to manually visualise the population and make a decision based on the dot plot. This was done by placing the channel of interest on the X-axis and on the Y-axis switching between FSC, SSC and the other two channels (FL1, FL2 or FL4) and placing quadrants to separate the populations. Once the correct percentage of positive expression was determined, the histogram was adjusted to accommodate the change and the statistics were updated. In some instances it was not clear what the decision should be in which case the marker was flagged and would be decided upon after further investigation at a later stage. Once this was completed for all wells in all channels, the statistics were exported to a Microsoft Excel spread sheet in preparation for statistical analysis.

2.7. Statistical analysis of flow cytometry data

Statistical analysis of the data included performing repeated measures ANOVA, paired t-tests and independent two-sample t-tests using R statistical software (Vienna, Austria).

2.7.1. Repeated measures ANOVA

This statistical analysis is used when there is a variable being measured two or more times in an experiment on the same experimental group. In the case of this study, the variable was each of the 252 markers, which were measured at three different time points in the same group of 33 people.

The repeated measures ANOVA is used to test whether there are any differences between the mean within a population.

2.7.2. Paired t-test

A paired t-test was used to determine if there was a significant difference between the values of two means of a related sample under the same conditions. In this case it was used to determine if there was a significant difference between time points e.g.

- Baseline (Dx) vs Week 4 (W4)
- Baseline (Dx) vs Week 24 (W24)
- Week 4 (W4) vs Week 24 (W24)

The null hypothesis is: $\mu_1 = \mu_2$ where the means are equal from any paired group. The paired t test is a more powerful test than the unpaired t test but only when there are matched samples.

2.7.3. Independent two-sample t-test

The three groups of samples in this study, TB patients, healthy community controls and other lung disease (OLD) controls, are not of equal size, which allows us to use the independent two-sample t-test, also known as the unequal variance t-test. In this case the Welch Approximate Degrees of Freedom (APDF) test was used with the hypothesis that there are equal means from two independent groups (55).   This test will allow us to detect if the means between any two groups are different e.g.

- TB patients at baseline vs healthy community controls
- TB patients at baseline vs other lung disease controls
- Healthy community controls vs other lung disease controls

2.7.4. Bonferroni correction

Due to the fact that the probability of finding at least one significant result by chance increases with the number of markers that are tested, it was necessary to apply the Bonferroni correction to the statistical analysis. This is a very stringent and conservative form of statistical analysis as it took 252 markers into consideration. Thus the Bonferroni correction was applied to limit the chances of obtaining false positive results when using a single set of data (56). Using this Bonferroni correction, if we let the test $\alpha = 0.05$, then we should only consider the markers with p-value $\leq \frac{0.05}{252} \approx 0.0002$.

2.7.5. Ingenuity pathway analysis (IPA) and QIucore heat maps

Ingenuity pathway analysis (IPA®,Qiagen Redwood City, www.qiagen.com/ingenuity) was performed on a selection of markers with the goal of identifying all potential biological pathways that changed during treatment as the relatively small study size of 33 patients and the strict Bonferroni correction may lead to false negative results. Surface markers that had a p-value <0.01 before the Bonferroni correction was applied were considered for IPA. Due to the uncertainty of demarcation between the negative and positive populations, markers that were flagged were excluded from IPA and heat map generation. The accession number for each marker was obtained from the UniProt (http://www.uniprot.org/) website (Table 3.7). Data input into the IPA software included a parameter for up- or down-regulation between time points and the resulting pathways were organised according to the number of molecules (surface markers) involved in the pathway as well as their p-values.

Based on the results from the repeated measures ANOVA, paired t-test and independent t-test, the markers were categorised and analysed as follows (Table 3.13):

- Repeated measures ANOVA (overall treatment response) - 17 markers
- Paired t-test TB diagnosis vs week 24 – 18 markers
- Paired t-test TB diagnosis vs week 4 – Five markers
- Paired t-test Week 4 vs week 24 – Eight markers

- Independent t-test TB diagnosis vs healthy community controls – 14 markers
- Independent t-test TB diagnosis vs OLD – 12 markers

Markers that were excluded from IPA analysis included flagged wells and in the case of duplicate wells, only one of the duplicates was included. In the case of CD45RB, CD45RO and CD15, an accession number for humans could not be sourced through the UniProt database thus were excluded. HLA included a cocktail of HLA-A, HLA-B and HLA-C which prevented the use of a single accession number. Since it could not be determined from this study which HLA was influencing the overall expression, it was decided to exclude it. Similarly MIC A/B was a combination of MIC A and MIC B and was excluded on the same rationale as HLA.

Heat maps were generated using the Qlucore Omics Explorer software (Lund, Sweden) on selected markers identified when comparing the change in expression between TB diagnosis and week 24 using repeated measures ANOVA analysis (Table 3.8). As for the IPA analysis, this was a proof-of-concept approach and we decided not to limit the analysis to the few markers that remained significantly different after the correction. All heat maps were obtained in an unbiased manner and were generated to create the following comparisons:

- TB patients at diagnosis, week 4 and week 24 (Figure 3.8) – 18 markers were used and showed a significant difference, especially between diagnosis and week 24. It appears that changes in the expression levels started changing from as early as week 4 however these changes were not statistically significant once the Bonferroni correction was applied.
- TB patients at diagnosis, week 24 and healthy community controls (Figure 3.11).
- TB patients at diagnosis, OLD controls and healthy community controls (Figure 3.12).

2.7.6. Association between expression of markers at diagnosis and study outcomes

Statistics were performed to determine the association between the expression of markers at diagnosis of disease and the following variables:

- Treatment outcome (definite cure, probable cure and not cured): Only comparisons between definite cure and not cured were performed as the clinical relevance of identifying a probably cure group are not yet clearly understood.
- Time to positivity (TTP): A simple correlation between marker expression at baseline and TTP was performed.
- Week 8 culture result: A comparison between the surface marker expression and the week 8 culture result was performed.
- Qualitative scan outcome: An association between the marker expression at diagnosis and the PET/CT outcome at EOT (Resolved, mixed and improved) was performed. A mixed response indicates that at least some lesions enlarge or show more intense uptake at EOT compared to diagnosis or that at least one new lesion has developed. An improved response indicates that all lesions with abnormal uptake have improved from diagnosis but that at least one lesion has abnormal isotope uptake. Resolved indicates that no lesions remain with increased uptake.
- New clinical groups by combination of the qualitative and quantitative scan outcomes: New outcome groups were formed by combining the original qualitative and quantitative groups to classify patients in clinical severity groups (table 2.7). Quantitative groups are indicated by the percentage of lung volume abnormal at EOT (Week 24), on both PET and CT. 0-1% = 1 (Best scan outcome); 1-2% = 2; 2-4% = 3; 4-6% = 4; >6% = 5 (worst outcome).

Table 2.7 Table of the new scan outcome groups

New group	Original group (Quantified scale 1-5)
Good	Resolved
	Improved with a quantitative score of 1
Moderate	Improved with a quantitative score of 2
	Mixed with a quantitative score of 1
Poor	Improved with a quantitative score of 3 or more
	Mixed with a quantitative score of 2 or more

Chapter 3: Results

3.1. Objective 1 - Optimisation of FACS^{TM} CAP procedures.

3.1.1. Objective 1.1: Assess the effect of cryopreservation on FACS^{TM} CAP performance.

Five healthy volunteers were recruited to determine the effect of cryopreservation on the expression of surface markers on PBMC's. The statistical analysis was performed according to the following criteria:

- Only surface markers that showed a difference in expression of 5% or more were chosen.
- Only surface markers that showed a difference in four or more patients were considered.
- Markers that were flagged, due to indeterminate analysis, were excluded from statistical analysis as well as the markers that exhibited a decrease in some patients and an increase in others.

Results of the comparison of surface markers between fresh and cryopreserved samples showed only five markers (Table 3.1) with a notable difference. Four markers (CD41a, CCR6, CD9 and CD62L) showed a down-regulation between fresh and frozen and one marker (CD7) showed an up-regulation. The outcome of this experiment showed that besides the five markers listed above; there was no notable difference when comparing the expression of surface markers between fresh and cryopreserved PBMCs. It was decided early in the project to continue with freshly isolated PBMCs due to insufficient cell concentrations found in the cryopreserved samples.

Table 3.1: Surface markers with up- or down-regulation when comparing fresh and cryopreserved PBMCs

Marker	Patient HC01		Patient HC02		Patient HC04		Patient HC05		Patient HC06		P-value
	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen	
CD41a	11.9	3.75	15.6	4.19	21.3	5.81	27.7	9.4	8.91	7.44	0.0202
CCR6	18.3	10.4	13.6	6.02	23.2	13.1	13	3.49	26.2	24.2	0.0067
CD9	27.3	19.4	33.1	23.6	34.7	27.6	37.3	19	18.4	20.7	0.069
CD62L	53.9	37.5	61.9	46.8	78.4	47.3	33.3	14	17.9	13.2	0.015
CD7	70.7	76.9	76.1	82.3	50.9	72.8	72.4	80.6	55.8	68.6	0.0203

3.1.2. Objective 1.2: Assess the effect of PBMC culture in the presence of *Mtb* antigen stimulation on marker expression in comparison to unstimulated PBMCs.

Five TB patients were recruited to determine the change in surface marker expression levels after stimulation of PBMC's with PPD. The statistical analysis was performed as outlined for the comparison of fresh and cryopreserved cells above (section 3.1.1).

The expression of surface markers was highly variable between patients. Markers such as CD41a, CD45Ra and CD61 were down-regulated when comparing stimulated with unstimulated PBMC's and expression for markers such as CD4v4, CD49a and CD62L were up-regulated in stimulated compared with unstimulated PBMC's (Table 3.2).

Table 3.2: Surface markers with up- or down-regulation when comparing unstimulated and PPD stimulated cells.

Marker	Patient S191		Patient S192		Patient S193		Patient S201		Patient S203		P-value
	Stim	Unstim	Stim	Unstim	Stim	Unstim	Stim	Unstim	Stim	Unstim	
CD41a	5.04	30.9	11.6	37.1	2.09	15.3	7.64	24.3	12.9	12.1	0.0302
CD45RA	64.9	74.9	58.2	57	0.71	57.8	34.3	39.8	72.9	87.9	0.1689
CD61	7.14	14.5	8.34	29.8	3.52	13	2.47	12.8	12.1	11.5	0.0532
CD4 v4	37	28.5	44.2	25.3	42.5	37.1	62.5	55.7	9.82	7.37	0.04
CD49a	32.1	16.1	3.95	2.49	9.58	1.9	15.7	5.13	9.38	4.34	0.03
CD62L	59.2	37.6	60.3	39	36	31.4	77.4	60.5	24.9	19.5	0.0205

It was noted that there was no significant change in the expression of activation markers when comparing unstimulated and PPD stimulated PBMC's (Table 3.3). The gating strategy for the analysis of these samples included the entire population of PBMCs where the PPD responsive T cells represent a small percentage. As only six markers showed a consistent change in expression between unstimulated and stimulated samples, it was decided to continue with the project using unstimulated PBMCs.

Table 3.3: List of common activation markers displaying no significant changes between PPD stimulated and unstimulated PBMCs

Marker	Patient S191		Patient S192		Patient S193		Patient S201		Patient S203		P-value
	Stim.	Unstim.	Stim.	Unstim.	Stim.	Unstim.	Stim.	Unstim.	Stim.	Unstim.	
HLA-DR	48.5	35.5	24.9	38.3	50.3	43.1	21.2	18.3	93.3	80.6	0.4076
CD69	0.25	2.01	7.91	9.97	2.35	0.84	1.42	0.89	1.01	21	0.334
CD25	1.14	1.51	2.61	9.26	0.34	1.03	1.91	7.59	1.96	1.86	0.1398
CD71	1.77	3.79	4.57	5.68	2.75	1.8	2.43	3.36	7.39	11.1	0.147
CD62L	37.6	59.2	39	60.3	31.4	36	60.5	77.4	19.5	24.9	0.0205

3.2. Objective 2: To assess the differential PBMC surface marker expression by FACS^{TM} CAP in TB patients during treatment.

All patients recruited into the study were analysed however, only 33 patients, for which there were three time points, were considered for the final statistical analysis. Nine other lung disease (OLD) and 11 healthy community controls were also included in the statistical analysis. The initial study design included an algorithm that could be used for automated analysis however due to the high variability of the data it would not have been accurate enough to use. Therefore all patient data was analysed manually using the FlowJo software by gating on the lymphocyte population. The isotype controls provided a cut-off between a positive and negative expression result although adjustments were made if necessary by relying on the profile of the expression in a dot plot using parameters two by two (FSC, SSC, FL1, FL2, FL4). In a case where the expression of a marker was unclear, the well was flagged and left to be decided after all patients were analysed and comparisons could be made. Wells that were still unclear were represented as flagged in the results tables and not included in further analysis.

3.2.1. Significant markers identified for overall treatment response (between diagnosis, week 4 and week 24).

Repeated measures ANOVA was performed on 252 markers and resulted in five markers with expression levels that were statistically significant between the time points (< 0.0002 after the Bonferroni correction was applied). The markers were CD120b, CD126, CD62L, CD48 and CD29. The expression levels of CD120b, CD126 and CD62L were significantly decreased during the course of treatment while CD48 and CD29 were both increased between diagnosis and end of treatment.

Of the 252 markers that were used, 29 had a p-value of <0.01 after the Bonferroni correction was applied (Table 3.4). A p-value of 0.01 was a random choice and allowed the investigation of a large number of markers with significant change or trends. Of the 29 markers, 24 were not significantly different; however, due to the small sample number and the application of the stringent Bonferroni correction,

these markers may have been falsely negative and may still be of biological significance.   A total of 11 markers were identified as flagged and while they were included in the initial results, they were excluded from further analysis due to their ability to erroneously skew results.   Kernel density graphs for selected markers demonstrate the distribution of expression when comparing the expression levels of surface markers between TB diagnosis and at the end of treatment.

Table 3.4: Selected markers from the repeated measures ANOVA analysis (p-values from repeated measures ANOVA test <0.01 before the Bonferroni correction).

Marker	Means					P-value	P-value*252	Flagged
	T0 (N=33)	W4 (N=33)	W24 (N=33)	Healthy (N=11)	Other (N=9)			
CD50	97.72	98.36	98.92	99.35	99.1	0.0005	0.1270	NO
CD48	96.88	97.6	98.75	99.37	98.18	0.0001	0.0298	NO
CD99	75.84	82.68	88.05	91.2	70.1	0.0011	0.2674	YES
CD58	61.63	68.23	70.5	75.51	56.14	0.0035	0.8865	NO
CD91	2.8	3.54	4.69	3.66	2.16	0.0077	1.9513	NO
CD4 v4	31.44	27.9	23.59	26.09	32.36	0.0055	1.3770	NO
CD244	35.27	38.75	49.06	48.62	28.16	0.0011	0.2790	YES
CD146	2.9	3.24	2.12	1.64	1.95	0.0051	1.2768	NO
CD120b	14.96	8.91	5.39	14.96	6.21	0.0000	0.0035	NO
NKG2D	45.4	50.89	52.99	51.26	39.01	0.0048	1.1999	YES
CD53	98.03	98.39	98.97	98.94	98.74	0.0018	0.4642	NO
CD45RB	97.34	98.05	98.62	99.05	98.53	0.0021	0.5251	NO
CD126	80.58	79.7	63.26	50.15	67.89	0.0000	0.0000	NO
F11 R	97.19	95.98	95.29	95.89	94.28	0.0017	0.4280	NO
CD210	22.48	28.88	40.69	53.38	24.45	0.0089	2.2381	YES
CD119	13.83	11.81	26.28	21.61	10.15	0.0005	0.1370	YES
CD29	89.88	91.79	95.25	95.96	94.09	0.0008	0.2054	NO
CD29	91.85	92.03	95.04	94.99	93.52	0.0001	0.0166	NO
CD29	91.09	92.63	95.26	96.24	95.28	0.0025	0.6389	NO
CD44	98.42	99.09	99.16	99.35	98.89	0.0083	2.0945	NO
CD11a	97.54	98.58	99	99.55	98.13	0.0006	0.1523	NO
CD212	6.86	4.23	18.82	29.01	17.98	0.0003	0.0677	YES
CD55	67.28	56.67	47.3	46.69	82.24	0.0004	0.0987	YES
CD4	35.56	30.85	27.05	30.71	37.7	0.0040	0.9985	NO
CD181	19.59	16.48	25.19	25.11	11.31	0.0005	0.1164	YES
CD81	98.18	98.3	99.16	99.3	99.38	0.0012	0.3013	NO
CD4	35	30.89	27.38	28.63	37.37	0.0074	1.8610	NO
CD62L	45.82	35.39	25.37	30.6	42.66	0.0000	0.0035	NO
CCR7	31.24	24.03	21.98	20.62	37.96	0.0053	1.3465	NO

A. CD126: TB (Dx), W4 and W24

B. CD120b: TB (Dx), W4 and W24

C. CD62L: TB (Dx), W4 and W24

D. CD48: TB (Dx), W4 and W24

Time

1:TB(T0)   2:TB(W4)   3:TB(W24)
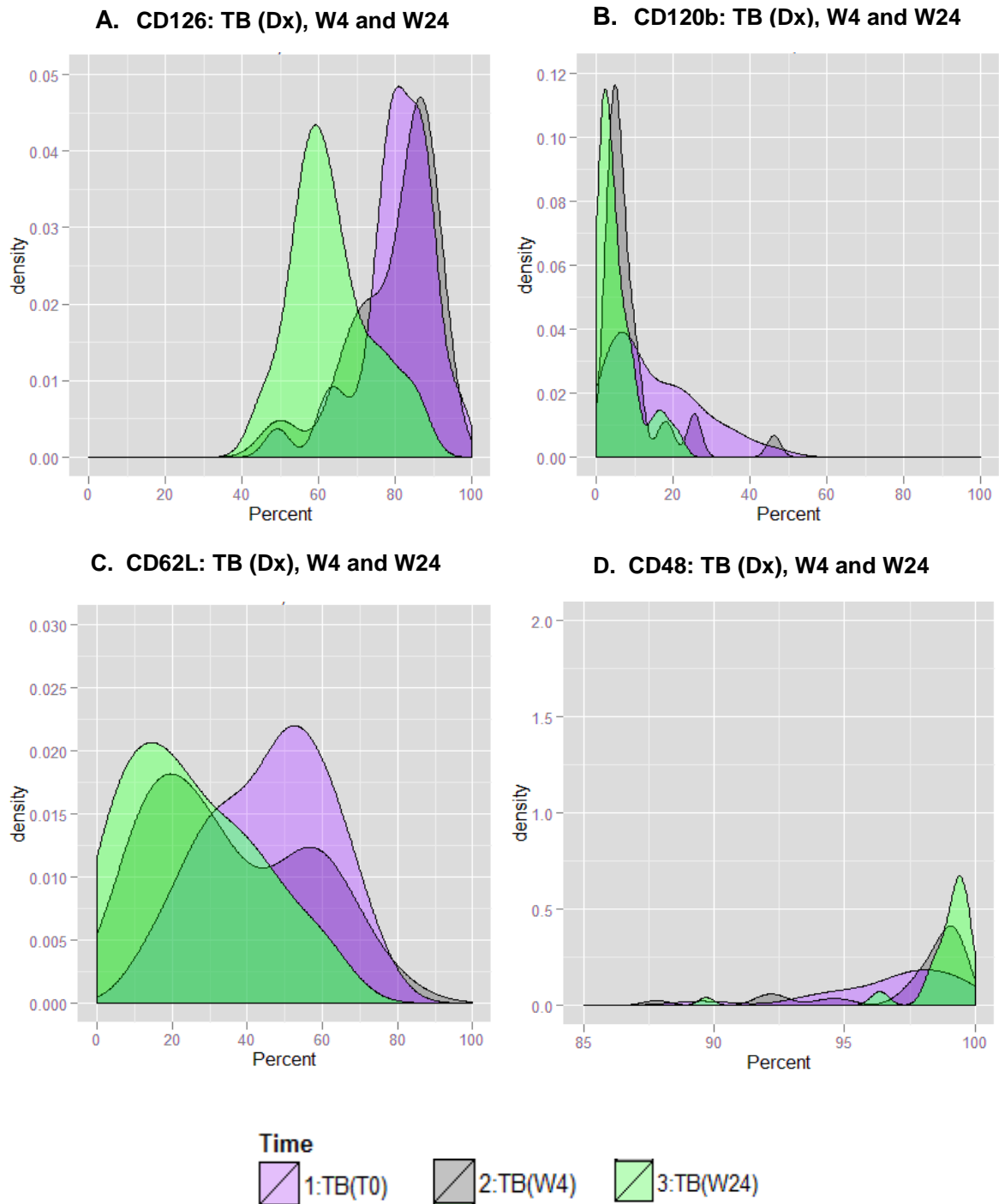
Figure 3.1: Kernel density graphs comparing the distribution of expression of markers between TB diagnosis, week 4 and week 24.

Distribution of expression of CD126 (a), CD120b (b), CD62L (c) and CD48 (d). PBMCs were isolated, stained and acquired on a flow cytometer to evaluate the expression of markers on the cells surface. The expression is denoted as a percentage of the total cells expressing the marker. The comparison was made between the expression levels at TB diagnosis, week 4 and week 24. A change in expression was considered significant if the p-value <0.0002 after the application of the Bonferroni correction.

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A. CD29: TB (Dx), W4 and W24

B. CD4: TB (Dx), W4 and W24

C. CD11a: TB (Dx), W4 and W24

D. F11 R: TB (Dx), W4 and W24

Time
1:TB(T0)   2:TB(W4)   3:TB(W24)
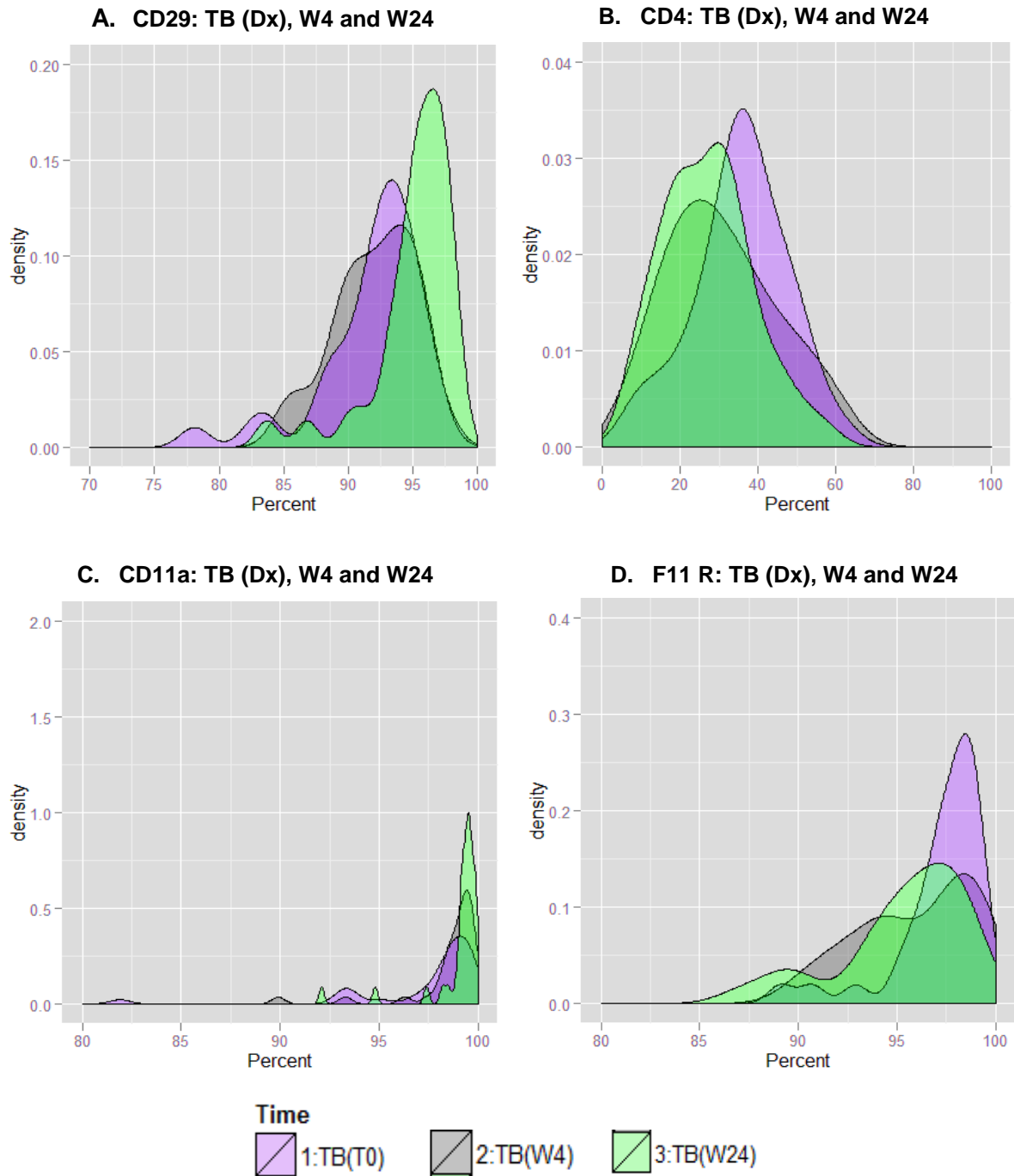
Figure 3.2: Kernel density graphs comparing the distribution of expression of markers between TB diagnosis, week 4 and week 24.

Distribution of expression of CD29 (a), CD4 (b), CD11a (c) and F11 R (d). PBMCs were isolated, stained and acquired on a flow cytometer to evaluate the expression of markers on the cells surface. The expression is denoted as a percentage of the total cells expressing the marker. The comparison was made between the expression levels at TB diagnosis, week 4 and week 24. A change in expression was considered significant if the p-value <0.0002 after the application of the Bonferroni correction.

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A. CCR7: TB (Dx), W4 and W24

B. CD58: TB (Dx), W4 and W24

Time

1:TB(T0)   2:TB(W4)   3:TB(W24)
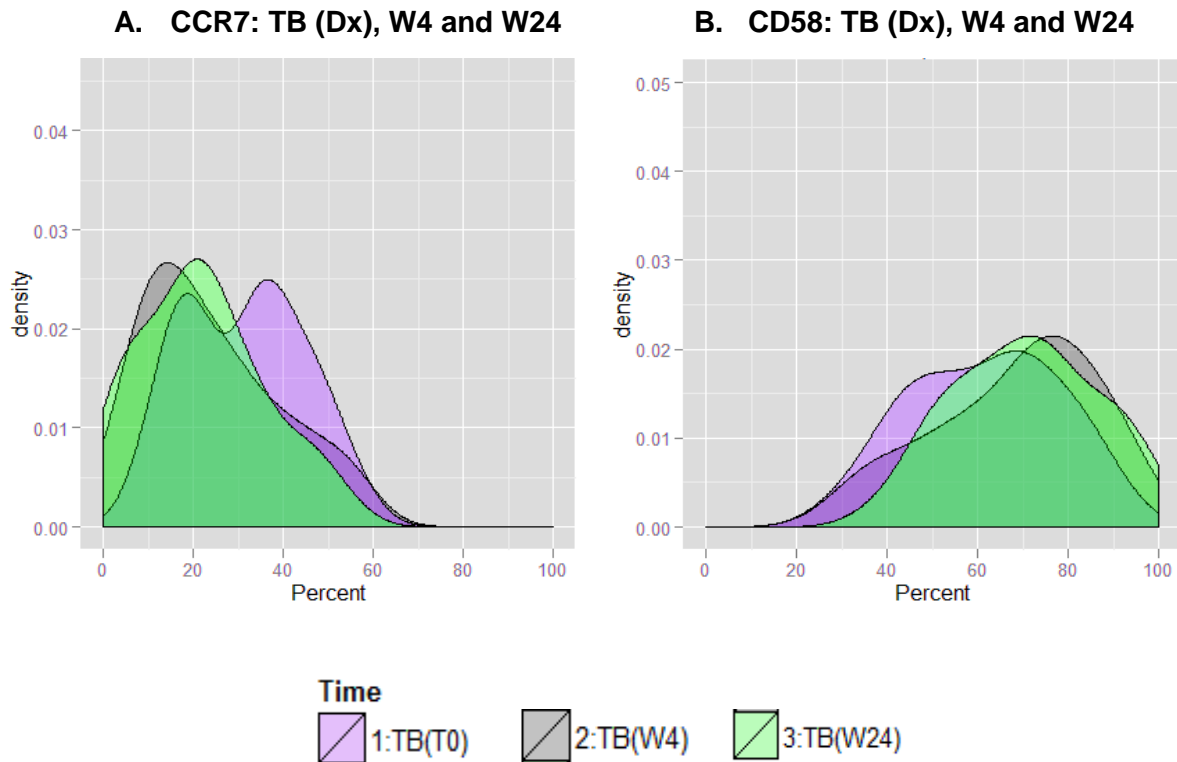
Figure 3.3: Kernel density graphs comparing the distribution of expression of markers between TB diagnosis, week 4 and week 24.

Distribution of expression of CCR7 (a) and CD58 (b). PBMCs were isolated, stained and acquired on a flow cytometer to evaluate the expression of markers on the cells surface. The expression is denoted as a percentage of the total cells expressing the marker. The comparison was made between the expression levels at TB diagnosis, week 4 and week 24. A change in expression was considered significant if the p-value <0.0002 after the application of the Bonferroni correction.

Coincidentally anti-CD4 and anti-CD126 were in the same well which allowed us to investigate the co-expression of CD4 and CD126 on cells at all three time points. CD126 was expressed on both CD4$^+$ and CD4$^-$ cells however, the significant down-regulation of CD126 occurred primarily on the CD4$^-$ population (p-value of 0.0001 before the Bonferroni correction).

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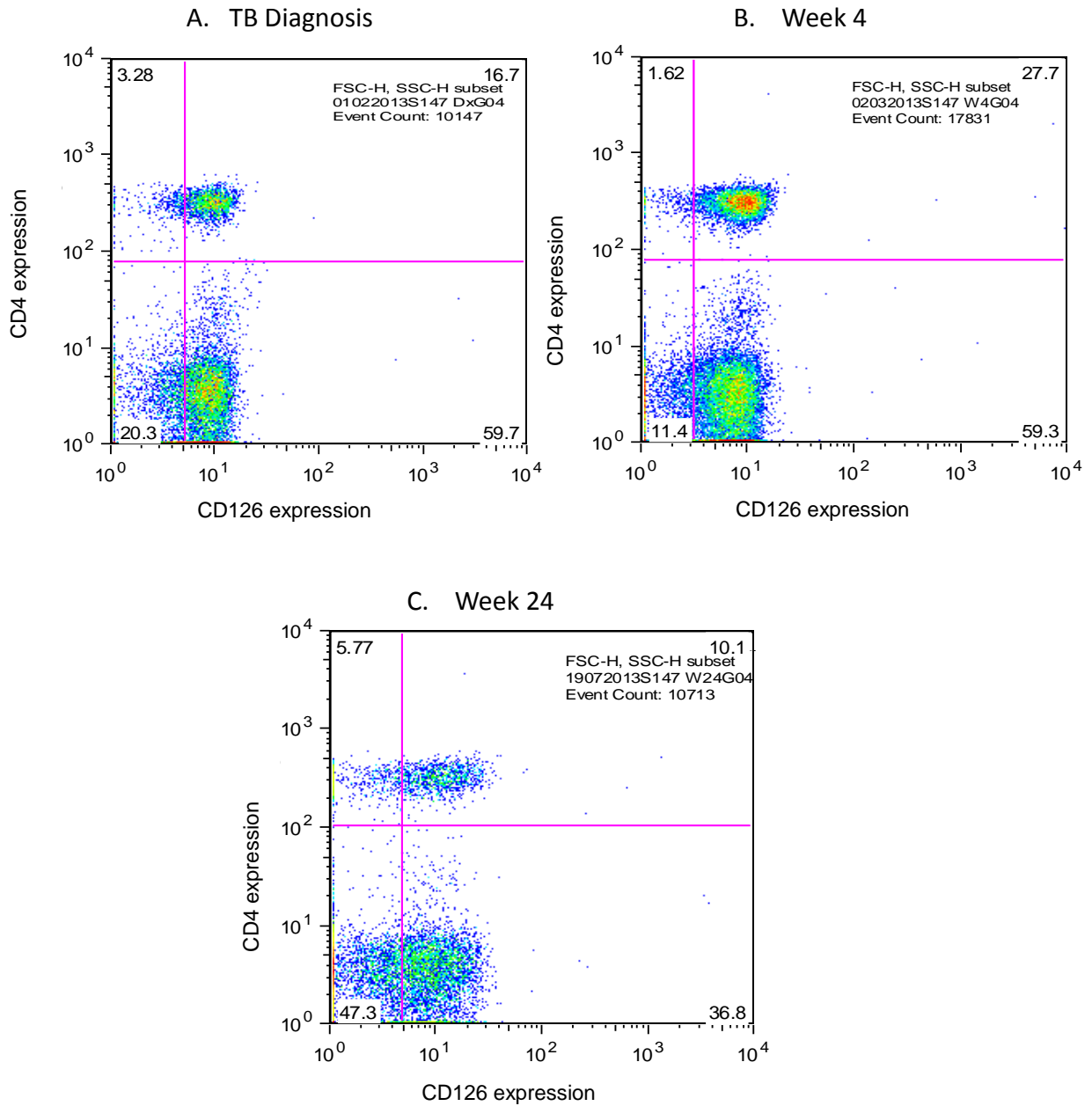
Figure 3.4: The co-expression of CD4 and CD126 in a TB patient (S147 Dx), at three time points.

The addition of both CD4 and CD126 in the same well allows the illustration of the co-expression of CD4 and CD126. It is evident that the significant down-regulation of CD126 occurs on CD4- cells. The X-axis indicates the expression of CD126 and the Y-axis shows the expression of CD4.

CD4 (Table 3.4) shows a high expression level before treatment in TB patients but then decreases after the start of treatment to eventually reach post-treatment levels comparable to those in healthy community controls. Similarly, CD8 and CD57 increase in expression after the start of treatment. Although these markers are not significantly different after the application of the Bonferroni correction, they may be significant biologically and deserve to be followed up with another study. Other markers of interest such as CCR7, CD127, CD27 and HLA-DR all show a trend of increased or decreased expression however this was not significant after the Bonferroni correction.

3.2.1.1. IPA pathways associated with significant markers in overall treatment response (between diagnosis, week 4 and week 24).

IPA was performed on 17 markers that had a p-value <0.01 before the Bonferroni correction was applied. Twenty three pathways had associations with at least two markers (Table 3.5). The top nine will be discussed. The first three pathways are illustrated in figures 3.5 – 3.7.

Table 3.5: Table of pathways associated with overall treatment response (between diagnosis and week 24)

Pathway	Number of markers involved
Leukocyte extravasation signalling	5
Crosstalk between Dendritic cells and Natural killer cells	4
Granulocyte adhesion and diapedesis	4
Caveolar-mediated endocytosis signalling	3
NF-κβ activation by viruses	3
Dendritic cell maturation	3
Hepatic fibrosis/Hepatic stellate cell activation	3
Role of osteoblasts, osteoclasts and chondrocytes in Rheumatoid Arthritis	3
T Helper cell differentiation	2

Leukocyte extravasation signalling
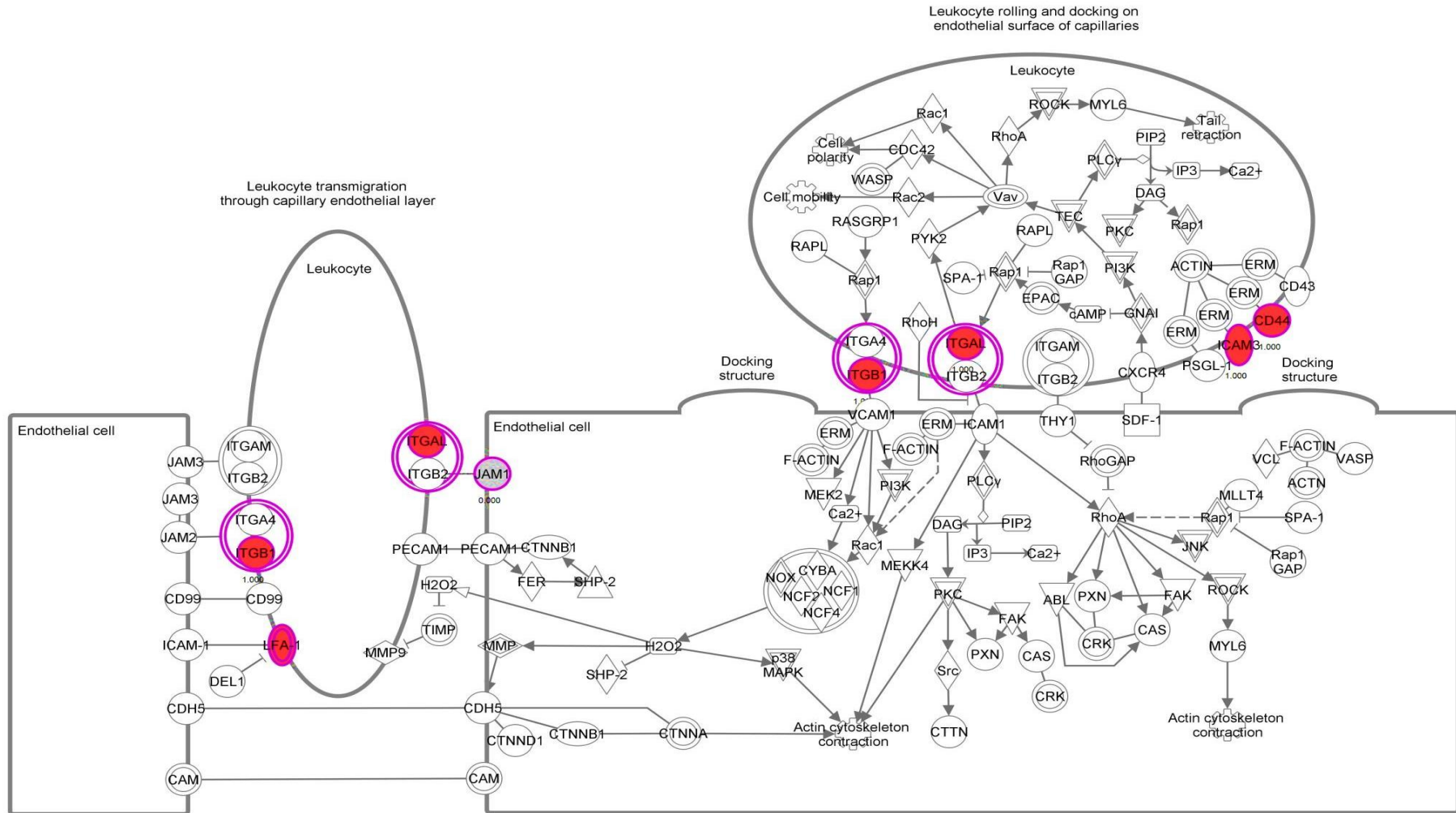
©**Figure 3.5: The leukocyte extravasation signalling pathway**

Leukocyte extravasation pathway illustrating the involvement of CD44, CD50, CD29, CD11a and F11-R. Markers highlighted as red were up-regulated at week 24.

Crosstalk between Dendritic cells and Natural killer cells
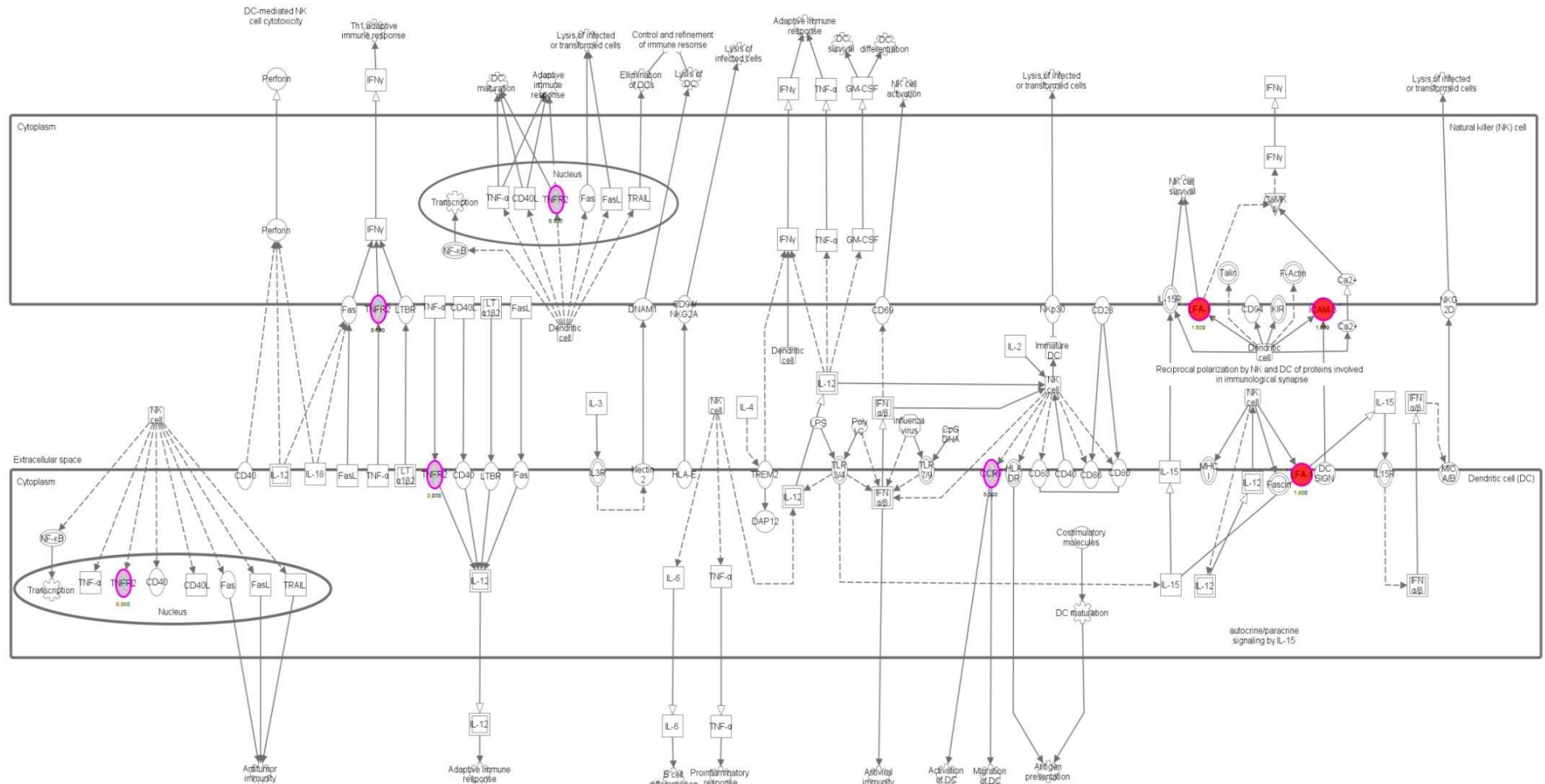
Figure 3.6: The crosstalk between Dendritic cells and Natural killer cells pathway

The crosstalk between Dendritic cells and Natural killer cells pathway illustrating the involvement of CD120b, CCR7, CD11a and CD50. The markers highlighted in red indicate an up-regulation at week 24 and the markers in grey are down-regulated at week 24

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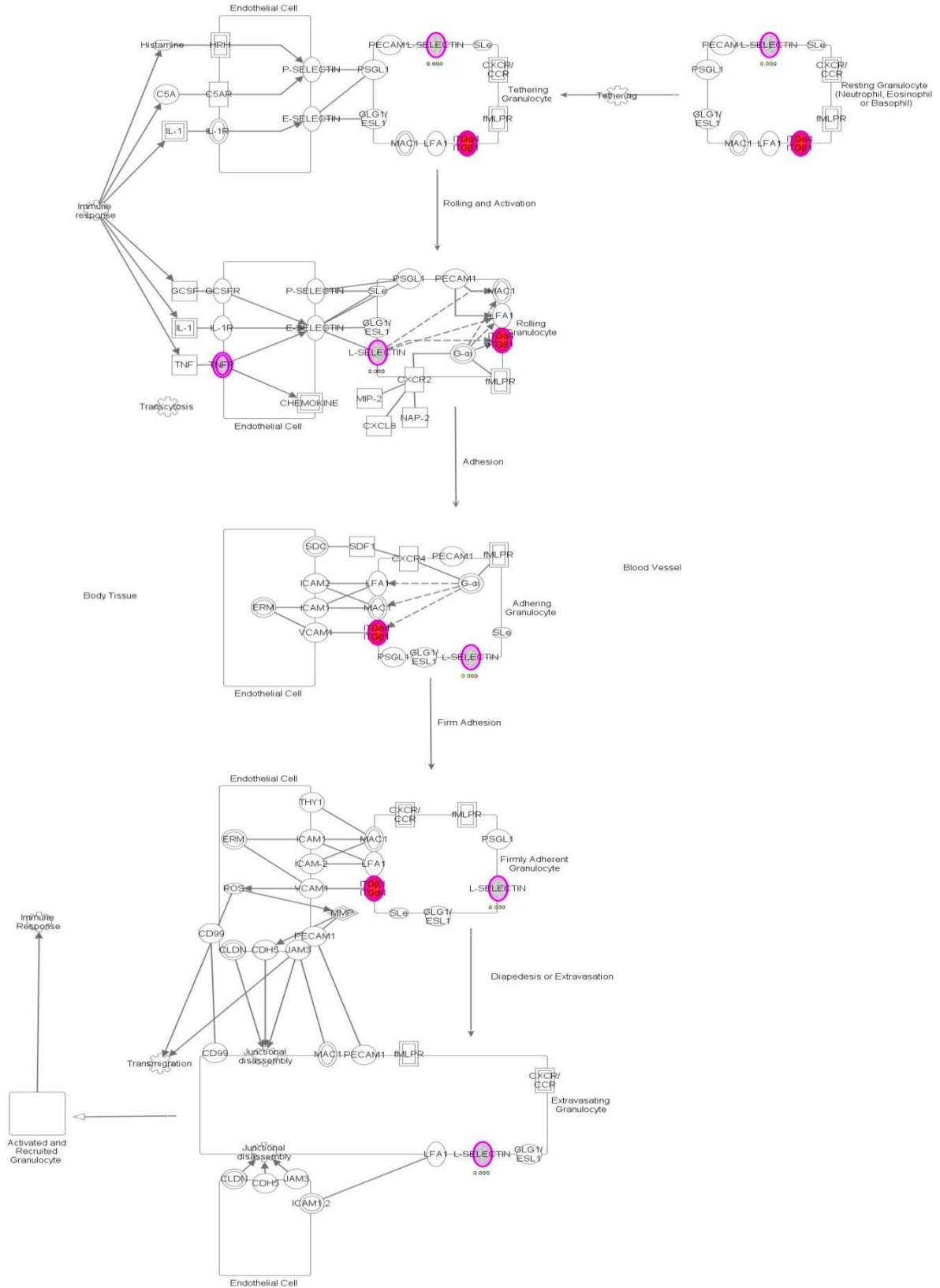
Granulocyte adhesion and diapedesis

 Figure 3.7: The granulocyte adhesion and diapedesis pathway

The granulocyte adhesion and diapedesis pathway illustrating the involvement of 4 markers. The markers highlighted in red indicate an up-regulation at week 24 and the markers in grey are down-regulated at week 24.

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3.2.2. Significant markers identified when comparing surface marker expression between two time points.

The paired t-test was applied to determine which markers would show a significant difference between two time points. The time points were compared as follows:

- Baseline (Dx) vs Week 4 (W4)
- Baseline (Dx) vs Week 24 (W24)
- Week 4 (W4) vs Week 24 (W24)

After the Bonferroni correction was applied, 41 markers showed a possible trend of change (p-value <0.01) (highlighted in blue in table 3.6). A comparison of marker expression between Dx and W4 did not yield any markers that were statistically different however, there were five markers that had a p-value <0.01 (Table 3.6). The comparison of expression between Dx and W24 resulted in five markers that were statistically different (CD120b, CD126, CD62L, CD29 and CD48) as well as 28 markers that had a p-value <0.01 (Table 3.6). Finally the comparison between W4 and W24 resulted in two markers whose expression was statistically different (CD120b and CD29) and 11 markers with a p-value <0.01 (Table 3.6). Thirteen markers were flagged as uncertain and two markers were repeated in the plate.

Table 3.6: Selected markers from paired t-test analysis (p-values from paired t-test <0.01)

	Diagnosis vs week 4		Diagnosis vs week 24		Week 4 vs week 24		Flagged
	P-value	P-val*252	P-value	P-val*252	P-value	P-val*252	
CD50	0.0140	3.5247	0.0015	0.3707	0.0462	11.6322	NO
CD48	0.0989	24.9149	0.0000	0.0030	0.0152	3.8403	NO
CD99	0.0458	11.5395	0.0010	0.2557	0.0538	13.5601	YES
CD58	0.0199	5.0110	0.0015	0.3782	0.3912	98.5902	NO
CD95	0.0976	24.6037	0.0010	0.2595	0.3213	80.9563	YES
CD91	0.1435	36.1675	0.0043	1.0818	0.0837	21.0827	NO
CD45RO	0.7374	185.8259	0.0295	7.4297	0.0045	1.1450	NO
CD4 v4	0.1510	38.0616	0.0007	0.1689	0.0945	23.8050	NO
CD85	0.8028	202.2980	0.0022	0.5566	0.0379	9.5403	YES
CD244	0.3593	90.5329	0.0003	0.0649	0.0132	3.3157	YES
CD15	0.5238	131.9869	0.0065	1.6495	0.0925	23.3068	NO
CD49c	0.0670	16.8798	0.0097	2.4484	0.3710	93.4987	YES
CD18	0.2153	54.2550	0.0011	0.2833	0.1019	25.6879	NO
CD146	0.3195	80.5190	0.0389	9.8070	0.0014	0.3442	NO
CD120b	0.0021	0.5268	0.0000	0.0079	0.0626	15.7659	NO
CD107a	0.0175	4.4043	0.0064	1.6169	0.4402	110.9292	NO
CD66	0.3107	78.3034	0.0038	0.9645	0.0854	21.5320	YES
NKG2D	0.0228	5.7406	0.0047	1.1859	0.3210	80.9033	YES
CD53	0.2058	51.8651	0.0008	0.2099	0.0154	3.8882	NO
CD45RB	0.0948	23.8970	0.0031	0.7818	0.0029	0.7318	NO
CD126	0.7083	178.4965	0.0000	0.0000	0.0000	0.0001	NO
F11 R	0.0004	0.0935	0.0020	0.5001	0.2701	68.0658	NO
CCR6	0.0911	22.9674	0.4048	101.9992	0.0085	2.1298	NO
CD210	0.1647	41.4951	0.0035	0.8699	0.0949	23.9250	YES
CD119	0.1478	37.2562	0.0111	2.7916	0.0030	0.7591	YES
CD29	0.2080	52.4121	0.0040	1.0102	0.0000	0.0005	NO
CD29	0.8300	209.1541	0.0005	0.1267	0.0000	0.0026	NO
CD29	0.2613	65.8401	0.0042	1.0507	0.0003	0.0755	NO
CD11a	0.0092	2.3179	0.0048	1.2046	0.0292	7.3567	NO
CD62P	0.8098	204.0718	0.0346	8.7124	0.0092	2.3105	NO
CD212	0.2105	53.0471	0.0082	2.0636	0.0011	0.2708	YES
CD55	0.0524	13.2061	0.0001	0.0329	0.0373	9.4036	YES
CD4	0.0650	16.3846	0.0014	0.3561	0.1324	33.3695	NO
CD181	0.1705	42.9602	0.0244	6.1414	0.0000	0.0038	YES
CD81	0.6803	171.4344	0.0007	0.1737	0.0030	0.7631	NO
CCR4	0.0044	1.0972	0.1311	33.0382	0.5449	137.3023	NO
CD4	0.0865	21.7936	0.0014	0.3414	0.1729	43.5593	NO
CD28	0.2122	53.4683	0.0059	1.4795	0.1934	48.7462	NO
CD62L	0.0081	2.0321	0.0000	0.0008	0.0324	8.1542	NO
CCR7	0.0134	3.3803	0.0014	0.3407	0.5311	133.8273	NO
CD172a	0.1211	30.5234	0.0091	2.2983	0.6765	170.4871	YES

3.2.2.1. IPA pathways associated with significant markers when comparing surface marker expression between two time points.

3.2.2.1.1. Comparison of marker expression between TB diagnosis and week 4

Five markers were analysed and resulted in four pathways with an association of two or more markers under investigation. The first ten pathways, associated with one or more surface markers, are listed in table 3.7.

3.2.2.1.2. Comparison of marker expression between TB diagnosis and week 24

Eighteen markers resulted in 33 pathways with an association of two or more markers under investigation. The first ten pathways, associated with one or more surface markers, are listed in table 3.7.

3.2.2.1.3. Comparison of marker expression between week 4 and week 24

Eight markers resulted in two pathways with an association of two or more markers under investigation. The first ten pathways, associated with one or more surface markers, are listed in table 3.7.

Table 3.7: List of the first ten pathways associated with the paired t-test and independent t-test data

	TB Dx vs W4	TB Dx vs W24	W4 vs W24	TB Dx vs healthy	TB Dx vs OLD
1	Granulocyte adhesion and diapedesis	Crosstalk between DC and NK cells	Granulocyte adhesion and diapedesis	Caveolar mediated endocytosis signalling	Crosstalk between DC and NK cells
2	Crosstalk between DC and NK cells	Granulocyte adhesion and diapedesis	Agranulocyte adhesion and diapedesis	Leukocyte extravasation signalling	DC maturation
3	Tight junction signalling	Caveolar mediated endocytosis signalling	Role of JAK family kinases in IL-6 type cytokine signalling	Agrin interaction and neuromuscular junctions	NF-κβ activation by viruses
4	Leukocyte extravasation signalling	NF- κβ activation by viruses	Semaphorin signalling in neurons	NF- κβ activation by viruses	Coagulation system
5	Tumoricidal function of hepatic NK cells	Leukocyte extravasation signalling	Regulation of cellular mechanics by Calpain protease	Reelin signalling in neurons	Role of macrophages, fibroblasts and endothelial cells in Rheumatoid arthritis
6	TNFR2 signalling	T helper cell differentiation	Actin nucleation by ARP-WASP Complex	Virus entry via endocytic pathways	Caveolar mediated endocytosis signalling
7	Induction of apoptosis by HIV-1	Agrin interaction and neuromuscular junctions	T helper cell differentiation	Paxilin signalling	PPAR signalling
8	T helper cell differentiation	Reelin signalling in neurons	Agrin interaction and neuromuscular junctions	PTEN signalling	Role of tissue factor in cancer
9	Agrin interaction and neuromuscular junctions	Communication between innate and adaptive immune cells	Macropinocytosis signalling	Granulocyte adhesion and diapedesis	Type II diabetes mellitus signalling
10	Caveolar mediated endocytosis signalling	Virus entry via endocytic pathways	TREM1 signalling	Integrin signalling	PTEN signalling

3.2.2.2. Heat map illustrating the change in expression between TB diagnosis, week 4 and week 24.

A comparison of 18 markers (Table 3.8) between TB patients at diagnosis, week 4 and week 24 showed a change of the expression levels of most of the markers especially between diagnosis and week 24. It appears that changes in the expression levels started changing from as early as week 4 however these changes were not statistically significant once the Bonferroni correction was applied.
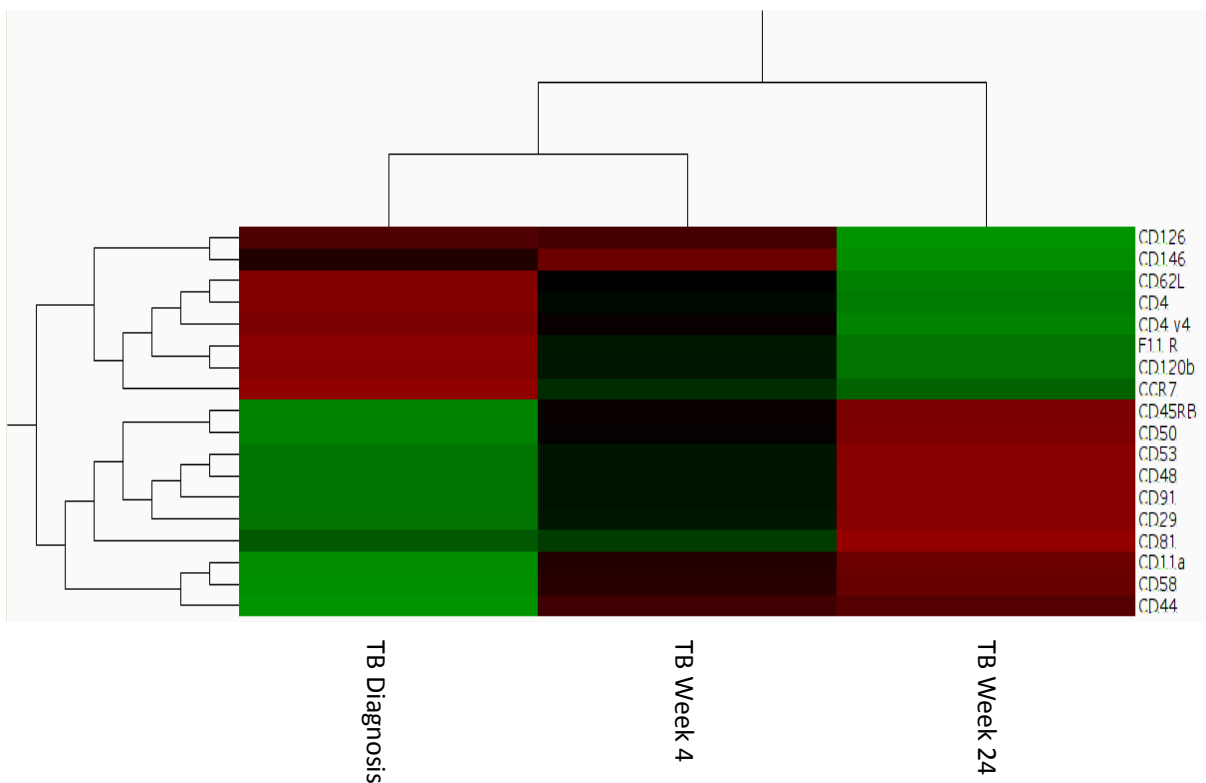
Figure 3.8: Heat map comparing expression of TB diagnosis, week 4 and week 24.

Heat map generated to compare the expression levels of markers between TB diagnosis, week 4 and week 24. An up-regulation is indicated by red and a down-regulation is indicated by green.

Table 3.8: List of surface markers used to generate Qlucore heat maps

Surface markers			
CD classification	**Alternative name**	**CD classification**	**Alternative name**
CD50	ICAM-3	CD126	IL-6R
CD48	BLAST-1	F11 R	JAM1
CD58	LFA-3	CD44	HCAM
CD91	LRP1	CD11a	ITGAL
CD4 v4	Clone L120	CD4	CD4
CD146	MCAM	CD81	TAPA-1
CD120b	TNFR2	CD62L	L-selectin
CD53	Tetraspanin-25	CCR7	CD127
CD45RB	CD45	CD29	ITGB1

Based on the results of the heat maps, it was decided to perform IPA on smaller groups of markers that showed a similar trend (up-regulation or down-regulation) to identify pathways associated with the markers that behaved in the same manner during treatment (Tables 3.8 and 3.9).   The top five pathways for each group had an association with two or more markers (Table 3.11).

Table 3.9: Markers up-regulated between diagnosis and week 24

Markers up-regulated between Dx and W24.	
CD45RB	CD58
CD11a	CD81
CD50	CD53
CD44	CD48
CD91	CD29

Table 3.10: Markers up-regulated between diagnosis and week 24

Markers down-regulated between diagnosis and week 24	
CD126	CCR7
CD146	CD62L
F11 R	CD4
CD120b	CD4 v4

Table 3.11: List of the top five Ingenuity pathways associated with the markers which showed an up-regulation (left) and down-regulation (right) when used to generate a heat map using the Qlucore Omics Explorer software.

Up-regulation between Dx and W24	Down-regulation between Dx and W24
Leukocyte extravasation signalling	Hepatic fibrosis/Hepatic stellate cell activation
Caveolar-mediated endocytosis signalling	T Helper cell differentiation
Agrin interactions at neuromuscular junctions	Communication between innate and adaptive immune cells
NF-κβ activation by viruses	Crosstalk between dendritic cells and natural killer cells
Reelin signalling in neurons	IL-6 signalling

3.2.3. Significant markers identified when comparing surface marker expression between two different patient groups.

When comparing the mean expression between TB patients (at diagnosis) with healthy community controls and other lung disease controls, it was assumed that the mean of all the groups was equal. A comparison between TB patients and healthy community controls using the independent two-sample t-test resulted in 23 markers with a p-value < 0.01; however, only four proved to be statistically different (CD48, CD18, CD126 and fMLPr).   Similarly, a comparison between TB patients and OLD controls and between healthy controls and OLD controls resulted in 13 and 9 markers respectively (highlighted in blue in table 3.12), with a p-value <0.01; however, after the Bonferroni correction was applied, only one marker was significant for the comparison of TB patients and OLD controls (CD220). CD220 had a negative expression level (<10% expression) and therefore was not considered biologically significant. CD126 was found to be significantly different and able to distinguish between TB patients before treatment (Dx) and healthy controls. CD48 and CD18 were both significantly different between TB diagnosis and healthy

controls; however, as the expression levels of these markers were both predominantly positive, the result was not biologically significant. It was noted that there is a more noticeable difference between TB patients before treatment and healthy controls than between TB patients and OLD controls and between OLD controls and healthy controls. Kernel density graphs for selected markers demonstrate the distribution of expression when comparing the expression levels of surface markers between TB patients at diagnosis, healthy community controls and OLD controls.

Table 3.12: Selected markers from the independent two-sample t-test analysis (p-values from independent two-sample t-test <0.01)

Marker	Means			TB diagnosis vs healthy control		TB diagnosis vs OLD		Healthy control vs OLD		Flagged
	T0 (N=33)	Healthy (N=11)	Other (N=9)	P-value	P-val*252	P-value	P-val*252	P-value	P-val*252	
CD6	63.19	75.83	60.07	0.0085	2.1364	0.5883	148.2557	0.0207	5.2261	YES
CD50	97.72	99.35	99.1	0.0015	0.3699	0.0014	0.3582	0.5342	134.6176	NO
CD48	96.88	99.37	98.18	0.0000	0.0020	0.0806	20.3086	0.0619	15.6102	NO
CD99	75.84	91.2	70.1	0.0074	1.8661	0.5774	145.4938	0.0606	15.2712	YES
CD147	98.05	99.27	98.62	0.0016	0.4057	0.2273	57.2877	0.1498	37.7410	NO
CD95	51.19	63.41	43.99	0.0344	8.6788	0.2422	61.0361	0.0100	2.5094	YES
CD64	2.12	2.03	1.11	0.8797	221.6964	0.0025	0.6322	0.1337	33.6808	NO
CD45	99.17	99.94	99.68	0.0022	0.5490	0.0523	13.1806	0.0508	12.7960	NO
CD66b	1.62	0.85	1.91	0.0040	1.0083	0.6935	174.7673	0.1724	43.4437	NO
CLA	8.12	8.15	5.43	0.9851	248.2381	0.0014	0.3554	0.0263	6.6380	NO
CD18	98.43	99.69	99.1	0.0001	0.0237	0.0414	10.4236	0.0150	3.7689	NO
HLA	99.44	99.95	99.74	0.0053	1.3377	0.1353	34.0876	0.0981	24.7268	NO
CD146	2.9	1.64	1.95	0.0011	0.2697	0.0330	8.3105	0.4290	108.1051	NO
CD120b	14.96	14.96	6.21	0.9994	251.8503	0.0028	0.7160	0.1115	28.0903	YES
CD107a	4.23	3.78	2.53	0.5763	145.2204	0.0030	0.7486	0.1527	38.4774	NO
CD109	56.6	29.96	46.04	0.0013	0.3356	0.1980	49.8940	0.0975	24.5774	YES
TGFBR2	6.73	2.77	3.54	0.0016	0.3931	0.0148	3.7415	0.3250	81.9115	NO
Ly b R	3	2.23	1.69	0.0931	23.4728	0.0040	1.0082	0.2671	67.3090	NO
MIC AB	6.55	2.77	5.49	0.0020	0.4941	0.5524	139.1988	0.1404	35.3703	NO
CD140a	1.74	1.1	1.23	0.0035	0.8741	0.0287	7.2410	0.5348	134.7670	NO
CD130	30.62	20.49	40.7	0.0274	6.9004	0.1417	35.7175	0.0090	2.2643	YES
CD150	33.57	38.92	24.02	0.2370	59.7252	0.0346	8.7309	0.0053	1.3341	YES
CD45RB	97.34	99.05	98.53	0.0013	0.3308	0.0268	6.7645	0.2854	71.9209	NO

Table 3.12: Selected markers from the independent two-sample t-test analysis continued

CD126	80.58	50.15	67.89	0.0000	0.0001	0.0118	2.9816	0.0029	0.7331	NO
CD16b	17.42	10.64	24.76	0.0110	2.7779	0.0523	13.1849	0.0007	0.1841	YES
CD141	2.22	1.36	1.22	0.0308	7.7700	0.0054	1.3610	0.6706	168.9887	NO
CD210	22.48	53.38	24.45	0.0064	1.6199	0.8096	204.0294	0.0217	5.4793	YES
CD220	1.34	1.29	0.68	0.8433	212.4995	0.0001	0.0303	0.0366	9.2171	NO
Le B4 R	3.53	2.23	2.32	0.0083	2.1008	0.0236	5.9396	0.8683	218.8242	NO
CD87	0.89	0.95	0.35	0.8944	225.3813	0.0017	0.4233	0.1792	45.1546	NO
fMLP r	17.43	7.23	10.84	0.0000	0.0071	0.0481	12.1132	0.1965	49.5056	YES
CD29	89.88	95.96	94.09	0.0032	0.8040	0.0234	5.9029	0.1881	47.4022	YES
CD29	91.09	96.24	95.28	0.0011	0.2791	0.0052	1.3176	0.3758	94.6920	YES
CD11a	97.54	99.55	98.13	0.0026	0.6471	0.6694	168.6998	0.2761	69.5675	NO
CD33	3.22	2.85	1.65	0.5451	137.3778	0.0014	0.3476	0.0319	8.0447	NO
CD55	67.28	46.69	82.24	0.0121	3.0421	0.0944	23.7903	0.0019	0.4864	YES
CD81	98.18	99.3	99.38	0.0136	3.4158	0.0007	0.1831	0.8399	211.6501	NO
CD20	13.56	19.12	18.21	0.0038	0.9567	0.1620	40.8345	0.7817	196.9960	NO
CCR9	23.3	10.46	7.55	0.0311	7.8410	0.0034	0.8485	0.5340	134.5687	YES
CD8	23.04	36.12	17.56	0.0201	5.0683	0.0418	10.5418	0.0024	0.6127	NO
CD235a	0.99	0.6	1.46	0.0425	10.6989	0.0752	18.9388	0.0012	0.3050	NO
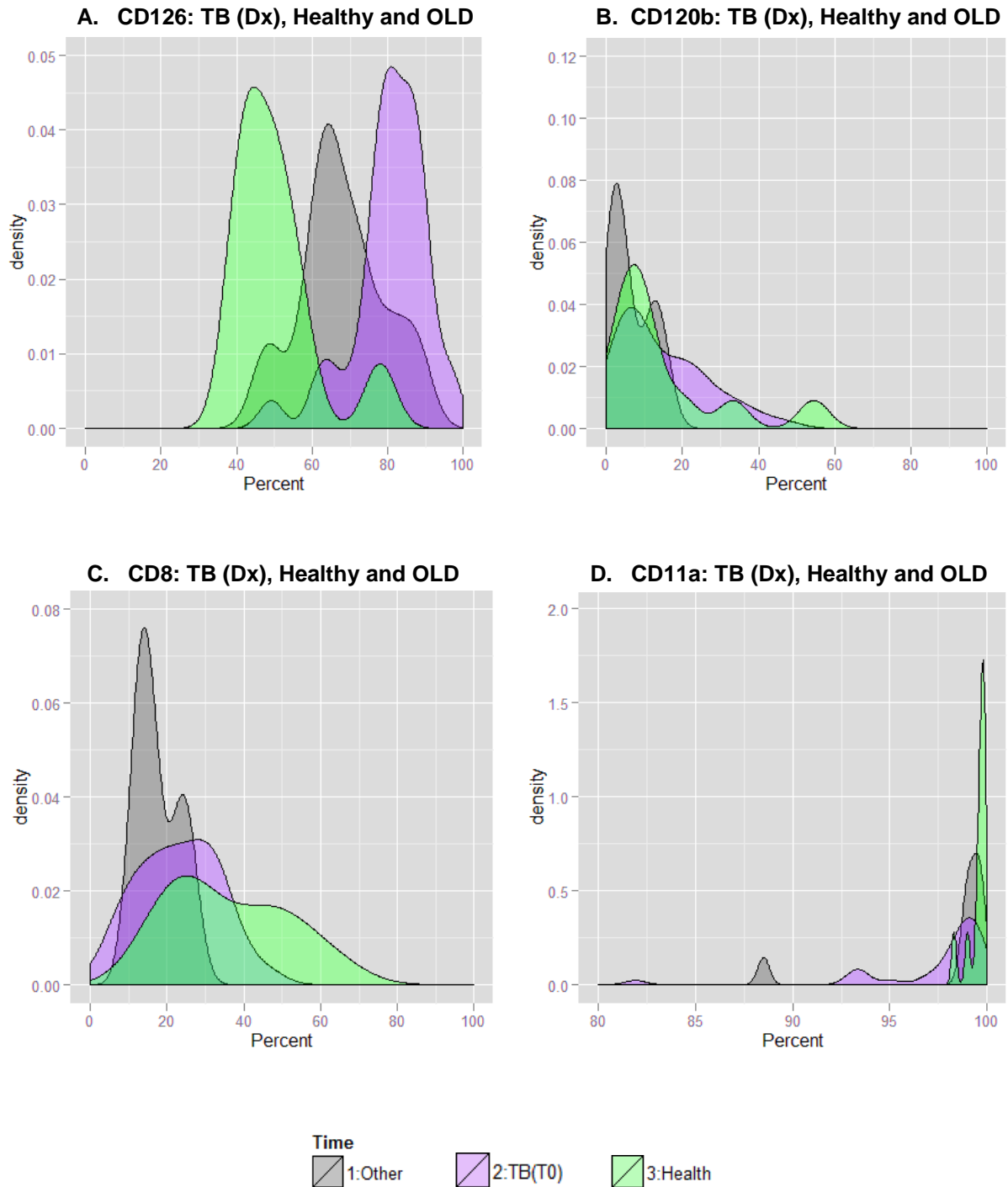
Figure 3.9: Kernel density graphs comparing the distribution of expression of markers between TB diagnosis, healthy controls and other lung disease controls (OLD).

Distribution of expression of CD126 (a), CD120b (b), CD8 (c) and CD11a (d). PBMCs were isolated, stained and acquired on a flow cytometer to evaluate the expression of markers on the cells surface. The expression is denoted as a percentage of the total cells expressing the marker. The comparison was made between the expression levels of TB patients at diagnosis, healthy community controls and OLD. A change in expression was considered significant if the p-value <0.0002 after the application of the Bonferroni correction.
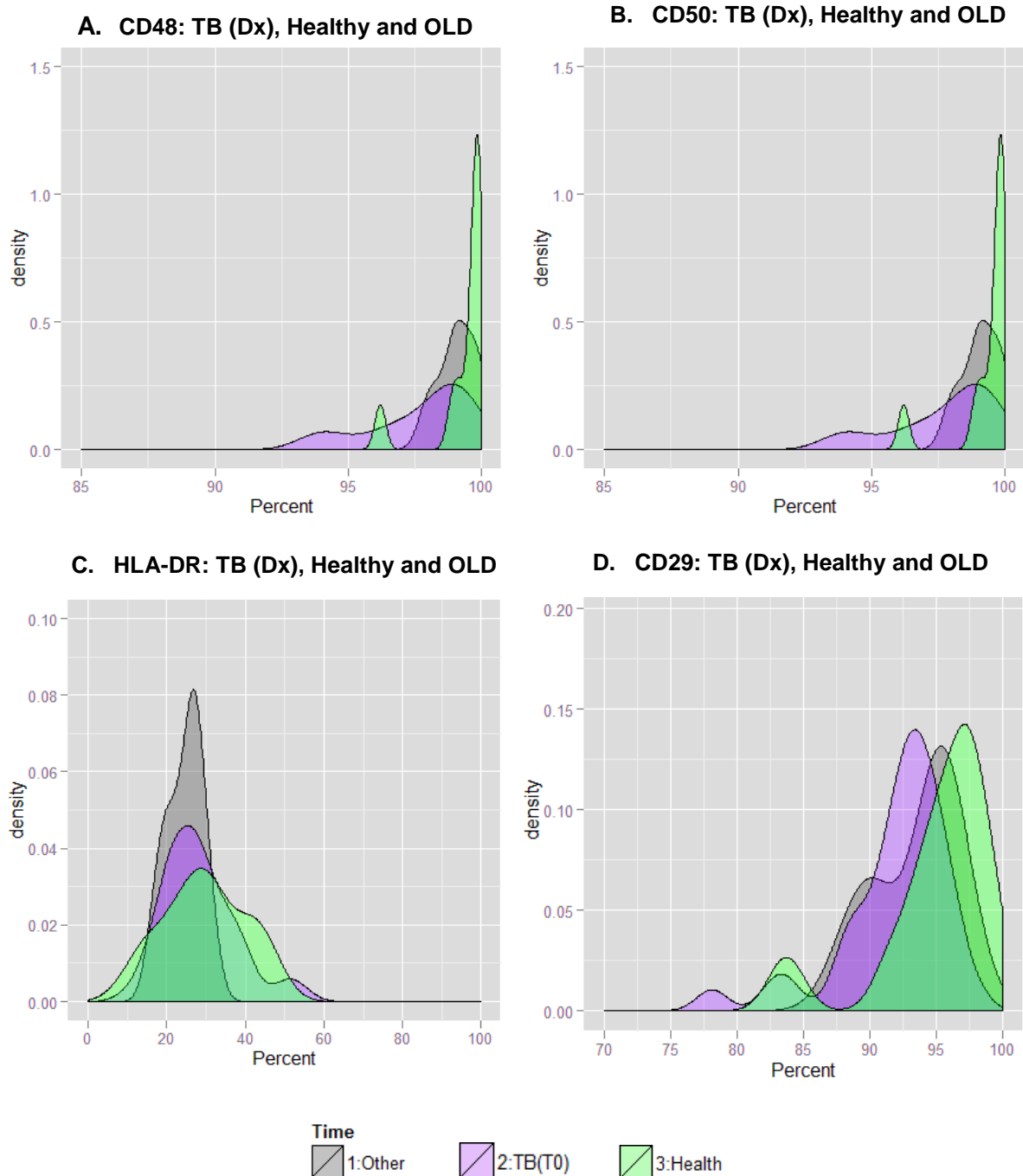
Figure 3.10: Kernel density graphs comparing the distribution of expression of markers between TB diagnosis, healthy controls and other lung disease controls (OLD).

Distribution of expression of CD48 (a), CD50 (b), HLA-DR (c) and CD29 (d). PBMCs were isolated, stained and acquired on a flow cytometer to evaluate the expression of markers on the cells surface. The expression is denoted as a percentage of the total cells expressing the marker. The comparison was made between the expression levels of TB patients at diagnosis, healthy community controls and OLD. A change in expression was considered significant if the p-value <0.0002 after the application of the Bonferroni correction.

3.2.3.1. IPA pathways associated with markers exhibiting a significant change in expression between two patient groups.

3.2.3.1.1. Comparison of marker expression between TB patients at diagnosis and healthy community controls

Fourteen markers resulted in 26 pathways with an association of two or more markers under investigation. The first ten pathways, associated with one or more surface markers, are listed in table 3.7.

3.2.3.1.2. Comparison of marker expression between TB patients at diagnosis and OLD controls

Twelve markers resulted in 15 pathways with an association of two or more markers under investigation. The first ten pathways, associated with one or more surface markers, are listed in table 3.7.

3.2.3.2. Heat map illustrating the change in expression between TB diagnosis, week 24 and healthy community controls.

A comparison between TB patients at diagnosis, EOT and healthy community controls showed a difference in expression levels for most markers. Week 24 and healthy community controls clustered together, sharing similar expression profiles compared to pre-treatment TB.
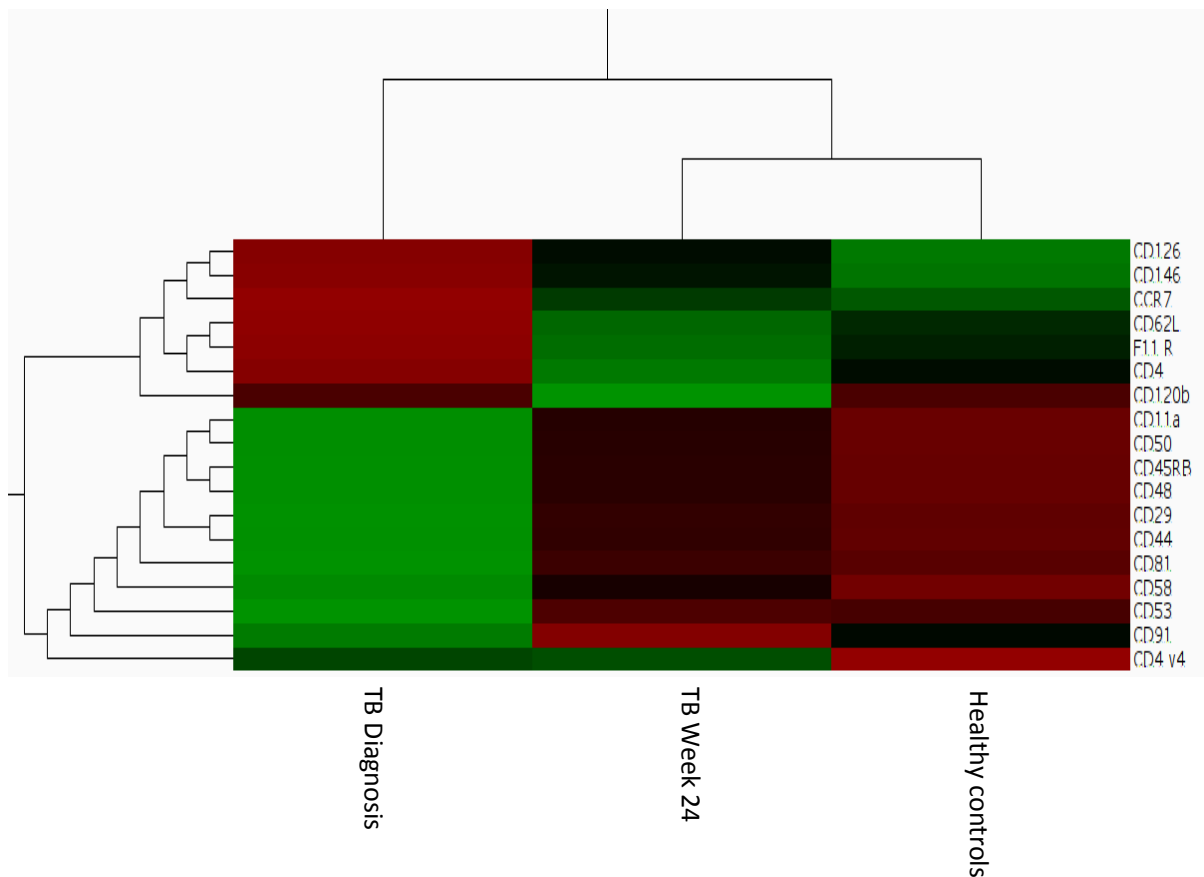
Figure 3.11: Heat map comparing expression of TB diagnosis, week 24 and healthy community controls.

Heat map generated to compare the expression levels of markers between TB diagnosis, week 24 and healthy controls. An up-regulation is indicated by red and a down-regulation is indicated by green.

3.2.3.3. Heat map illustrating the change in expression between TB diagnosis, OLD and healthy controls.

The comparison of the expression of markers between diagnosis and other lung disease showed a mixed profile where some markers showed an up-regulation in OLD compared with diagnosis and other markers showed a down-regulation while other markers remained unchanged or had little change. This also proved true when comparing OLD with healthy controls indicating a very different response to other lung diseases, such as pneumonia, than to TB.
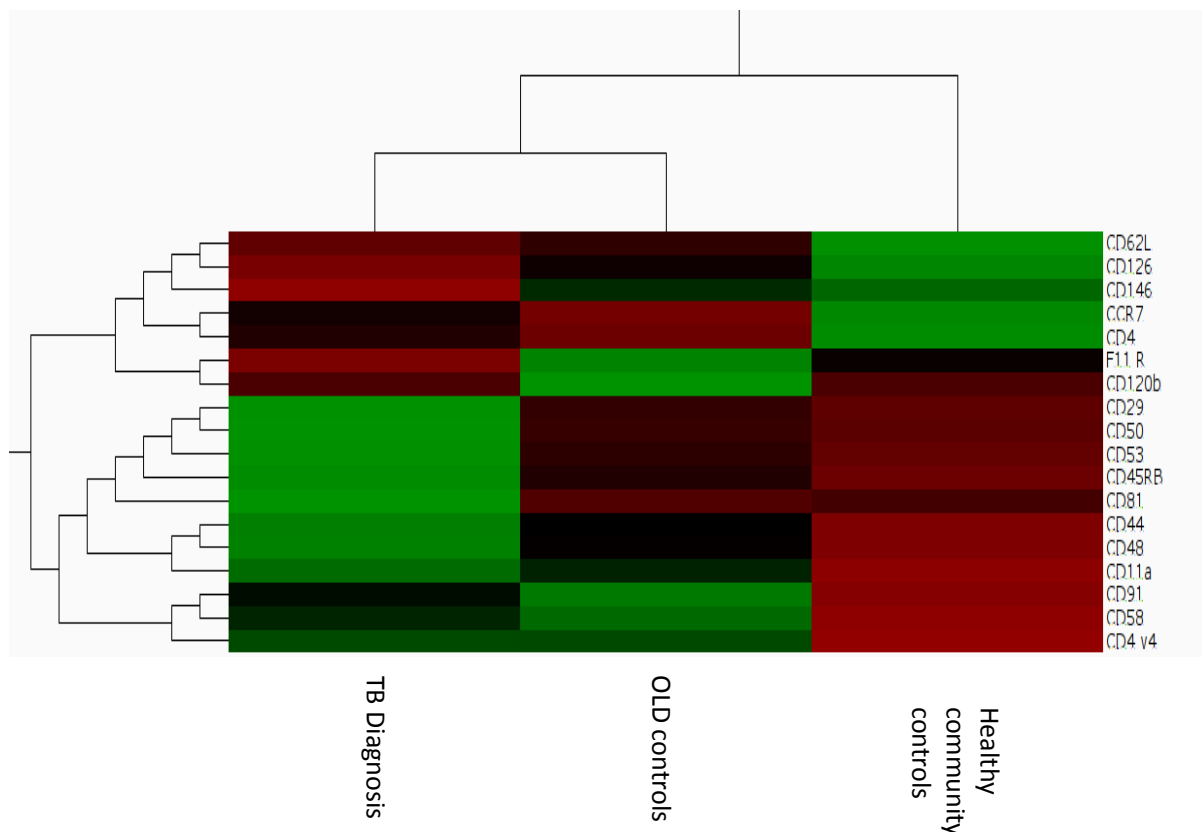
Figure 3.12: Heat map comparing expression of TB diagnosis, OLD and healthy community controls.

Heat map generated to compare the expression levels of markers between TB diagnosis, OLD controls and healthy community controls. An up-regulation is indicated by red and a down-regulation is indicated by green.

Table 3.13:. List of markers and their accession numbers used for IPA analysis

ANOVA		Paired t-test:		Paired t-test:		Paired t-test:		Independent t-test:		Independent t-test:	
TB Dx vs week 24		TB Dx vs week 24		TB Dx vs week 4		Week 4 vs week 24		TB Dx vs healthy		TB Dx vs OLD	
Marker:	Accession #:	Marker:	Accession #:	Marker:	Accession #:	Marker:	Accession #:	Marker:	Accession #:	Marker:	Accession #:
CD50	P32942	CD50	P32942	CD120b	P20333	CD146	P43121	CD50	P32942	CD50	P32942
CD48	P09326	CD48	P09326	F11 R	Q9Y624	CD126	P08887	CD48	P09326	CD64	P12314
CD58	P19256	CD58	P19256	CD11a	P20701	CCR6	P51684	CD147	P35613	CLA	Q8WTV0
CD91	Q07954	CD91	Q07954	CCR4	Q9ULM6	CD29	P05556	CD45	P08575	CD120b	P20333
CD4 v4	Q8IUX4	CD4 v4	Q8IUX4	CD62L	P14151	CD62P	P16109	CD66b	P31997	CD107a	P11279
CD146	P43121	CD18	P05107			CD81	P60033	CD18	P05107	Ly b R	P36941
CD120b	P20333	CD120b	P20333					CD146	P43121	CD141	P07204
CD53	P19397	CD107a	P11279					TGFBR2	P37173	CD220	P06213
CD126	P08887	CD53	P19397					CD140a	P16234	CD87	Q03405
F11 R	Q9Y624	CD126	P08887					CD126	P08887	CD29	P05556
CD44	P16070	F11 R	Q9Y624					Le B4 R	Q15722	CD33	P20138
CD11a	P20701	CD29	P05556					CD29	P05556	CD81	P60033
CD4	P01730	CD11a	P20701					CD11a	P20701		
CD81	P60033	CD81	P60033					CD20	P11836		
CD62L	P14151	CD4	P01730								
CCR7	P32248	CD28	P10747								
CD29	P05556	CD62L	P14151								
		CCR7	P32248								

3.2.4. Association between expression of markers at diagnosis and study outcomes

Due to the small sample size of this project, it was not possible to perform logistic regression and general discriminate analysis. Both parametric and non-parametric methods were used to verify the results.

3.2.4.1. Association between the expression of markers at diagnosis and the treatment outcome

Comparisons between definite cure and not cured were made and markers that were significant using ANOVA to compare the three treatment outcomes (definite cure, probably cure, not cured) were tested with the independent t-test. Due to the number of markers identified (six) by ANOVA and the application of the Bonferroni correction, a p-value < 0.008 was considered statistically significant. Five markers were significant when comparing the means between groups of patients classified as cured or not cured at the end of treatment (table 3.14).

Table 3.14: Table of significant markers when comparing cured and not cured treatment outcomes

Variable	Mean (definite cure)	Mean (not cured)	p-value
CD18	98.78462	66.21667	0.000888
CD11a	97.43077	63.45700	0.001188
CD50	97.63846	64.09667	0.001022
CD48	97.04231	67.30000	0.001115
CD53	97.94615	70.23333	0.001715

3.2.4.2. Association between the expression of markers at diagnosis and time to positivity (TTP)

No markers were correlated with the TTP when using a simple correlation. Scatter plots were used to identify markers showing a convincing linear relationship and were investigated for correlation with multiple linear regression models. The markers that were used in the multiple linear regression model were CD146, CD62P, CD4,

CD126, CD4v4, CD28 and CCR7 and none of these showed a significant linear relationship.

3.2.4.3. Association between expression of markers at diagnosis and week 8 culture results

Independent t-tests were used to compare marker expression with either a positive or negative week 8 culture results; however, none of the markers were significant.

3.2.4.4. Association between expression of markers at diagnosis and qualitative scan outcome

When comparing the expression of markers at diagnosis with the PET/CT outcome groups (resolved, mixed or improved), only two markers were significantly different. Further t-tests identified these markers as being significantly different between the mixed and improved groups with a p-value of 0.015 and 0.014 for CD45RO and CD4v4 respectively.

3.2.4.5. Association between markers and new clinical groups by combining qualitative and quantitative scan outcomes

New scan outcome groups were defined by combining the original qualitative and quantitative groups to stratify patients into clinical severity groups (table 2.7). There was no significant association found between the new scan outcome groups and the marker expression using ANOVA; however, non-parametric testing revealed CD18, CD11a, CD62P and CD81 had p-values <0.05 when comparing the good and poor outcome groups.

Chapter 4:   Discussion

Sputum smear microscopy and sputum culture are the only validated methods for monitoring treatment response in TB. Both methods have limitations such as a lack of sensitivity in smear microscopy or long turnaround time for culture results. This has resulted in a necessity for new treatment response markers.

Little is known about which PBMC surface markers change during the course of anti-TB therapy. Previous studies have identified a number of markers that, when used in combination, could provide potential serological biomarkers for outcome prediction in TB patients (57).   Djoba *et al.* demonstrated that serological markers such as CRP, sICAM-1, granzyme B, sLAG-3 and suPAR could potentially contribute to forming a predictive model to be used as biomarkers for treatment response (58).

Studies involving the detection of cytokine and chemokine concentrations in plasma from patients with pulmonary TB, pleural TB, other pleural diseases and healthy community controls revealed markers such as CXCL10/IP-10 and CCL3/MIP-1α were significantly increased in pleural TB patients compared with other pleural diseases making them potential plasma biomarkers for pleural TB disease (59).

This project investigated 252 markers simultaneously in PBMCs from actively infected TB patients at three time points in relation to treatment, healthy community controls and other lung disease controls. Five markers changed significantly from baseline to the end of treatment (EOT) in TB cases and four markers differentiated between TB diagnosis and healthy community controls. None of the markers showed a significant difference from TB baseline to week 4 on treatment or between active TB and other lung diseases; however, before the Bonferroni was applied each of these groups had five and 13 markers respectively that had a p-value <0.01 and could therefore still have a biological significance.

Comparison between TB diagnosis and week 4 did not yield any early treatment response markers (W4) that were significant after the Bonferroni correction; however, five markers (CD120b, CD126, CD62L, CD48 and CD29) were significantly different at EOT making them potential late treatment response markers.   Although CD48 and CD29 expression at baseline and EOT were significantly different, these

changes were very small and almost all the cells had a high positive expression and the biological and clinical relevance of these markers was therefore judged to be questionable. The four markers that differentiated between TB patients and healthy community controls were CD48, CD18, CD126 and fMLPr. As above, CD48 and CD18 both had high positive expression and very small differences deeming their significance as questionable. Statistically one in 20 markers are expected to show a significant difference by chance (P-value <0.05). Therefore, using the Bonferroni correction, and using an $\alpha = 0.05$ only markers with p-value $\leq \frac{0.05}{252} \approx 0.0002$ should be considered to limit false positive discovery. It is acknowledged that the Bonferroni correction is a highly stringent and conservative approach for adjustment for multiple tests and some researchers regard it as unhelpful (56). To prevent false negative discovery we therefore decided to include markers that did not withstand the Bonferroni correction but had a p value of <0.01. The markers listed in table 3.7 and table 3.8 for IPA and Qlucore respectively therefore includes all markers for which expression differences reached this p-value.

4.1. Objective 1 - Optimisation of FACS^{TM} CAP procedures.

4.1.1. Objective 1.1 - Comparison of surface marker expression between fresh and cryopreserved PBMCs.

Studies may require retrospective phenotypic and functional analysis of PBMCs from samples collected throughout the duration of a project. This makes cryopreservation a useful tool for storing live cells for prolonged periods of time. It has been shown that cell viability and the ability to proliferate and produce cytokines upon stimulation can be preserved when cryopreserving PBMCs (60).

One aspect of this project was to determine the effect of cryopreservation on surface marker expression of PBMC's for FACS^{TM} CAP on cryopreserved samples for future studies. PBMCs, from five healthy volunteers, were used to compare the expression of surface markers between freshly isolated PBMCs and cryopreserved PBMCs. Of the 252 markers that were included in this study, only five showed a notable difference between fresh and cryopreserved cells.   Markers that showed an

expression in only one or two samples were not considered as were markers that showed an increase in some patients but a decrease in others.   Of the markers that were significantly different between fresh and cryopreserved cells, four (CD41a, CCR6, CD9, CD62L) showed a down-regulation in their expression while only one marker (CD7) showed an up-regulation. Due to the small sample set for this aspect of the project, it was difficult to perform a strong statistical analysis to determine if the change in expression between fresh and cryopreserved cells was significant. Cryopreservation time for these samples ranged from four to eight weeks, a relatively short time when compared with long-term storage of PBMCs. Prolonged storage of PBMC's in liquid nitrogen may result in cells being more fragile and showing different expression levels of surface markers. The control samples were obtained from healthy volunteers therefore it is possible that a repeat of this experiment using TB patients could result in significantly different results due to the fragility of cells from TB patients. Costantini *et al.* demonstrated, in their comparison of fresh vs cryopreserved PBMCs, a marked decrease in the expression of CD62L in cryopreserved cells, which is in keeping with the results that we obtained (61).

When performing analysis on the flow cytometry data, it was noted that in four of the five samples the granulocyte population was diminished in the frozen samples confirming the poor recovery of these cells after freezing whereas the fifth had no granulocyte population.

The process of cryopreservation may lead to a loss of up to 50% of PBMCs and each plate required approximately 1×10^7 cells (total of 2×10^7 cells). It was soon evident that the concentration of PBMCs required to prepare duplicate plates would not be attainable in all the samples. It was decided to continue the project using only freshly isolated PBMCs to minimise the exclusion of samples due to insufficient cell concentrations.

4.1.2. Objective 1.2 - Comparison of surface marker expression between PPD stimulated and unstimulated PBMCs.

Purified protein derivative (PPD) is an extract of *Mtb* and is the antigen used in the Tuberculin skin test, a diagnostic test for detecting active or latent infection with *Mtb*

(62). Individuals who have had a previous encounter with *Mtb* or have a currently active infection will respond with a positive response noted by an induration of 10mm or more. In vitro stimulation of PBMCs with PPD results in the activation of T cells and the initiation of the Th1 cytokine response (63), mimicking the response of T cells to live *Mtb* .

Of the 252 surface markers, only six markers (CD41a, CD45RA, CD61, CD4 v4, Cd49a and CD62L) had an expression level that was consistently up-regulated or down-regulated after stimulation of PBMC's with PPD.

CD41a is a platelet glycoprotein receptor, which forms a part of the GPIIb/IIIa complex and is associated with normal platelet adhesion and aggregation. During the inflammatory response, platelets undergo a conformational change and function by forming clots during vascular injury (64). CD45RA is defined as a marker of naïve T cells and during stimulation of T cells, human CD45RA+ cells respond with a high frequency in primed individuals (65). The down-regulation of expression of CD45RA in PPD stimulated cells could be explained by this responsiveness to stimulation, which results after activation of T cells and an increased differentiation of naïve T cells.

The small number of significantly different markers might be explained by the fact that analysis was performed by gating on the total PBMC population in which TB specific T cells that respond to PPD stimulation represent a small percentage. It may also be due to the fact that these patients had an active TB infection, which could have resulted in the PBMC's already being maximally stimulated by the infection and thus not producing a further significant increase.

When evaluating the expression levels of activation markers, it was noted that the change in expression was not significantly different. Antas *et al.* observed an increase in expression of CD69 and CD25, both activation markers, when stimulated with PPD (66). However, the stimulation was performed over a longer period of time with the expression of CD69 and CD25 showing an up-regulation after 24 and 96 hours respectively (66). The lack of significant change in our samples may be attributed to the short incubation period or alternatively to the fact that our samples were from actively infected TB patients who may already have a maximally stimulated response prior to further PPD stimulation.

It was decided early in the project to continue the preparation of PBMCs without stimulation with PPD. This decision was made on the basis of cost and availability of PPD at the time that the project was being performed. The volume of PPD needed to stimulate 2×10^7 cells at a concentration of 10µg/mL for each sample at each time point meant that the cost of the PPD would be in excess of the provided budget. At the time that this decision was made, there was worldwide shortage of PPD, which resulted in a long delay in delivery which made it impractical to use in a time-sensitive study.

4.2. Objective 2: To assess the differential PBMC surface marker expression by FACS[TM] CAP in TB patients during treatment.

4.2.1. Markers with a significant change in expression after the Bonferroni correction.

4.2.1.1. CD126 (IL-6R)

IL-6 is a pleiotropic, pro-inflammatory cytokine, released by phagocytes, endothelial cells, fibroblasts, B cells and T cells, which has an increased presence in various diseases such as auto-immune and inflammatory conditions [67]. The biological activities associated with IL-6 can be grouped as follows:

- IL-6 belongs to the pro-inflammatory cytokine group, along with TNF and IL-1, responsible for initiating the early inflammatory response [68].
- IL-6 is involved in T and B cell promotion [67].
- IL-6 participates in haematopoiesis [69].
- It is the cytokine that induces the acute phase response [70].

The function of the acute-phase response is to neutralise invading pathogens and to assist in recovery after an injury to minimise tissue damage. The acute-phase response increases body temperature (fever), increases vascular permeability for

extravasation of leukocytes and induces production of acute-phase proteins (APP) by the liver (71). Some of the positive acute phase proteins include C-reactive protein, serum amyloid P (SAP), serum amyloid A (SAA), fibrinogen, alpha 1 antitrypsin, alpha 2 macroglobulin, ferritin, ceruloplasmin and haptoglobin (72). It has been shown that IL-6 is an important player in protection against *Mtb* and the presence of IL-6 is essential in the stimulation of a Th1 response after vaccination against *Mtb* (73). Singh *et al.* suggested that IL-6 could be used as a stand-alone diagnostic biomarker for TB or as part of a biomarker signature as they found significantly increased levels of IL-6 in mice when peritoneal macrophages were infected with *Mtb* (74). However, this approach could be problematic as IL-6 production would also occur in other infections, thereby decreasing specificity for TB.

The stimulation of IL-6 occurs through the interaction with two cell surface molecules: IL-6R (CD126) and gp130. IL-6 binds to membrane bound IL-6R and together the IL6/IL6R complex associates with gp130, a 130kDa polypeptide chain present on the surface of cells. IL-6R is membrane bound and is expressed predominantly by T cells, monocytes, activated B cells and neutrophils (75). gp130 is not specific to the IL-6R pathway and is found on all cells making it a common signal transducer which can associate with many specific receptors. This association of a specific receptor and a common signal transducer provides an explanation for the pleiotropy which is found amongst a number of different cytokine families (76). Soluble IL-6R (sIL-6R) is formed during one of two processes namely proteolysis (shedding) and translation from an alternatively spliced mRNA (77). The binding of IL-6/sIL-6R complex to bind gp130 is an example of trans-signalling and potentially allows the IL-6/sIL-6R complex to stimulate most cells in the body. Soluble gp130 (sgp130), present in higher concentrations in the blood than membrane gp130 (mgp130), acts as a natural inhibitor of IL-6 signalling by binding to the IL-6/sIL6R complex and preventing the complex from binding to membrane bound gp130 (mgp130) (78).

Although CD126 expression was higher in TB patients at diagnosis than week 4 and EOT, the difference was only statistically significant between diagnosis and EOT, suggesting limited utility as early treatment response biomarker, at least in univariate analysis. The increased expression level in TB patients is in keeping with the

increased inflammatory response during active disease with a subsequent decrease due to treatment. When comparing expression of CD126 of TB patients at diagnosis with healthy community controls and other lung disease controls, TB patients had higher levels than the other groups but only differences between TB cases and healthy community controls were statistically significant.

4.2.1.2. CD62L (L-selectin)

Once the human body encounters a pathogen lymphocytes must leave the peripheral circulation and be directed to secondary lymphoid organs where the antigens are presented (79). CD62L, also known as L-selectin, is a cell adhesion molecule found on the surface of lymphocytes, neutrophils, monocytes, eosinophils and haematopoietic stem cells (80). More specifically, L-selectin is involved in the differentiation of naïve T lymphocytes and their homing to the site of infection (81). The presence of L-selectin on the surface of naïve T cells allows the cells to exit the bloodstream, by attaching to activated endothelial cells, and entering the secondary lymph organs. The cells are returned to the blood stream a few hours later to continue their circulation through the body. During antigenic challenge, T cells are activated and L-selectin is shed.   The shedding of L-selectin occurs when leukocytes slow their velocity and roll along the endothelium in preparation of migration to the site of infection. The process of shedding serves to redirect leukocytes to the site of infection by preventing them from entering the secondary lymphoid tissue (82) (79).

A triphasic expression pattern of L-Selectin is initiated upon activation of T cells. The presence of an antigen results in the loss of approximately 70-90% of L-selectin within the first four hours as shedding occurs and T cells are re-directed to the site of infection. After 12 – 24 hours, T cells re-express L-Selectin three to four times greater than the starting expression and within five to seven days, the expression decreases again to either a very low or no expression of L-Selectin as cells differentiate into either central or effector memory T cells.   Sanchez-Garcia *et al.* showed that γδ T cells shed their L-Selectin upon activation of cells with *Mtb* but re-expressed L-Selectin after the antigen was removed indicating that the expression is regulated by the presence or absence of antigen (83). If the antigenic stimulation

persists, L-Selectin is expressed at low levels for an extended period of time (84). L-Selectin is a reliable surface marker for detecting memory cells and the presence or absence of the marker can differentiate the cells into either central memory cells (T_{cm}, CD62L+) or effector memory cells (T_{em}, CD62L-) (85).

The results of this study show an expression level of CD62L to be higher than that of healthy individuals but similar to patients with other lung diseases. The fact that T cell activation is not specific to only *Mtb* explains the similarity in expression levels of both TB diagnosis and other lung disease patients. The expression levels decreased at week 4 and continued to drop further at EOT to an expression level lower than healthy community controls. The low expression level at EOT could be attributed to the differentiation of T cells into a memory phenotype.

4.2.1.3. CD120b (TNFR2)

CD120b, also referred to as tumour necrosis factor receptor 2 (TNFR2), is a member of the tumour necrosis factor (TNF) superfamily. TNF is one of the primary initiators of the innate immune response and plays a vital role in the inflammation associated with bacterial, viral and parasitic infections (86). TNF interacts with TNFR1 and TNFR2 to optimally function during antigenic challenge. TNFR1 is expressed in low levels on most nucleated cells and contains a death domain through which the caspase pathway is activated, the NF-κβ pathway is promoted and apoptosis is initiated (87). TNFR1 is known to regulate TNF-induced inflammation and apoptosis (88). TNFR2 is preferentially expressed on the surface of lymphocytes and lacks a death domain. TNFR2 is typically activates the NF-κβ pathway with a subsequent activation of pro-survival target genes in the nucleus of cells thereby suppressing the immune system to auto-antigens and preventing immunopathology (89). TNFR2 serves as a co-stimulator during T cell activation by enhancing the response of lymphocytes to TCR-mediated signalling and promoting cell activation, proliferation and migration (90). Kim *et al.* showed that TNFR2 is vital in ensuring the survival of CD4+ and CD8+ T cells during the first few rounds of clonal expansion when responding to intracellular bacterial pathogens.

In this project, the expression of CD120b decreased during treatment with a significant change in expression between TB diagnosis and EOT. The change in expression at week 4 was significant before the Bonferroni correction but not after; however the small sample size and stringent correction for multiple comparisons should not preclude inclusion of this marker in future validation steps. Strikingly, expression at baseline is similar to that seen in healthy community controls and week 4 and week 24 expression is lower than in healthy community controls. During active TB infection there is an up-regulation of inflammatory cytokines and chemokines some of which initiate apoptosis and subsequent cell death. TNFR2, which is thought to have a pro-survival effect, could be up-regulated as a compensatory mechanism to moderate the effects of inflammation. It may be that the normal expression level of CD120b at baseline is inappropriately high for an infection that requires a strong pro-inflammatory response. Reduction after the onset of antibiotic treatment may be in keeping with the establishment of a more appropriate response. We do not know at which stage after completion of treatment, if ever, CD120b expression returns to normal levels.

4.2.2. IPA pathways and heat map data associated with the significant markers in overall treatment response (between diagnosis and week 24).

IPA is a web-based software platform that enables biological contextualization of research data by placing differentially expressed markers into the context of known biological pathways and disease associations. IPA was performed using the markers for which differences between groups or time points had a p-value <0.01 before Bonferroni correction, as only three markers remained significantly different after this correction for multiple comparisons. The canonical pathway generated by IPA showed 23 different pathways associated with two or more of the markers of interest of which the top nine pathways according to p-values and number of markers included into each pathway will be discussed briefly.

4.2.2.1. The pathway associated with Leukocyte extravasation signalling

The markers associated with the leukocyte extravasation signalling pathway include CD44, intercellular adhesion molecule 3 (ICAM3, CD50), integrin β1 (ITGβ1, CD29), ITGAL (Lymphocyte function-associated antigen 1, LFA-1, CD11a) and F11-R (Junctional adhesion molecule 1, JAM1).

Extravasation is an important contributor to the inflammatory process and haemostasis, which is largely mediated by adherence factors. Extravasation occurs when leukocytes migrate from the blood circulation through the endothelium and basement membrane towards the site of infection. This process is initiated by chemokines and other pro-inflammatory markers and begins when the leukocytes adhere to endothelial cells [91]. The leukocytes adhere and tether to the endothelium by recognising selectins on the surface of the endothelial cells, begin rolling along the vessel wall and slow down until they arrest and trans-endothelial migration occurs. This process is largely controlled by integrins and their ligands such as ITGβ1 (CD29), which serves as a membrane receptor involved in adherence and recognition during tissue repair and haemostasis [92].

Selectins (P, E and L) all form important roles in the adherence and capturing of leukocytes where P-selectin glycoprotein ligand-1 (PSGL1), a ligand for all three selectins, binds to the selectins inferring their function of adherence either to the endothelium (P and E-selectins) or for leukocyte – leukocyte binding during secondary capture (L-selectin). Secondary capture, mediated by L-selectin, occurs when a rolling leukocyte interacts with a free-flowing leukocyte, attaches to the endothelium and initiates rolling and adherence for accumulation migration to the site of infection [93]. CD44 is responsible for slowing the flow rate of the leukocytes thereby facilitating the attachment and migration of T cells. It has been suggested that elevated levels of CD44, in co-operation with shedding of L-selectin, inhibits the clearance of activated cells from the peripheral blood by secondary lymphoid tissue thereby also enhancing the migration of cells to the active site of inflammation [84]. LFA-1, a member of the β2 integrin family, is necessary to enhance firm adhesion of cells to activated endothelial cells [94]. Ligands such as JAM1 (F11R) can bind to ICAM3, a ligand found on the surface of leukocytes, and contribute to the adherence of leukocytes to the endothelium.

Our results indicate that these markers were significantly different when comparing TB diagnosis and EOT. Additionally the difference in expression between TB diagnosis and week 4 was significant before the Bonferroni correction for F11R and CD11a indicating a potential role as a constituent of an early treatment response biomarker signature. However, as these markers are involved in general inflammation and are not specific for *Mtb*, it is unlikely that these markers would take on a role as a key biomarker. The high expressions of the markers associated with this pathway bring their biological significance into question.

4.2.2.2. The pathway associated with crosstalk between dendritic cells and natural killer cells

The markers of interest associated with the crosstalk between dendritic cells and natural killer cells include TNFR2 (CD120b), CCR7, ITGAL (LFA-1, CD11a) and ICAM-3 (CD50).

DC's are antigen presenting cells that play an important role in the initiation of the immune response by inducing and stimulating CD4 and CD8 T cells necessary for the adaptive immune response. They reside in peripheral tissue and release cytokines and chemokines when a pathogen is detected [95]. Natural killer cells are involved in the innate immune response although they may share similar functions to cytotoxic T cells.   Their role in the initiation of the immune response involves the production of cytokines, primarily IFN-γ, which is one of the most important cytokines involved in the immune response. More recently it has become apparent that there is a crosstalk between DC's and NK's [96]. The mechanism of crosstalk occurs when DC's activate and stimulate NK's, which results in the subsequent production of IFN-γ, inducing the strong Th1 polarisation seen in *Mtb* infections [97].

In this pathway, TNFR2 and CCR7 are designated as having a role in the maturation and migration of DCs and the initiation of the adaptive immune response. In our analysis it was seen that CCR7 was down-regulated at EOT when compared with TB diagnosis and similar to healthy community controls at EOT, which is in keeping with a down-regulation of the immune response and it's components as the disease is resolved.

4.2.2.3. <u>The pathway associated with granulocyte adhesion and diapedesis</u>

The markers of interest associated with this pathway included L-selectin (CD62L), ITGAL (CD11a), ITGβ1 (CD29), TNFR2 (CD120b).

Granulocyte adhesion and diapedesis closely follows the leukocyte extravasation pathway described above where leukocytes are tethered, roll on and adhere to the endothelium in order to move between the endothelial cells and migrate to the site of infection.

L-selectin (CD62L) was down-regulated after treatment, which correlates with the resolution of disease as there would no longer be a need for the infiltration of leukocytes once an infection has been cleared. The changes in CD120b were discussed above.

ITGAL (CD11a) and ITGβ1 (CD29) were both significantly up-regulated after treatment but the very high expression levels at all three time points and in all groups (between 97.5 and 99.55% for CD11a and 91.85 and 95.04 for CD29) puts the clinical utility and biological significance in doubt.

4.2.2.4. <u>The pathway associated with caveolar mediated endocytosis signalling</u>

The markers associated with the caveolar mediated endocytosis signalling pathway are CD48, ITGAL (CD11a) and ITGβ1 (CD29). The ITGAL and ITGB1 were annotated as α-integrin and β-integrin respectively.

Endocytosis mediated by caveolae rafts is classified as clathrin-independent endocytosis. Caveolae appear as 50-80 μm invaginations in the plasma membrane of many cell types and their appearance and function are dependent on the cell type (98). Cell membranes are composed of phospholipids, cholesterols and sphingolipids. Rigid areas of membrane can form and move amongst the phospholipid bilayer regulating a variety of cellular functions and biological processes (99). Lipid rafts can associate with caveolin-1 to form caveolae which are capable of internalising potential pathogenic bacteria such as *Mtb*. Lipid rafts accumulate the signalling molecules necessary for the activation of lymphocytes as

well as playing an important role in the innate immune response. The role of lipid rafts in the immune response against *Mtb* is still largely unknown but it is thought that the mycobacterial 19 kDa lipo-protein mediated reactive oxygen species (ROS) formation allows toll-like receptor 2 translocation into the lipid rafts thereby promoting inflammation (100). It has also been shown that lipid rafts play a role in the induction of TNF- α and therefore contribute to the initiation of the innate immune response (101).

Upla *et al.* showed that α2β1 integrin is present in the raft-like membrane domains and the cluster formation of α2β1 integrin initiates the activation of endocytosis of caveolae therefore suggesting a novel mechanism of caveolae-mediated endocytosis (102). The up-regulation of α-integrin and β-integrin would therefore make sense as a compensatory mechanism in order to clear the *Mtb* infection via endocytosis. As CD48 can be found in the same fraction as caveolin, it is suggested that the up-regulation is due to its role in the bacterial receptor of caveolae in mast cells (103). The expression of CD48, and as discussed above ITGAL and ITGβ1, were all highly positive at all three time points and in all groups, once again bringing the biological relevance into question.

4.2.2.5. The pathway associated with NF-κβ activation by viruses

The markers associated with the NF-κβ activation by viruses pathway are CD4, ITGAL (CD11a) and ITGβ1 (CD29). In this pathway, ITGAL (CD11a) and ITGβ1 (CD29) are annotated as integrin α2β1.

Nuclear factor-κβ (NF- κβ) refers to a class of transcription factors that belong to the Rel family and play an important role in the initiation and promotion of inflammation as well as regulating the proliferation and survival of cells (104). Inflammation is characterised by the activation of both pro- and anti-inflammatory mediators present in tissue and blood cells. Cytokines, such as IL-1 and TNF-α, are released rapidly at the first sign of infection or tissue damage. The NF- κβ complex is initially inactive but may be activated in response to invading bacteria and viruses, other pro-inflammatory cytokines or stress-inducing agents (105). Two pathways exist for the activation of the NF-κβ pathway; namely, the canonical pathway and the alternative

pathway. The canonical pathway is activated by invading pathogens or the release of pro-inflammatory cytokines such as IL-1 and TNF-α while the alternative pathway is activated by cytokines from the TNF-family such as lymphotoxin β (106), CD40L, B cell activating factor and receptor activator of NF-κβ ligand (RANKL) (107). Certain viruses have the ability to activate NF- κβ to promote viral replication, prevent apoptosis and control the immune response. The activation of NF- κβ by viruses appears to occur via different mechanisms. In the case of HIV fusion of the viral and cell membrane activates NF- κβ with the assistance of CD4 (108) while other viruses such as cytomegalovirus (CMV) may function by α2β1 ligation (109). Lawrence *et al.* hypothesised that during resolution of disease, the NF- κβ pathway may be activated to express anti-inflammatory genes and induce apoptosis thereby playing a role in regulation of the inflammatory response (110).

The α2β1 integrin is known as the collagen-binding integrin which is expressed by Th17 cells. Although this study was not focussed on viruses and their activation of the NF- κβ pathway, there must be significant overlap with responses induced by *Mtb* and the change in α2β1 integrin expression could also be related to the Th17 response.

α2β1 integrin was in fact not up-regulated at week 24 but down-regulated at diagnosis of TB disease with expression of CD11a and CD29 returning to healthy community control level at week 24. This could be due to an increase in Th1 and Th2 cells, which would lead to a proportional decrease in Th17 cells or could be due to compartmentalisation of responses (peripheral blood versus site of disease).

4.2.2.6. The pathway associated with dendritic cell maturation

The markers involved in the dendritic cell maturation pathway include CD58, CCR7 and TNFR2 (CD120b).

Dendritic cells are potent antigen presenting cells and have the ability to stimulate naïve or memory T cells (95). The maturation of DC's forms a critical component in the initiation of the adaptive immune response and subsequent proliferation of

lymphocytes (111). DC's, in their immature form, reside in peripheral tissues and are capable of antigen capture. Upon stimulation, DC's migrate and mature to take on a role of antigen presentation and increased co-stimulatory molecule expression (95). Henderson *et al.* demonstrated that *Mtb* is phagocytosed by DCs suggesting a role of DCs in the early response to *Mtb*. They also showed that infection with *Mtb* resulted in an up-regulation of CD40, CD54, CD58, CD80 and MHC I and II molecules, indicative of maturation of DCs and antigen processing and presentation (112).

As previously discussed TNFR2 and CCR7, present on the surface of T cells and DCs, are involved in the maturation and migration of DCs as well as the stimulation of the adaptive immune response, all involved in the resolution of disease. As week 24 is post treatment and thus translates to cure, the need for an immune response is lessened resulting in the down-regulation of both markers (113).

4.2.2.7. The pathway associated with hepatic fibrosis and hepatic stellate cell activation

The markers associated with the Hepatic fibrosis/hepatic stellate cell activation pathway include IL-6R (CD126), CCR7 and TNFR2 (CD120b).

The hepatic stellate cell is a mesenchymal cell present in the liver and essential to hepatocellular function and liver fibrosis and repair. It has also been documented that the hepatic stellate cells are important in the development of the liver by playing a role in the formation of the intrahepatic bile ducts (114). Stellate cells have an ability to induce the infiltration of leukocytes, produce chemokines and express toll-like receptors (115), all necessary for inflammation. Additionally hepatic stellate cells are capable of producing chemo-attractants to attract neutrophils which contribute to the liver inflammation (116). Stellate cells are able to produce IL-6 when stimulated with TNF-α, IL-1β and lipopolysaccharide which in turn amplifies the acute phase response. The acute phase response is activated rapidly after tissue injury or trauma, infection or immunological disorders which may create an imbalance in homeostasis (117). The liver hepatocytes are responsible for the increased production of some blood proteins (acute phase proteins) such as C-reactive protein

(CRP), serum amyloid A and haptoglobin (118). Bacterial infections, such as *Mtb*, initiate a strong acute phase response which could explain the up-regulation of our markers at diagnosis with a subsequent down-regulation after treatment, albeit on peripheral blood T cells in this case, rather than on hepatic cells. Presumably similar stimuli that would up-regulate these markers on hepatic cells would also be able to achieve the same marker expression on T cells. The markers in this pathway all correlate with the liver's attempt to produce chemokines involved in the inflammatory process (119).

4.2.2.8. <u>The pathway associated with the role of osteoblasts, osteoclasts and chondrocytes in Rheumatoid Arthritis</u>

The markers associated with this pathway include TNFR2 (CD120b), LRP1 (CD91) and ITGβ1 (CD29).

Rheumatoid arthritis is a degenerative bone disease which results in inflammation in the soft tissue surrounding the affected joints. In healthy people, approximately 10% of bone mass is replaced each year and this is due to osteoclast and osteoblast activity.   Circulating blood monocytes have the ability to migrate to sites of injury or inflammation and differentiate into activated macrophages. However, in the case of a bone injury or trauma, the monocyte and macrophage precursors can also differentiate into osteoclasts (120).   There are even reports of trans-differentiation from monocytes into a number of cell types, including endothelial, osteoblasts, chondrocytes and hepatocyte-like cells (121).

As above, the markers measured in this study are all on T cells, but common effects of pro-inflammatory signals may be found on a range of cell types and in this case the changes seen in rheumatoid arthritis in response to inflammation may also be evident in inflammation due to TB.

4.2.2.9. <u>The pathway associated with T Helper cell differentiation</u>

The markers involved in T helper cell differentiation are TNFR2 (CD120b) and IL-6R (CD126).

It is a well-known fact that an infection with *Mtb* induces a T helper 1 response of which IFN-γ plays an important and central role. T helper cell subsets are in a dynamic balance, which has led to the assumption that a decrease in Th1 differentiation in *Mtb* is complemented by an increased Th2 differentiation (122).

Naïve T cells have the potential to differentiate into at least four types of T helper cells namely Th1, Th2, Th17 and Tregs. T cell precursors originate in the bone marrow from hematopoietic stem cells and travel to the thymus where they undergo maturation. Naïve T cells leave the thymus and enter the peripheral blood where they circulate until they encounter an antigen (123). Differentiation occurs when T cell precursors come into contact with antigen peptides complexed with MHC on the surface of antigen presenting cells (APCs).   Differentiation into lineage specific cells is dependent on the cytokine environment, types of APCs, the concentration of antigen as well as co-stimulatory molecules (124).

The activation of naïve T helper precursor cells requires three sets of receptors namely the T cell receptor (TCR), co-stimulatory receptors (CD28) and cytokine receptors. During activation, the TCR and CD28 co-stimulatory receptor are re-arranged into a complex between the antigen presenting cell and the T cell, known as the immunological synapse (IS).

<u>Th1 cell differentiation:</u> IL-12 and IFN-γ are two of the most important cytokines involved in Th1 cell differentiation. APCs secrete IL-12 upon activation which induces the production of IFN-γ by natural killer cells. Interferon gamma receptor (IFNGR) is expressed by the T helper precursor cells (Thps) and the formation of the IFNGR and IFN-γ complex initiates the Th1 differentiation providing a positive feedback loop (125). IL-12R is not present on the surface of the cells however it plays a critical role in the continuation of the differentiation of the Th1 response. A number of transcription factors are involved in the differentiation of the Th1 cell subset such as T-box transcription factor (T-bet), Signal transducer and activator of

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transcription 1 (STAT1) and STAT4 as well as the inhibitory transcription factor GATA3 . Although these transcription factors all have their own signalling pathway, they also work together for effective Th1 differentiation.   Animal studies have shown that a deficiency in IL-12, IL12R, signal transducer and activator of transcription protein (STAT) 4 and T-bet produce a defective Th1 response and increased susceptibility to *Mtb* (126). Literature also shows that *Mtb h*as the ability to inhibit the production of IL-12 thereby inhibiting the production of Th1 cells necessary to fight the infection.   *Mtb* infected cells have a tendency to increase the production of transforming growth factor β (TGF- β) which plays an instrumental role in the differentiation of Tregs which thus inhibit protective responses initiated by the host (127). Unfortunately the data on IL-12R (CD212) and IFN-γR (CD119) was not interpretable in this study due to difficulty in determining the appropriate cut-off for positivity on the FACS plots.

TNFR2 showed a down-regulation in expression post-treatment, as discussed previously. TNF is important for the recruitment of leukocytes and macrophages to the site of infection and for the maintenance of granulomas and therefore a decrease in this TNFR expression, that has anti-inflammatory effects, could indicate a resetting of the TNF-associated pro- and anti-inflammatory balance as the disease resolves.

Th2 cell differentiation: IL-4 and IL-2 are the most important cytokines and undergo stimulation in their production to initiate Th2 cell differentiation. IL4R binds to its ligand which results in an activation of STAT6 which is the main transcription factor associated with differentiation and induces an up-regulation of GATA3. GATA3 is considered to be the master regulator of Th2 differentiation and works in conjunction with STAT6 to regulate the Th2 genes. It was shown that mice that are deficient in GATA3 have a tendency to produce a Th1 lineage of cells instead of Th2 (128). IL-6 is known to enhance the Th2 response by increasing the production of IL-4 by naïve T cells while simultaneously inhibiting the Th1 response by interfering with STAT1 activation (129).

Other T cell phenotypes: Th17, Treg and T follicular cell differentiation: The major signalling cytokines involved in Th17 cell differentiation are IL-6, IL-21, IL-23 and TGF-β with retinoic acid receptor-related orphan receptor gamma-T (RORγt) being

the master regulator. TGF-β plays a role in the development of regulatory T cells (Tregs). If present in high concentrations and alone, it can skew the cell differentiation to a Treg lineage. However, at low concentrations and in the presence of IL-6, it will initiate Th17 differentiation while simultaneously increasing the production of IL-21 and IL23R, both of which are important in the subsequent phases of Th17 differentiation. TGF-β in the presence of IL-6 leads to the activation of RORγt which induces the production of IL-17A and IL-17F (130). We found a higher expression of CD126 (IL-6R) in TB patients at diagnosis than at EOT, healthy community controls or OLD controls, which is indicative of an increase in the production and differentiation of Th17 cells during active TB. The cytokines produced by Th17 cells are pro-inflammatory and their importance in the control of TB has previously been demonstrated (37).

Tfh cells are a subset of CD4 T cells that provides assistant in the formation of B cells. The primary cytokines involved in the induction of Tfh production are IL-6 and IL-21 although it still remains unclear of the exact role each of these cytokines play in Tfh development. Eto *et al.* showed that a deficiency in both IL-6 and IL-21 resulted in a decreased differentiation of Tfh cells however an absence of only one cytokine had no effect (131). B cells may have a regulatory role in the immune response to intracellular pathogens such as *Mtb*. B cells may form aggregates in *Mtb* infected lung and regulate the formation of granulomas, cytokine production and the activation of T cells. Thus a down-regulation of IL-6R post-treatment may indicate a decrease in the Tfh cell differentiation due to the clearance of *Mtb* and therefore a diminished need for B cell production.

4.2.3. IPA pathways and heat map data associated with significant markers when comparing surface marker expression between two timepoints (Dx - week 4, Dx - week 24 and week 4 - week 24).

When performing IPA on markers with a p-value <0.01 before the Bonferroni correction for the paired t-test data, it was noted that there were a number of different pathways when comparing groups as outlined in section 3.2.2.1. The pathways identified in the TB diagnosis versus week 24 analysis correlate closely

with the pathways associated with the overall treatment response pathways in section 4.3. The markers that changed significantly between diagnosis and week 4 represent pathways associated with communication between innate and adaptive immune cells. The granulocyte adhesion and diapedesis, leukocyte extravasation, crosstalk between DC and NK cells, TNFR2 signalling, T helper cell differentiation tight junction signalling pathways were all involved in early treatment changes when there is an influx of leukocytes and immune cells to the site of infection as well as communication and crosstalk of immune constituents of innate and adaptive immune response signalling. Changes in these pathways, some increased and others decreased, point towards a reprogramming of the immune system as antibiotics decrease bacterial burden. The pathways associated with later treatment changes (between week 4 and EOT time points) overlapped strongly with early treatment changes although pathways associated with apoptosis and clearance of pathogens also became evident. The granulocyte and agranulocyte adhesion and diapedesis, semaphorin signalling in neurons, regulation of cellular mechanics by Calpain protease, T helper cell differentiation and TREM1 signalling pathways all support the inflammatory response and migration of immune cells to the site of infection while the macropinocytosis signalling and role of JAK family kinases in IL-6 type cytokine signalling pathways play a role in antigen presentation and interactions of the humoral and cellular mediated adaptive immune response.

Qlucore Omics Explorer heat maps provide an interactive visualisation of large multivariate data sets assisting in the identification of patterns. In this project, the expression of surface markers was compared for three time points and participant groups and the changes were depicted as either red, to denote an up-regulation, or green, to denote a down-regulation, of expression. The varying shades of either red or green indicate the degree of either the up- or down-regulation. Black boxes indicate that there was no change relative to the mean expression of the entire data set. Heat maps were generated using the surface markers that had a p-value <0.01 before the Bonferroni correction.

4.2.3.1. Comparison of marker expression between TB patients at diagnosis, week 4 and week 24

A heat map (Figure 3.8) showing the comparison between the time points of TB patients at diagnosis, week 4 and EOT revealed a trend for differences in expression of the markers, with both increased and decreased expression after initiation of treatment. When performing IPA on the clusters of up- and down-regulated markers, it was evident that the markers in each cluster are associated with pathways that are involved in the initiation of the immune response and migration cells to the site of infection.

4.2.4. IPA pathways and heat map data associated with significant markers when comparing surface marker expression between two patient groups (TB diagnosis, healthy community controls and other lung disease controls).

When comparing IPA results it was noted that the NF-κβ activation by viruses, caveolar mediated endocytosis signalling and PTEN signalling pathways were the common ones involved with the markers differentiating between TB, healthy community controls and other lung disease controls. Pathways (leukocyte extravasation signalling, Paxilin signalling, granulocyte adhesion and diapedesis and integrin signalling) associated with the comparison between TB and healthy community controls involved those that are directly related to adhesion and migration of cells through the endothelium and thus affiliated with early inflammation and initiation of the innate immune response. Pathways associated with the differences between TB patients and other lung disease controls include those that are involved in innate responses such as crosstalk between DC and NKs and DC maturation. The coagulation system and role of tissue factor in cancer pathways are both related to the release of tissue factor by monocytes in early inflammation in an attempt to prevent the spread of infection. This occurs in the early stages of inflammation, simultaneously with the production of pro-inflammatory cytokines and the recruitment of immune cells (132). The PPAR signalling pathway and type II diabetes mellitus signalling pathways are related to general chronic inflammation and the

differentiation and development of cells, neither of which are directly related to TB but rather a broad spectrum of chronic inflammatory diseases.   While there are a number of pathways highlighted as different between TB and healthy community controls and TB and OLD controls, most of the pathways are related to early inflammatory responses and the innate immune response. There is a strong overlap between the pathways that differ between groups.

4.2.4.1. Comparison of marker expression between TB patients at diagnosis, week 24 and healthy community controls

The heat map (Figure 3.11) shows that marker expression of week 24 and healthy community controls cluster together and are separate from the diagnosis time point, implying that patients have moved towards a healthy marker expression profile after treatment.

4.2.4.2. Comparison of marker expression between TB patients at diagnosis, OLD controls and healthy community controls

The heat map (Figure 3.12) generated showed that the two disease groups cluster together and are separate from the healthy community control group.

4.2.5. Association between expression of markers at diagnosis and study outcomes

Due to the small sample size of this project, it was not possible to perform logistic regression and general discriminate analyses. Parametric and non-parametric methods were performed to verify data and in most cases the results were the same. The standard deviations are large indicating that a more extensive validation study is necessary in order to draw any conclusions from this statistical data.

4.3. Summary

Biomarkers, or biological markers, are defined as characteristics of normal biological function, pathological processes or pharmacological responses to therapeutic interventions which can be objectively, accurately and reproducibly measured (134). The WHO states that a biomarker may constitute any parameter that can be measured and reflects an interaction between a biological system and a potential chemical, physical or biological hazard (135).

Limitations in diagnosis and monitoring treatment response have inspired research groups around the world to identify host biomarkers which are capable of early diagnosis of TB, providing correlates of risk of TB, providing correlates of protection against active TB disease and for determining the response of a patient to treatment (3). An optimal biomarker for TB should be able to differentiate between latent or active infection, monitor treatment response and reliably predict a clinical outcome of actively infected patients (3). Currently there is no single biomarker which is able to monitor treatment response to TB chemotherapy; however, a more feasible idea would be to create a biomarker panel or signature made up of a number of measurable characteristics that could specifically and sensitively monitor treatment response from as early as four weeks.

This project looked at 252 cell surface markers in PBMCs of actively infected TB patients as well as healthy community controls and other lung disease controls. Using the very stringent Bonferroni correction, we identified five markers that changed significantly during the course of treatment in TB patients. Changes of two markers were not deemed as biologically significant as the mean expression was predominantly positive through the course of treatment and it is unlikely that the very minor change in expression is biologically relevant or useful from a biomarker perspective. Three markers, CD62L, CD120b and CD126 were significantly down-regulated between diagnosis of TB and EOT. All three markers play important roles in the inflammatory response to bacterial, viral or parasitic pathogens as well as the control of auto-immunity. It is possible that some of the changes seen may be due to

changes in cell populations in the circulation during the inflammatory response, changes in the surface protein regulation of a specific cell type or even as a result of changes in a specific marker expression in several cell types. It is possible that some changes in surface marker expression may be missed or understated due to the up-regulation of a marker in one cell type but a down-regulation of the same marker in another cells type resulting in a net effect of no change. This may due to the fact that multiple cell types may express the same marker and differentiation between these possibilities would be difficult.

The analysis of 252 markers on the surface of peripheral blood mononuclear cells has been made possible using the FACS^{TM} CAP technology designed by Becton Dickinson Biosciences. The present study is the first to evaluate the utility of this technology in a clinical study of this magnitude and a first evaluation in the heterogeneous sample type, PBMCs, right after isolation from whole blood. The work highlights some critical aspects of the FACS^{TM} CAP technology:

- Feasibility for biomarker discovery.
- Technically relatively demanding due to the need for PBMC isolation. Future studies will evaluate simplified approaches like testing of whole blood.
- The need for manual rather than automated analysis in a heterogeneous sample type like PBMCs due to difficulty in obtaining standard gating. This is a serious concern as this process is very labour intensive and requires relatively advanced training.
- The need for validation experiments through selection of the most promising markers for properly powered studies to confirm candidate marker performance.
- The need for further optimisation of cryopreserved PBMC samples, which was difficult to achieve due to the loss of cells in the cryopreservation process with resultant low cell concentrations.
- The need for further optimisation of stimulation assays as PPD stimulation alone did not appear promising (only three markers showed up-regulation). Antigen concentrations need to be titrated and different antigens should be investigated.
- The marker expression between TB at diagnosis and other lung diseases only showed a trend for differences after Bonferroni correction. However, the small

number of participants (only 9 other lung diseases) may have precluded the discovery of truly differentially expressed markers. Furthermore, a lack of specificity may not be a major obstacle in the evaluation of TB treatment response, particularly in clinical trials of new drugs, as biomarkers always have to be interpreted in a proper clinical context and other diseases would have been largely excluded in a properly conducted clinical trial.

4.4. Conclusion

The FACS™ CAP technology allowed the identification of three promising cell surface markers that require further validation as biomarker candidates for TB treatment response evaluation. This will be achieved through investigation of these and the most promising other markers that showed a trend for differences after Bonferroni correction in a candidate biomarker project of a new cohort of TB patients undergoing treatment.

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